

Second Edition

Epigenetic Cancer Therapy

Translational Epigenetics Series

Edited by Steven G. Gray



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Second Edition

Edited by

Steven G. Gray

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Introduction

1

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Chapter outline

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1 Introduction

Epigenetics has come a long way from its earliest incarnations. But how do we now define this phenomenon? The classical definition coined by Waddington in 1942 used a phrase “epigenetic landscape” to describe how genes might interact with their surroundings to produce a phenotype [1,2]. Commonly used definitions nowadays posit epigenetics as a “stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” [3], or as “chromatin-based events that regulate DNA-templated processes” [4]. A consensus definition still remains elusive.

Critically, it is now well established that aberrant epigenetics are a common feature in cancer [5]. There are currently four well-described mechanisms that underpin epigenetic regulation. They are: DNA CpG methylation, histone posttranslational modifications (PTMs), histone variants, and noncoding RNA (ncRNA) [6]. However, the issue of ncRNA being a true epigenetic mechanism is controversial. For example, the journal *Clinical Epigenetics* describes the issue as “Differential RNA expression levels (coding or non-coding) or RNA modifications cannot be considered as part of epigenetics, and this includes levels of the RNA modifying enzymes or readers,” with associated strict criteria for publication [7].

All four work individually or together to elicit what is often described as the “Epigenetic code.” Generally speaking, this is described as the “epigenome,” and large research programs have been

undertaken (e.g., the ENCODE Project Consortium) to generate epigenetic “roadmaps” or “blue-prints” of human health and disease including cancer [8].

Given the scale of advances in the field of epigenetics in recent years, this revised volume has been re-designed to reflect these advances and has been restructured accordingly.

2 Introduction to the area (key concepts)

In beginning the formulation for this revised volume it was felt that as this book is aimed not only at students but also at scientists, medics, and researchers, it would be critical to have a significant introductory section, allowing the reader to become acquainted with the known mechanisms by which epigenetic regulation of gene expression is achieved and how epigenetic dysregulation is a common feature in cancer [9,10].

As a concept the “idea” of an “epigenetic code” has simplified the way we can describe the mechanistic elements that the cell uses to recognize and elicit epigenetic or aberrant epigenetic regulation in a cancer cell. Generally speaking, there are three “types” of regulatory protein known as “readers,” “writers,” or “erasers” of the epigenetic code. These are most generally associated with proteins that interact with histones through histone PTMs, although some have suggested a fourth category “remodeler,” while proteins, such as DNA methyltransferases, which modify DNA, have also been classified as “writers” [11].

Five main mechanisms allow the epigenetic machinery to regulate expression through: (1) DNA methylation; (2) chromatin accessibility; (3) histone modifications; (4) DNA–protein interactions; and (5) chromatin tridimensional architecture [12].

DNA methylation was one of the first described epigenetic events, and the suggestion that methylases could act as oncogenic agents was first postulated in 1964 by Srinivasan and Borek [13]. Over the next 50 years our understanding of DNA methylation has dramatically changed, with the recent identification of additional methylation states, such as hydroxymethylation [14]. In this revised edition, the roles of DNA methylation and hydroxymethylation in cancer are discussed in detail, how mutations in epigenetic regulators are involved with the fidelity of DNA methylation maintenance and how DNA methylation may be a driving process during tumorigenesis, and discuss the current available methodologies by which researchers can investigate the landscape of DNA methylation and hydroxymethylation in the laboratory setting.

We have known about histone posttranslational mechanisms, such as histone acetylation, since the 1960s [15], but the notion of a “Histone Code” was not formulated until the turn of the century by Jenuwein and Allis [16]. The histone code basically involves mechanisms by which cells mark histones in chromatin using PTMs to regulate gene expression allowing the various readers, writers, and erasers allude to above to add/remove/read and elicit epigenetic regulation. In this revised edition, a dedicated chapter describes the key concepts surrounding these three classes of proteins, with discussions on the key elements in the complex interplay of epigenetic proteins in the regulation of gene transcription, and how defects in these systems can contribute to cancer initiation and progression. On top of the existing histones, a newly emerging area of importance is oncohistones [17,18]. The importance of these mutated histones in cancer is obvious from their name, and a new chapter in this revised edition introduces the reader to this important topic.

Despite the ongoing issue of ncRNAs as an epigenetic mechanism, their potential role in epigenetic-based processes, such as gene regulation, needs to be considered. One class of ncRNAs is called microRNAs, or miRNAs (and include a special subclass called epi-miRNAs) [19], while another is long noncoding RNAs (lncRNAs) [20,21]. The roles of both in cancer are discussed in detail, their biogenesis, mechanism of action, their role in cancer initiation, promotion, and progression, and their potential as epigenetic anticancer therapeutics. Other emerging classes not currently discussed in this new edition (but which the reader should be aware of include enhancer RNAs [22] and circular RNAs) [23,24].

RNA itself can be modified in ways that essentially mimic those observed for epigenomics and have led to a term frequently found in the literature “epitranscriptomics” [25], a subject often viewed as controversial within the epigenetics community. Despite the controversy, epitranscriptomic changes are also observed in cancer [26,27], and for inclusivity a chapter devoted to this has been added to this revised volume, as the role of epigenetic modification of RNA, while in its infancy, demonstrates the potential importance with respect to elucidating the functional role of RNA methylation with regard to cancer. Whether it can truly be considered a form of epigenetic regulation remains to be resolved however.

The emergence of next-generation sequencing (NGS) and large-scale epigenetic mapping projects have consequently led to a wealth of epigenetic data [8]. For many researchers, this represents a technical challenge as to how to integrate and interrogate this effectively, and to this end, an updated chapter discussing these issues has been extensively re-written to introduce the reader to these challenges.

Other emerging concepts in this revised edition introduce the reader to subjects such as the potential use of synthetic biology to examine epigenetic mechanisms [28,29]. And a chapter has been included to introduce the reader as to how environmental aspects, such as pollution [30] may play a potential role on cellular epigenetic regulation that may increase cancer risk.

3 Epigenetics and cancer

Having discussed the basics, the next section deals with a series of actual cancer settings. No one cancer is the same and so individual chapters on various important cancers have been revised and updated to discuss the roles of aberrant epigenetics within particular tumor types and describe the recent advances in our knowledge regarding the potential role of epigenetic targeting agents in these cancers. Finally, the key currently identified potential epigenetic targets/biomarkers for therapy have been discussed in detail for each cancer type.

4 Targeting aberrant epigenetics

It was not until 2004–06 that drugs targeting DNA methyltransferases were finally approved by the FDA for the treatment of myelodysplastic syndrome [31–33]. Since the development and approval of demethylating agents, such as Dacogen and Vidaza, drugs, such as histone deacetylase inhibitors (e.g., Vorinostat and Romidepsin), have also received regulatory approval for the

treatment of myelodysplastic syndrome and cutaneous T-cell lymphoma [34,35]. Since then several other HDACi (Belinostat, Panobinostat, Chidamide) have been approved for use in various countries and settings [36–39].

Despite this significant challenges remain regarding novel therapies targeting the epigenetic machinery, with few new approvals to date. The revised edition includes chapters covering many of these new therapeutic targets and is aimed at introducing the reader to various aspects with respect to the development of new and emerging epigenetic therapeutics themselves.

5 Issues to overcome/areas of concern

One of the major drawbacks encountered with epigenetic therapies has been the issue of ineffectively low concentrations within the context of solid tumors [40]. But these are not the only issues of concern. [Section 4](#) of the revised edition *Epigenetic Cancer Therapy* highlights to the reader some potential areas or issues that may also be important to consider when epigenetically targeting cancer.

One such critical issue with respect to epigenetic targeting concerns the actual compositional makeup of the tumor itself. It is now becoming apparent that intratumoral heterogeneity (ITH) and epigenetic ITH are major issues affecting tumor makeup [41–44]. Obviously, this has major implications for both standard therapies as well as epigenetic therapies, and in this revised edition (epi) ITH is discussed in detail.

Leading on from ITH (and epiITH), given that single-cell sequencing is now a commonly used methodology in the study of epigenetics [45,46], this may add further complexity to all large-scale NGS studies [12], and the complexity and challenges for single-cell epigenetic studies have been summarized for the reader in a new and separate chapter for this edition.

Other common concerns with respect to epigenetics and cancer arise at many levels. One such relates to the ability of cancer cells to evolve resistance to DNA damaging agents, such as cisplatin, and adds additional clinical evidence with respect to how epigenetic targeting agents may play a future role in the clinical management of DNA damaging therapeutic regimens.

In the final chapter of this section, we re-explore the role of epigenetics in cancer stem cells, and how these can potentially play a role in cancer drug resistance [47,48], and the role of “stemness” within the context of both epigenetics and cancer is covered in depth.

6 Future directions: translation to the clinic

In the final section of this revised edition, the reader is introduced to how the field of epigenetic therapy may evolve in the near future, particularly how we may achieve personalized epigenetic therapy.

In this edition, we re-explore and update areas previously covered, and two chapters discuss how epigenetic analysis can be used in both chemosensitivity testing and triaging of patients to appropriate treatment arms and/or to increase the numbers of patients suitable for personalized therapy. A new topic that has also been described centers on the issue of “epigenetic priming,” whether

or not low-dose targeting of the epigenetic machinery can be used to “prime” a cancer for an improved response to other therapies [49–51]. In the final two chapters, we explore how epigenetics can be incorporated into newer technologies, such as CRISPR editing [28,29,52,53].

The wealth of data emerging regarding both the aberrant epigenetics underpinning cancer combined with the exciting new developments with respect to therapeutically targeting cancers through inhibition of the epigenetic regulatory machinery has thrust epigenetics to the forefront of cancer research. By providing this comprehensive volume of how our understanding of epigenetics and epigenetic cancer therapy continues to evolve evolving, I hope that readers of this revised edition will identify or gain benefit for their studies in the treatment of cancer. I would also like to thank all of the contributors for the effort they have put in and for their time and patience.

References

- [1] Waddington CH. The epigenotype. *Int J Epidemiol* 2012;41(1):10–13.
- [2] Waddington C.H. Epigenetics and evolution. *Symp Soc Exp Biol* 1953.
- [3] Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. *Genes Dev* 2009;23(7):781–3.
- [4] Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell* 2012;150(1):12–27.
- [5] Darwiche N. Epigenetic mechanisms and the hallmarks of cancer: an intimate affair. *Am J Cancer Res* 2020;10(7):1954–78.
- [6] Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet* 2016;17(8):487–500.
- [7] Epigenetics C. Editorial policy on submissions involving RNAs. <<https://clincalepigeneticsjournalbiomedcentralcom/about>>; 2022.
- [8] Moore JE, Purcaro MJ, Pratt HE, Epstein CB, Shores N, Adrian J, et al. Expanded encyclopaedias of DNA elements in the human and mouse genomes. *Nature* 2020;583(7818):699–710.
- [9] Flavahan WA, Gaskell E, Bernstein BE. Epigenetic plasticity and the hallmarks of cancer. *Science* 2017;357(6348).
- [10] Baylin SB, Jones PA. Epigenetic determinants of cancer. *Cold Spring Harb Perspect Biol* 2016;8(9).
- [11] Chen JF, Yan Q. The roles of epigenetics in cancer progression and metastasis. *Biochem J* 2021;478(17):3373–93.
- [12] Casado-Pelaez M, Bueno-Costa A, Esteller M. Single cell cancer epigenetics. *Trends Cancer* 2022;
- [13] Srinivasan PR, Borek E. Enzymatic alteration of nucleic acid structure. *Science* 1964;145(3632):548–53.
- [14] Tsionplis NJ, Bailey DW, Chiou LF, Wissink FJ, Tsagaratou A. TET-mediated epigenetic regulation in immune cell development and disease. *Front Cell Dev Biol* 2020;8:623948.
- [15] Verdin E, Ott M. 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat Rev Mol Cell Biol* 2014;.
- [16] Jenuwein T, Allis CD. Translating the histone code. *Science* 2001;293(5532):1074–80.
- [17] Bagert JD, Mitchener MM, Patriotis AL, Dul BE, Wojcik F, Nacev BA, et al. Oncohistone mutations enhance chromatin remodeling and alter cell fates. *Nat Chem Biol* 2021;17(4):403–11.
- [18] Amatori S, Tavolaro S, Gambardella S, Fanelli M. The dark side of histones: genomic organization and role of oncohistones in cancer. *Clin Epigenetics* 2021;13(1):71.
- [19] Papadimitriou MA, Panoutsopoulou K, Pilala KM, Scorilas A, Avgeris M. Epi-miRNAs: modern mediators of methylation status in human cancers. *Wiley Interdiscip Rev RNA* 2022;e1735.

- [20] Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet* 2016;17(1):47–62.
- [21] Ponting CP, Haerty W. Genome-wide analysis of human long noncoding RNAs: a provocative review. *Annu Rev Genomics Hum Genet* 2022;.
- [22] Adhikary S, Roy S, Chacon J, Gadao SS, Das C. Implications of enhancer transcription and eRNAs in cancer. *Cancer Res* 2021;81(16):4174–82.
- [23] Greene J, Baird AM, Brady L, Lim M, Gray SG, McDermott R, et al. Circular RNAs: biogenesis, function and role in human diseases. *Front Mol Biosci* 2017;4:38.
- [24] Harper KL, Mottram TJ, Whitehouse A. Insights into the evolving roles of circular RNAs in cancer. *Cancers (Basel)* 2021;13(16).
- [25] Murakami S, Jaffrey SR. Hidden codes in mRNA: control of gene expression by m(6)A. *Mol Cell* 2022;82(12):2236–51.
- [26] Barbieri I, Kouzarides T. Role of RNA modifications in cancer. *Nat Rev Cancer* 2020;20(6):303–22.
- [27] Primac I, Penning A, Fuks F. Cancer epitranscriptomics in a nutshell. *Curr Opin Genet Dev* 2022;75:101924.
- [28] Bloomer H, Khirallah J, Li Y, Xu Q. CRISPR/Cas9 ribonucleoprotein-mediated genome and epigenome editing in mammalian cells. *Adv Drug Deliv Rev* 2022;181:114087.
- [29] Huerne K, Palmour N, Wu AR, Beck S, Berner A, Siebert R, et al. Auditing the editor: a review of key translational issues in epigenetic editing. *Crispr J* 2022;5(2):203–12.
- [30] Li S, Chen M, Li Y, Tollesfson TO. Prenatal epigenetics diets play protective roles against environmental pollution. *Clin Epigenetics* 2019;11(1):82.
- [31] Gore SD, Jones C, Kirkpatrick P. Decitabine. *Nat Rev Drug Discov* 2006;5(11):891–2.
- [32] Issa JP, Kantarjian HM, Kirkpatrick P. Azacitidine. *Nat Rev Drug Discov* 2005;4(4):275–6.
- [33] Kaminskas E, Farrell A, Abraham S, Baird A, Hsieh LS, Lee SL, et al. Approval summary: azacitidine for treatment of myelodysplastic syndrome subtypes. *Clin Cancer Res* 2005;11(10):3604–8.
- [34] Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. *Oncologist* 2007;12(10):1247–52.
- [35] Campas-Moya C. Romidepsin for the treatment of cutaneous T-cell lymphoma. *Drugs Today (Barc)* 2009;45(11):787–95.
- [36] Pojani E, Barlocco D. Romidepsin (FK228), a histone deacetylase inhibitor and its analogues in cancer chemotherapy. *Curr Med Chem* 2021;28(7):1290–303.
- [37] Lee HZ, Kwitkowski VE, Del Valle PL, Ricci MS, Saber H, Habtemariam BA, et al. FDA approval: belinostat for the treatment of patients with relapsed or refractory peripheral T-cell lymphoma. *Clin Cancer Res* 2015;21(12):2666–70.
- [38] Banerjee S, Adhikari N, Amin SA, Jha T. Histone deacetylase 8 (HDAC8) and its inhibitors with selectivity to other isoforms: an overview. *Eur J Med Chem* 2019;164:214–40.
- [39] Laubach JP, Moreau P, San-Miguel JF, Richardson PG. Panobinostat for the treatment of multiple myeloma. *Clin Cancer Res* 2015;21(21):4767–73.
- [40] Zi Y, Yang K, He J, Wu Z, Liu J, Zhang W. Strategies to enhance drug delivery to solid tumors by harnessing the EPR effects and alternative targeting mechanisms. *Adv Drug Deliv Rev* 2022;188:114449.
- [41] Guo M, Peng Y, Gao A, Du C, Herman JG. Epigenetic heterogeneity in cancer. *Biomark Res* 2019;7:23.
- [42] Swanton C, Beck S. Epigenetic noise fuels cancer evolution. *Cancer Cell* 2014;26(6):775–6.
- [43] Easwaran H, Tsai HC, Baylin SB. Cancer epigenetics: tumor heterogeneity, plasticity of stem-like states, and drug resistance. *Mol Cell* 2014;54(5):716–27.
- [44] Landau DA, Clement K, Ziller MJ, Boyle P, Fan J, Gu H, et al. Locally disordered methylation forms the basis of intratumor methylome variation in chronic lymphocytic leukemia. *Cancer Cell* 2014;26(6):813–25.

- [45] Ogbeide S, Giannese F, Mincarelli L, Macaulay IC. Into the multiverse: advances in single-cell multiomic profiling. *Trends Genet* 2022;38(8):831–43.
- [46] Preissl S, Gaulton KJ, Ren B. Characterizing cis-regulatory elements using single-cell epigenomics. *Nat Rev Genet* 2022;.
- [47] Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010;141(1):69–80.
- [48] Heery R, Finn SP, Cuffe S, Gray SG. Long non-coding RNAs: key regulators of epithelial-mesenchymal transition, tumour drug resistance and cancer stem cells. *Cancers (Basel)* 2017;9(4).
- [49] Wang Y, Tong C, Dai H, Wu Z, Han X, Guo Y, et al. Low-dose decitabine priming endows CAR T cells with enhanced and persistent antitumour potential via epigenetic reprogramming. *Nat Commun* 2021;12(1):409.
- [50] Lu Z, Zou J, Li S, Topper MJ, Tao Y, Zhang H, et al. Epigenetic therapy inhibits metastases by disrupting premetastatic niches. *Nature* 2020;579(7798):284–90.
- [51] Tsai HC, Li H, Van Neste L, Cai Y, Robert C, Rassool FV, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer Cell* 2012;21(3):430–46.
- [52] Belk JA, Yao W, Ly N, Freitas KA, Chen YT, Shi Q, et al. Genome-wide CRISPR screens of T cell exhaustion identify chromatin remodeling factors that limit T cell persistence. *Cancer Cell* 2022;40(7):768–786.e7.
- [53] Bashor CJ, Hilton IB, Bandukwala H, Smith DM, Veiseh O. Engineering the next generation of cell-based therapeutics. *Nat Rev Drug Discov* 2022;1–21.

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PART

Introduction and key
concepts

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Methylation and hydroxymethylation in cancer

2

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1 Introduction

Cancer cells evolve traits to promote their own survival and progression of the cancer disease by the accumulation of mitotically heritable genetic and epigenetic aberrations causing deregulation of, for example, cell cycling, DNA damage response, differentiation, apoptosis, and cell adhesion. Most of these traits involve epigenetic mechanisms that affect the expression of genes that in turn

promote cell proliferation or survival. Epigenetic abnormalities are common throughout all cancers and can be targeted to reprogram cancer cells. One of the most frequent classes of genes found to be mutated is the epigenetic regulators, providing a link between genetic alterations and epigenetic changes in cancer. Detection and functional characterization of epigenetic marks and regulators, and correlation of these findings to the pathogenesis and clinical outcome of specific diseases, form basis for the development of novel and targeted treatment modalities.

This chapter reviews aberrant DNA methylation and hydroxymethylation patterns in cancer, the role of epigenetic regulators involved in DNA methylation maintenance fidelity, and the driving events in the process of DNA methylation in tumorigenesis. To understand the aberrant DNA modifications in cancer an overview of DNA modifications in normal cells is provided as well.

2 Epigenetics

Epigenetics is defined as heritable changes in patterns of gene expression and function, which create a new phenotype but without a corresponding change in the DNA sequence [1]. Traditionally, epigenetic marks have been broadly classified into three groups: direct modification of the DNA (primarily cytosine methylation and hydroxymethylation), posttranslational modifications of histone proteins, and positioning of nucleosomes along the DNA. These together make up what is referred to as the epigenome. With the advent of genome-wide studies, our understanding of the epigenome is rapidly growing. The epigenome is involved in most cellular functions, including transcription, replication, and DNA repair. The epigenetic modifications gracefully combine forces to direct and regulate cellular fate. Failure of the epigenome to function properly can result in inappropriate activation/inhibition of genes that have been shown to be initiators and drivers of cancer, right alongside and in combination with genetic aberrations [2,3].

2.1 Chromatin structure

Every single human cell contains about $1\frac{1}{2}$ m of genomic DNA, consisting of approximately 3 billion base pairs, packed inside a small nucleus folded in chromatin with different levels of packaging. Chromatin is organized in repeating units of nucleosomes, each of which is a complex of 146 bp of double-stranded DNA wrapped around an octamer protein structure consisting of two subunits of each of the histone proteins H2A, H2B, H3, and H4 [4]. A fifth main histone protein, histone H1, localizes to internucleosomal DNA and is also named the linker histone. Additional histone variants that can be incorporated into nucleosomes are also reported in eukaryotes, such as the H2A.Z found in nucleosomes bordering the nucleosome-depleted regions (NDRs) at the transcription start sites (TSS) of active genes [5].

Numerous nonhistone proteins, such as transcription factors, polymerases, and other enzymes, bind to internucleosomal DNA and NDRs. Due to spatial organization and gene regulatory function, nucleosomes are folded in a complex manner to eventually form a chromosome. The template for transcription is chromatin and the structure of chromatin in mammalian cells changes dynamically, enforced by transcriptional needs. It may exist in a condensed, transcriptionally silent form, called heterochromatin, or in less condensed chromatin, named euchromatin, with a “beads-on-a-

string” conformation that is accessible for the transcriptional machinery. DNA modifications, post-translational histone modifications, and nucleosome remodeling operate in a dynamic way to change the chromatin structure.

2.2 Methylation in cellular homeostasis

DNA methylation is a chemical modification that plays an important role in the regulation of gene expression, genomic imprinting, X chromosome inactivation, transposon silencing, and genome stability. In mammalian DNA, methylation takes place symmetrically at the carbon-5 position of cytosines (5-methylcytosines, 5mC) preceding guanines, so-called CpG dinucleotides, where “p” refers to the phosphodiester bond in the DNA backbone.

2.2.1 Genomic distribution of DNA methylation

To understand the role of DNA methylation in cellular function, one must take into consideration the CpG distribution within the genome. CpG sites are rare (the observed frequency of 1% CpG dinucleotides is only ~20% of expected), which is believed to be caused by the spontaneous deamination of 5mC into thymine [6]. Thus, mammals have roughly fivefold fewer CpG dinucleotides than expected from the nucleotide composition of their genome. Interestingly, the CpG distribution genome wide is nonrandom. Large areas of the genome are only sparsely punctuated by CpG sites, and these are in turn heavily methylated. These CpG-poor oceans are interrupted by short, CpG-rich regions termed CpG islands [7]. These islands are defined as >0.5-kb stretches of DNA with a G + C content $\geq 55\%$. Based on the deamination rates of 5mC, it can be speculated how CpG islands exist. While they are most likely maintained through evolution, one explanation may be that CpG islands are rarely methylated, or only transiently methylated in the germ line, hence avoiding conversion into thymine [8].

CpG islands preferentially locate to the promoter/5' region of genes and 60% of human promoters have associated CpG islands. Promoter CpG islands tend to remain unmethylated during development and in normal somatic tissues, except for a few (~6%) that become methylated in a tissue-specific manner during early development [9]. For example, developmentally important genes may be tissue-specifically methylated in the somatic, differentiated tissue. Furthermore, X-chromosome inactivation and genomic imprinting are normal methylation events coordinated during development that occur at specific CpG islands and imprinting-associated differentially methylated regions, respectively. Lastly, CpG islands covering repetitive elements assumed to have evolved from parasitic elements are also highly methylated and help maintain chromosomal stability by inhibiting the transposition of these elements [10].

Regions with lower CpG density that lie within close proximity (~2 kb) of CpG islands, demarking areas between oceans and islands, are termed CpG island shores (Figure 2.1 and Box 2.1) [11]. Shore methylation also correlates with gene silencing; most tissue-specific methylation does not occur at islands but at CpG island shores, and differentially methylated shore regions can sufficiently distinguish different somatic tissues. Sequences (~2 kb) that border the shores are referred to as shelves. Canyons are regions (≥ 3.5 kb), with low methylation density that are distinct from islands and shores but contained within their boundaries, which span conserved domains that frequently bind transcription factors [12].

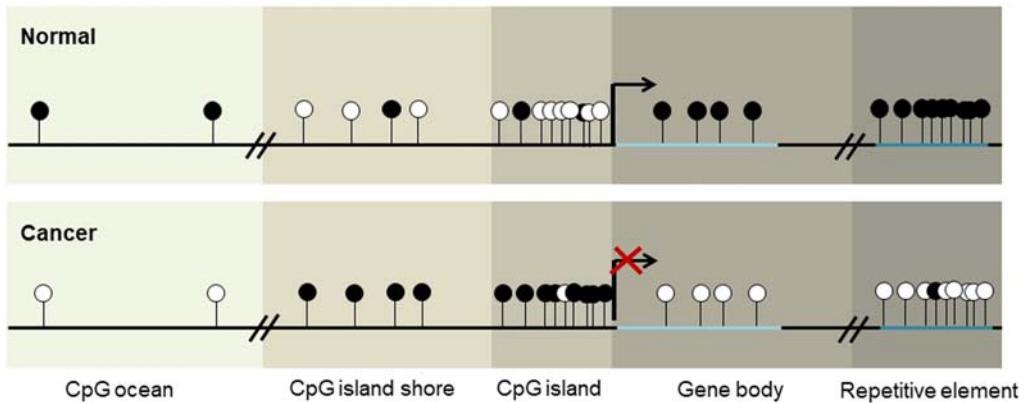


FIGURE 2.1 Distribution of CpG sites within the genome.

The top panel illustrates an overview of the CpG distribution and transcriptional effects of CpG methylation in normal cells, while the bottom panel illustrates alterations in cancer. CpG islands are typically located in the promoter region, 5' to the transcription start site (TSS) and are regions of high CpG density. These remain unmethylated in normal cells but become aberrantly methylated in cancer. Bordering CpG islands are CpG shores. These have a lower CpG density and can cover promoter and/or enhancer elements. Large genomic areas with low CpG density are named CpG oceans and comprise most of the genome. Repetitive elements and gene bodies have higher densities of CpG's and are methylated in normal cells. White lollipops: unmethylated CpG sites (5 C). Black lollipops: methylated CpG sites (5mC).

BOX 2.1 The DNA methylation landscape and related terms.

CpG islands

- Short CpG rich areas defined as >0.5kb stretches of DNA with a G+C content $\geq 55\%$. Typically located in the promoter region, 5' untranslated region to the TSS.

CpG shores

- Regions with lower CpG density that lie within the 2 kb up- and downstream of a CpG island.

CpG shelves

- 2 kb regions that border CpG shores.

CpG canyon

- Large regions (>3.5kb) of low methylation that span conserved domain that frequently bind transcription factors.

CpG ocean

- Regions with low methylation and not characterized in any of the above.

Gene body

- The entire gene from transcription startsite to the end of transcript.

Gene desert

- Regions with very few, if any genes in a 500 kb region.

Lastly, it should be mentioned that promoter and enhancer regions with CpG-rich regions that do not meet the CpG island criteria, or are categorized into any of the above, also show an inverse correlation between DNA methylation status and gene expression [13].

2.2.2 Functional role of DNA methylation

In normal cells, most CpG sequences in the genome are methylated, but CpG islands and CpG island shores are exceptionally hypomethylated. Many of these hypomethylated regions of DNA function as elements that regulate gene expression, such as promoters and enhancers. Unmethylated islands at promoters correspond to either active transcription or a poised state, where genes can be expressed if the appropriate cellular cues are present. CpG sites in promoter-associated CpG islands are often less than 10% methylated while distal regulatory sequences, such as enhancers, are commonly marked by levels of 5mC ranging from 10% to 50% [14,15]. The mechanism that protects CpG island promoters from methylation does not involve sequestration of the promoters in condensed chromatin since unmethylated CpG-rich sequences in nuclei show accessibility to diffusible factors, such as DNase I [16].

Although the mechanisms that protect most CpG island promoters from de novo methylation are not understood, a specific class of CpG island promoters is protected from de novo methylation by the multidomain chromosomal protein, FBXL10, bound by the polycomb-repressive complexes (PRC) 1 and 2. In the absence of FMXL10, PRC-bound promoters undergo de novo methylation with concomitant silencing of gene expression [17].

The functional consequence of DNA methylation at CpG islands/shores in gene promoters/enhancers is the inhibition of gene expression, while unmethylated promoter regions are permissive for transcription. Transcriptional inhibition occurs as a consequence of numerous factors. First, the methyl-group itself can sterically block the binding of transcription factors to the promoter [18]. Second, methyl-CpG-binding proteins are recruited to methylated DNA, and these in turn bind chromatin-remodeling complexes that further compact the area, making it inaccessible (Figure 2.2). In a similar fashion, unmethylated CpG sites bind different proteins, which recruit histone methyl-transferases (HMTs) that mark the chromatin with active marks [18]. It has also been shown that DNA methylation affects and directs RNA splicing [19]. Although promoter methylation shows a negative correlation with transcription, early studies of intragenic DNA methylation posited the opposite finding [20]. It is also believed that the gene body methylation inhibits spurious initiation of transcription within the gene body. Certain active genes are more methylated in gene bodies than repressed genes; however, this correlation appears to be tissue specific [20].

2.2.3 DNA methyltransferases

DNA methylation is catalyzed by a group of enzymes collectively named DNA methyltransferases (DNMTs). DNMTs covalently modify the carbon-5 position of cytosine residues, using S-adenosyl methionine as a methyl donor. DNMTs are divided into maintenance (DNMT1) and *de novo* methyltransferases (DNMT3A and DNMT3B). There is also a catalytically inactive DNMT, DNMT3L, which interacts with and stimulates the activity of DNMT3A and DNMT3B, specifically in the germline [21]. Both *de novo* DNA methylation and maintenance DNA methylation are important for normal development. During development, the *de novo* DNMTs are highly expressed and establish the methylation patterns independently of replication. Through differentiation, they are downregulated, and DNMT1 takes over and ensures that DNA methylation patterns are copied

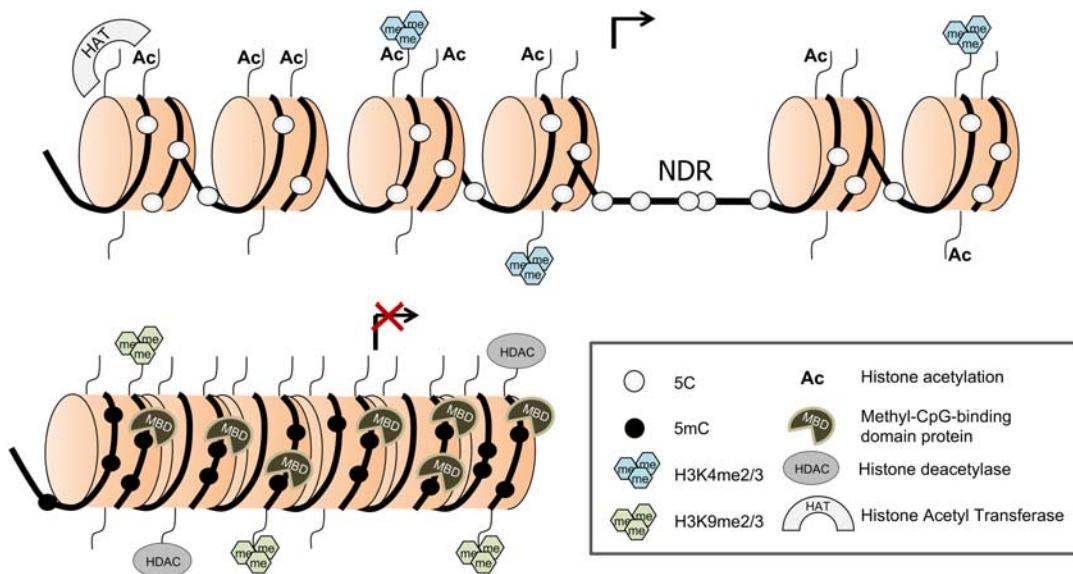


FIGURE 2.2 Methylation of CpG islands in gene promoters relates to transcriptional inactivity.

Methylation of CpGs in the promoters of genes relates to transcriptional inactivity, demonstrated by the observation that several proteins involved in transcriptional repression bind to methylated CpGs, but not to unmethylated CpGs. One such family of proteins is the highly conserved methyl-CpG-binding domain (MBD) proteins. When the MBD proteins (MBD1, MBD2, MBD4, and MeCP2) bind to methylated CpGs, histone deacetylases (HDAC), histone methyltransferases, and ATP-dependent chromatin remodeling complexes are recruited to the methylated DNA leading to a closed chromatin structure and transcriptional repression. Histone acetyl transferases (HAT) acetylate lysine amino acids on histone proteins and regulates gene expression by opening or closing the chromatin structure. In most cases, histone acetylation activates transcription.

and stably inherited to daughter cells. Hence, DNMT1 is highly expressed during S phase and has a strong affinity ($30-40 \times$) toward hemi-methylated DNA. However, studies have shown that the *de novo* DNA methyltransferases are also required for maintenance methylation in human embryonic stem cells (ESCs). DNMT1 itself is not sufficient for maintaining DNA methylation since a gradual loss of methylation occurs in subsequent cell divisions in DNMT3A and DNMT3B knockout ESCs [22]. It is suggested that the three enzymes cooperate to maintain DNA methylation at densely methylated regions, repetitive elements, and imprinted genes and that the cooperativity of these three enzymes may ensure that the fidelity of methylation patterns is maintained.

In addition, it has become clear that multiple additional regulatory inputs, especially those mediated by the interaction with the multidomain protein ubiquitin-like with PHD and ring finger domains 1 (UHRF1), are required *in vivo* to ensure stable maintenance methylation through mitotic divisions [23].

An intriguing question is how DNA methylation is targeted to specific sites in the genome. In plants, RNA interference is a dominant mechanism, but only few examples have been observed in humans. There are multiple theories, and these are reviewed elsewhere, but the most convincing studies show that other epigenetic factors (e.g., histone modifications) recruit the *de novo* DNMTs

to specific genes and that the underlying DNA sequence also guides DNMTs [24,25]. Conversely, CpG islands may be protected from methylation through R-loop formations coupled with GC strand asymmetry and through active histone marks in the vicinity, directly blocking DNMT access to the DNA [26].

2.3 DNA demethylation in cellular homeostasis

For decades, methylation of cytosines was thought to be a stable modification. Since 2009, several studies have revealed that the ten-eleven translocation (TET) family of Fe^{2+} - and α -ketoglutarate (α -KG)-dependent dioxygenases are involved in active demethylation of DNA by catalyzing the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) and further oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), that is excised by thymine DNA glycosylase (TDG) and replaced by unmodified cytosine through base excision repair (BER) (Figure 2.3)

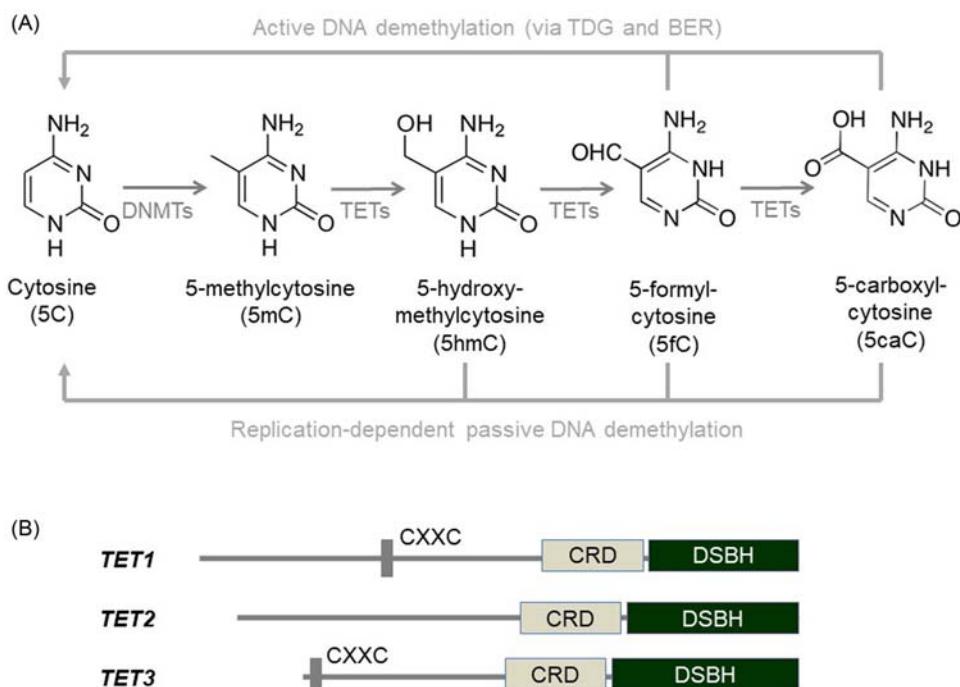


FIGURE 2.3 DNA demethylation and TET proteins.

(A) All three TET enzymes have a core catalytic domain containing a highly conserved cysteine-rich domain (CRD), a double-stranded β -helix (DSBH) domain and binding sites for Fe^{2+} and α -ketoglutarate (α -KG). *TET1* and *TET3* also contain a DNA-binding CXXC zinc finger domain. (B) All three TET enzymes oxidize the methyl group at the fifth carbon position of cytosines (5mC), that have been incorporated by the DNMTs, to 5hmC. TET proteins can further oxidize 5hmC to 5fC and 5caC, which can be removed by thymine DNA glycosylase (TDG) and replaced by unmodified cytosine (5C) through base excision repair (BER).

[27–29]. Besides catalyzing active DNA demethylation, TET enzymes are shown to be involved in passive DNA demethylation, since the DNMT1/UHRF1 complex has a much lower affinity for 5mC, 5fC, or 5caC and thus the cytosine on the newly synthesized strand is not methylated leading to replication-dependent passive DNA demethylation [30].

There are three TET proteins, TET1, TET2, and TET3. The core catalytic domain of all enzymes (i.e., the region responsible for CpG recognition, substrate preference, and catalytic activity) contains a highly conserved cysteine-rich domain, a double-stranded β -helix (DSBH) domain and binding sites for Fe^{2+} and $\alpha\text{-KG}$ (Figure 2.3). TET1 and TET3 also contain a CXXC zinc finger domain that can bind DNA. All three TET enzymes oxidize 5mC—the methyl group at the fifth carbon position of cytosines that have been incorporated by the DNMTs—to 5hmC.

For full catalytic activity of the TET enzymes, oxygen and the cofactors Fe^{2+} and $\alpha\text{-KG}$ must be present (Figure 2.4). Also, ascorbate, the reduced form of vitamin C, has an important role as an indirect cofactor for the TET and Jumonji (JMJC) family of dioxygenase enzymes *via* its properties as a reducing agent of iron [31,32]. In the presence of ascorbate, iron is reduced to Fe^{2+} from Fe^{3+} and thereby restores the catalytic activity of dioxygenases (Figure 2.4). *In vitro* studies have confirmed the enhanced TET activity induced by ascorbate and the inability of other antioxidants to elicit the same effect [31,33,34]. Besides Fe^{2+} and ascorbate, TET and JHDM enzymes utilize α -ketoglutarate ($\alpha\text{-KG}$, also called 2-oxoglutarate) as a cosubstrate for full catalytic activity. $\alpha\text{-KG}$ is converted from isocitrate—both important components of the citric acid cycle—by the isocitrate dehydrogenases (cytosolic IDH1 and mitochondrial IDH2; see Section 5.3 and Figure 2.5).

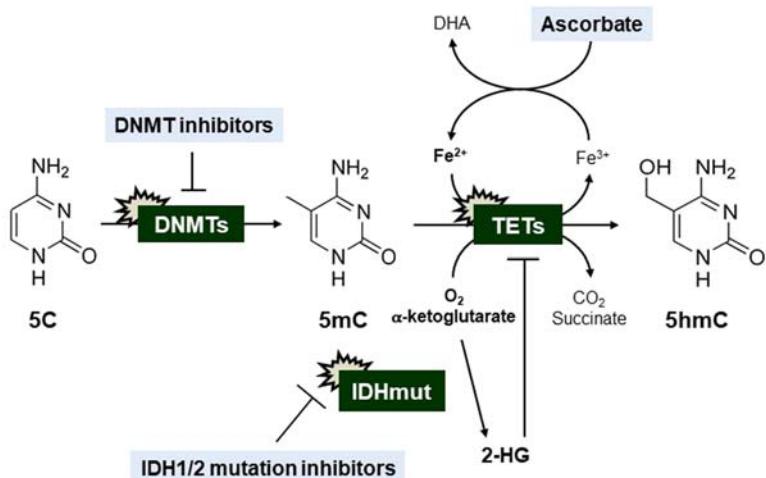


FIGURE 2.4 Mutations in genes encoding DNA methylation regulators affect genomic 5mC or 5hmC levels that can be manipulated by cofactors, unspecific or mutation specific drugs.

Ascorbate is an indirect cofactor of TET enzymes as a reducing agent of iron. The DNMT interfering drugs azacytidine and decitabine form a covalent bond with the DNMT enzymes, thereby preventing them from further methyltransferase activity. The IDH1 inhibitor, ivosidenib, and the IDH2 inhibitor, inasidenib, normalizes the inhibitory effects of 2-hydroxyglutamate (2-HG) on TET enzymes. 5mC, methylcytosine; 5hmC, 5-hydroxymethylcytosine.

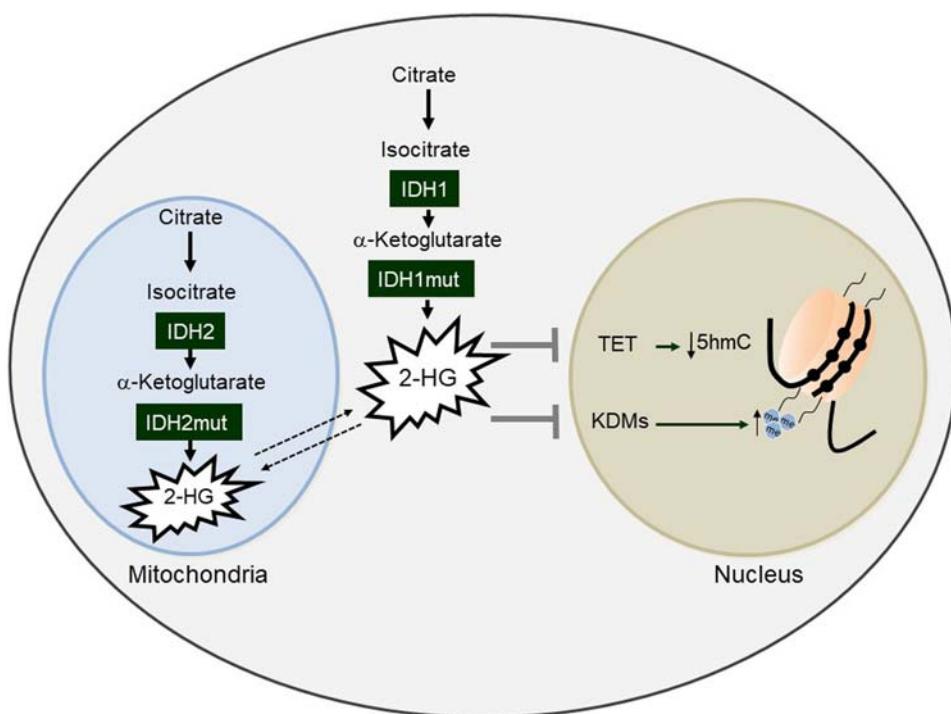


FIGURE 2.5 IDH1/2 mutations result in the synthesis of 2-HG, a competitive inhibitor of α-KG.

α -Ketoglutarate (α -KG) is converted from isocitrate by isocitrate dehydrogenases (cytosolic IDH1 and mitochondrial IDH2, both important components of the citric acid cycle). *IDH1/2* missense mutations alter the activity of the enzymes so that α -KG is reduced to 2-hydroxyglutarate (2-HG) resulting in impairment of enzymes that require α -KG as a cofactor, such as the TET proteins and the JMJC family of histone lysine demethylases (KDMs).

The central roles of the TETs in human cancers are underscored by findings that these genes—and genes important for regulating their cofactors—are frequently mutated in hematological and other cancers [35].

2.4 DNA hydroxymethylation in cellular homeostasis

5hmC is a normal constituent of mammalian DNA and forms slowly during the first 30 h following DNA synthesis in cultured cells [36]. 5hmC is, however, considerably less abundant than 5mC with less than 1% of 5mC levels in most cell types. However, the 5hmC content varies during development and cell differentiation. In ESCs and cerebellar Purkinje neurons 5hmC levels are as high as 5% and 40% of 5mC levels, respectively [27,28]. Interestingly, global 5hmC declines with age in human blood cells and associates with X-chromosome inactivation skewing and reduced telomere length [37]. In contrast, 5hmC levels are increased in adult brain relative to fetal brain [38].

Given the very low 5hmC content in mammalian DNA, highly sensitive detection methods are required for quantitative evaluation of 5hmC content. Also, DNA modifications are not preserved during PCR amplification and the standard methods used in the analysis of DNA methylation, such as bisulfite- or restriction enzyme-based techniques, are incapable of discriminating 5hmC and 5mC. There are, however, a steadily increasing number of methods available for specific detection of 5hmC either globally, genome wide, or at single bases, as reviewed elsewhere [39,40].

Genome-wide mapping studies have shown that 5hmC and the TET proteins are enriched in the gene bodies, promoters, and enhancers of transcriptionally active genes, indicating that 5hmC has specific biological roles [41,42]. 5hmC levels in promoters and gene bodies are typically positively correlated with gene expression [37,43]. 5hmC levels in enhancers are often cell type specific and positively correlated with active enhancer histone marks, such as H3K4me1 and H3K27ac [44].

The regulatory functions of 5hmC and TET1 in ESCs have been confirmed by several studies using affinity-based approaches. Unique genomic distribution patterns of TET1 have been mapped to TSS of CpG-rich promoters and within genes [45,46]. 5hmC is enriched at CpG islands with low to medium GC-content, at promoters with intermediate CpG density, at promoters with the bivalent histone marks (H3K4me3 and H3K27me3), and at intergenic cis-regulatory elements, such as active enhancers and transcription factor-binding sites [45,46]. Tet-assisted bisulfite and oxidative bisulfite sequencing enabling quantitative sequencing at base resolution level has revealed the distribution of 5hmC around and not within transcription factor-binding sites in ESCs [47] and at 5' splice sites in the brain suggesting a role in the regulation of splicing [38]. Furthermore, in human brain, 5hmC is enriched at poised enhancers and negatively correlated with the H3K27me3 and H3K9me3 enriched genomic regions.

Also in brain tissue, methyl-CpG-binding protein 2 (MeCP2) is identified as the major 5hmC-binding protein and binds 5hmC- and 5mC-containing DNA with similar high affinities. On a mechanistic level, abnormal 5hmC impacts chromatin structure by interrupting the interaction of 5hmC-specific binding proteins [48].

Genome-wide profiling studies indicate that the majority of 5hmC are stable epigenetic marks and thus not only intermediate products of active DNA demethylation [36]. In line with this, a comprehensive study of the 5hmC landscape in 19 human tissues show that 5hmC levels are highly tissue-specific and serves as fundamental regulatory elements affecting tissue-specific gene expression [49]. This, together with the fact that the 5hmC level undergoes highly dynamic changes during development and differentiation [50,51], suggest that 5hmC plays a critical role in developmental processes and that dysregulation of genomic 5hmC may be involved in tumorigenesis.

3 DNA methylation patterns in cancer

The DNA methylation landscape is profoundly disturbed in cancer cells compared to their normal counterparts. Cancer cells are characterized by global hypomethylation together with focal *de novo* promoter hypermethylation [1]. These observations have been made more than three decades ago, where initial studies showed hypomethylation of repetitive elements in both cell lines and primary tumors, and hypermethylation of promoter CpG islands, including those of tumor suppressor genes. While these observations still hold true, recent epigenome-wide studies have shown that alterations

in the DNA methylome of cancer cells are far more complex. The underlying initiating mechanisms of cancer-specific methylation changes are still largely unclear, but it is apparent that they occur early in tumorigenesis and contribute to both cancer initiation and progression [52].

3.1 Hypermethylation in cancer

As mentioned above, cancer methylomes exhibit focal regions of hypermethylation, frequently in transcriptional regulatory elements, such as promoters and enhancers of genes, including tumor suppressor genes [1]. Early studies have primarily revealed cancer-specific methylation of CpG islands in gene promoters causing silencing of the associated gene. The list of aberrantly methylated genes in cancer is steadily growing, including hundreds of genes affecting major cellular pathways, such as cell cycle control (*p15^{INK4B}*, *p16^{INK4A}*, *RB*), apoptosis (*TMS1*, *DAPK1*, *SFRP1*) and DNA repair (*MGMT*, *BRCA1*, *hMLH1*). Silencing of genes in DNA repair pathways will further propagate the carcinogenic state by allowing cells to accumulate additional genetic lesions. Also, silencing of transcription factors will indirectly silence or downregulate a large number of other genes [53]. Hypermethylated promoters undergo silencing, presumably by transcriptional repressors and histone-modifying enzymes that are recruited in a methylated DNA-binding (MBD) protein-dependent manner. This is exemplified by the finding that the NuRD complexes containing MBD2 binds to the p14/p16 locus and regulates gene silencing in human cancer cells [54]. High expression of UHRF1, a central factor in the maintenance of DNA methylation, is also critical for the suppression of tumor suppressor genes via hypermethylation of promoter regions in human colorectal cancer cells [55]. UHRF1 depletion results in significant promoter demethylation, gene upregulation, and suppression of multiple oncogenic properties of human colorectal cancer cells, suggesting that DNA methylation-mediated regulation of gene expression is important for maintaining the properties of cancer.

Worth mentioning is also the mutagenic properties of 5mC, since the spontaneous deamination of methylated cytosine to thymine described previously may result in point mutations if remained impaired [56]. More than 30% of all germline and almost half of all somatic *TP53* mutations occur at methylated CpGs. Sequencing studies showed that 70% of tier 1 mutations in acute myeloid leukemia (AML) comprised 5mC-T transitions [57]. Furthermore, 5mC may be involved in the mutagenic effect of exogenous carcinogens from cigarette smoke by promoting formation of adducts on the subsequent guanine and thus the conversion of guanine to thymine [56].

Interestingly, hypermethylation events at CpG island shores and CpG-rich distal regions (e.g., enhancers) may also be cancer-specific alterations, which in some studies correlate more closely with gene expression [58,59].

Some oncogenes have been reported to be activated by DNA hypermethylation within gene bodies. Su et al. [60] reported that many gene bodies of Homeobox genes are hypermethylated in cancer cells and their levels are associated with gene expression. They also demonstrated that gene expression was significantly increased when they used dCas9-SunTag-DNMT3A to introduce DNA methylation into the gene body of *DLX1*, one of the homeobox genes. Moreover, a comparison between gene expression and DNA methylation levels in chronic lymphocytic leukemia (CLL) cases showed a significant positive correlation between differentially methylated CpGs only within gene bodies and gene expression [61]. Aberrant DNA methylation within gene bodies may have an indirect effect on gene expression or isoform expression by altering RNA splicing [19].

It is apparent that methylation patterns are tumor specific and can be used as biomarkers to stratify tumors into subtypes according to their distinct methylation profiles. In the recent years DNA methylation analysis of cell-free DNA has emerged as a noninvasive approach for cancer detection [62].

Initially, the causal relevance for epigenetic alterations in cancer was questioned, and it has been suggested that these are merely passengers and not drivers of carcinogenesis. This notion has however been disproven based on a number of observations. First, hypermethylation of tumor suppressors serves as an alternative mechanism to mutation in Knudson's two hit hypothesis [56]. As seen for both *BRCA1* in breast cancer and *CDKN2A* in lung cancer, methylation of the promoter is mutually exclusive to any mutational or structural inactivation events [63,64]. Second, Carvalho et al. has shown that DNA methylation is a driver of tumorigenesis [65]. These data suggest that cancer cells become addicted to epigenetic alterations and these are essential for cancer cell survival. Moreover, tumors show hypermethylation as an early event in carcinogenesis, which is supported by the finding of the so-called "field effect" where adjacent normal tissues also harbor altered DNA methylation patterns [66].

Issa et al. demonstrated that there was a distinct subset of colorectal cancers with extensive hypermethylation of a subset of CpG islands that remained unmethylated in other colorectal tumors, a phenomenon termed "CpG island methylator phenotype" (CIMP) [67]. The efforts of the cancer genome atlas network have identified CIMPs in breast and endometrial cancers, glioblastomas and acute myeloid leukemias, but not in serous ovarian, squamous lung or renal kidney tumors [68]. Finally, regions subjected to cancer-associated DNA methylation changes comprise short interspersed or clustered regions as well as long blocks in so-called long-range epigenetic silencing [69]. Such phenotypes highly indicate that methylation events in cancer are not random and occur through coordinated mechanisms.

The question remains, why some regions become methylated, and others do not? It is commonly accepted that genetic changes in cancer occur randomly and are maintained through selection. A similar model has been proposed for epigenetic alterations; hypermethylation events are random, stochastic events that then are selected for because they are advantageous for cell survival. This would explain how cancers have been stratified in subtypes according to their distinct methylation profiles. While this is still believed to hold true, several recent studies have added to the complexity of cancer methylome establishment.

An initial study indicated that *de novo* methylation in cancer, as during development, may partially be determined by an instructive mechanism that recognizes specifically marked regions in the genome [70]. This group utilized DNA methyl-specific antibodies coupled with microarray analysis to investigate the genome-wide *de novo* methylation found in colon and prostate cancer cells. The authors found that only ~15% of the genes methylated in cancer samples are actively transcribed in normal tissue and that these are already inactivated by methylation in precancerous tissue. Collectively, these observations led the authors to suggest that much of the *de novo* methylation observed in cancer is not necessarily the result of growth selection but may instead occur in an instructive manner. The following year, three groups reported that genes that become aberrantly methylated in cancer are Polycomb group targets in ESCs [71–73]. Their data suggested that cancer cells target *de novo* methylation by taking advantage of a preexisting epigenetic repression program, namely the PRC2 mediated H3K27me3 mark, that is, genes that are already silenced are consistently targeted for cancer-specific methylation. This would explain why genes that do not

necessarily confer a growth advantage become methylated. Notably, many CIMP loci are known polycomb targets [74]. Thus the cancer cell epigenome is in part determined by cell of origin, as well as passenger events at genes that are not required for that particular cancer [73]. Finally, in some instances, fusion proteins can misdirect DNMTs to genes, thereby causing their silencing [75].

In contrast to the above, Spencer et al. [76] suggest that CpG island hypermethylation in AML is a consequence of rapid cellular proliferation and not a pathogenic event in the development of AML. DNMT3A mediated CpG hypermethylation occurs in nonleukemic cells in response to cytokine-induced proliferation.

3.2 Hypomethylation in cancer

In addition to regions of hypermethylation, cancer cells display marked loss of DNA methylation genome-wide (20–60% less 5mC). This hypomethylation occurs at multiple genomic sites, including CpG islands at repetitive regions and transposable elements, CpG-poor promoters, CpG island shores, introns and in gene deserts (typically same area as CpG oceans) [11]. The consequence of DNA hypomethylation at repetitive regions is genomic instability that in turn promotes chromosomal rearrangements and copy number changes. Demethylation of transposable elements also increases genomic instability, and their transposition can in turn inactivate other genes [10]. Some transposable elements drive oncogene expression in cancer, mediated by a process called onco-exaptation [77,78]. Although rare, hypomethylation occurring at promoters of known oncogenes results in their expression and further exacerbation of the carcinogenic state. Zhao et al. found multiple new hypomethylated intergenic regions associated with gene expression near oncogenic driver genes, *AR*, *MYC*, and *ERG*, specifically in prostate cancer cells [79]. Many of these regions contain binding sequences for transcriptional factors and colocalize with a mark of enhancer, H3K27Ac.

Hypomethylation events can also cause loss of imprinting (LOI). The most common example is *IGF2*, where LOI at the paternal allele has been reported in a large number of cancers, including breast, liver, lung, and colon cancers [80]. Finally, demethylation events at enhancers may affect transcriptional rate, while demethylation of gene bodies may affect RNA splicing [81]. The exact mechanisms by which global DNA methylation is lost from the cancer epigenome is not yet fully understood. A possibility is that many regions of DNA hypomethylation could be tied to broad shifts in chromatin organization or result from mutations in chromatin regulators that promote the active or passive process of removing DNA methylation and affect DNA methylation homeostasis.

3.3 DNA hydroxymethylation in cancer

The first study to investigate 5hmC levels in cancer was performed in AML patient cells by Ko et al. who has investigated the functional consequence of *TET2* mutations. The authors have found relatively lower levels of 5hmC in bone marrow samples from patients with *TET2* mutations comparing to *TET2* wild type [82]. Several studies have shown global loss of 5hmC in a variety of human solid tumors (breast, colon, gastric, liver, lung, melanoma, brain, and prostate cancers) compared with the normal surrounding tissue [51,83,84]. The decrease in 5hmC is often associated with downregulation of the TETs or impaired activity of the TET enzymes (Section 4.2).

A significant reduction of 5hmC is found in colorectal and gastric cancers compared to the normal counterpart tissues, and the 5hmC reduction correlated with downregulation of TET1 [84,85]. In melanoma reduction of 5hmC is associated with downregulation of TET2 and IDH2 [86]. In breast and liver cancers, 5hmC and the expression levels of all three TETs are significantly reduced compared to matched benign tissues [84]. In most immortalized tumor cell cultures, 5hmC levels are reduced, and in *in vitro* experiments oncogene-induced cellular transformation has been linked to downregulation of TET1 [51,84,87].

Depletion of 5hmC in a variety of cancers has also been confirmed by the more specific and quantitative mass spectrometry methods. A four- to fivefold lower 5hmC content is found in hepatocellular carcinoma compared to normal tissues adjacent to the tumor and a significant correlation between 5hmC levels and tumor stage is seen [88]. In lung squamous cell cancers, two- to fivefold lower 5hmC compared to normal matched tissue is detected by RPLC-MS and in astrocytomas a strong depletion of 5hmC is observed; in some tumors a reduction of more than 30-fold has been detected compared to normal brain tissue [89]. There is no correlation between the levels of 5mC and 5hmC or with tumor stage or patient survival. In multiple myeloma, lower global 5hmC is found compared to normal plasma cells and is associated with disease severity and persists at enhancers of oncogenic regions [90]. In patients with myelodysplastic syndrome (MDS) and AML treated with azacytidine, increased levels of 5hmC relative to 5mC levels are observed in patients receiving 500 mg vitamin C daily compared to placebo [91]. As described above, vitamin C reduces iron and thereby increases TET activity. Thus, vitamin C supplementation might enhance the biological effect of azacytidine.

To summarize, a broad loss of global 5hmC occurs across many types of cancer and is related to gene expression [92,93]. Interestingly, it has been suggested that the loss of 5hmC is replication-dependent in mouse preimplantation embryos [94]. Since low 5hmC levels are also reported in liver adenomas as compared with normal liver tissue [84], one may speculate whether the loss of 5hmC documented in several cancers results from a replication-dependent passive process. In addition, all cytosine derivatives can induce C-to-T transition mutations, as first observed in *E. coli* cells, and thus 5hmC may also have potential mutagenic properties [95]. These findings, however, need to be validated in mammalian cells. Interestingly, both 5hmC levels and expression levels of *TET1* and *TET3* and components of the mismatch repair pathway correlate with elevated C-to-G transversion rates in various cancer genomes [36]. This suggests that 5hmC is associated with a distinct mutational burden and that the mismatch repair pathway is implicated in causing elevated transversion rates at 5hmC sites.

4 Aberrations of enzymes involved in DNA methylation homeostasis in cancer

The mechanisms that cause aberrant DNA methylation and DNA demethylation of specific gene promoters and other regulatory regions in cancer are largely unknown. Failed fidelity of DNA maintenance methylation or active DNA demethylation may be caused by aberrant expression or mutations of the enzymes involved in the homeostasis of CpG methylation, which will be elaborated on in the following.

4.1 DNA methyltransferases

Overexpression of the DNMTs has been correlated with an unfavorable prognostic outcome in several cancers. For example, in diffuse large B-cell lymphoma (DLBCL) overexpression of DNMT3B evaluated by immunohistochemistry is significantly correlated to advanced clinical stage, overall and progression free survival, and promoter hypermethylation of specific genes [96]. Whether the overexpression of DNMT3B is specific to DLBCL or a consequence of proliferation is not clarified.

Based on tumors with overexpression of DNMTs, especially DNMTs involved in *de novo* DNA methylation, it is suggested that DNMTs function as oncogenes by causing aberrant hypermethylation of tumor suppressor genes. However, inactivating mutations in DNMT3A are found to correlate with poor prognosis in myeloid malignancies and deletion of DNMT3A promotes tumor progression in a lung cancer mouse model, indicating its tumor suppressor function [97]. This correlates well with the recent finding that DNMT3A is essential for hematopoietic stem cell differentiation, where deletion of the gene caused both hyper- and hypomethylation events at promoters [98]. Interestingly, methylation differences between DNMT3A wild-type and mutant AML patient samples are limited [99,100]. Another group reported that in mice, Dmnt3a deficient tumors had altered, mainly loss of DNA methylation within gene bodies [101]. Jeong et al. showed that methylation at the edges of CpG canyons diminished in Dmnt3a-null mice hematopoietic stem cells, and genes that are typically dysregulated in human leukemias are enriched for canyon-associated genes [12]. These findings suggest that DNMT3A may maintain methylation at the boundaries of CpG canyons. Finally, microRNAs of the miR-29 family have been shown to be involved in the regulation of DNA methylation by targeting the DNA methyltransferases, DMNT3A, DNMT3B and DNMT1 [102].

It is thus evident that DNMT3A deregulation is important in hematopoietic malignancies, and future studies are likely to uncover the mechanisms involved in DNMT mediated tumor progression. Drugs interfering with DNMT activity are approved for clinical use, and several new drugs are currently in preclinical and clinical trials (Figure 2.4). The only class currently used routinely in the clinics are the nucleoside analogs, 5-aza-2'-deoxycytidine (5-Aza-CdR, decitabine) and 5-azacytidine (5-Aza-CR, azacytidine). Both drugs are initially phosphorylated by intracellular kinases [103]. 5-Aza-CR is incorporated preferentially into RNA; however, approximately 20% is converted by ribonucleotide reductase, and the phosphorylated forms are incorporated into DNA during replication. When incorporated into DNA, the drugs form a covalent bond with the DNMT, thereby trapping the enzyme and preventing it from further methyltransferase activity. This results in a passive demethylation of DNA in the subsequent cell cycles [103].

Azacytidine and decitabine are approved by the U.S. Food and Drug Administration (FDA) for treatment of MDS, chronic myelomonocytic leukemia (CMML) with 10–29% blasts in bone marrow, and AML with 20–30% blasts. European Medicines Agency (EMA) has approved the use of 5-Aza-CR for treatment of higher risk MDS, CMML with 10–29% blasts without myeloproliferative disorder, and AML with 20–30% blasts and multilineage dysplasia. Furthermore, azacytidine and aecitabine are approved by EMA for treatment of AML patients who ineligible for standard induction therapy because of coexisting conditions or age above 65 years. The combination of azacytidine and venetoclax, a small molecule inhibitor of the B-cell lymphoma 2 protein (BCL2), as treatment of patients with AML, who are ineligible for standard induction therapy, has been shown to be an effective treatment regimen leading to significant improvement of overall survival [104].

4.2 Ten-eleven translocation proteins

Genetic aberrations of *TET1* and, most commonly, *TET2* are found in many cancers, whereas mutations in *TET3* are rare. *TET1* was first identified as an MLL translocation partner in rare cases of AML and acute lymphoid leukemia (ALL) carrying the ten-eleven chromosomal translocation t(10;11) (q22;q23) leading to fusion of the *TET1* gene on chromosome 10q22 with the mixed-lineage leukemia gene (*MLL*) on chromosome 11q23 [105]. *TET1* is shown to be downregulated by promoter hypermethylation in hematopoietic cancers and recurrently mutated in multiple solid cancers, most frequently in skin, lung, gastrointestinal, and urogenital cancers [106,107]. Cimmino et al. has shown that deletion of *Tet1* in mice results in widespread genetic and epigenetic changes in hematopoietic stem cells and promotes the development of B-cell lymphoma in mice. The expression level of *TET1* varies among different cancers. *TET1* is specifically overexpressed in 40% of triple negative breast cancer and linked to hypomethylation and activation of cancer-specific oncogenic pathways [108]. In several other cancers, *TET1* is frequently downregulated [106,107].

TET2 is located on chromosome 4q24, a region that is commonly deleted or involved in chromosomal rearrangements in myeloid malignancies. Somatic *TET2* mutations are frequent in a variety of hematopoietic cancers, including myeloid malignancies (including myeloproliferative neoplasms, systemic mastocytosis, CMML, MDS, and AML) and lymphoid malignancies (T-cell and B-cell lymphomas) [109–112]. The highest frequency of *TET2* mutations is reported in CMML (35%–50%). *TET2* mutations are also frequently detected in T-cell lymphoma, with the highest frequency observed in peripheral T-cell lymphoma and angioimmunoblastic T-cell lymphoma (AITL) (38% and 47%, respectively) [111]. The observations that lymphoma-associated *TET2* mutations are found in common hematopoietic progenitors of the same patients [113] and that *Tet2*-deficient mice in addition to myeloid expansion develop increased proliferation of lymphoid cells [110] suggest that *TET2* mutations may be early events in lymphomagenesis.

Interestingly, in AITL, *TET2* mutations cooccur with mutations in *DNMT3A* and in the *RHOA* gene that encodes a Ras-related GTP-binding protein [114,115]. Furthermore, in contrary to the mutually exclusive occurrence of *IDH* and *TET2* mutations observed in myeloid malignancies, cooccurrence of *TET2* and *IDH2* mutation is observed in a significant proportion of patients with AITL [116]. The cooperative interactions of these mutations may drive the development of AITL, as reviewed in [117].

TET2 mutations have mainly been reported in hematological malignancies. In solid tumors, somatic alterations of *TET2* have so far only been reported in metastatic tissue of castration-resistant prostate cancer [118]. Furthermore, an increasing number of studies find downregulation of the *TET2* proteins in a variety of solid tumors. In the last decade several mechanisms regulating the expression levels of the TETs have been elucidated. One mechanism may be translational inhibition of *TET2* by miR-22 as reported in MDS [87]. Also in MDS, miR-9 and miR-34a indirectly control *TET2* by posttranscriptionally regulating the levels of the NAD-dependent deacetylase sirtuin-1 (SIRT1), which affect the function of the *TET2* protein at a posttranslational level [119]. Posttranslational acetylation of *TET2* has been shown to enhance the function of *TET2* in protecting against abnormal DNA methylation during oxidative stress [120]. In a recent study it has been shown that MYC directly regulates the expression of *TET1* and *TET2* in T-cell acute lymphoblastic leukemia [121].

The effect of *TET2* mutations on DNA methylation and hydroxymethylation patterns has been investigated in a variety of hematopoietic cancers and is somewhat controversial. In CMML, *TET2* mutations are associated with global hypermethylation as measured by the LINE-1 assay; however, DNA methylation levels of 10 promoter CpG islands that are frequently hypermethylated in myeloid leukemia do not differ in *TET2*-mutated versus wild-type samples [122]. Another study performing methylation profiling in CMML patients has shown that patients with *TET2* mutations contain more hypomethylated regions at differentially methylated CpG sites [123].

In AML, Figueroa et al. [124] found associations between *TET2* mutations and a hypermethylation signature that overlapped considerably with the methylation signature observed in *IDH1/IDH2* mutant AML. Ko et al. [82] showed a strong correlation between *TET2* mutations and low 5hmC levels and reported 2510 differentially hypomethylated regions in “ShmC low AML samples” (22 *TET2* mutated, 7 *TET2* wild type) in comparison with “ShmC high AML samples” (2 *TET2* mutated, 22 *TET2* wild type).

In DLBCL, distinct *TET2* mutations are associated with a hypermethylation signature, including genes involved in hematopoietic development and cancer [112]. Notably, 53.4% of the “*TET2* methylation signature genes” carry the bivalent H3K4me3/H3K27me3 silencing mark in human ESCs.

Recently it has been reported that TET deficiency in diverse cell types causes genome-wide hypomethylation in the heterochromatic compartment, concurrently with hypermethylation at the CpG promoter and enhancer sites in euchromatin, where TET proteins normally bind and function [125].

Mutations in *TET2* have also been reported in age-related clonal hematopoiesis with a frequency of approximately 10% in individuals over 80 years of age and shown to be associated with increased DNA methylation [126]. In a more recent study of elderly individuals with clonal hematopoiesis of indeterminate potential (CHIP) and patients with clonal cytopenia of undetermined significance (CCUS), *TET2* mutations are associated with hypermethylation at enhancer sites [127]. The hypermethylated sites are associated with leukocyte function, immune response, and myeloid differentiation and largely shared in CHIP, CCUS, and AML patients [127].

4.3 Isocitrate dehydrogenases

Somatic *IDH1* missense mutations at R132 and *IDH2* missense mutations at R172 or R140 have been reported in several malignancies [128]. The substituted residue at the three mutation sites is often associated with a particular cancer type. The mutations are most prevalent in grade II and III gliomas (70%), secondary glioblastomas (55–88%), certain cartilaginous and bone marrow tumors (20–80%), sinonasal undifferentiated carcinoma (35–80%), intrahepatic cholangiocarcinoma (6–30%), solid papillary carcinoma (> 77%), AML (15–30%), and AITLs (20%–30%). Mutations of *IDH1* and *IDH2* appear to be mutually; however, there are rare exceptions. Additional somatic *IDH1/2* variants have been detected in thyroid carcinomas (8–16%), osteoclastoma (25%), and Wilms tumor (10%) [128].

The *IDH1* point mutations at R132 and *IDH2* point mutations at R172 or R140 reside at the enzymes’ catalytic domains and alter the activity of the enzymes so that α -KG is converted to the oncometabolite, 2-HG, leading to increased intracellular and extracellular levels of 2-HG (Figures. 2.4 and 2.5) [129,130]. 2-HG is a competitive inhibitor of α -KG and enzymes that require α -KG as

a cofactor, such as the TET oxygenases and the JMJC family of histone demethylases therefore have impairment of their function in *IDH1/2*-mutated cells (Figure 2.5) [124,131]. Consistently, a significant increase in promoter hypermethylation has been observed in AMLs and gliomas carrying *IDH1/2* mutations [124,132]. Interestingly, *IDH1/2* mutations are present in 98% of glioma-CIMP positive tumors and associated with a distinct transcriptome, while none of the glioma-CIMP negative tumors have *IDH1/2* mutations. Furthermore, immortalized primary human astrocytes expressing mutant IDH1 (R132H) have increased production of 2-HG and hypermethylation of a substantial number of genes [132]. In a study by Jin et al., no difference in 5hmC levels was detected in *IDH1*-mutated versus *IDH1* wild-type gliomas [89]. This is in conflict with the observation from another study that shows significant downregulation of 5hmC levels in *IDH1*-mutant glioblastomas as assessed by immunohistochemistry [133].

Since α-KG is also a cofactor for the JMJC family, *IDH* mutations are associated with impairment of histone demethylation, resulting in differentiation blockage due to repression of lineage-specific differentiation genes [124,134]. Conditional knock-in mice engineered to express the *IDH1*(R132H) mutation in the myeloid compartment shows increased the number of hematopoietic progenitors and the development of splenomegaly, anemia, and extramedullary hematopoiesis. The cells of the myeloid lineage have changes in DNA methylation comparable to those observed in human *IDH1/2* mutant AML as well as hypermethylated histones [135].

Treatment of cancers carrying *IDH* mutations could thus potentially benefit from differentiation therapy (Figure 2.4). Enasidenib is a selective and orally available mutant *IDH2* inhibitor which has demonstrated efficacy both *in vitro* and *in vivo* by normalizing 2-HG levels and inducing myeloid differentiation in preclinical models and in patients with AML [136]. Ivosidenib is a selective and orally available mutant *IDH1* inhibitor with confirmed preclinical efficacy leading to robust 2HG inhibition and myeloid differentiation. Ivosidenib and enasidenib are approved by the FDA for relapsed/refractory AML carrying *IDH1* and *IDH2* mutations, respectively. Treatment with ivosidenib in combination with azacytidine is also approved by the FDA for newly diagnosed AML with *IDH1* mutation in patients who are 75 years or older or not fit for standard chemotherapy [137]. However, it appears that inhibition of the mut*IDH1/2* neomorphic activity may activate alternative oncogenic pathways and induce acquired resistance to *IDH* inhibitors [138,139].

In gliomas, *IDH1/2* mutations also have clinical importance since the presence of the *IDH1/2* mutations is required for the diagnosis of two adult diffuse glioma types astrocytomas and oligodendroglomas. Thus, *IDH1/2* mutations are early driver mutations and have prognostic importance [140]. The mutant *IDH1/2* inhibitors have entered clinical trials for patients with gliomas with *IDH1/2* mutations [140].

4.4 Succinate dehydrogenases

Gastrointestinal stromal tumors (GIST) without *KIT* mutations frequently harbor mutations of succinate dehydrogenase (*SDH*) subunit genes *SDHA*, *SDHB*, *SDHC*, or *SDHD*. *SDH* catalyzes the conversion of succinate to fumerate in the citric acid cycle downstream of *IDH2*. Accumulation of succinate in *SDH* deficient cells also leads to inhibition of α-KG-dependent dioxygenases, such as the TET proteins and the JMJDs [141]. Interestingly, two independent studies have shown a correlation between *SDH* mutations in GIST, a hypermethylation signature, and decreased levels of hydroxymethylation [142,143]. *Sdhb*-deficient mice have increased 5mC levels, decreased 5hmC

levels, and increased histone methylation levels in neuroendocrine chromaffin cells compared to *Sdhb* wild-type mice. Interestingly, an enhanced migratory capacity of chromaffin cells with *SDHB* loss is observed *in vitro*, which is reversible by treatment with a DNMT inhibitor. Germline *SDH* mutations have also been reported in phaeochromocytoma-paraganglioma syndrome as well as in sporadic cases [144].

The discoveries of the mutations in the metabolic enzymes, IDH and SDH, and the effect of these mutations on the epigenome, strongly support a connection between metabolic disruption and epigenetic aberrations in cancer.

5 Conclusions

While DNA methylation has been implicated in numerous biological processes and aberrant DNA methylation patterns are considered as a hallmark of cancer, the recognition of biological significance for DNA hydroxymethylation in cancer is emerging. DNA hydroxymethylation is an intermediate in the process of DNA demethylation but is also a stable epigenetic mark with a proposed function in transcriptional regulation, cell identity, and developmental processes. Global as well as locus-specific loss of DNA hydroxymethylation has been reported in several cancers in comparison with normal counterpart tissue. The loss of 5hmC in cancer may be associated with the altered expression levels or activities of TET enzymes, due to replication-dependent passive demethylation, and an increased mutational burden (C-to-T transition mutations) at hydroxymethylated cytosines. The depletion of DNA hydroxymethylation in cancer is a novel prognostic biomarker for distinct cancers.

Furthermore, emerging evidence based on high-throughput and genome-wide analysis of DNA methylation points toward that DNA hyper- and hypomethylation is not that tightly limited to specific genomic boundaries as previously recognized but found throughout the genome. Hypermethylation events at CpG island shores and CpG-rich distal regions (e.g., enhancers) have also been associated with cancer and may correlate more closely with gene expression. In addition, the underlying mechanisms that direct DNA methylation to specific gene promoters in cancer is largely unknown. They may involve failed fidelity of DNA maintenance methylation caused by aberrant expression or mutations of the enzymes involved in the homeostasis of CpG methylation.

An obstacle in studies of DNA modifications is the requirement of experimental treatment of the DNA to preserve the information on DNA modifications. Future studies using third-generation sequencing techniques will allow detection of DNA modifications without manipulation and may thus contribute further to the genomic mapping of DNA modifications in cancer.

List of abbreviations

AITL	Angioimmunoblastic T-cell lymphoma
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
5-Aza-CR	5-Azacytidine
5-Aza-CdR	5-Aza-2'-deoxycytidine

BER	Base excision repair
BRCA1	Breast cancer 1, early onset
C	Cytosine
caC	Carboxylcytosine
CCUS	Clonal cytopenia of undetermined significance
CDKN2	Cyclin-dependent kinase inhibitor 2A
CHIP	Clonal hematopoiesis of indeterminate potential
CIMP	CpG island methylator phenotype
CLL	Chronic lymphocytic leukemia
CMML	Chronic myelomonocytic leukemia
CMS	Cytosine 5-methylenesulfonate
CpG	Cytosine–phosphate–guanine
DAPK1	Death-associated protein kinase 1
DLBCL	Diffuse large B-cell lymphoma
DLX1	Distal-less homeobox 1
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferases
EMA	European medicines agency
ESCs	Embryonic stem cells
EZH2	Enhancer of Zeste homolog 2
FBXL10	F-box and leucine-rich repeat protein 10
fC	Formylcarboxyl
FDA	U.S. Food and Drug Administration
G	Guanine
GIST	Gastrointestinal stromal tumors
2-HG	2-Hydroxyglutarate
hmC	Hydroxymethylcytosine
HMT	Histone methyltransferase
IDH1/2	Isocitrate dehydrogenase 1/2
IGF2	Insulin-like growth factor 2
JMJC	Jumonji C family protein
α-KG	α-Ketoglutarate
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LC–MS	Liquid chromatography–mass spectrometry
LINE-1	Long interspersed elements 1
LOI	Loss of imprinting
Mc	Methylcytosine
MDS	Myelodysplastic syndrome
MeCP2	Methyl-CpG-binding protein 2
MGMT	Methyl-CpG-binding protein 2
NDR	O-6-Methylguanine-DNA methyltransferase
NGS	Nucleosome-depleted regions
p15^{INK4B}	Cyclin-dependent kinase 4 inhibitor B
p16^{INK4A}	Cyclin-dependent kinase 4 inhibitor A
PCR	Polymerase chain reaction
PRC2	Polycomb-repressive complex 2
RB	Retinoblastoma tumor suppressor 2
RNA	Ribonucleic acid

RPLC	Reversed-phase liquid chromatography
SDH	Succinate dehydrogenase
SFRP1	Secreted frizzled-related protein 1
T	Thymine
TDG	Thymine-DNA glycosylase
TET	Ten-eleven translocation
TMS1	Target of methylation-induced silencing
TP53	Tumor protein p53
TSS	Transcription start site
U	Uracil
UHRF1	Ubiquitin-like with PHD and ring finger domains 1

References

- [1] Baylin SB, Jones PA. Epigenetic determinants of cancer. *Cold Spring Harb Perspect Biol* 2016;8:9.
- [2] You JS, Jones PA. Cancer genetics and epigenetics: two sides of the same coin? *Cancer Cell* 2012;22(1):9–20.
- [3] Shen H, Laird PW. Interplay between the cancer genome and epigenome. *Cell* 2013;153(1):38–55.
- [4] Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 1997;389(6648):251–60.
- [5] Kelly TK, Miranda TB, Liang G, et al. H2A.Z maintenance during mitosis reveals nucleosome shifting on mitotically silenced genes. *Mol Cell* 2010;39(6):901–11.
- [6] Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012;13(7):484–92.
- [7] Bird A, Taggart M, Frommer M, Miller OJ, Macleod D. A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. *Cell* 1985;40(1):91–9.
- [8] Smallwood SA, Tomizawa S, Krueger F, et al. Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nat Genet* 2011;43(8):811–14.
- [9] Straussman R, Nejman D, Roberts D, et al. Developmental programming of CpG island methylation profiles in the human genome. *Nat Struct Mol Biol* 2009;16(5):564–71.
- [10] Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. *Nat Rev Genet* 2008;9(6):465–76.
- [11] Doi A, Park IH, Wen B, et al. Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet* 2009;41(12):1350–3.
- [12] Jeong M, Sun D, Luo M, et al. Large conserved domains of low DNA methylation maintained by Dnmt3a. *Nat Genet* 2014;46(1):17–23.
- [13] Han H, Cortez CC, Yang X, Nichols PW, Jones PA, Liang G. DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. *Hum Mol Genet* 2011;20(22):4299–310.
- [14] Schultz MD, He Y, Whitaker JW, et al. Human body epigenome maps reveal noncanonical DNA methylation variation. *Nature* 2015;523(7559):212–16.
- [15] Ziller MJ, Gu H, Müller F, et al. Charting a dynamic DNA methylation landscape of the human genome. *Nature* 2013;500(7463):477–81.
- [16] Edwards JR, O'Donnell AH, Rollins RA, et al. Chromatin and sequence features that define the fine and gross structure of genomic methylation patterns. *Genome Res* 2010;20(7):972–80.

- [17] Boulard M, Edwards JR, Bestor TH. FBXL10 protects Polycomb-bound genes from hypermethylation. *Nat Genet* 2015;47(5):479–85.
- [18] Deaton AM, Bird A. CpG islands and the regulation of transcription. *Genes Dev* 2011;25(10):1010–22.
- [19] Gelfman S, Cohen N, Yearim A, Ast G. DNA-methylation effect on cotranscriptional splicing is dependent on GC architecture of the exon-intron structure. *Genome Res* 2013;23(5):789–99.
- [20] Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S. Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. *Nat Genet* 2007;39(1):61–9.
- [21] Ooi SK, Qiu C, Bernstein E, et al. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature* 2007;448(7154):714–17.
- [22] Liang G, Chan MF, Tomigahara Y, et al. Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Mol Cell Biol* 2002;22(2):480–91.
- [23] Bostick M, Kim JK, Estève PO, Clark A, Pradhan S, Jacobsen SE. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science* 2007;317(5845):1760–4.
- [24] Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet* 2009;10(5):295–304.
- [25] Lienert F, Wirbelauer C, Som I, Dean A, Mohn F, Schübeler D. Identification of genetic elements that autonomously determine DNA methylation states. *Nat Genet* 2011;43(11):1091–7.
- [26] Ginno PA, Lott PL, Christensen HC, Korf I, Chédin F. R-loop formation is a distinctive characteristic of unmethylated human CpG island promoters. *Mol Cell* 2012;45(6):814–25.
- [27] Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 2009;324(5929):929–30.
- [28] Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009;324(5929):930–5.
- [29] Ito S, Shen L, Dai Q, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 2011;333(6047):1300–3.
- [30] Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. *Nature* 2013;502(7472):472–9.
- [31] Hore TA, von Meyenn F, Ravichandran M, et al. Retinol and ascorbate drive erasure of epigenetic memory and enhance reprogramming to naïve pluripotency by complementary mechanisms. *Proc Natl Acad Sci U S A* 2016;113(43):12202–7.
- [32] Tsukada Y, Fang J, Erdjument-Bromage H, et al. Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 2006;439(7078):811–16.
- [33] Minor EA, Court BL, Young JI, Wang G. Ascorbate induces ten-eleven translocation (Tet) methylcytosine dioxygenase-mediated generation of 5-hydroxymethylcytosine. *J Biol Chem* 2013;288(19):13669–74.
- [34] Liu M, Ohtani H, Zhou W, et al. Vitamin C increases viral mimicry induced by 5-aza-2'-deoxycytidine. *Proc Natl Acad Sci U S A* 2016;113(37):10238–44.
- [35] Rasmussen KD, Helin K. Role of TET enzymes in DNA methylation, development, and cancer. *Genes Dev* 2016;30(7):733–50.
- [36] Bachman M, Uribe-Lewis S, Yang X, Williams M, Murrell A, Balasubramanian S. 5-Hydroxymethylcytosine is a predominantly stable DNA modification. *Nat Chem* 2014;6(12):1049–55.
- [37] Buscarlet M, Tessier A, Provost S, Mollica L, Busque L. Human blood cell levels of 5-hydroxymethylcytosine (5hmC) decline with age, partly related to acquired mutations in TET2. *Exp Hematol* 2016;44(11):1072–84.
- [38] Wen L, Li X, Yan L, et al. Whole-genome analysis of 5-hydroxymethylcytosine and 5-methylcytosine at base resolution in the human brain. *Genome Biol* 2014;15(3):R49.

- [39] Zeng H, He B, Yi C. Compilation of modern technologies to map genome-wide cytosine modifications in DNA. *Chembiochem* 2019;20(15):1898–905.
- [40] Wu H, Zhang Y. Charting oxidized methylcytosines at base resolution. *Nat Struct Mol Biol* 2015;22(9):656–61.
- [41] Wu H, D’Alessio AC, Ito S, et al. Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. *Genes Dev* 2011;25(7):679–84.
- [42] Xu Y, Wu F, Tan L, et al. Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. *Mol Cell* 2011;42(4):451–64.
- [43] Ficz G, Branco MR, Seisenberger S, et al. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 2011;473(7347):398–402.
- [44] Han D, Lu X, Shih AH, et al. A highly sensitive and robust method for genome-wide 5hmC profiling of rare cell populations. *Mol Cell* 2016;63(4):711–19.
- [45] Williams K, Christensen J, Pedersen MT, et al. TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 2011;473(7347):343–8.
- [46] Pastor WA, Pape UJ, Huang Y, et al. Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. *Nature* 2011;473(7347):394–7.
- [47] Yu M, Hon GC, Szulwach KE, et al. Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. *Cell* 2012;149(6):1368–80.
- [48] Mellén M, Ayata P, Dewell S, Kriaucionis S, Heintz N. MeCP2 binds to 5hmC enriched within active genes and accessible chromatin in the nervous system. *Cell* 2012;151(7):1417–30.
- [49] He B, Zhang C, Zhang X, et al. Tissue-specific 5-hydroxymethylcytosine landscape of the human genome. *Nat Commun* 2021;12(1):4249.
- [50] Song CX, Clark TA, Lu XY, et al. Sensitive and specific single-molecule sequencing of 5-hydroxymethylcytosine. *Nat Methods* 2011;9(1):75–7.
- [51] Haffner MC, Chaux A, Meeker AK, et al. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. *Oncotarget* 2011;2(8):627–37.
- [52] Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006;7(1):21–33.
- [53] Akiyama Y, Watkins N, Suzuki H, et al. GATA-4 and GATA-5 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer. *Mol Cell Biol* 2003;23(23):8429–39.
- [54] Stirzaker C, Song JZ, Ng W, et al. Methyl-CpG-binding protein MBD2 plays a key role in maintenance and spread of DNA methylation at CpG islands and shores in cancer. *Oncogene* 2017;36(10):1328–38.
- [55] Kong X, Chen J, Xie W, et al. Defining UHRF1 domains that support maintenance of human colon cancer DNA methylation and oncogenic properties. *Cancer Cell* 2019;35(4):633–48.
- [56] Grønbæk K, Hother C, Jones PA. Epigenetic changes in cancer. *Apmis* 2007;115(10):1039–59.
- [57] Walter MJ, Shen D, Ding L, et al. Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med* 2012;366(12):1090–8.
- [58] Aran D, Hellman A. DNA methylation of transcriptional enhancers and cancer predisposition. *Cell* 2013;154(1):11–13.
- [59] Aran D, Sabato S, Hellman A. DNA methylation of distal regulatory sites characterizes dysregulation of cancer genes. *Genome Biol* 2013;14(3):R21.
- [60] Su J, Huang YH, Cui X, et al. Homeobox oncogene activation by pan-cancer DNA hypermethylation. *Genome Biol* 2018;19(1):108.
- [61] Kulis M, Heath S, Bibikova M, et al. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nat Genet* 2012;44(11):1236–42.

- [62] Barefoot ME, Loyfer N, Kiliti AJ, McDeed APt, Kaplan T, Wellstein T. Detection of cell types contributing to cancer from circulating, cell-free methylated DNA. *Front Genet* 2021;12:671057.
- [63] The Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature* 2011;474(7353):609–15.
- [64] The Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. *Nature* 2012;489(7417):519–25.
- [65] De Carvalho DD, Sharma S, You JS, et al. DNA methylation screening identifies driver epigenetic events of cancer cell survival. *Cancer Cell* 2012;21(5):655–67.
- [66] Tabby R, Issa JP. Cancer epigenetics. *CA Cancer J Clin* 2010;60(6):376–92.
- [67] Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 1999;96(15):8681–6.
- [68] Weisenberger DJ. Characterizing DNA methylation alterations from The Cancer Genome Atlas. *J Clin Investig* 2014;124(1):17–23.
- [69] Coolen MW, Stirzaker C, Song JZ, et al. Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. *Nat Cell Biol* 2010;12(3):235–46.
- [70] Keshet I, Schlesinger Y, Farkash S, et al. Evidence for an instructive mechanism of de novo methylation in cancer cells. *Nat Genet* 2006;38(2):149–53.
- [71] Ohm JE, McGarvey KM, Yu X, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet* 2007;39(2):237–42.
- [72] Schlesinger Y, Straussman R, Keshet I, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet* 2007;39(2):232–6.
- [73] Sproul D, Kitchen RR, Nestor CE, et al. Tissue of origin determines cancer-associated CpG island promoter hypermethylation patterns. *Genome Biol* 2012;13(10):R84.
- [74] Widschwendter M, Fiegl H, Egle D, et al. Epigenetic stem cell signature in cancer. *Nat Genet* 2007;39(2):157–8.
- [75] Di Croce L, Raker VA, Corsaro M, et al. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* 2002;295(5557):1079–82.
- [76] Spencer DH, Russler-Germain DA, Ketkar S, et al. CpG island hypermethylation mediated by DNMT3A is a consequence of AML progression. *Cell* 2017;168(5):801–816.e13.
- [77] Babaian A, Mager DL. Endogenous retroviral promoter exaptation in human cancer. *Mob DNA* 2016;7:24.
- [78] Jang HS, Shah NM, Du AY, et al. Transposable elements drive widespread expression of oncogenes in human cancers. *Nat Genet* 2019;51(4):611–17.
- [79] Zhao SG, Chen WS, Li H, et al. The DNA methylation landscape of advanced prostate cancer. *Nat Genet* 2020;52(8):778–89.
- [80] Ito Y, Koessler T, Ibrahim AE, et al. Somatically acquired hypomethylation of IGF2 in breast and colorectal cancer. *Hum Mol Genet* 2008;17(17):2633–43.
- [81] Ehrlich M, Lacey M. DNA hypomethylation and hemimethylation in cancer. *Adv Exp Med Biol* 2013;754:31–56.
- [82] Ko M, Huang Y, Jankowska AM, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* 2010;468(7325):839–43.
- [83] Johnson KC, Houseman EA, King JE, von Herrmann KM, Fadul CE, Christensen BC. 5-Hydroxymethylcytosine localizes to enhancer elements and is associated with survival in glioblastoma patients. *Nat Commun* 2016;7:13177.
- [84] Yang H, Liu Y, Bai F, et al. Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. *Oncogene* 2013;32(5):663–9.

- [85] Neri F, Dettori D, Incarnato D, et al. TET1 is a tumour suppressor that inhibits colon cancer growth by derepressing inhibitors of the WNT pathway. *Oncogene* 2015;34(32):4168–76.
- [86] Lian CG, Xu Y, Ceol C, et al. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell* 2012;150(6):1135–46.
- [87] Song SJ, Ito K, Ala U, et al. The oncogenic microRNA miR-22 targets the TET2 tumor suppressor to promote hematopoietic stem cell self-renewal and transformation. *Cell Stem Cell* 2013;13(1):87–101.
- [88] Chen ML, Shen F, Huang W, et al. Quantification of 5-methylcytosine and 5-hydroxymethylcytosine in genomic DNA from hepatocellular carcinoma tissues by capillary hydrophilic-interaction liquid chromatography/quadrupole TOF mass spectrometry. *Clin Chem* 2013;59(5):824–32.
- [89] Jin SG, Jiang Y, Qiu R, et al. 5-Hydroxymethylcytosine is strongly depleted in human cancers but its levels do not correlate with IDH1 mutations. *Cancer Res* 2011;71(24):7360–5.
- [90] Alberge JB, Magrangeas F, Wagner M, et al. DNA hydroxymethylation is associated with disease severity and persists at enhancers of oncogenic regions in multiple myeloma. *Clin Epigenetics* 2020;12(1):163.
- [91] Gillberg L, Ørskov AD, Nasif A, et al. Oral vitamin C supplementation to patients with myeloid cancer on azacitidine treatment: Normalization of plasma vitamin C induces epigenetic changes. *Clin Epigenetics* 2019;11(1):143.
- [92] Ficz G, Gribben JG. Loss of 5-hydroxymethylcytosine in cancer: cause or consequence? *Genomics* 2014;104(5):352–7.
- [93] Pfeifer GP, Xiong W, Hahn MA, Jin SG. The role of 5-hydroxymethylcytosine in human cancer. *Cell Tissue Res* 2014;356(3):631–41.
- [94] Inoue A, Zhang Y. Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. *Science* 2011;334(6053):194.
- [95] Xing XW, Liu YL, Vargas M, et al. Mutagenic and cytotoxic properties of oxidation products of 5-methylcytosine revealed by next-generation sequencing. *PLoS One* 2013;8(9):e72993.
- [96] Amara K, Ziadi S, Hachana M, Soltani N, Korbi S, Trimeche M. DNA methyltransferase DNMT3b protein overexpression as a prognostic factor in patients with diffuse large B-cell lymphomas. *Cancer Sci* 2010;101(7):1722–30.
- [97] Chen BF, Chan WY. The de novo DNA methyltransferase DNMT3A in development and cancer. *Epigenetics* 2014;9(5):669–77.
- [98] Challen GA, Sun D, Jeong M, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet* 2011;44(1):23–31.
- [99] Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* 2010;363(25):2424–33.
- [100] Yan XJ, Xu J, Gu ZH, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet* 2011;43(4):309–15.
- [101] Raddatz G, Gao Q, Bender S, Jaenisch R, Lyko F. Dnmt3a protects active chromosome domains against cancer-associated hypomethylation. *PLoS Genet* 2012;8(12):e1003146.
- [102] Garzon R, Liu S, Fabbri M, et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood* 2009;113(25):6411–18.
- [103] Cortez CC, Jones PA. Chromatin, cancer and drug therapies. *Mutat Res* 2008;647(1–2):44–51.
- [104] DiNardo CD, Jonas BA, Pullarkat V, et al. Azacitidine and venetoclax in previously untreated acute myeloid leukemia. *N Engl J Med* 2020;383(7):617–29.
- [105] Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y. LCX leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). *Cancer Res* 2002;62(14):4075–80.

- [106] Liu W, Wu G, Xiong F, Chen Y. Advances in the DNA methylation hydroxylase TET1. *Biomark Res* 2021;9(1):76.
- [107] Cimmino L, Dawlaty MM, Ndiaye-Lobry D, et al. TET1 is a tumor suppressor of hematopoietic malignancy. *Nat Immunol* 2015;16(6):653–62.
- [108] Good CR, Panjarian S, Kelly AD, et al. TET1-mediated hypomethylation activates oncogenic signaling in triple-negative breast cancer. *Cancer Res* 2018;78(15):4126–37.
- [109] Solary E, Bernard OA, Tefferi A, Fuks F, Vainchenker W. The Ten-Eleven Translocation-2 (TET2) gene in hematopoiesis and hematopoietic diseases. *Leukemia* 2014;28(3):485–96.
- [110] Quivoron C, Couronné L, Della Valle V, et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. *Cancer Cell* 2011;20(1):25–38.
- [111] Lemonnier F, Couronné L, Parrens M, et al. Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. *Blood* 2012;120(7):1466–9.
- [112] Asmar F, Punj V, Christensen J, et al. Genome-wide profiling identifies a DNA methylation signature that associates with TET2 mutations in diffuse large B-cell lymphoma. *Haematologica* 2013;98(12):1912–20.
- [113] Viguié F, Aboura A, Bouscary D, et al. Common 4q24 deletion in four cases of hematopoietic malignancy: early stem cell involvement? *Leukemia* 2005;19(8):1411–15.
- [114] Couronné L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. *N Engl J Med* 2012;366(1):95–6.
- [115] Sakata-Yanagimoto M, Enami T, Yoshida K, et al. Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. *Nat Genet* 2014;46(2):171–5.
- [116] Odejide O, Weigert O, Lane AA, et al. A targeted mutational landscape of angioimmunoblastic T-cell lymphoma. *Blood* 2014;123(9):1293–6.
- [117] Van Arnam JS, Lim MS, Elenitoba-Johnson KSJ. Novel insights into the pathogenesis of T-cell lymphomas. *Blood* 2018;131(21):2320–30.
- [118] Nickerson ML, Im KM, Misner KJ, et al. Somatic alterations contributing to metastasis of a castration-resistant prostate cancer. *Hum Mutat* 2013;34(9):1231–41.
- [119] Sun J, He X, Zhu Y, et al. SIRT1 activation disrupts maintenance of myelodysplastic syndrome stem and progenitor cells by restoring TET2 function. *Cell Stem Cell* 2018;23(3):355–369.e9.
- [120] Zhang YW, Wang Z, Xie W, et al. Acetylation enhances TET2 function in protecting against abnormal DNA methylation during oxidative stress. *Mol Cell* 2017;65(2):323–35.
- [121] Poole CJ, Lodh A, Choi JH, van Riggelen J. MYC deregulates TET1 and TET2 expression to control global DNA (hydroxy)methylation and gene expression to maintain a neoplastic phenotype in T-ALL. *Epigenetics Chromatin* 2019;12(1):41.
- [122] Yamazaki J, Taby R, Vasanthakumar A, et al. Effects of TET2 mutations on DNA methylation in chronic myelomonocytic leukemia. *Epigenetics* 2012;7(2):201–7.
- [123] Pérez C, Martínez-Calle N, Martín-Subero JI, et al. TET2 mutations are associated with specific 5-methylcytosine and 5-hydroxymethylcytosine profiles in patients with chronic myelomonocytic leukemia. *PLoS One* 2012;7(2):e31605.
- [124] Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* 2010;18(6):553–67.
- [125] López-Moyado IF, Tsagaratou A, Yuita H, et al. Paradoxical association of TET loss of function with genome-wide DNA hypomethylation. *Proc Natl Acad Sci U S A* 2019;116(34):16933–42.
- [126] Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* 2014;371(26):2488–98.

- [127] Tulstrup M, Soerensen M, Hansen JW, et al. TET2 mutations are associated with hypermethylation at key regulatory enhancers in normal and malignant hematopoiesis. *Nat Commun* 2021;12(1):6061.
- [128] Hvinden IC, Cadoux-Hudson T, Schofield CJ, McCullagh JSO. Metabolic adaptations in cancers expressing isocitrate dehydrogenase mutations. *Cell Rep Med* 2021;2(12):100469.
- [129] Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* 2009;462(7274):739–44.
- [130] Ward PS, Patel J, Wise DR, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* 2010;17(3):225–34.
- [131] Chowdhury R, Yeoh KK, Tian YM, et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. *EMBO Rep* 2011;12(5):463–9.
- [132] Turcan S, Rohle D, Goenka A, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature* 2012;483(7390):479–83.
- [133] Xu W, Yang H, Liu Y, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α -ketoglutarate-dependent dioxygenases. *Cancer Cell* 2011;19(1):17–30.
- [134] Lu C, Ward PS, Kapoor GS, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* 2012;483(7390):474–8.
- [135] Sasaki M, Knobbe CB, Munger JC, et al. IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. *Nature* 2012;488(7413):656–9.
- [136] Amatangelo MD, Quek L, Shih A, et al. Enasidenib induces acute myeloid leukemia cell differentiation to promote clinical response. *Blood* 2017;130(6):732–41.
- [137] Issa GC, DiNardo CD. Acute myeloid leukemia with IDH1 and IDH2 mutations: 2021 treatment algorithm. *Blood Cancer J* 2021;11(6):107.
- [138] Intlekofer AM, Shih AH, Wang B, et al. Acquired resistance to IDH inhibition through trans or cis dimer-interface mutations. *Nature* 2018;559(7712):125–9.
- [139] Harding JJ, Lowery MA, Shih AH, et al. Isoform switching as a mechanism of acquired resistance to mutant isocitrate dehydrogenase inhibition. *Cancer Discov* 2018;8(12):1540–7.
- [140] Gatto L, Franceschi E, Tosoni A, et al. IDH inhibitors and beyond: the cornerstone of targeted glioma treatment. *Mol Diagn Ther* 2021;25(4):457–73.
- [141] Janeway KA, Kim SY, Lodish M, et al. Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. *Proc Natl Acad Sci U S A* 2011;108(1):314–18.
- [142] Killian JK, Kim SY, Miettinen M, et al. Succinate dehydrogenase mutation underlies global epigenomic divergence in gastrointestinal stromal tumor. *Cancer Discov* 2013;3(6):648–57.
- [143] Mason EF, Hornick JL. Succinate dehydrogenase deficiency is associated with decreased 5-hydroxymethylcytosine production in gastrointestinal stromal tumors: implications for mechanisms of tumorigenesis. *Mod Pathol* 2013;26(11):1492–7.
- [144] van Nederveen FH, Gaal J, Favier J, et al. An immunohistochemical procedure to detect patients with paraganglioma and phaeochromocytoma with germline SDHB, SDHC, or SDHD gene mutations: a retrospective and prospective analysis. *Lancet Oncol* 2009;10(8):764–71.

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Writers, erasers, and readers of DNA and histone methylation marks

3

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1 Introduction

In eukaryotic cells, genetic material is packaged into chromatin. The basic structural unit of chromatin is the nucleosome, which consists of ~146 bp of DNA wrapped around a histone octamer (two copies each of the core histones H2A, H2B, H3, and H4). Multiple residues of core histones, mostly on their N-terminal tails, are subject to various posttranslational modifications (PTMs). These PTMs, in combination with DNA modifications, form the epigenetic code, which modulates chromatin compaction and gene expression. Cytosine methylation at the C5 position (5-methylcytosine, 5mC) is the predominant DNA modification in mammals. 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), which serve as intermediates of demethylation ([Figure 3.1A](#)). Another DNA modification, adenine methylation at the N6 position (6mA), is abundant in prokaryotes and some lower eukaryotes. The presence of low levels of 6mA in mammalian cells has been recently reported but remains a controversial issue (this chapter will not cover 6mA, and DNA methylation refers to 5mC hereafter). Dozens of histone PTMs have been identified, including methylation, acetylation, phosphorylation, sumoylation, ubiquitylation, and ADP-ribosylation. A wide variety of enzymes have evolved that modify specific amino acids and nucleotide bases (“writers”), along with enzymes to remove these marks (“erasers”). Cells also express a diverse complement of proteins that bind chromatin modifications (“readers”), providing the ability to sense the chromatin state. In this chapter, we summarize key mammalian proteins involved in writing, erasing, and reading DNA methylation and histone methylation, which primarily occurs on lysine and arginine residues ([Figure 3.1B and C](#)). We also discuss the relevance of these epigenetic regulators in cancer. Albeit not the focus of this chapter, nonhistone PTMs are also widespread and have important functions.

2 DNA methylation writers, erasers, and readers

DNA methylation, occurring mostly in the context of CpG dinucleotides, is correlated with chromatin compaction and gene silencing. In general, repetitive sequences, such as satellite repeats in centromeric and pericentromeric regions and transposable elements dispersed throughout the genome, and gene bodies, especially exons, are highly methylated, whereas CpG islands (CGIs), that is, 500–2000-bp GC-rich sequences that are frequently associated with gene promoters, are usually devoid of DNA methylation. DNA methylation plays critical roles in a variety of biological processes, including genomic imprinting, X chromosome inactivation, and maintenance of genome integrity. DNA methylation patterns and levels are regulated by the opposing effects of the DNA methylation and demethylation machineries, including DNA methyltransferases (DNMTs) and the ten-eleven translocation (TET) family of 5mC dioxygenases, and the biological effects of 5mC are mediated, in part, by methyl-CpG-binding proteins ([Figure 3.1A](#) and [Table 3.1](#)).

2.1 DNMTs

The DNMT family consists of DNMT1, DNMT3A, and DNMT3B, as well as the catalytically inactive DNMT3L ([Table 3.1](#)). DNMT1 functions as the major maintenance methyltransferase (MTase), ensuring

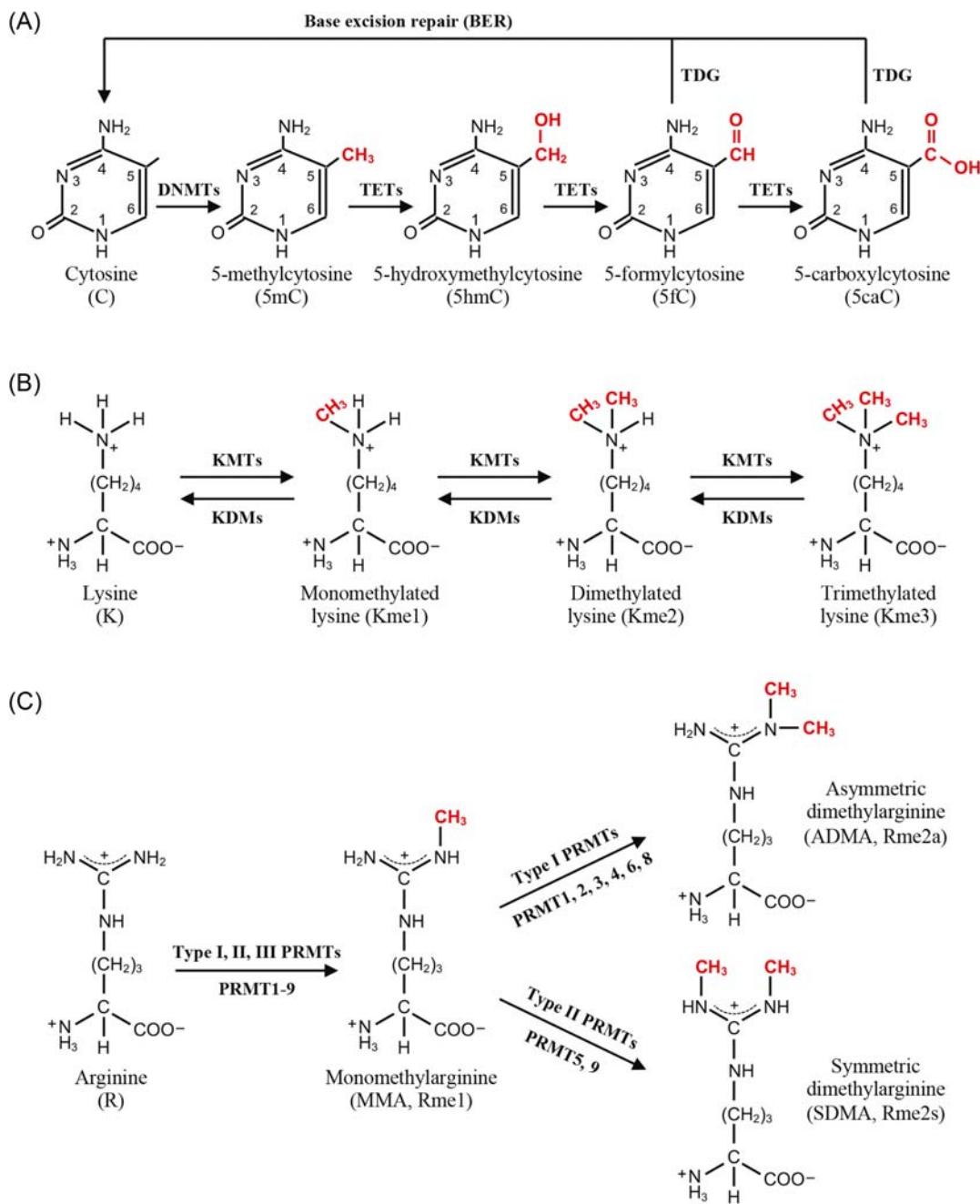


FIGURE 3.1

DNA (cytosine) and protein (lysine and arginine) methylation. (A) Cytosine can be methylated by DNA methyltransferases (DNMTs), generating 5-methylcytosine (5mC), which can be oxidized by the ten-eleven translocation (TET) family of 5mC dioxygenases, converting to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5fC and 5caC can be excised by thymine DNA glycosylase (TDG), followed by base excision repair (BER) to achieve active demethylation. (B) Lysine (K) can be mono-, di-, and trimethylated by lysine methyltransferases (KMTs) and demethylated by lysine demethylases (KDMs). (C) Arginine (R) can be mono- and asymmetrically or symmetrically dimethylated by different protein arginine methyltransferases (PRMTs). No dedicated arginine demethylases have been identified.

TABLE 3.1 DNMTs, TETs, and methyl-CpG-binding proteins and their links to human diseases.

Protein	Major functions in mouse	Human diseases caused by mutations	References
DNMT1	Maintenance DNA MTase; essential for embryonic development	HSAN IE, ADCA-DN	[1,2]
DNMT3A	<i>De novo</i> DNA MTase; essential for establishing germline methylation, including genomic imprints, and spermatogenesis	AML, Tatton-Brown-Rahman syndrome, microcephalic dwarfism	[3–7]
DNMT3B	<i>De novo</i> DNA MTase; essential for establishing embryonic methylation patterns and embryonic development; some inactive isoforms (e.g., DNMT3B3) may act as cofactors of DNMT3B	ICF syndrome (also caused by mutations in ZBTB24, CDCA7, and HELLS)	[3,8–11]
DNMT3C	<i>De novo</i> DNA MTase (present only in rodents); methylates and silences evolutionarily young retrotransposons during spermatogenesis		[12]
DNMT3L	Cofactor of DNMT3A; essential for establishing germline methylation, including genomic imprints, and spermatogenesis		[13–17]
TET1	5mC dioxygenase; initiates active demethylation; highly expressed in PGCs, mESCs, and some somatic tissues	AML carrying [t(10;11)(q22;q23)] fusion	[18–20]
TET2	5mC dioxygenase; initiates active demethylation; pleiotropic roles during hematopoiesis	Myeloid malignancies	[21–23]
TET3	5mC dioxygenase; initiates active demethylation of paternal genome during early embryogenesis		[24]
MeCP2	5mC-binding protein; transcriptional repressor; required for normal neural functions	Rett syndrome	[25–27]
MBD1	5mC-binding protein; KO mice: viable		[28]
MBD2	5mC-binding protein; subunit of Mi-2/NuRD complex; KO mice: viable, with T-cell defects		[28]
MBD3	Unable to bind 5mC; subunit of Mi-2/NuRD complex; essential for embryonic development		[28]
MBD4	5mC-binding protein; involved in DNA mismatch repair; not essential for development		[28]
MBD5	5mC-binding protein? KO mice: postnatal lethality	2q23.1 microdeletion syndrome	[28,29]
MBD6	5mC-binding protein?		[28]
KAISO/ ZBTB33	5mC-binding protein? KO mice: viable		[28]
ZFP57	5mC-binding protein; required for maintaining genomic imprints and embryonic development	Transient neonatal diabetes	[30–32]
ZFP445/ ZNF445	5mC-binding protein; involved in maintaining genomic imprints		[33]
WT1	5mC- and 5caC-binding protein; transcription factor; required for embryonic development	Wilms tumor	[34]
UHRF1	Cofactor of DNMT1; preferentially bind hemimethylated DNA; essential for embryonic development		[35]
UHRF2	5hmC-binding protein; not essential for development		[36]

ADCA-DN, *Autosomal dominant cerebellar ataxia, deafness and narcolepsy*; AML, *acute myeloid leukemia*; HSAN IE, *hereditary sensory and autonomic neuropathy with dementia and hearing loss type IE*; ICF, *immunodeficiency, centromeric instability, and facial anomalies*.

faithful propagation of DNA methylation patterns through cell divisions [1]. UHRF1, a multidomain protein, is an essential accessory factor of maintenance methylation by directing DNMT1 to hemimethylated CpG sites generated during DNA replication [35]. DNMT3A and DNMT3B act primarily as *de novo* MTases to establish DNA methylation patterns [3]. Genetic studies demonstrate that DNMT3A is required for germline methylation, including the establishment of genomic imprinting, whereas DNMT3B is the major enzyme responsible for establishing methylation patterns during embryonic development [3,4]. The rodent genome also carries a *Dnmt3b* duplicated gene, initially thought to be a pseudogene but subsequently found to be functional and renamed *Dnmt3c* [12]. DNMT3C is expressed in male germ cells and methylates evolutionarily young retrotransposons during spermatogenesis [12]. DNMT3L serves as an accessory factor of DNMT3A in the germline; the phenotype of *Dnmt3L* knockout (KO) mice, that is, failure to establish maternal methylation imprints and spermatogenesis defect [13,14], is almost identical to that of mice with germline-specific deletion of *Dnmt3a* [4]. DNMT3L has been shown to stimulate DNMT3A/3B activity, stabilize DNMT3A protein, and recruit DNMT3A to chromatin regions where lysine 4 of histone H3 (H3K4) is unmethylated [15–17]. Recent work shows that the catalytically inactive DNMT3B3 isoform (due to alternative splicing) preferentially enhances DNMT3B-mediated methylation, suggesting that DNMT3B3 and perhaps the structurally similar DNMT3B6 serve as accessory factors of DNMT3B during embryogenesis and in somatic tissues [8], similar to the effect of DNMT3L on DNMT3A in the germline.

While the current model that DNMT3A/3B and DNMT1 carry out *de novo* and maintenance methylation, respectively, has served the field well, it seems to be overly simplified. Mouse embryonic stem cells (mESCs) deficient for both *Dnmt3a* and *Dnmt3b* show gradual loss of global methylation, indicating the involvement of DNMT3A/3B in maintenance methylation as well [37]. On the other hand, recent studies suggest that DNMT1 also has *de novo* MTase activity, although such activity may be suppressed by other factors [38,39].

2.2 TETs

Despite the commonly used term “DNA demethylation,” there exist no “demethylases” that directly cleave the methyl group from 5mC. However, demethylation can be achieved via two mechanisms: (1) DNA replication-dependent dilution of 5mC due to deficient/insufficient maintenance methylation (passive demethylation) and (2) enzyme-mediated removal of 5mC, followed by replacement with unmodified cytosine (active demethylation). During the mammalian life cycle, there are two genome-wide demethylation events, occurring during early embryogenesis and in primordial germ cells (PGCs), with the involvement of both passive and active mechanisms [40]. Global or locus-specific demethylation also occurs in other biological contexts. For example, in 2C-like cells (a small population of transient totipotent cells in cultured mESCs), ZSCAN4-induced UHRF1 and DNMT1 degradation leads to global (passive) demethylation, which is required for telomere extension [41].

The TET family of 5mC dioxygenases—TET1, TET2, and TET3—initiates active demethylation by converting 5mC to oxidized derivatives 5hmC, 5fC, and 5caC [18,21,22,42]. 5fC and 5caC can be excised by thymine DNA glycosylase (TDG), followed by base excision repair (BER) to replace them with unmodified cytosines [42] (Figure 3.1A). It is worth mentioning that 5hmC, 5fC, and 5caC can also be passively diluted as a result of DNA replication. The different expression patterns of TET proteins contribute to their functional specificities during epigenetic reprogramming in early embryos and PGCs. TET3 is highly expressed in oocytes and, after fertilization, is essential

for the active erasure of paternally inherited 5mC marks in the zygote [24], whereas TET1 and TET2 are expressed in PGCs and are involved in 5mC erasure at specific loci, including imprinting control regions (ICRs), CGIs of the inactive X chromosome in females, and germline-specific genes [19] (Table 3.1). Interestingly, recent evidence suggests that downregulation of TET3 in oocytes mediates maternal inheritance of glucose intolerance due to insufficient demethylation and aberrant silencing of insulin secretion genes on paternal alleles [43]. The three TET proteins show overlapping and distinct expression patterns and functions during development and in stem cells and somatic tissues [44].

In addition to being intermediates of DNA demethylation, 5hmC, 5fC and 5caC may serve as epigenetic marks. However, their abundance (especially 5fC and 5caC) is low in most cell types, and most of the reported “readers” remain to be validated. Thus further studies are necessary to establish the significance of these 5mC oxidized derivatives as components of the epigenetic code.

2.3 Methyl-CpG-binding proteins

5mC can directly block transcription factors from binding DNA. It can also be recognized by methyl-CpG-binding proteins, which recruit other factors to induce chromatin compaction. There are three families of proteins that bind 5mC: methyl-CpG-binding domain (MBD) proteins, zinc finger (ZNF) proteins, and SET and RING finger-associated (SRA) domain proteins. Some of these proteins have also been reported to recognize 5hmC, 5fC, and/or 5caC.

The MBD family consists of MeCP2 and MBD1–6. With the exception of MBD3, which contains sequence variations in its MBD domain, MBD proteins recognize methylated CpG sites, with the flanking sequences modulating the binding specificity and affinity. MeCP2 and MBD3 may also bind 5hmC [45,46]. In addition to the MBD domain, MBD proteins contain various protein–protein interaction domains, which recruit distinct corepressor complexes. For example, MeCP2 interacts with the Sin3A complex, MBD1 with SETDB1, SUV39H1, and HP1, and MBD2 and MBD3, in a mutually exclusive manner, with the Mi-2/NuRD complex. MBD4 contains a glycosylase domain and is involved in DNA mismatch repair. While work on MBD5 and MBD6 is limited, they have been reported to interact with the PR-DUB Polycomb protein complex [47].

ZNF proteins that have been reported to bind methylated DNA include ZBTB proteins (ZBTB33/KAISO, ZBTB4, and ZBTB38) and KRAB domain-containing proteins (ZFP57 and ZFP445/ZNF445). ZBTB proteins contain a BTB/POZ domain, which usually recruits histone deacetylase (HDAC)-containing complexes to repress transcription [48]. ZFP57 and ZFP445 act together to specifically recognize a methylated hexanucleotide sequence (TGC^mCGC) present in ICRs and protect DNA methylation imprints against the wave of demethylation that erases most 5mC marks during early embryogenesis [30,33]. Other ZNF proteins, including ERG1 and WT1, may also exert some of their functions by recognizing 5mC and/or oxidized derivatives [34].

The SRA family has two members: UHRF1 and UHRF2. UHRF1, via its SRA domain, specifically recognizes hemimethylated DNA and is essential for DNMT1-mediated maintenance methylation [35]. UHRF2 is not an essential gene. There is evidence that the SRA domain of UHRF2 is a 5hmC reader domain [36].

In addition to 5mC readers, some proteins specifically recognize unmethylated CpG sites. Prominent recent examples include BEND3, which binds an unmethylated CpG-containing motif

and prevents the premature activation of bivalent genes during differentiation [49], and QSER1, which protects DNA methylation valleys (or canyons) and bivalent promoters from DNMT3-mediated *de novo* methylation [50].

3 Histone lysine methylation writers, erasers, and readers

Numerous evolutionarily conserved lysine residues in core histones are subject to methylation, with H3K4, H3K9, H3K27, H3K36, H4K20 (on N-terminal tails), and H3K79 (within globular domain) being the canonical methylation sites. Each lysine can be mono-, di-, or trimethylated (Figure 3.1B). Histone lysine methylation can signal activation or repression, depending on the sites and degree of methylation. In general, H3K4, H3K36, and H3K79 methylation marks are considered active modifications, whereas H3K9, H3K27, and H4K20 methylation marks are associated with repressive chromatin. H3K4me3 is a hallmark of promoters with particular enrichment at the transcriptional start sites (TSSs), while H3K4me1 is enriched in enhancers. The gene bodies of active genes contain multiple marks, including H3K79me2/3, which peak toward the 5' end of the coding region, and H3K36me2/3, which are more abundant toward the 3' end. Conversely, inactive promoters are often marked by H3K27me3 or H3K9me3. Epigenetic hallmarks of constitutive heterochromatin include H3K9me3 and H4K20me3. In stem cells, a subset of promoters contain both the active H3K4me3 and the repressive H3K27me3 marks. These bivalent promoters are considered poised for differentiation that can be rapidly activated or repressed during differentiation [51]. Over the last two decades, great progress has been made in identifying and characterizing the lysine MTases (KMTs), lysine demethylases (KDMs), and effector proteins that recognize specific methyllysine residues (Figure 3.1B and Table 3.2).

3.1 KMTs

With the exception of DOT1L (KMT4) and KMT9, all known KMTs contain the Su(var)3–9, Enhancer-of-zeste and Trithorax (SET) domain [52,69]. In general, KMTs show strong preferences for particular lysine residues (Table 3.2). KMT1 proteins are specific for H3K9, KMT2 proteins and SET7/9 (KMT7) for H3K4, most KMT3 proteins for H3K36, KMT5 proteins for H4K20, and KMT6 proteins for H3K27. Several members of the PRDF1 and RIZ1 homology domain (PRDM) family, characterized by the presence of a SET-like domain (known as PR domain) and a variable array of C₂H₂ ZNFs, have been shown to have lysine MTase activity. These proteins, forming the KMT8 subgroup, appear to be less specific [67]. DOT1L and KMT9 are members of the seven-beta-strand (7 β S) MTase family, which specifically catalyze H3K79me1/2/3 and H4K12me1 marks, respectively [52,69]. KMTs are also specific for the number of methyl groups added (Table 3.2). For example, SETD8 (KMT5A) converts unmethylated H4K20 to H4K20me1, which is further converted to H4K20me2/3 by SUV420H1/H2 (KMT5B and KMT5C). There are multiple H3K36 MTases, and only SETD2 (KMT3A) can catalyze H3K36me3, while the others are restricted to mono- and/or di-methylation [52]. The H3K9 MTases show varying specificities for genomic regions: SUV39H1/H2 (KMT1A and KMT1B) convert H3K9me1 to H3K9me2/3 in constitutive heterochromatin [53], G9A/EHMT2 (KMT1C) and GLP/EHMT1 (KMT1D) form a heterodimer that catalyzes H3K9me1/2 in euchromatin to repress gene expression [80], and

TABLE 3.2 KMTs and KDMs and their relevance to human diseases.

Protein	Other names	Substrate specificity and major mammalian functions	Relevance to cancer and other diseases	References
KMT1A	SUV39H1	Converts H3K9me1 to H3K9me2/3 in constitutive heterochromatin; not essential for mouse development	Various cancers	[52–55]
KMT1B	SUV39H2	Converts H3K9me1 to H3K9me2/3 in constitutive heterochromatin; not essential for mouse development; KMT1A/1B DKO mice: increased tumor risk and impaired fertility	Lung, colorectal, and gastric cancers	[52,54,55]
KMT1C	G9A/EHMT2	Forms heterodimer with KMT1D to catalyze H3K9m2 in euchromatin; essential for mouse embryonic development	Various cancers	[52,54,55]
KMT1D	GLP/EHMT1	Forms heterodimer with KMT1C to catalyze H3K9m2 in euchromatin; essential for mouse embryonic development	Kleefstra-1 syndrome, glioma, hematologic malignancies	[52,54,55]
KMT1E	SETDB1/ESET	Catalyzes H3K9me2/3 in euchromatin; essential for early embryogenesis and germ cell development in mice	Various cancers	[52,54,55]
KMT1F	SETDB2/CLL8	Converts H3K9me1/2 to H3K9me3; involved in innate and adaptive immunity, inflammation and T-cell differentiation	Hematologic malignancies, gastric cancer	[54,55]
KMT2A	MLL1	Mainly deposits H3K4me3 in promoters; required for mouse embryonic development and hematopoiesis	MLL-rearranged leukemia, Wiedemann-Steiner syndrome	[52,56]
KMT2B	MLL2	Mainly deposits H3K4me3 in promoters; essential for mouse embryogenesis	Dystonia, various cancers	[52,56]
KMT2C	MLL3	Deposits H3K4me1/2 in enhancers; essential for mouse embryogenesis	Kleefstra-2 syndrome, various cancers	[52,56]
KMT2D	MLL4	Deposits H3K4me1/2 in enhancers; essential for mouse embryogenesis	Kabuki syndrome, various cancers	[52,56]
KMT2F	SETD1A	Catalyzes H3K4me2/3 at TSSs; essential for mouse embryogenesis	Schizophrenia, various cancers	[52,56]
KMT2G	SETD1B	Catalyzes H3K4me2/3 at TSSs; essential for mouse embryogenesis	12q24.31 microdeletion syndrome, various cancers	[52,56]
KMT2H	ASH1L	Catalyzes H3K36me2; essential for mouse embryogenesis; involved in hematopoiesis	Tourette syndrome, leukemia, breast, and pancreatic cancers	[52,57]
KMT3A	SETD2	Catalyzes H3K36me3; essential for mouse development; involved in osteogenesis, myogenesis, germ cell development and hematopoiesis	Luscan-Lumish syndrome, various cancers	[52,58]
KMT3B	NSD1	Catalyzes H3K36me1/2; essential for mouse embryogenesis	Sotos syndrome, Beckwith-Wiedemann syndrome, hematologic malignancies, neuroblastoma	[52,58]
KMT3C	SMYD2	Catalyzes H3K36me2 and H3K4me1; cardiac KO mice: no phenotype	Pancreatic and gastric cancers	[58,59]
KMT3D	SMYD1	Catalyzes H3K4me2/3; involved in cardiac and skeletal muscle physiology		[59,60]
KMT3E	SMYD3	Catalyzes H3K4me2/3 and H4K20me3; involved in cardiac and skeletal muscle physiology	Liver, colon, and breast cancers	[59,60]
KMT3F	NSD3/WHSC1L1	Catalyzes H3K36me1/2; involved in neural crest migration and retinal network formation	Hematologic malignancies, breast and lung cancers	[52,58]

TABLE 3.2 KMTs and KDMs and their relevance to human diseases. *Continued*

Protein	Other names	Substrate specificity and major mammalian functions	Relevance to cancer and other diseases	References
KMT3G	NSD2/ WHSC1	Catalyzes H3K36me1/2; KO mice: postnatal lethality; involved in B cell development	Wolf–Hirschhorn syndrome, various cancers	[52,58]
KMT4	DOT1L	Catalyzes H3K79me1/2/3; essential for mouse development; involved cardiac development and hematopoiesis	MLL-rearranged leukemia	[52,58,61]
KMT5A	SET8/ SETD8	Catalyzes H4K20me1; essential for mouse embryogenesis	Breast cancer	[52,58]
KMT5B	SUV420H1	Converts H4K20me1 to H4K20me2/3; KO mice: postnatal lethality, short stature	Intellectual disability, various cancers	[52,62]
KMT5C	SUV420H2	Converts H4K20me1 to H4K20me2/3; not essential for mouse development	Liver and lung cancers	[52,58,62]
KMT6A	EZH2	Catalyzes H3K27me2/3; essential for mouse embryogenesis	Weaver syndrome, lymphoma, and solid tumors	[52,63]
KMT6B	EZH1	Catalyzes H3K27me2/3 in stem cells; KO mice: viable, involved in hematopoiesis	Thyroid cancer	[52,64–66]
KMT7	SETD7/ SET7/ SET9	Catalyzes monomethylation of H3K4 and numerous nonhistone substrates; not essential for mouse development	Breast and prostate cancers	[60]
KMT8A	PRDM2/ RIZ1	Methylates H3K9; coactivator of estrogen and progesterone receptors	Various cancers	[67,68]
KMT8B	PRDM9	Catalyzes H3K4me3 and H3K36me3; required for meiosis and fertility	Various cancers	[52,67,68]
KMT9	C21orf127/ HEMK2/ N6AMT1	Forms a heterodimer with KMT9β (TRMT112) to catalyze H4K12me1	Prostate cancer	[69]
KDM1A	LSD1/ AOF2	Demethylates H3K4me1/2; essential for mouse embryonic development. LSD1n isoform demethylates H4K20me1 in neurons and is involved in learning and memory	Kabuki syndrome, prostate cancer	[58,70,71]
KDM1B	LSD2/ AOF1	Demethylates H3K4me1/2; KO mice: viable, females infertile due to defects in establishing maternal imprints	Lung cancer	[58,71,72]
KDM2A	JHDM1A/ FBXL11	Demethylates H3K36me1/2; essential for mouse embryonic development	Lung cancer	[58,71]
KDM2B	JHDM1B/ FBXL10	Demethylates H3K36me1/2; KO mice: postnatal lethality	Various cancers	[58,71]
KDM3A	JMD1A/ JHDM2A	Demethylates H3K9me1/2, putative RDM; KO mice: viable, obese	Various cancers	[58,71,73]
KDM3B	JMD1B/ JHDM2B	Demethylates H3K9me1/2, putative RDM; KO mice: postnatal lethality. KDM3A/3B DKO mice: embryonic lethality	Myeloid leukemia	[58,71,74]
KDM3C	JMD1C	Demethylates H3K9me1/2; KO mice: viable, males show reduced fertility	AML, esophageal cancer	[58,71]
KDM4A	JMD2A/ JHDM3A	Demethylates H3K9me2/3, H3K36me2/3; not essential for mouse development	Bladder and breast cancers	[58,71]
KDM4B	JMD2B	Demethylates H3K9me2/3, H3K36me2/3; not essential for mouse development	Peripheral nerve sheath tumor	[58,71]
KDM4C	JMD2C/ GASC1	Demethylates H3K9me2/3, H3K36me2/3; KO mice: viable; KDM4A/4C DKO mice: embryonic lethality	Various cancers	[58,71]

(Continued)

TABLE 3.2 KMTs and KDMs and their relevance to human diseases. *Continued*

Protein	Other names	Substrate specificity and major mammalian functions	Relevance to cancer and other diseases	References
KDM4D	JMJD2D	Demethylates H3K9me2/3; testis-enriched expression; not essential for mouse development	Various cancers	[58,71]
KDM4E	JMJD2E	Demethylates H3K9me2/3, putative RDM; functions poorly understood		[58,71,73]
KDM5A	JARID1A	Demethylates H3K4me2/3; KO mice: viable, with autism-like phenotype	Ankylosing spondylitis, leukemia, and various solid tumors	[58,71,75]
KDM5B	JARID1B/PLU1	Demethylates H3K4me1/2/3; essential for mouse embryogenesis	Various cancers	[58,71]
KDM5C	JARID1C/SMCX	Demethylates H3K4me2/3, putative RDM; X-linked; KO mouse: viable, with neurological deficits	Skeletal abnormalities, intellectual disability, cervical and renal cancers	[58,71,73,76]
KDM5D	JARID1D	Demethylates H3K4me2/3; Y-linked; expression restricted to testis, prostate and intestine	Prostate and renal cancers	[58,71]
KDM6A	UTX	Demethylates H3K27me2/3; X-linked; Female KO embryos die by E12.5, male KO mice viable with shorter lifespan	Kabuki syndrome, T-ALL, various solid tumors	[58,71]
KDM6B	JMJD3	Demethylates H3K27me2/3, putative RDM; KO mouse: postnatal lethality	Hodgkin's lymphoma	[58,71,73]
KDM6C	UTY	Demethylates H3K27me2/3 (reduced activity); Y-linked; KDM6A/6C DKO male mice: embryonic lethality	Prostate cancer	[58,71]
KDM7A	JHDM1D/KIAA1718	Demethylates H3K27me2; KO mice: viable, with anterior homeotic transformation of vertebral elements	Breast and prostate cancers	[58,71,77]
KDM7B	PHF8	Demethylates H3K9me1/2, H3K27me2, and H4K20me1; X-linked; KO mice: viable, with impaired learning and memory	Craniofacial abnormality, intellectual disability, various cancers	[58,71,78]
KDM7C	PHF2	Demethylates H4K20me3; KO mice: partial neonatal lethality, postnatal growth retardation with reduced adipose tissue	Breast cancer	[58,71,79]

T-ALL, *T-cell acute lymphoblastic leukemia*.

SETDB1/ESET (KMT1E) catalyzes H3K9me2/3 in euchromatin and is involved in gene repression, as well as proviral silencing in mESCs and early embryos [81]. Likewise, different MLL (KMT2) enzymes preferentially deposit H3K4 methylation marks in distinct gene regulatory elements (Table 3.2). KMTs are usually present in protein complexes [e.g., EZH2 (KMT6A) in polycomb-repressive complex 2 (PRC2)], and their enzymatic activity and specificity can be modified by other subunits.

3.2 KDMs

There are two classes of KDMs in mammals (Table 3.2). The flavin adenine dinucleotide (FAD)-dependent amine oxidase family comprises two members, LSD1 (KDM1A) and LSD2 (KDM1B), which are capable of demethylating H3K4me1/2, but not H3K4me3 (because of the chemical mechanism these

enzymes employ, they cannot act on trimethylated lysines) [70,72]. The other class of KDMs are jumonji C (JmjC) domain-containing, iron- and α -ketoglutarate (α KG)-dependent dioxygenases, which are capable of removing mono-, di-, and trimethyl marks [82,83]. The JmjC domain-containing family, with at least 30 members in human and mouse, is divided into seven subgroups based on JmjC domain homology and protein domain architecture (not all JmjC proteins have KDM activity). Like KMTs, KDM enzymes show specificity for both the site and degree of methylation. For example, JHDM1 (KDM2) proteins specifically demethylate H3K36me1/2, JHDM2 (KDM3) proteins specifically demethylate H3K9me1/2, whereas JMJD2 (KDM4) proteins demethylate both H3K9me2/3 and H3K36me2/3, but not H3K9me1 or H3K36me1. To date, no enzyme that is capable of demethylating H3K79 has been identified. Given that DOT1L preferentially methylates nucleosomal substrates over free histone H3, it is possible that the yet-to-be identified H3K79 demethylase(s) also targets only chromatinized H3K79 methylation. Biochemical assays using H3K79-methylated nucleosome substrates may facilitate the identification of the elusive demethylase(s).

3.3 Methyllysine-binding domains

Methylation does not change the electronic charge of the amino-acid side chain; the “meanings” of histone lysine methylation marks are mainly “interpreted” by effector molecules that specifically recognize the methylated sites. Multiple protein domains have been shown to bind methylated lysines, including plant homeodomain (PHD), ankyrin, ATRX-DNMT3-DNMT3L (ADD), bromo-adjacent homology (BAH), WD40 domains, and the “Royal Family” of domains, consisting of the Tudor, chromo, malignant brain tumor (MBT), and PWWP domains [58,84]. The substantial number and variety of domains that recognize methylated lysines demonstrates the functional importance of this modification. Each of these domains is present in numerous proteins; thus there are hundreds of potential methyllysine readers. Many of them are subunits of macromolecular complexes, which are critically involved in a variety of cellular processes [84].

Each methyllysine reader domain has specific binding characteristics, specifically recognizing certain lysines with a particular methylation status. For example, PHD fingers interact with H3K4me3, WD40 domains bind to multiple trimethylated lysines associated with repressive marks, and tandem Tudor domains (TTDs) recognize heterochromatic H3K9me3 mark in association with PHD fingers. Notably, many epigenetic writers and erasers also contain reader domains, including methyllysine-binding domains. For example, the PWWP domains in DNMT3A and DNMT3B contribute to the functional differences between the two *de novo* DNMTs. DNMT3B plays a major role in methylating gene bodies, because its PWWP domain recognizes H3K36me3, which is enriched in actively transcribed regions [85]. The DNMT3A PWWP domain shows dual recognition of H3K36me2 and H3K36me3, with a higher binding affinity toward H3K36me2, which directs DNMT3A to H3K36me2-containing intergenic regions for *de novo* methylation [86]. While lysine methylation usually creates binding sites for reader proteins, the presence of a particular methyl mark can interfere with protein binding in some cases. For example, the ADD domains of DNMT3A and DNMT3L interact with the N-terminal tail of histone H3, but H3K4 methylation disrupts this interaction [17,87]. The ligand specificity of the various domains is mainly determined by two factors: (1) the conformation of the binding pocket provides for either specific domain–ligand binding to enhance interaction or steric hindrance to inhibit strong interaction with certain ligands and (2) the amino acids surrounding the modified lysine can interact with the reader protein, which can positively or negatively affect binding.

4 Arginine methylation writers, erasers, and readers

Arginine methylation is a widespread PTM that occurs on a variety of substrates, including histones, RNA-binding proteins, and many other proteins. Over 30 methylation sites on core histones have been identified, with the common ones including H2AR3, H2AR29, H2BR29, H2BR31, H2BR33, H3R2, H3R8, H3R17, H3R26, H3R42, H4R3, H4R17, and H4R19. While arginine methylation, which occurs on the guanidinium moiety (Figure 3.1C), does not change the net positive charge, it adds hydrophobicity to the side chain, thereby favoring interactions with aromatic cages, but also increases the bulkiness of the side chain and diminishes the hydrogen bonding capacity. Thus arginine methylation can alter protein-protein and protein-nucleic acid interactions and modulate fundamental cellular processes, such as gene transcription, RNA processing, signal transduction, and DNA damage response [88]. Arginine methylation is mediated by protein arginine methyltransferases (PRMTs), and methylated arginine residues can be selectively recognized by proteins with specific reader domains [88,89] (Table 3.3). Although several JmjC proteins have been reported to demethylate methylarginine residues [73,74,76], further studies are necessary to validate their physiological significance as arginine demethylases (RDMs).

4.1 PRMTs

The PRMT family has nine members, PRMT1–9, which are divided into three types (types I, II, and III) based on the end products. All three types are capable of generating ω -N^G-monomethylarginine (MMA, me1), via the addition of one methyl group to one of the two terminal nitrogen atoms of the arginine amino acid. The type III enzyme PRMT7 does not modify the arginine further. The type I enzymes PRMT1, PRMT2, PRMT3, CARM1 (PRMT4), PRMT6, and PRMT8 further add a methyl group to the same methylated nitrogen atom previously methylated, generating ω -N^G,N^G-asymmetric dimethylarginine (ADMA, me2a). The type II enzymes PRMT5 and PRMT9 methylate the other, unmodified terminal nitrogen atom, generating ω -N^G,N^G-symmetric dimethylarginine (SDMA, me2s) (Figure 3.1C). PRMT1, the main type I enzyme, has a strong preference for the RGG/RG motif. PRMT5 is the primary type II enzyme, because PRMT9 has only one known substrate, SAP145, a splicing factor [94]. Unlike type I PRMTs, PRMT5 forms a complex with MEP50 (WDR77) and interacts with numerous substrate adaptors, which regulate substrate specificity. PRMT7, the sole type III enzyme, methylates substrates within an RxR consensus [88].

Histone arginine methylation has been implicated in both transcription activation and repression. Different methylation states at particular sites often have opposing effects. For example, PRMT1-mediated H4R3me2a leads to gene activation, whereas PRMT5-mediated H4R3me2s causes gene repression. PRMT5-mediated H2AR3me2s and H3R8me2s are also involved in gene repression. In addition, PRMT5 deposits H3R2me2s, an active mark. CARM1 functions mainly as a coactivator by asymmetrically dimethylating several histone arginine residues, including H3R17, H3R26, and H3R42. PRMT6 acts mainly as a transcription corepressor by asymmetrically dimethylating H3R2 and H2AR29. PRMT7-mediated H4R17me1 is involved in gene repression by enhancing allosteric PRMT5-mediated H4R3me2s. PRMT7 also monomethylates H2AR3 and H4R3 to regulate the expression of DNA repair enzymes [88,89].

TABLE 3.3 PRMTs and methylarginine-binding proteins and their relevance to human diseases.

Protein	Major activity and function in mammals	Relevance to cancer and other diseases	References
PRMT1	Main type I PRMT, catalyzes H4R3me2a, coactivator; essential for mouse embryonic development	Various cancers	[71,88–90]
PRMT2	Type I PRMT, catalyzes H3R8me2a; not essential for mouse development	Breast cancer	[89–91]
PRMT3	Type I PRMT, methylates ribosomal protein rpS2, no known histone substrate; localized in cytosol; KO mice: viable, smaller embryos	Breast cancer	[90,92]
PRMT4/ CARM1	Type I PRMT catalyzes H3R17me2a, H3R26me2a, and H3R42me2a, coactivator; KO mice: partial embryonic lethality, full neonatal lethality	Breast, prostate and colorectal cancers	[71,88–90]
PRMT5	Primary type II PRMT, catalyzes H3R2me2s, H3R8me2s, H4R3me2s, and H2AR3me2s, corepressor; essential for mouse embryonic development	Various cancers	[71,88–90]
PRMT6	Type I PRMT, catalyzes H3R2me2a, H3R42me2a, H4R3me2a, H2AR3me2a, and H2AR29me2a; not essential for mouse development	Bladder and lung cancer	[71,88–90]
PRMT7	Type III PRMT, catalyzes H4R17me1, H4R19me1, H2BR29me1, H2BR31me1, and H2BR33me1; KO mice: viable, reduced muscle-to-fat ratio	SBIDDS syndrome, breast and lung cancers	[71,88–90]
PRMT8	Type I PRMT, no known histone substrate; expressed in brain, localized in cytosol and plasma membrane; KO mice: viable, with abnormal behaviors	Ovarian, skin, and large intestine cancers	[90,93]
PRMT9/ FBXO11	Type II PRMT, catalyzes SAP145 R508me1/2s (only known substrate); involved in splicing	Intellectual disability	[94,95]
SMN	Binds ADMA and SDMA catalyzed by PRMT4 and PRMT5; involved in snRNP assembly and splicing; essential for mouse embryonic development	SMA	[89,96]
SPF30/ SMNDC1	Preferentially binds SDMA; required for spliceosome maturation		[89]
TDRD1	Binds SDMA on Milli; expressed only in germ cells; essential for spermatogenesis	Prostate and colorectal cancers	[89,97]
TDRD2/ TDRKH	Binds SDMA on Miwi and Miwi2; essential for spermatogenesis and piRNA biogenesis in germline	dHMN?	[89,98]
TDRD3	Binds ADMA catalyzed by PRMT1 and PRMT4, including H4R3me2a, H3R17me2s and RNAP II R1810me2a; forms complex with TOP3B to resolve R loops; KO mice: viable, with increased genome instability	Breast cancer	[89,99,100]
TDRD6	Binds SDMA on Miwi; required for spermatogenesis	APS1?	[89,101]
TDRD9	Binds SDMA on Miwi2; ATP-binding RNA helicase; expressed in germ cells; required for spermatogenesis	Lung and colorectal cancers	[97,102]
TDRD11 SND1	Binds SDMA on E2F1, coactivator; involved in spliceosome formation; ubiquitous expression, with high levels in liver and pancreas	Liver cancer	[89,103]

APS1, Autoimmune polyendocrine syndrome type 1; dHMN, distal hereditary neuropathy; SBIDDS, short stature, brachydactyly, intellectual developmental disability, and seizures; SMA, spinal muscular atrophy.

4.2 Putative RDMs

In contrast to KDMs, no dedicated RDMs have been identified, although some JmjC proteins have been shown to demethylate methylarginines. JMJD6 was initially reported to function as an RDM, but subsequent work suggested it to be a lysyl-5 hydroxylase instead [104]. JMJD1A (KDM3A), JMJD2E (KDM4E), JARID1C (KDM5C), and JMJD3 (KDM6B) are capable of demethylating methylarginines *in vitro* [73]. More recent studies show that JMJD1B (KDM3B) demethylates H4R3me2s and H3K9me2 to facilitate gene expression for the development of hematopoietic stem and progenitor cells and that JARID1C demethylates R170me2s of ULK1, a key regulator of autophagy [74,76]. As these putative RDMs also have KDM activity, it is challenging to dissect their physiological significance as arginine methylation erasers.

4.3 Methylarginine-binding proteins

Tudor domain, the founding member of the “Royal Family” of domains, is the best characterized domain that recognizes methylated arginine residues. In human, there are at least 36 proteins that harbor one or more Tudor domains. Some of these proteins have been demonstrated to bind methylarginine and/or methyllysine marks, with all of them having an aromatic cage to facilitate the methyl-dependent protein–protein interaction. Generally, the aromatic cage of the methylarginine binders is narrower than that of methyllysine binders [89].

Eight Tudor domain-containing proteins—SMN, SPF30, and TDRD1/2/3/6/9/11—have been reported to be methylarginine readers (Table 3.3). Tudor domains usually show preferences for different methylation states. For example, the Tudor domain of TDRD3 preferentially recognizes ADMA, including PRMT1 and CARM1 substrates, such as H4R3me2a, H3R17me2a, and R1810me2a on the C-terminal domain of RNA polymerase II, the Tudor domains of TDRD2 (TDRKH), TDRD6, and TDRD11 (SND1) bind SDMA, whereas the Tudor domain of SMN interacts with both ADMA and SDMA, including numerous CARM1 and PRMT5 substrates [89,103]. The functional importance of Tudor domain-mediated recognition of methyl marks is highlighted by the finding that SMN mutations, including point mutations in its Tudor domain, cause spinal muscular atrophy (SMA).

5 Interplay between different methylation marks

Early studies of epigenetic modifications dealt with their effects in isolation. However, chromatin can have multiple modifications at a given locus, which combine to determine the activity of that region. A prominent example is that the active H3K4me3 and repressive H3K27me3 marks act together to form bivalent promoters for silencing developmental genes while keeping them poised for activation in mESCs [51]. The deposition of a specific histone modification can affect the subsequent addition of other epigenetic marks. Furthermore, histone modifications at neighboring residues can influence their recognition by reader proteins. For instance, PRMT6-catalyzed H3R2me2a prevents some methyllysine readers from binding H3K4me3 and, conversely, PRMT5-catalyzed H3R2me2s enhances the interaction between H3K4me3 and some of its readers [89].

Crosstalk between DNA methylation and histone lysine methylation is extensive [105]. Genome-wide studies show an inverse correlation between H3K4 methylation and DNA methylation. This could

be attributable, at least in part, to the ability of H3K4 methylation to disrupt the interaction between the ADD domain of DNMT3A or DNMT3L and the N-terminal tail of histone H3 [17,87]. Indeed, erasure of H3K4 methylation by KDM1B, which is exclusively expressed in growing oocytes, is a prerequisite for DNMT3A/3L-mediated *de novo* methylation of maternally imprinted loci during oogenesis [72]. In general, H3K27 methylation and DNA methylation are also anticorrelated, with DNA methylation appearing to antagonize the recruitment of PRC2 in most cases [105]. In contrast, H3K9 methylation and DNA methylation are strongly associated, which work together to form heterochromatin to silence genes and retrotransposons. While the mechanisms involved are complex, components of the H3K9 methylation and DNA methylation machineries have been shown to interact, and H3K9me2/3 marks can serve as binding sites for the TTD of UHRF1, thereby facilitating DNMT1-mediated DNA methylation [35,105]. DNA methylation is also abundant in regions enriched with H3K36me2/3. The PWWP domains of DNMT3A and DNMT3B, by recognizing H3K36me2/3, play important roles in directing these enzymes to intergenic regions and gene bodies for *de novo* methylation [85,86]. In addition to removing H3K4 methylation, LSD1 demethylates and stabilizes DNMT1 [106], which represents another level of interplay between the histone lysine methylation and DNA methylation machineries.

The relationship between arginine methylation and DNA methylation is less well understood. A previous finding that PRMT5-catalyzed H4R3me2s is recognized by the ADD domain of DNMT3A and facilitates DNA methylation has been challenged and remains controversial [89]. Another study showed that PRMT6-mediated H3R2me2a disrupts the interaction between the UHRF1 PHD and histone H3, contributing to global DNA hypomethylation in cancer cells showing high levels of PRMT6 [107].

6 Relevance of DNA methylation and histone methylation in cancer

The importance of genetic alterations in cancer, including mutations that activate oncogenes and inactivate tumor suppressor genes, is well established. Studies in recent years have demonstrated that epigenetic dysregulation also plays crucial roles in the evolution of cancer and development of resistance to immune surveillance and therapy. In addition to altered expression, many epigenetic regulators, including writers, erasers, and readers involved in DNA and histone methylation, have been found to be mutated in cancer, thanks largely to whole genome sequencing studies.

6.1 DNA methylation and cancer

Cancer cells show aberrant DNA methylation patterns, including global hypomethylation and regional hypermethylation. Global hypomethylation, presumably due to insufficient maintenance methylation, is believed to contribute to genome instability. Regional hypermethylation, mainly caused by abnormal *de novo* methylation and/or deficient demethylation, is involved in silencing tumor suppressor genes. DNA methylation also plays an important role in the generation of mutations, as spontaneous deamination of 5mC results in C-to-T transition. Indeed, numerous mutations found in the p53 tumor suppressor is attributed to this mechanism.

Genetic alterations of important components of the DNA methylation and demethylation machineries have been identified in cancer. Somatic heterozygous mutations in *DNMT3A* are found in over 20% of patients with acute myeloid leukemia (AML) and, at lower frequencies, in other hematologic malignancies as well. The majority (~65%) of *DNMT3A* mutations in AML are

missense mutations affecting a single residue, R882, in the catalytic domain. This hotspot mutation not only severely diminishes the catalytic activity of the mutant protein but also exhibits a dominant-negative effect by inhibiting the activity of wild-type DNMT3A, resulting in moderate loss of methylation at many loci across the genome [5]. *TET1*, the founding member of the TET family, was named for its involvement of the ten-eleven translocation [t(10;11)(q22;q23)] in rare cases of leukemia, which results in the fusion of *TET1* gene, located on chromosome 10q22, with *MLL1* gene, located on chromosome 11q23 [20]. *TET2* is frequently mutated in hematologic malignancies. Its deficiency is likely required for both initiating myeloid transformation and maintenance of the malignant phenotype, as *TET2* restoration in a mouse model blocks aberrant self-renewal of hematopoietic stem and progenitor cells and progression of leukemia. *TET2* mutations also cooccur with *TET1* or *DNMT3A* mutations in acute B-lymphocytic leukemia and T-cell lymphoma [23]. Some solid tumors, albeit with no *TET* mutations, exhibit decreased 5hmC levels, which could be due to altered *TET* expression or mutations in regulatory genes, such as isocitrate dehydrogenases 1 and 2 (IDH1/2). IDH1/2 convert isocitrate to α KG, a cofactor of *TET* enzymes and the JmjC family of KDMs, but gain-of-function mutations lead to production of 2-hydroxyglutarate (2HG), an inhibitor of *TET*s and KDMs [108].

6.2 Histone methylation and cancer

Histone lysine methylation pathways are commonly altered in cancer, including genetic alterations in KMTs and/or KDMs that affect methylation at H3K4 (MLL family, JARID1A/1B), H3K27 (EZH2 and UTX), and H3K36 (SETD2 and NSD family) [52,58]. For example, *MLL1* was originally cloned as the gene associated with recurrent translocations of chromosome band 11q23 in a wide range of leukemias, including almost all cases of mixed lineage leukemia (MLL) and a subset of acute lymphoblastic leukemia (ALL) and AML. While in most cases with KMT or KDM mutations, cancers exhibit haploinsufficiency of the affected enzyme, *EZH2* activating mutations have been described. Wild-type EZH2 catalyzes H3K27 methylation, with monomethylation being the most efficient step of the reaction. However, certain lymphomas carry gain-of-function mutations that render the enzyme more efficient at catalyzing the addition of the trimethyl mark [52,58]. In addition to genetic alterations, aberrant expression of multiple KMTs and KDMs (e.g., SETDB1, LSD1) are observed in various types of cancer and has been associated with oncogenic functions. *DOTIL* is not mutated and usually not aberrantly expressed in cancer, but its activity and H3K79 methylation are important drivers of leukemogenesis in hematologic malignancies caused by rearrangements in the *MLL1* gene.

Overexpression, but not mutation, of *PRMT* genes is a common feature in cancer [90]. In fact, the only family member that to date has demonstrated appreciable mutation rates in cancer is *PRMT8*, where mutations have been identified in ovarian, skin, and colon cancers [90]. All other PRMT family members are overexpressed in tumors, and emerging mechanistic investigations are exploring their contribution to cancer. *PRMT1* is a component of the MLL complex that also exhibits acetyltransferase activity and is required for cellular transformation driven by this fusion protein. *PRMT2* is upregulated in breast cancer, with isoforms identified in these tumors that increase the estrogen receptor α (ER α) activity. *CARM1* has widespread roles in cancer, including an ability to serve as a coactivator in ER α -dependent breast cancer, which may explain its observed overexpression in a subset of breast cancers. Furthermore, at the molecular level, *CARM1* regulates transcription of the *CCNE1* (cyclin E1) gene, through histone methylation, and directly methylates the SWI/SNF subunit BAF155, leading to enhanced tumorigenesis. *PRMT5*

overexpression in hematologic malignancies causes downregulation of RB family proteins. PRMT5 also directly methylates the tumor suppressors p53 and PDCD4, affecting the activity of both proteins. Furthermore, PRMT5 enhances tumorigenicity and glycolysis in pancreatic cancers via the FBW7–cMyc axis. PRMT6 expression is increased in a variety of cancers and contributes to oncogenesis, perhaps in part due to the global DNA hypomethylation induced by disassociation of UHRF1 to chromatin [107].

7 Regulators of DNA and histone methylation as therapeutic targets

Unlike genetic alterations, most, if not all, epigenetic changes are reversible, making epigenetic therapy an attractive therapeutic approach for cancer. Indeed, several “epigenetic drugs,” including DNMT, HDAC, and EZH2 inhibitors, have been approved by the US Food and Drug Administration (FDA). The nucleoside analogs 5-azacytidine (Vidaza) and 5-aza-2'-doxycytidine (Decitabine, Dacogen) incorporate into DNA, where they trap DNMTs, resulting in DNA hypomethylation. These demethylating agents were approved for the treatment of myelodysplastic syndrome (MDS), AML, and chronic myelomonocytic leukemia (CMLL). The HDAC inhibitors Vorinostat (Zolinza) and Romidepsin (Istodax) were approved for cutaneous T-cell lymphoma (CTCL). Another HDAC inhibitor Belinostat (Bleedodaq) was subsequently approved for relapsed or refractory peripheral T-cell lymphoma (PTCL). Recently the EZH2 inhibitor Tazemetostat was approved for treating epithelioid sarcoma, a rare cancer, and relapsed or refractory follicular lymphoma with *EZH2* mutation.

Tazemetostat and other EZH2 inhibitors are being or having been evaluated for safety and efficacy in clinical trials covering a wide range of other cancer types. Given that EZH1 and EZH2 can both catalyze H3K27 methylation, dual EZH1 and EZH2 inhibitors have been developed, which have been shown to suppress H3K27 methylation more strongly than EZH2 inhibitors alone and have higher anti-tumor activity against several hematologic malignancies in preclinical models. Furthermore, compounds have been developed that selectively block the interaction between the PRC2 subunit EED and H3K27me3, which show similar antitumor effects as EZH2 inhibitors and, importantly, are also effective against cells that have acquired resistance to EZH2 inhibitors [109]. DOT1L is another KMT that has been actively pursued as a therapeutic target. Both *S*-adenosylmethionine (SAM)-competitive and non-SAM-competitive DOT1L inhibitors have been developed (SAM is the universal methyl donor). In clinical trials, Pinometostat, a SAM-competitive DOT1L inhibitor, showed acceptable safety and pharmacodynamics, as well as a moderate reduction in H3K79me2 at genes targeted by MLL fusion proteins. Preclinical studies support the use of Pinometostat as a combination therapy with existing standard-of-care drugs for AML, including DNMT inhibitors. There is also evidence that Pinometostat might be effective in AML with *DNMT3A* mutations [109]. Menin is an interacting partner of MLL proteins and plays an important role in MLL chromatin docking, including localization of MLL fusion proteins to chromatin. Inhibitors that block the MLL-Menin interaction are being evaluated in clinical trials for acute leukemias [109]. Emerging evidence suggests that KDMs are promising therapeutic targets as well. At present, several LSD1 inhibitors are in clinical trials for various types of cancer [110].

Several clinical-grade small-molecule PRMT inhibitors have been developed. Some of them specifically inhibit a particular enzyme, for example, PRMT3, CARM1, PRMT5, PRMT6, or PRMT7, whereas others target multiple PRMTs, such as type I PRMTs, CARM1, PRMT6, PRMT5, and PRMT7 [88,111]. In addition to inhibition of the catalytic activities of PRMTs, PRMT5-substrate adaptor interaction

inhibitors and a PRMT5 degrader using a proteolysis-targeting chimera (PROTAC) have recently been generated [88]. The availability of PRMT inhibitors has allowed the research community to define tumor type vulnerability. For example, PRMT5 inhibition has recently emerged as a potential therapy against methylthioadenosine phosphorylase (MTAP)-deficient cancers, in which the accumulation of the substrate 5'-methylthioadenosine (MTA) inhibits PRMT5 activity, thus sensitizing the cells to further PRMT5 inhibition. The *MTAP* gene is frequently lost due to its proximity to the commonly deleted tumor suppressor gene *CDKN2A*. So far, inhibitors targeting PRMT5 and type I PRMTs have entered clinical trials for patients with hematologic malignancies or advanced solid tumors, including MTAP-deleted tumors [111].

The DNA demethylating agents 5-azacytidine and 5-aza-2'-doxycytidine show substantial DNA damage and cellular toxicity and are ineffective in treating solid tumors. These drawbacks have led to a persistent search for nonnucleoside DNMT inhibitors. Although multiple such inhibitors have been evaluated, none of them turned out to be specific for DNMT1 or DNMT3 with a clear translation from *in vitro* to *in vivo* activity. Recently GlaxoSmithKline reported a new class of reversible DNMT1-selective inhibitors, which were superior to 5-aza-2'-doxycytidine for tumor regression in a mouse model of AML [112].

8 Conclusion

A complex network, involving a variety of proteins, is required in order to maintain the desired gene expression programs; defects in these pathways can result in cancer. While great progress has been made in identifying the writers, erasers, and readers of various epigenetic marks, knowledge about the roles of many of these proteins in physiological and pathological processes, including tumorigenesis, is still limited. We expect that the expansion of novel technologies for genetic manipulations and for studying chromatin dynamics will lead to new and exciting discoveries in the field. Emerging evidence demonstrates that a wide variety of human malignancies coopt these pathways to provide a selective growth advantage. These observations, in combination with an ever-expanding dataset derived from whole genome sequencing of patient tumors to identify key driver mutations, should allow for the personalized treatment of cancer patients. In addition to the epigenetic therapies that have been approved and are being evaluated in clinical trials, as described in this chapter, more epigenetic regulators are being targeted, with some inhibitors showing promising therapeutic effects, in preclinical studies. It is generally believed that targeting multiple cancer-related pathways would have stronger therapeutic benefits than modifying any single pathway. We expect most epigenetic drugs to be used in combination with other therapies to enhance efficacy, reduce toxicity, and/or minimize the development of resistance.

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List of abbreviations

2HG	2-Hydroxyglutarate
5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
6mA	N(6)-Methyladenine
ADCA-DN	Autosomal dominant cerebellar ataxia, deafness, and narcolepsy
ADD	ATRX-DNMT3-DNMT3L
ADMA	$\omega\text{-}N^G,N^G$ -Asymmetric dimethylarginine
αKG	α -Ketoglutarate
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APS1	Autoimmune polyendocrine syndrome type 1
BAH	Bromo-adjacent homology
BER	Base excision repair
CGI	CpG island
CMLL	Chronic myelomonocytic leukemia
CpG	Cytosine–guanine dinucleotide
CTCL	Cutaneous T-cell lymphoma
dHMN	Distal hereditary neuropathy
DNMT	DNA methyltransferase
ERα	Estrogen receptor α
ESC	Embryonic stem cell
FAD	Flavin adenine dinucleotide
FDA	Food and Drug Administration
HDAC	Histone deacetylase
HSAN IE	Hereditary sensory and autonomic neuropathy with dementia and hearing loss type IE
ICF	Immunodeficiency, centromeric instability, and facial anomalies
ICR	Imprinting control region
IDH1/2	Isocitrate dehydrogenases 1 and 2
JmjC	Jumonji C
KDM	Lysine demethylase
KMT	Lysine methyltransferase
KO	Knockout
KRAB	Krüppel associated box
MDS	Myelodysplastic syndrome
MBD	Methyl-CpG-binding domain
MBT	Malignant brain tumor
MLL	Mixed lineage leukemia
MMA	$\omega\text{-}N^G$ -Monomethylarginine
MTA	Methylthioadenosine
MTAP	Methylthioadenosine phosphorylase
MTase	Methyltransferase
NuRD	Nucleosome remodeling deacetylase
PGC	Primordial germ cell

PHD	Plant homeodomain
PRC2	Polycomb-repressive complex 2
PR-DUB	Polycomb-repressive deubiquitinase
PRMT	Protein arginine methyltransferase
PROTAC	Proteolysis-targeting chimera
PTCL	Peripheral T-cell lymphoma
PTM	Posttranslational modification
PWWP	Proline–tryptophan–tryptophan–proline
RDM	Arginine demethylase
RING	Really Interesting New Gene
SAM	S-Adenosylmethionine
SBIDDS	Short stature, brachydactyly, intellectual developmental disability, and seizures
SDMA	ω -N ^G ,N ^G -Symmetric dimethylarginine
SET	Su(var)3-9, Enhancer-of-zeste and Trithorax
SMA	Spinal muscular atrophy
SRA	SET and RING finger associated
T-ALL	T-cell acute lymphoblastic leukemia
TDG	Thymine DNA glycosylase
TET	Ten-eleven translocation
TSS	Transcriptional start site
TTD	Tandem tudor domain
ZBTB	Zinc finger and BTB domain containing
ZNF	Zinc finger

References

- [1] Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 1992;69(6):915–26.
- [2] Moghadam KK, Pizza F, La Morgia C, et al. Narcolepsy is a common phenotype in HSAN IE and ADCA-DN. *Brain* 2014;137(Pt 6):1643–55.
- [3] Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999;99(3):247–57.
- [4] Kaneda M, Okano M, Hata K, et al. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 2004;429(6994):900–3.
- [5] Yang L, Rau R, Goodell MA. DNMT3A in haematological malignancies. *Nat Rev Cancer* 2015;15(3):152–65.
- [6] Tatton-Brown K, Seal S, Ruark E, et al. Mutations in the DNA methyltransferase gene DNMT3A cause an overgrowth syndrome with intellectual disability. *Nat Genet* 2014;46(4):385–8.
- [7] Heyn P, Logan CV, Fluteau A, et al. Gain-of-function DNMT3A mutations cause microcephalic dwarfism and hypermethylation of Polycomb-regulated regions. *Nat Genet* 2019;51(1):96–105.
- [8] Zeng Y, Ren R, Kaur G, et al. The inactive Dnmt3b3 isoform preferentially enhances Dnmt3b-mediated DNA methylation. *Genes Dev* 2020;34(21–22):1546–58.
- [9] Xu GL, Bestor TH, Bourc'his D, et al. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* 1999;402(6758):187–91.
- [10] de Greef JC, Wang J, Balog J, et al. Mutations in ZBTB24 are associated with immunodeficiency, centromeric instability, and facial anomalies syndrome type 2. *Am J Hum Genet* 2011;88(6):796–804.
- [11] Thijssen PE, Ito Y, Grillo G, et al. Mutations in CDCA7 and HELLS cause immunodeficiency-centromeric instability-facial anomalies syndrome. *Nat Commun* 2015;6:7870.

- [12] Barau J, Teissandier A, Zamudio N, et al. The DNA methyltransferase DNMT3C protects male germ cells from transposon activity. *Science* 2016;354(6314):909–12.
- [13] Bourc'his D, Xu GL, Lin CS, Bollman B, Bestor TH. Dnmt3L and the establishment of maternal genomic imprints. *Science* 2001;294(5551):2536–9.
- [14] Hata K, Okano M, Lei H, Li E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development* 2002;129(8):1983–93.
- [15] Suetake I, Shinozaki F, Miyagawa J, Takeshima H, Tajima S. DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. *J Biol Chem* 2004;279(26):27816–23.
- [16] Veland N, Lu Y, Hardikar S, et al. DNMT3L facilitates DNA methylation partly by maintaining DNMT3A stability in mouse embryonic stem cells. *Nucleic Acids Res* 2019;47(1):152–67.
- [17] Ooi SK, Qiu C, Bernstein E, et al. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature* 2007;448(7154):714–17.
- [18] Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009;324(5929):930–5.
- [19] Seisenberger S, Andrews S, Krueger F, et al. The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Mol Cell* 2012;48(6):849–62.
- [20] Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR. TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). *Leukemia* 2003;17(3):637–41.
- [21] Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* 2010;466(7310):1129–33.
- [22] Ito S, Shen L, Dai Q, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 2011;333(6047):1300–3.
- [23] Kunimoto H, Nakajima H. TET2: a cornerstone in normal and malignant hematopoiesis. *Cancer Sci* 2021;112(1):31–40.
- [24] Gu TP, Guo F, Yang H, et al. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. *Nature* 2011;477(7366):606–10.
- [25] Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet* 1999;23(2):185–8.
- [26] Guy J, Hendrich B, Holmes M, Martin JE, Bird A. A mouse MeCP2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat Genet* 2001;27(3):322–6.
- [27] Chen RZ, Akbarian S, Tudor M, Jaenisch R. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet* 2001;27(3):327–31.
- [28] Shimbo T, Wade PA. Proteins that read DNA methylation. *Adv Exp Med Biol* 2016;945:303–20.
- [29] Talkowski ME, Mullegama SV, Rosenfeld JA, et al. Assessment of 2q23.1 microdeletion syndrome implicates MBD5 as a single causal locus of intellectual disability, epilepsy, and autism spectrum disorder. *Am J Hum Genet* 2011;89(4):551–63.
- [30] Quenneville S, Verde G, Corsinotti A, et al. In embryonic stem cells, ZFP57/KAP1 recognize a methylated hexanucleotide to affect chromatin and DNA methylation of imprinting control regions. *Mol Cell* 2011;44(3):361–72.
- [31] Li X, Ito M, Zhou F, et al. A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. *Dev Cell* 2008;15(4):547–57.
- [32] Mackay DJ, Callaway JL, Marks SM, et al. Hypomethylation of multiple imprinted loci in individuals with transient neonatal diabetes is associated with mutations in ZFP57. *Nat Genet* 2008;40(8):949–51.
- [33] Takahashi N, Coluccio A, Thorball CW, et al. ZNF445 is a primary regulator of genomic imprinting. *Genes Dev* 2019;33(1–2):49–54.
- [34] Hashimoto H, Olanrewaju YO, Zheng Y, Wilson GG, Zhang X, Cheng X. Wilms tumor protein recognizes 5-carboxylcytosine within a specific DNA sequence. *Genes Dev* 2014;28(20):2304–13.

- [35] Mancini M, Magnani E, Macchi F, Bonapace IM. The multi-functionality of UHRF1: epigenome maintenance and preservation of genome integrity. *Nucleic Acids Res* 2021;49(11):6053–68.
- [36] Zhou T, Xiong J, Wang M, et al. Structural basis for hydroxymethylcytosine recognition by the SRA domain of UHRF2. *Mol Cell* 2014;54(5):879–86.
- [37] Chen T, Ueda Y, Dodge JE, Wang Z, Li E. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol Cell Biol* 2003;23(16):5594–605.
- [38] Li Y, Zhang Z, Chen J, et al. Stella safeguards the oocyte methylome by preventing de novo methylation mediated by DNMT1. *Nature* 2018;564(7734):136–40.
- [39] Haggerty C, Kretzmer H, Riemenschneider C, et al. Dnmt1 has de novo activity targeted to transposable elements. *Nat Struct Mol Biol* 2021;28(7):594–603.
- [40] Zeng Y, Chen T. DNA methylation reprogramming during mammalian development. *Genes (Basel)* 2019;10(4):257.
- [41] Dan J, Rousseau P, Hardikar S, et al. Zscan4 inhibits maintenance DNA methylation to facilitate telomere elongation in mouse embryonic stem cells. *Cell Rep* 2017;20(8):1936–49.
- [42] He YF, Li BZ, Li Z, et al. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 2011;333(6047):1303–7.
- [43] Chen B, Du YR, Zhu H, et al. Maternal inheritance of glucose intolerance via oocyte TET3 insufficiency. *Nature* 2022;605(7911):761–6.
- [44] Yang J, Bashkenova N, Zang R, Huang X, Wang J. The roles of TET family proteins in development and stem cells. *Development* 2020;147(2):dev183129.
- [45] Mellen M, Ayata P, Dewell S, Kriaucionis S, Heintz N. MeCP2 binds to 5hmC enriched within active genes and accessible chromatin in the nervous system. *Cell* 2012;151(7):1417–30.
- [46] Yildirim O, Li R, Hung JH, et al. Mbd3/NURD complex regulates expression of 5-hydroxymethylcytosine marked genes in embryonic stem cells. *Cell* 2011;147(7):1498–510.
- [47] Baymaz HI, Fournier A, Laget S, et al. MBD5 and MBD6 interact with the human PR-DUB complex through their methyl-CpG-binding domain. *Proteomics* 2014;14(19):2179–89.
- [48] Perez-Torrado R, Yamada D, Defossez PA. Born to bind: the BTB protein-protein interaction domain. *Bioessays* 2006;28(12):1194–202.
- [49] Zhang J, Zhang Y, You Q, et al. Highly enriched BEND3 prevents the premature activation of bivalent genes during differentiation. *Science* 2022;375(6584):1053–8.
- [50] Dixon G, Pan H, Yang D, et al. QSER1 protects DNA methylation valleys from de novo methylation. *Science* 2021;372(6538):eabd0875.
- [51] Bernstein BE, Mikkelsen TS, Xie X, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 2006;125(2):315–26.
- [52] Husmann D, Gozani O. Histone lysine methyltransferases in biology and disease. *Nat Struct Mol Biol* 2019;26(10):880–9.
- [53] Rea S, Eisenhaber F, O’Carroll D, et al. Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* 2000;406(6796):593–9.
- [54] Paddeken J, Methot SP, Gasser SM. Establishment of H3K9-methylated heterochromatin and its functions in tissue differentiation and maintenance. *Nat Rev Mol Cell Biol* 2022;23(9):623–40.
- [55] Saha N, Muntean AG. Insight into the multi-faceted role of the SUV family of H3K9 methyltransferases in carcinogenesis and cancer progression. *Biochim Biophys Acta Rev Cancer* 2021;1875(1):188498.
- [56] Rao RC, Dou Y. Hijacked in cancer: the KMT2 (MLL) family of methyltransferases. *Nat Rev Cancer* 2015;15(6):334–46.
- [57] Zhang C, Xu L, Zheng X, Liu S, Che F. Role of Ash1l in Tourette syndrome and other neurodevelopmental disorders. *Dev Neurobiol* 2021;81(2):79–91.

- [58] Hyun K, Jeon J, Park K, Kim J. Writing, erasing and reading histone lysine methylations. *Exp Mol Med* 2017;49(4):e324.
- [59] Tracy C, Warren JS, Szulik M, et al. The Smyd family of methyltransferases: role in cardiac and skeletal muscle physiology and pathology. *Curr Opin Physiol* 2018;1:140–52.
- [60] Yang L, Jin M, Jeong KW. Histone H3K4 methyltransferases as targets for drug-resistant cancers. *Biology (Basel)* 2021;10(7):581.
- [61] Alexandrova E, Salvati A, Pecoraro G, et al. Histone methyltransferase DOT1L as a promising epigenetic target for treatment of solid tumors. *Front Genet* 2022;13:864612.
- [62] Gabellini D, Pedrotti S. The SUV4–20H histone methyltransferases in health and disease. *Int J Mol Sci* 2022;23(9):4736.
- [63] Kim KH, Roberts CW. Targeting EZH2 in cancer. *Nat Med* 2016;22(2):128–34.
- [64] Shen X, Liu Y, Hsu YJ, et al. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. *Mol Cell* 2008;32(4):491–502.
- [65] Hidalgo I, Herrera-Merchan A, Ligos JM, et al. Ezh1 is required for hematopoietic stem cell maintenance and prevents senescence-like cell cycle arrest. *Cell Stem Cell* 2012;11(5):649–62.
- [66] Calebiro D, Grassi ES, Eszlinger M, et al. Recurrent EZH1 mutations are a second hit in autonomous thyroid adenomas. *J Clin Invest* 2016;126(9):3383–8.
- [67] Di Tullio F, Schwarz M, Zorgati H, Mzoughi S, Guccione E. The duality of PRDM proteins: epigenetic and structural perspectives. *FEBS J* 2022;289(5):1256–75.
- [68] Sorrentino A, Federico A, Rienzo M, et al. PR/SET domain family and cancer: novel insights from the Cancer Genome Atlas. *Int J Mol Sci* 2018;19(10):3250.
- [69] Metzger E, Wang S, Urban S, et al. KMT9 monomethylates histone H4 lysine 12 and controls proliferation of prostate cancer cells. *Nat Struct Mol Biol* 2019;26(5):361–71.
- [70] Shi Y, Lan F, Matson C, et al. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 2004;119(7):941–53.
- [71] Jambhekar A, Dhall A, Shi Y. Roles and regulation of histone methylation in animal development. *Nat Rev Mol Cell Biol* 2019;20(10):625–41.
- [72] Ciccone DN, Su H, Hevi S, et al. KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. *Nature* 2009;461(7262):415–18.
- [73] Walport LJ, Hopkinson RJ, Chowdhury R, et al. Arginine demethylation is catalysed by a subset of JmjC histone lysine demethylases. *Nat Commun* 2016;7:11974.
- [74] Li S, Ali S, Duan X, et al. JMJD1B demethylates H4R3me2s and H3K9me2 to facilitate gene expression for development of hematopoietic stem and progenitor cells. *Cell Rep* 2018;23(2):389–403.
- [75] El Hayek L, Tuncay IO, Nijem N, et al. KDM5A mutations identified in autism spectrum disorder using forward genetics. *Elife* 2020;9:e56883.
- [76] Li J, Zhang T, Ren T, et al. Oxygen-sensitive methylation of ULK1 is required for hypoxia-induced autophagy. *Nat Commun* 2022;13(1):1172.
- [77] Higashijima Y, Nagai N, Yamamoto M, et al. Lysine demethylase 7a regulates murine anterior-posterior development by modulating the transcription of Hox gene cluster. *Commun Biol* 2020;3(1):725.
- [78] Chen X, Wang S, Zhou Y, et al. Phf8 histone demethylase deficiency causes cognitive impairments through the mTOR pathway. *Nat Commun* 2018;9(1):114.
- [79] Okuno Y, Ohtake F, Igarashi K, et al. Epigenetic regulation of adipogenesis by PHF2 histone demethylase. *Diabetes* 2013;62(5):1426–34.
- [80] Shinkai Y, Tachibana M. H3K9 methyltransferase G9a and the related molecule GLP. *Genes Dev* 2011;25(8):781–8.
- [81] Matsui T, Leung D, Miyashita H, et al. Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* 2010;464(7290):927–31.

- [82] Tsukada Y, Fang J, Erdjument-Bromage H, et al. Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 2006;439(7078):811–16.
- [83] Whetstone JR, Nottke A, Lan F, et al. Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. *Cell* 2006;125(3):467–81.
- [84] Cornett EM, Ferry L, Defossez PA, Rothbart SB. Lysine methylation regulators moonlighting outside the epigenome. *Mol Cell* 2019;75(6):1092–101.
- [85] Baubec T, Colombo DF, Wirbelauer C, et al. Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. *Nature* 2015;520(7546):243–7.
- [86] Weinberg DN, Papillon-Cavanagh S, Chen H, et al. The histone mark H3K36me2 recruits DNMT3A and shapes the intergenic DNA methylation landscape. *Nature* 2019;573(7773):281–6.
- [87] Otani J, Nankumo T, Arita K, Inamoto S, Ariyoshi M, Shirakawa M. Structural basis for recognition of H3K4 methylation status by the DNA methyltransferase 3A ATRX-DNMT3-DNMT3L domain. *EMBO Rep* 2009;10(11):1235–41.
- [88] Xu J, Richard S. Cellular pathways influenced by protein arginine methylation: Implications for cancer. *Mol Cell* 2021;81(21):4357–68.
- [89] Gayatri S, Bedford MT. Readers of histone methylarginine marks. *Biochim Biophys Acta* 2014;1839(8):702–10.
- [90] Yang Y, Bedford MT. Protein arginine methyltransferases and cancer. *Nat Rev Cancer* 2013;13(1):37–50.
- [91] Yoshimoto T, Boehm M, Olive M, et al. The arginine methyltransferase PRMT2 binds RB and regulates E2F function. *Exp Cell Res* 2006;312(11):2040–53.
- [92] Swiercz R, Cheng D, Kim D, Bedford MT. Ribosomal protein rpS2 is hypomethylated in PRMT3-deficient mice. *J Biol Chem* 2007;282(23):16917–23.
- [93] Kim JD, Park KE, Ishida J, et al. PRMT8 as a phospholipase regulates Purkinje cell dendritic arborization and motor coordination. *Sci Adv* 2015;1(11):e1500615.
- [94] Yang Y, Hadjikyriacou A, Xia Z, et al. PRMT9 is a type II methyltransferase that methylates the splicing factor SAP145. *Nat Commun* 2015;6:6428.
- [95] Jansen S, van der Werf IM, Innes AM, et al. De novo variants in FBXO11 cause a syndromic form of intellectual disability with behavioral problems and dysmorphisms. *Eur J Hum Genet* 2019;27(5):738–46.
- [96] Schrank B, Gotz R, Gunnersen JM, et al. Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proc Natl Acad Sci USA* 1997;94(18):9920–5.
- [97] Mo HY, Choi EJ, Yoo NJ, Lee SH. Mutational alterations of TDRD 1, 4 and 9 genes in colorectal cancers. *Pathol Oncol Res* 2020;26(3):2007–8.
- [98] Miura S, Kosaka K, Nomura T, et al. TDRKH is a candidate gene for an autosomal dominant distal hereditary motor neuropathy. *Eur J Med Genet* 2019;62(12):103594.
- [99] Yang Y, McBride KM, Hensley S, Lu Y, Chedin F, Bedford MT. Arginine methylation facilitates the recruitment of TOP3B to chromatin to prevent R loop accumulation. *Mol Cell* 2014;53(3):484–97.
- [100] Morettin A, Paris G, Bouzid Y, et al. Tudor domain containing protein 3 promotes tumorigenesis and invasive capacity of breast cancer cells. *Sci Rep* 2017;7(1):5153.
- [101] Bensing S, Fetissov SO, Mulder J, et al. Pituitary autoantibodies in autoimmune polyendocrine syndrome type 1. *Proc Natl Acad Sci USA* 2007;104(3):949–54.
- [102] Shoji M, Tanaka T, Hosokawa M, et al. The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. *Dev Cell* 2009;17(6):775–87.
- [103] Wright T, Wang Y, Bedford MT. The role of the PRMT5-SND1 axis in hepatocellular carcinoma. *Epigenomes* 2021;5(1) epigenomes5010002.
- [104] Webby CJ, Wolf A, Gromak N, et al. Jmjd6 catalyses lysyl-hydroxylation of U2AF65, a protein associated with RNA splicing. *Science* 2009;325(5936):90–3.

- [105] Li Y, Chen X, Lu C. The interplay between DNA and histone methylation: molecular mechanisms and disease implications. *EMBO Rep* 2021;22(5):e51803.
- [106] Wang J, Hevi S, Kurash JK, et al. The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. *Nat Genet* 2009;41(1):125–9.
- [107] Veland N, Hardikar S, Zhong Y, et al. The arginine methyltransferase PRMT6 regulates DNA methylation and contributes to global DNA hypomethylation in cancer. *Cell Rep* 2017;21(12):3390–7.
- [108] Losman JA, Koivunen P, Kaelin Jr. WG. 2-Oxoglutarate-dependent dioxygenases in cancer. *Nat Rev Cancer* 2020;20(12):710–26.
- [109] Bhat KP, Umit Kaniskan H, Jin J, Gozani O. Epigenetics and beyond: targeting writers of protein lysine methylation to treat disease. *Nat Rev Drug Discov* 2021;20(4):265–86.
- [110] Lv YX, Tian S, Zhang ZD, Feng T, Li HQ. LSD1 inhibitors for anticancer therapy: a patent review (2017-present). *Expert Opin Ther Pat* 2022;32(9):1027–42.
- [111] Wu Q, Schapira M, Arrowsmith CH, Barsyte-Lovejoy D. Protein arginine methylation: from enigmatic functions to therapeutic targeting. *Nat Rev Drug Discov* 2021;20(7):509–30.
- [112] Pappalardi MB, Keenan K, Cockerill M, et al. Discovery of a first-in-class reversible DNMT1-selective inhibitor with improved tolerability and efficacy in acute myeloid leukemia. *Nat Cancer* 2021;2(10):1002–17.

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Oncohistones

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1 Introduction

Dimerized pairs of core histone proteins (H2A–H2B and H3–H4) form the nucleosome particle which is then wrapped by 147 bp of DNA. The repeating nucleosome cores are stabilized by the linker histone H1 to further compact linear DNA [1]. As well as canonical histones (H2A, H2B, H3, and H4), we also have noncanonical histones (core histone variants). Canonical histones are replication dependent (only expressed in the S phase) and encoded by multicity gene families whereas noncanonical histones are expressed independently of replication and are encoded by one or two independent genes [2,3]. Overall, histones are highly conserved across species, and the genes encoding the canonical histones are located in four clusters encompassing a total of 72 histone genes: 6 genes for histone H1, 16 genes for histone H2A, 22 genes for histone H2B, and 14 genes for histone H4 [4]. Histone H1 is the most diverse of all histones with at least 10 isoforms in

humans while H2A variants are considered to be the most diverse family of the core histone proteins with substantial variation in their amino- and carboxy-terminal tail regions [3]. Minor sequence differences (a few amino acid substitutions) are observed in the H2B and H3 variants while only one H4 isoform (the canonical H4) has been identified so far [3]. The incorporation of histone variants into chromatin can have either profound or relatively modest effects. However, just like canonical histones, deregulation of histone variant expression and deposition has been implicated in both developmental syndromes and cancer [5].

Prior to the discovery of oncohistones, histones had been linked to tumorigenesis mainly through alterations in posttranslational modifications (PTMs) and through the enzymes that regulate these modifications [6]. However, in January 2012, Schwartzentruber et al. [7] identified high-frequency, somatic point mutations in histone-coding genes capable of driving oncogenesis. Thus the term “oncohistones” refers to mutations in histone-coding genes capable of driving/progressing tumorigeneses [2,8]. In fact, some histone mutations have also been shown to exert a dominant, gain-of-function effect such as in the case of pediatric high-grade gliomas (pHGG) where all histone H3 mutations were heterozygous [9]. A notable feature of these mutations is that they often occur on or near residues that are highly modified with PTMs, such as H3K27 and H3K36 [9,10]. As such, any mutations in these histone residues means that the site cannot be modified by PTM enzymes resulting in changes to genome stability and gene expression [3].

In the following sections, we describe the current known “Oncohistones,” their effects on epigenetic regulation and potential role in tumorigenesis.

2 Histone H1 in tumorigenesis

H1 linker histones are encoded by ten different genes, five of which (*H1 A–E*) are expressed in a replication-dependent manner [11]. These histones act as transcriptional repressors by limiting chromatin accessibility [12] and are depleted from actively transcribed domains [13]. However, recurrent H1 mutations have been found to occur in approximately 30%–40% of diffuse large B-cell lymphomas (DLBCL), ~30% of follicular lymphomas (FL) and ~50% of Hodgkin lymphomas (HL) [11,14–18].

FL is a common, indolent B-cell lymphoma with an incidence rate of approximately 14,000 in the United States in 2016 [19]. On a genetic level, FL is characterized by a balanced chromosomal translocation t(14;18), occurring in about 90% of cases, resulting in deregulated expression of *BCL2* while other cases lack this translocation [20]. At a genomic level, FL is characterized by loss of heterozygosity (LOH) and acquired genomic copy number aberrations as well as acquired uniparental disomy/copy neutral LOH [21–23]. However, in recent years an increasing amount of attention has been turned to gene mutations in histones and their role in FL tumorigenesis.

In one study utilizing massively parallel exome sequencing and high-resolution SNP 6.0 array profiling of 114 FL cases discovered that 27% of cases carried mutations in *HIST1H1 B-E* [16]. Overall, the authors identified 42 somatically acquired mutations and 8 somatically acquired synonymous nucleotide changes in 1 of 4 *HIST1H1* genes (*HIST1H1 B-E*; *HIST1H1A* was not mutated) as well as 7 germline mutations [16]. Many of these mutations were missense with most restricted to the C-terminus of the proteins while occasional indels (insertion or deletion of bases in the

genome) and nonsense mutations were identified as well [16]. While Li et al. [16] did not explore the implications of these mutations on tumorigenesis, Okosun et al. discovered a link between H1 gene mutations and FL transformation to DLBCL. Okosun et al. [15] observed mutations affecting at least one H1 gene in 28% of their series, with *HIST1H1C* and *HIST1H1E* being the most frequently mutated. Most of these mutations were missense and clustered within the highly conserved globular domain, targeting residues directly involved in DNA binding [15]. The authors determined that these mutations most likely lead to a loss-of-function phenotype by reducing the binding affinity and residence time of these H1 subtypes in chromatin which affects chromatin compaction and specific gene regulation.

On the other hand, DLBCL is an aggressive non-Hodgkin lymphoma with an estimated incidence of 7 per 100,000 in the United States [24]. DLBCL has two distinct molecular subtypes—germinal center B cell (GCB) and activated B cell (ABC) with about 15% of patients remaining unclassifiable. These subtypes are driven by different intracellular oncogenic signaling pathways while histone modifications differ very little [25]. Whole-exome sequencing of patient samples with primary DLBCL (paired tumor and germline) revealed 59 nonsynonymous and 35 synonymous mutations among 31 histone H1 proteins in 69% patients [26]. Furthermore, additional whole exome sequencing studies have identified mutations in histone protein H1 or core protein H2 as being common in DLBCL [18,27].

While these initial studies did not explore the functional significance of these mutations, a recent study conducted by Yusufova et al. did [11]. The authors established that loss-of-function mutations of H1 drive malignant transformation in GCB-DLBCL acting primarily as a tumor suppressor. When mutated histone H1 drives malignant transformation via epigenetic reprogramming and derepression of developmentally silenced genes through 3D genome reorganization [11].

3 H2 histone mutations in tumorigenesis

Across all cancer types, 2.1% of sequenced patients (864 out of 40,317) have been found to have mutations genes encoding H2A with the most frequently mutated cancers being bladder cancer, endometrial carcinomas, head and neck carcinomas, esophagogastric adenocarcinomas and cervical squamous cell carcinomas [28]. Moreover, multiple mutations have also been identified in the histone genes *HIST1H2AC* and *HIST1H2AM* in FL [16], and in *HIST2H2BE* in DLBCL [18]. The most frequently mutated amino-acid residue is glutamate 121 to lysine (E121K) [28]. However, the implications of these mutations on tumorigenesis have yet to be elucidated.

H2A.Z is a highly conserved histone H2A variant that shares 60% similarity with the canonical histone [29]. This variant has two isoforms, H2A.Z.1 and H2A.Z.2, encoded by *H2AZ1* and *H2AZ2* genes and regulated by independent promoters [30]. Of these, the H2A.Z.1 isoform has been found to have a cancer-associated arginine 80 to cysteine (R80C) mutation which causes the destabilization of the nucleosome while the colony formation ability of these cells is slightly enhanced [31].

Histone H2B on the other hand is frequently mutated and roughly 3% of sequenced patient samples contained a mutation in *H2B* with endometrial carcinomas being the most frequently mutated [28]. The authors identified H2B glutamate 76 to lysine (H2B-E76K) as the most commonly

occurring missense mutation, the occurrence of which often coincides with gain-of-function mutations of oncogenes, such as *RAS* and *PIK3CA* [28], and more recent estimates suggest that this mutation occurs in 0.105% of cancer patients overall [32]. While the H2B-E76K mutation is not a driver mutation, it does contribute to the progression of cancer by inhibiting histone octamer formation by destabilizing H2B interactions with H4 [28,32]. Indeed, biochemical analyses revealed that the H2B-E76K mutation reduces the interaction between H2A–H2B and H3–H4 resulting in a destabilized nucleosome [31], and in the absence of DNA, H2A-H2BE76K dimers cannot be assembled onto the H3–H4 tetramer to form an octamer [28,31,32]. However, this mutant is effectively incorporated into chromatin in cells, and the nucleosomal instability induced by the H2B-E76K incorporation contributes to the oncogenic transformation of cells [31].

Moreover, H2B E76K mutations have been shown to have a significant effect on the transcriptome and cellular phenotypes of breast cancer cells [33]. In spite of the destabilizing effects of the mutation, both nucleosomal occupancy and chromatin accessibility appeared unaltered in H2B E76K-enriched regions [33]. However, the study did observe a positive correlation between H2B E76K enrichment and elevated gene expression. The relationship between H2B E76K and transcription showed that polymerase II (Pol II) progressed more efficiently on H2B E76K-enriched *ADAM19* [33]. The authors proposed a model in which the H2B E76K mutant histone preferentially localizes to *ADAM19*, subsequently promoting transcription by facilitating H2A–H2B dimers displacement/exchange during Pol II progression [32,33]. This study determined that the H2B E76K-enriched gene *ADAM19* is indeed upregulated H2B E76K mutant cells and contributed to their enhanced colony formation ability, an effect that was reversed upon *ADAM19* knockdown [33].

Histone H2B glycine 53 to aspartic acid (G53D) missense mutations have also been found to occur in 6.8% (10 out of 146) pancreatic ductal adenocarcinoma (PDAC) patients [34]. Of note, this mutation was also found in glioblastoma multiforme (1%) and lung squamous cell carcinoma (1.1%) [32]. This is a novel cancer-promoting mutation capable of enhancing transcription elongation *in vitro* by weakening the interaction between nucleosomal DNA and the histone octamer [34]. As well as affecting DNA-histone octamer interactions, H2BG53D also elevates the expression of cancer associated genes and leads to enhanced oncogenic properties in PDAC [32,35].

A number of other histone H2 mutations have been described [32], and while the effects of histone H2 mutations on nucleosomal and chromatin remodeling has been recently reviewed [36] their functional relevance in carcinogenesis has yet to be elucidated. However, a recent report has shown that the oncohistone mutation in H2BD51 is at a key site for ADB-ribosylation by PARP-1 in wild-type histones, but which cannot be ribosylated when mutated. Functionally, if mutated this results in the inhibition of p300-mediated lysine acetylation of H2B, coupled with significant alterations to chromatin accessibility at gene regulator elements (promoters and enhancers). Furthermore, while this mutation was found to enhance tumor growth in a xenograft model, it did result in any enhanced resistance to standard PARPi [37].

4 H3 histones in tumorigenesis (including histone H3 variants)

Initially, mutations in the genes encoding histone H3 were discovered in pediatric gliomas [7,38], an association that has since been confirmed by many other studies [39–42]. In fact, 19.3% of

high-grade gliomas have been found to have mutations in histone H3 [28]. However, the list of cancers known to carry H3 mutations has progressively lengthened to include other cancers, such as bladder cancer, esophageal squamous cell carcinoma, endometrial carcinoma, nonmelanoma skin cancers, chondrosarcoma, osteosarcoma, and acute myeloid leukemia [43–49]. Intriguingly, in cancer only 1 of the 30 known alleles of histone H3 is mutated [50].

Many mutations of histone H3 have been identified and while the most studied mutations, such as K27M, K36M, and various G34W (discussed in greater detail below), localize to the N-terminal domain, there are several residues in the globular domain, such as E97K, E105K and R131C that have been found to be mutated at similar or even higher rates [44]. Indeed, the most frequently mutated amino-acid residue in the canonical histone *H3* genes is E105 [28]. Also of note, although few mutations have been cataloged for the centrosome specific H3 histone CENPA, two mutations, R69C/L and R130Q/W/L, were among the most frequent [28].

5 K27M

The histone H3 lysine 27 to methionine (K27M) mutation is of particular interest as it is the only histone mutation recognized by the World Health Organization as a marker for tumor classification [51]. The *H3F3A* gene that encodes H3.3 uses the sequence AAG to encode lysine at position 27 but this sequence is frequently mutated to ATG (K→M) in cancers [10,28,44]. Although more than half of the genes encoding canonical histone H3.1 and H3.2 use AAG to encode K27, the K27M missense mutation is not observed frequently among these proteins, suggesting that the significance of the H3K27M mutation may be specific to histone H3.3 function [28]. However, unlike many other histone mutations, this mutation does not appear to affect the structure of the nucleosome [31].

Pediatric high-grade gliomas (pHGGs) are epigenetically dysregulated tumors with a dismal prognosis [52,53]. Glioblastoma multiforme (GBM) and diffuse midline glioma (DMG, formerly known as diffuse intrinsic pontine glioma) are a subset of pHGGs found to harbor mutations in genes encoding the histone H3.3. In these gliomas, mutations in histone H3.3 were much more prevalent in pediatric settings with 19%–31% of GBM samples and 60% of DMG samples containing recurrent K27M mutation in *H3F3A* [7,38]. As well as this, DMG can also contain mutations in histone H3.1 encoding *HIST1H3B* and *HIST1H3C* [38,54]. The H3 K27M mutations in DMGs were found to also be associated with mutations in TP53, PDGFRA, ACVR1 and BCOR [55]. Moreover, the two histone H3 variants were found to drive two distinct oncogenic programs potentially having specific therapeutic targets [54], and to have prognostic value in both children and young adults [56].

pHGGs with the K27M mutation display lower amounts of H3K27 di- and trimethylation (H3K27me2 and H3K27me3) with an increase in acetylated H3K27 (H3K27ac) and DNA hypomethylation [57–62]. The reduction in the repressive histone mark H3K27me3, and consequently the loss in gene silencing, is caused by K27M acting as a dominant-negative inhibitor of EZH2, a writer subunit of the Polycomb repressive complex 2 (PCR2) which is a necessary complex in H3K27 methylation [57–60]. The inhibition of H3K27me3 only occurs when H3K27M oncohistones are deposited into chromatin and then only when expressed in cycling cells [63]. This suggests that oncohistones inhibit EZH2 as chromatin patterns are being duplicated in proliferating cells,

predisposing them to tumorigenesis [63]. The reduction in H3K27me3 levels also occurs within superenhancers, which subsequently exhibit perturbed transcriptional function [64]. Fang et al. [65] demonstrated that H3.3K27M mutant proteins sequestered the PRC2 complex at “transcriptionally poised” promoters causing epigenetic reprogramming, and resulting in epigenetic silencing of specific tumor suppressors, such as Wilms Tumor 1 (WT-1), in the tumorigenic process [65]. This potential link with “poised” promoters was further shown in a study demonstrated that the loss of H3K27me3 associated with this mutation regulates a subset of differentiation genes through which it could contribute to tumor phenotype and growth [66]. In addition to this subset of differentiation genes, this mutation has been shown to repress the p16/ink4a (CDKN2A) locus encoding two additional important tumor suppressors resulting in accelerated tumor growth [67]. Furthermore, knockdown of the K27M mutation in DMG xenografts restored K27M-dependent loss of H3K27me3 and resulted in delayed tumor growth [66]. Most recently, a study on the effect of the K27M mutation on combinatorial epigenetic patterns found that the H3K27M mutation was associated not only with preferential binding of the PRC2 complex but also found a direct interaction with MLL1, leading to genome-wide redistribution of H3K4me3. This mutant-mediated deregulation results in unbalanced “bivalent” chromatin, potentially leading to a poorly differentiated cellular state in these tumors [68].

The histone variant H3.3 K27M (H3.3K27M) mutation is a defining characteristic of diffuse intrinsic pontine glioma (DIPG)/diffuse midline glioma (DMG). Oligodendrocyte progenitor cells (OPCs) in the brainstem are considered to be candidate cells-of-origin for DMG. An animal study of DMG which compared two progenitor cells (Olig2-expressing versus Nestin-expressing) determined that the tumorigenic effects of H3.3K27M are cell-of-origin dependent, with H3.3K27M being more oncogenic in Nestin + cells than in Olig2 + cells [69], and could potentially reflect the situation previously observed where expression of pre-EMT genes was increased in the H3K27M tumors as compared to non-K27M tumors [70]. Using CRISPR to generate isogenic glioma cell lines which were either wild type or H3.3K27M, Lewis et al. [71] found that H3.3K27M cells have unique accessible chromatin at regions corresponding to neurogenesis, NOTCH, and neuronal development pathways with corresponding gene overexpression. Of these, ASCL1 and NEUROD1 were identified as key altered neurodevelopmental factors, and it is interesting to note that these factors have been associated with EMT and drug resistance in another cancer with neuroendocrine features small cell lung cancer (SCLC) [72].

In addition to these identified oncogenic pathways, two recent studies have shown that H3.3K27M mutation are associated with genomic instability through derepression of transposable elements and an increase in mitotic abnormalities [73,74].

6 K36M

Recurrent histone H3 lysine 36 to methionine (H3K36M) or isoleucine (H3K36I) mutations have been identified in chondroblastomas, undifferentiated sarcomas, head and neck squamous cell carcinomas and pediatric gliomas [45,49,75–78]. Indeed, in 95% of chondroblastomas, K36M mutations were identified mainly in *H3F3B* with one mutation identified in *H3F3A* [45]. Due to the prevalence of this mutation in chondroblastomas, many studies to date have used chondroblastomas as a model to investigate the mechanisms and effects of this mutation on oncogenesis.

Histone H3K36 mutations drive oncogenesis by deregulating epigenetic mechanisms. Specifically, the presence of the K36M mutation leads to a marked decrease in H3K36 di- and trimethylation (H3K36me2/3) while an increase in H3K27me3 is observed in intergenic regions previously devoid of K27 methylation [43]. Indeed, this study demonstrates that H3K36M is a potent inhibitor of SETD2 and NSD2, methyltransferases that catalyze the methylation of H3K36. The mutant histone affects the binding and inhibits the catalytic activity of these methyltransferases by altering the hydrophobic pocket responsible for methylation [79,80]. Cryo-Electron Microscopy (Cryo-EM) of SETD2 bound at the nucleosomal level showed that SETD2 was strongly attached to nucleosomes with the H3.3K36M mutation but not to wild-type nucleosomes suggesting that under normal circumstances SETD2 is rapidly released from wild-type nucleosomes during transcriptional elongation, but if mutated, the oncogenic H3.3K36M binds and impairs SETD2 release which may affect the movement of RNA polymerase II during this process [81].

Furthermore, it has also been noted that the activity of the PRC1 complex that binds to H3K27me3 and suppresses gene expression spreads to these intergenic regions and a model has been postulated whereby “dilution” of the PRC1 complex away from repressed gene loci leads to ectopic gene expression contributing to the differentiation blockade by H3.3 K36M [43].

Mutated H3.3K36M has also been associated with the derepression of transposable elements which could potentially lead to chromosomal instability [74]. Additional research which compared H3K36 methylation in chondroblastoma cells which were either mutant for H3.3K36M or H3.1K36M found that each mutation altered different subsets of genes [82], with H3K36me2 levels enriched at gene bodies, as well as intergenic regions, while H3K36me3 was enriched at gene bodies only. In H3.3K36M mutant cells, H3K27me3 is significantly increased in the intergenic regions, with over 50% of the H3K27me3 peaks being in unique regions, in contrast to H3.1K36M mutant cells, where less than 5% of the H3K27me3 peaks were unique [82]. Because H3K27me3 in intergenic regions is correlated to the activation of enhancers, further analysis identified that both H3.3K36M and H3.1K36M mutant proteins inactivate different enhancers associated with their respective genomes [82], resulting in tumorigenic effects for H3.3K36M and H3.1K36M that links reprogramming of enhancer landscapes in a cell context-dependent manner [82,83]. In this regard, the cellular context is important as the H3.3K27M mutation will induce apoptosis in differentiated cells whereas its expression will promote proliferation in embryonic stem cells [84]. Moreover, the timing of mutant protein expression appears to be critical with respect to tumor occurrence [82,85,86]. In a separate development, Hawkins and colleagues have demonstrated in a transgenic mouse expressing H3.3K27M in diverse progenitor cell populations, that a key pathway early event in H3.3K27M driven tumorigenesis is the activation of the RAS/MYC axis, which can be targeted directly using Omomyc, a peptide-based dominant negative MYC inhibitor, or indirectly by using MEK or AKT inhibitors, such as cobimetinib (MEK inhibitor) or A443654 (pan-AKT inhibitor) [87].

7 G34

In 92% of giant cell tumors of bone (GCTB), glycine 34 to tryptophan (G34W) as well as one glycine 34 to leucine (G34L) mutation have been discovered exclusively in *H3F3A* [45]. A further

14% of nonbrainstem pediatric glioblastoma cases were found to have a glycine 34 to arginine (G34R) mutation in *H3F3A* as well [38,78]. Residue G34 mutations are capable oncogenic drivers. Indeed, knock-down of the G34W mutation *in vitro* caused a significant decrease in cell proliferation, migration and colony formation capacity in GCTB while removal of G34W *in vivo* prevented tumor formation [88,89]. It is likely that G34 mutants drive tumorigenesis as a consequence of a global epigenetic remodeling process initiated by the *in cis* effects of this mutation in the stromal cell of origin [89].

While posttranslational modifications (PTMs) do not occur on the G34 residue, G34 is in close proximity to sites where PTMs, such as methylation, frequently occur. In fact, when located on the same histone tail, mutations at G34 (G34W/L) result in a decrease in H3K36me3 levels with a concomitant redistribution of H3K27me3 from intergenic to active genic regions normally enriched for H3.3 as well as G34W [89,90]. In intergenic regions, H3K27me3 loss is replaced by H3K36me2 or H3K9me3 in areas vacated by PRC2 [89]. The reduction in genic H3K36me3 levels is the result of mutant G34-induced steric hindrance of the catalytic groove of SETD2 [80]. By blocking SETD2-mediated H3K36 methylation at active enhancers, the G34 mutant promotes aberrant PRC2-mediated redistribution of H3K27me2/3 on nucleosomes [91]. In intergenic regions, H3K27me3 loss is replaced by H3K36me2 or H3K9me3 in areas vacated by PRC2 [89]. In addition, G34-mutated oncohistones show a net gain of H3K27me3 levels [59,90,92]. Furthermore, G34 mutations have been suggested to impede H3.3K36 access by causing the complete loss of KDM2A, a H3K36me2 demethylase [93] as well as inhibiting members of the KDM4 H3K9/K36 demethylase family, resulting in increased H3K36me3 and H3K9me3 at select loci [94]. The G34W mutation has also recently been shown to have a gain-of-function effect on the H3K36me2 methyltransferase NSD1 [95].

Two recent studies utilizing stem cell models to study the role of histone H3G34R in gliomagenesis have shown that while the G34R mutation is not strongly oncogenic by itself, additional mutations of key drivers were required for tumorigenesis [96,97] and identified that amplification of PDGFRA [97], loss or inactivation of p53 [96,97] or mutation of ATRX [96] were necessary for oncogenesis [96–98]. In support of these observations, altered expression of PDGFRA has previously been linked to H3G34R in gliomagenesis [99], while alterations to p53 and ATRX have well established roles in gliomagenesis [100]. Intriguingly, ATRX-deficient gliomas have been shown to be sensitive to PDGFR inhibitors [101] raising the possibility that patients with the H3G34R mutation may be suitable for therapy with PDGFRi.

The two stem cell studies also identified alterations to gene splicing involving the key splicing factor ZMYND11 [96,97]. In this regard, Funato et al. [96] demonstrated that upregulated alternative splicing events were enriched for exon skipping and reduced intron retention, consistent with previous observations for both G34R and ZMYND11 playing roles in these areas [92,102–104].

Functionally, this led to enhanced expression of members of the Notch signaling pathway in particular identifying NOTCH2NL as a key upregulated member and functionally demonstrating its role in gliomagenesis [96].

In a similar vein an earlier study identified that the G34R-mutated histones also interacted with ZMYND8 (RACK7) resulting in the suppression of transcription of CIITA, the master regulator of MHC (major histocompatibility complex) class II molecules, and concomitant suppression of MHC class II molecule expression and transport [105]. However, a role for ZMYND8 in RNA splicing has yet to be described [106].

8 H4 histones in tumorigenesis

Histone H4 mutations have been identified in various cancers, such as endometrial carcinomas, bladder urothelial carcinomas, head and neck squamous cell carcinomas, esophagogastric carcinomas, as well as colorectal carcinomas [28]. Furthermore, a mutation of unknown significance in *HIST1H4* has been identified in FL [16]. Overall, 1.7% of samples contained mutations in 14 canonical histone H4 paralogs while the most frequently observed mutation across these paralogs was arginine to cysteine at position 3 (H4 R3C) [28] followed by L49F, S1C, and K79N (10). While the impact of H4 mutations on oncogenesis has yet to be elucidated, there are studies to suggest that H4 mutations can cause developmental syndromes as well as have an impact on cellular functions, such as nucleotide excision repair and chromatin compaction [107–110].

While little is known of the mechanisms by which H4 mutations could affect oncogenesis, some possible mechanisms have been proposed based on existing knowledge of the functional roles of amino acid residues, both mutant and wild-type. For example, similarly to H2B E76K, mutations in H4 residues R92 and D68 have been postulated to affect oncogenesis by destabilizing nucleosomes as the H2B residue E76 engages with H4 residues R92 and D68 in a hydrogen bonding network [10].

N-terminal acetyltransferases (NATs) are a highly conserved acetyltransferases that play significant roles in N-terminal acetylation of proteins. Of these NatD has been identified as a family member which selectively targets histones H4 and H2A [111]. Functionally, a role for NatD has been described in lung cancer, where it is both upregulated and its activity has been shown to antagonize histone H4 serine 1 phosphorylation, and it functions as a crucial epigenetic regulator of cell invasion [112]. More recently the effect of oncohistone mutations in histone H2 and H4 (the specific substrates of NatD) has been investigated, and have shown that the oncohistone mutations at Ser1 and Gly2 of H4 and H2A strongly decreased by over 100-fold the catalytic efficiency and substrate recognition by NatD [113], and further examined the functional crosstalk between local PTMs of histones and oncohistones, where phosphorylation of H4S1 suppresses N α -acetylation by over 100-fold, while di-methylation of H4R3 lead to a 10–20 fold decrease in the turnover number of NatD activity, while G4S and H4R3me2s demonstrated the strongest inhibition on NatD-catalyzed acetylation [113].

9 Oncohistone mimics

EZHIP is a protein that has very restricted expression limited only to the testes, ovaries and embryo-surrounding tissues of placental mammals, and is known to functionally interact with the PRC2 complex and inhibit its function [78,114,115]. Two papers linked high expression of EZHIP with childhood posterior fossa group A ependymomas (PFAs) [116,117]. Critically, Jain et al. found that a region of EZHIP comprising a stretch of 12 amino acids is highly similar to the sequence that contains the methionine 27 residues in oncohistone H3K27M and suggested that this protein functions as an oncohistone mimic [117,118]. Functional studies of EZHIP via multiple approaches (metabolic analyses, single-cell RNA sequencing and noninvasive metabolic imaging of patients) identified alterations to both the glycolytic pathway and the tricarboxylic acid cycle, and further showed that metformin could effectively be used to lower EZHIP levels and demonstrate

potential therapeutic efficacy in patient derived xenografts [119], but other potential therapeutic avenues, such as PARP inhibitors, have recently been linked to EZHIP [114].

10 Can we target oncohistones effectively?

Given the fact that many oncohistones exhibit a superdominant behavior whereby small proportions of mutated histones exert significant effects in the presence of excess wild-type histones [2], the potential to either specifically target oncohistones or identify therapeutic opportunities for individualizing cancer therapy based on oncohistone mutation status is becoming increasingly more likely. A comprehensive functional analysis of oncohistones utilizing a DNA barcoded mononucleosome library and a humanized yeast library has been developed which may assist in this process [120].

11 Targeting oncohistone-altered pathways

As mentioned in previous sections several potential therapeutic possibilities have already been identified (e.g., metformin, PDGFRi, MEK inhibitors), suggesting that as our knowledge increases with respect to the pathways associated with oncohistone, that at the very least patients could be stratified for individualized therapy and in the following section we shall explore these and other possibilities.

One area that continues to be linked to oncohistones involves cellular metabolism, and a recent publication has identified decreased expression of MAT2A as a key element in H3K27M-mutated DMG gliomas. The decreased expression of MAT2A is caused by compromised methionine metabolism and feeds into a negative feedback loop induced by the metabolite decarboxylated S-adenosyl methionine. The net effect is to potentially render patient's sensitive to methionine-restricted diets, which was confirmed in various *in vivo* models [121]. Another study using an *in vitro* chemical screening approach identified STAT3 inhibitors as having strong cytotoxic activity. Detailed analysis identified that one form of activated STAT3, phospho-tyrosine-705 STAT3 (pSTAT3 (Y705)), was selectively upregulated in H3K27M-mutant cell lines and this was subsequently validated in clinical specimens both in patient samples and in circulating plasma extracellular vesicles taken from patients with H3K27M-mutant DMG. Using WP1066, a STAT3 pathway inhibitor currently in clinical use for pediatric brain tumors, the authors treated patient-derived intracranial xenografts and observed increased OS and a stasis of tumor growth [122]. The ability to detect monitor pSTAT3(Y705) in patient blood potentially paves the way for its utility as blood based biomarker to monitor for patient response to therapy.

The angiogenesis inhibitor Bevacizumab has also proven to have some effect in H3K27M-mutated spinal cord DMGs, and while its mode of action may not directly target the tumors, it may alleviate edema in these patients [123]. Another off-target therapeutic opportunity has been identified in DMGs whereby inhibition of EZH2 with GSK126, was found to potentiate sensitivity to cholesterol metabolism inhibitors (statins). In this regard, in an animal model noncytotoxic low-doses of both GSK126 and Atorvastatin were found to synergize and showed significant growth inhibitory effects, whereas single treatments were ineffective [124]. Interestingly, in a chick embryo

chorioallantoic membrane model, combined low-dose therapy showed an antiangiogenic effect, which was not seen for individual treatments [124], and provides further support for the potential use of antiangiogenic agents, such as bevacizumab, in the treatment option for DMG.

Imipridone (ONC201) is a small-molecule inhibitor that selectively acts as an antagonist of the Dopamine Receptor D₂ (DRD2), and in early clinical trials, it was found to have potential activity against H3.3K27M-mutated gliomas [125]. How this drug was eliciting its effects has taken some time but an initial observation suggesting that this molecule targeted the mitochondrial serine protease caseinolytic protease proteolytic subunit (ClpP) [126] and more recently confirmed in a separate study [127]. ClpP (mRNA and protein) expression is significantly ($P < 0.01$) associated with glioma grade [127], and it has been hypothesized that gliomas may be particularly sensitive to ClpP activation due to its “over-expression, their sensitivity to the downstream metabolic effects, or perhaps some degree of both” [125]. Combining ONC201 or ONC206 with other agents may also yield synergistic activities similar to that seen for GSK126 and statins, and indeed a recent study by Borsuk et al. [128] found that combinatorial therapies of histone deacetylase inhibitors synergized with imipridones to elicit synergistic cell death which may have clinical trial potential in H3K27M-mutated glioma.

Finally, a study from a phase I clinical trial of ONC201 in H3K27M-mutated DMG (NCT03416530) examined whether levels of H3K27M in circulating free-DNA (cfDNA) measured as a variant allele fraction (VAF) had any utility as a candidate biomarker to supplement radiographic monitoring of patient response. The results of this study found that changes in H3.3K27M VAF over time correlated with prolonged progression-free survival (PFS) and suggest additional clinical utility in terms of monitoring patient response and/or to differentiate between possible pseudo-progression and pseudo-response [129].

Despite the identification of such candidate therapies, it will be particularly important to identify in detail why these agents have preferential activity against oncohistone-mutated cancers.

12 Car-T-mediated targeting of oncohistone mutated cancer

H3K27M-mutated gliomas have been shown to overexpress a cell surface antigen disialoganglioside GD2 [130]. Subsequently, a CAR-T-based approach was used by these authors to demonstrate that these tumors could be effectively targeted both *in vitro* and *in vivo* [130] and led to the initiation of a first-in-human phase I clinical trial (NCT04196413). The clinical outcomes from the first four patients treated in this trial have recently been presented, and while patients developed symptoms of on-tumor neurotoxicity, they did not manifest any signs or symptoms of on-target, off-tumor toxicity [131], and three of four patients derived radiographic and clinical benefit [131].

13 Role of crispr in targeting oncohists?

Is there a role for CRISPR/Cas gene editing in targeting oncohists? Genome editing holds great potential for cancer treatment due to its ability to precisely inactivate or repair cancer-related genes, but significant challenges remain particularly in the area of precise delivery of CRISPR/Cas to solid

tumors for efficient cancer therapy [132]. Several studies have utilized CRISPR to examine the functional roles of oncohistones themselves [33,73,133–135], but to our knowledge any use of this technology to specifically target oncohistone-mutated cancers has yet to be demonstrated.

14 Conclusions

A large body of evidence now seems to suggest that the “histone mutational landscape converges at critical functional and structural interfaces, raising the possibility that disparate histone mutations may function by shared mechanisms” [120]. This may lead to the identification of common target pathways (e.g., metabolism which appears to be a common feature affected is several oncohistone studies). In this regard, as our knowledge increases concerning the mechanisms/pathways/targets affected by oncohistones, it may be possible to consider these as similar to actionable oncogenic driver mutations, such as KRAS [136], or other actionable driver mutations [137], and target these accordingly [92]. In addition, potentially either cfDNA/cfRNA of oncohistone-mutated DNA [129], or targets affected by oncohistones could also be utilized as companion biomarker(s) for either monitoring treatment outcome, or stratifying patients to appropriate therapy [138]. Nevertheless, a full understanding of the biological changes elicited in tumors by oncohistones will be required to develop personalized therapeutic regimens for treating patients with oncohistone mutations effectively and is exemplified by the development of effective CAR-T-derived therapy to treat H3K27M-mutated gliomas [131].

References

- [1] Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 1997;389(6648):251–60.
- [2] Flaus A, Downs JA, Owen-Hughes T. Histone isoforms and the oncohistone code. *Curr Opin Genet Dev* 2021;67:61–6.
- [3] Maze I, Noh KM, Soshnev AA, Allis CD. Every amino acid matters: essential contributions of histone variants to mammalian development and disease. *Nat Rev Genet* 2014;15(4):259–71.
- [4] Singh R, Bassett E, Chakravarti A, Parthun MR. Replication-dependent histone isoforms: a new source of complexity in chromatin structure and function. *Nucleic Acids Res* 2018;46(17):8665–78.
- [5] Martire S, Banaszynski LA. The roles of histone variants in fine-tuning chromatin organization and function. *Nat Rev Mol Cell Biol* 2020;21(9):522–41.
- [6] Yuen BT, Knoepfler PS. Histone H3.3 mutations: a variant path to cancer. *Cancer Cell* 2013;24(5):567–74.
- [7] Schwartzenbuber J, Korshunov A, Liu XY, et al. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature* 2012;482(7384):226–31.
- [8] Qiu L, Hu X, Jing Q, Zeng X, Chan KM, Han J. Mechanism of cancer: oncohistones in action. *J Genet Genomics* 2018;45(5):227–36.
- [9] Diaz AK, Baker SJ. The genetic signatures of pediatric high-grade glioma: no longer a one-act play. *Semin Radiat Oncol* 2014;24(4):240–7.
- [10] Nacev BA, Feng L, Bagert JD, et al. The expanding landscape of ‘oncohistone’ mutations in human cancers. *Nature* 2019;567(7749):473–8.

- [11] Yusufova N, Kloetgen A, Teater M, et al. Histone H1 loss drives lymphoma by disrupting 3D chromatin architecture. *Nature* 2021;589(7841):299–305.
- [12] Fan Y, Nikitina T, Zhao J, et al. Histone H1 depletion in mammals alters global chromatin structure but causes specific changes in gene regulation. *Cell* 2005;123(7):1199–212.
- [13] Cao K, Lailler N, Zhang Y, et al. High-resolution mapping of h1 linker histone variants in embryonic stem cells. *PLoS Genet* 2013;9(4):e1003417.
- [14] Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature* 2011;476(7360):298–303.
- [15] Okosun J, Bödör C, Wang J, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. *Nat Genet* 2014;46(2):176–81.
- [16] Li H, Kaminski MS, Li Y, et al. Mutations in linker histone genes HIST1H1 B, C, D, and E; OCT2 (POU2F2); IRF8; and ARID1A underlying the pathogenesis of follicular lymphoma. *Blood* 2014;123(10):1487–98.
- [17] Soshnev AA, Allis CD, Cesarman E, Melnick AM. Histone H1 mutations in lymphoma: a link(er) between chromatin organization, developmental reprogramming, and cancer. *Cancer Res* 2021;81(24):6061–70.
- [18] Chapuy B, Stewart C, Dunford AJ, et al. Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes. *Nat Med* 2018;24(5):679–90.
- [19] Cerhan JR. Epidemiology of follicular lymphoma. *Hematol Oncol Clin North Am* 2020;34(4):631–46.
- [20] Leich E, Ott G, Rosenwald A. Pathology, pathogenesis and molecular genetics of follicular NHL. *Best Pract Res Clin Haematol* 2011;24(2):95–109.
- [21] Cheung KJ, Delaney A, Ben-Neriah S, et al. High resolution analysis of follicular lymphoma genomes reveals somatic recurrent sites of copy-neutral loss of heterozygosity and copy number alterations that target single genes. *Genes Chromosomes Cancer* 2010;49(8):669–81.
- [22] Fitzgibbon J, Iqbal S, Davies A, et al. Genome-wide detection of recurring sites of uniparental disomy in follicular and transformed follicular lymphoma. *Leukemia* 2007;21(7):1514–20.
- [23] Ross CW, Ouillette PD, Saddler CM, Shedd KA, Malek SN. Comprehensive analysis of copy number and allele status identifies multiple chromosome defects underlying follicular lymphoma pathogenesis. *Clin Cancer Res* 2007;13(16):4777–85.
- [24] Horvat M, Zadnik V, Južnič Šetina T, et al. Diffuse large B-cell lymphoma: 10 years' real-world clinical experience with rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisolone. *Oncol Lett* 2018;15(3):3602–9.
- [25] Danilov AV, Magagnoli M, Matasar MJ. Translating the biology of diffuse large B-cell lymphoma into treatment. *Oncologist* 2022;27(1):57–66.
- [26] Lohr JG, Stojanov P, Lawrence MS, et al. Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. *Proc Natl Acad Sci U S A* 2012;109(10):3879–84.
- [27] Morin RD, Mungall K, Pleasance E, et al. Mutational and structural analysis of diffuse large B-cell lymphoma using whole-genome sequencing. *Blood* 2013;122(7):1256–65.
- [28] Bennett RL, Bele A, Small EC, et al. A mutation in histone H2B represents a new class of oncogenic driver. *Cancer Discov* 2019;9(10):1438–51.
- [29] Zlatanova J, Thakar A. H2A.Z: view from the top. *Structure* 2008;16(2):166–79.
- [30] Matsuda R, Hori T, Kitamura H, Takeuchi K, Fukagawa T, Harata M. Identification and characterization of the two isoforms of the vertebrate H2A.Z histone variant. *Nucleic Acids Res* 2010;38(13):4263–73.
- [31] Arimura Y, Ikura M, Fujita R, et al. Cancer-associated mutations of histones H2B, H3.1 and H2A.Z.1 affect the structure and stability of the nucleosome. *Nucleic Acids Res* 2018;46(19):10007–18.
- [32] Wan YCE, Chan KM. Histone H2B mutations in cancer. *Biomedicines* 2021;9(6).
- [33] Kang TZE, Zhu L, Yang D, et al. The elevated transcription of ADAM19 by the oncohistone H2BE76K contributes to oncogenic properties in breast cancer. *J Biol Chem* 2021;296:100374.

- [34] Wan YCE, Leung TCS, Ding D, et al. Cancer-associated histone mutation H2BG53D disrupts DNA-histone octamer interaction and promotes oncogenic phenotypes. *Signal Transduct Target Ther* 2020;5(1):27.
- [35] Wan YCE, Liu J, Zhu L, et al. The H2BG53D oncohistone directly upregulates ANXA3 transcription and enhances cell migration in pancreatic ductal adenocarcinoma. *Signal Transduct Target Ther* 2020;5(1):106.
- [36] Dao HT, Pham LTD. Acidic patch histone mutations and their effects on nucleosome remodeling. *Biochem Soc Trans* 2022;50(2):907–19.
- [37] Huang D, Camacho CV, Martire S, et al. Oncohistone mutations occur at functional sites of regulatory ADP-ribosylation. *Cancer Res* 2022.
- [38] Wu G, Broniscer A, McEachron TA, et al. Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. *Nat Genet* 2012;44(3):251–3.
- [39] Buczkowicz P, Hoeman C, Rakopoulos P, et al. Genomic analysis of diffuse intrinsic pontine gliomas identifies three molecular subgroups and recurrent activating ACVR1 mutations. *Nat Genet* 2014;46(5):451–6.
- [40] Fontebasso AM, Papillon-Cavanagh S, Schwartzenbuber J, et al. Recurrent somatic mutations in ACVR1 in pediatric midline high-grade astrocytoma. *Nat Genet* 2014;46(5):462–6.
- [41] Morgan MA, Shilatifard A. Medicine. (Poly)combing the pediatric cancer genome for answers. *Science* 2013;340(6134):823–4.
- [42] Wu G, Diaz AK, Paugh BS, et al. The genomic landscape of diffuse intrinsic pontine glioma and pediatric non-brainstem high-grade glioma. *Nat Genet* 2014;46(5):444–50.
- [43] Lu C, Jain SU, Hoelper D, et al. Histone H3K36 mutations promote sarcomagenesis through altered histone methylation landscape. *Science* 2016;352(6287):844–9.
- [44] Amatori S, Tavolaro S, Gambardella S, Fanelli M. The dark side of histones: genomic organization and role of oncohistones in cancer. *Clin Epigenetics* 2021;13(1):71.
- [45] Behjati S, Tarpey PS, Presneau N, et al. Distinct H3F3A and H3F3B driver mutations define chondroblastoma and giant cell tumor of bone. *Nat Genet* 2013;45(12):1479–82.
- [46] Gessi M, Capper D, Sahm F, et al. Evidence of H3 K27M mutations in posterior fossa ependymomas. *Acta Neuropathol* 2016;132(4):635–7.
- [47] Kleinschmidt-DeMasters BK, Donson A, Foreman NK, Dorris K. H3 K27M mutation in gangliogliomas can be associated with poor prognosis. *Brain Pathol* 2017;27(6):846–50.
- [48] Lehnertz B, Zhang YW, Boivin I, et al. H3(K27M/I) mutations promote context-dependent transformation in acute myeloid leukemia with RUNX1 alterations. *Blood* 2017;130(20):2204–14.
- [49] Papillon-Cavanagh S, Lu C, Gayden T, et al. Impaired H3K36 methylation defines a subset of head and neck squamous cell carcinomas. *Nat Genet* 2017;49(2):180–5.
- [50] Lowe BR, Yadav RK, Henry RA, et al. Surprising phenotypic diversity of cancer-associated mutations of Gly 34 in the histone H3 tail. *Elife* 2021;10.
- [51] Louis DN, Perry A, Wesseling P, et al. The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. *Neuro Oncol* 2021;23(8):1231–51.
- [52] Vuong HG, Le HT, Ngo TNM, et al. H3K27M-mutant diffuse midline gliomas should be further molecularly stratified: an integrated analysis of 669 patients. *J Neurooncol* 2021;155(3):225–34.
- [53] Metselaar DS, du Chatinier A, Stuiver I, Kaspers GJL, Hulleman E. Radiosensitization in pediatric high-grade glioma: targets, resistance and developments. *Front Oncol* 2021;11:662209.
- [54] Castel D, Philippe C, Calmon R, et al. Histone H3F3A and HIST1H3B K27M mutations define two subgroups of diffuse intrinsic pontine gliomas with different prognosis and phenotypes. *Acta Neuropathol* 2015;130(6):815–27.

- [55] Lowe BR, Maxham LA, Hamey JJ, Wilkins MR, Partridge JF. Histone H3 mutations: an updated view of their role in chromatin deregulation and cancer. *Cancers (Basel)* 2019;11(5).
- [56] Enomoto T, Aoki M, Hamasaki M, et al. Midline glioma in adults: clinicopathological, genetic, and epigenetic analysis. *Neurol Med Chir (Tokyo)* 2020;60(3):136–46.
- [57] Bender S, Tang Y, Lindroth AM, et al. Reduced H3K27me3 and DNA hypomethylation are major drivers of gene expression in K27M mutant pediatric high-grade gliomas. *Cancer Cell* 2013;24(5):660–72.
- [58] Chan KM, Fang D, Gan H, et al. The histone H3.3K27M mutation in pediatric glioma reprograms H3K27 methylation and gene expression. *Genes Dev* 2013;27(9):985–90.
- [59] Lewis PW, Müller MM, Koletsky MS, et al. Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma. *Science* 2013;340(6134):857–61.
- [60] Venneti S, Garimella MT, Sullivan LM, et al. Evaluation of histone 3 lysine 27 trimethylation (H3K27me3) and enhancer of Zest 2 (EZH2) in pediatric glial and glioneuronal tumors shows decreased H3K27me3 in H3F3A K27M mutant glioblastomas. *Brain Pathol* 2013;23(5):558–64.
- [61] Harutyunyan AS, Chen H, Lu T, et al. H3K27M in gliomas causes a one-step decrease in H3K27 methylation and reduced spreading within the constraints of H3K36 methylation. *Cell Rep* 2020;33(7):108390.
- [62] Harutyunyan AS, Krug B, Chen H, et al. H3K27M induces defective chromatin spread of PRC2-mediated repressive H3K27me2/me3 and is essential for glioma tumorigenesis. *Nat Commun* 2019;10(1):1262.
- [63] Sarthy JF, Meers MP, Janssens DH, et al. Histone deposition pathways determine the chromatin landscapes of H3.1 and H3.3 K27M oncohistones. *Elife* 2020;9.
- [64] Chen KY, Bush K, Klein RH, et al. Reciprocal H3.3 gene editing identifies K27M and G34R mechanisms in pediatric glioma including NOTCH signaling. *Commun Biol* 2020;3(1):363.
- [65] Fang D, Gan H, Cheng L, et al. H3.3K27M mutant proteins reprogram epigenome by sequestering the PRC2 complex to poised enhancers. *Elife* 2018;7.
- [66] Silveira AB, Kasper LH, Fan Y, et al. H3.3 K27M depletion increases differentiation and extends latency of diffuse intrinsic pontine glioma growth in vivo. *Acta Neuropathol* 2019;137(4):637–55.
- [67] Cordero FJ, Huang Z, Grenier C, et al. Histone H3.3K27M represses p16 to accelerate gliomagenesis in a murine model of DIPG. *Mol Cancer Res* 2017;15(9):1243–54.
- [68] Furth N, Algranati D, Dassa B, et al. H3-K27M-mutant nucleosomes interact with MLL1 to shape the glioma epigenetic landscape. *Cell Rep* 2022;39(7):110836.
- [69] Tomita Y., Shimazu Y., Somasundaram A., et al. A novel mouse model of diffuse midline glioma initiated in neonatal oligodendrocyte progenitor cells highlights cell-of-origin dependent effects of H3K27M. *Glia*; 2022.
- [70] Sanders LM, Cheney A, Seninge L, et al. Identification of a differentiation stall in epithelial mesenchymal transition in histone H3-mutant diffuse midline glioma. *Gigascience* 2020;9:12.
- [71] Lewis NA, Klein RH, Kelly C, Yee J, Knoepfler PS. Histone H3.3 K27M chromatin functions implicate a network of neurodevelopmental factors including ASCL1 and NEUROD1 in DIPG. *Epigenetics Chromatin* 2022;15(1):18.
- [72] Allison Stewart C, Tong P, Cardnell RJ, et al. Dynamic variations in epithelial-to-mesenchymal transition (EMT), ATM, and SLFN11 govern response to PARP inhibitors and cisplatin in small cell lung cancer. *Oncotarget* 2017;8(17):28575–87.
- [73] Bočkaj I, Martini TEI, de Camargo Magalhães ES, et al. The H3.3K27M oncohistone affects replication stress outcome and provokes genomic instability in pediatric glioma. *PLoS Genet* 2021;17(11):e1009868.
- [74] Chaouch A, Berlandi J, Chen CCL, et al. Histone H3.3 K27M and K36M mutations de-repress transposable elements through perturbation of antagonistic chromatin marks. *Mol Cell* 2021;81(23):4876–4890.e7.

- [75] Kernohan KD, Grynspan D, Ramphal R, et al. K36M mutation in a congenital-onset soft tissue neoplasm. *Pediatr Blood Cancer* 2017;64(12).
- [76] Klein BJ, Krajewski K, Restrepo S, Lewis PW, Strahl BD, Kutateladze TG. Recognition of cancer mutations in histone H3K36 by epigenetic writers and readers. *Epigenetics* 2018;13(7):683–92.
- [77] Snuderl M, Dolgalev I, Heguy A, et al. Histone H3K36I mutation in a metastatic histiocytic tumor of the skull and response to sarcoma chemotherapy. *Cold Spring Harb Mol Case Stud* 2019;5:5.
- [78] Krug B, Harutyunyan AS, Deshmukh S, Jabado N. Polycomb repressive complex 2 in the driver's seat of childhood and young adult brain tumours. *Trends Cell Biol* 2021;31(10):814–28.
- [79] Fang D, Gan H, Lee JH, et al. The histone H3.3K36M mutation reprograms the epigenome of chondroblastomas. *Science* 2016;352(6291):1344–8.
- [80] Zhang Y, Shan CM, Wang J, Bao K, Tong L, Jia S. Molecular basis for the role of oncogenic histone mutations in modulating H3K36 methylation. *Sci Rep* 2017;7:43906.
- [81] Liu Y, Zhang Y, Xue H, et al. Cryo-EM structure of SETD2/Set2 methyltransferase bound to a nucleosome containing oncohistone mutations. *Cell Discov* 2021;7(1):32.
- [82] Zhang Y, Fang D. The incorporation loci of H3.3K36M determine its preferential prevalence in chondroblastomas. *Cell Death Dis* 2021;12(4):311.
- [83] Nagaraja S, Quezada MA, Gillespie SM, et al. Histone variant and cell context determine H3K27M reprogramming of the enhancer landscape and oncogenic state. *Mol Cell* 2019;76(6):965–980.e12.
- [84] Funato K, Major T, Lewis PW, Allis CD, Tabar V. Use of human embryonic stem cells to model pediatric gliomas with H3.3K27M histone mutation. *Science* 2014;346(6216):1529–33.
- [85] Pathania M, De Jay N, Maestro N, et al. H3.3(K27M) cooperates with Trp53 loss and PDGFRA gain in mouse embryonic neural progenitor cells to induce invasive high-grade gliomas. *Cancer Cell* 2017;32(5):684–700.e9.
- [86] Larson JD, Kasper LH, Paugh BS, et al. Histone H3.3 K27M accelerates spontaneous brainstem glioma and drives restricted changes in bivalent gene expression. *Cancer Cell* 2019;35(1):140–155.e7.
- [87] Pajovic S, Siddaway R, Bridge T, et al. Epigenetic activation of a RAS/MYC axis in H3.3K27M-driven cancer. *Nat Commun* 2020;11(1):6216.
- [88] Fellenberg J, Sähr H, Mancarella D, et al. Knock-down of oncohistone H3F3A-G34W counteracts the neoplastic phenotype of giant cell tumor of bone derived stromal cells. *Cancer Lett* 2019;448:61–9.
- [89] Khazaei S, De Jay N, Deshmukh S, et al. G34W promotes growth and impedes differentiation of osteoblast-like mesenchymal progenitors in giant cell tumor of bone. *Cancer Discov* 2020;10(12):1968–87.
- [90] Shi L, Shi J, Shi X, Li W, Wen H. Histone H3.3 G34 mutations alter histone H3K36 and H3K27 methylation in cis. *J Mol Biol* 2018;430(11):1562–5.
- [91] Jain SU, Khazaei S, Marchione DM, et al. Histone H3.3 G34 mutations promote aberrant PRC2 activity and drive tumor progression. *Proc Natl Acad Sci U S A* 2020;117(44):27354–64.
- [92] Deshmukh S, Ptack A, Krug B, Jabado N. Oncohistones: a roadmap to stalled development. *FEBS j* 2022;289(5):1315–28.
- [93] Cheng Z, Cheung P, Kuo AJ, et al. A molecular threading mechanism underlies Jumonji lysine demethylase KDM2A regulation of methylated H3K36. *Genes Dev* 2014;28(16):1758–71.
- [94] Voon HPJ, Udugama M, Lin W, et al. Inhibition of a K9/K36 demethylase by an H3.3 point mutation found in paediatric glioblastoma. *Nat Commun* 2018;9(1):3142.
- [95] Bröhm A, Schoch T, Grünberger D, et al. The H3.3 G34W oncohistone mutation increases K36 methylation by the protein lysine methyltransferase NSD1. *Biochimie* 2022;198(86–91).

- [96] Funato K, Smith RC, Saito Y, Tabar V. Dissecting the impact of regional identity and the oncogenic role of human-specific NOTCH2NL in an hESC model of H3.3G34R-mutant glioma. *Cell Stem Cell* 2021;28(5):894–905.e7.
- [97] Bressan RB, Southgate B, Ferguson KM, et al. Regional identity of human neural stem cells determines oncogenic responses to histone H3.3 mutants. *Cell Stem Cell* 2021;28(5):877–893.e9.
- [98] Klein RH, Knoepfler PS. Stem cell models help crack regional oncohistone codes driving childhood gliomas. *Cell Stem Cell* 2021;28(5):785–7.
- [99] Chen CCL, Deshmukh S, Jessa S, et al. Histone H3.3G34-mutant interneuron progenitors co-opt PDGFRA for gliomagenesis. *Cell* 2020;183(6):1617–1633.e22.
- [100] Kannan S, Murugan AK, Balasubramanian S, Munirajan AK, Alzahrani AS. Gliomas: genetic alterations, mechanisms of metastasis, recurrence, drug resistance, and recent trends in molecular therapeutic options. *Biochem Pharmacol* 2022;201:115090.
- [101] Pladevall-Morera D, Castejón-Grifñán M, Aguilera P, et al. ATRX-deficient high-grade glioma cells exhibit increased sensitivity to RTK and PDGFR inhibitors. *Cancers (Basel)* 2022;14(7).
- [102] Guo R, Zheng L, Park JW, et al. BS69/ZMYND11 reads and connects histone H3.3 lysine 36 trimethylation-decorated chromatin to regulated pre-mRNA processing. *Mol Cell* 2014;56(2):298–310.
- [103] Wen H, Li Y, Xi Y, et al. ZMYND11 links histone H3.3K36me3 to transcription elongation and tumour suppression. *Nature* 2014;508(7495):263–8.
- [104] Lim J, Park JH, Baude A, et al. The histone variant H3.3 G34W substitution in giant cell tumor of the bone link chromatin and RNA processing. *Sci Rep* 2017;7(1):13459.
- [105] Jiao F, Li Z, He C, et al. RACK7 recognizes H3.3G34R mutation to suppress expression of MHC class II complex components and their delivery pathway in pediatric glioblastoma. *Sci Adv* 2020;6(29):eaba2113.
- [106] Chen Y, Tsai YH, Tseng SH. Regulation of ZMYND8 to treat cancer. *Molecules* 2021;26:4.
- [107] Chen Q, Yang R, Korolev N, Liu CF, Nordenskiöld L. Regulation of nucleosome stacking and chromatin compaction by the histone H4 N-terminal tail-H2A acidic patch interaction. *J Mol Biol* 2017;429(13):2075–92.
- [108] Selvam K, Rahman SA, Li S. Histone H4 H75E mutation attenuates global genomic and Rad26-independent transcription-coupled nucleotide excision repair. *Nucleic Acids Res* 2019;47(14):7392–401.
- [109] Tessadori F, Giltay JC, Hurst JA, et al. Germline mutations affecting the histone H4 core cause a developmental syndrome by altering DNA damage response and cell cycle control. *Nat Genet* 2017;49(11):1642–6.
- [110] Tessadori F, Rehman AU, Giltay JC, et al. A de novo variant in the human HIST1H4J gene causes a syndrome analogous to the HIST1H4C-associated neurodevelopmental disorder. *Eur J Hum Genet* 2020;28(5):674–8.
- [111] Magin RS, Liszczak GP, Marmorstein R. The molecular basis for histone H4- and H2A-specific amino-terminal acetylation by NatD. *Structure* 2015;23(2):332–41.
- [112] Ju J, Chen A, Deng Y, et al. NatD promotes lung cancer progression by preventing histone H4 serine phosphorylation to activate Slug expression. *Nat Commun* 2017;8(1):928.
- [113] Ho YH, Huang R. Effects of oncohistone mutations and PTM crosstalk on the N-terminal acetylation activities of NatD. *ACS Chem Biol* 2022.
- [114] Jenseit A, Camgöz A, Pfister SM, Kool M. EZHIP: a new piece of the puzzle towards understanding pediatric posterior fossa ependymoma. *Acta Neuropathol* 2022;143(1):1–13.
- [115] Aranda S, Di Croce L. Inhibitory protein puts a lid on an epigenetic marker. *Nature* 2019;573(7772):38–9.

- [116] Hübner JM, Müller T, Papageorgiou DN, et al. EZHIP/CXorf67 mimics K27M mutated oncohistones and functions as an intrinsic inhibitor of PRC2 function in aggressive posterior fossa ependymoma. *Neuro Oncol* 2019;21(7):878–89.
- [117] Jain SU, Do TJ, Lund PJ, et al. PFA ependymoma-associated protein EZHIP inhibits PRC2 activity through a H3 K27M-like mechanism. *Nat Commun* 2019;10(1):2146.
- [118] Jain SU, Rashoff AQ, Krabbenhoft SD, et al. H3 K27M and EZHIP impede H3K27-methylation spreading by inhibiting allosterically stimulated PRC2. *Mol Cell* 2020;80(4):726–735.e7.
- [119] Panwalkar P, Tamrazi B, Dang D, et al. Targeting integrated epigenetic and metabolic pathways in lethal childhood PFA ependymomas. *Sci Transl Med* 2021;13(614):eabc0497.
- [120] Bagert JD, Mitchener MM, Patriotis AL, et al. Oncohistone mutations enhance chromatin remodeling and alter cell fates. *Nat Chem Biol* 2021;17(4):403–11.
- [121] Golbourn BJ, Halbert ME, Halligan K, et al. Loss of MAT2A compromises methionine metabolism and represents a vulnerability in H3K27M mutant glioma by modulating the epigenome. *Nat Cancer* 2022.
- [122] Zhang L, Neswick CL, Day CA, et al. STAT3 is a biologically relevant therapeutic target in H3K27M-mutant diffuse midline glioma. *Neuro Oncol* 2022.
- [123] Yabuno S, Kawauchi S, Umakoshi M, et al. Spinal cord diffuse midline glioma, H3K27M- mutant effectively treated with bevacizumab: a report of two cases. *NMC Case Rep J* 2021;8(1):505–11.
- [124] Rahal F, Capdevielle C, Rousseau B, et al. An EZH2 blocker sensitizes histone mutated diffuse midline glioma to cholesterol metabolism inhibitors through an off-target effect. *Neurooncol Adv* 2022;4(1):vdac018.
- [125] Purow B. ONC201 and ONC206: metabolically ClipPing the wings of diffuse midline glioma. *Neuro Oncol* 2022.
- [126] Graves PR, Aponte-Collazo LJ, Fennell EMJ, et al. Mitochondrial protease ClpP is a target for the anti-cancer compounds ONC201 and related analogues. *ACS Chem Biol* 2019;14(5):1020–9.
- [127] Przystal JM, Cosentino CC, Yadavilli S, et al. Imipridones affect tumor bioenergetics and promote cell lineage differentiation in diffuse midline gliomas. *Neuro Oncol* 2022.
- [128] Borsuk R, Zhou L, Chang WI, et al. Potent preclinical sensitivity to imipridone-based combination therapies in oncohistone H3K27M-mutant diffuse intrinsic pontine glioma is associated with induction of the integrated stress response, TRAIL death receptor DR5, reduced ClpX and apoptosis. *Am J Cancer Res* 2021;11(9):4607–23.
- [129] Cantor E, Wierzbicki K, Tarapore RS, et al. Serial H3K27M cell-free tumor DNA (cf-tDNA) tracking predicts ONC201 treatment response and progression in diffuse midline glioma. *Neuro Oncol* 2022.
- [130] Mount CW, Majzner RG, Sundaresh S, et al. Potent antitumor efficacy of anti-GD2 CAR T cells in H3-K27M(+) diffuse midline gliomas. *Nat Med* 2018;24(5):572–9.
- [131] Majzner RG, Ramakrishna S, Yeom KW, et al. GD2-CAR T cell therapy for H3K27M-mutated diffuse midline gliomas. *Nature* 2022;603(7903):934–41.
- [132] Zhang D, Wang G, Yu X, et al. Enhancing CRISPR/Cas gene editing through modulating cellular mechanical properties for cancer therapy. *Nat Nanotechnol* 2022.
- [133] Fang D, Wang H, Zhang Z. Probing the function of oncohistones using mutant transgenes and knock-in mutations. *Methods Mol Biol* 2018;1832:339–56.
- [134] Sangatsuda Y, Miura F, Araki H, et al. Base-resolution methylomes of gliomas bearing histone H3.3 mutations reveal a G34 mutant-specific signature shared with bone tumors. *Sci Rep* 2020;10(1):16162.
- [135] Rajagopalan KN, Chen X, Weinberg DN, et al. Depletion of H3K36me2 recapitulates epigenomic and phenotypic changes induced by the H3.3K36M oncohistone mutation. *Proc Natl Acad Sci U S A* 2021;118(9).

- [136] Asimgil H, Ertetik U, Çevik NC, et al. Targeting the undruggable oncogenic KRAS: the dawn of hope. *JCI Insight* 2022;7(1).
- [137] König D, Savic Prince S, Rothschild SI. Targeted therapy in advanced and metastatic non-small cell lung cancer. An update on treatment of the most important actionable oncogenic driver alterations. *Cancers (Basel)* 2021;13(4).
- [138] Valla V, Alzabin S, Koukoura A, Lewis A, Nielsen AA, Vassiliadis E. Companion diagnostics: state of the art and new regulations. *Biomark Insights* 2021;16:11772719211047763.

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microRNA, epi-microRNA, and cancer

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1 miRNA biogenesis and functionality

microRNAs (miRNAs) are a species of the nonprotein-coding RNA family, represented by short, single-stranded RNA approximately 18–22 nucleotides in length [1]. miRNAs are regulators of gene expression at a posttranscriptional level, and newer miRNA functions are being identified [1]. In combination with an RNA-induced silencing complex (RISC), miRNAs specifically target complementary messenger RNA (mRNA) transcripts, typically via imperfect complementary base pairing, and repress their translation [1,2]. The role of miRNA as bystanders and effectors within the epigenetic landscape of the cell is becoming apparent [3]. In diseases, such as cancer, the complex relationship between miRNA and epigenetics is uncovering a new understanding of cancer cell biology.

Lee et al. [4] identified the first miRNA in *Caenorhabditis elegans*. The *lin-4* gene encodes two small nonprotein-coding RNA transcripts approximately 22 and 61 nucleotides in length, and is essential for postembryonic development in *C. elegans* [4]. The sequences of the RNA transcripts have complementarity to the 3'-UTR (3' untranslated region) of the *lin-14* mRNA and bind to the mRNA via antisense RNA:RNA interactions, repressing mRNA translation, resulting in downregulation of LIN-14 protein levels [4–6]. A second miRNA was identified in *C. elegans*. The *let-7* gene encodes a 21-nucleotide RNA transcript with complementarity to the 3'-UTR of five heterochronic genes involved in normal development: *lin-14*, *lin-28*, *lin-41*, *lin-42*, and *daf-12* [7,8]. Expression of *lin-4* and *let-7* is essential in postembryonic development and developmental timing within *C. elegans*. In 2001 numerous small nonprotein-coding RNAs were identified and were collectively termed “miRNA” [9–11], including 21 novel human miRNAs, miR-1 to miR-33 [9]. Intensified research efforts identified additional miRNAs in mammals, fish, worms, flies, and plants [1]. Over 2000 human miRNA precursor sequences have been identified, but the functionality of most miRNA is still unknown.

Genes encoding miRNA are located throughout the genome as individual genes, polycistrons, or within introns of pre-mRNA [1]. miRNA genes located within introns of pre-mRNA are ideally located for translational repression of their mRNA host [1]. Polycistronic miRNA genes encode a cluster of miRNA precursors, which are transcribed as a single transcript then processed into individual mature miRNAs that may have related or nonrelated functions [1]. miRNA genes are transcribed in the nucleus by RNA polymerase II or III, most often RNA polymerase II, producing a single-stranded RNA transcript 1–7 kb in length [12,13] (Figure 5.1).

The primary-miRNA (pri-miRNA) transcript folds into an imperfect hairpin structure as a result of Watson–Crick base pairing and is processed in the nucleus by Drosha and DGCR [14,15]. The 5' and 3' ends of the hairpin structure are asymmetrically cleaved producing a precursor-miRNA (pre-miRNA), which is exported to the cytoplasm via exportin-5 [16]. In the cytoplasm, Dicer and TRBP cleave the loop structure off the hairpin, forming the miRNA duplex, termed “miR-5p/-3p” (formerly known as miRNA-miRNA*) [17,18]. The relative thermodynamic stability of the strands determines which arm of the duplex will be incorporated into RISC as the mature miRNA, while the passenger strand (miRNA*) is subsequently degraded [17,19]. Argonaute (Ago) proteins constitute the major functional element of RISC. In mammals, there are four Ago proteins, all of which are capable of repressing mRNA translation as part of the RISC, but only Ago2 is able to directly cleave the mRNA target [20]. The piwi-argonaute-zwille (PAZ) domain of the Ago protein binds the 2-nucleotide overhang at the 3' end of the mature miRNA strand [19]. The duplex then unwinds and the 5' end of the mature miRNA strand is bound by the Ago MID (middle) domain [1]. Exposed bases of the mature miRNA within RISC bind to target mRNA sequences via complementary base pairing [17]. The mRNA may be regulated by an RNAi mechanism involving direct cleavage and degradation by RISC, or repression of mRNA translation, which is most frequently the case in mammals [1].

Within the target mRNA, the “seed site” is a sequence of approximately seven nucleotides and is essential for miRNA binding, which are highly conserved between species [21–23]. The mRNA seed site is frequently, but not exclusively, located in the 3'-UTR. The “seed region” at the 5' end of the miRNA binds the seed site in the mRNA [21,24]. The 5'-UTR and coding sequence of the mRNA can also contain “seed sites”; the miRNA RISC complex can potentially bind to any region of the mRNA [24,25]. The general consensus is that the 3'-UTR is the most accessible region of

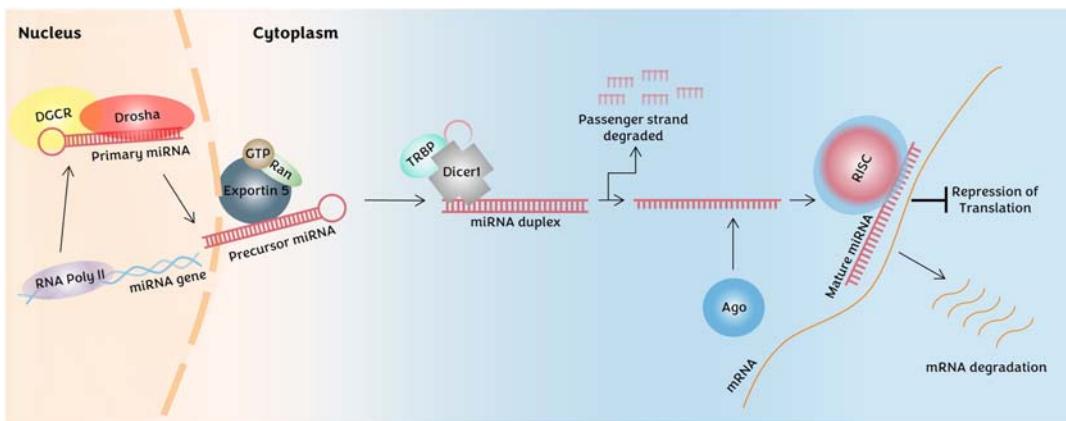


FIGURE 5.1 miRNA biogenesis.

miRNA genes are transcribed in the nucleus by RNA polymerase II to produce a long, single-stranded RNA transcript. The primary-miRNA (pri-miRNA) folds into a hairpin structure and is processed by Drosha and DGCR, whereby the 5' and 3' ends of the hairpin structure are asymmetrically cleaved to produce the precursor-miRNA (pre-miRNA). The pre-miRNA is exported from the nucleus to the cytoplasm via exportin-5 and Ran-GTP on the nuclear membrane and is further processed in the cytoplasm by Dicer and TRBP. The loop of the hairpin structure is cleaved to produce the miRNA duplex. Within the miRNA duplex one arm is the passenger strand and the other is the mature (guide) miRNA. The passenger strand is degraded while the mature strand complexes with Argonaute (Ago) proteins. The Ago proteins constitute the major functional element of the RISC. The exposed bases of the mature miRNA bind to complementary target mRNA sequences, typically via imperfect complementary base pairing. Subsequently, translation of the mRNA is repressed, thereby downregulating expression of the target at protein level.

the mRNA, as RISC has less competition binding to the mRNA furthest away from the ribosome and translational machinery [1,24,26]. Furthermore, regulated target mRNAs generally have longer 3'-UTR compared to ubiquitously expressed genes which tend to have shorter 3'-UTRs that are depleted of miRNA-binding sites [27]. A mature miRNA within the RISC guides the complex toward complementary mRNA targets, stringent seed sites have perfect Watson–Crick base pairing between the mRNA and miRNA [24]. However, the RISC can also tolerate G:U wobble and mismatch binding between the miRNA seed region and mRNA seed site [24]. In mammals, miRNA:mRNA binding is generally the result of imperfect complementary base pairing, while in plants near-perfect complementary base pairing is most common [1]. The imperfect nature of miRNA target binding enables a single miRNA to target multiple mRNA targets, leading to a degree of redundancy between miRNAs [28]. This variation and flexibility in miRNA:mRNA binding can make it difficult to predict mRNA targets using bioinformatic tools [24].

Once incorporated into the RISC, the mature miRNA downregulates target gene expression at the posttranscriptional level in which the mechanisms can be broadly divided into two categories: translational repression and mRNA degradation [29]. In metazoans, mRNA targets are typically translationally repressed by their regulating miRNA due to the imperfect base pairing between the miRNA and mRNA as opposed to mRNA cleavage [1]. Experimental evidence in mammals has demonstrated that the levels

of target mRNA remain unchanged upon miRNA targeting, but a decrease in protein expression is observed [1,4]. Studies also demonstrate that miRNA binding to targets can frequently result in degradation, with the miRNA promoting translational quiescence, followed by degradation of the target as a secondary consequence. The exact mechanism of translational repression is unclear; the RISC complex may repress translation at the initiation or postinitiation stage, or both [29]. Alternatively, mRNA targets can be guided by the RISC into processing (P)-bodies, sequestering them from ribosomes and the translational machinery [19,29]. Endonucleases can subsequently enter P-bodies and degrade the sequestered mRNAs, or mRNAs can later be released back into the cytoplasm for translation if protein levels decrease below the requirements of the cell, thus demonstrating miRNA-mediated repression is reversible [30,31]. Near-perfect base pairing between the miRNA:mRNA is associated with direct cleavage of the mRNA by the RISC [1]. miRNAs are also destabilized by the gradual shortening of the poly A tail, causing mRNA degradation by progressive decay catalyzed by the exosome or degradation by endonucleases [32].

Generally, miRNAs are recognized as functioning to downregulate gene expression; however, there is evidence of further functional roles of miRNAs, including links between miRNAs and epigenetics [3]. The expression of miRNA genes is regulated by their epigenetic status and miRNAs are known to have specific epigenetic functions [3]. Although miRNAs are processed and function in the cytoplasm, there is evidence that mature miRNAs are associated with Ago proteins found in the nucleus [33,34]. The miRNAs in the nucleus are reported to have epigenetic functions, such as modulating mRNA splicing and targeting gene promoters to activate or repress transcription [33,34].

miRNAs account for approximately 1% of the genome and were previously estimated to regulate approximately 30% of genes [1]; this has now been revised to approximately 60% [35]. Different cell types have specialized functions and express a specific set of genes related to the function of the cell; this is reflected in tissue-specific miRNA expression profiles [36]. Disrupting the highly complex miRNA regulatory network within the cell can induce abnormal cell behavior and disease initiation or progression [37], and as such, dysregulated miRNA expression is a common feature in human diseases, especially cancer.

2 miRNA in cancer biology

miRNAs are involved in all pathways and cellular processes within the cell, hence it is not surprising that miRNA dysregulation is viewed as a fundamental feature of cancer and is considered instrumental in the acquisition of the hallmarks of cancer, such as invasion, angiogenesis, evasion of apoptosis as well as being key drivers within newer hallmarks, such as phenotypic plasticity [38,39]. Tumors most often have a reduced level of mature miRNA, due to the loss of genetic material, alterations to the machinery associated with biogenesis, and epigenetic silencing [40]. It is proposed that cancer-associated miRNAs either have an oncogenic or tumor-suppressive activity [41], depending on tissue type and location of the cancer. The link between miRNA and cancer was first established by Calin et al. [42] who reported the deletion or downregulation of miR-15a and miR-16-1 encoded at the 13q14 loci in a majority of B-cell chronic lymphocytic leukemia cases. It was later reported that the alterations in expression of miRNA in various cancers could be critical to the understanding of cancer pathophysiology [43]. Interestingly, Calin et al. [43] revealed that many miRNAs are encoded at fragile sites and within common breakpoint regions in the genome, thus increasing their susceptibility to mutation and deletion.

As miRNAs play a significant role in the regulation of many aspects of cellular machinery, it has been suggested that the deregulation of these small noncoding molecules could substantially affect the cell and its progression through the cell cycle. The frequently altered miRNA, miR-31, is disrupted in opposing directions in a wide range of tumor types [44], for example, in mesothelioma it has been established that miR-31 is significantly downregulated, effecting the expression of PPP6C, a pro-survival phosphatase [45], however it is also noted that overexpression of miR-31 leads to an increase in transportome-regulated chemoresistance [46]. Conversely, the same miRNA within the colorectal cancer microenvironment is highly upregulated across all stages of the disease [47].

The multiple component miRNA polycistron miR-17~92 associated with tumor formation was discovered by He et al. [48] and was found to be amplified in B-cell lymphomas in both cell line studies and samples of tumor tissue. Suarez et al. [49] concluded that the cluster carries out pleiotropic functions and modifies postnatal angiogenesis in response to vascular factors, such as vascular endothelial growth factor (VEGF). The cluster has also been shown to promote carcinogenesis by altering cell cycle phase distribution, as in Sylvestre et al. [50]. MiR-17~92 is activated by members of the E2F family, which stimulate several S phase genes, including thymidine kinase. E2F1, E2F2, and E2F3 are all modulated by the miR-17~92 cluster, via their 3'-UTR-binding sites. In addition, overexpression of a member of the cluster, miR-20a, decreased apoptosis in a prostate cancer cell line whilst the inhibition of miR-20a produced an increase in cell death, thus potentially elucidating some of the oncogenic capabilities of the miR-17~92 cluster. The study suggested that the autoregulation between E2F1–3 and miR-20a may contribute to the regulation of apoptotic events and proliferation [50].

Gao et al. [51] investigated miR-184 for regulatory functions within hepatocellular carcinoma (HCC). Using the inositol polyphosphate phosphatase-like 1 (INPPL1) insulin regulator as a recognized target, miR-184 was found to be central in HCC cell proliferative activity, and silencing of miR-184 lead to the overexpression of INPPL1. The miRNA was also allied to the inhibition of caspases 3 and 7, suggesting a role in the evasion of apoptosis [51].

In pancreatic cancer, miR-106a is highly expressed in tumor tissue and in four cell lines, one of which, SW-1990, a highly invasive line [52]. In cells transfected with a miR-106a mimic, tumor cell growth was stimulated, whereas an miR-106a inhibitor decreased cell viability [52]. Mace et al. [53] investigated miRNA in relation to pancreatic tumor cells under hypoxia; findings suggested that miR-21 was induced via hypoxia-inducible factor-1 α upregulation, and miR-21 overexpression promoted the evasion of apoptosis in a hypoxic environment.

Let-7a, a tumor suppressor miRNA, is lost in malignant melanoma, where it has been demonstrated to regulate integrin β_3 expression [54]. The integrin β_3 subunit $\alpha v \beta_3$ family of adhesion receptors are involved in the transition from dysplastic nevi to tumorigenic melanomas, and overexpression has been linked to increased cellular motility [55]. miR-143 and miR-145, located at the 5q33 fragile site, represent tumor-suppressive miRNA [56]. The miR-143~145 cluster is downregulated in many cancers, suggesting a “protective” role for the miRNA. miR-143 and miR-145 are recurrently coordinately downregulated in endometrial cancer, with a connection made between downregulation of miR-143/~145 and overexpression of DNA methyltransferase (DNMT) 3B [57]. The DNMT group contributes to the coordination of mRNA expression in normal tissues and overexpression in many tumors [58]. In addition, miR-29 has been identified to target DNMT2A and DNMT3B, which have been found to be upregulated in lung carcinoma [59]. The miR-29 family has been identified as being upregulated in induced and replicative senescence, and functions to inhibit DNA synthesis and repress the *B-Myb* oncogene in combination with Rb within a tumorigenic cell [60,61].

The well-established hallmarks of cancer are integrally linked with miRNA expression, and indeed hundreds of miRNAs have been found to be novel regulators of these distinctive carcinogenic hallmarks. miR-519 has demonstrated the ability to inhibit proliferation in cervical, colon, and ovarian cell lines, through one of its targets, HuR, an RNA-binding protein [62]. Furthermore, the RhoA pathway can be modulated through miR-146a in prostate cancer [63]. Resulting in down-regulation of the serine/threonine protein kinase ROCK1, leading to the dysregulation of the actin cytoskeleton [63], and alterations in cellular motility. The expression of miR-34a correlates with p53 expression, and can be termed a regulator of apoptosis [64]. The cluster of miR-290 can directly regulate the DNMT expression in Dicer1-null cells, which indirectly affects telomere integrity and length, thus implying significance in the regulation of replicative potential [65]. An example relating to angiogenesis regulation of miR-378, which binds to the 3'-UTR region of VEGF, which promotes neovascularization [66]. Other miRNAs, such as miR-10b and miR-23a, are implicated in the regulation of invasion and genomic stability, respectively [67].

miRNAs are novel therapeutic targets and promising cancer biomarkers with potential applications in diagnostics, prognostic, tumor staging, patient response to treatment, and determination of developmental lineages and clinical subtypes [68]. However, the functional roles of many miRNAs are yet to be determined. More specifically, miRNAs may play a fundamental role in the epigenetic changes observed within carcinogenesis, and understanding the dysregulation of these small, non-coding molecules could be imperative in our understanding of cancer biology [69].

3 miRNA: an epigenetic perspective

miRNAs are directly and indirectly linked with epigenetics [3]. The indirect link involves the regulation of miRNA expression via epigenetic modifications, namely DNA methylation and chromatin remodeling, which ultimately determine the accessibility of miRNA genes for transcription [70]. Conversely, miRNAs are directly linked to epigenetics in one of two ways, firstly epi-miRNAs (epigenetic-miRNA) control the expression of the epigenetic machinery via translational repression [71]. Secondly, miRNAs have emerging roles as functional epigenetic components that modulate gene expression at the transcriptional level by interacting with gene promoter regions and the transcriptional machinery, to positively or negatively regulate gene expression [72]. Epigenetics and miRNAs are entwined in a complex circuit and modulate the expression of vast numbers of genes within the genome (Figure 5.2).

3.1 Epigenetic alteration of miRNA expression

Analysis of miRNA genes suggests that almost half of the sequences are associated with CpG islands, and thus are liable to epigenetic regulation [73,74]. Histone modification controls DNA accessibility by manipulating chromatin states in order for gene expression to be controlled throughout various developmental stages of life [75]. The loci of a number of miRNA genes being so closely located to CpG islands reveals how they would be subject to DNA methylation; this is a major route by which miRNA expression of some primary-miRNA transcripts is regulated [76], but it has limited impact on the control of mature miRNA levels. DNA methylation decreases in many

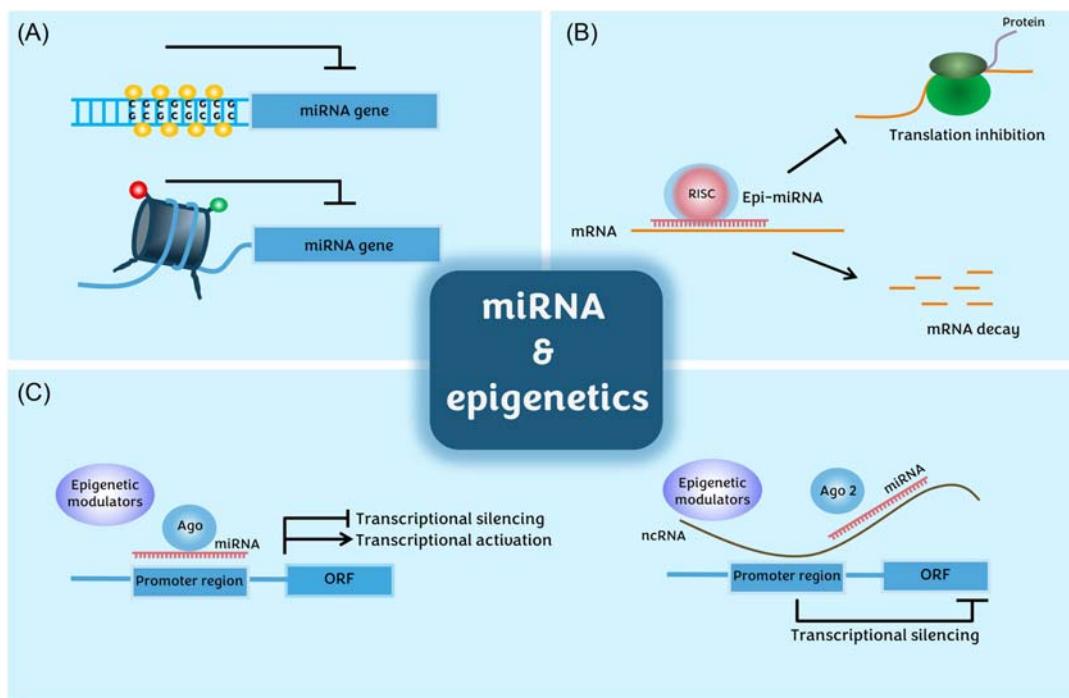


FIGURE 5.2 Epigenetics and miRNA.

(A) Epigenetic alteration of miRNA expression. Both DNA methylation (*top*) and histone modification (*bottom*) are adopted in the regulation of miRNA gene expression within many different cancers. Susceptibility to methylation has been allied with miRNA gene proximity to CpG islands. (B) Epigenetic-miRNA. Epi-miRNAs are miRNAs that specifically regulate the expression of genes encoding epigenetic machinery. The RISC complex with the epi-miRNA is guided to complementary mRNA targets, which are subsequently degraded or translationally repressed. (C) miRNA with epigenetic function. miRNAs are functional components of the epigenetic machinery. In collaboration with Ago proteins and components of the epigenetic machinery, miRNAs are capable of inducing transcriptional activation and silencing. The miRNA interacts with the promoter region of genes and controls gene expression much like a transcription factor. Alternatively, the miRNA may recruit additional epigenetic machinery, which alter the epigenetic status of the gene (*left*). An alternative mechanism involves noncoding RNA (ncRNA) produced from the promoter region of a gene which serves as the miRNA target. The ncRNA intermediate recruits the complementary miRNA to the promoter region of the gene. Additional epigenetic machinery and Ago2 are subsequently recruited to silence transcription (*right*).

tissue types during aging, and it is suggested that DNMT3b activity is increased with age, which feeds back into examples, such as the miR-143~145 cluster, as discussed previously [57,77].

There have been significant studies relating to epigenetic regulation of miRNA expression in various cancers. Using DNMT inhibitors and HDAC inhibitors, it has been observed that there are a range of effects on miRNA expression in renal cell carcinoma [78]. Upregulation of miR-9-1,

miR-642, miR-95, and miR-184 has been identified via microarray and qPCR validation, and upon further investigation it was identified that these miRNAs are located within, or near to, a CpG island [78]. Post addition of DNMT inhibitors, it was observed that expression of miR-9-1 and miR-642 was restored, indicating that the miRNAs were downregulated by a DNA methylation mechanism. Overall, the inhibition of DNA methylation initiated the reexpression of previously silenced miRNAs with recognized tumor suppressor functions [78].

Within colorectal cancer, the methylation status of miR-9-1, miR-129-2, and miR-137 has been analyzed in matched tumor and normal (disease-free) adjacent tissue samples. DNA methylation of miR-137 was apparent in all primary tumor samples, with the matched normal tissue having 23% miR-137 methylation; it was concluded that colorectal mucosal defects correlated with anomalous miRNA methylation. In addition to the evident variations in methylation, treatment with HDAC inhibitor restored the expression of miR-9-1, miR-129-2, and miR-137, supporting the hypothesis that miRNA silencing is induced by histone modifications. Both DNA hypermethylation and histone modification have been illustrated as being contributors to the transcriptional downregulation of specific miRNAs in colorectal cancer [79].

Epithelial ovarian cancer is also subject to epigenetic control of miRNA expression [80]. Three genomic loci, on chromosome 14, 19, and X, harbor 25 miRNAs significantly downregulated in ovarian cancer. Using ovarian cancer cell lines, it has been established that treatment with DNMT inhibitors and HDAC inhibitors restored expression of miR-34b, a tumor suppressor miRNA regulated by p53 [80]. Additionally, epigenetic mechanisms may also alter expression of miRNA genes in prostate cancer. For example, miR-205, miR-21, and miR-196b have been established to be downregulated through promoter methylation [81]. Conversely, miR-615 has been identified to be activated through epigenetic means in prostate cancer cell lines [82].

The prevalence of miRNAs that illustrate varying expression due to epigenetic regulation suggests that there is a complex relationship underpinning the regulation of miRNAs that mainly represses the promoter region of tumor suppressor miRNA genes; however, there is evidence of the recruitment of miRNA “activators,” as in prostate carcinogenesis. It has been proposed, as in Hulf et al., that changes at the pri-miRNA transcript level compared with alterations in mature miRNA expression can allow for the detection of functionally relevant miRNAs that are epigenetically regulated [82]. The study of epigenetic mechanisms governing miRNA expression may provide further answers as to how the cancer cell has a selective advantage over normal tissue, and aid in the development of both novel diagnostics and therapeutics. In another dimension to this concept, certain miRNAs, termed epi-miRNAs, can themselves modulate the epigenetic machinery, which alludes to a circular feedback loop, demonstrating an even greater depth of complexity in miRNA biology [73].

3.2 Epi-miRNA

Epi-miRNAs modulate the expression of genes encoding the epigenetic machinery, such as DNMTs, HDACs, and polycomb group (PcG) proteins [3]. The genes encoding epi-miRNA are also subject to epigenetic regulation via DNA methylation and chromatin remodeling. Dysregulation of these intricate networks within normal cells can contribute toward the initiation and promotion of cancer and may provide novel therapeutic targets.

The miR-29 family members were the first epi-miRNAs to be identified [59]. The *de novo* DNMTs, DNMT3A and DNMT3B, are translationally repressed by the miR-29 family

(miR-29a, miR-29b, and miR-29c). Binding sites within the 3'-UTRs of DNMT3A and DNMT3B were identified and validated in lung cancer cell lines, and miR-29 expression was inversely correlated with DNMT3A and DNMT3B in lung cancer tissues. In addition to the *de novo* DNMTs, miR-29 was shown to indirectly repress DNMT1 in acute myeloid leukemia [83]. Ectopic expression of miR-29 in cancer cell lines resulted in global DNA hypomethylation, reexpression of tumor suppressor genes, and inhibition of tumorigenesis both *in vitro* and *in vivo* [59,83]. However, an additional layer of complexity has recently developed. As well as regulating DNMTs, the miR-29 family also regulates the DNA demethylation machinery [84]. The tet methylcytosine dioxygenase (TET) protein family, in conjunction with thymine DNA glycosylase (TDG), modulates DNA demethylation [84]. The TET1 and TDG genes are both targets of miR-29 translational repression in lung cancer cell lines [84]. These recent developments suggest miR-29 is responsible for maintaining the existing DNA methylation status in normal cells by modulating the activity of both DNMTs and demethylases. Restoring endogenous miR-29 expression has the potential to repress *de novo* methylation, maintain cellular methylation status, and release hypermethylated tumor suppressor genes.

Other epi-miRNAs known to regulate the DNA methylation machinery include miR-152, miR-301, and miR-148 [85]. Interestingly, miR-148 expression is regulated by methylation and is itself a regulator of the *DNMT3B* gene [3,85]. The miR-148 binding site is located within the protein-coding region of the DNMT3B splice variants DNMT3B1, DNMT3B2, and DNMT3B4. The binding site is conserved between these splice variants; however, the binding site is absent in the *DNMT3B3* gene which is therefore resistant to miR-148 repression [85]. The abundance of DNMT3B splice variants is modulated by miR-148 and expression of miR-148 is regulated by DNA methylation, suggesting a complex regulatory feedback mechanisms exist between miRNA and the epigenetic machinery.

PcG proteins mediate gene silencing through histone modifications and chromatin remodeling. The PRC1 and PRC2 complexes are multidomain protein complexes consisting of PcG proteins. Expression of the PcG proteins is regulated by miRNA and the PRC complexes in turn modulate miRNA expression [86]. Enhancer of Zeste Homolog 2 (EZH2) is a histone methyltransferase, and the catalytic subunit of the PRC2 complex. EZH2 contributes to epigenetic silencing via tri-methylation of the core histone H3 lysine 27 (H3K27me3). Overexpression of EZH2 in solid tumors is associated with enhanced tumorigenesis and metastasis [86]. In a prostate cancer study, miR-101 was inversely correlated with EZH2 protein levels, and miR-101 expression decreased during cancer progression [87]. Hence, miR-101 was identified as a tumor suppressor in prostate cancer and was also downregulated in bladder, lung, breast, and colorectal cancers [86,88–90].

Furthermore, DNMT3B has been identified as a target of miR-101 repression [91]. Restoring miR-101 may attenuate the oncogenic effects of EZH2 overexpression and return the epigenetic status of tumor cells to a more normal state.

3.3 miRNA with epigenetic functions

Evidence suggesting miRNAs also function to regulate gene expression at the transcriptional level within the nucleus has been well reviewed [34,92]. High levels of miRNA and Ago proteins are reported to exist within the nucleus [34,93]. Emerging evidence suggests that miRNAs in collaboration with Ago proteins and other noncoding RNA species regulate gene transcription [34]. miRNAs in the nucleus may function like transcription factors to initiate gene expression, while, alternatively, miRNA binding at the promoter region may induce changes in chromatin structure or recruit other RNA species or proteins to induce transcription activation [72,94].

The first direct epigenetic modification induced by a miRNA was initially identified via a bioinformatics approach [94]. Gene promoter regions were scanned for complementarity to miR-373, thereby identifying 80% sequence complementarity between miR-373 and the promoter regions of the genes encoding E-Cadherin and CSDC2 (cold shock domain-containing protein C2) [94]. Ectopic expression of pre-miR-373 and mature miR-373 both induced target expression *in vitro*. The mechanism by which miR-373 can regulate transcription initiation remains unclear. Other miRNAs demonstrated to positively regulate gene expression include miR-744, miR-1186, and miR-466d-3p [95]. These miRNAs have high promoter complementarity with the *CCNB1* gene. Ectopic expression of miR-744, miR-1186, and miR-466d-3p induces cyclin B1 expression from the *CCNB1* gene *in vitro* and silencing endogenous miR-744 decreases cyclin B1 levels [95]. Furthermore, miR-744 increases RNA polymerase II and histone H3 lysine 4 tri-methylation (H3K4me3) at the *CCNB1* transcriptional start site [95]. In addition, Ago1 is localized to the promoter region and is proposed to recruit chromatin-modifying proteins to activate transcription [95]. *In vivo*, miR-747 and miR-1186 overexpression initially promotes tumor growth through *CCNB1* gene expression; however, long-term exposure results in chromosomal instability and inhibits tumor growth [95].

Alternatively, miRNAs can epigenetically silence transcriptional activation of genes [72]. miR-320 is encoded within the promoter region of the cell cycle gene *POLR3D* in the antisense orientation, and mature miR-320 has inherent complementarity to its target [96]. Acting in a *cis* orientation, miR-320 is transcribed from the promoter region and processed to the mature form, which silences the *POLR3D* gene by recruiting Ago1 and EZH2, thereby inducing epigenetic transcriptional gene silencing [96]. miRNA mimics have been employed to determine the mechanism by which miR-423-5p silences transcription of the human progesterone receptor (PR) [97]. Despite low complementarity between miR-423-5p and the promoter region of the PR gene, the miR-423-5p mimic silences transcription, acting in a *trans* orientation [97]. Noncoding RNA (ncRNA) transcribed from the PR promoter served as the miR-423-5p target, effectively recruiting miR-423-5p to the promoter region of the PR gene. In contrast to other studies, Ago2, as opposed to Ago1, is localized to the promoter suggesting an alternative mechanism for transcriptional gene silencing depending on the sequence complementarities between the miRNA and promoter region [97]. In addition, decreased localization of RNA polymerase II at the promoter region and increased histone H3 lysine 9 dimethylation (H3K9me2) induced epigenetic transcriptional silencing [97].

In the context of epigenetics, not only is the expression of certain miRNAs modulated by the epigenetic status of their encoding genes, but miRNAs themselves also have specific epigenetic functions and are able to modulate the epigenetic landscape within the cell [3]. Undoubtedly, miRNA and epigenetics are intertwined and dysregulation of the miRNA epigenetic circuit is both cause and consequence in cancer initiation and progression [3]. The extensive feedback loops and collaborations that exist between miRNAs and epigenetics suggest that a fragile balance must be maintained for the normal function of a cell.

4 miRNA epigenetic therapy

The goal in developing miRNA-modulating therapeutic drugs is to return miRNA expression to levels found in nondiseased cells. The attraction of this strategy is that it has the potential to restore control over many coregulated pathways with a single agent. An overview of the currently available strategies for miRNA inhibition or replacement is provided in Table 5.1. In essence, miRNA

Table 5.1 Methods for modulating miRNA expression levels *in vivo*.

Method	Description	Mechanism
Inhibition		
AntimiRs	Single-stranded AS-ODN complimentary to an miR target, modified by LNA substitutions; usually 13–22 nucleotides in length	High-affinity duplex formation, leading to RNaseH-mediated degradation of target miRNA
Tiny LNAs	Similar to antimiRs in composition, but much shorter (8 nucleotides); target seed sequence of individual miRNAs or families	As above, but may target multiple miRNA
AntagomirRs	Single-stranded AS-ODN complimentary to the miR target, modified by 2' O-Me groups; usually 22 or 23 nucleotides in length	Bind single miRNA target, but mechanism not fully understood
Sponges	Expressed transcripts with multiple tandem miRNA-binding sites; requires viral/nonviral delivery vehicle	Act as decoys for target miRNA(s), reducing regulation (and increasing expression) of true mRNA targets
Replacement		
miRNA mimic	Double-stranded synthetic RNAs, with or without modification, of sequence related to mature miRNAs; requires delivery vehicle	Mimic postdicer miRNA activity via incorporation into RISC and regulation of target mRNAs
Expression constructs	Plasmid or virus-encoded expression construct; former requires nonviral delivery vehicle	Expressed miRNAs enter miRNA processing pathway in the nucleus; exported into the cytoplasm and incorporated into RISC
Small molecules	Transcriptional activators (decitabine) or processing modulators (enoxacin)	Drugs that nonspecifically alter expression or RNA processing, leading to upregulation of miRNA levels

inhibition borrows heavily from antisense technology, whereas miRNA replacement mirrors the gene therapy techniques previously developed for RNAi-based therapeutic approaches. In addition, miRNA-specific approaches, such as sponges and small-molecule modulators of miRNA biogenesis, are available. Nucleic acid-based methods offer the most specific methods, and effective control of miRNA levels can be achieved but, as for antisense and gene therapy, the translation of *in vitro* results into *in vivo* efficacy is heavily dependent on delivery.

4.1 miRNA inhibition in cancer

In order to reduce overexpressed oncogenic miRNAs, several overlapping strategies can be employed, all of which are based on sequence-specific inhibitors that lead to sequestration or induced cleavage of the target. In a broad sense, miRNA inhibitors can be grouped into synthetic antisense oligonucleotides (so-called antagomirRs or antimiRs) and expressed decoys (known as sponges). Direct miRNA inhibitors are essentially antisense oligonucleotides (AS-ODN) complementary to the mature miRNA target, which are generally chemically modified to improve activity, stability (resistance to nucleases), and delivery [98] (Figure 5.3). Typically, these AS-ODN inhibitors are administered without delivery vehicle when used *in vivo*, although conjugation to cholesterol and other functional groups has been used to improve uptake [99].

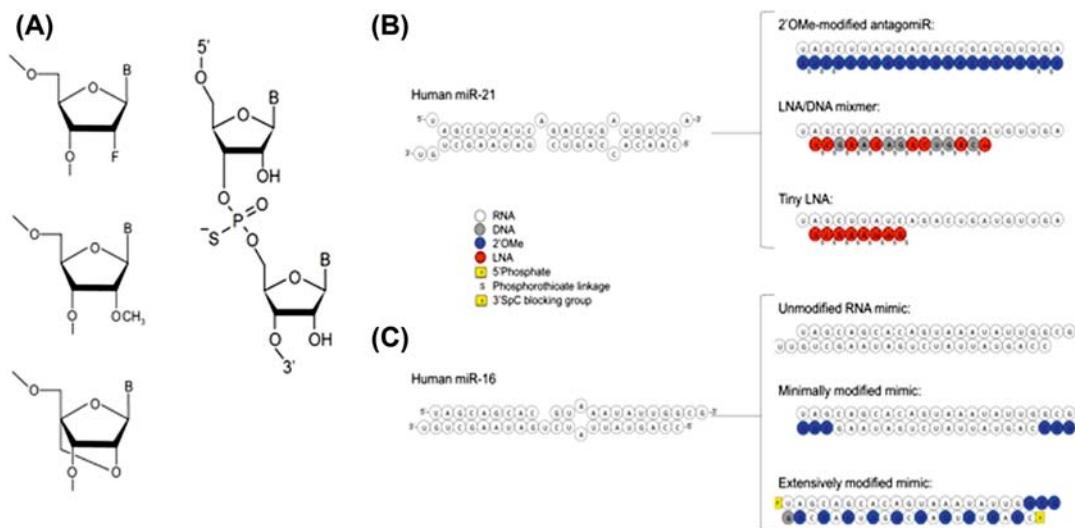


FIGURE 5.3 Common chemical modification strategies for synthetic miRNA inhibitors and mimics.

(A) Modified sugars commonly used in synthetic oligonucleotides include 2' F (*top left*), 2' O-Me (*middle left*), and LNA ribose modifications, all of which can impart resistance to nucleases and/or prevent off-target effects and immune stimulation. Introducing phosphorothioate (PS) linkages (*right*) between nucleotides also provides nuclease resistance. (B) Common types of miRNA inhibitors are shown, using the human miR-21 sequence as target. Antagomirs (*top*) consist of a fully 2' O-Me-modified oligo, with terminal PS; LNA/DNA mixmers (*middle*) are, as the name suggests, a mix of LNA-modified ribose and deoxyribose nucleotides, and are usually around 15–22 nucleotides; tiny LNAs (*bottom*) are fully LNA-modified oligos specific for the seed sequence, and have a minimum length of eight nucleotides. Both mixmers and tiny LNAs have fully PS-modified backbones. (C) Examples of mimics of the human miR-16 sequence are shown. An unmodified double-stranded RNA (*top*), with fully complementary sense strand is active but can give rise to off-target effects and stimulation of the innate immune system. More common are minimally (*middle*) or extensively (*bottom*) modified mimics. (NB: All examples are illustrative only and have not necessarily been tested for activity.)

Typical sugar modifications include 2' O-Me and locked nucleic acid (LNA) substitutions, both of which increase binding affinity and nuclease resistance of the AS-ODN. Inhibitors with 2' O-Me modification (consisting of a substitution of the 2' hydroxyl with a methoxy moiety), so called “antagomirs,” were one of the first to be shown to inhibit miRNA activity [99]. With only moderate affinity for the cognate sequence, antagomir needs to be used at relatively high concentrations. In the case of LNA substitutions, the 2' O, 4' C-methylene bridge causes the nucleotide to be locked in an RNA-mimicking conformation, and the resulting antimirs show greatly increased affinity for target miRNAs. In addition, this increased affinity enables LNA-based antimirs of much shorter length to maintain activity. This has been exploited in the case of so-called Tiny LNAs, 8-nucleotide long AS-ODN, that inhibit either single miRNAs or miRNA families via interaction with the seed sequence in the miRNA target [100]. Finally, changes in the phosphodiester backbone also impart desirable properties to AS-ODN inhibitors. Most commonly used is the phosphorothioate modification, consisting of replacement of a nonbridging oxygen atom with sulfur [98]. See Figure 5.3.

Now that they have become a very common reagent in cancer research, the use of miRNA inhibitors *in vivo* with the aim of developing anticancer therapies is also increasing. Examples of oncogenic miRNAs that have been targeted by inhibitors include the well-characterized oncomiRs miR-21, miR-155, the miR-17~92 cluster, and others. While a comprehensive review of all *in vivo* studies is beyond the scope of this review, a brief overview is provided below. One of the first oncogenic miRNAs identified, miR-21, is commonly upregulated in cancer, and has been linked to inhibition of a range of tumor suppressor genes [101] and drug resistance [102]. While experimental inhibition of miR-21 via genetic knockout or transfection of inhibitors prior to implantation leads to tumor growth inhibition and has revealed details of the function of miR-21 (and other miRNAs), these methods are of limited clinical relevance. Several groups, however, have employed miR-21 inhibitors of various types using various routes of administration in preclinical xenograft models. For example, direct intratumoral injection of antimir-21 was able to inhibit growth of glioblastoma [103,104], multiple myeloma [105], and tongue squamous cell carcinoma tumors [106], while systemic delivery via intraperitoneal injection resulted in reduced growth of subcutaneous breast [107] or colon cancer xenografts [108]. This effect was enhanced by coadministration of an miR-181b-1 antimir [108]. There is also evidence that the inhibition of miR-21 following intratumoral injection has antiangiogenic effects, further hampering tumor growth [107].

Further studies have reported silencing of additional cancer-associated miRNAs with systemic antisense treatment in tumor models in mice. Intratumoral injections of a cholesterol-conjugated antagomiR of miR-17-5p resulted in striking inhibition of xenografts derived from *MYCN*-amplified neuroblastoma cells [109]. In a breast cancer model, miR-19a was able to reduce tumor growth, and antimir treatment was synergistic in effect when combined with taxol [110]. Employing a tiny LNA approach, allograft tumors in a model of medulloblastoma were treated by intravenous injection of 8-mer LNA AS-ODN targeting either the miR-17 or miR-19 seed sequences [111], resulting in a decrease in tumor growth. In line with its pro-metastatic role, intravenous administration of a miR-10b antagomiR led to a reduction in breast cancer metastases with minimal effects on the primary tumor [112]. In a neuroblastoma model, intraperitoneal injection with anti-miR-380 led to a greatly reduced tumor mass [113].

The oncomiR miR-155 has also been silenced *in vivo*. Intratumoral injection of anti-miR-155 reduced tumor growth in subcutaneous breast cancer xenografts [114], while a tiny LNA antimir strategy was successful in B-cell lymphoma [115]. Interestingly, and although not a cancer-related study, it was recently shown that tiny LNAs targeting the seed sequence could effectively inhibit miR-33a/b in nonhuman primates [116], paving the way for similar approaches targeting cancer-associated miRNA families in patients.

In addition, miRNA decoys—known as sponges—have also been used to inhibit oncomiR function. These sponges consist of expression constructs in which the RNA has multiple miRNA target sites, which “mop up” the miRNA thereby limiting regulation of *bona fide* targets [117]. Such a strategy has been used to demonstrate that the metastasis-reducing effects of miR-10b inhibition are tumor cell specific, and not due to effects on the host microenvironment [112]. Similarly, a miR-9 sponge reduced the levels of miR-9, and subsequent development of metastases, by half in the same model [112]. Despite their utility in experimental systems *in vitro* and *in vivo*, the application of sponges in a clinical setting will face greater hurdles, as they require access to the nucleus for expression. Nevertheless, long-term depletion of miR-122 was achieved in the mouse liver using an AAV-driven sponge construct [118], suggesting that such a strategy may be useful in HCC.

4.2 miRNA replacement in cancer

A number of miRNA families with tumor suppressor activity are downregulated in cancer. These tend to be those most conserved throughout evolution (e.g., let-7, miR-15/16, miR-34, and miR-200), which control pathways essential for development and cell proliferation. Restoring the miRNA levels thus enables control over these growth-promoting pathways to be re-exerted. Experimentally, this can be achieved using miRNA expression vectors (either plasmid or virus encoded), but therapeutic applications have generally employed synthetic miRNA mimics complexed with a variety of liposomal and/or nanoparticle-based delivery vehicles.

In early experiments, mimics were designed with structures (and sequences) equivalent to the endogenous form produced by Dicer processing, where guide and passenger strand are not fully homologous. In contrast, more recent designs have used artificial passenger strands with complete homology [119]. Although this introduces a nonnatural sequence into the cells, off-target effects can be avoided through chemical modification (see below). Furthermore, in common with small-interfering RNAs (siRNAs), mimic duplexes are effective with overhangs, or as blunt-ended double-stranded RNAs.

In addition to duplex structure, chemical modification of miRNA mimics has also borrowed heavily from strategies used for siRNA. A detailed discussion of these approaches is beyond the scope of the current review, but in brief, 2' O-Me and LNA substitutions can be included without loss of miRNA function (Figure 5.3, and reviewed for siRNA [120] and miRNA [119]). It should also be kept in mind that many studies using mimics *in vivo* use one of the various proprietary modification strategies found in off-the-shelf reagents. An important point to note is that modifications of the passenger strands of miRNA mimics are tolerated more readily than those of the mature miRNA (guide) strand. Typically, modifications in and around the seed sequence of the guide strand are avoided. Also, as the goal with miRNA mimics is generally to prevent misleading of the passenger strand, this can be terminally modified with 2' O-Me to prevent loading, while at the same time avoiding phosphorothioate and LNA modifications will permit nuclease degradation of the passenger strand following duplex unwinding and the loading of the mature miRNA into the RISC [119].

As in the case of inhibitors, synthetic (or expressed) mimics of tumor suppressor miRNAs can be introduced into tumor cells before implantation to investigate effects on tumor growth but again, this is of limited clinical relevance. Here, we will consider only mimics that have been used as a therapeutic approach in preclinical tumor models. The first study of miR-34a mimics as a therapeutic approach focused on a lung cancer model and demonstrated that both intratumoral and intravenous administrations of lipid-formulated mimic could inhibit xenograft growth [121]. Of relevance to therapeutic applications, the mimics also had effects in cancer cells with normal miR-34a expression [121]. The same miR-34a formulation was later shown to inhibit autochthonous lung tumors [122] and to reduce the growth of orthotopic prostate xenografts and the incidence of lung metastases derived from these tumors [123]. As the miR-34a mimic was able to inhibit the function of the cancer stem cell population [123], this underlines the importance of this miRNA and its relevance as a therapeutic target.

The first study to target miR-16 as a therapeutic approach used atelocollagen to systemically deliver a miR-16 mimic in a metastatic prostate cancer model [124]. This reduced overall tumor burden by decreasing the number of metastases. Similarly, in a model of multiple myeloma,

treatment with either miR-15a or miR-16 formulated in neutral lipid emulsion was able to greatly inhibit tumor formation following intravenous injection of tumor cells [125]. Finally, restoring miR-16 levels in a malignant pleural mesothelioma model using a miR-16 mimic encapsulated in minicells was able to strongly inhibit tumor growth [126]. As predicted targets of the miR-15/16 family include VEGF, inhibition of angiogenesis may play a role in tumor growth inhibition, as suggested by the reduced number of vessels in miR-15a-treated tumors [125].

4.3 Small-molecule-based miRNA modulation

A number of drugs have been shown to have effects on miRNA expression, although in most cases these are not miRNA-specific effects. Like protein-coding genes, many miRNAs are epigenetically silenced in cancer, and this can be reversed by demethylating agents [70]. For example, decitabine was shown to produce upregulation of the expression of epigenetically silenced miRNAs and was used to identify three miRNAs commonly silenced in metastatic cancer [127]. Small molecules that modulate RNA processing can also affect levels of mature miRNAs. The antibacterial fluoroquinolone enoxacin binds the protein TARRNA-binding protein 2, a partner of Dicer, and increases the levels of several mature miRNAs, including miR-16, and leads to growth inhibition in a range of cancer cells [128]. Likewise, curcumin derivatives have been shown to alter the expression of miR-21 [129,130] and other selected miRNAs [130] both *in vitro* and *in vivo*, possibly due to inhibition of NF- κ B signaling [130]. Similarly, other agents, such as kinase inhibitors, which alter transcription factor expression, also contribute to miRNA regulation but are beyond the scope of this chapter.

4.4 miRNA therapy in clinical trials

Although in recent years many preclinical studies have shown that delivery of miRNA inhibitors or mimics is able to reduce tumor growth *in vivo*, translation into clinical application in humans remains challenging, and only few candidates have entered clinical trials.

The first miRNA inhibitor to enter clinical trial was *Miravirsen* (Santaris Pharma), an LNA-based antimiR that sequesters miR-122, which is an essential factor for HCV replication in the liver. In completed clinical trials, it has proved to be well tolerated and effective in reducing HCV titers [131]. Additional inhibitors that have entered clinical trial have mainly been developed by Regulus Therapeutics, who following positive preclinical data have initiated clinical trials for *RG-101* (miR-122 inhibitor) for hepatitis infection, *RG-012* (miR-21 inhibitor) for Alport syndrome (genetic kidney disorder), and *RGLS4326* (miR-17 inhibitor) for polycystic kidney disease. While the clinical trial for *RG-101* has been terminated due to occurrence of hyperbilirubinemia in patients treated with the inhibitor, phase I trials of *RG-012* and *RGLS4326* have been completed with *RG-012* (*Lademirsen*) moving forward to a phase II trial (NCT02855268) for continued assessment of safety and tolerability as well as efficacy of subcutaneous injections of the inhibitor. Another inhibitor designed to be delivered by subcutaneous injection is the miR-92 inhibitor *MRG-110* by Miragen Therapeutics. In the context of wound healing, MRG-110 is currently evaluated in a phase I clinical trial (NCT03603431) of intradermal injection in healthy volunteers. Furthermore, a dose-escalation phase I trial (European Clinical Trials Database [EudraCT] No. 2017-004180-12) of intravenous injection on healthy volunteers has been completed and shown that both miR-92 and several of its target genes were downregulated in peripheral blood cells following administration of

the inhibitor [132]. In the context of cancer, Miragen Therapeutics was evaluating *Cobomarsen* (RG-106, a miR-155 inhibitor) in patients with cutaneous T-cell lymphoma; however, the trial (NCT03713320) was terminated early due to business reasons. Further inhibitors in clinical development by Miragen have recently been reviewed by Chakraborty et al. [133].

The first miRNA replacement therapy that entered clinical trial was *MRX34*, a miR-34 mimic developed by MiRNA Therapeutics (now Synlogic). Following initially very positive signs from the multicentre phase I trial [134], in 2016, the trial (NCT01829971) had to be suspended due to occurrence of five immune-related serious adverse events (SAEs). Nevertheless, data available from the trial prior to termination showed successful upregulation of miR-34 in the tumors as well as downregulation of miR-34 target genes in white blood cells of the treated patients [135]. The reasons underlying the SAEs are not yet fully understood; however, the same liposomal carrier had been successfully used in a previous study [136], leading the authors to speculate that either the specific activity of the miR-34 mimic or an inflammatory effect of the mimic formulation was responsible for the SAEs. Another miRNA replacement therapy evaluated in a completed phase I clinical trial is delivery of so-called “TargomiR,” a miR-16 mimic to patients with malignant pleural mesothelioma (NCT02369198). Following promising preclinical data [126], the MesomiR-1 trial evaluated safety and tolerability of miR-16 mimic containing bacterially derived minicells (developed by EnGeneIC Ltd), which were coated with bispecific antibodies against EGFR to achieve targeting of EGFR overexpressing tumor cells. The dose-escalation study revealed a maximum tolerated dose of 5×10^9 TargomiRs once weekly, which was associated with an acceptable toxicity profile [137]. In addition, the authors reported preliminary efficacy data from this phase I study, revealing stable disease being achieved in 68% of the treated mesothelioma patients. Interestingly, the study also reported one patient with a partial response, and a “complete” metabolic response on PET-CT scan 8 weeks after start of treatment [138]. Although at present no other miRNA replacement therapies are in clinical trial, the encouraging data from the MesomiR-1 trial highlight the suitability of miRNA replacement strategies for clinical use, and further trials are eagerly awaited.

5 Future perspectives

Both preclinical and clinical studies have shown that targeting aberrant miRNA expression has great promise for cancer therapy. However, optimism in the field is tempered somewhat by the continuing difficulties in achieving controlled delivery, and the various off-target effects related to stimulation of the innate immune system and cell-type specific (opposing) roles of miRNAs in different cells. Furthermore, it should be kept in mind that miRNAs with oncogenic functions in one cancer type can be tumor suppressive in another [139], and also that dysregulated miRNAs expression in cancer can be changed in the opposite direction in other diseases (e.g., miR-15 [140] and miR-34 [141]). Nevertheless, first results from phase I clinical trials have shown that miRNA therapeutics can be successful in humans. While delivery of miRNAs remains one of the major challenges (reviewed in [142,143]), we can hope that the advancements in RNA-based therapeutics achieved as a result of the development of vaccines against the SARS-CoV-2 virus, will also positively influence the development of miRNA-based therapeutics.

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List of abbreviations

AGO	Argonaute
AS-ODN	Antisense oligonucleotides
CpG	CG sites
DGCR	DiGeorge Syndrome Chromosome Region
DMNT	DNA Methyl Transferase
GTP	Guanosine triphosphate
LNA	Locked nucleic acid
ncRNA	Noncoding RNA
mRNA	Messenger RNA
miRNA	microRNA
PAZ	Piwi-argonaute-zwille
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
TRBP	Transactivation response element RNA-binding protein
UTR	Untranslated region

References

- [1] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116(2):281–97.
- [2] He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004;5 (7):522–31.
- [3] Malumbres M. miRNAs and cancer: an epigenetics view. *Mol Asp Med* 2013;34(4):863–74.
- [4] Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993;75(5):843–54.
- [5] Wightman B, Bürglin TR, Gatto J, Arasu P, Ruvkun G. Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes Dev* 1991;5(10):1813–24.
- [6] Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* 1993;75(5):855–62.
- [7] Reinhart BJ, Slack FJ, Basson M, et al. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 2000;403(6772):901–6.
- [8] Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, Ruvkun G. The lin-41 RBCC gene acts in the *C. elegans* heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol Cell* 2000;5(4):659–69.
- [9] Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science* 2001;294(5543):853–8.
- [10] Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* 2001;294(5543):858–62.

- [11] Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* 2001;294(5543):862–4.
- [12] Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* 2006;13(12):1097–101.
- [13] Lee Y, Kim M, Han J, et al. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* 2004;23(20):4051–60.
- [14] Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* 2004;18(24):3016–27.
- [15] Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J* 2002;21(17):4663–70.
- [16] Bohnsack MT, Czaplinski K, Gorlich D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* 2004;10(2):185–91.
- [17] Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 2003;115(2):199–208.
- [18] Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev* 2001;15(20):2654–9.
- [19] Peters L, Meister G. Argonaute proteins: mediators of RNA silencing. *Mol Cell* 2007;26(5):611–23.
- [20] Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 2004;15(2):185–97.
- [21] Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell* 2003;115(7):787–98.
- [22] Pasquinelli AE, Reinhart BJ, Slack F, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 2000;408(6808):86–9.
- [23] Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009;19(1):92–105.
- [24] Saito T, Saetrom P. MicroRNAs-targeting and target prediction. *N Biotechnol* 2010;27(3):243–9.
- [25] Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci U S A* 2007;104(23):9667–72.
- [26] Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* 2007;27(1):91–105.
- [27] Stark A, Brennecke J, Bushati N, Russell RB, Cohen SM. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution. *Cell* 2005;123(6):1133–46.
- [28] Lim LP, Lau NC, Garrett-Engele P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 2005;433(7027):769–73.
- [29] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008;9(2):102–14.
- [30] Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 2006;125(6):1111–24.
- [31] Brengues M, Teixeira D, Parker R. Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science* 2005;310(5747):486–9.
- [32] Parker R, Song H. The enzymes and control of eukaryotic mRNA turnover. *Nat Struct Mol Biol* 2004;11(2):121–7.
- [33] Younger ST, Pertsemlidis A, Corey DR. Predicting potential miRNA target sites within gene promoters. *Bioorg Med Chem Lett* 2009;19(14):3791–4.
- [34] Huang V, Li LC. miRNA goes nuclear. *RNA Biol* 2012;9(3):269–73.
- [35] Shu J, Silva BVRe, Gao T, Xu Z, Cui J. Dynamic and modularized microRNA regulation and its implication in human cancers. *Sci Rep* 2017;7(1):13356.

- [36] Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435(7043):834–8.
- [37] Li M, Li J, Ding X, He M, Cheng SY. microRNA and cancer. *Aaps j* 2010;12(3):309–17.
- [38] Hanahan D. Hallmarks of cancer: new dimensions. *Cancer Discovery* 2022;12(1):31–46.
- [39] Lionetti MC, Cola F, Chepizhko O, et al. MicroRNA-222 regulates melanoma plasticity. *J Clin Med* 2020;9(8):2573.
- [40] Jansson MD, Lund AH. MicroRNA and cancer. *Mol Oncol* 2012;6(6):590–610.
- [41] Vandenboom Ii TG, Li Y, Philip PA, Sarkar FH. MicroRNA and cancer: tiny molecules with major implications. *Curr genomics* 2008;9(2):97–109.
- [42] Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* 2002;99(24):15524–9.
- [43] Calin GA, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* 2006;66(15):7390–4.
- [44] Laurila EM, Kallioniemi A. The diverse role of miR-31 in regulating cancer associated phenotypes. *Genes Chromosom Cancer* 2013;.
- [45] Ivanov SV, Goparaju CM, Lopez P, et al. Pro-tumorigenic effects of miR-31 loss in mesothelioma. *J Biol Chem* 2010;285(30):22809–17.
- [46] Moody HL, Lind MJ, Maher SG. MicroRNA-31 Regulates Chemosensitivity in Malignant Pleural Mesothelioma. *Mol Ther Nucleic Acids* 2017;8:317–29.
- [47] Cekaite L, Rantala JK, Bruun J, et al. MiR-9, -31, and -182 deregulation promote proliferation and tumor cell survival in colon cancer. *Neoplasia* 2012;14(9):868–79.
- [48] He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. *Nature* 2005;435(7043):828–33.
- [49] Suarez Y, Fernandez-Hernando C, Yu J, et al. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. *Proc Natl Acad Sci U S A* 2008;105(37):14082–7.
- [50] Sylvestre Y, De Guire V, Querido E, et al. An E2F/miR-20a autoregulatory feedback loop. *J Biol Chem* 2007;282(4):2135–43.
- [51] Gao B, Gao K, Li L, Huang Z, Lin L. miR-184 functions as an oncogenic regulator in hepatocellular carcinoma (HCC). *Biomed Pharmacother* 2014;68(2):143–8.
- [52] Li P, Xu Q, Zhang D, et al. Upregulated miR-106a plays an oncogenic role in pancreatic cancer. *FEBS Lett* 2014;588(5):705–12.
- [53] Mace TA, Collins AL, Wojcik SE, Croce CM, Lesinski GB, Bloomston M. Hypoxia induces the overexpression of microRNA-21 in pancreatic cancer cells. *J Surg Res* 2013;184(2):855–60.
- [54] Müller DW, Bosserhoff AK. Integrin beta 3 expression is regulated by let-7a miRNA in malignant melanoma. *Oncogene* 2008;27(52):6698–706.
- [55] Dang D, Bamburg JR, Ramos DM. Alphavbeta3 integrin and cofilin modulate K1735 melanoma cell invasion. *Exp Cell Res* 2006;312(4):468–77.
- [56] Koturbash I, Zemp FJ, Pogribny I, Kovalchuk O. Small molecules with big effects: The role of the microRNAome in cancer and carcinogenesis. *Mutat Res Genet Toxicol Environ Mutagen* 2011;722(2):94–105.
- [57] Zhang X, Dong Y, Ti H, et al. Down-regulation of miR-145 and miR-143 might be associated with DNA methyltransferase 3B overexpression and worse prognosis in endometrioid carcinomas. *Hum Pathol* 2013;44(11):2571–80.
- [58] Robertson KD, Uzvolgyi E, Liang G, et al. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res* 1999;27(11):2291–8.

- [59] Fabbri M, Garzon R, Cimmino A, et al. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci U S A* 2007;104(40):15805–10.
- [60] Martinez I, Cazalla D, Almstead LL, Steitz JA, DiMaio D. miR-29 and miR-30 regulate B-Myb expression during cellular senescence. *Proc Natl Acad Sci U S A* 2011;108(2):522–7.
- [61] Wang Y, Zhang X, Li H, Yu J, Ren X. The role of miRNA-29 family in cancer. *Eur J Cell Biol* 2013;92(3):123–8.
- [62] Abdelmohsen K, Srikanth S, Kuwano Y, Gorospe M. miR-519 reduces cell proliferation by lowering RNA-binding protein HuR levels. *Proc Natl Acad Sci U S A* 2008;105(51):20297–302.
- [63] Zhou X, Wei M, Wang W. MicroRNA-340 suppresses osteosarcoma tumor growth and metastasis by directly targeting ROCK1. *Biochem Biophys Res Commun* 2013;437(4):653–8.
- [64] Chen X, Zhang Y, Yan J, Sadiq R, Chen T. miR-34a suppresses mutagenesis by inducing apoptosis in human lymphoblastoid TK6. *Cell Mutat Res Genet Toxicol Environ Mutagen* 2013;758(1–2):35–40.
- [65] Benetti R, Gonzalo S, Jaco I, et al. A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. *Nat Struct Mol Biol* 2008;15(3):268–79.
- [66] Hua Z, Lv Q, Ye W, et al. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. *PLoS One* 2006;1(1):e116.
- [67] Ruan K, Fang X, Ouyang G. MicroRNAs: novel regulators in the hallmarks of human cancer. *Cancer Lett* 2009;285(2):116–26.
- [68] Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. *Nat Rev Clin Oncol* 2014;11(3):145–56.
- [69] Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* 2009;10(10):704–14.
- [70] Baer C, Claus R, Plass C. Genome-wide epigenetic regulation of miRNAs in cancer. *Cancer Res* 2013;73(2):473–7.
- [71] Yao Q, Chen Y, Zhou X. The roles of microRNAs in epigenetic regulation. *Curr Opin Chem Biol* 2019;51:11–17.
- [72] Li LC. Chromatin remodeling by the small RNA machinery in mammalian cells. *Epigenetics* 2014;9(1):45–52.
- [73] Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol* 2009;27(34):5848–56.
- [74] Weber B, Stremann C, Brueckner B, Lyko F. Methylation of human microRNA genes in normal and neoplastic cells. *Cell Cycle (Georgetown, Tex)* 2007;6(9):1001–5.
- [75] Wang Z, Yao H, Lin S, et al. Transcriptional and epigenetic regulation of human microRNAs. *Cancer Lett* 2013;331(1):1–10.
- [76] Han L, Witmer PD, Casey E, Valle D, Sukumar S. DNA methylation regulates MicroRNA expression. *Cancer Biol Ther* 2007;6(8):1284–8.
- [77] Huidobro C, Fernandez AF, Fraga MF. Aging epigenetics: causes and consequences. *Mol Asp Med* 2013;34(4):765–81.
- [78] Schifffgen M, Schmidt DH, von Rücker A, Müller SC, Ellinger J. Epigenetic regulation of microRNA expression in renal cell carcinoma. *Biochem Biophys Res Commun* 2013;436(1):79–84.
- [79] Bandres E, Agirre X, Bitarte N, et al. Epigenetic regulation of microRNA expression in colorectal cancer. *Int J Cancer* 2009;125(11):2737–43.
- [80] Zhang L, Volinia S, Bonome T, et al. Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. *Proc Natl Acad Sci U S A* 2008;105(19):7004–9.
- [81] Jerónimo C, Bastian PJ, Bjartell A, et al. Epigenetics in prostate cancer: biologic and clinical relevance. *Eur Urol* 2011;60(4):753–66.

- [82] Hulf T, Sibbritt T, Wiklund E, et al. Discovery pipeline for epigenetically deregulated miRNAs in cancer: integration of primary miRNA transcription. *BMC Genomics* 2011;12(1):54.
- [83] Garzon R, Liu S, Fabbri M, et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood* 2009;113(25):6411–18.
- [84] Morita S, Horii T, Kimura M, Ochiya T, Tajima S, Hatada I. miR-29 represses the activities of DNA methyltransferases and DNA demethylases. *Int J Mol Sci* 2013;14(7):14647–58.
- [85] Duursma AM, Kedde M, Schrier M, le Sage C, Agami R. miR-148 targets human DNMT3b protein coding region. *RNA* 2008;14(5):872–7.
- [86] Cao Q, Mani RS, Ateeq B, et al. Coordinated regulation of polycomb group complexes through microRNAs in cancer. *Cancer Cell* 2011;20(2):187–99.
- [87] Varambally S, Cao Q, Mani RS, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science* 2008;322(5908):1695–9.
- [88] Friedman JM, Liang G, Liu CC, et al. The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. *Cancer Res* 2009;69(6):2623–9.
- [89] Kleer CG, Cao Q, Varambally S, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc Natl Acad Sci U S A* 2003;100(20):11606–11.
- [90] Varambally S, Dhanasekaran SM, Zhou M, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 2002;419(6907):624–9.
- [91] Wei X, Xiang T, Ren G, et al. miR-101 is down-regulated by the hepatitis B virus x protein and induces aberrant DNA methylation by targeting DNA methyltransferase 3A. *Cell Signal* 2013;25(2):439–46.
- [92] Stavast CJ, Erkeland SJ. The non-canonical aspects of microRNAs: many roads to gene regulation. *Cells* 2019;8(11).
- [93] Hwang HW, Wentzel EA, Mendell JT. A hexanucleotide element directs microRNA nuclear import. *Science* 2007;315(5808):97–100.
- [94] Place RF, Li LC, Pookot D, Noonan EJ, Dahlia R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A* 2008;105(5):1608–13.
- [95] Huang V, Place RF, Portnoy V, et al. Upregulation of cyclin B1 by miRNA and its implications in cancer. *Nucleic Acids Res* 2012;40(4):1695–707.
- [96] Kim DH, Saetrom P, Snøve Jr. O, Rossi JJ. MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A* 2008;105(42):16230–5.
- [97] Younger ST, Corey DR. Transcriptional gene silencing in mammalian cells by miRNA mimics that target gene promoters. *Nucleic Acids Res* 2011;39(13):5682–91.
- [98] Hydbring P, Badalian-Very G. Clinical applications of microRNAs. *F1000Res* 2013;2:136.
- [99] Stenvang J, Petri A, Lindow M, Obad S, Kauppinen S. Inhibition of microRNA function by antimiR oligonucleotides. *Silence* 2012;3(1):1.
- [100] Obad S, dos Santos CO, Petri A, et al. Silencing of microRNA families by seed-targeting tiny LNAs. *Nat Genet* 2011;43(4):371–8.
- [101] Pan X, Wang ZX, Wang R. MicroRNA-21: a novel therapeutic target in human cancer. *Cancer Biol Ther* 2010;10(12):1224–32.
- [102] Hong L, Han Y, Zhang Y, et al. MicroRNA-21: a therapeutic target for reversing drug resistance in cancer. *Expert Opin Ther Targets* 2013;17(9):1073–80.
- [103] Zhou X, Ren Y, Moore L, et al. Downregulation of miR-21 inhibits EGFR pathway and suppresses the growth of human glioblastoma cells independent of PTEN status. *Lab Invest* 2010;90(2):144–55.
- [104] Wang YY, Sun G, Luo H, et al. MiR-21 modulates hTERT through a STAT3-dependent manner on glioblastoma cell growth. *CNS Neurosci Ther* 2012;18(9):722–8.

- [105] Leone E, Morelli E, Di Martino MT, et al. Targeting miR-21 inhibits in vitro and in vivo multiple myeloma cell growth. *Clin Cancer Res* 2013;19(8):2096–106.
- [106] Li J, Huang H, Sun L, et al. MiR-21 indicates poor prognosis in tongue squamous cell carcinomas as an apoptosis inhibitor. *Clin Cancer Res* 2009;15(12):3998–4008.
- [107] Zhao D, Tu Y, Wan L, et al. In vivo monitoring of angiogenesis inhibition via down-regulation of mir-21 in a VEGFR2-luc murine breast cancer model using bioluminescent imaging. *PLoS One* 2013;8(8):e71472.
- [108] Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Mol Cell* 2010;39(4):493–506.
- [109] Fontana L, Fiori ME, Albini S, et al. Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS One* 2008;3(5):e2236.
- [110] Liang Z, Li Y, Huang K, Wagar N, Shim H. Regulation of miR-19 to breast cancer chemoresistance through targeting PTEN. *Pharm Res* 2011;28(12):3091–100.
- [111] Murphy BL, Obad S, Bihannic L, et al. Silencing of the miR-17~92 cluster family inhibits medulloblastoma progression. *Cancer Res* 2013;73(23):7068–78.
- [112] Ma L, Reinhardt F, Pan E, et al. Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. *Nat Biotechnol* 2010;28(4):341–7.
- [113] Swarbrick A, Woods SL, Shaw A, et al. miR-380-5p represses p53 to control cellular survival and is associated with poor outcome in MYCN-amplified neuroblastoma. *Nat Med* 2010;16(10):1134–40.
- [114] Zheng SR, Guo GL, Zhai Q, Zou ZY, Zhang W. Effects of miR-155 antisense oligonucleotide on breast carcinoma cell line MDA-MB-157 and implanted tumors. *Asian Pac J Cancer Prev* 2013;14(4):2361–6.
- [115] Zhang Y, Roccaro AM, Rombaoa C, et al. LNA-mediated anti-miR-155 silencing in low-grade B-cell lymphomas. *Blood* 2012;120(8):1678–86.
- [116] Rottiers V, Obad S, Petri A, et al. Pharmacological inhibition of a microRNA family in nonhuman primates by a seed-targeting 8-mer antimir. *Sci Transl Med* 2013;5(212):212ra162.
- [117] Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods* 2007;4(9):721–6.
- [118] Xie J, Ameres SL, Friedline R, et al. Long-term, efficient inhibition of microRNA function in mice using rAAV vectors. *Nat Methods* 2012;9(4):403–9.
- [119] Henry JC, Azevedo-Pouly AC, Schmittgen TD. MicroRNA replacement therapy for cancer. *Pharm Res* 2011;28(12):3030–42.
- [120] Rettig GR, Behlke MA. Progress toward in vivo use of siRNAs-II. *Mol Ther* 2012;20(3):483–512.
- [121] Wiggins JF, Ruffino L, Kelnar K, et al. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. *Cancer Res* 2010;70(14):5923–30.
- [122] Trang P, Wiggins JF, Daige CL, et al. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol Ther* 2011;19(6):1116–22.
- [123] Liu C, Kelnar K, Liu B, et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nat Med* 2011;17(2):211–15.
- [124] Takeshita F, Patrawala L, Osaki M, et al. Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. *Mol Ther* 2010;18(1):181–7.
- [125] Sun CY, She XM, Qin Y, et al. miR-15a and miR-16 affect the angiogenesis of multiple myeloma by targeting VEGF. *Carcinogenesis* 2013;34(2):426–35.
- [126] Reid G, Pel ME, Kirschner MB, et al. Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma. *Ann Oncol* 2013;24(12):3128–35.
- [127] Lujambio A, Calin GA, Villanueva A, et al. A microRNA DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci U S A* 2008;105(36):13556–61.

- [128] Melo S, Villanueva A, Moutinho C, et al. Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing. *Proc Natl Acad Sci U S A* 2011;108(11):4394–9.
- [129] Roy S, Yu Y, Padhye SB, Sarkar FH, Majumdar AP. Difluorinated-curcumin (CDF) restores PTEN expression in colon cancer cells by down-regulating miR-21. *PLoS One* 2013;8(7):e68543.
- [130] Yang CH, Yue J, Sims M, Pfeffer LM. The curcumin analog EF24 targets NF- κ B and miRNA-21, and has potent anticancer activity in vitro and in vivo. *PLoS One* 2013;8(8):e71130.
- [131] Janssen HL, Reesink HW, Lawitz EJ, et al. Treatment of HCV infection by targeting microRNA. *N Engl J Med* 2013;368(18):1685–94.
- [132] Abplanalp WT, Fischer A, John D, et al. Efficiency and target derepression of anti-miR-92a: results of a first in human study. *Nucleic Acid Ther* 2020;30(6):335–45.
- [133] Chakraborty C, Sharma AR, Sharma G, Lee SS. Therapeutic advances of miRNAs: a preclinical and clinical update. *J Adv Res* 2021;28:127–38.
- [134] Beg MSBM, Sachdev J, Hong DS, Smith S, Bader A, Stoudemire J, et al. Multicenter phase I study of MRX34, a first-in-class microRNA miR-34 mimic liposomal injection. In: AACR annual meeting, San Diego, April 5–9; 2014.
- [135] Hong DS, Kang YK, Borad M, et al. Phase 1 study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours. *Br J Cancer* 2020;122(11):1630–7.
- [136] Tolcher AW, Rodriguez WV, Rasco DW, et al. A phase 1 study of the BCL2-targeted deoxyribonucleic acid inhibitor (DNAi) PNT2258 in patients with advanced solid tumors. *Cancer Chemother Pharmacol* 2014;73(2):363–71.
- [137] van Zandwijk N, Pavlakis N, Kao SC, et al. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *Lancet Oncol* 2017;18(10):1386–96.
- [138] Kao SC, Fulham M, Wong K, et al. A significant metabolic and radiological response after a novel targeted microRNA-based treatment approach in malignant pleural mesothelioma. *Am J Respir Crit Care Med* 2015;191(12):1467–9.
- [139] Ling H, Fabbri M, Calin GA. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. *Nat Rev Drug Discov* 2013;12(11):847–65.
- [140] Hullinger TG, Montgomery RL, Seto AG, et al. Inhibition of miR-15 protects against cardiac ischemic injury. *Circ Res* 2012;110(1):71–81.
- [141] Bernardo BC, Gao XM, Winbanks CE, et al. Therapeutic inhibition of the miR-34 family attenuates pathological cardiac remodeling and improves heart function. *Proc Natl Acad Sci U S A* 2012;109(43):17615–20.
- [142] Forterre A, Komuro H, Aminova S, Harada M. A comprehensive review of cancer microRNA therapeutic delivery strategies. *Cancers (Basel)* 2020;12(7).
- [143] Momin MY, Gaddam RR, Kravitz M, Gupta A, Vikram A. The challenges and opportunities in the development of microRNA therapeutics: a multidisciplinary viewpoint. *Cells* 2021;10(11).

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Long noncoding RNA in human cancers: to be or not to be, that is the question

6

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1 IncRNAs in cancer have come of age

Although already discovered in the 1990s, lncRNAs [1–3], now defined as transcripts >200 nucleotides lacking an open reading frame (ORF) and thus not translated [4], were at first classified as “evolutionary junk.” Therefore the discovery and annotation of these transcripts has been very limited in the pregenomic era.

The first two decades of the 21th century, however, have been marked by the revelation that the fraction of protein-coding genes in the human genome is only about 1.2% [5–8]. These findings and the advent of high-throughput sequencing technologies have fostered the interest toward lncRNAs and enriched their catalog, although the dissection of their functional roles is still lagging behind [9–12]. In the last two decades the rapid discovery of novel lncRNAs in many species led to the proposition of multiple molecular mechanisms through which lncRNAs may exert their functions, thus adding an unprecedented layer of complexity in gene expression regulation, but also generating a lot of skepticism around their physiological relevance. In addition, driven mostly by

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the field of transcription and epigenetics, the characterization of lncRNAs has been biased for a long time, toward nuclear transcripts.

To date, as reported in the latest human genome release GRCh38 (GENCODE version 40), 53,029 transcripts are annotated in the human genome as bona fide lncRNAs, generated by 17,748 validated and 1057 “To be Experimentally Confirmed” (TEC) lncRNA genes. Being generally dispensable for most physiological processes and predominantly primate-specific, these transcripts have so far raised more questions than answers. Recently, a general model has been proposed to explain the profound effect exerted by nonconserved and lowly expressed lncRNAs in cancer [13]. This model posits that lncRNAs overcome the limitations of stoichiometry by regulating condensate formation and thus places this class of non-coding RNAs as crucial and direct mediators of the tumor microenvironmental cues [13].

Many classifications have been proposed for lncRNAs, based on the genomic localization, sense of transcription, localization of modulated genes, and/or molecular function [9,11,14,15]. However, all these classifications become blurry when considering the complexity and dynamics of the cancer genome. Recent work from the Delattre group for instance has shown that in Erwing sarcoma, chimeric transcription factors drive the expression of neogenes [16]. In addition, although the magnitude of this phenomenon and its physiological relevance need to be further clarified, several studies indicate that small peptides can arise from lncRNA transcripts [16–21]. Considering all the above, a revision of the current artificial definitions of (non)coding genes and (non)coding transcripts is now not only logical but also necessary.

Beyond all controversies, however, the pathogenic role of lncRNAs have been described in both tumor onset and progression [15,22,23]. In addition, in line with their participation to multiple cellular processes (e.g., cell proliferation, differentiation, apoptosis, stemness, and migration), several lncRNAs have been reported as altered or mutated in multiple pathological conditions [22,24–26]. A clear relevance for lncRNAs as diagnostic and/or prognostic markers has been indicated by omics-data (e.g., from TCGA Consortium, Cancer LncRNA Census, and TANRIC platform), revealing sequence and/or expression alterations in cancer-specific lncRNAs and their association with different cancer hallmarks, and confirmed by the implementation of their use in the clinic (e.g., *PCA3*; *PCAT-1*) [15,22–24,27–29].

In this chapter, we will focus on the relatively unexplored universe of lncRNAs interacting with (and/or regulating) the protein synthesis machinery, as this specific process is of outmost importance not only for tumorigenesis but also for cancer progression. Furthermore, we will also discuss how lncRNAs inspired and directed therapies may provide a cancer-specific strategy to target this process for cancer treatment.

2 Regulation of rRNA biogenesis

Ribosome biogenesis, one of the most energy consuming process in the cell, begins in the nucleolus with the transcription of the rRNAs, and requires the coordinated activity of all the three polymerases and of a large number of transcription factors, nucleases and small noncoding RNAs that contribute to the processing and maturation of the rRNAs [30].

Growing evidence indicates that lncRNAs may positively or negatively modulate ribosome biogenesis in the nucleoli at different steps: (1) by affecting the nucleolar structure, (2) by affecting rRNA and ribosomal protein transcription, or (3) by affecting rRNA maturation and ribosomal protein assembly [31].

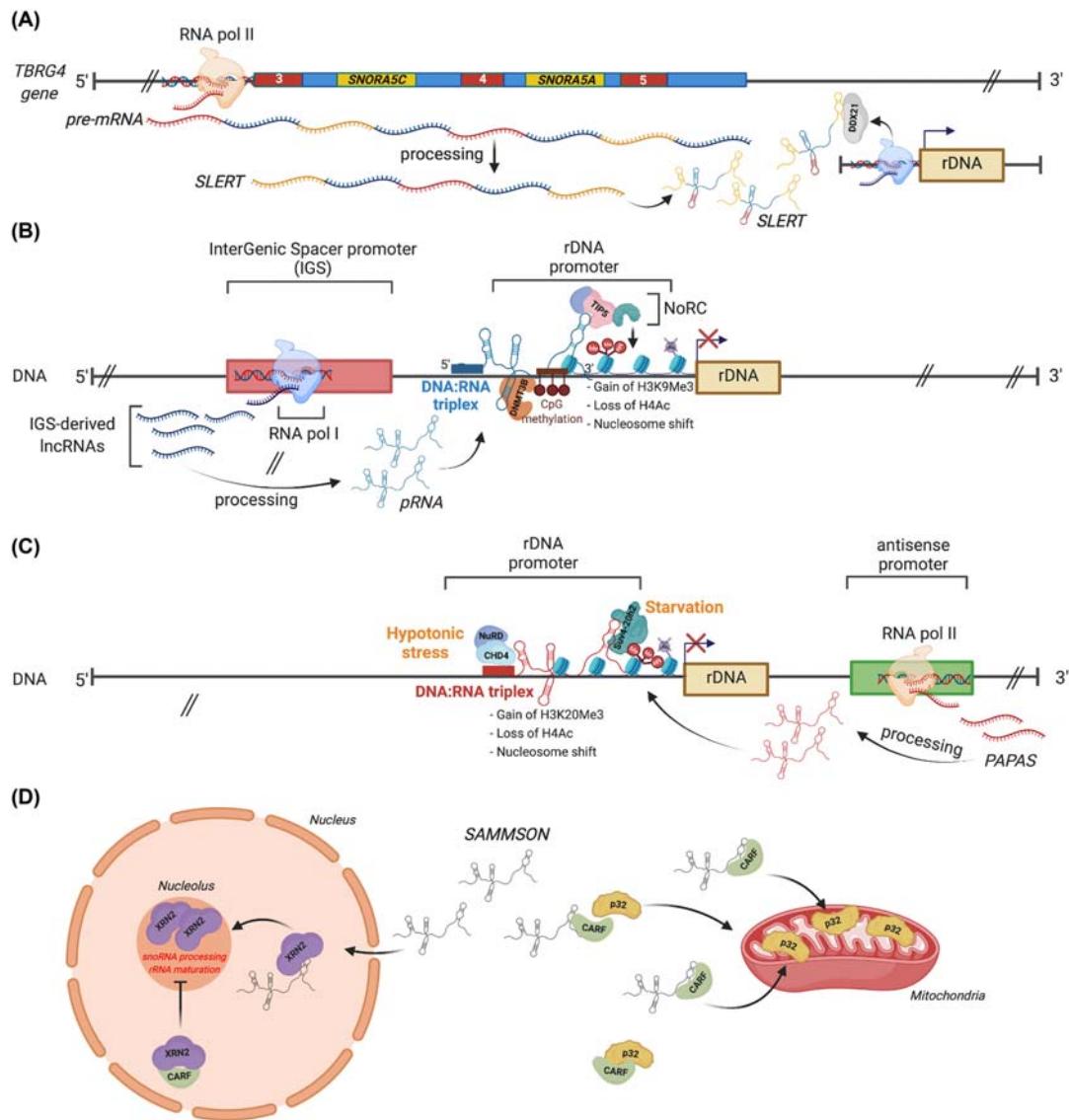
Among them, lncRNAs transcribed from the intergenic spacer sequences (IGS) that separate rDNA gene clusters play a major role in dictating nucleolus structural integrity and modulating epigenetically rDNA transcription. Acting through protein binding (or sequestration), these lncRNAs are involved in chromatin modifications, as well as in the formation and modulation of small nuclear/nucleolar ribonucleoproteins and in translation [32]. In the following paragraphs, we will focus on lncRNAs displaying a positive or negative activity on rDNA transcription as well as rRNA processing.

2.1 Positive regulators of rDNA transcription and rRNA processing

One of the first hints that RNA pol II-transcribed lncRNAs may have a positive role on the modulation of rRNA biogenesis came from the identification of rDNA-derived spliced polyadenylated transcripts termed *DISNOR187* and *DISNOR238* [33]. In 2013 a distal flanking sequence (DJ) at the telomeric side of the acrocentric chromosomes was shown to be transcriptionally active, rather than just a passive heterochromatic region [33]. In particular, two distinct active promoters have been identified, 187- and 238-kb upstream of the rDNA promoter, leading to the production of *DISNOR187* and *DISNOR238*, respectively. Depletion of these transcripts in human cells activates the nucleolar stress response pathway and suppresses the transcription of rDNA. However, to date, nothing is known about their mechanism of action on rRNA biogenesis and genome stability, as well as their connection to human diseases, including cancer [34].

Among the lncRNAs involved in the positive modulation of rDNA transcription, the SnoRNA-ended lncRNA that Enhances preribosomal RNA Transcription (*SLERT*) probably represents the most relevant example. This 694-nt-long transcript was identified for its high expression in human embryonic stem cells as a H/ACA small nucleolar RNA (snoRNA) originating from the splicing of an intron of the Transforming Growth Factor Beta Regulator 4 (*TBRG4*) pre-mRNA [35] (Figure 6.1A). From a structural point of view, *SLERT* contains the sequences of two snoRNAs at the 5' (*SNORA5C*) and 3' (*SNORA5A*) ends, required for its nucleolar translocation. Mechanistically, *SLERT* binds the DEAD-box RNA helicase 21 (DDX21), and inhibitor of rDNA transcription. In the nucleolus, DDX21 forms ring-shaped structures coupled to RNA polymerase I. Interestingly, the binding of *SLERT* to DDX21 ATP domain alters protein conformation to enlarge DDX21 rings and loosens the interaction with RNA pol I subunits, thus promoting rDNAs' transcription [35,36] (Figure 6.1A). In line with the need of tumor cells for *de novo* protein synthesis and increased ribosome biogenesis, the oncogenic role of *SLERT* has been demonstrated by its depletion and overexpression in cells and xenograft models, and *SLERT/DDX21* axis has been proposed as a potential target for anticancer drugs [35].

Another class of lncRNAs that recently emerged as positive regulators of rDNA transcription are the Alu RNAs. These lncRNAs are actively transcribed from Alu elements – particularly abundant in rDNA genes [37] – that belong to the class of retrotransposons and constitute about 10% of the entire human genome. The presence of repeats and of recombination sites in the flanking sequences of Alu elements [38] makes these regions hotspots of genetic instability in cells and a

**FIGURE 6.1** IncRNAs involved in rRNA biogenesis.

In the nucleolus—where ribosome biogenesis takes place starting from rDNA transcription—distinct IncRNAs contribute to processing/maturation of rRNAs. (A) Schematic representation of the contribution of *SLERT* IncRNA on the regulation of rDNA transcription. Processed upon the splicing of the intron 3 of *TBRG4* pre-mRNA, *SLERT* is characterized by the presence of snoRNA sequences (depicted in yellow) at its ends. Its binding to DDX21 (in gray)—an helicase that generally impedes RNA pol I (in cyan) to actively transcribe

(Continued)

major cause of malignant transformation, especially through the disruption of tumor suppressor genes promoters and coding regions [39]. Interestingly, Alu RNAs are highly abundant in nucleoli and may be required for the maintenance of the nucleolar structure [40,41]. Notably, Alu RNA silencing is associated with decreased amounts of rRNA and with the dispersion of some essential nucleolar proteins, including nucleolin and RNA pol I [40]. Lastly, the Survival Associated Mitochondrial Melanoma-specific Oncogenic Noncoding RNA (*SAMMSON*) was shown to coordinately boost rRNA biogenesis in the nucleus and in the mitochondria during melanomagenesis, to increase cancer cell fitness [42]. Toward this, *SAMMSON* interacts with three major regulators of rRNA maturation in both compartments, namely 5'-3' eXoRiboNuclease 2 (XRN2), Collaborator of ARF (CARF) and p32. As CARF negatively regulates XRN2 localization to nucleoli where it contributes to the maturation of the 18S rRNA, *SAMMSON*-mediated sequestration of CARF in the cytoplasm increases the processing of the 18S rRNA [42]. In addition, on the cytosolic side, *SAMMSON* promotes and aberrant interaction between CARF and p32, overall promoting p32 localization to the mitochondria and mitochondrial rRNA maturation [42]. In keeping with this, pharmacological inhibition of *SAMMSON* have been shown to deliver efficient antimelanoma responses alone or in combination with MAPKi [43].

2.2 Negative regulators of rDNA transcription and rRNA processing

LncRNA can also regulate negatively transcription and maturation of rRNAs. Promoter-associated RNAs, or *pRNAs*, are 250–300 nucleotide long lncRNAs transcribed from IGS by RNA pol I from an alternative promoter 2 kb upstream of the main rDNA promoter and processed by the exosome complex 8 [44–46]. The helicase DHX9 was shown to be a crucial factor in *pRNAs* processing [47]. A typical *pRNA* exerts a dual function on rDNA transcription mediated by the 5' and 3'

-
- ◀ rDNA—causes a conformational change in this complex allowing rRNA transcription by Pol I. (B) Transcribed from the IGS promoter (dark pink box), *pRNA* (in blue) plays a dual role on the transcription of rDNA, thanks to 5' and 3' ends displaying distinct features. Forming a DNA:RNA triplex, *pRNA* recruits DNMT3b (in orange) at the rDNA promoter increasing CpG islands' methylation and causing transcriptional inhibition. Moreover, through its 3' end stem-loop structure *pRNA* interacts with TIP5 (in light pink) and engages the nucleolar remodeling complex (whose components are depicted in violet and dark green) close to rDNA promoter inducing NoRC-dependent heterochromatin formation and nucleosome shift and impeding pol I to access rDNA promoter. (C) Transcribed from a promoter antisense to the rDNA gene cluster, the lncRNA *PAPAS* (in red) inhibits pre-rRNA synthesis under starvation by binding Suv4–20h2—an histone methyltransferase (depicted in dark green)—that induces H4K20Me3 and chromatin repression. Moreover, upon hypotonic stress, *PAPAS* binds CHD4 (cyan) and the NuRD complex (blue) inducing histone deacetylation and nucleosome shift which in turn inhibit rDNA transcription. (D) *SAMMSON* (depicted in black)—aberrantly expressed in melanoma—sequesters in the cytoplasm of melanoma cells with CARF (green), an RNA-binding protein that (in normal cells) limits the access of XRN2 (violet) to the nucleoli. High *SAMMSON* expression in tumor cells promotes the interaction of CARF with p32 in the cytoplasm at the expense of CARF/XRN2 interaction; in turn, it favors the mitochondrial localization of p32 (right panel) and the nucleolar localization of XRN2 (left panel). Hence, *SAMMSON* promotes snoRNA processing and pre-rRNA maturation (and thus protein synthesis) in both cytosol and mitochondria.

sequence, respectively. Specifically, the 5' end of *pRNAs* forms a DNA:RNA triplex with a regulatory element of the rDNA promoter, causing the transcriptional silencing of rRNA genes via the redirection of DNMT3b to CpG islands in the proximity of rDNA promoter [44,48] (Figure 6.1B, left panel). In addition, by forming a stem-loop structure at its 3' end, *pRNAs* can interact with Transcription termination factor I-interacting protein 5 (TIP5), one of the Nucleolar Remodeling Complex (NoRC) components. Hence, by acting as a scaffold, *pRNA* lncRNAs engage this repressive complex close to rDNA promoter inducing NoRC-dependent heterochromatin formation. Indeed, *pRNA*-NoRC complex increases heterochromatic histone marks (H3K9me3 and H4K20me3), while decreasing H3K4me3 (euchromatic mark) and induces nucleosome shift, which in turn impedes RNA pol I to access the rDNA promoter [45] (right panel). As for other IGS-derived lncRNAs, sequence variations in the genomic region encompassing *pRNAs* have been negatively correlated with its expression levels in cancer cells and associated with malignant transformation in lung [49,50]. In line with their role in keeping genomic stability through heterochromatin formation at telomeres and centromeres [52], *pRNA* lncRNAs are likely to act as tumor suppressors.

Among the IGS-derived transcripts, it is worth mentioning the Promoter And Pre-rRNA Antisense (*PAPAS*) lncRNA. This lncRNA is transcribed by RNA pol II antisense to the rDNA gene clusters and has sequence complementary both to the rDNA promoter and the rDNA coding region. Following its induction during starvation, *PAPAS* inhibits pre-rRNA synthesis [52] (Figure 6.1C) by guiding the histone methyltransferase Suv4–20h2 to rDNA genes, thus causing an increase of H4K20Me3 and the compaction of chromatin that becomes inaccessible to RNA pol I [52] (Figure 6.1C). *PAPAS* overexpression upon hypotonic stress instead, inhibits rDNA transcription, via a distinct mechanism. In hypotonic conditions, *PAPAS* interacts with the heat-shock ATPase CHD4 and the adenosine triphosphatase subunit of the Nucleosome Remodeling and Deacetylase (NuRD) complex [53], causing histones' deacetylation and a 24-nt downstream shift of the promoter-bound nucleosome in a position that is refractory to transcription initiation [54] (Figure 6.1C). *PAPAS* has been recently found overexpressed in blood and tumor biopsies of patients with hepatocellular carcinoma [55] and triple-negative breast cancer [56]. Its overexpression *in vitro* has been associated with miR-188-5p downregulation and HCC cell proliferation [55], as well as with the repression of miR-34a and reduced cell migration/invasion of breast cancer cells [56]. A tumor suppressive effect of *PAPAS*—through the downregulation of *HOTTIP* lncRNA—has been recently reported in papillary thyroid carcinomas [57].

Environmental stress, and particularly the induction of heat shock proteins, has been positively correlated with the increase of three other 300-400-nucleotide-long ribosomal IGS lncRNAs enriched in (TC)_n or (GA)_n simple repeats [58]. As the sites of active transcription of these lncRNA are located at about 16, 22, and 28 kb from rDNA, they have been named *IGS16*, *IGS22*, and *IGS28*, respectively. The overexpression of these transcripts is associated with transcriptionally inactive nucleoli (e.g., for *IGS16* and *IGS22*) or, in the case of *IGS28* accumulation, with the loss of nucleolar structure. These IGS lncRNAs interact with the nucleolar detention signal of several proteins, including cell cycle regulators and chaperons in the nucleolus causing the formation of “nucleolar caps” and leading to the inhibition of rRNA synthesis [59]. Due to their involvement in DNA replication under stress conditions they may be among the first targets in the oncogenic process [32]. Noteworthy, another 10-kb-long lncRNA is actively transcribed by RNA pol I from the 28-kb IGS region, that is, the Pyrimidine-rich Non-Coding Transcript [60] (*PNCTR*). Although

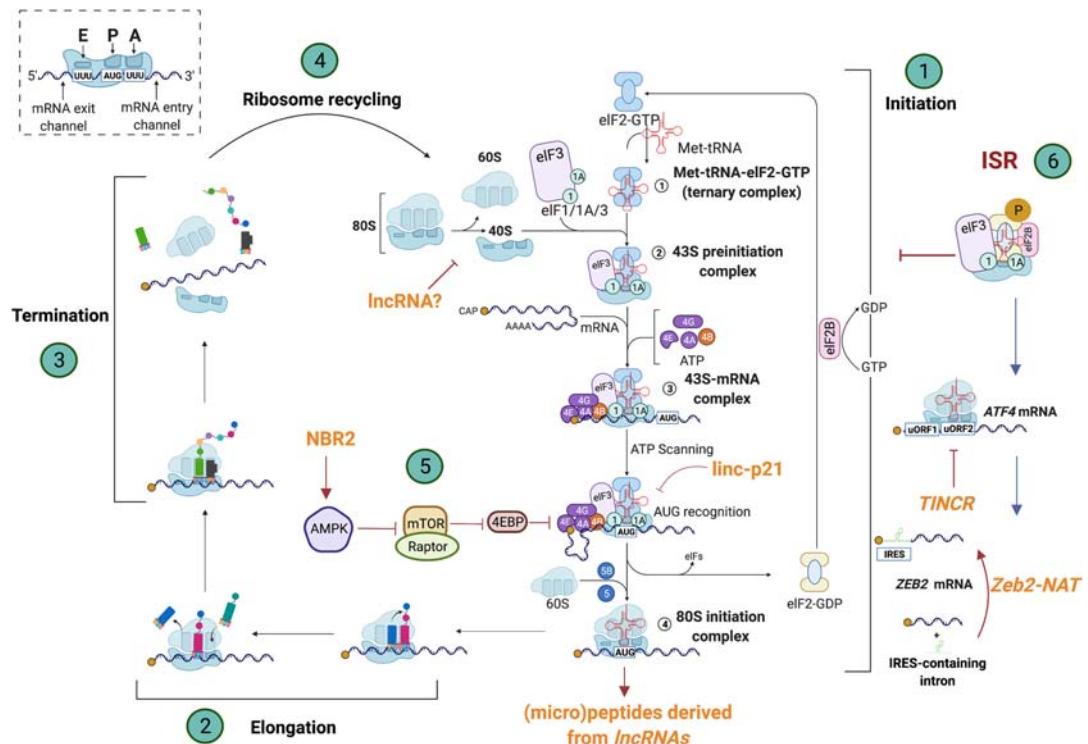


FIGURE 6.2 IncRNA involved in translation.

① Translation starts with the incorporation of the recruitment of the small ribosomal subunit (40S) to a new 43S preinitiation complex: a new Met-tRNA bound to the ternary complex (formed by eIF2-GTP), together with eIF1, eIF1A, eIF3, eIF5, and 40S base pair with the rRNA at the P-site. After the formation of the 43S preinitiation complex, the 5' cap region of the mRNA is recognized in an ATP-dependent manner by eIF4F and eIF4B, leading the 43S complex to bind this mRNA. The new 43S-mRNA complex starts the scan of the 5' UTR to 3' direction, until the recognition of the initiation codon AUG. The eIFs are then released, eIF5 hydrolyzes the GTP bound to the eIF2, with the release of Pi and eIF2-GDP. The large ribosomal subunit (60S) can then join the complex to form a fully mature ribosome (80S), leading to progression into the elongation phase. ② In this phase, new tRNAs, complementary to the mRNA codons, are recruited in the P site of the 80S and induce the elongation of the nascent polypeptide, until a stop codon occurs. ③ At this point, the peptide is released, and the ribosomal subunits disassemble from the mRNA, ④ ready again to be recycled in a new initiation process. A well-established tumor suppressor IncRNA, lincRNA-p21 base pairs with *CTNNB1* and *JUNB* mRNA and causes their association with the translational repressors RCK and FMRP, thus leading to the attenuation of their translation. ⑤ In condition of stress, when the cell needs to spare energy CAP-dependent translation is shut down via the inhibition of mTOR by the activation of AMPK that inhibits the mTOR pathway. The IncRNA NBR2 reduce translation by binding to AMPK and enhancing its kinase activity. Downregulation of NBR2 leads to unchecked cell cycle with consequent increase in tumor development and bad prognosis. ⑥ Several intracellular and extracellular stimuli (e.g., viral infection and endoplasmic reticulum stress) induce the ISR. During this response, cap-dependent translation is abolished by stabilization of the phosphorylated form of eIF2alpha, which cannot be incorporated anymore in the ternary complex. In melanoma, the IncRNA TINCR binds to *ATF4* mRNA and other ISR mRNAs blocking their translation and impairing metastatic progression. Upon induction of EMT, the IncRNA Zeb2-NAT binds Zeb2 RNA and lead to inclusion of an IRES in the transcript which leads to translation of this mRNA.

PNCTR has a relevant role in cancer cells by inhibiting apoptosis, its activity has to be ascribed rather to mRNA splicing than to rRNA biogenesis [60].

Lastly, in principle, lncRNAs may also increase or impair ribosome assembly, even though, to the best of our knowledge, no transcript with this function has been reported yet (Figure 6.2).

3 Regulation of translation in cancer

Protein synthesis is a complex multistep process that starts in the nucleus with ribosome biogenesis and terminates in the cytoplasm where the mRNA translation takes place. Quantitative and qualitative reprogramming of translation are required for tumor initiation, to provide highly dividing cells with sufficient energy and essential metabolites, and during progression and drug responses to survive the hostile environment [61,62]. Consistent with above, all the major oncogenic and tumor suppressive pathway in the cell intersect to regulate this process (e.g., MYC and PTEN signaling) [63].

In response to endogenous and exogenous stimuli, different oncogenic pathways, including PI3K-AKT, RAS-MAPK converge to sustain protein synthesis [5,6]. Importantly, genetic alteration in various components of the translational machinery synergizing with the oncogenic signaling has been detected in cancer [63].

Not surprisingly, numerous ribosome biogenesis and translation inhibitors are already in use in the clinic for the treatment of cancer [63].

Translation can be divided in four main steps: initiation, elongation, termination, and ribosome recycling. With only ~0.5–3.6 initiations per minutes against 3–10 amino acid per second during elongation [62,64,65], initiation can be considered the rate limiting step of the whole translation process. Therefore it is also the step that is most tightly controlled. Most mRNA in normal conditions are translated by the so-called CAP-dependent translation. During this process, the eukaryotic initiation factor 4F (eIF4F) recognizes and binds to the 5' cap structure on the mRNA, leading to the recruitment of the 40S ribosomal subunit to the 5' end of the mRNA to form a 43S complex, comprising the 40S, eIF5, eIF3, eIF1, eIF1A, eIF2 and the Met-tRNA_i. Next, the 40S subunit scans down the mRNA in a 5'-3' direction until a start codon is encountered, thus leading to the recruitment of the 60S subunit to form a full ribosome (80S), and to the start of protein synthesis. However, in condition of stress, when the cell needs to spare energies and preserve genome integrity, CAP-dependent translation is shut down via the activation of AMP-activated protein Kinase (AMPK) that inhibits the mammalian target of rapamycin (mTOR) pathway [66]. Simultaneously, to ensure the synthesis of a minimum pool of proteins essential for survival and/or to overcome the stress, alternative translation mechanisms become active. Those alternative mechanisms, independent of one or more initiation factors, rely mostly on structural motifs (e.g., Internal Ribosome Entry Sites or IRES and other 5' RNA structures) or on alternative initiation sites (e.g., upstream IRF or uORFs) for ribosome recruitment and translational regulation. Although not validated yet, the possibility that some lncRNAs may become integral part of the ribosome and drive the translation of specific mRNAs have also been proposed [67].

While high translational levels, sustained by the activation of oncogenes, are required to support unrestrained growth during tumor initiation, translation rewiring through activation of CAP-

independent mechanisms such the activation of the Integrated Stress Response (ISR) are mostly associated with tumor progression and therapy resistance [68–70], as they foster the generation of quiescent drug-tolerant cell subpopulations [71]. An intriguing exception is the finding that in non-melanoma skin cancer uORFs could fuel tumor growth by acting as translation activators [72]. Regulation of CAP-dependent and alternative translation via lncRNAs has been already reported. A few key examples are reported in the following paragraph.

4 Regulation of translation by lncRNAs

Regulation of translation by lncRNAs can occur at all steps during the process. For instance, lncRNAs can indirectly affect translation by regulating the activity of the upstream signaling cascades. A key example of such a lncRNA is represented by neighbor of *BRCA1* gene 2 (*NBR2*) that regulates AMPK. Upon energy imbalance *NBR2* binds to AMPK and enhances its kinase activity, thus leading to the attenuation of translation [73]. In keeping with this, *NBR2* low expression correlates with bad prognosis in some human cancers and its downregulation leads to unchecked cell cycling and increased tumor development *in vivo* [73]. LncRNAs, however, can also recruit specific transcripts to the ribosomes via sense-antisense partial base-pairing [74] and/or via the recruitment of specific RNABPs to the mRNA. This last case is exemplified by the human lncRNA lincRNA-p21, a transcript with tumor suppressive functions, known to be associated with ribosomes. In human cervical carcinoma (HeLa) cells lincRNA-p21 interact via imperfect base-pairing with *CTNNB1* and *JUNB* mRNA and stabilizes them. The binding of lincRNA-p21 triggers the association of these mRNAs with the translational repressors RCK and FMRP, leading to the attenuation of their translation [75]. Another lncRNA directly interacting with mRNAs to regulate their translation is Tissue Differentiation-Inducing Non-Protein Coding RNA (*TINCR*) [76]. In melanoma, *TINCR* recognizes the mRNA of the Activating Transcription Factor 4 (*ATF4*) and other ISR players, possibly through base pair recognition, thus preventing their translation. As *ATF4* activation is an important step toward melanoma phenotype switching and a driver of invasion [68], overexpression of *TINCR* *in vivo* in PDX models decreases metastasis formation and overcomes resistance to MAPK inhibition [76]. Another intriguing example of how lncRNA regulates translation of target gene and induce tumor progression is represented by the lncRNA Zeb2-Natural Antisense Transcript (*Zeb2-NAT*). Zeb2 is a key regulator of epithelial-to-mesenchymal transition (EMT) not only during physiological development but also in cancer progression [77]. Zeb2 is translated thanks to an IRES present in a 5' intron, that is spliced out in normal conditions. Upon induction of the EMT, Zeb2-NAT base pairs to Zeb2 RNA mask the intron–exon boundary, leading to intron retention and expression of the IRES. Translation of Zeb2 through this mechanism leads to E-cadherin repression and EMT [78].

4.1 Dismantling the dogmas: coding–noncoding RNA in cancer

Considering that most lncRNAs are transcribed by the RNA polymerase II and thus share many features with mRNAs, one unresolved question is what are the key features that allow them to escape translation even if they interact with the ribosomes. One obvious answer is that lncRNAs

are, generally speaking, less abundant than protein-coding genes and thus they have to compete with many abundant mRNA to be translated. In addition, evidence is emerging that although rare, translation of lncRNAs into (micro)peptides actually occurs in specific situations and may be altered during tumorigenesis [67]. Some of the previously mentioned neo transcripts identified in Erwing sarcoma for instance, are translated into proteins [16]. In addition, certain stress responses, occurring when the biosynthetic processes are shut down (e.g., the induction of the ISR), may favor the transcription/stability of aberrant transcripts and/or the binding to the ribosome and translation of less abundant non-coding RNA species.

Importantly, lncRNAs with coding potential and lncRNA-derived (micro)peptides have been found also into extracellular vesicles and thus they can be exchanged with the tumor microenvironment [18]. All together these findings open up to a re-evaluation of the current theories about (lnc)RNA emergence and function, particularly in cancer where the cells are subjected to a high evolutionary pressure that accelerates the selection of neo features. This new dimension unravels novel exciting possibilities for therapeutic intervention.

4.2 Targeting translation in cancer: the lesson of lncRNAs

It is now established that lncRNAs are of extraordinary diagnostic and prognostic value as cancer biomarkers. For instance, several patents have been issued based on the expression analysis of lncRNAs, such as Metastasis-Associated Lung Adenocarcinoma Transcript 1 (*MALAT1*; CN104498495) and Prostate CAncer gene 3 (*PCA3*; US8551699B2), proposed as prognostic and diagnostic biomarkers in prostate cancer. Similarly, *MALAT1* (CN105586399A) and HOX Transcript Antisense Intergenic RNA (*HOTAIR*; CN105586399A) quantification have been proposed in gastric cancer.

Given their cancer-specific expression and the variety cancer-relevant processes they have been implicated in, lncRNAs are also promising therapeutic targets. In this sense, different strategies can be adopted to modulate their expression. For instance, the targeting of lncRNAs can be based on transcriptional, post-transcriptional or steric inhibitions, aiming to modulate the expression, the stability or the processing of lncRNAs, as well as the formation of secondary structure or protein interactions. Moreover, methodological advancements have largely improved *in vitro* RNA transcription [79,80] and delivery systems, for reaching high efficiency integration and high-fidelity expression of lncRNAs both for *in vitro* assays and for increasing their expression *in vivo* [81]. These technical advancements, also in light of the rapid progression in the design and use of RNA vaccines occurred in the last years, will likely prompt the future research efforts toward the adoption of injection-based strategies to overexpress lncRNAs with relevant anticancer activities (e.g., tumor suppressors) or those showing the potential to modulate specific cellular processes (including ribosome biogenesis and translation).

Several loss-of-function studies are based on RNA interference (RNAi) approaches, whose large employment is justified by their versatility and easy use. The silencing of lncRNAs is commonly performed by double-stranded small-interfering RNAs (dsRNAs), whose mechanism of action involves Dicer and RISC complexes, as well as Argonaute 2 (Ago2) protein [82]. Although RNAi-mediated targeting mainly acts at post-transcriptional level, transcriptional gene silencing may also be induced [83,84]. Some additional chemical modifications (e.g., 2'-O methyl sugar residues and phosphorothioate bonds at the 3'-end) have been used to reduce their susceptibility to enzymatic

degradation [85]. Even so, widely used for analyzing the molecular and functional effects of lncRNAs' silencing in cancer cell lines, the use of RNAi-based approaches found more limitations for *in vivo* studies. For several lncRNAs, siRNA-mediated knockdown has been instrumental for assessing their oncogenic or oncosuppressor activities, as well as to clarifying the mechanisms of action and target genes. For instance, the knockdown by siRNAs of *Colon Cancer Associated Transcript 1 (CCAT1)* lncRNA—performed both *in vitro* human cells and in mouse xenografts—revealed its oncogenic properties and suggested its targeting as promising approach for increasing the survival in colon cancer [86]. Moreover, a similar outcomes have been obtained by knocking down with siRNAs the oncogenic lncRNA *MALAT1* in breast cancer [87,88] and the Cytosolic Oncogenic antisense to MET Transcript (*COMETT*) in thyroid cancer cells [89]. Particularly, *MALAT1* inhibition is sufficient to block metastatic capacity of breast [87,88] and *COMETT* knock-down reduces oncogenic properties of thyroid cancer cells and increases their sensitivity to B-raf inhibitors [89].

A valid alternative approach to RNAi, able to transcriptionally modulate lncRNAs, is represented by the antisense oligonucleotides (ASOs). ASOs are DNA-based single strand oligos that engage RNase-H-mediated degradation of target RNA, acting also on nascent transcripts and able to efficiently silence even nuclear lncRNAs [90,91]. Furthermore, additional modifications (e.g., 2'-O-methoxyethylation, S-constrained ethylation, and locked nucleic acids) have been inserted for improving RNase-H-mediated cleavage, ASOs' stability and specificity [92]. Notably, fully-modified ASOs—including morpholinos and splice-switching oligos—are able to induce a steric inhibition, blocking contact interfaces between lncRNA, proteins or splicing regulatory elements [93–95]. Thus the long half-life, high resistance to endonucleases, low toxicity and the locally containment in the application site, confer to ASOs a marked potential for clinical use [91,96,97], as also confirmed by the ASO-based FDA-approved compound for the treatment of spinal muscular atrophy [98]. Thus their use *in vivo* has been successfully adopted for studying lncRNAs in preclinical settings [90,92,97]. For instance, ASOs-mediated silencing, of *SAMMSON*, both *in vitro* and in patient-derived xenograft models, indicated that its inhibition reduces cutaneous melanoma growth and results in tumor regression in combination with MAPK inhibition [43]. Additionally its silencing can also impair the growth and viability of uveal melanomas [99]. Similarly, among several lncRNAs, ASO-mediated knockdown has been instrumental for assessing the role of *MALAT1* and 20 mammary tumor-associated RNAs (*MaTARs*) in breast cancer metastasis [87,100], as well as of the lncRNA HOXA10 antisense RNA (*HOXA10-AS*) in oral squamous cell carcinoma [101] and of a novel lncRNA—AC104041.1—in head and neck squamous carcinoma [102]. Moreover, the ASO-mediated targeting of *MALAT1* in human lung cancer cells and murine xenografts further confirmed the beneficial effects in terms of metastasis reduction [103].

The high number of studies based on RNAi- or ASO-mediated knockdown of lncRNAs has been increasingly revealing the feasibility to adopt these approaches in cancer therapy. Accordingly, the therapeutic potential of RNA-based molecules has been clearly demonstrated by FDA and/or the European Medicines Agency (EMA) approval for 11 siRNAs- or ASOs-based therapeutics able to modulate gene expression or pre-mRNA splicing [104,105], further suggesting that the inclusion of lncRNA-based therapeutics in the clinic is approaching. Moreover, lncRNAs have also inspired clinically relevant targeting approaches for the modulation of mRNAs translation e/o stability. For instance, synthetic *in vitro* transcribed lncRNAs (*SINEUPs*) may be designed for targeting antisense sequence increasing the translation of sense mRNAs and ribozymes or

deoxyribozymes catalyzing protein-independent RNA cleavages [106–112]. Thus, the modularity and the ability of *SINEUPs* to *trans*-enhance protein synthesis make feasible to design effective *SINEUPs*-based therapeutics [111] able to compensate haploinsufficient genes or enhancing “protective” protein functions, including those compromised by the alteration of lncRNAs.

Lastly, the advent of genome editing approaches based on the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Associated protein 9 (Cas9) system, provides further interesting opportunities for genomic modulation of lncRNAs [113–116]. In its simplest forms, the type II CRISPR-system modulates the target genes taking advantage of the precision of a chimeric single-guide RNA (sgRNA) able to orientate the nuclease Cas9 — acting also as helicase—to specific genomic sites [113]. Different systems are based on the generation and repair of double-stranded breaks (DSB), adopting, for example, excision approaches (CRISPRn excision), nonhomologous end joining (CRISPRn mutagenesis) or homology-directed repairs (CRISPRn homologous recombination) [116–118].

However, it has been estimated that only 38% of lncRNAs can be specifically targeted by classical CRISPR/Cas9 systems [118]. Indeed, the incomplete annotation of their promoters, interaction motifs and the complexity of their structure and mechanisms of actions pose some limitations to the efficacy of standard CRISPR-based approaches. For instance, the use of the CRISPRn method requires a complete knowledge of the motifs, whereas the adoption of HDR-based CRISPR approaches needs the precise knowledge of the sequences and the exclusion of promoters’ overlap which can lead to “off-target” effects. However, further opportunities have been provided by innovative CRISPR approaches overcoming classical editing [89,116], such as the Double Excision CRISPR KnockOut (DECKO) system—based on the combination of CRISPR interference (CRISPRi) and “Dead”-Cas9 (dCas9)—or CRISPR inhibition (CRISPRi) and CRISPR activation (CRISPRa) systems, originated by the fusion of Cas9 with specific effector domains (e.g., the Krüppel-associated box domain or activation domains of VP64, p65) [29,89,116,118–121]. Notably, CRISPR/Cas9 systems—both standard and based on the combination with specific effectors for epigenetic silencing—have been adopted for silencing different lncRNA, such as *MALAT1* [122–124], whereas the CRISPR-mediated tagging and regulation of lncRNAs (CTRL) method has been successfully used for inducing the upregulation of *HOTAIR*, *DICER1-AS1* and *PTENP1*, among others [116,125]. Moreover, lncRNA targeting has been induced by CRISPR systems using different Cas enzymes, such as the CRISPR/Cas13 systems (class 2, type VI) adopted for targeting very long intergenic noncoding (vlnc) RNAs and labeling *NEAT1* lncRNA [126–128].

Thus, the possibility to target lncRNAs at different functional levels and the rapid advancements in methodology and technological approaches will provide a wide range of lncRNA-based therapeutic approaches for various pathological conditions, including human cancers in which the pathogenic relevance of lncRNA is by this time undisputed.

5 Conclusion and perspective

As highlighted previously, inhibition of translation and translation rewiring are emerging as promising therapeutic strategies in cancer. However, targeting these processes specifically in cancer cells, still represents a challenge as it often results into the development of unwanted toxicities (see for

instance elongation inhibitors and PI3Ki [129]). As more and more lncRNAs communicating with the translation machinery are discovered and characterized, the resulting knowledge may be exploited for the development of better anticancer drugs. The existence of lncRNAs not detectable in normal tissues, able to regulate ribosome biogenesis (e.g., *SAMMSON*) and/or to control a defined translational program (e.g., *TINCR*, *LISR*), will lead to the design of cancer-selective therapies able to overcome toxicities resulting from noncancer cell targeting.

In addition, the possibility to artificially customize ribosomes to execute a specific gene expression program by specific lncRNAs and/or the enforcement of specific noncoding transcripts translation is a promising field for the development of novel immunotherapies. Finally, in this chapter we covered only cytosolic translation. However, as mitochondrial translation [71,130,131] and metabolism are emerging as essential pathways in cancer progression and therapy resistance, a deeper knowledge of the mitochondrial translational machinery and its lncRNA-mediated communication [132] with the cell nucleus would certainly reveal new targeting opportunities (Table 6.1).

TABLE 6.1 LncRNAs in ribosome biogenesis and translation regulation.

Name	Function	Interactors	References
Neighbor of BRCA1 and 2 (<i>NBR2</i>) <i>lincRNA-p21</i>	Enhancement of AMPK activity Proliferation, cell cycle, metabolism, and reprogramming. CTNNB1 and JUNB translation	AMPK <i>CTNNB1JUNB</i>	[73] [75]
Tissue differentiation-inducing noncoding RNA (<i>TINCR</i>)	Inhibition of melanoma metastatic spreading	ATF4 and ISR mRNA	[68,76]
Natural antisense RNA ZEB2 (<i>Zeb2-NAT</i>)	Alternative splicing of <i>Zeb2</i>	<i>Zeb2</i>	[78]
Ribosome InterGenic Spacer (<i>IGS</i>)	Protein immobilization in the nucleolus	rDNA	[58,59]
SnoRNA-ended lncRNA that enhances preribosomal RNA transcription (<i>SLERT</i>)	Promotion of pre-rRNA transcription	DDX21(DEAD-box RNA helicase 21)	[35,36]
<i>Alu</i> RNAs	Maintenance of the nucleolar structure	NCL (Nucleolin)	[39,40]
Survival associated mitochondrial melanoma-specific oncogenic noncoding RNA (<i>SAMMSON</i>)	rRNA maturation	XRN2, p32CARF	[42,43]
Promoter-associated RNAs (<i>pRNAs</i>)	Silencing of rDNA transcription	rDNAs, TIP5	[44,45]

(Continued)

TABLE 6.1 LncRNAs in ribosome biogenesis and translation regulation. *Continued*

Name	Function	Interactors	References
Promoter And Pre-rRNA AntiSense (<i>PAPAS</i>)	rDNA silencing	Histone methyltransferase Suv4–20h2Heat-shock ATPase CHD4	[52–54]
Metastasis-associated lung adenocarcinoma transcript 1 (<i>MALAT1</i>)	Transcriptional regulator of gene involved in cancer metastasis, cell migration and cell cycle regulation	hnRNPs, splicing factors, chromatin remodeler, and RNA editing enzymes	[87,88,103]
Prostate CAncer gene 3 (<i>PCA3</i>)	Control of prostate cancer and cell survival	Cancer-related miRNAs	[133–135]
HOT transcript antisense intergenic RNA (<i>HOTAIR</i>)	Triggering EMT and the acquisition of stemness	Polycomb Repressive Complex 2 (PRC2) and Lysine-Specific Demethylase 1 (LSD1) complexes	[136,137]
Colon cancer-associated transcript 1 (<i>CCAT1</i>)	Promote tumor formation	CCCT-binding factor (CTCF) polycomb repressive complex 2 (PRC2) suppressor of variegation 3–9 homolog 1 (SUV39H1)	[86]

References

- [1] Brannan CI, et al. The product of the H19 gene may function as an RNA. *Mol Cell Biol* 1990;10(1):28–36.
- [2] Brockdorff N, et al. The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell* 1992;71(3):515–26.
- [3] Brown CJ, et al. The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell* 1992;71(3):527–42.
- [4] Rinn JL, et al. The transcriptional activity of human Chromosome 22. *Genes Dev* 2003;17(4):529–40.
- [5] Lander ES, et al. Initial sequencing and analysis of the human genome. *Nature* 2001;409(6822):860–921.
- [6] Venter JC, et al. The sequence of the human genome. *Science* 2001;291(5507):1304–51.
- [7] Johnson JM, et al. Dark matter in the genome: evidence of widespread transcription detected by microarray tiling experiments. *Trends Genet* 2005;21(2):93–102.
- [8] Birney E, et al. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 2007;447(7146):799–816.
- [9] Derrien T, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res* 2012;22(9):1775–89.
- [10] Moran VA, Perera RJ, Khalil AM. Emerging functional and mechanistic paradigms of mammalian long non-coding RNAs. *Nucleic Acids Res* 2012;40(14):6391–400.
- [11] Ma L, Bajic VB, Zhang Z. On the classification of long non-coding RNAs. *RNA Biol* 2013;10(6):925–33.
- [12] Lowe R, et al. Transcriptomics technologies. *PLoS Comput Biol* 2017;13(5):e1005457.
- [13] Adnane S, Marino A, Leucci E. LncRNAs in human cancers: signal from noise. *Trends Cell Biol* 2022.

- [14] Fabbri M, et al. Decrypting noncoding RNA interactions, structures, and functional networks. *Genome Res* 2019;29(9):1377–88.
- [15] Aprile M, et al. LncRNAs in Cancer: From garbage to Junk. *Cancers (Basel)* 2020;12(11).
- [16] Vibert J, et al. Oncogenic chimeric transcription factors drive tumor-specific transcription, processing, and translation of silent genomic regions. *Mol Cell* 2022.
- [17] Ruiz-Orera J, et al. Long non-coding RNAs as a source of new peptides. *Elife* 2014;3:e03523.
- [18] Almeida A, et al. Urinary extracellular vesicles contain mature transcriptome enriched in circular and long noncoding RNAs with functional significance in prostate cancer. *J Extracell Vesicles* 2022;11(5):e12210.
- [19] Pang Y, Mao C, Liu S. Encoding activities of non-coding RNAs. *Theranostics* 2018;8(9):2496–507.
- [20] Wu P, et al. Emerging role of tumor-related functional peptides encoded by lncRNA and circRNA. *Mol Cancer* 2020;19(1):22.
- [21] Xing J, et al. LncRNA-encoded peptide: functions and predicting methods. *Front Oncol* 2020;10:622294.
- [22] Huarte M. The emerging role of lncRNAs in cancer. *Nat Med* 2015;21(11):1253–61.
- [23] Chi Y, et al. Long non-coding RNA in the pathogenesis of cancers. *Cells* 2019;8(9).
- [24] Niland CN, Merry CR, Khalil AM. Emerging roles for long non-coding RNAs in cancer and neurological disorders. *Front Genet* 2012;3:25.
- [25] Fang Y, Fullwood MJ. Roles, functions, and mechanisms of long non-coding RNAs in cancer. *Genomics Proteom Bioinforma* 2016;14(1):42–54.
- [26] Gao Y, et al. Comprehensive characterization of somatic mutations impacting lncRNA expression for pan-cancer. *Mol Ther Nucleic Acids* 2019;18:66–79.
- [27] Li J, et al. TANRIC: an interactive open platform to explore the function of lncRNAs in cancer. *Cancer Res* 2015;75(18):3728–37.
- [28] Carlevaro-Fita J, et al. Cancer LncRNA Census reveals evidence for deep functional conservation of long noncoding RNAs in tumorigenesis. *Commun Biol* 2020;3(1):56.
- [29] The ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium. Pan-cancer analysis of whole genomes. *Nature* 2020;578(7793):82–93.
- [30] Ruggero D. Revisiting the nucleolus: from marker to dynamic integrator of cancer signaling. *Sci Signal* 2012;5(241):pe38.
- [31] Verheyden Y, Goedert L, Leucci E. Control of nucleolar stress and translational reprogramming by lncRNAs. *Cell Stress* 2018;3(1):19–26.
- [32] Smirnov E, Chmúřčíková N, Cmarko D. Human rDNA and cancer. *Cells* 2021;10(12).
- [33] Floutsakou I, et al. The shared genomic architecture of human nucleolar organizer regions. *Genome Res* 2013;23(12):2003–12.
- [34] van Sluis M, et al. Human NORs, comprising rDNA arrays and functionally conserved distal elements, are located within dynamic chromosomal regions. *Genes Dev* 2019;33(23–24):1688–701.
- [35] Xing YH, et al. SLERT regulates DDX21 rings associated with Pol I transcription. *Cell* 2017;169(4):664–678.e16.
- [36] Wu M, et al. lncRNA SLERT controls phase separation of FC/DFCs to facilitate Pol I transcription. *Science* 2021;373(6554):547–55.
- [37] Gonzalez IL, Petersen R, Sylvester JE. Independent insertion of Alu elements in the human ribosomal spacer and their concerted evolution. *Mol Biol Evol* 1989;6(4):413–23.
- [38] Kupriyanova NS, et al. Non-canonical ribosomal DNA segments in the human genome, and nucleoli functioning. *Gene* 2015;572(2):237–42.
- [39] Zhang W, et al. Alu distribution and mutation types of cancer genes. *BMC Genomics* 2011;12:157.
- [40] Caudron-Herger M, et al. Alu element-containing RNAs maintain nucleolar structure and function. *EMBO J* 2015;34(22):2758–74.

- [41] Zhang XO, Gingeras TR, Weng Z. Genome-wide analysis of polymerase III-transcribed Alu elements suggests cell-type-specific enhancer function. *Genome Res* 2019;29(9):1402–14.
- [42] Vendramin R, et al. SAMMSON fosters cancer cell fitness by concertedly enhancing mitochondrial and cytosolic translation. *Nat Struct Mol Biol* 2018;25(11):1035–46.
- [43] Leucci E, et al. Melanoma addiction to the long non-coding RNA SAMMSON. *Nature* 2016;531(7595):518–22.
- [44] Mayer C, et al. Intergenic transcripts regulate the epigenetic state of rRNA genes. *Mol Cell* 2006;22(3):351–61.
- [45] Santoro R, et al. Intergenic transcripts originating from a subclass of ribosomal DNA repeats silence ribosomal RNA genes in trans. *EMBO Rep* 2010;11(1):52–8.
- [46] Bierhoff H, et al. Noncoding transcripts in sense and antisense orientation regulate the epigenetic state of ribosomal RNA genes. *Cold Spring Harb Symp Quant Biol* 2010;75:357–64.
- [47] Leone S, et al. The RNA helicase DHX9 establishes nucleolar heterochromatin, and this activity is required for embryonic stem cell differentiation. *EMBO Rep* 2017;18(7):1248–62.
- [48] Mayer C, Neubert M, Grummt I. The structure of NoRC-associated RNA is crucial for targeting the chromatin remodelling complex NoRC to the nucleolus. *EMBO Rep* 2008;9(8):774–80.
- [49] Ohashi R, et al. Frequent germline and somatic single nucleotide variants in the promoter region of the ribosomal RNA Gene in Japanese lung adenocarcinoma patients. *Cells* 2020;9(11).
- [50] Shiao YH, et al. An intergenic non-coding rRNA correlated with expression of the rRNA and frequency of an rRNA single nucleotide polymorphism in lung cancer cells. *PLoS One* 2009;4(10):e7505.
- [51] Postepska-Igielska A, et al. The chromatin remodelling complex NoRC safeguards genome stability by heterochromatin formation at telomeres and centromeres. *EMBO Rep* 2013;14(8):704–10.
- [52] Bierhoff H, et al. Quiescence-induced LncRNAs trigger H4K20 trimethylation and transcriptional silencing. *Mol Cell* 2014;54(4):675–82.
- [53] Zhao Z, et al. Heat shock represses rRNA synthesis by inactivation of TIF-IA and lncRNA-dependent changes in nucleosome positioning. *Nucleic Acids Res* 2016;44(17):8144–52.
- [54] Zhao Z, et al. lncRNA-induced nucleosome repositioning reinforces transcriptional repression of rRNA genes upon hypotonic stress. *Cell Rep* 2016;14(8):1876–82.
- [55] Ma J, et al. LncRNA PAPAS promotes hepatocellular carcinoma by interacting with miR-188-5p. *J Cell Biochem* 2019;120(8):13494–500.
- [56] Kong Y, Geng C, Dong Q. LncRNA PAPAS may promote triple-negative breast cancer by downregulating miR-34a. *J Int Med Res* 2019;47(8):3709–18.
- [57] Xiao J, et al. Long non-coding (lnc)RNA PAPAS overexpression inhibits tumor growth in papillary thyroid carcinoma by downregulating lncRNA HOTTIP. *Oncol Lett* 2020;19(3):2281–5.
- [58] Jacob MD, et al. Environmental cues induce a long noncoding RNA-dependent remodeling of the nucleolus. *Mol Biol Cell* 2013;24(18):2943–53.
- [59] Audas TE, Jacob MD, Lee S. Immobilization of proteins in the nucleolus by ribosomal intergenic spacer noncoding RNA. *Mol Cell* 2012;45(2):147–57.
- [60] Yap K, et al. A short tandem repeat-enriched RNA assembles a nuclear compartment to control alternative splicing and promote cell survival. *Mol Cell* 2018;72(3):525–540.e13.
- [61] Hershey JWB, Sonenberg N, Mathews MB. Principles of translational control. *Cold Spring Harb Perspect Biol* 2019;11(9).
- [62] Yan X, et al. Dynamics of translation of single mRNA molecules in vivo. *Cell* 2016;165(4):976–89.
- [63] Xu Y, Ruggero D. The role of translation control in tumorigenesis and its therapeutic implications. *Annu Rev Cancer Biol* 2020;4(1):437–57.
- [64] Wu B, et al. Translation dynamics of single mRNAs in live cells and neurons. *Science* 2016;352(6292):1430–5.

- [65] Morisaki T, et al. Real-time quantification of single RNA translation dynamics in living cells. *Science* 2016;352(6292):1425–9.
- [66] Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* 2012;13(4):251–62.
- [67] Cinque S, et al. The cancer-specific lncRNA LISR customizes ribosomes to suppress anti-tumour immunity. *bioRxiv* 2023. Available from: <https://doi.org/10.1101/2023.01.06.523012>.
- [68] Falletta P, et al. Translation reprogramming is an evolutionarily conserved driver of phenotypic plasticity and therapeutic resistance in melanoma. *Genes Dev* 2017;31(1):18–33.
- [69] van Galen P, et al. Integrated stress response activity marks stem cells in normal hematopoiesis and leukemia. *Cell Rep* 2018;25(5):1109–1117.e5.
- [70] Donati G, et al. Targeting mitochondrial respiration and the BCL2 family in high-grade MYC-associated B-cell lymphoma. *Mol Oncol* 2022;16(5):1132–52.
- [71] Vendramin R, et al. Activation of the integrated stress response confers vulnerability to mitoribosome-targeting antibiotics in melanoma. *J Exp Med* 2021;218(9).
- [72] Sendoel A, et al. Translation from unconventional 5' start sites drives tumour initiation. *Nature* 2017;541(7638):494–9.
- [73] Liu X, et al. LncRNA NBR2 engages a metabolic checkpoint by regulating AMPK under energy stress. *Nat Cell Biol* 2016;18(4):431–42.
- [74] Carrieri C, et al. Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature* 2012;491(7424):454–7.
- [75] Yoon JH, et al. LincRNA-p21 suppresses target mRNA translation. *Mol Cell* 2012;47(4):648–55.
- [76] Melixetian M, et al. Long non-coding RNA TINCR suppresses metastatic melanoma dissemination by preventing ATF4 translation. *EMBO Rep* 2021;22(3):e50852.
- [77] Brabletz T, et al. EMT in cancer. *Nat Rev Cancer* 2018;128–34 England.
- [78] Beltran M, et al. A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. *Genes Dev* 2008;22(6):756–69.
- [79] Pardi N, et al. In vitro transcription of long RNA containing modified nucleosides. *Methods Mol Biol* 2013;969:29–42.
- [80] Weissman D, et al. HPLC purification of in vitro transcribed long RNA. *Methods Mol Biol* 2013;969:43–54.
- [81] Zhang Y, et al. Overexpression of lncRNAs with endogenous lengths and functions using a lncRNA delivery system based on transposon. *J Nanobiotechnol* 2021;19(1):303.
- [82] Hannon GJ, Rossi JJ. Unlocking the potential of the human genome with RNA interference. *Nature* 2004;431(7006):371–8.
- [83] Golding MC, et al. Depletion of Kcnq1ot1 non-coding RNA does not affect imprinting maintenance in stem cells. *Development* 2011;138(17):3667–78.
- [84] Stojic L, et al. Transcriptional silencing of long noncoding RNA GNG12-AS1 uncouples its transcriptional and product-related functions. *Nat Commun* 2016;7:10406.
- [85] Morrissey DV, et al. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol* 2005;23(8):1002–7.
- [86] Kim T, et al. Long-range interaction and correlation between MYC enhancer and oncogenic long non-coding RNA CARLo-5. *Proc Natl Acad Sci U S A* 2014;111(11):4173–8.
- [87] Arun G, et al. Differentiation of mammary tumors and reduction in metastasis upon Malat1 lncRNA loss. *Genes Dev* 2016;30(1):34–51.
- [88] Jadaliha M, et al. Functional and prognostic significance of long non-coding RNA MALAT1 as a metastasis driver in ER negative lymph node negative breast cancer. *Oncotarget* 2016;7(26):40418–36.
- [89] Esposito R, et al. Hacking the cancer genome: profiling therapeutically actionable long non-coding RNAs using CRISPR-Cas9 screening. *Cancer Cell* 2019;35(4):545–57.

- [90] Allerson CR, et al. Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *J Med Chem* 2005;48(4):901–4.
- [91] Scoles DR, Minikel EV, Pulst SM. Antisense oligonucleotides: a primer. *Neurol Genet* 2019;5(2):e323.
- [92] Yoo BH, et al. 2'-O-methyl-modified phosphorothioate antisense oligonucleotides have reduced non-specific effects in vitro. *Nucleic Acids Res* 2004;32(6):2008–16.
- [93] Blum M, et al. Morpholinos: antisense and sensibility. *Dev Cell* 2015;35(2):145–9.
- [94] Havens MA, Hastings ML. Splice-switching antisense oligonucleotides as therapeutic drugs. *Nucleic Acids Res* 2016;44(14):6549–63.
- [95] Godfrey C, et al. Delivery is key: lessons learnt from developing splice-switching antisense therapies. *EMBO Mol Med* 2017;9(5):545–57.
- [96] Scoles DR, Pulst SM. Oligonucleotide therapeutics in neurodegenerative diseases. *RNA Biol* 2018;15(6):707–14.
- [97] Smith CIE, Zain R. Therapeutic oligonucleotides: state of the art. *Annu Rev Pharmacol Toxicol* 2019;59:605–30.
- [98] Neil EE, Bisaccia EK. Nusinersen: a novel antisense oligonucleotide for the treatment of spinal muscular atrophy. *J Pediatr Pharmacol Ther* 2019;24(3):194–203.
- [99] Dewaele S, et al. The long non-coding RNA SAMMSON is essential for uveal melanoma cell survival. *Oncogene* 2022;41(1):15–25.
- [100] Diermeier SD, et al. Mammary tumor-associated RNAs impact tumor cell proliferation, invasion, and migration. *Cell Rep* 2016;17(1):261–74.
- [101] Yan X, et al. Silencing lncRNA HOXA10-AS decreases cell proliferation of oral cancer and HOXA10-antisense RNA can serve as a novel prognostic predictor. *J Int Med Res* 2020;48(8):300060520934254.
- [102] Li M, et al. Antisense oligonucleotides targeting lncRNA AC104041.1 induces antitumor activity through Wnt2B/β-catenin pathway in head and neck squamous cell carcinomas. *Cell Death Dis* 2020;11(8):672.
- [103] Gutschner T, et al. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. *Cancer Res* 2013;73(3):1180–9.
- [104] Forbes DC, Peppas NA. Oral delivery of small RNA and DNA. *J Control Rel* 2012;162(2):438–45.
- [105] Winkle M, et al. Noncoding RNA therapeutics—challenges and potential solutions. *Nat Rev Drug Discov* 2021;20(8):629–51.
- [106] Franzen S. Expanding the catalytic repertoire of ribozymes and deoxyribozymes beyond RNA substrates. *Curr Opin Mol Ther* 2010;12(2):223–32.
- [107] Modarresi F, et al. Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. *Nat Biotechnol* 2012;30(5):453–9.
- [108] Fatemi RP, Velmeshev D, Faghihi MA. De-repressing lncRNA-targeted genes to upregulate gene expression: focus on small molecule therapeutics. *Mol Ther Nucleic Acids* 2014;3(11):e196.
- [109] Takahashi H, et al. Identification of functional features of synthetic SINEUPs, antisense lncRNAs that specifically enhance protein translation. *PLoS One* 2018;13(2):e0183229.
- [110] Takahashi H, Carninci P. Widespread genome transcription: new possibilities for RNA therapies. *Biochem Biophys Res Commun* 2014;452(2):294–301.
- [111] Espinoza S, et al. SINEUPs: a novel toolbox for RNA therapeutics. *Essays Biochem* 2021;65(4):775–89.
- [112] Zucchielli S, et al. SINEUPs: A new class of natural and synthetic antisense long non-coding RNAs that activate translation. *RNA Biol* 2015;12(8):771–9.
- [113] Ran FA, et al. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 2013;8(11):2281–308.
- [114] Wang T, et al. Genetic screens in human cells using the CRISPR-Cas9 system. *Science* 2014;343(6166):80–4.

- [115] Mali P, et al. RNA-guided human genome engineering via Cas9. *Science* 2013;339(6121):823–6.
- [116] Awwad DA. Beyond classic editing: innovative CRISPR approaches for functional studies of long non-coding RNA. *Biol Methods Protoc* 2019;4(1):bpz017.
- [117] Shalem O, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 2014;343(6166):84–7.
- [118] Goyal A, et al. Challenges of CRISPR/Cas9 applications for long non-coding RNA genes. *Nucleic Acids Res* 2017;45(3):e12.
- [119] Aparicio-Prat E, et al. DECKO: Single-oligo, dual-CRISPR deletion of genomic elements including long non-coding RNAs. *BMC Genomics* 2015;16:846.
- [120] Gilbert LA, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013;154(2):442–51.
- [121] Gilbert LA, et al. Genome-scale CRISPR-mediated control of gene repression and activation. *Cell* 2014;159(3):647–61.
- [122] Liu Y, et al. Biallelic insertion of a transcriptional terminator via the CRISPR/Cas9 system efficiently silences expression of protein-coding and non-coding RNA genes. *J Biol Chem* 2017;292(14):5624–33.
- [123] Chen W, et al. CRISPRInc: a manually curated database of validated sgRNAs for lncRNAs. *Nucleic Acids Res* 2019;47(D1):D63–d68.
- [124] Janga H, et al. Cas9-mediated excision of proximal DNaseI/H3K4me3 signatures confers robust silencing of microRNA and long non-coding RNA genes. *PLoS One* 2018;13(2):e0193066.
- [125] Cheng TL, Qiu Z. Long non-coding RNA tagging and expression manipulation via CRISPR/Cas9-mediated targeted insertion. *Protein Cell* 2018;8:20–5.
- [126] Abudayyeh OO, et al. RNA targeting with CRISPR-Cas13. *Nature* 2017;550(7675):280–4.
- [127] Yang LZ, et al. Dynamic imaging of RNA in living cells by CRISPR-Cas13 systems. *Mol Cell* 2019;76(6):981–997.e7.
- [128] Xu D, et al. A CRISPR/Cas13-based approach demonstrates biological relevance of vline class of long non-coding RNAs in anticancer drug response. *Sci Rep* 2020;10(1):1794.
- [129] Vanhaesbroeck B, et al. PI3K inhibitors are finally coming of age. *Nat Rev Drug Discov* 2021;20(10):741–69.
- [130] Skrtić M, et al. Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell* 2011;20(5):674–88.
- [131] D’Andrea A, et al. The mitochondrial translation machinery as a therapeutic target in Myc-driven lymphomas. *Oncotarget* 2016;7(45):72415–30.
- [132] Vendramin R, Marine JC, Leucci E. Non-coding RNAs: the dark side of nuclear-mitochondrial communication. *EMBO J* 2017;36(9):1123–33.
- [133] Bussemakers MJ, et al. DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. *Cancer Res* 1999;59(23):5975–9.
- [134] Guo S, et al. LncRNA PCA3 promotes antimony-induced lipid metabolic disorder in prostate cancer by targeting MIR-132-3 P/SREBP1 signaling. *Toxicol Lett* 2021;348:50–8.
- [135] Lemos AE, et al. PCA3 long noncoding RNA modulates the expression of key cancer-related genes in LNCaP prostate cancer cells. *Tumour Biol* 2016;37(8):11339–48.
- [136] Jarroux J, et al. HOTAIR lncRNA promotes epithelial-mesenchymal transition by redistributing LSD1 at regulatory chromatin regions. *EMBO Rep* 2021;22(7):e50193.
- [137] Gupta RA, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 2010;464(7291):1071–6.

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The emerging roles of epitranscriptomic marks in cancer

7

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1 Introduction

The central dogma of molecular biology states that DNA is transcribed as RNA that translates into protein, in which mRNA carries the genetic information while ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) play important roles in protein translation. However, recent data have shown that the human genome is much more complex than originally anticipated and in fact >80% of the genome is transcribed to produce thousands of noncoding RNAs, including microRNAs (miRNAs), piwi-interacting RNA (piRNA), small Cajal body-specific RNAs (scaRNAs), small nucleolar RNAs (snoRNAs), and long noncoding RNAs (lncRNAs). Recent advances in high-throughput methods [e.g., mass spectrometry, next-generation sequencing, especially RNA sequencing (RNA-seq)] have added more complexity to the regulatory roles of RNAs by uncovering many modifications in RNAs, which has emerged as a new field of study called epitranscriptomics [1,2]. To date, there

TABLE 7.1 List of writers, readers, and erasers of epitranscriptomic marks described in this chapter.

Modification	Writers	Readers	Erasers
m ⁶ A	METTL3, METTL4, WTAP, VIRMA (KIAA1429), METTL16, RBM15/15B, ZC3H13	YTHDF1–3, YTHDC1, YTHDC2, HNRNPC, RBMX (HNRNPG), IGF2BP1–3	ALKBH5, FTO
A-to-I RNA editing	ADAR1–3	Unknown	Unknown
m ⁵ C	NSUN1–7, TRDMT1 (DNMT2)	ALYREF, YBX1, RAD52	TET1–3
m ¹ A	TRMT6/61A, TRMT61B, TRMT10C, NML	YTHDC1, YTHDF1–3	FTO, ALKBH1, ALKBH3
m ⁷ G	METTL1, WBSCR22, RNMT	eIF4F complex	Unknown
2'-O-Me	FBL, NOP56, NOP58	Unknown	Unknown
Ψ	PUS1–10, PUS7L, RPUSD1–4, DKC1	Unknown	Unknown
C-to-U RNA editing	APOBEC1, APOBEC3G, A1CF, RBM47	Unknown	Unknown
m ³ C	METTL6, METTL8	Unknown	ALKBH1, ALKBH3

are over 170 RNA modifications known across species [3], which include the most-well-studied *N*⁶-methyladenosine (m⁶A), A-to-I RNA editing, 2'-O-methylation (2'-O-Me), *N*¹-methyladenosine (m¹A), 3-methylcytidine (m³C), 5-methylcytosine (m⁵C), *N*⁷-methylguanosine (m⁷G), and pseudouridylation (Ψ) to name a few. In general, the terminology used in epitranscriptomics (e.g., writers, readers, and erasers; Table 7.1 [4–6]) is largely derived from the well-established field of epigenetics. In the following, epitranscriptomic marks investigated in tumorigenesis will be discussed to provide an overview of epitranscriptomics in cancer.

2 *N*⁶-methyladenosine in cancer

m⁶A is methylation of adenosine (A) at its nitrogen-6 position, and it is found prevalently in mRNAs [7]. It is by far the most well-studied epitranscriptomic marks in recent years, and there are over 1500 articles published about m⁶A in cancer (PubMed search performed on January 2, 2022). As there are excellent review articles published about m⁶A in cancer [8–10], this section focuses only on findings published since 2021.

The Wnt/β-catenin signaling pathway is an important regulator of development and cell differentiation. Its dysregulation is linked to several types of cancers; yet, its mechanism of action remains to be poorly understood [11]. To this end, m⁶A marks may hold a key to understand this pathway better. For example, the m⁶A eraser, FTO, demethylates the *PJA2* (an E3 ubiquitin ligase) mRNA to reduce its mRNA decay through the m⁶A reader, YTHDF2. This cascade of events represses Wnt signaling, thereby inhibiting tumorigenesis in pancreatic cancer [12]. Yet another study showed that the expression of FTO itself is regulated by Wnt signaling, where the expression of FTO is downregulated in lung adenocarcinoma tissues compared to adjacent normal tissue [13].

Mechanistically, the promoter region of *FTO* is bound by the EZH2/β-catenin complex, which, in turn, enhances the m⁶A levels in many mRNAs, including the oncogene *MYC*. The increased m⁶A marks on the *MYC* mRNA result in more *MYC* protein, which increases glycolysis and tumorigenesis in lung cancer, suggesting that m⁶A marks are dynamically regulated by Wnt signaling.

Compared to normal neural stem cells (NSCs), m⁶A marks are more common in glioblastoma stem cells (GSCs), while GSCs display preferential expression of YTHDF2 [14]. YTHDF2 stabilize *MYC* and *VEGFA* mRNAs in GSCs in a m⁶A-dependent manner. IGFBP3 is identified as a downstream effector of the YTHDF2-MYC axis in GSCs, supporting cell proliferation, migration, and invasion via regulating the activity IGF2, which further activates PI3K/Akt signaling. Thus YTHDF2-MYC-IGFBP3 axis could be a specific target in glioblastoma treatment.

MiRNAs are small noncoding RNAs (ncRNAs) of ~22 nucleotides (nt) in length that function as posttranscriptional regulators to inhibit translation by binding to the 3'-untranslated regions (UTR) of mRNAs. Their functions in cancer are highly studied, and miRNAs are frequently dysregulated in tumorigenesis [15]. MiRNAs are also modified by m⁶A enzymes [16], impacting their biogenesis and functions. For example, the m⁶A writer, METTL3, methylates *miR-1246*, which targets *PEG3* mRNA to induce cell growth in nonsmall-cell lung cancer (NSCLC) cells. Besides miRNAs being m⁶A marked, miRNAs can also target m⁶A enzymes. For example, the m⁶A reader, YTHDF2, binds m⁶A modified *GSK3β* to degrade its mRNA, which ultimately regulates the stability of β-catenin leading to cell proliferation [17]. In colorectal cancer (CRC), the expression of *miR-6125* is downregulated, which correlates with tumor size and prognosis in CRC patients. When *miR-6125* is overexpressed in CRC cells, *miR-6125* binds the 3'-UTR of *YTHDF2* mRNA to inhibit its translation, leading to cell cycle arrest at G0/G1 phase by downregulating the expression of Cyclin D1 [17], suggesting that *miR-6125* could be a therapeutic target for CRC by modulating the oncogenic YTHDF2.

Long noncoding RNA (lncRNA) is a collective term to describe any ncRNAs whose lengths are longer than 200 nt. Within the last decade, the number of studies identifying and describing the functions of lncRNAs has exploded as lncRNAs can bind other macromolecules (DNA, RNA, and proteins) to regulate various cellular processes, including in cancer [18]. High-throughput screening studies have shown that lncRNAs have m⁶A marks, which affect their functions by altering the sequence, structure, and binding to macromolecules [19]. One of the most well-studied oncogenic lncRNA, *MALAT1*, has m⁶A marks, which are regulated by the m⁶A writer, METTL3. In glioma, the expression of METTL3 is associated with malignancy and poor prognosis in isocitrate dehydrogenase (IDH)-wild-type gliomas, but not IDH-mutation gliomas [20]. Mechanistically, METTL3 methylates *MALAT1*, and thereby enhances its stability via binding of the RNA-binding protein, HuR (encoded by the *ELAVL1* gene), leading to the activation of NF-κB [20]. The opposite effect is reported in bladder cancer, where the m⁶A eraser, FTO, demethylates 5'-end of *MALAT1* to evade its degradation pathway via the m⁶A reader, YTHDF2. The prolonged presence of *MALAT1* allows it to bind *miR-384* as a miRNA sponge to modulate the expression of the *miR-384* target, *MAL2*, thereby promoting bladder cancer proliferation [21]. A similar mechanism, in which YTHDF2 degrades m6A-marked lncRNAs, is also observed in another well-known lncRNA, *NEAT1*. The m6A writer, METTL14, methylates the short isoform of *NEAT1*, *NEAT1_I*, to promote binding of YTHDF2, which facilitates degradation of *NEAT1_I* [22]. In renal cell carcinoma (RCC), the expression of METTL14 is downregulated, which contributes to the stabilization of *NEAT1_I*, leading to RCC cell growth and metastasis [22].

Circular RNAs (circRNAs) are generated by backsplicing events of exons and/or introns of both protein-coding and ncRNA genes [23]. Because circRNAs lack free ends necessary for exonuclease-mediated degradation, they are relatively stable in circulation, which makes them attractive diagnostic biomarkers in many diseases, including cancer [24]. Recent studies have shown that circRNAs also possess m⁶A marks, where m⁶A enzymes regulate the biogenesis of circRNAs and possibly their functions [25]. For example, *circCPSF6* is generated by backsplicing of exons 2 and 9 of the host gene, *CPSF6*, in a head-tail manner, consisting of eight exons without introns [26]. It is highly upregulated in hepatocellular carcinoma (HCC) subjects, whose expression patterns are associated with poor prognostic of HCC patients. This circRNA is demethylated by the m⁶A eraser, ALKBH5, whose m⁶A marks are recognized by the m⁶A reader, YTHDF2, for its degradation. Mechanistically, *circCPSF6* competitively binds the RNA-binding protein, PCBP2, to interfere with its binding to the *YAP1* mRNA, thereby enhancing the mRNA stability of *YAP1* leading to HCC malignancy [26]. As aberrant RNA splicing (also known as mis-splicing) in cancer cells is common [27], more circRNAs in relation with m⁶A marks will be discovered and reported, which will further elucidate the disease mechanisms in cancer.

3 A-to-I RNA editing in cancer

The target of m⁶A, which is the nitrogen-6 position of A, can also be modified, if unmethylated [28]. This modification is called adenosine-to-inosine (A-to-I) RNA editing, where the RNA editing enzymes, adenosine deaminase acting on RNAs (ADARs), bind double-stranded RNAs (as in the case of repetitive elements, such as ALU repeats) to catalyze a A-to-I hydrolytic deamination reaction [29]. I is recognized as guanosine (G) by translational and splicing machineries as well as the reverse transcription reaction; thus it is possible to detect A-to-G changes in RNA-seq reads when these reads are mapped to the reference genome [30]. As there are several computational programs available to detect RNA editing sites in RNA-seq data (comprehensively reviewed here [31]), a number of screening studies have been conducted, including those in cancer [32–34]. RNA-edited sites differ greatly among cancer types and are found mainly in 3'-UTRs and introns [34] but also in protein-coding regions, resulting in changes in the amino acid sequence [35].

Because over a million RNA editing sites have been reported in the human transcriptome [36], with changes in numbers depending on the cancer type and disease progression [34], many editing sites in oncogenic transcripts and their effects on tumorigenesis have been reported. For example, editing of *AZIN1* (a member of antizyme inhibitor family) enhances tumorigenesis and promotes cancer stem cell-like features in CRC [37]. Another study showed that editing of *SLC22A3* (a metastasis-suppressor gene) results in disruption of its suppressing action of ACTN4-mediated actin crosslinking and filopodia formation promoting cell adhesion in esophageal cancer [38].

A previous study reported reduced RNA editing (hypoediting) in brain tumors [32]; however, it is not clear how such hypoediting affects tumorigenesis. Galeano et al. reported that impaired editing of ADAR2 in glioblastoma multiforme is linked to glioblastoma pathogenesis as ADAR2 suppresses tumor growth via editing of the cell cycle control gene, *CDC14B*, that regulates the Skp2/p21/p27 pathway [39]. Another study reported that DAP3 (a member of the death-associated protein family) suppresses the editing activity of ADAR2 by interacting with its deaminase domain [40].

As editing occurs cotranscriptionally with pre-mRNA splicing [41], the effects of editing can also be seen in splicing patterns, such as in the splicing factor *HNRPLL*, where editing generates a novel splicing variant of this gene enhancing tumor cell survival by regulating growth-related genes (e.g., *CCND1* and *TGFBR1*) [42]. A few examples provided above clearly indicate that A-to-I RNA editing can play a major role in cancer.

4 5-Methylcytosine in cancer

The m⁵C marks can be found in both DNA and RNA, whose marks are regulated by different set of enzymes (Table 7.1). m⁵C marks are found abundantly in rRNAs and tRNAs, however, recent studies have also uncovered the presence of m⁵C in mRNAs and ncRNAs that are differentially regulated in cancers [43]. Mechanistically, the m⁵C writer, NSUN2, upregulates the transcription factor TEAD1 to enhance the proliferation and invasion of hypopharyngeal squamous cell carcinoma [44]. Furthermore, NSUN2 methylates *GRB2* to stabilize its mRNA via binding of RNA-binding protein, LIN28B [45]. Upregulation of GRB2 activates the oncogenic PI3K/AKT and ERK/MAPK signaling in esophageal squamous cell carcinoma. Besides protein-coding genes, NSUN2 also methylates the lncRNA *H19*, which binds the oncoprotein G3BP1. By competitive binding of G3BP1 to *MYC*, methylated *H19* may promote the accumulation of *MYC* leading to proliferation and cell-cycle progression in HCC [46].

Besides NSUN2, other m⁵C writers also regulate tumorigenesis. For example, the RNA methyltransferase TRDMT1 (DNMT2) is recruited to DNA damage sites triggered by reactive oxygen species to regulate DNA damage code at the mRNA level [47]. When *TRDMT1* is silenced, repair of DNA double-stranded breaks via homologous recombination (HR) is impaired, leading to increased sensitivity to radiotherapy and PARP inhibitors (PARPi; i.e., Olaparib). This process is regulated in part by the novel m⁵C reader, RAD52. Based on these findings, the authors propose that inhibiting TRDMT1 may represent a therapeutic strategy to target PARPi-resistant, HR-deficient tumors [47].

Another example is the m⁵C reader, ALYREF, which binds to m⁵C sites in the 3'-UTR of the glycolytic enzyme, *PKM2*, to stabilize its mRNA, thereby promoting bladder cancer cell proliferation [48]. Taken together, the actions of m⁵C enzymes are important in tumorigenesis and could also be potential targets of anticancer therapies.

5 *N*⁷-methylguanosine in cancer

tRNAs are key players in translation, and have recently been shown to exhibit a dynamic lifecycle involving epitranscriptomics, especially in cancer [49]. One such modification is m⁷G, which is a methylation of nitrogen-7 position of guanosine (G). The methyltransferase complex METTL1/WDR4 is upregulated in cancer, and correlates with poor patient survival [29,50]. This increased expression of METTL1 in cancer may induce tumorigenesis by enhancing m⁷G modification on tRNAs, which, in turn, increases the translation of mRNAs, especially those involved in cell cycle regulation and the epidermal growth factor receptor pathway [50–52]. METTL1 also methylates

miRNAs (e.g., *let-7e-5p* [53]) to regulate tumorigenesis, which highlights the diverse functions of m⁷G marks in cancer.

The eukaryotic initiation factor 4F (eIF4F) is a protein complex that facilitates the recruitment of ribosomes to mRNAs to initiate translation. Its dysregulation has been linked to tumorigenesis, and it is thus highly studied in many cancers [54]. The eIF4F complex specifically binds to the m⁷G marks at the 5'-cap of mRNAs as well as those of ncRNAs [55]. A previous study showed that resistance to anti-BRAF, and -MEK inhibitors, or the combination of two inhibitors in BRAF (V600)-mutant tumors is associated with an increase in the eIF4F complex formation [56]. Based on this study and others, several small-molecule inhibitors of the eIF4F complex are being tested as anticancer drugs [54], suggesting that understanding of m⁷G marks is important for development of new cancer therapies.

6 2'-O-methylation in cancer

snoRNAs are regulatory ncRNAs that modify rRNAs and small nuclear RNAs. There are two major classes of snoRNAs; both of which are involved in two separate epitranscriptomic marks: (1) C/D box snoRNAs for 2'-O-Me and (2) H/ACA box snoRNAs for pseudouridylation, which will be discussed in the next section. Dysregulation of many snoRNAs is linked to tumorigenesis [57]. For example, the C/D box snoRNA, *SNORD42A*, regulates ribosomal biogenesis via 2'-O-Me at uridine 116 of 18S rRNA, influencing proliferation of leukemic cells by regulating the overall protein synthesis [58]. Similarly, ablation of either C/D box snoRNA *SNORD14D* or *SNORD35A* inhibits leukemia self-renewal potential [59].

Besides snoRNAs, 2'-O-Me marks can also be found in other ncRNAs in cancer. For example, in NSCLC, the oncomiR, *miR-21-5p*, is 2'-O-methylated at its 3'-terminal by the action of the methyltransferase HENMT1 [60]. Compared to unmethylated *miR-21-5p*, the 2'-O-methylated *miR-21-5p* is more stable and associates tightly with Argonaute-2. Another example is the lncRNA *ZFAS1*, which encodes a cluster of C/D box *SNORD12* (*SNORD12*, *SNORD12B*, and *SNORD12C*). This lncRNA binds the 2'-O-Me writer, NOP58, to enhance proliferation and to inhibit apoptosis in CRC [61], further highlighting the importance of 2'-O-Me in cancer.

7 Pseudouridylation in cancer

The first epitranscriptomic mark discovered [62], pseudouridylation (Ψ), has received considerable attention in drug development, especially in the case of mRNA vaccines (e.g., COVID-19 mRNA vaccines). Originally discovered in the tRNA dihydrouridine and anticodon stem-loops, the importance of Ψ in cancers is well documented. For example, DKC1 is a pseudouridine synthase, whose mutations are associated with ribosomal dysfunction and regulation of the tumor suppressor p27 [63]. Its upregulation is associated with poor prognosis in breast, liver, lung, and prostate cancer [64]. DKC1 is upregulated in glioma cells and promotes cell cycle progression and motility via upregulating N-cadherin, HIF1A, and MMP-2 [65]. DKC1 encodes dyskerin, which is a nucleolar-localized protein that functions in two distinct complexes: (1) ribonuclear protein complexes

involved in ribosome biogenesis and (2) the telomerase holoenzyme for telomerase stabilization and maintenance. Both processes are linked to tumorigenesis [66], suggesting that dyskerin is a potential therapeutic target [67].

8 *N¹-methyladenosine in cancer*

As with most epitranscriptomic marks, m¹A was initially discovered in rRNAs and tRNAs, but recent research indicates the presence of m¹A in other types of RNAs as well [68]. In the nucleus, m¹A modifications are catalyzed by methyltransferase 6/61A (TRMT6-TRMT61A) and are erased by ALKBH1 and ALKBH3 [69]. Furthermore, m¹A marks are also present in human mitochondria, which affect mitochondrial transcript processing [70]. In breast and ovarian cancers, the m¹A eraser, ALKBH3, demethylates the 5'-UTR near the translation initiation site of the cytokine *CSF-1* mRNA to regulate its mRNA stability [71]. Another study reported that ALKBH3 promotes cancer progression by regulating the generation of tRNA-derived small RNAs [72], which are cleaved from tRNAs under stress [73]. Similarly, another m¹A eraser, ALKBH1, is also shown to regulate the tRNA cleavage to generate small RNAs in B35 rat neuroblastoma cell line [73], underscoring the important functions of m¹A in tRNA regulation in cancer. The low expression of ALKBH1 is related to the poor survival of patients with pancreatic adenocarcinoma, and its expression level has important clinical significance. Mechanistically, ALKBH1 acts through mTOR and ErbB signaling pathway in the development of pancreatic cancer [74].

9 *3-Methylcytidine in cancer*

m³C occurs mostly in tRNAs. A previous study demonstrated that the m³C writers METTL2 and 6 modify tRNAs, while another m³C writer, METTL8, modifies only mRNAs [75]. However, more recently METTL8 was shown to localize in mitochondria and to target mitochondrial tRNAs (mt-tRNA) in human cells, affecting respiratory chain activities [76]. METTL8 is upregulated in several types of cancers, and high level of METTL8 was shown to negatively correlate with survival in patients with highly aggressive pancreatic cancer [76]. Mechanistically, another study shows that SUMOylated METTL8 promotes tumorigenesis by influencing the formation of R-loops [77], which are DNA-DNA:RNA hybrid structures that affect genome stability in cancer [78].

10 *Epitranscriptomics in diagnostics and therapeutics*

As more and more dysregulation of epitranscriptomic marks in diseased conditions compared to healthy are being discovered, the field of epitranscriptomics is moving toward potential therapeutic usages of these marks.

10.1 Epitranscriptomic marks as diagnostic biomarkers

As mentioned above, the numbers of epitranscriptomic sites differ significantly in tumors compared to the healthy tissues as well as among different cancer types. The changes in epitranscriptomic sites can also be used as diagnostic biomarkers to detect a certain disease and its status. To this end, differential epitranscriptomic marks on miRNAs have been reported. The reason for focusing on miRNAs is in line with the current trend of exploring miRNAs as biomarkers based on the stability of circulating miRNAs in the blood [79]. For example, m⁶A methylation of *miR-200b-3p* is associated with glioblastoma multiforme patients with a poor survival [80]. Another example is m⁵C-methylated *miRNA-181a-5p*, which is associated with poor prognosis of glioblastoma multiforme patients [81]. As the techniques to detect specific epitranscriptomic marks improve, more efforts will be spent on discovering differential epitranscriptomic marks, which may be able to increase the accuracy of prognostics as in the case of methylation of *miR-17-5p* in serum samples to distinguish early pancreatic cancer patients from the healthy controls using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [82].

10.2 Therapeutic tools to correct mutations

DNA mutations drive cancers. Thus the rise of CRISPR technology has inspired a series of therapeutic approaches to alter the defective genomic sequences. Yet, this technology suffers from several drawbacks, including introduction of bacterial-derived proteins (i.e., Cas9) causing immune reactions in the host (i.e., humans), DNA damage leading to cellular apoptosis, and off-target effects [83]. To solve these problems, RNA-based therapeutic strategies are increasingly being developed. RNA is an attractive candidate compared to DNA because: (1) no permanent changes to the patient's genome as only RNA sequences will be altered; (2) less immunogenic, if endogenous proteins (e.g., ADARs) are used; and (3) very specific binding sites. The last part is highlighted in a recent method, named RNA Editing for Programmable A-to-I Replacement (REPAIR), in which a ADAR2 deaminase domain is fused to CRISPR-Cas13b enzyme to perform A-to-I conversion in RNA transcripts [84]. A similar approach was used for another RNA editing strategy, cytidine to uridine (C-to-U) RNA editing. Compared to A-to-I RNA editing, it is less common in mammals, but highly active in plants [85]. This approach is called RNA Editing for Specific C-to-U Exchange (RESCUE) [86]. Inspired by the recent development of mRNA-based vaccines, more efforts are underway to test RNA-based therapeutics, including the genetic manipulation in cancer patients at the level of RNA instead of genomic DNA.

11 Conclusions

In this chapter, we describe the current status of epitranscriptomics in relation to cancer. As it was the case for epigenetics, the field of epitranscriptomics is rapidly expanding as new experimental methods are developed. Recently, the first-in-class catalytic inhibitor of *METTL3*, STM2457, was shown to reduce tumor growth in acute myeloid leukemia and to increase in myeloid differentiation and apoptosis [43]. Another example is the PUS7 inhibitor compound C17 (NSC107512) suppressed tRNA pseudouridylation and glioblastoma tumorigenesis in mice [87]. Given the explosion

in the development of RNA medicines, highlighted by the COVID-19 mRNA vaccines, as well as recent discoveries in RNA modifications and their role in cancer, diagnostics and prognostics as well as therapeutics epitranscriptomics, will be available in near future; not only in basic biology but also expanded usages in therapeutic purposes, including diagnostic and prognostic as well as potential therapeutic approaches, especially in cancers.

List of abbreviations

2'-O-Me	2'-O-Methylation
m³C	3-Methylcytidine
m⁵C	5-Methylcytosine
CRC	Colorectal cancer
GSC	Glioblastoma stem cells
HCC	Hepatocellular carcinoma
HR	Homologous recombination
IDH	Isocitrate dehydrogenase
lncRNAs	Long noncoding RNAs
NSC	Neural stem cells
ncRNAs	Noncoding RNAs
m¹A	N ¹ -Methyladenosine
m⁶A	N ⁶ -Methyladenosine
m⁷G	N ⁷ -Methylguanosine
PARPi	PARP inhibitors
piRNA	Piwi-interacting RNA
Ψ	Pseudouridylation
RCC	Renal cell carcinoma
RNA-seq	RNA sequencing
rRNAs	Ribosomal RNAs
scaRNAs	Small Cajal body-specific RNAs
snRNAs	Small nucleolar RNAs
tRNAs	Transfer RNAs
UTRs	Untranslated regions

References

- [1] Roundtree IA, He C. RNA epigenetics—chemical messages for posttranscriptional gene regulation. *Curr Opin Chem Biol* 2016;30:46–51.
- [2] Saleto Y, Meyer K, Korlach J, Vilfan ID, Jaffrey S, Mason CE. The birth of the Epitranscriptome: deciphering the function of RNA modifications. *Genome Biol* 2012;13(10):175.
- [3] Boccalotto P, Machnicka MA, Purta E, et al. MODOMICS: a database of RNA modification pathways. 2017 update. *Nucleic Acids Res* 2018;46(D1):D303–7.
- [4] Barbieri I, Kouzarides T. Role of RNA modifications in cancer. *Nat Rev Cancer* 2020;20(6):303–22.
- [5] Kudrin P, Meierhofer D, Vågbø CB, Ørom UAV. Nuclear RNA-acetylation can be erased by the deacetylase SIRT7. *bioRxiv* 2021; 2021.04.06.438707.
- [6] Esteve-Puig R, Bueno-Costa A, Esteller M. Writers, readers and erasers of RNA modifications in cancer. *Cancer Lett* 2020;474:127–37.

- [7] Fu Y, Dominissini D, Rechavi G, He C. Gene expression regulation mediated through reversible m(6)A RNA methylation. *Nat Rev Genet* 2014;15(5):293–306.
- [8] Wang T, Kong S, Tao M, Ju S. The potential role of RNA N6-methyladenosine in Cancer progression. *Mol Cancer* 2020;19(1):88.
- [9] Chen XY, Zhang J, Zhu JS. The role of m(6)A RNA methylation in human cancer. *Mol Cancer* 2019;18(1):103.
- [10] Sun T, Wu R, Ming L. The role of m6A RNA methylation in cancer. *Biomed Pharmacother* 2019;112:108613.
- [11] Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. *Oncogene* 2017;36(11):1461–73.
- [12] Zeng J, Zhang H, Tan Y, Wang Z, Li Y, Yang X. m6A demethylase FTO suppresses pancreatic cancer tumorigenesis by demethylating PJA2 and inhibiting Wnt signaling. *Mol Ther Nucleic Acids* 2021;25:277–92.
- [13] Yang X, Shao F, Guo D, et al. WNT/beta-catenin-suppressed FTO expression increases m(6)A of c-Myc mRNA to promote tumor cell glycolysis and tumorigenesis. *Cell Death Dis* 2021;12(5):462.
- [14] Dixit D, Prager BC, Gimple RC, et al. The RNA m6A reader YTHDF2 maintains oncogene expression and is a targetable dependency in glioblastoma stem cells. *Cancer Discov* 2021;11(2):480–99.
- [15] Peng Y, Croce CM. The role of MicroRNAs in human cancer. *Signal Transduct Target Ther* 2016;1:15004.
- [16] Berulava T, Rahmann S, Rademacher K, Klein-Hitpass L, Horsthemke B. N6-adenosine methylation in MiRNAs. *PLoS One* 2015;10(2):e0118438.
- [17] Li H, Zhang N, Jiao X, et al. Downregulation of microRNA-6125 promotes colorectal cancer growth through YTHDF2-dependent recognition of N6-methyladenosine-modified GSK3beta. *Clin Transl Med* 2021;11(10):e602.
- [18] Huarte M. The emerging role of lncRNAs in cancer. *Nat Med* 2015;21(11):1253–61.
- [19] He RZ, Jiang J, Luo DX. The functions of N6-methyladenosine modification in lncRNAs. *Genes Dis* 2020;7(4):598–605.
- [20] Chang YZ, Chai RC, Pang B, et al. METTL3 enhances the stability of MALAT1 with the assistance of HuR via m6A modification and activates NF-kappaB to promote the malignant progression of IDH-wildtype glioma. *Cancer Lett* 2021;511:36–46.
- [21] Tao L, Mu X, Chen H, et al. FTO modifies the m6A level of MALAT and promotes bladder cancer progression. *Clin Transl Med* 2021;11(2):e310.
- [22] Liu T, Wang H, Fu Z, et al. Methyltransferase-like 14 suppresses growth and metastasis of renal cell carcinoma by decreasing long noncoding RNA NEAT1. *Cancer Sci* 2021;.
- [23] Kristensen LS, Andersen MS, Stagsted L, Ebbesen KK, Hansen TB, Kjems J. The biogenesis, biology and characterization of circular RNAs. *Nat Rev Genet* 2019;20(11):675–91.
- [24] Su M, Xiao Y, Ma J, et al. Circular RNAs in cancer: emerging functions in hallmarks, stemness, resistance and roles as potential biomarkers. *Mol Cancer* 2019;18(1):90.
- [25] Zhang L, Hou C, Chen C, et al. The role of N(6)-methyladenosine (m(6)A) modification in the regulation of circRNAs. *Mol Cancer* 2020;19(1):105.
- [26] Chen Y, Ling Z, Cai X, et al. Activation of YAP1 by N6-methyladenosine-modified CircCPSF6 drives malignancy in hepatocellular carcinoma. *Cancer Res* 2021;.
- [27] Dong X, Chen R. Understanding aberrant RNA splicing to facilitate cancer diagnosis and therapy. *Oncogene* 2020;39(11):2231–42.
- [28] Xiang JF, Yang Q, Liu CX, Wu M, Chen LL, Yang L. N(6)-Methyladenosines modulate A-to-I RNA editing. *Mol Cell* 2018;69(1):126–35 e6.
- [29] Uchida S, Jones SP. RNA editing: unexplored opportunities in the cardiovascular system. *Circ Res* 2018;122(3):399–401.

- [30] John D, Weirick T, Dimmeler S, Uchida S. RNAEditor: easy detection of RNA editing events and the introduction of editing islands. *Brief Bioinform* 2017;18(6):993–1001.
- [31] Diroma MA, Ciaccia L, Pesole G, Picardi E. Elucidating the editome: bioinformatics approaches for RNA editing detection. *Brief Bioinform* 2019;20(2):436–47.
- [32] Paz N, Levanon EY, Amariglio N, et al. Altered adenosine-to-inosine RNA editing in human cancer. *Genome Res* 2007;17(11):1586–95.
- [33] Wang Y, Xu X, Yu S, et al. Systematic characterization of A-to-I RNA editing hotspots in microRNAs across human cancers. *Genome Res* 2017;27(7):1112–25.
- [34] Han L, Diao L, Yu S, et al. The genomic landscape and clinical relevance of A-to-I RNA editing in human cancers. *Cancer Cell* 2015;28(4):515–28.
- [35] Peng X, Xu X, Wang Y, et al. A-to-I RNA editing contributes to proteomic diversity in cancer. *Cancer Cell* 2018;33(5):817–828.e7.
- [36] Bazak L, Haviv A, Barak M, et al. A-to-I RNA editing occurs at over a hundred million genomic sites, located in a majority of human genes. *Genome Res* 2014;24(3):365–76.
- [37] Shigeyasu K, Okugawa Y, Toden S, et al. AZIN1 RNA editing confers cancer stemness and enhances oncogenic potential in colorectal cancer. *JCI Insight* 2018;3(12).
- [38] Fu L, Qin YR, Ming XY, et al. RNA editing of SLC22A3 drives early tumor invasion and metastasis in familial esophageal cancer. *Proc Natl Acad Sci U S A* 2017;114(23):E4631–40.
- [39] Galeano F, Rossetti C, Tomaselli S, et al. ADAR2-editing activity inhibits glioblastoma growth through the modulation of the CDC14B/Skp2/p21/p27 axis. *Oncogene* 2013;32(8):998–1009.
- [40] Han J, An O, Hong H, et al. Suppression of adenosine-to-inosine (A-to-I) RNA editome by death associated protein 3 (DAP3) promotes cancer progression. *Sci Adv* 2020;6(25):eaba5136.
- [41] Licht K, Kapoor U, Amman F, et al. A high resolution A-to-I editing map in the mouse identifies editing events controlled by pre-mRNA splicing. *Genome Res* 2019;29(9):1453–63.
- [42] Chen YT, Chang IY, Liu H, et al. Tumor-associated intronic editing of HNRPLL generates a novel splicing variant linked to cell proliferation. *J Biol Chem* 2018;293(26):10158–71.
- [43] Xue C, Zhao Y, Li L. Advances in RNA cytosine-5 methylation: detection, regulatory mechanisms, biological functions and links to cancer. *Biomark Res* 2020;8:43.
- [44] Chen L, Ding J, Wang B, et al. RNA methyltransferase NSUN2 promotes hypopharyngeal squamous cell carcinoma proliferation and migration by enhancing TEAD1 expression in an m(5)C-dependent manner. *Exp Cell Res* 2021;404(2):112664.
- [45] Su J, Wu G, Ye Y, et al. NSUN2-mediated RNA 5-methylcytosine promotes esophageal squamous cell carcinoma progression via LIN28B-dependent GRB2 mRNA stabilization. *Oncogene* 2021;40(39):5814–28.
- [46] Sun Z, Xue S, Zhang M, et al. Aberrant NSUN2-mediated m(5)C modification of H19 lncRNA is associated with poor differentiation of hepatocellular carcinoma. *Oncogene* 2020;39(45):6906–19.
- [47] Chen H, Yang H, Zhu X, et al. m(5)C modification of mRNA serves a DNA damage code to promote homologous recombination. *Nat Commun* 2020;11(1):2834.
- [48] Wang JZ, Zhu W, Han J, et al. The role of the HIF-1alpha/ALYREF/PKM2 axis in glycolysis and tumorigenesis of bladder cancer. *Cancer Commun (Lond)* 2021;41(7):560–75.
- [49] Miano V, Codino A, Pandolfini L, Barbieri I. The non-coding epitranscriptome in cancer. *Brief Funct Genomics* 2021;20(2):94–105.
- [50] Chen Z, Zhu W, Zhu S, et al. METTL1 promotes hepatocarcinogenesis via m(7) G tRNA modification-dependent translation control. *Clin Transl Med* 2021;11(12):e661.
- [51] Orellana EA, Liu Q, Yankova E, et al. METTL1-mediated m(7)G modification of Arg-TCT tRNA drives oncogenic transformation. *Mol Cell* 2021;81(16):3323–3338.e14.
- [52] Dai Z, Liu H, Liao J, et al. N(7)-Methylguanosine tRNA modification enhances oncogenic mRNA translation and promotes intrahepatic cholangiocarcinoma progression. *Mol Cell* 2021;81(16):3339–3355.e8.

- [53] Pandolfini L, Barbieri I, Bannister AJ, et al. METTL1 promotes let-7 microRNA processing via m7G methylation. *Mol Cell* 2019;74(6):1278–1290.e9.
- [54] Malka-Mahieu H, Newman M, Desaubry L, Robert C, Vagner S. Molecular pathways: the eIF4F translation initiation complex-new opportunities for cancer treatment. *Clin Cancer Res* 2017;23(1):21–5.
- [55] Culjkovic-Kraljacic B, Skrabaneck L, Revuelta MV, et al. The eukaryotic translation initiation factor eIF4E elevates steady-state m(7)G capping of coding and noncoding transcripts. *Proc Natl Acad Sci U S A* 2020;117(43):26773–83.
- [56] Boussemart L, Malka-Mahieu H, Girault I, et al. eIF4F is a nexus of resistance to anti-BRAF and anti-MEK cancer therapies. *Nature* 2014;513(7516):105–9.
- [57] Williams GT, Farzaneh F. Are snoRNAs and snoRNA host genes new players in cancer? *Nat Rev Cancer* 2012;12(2):84–8.
- [58] Pauli C, Liu Y, Rohde C, et al. Site-specific methylation of 18S ribosomal RNA by SNORD42A is required for acute myeloid leukemia cell proliferation. *Blood* 2020;135(23):2059–70.
- [59] Zhou F, Liu Y, Rohde C, et al. AML1-ETO requires enhanced C/D box snoRNA/RNP formation to induce self-renewal and leukaemia. *Nat Cell Biol* 2017;19(7):844–55.
- [60] Liang H, Jiao Z, Rong W, et al. 3'-Terminal 2'-O-methylation of lung cancer miR-21-5p enhances its stability and association with Argonaute 2. *Nucleic Acids Res* 2020;48(13):7027–40.
- [61] Wu H, Qin W, Lu S, et al. Long noncoding RNA ZFAS1 promoting small nucleolar RNA-mediated 2'-O-methylation via NOP58 recruitment in colorectal cancer. *Mol Cancer* 2020;19(1):95.
- [62] Cohn WE, Volkin E. Nucleoside-5'-phosphates from ribonucleic acid. *Nature* 1951;167(4247):483–4.
- [63] Bellodi C, Krasnykh O, Haynes N, et al. Loss of function of the tumor suppressor DKC1 perturbs p27 translation control and contributes to pituitary tumorigenesis. *Cancer Res* 2010;70(14):6026–35.
- [64] Guerrieri AN, Zacchini F, Onofrillo C, et al. DKC1 overexpression induces a more aggressive cellular behavior and increases intrinsic ribosomal activity in immortalized mammary gland cells. *Cancers (Basel)* 2020;12(12).
- [65] Miao FA, Chu K, Chen HR, et al. Increased DKC1 expression in glioma and its significance in tumor cell proliferation, migration and invasion. *Invest N Drugs* 2019;37(6):1177–86.
- [66] Ruggero D, Grisendi S, Piazza F, et al. Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. *Science* 2003;299(5604):259–62.
- [67] Penzo M, Guerrieri AN, Zacchini F, Trere D, Montanaro L. RNA pseudouridylation in physiology and medicine: for better and for worse. *Genes (Basel)* 2017;8(11).
- [68] Xiong X, Li X, Yi CN. (1)-methyladenosine methylome in messenger RNA and non-coding RNA. *Curr Opin Chem Biol* 2018;45:179–86.
- [69] Zhang C, Jia G. Reversible RNA Modification N(1)-methyladenosine (m(1)A) in mRNA and tRNA. *Genomics Proteom Bioinforma* 2018;16(3):155–61.
- [70] Ali AT, Idaghdour Y, Hodgkinson A. Analysis of mitochondrial m1A/G RNA modification reveals links to nuclear genetic variants and associated disease processes. *Commun Biol* 2020;3(1):147.
- [71] Woo HH, Chambers SK. Human ALKBH3-induced m(1)A demethylation increases the CSF-1 mRNA stability in breast and ovarian cancer cells. *Biochim Biophys Acta Gene Regul Mech* 2019;1862(1):35–46.
- [72] Chen Z, Qi M, Shen B, et al. Transfer RNA demethylase ALKBH3 promotes cancer progression via induction of tRNA-derived small RNAs. *Nucleic Acids Res* 2019;47(5):2533–45.
- [73] Rashad S, Han X, Sato K, et al. The stress specific impact of ALKBH1 on tRNA cleavage and tiRNA generation. *RNA Biol* 2020;17(8):1092–103.
- [74] Zheng Q, Yu X, Zhang Q, He Y, Guo W. Genetic characteristics and prognostic implications of m1A regulators in pancreatic cancer. *Biosci Rep* 2021;41(4).
- [75] Xu L, Liu X, Sheng N, et al. Three distinct 3-methylcytidine (m(3)C) methyltransferases modify tRNA and mRNA in mice and humans. *J Biol Chem* 2017;292(35):14695–703.

- [76] Scholler E, Marks J, Marchand V, et al. Balancing of mitochondrial translation through METTL8-mediated m(3)C modification of mitochondrial tRNAs. *Mol Cell* 2021;81(23):4810–4825.e12.
- [77] Zhang LH, Zhang XY, Hu T, et al. The SUMOylated METTL8 induces R-loop and tumorigenesis via m3C. *iScience* 2020;23(3):100968.
- [78] Wells JP, White J, Stirling PC. R loops and their composite cancer connections. *Trends Cancer* 2019; 5(10):619–31.
- [79] Condrat CE, Thompson DC, Barbu MG, et al. miRNAs as biomarkers in disease: latest findings regarding their role in diagnosis and prognosis. *Cells* 2020;9(2).
- [80] Briand J, Serandour AA, Nadaradjane A, et al. N6-adenosine methylation of miRNA-200b-3p influences its functionality and is a theranostic tool. *Mol Ther Nucleic Acids* 2020;22:72–83.
- [81] Cheray M, Etcheverry A, Jacques C, et al. Cytosine methylation of mature microRNAs inhibits their functions and is associated with poor prognosis in glioblastoma multiforme. *Mol Cancer* 2020;19(1):36.
- [82] Konno M, Koseki J, Asai A, et al. Distinct methylation levels of mature microRNAs in gastrointestinal cancers. *Nat Commun* 2019;10(1):3888.
- [83] Jiang L, Ingelshed K, Shen Y, et al. CRISPR/Cas9-induced DNA damage enriches for mutations in a p53-linked interactome: implications for CRISPR-based therapies. *Cancer Res* 2022;82(1):36–45.
- [84] Cox DBT, Gootenberg JS, Abudayyeh OO, et al. RNA editing with CRISPR-Cas13. *Science* 2017; 358(6366):1019–27.
- [85] Vu LT, Tsukahara T. C-to-U editing and site-directed RNA editing for the correction of genetic mutations. *Biosci Trends* 2017;11(3):243–53.
- [86] Abudayyeh OO, Gootenberg JS, Franklin B, et al. A cytosine deaminase for programmable single-base RNA editing. *Science* 2019;365(6451):382–6.
- [87] Cui Q, Yin K, Zhang X, et al. Targeting PUS7 suppresses tRNA pseudouridylation and glioblastoma tumorigenesis. *Nat Cancer* 2021;2(9):932–49.

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Epigenomic profiling at genome scale: from assays and analysis to clinical insights

8

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1 Introduction

Over the past decade, the decreasing cost of epigenome-wide profiling techniques, and in particular of high-throughput sequencing, has led to a leap in our understanding of the role of epigenetics in cancer. These advances have helped to highlight the central role of epigenomics in the silencing of

tumor suppressors and activation of oncogenes [1] and have demonstrated that drivers of cancer progression often affect epigenetic regulators, resulting in global changes to the epigenome and associated dysregulation of downstream biological processes [2,3]. Based on these discoveries, numerous anticancer drugs targeting epigenomic regulators have already been developed and are currently on the market [4,5], with many more currently in development.

Epigenomic profiling has also been hugely informative in the annotation of the noncoding regions of the genome, with different epigenomic marks having been shown to associate with distinct genomic regions, providing evidence for, and aiding in the interpretation of functional effects of genetic variants occurring at those loci.

One limitation of so-called “bulk” epigenomic profiling approaches in cancer is that the resultant epigenomic signals represent an average across all of the heterogenous cell types present within a tumor. While computational approaches have been developed to deconvolve this signal [6,7], new single-cell epigenomic assays have recently been developed which aim to improve our understanding of the role and importance of epigenetic heterogeneity within a tumor, including in determining clonal identity, phenotypic plasticity, the role of the tumor microenvironment, cell-to-cell signaling, metastatic potential, and resistance to treatment [8].

In this chapter, we briefly review some of the most commonly used assays that allow us to study the epigenome. Following the description of experimental assays, we discuss statistical and computational methods for their analysis, before providing two use cases that illustrate the types of analyses described. Code for these analyses is provided in publicly available github repositories, with the hope that the reader will be able to apply similar epigenomic analysis in their own research.

2 Epigenomic profiling methods and the data generated by large-scale epigenomic projects

The epigenome of a cell consists of many interdependent layers, including three-dimensional chromosomal conformation, chromatin accessibility, DNA methylation and hydroxymethylation, histone tail modifications and alternative types of histones, and the expression of small and long noncoding RNAs, along with other transcripts involved in the regulation of epigenomic signals. Many experimental procedures have been designed to probe each of these elements of the epigenome. Such methods have varying degrees of resolution, throughput and coverage, as well as unique biases that must be accounted for. In this section, we will briefly discuss some of the most widely used epigenomic profiling methods; a summary of these methods is provided in [Table 8.1](#), for additional information, readers are directed to relevant links provided in that table. We also describe several important publicly available resources and datasets generated using these methods.

2.1 Genome-wide methylation

Methylation of cytosine bases in the DNA (DNA methylation) is the most widely studied portion of the epigenetic landscape of human cells. In mammalian cells, DNA methylation is found almost exclusively at CpG loci (a cytosine followed by a guanine). It has been shown that methylation

Table 8.1 Summary of epigenomic methods for bulk tissues.

Method	Description	Pros	Cons	References
Methylation				
Infinium Methylation EPIC BeadChip	Method uses bisulfite conversion followed by detection of methylation status based on differentially labeled Cy3 or Cy5 using bead array containing probe for ~850 K individual CpGs	Detection of methylation status at single nucleotide resolution at ~850K individual CpGs	Covers only 30% of the human methylome and has suboptimal coverage of regulatory elements	[9]
MeDIP-seq	Method uses an antibody to immunoprecipitate methylated DNA or hydroxymethylated-specific regions. Sequencing of immunoprecipitated DNA determines methylation or hydroxymethylation status	Comprehensive methylome coverage at fraction of the cost and can specifically detect 5mC and 5hmC based on antibody used	Resolution is 100–300 bp	[10]
MRE-seq	Method uses methlysensitive restriction enzyme (MRE) to cut unmethylated cytosine within their recognition sequence. Sequencing of size selected fragments detects unmethylated CpGs that can be used to determine relative methylation status of other CpGs	Method is reliable, inexpensive, and can detect methylation status of >1 million CpGs or 65% of CpG islands at single nucleotide resolution. Can be combined with MeDIP-seq to produce genome-wide CpG methylation measurement at high coverage and high resolution	Only assays CpG islands that contain the restriction enzyme recognition sequence. This only represents 1–2% of the genome and 7% of total CpGs	[11,12]
RRBS	Method uses methylation insensitive restriction enzyme to digest DNA and select fragments containing CpG dinucleotides. This is followed by bisulfite treatment and sequencing	Retains the advantage of WGBS while reducing the cost. Provides single base pair resolution, captures 90% of CpG islands, determines methylation status at non-CpG sites, and provides information regarding strand and allele-specific methylation	Fragments selected are biased based on restriction enzyme used and cannot distinguish DNA methylation from hydroxymethylation	[13,14]
WGBS	DNA sonication is followed by bisulfite treatment. Sequencing of bisulfite treated DNA fragments detects methylation status at single base pair level	Provides single CpG resolution of DNA methylation and provides information regarding strand and allele-specific methylation	Costly since many reads do not contain CpG dinucleotides. Cannot distinguish DNA methylation from hydroxymethylation	[15,16]

(Continued)

Table 8.1 Summary of epigenomic methods for bulk tissues. *Continued*

Method	Description	Pros	Cons	References
Histone modifications				
ChIP-seq	Method uses a specific antibody to detect histone modification and immunoprecipitate the DNA fragments. Histone modified chromatin sites are determined by sequencing the immunoprecipitated DNA	Inexpensive, provides higher resolution maps than ChIP-chip and better signal-to-noise ratios	Chromatin maps rely upon availability of specific antibodies	[17]
CUT&RUN	Method uses antibodies specific to target protein-DNA complexes on native chromatin and cleaves them for analysis by qPCR or next-gen sequencing using micrococcal nuclease	More cost effective than ChIP-seq and requires only ~1/10th the sequencing depth as ChIP-seq	Also relies on the availability of specific antibodies	[18]
DamID-seq	Method based on expression of a fusion protein consisting of <i>E. coli</i> deoxyadenosine methylase (Dam) and the protein (histone modification) of interest. This allows Dam to methylate the adenine base in GATC motifs near the protein of interest's binding sites which can then be amplified and detected	Does not rely on availability of antibodies	Limited by GATC sequence occurrences and need for transgenic cells	[19]
Chromatin accessibility				
DNase-seq	Method uses DNase I enzyme to digest DNase I hypersensitive sites and detect open chromatin regions by sequencing DNase I digested DNA fragments	Provides high-resolution mapping of open chromatin regions across the genome	DNase I optimal concentration for digestion varies from sample to sample, so each sample needs to be individually titrated	[20,21]
FAIRE-seq	Method uses formaldehyde to crosslink cells or dissociated tissues which are then lysed and sonicated. The sheared fragments representing regions of nucleosome-depleted DNA are then isolated and sequenced	Does not require DNase enzyme optimization	Cannot identify regulatory proteins bound to DNA	[22]

ATAC-seq	Method uses a hyperactive form of Tn5 transposase to insert sequencing adapters into accessible regions of chromatin which are then purified, amplified, and sequenced	Has low input material requirement and is faster and more sensitive than alternative assays	Fragments tagged by incorrectly oriented adaptors are lost during PCR and reads are prone to contamination from mitochondrial DNA	[23]
Nuclear organization				
Chromosomal conformation capture (Hi-C)	Method works by cross-linking and ligating physically interacting genomic regions to generate a contact map of DNA interactions	Characterizes the three-dimensional conformation of genomic DNA in the nucleus	Not well-suited for profiling short-distance interactions	[24]
ChIA-PET	This method combines ChIP-seq and Hi-C by using antibodies to pull down protein of interest along with cross-linked DNA fragments which are then ligated to detect DNA regions that interact via a protein of interest	Can globally detect a large number of both short and long-range chromatin interactions	Requires a large amount of starting material and also a known protein of interest and specific antibodies	[25]
Gene expression				
RNA-seq	Method uses polyT fractionated or RNA fragments to convert to a library of cDNAs, which are PCR amplified and sequenced. Different libraries need to be constructed for the type of RNA quantified (small RNA, <200 bp, or large RNA, >200 bp) based on size selection	Genome-wide snapshot of transcript level in the sample of coding and noncoding regions	Transcriptomic profile with polyT-based fractionation does not include several non-polyT-based noncoding transcripts. Requires significant depth to detect expression of noncoding RNAs	[26]

profiles are deeply involved in the regulation of gene expression, cellular differentiation, and embryonic development. The disruption of such patterns has been associated with many human pathologies, including cancer, neurodevelopmental disorders, and neurodegenerative disorders. The most comprehensive method for detecting DNA methylation is whole-genome bisulfite sequencing [15,16]. This method involves treating purified and fragmented DNA with the chemical bisulfite, which converts unmethylated cytosines to uracils while methylated cytosines are protected from bisulfite treatment and are not converted. DNA fragments are then PCR amplified and sequenced, and reads are mapped to a reference genome. The methylation level at each cytosine is determined by comparing the number of reads with a “C” base call at that position to the number with a “T” base call for the same position (uracils are converted to thymines after PCR amplification). This technique has genome-wide coverage and single base resolution, however, the high depth of sequencing necessary to achieve high accuracy of methylation calls means that this technique can be costly compared to other approaches, both in terms of sequencing and in terms of computational time and resources required to process the data. Other techniques sacrifice the coverage of all cytosines in the genome for a decreased cost and increased throughput. Examples of such techniques are reduced representation bisulfite sequencing (RRBS) and the popular Infinium Human Methylation 450 (450 K) and more recent EPIC (850 K) BeadChip arrays. These techniques also use bisulfite treatment to distinguish between methylated and unmethylated cytosines. However, in RRBS, instead of sequencing the entire genome, a combination of restriction enzyme treatment and size selection is applied to DNA in order to enrich for genomic regions with high CpG density before sequencing. In the case of the BeadChip arrays, instead of sequencing, a microarray containing probes designed to assess the methylation status of selected CpGs distributed throughout the genome is used.

Also, worth mentioning are methods that achieve a decreased cost and do not involve bisulfite treatment. Examples of this type of method are methylated DNA immunoprecipitation sequencing (MeDIP-seq) and methylation-sensitive restriction enzyme sequencing (MRE-seq). In MeDIP-seq, antibodies that recognize methylated CpGs are used to immunoprecipitate and enrich for DNA fragments containing methylated CpGs. After sequencing and mapping of reads, genomic regions with high levels of methylated CpGs will have many reads mapped to them, while regions with low levels of CpG methylation will have few or no reads mapped to them. On the contrary, MRE-seq uses methylation-sensitive restriction enzymes and size selection of DNA fragments to enrich for regions with unmethylated CpGs. This makes MeDIP-seq and MRE-seq highly complementary and the output of these two methods have been combined to estimate a single base resolution of methylation profile [27,28].

Many recent studies have also recognized the hydroxymethylation of cytosines (5hmC) as an important component of epigenomic signatures in cancer [29–31] and their potential as biomarkers; the authors in [32], for example, provide evidence that hydroxymethylation changes in circulating cell-free DNA show great promise for the early detection of pancreatic cancer. Assays similar to those described above have also been developed to study this epigenetic mark [33].

2.2 Alternative histones and histone modifications

Histones are found in complexes called nucleosomes. Each nucleosome is comprised of eight histones (usually two copies of H2A, H2B, H3, and H4) bound by 147 bp of DNA. Many chemical

modifications can be found in the tails of the histones, including, but are not limited to acetylation, mono-, di-, or tri-methylation and ubiquitination. Alternative versions of specific histones can be observed in the nucleosomes, with specific histone variants, termed “oncohistones,” present at increased rates in a variety of cancers, including head and neck cancers, gliomas and sarcomas [34]. The identification of genomic regions harboring modified or alternative histones can be carried out using chromatin immunoprecipitation (ChIP) studies with antibodies for the specific histone modification of interest. While early studies used microarrays after the immunoprecipitation step to identify genome-wide histone modifications (ChIP-Chip), ChIP-seq is currently the most widely used approach. With ChIP-seq, the DNA fragments bound to the modified histone are isolated, sequenced and then mapped to a reference genome. Regions with a large number of mapped reads (“peaks”) correspond to locations where DNA was wrapped around histones carrying the specific epigenetic mark of interest.

2.3 Chromatin accessibility

The genomic DNA in the nucleus is generally highly compacted, however, different regions of the genome can be in more or less compact states. Active and regulatory (promoters, enhancers) regions tend to be in a less compact state, making it possible for transcription machinery to recognize DNA motifs and bind to those regions. A commonly used assay to profile chromatin accessibility genome-wide is DNase I hypersensitive site sequencing (DNase-seq) which digests genomic DNA with low levels of the DNase I enzyme. Genomic regions that are in a less condensed state will have a higher rate of digestion. Biotinylated linkers are then bound to the digested ends of the DNA fragments, which are then purified and sequenced. As with ChIP-seq, reads are then mapped to a reference genome and peaks are identified, which, in this case, correspond to regions with greater chromatin accessibility.

A more recent approach, combining elements of both DNase-Seq and ChIP-seq is ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) [23]. This assay uses a hyperactive form of Tn5 transposase to insert sequencing adapters into accessible regions of chromatin, providing information simultaneously about chromatin accessibility, transcription factor binding sites, and nucleosome occupancy.

2.4 Chromatin conformation

The assays and data types mentioned so far provide only a one-dimensional view of the epigenomic landscape of a cell, the three-dimensional conformation of the genomic DNA in the nucleus however, is highly significant in determining the behavior of a cell [35], is cell-type specific and can be disrupted in disease states. This three-dimensional architecture of the genome can be profiled through chromosomal conformation capture assays. The Hi-C [24], as well as newer related methods, generates genome-wide profiles of DNA interactions by first cross-linking chromatin, then fragmenting, biotinylating, and re-ligating DNA fragments from interacting genomic regions. Sequencing using the paired-end approach, the two ends of the same DNA fragment in a Hi-C library will come from regions that were adjacent at the time of cross-linking. Each end of the fragment is mapped to the reference genome, with the number of paired-end reads that map to each pair of genomic regions used as a measure of the level of interaction between those regions. The end result is a representation of genome-wide chromatin structural features in the form of an interaction matrix or contact map, which provides

multiresolution insight into the *cis*- and *trans*-interactions between genomic elements. A recent example of the use of a variant of Hi-C (termed *in situ* HiC) [36] is given by the authors in [37], who provide evidence of widespread changes in chromatin architecture in T-cell acute lymphoblastic leukemia (T-ALL), including a *trans*-activating domain (TAD) fusion event, which results in increased enhancer-promoter interactions for the MYC oncogene.

2.5 Gene expression including small and noncoding RNAs

Transcription of both coding and noncoding genes is closely intertwined with epigenomic states. Many assays have been developed to profile the transcriptome; however, RNA-seq is the most commonly used. This method begins with the extraction of RNA from a population of cells or from a single cell. Many methods exist for RNA extraction, and the chosen method will depend on the type of RNA species that one is interested in profiling. In the case of polyadenylated transcripts, the extraction is followed by reverse transcription of RNA into cDNA, followed by sequencing. Due to the many variations of this method, we do not provide further details on this technique here, but instead refer the reader to a recent in-depth review on this topic [38].

2.6 Single-cell methods

Recent years have seen an exponential increase in the generation of single-cell data with new studies routinely producing data on hundreds of thousands of individual cells [39]. These data provide a higher resolution perspective of cell states than bulk methods and has been used to characterize rare cell populations and resolve cell–cell heterogeneity. In the context of cancer, studies have made use of single-cell techniques to understand cell-to-cell signaling mechanisms between tumor, immune, and other stromal cells that make up the tumor microenvironment and has led to better understanding of immunotherapy-based treatments [40]. While the cost of generating single-cell data means that studies are typically limited to small numbers of samples, there have been continued improvements in multiplexing and sequencing technologies, as well as large scale efforts to integrate single-cell data from distinct experiments. This has led to the development of reference atlases that encompass data from multiple different modalities, patients, and disease conditions.

The majority of single-cell assays follow a similar workflow—first, the tissue sample is dissociated and individual cells are isolated, followed by compartmentalization (in droplets or wells, for example) and lysis of cells. In commonly used protocols, such as the 10X Genomics Chromium sequencing assay, sequencing reads are tagged with a cellular barcode, indicating which cell the read originated from, as well as a unique molecular identifier (UMI) to allow for the collapsing of any PCR duplicates.

Using these techniques for isolating and labeling individual cells, many of the epigenome profiling methods that have been developed for bulk samples, such as bisulfite sequencing, ChIP-seq and ATAC-seq, have been adapted for profiling single cells. With the exception of the transcriptome, the epigenome of single cells can be challenging to profile because there are only two copies of genomic DNA in a single diploid cell on which epigenetic information is scattered, in contrast to dozens of copies of mRNA molecules. However, technological advancements have made it possible to robustly and accurately profile several layers of the epigenome at the single-cell level, including gene expression, DNA methylation, chromatin accessibility, histone modifications, and nuclear organization.

For single-cell methylome profiling, bisulfite-conversion-dependent and independent methods have been developed, with newer methods introducing improvements to mitigate DNA loss [41]. Perhaps the most commonly used method for single-cell epigenomic profiling is scATAC-seq, for characterizing chromatin states in single cells. Multiple protocols have been published for implementing ATAC-seq at the single-cell level, which make use of either a combinatorial indexing [42], droplet-based [43] (commercialized by 10X Genomics) or well-based approach for isolating and labeling single cells [44]. With regard to profiling of histone modifications, several protocols have been published including a droplet-based scChIP-seq protocol which was initially developed in [45] and improved upon in [46]. Other single-cell histone modification profiling techniques include single-cell DNA adenine methyltransferase identification (scDamID) [47] and a CUT&TAG approach that has been adapted for single cell [48]. Conventional chromosome conformation capture methods, such as Hi-C, have also been adapted for single-cell assays to reveal 3D nuclear organization and cell–cell variability in chromosome structure at the single-cell level [49].

The methods mentioned above are routinely combined in studies that characterize multiple layers of the epigenome [50]. For example, scRNA-seq is commonly combined with scATAC-seq to characterize gene expression and chromatin accessibility dynamics in tandem. Several methods have also been developed to profile multiple epigenomic layers simultaneously in the same individual cells. For example, it is possible to simultaneously measure the transcriptome along with the DNA methylome, chromatin accessibility and cell surface epitopes in single cells [51–58]. More advanced methods have also been developed to simultaneously profile multiple epigenomic layers in single cells, including scNOMe-seq, which measures chromatin accessibility, DNA methylation, and nucleosome phasing [59], and scCOOL-seq, which can measure chromatin state, DNA methylation, and copy number variation in single cells [60].

Finally, recent years have also seen a growing number of single-cell spatial epigenomic profiling protocols. The techniques mentioned above all begin by dissociating a tissue sample into a cell suspension and so information about the spatial organization of cells in the tissue is lost. Platforms, such as spatial transcriptomics [61] and MERFISH [62], allow for the preservation of cell-cell organization in original tissue contexts. A handful of methods have been proposed that take advantage of these platforms to profile the epigenome while preserving the spatial structure of the tissue including sciMAP-ATAC [63] and spatial ATAC [64] for spatial mapping of chromatin accessibility and Spatial-CUT&Tag for spatially resolved chromatin modification profiling [65].

Although there are challenges associated with single-cell epigenomic profiling assays, such as the high cost, data sparsity, and significant batch effects between samples, improvements to sequencing technologies are continuously being introduced to make these assays more affordable and a plethora of computational tools and pipelines have been developed to combat technical issues using imputation and batch effect correction [66,67]. Some of the most common tools for analyzing single-cell data are discussed in Section 3.4, for more detailed information on single-cell protocols the reader is directed to [68,69].

2.7 Epigenomic projects and publicly available epigenomic data

Numerous national and international epigenomics projects and consortia have generated and analyzed a large number of complete epigenomes from normal and cancer cell types. As of the 2020–10 release, the IHEC data portal, for example, contains over 1300 complete epigenome and over 7500 datasets

Table 8.2 Summary of large-scale epigenome projects.

Projects	Description	Data portal	References
Roadmap Epigenomics Project	Generated reference human epigenome profiles across >100 different cell types and tissues	http://www.roadmapepigenomics.org/	[70,71]
International Human Epigenome Consortium (IHEC)	Extends the efforts of the Roadmap Epigenomics Project to generate 1000 reference maps of human epigenome	http://ihec-epigenomes.org	[72,73]
The Cancer Genome Atlas (TCGA)	Perform comprehensive genomic and epigenomic characterization in more than 20 types of human cancers	http://cancergenome.nih.gov/	[2,74]
International Cancer Genome Consortium (ICGC)	Extends the TCGA project to generate comprehensive catalogs of genomic and epigenomic characterization of more than 50 different cancer types	http://dcc.icgc.org	[72]
Encyclopedia of DNA Elements (ENCODE)	Determines functional elements in the human genome by generating transcription factor and maps of chromatin modifications	http://encodeproject.org/	[75]
BLUEPRINT	European project that aims to provide reference epigenomes from hematopoietic cell lineages	https://www.blueprint-epigenome.eu/	[76]

from across the NIH Roadmap, ENCODE, Canadian Epigenetics, Environment and Health Research Consortium (CEEHRC), BLUEPRINT, AMED-CREST (Japan Agency for Medical Research and Development), and EpiHK projects. These datasets, which can be explored, interrogated, visualized, and downloaded, span a wide range of tissues and cell types from mouse and human samples, and include histone mark (H3K27ac, H3K27me3, H3K36me3, H3K4me1, H3K9me3), WGBS, and transcriptomic data. Data from TCGA available through the NCI's Genomic Data Common portal (<https://portal.gdc.cancer.gov/>) include methylation array data and miRNA-seq data from more than 13,000 cases, as well as ATAC-seq data for more than 400 cases, while the ICGC Data Portal (<https://dcc.icgc.org/>) contains bisulfite sequencing data for 86 Ewing sarcoma patients. Table 8.2 summarizes some of these projects and provides a description of their goals.

In addition to making large amounts of data publicly available, these projects have made significant contributions to the standardization of protocols, data processing pipelines, and analytical tools used to mine epigenomic data, including guidelines on minimum criteria for reference epigenomes. Furthermore, they have significantly improved the metadata standards used for epigenomic data, a hugely significant step for the ability to share data and perform integrative epigenomic analyses.

3 Tools and analyses

In this section, we discuss analytical approaches and statistical considerations for some of the assays previously described. As with epigenomic profiling assays, new computational approaches are constantly being developed and finding “gold-standard” tools and pipelines can be difficult.

While a number of command-line and graphical user interface-based applications are available, statistical analysis of large-scale epigenomic datasets is often carried out using the R statistical programming language [77], leveraging Bioconductor's rich collection of packages and functionality [78]. The use of software containers, such as Docker [79] and Singularity [80], are strongly encouraged as these can help to ensure reproducibility of results from data analysis pipelines by packaging data, applications, and associated scripts into shareable images from which multiple identical container instances can be launched. Finally, the size of datasets involved can often require the use of local high-performance computing (HPC) or cloud computing resources for their analysis, particularly for the use of machine-learning models which can be computationally costly to train.

3.1 Detecting differential methylation

The first step in the analysis of methylation data is preprocessing. For sequencing-based approaches, such as WGBS and RRBS, this will involve first checking the quality of the raw read data. FASTQC [81] is a popular tool which provides information on a per-base quality scores, sequence GC content, k-mer analysis and a number of other useful quality measures. FASTQC information across multiple samples can be easily aggregated into an interactive HTML report using Multiqc [82]. Depending on QC results, reads may need to be trimmed and realigned. Output from methylation-aware aligners, such as Bismark [83] and bwa-meth [84], are further QC'ed (to examine coverage and confirm the presence of an expected bimodal distribution in methylation percentages) and are then used as input to differential methylation calling tools. For array-based approaches, ChAMP [85] and Minfi [86] are popular Bioconductor packages that provide a wide range of functionality for the analysis of DNA methylation data from 450 K or EPIC arrays. Preprocessing methods here include probe QC and filtering (e.g., removal of probes with low detection values, probes missing values from a large number of samples, or SNP-related probes), imputation of missing data where possible, and normalization, for which a number of different approaches are available including quantile normalization and variants thereof.

Depending on how samples were processed, batch correction is often a vital aspect of sample preprocessing. Batch effects refer to the systematic technical noise introduced for example, by using a particular set of reagents, on a particular machine, at a particular date/time. This technical noise may mask biological signal, leading to inaccurate results and conclusions, or, if samples from different experimental groups are processed as different batches, may greatly confound statistical analysis. Common approaches to remove or correct for batch effects include incorporation of batches as random effect in linear mixed models when testing for differential methylation, or adjustment of methylation values based on latent factors. Popular approaches for batch correction include the Combat [87] and surrogate variable analysis [88] tools.

Once the data have been preprocessed, exploratory data analysis is typically carried out as an initial assessment of how similar samples are based on their methylation profiles; this can help to identify potential outlier samples, which may need to be excluded from the analysis. Given the high-dimensional nature of genome-wide epigenomic profiling (hundreds of thousands of data points per sample), visualization is usually carried out on a lower-dimensional (2D/3D) representation of the data based on principal components analysis, multidimensional scaling, or some other dimensionality reduction technique. Pairwise sample-to-sample correlation can be calculated, and within- and between-sample group similarity can be assessed using clustering approaches. Many

algorithms are available to cluster objects based on similarity between their associated features; common examples are hierarchical clustering, k-means clustering, and k-nearest neighbor clustering. The choice of clustering algorithm, the similarity metric used to compare the features associated with each object, and other input parameters may all affect the final result of this type of analysis and the choice of such parameters needs to be carefully made on a case-by-case basis, as no single parameter set is likely to prove optimal across all experiments. Clustering is an unsupervised machine learning (ML) approach, with the algorithms having no a priori information about sample classes or groupings; in a later section we contrast this with supervised classification approaches. In the context of cancer epigenomics, cluster analysis has been successfully applied to many different situations, including for defining molecular subtypes based on their gene expression, methylation, or other epigenomic profiles [5,89,90]. These identified subtypes have been shown to be strongly associated with survival and can be used to stratify patients or determine treatment plans. Once sample groups have been defined in this way, it is then possible to determine the most likely group to which a new sample belongs.

Differential methylation (determined for a given effect size and threshold for statistical significance) can be tested at either individual CpGs, known as differentially methylated positions, or DMPs, or across a number of DMPs within a defined range, known as a differentially methylated regions, or DMRs. For DMPs, statistical approaches may be count-based, using, for example, Fisher's exact test for simple treatment/control designs, or may use a regression-based approach, for example, Methylkit [91], which offers a more flexible and powerful model when biological replicates and covariates need to be considered. For DMRs, smoothing approaches, such as Bsmooth [92], may be employed to borrow information across adjacent CpGs, or alternatively, the binary segmentation implemented by metilene [93] has demonstrated both computational efficiency for large sample numbers and high sensitivity in detecting differential methylation at region-level without the need for individual DMP calls.

Having identified statistically significant changes in methylation at DMP and/or DMR level, the next step is to provide annotations for the genomic loci at which these changes are observed, so that results can be appropriately interpreted based on the experimental design. Annotations from Refseq, Genbank, and Ensembl can all be accessed via the Bioconductor AnnotationHub package [94] and provide a relatively straightforward way to identify methylation changes that occur in the promoter regions or regions overlapping a gene. Determining which genes may be affected when methylation changes are observed in noncoding regions, however, is not as straightforward. The GREAT tool tries to address this issue by allowing the user to define both proximal and distal "regulatory domains" for each gene and then assigning an annotation to each of the inputs (DMPs/DMRs) based on genomic distance from the input to these regulatory domains [95]. An enrichment analysis using gene-sets from multiple biological databases is then carried out to determine if differences observed in methylation signals occur at a statistically "unusual" level in any specific biological functions or pathways. The rGREAT Bioconductor package [96] provides a programmatic interface to the GREAT tool for easy incorporation into analytical pipelines.

Finally, downstream analysis may also include the integration of methylation profiling data with other genomic data; gene expression data, for example, can be combined with methylation data to identify methylation quantitative trait loci (meQTLs), regions of the genome where a change in methylation status is proven to elicit a corresponding change in expression for the gene in question.

3.2 Epigenome-wide association studies

In the last two decades, genome-wide association studies (GWAS) have proven very successful in using large-scale population-level data to identify associations between genomic variation (primarily single-nucleotide polymorphisms, or SNPs) and phenotypes of interest. Epigenome-wide association studies (EWAS) have similarly emerged in recent years as a powerful method for understanding the epigenomic variability associated with health, disease, and response to environmental factors. The EWAS Open Platform (<https://ngdc.cncb.ac.cn/ewas/>) [97] is a comprehensive knowledge base and analysis suite that integrates EWAS Atlas [98], EWAS Data Hub [99], and EWAS Toolkit, the latter providing a web-based toolkit for epigenome-wide association studies. EWAS Open Platform includes information from databases, such as Methbank [100], scMethbank [101] (single-cell whole-genome methylation maps for human and mouse), and many others, resulting in information on a total of more than 600,000 EWAS associations from over 950 publications covering a variety of diseases, phenotypes, and environmental factors. It also provides access to normalized DNA methylation array data (primarily from 450 K and EPIC arrays) from GEO, TCGA, ENCODE, and ArrayExpress, covering more than 100,000 samples, across more than 1000 tissues/cell types and more than 600 diseases. These epigenomic associations can help to explain some of the phenotypic differences observed between individuals that cannot be explained by genetic variation alone. Importantly, from a clinical perspective, the epigenome is easier to modify through changes in lifestyle meaning that associations identified between disease phenotypes and the epigenome may provide greater potential for new interventions or preventative strategies than those identified by GWAS.

Despite the great potential of EWAS for human health and disease, there remain many challenges when performing this type of experiment. Studying epigenetic changes within hard-to-obtain tissues and cell types is difficult, so whole blood is most commonly used as the source material. Blood, however, may not be a reliable proxy for epigenetic changes in the primary tissue of interest and also contains a heterogeneous mix of cell types which can make accurate interpretation of results more challenging. While there remain limited options in the former case, approaches for dealing with the latter are to either purify the cell type of interest prior to the analysis, to perform cell-type-specific deconvolution of methylation signals, or to perform single-cell methylation profiling. However, as previously mentioned, single-cell approaches are typically more costly and may be less-well developed, making them unfeasible for large-scale EWAS studies. Also, of key importance is the question of causality. Epigenetic variations can both cause and be caused by disease, making it difficult to identify epigenetic dysregulation as an underlying disease mechanism without, for example, longitudinal data which demonstrate a “normal” epigenetic profile prior to disease onset.

Multiple sources of bias and/or confounding can also have an effect on the results of an EWAS study. The epigenetic profile of an individual can be affected by age, gender, exposure to environmental factors, and by the genetic background of that individual. Therefore the population structure of the subjects involved in an EWAS should be carefully considered, and ideally, one should either select a population that is largely homogeneous in terms of these characteristics, or profile a population large enough to allow stratification or statistical correction for such factors.

The first step in an EWAS study is to identify DMPs and DMRs (as described in the previous section). Statistical analyses are then applied to the dataset in order to identify associations between methylation and the phenotype of interest. The most basic way of performing such analysis is by

using univariate tests of association, such as t-tests or linear regressions in a site-by-site manner. CpGaasoc [102] is an R package which allows a user to test for association between CpG methylation and a phenotype of interest using mixed effect linear models to account for, for example, chip/batch effect; it also supports quality filtering and the generation of QQ, Manhattan, and scatter plots to visualize results.

Measurements of DNA methylation at the single site level can be noisy; therefore since methylation statuses tend to be similar among nearby CpGs, many approaches perform association testing focusing on changes in the level of methylation across longer regions of the genome. This generally leads to greatly improved specificity, however, if a true association does involve only an individual CpG site, these methods may miss it. Note that either in a site-by-site approach or approaches focusing on longer regions, multiple hypothesis testing correction is essential and in general, false discovery rate-based correction is recommended for this type of high-throughput study as it provides reasonable control of type I errors without being overly stringent. Despite our best efforts to avoid them, such studies will always find at least a few spurious associations. Therefore once one has identified regions demonstrating statistically significant association with the phenotype, it is important to interpret the possible functional effect of such associations, as well as to validate the identified associations in an independent dataset, ideally using a different type of methylation profiling assay. This helps to provide confidence that the observed results are reproducible and less likely to be due to technical biases specific to the assay originally used. For a more in depth discussion and additional recommendations on EWAS, the reader is referred to [103].

3.3 Analysis of chromatin and histone states

When exploring chromatin accessibility (ATAC-seq) and histone modifications (ChIP-seq), the primary task is that of peak calling, which involves identifying regions of the genome showing an enrichment of reads relative to an expected number of reads under a background distribution (commonly, a Poisson distribution). There are a number of tools available for peak calling, including SPP [104], SICER [105], and the more recent normR Bioconductor package [106]. Here however, we focus on the popular MACS2 [107] tool (for a recent review of peak calling algorithms, readers are referred to [108]). MACS2 works by using a sliding window approach to compare the signal within a fixed-size window in the ChIP sample (sample treated with ChIP antibody) to a background signal, which is estimated from an input or control sample (sample without ChIP antibody). While it is possible to call peaks without a control, this is not recommended as controls can help to mitigate bias due to GC content, genome mappability, and DNA repeats.

An important step in conducting an analysis of ChIP-seq or ATAC-seq data is to assess the quality of candidate peaks. The ENCODE consortium established a number of useful guidelines and statistical measures for determining peak quality, including metrics such as fraction of reads in peaks scores, the values of which typically correlate positively with the number of called peaks/regions, relative and normalized strand cross-correlation, which quantify the correlation between read densities on the +ve and -ve strands, and the irreproducible discovery rate (IDR), which uses pseudoreplicates to measure consistency between called peaks.

Another issue that must be considered when applying peak calling algorithms to epigenomic datasets is that different epigenomic marks have distinct patterns of distribution across the genome. Applying the same statistical framework of peak detection to all different classes of epigenomic

marks can result in biological misinterpretation [109,110]. Epigenomic marks can be categorized into three classes: point-source, broad-source, and mixed-source [110]. Point-source factors yield punctate signals and are typically obtained from transcription factors or from localized histone modifications, such as H3K4me1 and H3K4me3, which are active chromatin marks localized at enhancers and transcription start sites. Broad-source factors are associated with large genomic domains, result in broad peak signals and are typically observed in H3K36me3 and H3K9me3 modifications which are involved in chromatin elongation and repression. Finally, mixed-source factors bind at small genomic domains in some parts of the genome and broader domains in others, examples include RNA polymerase II and H3K27me3 modification. MACS2 can detect both narrow and broad peaks as can the other tools mentioned above.

Once a set of high-confidence peaks has been generated, a common downstream task is to identify differential enrichment between two conditions, for example, between different treatments or timepoints. Again, multiple tools exist for this task; however, the recently released DiffChIP R package [111], which is based on limma, demonstrates low false positive rates and high sensitivity compared to alternative methods. The final step of peak analysis is to associate peak regions with genomic annotations. As with changes in methylation levels, the AnnotationHub package and GREAT/rGREAT tools can be used for annotation and enrichment analysis.

Peak calling algorithms, such as the ones described in the previous section, can be used to identify and annotate regions harboring individual chromatin marks (e.g., H3K4me1 marking active enhancer regions and H3K4me3 marking active promoter regions); the cooccurrence of multiple marks, however, is highly relevant for the activity of multiple genomic elements, making annotations combining multiple epigenomic marks far more informative. Most approaches to annotating the genome based on multiple epigenomic marks begin by identifying recurrent patterns of epigenomic signals that consistently appear throughout the genome. To accomplish this, one usually divides the genome into nonoverlapping windows of a fixed size and groups them based on their multimark epigenomic profile. Spark is one such tool that uses k-means clustering to identify groups of genomic regions with similar patterns of epigenomic signals [112]. The ChromHMM tool, on the other hand, trains a Hidden Markov Model with a specified number of states such that each state in the model will be associated with a particular combination of epigenomic signals [113]. The chromatin states can then be associated with particular genomic annotations based on their overlap. Once the possible chromatin states are defined, new genomic regions can be classified into these different states based on their epigenomic profiles. This approach can also be extended to epigenomic profiling of different cell types and projects, such as the NIH Epigenomics Roadmap consortium, ENCODE, and IHEC, have already profiled a wide range of cell types using the methods described above, greatly improving our understanding of the noncoding regions of the genome.

3.4 Single-cell methods

The beginning of any single-cell analysis is to align and quantify the data. Which tool you use for this step will depend on which sequencing assay has been used. The CellRanger program developed by 10X Genomics can only be used for preprocessing of data sequenced using their Chromium assay whereas other tools, such as the kallisto bustools workflow [114], are more versatile and can be used with data from any UMI-based sequencing technology.

Following on from preprocessing, there are several tools that can be used to perform downstream analysis of single-cell data. The Signac R package is one such tool which builds upon the popular Seurat R package for analysis of scRNA-seq data, to enable analysis of single-cell chromatin data [115]. Steps in a typical single-cell analysis workflow may include calling peaks, quality control to remove any cells that may have been damaged/lysed prior to sequencing, normalization, dimensionality reduction, visualization, and clustering of cells, calculation of differential activity between conditions, and integration of data from different samples/conditions/modalities.

Data integration remains a challenge in single-cell data analysis, particularly integration of data from different modalities, which is usually vital in single-cell epigenomic analysis because, as mentioned above, studies typically measure multiple epigenomic layers simultaneously. Tools, such as Multi-Omics Factor Analysis (MOFA), have been developed for this application and make it possible to perform comprehensive and scalable integration of single-cell multimodal data [116].

3.5 Machine learning

Machine learning refers to the ability of an algorithm to learn from data without being explicitly programmed. ML approaches are particularly well-suited to finding patterns in complex, high-dimensional datasets and can be split into two broad types—unsupervised learning, where the goal is to find patterns or relationships in unlabeled data, and supervised learning, where known sample labels are used to train the algorithm. We have previously described clustering of samples based on their epigenomic profiles as a type of unsupervised learning; classification is a type of supervised learning approach where the algorithm learns a mapping between sample features (e.g., methylation values) and group labels—this can be for either binary (e.g., cancer vs. normal, treated vs. untreated, responder vs. nonresponder) or multiclass (e.g., breast cancer subtype) problems. To train the model, data is split into training and test subsets, with the former used to build the predictive model and the latter used to assess how its predictive performance generalizes to unseen samples. Model performance can be assessed using a variety of measures, such as classification accuracy (correct class label predictions on the test set), a confusion matrix (correct and incorrect class label predictions across different sample groups), or area under the receiver operating characteristic curve.

Once a classifier has been adequately trained, it will be able to predict class membership for new samples, providing the ability to stratify patients based on epigenomics profiles and enabling precision medicine approaches [117]. A key component of supervised classification is the selection (or, depending on the approach, automated identification) of sample features that are important for distinguishing between classes. Relative importance of selected features can then be used both to construct a biomarker signature for the experimental groups being studied and to highlight previously unknown biological mechanisms. Common examples of supervised classification approaches are support vector machines, or SVMs [118], and decision tree approaches, such as random forest [119] or gradient-boosted trees [120].

Artificial neural networks (ANNs), or simply neural networks, are a powerful and flexible class of ML models that can perform both supervised and unsupervised learning. Inspired by biological neural networks, they are composed of interconnected layers of neurons which process input signals to produce an output. During training, the weights of the connections between neurons are adjusted which results in the trained network learning an internal representation of the input data. Deep

learning refers to a subclass of neural network-based approaches which has become increasingly important in recent years. In deep learning, the network architecture contains a large number of layers, allowing the model both to construct more complex representations of the data and, in the case of supervised deep learning, to automatically select features which are important for the predictive performance [21].

While ML approaches offer enormous potential in the domain of epigenomic data analysis, there are some important considerations for their use. To train ML models, a large amount of data is typically required, which, depending on the problem domain, may need to be labeled and should typically include balanced representation of sample classes. Most ML algorithms include hyperparameters that will modify the way in which the algorithm learns; these require tuning for the specific dataset at hand as part of the sometimes already very time-consuming model training process. In the case of neural network and deep learning models in particular, the computational complexity increases considerably and efficient training often requires the use of specialized hardware, such as GPUs. Finally, model interpretability is also a key concern. So-called black-box models that offer little or no insight into how predictions were made will be of limited clinical value, making explainable AI (XAI) a very active area of current research.

For a recent review on the use of ML in clinical epigenetics, readers are referred to [122,123].

3.6 Case studies

3.6.1 Case study 1: a multiomics signature for prostate cancer (https://github.com/BarryDigby/epi_chap)

3.6.1.1 Background

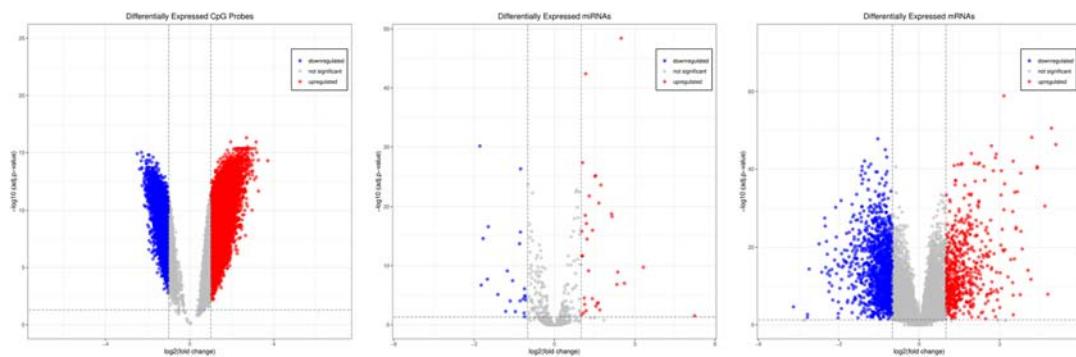
Prostate cancer (PCa) is one of the leading causes of mortality amongst males, second only to non-melanoma skin cancer. In addition to genomic alterations, epigenetic modifications, such as DNA methylation, have been reported to play a role in PCa formation and persistence, providing a novel avenue for biomarker generation for the early detection, progression and survival evaluation of PCa [124]. Furthermore, aberrant DNA hypermethylation has been found in many cancer types, negatively regulating the transcription of downstream microRNAs (miRNAs) and genes within the transcriptome [125]. In this study, we perform an integrative analysis to identify a highly correlated multiomics signature discriminating PCa samples from matched normal samples using methylation array, miRNA and gene expression datasets from TCGA.

3.6.1.2 Dataset

All methylation IDAT files, miRNA and gene expression files for TCGA-PRAD were downloaded using the GDC portal (GDC version 32 release date March 22, 2022). To both reduce the computational workload and facilitate a balanced target class for classification, matched tumor-normal samples from the same patient for which there exists methylation, miRNA and gene expression data were considered for the analysis ($n = 68$).

3.6.1.3 Approach

Methylation data was processed using the minfi package [86]: probes were normalized using the preprocessFunnorm function [126] and all probes overlapping SNPs were removed. Raw signal

**FIGURE 8.1**

Volcano plots of differentially methylated CpG probes, differentially expressed miRNAs and differentially expressed genes displaying global expression trends in the TCGA-PRAD cohort. Please check the online version for the color image.

intensities were converted to M-values for downstream statistical analysis [127]. Gene expression counts were filtered to include protein-coding genes prior to normalization and transformation using DESeq2 and the varianceStabilizingTransformation function, respectively [128]. miRNA datasets were processed in a similar manner to gene expression data.

Differentially methylated probe analysis, miRNA and gene expression analysis were performed using limma and DESeq2, respectively, to reduce the number of input features for the classification model. After applying a log₂ fold change cutoff of $+/-1$ and adjusted *P*-value threshold of $<.05$, there remained 58 miRNAs and 2831 differentially expressed genes. A log₂ fold change cutoff of $+/-2$ and an adjusted *P*-value threshold of $<.05$ were used to identify differentially methylated probes. Unannotated probes were discarded, producing a robust set of 6810 differentially methylated probes (Figure 8.1).

The Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DIABLO) method, developed by the authors of mixOmics [129] was used to extract correlated information between multiomics datasets, identify key omics variables (CpG, miRNA, mRNA) and extract discriminant features for the phenotypes of interest [130]. Briefly, DIABLO uses projection to Latent Structure models (PLS), extending both sparse PLS-Discriminant Analysis (sPLS-DA) and generalized canonical correlation analysis to provide a supervised framework for classification of new samples. A training set was constructed from the TCGA-PRAD dataset ($n = 48$) and used as input to DIABLO. Both the optimal number of components and biomarker panel size (features) were identified using a grid search approach, where the classification performance using a five-fold cross-validation repeated 20 times was utilized for all combinations, arriving at a combined panel size of 10 CpGs, 5 miRNAs, and 45 mRNA.

In Figure 8.2, we can see that CpG probes demonstrate the best discriminative ability between prostate tumor and prostate normal samples. The individual contribution of loadings in the biomarker panel can be viewed in Figure 8.3, and viewed as a global snapshot of expression in the clustered image map in Figure 8.4.

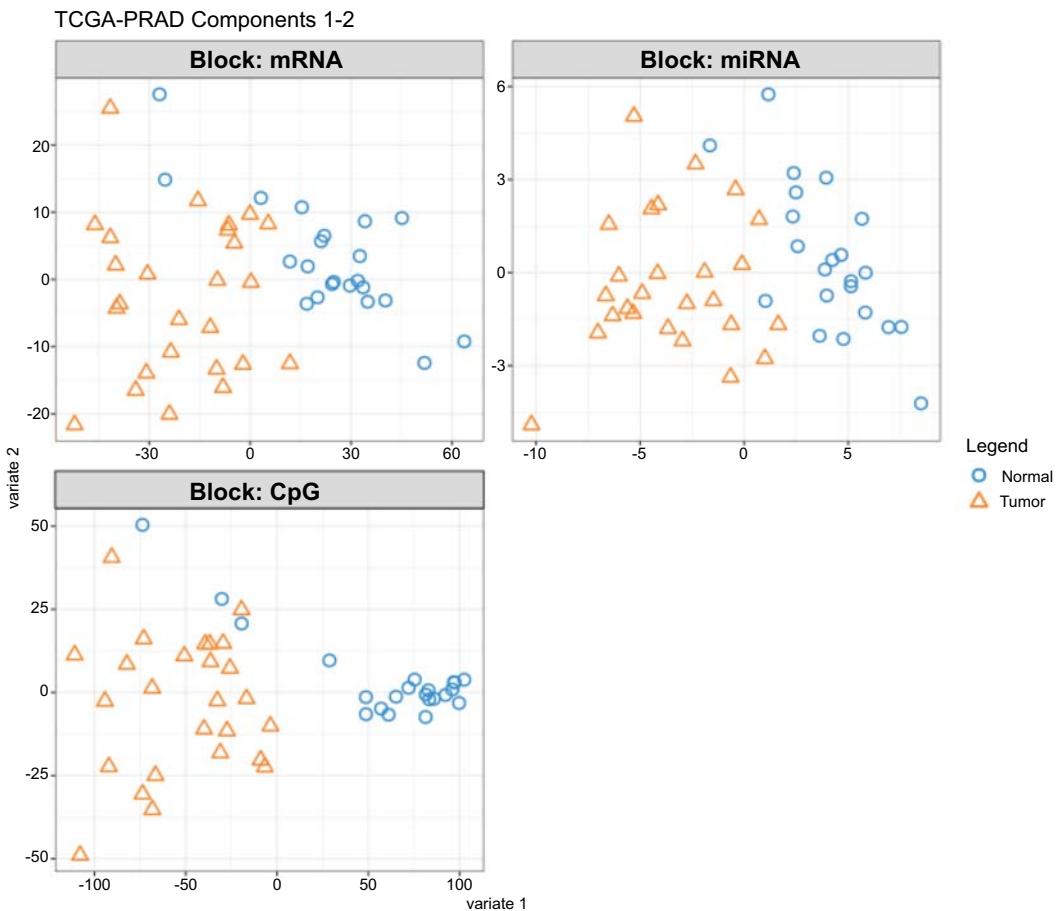


FIGURE 8.2

Individual samples from multiblock SPLS-DA projected onto feature space spanned by components 1 and 2 for CpG, miRNA and mRNA blocks. “Block: CpG” demonstrates the best clustering/separation in terms of sample class, indicating highly variable methylation patterns between tumor and normal prostate cancer patients.

3.6.1.4 Results

The biomarker panel derived from the multiomics TCGA-PRAD dataset exhibited a global accuracy of 95% (incorrectly classified one normal as tumor). As can be observed in [Figure 8.5](#), the classification accuracy achieved by the methylation biomarker panel alone achieved an accuracy of 100%, demonstrating the utility of DNA methylation as a stable independent biomarker capable of stratifying TCGA-PRAD samples ([Table 8.3](#)).

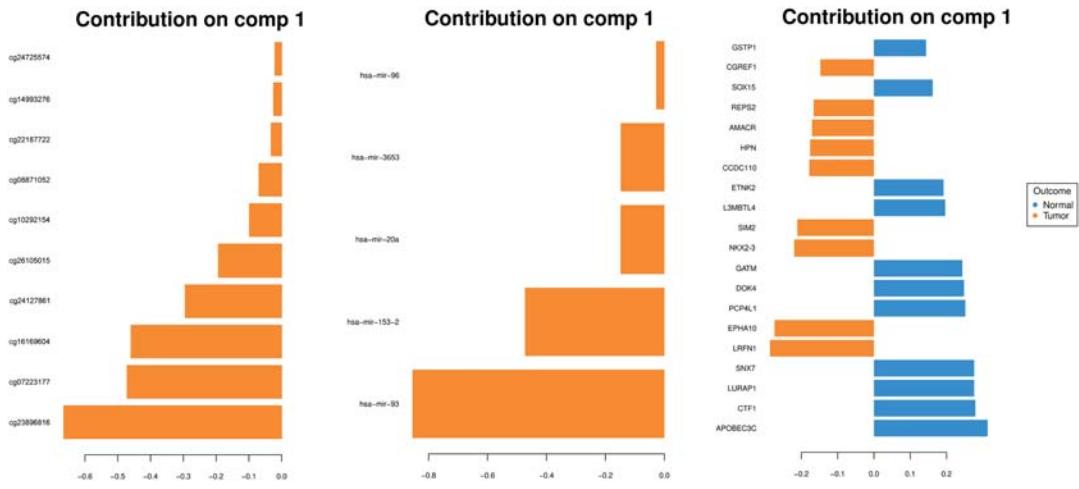


FIGURE 8.3

Candidate loading coefficient weights from component 1 extracted for further validation tests delineating tumor-normal TCGA-PRAD samples.

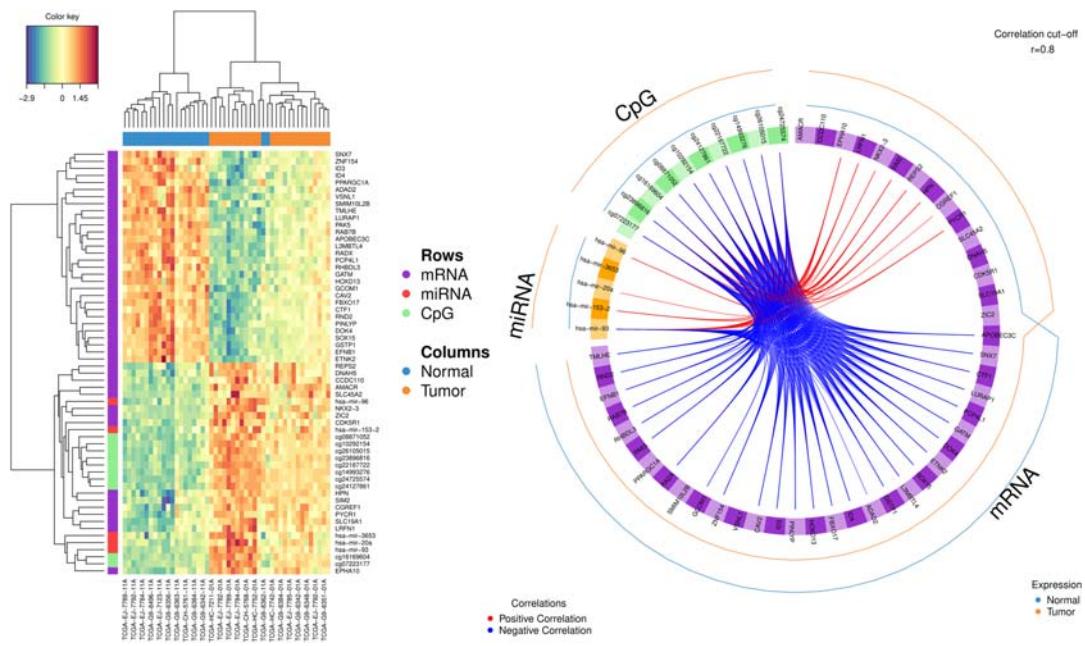
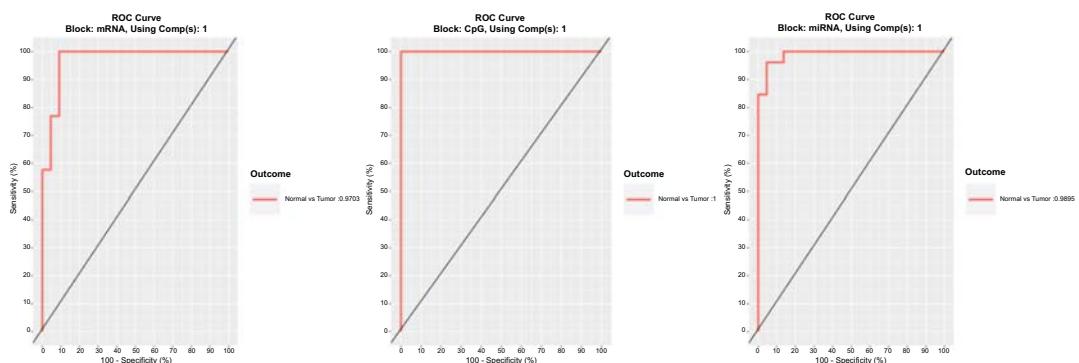


FIGURE 8.4

Clustered Image Map (CIM) and Circos plot for the variables selected by multiblock sPLS-DA performed on TCGA-PRAD on component 1. The CIM plot displays excellent separation between tumor versus normal using unsupervised clustering (Euclidean distance and complete linkage). The circos plot shows a CpG signature that is highly expressed in tumor samples (outer orange layer), exhibiting a strong negative correlation ($>r = -0.8$, inner links) with genes lowly expressed in prostate cancer, hinting at a possible regulatory effect on gene expression in prostate cancer. Please check the online version for the color image.

**FIGURE 8.5**

Receiver operating characteristic (ROC) curve for each sPLS-DA block on the test partition of the dataset. The CpG block displayed the highest performance with 100% accuracy, correctly classifying each of the tumor and normal samples.

Table 8.3 Differentially methylated probes.

Probes	Chr	Position	Relation to CpG Island	Gene	Gene region	LogFC	Adj. P-value
cg24725574	chr6	30006940	Island	HLA-J	Body	4.566	1.571E – 16
cg24127861	chr14	24171738	N_Shore	REC8	TSS1500	2.487	3.89E – 16
cg08871052	chr2	42047479	OpenSea	PKDCC	TSS1500	3.434	4.763E – 16
cg14993276	chr19	10286966	Island	ICAM4	TSS200	3.662	7.468E – 16
cg22187722	chr7	29146349	OpenSea	CPVL	5'UTR;1stExon	2.98	1.173E – 15
cg23896816	chr22	39077248	OpenSea	APOBEC3G	5'UTR;1stExon	2.589	4.432E – 15
cg26105015	chr3	123026252	N_Shore	SEMA5B	5'UTR	2.767	8.708E – 15
cg16169604	chr2	130727913	S_Shore	GPR148	TSS1500	2.746	9.327E – 15
cg07223177	chrX	46574763	Island	CHST7	1stExon	2.14	2.05E – 13
cg10292154	chr16	27450124	OpenSea	IL21R	3'UTR	3.602	2.095E – 13

3.6.2 Case study 2: a multiomics approach to characterizing the immune cell landscape in renal cell carcinoma (https://github.com/Sarah145/epigenomics_case_study)

3.6.2.1 Background

Clear cell renal cell carcinoma (ccRCC) is a common genitourinary tumor in which the immune microenvironment is known to play an important role in the pathogenesis and therapeutic response of the disease. Although ccRCC is responsive to immune-based therapies, most patients go on to develop drug resistance [131] and so it is essential to understand the developmental and functional states of immune cells in patients in order to develop novel and improved therapies. The objective of this case study is to characterize the regulatory landscape of the immune cell compartment in ccRCC to determine if there is potential to guide therapeutic strategies.

3.6.2.2 Dataset

In this case study we analyze single-cell ATAC-seq (scATAC-seq) and single-cell RNA-seq (scRNA-seq) data from a patient with ccRCC. The samples were obtained from tumor tissue (RCC) and adjacent normal tissue (adjNorm) and cells were sorted prior to sequencing so only CD45+ cells are included. The samples were sequenced using the 10X Genomics Chromium solutions for scATAC and scRNA sequencing and were generated as part of a study recently published in Nature Cancer [132].

3.6.2.3 Analysis approach

The approach for this case study follows a typical workflow for a scATAC-seq and scRNA-seq analysis. We begin by calling peaks in genomic regions in the scATAC-seq data and clustering cells based on their chromatin accessibility landscape and then integrating with scRNA-seq data to improve annotation of cell types and relate chromatin accessibility to gene expression. The majority of the analysis is performed in R using the Seurat and Signac packages [115,133] and the homer command line tool was used to perform motif enrichment [134].

3.6.2.4 Results

The scATAC-seq dataset consists of 4843 immune cells—3179 from tumor tissue and 1664 from adjacent normal tissue. MACS3 was used to perform peak calling on the scATAC-seq fragments and identified 157,131 peaks.

UMAP was used to reduce the dimensionality of the dataset and visualize the cells (Figure 8.6A). Cells were clustered based on their chromatin accessibility using the Leiden community detection algorithm which detected 10 clusters with distinct chromatin accessibility profiles. To classify each cluster, chromatin accessibility was used to predict gene activity scores and for each cluster, the genes with the top activity scores were used to estimate the cell type (Figure 8.6B).

Clusters 1 and 7 are made up of NK cells and had high activity scores for typical NK cell marker genes, such as KLRF1 and GNLY (Figure 8.6E). Cluster 1 NK cells were present in both the tumor and normal sample; however, cluster 7 NK cells were only present in the tumor sample (Figure 8.6C).

These cells had high activity scores for genes, such as KIR2DL4, which has been associated with NK cellular senescence [135]. Cluster 5 cells also show high activity scores for NK-cell- and T-cell-related genes (CD5, CD70) and so were labeled as cytotoxic cells.

Clusters 2 and 3 had high activity scores for T-cell-specific genes, such as CD8B and CD27. Similar to the NK cells—there were two distinct clusters of T cells, one identified in both tumor and normal and the other identified mostly in tumor (Figure 8.6C). The tumor-specific T cells had higher activity scores for genes, such as CTLA4, which is a marker of T-cell exhaustion, suggesting that these T cells may be dysfunctional. A small cluster of cells that also showed high activity of genes related with regulatory T-cell functions (e.g., FOXP3) was also identified (cluster 10). This cluster was only present in the tumor sample.

Cluster 4 showed high activity for genes typically expressed in phagocytic cells and is likely made up of a combination of monocytes/macrophages and dendritic cells. GO terms related to phagocytosis were enriched among genes with the top activity scores in this cluster (Figure 8.6F).

Cluster 9 was easily identified as B cells, given its high activity scores for genes, such as MS4A1, and enrichment of GO terms, such as “B-cell activation.”

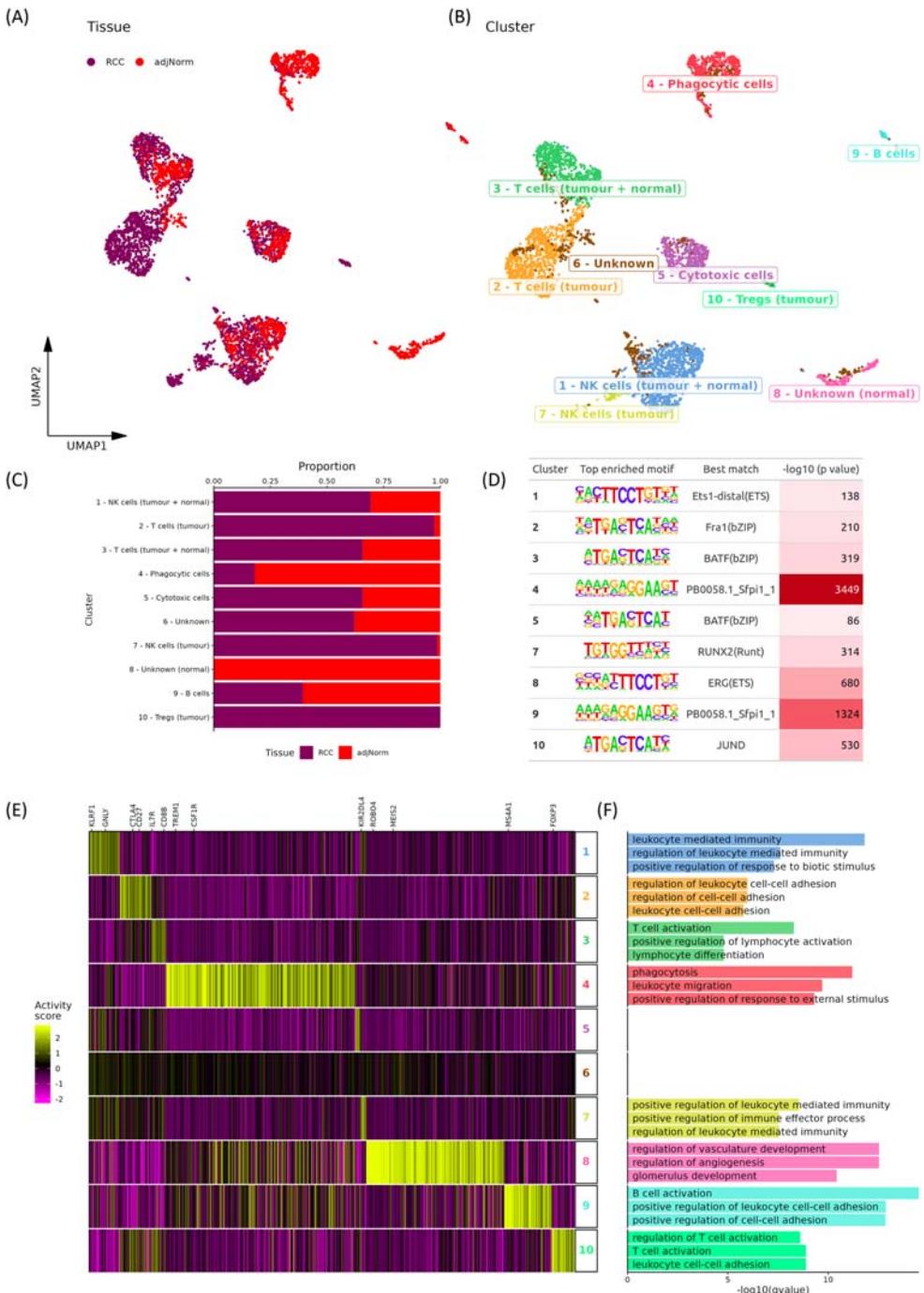


FIGURE 8.6

scATAC-seq identifies 10 clusters with distinct chromatin accessibility profiles which correspond to different immune cell types. UMAP of scATAC-seq data colored by tissue of origin (A) and cluster (B). (C) Proportion of cells from each tissue in each cluster. (D) Top enriched motif in each cluster. (E) Activity of differentially accessible genes in each cluster. (F) GO terms enriched among differentially accessible genes in each cluster. Please check the online version for the color image.

Finally, clusters 6 and 8 could not be labeled as specific cell types due to ambiguous activity scores for marker genes of different cell types. Cluster 6 did not have any significantly differentially accessible genes and also is widely dispersed and does not form a distinct cluster on the UMAP graph. These could potentially be poor quality cells. Cells in cluster 8 showed upregulated accessibility of genes that are associated with both endothelial cells (e.g., ROBO4) and myeloid lineage cells (e.g., MEIS2). These are potentially a mixture of myeloid cells with some contamination from endothelial cells.

To improve the annotation of the scATAC-seq data and enhance functional characterization of immune cells in ccRCC, the scATAC-seq data was integrated with scRNA-seq data from the same patient and the same tissues (RCC, adjacent normal). The scRNA-seq dataset consists of 18,429 cells (8674 from tumor tissue and 9755 from adjacent normal tissue), with gene expression values for 17,152 genes. Similarly to above, UMAP was used to visualize the cells (Figure 8.7A) and cells were clustered using the Leiden community detection algorithm, this time on their gene expression profile. The detected clusters were then given cell type labels based on the expression of marker genes for specific immune cell types (Figure 8.7B and C).

The scATAC-seq data were integrated with the scRNA-seq data using the Signac's functions for cross-modality integration and label transfer. Briefly, shared correlation patterns in the gene activity matrix of the scATAC-seq dataset and scRNA-seq dataset are identified to match biological states across the two modalities. A classification score for each scRNA-seq-defined cluster label is returned for each cell in the scATAC-seq dataset. In general, the predicted cell type labels lined up well with the original cluster labels but provided more clarity for the T cell subsets (e.g., CD4, CD8 T cells) and different types of monocytes (Figure 8.7D).

Cells in the scATAC-seq dataset were then grouped by predicted cell type and for each cell type, differentially accessible peaks were identified between the RCC and adjNorm samples. The CD8 memory T cells showed significant changes in accessibility of several genes related to T-cell dysregulation (CTLA4, LAYN and TOX) [136,137]. Several peaks were identified in these genes in the RCC samples but not in the adjNorm samples (Figure 8.6E). The increased accessibility appears to correlate with increased mRNA expression of these genes in CD8 memory T cells in the scRNA-seq data (Figure 8.6F). The combined evidence from the scATAC-seq and scRNA-seq data suggests that CD8 memory T cells in ccRCC are dysfunctional and exhausted and could be a potential immunotherapeutic target in ccRCC.

3.6.2.5 Discussion

As can be observed in Figure 8.6 above, scATAC-seq was able to accurately characterize the chromatin accessibility profile of immune cells and identified clusters of tumor-specific cells, along with their predicted regulatory transcription factors, that may represent therapeutic targets in ccRCC. The integration of the scATAC-seq data with scRNA-seq data from the same tissue enabled a higher resolution classification of cell types in the scATAC-seq dataset and provided further evidence of T cell dysregulation in ccRCC. In particular, CD8 memory T cells are affected by the disease and display markers of T-cell exhaustion at the level of both chromatin accessibility and gene expression. This case study demonstrates the potential utility of single-cell multiomic data in immune-based stratification of cancer patients and in guiding therapeutic strategies to overcome resistance to immune based cancer therapies.

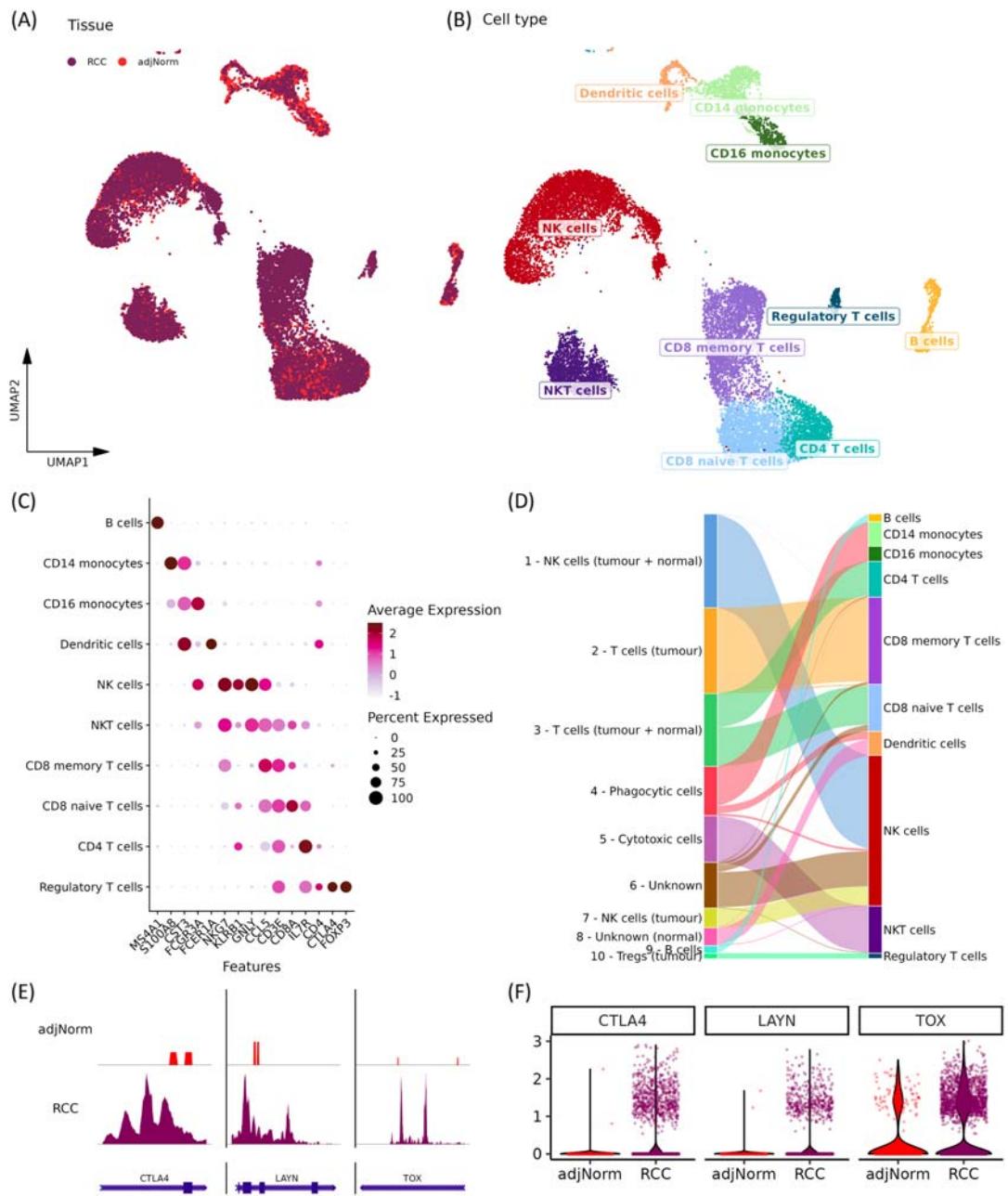


FIGURE 8.7

Integration of scRNA-seq and scATAC-seq data improves cell type annotation and identifies dysregulated genes in ccRCC. UMAP of scRNA seq data colored by tissue of origin (A) and cell type (B). (C) Expression of cell type marker genes in each cell type in the scRNA-seq data. (D) Comparison of original annotations (left) for cells in the scATAC-seq dataset with predicted annotations (right) after integrating with scRNA-seq data. (E) Chromatin accessibility profiles in cells labeled “CD8 memory T cells” at regions corresponding to dysfunctional T-cell genes (CTLA4, LAYN, TOX). (F) Normalized expression values (RNA) of dysfunctional T-cell genes in CD8 memory T cells. Please check the online version for the color image.

4 Conclusion

The past decade has brought significant improvements to high-throughput epigenomic profiling techniques, leading to a leap in our understanding of the role of epigenomics in cancer, including the identification of epigenomic biomarkers for disease subtypes, progression, and treatment response, as well as the development of epigenetic modifiers as novel therapeutic interventions. In this chapter, we have introduced some of the high-throughput assays that can be used to generate these large-scale epigenomic datasets, as well as discussing some of the current statistical and computational tools that can be used for their analysis. Our survey however, is by no means comprehensive, and will no doubt quickly become outdated in this fast moving field as new assays are developed, particularly in the area of single-cell epigenomics. These developments will further drive the need for novel computational approaches including ML and deep learning, not only for epigenomic data, but also to integrate diverse and heterogeneous datasets to provide a systems-level understanding of the complex interactions between genomic, epigenomic, transcriptomic, proteomic, and metabolomic drivers of cancer.

References

- [1] Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;128(4):683–92.
- [2] Garraway LA, Lander ES. Lessons from the cancer genome. *Cell*. 2013;153(1):17–37.
- [3] Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell*. 2012;150(1):12–27.
- [4] Ntziachristos P, Tsirigos A, Welstead G, Trimarchi T, Bakogianni S, Xu L. Contrasting roles of histone H3 lysine 27 demethylases in acute lymphoblastic leukaemia. *Nature* 2014;514(7523):513–17.
- [5] Mack S.C., Witt H., Piro R.M., Gu L., Zuyderduyn S., Stütz A.M.. Epigenomic alterations define lethal CIMP-positive ependymomas of infancy.
- [6] Arneson D, Yang X, Wang K. MethylResolver—a method for deconvoluting bulk DNA methylation profiles into known and unknown cell contents. *Commun Biol* 2020;3(1):1–13.
- [7] Salas LA, Zhang Z, Koestler DC, Butler RA, Hansen HM, Molinaro AM, et al. Enhanced cell deconvolution of peripheral blood using DNA methylation for high-resolution immune profiling. *Nat Commun* 2022;13:761.
- [8] Casado-Pelaez M., Bueno-Costa A., Esteller M.. Single cell cancer epigenetics. *Trends Cancer* 2022 [cited August 3, 2022]. <<https://www.sciencedirect.com/science/article/pii/S2405803322001339>>.
- [9] Pidsley R, Zotenko E, Peters TJ, Lawrence MG, Risbridger GP, Molloy P, et al. Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. *Genome Biol* 2016;17(1):208.
- [10] Pomraning KR, Smith KM, Freitag M. Genome-wide high throughput analysis of DNA methylation in eukaryotes. *Methods San Diego Calif* 2009;47(3):142–50.
- [11] Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, et al. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat Biotechnol* 2009;27(4):361–8.
- [12] Li D, Zhang B, Xing X, Wang T. Combining MeDIP-seq and MRE-seq to investigate genome-wide CpG methylation. *Methods San Diego Calif* 2015;72:29–40.
- [13] Meissner A, Gnirke A, Bell GW, Ramsahoye B, Lander ES, Jaenisch R. Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. *Nucleic Acids Res* 2005;33(18):5868–77.

- [14] Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, et al. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature*. 2008;454(7205):766–70.
- [15] Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, et al. Dynamic changes in the human methylome during differentiation. *Genome Res* 2010;20(3):320–31.
- [16] Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, et al. Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* 2011;471(7336):68–73.
- [17] Park PJ. ChIP-seq: advantages and challenges of a maturing technology. *Nat Rev Genet* 2009;10(10):669–80.
- [18] Skene PJ, Henikoff S. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. In: Reinberg D, editor. *eLife*, 6. 2017. p. e21856.
- [19] Wu F, Olson BG, Yao J. DamID-seq: genome-wide mapping of protein-DNA interactions by high throughput sequencing of adenine-methylated DNA fragments. *J Vis Exp JoVE* 2016;107:53620.
- [20] Boyle AP, Davis S, Shulha HP, Meltzer P, Margulies EH, Weng Z, et al. High-resolution mapping and characterization of open chromatin across the genome. *Cell*. 2008;132(2):311–22.
- [21] Song L, Crawford GE. DNase-seq: a high-resolution technique for mapping active gene regulatory elements across the genome from mammalian cells. *Cold Spring Harb Protoc* 2010;2010(2) pdb.prot5384.
- [22] Giresi PG, Kim J, McDaniell RM, Iyer VR, Lieb JD. FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res* 2007;17(6):877–85.
- [23] Buenrostro JD, Wu B, Chang HY, Greenleaf WJ. ATAC-seq: a method for assaying chromatin accessibility genome-wide. *Curr Protoc Mol Biol* 2015;109:21.29.1–21.29.9.
- [24] van Berkum NL, Lieberman-Aiden E, Williams L, Imakaev M, Gnirke A, Mirny LA, et al. Hi-C: a method to study the three-dimensional architecture of genomes. *J Vis Exp* 2010;39:1869.
- [25] Fullwood MJ, Liu MH, Pan YF, Liu J, Xu H, Mohamed YB, et al. An oestrogen-receptor- α -bound human chromatin interactome. *Nature*. 2009;462(7269):58–64.
- [26] Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009;10(1):57–63.
- [27] Harris R, Wang T, Coarfa C, Nagarajan RP, Hong C, Downey SL. Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. *Nat Biotechnol* 2010;28(10):1097–105.
- [28] Stevens M, Cheng JB, Li D, Xie M, Hong C, Maire CL. Estimating absolute methylation levels at single-CpG resolution from methylation enrichment and restriction enzyme sequencing methods. *Genome Res* 2013;23(9):1541–53.
- [29] Gambichler T, Steuke AK, Schmitz L, Stockfleth E, Becker Jc. Altered hydroxymethylation in cutaneous squamous cell carcinoma and keratoacanthoma. *Br J Dermatol* 2020;183(5):955–7.
- [30] Zhu H, Zhu H, Tian M, Wang D, He J, Xu T. DNA methylation and hydroxymethylation in cervical cancer: diagnosis, prognosis and treatment. *Front Genet* 2020;11:347.
- [31] Xu T, Gao H. Hydroxymethylation and tumors: can 5-hydroxymethylation be used as a marker for tumor diagnosis and treatment? *Hum Genomics* 2020;14:15.
- [32] Guler GD, Ning Y, Ku CJ, Phillips T, McCarthy E, Ellison CK, et al. Detection of early stage pancreatic cancer using 5-hydroxymethylcytosine signatures in circulating cell free DNA. *Nat Commun* 2020;11(1):5270.
- [33] Yu M, Hon GC, Szulwach KE, Song CX, Jin P, Ren B. Tet-assisted bisulfite sequencing of 5-hydroxymethylcytosine. *Nat Protoc* 2012;7(12):2159–70.
- [34] Nacev BA, Feng L, Bagert JD, Lemiesz AE, Gao J, Soshnev A, et al. The expanding landscape of ‘oncohistone’ mutations in human cancers. *Nature*. 2019;567(7749):473–8.
- [35] Jost D, Carrivain P, Cavalli G, Vaillant C. Modeling epigenome folding: formation and dynamics of topologically associated chromatin domains. *Nucleic Acids Res* 2014.

- [36] Johanson TM, Allan RS. In situ HiC. *Methods Mol Biol* Clifton NJ 2022;2458:333–43.
- [37] Kloetgen A, Thandapani P, Ntziachristos P, Ghebrechristos Y, Nomikou S, Lazaris C, et al. Three-dimensional chromatin landscapes in T cell acute lymphoblastic leukemia. *Nat Genet* 2020;52(4):388–400.
- [38] Ura H, Togi S, Niida Y. A comparison of mRNA sequencing (RNA-Seq) library preparation methods for transcriptome analysis. *BMC Genomics* 2022;23(1):303.
- [39] Svensson V, da Veiga Beltrame E, Pachter L. A curated database reveals trends in single-cell transcriptomics. *Databse*. 2020;2020:baaa073.
- [40] Ma A, Xin G, Ma Q. The use of single-cell multi-omics in immuno-oncology. *Nat Commun* 2022;13(1):2728.
- [41] Ahn J, Heo S, Lee J, Bang D. Introduction to single-cell DNA methylation profiling methods. *Biomolecules*. 2021;11(7):1013.
- [42] Cusanovich DA, Daza R, Adey A, Pliner H, Christiansen L, Gunderson KL, et al. Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science* 2015;348(6237):910–14.
- [43] Buenrostro JD, Wu B, Litzenburger UM, Ruff D, Gonzales ML, Snyder MP, et al. Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature* 2015;523(7561):486–90.
- [44] Mezger A, Klemm S, Mann I, Brower K, Mir A, Bostick M, et al. High-throughput chromatin accessibility profiling at single-cell resolution. *Nat Commun* 2018;9(1):3647.
- [45] Rotem A, Ram O, Shores N, Sperling RA, Goren A, Weitz DA, et al. Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. *Nat Biotechnol* 2015;33(11):1165–72.
- [46] Grosselin K, Durand A, Marsolier J, Poitou A, Marangoni E, Nemati F, et al. High-throughput single-cell ChIP-seq identifies heterogeneity of chromatin states in breast cancer. *Nat Genet* 2019;51(6):1060–6.
- [47] Kind J, Pagie L, de Vries SS, Nahidazar L, Dey SS, Bienko M, et al. Genome-wide maps of nuclear lamina interactions in single human cells. *Cell*. 2015;163(1):134–47.
- [48] Bartosovic M, Kabbe M, Castelo-Branco G. Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues. *Nat Biotechnol* 2021;39(7):825–35.
- [49] Nagano T, Lubling Y, Stevens TJ, Schoenfelder S, Yaffe E, Dean W, et al. Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. *Nature* 2013;502(7469):59–64.
- [50] Zhu C, Preissl S, Ren B. Single-cell multimodal omics: the power of many. *Nat Methods* 2020;17(1):11–14.
- [51] Angermueller C, Clark SJ, Lee HJ, Macaulay IC, Teng MJ, Hu TX, et al. Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. *Nat Methods* 2016;13(3):229–32.
- [52] Hou Y, Guo H, Cao C, Li X, Hu B, Zhu P, et al. Single-cell triple omics sequencing reveals genetic, epigenetic, and transcriptomic heterogeneity in hepatocellular carcinomas. *Cell Res* 2016;26(3):304–19.
- [53] Hu Y, An Q, Guo Y, Zhong J, Fan S, Rao P, et al. Simultaneous profiling of mRNA transcriptome and DNA methylome from a single cell. *Methods Mol Biol* 2019;1979:363–77.
- [54] Hu Y, Huang K, An Q, Du G, Hu G, Xue J, et al. Simultaneous profiling of transcriptome and DNA methylome from a single cell. *Genome Biol* 2016;17(1):88.
- [55] Cao J, Cusanovich DA, Ramani V, Aghamirzaie D, Pliner HA, Hill AJ, et al. Joint profiling of chromatin accessibility and gene expression in thousands of single cells. *Science*. 2018;361(6409):1380–5.
- [56] Liu L, Liu C, Quintero A, Wu L, Yuan Y, Wang M, et al. Deconvolution of single-cell multi-omics layers reveals regulatory heterogeneity. *Nat Commun* 2019;10(1):470.
- [57] Zhu C, Yu M, Huang H, Juric I, Abnousi A, Hu R, et al. An ultra high-throughput method for single-cell joint analysis of open chromatin and transcriptome. *Nat Struct Mol Biol* 2019;26(11):1063–70.
- [58] Swanson E, Lord C, Reading J, Heubeck AT, Genge PC, Thomson Z, et al. Simultaneous trimodal single-cell measurement of transcripts, epitopes, and chromatin accessibility using TEA-seq. *eLife* 2021;10:e63632.

- [59] Pott S. Simultaneous measurement of chromatin accessibility, DNA methylation, and nucleosome phasing in single cells. *eLife.*, 6. 2017. p. e23203.
- [60] Guo F, Li L, Li J, Wu X, Hu B, Zhu P, et al. Single-cell multi-omics sequencing of mouse early embryos and embryonic stem cells. *Cell Res* 2017;27(8):967–88.
- [61] Ståhl PL, Salmén F, Vickovic S, Lundmark A, Navarro JF, Magnusson J, et al. Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science* 2016;353(6294):78–82.
- [62] Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* 2015;348(6233):aaa6090.
- [63] Thornton CA, Mulqueen RM, Torkenczy KA, Nishida A, Lowenstein EG, Fields AJ, et al. Spatially mapped single-cell chromatin accessibility. *Nat Commun* 2021;12(1):1274.
- [64] Llorens-Bobadilla E, Zamboni M, Marklund M, Bhalla N, Chen X, Hartman J, et al. Chromatin accessibility profiling in tissue sections by spatial ATAC. *bioRxiv* 2022; 2022.07.27.500203.
- [65] Deng Y, Bartosovic M, Kukanja P, Zhang D, Liu Y, Su G, et al. Spatial-CUT&Tag: spatially resolved chromatin modification profiling at the cellular level. *Science* 2022;375(6581):681–6.
- [66] Hou W, Ji Z, Ji H, Hicks SC. A systematic evaluation of single-cell RNA-seq imputation methods. *Genome Biol* 2020;21(1):218.
- [67] Chazarra-Gil R, van Dongen S, Kiselev VY, Hemberg M. Flexible comparison of batch correction methods for single-cell RNA-seq using BatchBench. *Nucleic Acids Res* 2021;49(7):e42.
- [68] Slovin S, Carissimo A, Panariello F, Grimaldi A, Bouché V, Gambardella G, et al. Single-cell RNA sequencing analysis: a step-by-step overview. *Methods Mol Biol* 2021;2284:343–65.
- [69] Mehrmohamadi M, Sepehri MH, Nazer N, Norouzi MR. A comparative overview of epigenomic profiling methods. *Front Cell Dev Biol* 2021;9:714687.
- [70] Bernstein BE, Stamatoyannopoulos JA, Costello JF, Ren B, Milosavljevic A, Meissner A, et al. The NIH roadmap epigenomics mapping consortium. *Nat Biotechnol* 2010;28(10):1045–8.
- [71] Satterlee JS, Chadwick LH, Tyson FL, McAllister K, Beaver J, Birnbaum L, et al. The NIH common fund/roadmap epigenomics program: successes of a comprehensive consortium. *Sci Adv* 2019;5(7): eaaw6507.
- [72] International Cancer Genome ConsortiumHudson TJ, Anderson W, Artez A, Barker AD, Bell C, et al. International network of cancer genome projects. *Nature* 2010;464(7291):993–8.
- [73] Stunnenberg HG, Abrignani S, Adams D, Almeida M, de, Altucci L, Amin V, et al. The International human epigenome consortium: a blueprint for scientific collaboration and discovery. *Cell* 2016;167(5):1145–9.
- [74] Weinstein JN, Collisson EA, Mills GB, Shaw KM, Ozenberger BA, Ellrott K, et al. The Cancer Genome Atlas Pan-Cancer Analysis Project. *Nat Genet* 2013;45(10):1113–20.
- [75] ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature* 2012;489(7414):57–74.
- [76] Martens JHA, Stunnenberg HG. BLUEPRINT: mapping human blood cell epigenomes. *Haematologica* 2013;98(10):1487–9.
- [77] R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2021. <<https://www.R-project.org/>>.
- [78] Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004;5(10):R80.
- [79] Merkel D. Docker: lightweight linux containers for consistent development and deployment. *Linux J* 2014;2014(239):2.
- [80] Kurtzer GM, Sochat V, Bauer MW. Singularity: Scientific containers for mobility of compute. *PLoS One* 2017;12(5):e0177459.
- [81] Andrews S. FastQC: a Qual Control Tool High Throughput Sequence Data;. 2010. Available from: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.

- [82] Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 2016;32(19):3047–8.
- [83] Krueger F, Andrews SR. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* 2011;27(11):1571–2.
- [84] Pedersen B.S., Eyring K., De S., Yang I.V., Schwartz D.A.. Fast accurate alignment long bisulfite-seq reads. arXiv; 2014. <<https://arxiv.org/abs/1401.1129>>.
- [85] Morris TJ, Butcher LM, Feber A, Teschendorff AE, Chakravarthy AR, Wojdacz TK, et al. ChAMP: 450k chip analysis methylation pipeline. *Bioinformatics* 2014;30(3):428–30.
- [86] Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics* 2014;30(10):1363–9.
- [87] Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 2007;8(1):118–27.
- [88] Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 2012;28(6):882–3.
- [89] Kulis M, Heath S, Bibikova M, Queirós AC, Navarro A, Clot G. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nat Genet* 2012;44(11):1236–42.
- [90] Salhia B, Kiefer J, Ross JTD, Metapally R, Martinez RA, Johnson KN. Integrated genomic and epigenomic analysis of breast cancer brain metastasis. *PLoS One* 2014;9(1).
- [91] Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, et al. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biol* 2012;13(10):R87.
- [92] Hansen KD, Langmead B, Irizarry RA. BSsmooth: from whole genome bisulfite sequencing reads to differentially methylated regions. *Genome Biol* 2012;13(10):R83.
- [93] Jühlung F, Kretzmer H, Bernhart SH, Otto C, Stadler PF, Hoffmann S. metilene: Fast and sensitive calling of differentially methylated regions from bisulfite sequencing data *Genome Res* 2015;[cited 2022 Aug 3]. Available from: <https://genome.cshlp.org/content/early/2015/12/24/gr.196394.115>.
- [94] Morgan M, Shepherd L. AnnotationHub: Client access AnnotationHub Resour 2022;
- [95] McLean C, Bristor D, Hiller M, Clarke S, Schaar B, Lowe C. GREAT improves functional interpretation of cis-regulatory regions. *Nat Biotechnol* 2010;28(5):495–501.
- [96] Gu Z, Hübschmann D. rGREAT: an R/Bioconductor package for functional enrichment on genomic regions. *bioRxiv* 2022; 2022.06.05.494877.
- [97] Xiong Z, Yang F, Li M, Ma Y, Zhao W, Wang G, et al. EWAS Open Platform: integrated data, knowledge and toolkit for epigenome-wide association study. *Nucleic Acids Res* 2022;50(D1):D1004–9.
- [98] Li M, Zou D, Li Z, Gao R, Sang J, Zhang Y, et al. EWAS Atlas: a curated knowledgebase of epigenome-wide association studies. *Nucleic Acids Res* 2019;47(D1):D983–8.
- [99] Xiong Z, Li M, Yang F, Ma Y, Sang J, Li R, et al. EWAS Data Hub: a resource of DNA methylation array data and metadata. *Nucleic Acids Res* 2020;48(D1):D890–5.
- [100] Li R, Liang F, Li M, Zou D, Sun S, Zhao Y, et al. MethBank 3.0: a database of DNA methylomes across a variety of species. *Nucleic Acids Res* 2018;46(D1):D288–95.
- [101] Zong W, Kang H, Xiong Z, Ma Y, Jin T, Gong Z, et al. scMethBank: a database for single-cell whole genome DNA methylation maps. *Nucleic Acids Res* 2022;50(D1):D380–6.
- [102] Barfield RT, Kilaru V, Smith AK, Conneely KN. CpGassoc: an R function for analysis of DNA methylation microarray data. *Bioinformatics* 2012;28(9):1280–1.
- [103] Campagna MP, Xavier A, Lechner-Scott J, Maltby V, Scott RJ, Butzkueven H, et al. Epigenome-wide association studies: current knowledge, strategies and recommendations. *Clin Epigenetics* 2021;13(1):214.

- [104] Kharchenko PV, Tolstorukov MY, Park PJ. Design and analysis of ChIP-seq experiments for DNA-binding proteins. *Nat Biotechnol* 2008;26(12):1351–9.
- [105] Xu S, Grullon S, Ge K, Peng W. Spatial clustering for identification of ChIP-enriched regions (SICER) to map regions of histone methylation patterns in embryonic stem cells. *Methods Mol Biol* 2014;1150:97–111.
- [106] Helmuth J, Li N, Arrigoni L, Gianmoena K, Cadenas C, Gasparoni G, et al. normR: regime enrichment calling for ChIP-seq data. *bioRxiv* 2016;. Available from: <https://doi.org/10.1101/082263>.
- [107] Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* 2008;9(9):R137.
- [108] Thomas R, Thomas S, Holloway AK, Pollard KS. Features that define the best ChIP-seq peak calling algorithms. *Brief Bioinform* 2017;18(3):441–50.
- [109] Pepke S, Wold B, Mortazavi A. Computation for ChIP-seq and RNA-seq studies. *Nat Methods* 2009;6 (11 Suppl.).
- [110] Landt SG, Marinov GK, Kundaje A, Kheradpour P, Pauli F, Batzoglou S. ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. *Genome Res* 2012;22(9):1813–31.
- [111] Chen Y, Chen S, Lei EP. DiffChIP: a differential peak analysis method for high throughput sequencing data with biological replicates based on limma. *Bioinformatics* 2022;btac498.
- [112] Nielsen C, Younesy H, O'Geen H, Xu X, Jackson A, Milosavljevic A. Spark: a navigational paradigm for genomic data exploration. *Genome Res* 2012;22(11):2262–9.
- [113] Ernst J, Kellis M. ChromHMM: automating chromatin-state discovery and characterization. *Nat Methods* 2012;9(3):215–16.
- [114] Melsted P, Booshaghi AS, Liu L, Gao F, Lu L, Min KH, et al. Modular, efficient and constant-memory single-cell RNA-seq preprocessing. *Nat Biotechnol* 2021;39(7):813–18.
- [115] Stuart T, Srivastava A, Madad S, Lareau CA, Satija R. Single-cell chromatin state analysis with Signac. *Nat Methods* 2021;18(11):1333–41.
- [116] Argelaguet R, Arnol D, Bredikhin D, Deloro Y, Velten B, Marioni JC, et al. MOFA + : a statistical framework for comprehensive integration of multi-modal single-cell data. *Genome Biol* 2020;21(1):111.
- [117] Orozco JJ, Knijnenburg TA, Manughian-Peter AO, Salomon MP, Barkhoudarian G, Jalas JR, et al. Epigenetic profiling for the molecular classification of metastatic brain tumors. *Nat Commun* 2018;9 (1):4627.
- [118] Huang S, Cai N, Pacheco PP, Narrandes S, Wang Y, Xu W. Applications of support vector machine (SVM) learning in cancer genomics. *Cancer Genomics Proteom* 2018;15(1):41–51.
- [119] Toth R, Schiffmann H, Huber-Magg C, Büscheck F, Höflmayer D, Weidemann S, et al. Random forest-based modelling to detect biomarkers for prostate cancer progression. *Clin Epigenetics* 2019;11(1):148.
- [120] Ma B, Chai B, Dong H, Qi J, Wang P, Xiong T, et al. Diagnostic classification of cancers using DNA methylation of paracancerous tissues. *Sci Rep* 2022;12(1):10646.
- [121] Shrestha A, Mahmood A. Review of deep learning algorithms and architectures. *IEEE Access* 2019;7:53040–65.
- [122] Arslan E, Schulz J, Rai K. Machine learning in epigenomics: insights into cancer biology and medicine. *Biochim Biophys Acta Rev Cancer* 2021;1876(2):188588.
- [123] Rauschert S, Raubenheimer K, Melton PE, Huang RC. Machine learning and clinical epigenetics: a review of challenges for diagnosis and classification. *Clin Epigenetics* 2020;12(1):51.
- [124] Sugiura M, Sato H, Kanesaka M, Imamura Y, Sakamoto S, Ichikawa T, et al. Epigenetic modifications in prostate cancer. *Int J Urol Jpn Urol Assoc* 2021;28(2):140–9.
- [125] Aure MR, Fleischer T, Bjørklund S, Ankil J, Castro-Mondragon JA, Bathen TF, et al. Crosstalk between microRNA expression and DNA methylation drives the hormone-dependent phenotype of breast cancer. *Genome Med* 2021;13(1):72.

- [126] Fortin JP, Labbe A, Lemire M, Zanke BW, Hudson TJ, Fertig EJ, et al. Functional normalization of 450k methylation array data improves replication in large cancer studies. *Genome Biol* 2014;15(11).
- [127] Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinf* 2010;11(1):1–9.
- [128] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15(12):1–21.
- [129] Rohart F, Gautier B, Singh A, Cao KAL. mixOmics: An R package for ‘omics feature selection and multiple data integration. *PLoS Comput Biol* 2017;13(11).
- [130] Singh A, Shannon CP, Gautier B, Rohart F, Vacher M, Tebbutt SJ, et al. DIABLO: an integrative approach for identifying key molecular drivers from multi-omics assays. *Bioinformatics*. 2019;35(17):1367–4803.
- [131] Braun DA, Bakouny Z, Hirsch L, Flippot R, Van Allen EM, Wu CJ, et al. Beyond conventional immune-checkpoint inhibition—novel immunotherapies for renal cell carcinoma. *Nat Rev Clin Oncol* 2021;18(4):199–214.
- [132] Kourtis N, Wang Q, Wang B, Oswald E, Adler C, Cherravuru S, et al. A single-cell map of dynamic chromatin landscapes of immune cells in renal cell carcinoma. *Nat Cancer* 2022;3(7):885–98.
- [133] Hao Y, Hao S, Andersen-Nissen E, Mauck WM, Zheng S, Butler A, et al. Integrated analysis of multi-modal single-cell data. *Cell*. 2021;184(13):3573–3587.e29.
- [134] Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* 2010;38(4):576–89.
- [135] Rajagopalan S, Long EO. Cellular senescence induced by CD158d reprograms natural killer cells to promote vascular remodeling. *Proc Natl Acad Sci U S A* 2012;109(50):20596–601.
- [136] Pan JH, Zhou H, Cooper L, Huang JL, Zhu SB, Zhao XX, et al. LAYN is a prognostic biomarker and correlated with immune infiltrates in gastric and colon cancers. *Front Immunol* 2019;10:6.
- [137] Liang C, Huang S, Zhao Y, Chen S, Li Y. TOX as a potential target for immunotherapy in lymphocytic malignancies. *Biomark Res* 2021;9(1):20.

Environmental pollution, epigenetics, and cancer

9

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1 Introduction

1.1 Pollution

Earth's human population has increased in number from around 1 billion at the time of the 18th century Industrial Revolution, to a projected 8 billion by the year 2023. This continued growth of human civilization has resulted in a level of activity from industry, manufacturing, agriculture, and power generation that is placing ever-mounting pressure on our Earth system: the planet's interacting physical, chemical, and biological processes [1,2]. We are now existing outside of the historical environmental limits within which humanity has, until now, safely operated, dubbed our *planetary boundaries* [1–3]. Not only does unsustainable human activity threaten to eventually destabilize the Earth System as a whole, but also it poses multiple serious risks to human health [4–7]. As a consequence of our activities, both toxic and nontoxic industrial and commercial pollutants are constantly being produced and released into our atmosphere, water sources, and soil, having both direct and indirect deleterious effects on human health [8]. The biggest contributors to pollution are the industries related to manufacturing, agriculture, and fuel refining, as well as those surrounding medicine, pharmaceutics, and sanitation [9].

Pollution is broadly defined as “the presence or introduction into the environment, especially as a result of human activity, of harmful or poisonous substances.” Pollution can be categorized by its production source—industrial, agricultural, naturally occurring—by the environmental medium in which it is carried—air, water, soil—or by the point of entry to and effects on the human body—respiratory/dermal entry, neurotoxic/cardiotoxic effects. For the purposes of this chapter, pollutants will be categorized according to their composition under the following headings: *particulate matter*, *gaseous pollutants*, *heavy metals*, *persistent organic pollutants*, and *others*.

1.2 Health effects of pollution

Pollution is the largest environmental cause of disease and premature death in the world [6]. In 2017 it was estimated that 8.3 million premature deaths were due to pollution-related diseases, that is 15% of global deaths that year [7,10]. Pollution was further responsible for an estimated 275 million disability-adjusted life years (DALYs). Notably, these are conservative estimates as many known toxins had not yet been included in analyses. As such, the true burden of pollution on health is likely much greater than we can currently appreciate [6].

The following extract, from the *2017 Lancet Commission on Pollution and Health*, highlights the severity of the issue:

Diseases caused by pollution were responsible for an estimated 9 million premature deaths in 2015—16% of all deaths worldwide—three times more deaths than from AIDS, tuberculosis, and malaria combined and 15 times more than from all wars and other forms of violence. [6]

Air pollution is the biggest contributor to the global disease burden of pollution. In 2012, one in every nine deaths globally was the result of an air pollution-related condition [11]. In 2015 ambient and household air pollution were believed to have caused 6.4 million deaths globally [12].

Household air pollution is a major risk factor for morbidity and mortality in low- and middle-income countries [12,13]. Over 3 billion people worldwide rely on the burning of solid fuels, such as wood, animal dung, charcoal, crop residues, and coal, for their everyday activities, such as cooking, space heating, room lighting, and more [14]. Household air pollution consists of both toxic organic and inorganic chemicals released during the burning of these solid fuels in the home, as well as the volatile organic compounds (VOCs) which slowly leach from detergents, paints, and varnishes used around the house [15,16]. The PM and gaseous air pollution created in such households was responsible for 2.8 million deaths and 85.6 million DALYs in 2015 [12]. The most common health effects related to household air pollution include acute lower respiratory infections in children and COPD, lung cancer, ischemic heart disease and cerebrovascular diseases, such as stroke in adults [17].

Ambient air pollution, which generally refers to the conditions in outdoor spaces, is influenced by industrial outputs, natural disasters, and traffic-related air pollution (TRAP). In urban areas, traffic-related vehicle exhaust fumes represent a significant contributor to air pollution. TRAP is a composite of multiple pollution types which are produced as a consequence of the combustion of fossil fuels in cars and trucks. It contains both ultrafine PM and gaseous components, including black carbon, ozone, nitrogen dioxide, sulfur dioxide, VOCs, and polycyclic aromatic hydrocarbons [18–20]. Studies have shown significant reduction in lung function in individuals living close to busy roads as compared to individuals living greater distances from such roads [21,22]. TRAP has also been associated with both benign and malignant lung tumors in animals and there is a well-established association between TRAP and lung cancer risk in humans [23]. One Spanish study also demonstrated an association between long-term PAH and diesel exhaust emission exposure and bladder cancer incidence and mortality [24]. TRAP has also been associated with a number of cancer types, including colorectal and gastric cancers [25,26].

Children are known to be highly sensitive to the effects of TRAP due to their rapidly developing lungs and immune systems. TRAP exposure in childhood has been linked with decreased lung function, increased asthma incidence and exacerbation and airway inflammation [27,28].

In 2019 ambient PM of less than $2.5\text{ }\mu\text{m}$ in diameter was ranked as one of the biggest global contributors to DALYs in the Global Burden of Diseases Study (GBDS). This was the first in the GBDS series to acknowledge the role of PM in contributing to shorter gestational periods and lower birth weights in humans, which increased its disease burden score [29,30]. Furthermore, TRAP is also known to produce systemic effects. For example, children living in areas with greater TRAP have been shown to have higher body mass indices, potentially as a consequence of metabolic changes induced by pollution exposure [31].

The duration and accumulation of pollution exposure also impact the health outcomes of exposed individuals. In a large cohort study of US transport industry personnel, among workers who had regular exposure to diesel engine exhausts a 20–40% increased cancer risk was observed. There were also statistically significant positive trends between risk and longer duration of employment. Notably, an approximate twofold increase in risk of developing lung cancer was seen after 20 years of employment, supporting the association between increased health risk and chronic and

cumulative pollutant exposure [23]. A number of animal studies have also demonstrated that long-term pollution exposure is linked with epigenetic, such as in changes in inflammatory gene methylation patterns and microRNA expression [32–34].

2 Pollutants

2.1 Particulate matter

Particulate matter (PM) refers to a type of air pollution composed of fragments of material on the microscale. It is a complex mixture of both suspended solid particles and liquid droplets which vary in size and composition [35]. PM is generally classified by its size and aerodynamic diameter, which is measured in micrometers. “PM10” denotes PM which is $<10\text{ }\mu\text{m}$ in diameter, while PM2.5 particles and PM0.1 particles are <2.5 and $<0.1\text{ }\mu\text{m}$ in diameter, respectively. These sizes are also referred to as coarse, fine, and ultrafine PM, respectively [36].

The impact of a specific PM on the body depends in large part on particle size. When inhaled, most coarse PM is readily removed from the respiratory tract by mucociliary clearance [37]. Fine PM, on the other hand, can travel to the alveoli where it is retained by the lung parenchyma and can enter the bloodstream [38]. Ultrafine PM can penetrate further, through both respiratory mucosae and pulmonary barriers, where it can then become internalized by lung cells, such as alveolar macrophages. From this point, the ultrafine PM can enter the bloodstream, facilitating systemic exposure to the pollutant [36,39].

In addition to size, the chemical and structural composition of PM plays an important role in its toxicity. Common substances that make up PM pollution include nitrates, sulfates, elemental and organic carbons, absorbed metals, organic compounds, such as polyaromatic hydrocarbons (PAHs), and biological compounds, such as endotoxins and cell fragments [40].

The specific surface area of fine PM allows for the binding of toxic compounds, such as transition metals and PAHs. These compounds attach to the surface of PM during combustion and can be carried deep into the lungs. PM2.5 and PM0.1 are more closely related to the adverse health effects of air pollution than larger particles because they can enter the bloodstream, and their specific surface area allows for greater binding of toxic compounds [36,39].

The harm of PM exposure is not only mediated by direct organ contact with the pollutant. PMs can induce systemic inflammation affecting organs far from the initial site of contact. For example, repeated exposure to PM causes both lipid peroxidation and the depletion of antioxidants, which creates a state of oxidative stress; a disruption in the equilibrium between pro- and antioxidants which leads to cell damage and death, as well as the activation of proinflammatory signaling pathways [36,41]. This activation of inflammation induces leukocytes, adhesion proteins, clotting factors, cytokines, and inflammatory mediators, which can cause damage to vascular endothelium [42]. Repeated exposure to PMs, and subsequent inflammation, therefore contributes to a number of cardiovascular conditions [40].

Epigenetic changes have been linked with the gene dysregulation and cellular responses induced by air pollution exposure, particularly ambient PM exposure. PM10 mediated responses in the lungs are believed to be influenced through chromatin remodeling by histone acetylation. This was suggested by Gilmour et al. who showed that cytokine release promoted by PM10 exposure is

enhanced by cotreatment with a histone deacetylase inhibitor [43]. Exposure to ambient PM10 has also been negatively associated with levels of histone 3 lysine 27 tri-methylation (H3K27me3) and histone 3 lysine 36 tri-methylation (H3K36me3) in the blood [44].

H3K27me3 is associated with gene inactivation and is maintained by histone methyltransferases, such as the polycomb group protein enhancer of zeste homolog 2 (EZH2). Studies in mice have shown negative associations between EZH2 expression and traffic-related PM10 exposure [45]. Hence, PM10 exposure can result in depletion of H3K27me3 due to decreased DNA methylation via EZH2 [46]. It has been demonstrated that H3K27me3 plays a role in the immune response to cancer and that H3K27me3 levels are decreased in response to PM10 exposure [44,47]. Furthermore, H3K27me3 is associated with tumor suppressor gene promoters that are hypermethylated in cancer cells [48]. Decreased H3K27me3 levels have also been established as a premarker of aberrant DNA methylation involved in the formation of a field defect, wherein normal tissue becomes predisposed to carcinogenesis [49].

H3K36me3 loss has been linked with poorer outcomes in both renal cell and liver cancers [50,51]. This histone modification is believed to play a tumor suppressive role in the cell and does so by facilitating mismatch repair during the cell cycle [52]. This has led to H3K36me3 being suggested as a potential biomarker for early detection and therapeutic targeting in cancer [53].

Furthermore, in a 2015 study, Liu et al. demonstrated global elevation of enhancer-associated histone 3 lysine 27 acetylation (H3K27ac) in individuals exposed to high levels of PM2.5 [54]. H3K27ac is a recognized marker of active regulatory histone modification and is believed to separate active enhancers from their inactive counterparts [55]. In the study, the genes found to be associated with H3K27ac were those involved in the activation of cellular responses to wounding and immune response. Overall, PM2.5 exposure is believed to enhance global gene activity, with changes in H3K27ac modification loci affecting local gene expression, particularly those involved in immune response and inflammation, and potentially contributing to a variety of disease states [54]. Furthermore, in a study of electric-furnace steel plant workers with well-documented PM exposure, Bollati et al. found consistent elevation of the microRNAs miR-222 and miR-21 in the peripheral blood leukocytes of participants following occupational PM exposure [56].

In an experiment by Jardim et al., bronchial epithelial cells which were exposed to diesel exhaust particles over a period of 24 hours showed dramatic changes in microRNA expression profile. Of the 313 detectable microRNAs investigated in the study, 197 were either up- or downregulated by 1.5-fold. These microRNA expression alterations may be involved in inflammation regulation and oncogenic pathways [57].

In a 2016 evaluation, the International Agency for Research on Cancer classified both outdoor air pollution and airborne PM as human carcinogens [58]. In this study, exposure to these pollutants were observed to cause lung cancer and have a positive association with bladder cancer. Earlier studies had also established associations between ambient air pollution and pancreatic cancer, cancers of the upper digestive tract and digestive accessory organs, colorectal cancer, cancers of the brain, cervical cancer, breast cancer, hepatocellular carcinoma, childhood leukemia and prostate cancer [25,32,34–40].

An association between PM2.5 and PM10 exposure and lung cancer has been established in a number of studies [59]. A study in São Paulo, Brazil, investigating the impact of PM exposure on

cancer incidence and mortality showed an association between PM10 exposure and cancers of the skin, lungs, thyroid, larynx and bladder [60]. Furthermore, the American Cancer Society's prospective Cancer Prevention Study II, which followed 623,048 participants over a period of 22 years found an association between PM2.5 and death from kidney and bladder cancers [61].

Variations in national pollution levels can cause major impacts on the health outcomes of varying demographics of a population. For example, in China PM2.5 contributed to 1.1 million deaths in 2015. The impact of this varied greatly between provinces however, with PM2.5 contributing to 132.1 deaths per 100,000 people in Qinghai but contributing to only 40.6 deaths per 100,000 people in Hong Kong. Similarly, in the USA, where PM2.5 contributed to 88,400 death, large variation was seen in PM2.5 attributed deaths between states. Death rates varied from 27.1 per 100,000 being attributed to PM2.5 in Mississippi to 8.1 deaths per 100,000 in Hawaii. This variation relates to varying levels and characteristics of air pollution between locations [12].

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As the principle organ exposed, the impact of air pollution on the epigenetic profile of the lungs has always been of particular interest.

2.2 Gaseous pollutants

Gaseous pollutants can be categorized as either primary or secondary. Common primary gaseous pollutants include sulfur dioxide, nitrogen dioxide (NO_2), and VOCs. These are pollutants which are directly released into the atmosphere, usually due to combustion of fossil fuels for industry or transport [35,36]. Secondary pollutants, on the other hand, form within the atmosphere largely from primary pollutants. For example, highly reactive ground level ozone (O_3) is formed in a reaction between NO_2 and PAHs when they are exposed to heat and sunlight in the atmosphere. The extent of tissue damage caused by gaseous air pollutants depends on their water solubility, concentration, ability to oxidize tissue and the susceptibility of the individual breathing it in. SO_2 , for example, is highly water soluble and largely causes damage to the upper airways and skin. NO_2 and O_3 , on the other hand, are not as water soluble and can thus penetrate deeper into the body, entering the bloodstream where they interact with hemoglobin [36]. This explains why although gaseous pollutants primarily effect the lungs, they are also believed to induce hematological problems and are linked to certain types of cancer [35].

2.2.1 Sulfur dioxide (SO_2)

SO_2 is released into the atmosphere both naturally from volcanoes and as a by-product of industrial copper extraction. In addition to being carried in the air, its high aqueous solubility allows it to be incorporated into clouds to form acid rain, which then enters into groundwater sources and soil. Contact with SO_2 is largely in the skin, gastrointestinal tract, and upper airways as it is

dissolved in water [36]. SO₂ has shown the ability to hyper-methylate DNA in *Arabidopsis* tissue models [62].

2.2.2 Nitrogen dioxide (NO₂)

NO₂ is produced from the combustion of fossil fuels and as a by-product of fertilizer manufacturing. It also presents an occupational hazard for agricultural workers as it is produced by microorganisms during the decomposition of grain. NO₂ is absorbed into the bloodstream through epithelia, where it can then go on to produce harmful metabolites in the form of reactive nitrogen species, leading to oxidative stress [36].

A 2018 study showed an association between both long- and short-term exposure to NO₂ and alteration of DNA methylation in the Foxp3 gene [19]. The FOXP3 protein is an immunoregulator affecting the function of regulatory T cells. An excess of these cells can allow cancer cells to evade the immune system [63]. Another 2019 study observed 45 differently methylated probes and 57 differently methylated regions related to NO₂ exposure in a clinical study on air pollution [64].

The gaseous pollutants NO₂ and O₃ have been experimentally linked to altered telomere length, expression of genes involved in DNA damage and repair, inflammation, immune response, oxidative stress and epigenetic effects. These cellular changes are all known risk factors for cancer [65]. Furthermore, NO₂ levels have been positively associated with colorectal cancer mortality [61]. Another factor that influences the health impact of pollution is the concentration of the exposure. There are a number of epidemiological studies verifying this. For example, in a 2010 case-control study comparing 383 cases with 416 controls, Crouse et al. reported a positive association between incidence of postmenopausal breast cancer and NO₂ concentrations [66]. This was achieved using a land use regression model to predict NO₂ concentrations across Montreal, Canada. The study found that for every 5 parts per billion increase in NO₂ concentration, there was a 25% increased risk for postmenopausal breast cancer.

2.2.3 Ozone (O₃)

O₃ in the Earth's stratosphere serves a vital protective function in absorbing UV light from the sun. It is a secondary pollutant formed in a reaction between NO₂ and PAHs when they are exposed to heat and sunlight in the atmosphere. "Ground O₃" is formed when these substances occur in too high levels closer to the ground. Fry et al. showed that exposure to 0.4 parts per million of O₃ for two hours induced a significant increase in the expression of 10 miRNAs in human subjects. These were miR-132, miR-143, miR-145, miR-199a, miR-199b-5p, miR-222, miR-223, miR-25, miR-424, and miR-582-5p [67].

2.2.4 Trichloroethylene

Trichloroethylene (TCE) is synthetic VOC used as an industrial solvent. It enters the environment via factory wastewater, where it can then evaporate into the atmosphere. TCE exposure is associated with renal cell carcinoma and non-Hodgkin lymphoma. Contact with humans is generally via the ingestion of contaminated water or via inhalation during TCE vapor intrusion into enclosed spaces [68]. A study using murine models showed increased DNA methylation in gene promoters associated with the proper functioning of CD4-positive T cells.

2.2.5 Methyl *tert*-butyl ether

Methyl *tert*-butyl ether (MTBE) is a synthetic VOC which is used as a fuel additive and antiknocking agent in motor gasoline. It is released into the atmosphere as fumes during petrol filling, and can leak from faulty fuel storage tanks and subsequently dissolve into groundwater.

Entry into the human body is typically by inhalation during fueling or by ingestion when dissolved in contaminated water sources.

MTBE has been banned in the United States since 1972 over concerns about potential toxicity, but is still used as a fuel additive in Europe, the Middle East, Africa, Asia, and Latin America [69]. Hypomethylation of LINE1-Ta and ALU-YD6 has been observed in petrol station workers who tested positive for MTBE in their urine [70].

2.2.6 Chloroform

Chloroform is a synthetic VOC. It enters into the atmosphere after being formed in chlorinated water sources, such as drinking water, wastewater, and swimming pools. It is also an industry by-product in manufacturing, waste management, and sanitation. Entry into the body is via inhalation when it becomes volatilized into the air. Murine models of chloroform exposure showed global DNA hypomethylation, and a subsequent increase in the level of expression of the proto-oncogene c-MYC, which is commonly associated with renal and hepatocarcinoma [9,71].

2.2.7 Benzene

Benzene is a volatile aromatic compound naturally emitted into the atmosphere from forest fires and volcanoes, as well as due to human activity from the burning of fossil fuels and tobacco. It is used in the process of manufacturing nylon, plastics, rubbers, and other synthetic materials, and typically volatilizes into the atmosphere or enters the water table through wastewater. The leaching of benzene from paint and varnish is a contributor to household pollution [9,72].

Low level exposure to benzene has been shown to induce hypomethylation of Alu-DNA and hypermethylation of cyclin-dependent kinase inhibitors. A link between the development of acute myeloid leukemia (AML) and lymphoma and benzene poisoning was made based on the hypomethylation of promotor regions for p15 and p16 tumor suppressor genes. Additionally, cases of chronic benzene poisoning have been associated with the dysregulation of 13 different miRNAs when compared to a healthy control population [72–74]. Air pollution has even been associated with childhood cancers, such as leukemia and astrocytoma [35, 47]. These cancers are associated with both prenatal and early childhood exposure to PM2.5 and benzene from vehicle exhaust fumes.

AML is a cancer type that has been consistently associated with airborne benzene exposure [72]. A progressive decline in global genomic DNA methylation levels has been identified in the development of AML, as well as a number of other cancer types [75]. Other DNA methylation aberrations characteristic of AML include gene-specific hypermethylation or hypomethylation and loss of imprinting [72]. In AML, gene-specific hypermethylation exhibits a specific pattern, with the tumor suppressor gene p15 commonly methylated and thus inactivated [76]. The underlying mechanism of these methylation aberrations is believed to be genome-wide epigenetic instability [77].

2.2.8 Acetaldehyde

Acetaldehyde is an aldehyde commonly produced by plants and found in bread, coffee, and ripened fruit. It is released into the atmosphere as a result of industrial manufacturing and fossil fuel combustion. Acetaldehyde is a toxic metabolite of ethanol, and is a major contributor to the phenomenon of the hangover. Exogenous acetaldehyde enters the body via inhalation from contaminated air. The epigenetic effects of acetaldehyde exposure include histone H3 acetylation at Lys9 and phosphorylation of histone H3 at Ser10 and 28 [78,79]

2.3 Heavy metals

Heavy metals pollutants include lead, mercury, silver, cadmium, nickel, chromium and several others. These metals enter the atmosphere from a number of sources, including combustion, human industrial activities, and wastewater discharges. While the body does require trace amounts of some of these elements to maintain certain metabolic reactions, elevated concentrations of these metals can quickly become toxic. The danger posed by heavy metals to human health results from their bioaccumulation, wherein they are taken in and stored at a rate much faster than they are metabolized or excreted [35].

2.3.1 Nickel

Nickel is a transition element capable of forming water soluble compounds. It is used in metal manufacturing and found in batteries, medical devices, coins, jewelry, and many items which end up in landfills. This metal usually enters into the environment via wastewater or leaching from landfill, leading to contamination of soil and groundwater. Nickel dust from refining and manufacture can also travel in the air as PM. Human exposure is via ingestion or inhalation [80].

In vivo and *in vitro* studies have shown both an increase gene silencing and chromosome condensation associated with nickel-induced DNA methylation. These changes are due to the ability of nickel ions to substitute for magnesium ions in the phosphate backbone of DNA.

[81]. Nickel has also been shown to affect histones by reducing demethylase activity, increasing global H3K9 mono- and di-methylation, decreasing acetylation of histones 2A, 2B, 3, and 4, as well as affecting histone ubiquitination [82–85]

2.3.2 Cadmium

Cadmium is a transition metal produced as a by-product of zinc manufacturing, as well as being released from tobacco smoke, factories, mines, and agricultural activity into water sources, soil, and the atmosphere. It bioaccumulates with a biological half-life of 25–30 years, and is found in high levels in shellfish and rice. Exposure to humans is via ingestion of contaminated food or water, or via inhalation of contaminated air, and has been linked to cancer of the breast, lung, prostate, nasopharynx, pancreas, and kidneys [9,86,87].

Cadmium depletes levels of S-adenosyl methionine (SAM) which is a methyl donor key to the functioning of DNA methyltransferases [88]. Cadmium-induced DNA hypermethylation has been shown to induce malignant transformation of prostate epithelial cells *in vitro*. The epigenetic modification patterns induced by cadmium have been linked to their ability to sequester methyl groups from SAM, leading to methylome changes due to the limiting of DNA methyltransferase activity

[89]. Cadmium inhibition of histone demethylase has been proposed as being due to cadmium's ability to replace zinc in the core of the enzyme [90].

2.3.3 Chromium

Chromium has two common valence states: trivalent chromium and hexavalent chromium. Hexavalent chromium (Cr(VI)) is extremely water soluble and so poses more of a threat as a pollutant as it is more environmentally mobile. Cr(VI) can also volatilize from water sources into the atmosphere. It is released into the environment in wastewater from industrial processing and manufacturing facilities. Human exposure is primarily through inhalation as well as ingestion of contaminated drinking water [91]. Long-term inhalation of Cr(VI) has been shown to cause pulmonary tumorigenesis in humans [92].

Cr(VI) causes hypermethylation of the CDKN2A gene which codes for the tumor suppressor protein p16, with Cr(VI) inversely correlated to p16 mRNA levels. Cr(VI) was also shown to methylate regions of the DNA repair genes hMLH1/HOGG1/MGMT/RAD53, as well as the tumor suppressor gene APC [93]. Histone acetylation, phosphorylation, methylation, and ubiquitination have also been recorded [85,94]. Furthermore, chromate, a chromium-containing salt, causes global DNA hypomethylation [95].

2.3.4 Lead

Lead is an elemental metal with neurotoxic properties. It is released into the environment from industrial mining, manufacturing and smelting activities, and recycling plants. Historically lead was included in motor gasoline, which has left significant legacy pollution in soil. Furthermore, some older water pipes are still made of lead. Human exposure is typically through ingestion of contaminated water [86]. Lead has been identified as a chemical which is capable of impacting the children of organisms which have been exposed to it, resulting in identifiable trans-generationally inherited epigenetic marks on the methylome [96].

2.3.5 Mercury

Mercury is a liquid-at-room-temperature elemental metal and a well-documented carcinogen. It is released into the atmosphere as a by-product in coal burning power plants and is present in soil and water due to leaching from landfills containing archaic technologies incorporating mercury, such as thermometers and batteries. Mercury bioaccumulates in marine organisms as methylmercury, and consumption of contaminated seafood is the primary route of exposure to humans. Mercury exposure has been associated with global DNA hypomethylation in *in vivo* neural tissue, as well as RND2 hypermethylation in murine embryonic stem cells [9,86,97–99].

2.3.6 Arsenic

Arsenic is a toxic metalloid which is naturally occurring in the environment, but is also released in unnatural amounts in wastewater from industrial and agricultural activity, and as fumes from coal burning power plants. Exposure to humans is through ingestion and inhalation of contaminated water and seafood, and air respectively. Arsenic is associated with cancer of the bladder, kidneys, liver, prostate, and lung [9,86,100].

Epigenetic investigations have shown that arsenic causes inhibition of p16 and p53 tumor suppressor proteins via DNA hypermethylation, as well as showing an increase in LINE-1 methylation

in females [76,86,101]. Arsenic also reduces the pool of SAM molecules needed for the proper functioning of DNA methyltransferase enzymes. This leads to dysregulation of DNA methylation and has been shown to upregulate oncogenes and downregulate tumor-suppressor genes [102,103].

Arsenic has also been shown to cause decreased H3 acetylation in histones linked to renal cell carcinoma, as well as altered histone phosphorylation, methylation, and ubiquitination [9,85,94,104].

2.4 Persistent organic pollutants

Persistent organic pollutants are a group of toxic chemicals that persist in the environment for long periods of time. These compounds were incorporated into technology in the mid-20th century before their harmful effects were known. Although many have been banned for almost 50 years, their resistance to degradation means that they still persist and bioaccumulate in plant and animal populations today, where they bind stably to lipids to avoid excretion. Biomagnification of these pollutants can thus occur wherein they accumulate as they move up through the food chain. Such pollutants include pesticides, dioxins, and polychlorinated biphenyls. These compounds enter the air due to incomplete combustion or burning of chlorine containing materials, such as plastics. They often contaminate plants as pesticides or in dust and thus enter the food chain where they bioaccumulate, stably bound to lipids [35].

2.4.1 *Dichlorodiphenyltrichloroethane and dichlorodiphenyldichloroethylene*

In the 20th century dichlorodiphenyltrichloroethane (DDT) was used as an industrial pesticide, but is now banned in the West due to concerns about toxicity. However, it is still used extensively in developing nations. DDT bioaccumulates in plants and animals in the food chain so there is still a legacy of pollution even in countries where it has been banned. DDT, and its similarly toxic breakdown product dichlorodiphenyldichloroethylene (DDE), are fat soluble and can only be excreted via lactation.

DDT and DDE are associated with an increased risk of breast cancer, and have been shown to exhibit transgenerational epigenetic effects on the children of the organisms originally exposed, including promoting the development of adult onset obesity. DDT has been shown to exhibit hypermethylation of H3K27me3 in histones, as well as producing alterations in ncRNA and DNA methylation in sperm cells [105–107].

2.4.2 *Polybrominated diphenyl ethers*

Polybrominated diphenyl ethers (PBDEs) are organobromine compounds commonly utilized as flame retardants, being incorporated into building materials, plastics, and electronics. PBDE is released into the environment through industrial wastewater, sewage, as well as being found in dairy and beef products. PBDE has been shown to dysregulate both histones and their regulatory pathways [108,109].

2.4.3 *Perfluorooctanoic acid*

Perfluorooctanoic acid (PFOA) is a synthetic organo-fluorine typically used as an industrial surfactant and is incorporated into textiles, carpets, and used in fire fighting. It was listed as an emergent

contaminant in 2014 by the EPA. PFOA has been shown increase DNA methylation globally as well as modify histones. It has also been implicated in the progression of breast cancer [110,111].

2.4.4 Benzo[a]pyrene

Benzo[a]pyrene is a polycyclic aromatic hydrocarbon released into the atmosphere naturally from forest fires and volcanic eruptions, and is also released due to the burning of fossil fuels and wood. Benzo[a]pyrene is also present in roasted organic consumables, such as coffee beans. Inhaled atmospheric benzo[a]pyrene has been implicated in the development of lung cancer [112,113].

Studies have shown that exposure to benzo[a]pyrene interferes with mitochondria and impacts microRNAs and their gene targets, as well as affecting the expression of several antitumorigenesis related epigenetic modifiers. It is also implicated in the hypomethylation of both mitochondrial and nuclear DNA, as well as histone tail methylation, altering chromatin structure [113].

2.5 Others

2.5.1 Bisphenol A

Bisphenol A (BPA) is a synthetic endocrine-disrupting chemical. It is used as a feedstock material in plastic and epoxy manufacturing and is now ubiquitous in the environment. BPA is detectable in the serum and urine of the majority of people. BPA has been linked to the pathogenesis of both prostate and breast cancer [60].

BPA exposure *in utero* was shown to induce DNA hypomethylation in HOXA10 gene promoter-region CpG sites. Reduced levels of Hoxa10 protein has been associated with increased tumor differentiation in prostate cancer [114,115]. In addition, BPA has been shown to induce hypermethylation of LAMP3 promoter regions, reducing its expression. Conversely, LAMP3 over-expression has been associated with both increased differentiation in oral squamous cell carcinoma tissue samples and tamoxifen resistance in breast cancer cell cultures [116,117]. BPA has also been shown to affect miRNA expression levels, with various miRNA reported to be downregulated [118].

2.5.2 Aflatoxin B1

Aflatoxin B1 is a toxin produced by Aspergillus fungus present on peanuts, corn, and other grains. Exposure to humans is via ingestion of contaminated foods. Aflatoxin B1 has been linked to hepatocellular carcinoma, and has also been shown to increase both DNA hypermethylation and histone deacetylation at F2A1 and F3 [119,120].

2.5.3 Radon

Radon is a radioactive, gaseous chemical element. It is found naturally in rock, from where it can either be released into the atmosphere or in to groundwater. Radon can also adhere to PM, allowing it to be inhaled and internalized into the lung parenchyma. It is the highest cause of lung cancer in nonsmoking populations [121]. In terms of epigenetics, radon has been associated with different exposure-dependent DNA methylation changes across various age groups, including neonates, 7 year olds, teenagers, and pregnant mothers [122].

2.5.4 Micoplastics

Micoplastics (MPs) are emerging as a major cause for concern with regard to their impact on human health. Since the beginning of widespread plastics use in the 1950s, nearly ten billion metric tonnes of virgin plastics have been produced globally, of which only around 30% are still in use today [123].

Due to the synthetic nature and durability of plastics, they have very poor biodegradation rates [124]. Therefore rather than decomposing at any appreciable rate, these waste products age, weakening and fragmenting over time through exposure to UV light, embrittlement, biological factors, and physical abrasion [125–127]. Thus in time plastics are broken down into smaller and smaller pieces, eventually becoming fragments with diameters less than 5 millimeter [123,128]. These are known as secondary MPs. Further to MP formation secondary to macro-plastics breakdown, some MPs originate directly from product manufacturing. These are known as primary MPs and include microbeads such as those often used in cosmetic products and industrial abrasives, such as “sandblasting” media [129,130].

Both primary and secondary MPs are now ubiquitous throughout our environments. They have been observed on land, in rivers and oceans, in coastal sands, on agricultural lands, in urban areas, in sea salt, on mountain tops and significant amounts of MPs have even been observed frozen in Arctic Sea ice [131–133].

Human beings come into regular contact with MPs. These particles are frequently present in our food and beverages, particularly our drinking water, and studies have even shown the presence of MPs in human stool [134–136]. Studies have also suggested that the average daily inhalation of MPs is between 97–170 particles per person [137].

Another concern associated with inhalation of MPs is that, much like other PM pollutants, due to their large specific surface areas and hydrophobicity, other, more harmful, pollutants can adsorb onto the surface of these particles [138]. Plastics often contain a variety of purposefully or unintentionally added chemicals [139]. These adsorbed compounds and chemical additives have the potential to amplify the toxicity and adverse effects of MPs. Some studies have even observed the release of toxic monomers and additives, which are associated with cancer and reproductive abnormalities, from MPs [140,141].

In the assessment of the health impact of MPs, a major focus has been on the effects of MP ingestion on the gastrointestinal system, with several studies having demonstrated adverse effects of MPs on the digestive systems of numerous living organisms [142–144]. The adverse effects of MP air pollution exposure are not yet characterized; however, epidemiological studies have demonstrated adverse effects in the lungs of individuals working in the flocking and textile industries [145–148]. These adverse effects have been attributed to long-term high occupational exposure to airborne MPs and include pathologies, such as asthma, pulmonary fibrosis, interstitial lung disease, pneumothorax, and chronic bronchitis. Further investigations are required and underway to establish the full extent to and mechanisms by which MPs effect human health.

3 Conclusion

The world we inhabit now is very different to the one in which our physiology evolved to live. As a result of human progress and technological advancement, our environment now contains a

plethora of toxic pollutants in concentrations that are harmful to our bodies in ways that we are familiar with, as well as in ways that we do not yet understand. Modern pollution is so ubiquitous in the environment that MPs are now being used as an archeological gold standard for stratigraphic identification of this period of history, the Anthropocene [133].

Historic public health initiatives against pollution in the West, such as the banning of the pesticide DDT or the removal of lead from motor gasoline, need to set the example for future policy action regarding harmful chemicals across all societies. Furthermore, as we identify more toxic and epigenetic effects of pollutants in our daily lives, a concerted effort needs to be made to lobby the industries and practitioners responsible for the exposure of our populations to these toxic substances.

Understanding the epigenetic effects that these pollutants have on human physiology will help to fight the fire of pollution-related disease through the development of new clinical therapies, but it is imperative that the fundamental production and release of PM, gaseous pollutants, heavy metals, persistent organic pollutants, and many other compounds into our environment be tackled if we are to see any real change in their resultant disease burden.

And yet our understanding of its true magnitude—it's devastating relationship to human and planetary health; its immense drain on national budgets; its sheer volume—remains limited, occluded by vested interests and overtaxed political infrastructures, and lost among the compartmentalized foci of individual ministries and government agencies.

—From “Global, Regional, and Country Analysis December 2019” by the Global Alliance on Health and Pollution

References

- [1] Steffen W, et al. Trajectories of the Earth System in the Anthropocene. *Proc Natl Acad Sci U S A* 2018;115(33):8252–9.
- [2] Steffen W, et al. Planetary boundaries: Guiding human development on a changing planet. *Science* 2015;347(6223):1259855.
- [3] Rockström J, et al. Planetary boundaries: exploring the safe operating space for humanity. *Ecol Soc* 2009;14(2).
- [4] Watts N, et al. The 2019 report of The Lancet Countdown on health and climate change: ensuring that the health of a child born today is not defined by a changing climate. *Lancet* 2019;394 (10211):1836–78.
- [5] Watts N, et al. The Lancet Countdown on health and climate change: from 25 years of inaction to a global transformation for public health. *Lancet* 2018;391(10120):581–630.
- [6] Landrigan PJ, et al. The Lancet Commission on pollution and health. *Lancet* 2018;391(10119):462–512.
- [7] Fuller R SK, Hanrahan D. Pollution and health metrics. Global Alliance on Health and Pollution; 2019.
- [8] Marcantonio R, et al. Global distribution and coincidence of pollution, climate impacts, and health risk in the Anthropocene. *PLoS One* 2021;16(7):e0254060.
- [9] Sharavanan VJ, et al. Pollutants inducing epigenetic changes and diseases. *Environ Chem Lett* 2020;18 (2):325–43.
- [10] GBD 2017 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories. *Lancet* 2017;390(10104):1361–460.

- territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2018;392(10159):1789–1858.
- [11] World Health Organization. Ambient air pollution: a global assessment of exposure and burden of disease. World Health Organization; 2016.
 - [12] Cohen AJ, et al. Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015. Lancet 2017;389(10082):1907–18.
 - [13] Chafe ZA, et al. Household cooking with solid fuels contributes to ambient PM_{2.5} air pollution and the burden of disease. Environ Health Perspect 2014;122(12):1314–20.
 - [14] World Health Organisation. WHO indoor air quality guidelines: household fuel combustion. Executive summary; 2014.
 - [15] National Center for Health Statistics <<https://www.cdc.gov/nchs/>> (accessed 10.01.2023).
 - [16] Apte K, Salvi S. Household air pollution and its effects on health. F1000Research 2016;5:F1000.
 - [17] World Health Organization. Global Health Observatory (GHO) data: the data repository. <<https://apps.who.int/gho/data/node.home>> (accessed 10.01.2023).
 - [18] Rider CF, Carlsten C. Air pollution and DNA methylation: effects of exposure in humans. Clin Epigenetics 2019;11(1):131.
 - [19] Prunicki M, et al. Exposure to NO₂, CO, and PM_{2.5} is linked to regional DNA methylation differences in asthma. Clin Epigenetics 2018;10(1):2.
 - [20] Health Effects Institute. Traffic-related air pollution: a critical review of the literature on emissions, e., and health effects. HEI Special Report 17. Boston: Health Effects Institute; 2010. <<https://www.healtheffects.org/publication/traffic-related-air-pollution-critical-review-literature-emissions-exposure-and-health>> (accessed 10.01.2023)
 - [21] Gauderman WJ, et al. Effect of exposure to traffic on lung development from 10 to 18 years of age: a cohort study. Lancet 2007;369(9561):571–7.
 - [22] Brunekreef B, et al. Air pollution from truck traffic and lung function in children living near motorways. Epidemiology 1997;8(3):298–303.
 - [23] IARC. Monographs on the evaluation of carcinogenic risks to humans: diesel and gasoline engine exhausts and some nitroarenes; 2014.
 - [24] Castaño-Vinyals G, et al. Air pollution and risk of urinary bladder cancer in a case-control study in Spain. Occup Environ Med 2008;65(1):56–60.
 - [25] Kachuri L, et al. Workplace exposure to diesel and gasoline engine exhausts and the risk of colorectal cancer in Canadian men. Environ Health 2016;15:4.
 - [26] Chiu HF, et al. Traffic air pollution and risk of death from gastric cancer in Taiwan: petrol station density as an indicator of air pollutant exposure. J Toxicol Environ Health A 2011;74(18):1215–24.
 - [27] Chen Z, et al. Chronic effects of air pollution on respiratory health in Southern California children: findings from the Southern California Children's Health Study. J Thorac Dis 2015;7(1):46–58.
 - [28] Bowatte G, et al. The influence of childhood traffic-related air pollution exposure on asthma, allergy and sensitization: a systematic review and a meta-analysis of birth cohort studies. Allergy 2015;70(3):245–56.
 - [29] GBD 2019 Risk Factors Collaborators. Global burden of 87 risk factors in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet 2020;396(10258):1223–49.
 - [30] GBD 2015 Risk Factors Collaborators. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet 2016;388(10053):1659–1724.
 - [31] Jerrett M, et al. Traffic-related air pollution and obesity formation in children: a longitudinal, multilevel analysis. Environ Health 2014;13:49.

- [32] Liu J, et al. Combined inhaled diesel exhaust particles and allergen exposure alter methylation of T helper genes and IgE production in vivo. *Toxicol Sci* 2008;102(1):76–81.
- [33] Izzotti A, et al. Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. *FASEB J* 2009;23(3):806–12.
- [34] Farraj AK, et al. ST depression, arrhythmia, vagal dominance, and reduced cardiac micro-RNA in particulate-exposed rats. *Am J Respir Cell Mol Biol* 2011;44(2):185–96.
- [35] Kampa M, Castanas E. Human health effects of air pollution. *Environ Pollut* 2008;151(2):362–7.
- [36] Schraufnagel DE, et al. Air pollution and noncommunicable diseases: a review by the forum of international respiratory societies' environmental committee, part 2: air pollution and organ systems. *Chest* 2019;155(2):417–26.
- [37] Pinkerton KE, et al. Distribution of particulate matter and tissue remodeling in the human lung. *Environ Health Perspect* 2000;108(11):1063–9.
- [38] Churg J, Brauer M. Human lung parenchyma retains PM_{2.5}. *Am J Respir Crit Care Med* 1997;155(6):2109–11.
- [39] Feng S, et al. The health effects of ambient PM_{2.5} and potential mechanisms. *Ecotoxicol Environ Saf* 2016;128:67–74.
- [40] Brook RD, et al. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation* 2004;109(21):2655–71.
- [41] Madl AK, et al. Nanoparticles, lung injury, and the role of oxidant stress. *Annu Rev Physiol* 2014;76:447–65.
- [42] Tamagawa E, et al. Particulate matter exposure induces persistent lung inflammation and endothelial dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2008;295(1):L79–85.
- [43] Gilmour PS, et al. Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. *Am J Physiol Lung Cell Mol Physiol* 2003;284(3):L533–40.
- [44] Zheng Y, et al. Traffic-derived particulate matter exposure and histone H3 modification: a repeated measures study. *Environ Res* 2017;153:112–19.
- [45] Miousse IR, et al. Epigenetic alterations induced by ambient particulate matter in mouse macrophages. *Environ Mol Mutagen* 2014;55(5):428–35.
- [46] Viré E, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006;439(7078):871–4.
- [47] Raaschou-Nielsen O, et al. Air pollution from traffic and cancer incidence: a Danish cohort study. *Environ Health* 2011;10:67.
- [48] Schlesinger Y, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet* 2007;39(2):232–6.
- [49] Takeshima H, et al. Induction of aberrant trimethylation of histone H3 lysine 27 by inflammation in mouse colonic epithelial cells. *Carcinogenesis* 2012;33(12):2384–90.
- [50] Tamagawa H, et al. Global histone modification of H3K27 correlates with the outcomes in patients with metachronous liver metastasis of colorectal cancer. *Eur J Surg Oncol* 2013;39(6):655–61.
- [51] Ho TH, et al. Loss of histone H3 lysine 36 trimethylation is associated with an increased risk of renal cell carcinoma-specific death. *Mod Pathol* 2016;29(1):34–42.
- [52] Li F, et al. The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutS α . *Cell* 2013;153(3):590–600.
- [53] Li GM. Decoding the histone code: Role of H3K36me3 in mismatch repair and implications for cancer susceptibility and therapy. *Cancer Res* 2013;73(21):6379–83.
- [54] Liu C, et al. Characterization of genome-wide H3K27ac profiles reveals a distinct PM_{2.5}-associated histone modification signature. *Environ Health* 2015;14:65.
- [55] Creyghton MP, et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci U S A* 2010;107(50):21931–6.

- [56] Bollati V, et al. Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes. *Environ Health Perspect* 2010;118(6):763–8.
- [57] Jardim MJ, et al. Disruption of microRNA expression in human airway cells by diesel exhaust particles is linked to tumorigenesis-associated pathways. *Environ Health Perspect* 2009;117(11):1745–51.
- [58] International Agency for Research on Cancer. Monographs on the evaluation of carcinogenic risks to humans. Outdoor air pollution. Lyon, France: International Agency for Research on Cancer; 2016.
- [59] Cui P, et al. Ambient particulate matter and lung cancer incidence and mortality: a meta-analysis of prospective studies. *Eur J Public Health* 2014;25(2):324–9.
- [60] Yanagi Y, Assunção JV, Barrozo LV. The impact of atmospheric particulate matter on cancer incidence and mortality in the city of São Paulo, Brazil. *Cad Saude Publica* 2012;28(9):1737–48.
- [61] Turner MC, et al. Ambient air pollution and cancer mortality in the cancer prevention study II. *Environ Health Perspect* 2017;125(8):087013.
- [62] Yi H, Li L.. DNA methylation changes in response to sulfur dioxide stress in arabidopsis plants. *Proc Environ Sci* 18:37–42.
- [63] Zhang L, Zhao Y. The regulation of Foxp3 expression in regulatory CD4(+)CD25(+)T cells: multiple pathways on the road. *J Cell Physiol* 2007;211(3):590–7.
- [64] Lee MK, et al. Genome-wide DNA methylation and long-term ambient air pollution exposure in Korean adults. *Clin Epigenetics* 2019;11(1):37.
- [65] DeMarini DM. Genotoxicity biomarkers associated with exposure to traffic and near-road atmospheres: a review. *Mutagenesis* 2013;28(5):485–505.
- [66] Crouse DL, et al. Postmenopausal breast cancer is associated with exposure to traffic-related air pollution in Montreal, Canada: a case-control study. *Environ Health Perspect* 2010;118(11):1578–83.
- [67] Fry RC, et al. Air toxics and epigenetic effects: ozone altered microRNAs in the sputum of human subjects. *Am J Physiol Lung Cell Mol Physiol* 2014;306(12):L1129–37.
- [68] Gilbert KM, et al. Chronic exposure to water pollutant trichloroethylene increased epigenetic drift in CD4 + T cells. *Epigenomics* 2016;8(5):633–49.
- [69] Badra J, et al. Understanding of the octane response of gasoline/MTBE blends. *Fuel* 2022;318:123647.
- [70] Rota F, et al. Epigenetic and transcriptional modifications in repetitive elements in petrol station workers exposed to benzene and MTBE. *Int J Environ Res Public Health* 2018;15(4):735.
- [71] Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological profile for chloroform. Atlanta, GA: Public Health Service, U.S. Department of Health and Human Services; 1997.
- [72] Bollati V, et al. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res* 2007;67(3):876–80.
- [73] Xing C WQ, Li B, Tian H, Ni Y, Yin S, Li G. Methylation and expression analysis of tumor suppressor genes p15 and p16 in benzene poisoning. *Chem Biol Interact* 2010;184:306–9.
- [74] Bai W, et al. Aberrant miRNA profiles associated with chronic benzene poisoning. *Exp Mol Pathol* 2014;96(3):426–30.
- [75] Lübbert M, et al. A switch toward demethylation is associated with the expression of myeloperoxidase in acute myeloblastic and promyelocytic leukemias. *Blood* 1992;80(8):2066–73.
- [76] Melki JR, Vincent PC, Clark SJ. Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. *Cancer Res* 1999;59(15):3730–40.
- [77] Timp W, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. *Nat Rev Cancer* 2013;13(7):497–510.
- [78] Uebelacker M, Lachenmeier DW. Quantitative determination of acetaldehyde in foods using automated digestion with simulated gastric fluid followed by headspace gas chromatography. *J Automated Methods Manag Chem* 2011;2011:907317.
- [79] Shukla S.D., et al. Acetaldehyde alters MAP kinase signalling and epigenetic histone modifications in hepatocytes. *Novartis Found Symp*, 2007;285:217–24; discussion 224–8.

- [80] Genchi G, et al. Nickel: Human Health and Environmental Toxicology. *Int J Environ Res Public Health* 2020;17(3):679.
- [81] Lee YW, et al. Carcinogenic nickel silences gene expression by chromatin condensation and DNA methylation: a new model for epigenetic carcinogens. *Mol Cell Biol* 1995;15(5):2547–57.
- [82] Chen H, et al. Nickel ions increase histone H3 lysine 9 dimethylation and induce transgene silencing. *Mol Cell Biol* 2006;26(10):3728–37.
- [83] Golebiowski F, Kasprzak KS. Inhibition of core histones acetylation by carcinogenic nickel(II). *Mol Cell Biochem* 2005;279(1–2):133–9.
- [84] Jose CC, et al. Nickel-induced transcriptional changes persist post exposure through epigenetic reprogramming. *Epigenetics Chromatin* 2019;12(1):75.
- [85] Ke Q, et al. Alterations of histone modifications and transgene silencing by nickel chloride. *Carcinogenesis* 2006;27(7):1481–8.
- [86] Martin EM, Fry RC. Environmental influences on the epigenome: exposure- associated DNA methylation in human populations. *Annu Rev Public Health* 2018;39:309–33.
- [87] Genchi G, et al. The effects of cadmium toxicity. *Int J Environ Res Public Health* 2020;17(11).
- [88] Geng HX, Wang L. Cadmium: toxic effects on placental and embryonic development. *Environ Toxicol Pharmacol* 2019;67:102–7.
- [89] Benbrahim-Tallaa L, et al. Tumor suppressor gene inactivation during cadmium-induced malignant transformation of human prostate cells correlates with overexpression of de novo DNA methyltransferase. *Environ Health Perspect* 2007;115(10):1454–9.
- [90] Xiao C, et al. Cadmium induces histone H3 lysine methylation by inhibiting histone demethylase activity. *Toxicol Sci* 2015;145(1):80–9.
- [91] Prasad S, et al. Chromium contamination and effect on environmental health and its remediation: a sustainable approaches. *J Environ Manage* 2021;285:112174.
- [92] Cabral Pinto MMS, et al. An inter-disciplinary approach to evaluate human health risks due to long-term exposure to contaminated groundwater near a chemical complex. *Exposure Health* 2020;12(2):199–214.
- [93] Chen QY, et al. Molecular and epigenetic mechanisms of Cr(VI)-induced carcinogenesis. *Toxicol Appl Pharmacol* 2019;377:114636.
- [94] Schnekenburger M, Talaska G, Puga A. Chromium cross-links histone deacetylase 1-DNA methyltransferase 1 complexes to chromatin, inhibiting histone-remodeling marks critical for transcriptional activation. *Mol Cell Biol* 2007;27(20):7089–101.
- [95] Wang TC, et al. Oxidative DNA damage and global DNA hypomethylation are related to folate deficiency in chromate manufacturing workers. *J Hazard Mater* 2012;213–214:440–6.
- [96] Sen A, et al. Multigenerational epigenetic inheritance in humans: DNA methylation changes associated with maternal exposure to lead can be transmitted to the grandchildren. *Sci Rep* 2015;5:14466.
- [97] Cardenas A, et al. Differential DNA methylation in umbilical cord blood of infants exposed to mercury and arsenic in utero. *Epigenetics* 2015;10(6):508–15.
- [98] Richard Pilsner J LA, Nam DH, Letcher RJ, Sonne C, Dietz R, et al. Mercury-associated DNA hypomethylation in polar bear brains via the LUMINOMETRIC Methylation Assay: a sensitive method to study epigenetics in wildlife. *Mol Ecol* 2009;307–14.
- [99] Arai Y, et al. Epigenetic assessment of environmental chemicals detected in maternal peripheral and cord blood samples. *J Reprod Dev* 2011;57(4):507–17.
- [100] Martinez VD, et al. Arsenic exposure and the induction of human cancers. *J Toxicol* 2011;2011:431287.
- [101] Hossain K, et al. Chronic exposure to arsenic, LINE-1 hypomethylation, and blood pressure: a cross-sectional study in Bangladesh. *Environ Health* 2017;16(1):20.

- [102] Csanaky I, Németi B, Gregus Z. Dose-dependent biotransformation of arsenite in rats—not S-adenosylmethionine depletion impairs arsenic methylation at high dose. *Toxicology* 2003;183(1):77–91.
- [103] Coppin JF QW, Waalkes MP. Interplay between cellular methyl metabolism and adaptive efflux during oncogenic transformation from chronic arsenic exposure in human cells. *J Biol Chem* 2008;19342–50.
- [104] Jensen TJ, et al. Epigenetic remodeling during arsenical-induced malignant transformation. *Carcinogenesis* 2008;29(8):1500–8.
- [105] Cohn BA, Cirillo PM, Terry MB. DDT and breast cancer: prospective study of induction time and susceptibility windows. *J Natl Cancer Inst* 2019;111(8):803–10.
- [106] Skinner MK, et al. Ancestral dichlorodiphenyltrichloroethane (DDT) exposure promotes epigenetic transgenerational inheritance of obesity. *BMC Med* 2013;11(1):228.
- [107] Ben Maamar M, et al. Epigenetic transgenerational inheritance of altered sperm histone retention sites. *Sci Rep* 2018;8(1):5308.
- [108] Poston RG, Saha RN. Epigenetic effects of polybrominated diphenyl ethers on human health. *Int J Environ Res Public Health* 2019;16(15).
- [109] Mackintosh SA, et al. Review on the occurrence and profiles of polybrominated diphenyl ethers in the Philippines. *Environ Int* 2015;85:314–26.
- [110] Pierozan P, Cattani D, Karlsson O. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) induce epigenetic alterations and promote human breast cell carcinogenesis in vitro. *Arch Toxicol* 2020;94(11):3893–906.
- [111] Nicole W. PFOA and cancer in a highly exposed community: new findings from the C8 science panel. *Environ Health Perspect* 2013;121(11–12):A340.
- [112] Defoix C, et al. Environmental pollutant benzo[a]pyrene impacts the volatile metabolome and transcriptome of the human gut microbiota. *Front Microbiol* 2017;8:1562.
- [113] Bhargava A, et al. Mapping the mitochondrial regulation of epigenetic modifications in association with carcinogenic and noncarcinogenic polycyclic aromatic hydrocarbon exposure. *Int J Toxicol* 2020;39(5):465–76.
- [114] Weng Y-I, et al. Epigenetic influences of low-dose bisphenol A in primary human breast epithelial cells. *Toxicol Appl Pharmacology* 2010;248(2):111–21.
- [115] Hatanaka Y, et al. HOXA10 expression profiling in prostate cancer. *Prostate* 2019;79(5):554–63.
- [116] Lu J, et al. Clinical significance and prognostic value of the expression of LAMP3 in oral squamous cell carcinoma. *Dis Markers* 2017;2017:1218254.
- [117] Nagelkerke A, et al. LAMP3 is involved in tamoxifen resistance in breast cancer cells through the modulation of autophagy. *Endocr Relat Cancer* 2014;21(1):101–12.
- [118] Cho H, et al. A relationship between miRNA and gene expression in the mouse Sertoli cell line after exposure to bisphenol A. *BioChip J* 2010;4(1):75–81.
- [119] Rushing BR, Selim MI. Aflatoxin B1: a review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. *Food Chem Toxicol* 2019;124:81–100.
- [120] Dai Y, et al. Aflatoxin B1-induced epigenetic alterations: an overview. *Food Chem Toxicol* 2017;109:683–9.
- [121] Garcia-Rodriguez JA. Radon gas—the hidden killer: what is the role of family doctors? *Can Fam Phys* 2018;64(7):496–501.
- [122] de Vocht F, et al. Residential exposure to radon and DNA methylation across the lifecourse: an exploratory study in the ALSPAC birth cohort. *Wellcome Open Res* 2019;4:3.
- [123] Geyer R, Jambeck JR, Law KL. Production, use, and fate of all plastics ever made. *Sci Adv* 2017;3(7):e1700782.
- [124] Barnes DK, et al. Accumulation and fragmentation of plastic debris in global environments. *Philos Trans R Soc Lond B Biol Sci* 2009;364(1526):1985–98.

- [125] Song YK, et al. Combined effects of UV exposure duration and mechanical abrasion on microplastic fragmentation by polymer type. *Environ Sci Technol* 2017;51(8):4368–76.
- [126] Thompson RC, et al. Lost at sea: where is all the plastic? *Science* 2004;304(5672):838.
- [127] Gregory MR. Accumulation and distribution of virgin plastic granules on New Zealand beaches. *N Z J Mar Freshwater Res* 1978;12(4):399–414.
- [128] Schmid C, Cozzarini L, Zambello E. Microplastic's story. *Mar Pollut Bull* 2021;162:111820.
- [129] Gregory MR. Plastic 'scrubbers' in hand cleansers: a further (and minor) source for marine pollution identified. *Mar Pollut Bull* 1996;32(12):867–71.
- [130] Andrade AL. Microplastics in the marine environment. *Mar Pollut Bull* 2011;62(8):1596–605.
- [131] Browne MA, Galloway T, Thompson R. Microplastic—an emerging contaminant of potential concern? *Integr Environ Assess Manag* 2007;3(4):559–61.
- [132] Brahney J, et al. Plastic rain in protected areas of the United States. *Science* 2020;368(6496):1257–60.
- [133] Zalasiewicz J, et al. The geological cycle of plastics and their use as a stratigraphic indicator of the Anthropocene. *Anthropocene* 2016;13:4–17.
- [134] World Health Organisation. Microplastics drinking-water. 2019.
- [135] Barboza LGA, et al. Marine microplastic debris: an emerging issue for food security, food safety and human health. *Mar Pollut Bull* 2018;133:336–48.
- [136] Schwabl P, et al. Detection of various microplastics in human stool: a prospective case series. *Ann Intern Med* 2019;171(7):453–7.
- [137] Cox KD, et al. Human consumption of microplastics. *Environ Sci Technol* 2019;53(12):7068–74.
- [138] Shi Q, et al. Combined cytotoxicity of polystyrene nanoplastics and phthalate esters on human lung epithelial A549 cells and its mechanism. *Ecotoxicol Environ Saf* 2021;213:112041.
- [139] Groh KJ, et al. Overview of known plastic packaging-associated chemicals and their hazards. *Sci Total Environ* 2019;651(Pt 2):3253–68.
- [140] vom Saal FS, Hughes C. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. *Environ Health Perspect* 2005;113(8):926–33.
- [141] Garrigós MC, et al. Determination of residual styrene monomer in polystyrene granules by gas chromatography-mass spectrometry. *J Chromatogr A* 2004;1061(2):211–16.
- [142] Karbalaei S, et al. Occurrence, sources, human health impacts and mitigation of microplastic pollution. *Environ Sci Pollut Res Int* 2018;25(36):36046–63.
- [143] Wang J, et al. The behaviors of microplastics in the marine environment. *Mar Environ Res* 2016;113:7–17.
- [144] Yamashita R, et al. Physical and chemical effects of ingested plastic debris on short-tailed shearwaters, *Puffinus tenuirostris*, in the North Pacific Ocean. *Mar Pollut Bull* 2011;62(12):2845–9.
- [145] Pimentel JC, Avila R, Lourenço AG. Respiratory disease caused by synthetic fibres: a new occupational disease. *Thorax* 1975;30(2):204–19.
- [146] Atis S, et al. The respiratory effects of occupational polypropylene flock exposure. *Eur Respir J* 2005;25(1):110–17.
- [147] Eschenbacher WL, et al. Nylon flock-associated interstitial lung disease. *Am J Respir Crit Care Med* 1999;159(6):2003–8.
- [148] Kern DG, et al. Flock worker's lung: broadening the spectrum of clinicopathology, narrowing the spectrum of suspected etiologies. *Chest* 2000;117(1):251–9.

Synthetic biology and cell engineering—deriving new insights into cancer epigenetics 10

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Chapter outline

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1 Introduction: an overview of epigenetic engineering

Dynamic chromatin-mediated epigenetic states have been linked to processes, including misregulated differentiation, proliferation, invasion, and other phenotypes, that support cancer aggressiveness. Therefore much effort has been invested in developing novel reporters and probes built from nucleic acids and proteins that can precisely track chromatin features such as **methylated DNA** and post-translational modifications on **histones** in living cancer cells. These reporters and probes can be modified to act upon chromatin in a site-specific or region-specific manner, for example, at single genes or enhancer domains, respectively. The ability to manipulate chromatin features, and consequently the associated gene expression states, has implications to expand the epigenetic therapy toolbox beyond current FDA-approved inhibitors of **chromatin-modifying enzymes (CMEs)**. However, substantial barriers such as inefficient delivery of synthetic nucleic acids and proteins into various cancer cell types and solid tumors *in vivo* place therapeutic applications of new molecular tools far out on the horizon [1,2]. In the meantime, these tools provide a powerful means to perform basic research experiments that cannot be accomplished with epigenetic drugs or small interfering RNAs that are easier to deliver into cells and tumors.

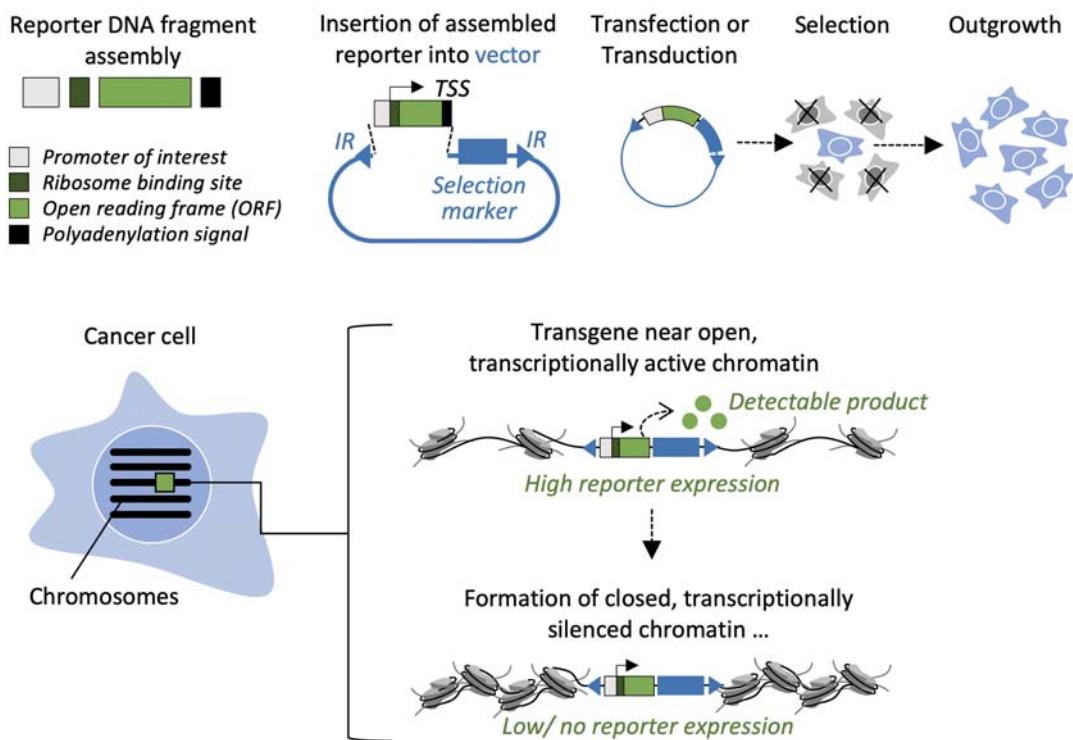
Epigenetic engineering, the practice of developing tools to precisely sense and/or manipulate chromatin, is an approach that has been developed by scientists within the traditional chromatin

research community, and recently by scientists from the synthetic biology community. Traditional chromatin researchers have designed reporters primarily to observe changes within the context of the natural chromatin machinery of cancer cells. Synthetic biologists approach cancer epigenetics from an engineering perspective, for instance by using pared-down DNA sequences and protein modules as building blocks to generate artificial epigenetic regulation and to produce empirical data for mathematical modeling.

2 Genetic reporters: synthetic genes to monitor transcriptional regulation

To map chromatin features, such as DNA methylation (meCpG) and **histone posttranslational modifications** at specific genes and across the entire genome, scientists typically extract chromatin from cancer cells and analyze the material with processes, such as bisulfite sequencing, chromatin immunoprecipitation (ChIP), and ATAC-seq. However, investigations of chromatin dynamics and their impact on gene transcription in living cells are only possible with cell engineering approaches, such as the chromosomal transgenic **reporter**. A **transgene** is recombinant DNA that is built *in vitro* with molecular cloning techniques, includes the minimum components for gene expression, that is, promoter, ribosome binding sequence, **open reading frame (ORF)**, and polyadenylation signal, and is artificially introduced into the chromosomal DNA of a living cell (Figure 10.1). Work with model organisms, such as fruit flies (*Drosophila*), has established that once a transgene has been inserted into a chromosome, the activity of the promoter and therefore the level of expression (transcription) from the transgene can be influenced epigenetically by the surrounding chromatin environment [3–7], a phenomenon known as position effect variegation. This phenomenon has been adapted to monitor changes in chromatin states in human cells, including cancer-derived cell lines.

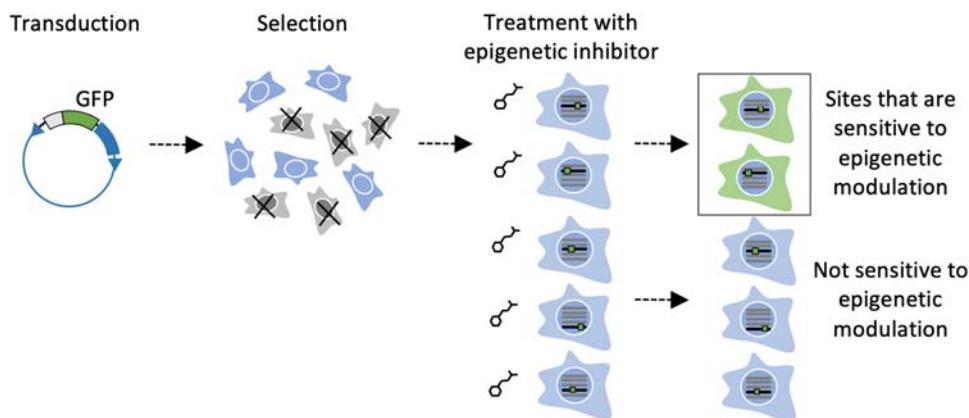
Transgenes are useful for investigating specific promoters that are known to adopt different epigenetic states in cancer cells. The constructed transgene typically carries an antibiotic resistance marker and is introduced into a cultured cell line via transfection or viral transduction (Figure 10.1). Then, the cells are grown under selection with the appropriate antibiotic, and a clonal line that carries a single transgene at a single locus is isolated for use in experiments. For instance, a transgene containing the promoter region of the glioblastoma-overexpressed *SNHG12* gene placed upstream of a luciferase ORF was used to verify that DNMT1 could methylate the promoter and reduce transcription [8]. This discovery suggests that loss of DNMT1 activity may underlie the increased levels of *SNHG12* expression in glioblastoma. In a prostate cancer-focused study, scientists used a transgenic reporter to understand how the tumor-suppressor gene *DEFB1* is downregulated [9]. A *DEFB1* promoter region containing fourteen CpG sites was placed upstream of a luciferase ORF. The promoter was artificially methylated *in vitro* with purified methyltransferase enzymes and was then introduced into nonprostate cancer cells (HEK293T). Reduced expression compared to a nonmethylated control demonstrated that promoter methylation, even in a nonprostate cancer cell, was sufficient to silence the *DEFB1* promoter. A similar approach was used to study the causal relationship between DNA methylation and activity of the *Tnf* promoter in colon cancer [10]. In a study on the general effect of DNA hypomethylating drug decitabine (DAC) on

**FIGURE 10.1**

Genetic reporters: general design, construction, and application. A promoter of interest, a ribosome binding sequence, and an open-reading frame that encodes a detectable output (e.g., luciferase) are cloned into a commercially available or publicly shared expression vector that contains inverted repeat (IR) sequences and an antibiotic selection marker. *TSS* = transcription start site within the assembled reporter.

epigenetic states, scientists used a reporter that included a hypermethylated cytomegalovirus promoter and a green fluorescent protein (GFP) ORF in a SW48 colon cancer cell line [11]. DAC treatment resulted in varying (high and low) levels of GFP in individual cells. In the GFP-low subpopulation, the persistence of silencing-associated chromatin features, including H3K9 hypoacetylation, H3K27 trimethylation, and high **nucleosome** density, showed that hypomethylation after DAC is not sufficient for the stable activation of gene expression.

Recently other systems have been developed to analyze hundreds of thousands of cells in parallel without the need to isolate clonal transgenic cell lines. TRACE (transgene reporters across chromatin environments) randomly inserts GFP-encoding lentiviral transgenes into the chromosomes of cultured cells [12]. This system was used to identify transgene insert sites where GFP expression levels changed as a result of disrupting specific chromatin proteins that are also targets of interest for cancer therapy (Figure 10.2). TRACE was used to evaluate an epigenetic drug that inhibits LSD1 (lysine-specific demethylase 1A), and transcriptional repressor that is an important drug

**FIGURE 10.2**

Multiinsert reporter approaches for large scale parallel analysis. Platforms, such as TRACE (transgene reporters across chromatin environments) and TRIP (thousands of reporters integrated in parallel), can be used to compare differences in epigenetic regulation on different chromosomes and at different loci within chromosomes in a single experiment. In a study using TRACE, increased GFP signal (green cells) from the reporter transgene identified chromosomal sites that were sensitive to compounds that targeted chromatin proteins.

target for acute myeloid leukemia (AML) and other cancers [13]. When TRACE-engineered KBM-7 cells were treated with LSD1 inhibitor GSK2879552, transgene reporter GFP expression increased at a small fraction of chromosomal locations, only 981 of roughly 100,000 transgene insertion sites. Pharmacological inhibition of other leading drug targets EZH2 and EED, subunits of polycomb repressive complex 2 (PRC2), did not relieve silencing whereas genetic knockdown of a different member of PRC2, SUZ12, increased transgene reporter GFP levels. This result suggests that the development of inhibitors of SUZ12 might improve the efficacy of epigenetic therapy for CML. Another multitransgene insertion platform called TRIP (Thousands of Reporters Integrated in Parallel) has been used to determine how different chromatin structures across the genome influence transgene expression in mouse embryonic stem cells [14] and has the potential to be adapted to cancer studies.

3 Protein reporters: engineered proteins to track chromatin features in cancer cells

Traditionally, antibodies have been used to “paint” regions in fixed cells that are enriched for specific epigenetics marks, such as histone posttranslational modifications (histone PTMs), DNA methylation (meCpG), and nonhistone chromatin proteins. Generally, functional antibodies are difficult to deliver into living cells. However, other proteins, such as **histone PTM-binding domains** and **methyl-DNA-binding domains**, herein collectively called **chromatin-binding domains (CBDs)**,

are naturally expressed in cells in a family of proteins known as chromatin readers, or reader-effectors. Cell engineers have isolated the minimal reader-effectors sequences required to selectively bind specific histone PTMs and meCpG and fused these with fluorescent proteins to paint chromatin regions in living cells. This is achieved by constructing a recombinant DNA ORF that includes the CBD in-frame with a detectable protein tag, such as a fluorescent protein, and a strong nuclear localization signal (NLS) (Figure 10.3). The recombinant ORF is then included in a transgene so that once it is transfected or transduced, cells express the protein reporter which enters the nucleus and accumulates at chromatin regions that bear the target epigenetic mark. These regions can be visualized as bright areas or punctae in the nucleus, similar to immunofluorescence cytology (IFC) but without fixation and staining of the cells. The protein reporter is trans-acting; therefore a stably integrated transgene is not necessary, and selection and outgrowth of a clonal cell line is optional.

Transgene-expressed protein reporters are useful for tracking gains and losses of histone marks at subnuclear scale [15–18]. Protein reporters with CBDs that bind H3K9me3 and DNA methylation (meCpG) were used to determine that DNA methylation, but not H3K9me3, was redistributed in the nuclei of breast cancer MCF7 cells as they became resistant to tamoxifen [19]. Other protein reporters that have been tested and validated in model mammalian cell lines, such as immortalized

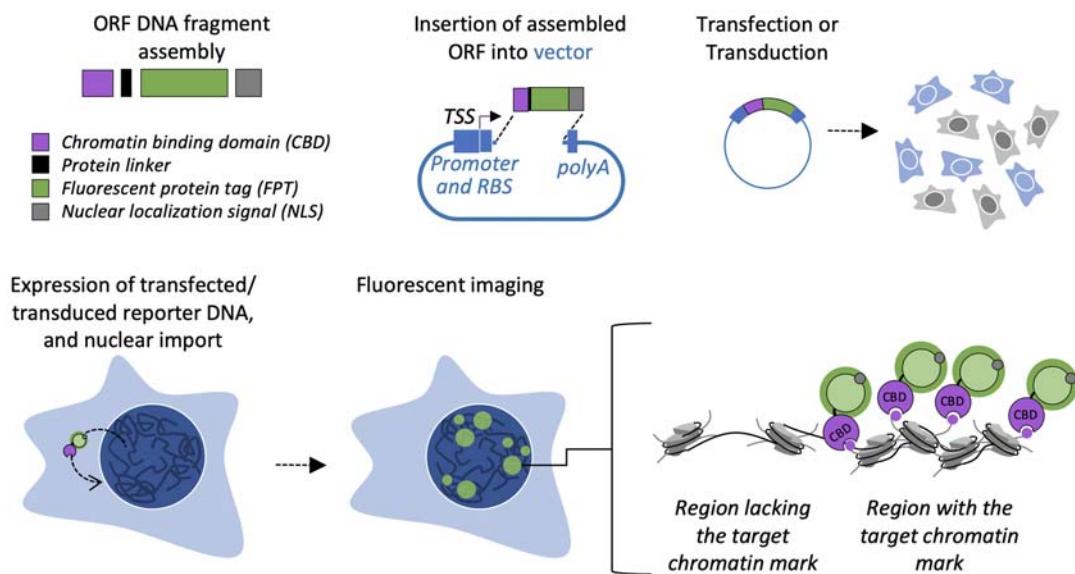


FIGURE 10.3

Protein reporters: design, construction, and application. An open-reading frame is built from a chromatin-binding domain (CBD), protein linker (e.g., glycine-serine repeat), fluorescent protein tag (FPT), and nuclear localization signal and cloned into an expression vector that contains a promoter and ribosome binding sequence (upstream) and a poly-adenylation signal (downstream). TSS = transcription start site. Once the protein reporter is expressed from transfected DNA, it is imported into the nucleus and accumulates at regions that bear the target chromatin modification.

human cell lines and mouse embryonic stem cells, have the potential to be used for cancer research. For instance, one could investigate the effect of epigenetic drugs, such as histone acetyltransferase inhibitors, deacetylase inhibitors, and methyltransferase inhibitors, by monitoring changes in the distribution of protein probes that recognize acetylation of histone H3K4 (probe BRD-GFP) [17], or methylation of histone H3K27 and H3K4 (chromatin-sensing multivalent probes) [15] throughout the nucleus. It is also possible to monitor changes in chromatin marks at specific subregions such as telomeres and pericentromeric DNA by using combinations of chromatin-binding probes and DNA-binding **fusion proteins**, a technology called bimolecular anchor detector system [16]. This system was used to track DNA methylation and histone H3K9 methylation specifically at repetitive DNA sequences. In cases where a highly specific CBD is not available for a chromatin modification of interest, modification-specific intracellular antibodies, or "mintbodies," offer an alternative. Transgene-encoded protein probes built from mintbodies and fluorescent proteins have been used to detect acetylated histone H3K9 and monomethylated histone H4K20 in human cell lines [20,21].

Protein reporters can also be used to biochemically tag protein interactions in living cells [22]. CBDs have been engineered to include a photo-sensitive amino acid that upon exposure to ultraviolet light rapidly forms a covalent bond between the binding domain (BRD4 or CBX1) and the cognate histone PTM (histone acetyl lysine or histone H3K27me3, respectively). The covalent bond stabilizes the reader-histone interaction so that complexes can be extracted and analyzed by ChIP, western blot, or liquid chromatography-mass spectrometry [23–25]. Reporters built from CBDs fused to a biotin ligase were used to tag proteins within a 35-nm radius of histone H3K27me3, H3K9me3, H3K4me3, and meCpG in mouse stem cells [26]. These protein reporters enable the capture of weak and transient interactions between chromatin binding proteins and their target epigenetic marks at high temporal precision. So far these novel systems have been used in model cell lines, including human HEK293 and mouse embryonic stem cells, and can potentially be used to investigate chromatin complexes in cancer cells. The chromatin modifications that can be detected by current protein reporters, including meCpG, histone acetylation, H3K4me3, H3K9me3, and H3K27me3, have been the focus of research for a wide variety of cancer subtypes, as discussed in other chapters.

One critical limitation of protein reporters is that the engineered CBDs are not suitable for all cancer-relevant chromatin marks. For instance, the oncohistone H3K27M is not currently known to be specifically bound by a “reader” protein domain. Furthermore, histone deacetylase and demethylase activity produce unmodified histone residues that are not specifically recognized by well-characterized CBDs. This gap could be addressed by exploring the use of recently reported proteins that bind nonacetylated histone H3, such as the ZZ domain of p300 and ZZZ3 [27,28], or continued development of histone-specific mintbodies that show no cross-reactivity with other chromatin proteins.

4 Epigenome editing: precise modification of chromatin

Epigenome editing is a cell engineering technique that generates or removes covalent modifications of chromatin, DNA methylation and histone posttranslational modifications, at specific

locations within the genome in living cells [29]. This is achieved by using an epigenome editor fusion protein which typically contains a CME and a targeting module that binds DNA at clinically important therapeutic genes [30], intergenic enhancer elements [31], pericentromeric chromatin [32], and other genomic loci [33–35]. To express epigenome editor fusion proteins in cancer cells, the scientist must build a recombinant DNA ORF that includes a sequence-specific DNA-targeting module, the core functional domain of a CME, and a strong NLS (Figure 10.4). A detectable fluorescent tag is often included so that cells can be quickly visually inspected to ensure that the protein is being expressed. The recombinant ORF is cloned into a transgene vector and transfected or transduced into cells. After the epigenome editor proteins are expressed, they enter the nucleus and bind the target site and the chromatin-modifying module generates modifications close to the binding site. As is the case for protein reporters (Figure 10.3) epigenome editors are

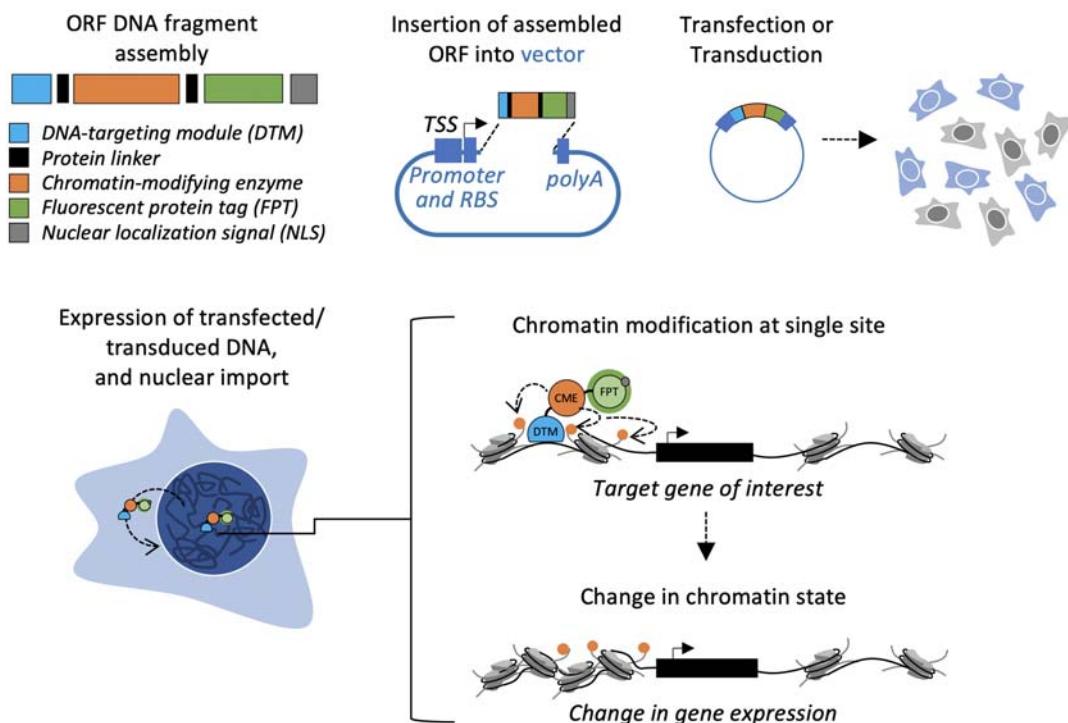


FIGURE 10.4

Epigenome editing: design, construction, and use of epigenome editor fusion proteins. An open-reading frame (ORF) is built from a DNA-targeting module (DTM), protein linker (e.g., glycine-serine repeat), chromatin-modifying enzyme (CME), fluorescent protein tag (FPT), and nuclear localization signal (NLS) and cloned into an expression vector that contains a promoter and ribosome binding sequence (upstream) and a polyadenylation signal (downstream). TSS = transcription start site. Once the protein reporter is expressed from transfected DNA, it is imported into the nucleus, binds at a site that is recognized by the DTM, and remodels nearby chromatin according to the enzymatic activity of the CME.

trans-acting, therefore a stably integrated transgene is not necessary. However, in certain cases where controlled levels of the epigenome editor proteins is desired, for example, for timed assays or to prevent toxic overexpression, scientists often use a stably integrated single transgene insert under the control of a chemically inducible promoter.

Several types of DNA-targeting modules have been used for epigenome editing in human cancer cells, including bacterial LexA, yeast Gal4, mammalian zinc finger “ZF,” mammalian Transcription activator-like effector “TALE,” and bacterial CRISPR-derived dead-Cas9 (dCas9) complexed with DNA-specific guide RNA (gRNA) [36]. Modules are typically selected based on their specific advantages over others (see Table 1 in [2]). For instance, while 20-base pair gRNAs are easier to design and less expensive to build than DNA-binding proteins like ZFs and TALEs, TALEs have shown superior performance as targeting modules within chromatin and as regulators in genetic circuits compared to dCas9/gRNA [37–39].

CMEs that have been used for epigenome editing in cancer cells catalyze several different modifications, including DNA methylation and demethylation, histone methylation and demethylation, and histone deacetylation (see Table 2 in Falahi et al. [2]). A TALE-TET1 (ten-eleven translocation hydroxylase) fusion has been used to demethylate and transcriptionally activate *RHOXF2* in the Chronic Myelogenous Leukemia cell line K562 [40]. Stolzenburg et al. used a ZF-DNMT3A (DNA methyltransferase 3A) fusion to silence the *SOX2* oncogene in a breast cancer cell line (MCF7), and silencing was maintained over several cell divisions *in vitro* and in mouse MCF7 tumor xenografts [41]. In another study a ZF-SKD (G9a, SUV39-H1/super KRAB domain) was used to generate H3K9me2 and transcriptional silencing at the *HER2/neu* gene in ovarian (SKOX3) and breast (SKBR3 and MDA-MB-231) cancer cell lines [42]. These and other studies [29] demonstrated that epigenome editing enables precise and controlled manipulation of chromatin marks at specific DNA sites within chromatin. This level of precision is not possible with epigenetic drugs or genetic knockdown because these approaches act upon CMEs and chromatin-binding proteins that regulate multiple target genes. Still, one limitation of targeted CMEs is that the number and local distribution of modifications cannot be controlled. For instance, two proximal nucleosomes, two identical histone tails within a nucleosome, or multiple CpG base pairs may be affected in an all-or-none manner.

Chromatin marks can be installed with greater precision by using a combination of gene editing (DNA cutting and repair) and epigenetic approaches. A system called HARDEN (Homology Assisted Repair Dependent Epigenetic eNgeniering) uses a Cas9 endonuclease to cut DNA, and the double stranded break is repaired with a premethylated DNA fragment that has been transfected into the cells [43]. So far, HARDEN has been used to silence the expression of neurodegenerative disease genes *C9orf72* and *APP* to generate a patient-derived induced pluripotent stem cell model of amyotrophic lateral sclerosis and frontotemporal dementia that reflects DNA methylation patterns in patients. In cancer research, HARDEN could be used to introduce sequences with specific combinations and distributions of meCpG's near promoters of interest to experimentally investigate how the position of DNA methylation affects transcription.

Epigenome editors can be used to alter chromatin marks *de novo*, enabling scientists to determine causal relationships between chromatin marks and downstream processes, such as transcriptional regulation. In the examples described above, epigenome editors were used to establish that gene expression was a consequence of DNA methylation and histone modification states in leukemia, breast, and ovarian cell lines. Another significant insight that has come from epigenome

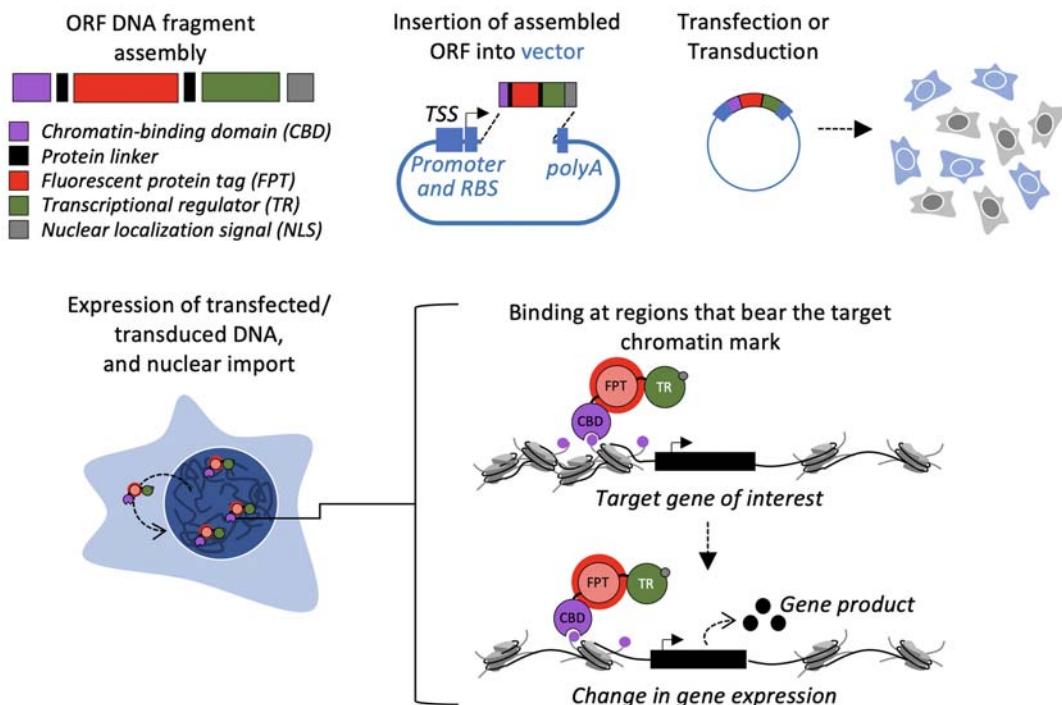
editing is the transient nature of *de novo* chromatin marks in specific contexts. Specifically, Kungulovski et al. showed that 5meC or H3K9me3 generated by transient activity of ZF-DNMT or ZF-HMT, respectively, were eventually lost in ovarian cancer cells [44].

5 Epigenome actuation: streamlined chromatin-binding regulators of transcription

Epigenome actuation is a new method in epigenetic engineering that uses synthetic reader-actuator proteins to control gene transcription [22]. In contrast to epigenome editing, which alters histone modifications and DNA methylation at specific sites, epigenome actuation operates downstream of the editing step by sensing (binding to) the chromatin marks and then regulating gene expression. This gene regulation step is usually mediated by the cell's own proteins, including DNA-binding transcription factors and chromatin-binding reader-effectors. However, the regulation machinery in cancer cells is often dysfunctional, for example, the SWI/SNF remodeling complex is mutated in 25% of cancers [45] and might inhibit the effects of epigenome editing. Synthetic reader-actuators have transcription factor activity, and therefore can be used to compensate for deficient regulation machinery. Furthermore, synthetic reader-actuators can be used as simplified models to derive insights into the function of natural reader-effector proteins. Natural reader-effectors, which are often mutated and misregulated in cancers, often have large unstructured regions and scaffold multiprotein complexes which make it challenging to perform experimental manipulations to distinguish the roles of each protein and subdomain in transcriptional regulation. To deconvolute the mechanism of chromatin signal transduction (chromatin binding followed by gene regulation) synthetic reader-effectors can be used to determine which domains are necessary and/or sufficient to switch gene expression states within different chromatin regions.

Synthetic reader-actuators are similar to protein probes (see Figure 10.3), except that synthetic reader-actuators also include a transcriptional regulator (TR), such as an activation domain (e.g., VP64) or repression domain (e.g., KRAB). The recombinant DNA ORF is constructed from a CBD, a detectable protein tag such, as a fluorescent protein, a TR, and a strong NLS, and the assembled ORF is cloned into an expression vector (Figure 10.5). After delivery of the vector into cells, the expressed synthetic reader-actuator protein enters the nucleus, accumulates at chromatin regions that bear the target epigenetic mark, and regulates nearby genes through the activity of the TR. Synthetic reader-actuators can be expressed from transiently transfected DNA, or from a stably integrated transgene. The latter option, with the use of a chemically inducible promoter, allows the scientist to control the timing and levels of synthetic reader-actuator expression. This can be especially important if the protein is expected to have a strong and immediate impact on gene expression and cell phenotype.

Epigenome actuation has been used to identify genes that can be activated in hypersilenced chromatin in cancer cells. A fusion called the polycomb transcription factor (PcTF) containing a H3K27me3-specific CBD and a transcriptional activator (VP64) that is known to interact with the transcriptional preinitiation complex (PIC) was used in breast cancer cells (MCF&, BT-474, and BT-549) [46] to coactivate immune signaling genes that are known to be repressed by H3K27me3 and polycomb complexes [47]. In earlier work, this synthetic reader-effector was used to activate

**FIGURE 10.5**

Epigenome actuation: design, construction, and application of synthetic reader-actuators. An open-reading frame (ORF) is built from a chromatin-binding domain (CBD), protein linker (e.g., glycine-serine repeat), fluorescent protein tag (FPT), a transcriptional regulator (TR), and a nuclear localization signal. The ORF is cloned into an expression vector that contains a promoter and ribosome binding sequence (upstream) and a polyadenylation signal (downstream). TSS = transcription start site. Once the synthetic reader-actuator is expressed from transfected DNA, it translocates into the nucleus, accumulates at regions that carry the target chromatin modification, and regulates nearby promoters.

epigenetically repressed genes in blood cancer (K562), brain cancer (SK-N-SH), and bone cancer (U2OS) cell lines [48,49]. Pctf-mediated gene activation induced cell cycle arrest in U2OS cells [49]. These results suggest that stimulating the PIC at misregulated promoters can overcome transcriptional blockades at anticancer genes. Epigenome actuation followed by RNA-seq identified genes within repressed chromatin that maintain the capacity to become reactivated. This insight fills an important epigenetic therapy research gap where other interventions, such as pharmacological inhibition, genetic knockdown, and epigenome editing, generate incomplete or transient activation of epigenetically silenced genes [11,44]. The broader potential of reader-actuators for disease research is demonstrated by a recent study where MeCP2 protein that contained only the methyl-CpG-binding domain (MBD) and the NCoR/SMRT interaction domain (NID) was used to rescue normal neurological function in a MeCP2-deficient mouse model [50]. This result provided

mechanistic insight into the mutations that occur most frequently in the MBD and NID in Rett syndrome patients.

Each functional domain in a synthetic reader-actuator maintains its intrinsic activity in the context of the hybrid protein. Therefore combining different functions by linking together CBDs, TFs and protein tags is fairly straight-forward, as described in a recent **Golden Gate cloning** protocol [51]. Synthetic reader-actuators could be redesigned to bind methylated DNA and activate genes in cancer models where tumor suppressors are downregulated by repressor proteins, including MBD proteins in pancreatic cancer [52], breast cancer [53], and glioblastoma [54]. Other reconfigurations of the synthetic reader-actuator, such as CBDs that bind acetylated histones and TFs that repress transcription could be used to silence genes in cancer cells where oncogenes are upregulated by activator proteins. Examples include overexpressed bromodomain (BRD)-containing proteins in multiple myeloma, glioblastoma multiforme, small-cell lung cancer [55], prostate cancer [56], high-grade gliomas [57], and midline carcinoma [58,59] and overexpressed YEATS-containing proteins in nonsmall-cell lung cancer [60] and AML [61–63].

6 Conclusion

Cancer epigenetic research has been predominantly descriptive and correlative, where various chromatin features are mapped along the chromosomes of cancer cells (via ChIP, bisulfite sequencing, and ATAC-seq) and compared with gene expression states (e.g., RNA-seq data). These epigenomic and transcriptomic data fall short of providing mechanistic evidence for the causal relationship between chromatin modifications and gene expression; however, they are extremely important for identifying clinically relevant features in samples that are not amenable to cell engineering, for instance fresh and preserved patient tissues. Mechanistic insights can be gained by perturbing chromatin through pharmacological inhibition and genetic knockdown or overexpression of chromatin-remodeling proteins in living cells and in animal models. However, many chromatin proteins are not druggable, and altering the expression levels of these proteins can affect major, nonchromatin-related pathways where histone acetyltransferases, deacetylases, and methyltransferases modify cytoskeletal proteins, molecular chaperones, nuclear import factors [64], the mitotic spindle, and cytosolic signaling proteins [65]. Epigenetic engineering has produced tools derived from the chromatin machinery that enable more precise control of chromatin remodeling, as well as tools that generate read-out signals that track with chromatin states in living cells. Carefully designed experiments that use these tools have resolved the roles of specific chromatin modifications in modulating expression levels of genes in cancer cells. For instance, artificially methylated promoters in transgene reporters have provided direct evidence that aberrant DNA methylation, often observed in cancer cells, inhibits transcriptional activity [8–10]. Genetic reporter systems have also been used to show that pharmacological inhibition of different CMEs can generate changes in gene regulation that vary between individual genes or chromosomal loci [12] and between individual cells [11].

One outstanding question in cancer epigenetics that might be addressed by epigenetic engineering is how different mutated or misregulated chromatin proteins synergize to drive cancer gene regulation and oncogenic phenotypes. This question is particularly important for malignant gliomas, where chromatin regulators are most frequently mutated in the gliomas in children and young adults [66]. These mutated chromatin proteins represent distinct molecular functions and include

PPM1D (phosphatase) [67], histone H3 (nucleosome structure and transcription factor recruitment), SET2D (histone methyltransferase), ATRX (chromatin remodeler), and others [66]. It is not yet known whether these proteins act cooperatively or independently at chromosomal loci to misregulate genes in gliomas. An engineered system that artificially recruits these factors or their core functional domains to a target site (e.g., reporter gene) in glioma model cells could reveal whether these mutated proteins act in concert to alter gene transcription.

Another important and challenging question in cancer epigenetic therapy is the development of drug resistance. For instance, leukemia stem cells have been shown to develop resistance against the BET-inhibitor class of epigenetic drugs. Fong et al. reported that resistance was associated with reduced accumulation of BRD4 in chromatin, rather than through increased drug efflux or metabolism [68]. However, high levels of pro-cancer Wnt/β-catenin signaling genes were maintained, suggesting that an alternative chromatin mechanism might compensate for BRD4. Recent studies of triple negative breast cancer (TNBC) suggest that intratumoral heterogeneity at the individual cell level is driven in large part by epigenetic, rather than genetic differences. These differences influence levels of resistance to chemotherapy, the standard of care for TNBC. Protein probes could be used to track the dynamics of chromatin modifications as cells become resistant to treatment, as was demonstrated in a study of MCF7 cells [19].

In conclusion, established and emerging epigenetic engineering technologies are being used to derive important new mechanistic insights into cancer epigenetics. Epigenetic engineering in conjunction with descriptive and correlative research is an emerging research paradigm to resolve causal relationships between chromatin features, gene expression, and cell behavior [18]. This new knowledge is critical to effectively target aberrant chromatin in cancer and to realize the full potential of epigenetic cancer therapy.

Glossary

Chromatin-modifying enzymes Enzymes that covalently modify chromatin by catalyzing the attachment or removal of small molecules (e.g., acetyl, methyl, or phosphate groups) to histone residues or cytosines in DNA.

Methylated DNA Regions where cytosines at cytosine-guanine dinucleotides (5'-CpG-3') are methylated to form 5-methylcytosine. This modification, 5meCpG, is associated with transcriptional silencing when it appears at the promoter regions of genes.

Epigenome editing A cell engineering approach that uses synthetic chromatin-modifying enzymes fused to DNA-binding modules (epigenome editors) to alter chromatin features at specific genomic sites.

Epigenetic engineering A discipline where molecular components (typically nucleic acids and proteins) with well-defined intrinsic activity are used to develop tools to precisely sense and/or manipulate chromatin.

Epigenome actuation A cell engineering approach that uses streamlined reader-effector proteins (synthetic reader-effectors) to target sites that carry specific chromatin marks, and to regulate gene expression at those sites.

Epigenome engineering A specialized area within cellular engineering that focuses on using the chromatin machinery, for example, chromatin-modifying enzymes, chromatin marks, and reader-effectors, to investigate chromatin behavior and/or to manipulate cell states.

Fusion protein A hybrid protein product of cellularly expressed recombinant DNA that contains a continuous open-reading frame composed of a set of codons from one functional protein, followed in-frame by a set

of codons from another functional protein, etc. Several protein encoding regions can be combined, and a stop codon is placed at the very end of the final sequence.

Histones A family of basic proteins that associate with DNA to form nucleosomes, the core repeating subunits of chromatin.

Histone posttranslational modification (PTM) A covalent modification of a histone protein, including but not limited to methylation, acetylation, crotonylation, ubiquitination, and phosphorylation.

Mintbody A shorthand term for “modification-specific intracellular antibody,” which is a mimetic of a fluorescently labeled antigen binding fragment (Fab). Mintbodies are fusion proteins constructed from single chain variable fragments and fluorescent proteins.

Nucleosome The core repeating subunit of chromatin. A nucleosome includes two copies each of histones H2A, H2B, H3, and H4, 147 bp of DNA wrapped in 1.7 turns around a [H2A:H2B]2,H32:H42 core octamer.

Open-reading frame (ORF) A sequence of DNA that begins with a start codon at the 5' end (ATG) followed by a series of codons, and ends with a stop codon (UAA, UAG, UGA). In engineered reporters, an ORF typically encodes a readily detectable product, such as luciferase, a fluorescent protein, or a cell surface marker, that can be detected with an antibody for flow cytometry.

Chromatin-binding domain A domain within a chromatin reader or reader-effector protein that physically binds to methylated DNA or one or more specific histone posttranslational modifications.

Transgene Recombinant DNA that includes the minimum components for gene expression (i.e., promoter, ribosome binding sequence/5' untranslated region, open-reading frame, poly-adenylation signal) which is artificially introduced into the genome of a cell.

Transgenic reporter A synthetic gene that is used to determine whether transcription is increased or decreased by chromatin modifications at a promoter, or by the chromatin environment at a specific chromosomal site. A transgenic reporter consists of recombinant DNA that is built *in vitro* with molecular cloning techniques. It includes the minimum components for gene expression, such as a promoter, ribosome-binding sequence, open-reading frame (ORF), and poly-adenylation signal, and is artificially introduced into the chromosomal DNA of a living cell.

Abbreviations

CBD	Chromatin-binding domain
CME	Chromatin-modifying enzyme
NLS	Nuclear localization signal (or sequence)
ORF	Open-reading frame
PTM	Posttranslational modification

References

- [1] Ganesan A, Arimondo PB, Rots MG, Jeronimo C, Berdasco M. The timeline of epigenetic drug discovery: from reality to dreams. *Clin Epigenetics* 2019;11:174.
- [2] Falahi F, Sgro A, Blancafort P. Epigenome engineering in cancer: fairytale or a realistic path to the clinic? *Front Oncol* 2015;5:22.
- [3] Sun F-L, Haynes K, Simpson CL, et al. *cis*-Acting determinants of heterochromatin formation on *Drosophila melanogaster* chromosome four. *Mol Cell Biol* 2004;24:8210–20.
- [4] Cryderman DE, Morris EJ, Biessmann H, Elgin SC, Wallrath LL. Silencing at *Drosophila* telomeres: nuclear organization and chromatin structure play critical roles. *EMBO J* 1999;18:3724–35.

- [5] Sun FL, Cuaycong MH, Elgin SC. Long-range nucleosome ordering is associated with gene silencing in *Drosophila melanogaster* pericentric heterochromatin. *Mol Cell Biol* 2001;21:2867–79.
- [6] Wallrath LL, Elgin SC. Position effect variegation in *Drosophila* is associated with an altered chromatin structure. *Genes Dev* 1995;9:1263–77.
- [7] Sun FL, Cuaycong MH, Craig CA, Wallrath LL, Locke J, Elgin SC. The fourth chromosome of *Drosophila melanogaster*: interspersed euchromatic and heterochromatic domains. *Proc Natl Acad Sci U S A* 2000;97:5340–5.
- [8] Lu C, Wei Y, Wang X, et al. DNA-methylation-mediated activating of lncRNA SNHG12 promotes temozolomide resistance in glioblastoma. *Mol Cancer* 2020;19:28.
- [9] Lee J, Han JH, Jang A, Kim JW, Hong SA, Myung SC. DNA methylation-mediated downregulation of DEFB1 in prostate cancer cells. *PLoS One* 2016;11:e0166664.
- [10] Guo Y, Wu R, Gaspar JM, et al. DNA methylome and transcriptome alterations and cancer prevention by curcumin in colitis-accelerated colon cancer in mice. *Carcinogenesis* 2018;39:669–80.
- [11] Si J, Boumber YA, Shu J, et al. Chromatin remodeling is required for gene reactivation after decitabine-mediated DNA hypomethylation. *Cancer Res* 2010;70:6968–77.
- [12] Tchasonvnikarova IA, Marr SK, Damle M, Kingston RE. TRACE generates fluorescent human reporter cell lines to characterize epigenetic pathways. *Mol Cell* 2022;82:479–491.e7.
- [13] Zhang S, Liu M, Yao Y, Yu B, Liu H. Targeting LSD1 for acute myeloid leukemia (AML) treatment. *Pharmacol Res* 2021;164:105335.
- [14] Akhtar W, de Jong J, Pindyurin AV, et al. Chromatin position effects assayed by thousands of reporters integrated in parallel. *Cell* 2013;154:914–27.
- [15] Delachat AM-F, Guidotti N, Bachmann AL, et al. Engineered multivalent sensors to detect coexisting histone modifications in living stem cells. *Cell Chem Biol* 2018;25:51–56.e6.
- [16] Lungu C, Pinter S, Broche J, Rathert P, Jeltsch A. Modular fluorescence complementation sensors for live cell detection of epigenetic signals at endogenous genomic sites. *Nat Commun* 2017;8:649.
- [17] Sanchez OF, Mendonca A, Carneiro AD, Yuan C. Engineering recombinant protein sensors for quantifying histone acetylation. *ACS Sens* 2017;2:426–35.
- [18] Haynes KA. Chromatin research and biological engineering: an evolving relationship poised for new biomedical impacts. *Curr Op Syst Biol* 2019;14:73–81.
- [19] Zhao H, Lin LF, Hahn J, Xie J, Holman HF, Yuan C. Single-cell image-based analysis reveals chromatin changes during the acquisition of tamoxifen drug resistance. *Life* 2022;12. Available from: <https://doi.org/10.3390/life12030438>.
- [20] Sato Y, Kujirai T, Arai R, et al. A genetically encoded probe for live-cell imaging of H4K20 mono-methylation. *J Mol Biol* 2016;428:3885–902.
- [21] Sato Y, Mukai M, Ueda J, et al. Genetically encoded system to track histone modification in vivo. *Sci Rep* 2013;3:2436.
- [22] Franklin KA, Shields CE, Haynes KA. Beyond the marks: reader-effectors as drivers of epigenetics and chromatin engineering. *Trends Biochem Sci* 2022;47:417–32.
- [23] Arora S, Sappa S, Hinkelmann K, Islam K. Engineering a methyllysine reader with photoactive amino acid in mammalian cells. *Chem Commun* 2020;56:12210–13.
- [24] Wagner S, Sudhamalla B, Mannes P, et al. Engineering bromodomains with a photoactive amino acid by engaging ‘Privileged’ tRNA synthetases. *Chem Commun* 2020;56:3641–4.
- [25] Sudhamalla B, Dey D, Breski M, Nguyen T, Islam K. Site-specific azide-acetyllysine photochemistry on epigenetic readers for interactome profiling. *Chem Sci* 2017;8:4250–6.
- [26] Villaseñor R, Pfaendler R, Ambrosi C, et al. ChromID identifies the protein interactome at chromatin marks. *Nat Biotechnol* 2020;38:728–36.

- [27] Zhang Y, Mi W, Xue Y, Shi X, Kutateladze TG. The ZZ domain as a new epigenetic reader and a degradation signal sensor. *Crit Rev Biochem Mol Biol* 2019;54:1–10.
- [28] Zhang Y, Xue Y, Shi J, et al. The ZZ domain of p300 mediates specificity of the adjacent HAT domain for histone. *Nat Struct Mol Biol* 2018;25:841–9.
- [29] Rots MG, Jeltsch A. Editing the epigenome: overview, open questions, and directions of future development. *Methods Mol Biol* 2018;1767:3–18.
- [30] Mlambo T, Nitsch S, Hildenbeutel M, et al. Designer epigenome modifiers enable robust and sustained gene silencing in clinically relevant human cells. *Nucleic Acids Res* 2018;46:4456–68.
- [31] Li K, Liu Y, Cao H, et al. Interrogation of enhancer function by enhancer-targeting CRISPR epigenetic editing. *Nat Commun* 2020;11:485.
- [32] Yamazaki T, Hatano Y, Handa T, et al. Targeted DNA methylation in pericentromeres with genome editing-based artificial DNA methyltransferase. *PLoS One* 2017;12:e0177764.
- [33] Holtzman L, Gersbach CA. Editing the epigenome: reshaping the genomic landscape. *Annu Rev Genomics Hum Genet* 2018;19:43–71.
- [34] Nakamura M, Gao Y, Dominguez AA, Qi LS. CRISPR technologies for precise epigenome editing. *Nat Cell Biol* 2021;23:11–22.
- [35] Cano-Rodriguez D, Rots MG. Epigenetic editing: on the verge of reprogramming gene expression at will. *Curr Genet Med Rep* 2016;4:170–9.
- [36] Tekel SJ, Haynes KA. Molecular structures guide the engineering of chromatin. *Nucleic Acids Res* 2017;45:7555–70.
- [37] Jain S, Shukla S, Yang C, et al. TALEN outperforms Cas9 in editing heterochromatin target sites. *Nat Commun* 2021;12:606.
- [38] Lebar T, Jerala R. Benchmarking of TALE- and CRISPR/dCas9-based transcriptional regulators in mammalian cells for the construction of synthetic genetic circuits. *ACS Synth Biol* 2016;5:1050–8.
- [39] Gao X, Tsang JCH, Gaba F, Wu D, Lu L, Liu P. Comparison of TALE designer transcription factors and the CRISPR/dCas9 in regulation of gene expression by targeting enhancers. *Nucleic Acids Res* 2014;42:e155.
- [40] Maeder ML, Angstman JF, Richardson ME, et al. Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. *Nat Biotechnol* 2013;31:1137–42.
- [41] Stolzenburg S, Beltran AS, Swift-Scanlan T, Rivenbark AG, Rashwan R, Blancafort P. Stable oncogenic silencing in vivo by programmable and targeted de novo DNA methylation in breast cancer. *Oncogene*. 2015;34:5427–35.
- [42] Falahi F, Huisman C, Kazemier HG, et al. Towards sustained silencing of HER2/neu in cancer by epigenetic editing. *Mol Cancer Res* 2013;11:1029–39.
- [43] Cali CP, Park DS, Lee EB. Targeted DNA methylation of neurodegenerative disease genes via homology directed repair. *Nucleic Acids Res* 2019;47:11609–22.
- [44] Kungulovski G, Nunna S, Thomas M, Zanger UM, Reinhardt R, Jeltsch A. Targeted epigenome editing of an endogenous locus with chromatin modifiers is not stably maintained. *Epigenetics Chromatin* 2015;8:12.
- [45] Orlando KA, Nguyen V, Raab JR, Walhart T, Weissman BE. Remodeling the cancer epigenome: mutations in the SWI/SNF complex offer new therapeutic opportunities. *Expert Rev Anticancer Ther* 2019;19:375–91.
- [46] Olney KC, Nyer DB, Vargas DA, Wilson Sayres MA, Haynes KA. The synthetic histone-binding regulator protein Pctf activates interferon genes in breast cancer cells. *BMC Syst Biol* 2018;12:83.
- [47] Burr ML, Sparbier CE, Chan KL, et al. An evolutionarily conserved function of polycomb silences the MHC class I antigen presentation pathway and enables immune evasion in cancer. *Cancer Cell* 2019;36:385–401.e8.

- [48] Nyer DB, Daer RM, Vargas D, Hom C, Haynes KA. Regulation of cancer epigenomes with a histone-binding synthetic transcription factor. *NPJ Genom Med* 2017;2. Available from: <https://doi.org/10.1038/s41525-016-0002-3>.
- [49] Haynes KA, Silver PA. Synthetic reversal of epigenetic silencing. *J Biol Chem* 2011;286:27176–82.
- [50] Tillotson R, Selfridge J, Koerner MV, et al. Radically truncated MeCP2 rescues Rett syndrome-like neurological defects. *Nature* 2017;550:398–401.
- [51] Haynes KA, Priode JH. Rapid single-pot assembly of modular chromatin proteins for epigenetic engineering. *Methods Mol Biol* 2023;2599:191–214.
- [52] Xu J, Zhu W, Xu W, et al. Up-regulation of MBD1 promotes pancreatic cancer cell epithelial-mesenchymal transition and invasion by epigenetic down-regulation of E-cadherin. *Curr Mol Med* 2013;13:387–400.
- [53] Sapkota Y, Robson P, Lai R, Cass CE, Mackey JR, Damaraju S. A two-stage association study identifies methyl-CpG-binding domain protein 2 gene polymorphisms as candidates for breast cancer susceptibility. *Eur J Hum Genet* 2012;20:682–9.
- [54] Zhu D, Hunter SB, Vertino PM, Van Meir EG. Overexpression of MBD2 in glioblastoma maintains epigenetic silencing and inhibits the antiangiogenic function of the tumor suppressor gene BAI1. *Cancer Res* 2011;71:5859–70.
- [55] Lovén J, Hoke HA, Lin CY, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell* 2013;153:320–34.
- [56] Groner AC, Cato L, de Tribolet-Hardy J, et al. TRIM24 is an oncogenic transcriptional activator in prostate cancer. *Cancer Cell* 2016;29:846–58.
- [57] Green AL, DeSisto J, Flannery P, et al. BPTF regulates growth of adult and pediatric high-grade glioma through the MYC pathway. *Oncogene* 2020;39:2305–27.
- [58] Filippakopoulos P, Qi J, Picaud S, et al. Selective inhibition of BET bromodomains. *Nature* 2010;468:1067–73.
- [59] Grayson AR, Walsh EM, Cameron MJ, et al. MYC, a downstream target of BRD-NUT, is necessary and sufficient for the blockade of differentiation in NUT midline carcinoma. *Oncogene*. 2014;33:1736–42.
- [60] Mi W, Guan H, Lyu J, et al. YEATS2 links histone acetylation to tumorigenesis of non-small cell lung cancer. *Nat Commun* 2017;8:1088.
- [61] Wan L, Wen H, Li Y, et al. ENL links histone acetylation to oncogenic gene expression in acute myeloid leukaemia. *Nature* 2017;543:265–9.
- [62] Erb MA, Scott TG, Li BE, et al. Transcription control by the ENL YEATS domain in acute leukaemia. *Nature* 2017;543:270–4.
- [63] Daser A, Rabbits TH. Extending the repertoire of the mixed-lineage leukemia gene MLL in leukemogenesis. *Genes Dev* 2004;18:965–74.
- [64] Glazok MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. *Gene* 2005;363:15–23.
- [65] Sugeedha J, Gautam J, Tyagi S. SET1/MLL family of proteins: functions beyond histone methylation. *Epigenetics* 2021;16:469–87.
- [66] Phillips RE, Soshnev AA, Allis CD. Epigenomic reprogramming as a driver of malignant glioma. *Cancer Cell* 2020;38:647–60.
- [67] Akamandisa MP, Nie K, Nahta R, Hambardzumyan D, Castellino RC. Inhibition of mutant PPM1D enhances DNA damage response and growth suppressive effects of ionizing radiation in diffuse intrinsic pontine glioma. *Neuro Oncol* 2019;21:786–99.
- [68] Fong CY, Gilan O, Lam EYN, et al. BET inhibitor resistance emerges from leukaemia stem cells. *Nature* 2015;525:538–42.

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Epigenetics and
cancer

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Epigenetic targeted therapies in hematological malignancies

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1 Introduction

The biological process of blood cell development is a tightly controlled progression from multipotent hematopoietic stem cell (HSC) located in the bone marrow to fully differentiated mature blood cells circulating around the body. Hematopoiesis involves a carefully organized series of microenvironment changes coupled with transcriptional program activation and tightly regulated epigenetic processes which enable the step-by-step commitment of stem cells. Resulting mature blood cells provide critical roles in maintaining normal physiology, preventing or fighting infection and preventing bleeding. The traditional hematopoietic hierarchy simplifies a complex, dynamic process with inbuilt biases but does help to understand how numerous distinct malignant processes arise from within this system. When considering hematological malignancy, disorders are generally

classified as myeloid or lymphoid neoplasms depending on the cell of origin. Rarely, malignant disorders with features of both myeloid and lymphoid cells including mixed phenotype acute leukemia are observed. Many of these malignant phenotypes take the form of aggressive neoplasms with rapidly progressive disease resulting in mortality over several days, weeks, and/or months. Examples include acute leukemia, high-grade lymphomas, and advanced stages of plasma cell disorders (myeloma). Other malignant phenotypes present as chronic disorders, proliferating more slowly and ultimately causing harm over months and years if they ever do. Examples of these disorders include low-grade lymphomas, myeloproliferative neoplasms, and early stages of plasma cell dyscrasias. Figure 11.1 aims to demonstrate both the differentiation stages in normal hematopoiesis and the origins of commonly observed hematological malignancies.

A spectrum of malignant phenotypes is seen on the myeloid side. This ranges from the myeloproliferative neoplasms where an overproduction of mature myeloid cells is observed, myelodysplasia where the dysplasia within the stem and progenitor cell populations leads to ineffective hematopoiesis and acute myeloid leukemia where the rapid accumulation of immature blast cells quickly leads to bone marrow failure. In a normal healthy process, the development of blood cells is unchallenged, enabling normal commitment to mature functional cells. This process displays an appropriate responsiveness within the hematological system to the changing physiological demands for erythrocytes, leukocytes, and platelets that occur as the individual grows, fights off infections,

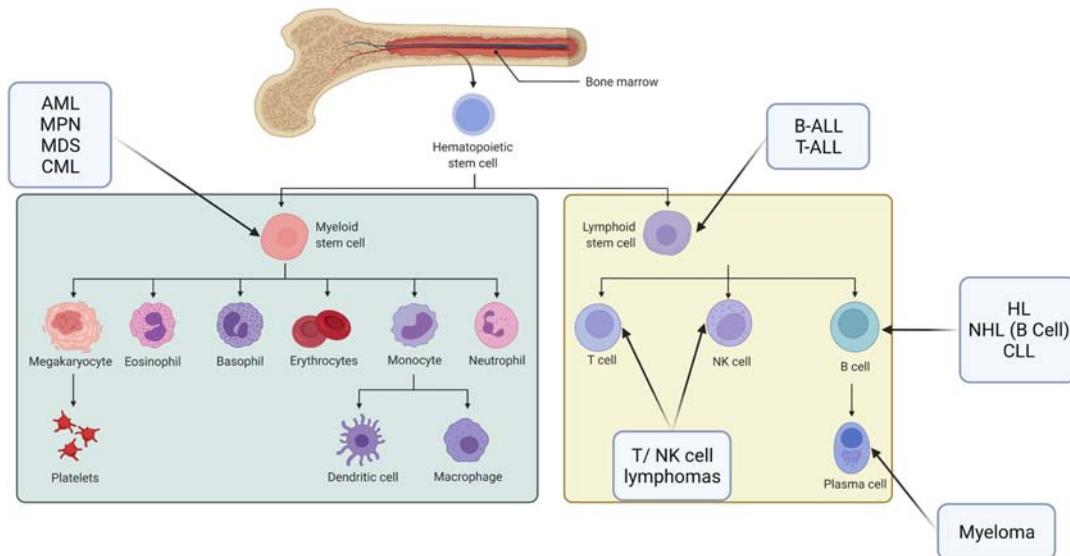


FIGURE 11.1

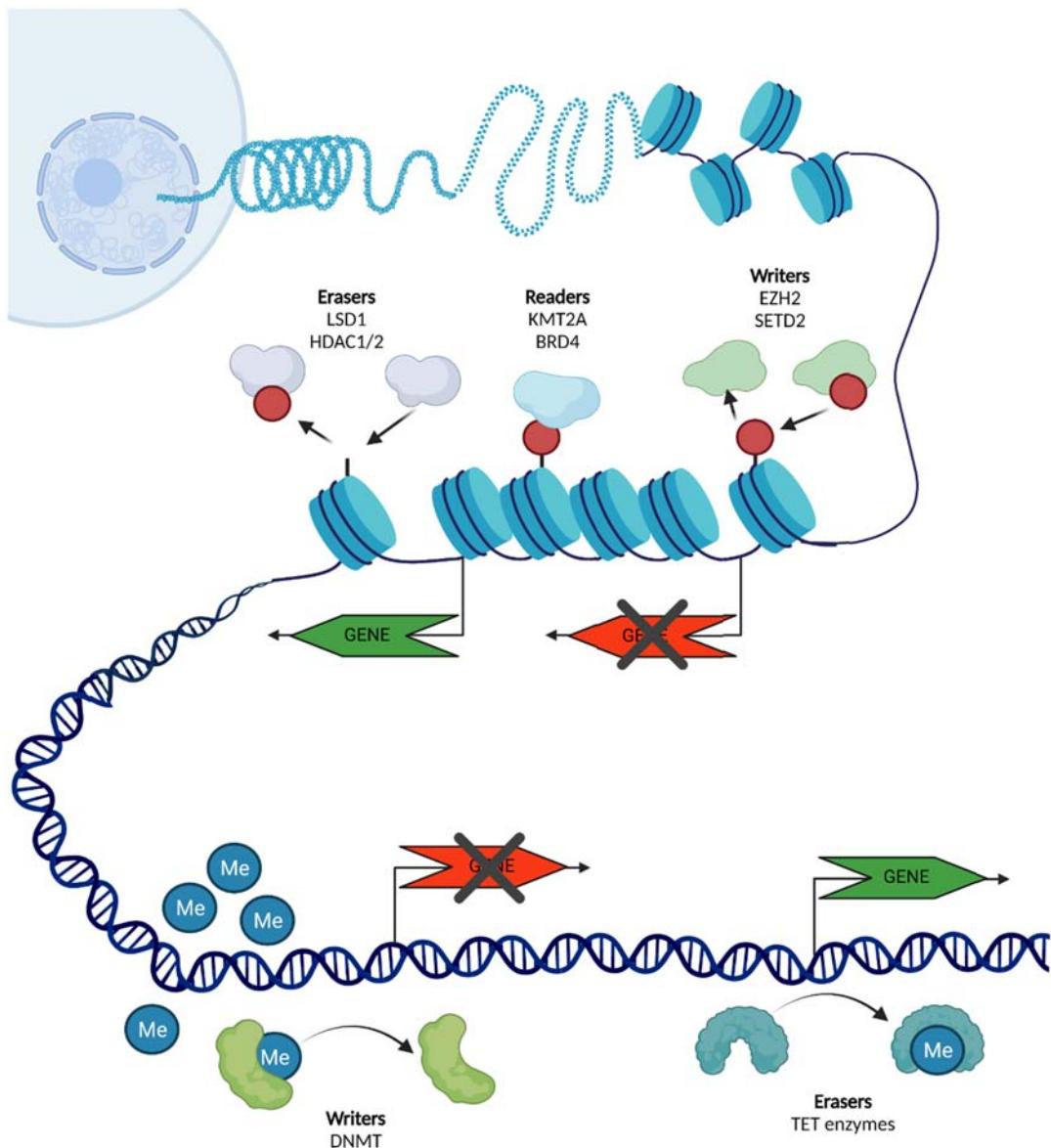
The process of hematopoiesis and originating sites of various blood disorders. Hematopoiesis is where blood cellular components are formed beginning primarily in the bone marrow before differentiating down either myeloid or lymphoid lineages before circulating around the body. Also highlighted are the primary sites where hematopoietic disorders are formed. AML: acute myeloid leukemia, MPN: myeloproliferative neoplasms, MDS: myelodysplastic syndromes, CML: chronic myeloid leukemia, B-ALL: B-cell acute lymphoblastic leukemia, T-ALL: T-cell acute lymphoblastic leukemia, HL: Hodgkin's lymphoma, NHL: non-Hodgkin's lymphoma, CLL: chronic lymphoblastic leukemia, T/NK cell lymphoma: T/natural killer cell lymphoma.

becomes pregnant, bleeds, or develops greater metabolic demands. However, during the process of leukemogenesis, these processes can be disrupted in multiple ways. A well-established theory which neatly summarized the development of many myeloid malignancies suggested they develop along a two-hit hypothesis. This model determined that there are mainly two classes of myeloid disease alleles which contribute to the transformation into leukemic blast cells. Class 1 alleles confer growth advantages through the activation of downstream signaling pathways driving proliferation; class 2 alleles mainly alter transcriptional targets which are of a primary focus in myelopoiesis resulting in a dysregulation of differentiation potential [1]. This theory predates the implementation of widespread use of next-generation sequencing technologies revealing new classes of mutations, which do not neatly fit into this model. In particular alterations targeting the proteins which act to regulate DNA methylation and posttranslational histone modifications [2]. These epigenetic processes are important intrinsic cellular mechanisms to maintain an appropriate transcriptional profile and it is not surprising that dysregulation brought either by a gain or loss of function is also a key contributor to the pathogenesis of many malignant myeloid phenotypes in hematology.

Dysregulation of normal epigenetic processes is also increasingly recognized in lymphoid neoplasms. The natural history of myeloma begins with premalignant monoclonal gammopathy of undetermined significance, it may then proceed through intermediate stages such as smoldering multiple myeloma before symptomatic myeloma develops which can progress further to plasma cell leukemia/extramedullary myeloma [3]. Although mutations in genes contributing to normal epigenetic processes are much less frequently observed, altered DNA methylation patterns are commonly observed [4]. As a broad group, lymphoma is considered the most common hematological disease; however, it is really a collection of very heterogeneous disorders arising predominantly from the neoplastic, clonal expansion of maturing B cells and in a much smaller number of cases, T or natural killer cell populations [5]. Once again epigenetic dysregulation both in the form of genetic mutations and dysregulation of methylation patterns is observed [6].

It is increasingly clear that epigenetic dysregulation is a feature across the board in hematological malignancy. Underlying the numerous malignant phenotypes observed are even more complex and numerous mechanisms by which these disorders may develop, challenging the hematologist and underlying the difficulties in employing a “one size fits all” treatment strategy for individuals with the same disease. In some cases, these epigenetic changes occur as a primary driver of the malignancy and in other cases as a secondary contributor. While current clinical treatment options are well defined for each of the highlighted disorders recent times have seen a shift toward the inclusion and reliance on understanding epigenetic control and regulation within these malignancies. Many epigenetic modifications remain reversible even when dysregulated. This potential plasticity provides an attractive therapeutic target.

The interplay of epigenetic modifications within cells facilitates a dynamic landscape of DNA methylation and histone modifications promoting the transcriptional expression or repression of genes. Examples of epigenetic regulatory processes include CpG island methylation along with the posttranslational modifications of histones by (de)methylation/(de)acetylation which promote chromatin conformation and in turn regulate expression of genes [7,8]. Within these epigenetic processes, proteins involved in chromatin regulation/remodeling are broadly categorized as readers, writers and erasers; dependent on their mechanism of action. Epigenetic writers catalyze the addition of chemical modifications to specific amino acids on histone tails and methylation of CpG islands on DNA. Epigenetic erasers remove these modifications while epigenetic readers recognize them and recruit larger chromatin remodeling complexes to further impact the alterations and dictate gene expression changes etc. [8–10]. Figure 11.2 demonstrates a broad overview of a number

**FIGURE 11.2**

The impact of epigenetic readers, writers and erasers on both histones and DNA within the hematopoietic context. Epigenetic writers catalyze the addition of chemical modifications to specific amino acids on histone tails and methylation of CpG islands on DNA therefore preventing gene transcription at both the histone and DNA levels. Epigenetic erasers remove these modifications thus allowing gene transcription to occur; while

(Continued)

of well characterized epigenetic regulatory processes implicated in normal hematopoiesis and malignancy. The role of epigenetic modifications in determining cell fate, self-renewal potential and differential trajectory has been observed in HSCs and therefore it makes sense that dysregulation of these processes can similarly impact a neoplastic clone derived from these cells.

This chapter will review the evolution of targeted epigenetic therapies into clinical use, while elucidating the mechanism of action for epigenetic alterations and exploring potential future directions for targeting the epigenome on a personalized level within these disorders.

2 Methylation as a clinical target in hematological disorders

2.1 DNA methylation

DNA methylation is controlled by the action of DNA methyltransferases (DNMTs), which catalyze the addition of a methyl group to CpG dinucleotides resulting in the creation of 5-methylcytosine (5-mC) [11]. Gene regulation is often controlled by the relationship between hypermethylation and hypomethylation.

Across the spectrum of myeloid malignancy, mutations in genes responsible for the methylation of DNA, including *DNMT3A*, *TET2*, and *IDH1/2*, are frequently observed [12]. Table 11.1 shows the relative frequencies observed. These same three genes are also observed to be mutated in T-cell lymphomas, in particular the angioimmunoblastic form, highlighting the potential for pathogenicity when DNA methylation is perturbed along the hematopoietic tree [13].

DNMT3A is one of the most commonly mutated *de novo* DNMT genes with numerous mutations identified across the gene [18]. *DNMT3A* mutations result in hypomethylation across CpG islands in AML and attenuate the hypermethylation of CpG islands that is seen in AML patients with wild-type (WT) *DNMT3A* [19]. Due to the impact of mutations within this gene conferring a poorer prognosis to AML patients it became an attractive therapeutic target and generated interest in the methylome. It is well understood that mutations in AML rarely occur alone; *DNMT3A* is known to be frequently associated with Nucleophosmin 1 (*NPM1*), fms-like tyrosine kinase 3 (*FLT3*) and isocitrate dehydrogenase 1/2 (*IDH1/2*). Patients displaying a collection of methylation altering mutations display higher bone marrow blast cell counts alongside shorter event free survival in AML [20].

IDH proteins facilitate the conversion of isocitrate to α -ketoglutarate (α -KG) with mutations in these genes causing the production of aberrant 2-hydroxyglutarate (2-HG) which competes with α -KG causing inhibition of the ten-eleven translocation methylcytosine dioxygenase 2 gene (*TET2*) [21]. Inhibition of *TET2* can lead to overexpression of 5-mC leading to the hypermethylation of genes and furthermore blocks cellular differentiation [22]. IDH1/2 mutations were defined by Parsons et al., in 2008 and Marcucci et al., in 2010, respectively [23,24]. Mutational hotspots in

- ◀ epigenetic readers recognize them and recruit larger chromatin remodeling complexes to further impact the alterations and dictate gene expression changes. Included are also examples of some well documented hematopoietic readers, writers, and erasers at the histone and DNA levels, however, not highlighted is the capability for some proteins to act in a dual function as writers and readers or erasers and readers.

Table 11.1 The Approximate Percentages of Patients with Mutations in Key DNA Methylation Genes

Gene	AML (%)	MDS (%)	MPN (%)	MDS/MPN Overlap (%)
<i>DNMT3A</i>	21	13	5	9
<i>TET2</i>	12	33	13	45
<i>IDH1</i>	8	3	1	—
<i>IDH2</i>	12	4	1	2

AML percentages from [14], MDS percentages from [15], MPN percentages from [16], and MDS/MPN from [17].

both genes differ with IDH1 displaying alterations at Arg132 while IDH2 hotspots occur at Arg140 more frequently than Arg172 [25]. In a similar manner, mutations within the *TET2* gene impair hydroxylation of 5-mC resulting in a dysregulated global methylome with loss of hydroxymethylation and DNA hypermethylation [22,26]. Restoration of *TET2* function can reverse the aberrant self-renewal of *TET2*-deficient cells and reduce DNA hypermethylation again highlighting the potential plasticity in the system [27].

2.2 Hypomethylating agents

2.2.1 Azacitidine

As mentioned in the previous edition of this chapter, hematological interest in epigenetics originated with azacitidine response for MDS patients [28]. Prior to the induction of targeted therapies MDS patients needing therapy underwent a regimen of high-dose chemotherapeutics alongside HSC/bone marrow transplants. These therapies often yielded disappointing results for patients. The development of azanucleosides (azacitidine/decitabine) in the 1960s offered promise to patients [29]. Azacitidine is classed as a cytidine analog and incorporates into RNA to inhibit metabolism and protein synthesis [30]. However, it was not until years later that the demethylation activity of azacitidine was uncovered [31]. In 1980 Taylor and Jones uncovered the ability of azacytidine to induce terminal differentiation; the team noted that azacitidine induced marked changes in the differentiation state of cultured mouse embryonic stem cells and furthermore inhibited the methylation of newly synthesized DNA [32]. Taylor and Jones found that the strand which contained azacitidine remained undermethylated during DNA synthesis following incorporation of the compound. Azacitidine asserts its effects through phosphorylation by a series of kinases resulting in azacitidine triphosphate which incorporates into RNA to disrupt RNA metabolism and protein synthesis. Azacitidine has the ability to form a derivative, azacitidine diphosphate, which is reduced by ribonucleotide reductase to form 5-aza-2'-deoxycytidine diphosphate which undergoes further phosphorylation to generate a triphosphate which is then incorporated into DNA to bind stoichiometrically to DNMTs causing hypomethylation of replicating DNA [33].

In view of these findings, a phase III clinical trial, CALGB 9221, was implemented as a multi-centre, randomized, open-label trial designed to compare the efficacy and safety of azacitidine against the standard of care therapy. Patient selection was randomized from any of the five sub-types of MDS. A crossover element was included for patients in the observation group after two to

four weeks cycle of supportive care if their disease progression met prespecified criteria. Therefore azacitidine effect on patient survival could not be directly studied due to the crossover element. Two further supporting trials were designed as multicentre single arm studies administering azacitidine via different methods; CALGB 8921 via subcutaneous injections and CALGB 8421 via intravenous injection. Endpoints from the studies were quantified as complete response (CR), complete normalization of blood cell counts, and bone marrow blast percentages for a minimum period of four weeks, and partial response (PR) defined as $\geq 50\%$ restoration from baseline blood cell count and a $\geq 50\%$ decrease in bone marrow blasts again for a minimum of four weeks. The response rate to azacitidine in all three studies was similar, the larger CALGB 9221 trial reported an overall response rate (ORR) (CR + PR) of 14.7%, 22/150, CALGB 8921 administered azacitidine through subcutaneous injections reported an ORR of 13.9%, 10/72, and the CALGB 8421 trial administered through intravenous injections recorded an ORR of 18.8%, 9/48, on average across the three trials the ORR to azacitidine was 15.2%, 41/270 [34–36]. Risk of leukemic transformation was reduced with a median time of 21 months in the treatment arm of CALGB 9221 and 12 months in the control arm. Overall around 60% of patients were observed to have some degree of improvement [37]. Follow-up checks on the patients demonstrated the clinical benefit of azacitidine treatment through long-lasting increases in blood cell counts and decreased bone marrow blast percentages, meaning blood transfusions were not necessary for the treated patients [38].

A number of additional but relatively small-scale trials have continued to evaluate the use of azacitidine in MDS in a single agent format. CR and PR rates remain modest and consistently below one-third of patients and ultimately survival benefits appear modest in this setting [35,39–41]. Mutations in genes responsible for dysregulated methylation including *TET2* may predict a better response to azacitidine [42]. Furthermore, azacitidine therapy does not appear to eradicate the leukemic stem cell population that is ultimately responsible for disease relapse [43]. Relapsed disease following on from azacitidine treatment failure is notoriously difficult to treat with outcomes in those progressing to secondary AML described as dismal [44].

The use of azacitidine in AML has followed on from the limited benefits that have been observed in MDS. The incidence of AML increases with age and accordingly many patients are unfit for the conventional high intensity chemotherapeutic regimens and stem cell transplant that offer the potential of cure at least in a subset of patients. For these individuals and those in whom the risk profile of their disease is unsuited to conventional intense treatment, azacitidine has become a regular option in the upfront treatment of patients. In older patients with low blast counts (20–30%), azacitidine improves survival (50% vs 16% at 2 years) and importantly reduces the number of inpatient days in comparison to conventional care [45]. In older patients with higher blast counts, a survival benefit has also been reported with azacitidine with median OS to be approximately 4 months longer and approx. 10% more individuals alive at 1 year [46]. Any form of disease stabilization has been associated with improved OS hence helping guide clinicians regarding the ongoing use of the therapy.

Oral azacitidine has also demonstrated a useful role in prolonging the remission of patients with AML. The QUAZAR AML-001 trial shows a significant benefit in OS (9.9 months) and relapse free survival (5.3 months) in comparison to placebo when azacitidine was used as a maintenance therapy. This benefit was independent of minimal residual disease (MRD) status with MRD negativity retained for longer and a higher rate of conversion from MRD positivity to negativity [47].

2.2.2 Decitabine

Decitabine is a deoxycytidine analog incorporated into DNA during the S phase of cell cycle resulting in the binding of methyltransferase causing inactivation. As mentioned above, decitabine was first synthesized in 1960s and was initially shown to cause cytotoxicity at higher doses. The advent of epigenetic understanding allowed studies to reassess decitabine as an epigenetic therapy. Upon this, the second mechanism of action was discovered highlighting the role decitabine has as a hypomethylating agent capable of reactivating silenced genes at lower doses; studies found a “optimal biologic” dosing regimen (at approximately 1/20th the original dose) which enabled the drug to be active with manageable side effects in patients with myeloid malignancies [48].

Normal progression of MDS results in inactivation of tumor suppressor genes through hypermethylation of DNA. Decitabine counters this effect; in a similar mechanism to azacitidine; through incorporation into DNA trapping DNMTs causing global hypomethylation which can be sustained resulting in the re-expression of tumor suppressor genes and activation of antitumor pathways [49]. Further studies assessing the effects of decitabine therapy on MDS patients reported high levels of apoptosis and death of neoplastic cells and furthermore a reduction in the proliferation ability of leukemic cells [50].

The results of trials examining decitabine monotherapy in MDS are strikingly similar to azacitidine. Combined rates of strict CR and PR definitions are less than a third of patients with around half experiencing some degree of hematological improvement. Survival benefits again appear modest at best while rates of AML transformation are reduced at early time points [51,52]. Use of decitabine in an AML maintenance phase has demonstrated a trend towards improved OS and DFS without reaching significance [53].

There are a number of challenges with hypomethylating agents in the MDS/AML setting and ORRs are certainly suboptimal from the patients’ perspective. Responses are generally achieved slowly, requiring many cycles of treatment and prolonged periods of supportive care. We also have not clearly defined who is more likely to benefit from this approach. Correlating disease response to epigenetic changes or transcriptional response has also proved challenging with inconsistent results. Relapse or progression is almost inevitable and increasingly challenging to manage effectively.

However, the effects that are observed have provided encouragement to build regimens combining additional therapeutic agents. In particular, the use of targeted therapies or pro-apoptotic agents are now routinely employed. Venetoclax is a *BCL-2* inhibitor which inhibits key antiapoptotic pathways activated in many malignant states [54]. In a study of 431 previously untreated patients who are ineligible for intensive therapy, treatment with azacitidine and venetoclax versus azacitidine alone demonstrated the composite CR outcome was achieved in 66.4% of patients in the azacitidine/venetoclax arm and only 28.2% in the azacitidine only arm. Median OS was improved by around 5 months after follow-up period of 20 months [55]. In retrospective analysis comparing the azacitidine/venetoclax combination with intensive chemotherapy at induction in AML, older age, adverse risk and *RUNX1* mutations were associated with a trend towards improved OS using this less intense approach [56]. This approach is quickly becoming standard of care approach for those unable to tolerate intensive treatment approaches.

Combination approaches using targeted agents alongside hypomethylating agents are also gathering interest. Early phase trials have shown potential benefits of the addition of *FLT3* inhibitors to

a hypomethylating agent in AML [57]. In advanced forms of MPN and in particular blast phase disease, the use of the *JAK* inhibitor ruxolitinib alongside azacitidine or decitabine has been trialed with some success. Fifty percentage of patients achieved a CR or PR with a favorable OS of 42% compared to historical cohorts seen for the azacitidine/ruxolitinib combination in one recent study [58]. Similarly, combinations of ruxolitinib and decitabine demonstrate overall responses rates of approximately 40% but with overall median survival still below 1 year [59,60]. Identifying the key synergistic combinations will continue to be an important focus in the coming years for these challenging to treat patient populations.

Table 11.2 summarizes where these agents are approved. Outside of the myeloid setting, hypomethylating agents have been investigated in a number of malignant contexts. Angioimmunoblastic T-cell lymphoma is of particular interest amongst the group of lymphoid malignancy. Sustained responses to azacitidine therapy have been observed in this group [61,62]. It is interesting that this lymphoid malignancy often arises in the context of clonal hematopoiesis with clonal populations in the HSC compartment exhibiting high rates of mutations in *TET2*, *DNMT3A*, or *IDH1/2* and a high rate of concurrent myeloid neoplasms demonstrating a similar pathogenesis to MDS. Beyond the uses documented in **Table 11.2**, there are preclinical and early phase clinical trials with azacitidine which reflects the interest in this drug across the entire spectrum of hematological malignancy. Preclinical studies suggests that azacitidine exposure in diffuse large B-cell lymphoma (DLBCL) cell line models and patients resulted in a sensitization of lymphoma cells to doxorubicin and that this was associated with the de-methylation of SMAD family member 1 (*SMAD1*) and upregulated expression of this gene [63]. The addition of oral azacitidine has recently been trialed in DLBCL and advanced follicular lymphoma (FL) as a “chemosensitizer,” with patients primed with oral azacitidine prior to and following the administration of R-CHOP chemotherapy, which is standard of care for these individuals. This approach demonstrated an ORR of 94% and a CR rate of 88% and an acceptable safety profile [64]. Whether this represents a real advantage over standard of care therapy remains to be established.

2.2.3 *IDH1/2* inhibitors

Ivosidenib and Enasidenib are inhibitors of *IDH1/2* respectively. Enasidenib (also called AG-221) was approved by the FDA in the United States as a first in class selective allosteric inhibitor for mutant *IDH2* proteins within relapsed or refractory AML [51]. Preclinical studies on enasidenib showed intracellular total serum 2-HG decreased by more than 90% while also reducing histone hypermethylation and enabling the restoration of myeloid differentiation [65–67]. Enasidenib confers differentiation effects onto cells and thus the drug is not deemed cytotoxic conferring lower rates of aplasia, neutropenia and thrombocytopenia when compared to commonly used chemotherapeutic agents [68].

Enasidenib was presented for clinical trials (NCT01915498) in AML patients. The study assessed 239 patients with relapsed or refractory AML, of which 113 were treated with enasidenib in a dose-escalation phase while 126 were treated with an expansion therapy. Results from the studies showed inhibition of mutant *IDH2* led to significant decreases in 2-HG levels to baseline similar to what is seen in healthy volunteers. Interestingly, efficacy differs depending on the *IDH2* mutation present, 2-HG levels were inhibited in up to 99% of patients harboring the R140 mutation while patients with the R172 mutation reported an 94% reduction in 2-HG levels [66]. Of the patients who took part in the study 20% achieved complete remission while the ORR for relapsed/refractory AML

Table 11.2 The Approved Indications for Hypomethylating Agents as Single Agents or Combination Therapies

Condition	Treatment Formulation	Single Agent Approvals	Combination Approvals
MDS	Azacitidine SC	<ul style="list-style-type: none"> – FDA approved for FAB myelodysplastic syndrome subtypes; RA, RARS, RAEB, RAEB-T – EMA approved for intermediate-2/high risk MDS in patients who cannot have HSCT 	
	Decitabine IV	FDA approved for all treated and previously treated <i>de novo</i> and secondary MDS of all FAB subtypes and intermediate-1, intermediate-2 and high risk.	
	Decitabine PO		FDA approved In combination with cedazuridine for all treated and previously treated <i>de novo</i> and secondary MDS with FAB subtypes (RA, RARS, RAEB) and intermediate-1, intermediate-2 and high risk.
AML	Azacitidine SC	EMA approved for patients who cannot have stem cell transplant and AML developed from MDS and bone marrow consists of 20%–30% blasts or AML where bone marrow has more than 30% blasts	<ul style="list-style-type: none"> – FDA approved in combination with venetoclax for newly diagnosed AML in patients 75 years or older or who have co-morbidities that preclude use of intensive induction therapy – EMA approved in combination with venetoclax in adults who cannot have intensive chemotherapy – FDA approved in combination with Ivosidenib for newly diagnosed with <i>IDH1</i> mutation in adults >75 years or who have co-morbidities precluding intensive induction chemotherapy
	Azacitidine PO	FDA approved for adult patients with AML achieving first complete remission or complete remission with incomplete blood count recovery and are not able to complete intensive curative therapy.	
	Decitabine IV	EMA approval for patients with newly diagnosed AML who are not eligible for standard induction therapy	
JMML	Azacitidine SC	FDA approved for pediatric patients aged 1 month and older with newly diagnosed JMML	

Table 11.2 The Approved Indications for Hypomethylating Agents as Single Agents or Combination Therapies *Continued*

Condition	Treatment Formulation	Single Agent Approvals	Combination Approvals
CMML	Azacitidine SC	<ul style="list-style-type: none"> – FDA approved for adult patients with CMML – EMA approved for CMML with 10%–29% marrow blasts without myeloproliferative disorder 	In combination with cedazuridine for intermediate 1/intermediate 2 AND high risk IPSS
	Decitabine IV	FDA approved in intermediate 1/intermediate 2 AND high risk IPSS	
	Decitabine PO		

FDA: US Food and Drug Administration, EMA: European Medicines agency, SC: subcutaneous, IV: intravenous, PO: oral, MDS: myelodysplasia, AML: acute myeloid leukemia, CMML: chronic myelomonocytic leukemia, JMML: juvenile myelomonocytic leukemia, FAB: French American British, RA: refractory anemia, RARS: refractory anemia with ringed sideroblasts, RAEB: refractory anemia with excess blasts, RAEB-T: refractory anemia with excess blasts in transformation, IPSS: International Prognostic Scoring System, HSCT: hematopoietic stem cell transplant.

patients was over 40% for those displaying R140 mutations and R172 carriers had achieved an ORR of over 53%.

The rates of differentiation syndromes resulting from enasidenib is around 10%, often occurring weeks after initiation and highlights the potential to induce differentiation from these epigenetically modifying therapies [66]. In a similar trial of Ivosidenib monotherapy, conducted mainly in the relapsed or refractory AML setting, the ORRs were 41.6% in IDH1 mutant patients with durations of responses between 6.5 and 9.3 months observed. Around one third of patients obtained transfusion independence in this study [69].

Combined with intensive chemotherapy, the addition of either of these targeted agents to individuals with the relevant mutation(s), composite CR rates were observed in approximately two thirds of patients for both drugs in a recent phase I trial [70]. Combining ivosidenib treatment with azacitidine in newly diagnosed AML patients with IDH1 mutations, deep and durable responses were observed with estimated one year survival rates of greater than 80% [71]. Some early preclinical work suggests a basis for potential synergy in this combination with enhanced antileukemic activity resulting from greater reductions in DNA methylation when combined [72].

2.3 Methylation of histone targets

Methylation of histone lysine residues is a major epigenetic modification; lysine methyltransferases (KMTs) can produce mono-, di-, or trimethylation of specific lysine residues. These posttranslational modifications along with acetylation and other less well characterized changes are critical epigenetic regulators; modifying, promoting or repressing chromatin structure and reader protein binding which can either enhance or repress expression of associated genetic material. For example, trimethylation of histone H3 lysine 4 (H3K4me3) is associated with active gene expression while

trimethylation of histone H3 lysine 27 (H3K27me3) is a repressive mark, associated with gene silencing [21]. Methylation of arginine residues is also increasingly recognized as an important histone posttranslational modification influencing gene expression [73].

The action of the Polycomb group of (PcG) proteins is well-characterized and provides a good example of how dysregulation of histone methylation impacts hematological malignancies. *EZH2* is a well-defined histone methyltransferase component of PcG proteins. *EZH2* forms part of the canonical Polycomb repressive complex 2 (*PRC2*), and canonically functions to enable the addition of a methyl group(s) onto H3K27 [74,75]. This methylation enables other PcG components, most notably, the polycomb repressive complex 1 (*PRC1*) to be recruited therefore contributing to gene repression and impacting stem cell lineage development and potency of the stem cell.

EZH2 is a particularly interesting gene in hematological malignancies. In non-Hodgkin's lymphomas, mutations are observed causing hyperactivity of the *EZH2*'s catalytic function. Occurring at tyrosine 641 (Y641) within the C-terminal SET domain causing gain of-function, these mutations are identified in 7.2% of FLs and 21.7% of DLBCLs derived from germinal center B cells [76]. This gain-of-function results in the hypermethylation of H3K27, altering the transcriptional program of the cell and reorganizing chromatin structure [77].

In contrast, although *EZH2* mutations are frequently observed in myeloid malignancy, they are loss-of-function mutations in this context. These mutations are observed in around 1%–2% of patients with de novo AML, around 13% of patients with patients with advanced forms of MPN and around 6% of MDS patients [78]. In the context of MPN, the presence of an *EZH2* mutation is a poor prognostic marker. It is much more frequently identified in myelofibrosis than the chronic forms of essential thrombocythemia and polycythemia vera and independently predicts shortened survival [79]. Loss of *EZH2* in murine MPN models synergistically contributes to the development of myelofibrosis alongside the *JAK2* V617F mutation. In MDS, loss of the long arm of chromosome 7 can involve the *EZH2* gene locus and the presence of an *EZH2* mutation are both associated with significantly worse survival [77].

EZH2 and the balance of H3K27me3 is therefore critical in hematological malignancy and either losing or gaining function in the wrong context is important in the pathogenesis of both myeloid malignancy and lymphoma respectively.

2.3.1 *EZH2* inhibition

Tazemetostat was the first *EZH2* inhibitor approved by the FDA for use in advanced/metastatic epithelioid sarcomas [80]. Tazemetostat targets both wild-type and mutant *EZH2* inducing cell cycle arrest resulting in apoptosis by specifically reducing H3K27me3 in cells [81]. Several specific potential outcomes have been explored with antilymphoma activity via the increased expression of chemokine (C–C motif) ligand 17 (CCL17)/thymus- and activation-regulated chemokine [82]. Gene set enrichment analysis (GSEA) carried out by the group show the promoter region of CCL17 showed H3K27 demethylation posttazemetostat treatment alongside concurrent CpG stimulation resulting in gene transcription activation [82]. Currently tazemetostat is in clinical development for other tumor types including DLBCL; along with a New Drug Application being granted for use in FL [80]. Approval was based using two cohorts encompassing *EZH2* mutant and *EZH2* wild type (NCT01897571); ORR and duration of response (DOR) were used to assess results of the study with ORR in 42 patients with 69% *EZH2* mutant, with 12% CRs, and 57% PR. The median DOR in these patients was 10.9 months. The ORR in 53 patients with *EZH2* wild type was 34%,

4% CRs, and 30% PRs. The median DOR was 13 months [83]. Numerous clinical trials are now ongoing investigating the role of tazemetostat and other *EZH2* inhibitors in B-cell non-Hodgkin's lymphomas [84]. As demonstrated in the myeloid malignancies, tipping the balance of H3K27me3 too far in the opposite direction is pathogenic in that context. FDA approval for tazemetostat was initially put on hold due to the case of T acute lymphoblastic lymphoma in a pediatric patient and there is a low risk of secondary malignancy using this approach.

2.3.2 Alternative lysine methylation targets

Outside of *EZH2* inhibition, there has been interest in the inhibition of other lysine methyltransferases across a range of hematological malignancies [85]. *DOT1L* inhibition has been shown to reduce H3K79 methylation and demonstrate activity in patients with relapsed/refractory AML or ALL with *KMT2A* (*MLL*) rearrangements. These rearrangements are common in both forms of acute leukemia and *DOT1L* recruitment to critical gene loci by chimeric *KMT2A* proteins resulting in enhanced H3K79 methylation and gene transcription which is an important component of the pathogenesis of these disorders [86]. Targeting menin, a scaffolding protein that permits the tethering of *KMT2A* to chromatin, has also demonstrated preclinical efficacy and is in clinical trials [85,87].

Inhibition of lysine specific demethylase 1 (*LSD1*) offers a potential therapeutic strategy using the opposite approach of targeting demethylation. *LSD1* specifically demethylates H3K4me1/2 and H3K9me1/2. Trials are ongoing across a range of myeloid malignancy including AML, MDS, MPN, and MDS/MPN overlap syndromes [88]. To date, no later stage clinical trials have reported on whether preclinical activity translates into clinical responses for single or combination therapy.

3 Acetylation as a clinical target in hematological disorders

The regulation of genes can also be controlled by the acetylation and deacetylation processes in which lysine residues residing on the tails of the core histones of nucleosomes are modified. The addition/removal of these modifications is moderated by the activities of histone acetyltransferases or histone deacetylases (HDACs). Acetylation is associated with increased transcriptional activity by the addition of an acetyl group causing reduced DNA and histone interactions [89]. The reduction of these interactions enables tightly packed chromatin to relax enabling the transcription machinery to access DNA. This process can be reversed by the activity of HDACs which remove the aforementioned acetyl group causing nucleosome structure to compact thus silencing gene expression [90,91]. In clinical progression for targeting acetylation within myeloid malignancies, HDACs have become the most common target. There are 11 defined human HDAC genes which can be broadly grouped into 3 classes: class 1 (HDAC1, 2, 3, and 8), class 2 (HDAC4, 5, 6, 7, 9, and 10), and class 4 (HDAC11). With a 3rd class of HDACs encompassing Sirt1–7 deacetylases proteins that are functionally unrelated to other HDAC proteins. It has been shown that *HDAC1* and 2 are often found expressed predominantly in nuclear localization within chromatin remodeling complexes acting within the cells, that is, msin3A, Nucleosome Remodeling and Deacetylation complex, and CoREST corepressor complexes [92,93]. The involvement of HDAC activity within hematological progression has been well-defined, where the overexpression of *HDAC5* and *HDAC7*

and lower expression of *HDAC4* are implicated in ALL, CML and AML [94]. Overexpression of *HDAC1* has also been associated with poor prognosis in myeloma [95]. The evolution of HDAC activity and expression in blood cancers have led to the development of a multitude of clinically approved drug therapies.

Bromodomain and extra terminal (BET) protein family consists of multiple epigenetic reader proteins (*BRD2*, *BRD3*, *BRD4*, and *BRDT*), which function to regulate gene transcription through binding of acetylated histones. BET proteins exert their action through bromodomains which act as a regulator of the protein-protein interactions, which control multiple cellular processes, such as transcription and chromatin remodeling [96]. BET proteins bind lysines that are acetylated and located within super-enhancer regions of DNA (regions contained within DNA that are enriched with repressive H3K27 acetylation marks) or within promoter gene regions that control the level of chromatin organization [97–99]. Within blood cancer *BRD1* is known to be disrupted in ALL with *BRD3* and *BRD4* being recently implicated in AML progression [100–102].

3.1 Histone deacetylation agents

3.1.1 HDAC inhibitors

Panobinostat is a pan-HDAC inhibitor approved in 2015 by the FDA for the treatment of myeloma when used in combination with bortezomib and dexamethasone. Panobinostat functions by regulating multiple cellular processes including transcription, differentiation, apoptosis, and cell cycle progression [103]. Within myeloma histone deacetylation, inhibition has a twofold action where DNA damage levels increase and apoptotic pathways are upregulated [104]. The approval of panobinostat was based on results from the PANORAMA1 study. The study showed that when panobinostat was administered alongside bortezomib, and dexamethasone resulted in a PFS of 11.99 months compared to 8.08 months in control groups. CR rate in treated groups was higher compared to control groups (60.7% vs 54.6%) [105]. Panobinostat treatment showed cytotoxicity against tumor cells while not affecting “normal” blood cells, highlighting the therapeutic potential of panobinostat. The use of panobinostat as a monotherapy has been evaluated in both solid tumors and other hematological malignancies; while showing little to no efficacy in solid tumors, there was activity in patients with relapsed or refractory Hodgkin’s lymphoma. Responses to panobinostat monotherapy have been observed in around 36% of patients with myelofibrosis and may be improved slightly with the addition of ruxolitinib [106,107].

Vorinostat (suberoylanilide hydroxamic acid) is a hydroxamic acid inhibitor that targets class 1 and 2 HDAC proteins. The discovery of vorinostat was the result of efforts to improve an existing first-generation hybrid polar compound used to induce differentiation of transformed cells before the anticancer activity of vorinostat was uncovered. Supporting work to characterize vorinostat reported pan-HDAC activity, which showed potent inhibition of HDAC1, 2, 3, 5, 6, 8, 9, 10, and 11 [108,109]. Vorinostat exerts antiproliferative effects involving the accumulation of acetylated histones enabling an open chromatin structure and transcriptional activation; *p21*, a strong cell cycle inhibitor, is strongly induced by vorinostat treatment and is commonly used as a marker of HDAC inhibition [110]. *p21* is involved in cellular processes, such as cell-cycle arrest, apoptosis, transcriptional regulation, senescence, and DNA repair. Vorinostat works to inhibit HDAC activity by binding to the catalytic pocket which induces the accumulation of hyperacetylated histones

impacting the expression of a limited number of genes; this results in transcriptional activation of some genes, along with repression of an equal or larger number of other genes. Within the treatment of hematological malignancies, vorinostat is being assessed in combination with chemotherapy or other biologic agents across a range of setting. FDA approval was granted for relapsed/refractory cutaneous T-cell lymphoma with an ORR observed in just under one-third of patients [111]. Romidepsin, an alternative HDACi, has demonstrated similar efficacy in this setting and is also FDA approved [112]. The VANTAGE008 study in myeloma demonstrated only a small improvement in PFS from 6.8 to 7.6 months for nonrefractory, previously treated relapsing myeloma patients treated with bortezomib in combination with vorinostat or placebo [113].

In a similar manner to the demethylating agents, the clinical responses to HDACi are relatively limited in most settings in a single agent form or for patients in an advanced disease stage. However, the potential benefits in combination therapies or earlier in the treatment algorithms are of significant interest. Numerous clinical trials are ongoing exploring the options for combination therapies based on HDACs across the spectrum of hematological malignancies [94,114].

3.1.2 JQ1/I-BET

There have been multiple novel BET inhibitors developed which showed mixed results with variable responses in hematological malignancies and solid tumors. The first two BET inhibitors were defined in 2010, I-BET and JQ1. I-BET is a benzodiazepine derivative which works to downregulate both transcription factors and proinflammatory genes [115]. JQ1 is a thienotriazolodiazepine described as the archetypal molecule of BET inhibitors. JQ1 functions to displace *BRD4* from chromatin which results in differentiation while also causing cell cycle arrest and induction of apoptotic pathways. Various pharmacological attributes of JQ1 have caused difficulty in the ability to translate preclinical studies into clinical application [102,116]. Research into BET inhibitors has highlighted their mechanism of action in targeting *MYC*. *MYC* encodes the *Myc* protein and promotes cellular growth and differentiation among other cellular processes. *Myc* is often deregulated in hematological malignancies making BET inhibition an attractive target in a range of malignant phenotypes. BET inhibition altered *Myc* function was based on *PTEFb* not being recruited to *c-Myc* during G1 phase of the cell cycle highlighting the critical role of *BRD4* within *c-Myc* transcription [117,118].

In models of myelofibrosis, BET inhibition attenuates NF-KB signaling demonstrating activity which is enhanced in combination with ruxolitinib [119]. This combination is now in clinical trials. In combination with ruxolitinib in JAKi naïve patients responses were seen in spleen volume in 63% of patients, with 59% reporting improved symptom burden [120]. Responses were lower when BET inhibition was given as monotherapy in the ruxolitinib refractory/intolerant setting [120]. The effect of BET inhibition on *MYC* makes it an attractive target in lymphoma; however, response rates of monotherapy are poor and trials to determine optimum synergistic combinations are ongoing.

4 Conclusion

The role of epigenetic dysregulation is becoming increasingly obvious across the range of hematological neoplasms. Figure 11.3 highlights the range of potential targets and epigenetic agents

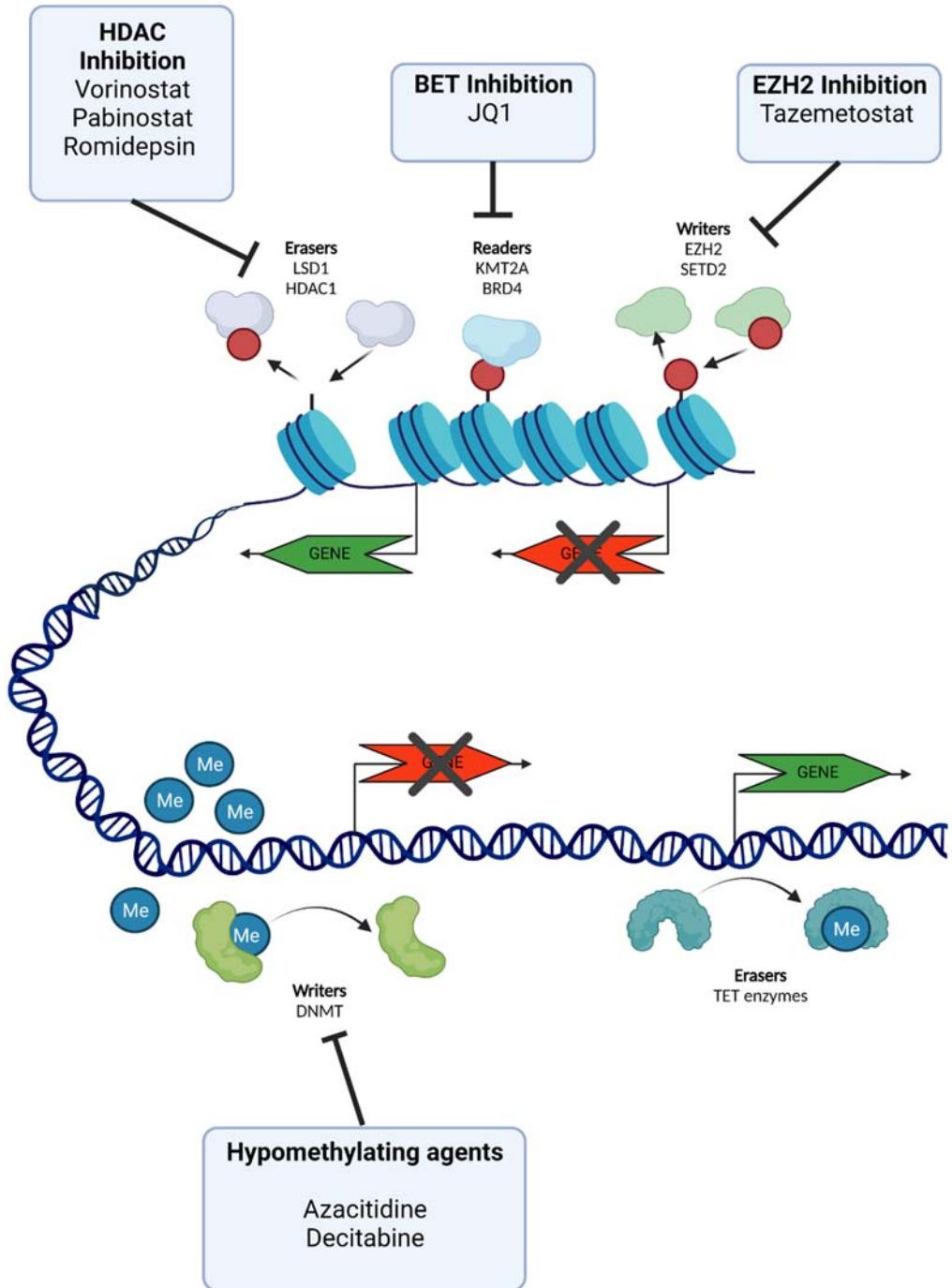


FIGURE 11.3

The compounds targeting epigenetic readers, writers and erasers on both histones and DNA within hematological disorders. Figure adapted from Figure two with the inclusion of drug compounds commonly used to target the highlighted epigenetic readers, writers and erasers within hematological malignancies.

currently under evaluation or in routine use in hematological malignancy. Just as epigenetic dysregulation can support and enhance the neoplastic features of a malignant cell, the plasticity and reversibility of many of these epigenetic modifications offer the potential for enhanced therapeutic benefits. The increasing availability of general epigenetic modifying therapies and targeted treatments has delivered some notable benefits for patients but there remains a long way to go for many. The NGS revolution has taught us much about these diseases from a genomic perspective and is helping to unravel the more complex and dynamic epigenetic changes that ultimately help to initiate and sustain these diseases. Understanding more about the nature of these changes and how current therapies affect these dynamic and evolving epigenetic landscapes is key to driving the next generation of epigenetic treatments.

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Figures created at biorender.com. [Figure 11.2](#) adapted from “Regulation of transcription in Eukaryotic cells” by biorender.com (2022)

References

- [1] Gilliland DG. Hematological malignancies. *Curr Opin Hematol* 2001;8(4):189–91 <https://journals.lww.com/co-hematology/Citation/2001/07000/Hematologic_malignancies.1.aspx>.
- [2] Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. *Nature* 2012;12 <<http://www.nature.com/reviews/cancer>>.
- [3] García-Ortiz A, Rodríguez-García Y, Encinas J, Maroto-Martín E, Castellano E, Teixidó J, et al. The role of tumor microenvironment in multiple myeloma development and progression. *Cancers* 2021;13(2):217. Available from: <https://www.mdpi.com/2072-6694/13/2/217/htm>.
- [4] Pawlyn C, Kaiser MF, Heuck C, Melchor L, Wardell CP, Murison A, et al. The spectrum and clinical impact of epigenetic modifier mutations in myeloma. *Clin Cancer Res* 2016;22(23):5783–94. Available from: <https://aacrjournals.org/clincancerres/article/22/23/5783/122583/The-Spectrum-and-Clinical-Impact-of-Epigenetic>.
- [5] Mugnaini EN, Ghosh N. Lymphoma. *Prim Care* 2016;43(4):661–75.
- [6] Pasqualucci L, Khiabanian H, Fangazio M, Vasishtha M, Messina M, Holmes AB, et al. Genetics of follicular lymphoma transformation. *Cell Rep* 2014;6(1):130–40.
- [7] Venney D, Mohd-Sarip A, Mills KI. The impact of epigenetic modifications in myeloid malignancies. *Int J Mol Sci* 2021;22(9):5013. Available from: <https://www.mdpi.com/1422-0067/22/9/5013/htm>.
- [8] Fong CY, Morison J, Dawson MA. Epigenetics in the hematologic malignancies. *Haematologica* 2014;99(12):1772–83. Available from: <https://pubmed.ncbi.nlm.nih.gov/25472952/>.
- [9] Hu D, Shilatifard A. Epigenetics of hematopoiesis and hematological malignancies. *Genes Dev* 2016;30(18):2021–41.
- [10] Esteller M. Molecular origins of cancer: epigenetics in cancer. *N Engl J Med* 2008;.
- [11] Yang X, Wong MPM, Ng RK. Aberrant DNA methylation in acute myeloid leukemia and its clinical implications. *Int J Mol Sci* 2019;.

- [12] Chan SM, Majeti R. Role of DNMT3A, TET2, and IDH1/2 mutations in pre-leukemic stem cells in acute myeloid leukemia. *Int J Hematol* 2013;98(6):648–57. Available from: <https://link.springer.com/article/10.1007/s12185-013-1407-8>.
- [13] Couronné L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. *N Engl J Med* 2012;366(1):95–6. Available from: <https://www.nejm.org/doi/10.1056/NEJMc1111708>.
- [14] Tyner JW, Tognon CE, Bottomly D, Wilmot B, Kurtz SE, Savage SL, et al. Functional genomic landscape of acute myeloid leukaemia. *Nature* 2018;562(7728):526–31. Available from: <https://ohsu.pure.elsevier.com/en/publications/functional-genomic-landscape-of-acute-myeloid-leukaemia>.
- [15] Haferlach T, Nagata Y, Grossmann V, Okuno Y, Bacher U, Nagae G, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia* 2014;28(2):241–7. Available from: <https://www.nature.com/articles/leu201336>.
- [16] Grinfeld J, Nangalia J, Baxter EJ, Wedge DC, Angelopoulos N, Cantrill R, et al. Classification and personalized prognosis in myeloproliferative neoplasms. *N Engl J Med* 2018;379(15):1416–30. Available from: <https://www.nejm.org/doi/full/10.1056/NEJMoa1716614>.
- [17] Palomo L, Meggendorfer M, Hutter S, Twardziok S, Ademà V, Fuhrmann I, et al. Molecular landscape and clonal architecture of adult myelodysplastic/myeloproliferative neoplasms. *Blood* 2020;136(16):1851–62. Available from: <https://pubmed.ncbi.nlm.nih.gov/32573691/>.
- [18] Brunetti L, Gundry MC, Goodell MA. DNMT3A in leukemia. *Cold Spring Harb Perspect Med* 2017;7(2).
- [19] Spencer DH, Russler-Germain DA, Ketkar S, Link DC, Dipersio JF, Ley Correspondence TJ, et al. CpG island hypermethylation mediated by DNMT3A is a consequence of AML progression article CpG island hypermethylation mediated by DNMT3A is a consequence of AML progression. *Cell* 2017;168:801–16. Available from: <https://doi.org/10.1016/j.cell.2017.01.021>.
- [20] Loghavi S, Zuo Z, Ravandi F, Kantarjian HM, Bueso-Ramos C, Zhang L, et al. Clinical features of de novo acute myeloid leukemia with concurrent DNMT3A, FLT3 and NPM1 mutations. *J Hematol Oncol* 2014;7(1):74. Available from: <http://jhoonline.biomedcentral.com/articles/10.1186/s13045-014-0074-4>.
- [21] Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting α -ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* 2010;17(3):225–34.
- [22] Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* 2010;18(6):553–67. Available from: <http://www.cell.com/article/S1535610810004836/fulltext>.
- [23] Parsons DW, Jones S, Zhang X, Lin JCH, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science* (80) 2008;321(5897):1807–12.
- [24] Marcucci G, Maharry K, Wu YZ, Radmacher MD, Mrózek K, Margeson D, et al. IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. *J Clin Oncol* 2010;28(14):2348–55.
- [25] Im AP, Sehgal AR, Carroll MP, Smith BD, Tefferi A, Johnson DE, et al. DNMT3A and IDH mutations in acute myeloid leukemia and other myeloid malignancies: associations with prognosis and potential treatment strategies. *Leukemia* 2014;1774–83.
- [26] Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* 2010;468(7325):839–43. Available from: <https://pubmed.ncbi.nlm.nih.gov/21057493/>.
- [27] Cimmino L, Dolgalev I, Wang Y, Yoshimi A, Martin GH, Wang J, et al. Restoration of TET2 function blocks aberrant self-renewal and leukemia progression. *Cell*. 2017;170(6):1079–1095.e20.
- [28] Prebet T, Gore SD. Development of epigenetic targeted therapies in hematological malignancies: from serendipity to synthetic lethality. *Epigenetic Cancer Ther* 2015;169–87. Available from: <https://jhu.pure.elsevier.com/en/publications/development-of-epigenetic-targeted-therapies-in-hematological-mal-3>.

- [29] Shen L, Kantarjian H, Guo Y, Lin E, Shan J, Huang X, et al. DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. *J Clin Oncol* 2010;28(4):605.
- [30] Paul TA, Bies J, Small D, Wolff L. Signatures of polycomb repression and reduced H3K4 trimethylation are associated with p15INK4b DNA methylation in AML. *Blood* 2010;115(15):3098–109. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20190193>.
- [31] Grövdal M, Khan R, Aggerholm A, Antunovic P, Astermark J, Bernell P, et al. Negative effect of DNA hypermethylation on the outcome of intensive chemotherapy in older patients with high-risk myelodysplastic syndromes and acute myeloid leukemia following myelodysplastic syndrome. *Clin Cancer Res* 2007;13(23):7107–12. Available from: <https://pubmed.ncbi.nlm.nih.gov/18056190/>.
- [32] Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 1980; 20(1):85–93. Available from: <https://pubmed.ncbi.nlm.nih.gov/6156004/>.
- [33] Christman JK. 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 2002;21(35):5483–95. Available from: <https://www.nature.com/articles/1205699>.
- [34] Santini V, Fenaux P, Mufti GJ, Hellström-Lindberg E, Silverman LR, List A, et al. Management and supportive care measures for adverse events in patients with myelodysplastic syndromes treated with azacitidine. *Eur J Haematol* 2010;85(2):130–8. Available from: <https://pubmed.ncbi.nlm.nih.gov/20394651/>.
- [35] Silverman LR, McKenzie DR, Peterson BL, Holland JF, Backstrom JT, Beach CL, et al. Further analysis of trials with azacitidine in patients with myelodysplastic syndrome: studies 8421, 8921, and 9221 by the Cancer and Leukemia Group B. *J Clin Oncol* 2006;24(24):3895–903. Available from: <https://pubmed.ncbi.nlm.nih.gov/16921040/>.
- [36] Cashen AF, Shah AK, Todt L, Fisher N, DiPersio J. Pharmacokinetics of decitabine administered as a 3-h infusion to patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). *Cancer Chemother Pharmacol* 2008;61(5):759–66. Available from: <https://pubmed.ncbi.nlm.nih.gov/17564707/>.
- [37] Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimir-Reissig R, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol* 2002;20(10):2429–40. Available from: <https://pubmed.ncbi.nlm.nih.gov/12011120/>.
- [38] Kaminskas E, Farrell AT, Wang Y-C, Sridhara R, Pazdur R. FDA drug approval summary: azacitidine (5-azacytidine, VidazaTM) for injectable suspension. *Oncologist* 2005;10(3):176–82. Available from: <https://academic.oup.com/oncolo/article/10/3/176/6386687>.
- [39] Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol* 2009;10(3):223–32. Available from: <https://pubmed.ncbi.nlm.nih.gov/19230772/>.
- [40] Prebet T, Sun Z, Figueroa ME, Ketterling R, Melnick A, Greenberg PL, et al. Prolonged administration of azacitidine with or without entinostat for myelodysplastic syndrome and acute myeloid leukemia with myelodysplasia-related changes: results of the US Leukemia intergroup trial E1905. *J Clin Oncol* 2014;32(12):1242–8.
- [41] Itzykson R, Thépot S, Quesnel B, Dreyfus F, Beyne-Rauzy O, Turlure P, et al. Prognostic factors for response and overall survival in 282 patients with higher-risk myelodysplastic syndromes treated with azacitidine. *Blood* 2011;117(2):403–11. Available from: <https://ashpublications.org/blood/article/117/2/403/28093/Prognostic-factors-for-response-and-overall>.
- [42] Cedena MT, Rapado I, Santos-Lozano A, Ayala R, Onecha E, Abaigar M, et al. Mutations in the DNA methylation pathway and number of driver mutations predict response to azacitidine in myelodysplastic syndromes. *Oncotarget* 2017;8(63):106948–61. Available from: <https://pubmed.ncbi.nlm.nih.gov/29291002/>.

- [43] Craddock C, Quek L, Goardon N, Freeman S, Siddique S, Raghavan M, et al. Azacitidine fails to eradicate leukemic stem/progenitor cell populations in patients with acute myeloid leukemia and myelodysplasia. *Leukemia* 2013;27(5):1028–36. Available from: <https://pubmed.ncbi.nlm.nih.gov/23223186/>.
- [44] Richardson DR, Green SD, Foster MC, Zeidner JF. Secondary AML emerging after therapy with hypomethylating agents: outcomes, prognostic factors, and treatment options. *Curr Hematol Malig Rep* 2021;16(1):97–111. Available from: <https://link.springer.com/article/10.1007/s11899-021-00608-6>.
- [45] Fenaux P, Mufti GJ, Hellström-Lindberg E, Santini V, Gattermann N, Germing U, et al. Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. *J Clin Oncol* 2010;28(4):562–9.
- [46] Dombret H, Seymour JF, Butrym A, Wierzbowska A, Selleslag D, Jang JH, et al. International phase 3 study of azacitidine vs conventional care regimens in older patients with newly diagnosed AML with >30% blasts. *Blood* 2015;126(3):291–9.
- [47] Roboz GJ, Ravandi F, Wei AH, Dombret H, Thol F, Voso MT, et al. Oral azacitidine prolongs survival of patients with AML in remission independently of measurable residual disease status. *Blood* 2022;139(14):2145–55. Available from: <https://pubmed.ncbi.nlm.nih.gov/34995344/>.
- [48] Jabbar E, Issa JP, Garcia-Manero G, Kantarjian H. Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. *Cancer* 2008;112(11):2341.
- [49] Tsai HC, Li H, Van Neste L, Cai Y, Robert C, Rassool FV, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer Cell* 2012;21(3):430–46. Available from: <https://pubmed.ncbi.nlm.nih.gov/22439938/>.
- [50] Wu L, Li X, Xu F, Chang C, He Q, Zhang Z, et al. Over-expression of RPL23 in myelodysplastic syndromes is associated with apoptosis resistance of CD34+ cells and predicts poor prognosis and distinct response to CHG chemotherapy or decitabine. *Ann Hematol* 2012;91(10):1547–54. Available from: <https://pubmed.ncbi.nlm.nih.gov/22580751/>.
- [51] Lübbert M, Suciu S, Baila L, Rüter BH, Platzbecker U, Giagounidis A, et al. Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: Final results of the randomized phase III study of the European organisation for research and treatment of cancer leukemia group and the German MDS study group. *J Clin Oncol* 2011;29(15):1987–96.
- [52] Oki Y, Kondo Y, Yamamoto K, Ogura M, Kasai M, Kobayashi Y, et al. Phase I/II study of decitabine in patients with myelodysplastic syndrome: a multi-center study in Japan. *Cancer Sci* 2012;103(10):1839–47. Available from: <https://onlinelibrary.wiley.com/doi/full/10.1111/j.1349-7006.2012.02386.x>.
- [53] Foran JM, Sun Z, Claxton DF, Lazarus HM, Arber DA, Rowe JM, et al. Maintenance decitabine (DAC) improves disease-free (DFS) and overall survival (OS) after intensive therapy for acute myeloid leukemia (AML) in older adults, particularly in FLT3-ITD-negative patients: ECOG-ACRIN (E-A) E2906 randomized study. *Blood* 2019;134(Supplement_1):115. Available from: https://ashpublications.org/blood/article/134/Supplement_1/115/426175/Maintenance-Decitabine-DAC-Improves-Disease-Free.
- [54] Mihalyova J, Jelinek T, Growkova K, Hrdinka M, Simicek M, Hajek R. Venetoclax: a new wave in hematooncology. *Exp Hematol* 2018;61:10–25. Available from: <https://pubmed.ncbi.nlm.nih.gov/29477371/>.
- [55] DiNardo CD, Jonas BA, Pullarkat V, Thirman MJ, Garcia JS, Wei AH, et al. Azacitidine and venetoclax in previously untreated acute myeloid leukemia. *N Engl J Med* 2020;383(7):617–29. Available from: <https://www.nejm.org/doi/full/10.1056/NEJMoa2012971>.
- [56] Cherry EM, Abbott D, Amaya M, McMahon C, Schwartz M, Rosser J, et al. Venetoclax and azacitidine compared with induction chemotherapy for newly diagnosed patients with acute myeloid leukemia. *Blood Adv* 2021;5(24):5565–73. Available from: <https://pubmed.ncbi.nlm.nih.gov/34610123/>.
- [57] Short NJ, Kantarjian H, Ravandi F, Dauer N. Emerging treatment paradigms with FLT3 inhibitors in acute myeloid leukemia. *Ther Adv Hematol* 2019;10:204062071982731.

- [58] Drummond MW, Gaskell C, Harrison C, Mead AJ, Yap C, Jackson AE, et al. Phazar: a phase ib study to assess the safety and tolerability of ruxolitinib in combination with azacitidine in advanced phase myeloproliferative neoplasms (MPN), including myelodysplastic syndromes (MDS) or acute myeloid leukaemia (AML) arising from MPN [ISRCTN16783472]. *Blood* 2020;136(Supplement 1):2–3. Available from: <https://ashpublications.org/blood/article/136/Supplement1/2/471090/Phazar-A-Phase-Ib-Study-to-Assess-the-Safety-and>.
- [59] Mascarenhas JO, Rampal RK, Kosiorek HE, Bhave R, Hexner E, Wang ES, et al. Phase 2 study of ruxolitinib and decitabine in patients with myeloproliferative neoplasm in accelerated and blast phase. *Blood Adv* 2020;4(20):5246–56. Available from: <https://ashpublications.org/bloodadvances/article/4/20/5246/469734/Phase-2-study-of-ruxolitinib-and-decitabine-in>.
- [60] Scandura JM, Roboz GJ, Moh M, Morawa E, Brenet F, Bose JR, et al. Phase 1 study of epigenetic priming with decitabine prior to standard induction chemotherapy for patients with AML. *Blood*. 2011; 118(6):1472–80.
- [61] Lemonnier F, Dupuis J, Sujobert P, Tournillhac O, Cheminant M, Sarkozy C, et al. Treatment with 5-azacytidine induces a sustained response in patients with angioimmunoblastic T-cell lymphoma. *Blood* 2018;132(21):2305–9. Available from: <https://pubmed.ncbi.nlm.nih.gov/30279227/>.
- [62] Gregory GP, Dickinson M, Yannakou CK, Wong J, Blombery P, Corboy G, et al. Rapid and durable complete remission of refractory AITL with azacitidine treatment in absence of TET2 mutation or concurrent MDS. *HemaSphere* 2019;3(2). Available from: <https://pubmed.ncbi.nlm.nih.gov/31723826/>.
- [63] Clozel T, Yang SN, Elstrom RL, Tam W, Martin P, Kormaksson M, et al. Mechanism-based epigenetic chemosensitization therapy of diffuse large B-cell lymphoma. *Cancer Discov* 2013;3(9):1002–19. Available from: <https://pubmed.ncbi.nlm.nih.gov/23955273/>.
- [64] Martin P, Bartlett NL, Chavez JC, Reagan JL, Smith SM, LaCasce AS, et al. Phase 1 study of oral azacitidine (CC-486) plus R-CHOP in previously untreated intermediate- to high-risk DLBCL. *Blood* 2022;139(8):1147–59. Available from: <https://ashpublications.org/blood/article/139/8/1147/476663/Phase-1-study-of-oral-azacitidine-CC-486-plus-R>.
- [65] Yen K., Travins J., Wang F., David M.D., Artin E., Straley K., et al. AG-221, a first-in-class ther target acute myeloid leukemia harboring oncogenic IDH2 mutatations. <<http://aacrjournals.org/cancerdiscovery/article-pdf/7/5/478/1845622/478.pdf>>.
- [66] Stein EM, DiNardo CD, Polleyea DA, Fathi AT, Roboz GJ, Altman JK, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. *Blood* 2017;130(6):722–31. Available from: <https://ash-publications.org/blood/article/130/6/722/36814/Enasidenib-in-mutant-IDH2-relapsed-or-refractory>.
- [67] Shih AH, Shank KR, Meydan C, Intlekofer AM, Ward P, Thompson CB, et al. AG-221, a small molecule mutant IDH2 inhibitor, remodels the epigenetic state of IDH2-mutant cells and induces alterations in self-renewal/differentiation in IDH2-mutant AML model in vivo. *Blood*. 2014;124(21):437.
- [68] Stein EM. IDH2 inhibition in AML: finally progress? *Best Pract Res Clin Haematol* 2015;28 (2–3):112–15. Available from: <https://pubmed.ncbi.nlm.nih.gov/26590767/>.
- [69] DiNardo CD, Stein EM, de Botton S, Roboz GJ, Altman JK, Mims AS, et al. Durable remissions with ivosidenib in IDH1-mutated relapsed or refractory AML. *N Engl J Med* 2018;378(25):2386–98. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29860938>.
- [70] Stein EM, DiNardo CD, Fathi AT, Mims AS, Pratz KW, Savona MR, et al. Ivosidenib or enasidenib combined with intensive chemotherapy in patients with newly diagnosed AML: a phase 1 study. *Blood* 2021;137(13):1792–803. Available from: <https://pubmed.ncbi.nlm.nih.gov/33024987/>.
- [71] DiNardo CD, Stein AS, Stein EM, Fathi AT, Frankfurt O, Schuh AC, et al. Mutant isocitrate dehydrogenase 1 inhibitor ivosidenib in combination with azacitidine for newly diagnosed acute myeloid leukemia. *J Clin Oncol* 2021;39(1):57–65. Available from: <https://pubmed.ncbi.nlm.nih.gov/33119479/>.

- [72] MacBeth KJ, Chopra VS, Tang L, Zheng B, Avanzino B, See WL, et al. Combination of azacitidine and enasidenib enhances leukemic cell differentiation and cooperatively hypomethylates DNA. *Exp Hematol* 2021;98(47-52):e6.
- [73] Blanc RS, Richard S. Arginine methylation: the coming of age. *Mol Cell* 2017;65(1):8–24. Available from: <https://pubmed.ncbi.nlm.nih.gov/28061334/>.
- [74] Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in polycomb-group silencing. *Science* (80) 2002;298(5595):1039–43.
- [75] Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. *Nature* 2011;469(7330):343–9. Available from: <https://www.nature.com/articles/nature09784>.
- [76] Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet* 2010;42(2):181–5. Available from: <https://pubmed.ncbi.nlm.nih.gov/20081860/>.
- [77] Sashida G, Iwama A. Multifaceted role of the polycomb-group gene EZH2 in hematological malignancies. *Int J Hematol* 2017;105(1):23–30. Available from: <https://link.springer.com/article/10.1007/s12185-016-2124-x>.
- [78] Rinke J, Chase A, Cross NCP, Hochhaus A, Ernst T. EZH2 in myeloid malignancies. *Cells* 2020;9(7):1639. Available from: <https://www.mdpi.com/2073-4409/9/7/1639/htm>.
- [79] Lundberg P, Karow A, Nienhold R, Looser R, Hao-Shen H, Nissen I, et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood* 2014;123(14):2220–8. Available from: <https://pubmed.ncbi.nlm.nih.gov/24478400/>.
- [80] Hoy SM. Tazemetostat: first approval. *Drugs* 2020;80(5):513–21. Available from: <https://link.springer.com/article/10.1007/s40265-020-01288-x>.
- [81] Knutson SK, Kawano S, Minoshima Y, Warholic NM, Huang KC, Xiao Y, et al. Selective inhibition of EZH2 by EPZ-6438 leads to potent antitumor activity in EZH2-mutant non-Hodgkin lymphoma. *Mol Cancer Ther* 2014;13(4):842–54. Available from: <https://pubmed.ncbi.nlm.nih.gov/24563539/>.
- [82] Yuan H, Nishikori M, Otsuka Y, Arima H, Kitawaki T, Takaori-Kondo A. The EZH2 inhibitor tazemetostat upregulates the expression of CCL17/TARC in B-cell lymphoma and enhances T-cell recruitment. *Cancer Sci* 2021;112(11):4604.
- [83] Morschhauser F, Tilly H, Chaidos A, McKay P, Phillips T, Assouline S, et al. Tazemetostat for patients with relapsed or refractory follicular lymphoma: an open-label, single-arm, multicentre, phase 2 trial. *Lancet Oncol* 2020;21(11):1433–42. Available from: <http://www.thelancet.com/article/S1470204520304411/fulltext>.
- [84] Duan R, Du W, Guo W. EZH2: A novel target for cancer treatment. *J Hematol Oncol* 2020;13(1):1–12. Available from: <https://jhoonline.biomedcentral.com/articles/10.1186/s13045-020-00937-8>.
- [85] Bhat KP, Ümit Kaniskan H, Jin J, Gozani O. Epigenetics and beyond: targeting writers of protein lysine methylation to treat disease. *Nat Rev Drug Discov* 2021;20(4):265–86. Available from: <https://www.nature.com/articles/s41573-020-00108-x>.
- [86] Stein EM, Garcia-Manero G, Rizzieri DA, Tibes R, Berdeja JG, Savona MR, et al. The DOT1L inhibitor pinometostat reduces H3K79 methylation and has modest clinical activity in adult acute leukemia. *Blood* 2018;131(24):2661–9. Available from: <https://ashpublications.org/blood/article/131/24/2661/37193/The-DOT1L-inhibitor-pinometostat-reduces-H3K79>.
- [87] Fiskus W, Boettcher S, Daver N, Mill CP, Sasaki K, Birdwell CE, et al. Effective Menin inhibitor-based combinations against AML with MLL rearrangement or NPM1 mutation (NPM1c). *Blood Cancer J* 2022;12(1):1–11. Available from: <https://www.nature.com/articles/s41408-021-00603-3>.
- [88] Fang Y, Liao G, Yu B. LSD1/KDM1A inhibitors in clinical trials: advances and prospects. *J Hematol Oncol* 2019;12(1):1–14. Available from: <https://jhoonline.biomedcentral.com/articles/10.1186/s13045-019-0811-9>.

- [89] Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev* 1998;12:599–606.
- [90] Zhang C, Zhong JF, Stucky A, Chen XL, Press MF, Zhang X. Histone acetylation: novel target for the treatment of acute lymphoblastic leukemia. *Clin Epigenetics* 2015;7(1).
- [91] Cheung P, Allis CD, Sassone-Corsi P. Signaling to Chromatin through Histone Modifications. *Cell*. 2000;103(2):263–71.
- [92] Yang XJ, Seto E. Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. *Curr Opin Genet Dev* 2003;13(2):143–53.
- [93] Wada T, Kikuchi J, Nishimura N, Shimizu R, Kitamura T, Furukawa Y. Expression levels of histone deacetylases determine the cell fate of hematopoietic progenitors. *J Biol Chem* 2009;284(44):30673–83. Available from: <http://www.jbc.org/article/S0021925820381217/fulltext>.
- [94] Wang P, Wang Z, Liu J. Role of HDACs in normal and malignant hematopoiesis. *Mol Cancer* 2020;19(1):1–21. Available from: <https://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-019-1127-7>.
- [95] Mithraprabhu S, Kalff A, Chow A, Khong T, Spencer A. Dysregulated Class I histone deacetylases are indicators of poor prognosis in multiple myeloma. *Epigenetics* 2014;9(11):1511–20. Available from: <https://www.tandfonline.com/doi/abs/10.4161/15592294.2014.983367>.
- [96] Sanchez R, Meslamani J, Zhou MM. The bromodomain: from epigenome reader to druggable target. *Biochim Biophys Acta* 2014;1839(8):676–85.
- [97] Devaiah BN, Lewis BA, Cherman N, Hewitt MC, Albrecht BK, Robey PG, et al. BRD4 is an atypical kinase that phosphorylates Serine2 of the RNA polymerase II carboxy-terminal domain. *Proc Natl Acad Sci U S A* 2012;109(18):6927–32. Available from: <http://www.pnas.org/cgi/doi/10.1073/pnas.1120422109>.
- [98] Jung M., Gelato K.A., Fernández-Montalván A., Siegel S., Haendler B. Targeting BET bromodomains for cancer treatment. *Epigenomics* 2015;7(3):487–501. <<https://www.futuremedicine.com/doi/abs/10.2217/epi.14.91>>.
- [99] Morgado-Pascual JL, Rayego-Mateos S, Tejedor L, Suarez-Alvarez B, Ruiz-Ortega M. Bromodomain and extraterminal proteins as novel epigenetic targets for renal diseases. *Front Pharmacol* 2019;10:1315.
- [100] Barbieri I, Cannizzaro E, Dawson MA. Bromodomains as therapeutic targets in cancer. *Brief Funct Genomics* 2013;12(3):219–30. Available from: <https://academic.oup.com/bfg/article/12/3/219/200677>.
- [101] Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature* 2011;478(7370):529–33. Available from: <https://www.nature.com/articles/nature10509>.
- [102] Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, et al. Selective inhibition of BET bromodomains. *Nature* 2010;468(7327):1067–73. Available from: <https://www.nature.com/articles/nature09504>.
- [103] Andreu-Vieyra CV, Berenson JR. The potential of panobinostat as a treatment option in patients with relapsed and refractory multiple myeloma. *Ther Adv Hematol* 2014;5(6):197–210. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25469210>.
- [104] Moore D. Panobinostat (Farydak): a novel option for the treatment of relapsed or relapsed and refractory multiple myeloma. *Pharm Ther* 2016;41(5):296.
- [105] Chun P. Histone deacetylase inhibitors in medical therapeutics. *Med Epigenetics* 2016;633–55.
- [106] Mascarenhas J, Sandy L, Lu M, Yoon J, Petersen B, Zhang D, et al. A phase II study of panobinostat in patients with primary myelofibrosis (PMF) and post-polycythemia vera/essential thrombocythemia myelofibrosis (post-PV/ET MF). *Leuk Res* 2017;53:13–19.
- [107] Mascarenhas J, Marcellino BK, Lu M, Kremyanskaya M, Fabris F, Sandy L, et al. A phase I study of panobinostat and ruxolitinib in patients with primary myelofibrosis (PMF) and post-polycythemia vera/essential thrombocythemia myelofibrosis (post-PV/ET MF). *Leuk Res* 2020;88:106272.

- [108] Wang H, Lim ZY, Zhou Y, Ng M, Lu T, Lee K, et al. Acy lurea connected straight chain hydroxamates as novel histone deacetylase inhibitors: synthesis, SAR, and in vivo antitumor activity. *Bioorg Med Chem Lett* 2010;20(11):3314–21.
- [109] Bradner JE, West N, Grachan ML, Greenberg EF, Haggarty SJ, Warnow T, et al. Chemical phylogenetics of histone deacetylases. *Nat Chem Biol* 2010;6(3):238–43. Available from: <https://pubmed.ncbi.nlm.nih.gov/20139990/>.
- [110] Uehara N, Yoshizawa K, Tsubura A. Vorinostat enhances protein stability of p27 and p21 through negative regulation of Skp2 and Cks1 in human breast cancer cells. *Oncol Rep* 2012;28(1):105–10. Available from: <https://pubmed.ncbi.nlm.nih.gov/22484732/>.
- [111] Olsen EA, Kim YH, Kuzel TM, Pacheco TR, Foss FM, Parker S, et al. Phase IIB multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous t-cell lymphoma. *J Clin Oncol* 2007;25(21):3109–15.
- [112] Piekarz RL, Frye R, Turner M, Wright JJ, Allen SL, Kirschbaum MH, et al. Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. *J Clin Oncol* 2009;27(32):5410–17.
- [113] Dimopoulos M, Siegel DS, Lonial S, Qi J, Hajek R, Facon T, et al. Vorinostat or placebo in combination with bortezomib in patients with multiple myeloma (VANTAGE 088): a multicentre, randomised, double-blind study. *Lancet Oncol* 2013;14(11):1129–40. Available from: <https://pubmed.ncbi.nlm.nih.gov/24055414/>.
- [114] José-Enériz ES, Gimenez-Camino N, Agirre X, Prosper F. HDAC inhibitors in acute myeloid leukemia. *Cancers* 2019;11(11):1794. Available from: <https://www.mdpi.com/2072-6694/11/11/1794/htm>.
- [115] Nicodeme E, Jeffrey KL, Schaefer U, Beinke S, Dewell S, Chung CW, et al. Suppression of inflammation by a synthetic histone mimic. *Nature* 2010;468(7327):1119–23. Available from: <https://www.nature.com/articles/nature09589>.
- [116] Moyer MW. First drugs found to inhibit elusive cancer target. *Nat Med* 2011;17(11):1325.
- [117] Moon KJ, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K. The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. *Mol Cell* 2005;19(4):523–34.
- [118] Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell*. 2011;146(6):904–17.
- [119] Kleppe M, Koche R, Zou L, van Galen P, Hill CE, Dong L, et al. Dual targeting of oncogenic activation and inflammatory signaling increases therapeutic efficacy in myeloproliferative neoplasms. *Cancer Cell* 2018;33(1):29–43. Available from: <https://pubmed.ncbi.nlm.nih.gov/29249691/>.
- [120] Mascarenhas J, Kremyanskaya M, Hoffman R, Bose P, Talpaz M, Harrison CN, et al. MANIFEST, a phase 2 study of CPI-0610, a bromodomain and extraterminal domain inhibitor (BETi), as monotherapy or “add-on” to ruxolitinib, in patients with refractory or intolerant advanced myelofibrosis. *Blood*. 2019;134(Supplement_1):670.

Epigenetic therapy in lung cancer 12

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1 Introduction

Significant improvements in the clinical outcome for lung cancer patients have been achieved over the past decade, and this is a result of the introduction of targeted therapies in genetically selected subpopulations of patients, as well as the unprecedented efficacy of immunotherapy. Despite this, only a small proportion of patients respond to immunotherapy and few patients achieve long-term survival. Several epigenetic modifications have been associated with the development and progression of many cancers, including lung cancer. The discovery of this link between epigenetic modifications and cancer fueled the development of agents to target changes in the epigenome. Many of these epigenetic agents have now been approved for use in diseases, most commonly in hematological malignancies. In addition, there is a growing body of research, including preclinical work and early-phase clinical trials, demonstrating the use of epigenetic-targeted drugs for the treatment of lung cancer.

2 Overview of lung cancer

Despite rapid advances in the field, lung cancer remains the leading cause of cancer death worldwide with 1.8 million cancer deaths in 2020 and is projected to remain so in developed and developing countries for decades to come [1,2]. Global 5-year survival is only 10%–20%, which is largely attributed to the late diagnosis associated with the disease.

Broad trends in lung cancer incidence over the past 25 years have been downward in men but, of concern, upward in women. This likely reflects changes in smoking patterns over time. While smoking is still overwhelmingly the leading preventable cause of cancer death, a small but appreciable number are due to exposure to asbestos and radon gas [3].

Lung cancer is broadly divided into nonsmall-cell lung cancer (NSCLC), comprising 85% of cases, and small-cell lung cancer (SCLC), making up the remaining 15%. However, the NSCLC designation is composed of many different subtypes with different pathophysiologies, presentations, and behaviors. Adenocarcinoma (AC) is the most common form of NSCLC, representing approximately one half of all lung cancers, followed by squamous cell carcinoma and large cell carcinoma.

Cancers that are identified early (stages I and II) can be cured with surgery, but this represents only 25%–30% of lung tumors [4]. For more locally advanced (stage III) cancers, treatment is typically combined chemotherapy and radiation with the intention of cure and can be followed by immunotherapy; however, the majority of these patients will eventually relapse [5].

Among those with metastatic (stage IV) cancers, treatments are typically aimed at controlling rather than curing the disease. Treatment for these cancers was traditionally based around chemotherapy but has become nuanced over the past 10 years. It is now known that up to 50% of ACs harbor genetic alterations, known as “driver mutations,” which may be treated with a targeted oral medication [6]. These treatments generally have higher response rates and less toxicity than standard “cytotoxic” chemotherapy.

The other major advance in the past decade has been the development of checkpoint inhibitors, also known as immunotherapy, which harness the body’s own immune system to target cancers. These agents can be effective either alone [7] or in combination with chemotherapy [8] in the treatment of metastatic NSCLC.

Despite these significant advances, 5-year survival rates in lung cancer remain poor and this represents an area of unmet need. This is attributable to resistance to targeted and nontargeted therapies, the underlying frailty of this population, and the substantial proportion of patients who are not eligible for targeted therapy or immunotherapy. Given these challenges, and the unique epigenetic background associated with lung cancer, increasing interest is devoted to the potential of epigenetic therapies in the management of lung cancer.

3 Epigenetic modifications in lung cancer

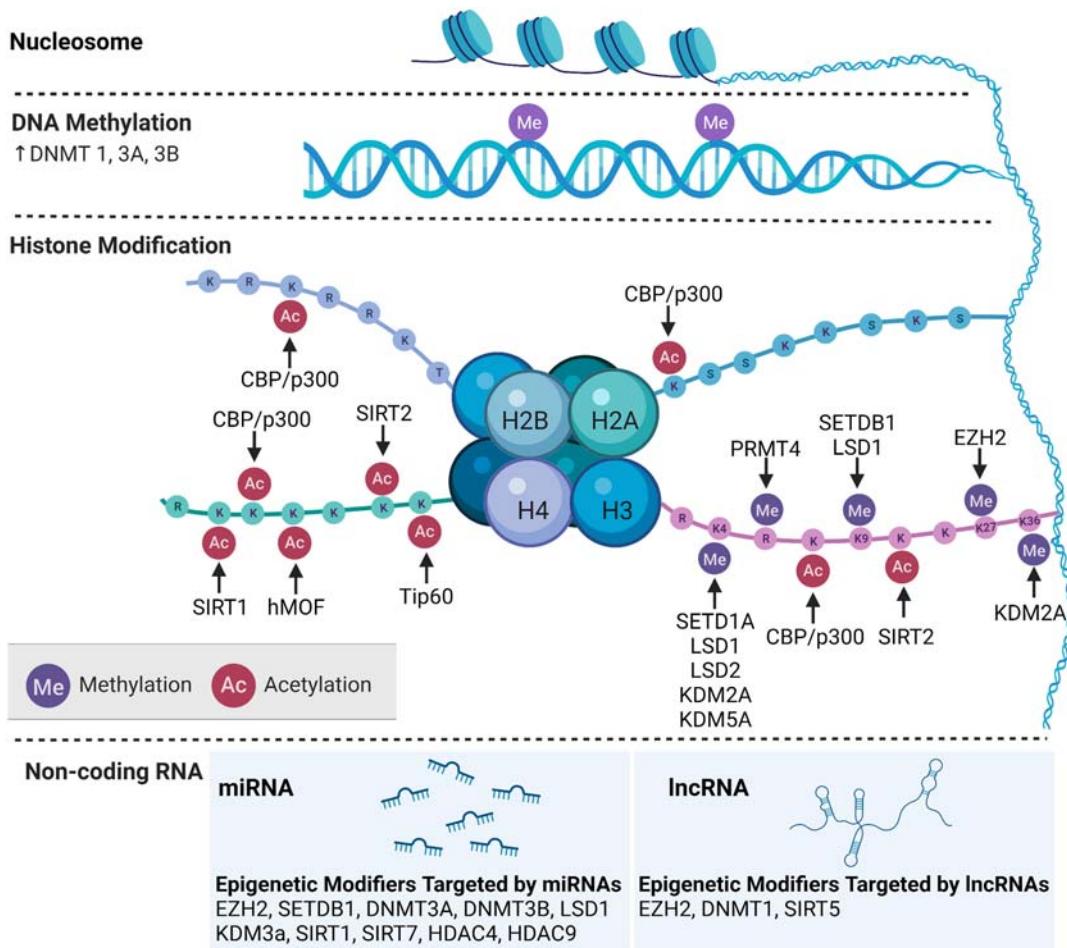
Dysregulation of the epigenome is frequently reported in lung cancer, leading to alterations in many cancer hallmarks, including proliferation, invasion, metastasis, apoptosis, and cell cycle regulation. Epigenetic modifications can be divided into three different processes; DNA methylation, histone modifications, and noncoding RNAs (ncRNAs) (Figure 12.1). These modifications influence many cellular processes, as well as the encoding of immune checkpoint proteins, tumor suppressors, or oncogenes in cancer.

3.1 DNA methylation

DNA methylation is a process in which methyl groups ($-CH_3$) from adenosylmethionine (SAM) are added to the 5' position of the pyrimidine ring of cytosines in the CpG dinucleotide called a CpG island. The hypermethylation of CpG-rich promoters result in silencing of gene transcription because the methylated CpGs can impair the binding of transcriptional factors and recruit repressive complexes [9,10]. DNA methylation is also associated with the inactivation of tumor suppressor genes in many cancers [11], including NSCLC [12]. This epigenetic modification is mediated by DNA methyltransferases (DNMTs).

All three DNMTs, DNMT1 [13,14], DNMT3A [15,16], and DNMT3B [17], are elevated in lung cancer. The main function of DNMT1 is maintenance of methylation patterns after DNA synthesis, while DNMT3A and DNMT3B are mainly *de novo* methylators of DNA [18,19]. High levels of DNMT1, DNMT3A and DNMT3B are associated with methylation status promotor changes in common lung cancer associated genes, such as fragile histidine triad diadenosine (*FHIT*), cyclin-dependent kinase inhibitor 2A (CDKN2A)/p16, and retinoic acid receptor beta (*RAR β*) [13]. Elevated levels of DNMT1 in lung cancer is associated with poor prognosis and drug resistance [20,21]. Downregulation of DNMT3A significantly inhibits the proliferation and G1/3 transition of lung cancer cells [22] and DNMT3A expression has the potential to be an independent marker in lung adenocarcinoma (LUAD) [23]. Transcriptional repression of the Rb/E2F pathway results in high levels of DNMT3B and downregulation of DNMT3B significantly reduces DNA methylation and suppresses tumor growth in lung xenograft models [15].

DNMT inhibitors (DNMTis) have been shown to enhance T-cell-mediated cytotoxicity of lung cancer cells. DNMTis alter the surface proteome of lung cancer cells, with an upregulation of multiple major histocompatibility complex (MHC)-unrestricted immune recognition molecules observed. DNMTi treatment of lung cancer cells enhances immune synapse formation between T cells and cancer cells to potentiate antitumor immunity [24]. See Table 12.1 for a full summary of the role of DNMTs in lung cancer.

**FIGURE 12.1** Epigenetic modifiers influencing lung cancer.

The three main processes of epigenetic modification, DNA methylation, histone modification, and noncoding RNAs influence lung cancer. DNMTs are elevated in lung cancer. Lysine methyltransferases, arginine methyltransferases, lysine demethylases, lysine acetyltransferases, and lysine deacetylases modify histones to influence gene expression. The two main categories of ncRNA influencing lung cancer are microRNAs (miRNA) and long ncRNAs (lncRNAs). Several ncRNA's target epigenetic modifiers with implications for lung cancer.

3.2 Histone modifications

Histones are divided into two groups; core histones H2A, H2B, H3, and H4 and linker histone H1. The most common histone modifications include methylation, acetylation and phosphorylation [28,29]. These modifications are mediated by “writers,” “readers,” and “erasers” at the histone tails.

TABLE 12.1 Altered DNA methyltransferases in lung cancer.

Epigenetic modifier-DNA methylation	Level in lung cancer	Significance	References
DNMT1	Elevated	Poor prognosis Drug resistance	[13,14,17,25–27]
DNMT3A	Elevated	Cell cycle ^a	[13,15,17,22,23]
DNMT3B	Elevated	Poor prognosis	[13,14,17]

^aIn cell line studies.

Writers include histone lysine methyltransferases (KMTs), histone acetylases (HATs), and histone arginine methyltransferases (PRMTs), amongst others. These operate enzymatically by catalyzing the addition of epigenetic marks onto histone tails. Readers, which recognize specific epigenetic marks and amplify their impact on DNA packaging, include proteins containing bromodomains, chromodomains and Tudor domains. Erasers, such as histone demethylases (KDMs) and histone deacetylases (HDACs), remove epigenetic marks [30]. Global histone H3 and histone H4 modification patterns, particularly increased methylation, is associated with poor prognosis in NSCLC patients [31].

3.2.1 Histone methylation

Histone methylation involves the addition of methyl groups to mainly lysine (K) and arginine (R) residues in the histone tails. The lysine residues can be mono-, di-, or trimethylated and the arginine residues are mono- or demethylated.

Lysine methylation can either activate or silence gene transcription, depending on the specific lysine position that is methylated [32]. The six major families of histone lysine methyltransferases (KMT1–6) exist either singly or within complexes. The majority of KMTs contain a SET domain [*Su(var)3–9*, *Enhancer of zeste* (*E(z)*), and *trithorax* (*trx*)], with one exception (KMT4/DOTL1) harboring a unique catalytic domain, DOT-1 [33]. Enhancer of zeste homolog 2 (EZH2/KMT6) alters gene expression through the trimethylation of lysine 27 in histone H3 (H3K27ME3) [34]. EZH2 is found to be elevated in lung cancer and is associated with poor prognosis [35–37]. The modifications induced by EZH2 repress gene expression and subsequently effect multiple biological processes, such as cell cycle progression [38], autophagy and apoptosis [39], DNA damage repair, cellular senescence [40], cell lineage determination, and relative signaling pathways [41]. EZH2's role in these pathways, along with its overactivity in cancer, has led to the development and clinical testing of specific EZH2 inhibitors. In January 2020, the first specific EZH2 inhibitor, tazemetostat, was approved by the Food and Drug Administration (FDA) for the treatment of metastatic epithelioid sarcoma [42]. In NSCLC, positive EZH2 expression correlates with larger tumor size and significantly shorter overall survival (OS) [43]. EZH2 has been shown to drive acquired resistance to chemotherapy in SCLC and combining an EZH2 inhibitor with standard of care controls SCLC in patient-derived xenograft models [44]. The formation of an immunosuppressive microenvironment is also associated with EZH2 activity [45], and disruption of EZH2 function in regulatory T cells (Tregs) leads to robust antitumor immunity [46,47]. EZH2 upregulation has shown to positively correlate with programmed death ligand 1 (PD-L1) levels and poor prognosis in lung cancer tissue, and ablation of EZH2 inhibits PD-L1 expression and delays lung cancer tumor growth *in vivo* [48].

The KMT2 family members, SETD1A/Set1A and SETD1B/Set1B are known to be essential for mammalian development [49,50]. Together, they are responsible for much of the tri-methylation of lysine 4 in histone H3 (H3K4ME3), which is associated with active transcription through interactions with effector proteins [51,52]. SETD1A is elevated in lung cancer tissue [53,54] and is associated with poor prognosis [55]. Through its critical epigenetic modifications, SETD1A drives cell proliferation [54] and epithelial – mesenchymal transition (EMT) [53] in lung cancer. Other members of the KMT2 family, which play roles in lung cancer include: KMT2C/MLL3 which has the potential to predict poor prognosis in plasma free circulating DNA of both operable and metastatic NSCLC patients; [56,57] KMT2D/MLL2/MLL4 is found to be frequently mutated in lung cancer [58] and loss of function of *Kmt2d* has shown to promote lung tumorigenesis and upregulate pro-tumorigenic programs, such as glycolysis *in vivo* [59].

There are many other KMTs which are elevated in lung cancer and result in poor prognosis for patients such as; SETDB1/KMT1E, which displays increased expression in NSCLC [60,61] and can enhance proliferation through regulation of the WNT–β-catenin pathway [60]; SMYD3, which plays a role in proliferation, migration, and invasion in NSCLC [62,63]; KMT1C/G9a/EHMT2 which promotes tumor cell invasion and migration through repressing caspase 1 (CASP1) expression [64]; KM5TA is highly expressed in LUAD and lung squamous cell carcinoma (LUSC) but it plays differential roles in each [65]; and SETD8 expression is correlated with primary tumor stage, lymph node metastases, and clinical stage, with its overexpression associated with cancer stemness-related genes and cell cycle-related genes in NSCLC [66]. KMTs which are downregulated in lung cancer include SETD7 [67] and SETD2 [68]. For a full summary on KMTs' role in lung cancer, see Table 12.2.

Arginine residues are also methylated on the histone tails and this process is mediated by PRMTs. Similar to lysine methylation, gene silencing or activation can occur following arginine methylation depending on the type of methylation on the histone tails. Elevated expression of PRMTs is observed in lung cancer and is largely associated with enhanced proliferation (Table 12.2). PRMTs are divided into two classes, type I and type II, depending on the nature of the modification induced [119]. PRMT1 [112], PRMT4/CARM1 [118], and PRMT6 [116] all belong to type I and are all elevated in lung cancer, with PRMT4/CARM1 also linked to poor prognosis [118]. It has been identified that PRMT6 has a noncatalytic role, in potentiating lung tumor progression via the alternate activation of tumor-associated macrophages (TAMs) [115]. PRMT7 belongs to the type II class of PRMTs and has been linked to the metastatic phenotype in NSCLC cancer cells [117].

Methylation is a reversible process and there are at least six families of KDMs, which remove the methyl from the lysine residues on the histone tails. Research into the role of many of these KDMs in lung cancer has intensified in recent years (Table 12.2). The KDM1 family includes lysine-specific histone demethylase 1 (LSD1)/KDM1A and lysine-specific histone demethylase 2 (LSD2)/KDM1B, both of which can demethylate H3K4ME2/ME1 but not H3K4ME3 [120,121], with LSD1 also demethylating H3K9 [122]. Overexpression of LSD1 is observed in NSCLC [87,123] and is associated with shorter survival [87], increased tumor progression and metastasis [90,124] and promotion of EMT [91]. JHDM1A (KDM2A) and JHDM1B (KDM2B) belong to the KDM2 family, which predominantly act on H3K36ME2/ME1 and H3K4ME3 [125]. KDM2A is elevated in NSCLC and the increased levels are associated with poor prognosis in patients [84]. The promotion of lung tumorigenesis through KDM2A is linked to its role in the epigenetic

TABLE 12.2 Altered histone methyltransferases and demethylases in lung cancer.

Epigenetic modifier-histone methylation	Level in lung cancer	Significance	References
Lysine methyltransferases			
KMT6/EZH2	Elevated	Poor prognosis	[35–37]
MMSET	Elevated	Proliferation ^a	[69]
SMYD3	Elevated	Growth ^a	[62,63]
WHSC1L1/NSD3	Elevated	Poor prognosis	[70–73]
KMT3C/SYMD2		Proliferation ^a	[74,75]
KMT1C/G9a/EHMT2	Elevated	Poor prognosis	[64]
KMT4/DOT1L	Elevated	Proliferation ^a	[76]
KMT1B/SUV39H2		Increased risk ^b	[77]
KMT8/RIZ1		Decreased risk ^b	[78]
KMT5A	Elevated	Poor prognosis	[65]
KMT2C/MLL3		Poor prognosis	[56,57]
KMT2D/MLL2/MLL4		Frequently mutated	[58,59]
SETD1A/Set1A	Elevated	Proliferation EMT	[53–55]
KMT1E/SETDB1	Elevated	Poor prognosis Proliferation ^a	[60,61]
SETD7	Downregulated	Poor prognosis Migration ^a Invasion ^a	[67]
GLP/EHMT1	Elevated	Cell cycle ^a	[79]
NSD2	Elevated		[80]
SETD2	Downregulated	EMT ^a	[68,81,82]
SETD8	Elevated	Poor prognosis	[66]
SET 7/9		Proliferation ^a	[83]
Lysine demethylases			
KDM2A/JHDM1a	Elevated	Poor prognosis	[84]
KDM5A/RBP2	Elevated	Metastasis	[85,86]
KDM1/LSD1	Elevated	Shorter survival	[87]
KDM4A/JMJD22A	Elevated	Oncogene-induced senescence ^a	[88]
		Metastasis	[88,92,93]
		Growth	
KDM5B/JAIRD1B	Elevated	Cell cycle ^a	[89]
KDM3A	Elevated	Proliferation ^a	[86]
KDM1/LSD1	Elevated	EMT	[90,91]
KDM5B/JAIRD1B	Elevated	Poor prognosis	
		Shorter overall survival	
		Proliferation	
		EMT ^a	[94,95]

(Continued)

TABLE 12.2 Altered histone methyltransferases and demethylases in lung cancer. *Continued*

Epigenetic modifier-histone methylation	Level in lung cancer	Significance	References
KDM3A	Upregulated		[96]
KDM6A/UTX	Elevated	EMT Metastasis ^a Poor prognosis	[97,98]
JMJD3/KDM6B	Elevated	EMT ^a Cell cycle Poor prognosis	[99–101]
KDM5A	Elevated	Proliferation ^a EMT ^a	[102]
KDM5C	Elevated	Growth Metastasis	[94,103]
JMJD1a	Elevated		[104]
LSD2	Elevated	Proliferation	[105]
KDM2B		EMT ^a	[106]
PHF8		Metastasis	[107]
JMJD2C	Elevated	Proliferation Poor prognosis	
		Migration Invasion	[108]
KDM4C	Elevated	Migration Invasion	[109]
JMJD2B	Elevated	Poor prognosis	[110]
Arginine methyltransferases			
PRMT1	Elevated	Proliferation ^a	[111,112]
PRMT5	Elevated	Growth ^a	[113,114]
PRMT6	Elevated	Proliferation ^a	
PRMT7	Elevated	Proliferation ^a	[112,115,116]
PRMT4/CARM1	Elevated	Invasion ^a	[117]
		Proliferation ^a	[118]
		Poor prognosis	

^aIn cell line studies.^bGene polymorphism.

regulation of the extracellular-signal-regulated kinase (ERK)1/2 signaling pathway [84], as well as cell-cycle associated genes and cell invasion-related genes [126]. KDM2B also been shown to play a role in lung tumorigenesis through its regulation of transforming growth factor-beta (TGF- β) induced EMT [106]. The KDM5 family consists of KDM5A, KDM5B, KDM5C and KDM5D, all of which can demethylate H3K4ME3/ME2. KDM5A/RBP2 is overexpressed in human lung cancer tissue [85] and overexpression of KDM5A is associated with enhanced EMT in lung cancer cells [86,102]. Elevated expression of KDM5B/JAIRD1B is also observed in lung cancer and is linked to shorter OS [94,127], and increased EMT and proliferation [95], which is modulated through the

decrease of p53 expression [128]. KDM6A/UTX from the KDM6 family is elevated in NSCLC patient tissue and associated with poor prognosis [129], by driving EMT, migration, and metastasis [97,98]. Other KDMs upregulated in lung cancer and associated with increased lung tumorigenesis include KDM3A [96,104,130], KDM4A/JMJD2A [88,92,93], KDM4B [110], KDM4C [108,109], and PHF8 [68,107]. For a full summary on KDMs role in lung cancer, see Table 12.2.

3.2.2 Histone acetylation

Histone acetylation involves the addition of an acetyl group from acetyl coenzyme A to the amino group of the lysine side chains. This neutralizes the positive charge of lysine and results in the activation of gene transcription by attenuating interactions between histones and DNA [131]. This process is carried out by HATs, of which there are three well studied groups; GNAT (HAT1, GCN₅, and PCAF), MYST (Tip60, MOF, MOZ, MORF, and HBO₁), and p300/CBP [132]. Various members of these groups are known to be elevated in lung cancer and are associated with a reduction in OS (Table 12.1).

Unlike other HATs, which target specific histones, p300 (KAT3B) and CBP (KAT3A) are capable of acetylating all four histones [133,134]. High p300 and CBP expression have been shown to be independent prognostic markers in SCLC [135]. p300 promotes migration and invasion via the induction of EMT in NSCLC cells [136], while acetylation of GATA3 by CBP has shown to inhibit cell migration and invasion in NSCLC cells [137]. Higher CBP levels are also reported in NSCLC patient tumors compared to normal tissue [138] and it is found to be mutated in a small subset of tumors [139]. KAT8 (hMOF) is responsible for acetylating histone H4 at lysine 16 (H4K16) and is a member of the MYST family of HATs. Upregulation of both KAT8 and H4K16 acetylation is observed in NSCLC tissue and is associated with poor prognosis [140,141]. KAT5/Tip60 is another member of the MYST family which is associated with lung tumorigenesis [142–144]. This enzyme plays a role in the repair of cisplatin induced DNA damage [142], as well as cell proliferation and migration [143]. Elevated steroid receptor coactivator-3 (SRC-3) levels is associated with poor prognosis in NSCLC [145] and SRC-3 increases pemetrexed resistance by decreasing chemotherapy-induced apoptosis via downregulating reactive oxygen species levels [146]. For a full summary of HATs role in lung cancer see Table 12.3.

Like methylation, acetylation is a reversible process, and the removal of the acetyl group from the lysine residue is mediated by a group of proteins HDACs. HDACs are divided into five groups; class I (HDAC1, 2, 3, and 8), IIa (HDAC4, 5, 7, and 9), IIb (HDAC6 and 10), and IV (HDAC11) are highly related in terms of structure and mechanism, and class III comprises the members of the Sirtuins (SIRT1–7), which differ as they require nicotinamide adenine dinucleotide (NAD⁺) as an enzymatic cofactor [172]. An association between all the HDACs has been linked to lung cancer; however, whether the levels are elevated or reduced depends on the specific HDAC (Table 12.3).

HDAC1 [151,152], HDAC2 [154], HDAC3 [155], HDAC10 [160,161], and HDAC11 [162] are all elevated in lung cancer with HDAC3 [155], HDAC10 [161], and HDAC11 [162] associated with poor prognosis. Reduced levels of some of the HDACs, including HDAC4, HDAC5, HDAC6, HDAC8, and HDAC9 are also reported in lung cancer [157]. Inhibition of HDAC11 significantly reduces self-renewal of cancer stem cells (CSCs) from NSCLC and the use of a highly selective HDAC11 inhibitor ablates the growth of drug-insensitive stem-like cells as well as therapy resistance lung cancer cells [162]. HDAC6 is positively associated with cisplatin resistance in NSCLC

TABLE 12.3 Altered histone acetylases and deacetylases in lung cancer.

Epigenetic modifier-histone acetylation	Level in lung cancer	Significance	References
Lysine acetyltransferases			
KAT3A/CBP	Elevated	Migration ^a Invasion Reduced overall survival	[135,137,138,147]
KAT3B/p300	Elevated	Reduced overall survival	[135,136]
KAT8/hMOF	Elevated	Migration ^a Poor prognosis	[140,141]
KAT2A/hGCN5	Elevated	Growth ^a Apoptosis ^a	[148,149]
KAT5/TIP60		DNA repair ^a Proliferation Migration	[142–144]
SRC-3	Elevated	Poor overall survival	[146]
KAT6B		Proliferation ^a	[150]
Histone deacetylases			
HDAC1	Elevated	Proliferation ^a	[151–153]
HDAC2	Elevated	Growth ^a Proliferation ^a	[154]
HDAC3	Elevated	Poor 5-year survival	[155]
HDAC4	Reduced	Poor prognosis	[156,157]
HDAC5	Reduced	Poor prognosis	[157]
HDAC6	Reduced	Poor prognosis	[157]
HDAC7		Poor prognosis	[157,158]
HDAC8	Reduced	Growth ^a	[157]
HDAC9	Reduced	Poor prognosis	[157,159]
HDAC10	Elevated	Proliferation Poor prognosis	[160,161]
HDAC11	Elevated	Poor prognosis	[162]
SIRT1	Elevated	Proliferation Prognosis	[163,164]
SIRT2	Reduced	Poor prognosis	[163,165]
SIRT3	Elevated	Poor prognosis	[166,167]
SIRT4	Elevated		[164]
SIRT5	Elevated		[164]
SIRT6	Reduced	Poor prognosis	[168–170]
SIRT7		Proliferation ^a	[171]

^aIn cell line studies.

[173] and it has been suggested that there is a ubiquitin-specific peptidase 10 (USP90)–HDAC6–cisplatin resistance axis in NSCLC [174]. HDAC inhibitors have multiple functions in immunomodulatory activates, including enhancing T-cell chemokine expression to augment response to PD-1 immunotherapy in lung cancer [175]. HDAC10 expression has shown to be positively correlated with the expression level of PD-L1 in NSCLC patients [161]. It has also been reported that HDAC6 inhibition promotes phenotypic changes that support enhanced T-cell activation and improved function of antigen presenting cells [176] and combining a HDAC6 inhibitor with chemotherapy promotes a robust antitumor response in NSCLC [177]. Within the Sirtuin family, there is also variation with expression levels, with SIRT1 [163], SIRT3 [178], SIRT4, and SIRT5 [164] displaying elevated levels of expression. SIRT6 has reported to be reduced and associated with poor prognosis in NSCLC patients [168]. However, there is also a study which reports a correlation between elevated SIRT6 expression in clinicopathological data and promotion of metastasis of NSCLC via the ERK1/2/MMP9 pathway [170]. For a full overview on HDACs role in lung cancer, see Table 12.3.

3.3 Noncoding RNAs

ncRNAs are functional RNAs, which are not translated into proteins and contain small ncRNAs and long noncoding RNAs (lncRNAs). Small ncRNAs are less than 200 bp in length, and lncRNAs are more than 200 bp in length [10].

The most widely studied small ncRNAs are microRNAs (miRNAs), which inhibit the translation of proteins through mediating the cleavage and degradation of messenger RNAs (mRNAs) by targeting the 3'-untranslated region (3'-UTR) [179]. miRNAs can regulate more than 30% of human genes involved in many cellular processes, such as proliferation, cell cycle arrest, differentiation, and apoptosis [10,180], and hence they play a major role in cancer. The oncogene EZH2 is a target of several miRNAs in lung cancer. miR-101-3p shows low expression in LUSC tissues and miR-101-3p can inhibit the viability, migration, invasion, and cell cycle of LUSC cells through inhibition of EZH2 [181]. The expression of miR-130-5p is significantly lower in LUAD and overexpression of miR-150-5p in lung cancer cells inhibits metastasis and invasion by targeting EZH2 [182]. The putative tumor suppressor, miR-124, displays lower expression in LUAD compared to matched normal tissue, and miR-124 is associated with an EMT phenotype in lung cancer cells via targeting of EZH2 [183]. miR-92b [184] and miR-4465 [185] also target EZH2 to inhibit proliferation and invasion in lung cancer cells. Overexpression of miR-26a in chemotherapy resistant lung cancer cells decreases the proliferation and increases the apoptosis rate of resistant cells *in vivo* and *in vitro*, and this is at least partially attributed to downregulation of EZH2 [186]. Other KMTs, such as SETDB1, are also targets of miRNAs. The miR-29 family (miR-29a, -29b, and -29c) are downregulated in NSCLC [187], and SETDB1 is targeted by miR-29s [188]. SETDB1 negatively regulates the expression of p53 and overexpression of SETDB1 downregulates the expression of miR-29s [188]. The miR-29 family also target DNMT3A and DNMT3B. Forced expression of miR-29s in lung cancer cells restores normal DNA methylation patterns and induces re-expression of tumor suppressor genes, such as FHIT and WW domain containing oxidoreductase (WWOX), leading to the inhibition of lung tumorigenesis *in vivo* [189]. DNMT3A has also shown to be a

target of miR-708-5p. The expression of miR-708-5p is significantly reduced in metastatic lung cancer samples [190] and NSCLC patients with high miR-708-5p expression show significantly better survival and lower recurrence [191]. miR-708-5p directly suppresses the translation of DNMT3A, resulting in upregulation of the tumor suppressor Cadherin 1 (CDH1) which leads to inhibition of lung cancer stemness through CDH1s reduction of Wnt/β-catenin signaling [191]. The suppression of KDMs by miRNAs, such as KDM3a by miR-449a [130] and LSD1 by miR-137 [192], has been shown to inhibit lung cancer development. Many of the HDACs are also regulated by miRNAs. SIRT1 is a direct target of miR-30a and miR-30a inhibits proliferation, invasion, and promotes apoptosis of lung cancer cells by inhibiting SIRT1 *in vitro* and *in vivo* [193]. SIRT7 is upregulated by miR-125a-5p, which leads to an increase in apoptosis in lung cancer cells to increase their radiosensitivity [194]. A negative correlation is observed between miR-520b and HDAC4 in lung cancer tissue and miR-520b decreases HDAC4 expression to control cell proliferation in lung cancer cells [156]. miR-509-3p exerts tumor-suppressive effects in NSCLC. HDAC9 is a direct target of miR-509-3p and the CBR3-AS1/miR-509-3p/HDAC9 pathway exerts tumor-promoting actions in NSCLC [195]. MiRNAs can also act as tumor suppressors by targeting immune checkpoint molecules [196], and it has been demonstrated that PD-L1 expression in NSCLC is negatively regulated by p53 via miR-34 [197].

lncRNAs are also known to have an impact on lung cancer and again there are many links with lncRNAs and EZH2. LINC00319 is upregulated in LUAD tissue and it is believed that LINC00319 contributes to lung tumor progression through reverse regulation of miR-450b-5p, and consequently activation of EZH2 [198]. The lncRNA, TP73-AS1, promotes NSCLC tumorigenesis through the regulation of miR-449a and activation of EZH2 [199]. LINC01088 [200], LINC00467 [201], and LINC0062 [202] can all promote lung cancer cell proliferation via binding to EZH2. Taurine upregulated gene1 (TUG1) is a lncRNA found to be overexpressed in SCLC tissue, and its expression is correlated with clinical stage and reduced survival time in SCLC [203]. TUG1 regulates the expression of LIM-kinase 2b via binding to EZH2 and this promotes cell growth and chemoresistance in SCLC [203]. DNMTs are also regulated by lncRNAs. The lncRNA, MIR210HG, recruits DNMT1 to inhibit CACNA2D2 expression and promote the tumorigenesis of NSCLC [204]. MIR210HG is highly expressed in NSCLC tissue and is associated with tumor stage and metastasis of patients [204]. LINC00337 also recruits DNMT1, resulting in the silencing of tissue inhibitor of metalloproteinases 2 (TIMP2), and consequently enhanced proliferation and invasion of NSCLC cells [205]. DNMT1 interacts with the lncRNA, nuclear-enriched abundant transcript 1 (NEAT1), to regulate cytotoxic T-cell infiltration in lung cancer via inhibition of cGAS/STING pathway, and NEAT1 is overexpressed in lung cancer tissue [206]. APCDD1L-AS1 is another lncRNA found to be upregulated in epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI)-resistant LUAD cells. This lncRNA sponges with miR-1322/miR-1972/miR-324-3p to remove the transcription inhibition of SIRT5 and inhibit the autophagic degradation of EGFR [207].

4 Environmental factors affecting the lung epigenome

There are many environmental factors which alter the lung epigenome and lead to the development of lung cancer. Exposure to cigarette smoke, asbestos, and radon gas is the three leading causes of lung cancer worldwide and all three result in alterations to the lung epigenome.

4.1 Cigarette smoking

Cigarette smoke contains over 60 known carcinogens [208] and it is responsible for more than 85% of lung cancer deaths. Chronic inflammation plays a significant role in the pathogenesis of smoking-induced lung cancer [209,210]. Lung tumors induced by smoking are recognized by genetic alterations in tumor suppressor genes and epigenetic changes, such as aberrant DNA methylation, altered histone acetylation, and aberrant miRNA expression [211]. An epigenome-wide meta-analysis of blood DNA methylation in 6685 new-borns from 13 studies showed roughly 6000 CpG sites differentially methylated as a result of smoking during pregnancy [212].

Upregulation of DNMTs is frequently associated with smoking in NSCLC [12,209]. Overexpression of DNMT1 is observed in lung cancer patients who smoke and is correlated with poor prognosis [213]. Nicotine-derived nitrosamine ketone (NNK), a chemical found in many tobacco products, induces the accumulation of DNMT1 and subsequent hypermethylation of the promoter of tumor suppressor genes, ultimately leading to tumorigenesis [213]. Characterization of epigenetic changes in the lungs of mice exposed to cigarette smoke revealed inflammation driven changes in cytosine methylation and hydroxymethylation patterns. These changes are indicative of an imbalance of DNA methylation/demethylation dynamics which leads to a shift in histone acetylation marks and gene expression patterns that could contribute to lung cancer [214]. Exposure of mice to NNK, leads to differential global methylation patterns of the lung tissue from week 2 versus week 4 of exposure, including in Adenylate Cyclase 6 (ADCY6), and Ras-related C3 botulinum toxin substrate 3 (Rac3) [215]. Significant differences in the methylation status of many genes have been observed between smokers and never smokers, such as Cytoplasmic Polyadenylation Element Binding (CPEB1), Cystatin E/M (CST6), Elastin Microfibril Interfacer (EMILIN2), Layillin (LAYN), and Marvel Domain Containing 3 (MARVELD3) [216], as well as TNF Receptor Superfamily Member 10c (TNFRSF10C), Basic Helix-Loop-Helix Family Member E22 (BHLHB5) and Boule Homolog (BOLL) [217]. Comparison of the genome-wide DNA methylation patterns between LUAD samples from smokers and nonsmokers identified that smoking-induced DNA methylations were mostly enriched in nuclear activities, including regulation of gene expression and chromatin remodeling [218].

4.2 Asbestos

Asbestos fibers are naturally occurring silicate mineral fibers used for insulation in buildings and are components in products, such as roofing shingles and water pipes. Asbestos exposure has been linked to lung cancer causation since the 1930s [219], and the exposure is associated with a five times increased risk of lung cancer [220]. Between 5% and 7% of all lung cancer cases in the world have high levels of asbestos linked to the disease, mainly due to occupational exposure [221].

Alterations in the expression of many miRNA are observed following exposure to asbestos. A study that examined the miRNA profile in NSCLC patients with asbestos-related and asbestos-unrelated NSCLC or with malignant pleural mesothelioma found an increased expression of miR-126 and miR-122 in asbestos-exposed patients [222]. A separate study which examined tumors from highly asbestos-exposed and nonexposed lung cancer patients identified thirteen asbestos related miRNAs. Overexpression of miR-148b, miR-374a, miR-241, Let-7D, miR-199b-5P, miR-331-3p, and miR-96, while under-expression of miR-939, miR-671-5p, miR-605, miR-1224-5p, and

miR-202 were reported [223]. An inverse correlation of specific target genes, such as growth arrest and DNA-damage-inducible protein (GADD454), latent transforming growth factor beta-binding protein 1 (LTBP1), FOSB, Neurocalcin Delta (NCALD), Calcium Voltage-Gated Channel Auxiliary (CACNA2D2), metastasis suppressor protein 1 (MTSS1), and Erythrocyte Membrane Protein Band 4.1 Like 3 (EPB41L3), were also identified [223].

Another epigenetic modification which is observed in asbestos-related lung cancer is DNA hypermethylation. There is a strong link between DNA hypermethylation and inflammation, and it is suggested that asbestos exposure contributes to lung cancer formation through this relationship [224]. Distinctive DNA methylation changes were observed in a genome-wide study of lung cancer tissue and paired normal lung from 28 asbestos exposed or nonexposed patients [225]. Differently methylated regions in genes, such as *RARB*, G-protein-coupled receptor 135 (GPR135), and Thyroid peroxidase (TPO), and differentially methylated CpGs in Neuroplastin (NPTN), Neuregulin 2 (NRG2), Glycosyltransferase 25 Domain Containing 2 (GLT25D2), and Transient Receptor Potential Cation Channel Subfamily C Member 3 (TRPC3), were significantly associated with asbestos exposure status in lung tumors [225]. Epigenetic inactivation of tumor suppressor genes, such as Ras association domain family member 1A (RASSF1A) and CDKN2A, has also been observed in lung cancer patients as a result of exposure to asbestos [226,227].

4.3 Radon gas

Radon is radioactive odorless gas occurring from the degradation of uranium from Earth's soil. Residential radon from soil is the second most important risk factor for the development of lung cancer, after smoking, and it accounts for 10% of cases [228].

Chronic exposure to radon results in the upregulation of miR-34a in human bronchial epithelial cells [229]. Long-term radon exposure is associated with increased CDKN2A and 0–6-methylguanine-DNA methyltransferase (MGMT) promoter methylation among Chinese miners [230]. It has been demonstrated in radon-induced lung tumors of rats that the locus containing the CDKN2A gene is frequently affected by DNA losses [231]. Exposure to plutonium, which is similar to radon, can induce CDKN2A gene inactivation through promoter methylation [232]. It was also demonstrated that this methylation of the CDKN2A gene displayed a striking dose response to plutonium, and workers with the highest exposure displayed a much greater risk of CDK2NA methylation in their tumors than controls [232]. Dysregulation of many lncRNAs are also linked to radon exposure and these lncRNAs play an important role in lung damage [233].

5 Epigenetic targeting of lung cancer

As our understanding of NSCLC pathophysiology becomes more textured, it is seen not as one disease but a collection of diseases with identifiable molecular abnormalities. This gives a basis for personalized medicine, in which treatments are tailored to these unique characteristics of an individual or their tumor [234]. Personalized medicine in lung cancer has had great success through the targeting of recognized driver alterations, such as mutations in EGFR [235,236] and fusions in anaplastic lymphoma kinase (ALK) [237,238]. Our understanding of the landscape of epigenetic

changes in lung cancer is evolving rapidly, and many of these changes are implicated in carcinogenesis, as described previously.

Parallel to this, several agents that target epigenetic changes have been approved in other disease settings, most commonly hematologic malignancies. For example, two DNMTis, decitabine, and 5-azacytidine [239] have FDA approval in the treatment of myelodysplastic syndrome. A further DNMTi, belinostat, was granted approval for the treatment of relapsed peripheral T-cell lymphoma [240]. Two HDACis, vorinostat [241] and romidepsin [242], have been approved in the management of cutaneous T-cell lymphoma. In the setting of relapsed or refractory acute myeloid leukemia (AML) with isocitrate dehydrogenase (IDH) mutations, the IDH inhibitors enasidenib [243] and ivosidenib [244] have approval.

A growing body of research has explored the use of epigenetic-targeted drugs of this kind in the setting of NSCLC. This research has included preclinical work and early-phase clinical trials. See Table 12.4 for a full list of clinical trials using epigenetic drugs in lung cancer.

5.1 Histone deacetylase inhibitors

Among epigenetic-targeted drugs in lung cancer, the largest area of preclinical and clinical research has been into HDAC inhibitors (HDACis). Mocetinostat (MGCD0103) is an isotype-specific, orally available benzamide which inhibits class I and IV HDAC isoforms. It has demonstrated dose-dependent inhibition of neoplastic growth in NSCLC xenograft models [245,246]. Mocetinostat also demonstrated synergistic cytotoxic effect when SCLC cell lines were exposed to mocetinostat combined with topoisomerase inhibitors [247]. This synergy is thought to be attributable to the interaction of HDAC1/2 with Topo II complexes. These preclinical findings led to subsequent evaluation in a phase I clinical trial. In this trial of 32 patients with solid tumors, including five with lung cancer, no objective responses were identified [248]. Five patients had stable disease, including one patient with lung cancer.

Mocetinostat has also been investigated in combination with cytotoxic agents and immunotherapy. A phase I clinical trial investigated the combination of mocetinostat with gemcitabine in the management of 25 patients with solid tumors [249], including two with lung cancer. Partial response was observed in two of 19 evaluable patients, but neither of the patients with lung cancer. A trial among NSCLC patients investigating the combination of nivolumab, an immune checkpoint inhibitor, with one of three agents, including mocetinostat, has completed recruiting (NCT02954991). One trial which aimed to evaluate the combination of mocetinostat with durvalumab, an immune checkpoint inhibitor, in patients with advanced solid tumors and NSCLC was terminated early (NCT02805660).

Vorinostat is a hydroxamic acid derivative that inhibits class I and II HDACs [250], and promotes cell cycle arrest and apoptosis. It has demonstrated antitumor activity against NSCLC cell lines alone [251], and increases carboplatin and paclitaxel activity against NSCLC cells when used in combination [252]. This synergy is likely due to vorinostat mediating an irreversible increase in DNA damage and a reversible increase in microtubule stability. These findings were brought forward to the clinical setting in several early phase trials. In a phase I trial of vorinostat with bortezomib in patients with solid tumors, one patient with NSCLC experienced a partial response [253]. However, when the combination was evaluated in a phase II trial of 18 patients with pretreated NSCLC, there were no objective responses and a 27.8% disease stability rate [254]. These included

TABLE 12.4 Clinical trials targeting the epigenome in lung cancer.

Drug	Epigenetic modifier class inhibitors	Additional drug	NCT/EU identifier	Status
Oral azacitidine	DNMT	Pembrolizumab Nab-paclitaxel, durvalumab Carboplatin or ABI-007 CC-223, erlotinib Romidepsin	NCT02546986 NCT02250326 NCT01478685 NCT01545947 NCT01537744	Active Active Completed Completed Completed
Azacitidine and entinostat	DNMT, HDAC	Nivolumab	NCT01928576 NCT01935947 NCT01886573	Recruiting Terminated Terminated
Azacitidine	DNMT	Pembrolizumab, epacadostat	NCT02959437	Terminated
Azacitidine (Vidaza)	DNMT		NCT02009436	Completed
Oral decitabine	DNMT	Nivolumab, tetrahydouridine Tetrahydouridine, pembrolizumab	NCT02664181 NCT03233724	Active Active
Decitabine	DNMT	MBG453 Genistein Valproic acid	NCT02608268 NCT01628471 NCT00084981	Active Completed Completed
Citarinostat	HDAC	Nivolumab	NCT02635061	Active
Entinostat	HDAC	Atezolizumab, carboplatin, etoposide Placebo Pembrolizumab Pembrolizumab	NCT04631029 NCT02897778 NCT02909452 NCT02437136	Active Completed Completed Unknown
Guadecitabine	DNMT	Pembrolizumab, mocetinostat Pembrolizumab Nivolumab, ipilimumab Carboplatin Tremelimumab, durvalumab	NCT03220477 NCT02998567 NCT04250246 NCT03913455 NCT03085849	Recruiting Active Not yet recruiting Active Completed
Mocetinostat	HDAC	Nivolumab, glesatinib, sitravatinib Durvalumab	NCT02954991 NCT02805660	Active Terminated
Panobinostat	HDAC	PDR001 PDR001 Pemetrexed Sorafenib Cisplatin, pemetrexed	NCT02890069 NCT03982134 NCT00907179 NCT01005797 NCT01336842	Active Terminated Terminated Completed Completed
Vorinostat	HDAC	Pembrolizumab Pembrolizumab Cisplatin, pemetrexed, radiation Iressa Paclitaxel, carboplatin	NCT02638090 NCT04357873 NCT01059552 NCT02151721 NCT01249443	Active Active Completed Unknown Terminated

TABLE 12.4 Clinical trials targeting the epigenome in lung cancer. *Continued*

Drug	Epigenetic modifier class inhibitors	Additional drug	NCT/EU identifier	Status
ORY-1001	Lysine demethylase	Etoposide	EUCTR2018-000469-35-ES	Authorized
Bomedemstat CC-90011	Lysine demethylase Lysine demethylase	Atezolizumab Cisplatin, etoposide, carboplatin, nivolumab Nivolumab	NCT05191797 NCT03850067 NCT04350463	Recruiting Recruiting
Tazemetostat	Histone methyltransferase		NCT03874455	Available
Abexinostat	HDAC	Pembrolizumab	NCT03590054	Active
MesomiR1	Mimic RNA		NCT02369198	Completed
Valproic acid	Histone acetylation	Vinorelbine, cisplatin	NCT01203735	Unknown status
Pivanex	HDAC		NCT00073385	Completed
Belinostat	HDAC	Cisplatin, etoposide	NCT00926640	Completed

a phase II randomized clinical trial which investigated the use of a combination of carboplatin, paclitaxel with either vorinostat or placebo among 94 patients with previously untreated NSCLC [255]. The use of vorinostat was associated with a significantly higher response rate (34 vs 12.5%) but no significant differences in progression-free survival (PFS) or OS. Vorinostat was associated with significantly higher levels of thrombocytopenia (18 vs 3%). However, when trialed as a single agent in a phase II study of 16 pretreated patients with NSCLC, no patients had objective responses [256].

Vorinostat has also been combined with targeted therapies, with the intention of re-sensitizing NSCLC cells to TKIs. In NSCLC cell lines with the C7979S EGFR mutation, typically resistant to EGFR TKIs, the addition of vorinostat to brigatinib significantly improved tumor sensitivity to the TKI [257]. In a phase I trial of NSCLC patients with EGFR mutations and TKI resistance mediated by BCL2-like-II (BIM) deletions, the use of vorinostat with gefitinib demonstrated a disease control rate of 83.3% and a median PFS of 5.2 months [258]. Two trials are currently recruiting to investigate the combination of vorinostat with pembrolizumab for patients with NSCLC and other tumors (NCT02638090 and NCT04357873).

Entinostat is a potent and selective inhibitor of class I and IV HDACs [259], which has been investigated for use in several malignancies, including breast [260] and lung cancers [261]. In three early phase trials of entinostat in patients with advanced solid malignancies, no NSCLC patients had objective responses, but all demonstrated histone acetylation in peripheral blood mononuclear cells (PBMCs) [261–263]. One of four NSCLC patients in one trial had disease stability for nine months. A phase II trial combined entinostat with erlotinib in the management of advanced NSCLC, with the rationale that entinostat can induce E-cadherin levels in NSCLC cells and sensitize them to EGFR-TKIs [264]. The combination did not improve 4-month PFS, the primary endpoint, but did improve OS in a subset with high E-cadherin levels (9.4 vs 5.4 months). Separately, a phase Ib/II single arm trial investigated the combination of entinostat and pembrolizumab in

patients with NSCLC [265]. This was guided by preclinical data supporting the role of HDACis in improving immune competency [266]. This did not meet the prespecified threshold for positivity in overall response rate (ORR), with 9.2% response, but benefit was greater in patients with elevated circulating monocytes at baseline. A further phase II trial recruiting at present, with a similar rationale to the previous, investigates the approach of epigenetic “priming” with entinostat and azacytidine (AZA), followed by nivolumab, in the management of advanced NSCLC (NCT01928576).

A pan-HDAC inhibitor, panobinostat, was investigated in a phase II trial of previously treated SCLC patients [267]. The study was prematurely closed due a lack of activity, although two patients had partial responses and three had prolonged stabilization of disease (10, 12, and 13 weeks).

Romidepsin, conversely, has approximately 10-fold specificity for HDAC class I and II versus HDAC class IV [268]. It was initially hypothesized that HDACi-induced chromatin relaxation would permit DNA damage with cytotoxic chemotherapy. However, this was not born out by a study involving the sequential exposing of SCLC cells to belinostat and romidepsin followed by cisplatin or etoposide [269]. Pretreatment with HDACis was not found to improve double-stranded DNA breaks, although simultaneous exposure of HDACis with cytotoxics had a synergistic effect. Romidepsin was also found to preferentially induce apoptosis in cultured lung cancer cells but not normal bronchial epithelia, when administered in sequence following azacytidine [270]. In the early phase clinical setting, romidepsin has been investigated in several trials. A phase I trial among patients with advanced or refractory neoplasms identified one partial response and eight with stable disease among 37 treated patients [271]. A trial of romidepsin in combination with AZA among 14 patients with solid tumors included one patient with NSCLC, but there were no objective responses [272]. A combination of erlotinib with romidepsin was investigated in a phase I trial of patients with pretreated advanced NSCLC, demonstrating disease control in seven of 10 evaluable patients but no objective responses [273]. It was found to increase histone acetylation status and inhibit EGFR phosphorylation. A further phase II trial of 19 NSCLC patients identified no objective responses but stable disease in nine patients [274]. Romidepsin was found to shift global gene expression profiles in these cells toward the profile of normal epithelium. Romidepsin was also investigated as a single agent in a phase II trial among patients with relapsed SCLC [275]. Among 16 patients, there were no objective responses and 3 had stable disease.

Pivanex (pivaloyloxymethyl butyrate) is a selective inhibitor of class I HDACs that has been demonstrated to inhibit clonogenicity of tumor cells in vitro, including NSCLC cells [276]. It was therefore trialed in the phase I setting among 28 patients with solid tumors. One patient, with squamous cell lung cancer, experienced a partial response [277]. A subsequent multicentre phase II trial evaluated Pivanex among 47 patients with refractory NSCLC [278]. Three patients (6%) had a partial response and 14 patients (30%) had stable disease for greater than 12 weeks, with median survival of 6.2 months. It has been hypothesized that the ability of Pivanex to induce tumor cell differentiation could be the basis for its association with prolonged survival but minimal regression.

5.2 DNA methyltransferase inhibitors

Hypermethylation of promoter regions is the most well-recognized of epigenetic changes to occur in tumors, present in most cancers and as previously described is associated with inappropriate transcriptional silencing of tumor suppressor genes [279]. The presence of DNA methylation is associated with shorter relapse free survival (RFS) in stage I NSCLC [280,281]. However, data regarding

the use of DNMTi in NSCLC is more limited than in the setting of HDACi use. In particular, the use of single agent DNMTis at doses sufficient for cytotoxicity is associated with complications, including prolonged cytopenias and subsequent loss of dose intensity [282], therefore much research in the area is preclinical or focused on combination therapy.

AZA is a cytosine analog, which substitutes nitrogen for carbon at the C5 position of the pyrimidine ring, that also inhibits DNMT and therefore prevents DNA methylation. Much preclinical research has addressed the optimal mechanism of drug delivery. In mouse models with NSCLC orthografts, it has been found that aerosolized [283] and intratracheal [284] delivery of AZA are associated with prolonged drug half-life and prolonged mouse survival respectively, compared to IV administration. In the case of intratracheal administration, the drug induced global demethylation of the epigenome (including lung cancer-associated genes, such as CXCL5, FOX, HOX, and GATA family transcription factors), at one-third of the comparable systemic dose.

AZA was combined with silibinin, a natural flavonolignan with anticancer efficacy, in a study of NSCLC cell lines [285]. Aggressive NSCLC with increased cell mobility is associated with repression of E-cadherin, and this drug combination was proposed as a means of modulating E-cadherin expression. This combination restored E-cadherin expression in NSCLC cells with an associated decrease in mobility and migratory potential. In this study, trichostatin A (TSA), a HDACi, was also combined with AZA and resulted in restored E-cadherin levels. AZA has been clinically investigated in at least 11 early phase trials of solid tumors, involving 573 patients overall, including 103 with lung cancer [286]. Among these, 8 (7.8%) had a response and 14 (13.6%) had stable disease.

5.3 BET inhibition

Bromodomain and Extra-Terminal domain (BET) proteins are epigenetic signaling factors, which can recognize acetylated lysine in chromatin and regulate lysine acetylation. BET inhibition is associated with suppression of B-cell lymphoma 2 (Bcl-2), MYC and MYCN expression [287]. In a preclinical study, 14 SCLC cell lines with expression of MYCL were exposed to JQ1, a BET inhibitor (BETi) [288]. Most SCLCs express MYCL, and MYCL is amplified in 10% of cases. All cell lines were found to be sensitive to JQ1 with growth inhibition and decreased expression of MYCL, MYCN and CDK6. By contrast, a second *in vitro* study also found SCLC cells to be sensitive to JQ1, but found this effect was mediated by regulation of achaete-scute family BHLH transcription factor 1 (ASCL1) gene expression and that there was no impact on MYC expression [289].

EGFR-TKI-mutant, osimertinib resistant NSCLC cell lines *in vitro* and in a xenograft mouse model [290] were subsequently examined in a preclinical study. BETs were found to be upregulated in osimertinib resistant cells compared to controls. The cancer cells were exposed to JQ1, TSA and vorinostat. JQ1 and TSA demonstrated synergistic growth-inhibitory effects, and downregulated BET and c-Myc expression. This may therefore represent a therapeutic approach for reversing osimertinib resistance. Conversely, Osimertinib combined with JQ1 has demonstrated efficacy against HER2-mutated NSCLC in a genetically modified mouse model [291].

5.4 EZH2 inhibitors

As mentioned previously, EZH2 is a lysine methyltransferase that is recurrently overexpressed in NSCLC and associated with a poor prognosis. It is associated with transcriptional silencing of

developmental gene networks. A study has demonstrated a causal role of EZH2 in NSCLC development, and demonstrated xenograft and mouse tumor regression in response to a novel EZH2 inhibitor (JQEZ5) [292]. It therefore represents a potential therapeutic approach for the NSCLC subset that overexpresses EZH2 without other driver mutations.

5.5 Histone demethylase inhibitors

Histone demethylase inhibitors (also known as lysine demethylase inhibitors) have attracted some interest in lung cancer research, and this has mostly focused on LSD1/KDM1A. As previously mentioned, its expression is upregulated in several tumor types, including NSCLC, where it is associated with a poor prognosis [293].

A cyclopropylamine-containing inhibitor of LSD1 has been shown to inhibit SCLC cell line growth *in vitro* and in mouse xenografts [294]. Those cell lines most susceptible, demonstrated DNA hypomethylation of a signature set of probes, which raises the possibility of using this as a biomarker of activity. In a similar study of an LSD1 inhibitor in NSCLC, investigators used the inhibitor HCl-2509 in LUAD cell lines and murine models [295]. Cell growth was significantly reduced, and it was demonstrated that the inhibitor interfered with EGFR downstream signaling. This was independent of underlying driver mutations.

The agent ORY-1001 (iademstat) is a potent and selective LSD1 inhibitor. It has been shown to suppress cell growth and induce apoptosis in NSCLC cell lines through controlling hexokinase expression [296]. A phase II trial investigated the combination of ORY-1001 with platinum-etoposide chemotherapy for patients with relapsed, extensive-stage SCLC [297]. Among 14 patients, 4 had partial response and 2 had prolonged stable disease. However, 11 had significant hematologic toxicity requiring dose adjustments and four patients died on trial.

5.6 Combination strategies

Combination approaches of epigenetic-targeted agents carry promise due to potential for greater effect with more limited toxicity. Several combinations of HDACi and DNMTi have been investigated.

AZA has been investigated in the preclinical setting with entinostat on KRAS/p53 comutated tumors in rats [298]. The combination was found to reduce tumor burden and cause epigenomic reprogramming, with increased expression of proapoptotic genes. AZA was combined with sodium phenylbutyrate [299] and sodium valproate [300] in phase I trials among solid tumor patients which included lung cancer. However, there was no clinical benefit among the lung cancer patients in these. The combination of AZA and entinostat brought promising results in a phase I/II trial of patients with recurrent NSCLC [301]. Among 42 patients, 1 had a complete response for 14 months, 1 had a partial response for 8 months, and 10 had stabilization of disease for at least 12 weeks. The median survival in the entire cohort was 6.4 months. Demethylation of 4 epigenetically silenced genes was detectable in blood samples, and was associated with significantly improved PFS and OS. In the case of the patient with a complete response, they had tumor-specific promoter hypermethylation in 3 of 4 genes in the primary tumor and nodes at baseline, typically associated with a poor prognosis, and then demethylation of all 3 with epigenetic therapy.

5-aza-2'-deoxycytidine (DAC), another DNMTi, has activity alone but its use has also been explored with several HDACis. It has demonstrated synergy with depsipeptide in inhibiting NSCLC proliferation in cell lines [302]. This inhibition was attributed to suppression of the removal of incorporated harmful nucleotide analogs from DNA. In addition, when a combination of DAC and sodium phenobutyrate was tested on NSCLC xenografts in mice, a significantly greater reduction in tumor bulk occurred than was seen with DAC alone [303]. Sodium phenobutyrate had no effect alone.

DAC was combined with valproate in the phase I setting for a trial of NSCLC patients, but the combination was unfortunately limited by dose-limiting neurotoxicity [304]. However, the combination was seen to reactivate hypermethylated genes.

6 Intratumor epigenetic heterogeneity and epigenetic therapies

Intratumor cellular heterogeneity (ITH) describes subpopulations of cancer cells with distinct phenotypic and molecular features within a single tumor [305]. These discrete populations can vary in motility, angiogenesis, proliferation and metastatic potential [306]. NSCLC is heterogeneous histologically, with such subtypes as AD and squamous cell carcinoma, but also heterogeneous molecularly, with diverse markers of differentiation [307]. ITH has been associated with poor outcomes. For example, hypoxic regions of tumor are more aggressive and less sensitive to treatment than normoxic regions [308].

An additional means of characterizing diversity in NSCLC is epigenetic heterogeneity. Epigenetic heterogeneity describes the epigenetic divergence of individual tumors (intertumor) or individual tumor cells (intratumor) from each other [309]. Therefore in genetically identical tumors, heterogeneity can instead be driven by diversity in transcriptional programs controlled by epigenetic regulators [310]. It has most commonly been analyzed by comparison of the methylome from spatially separated regions in the tumor [311], and techniques for this comparison have been refined in recent years with the introduction of the average pairwise ITH index (APITH) [312]. Intratumor epigenetic heterogeneity has been associated with shorter survival times in colorectal tumors [313], lymphomas [309] and also in LUADs [312].

ITH is also recognized as a driver for resistance mechanisms to cancer treatments in NSCLC, including therapies targeting EGFR, ALK and other molecular alterations [314]. This raises the question of what relevance intratumor epigenetic heterogeneity will have for the use of epigenetic-targeted therapies. It may offer new avenues of therapy, such as in reversing drug resistance. An interesting example is that of CSCs and other slowly replicating cell populations. These are less sensitive to traditional cytotoxic therapies targeting rapidly proliferating cells, which warrants an approach tailored to these subpopulations. HDACs are thought to be implicated in these resistance mechanisms. A study *in vitro* of chronic myelogenous leukemia stem cells demonstrated that a HDACi combined with imatinib could induce apoptosis in cells that were resistant to imatinib alone [315].

It is not only stem cells that can be targeted in this fashion. It has become clear that small populations of drug-tolerant cells, termed drug-tolerant persisters (DTPs), emerge in response to anticancer therapies in tumor cell lines. An altered chromatin state has been implicated in this persistence, which is driven by histone demethylase. These DTPs have been successfully suppressed with

HDACis, as was demonstrated in an EGFR-mutant NSCLC-derived cell line exposed to a HDACi [316]. In addition, in heterogeneous multiple myeloma, epigenetic therapy has been shown to reverse drug resistance [317].

However, it may also bring challenges. In the setting of ITH, it has been suggested that the existence of diverse subpopulations of cancer cells with varied resistance mechanisms will necessitate combination targeted approaches to effectively suppress tumor growth [318]. It is not yet clear what relevance epigenetic heterogeneity in lung cancer will have for epigenetic-targeted and combinatorial therapies, but there is clear potential for benefit.

7 Immunotherapy and epigenetics in lung cancer

The interface between epigenetics and immunotherapy is a promising and rapidly evolving field in lung cancer research. Epigenetics has relevance here due to the impact of epigenetic changes in tumor cells and immune cells; the potential of epigenetic modifications as biomarkers for immunotherapy; and the role of epigenetic-targeted drugs with immune checkpoint inhibitors (ICI) in cancer treatment.

Epigenetic changes in tumor cells can affect the growth of cancers. Hypermethylation of tumor suppressor genes, for example, is a common event leading to the development of tumors [319]. In addition, posttranslational modifications of histones control gene expression which underlies carcinogenesis [320]. Related to this, epigenetic mechanisms can affect antigen presentation. First, they can lead to re-expression of new antigens. For example, demethylation of cancer testis antigen (CTA) genes can lead to reactivation of CTAs on the surface of tumor cells, representing a potential target for ICI. Second, they can disrupt antigen presentation mechanisms, leading to impaired T-cell recognition of tumor cells. DNMT and HDAC can inhibit MHC class I expression, but this can be reversed through the use of DNMTi and HDACi [321].

Epigenetic changes can also alter immune cells. Among other changes, they can modulate immune cell chemokine expression to improve tumor infiltration. This was demonstrated in a study of various agents in combination with ICI on lung tumor models *in vitro* [175]. Romidepsin increased chemokine expression of multiple T-cell chemokines and enhanced response to ICI, including near-complete rejection in two models, as well as enhancing activation of tumor-infiltrating T cells. There are other mechanisms by which epigenetically targeted drugs can enhance immunity, including viral mimicry [322] and reversing T-cell exhaustion [323].

Given the significant role epigenetic changes have in cancer immunity, a natural question is whether there are identifiable epigenetic modifications which may serve as biomarkers. As they are typically blood samples and can be repeated at intervals, they represent an appealing, minimally invasive approach to diagnosing and monitoring malignancy. Several biomarkers have been proposed as potentially prognostic or predictive for ICI [324]. For example, CCL5 is a chemokine that is implicated in tumor-infiltrating lymphocyte infiltration in cancers. CCL5 is often epigenetically silenced, through DNA methylation but can be reactivated by decitabine. Its expression was associated with attenuated tumor growth in mice. A survival analysis subsequently performed on datasets correlated CCL5 expression with longer survival in lung cancer patients [325].

Finally, our understanding of the epigenetic changes wrought in tumor immune response has formed the basis for research into combinations of epigenetically targeted drugs with ICI in the

treatment of cancer. Preclinical studies were driven by the rationale that epigenetic agents could increase expression of immunomodulatory pathway genes and improve tumor antigen presentation. One study demonstrated that AZA use in NSCLC cell lines stimulated the expression of genes involved in antigen presentation and increased expression of PD-L1 [326]. They identified an “immune evasion” signature that recognized NSCLC subsets which may benefit from this AZA “priming” approach. The context for this study was an early phase trial of AZA in pretreated NSCLC [301]. While few responded to this treatment, it was found that a substantial number of those who progressed on AZA subsequently experienced a response to ICI. The preclinical work may provide a rationale for this response. A study of AZA with a CTLA-4 ICI in murine mesothelioma xenografts also identified antitumor activity [327].

In the clinical setting, a phase I/Ib trial investigated the combination of vorinostat with pembrolizumab among patients with ICI-naïve and ICI-resistant NCSLC [328]. Of 30 patients evaluable for response, 4 (13%) had partial response and 16 (53%) had stable disease. Among the pretreated cohort, 3 of 24 had partial response and 10 had stable disease. PD-L1 expression did not correlate with treatment benefit. This is being further investigated in a phase II clinical trial.

Another phase II trial investigated the combination of entinostat and pembrolizumab among patients with NSCLC previously treated with ICI [265]. Among 71 evaluable patients, 9% had a response, with median duration of response of 10.1 months. While greater benefit was seen among those with greater numbers of human leukocyte antigen-Dr isotype (HLA-Dr)_{hi} circulating classical monocytes at baseline, PD-L1 expression was once again not associated with benefit (although numbers were small). HLA-DR_{hi} is a primary antigen-presenting molecule of MHC class II, and therefore higher levels of HLA-DR_{hi} monocyte levels may represent a marker for sensitivity to this combination. As discussed in previous sections, there are numerous trials currently recruiting involving combinations of epigenetic agents and ICI in the management of lung malignancy.

8 Conclusions

Epigenetic regulation plays a clear and distinctive role in cancer initiation and progression. The approval of epigenetic-related drugs by the FDA demonstrates that targeting epigenetic alterations is a promising strategy for cancer treatment. The wealth of studies investigating the role of epigenetic modifiers in lung cancer has confirmed the role of these modifications in lung cancer proliferation, migration, and invasion. Both preclinical and early phase clinical studies have demonstrated antitumor effect with some of these epi-drugs in lung cancer. However, it is worth noting that much of the effect is achieved when these epi-drugs are employed in combination with other cancer therapeutic approaches, including chemotherapy. Finally, the evolving research investigating the interface between epigenetics and the immune microenvironment offers much promise of success stories involving the combination of epigenetic agents and immunotherapy for lung cancer patients.

References

- [1] Sung H, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71(3):209–49.

- [2] Rahib L, et al. Estimated projection of US cancer incidence and death to 2040. *JAMA Netw Open* 2021;4(4):e214708.
- [3] Darby S, et al. Radon in homes and risk of lung cancer: collaborative analysis of individual data from 13 European case-control studies. *BMJ* 2005;330(7485):223.
- [4] Howington JA, et al. Treatment of stage I and II non-small cell lung cancer: diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* 2013;143(5 Suppl):e278S–313S.
- [5] Consonni D, et al. Lung cancer prognosis before and after recurrence in a population-based setting. *J Natl Cancer Inst* 2015;107(6):djv059.
- [6] Barlesi F, et al. Routine molecular profiling of patients with advanced non-small-cell lung cancer: results of a 1-year nationwide programme of the French Cooperative Thoracic Intergroup (IFCT). *Lancet* 2016;387(10026):1415–26.
- [7] Reck M, et al. Five-year outcomes with pembrolizumab versus chemotherapy for metastatic non-small-cell lung cancer with PD-L1 tumor proportion score ≥ 50 . *J Clin Oncol* 2021;39(21):2339–49.
- [8] Gandhi L, et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. *N Engl J Med* 2018;378(22):2078–92.
- [9] Jasulionis MG. Abnormal epigenetic regulation of immune system during aging. *Front Immunol* 2018;9:197.
- [10] Yang Y, Wang Y. Role of epigenetic regulation in plasticity of tumor immune microenvironment. *Front Immunol* 2021;12:1013.
- [11] Bezu L, et al. Immunological effects of epigenetic modifiers. *Cancers* 2019;11(12):1911.
- [12] Al-Yozbaki M, et al. Targeting DNA methyltransferases in non-small-cell lung cancer. *Semin Cancer Biol* 2021.
- [13] Lin R-K, et al. Alteration of DNA methyltransferases contributes to 5' CpG methylation and poor prognosis in lung cancer. *Lung Cancer* 2007;55(2):205–13.
- [14] Xing J, et al. Expression of methylation-related genes is associated with overall survival in patients with non-small cell lung cancer. *Br J Cancer* 2008;98(10):1716–22.
- [15] Tang Y-A, et al. MDM2 overexpression deregulates the transcriptional control of RB/E2F leading to DNA methyltransferase 3A overexpression in lung cancer. *Clin Cancer Res* 2012;18(16):4325–33.
- [16] Wei W, et al. MicroRNA-9 enhanced radiosensitivity and its mechanism of DNA methylation in non-small cell lung cancer. *Gene* 2019;710:178–85.
- [17] Vallböhmer D, et al. DNA methyltransferases messenger RNA expression and aberrant methylation of CpG islands in non-small-cell lung cancer: association and prognostic value. *Clin Lung Cancer* 2006;8(1):39–44.
- [18] Issa J-PJ, et al. Increased cytosine DNA-methyltransferase activity during colon cancer progression. *J Natl Cancer Inst* 1993;85(15):1235–40.
- [19] Zhang J, et al. DNA methyltransferases in cancer: biology, paradox, aberrations, and targeted therapy. *Cancers* 2020;12(8):2123.
- [20] Tang H, et al. Upregulation of SPP1 is a marker for poor lung cancer prognosis and contributes to cancer progression and cisplatin resistance. *Front Cell Dev Biol* 2021;9:1109.
- [21] Sui C, et al. miR-148b reverses cisplatin-resistance in non-small cell cancer cells via negatively regulating DNA (cytosine-5)-methyltransferase 1 (DNMT1) expression. *J Transl Med* 2015;13(1):1–9.
- [22] Wei D, Yu G, Zhao Y. MicroRNA-30a-3p inhibits the progression of lung cancer via the PI3K/AKT by targeting DNA methyltransferase 3a. *OncoTargets Ther* 2019;12:7015–24.
- [23] Husni RE, et al. DNMT3a expression pattern and its prognostic value in lung adenocarcinoma. *Lung Cancer* 2016;97:59–65.
- [24] Weng RR, et al. Epigenetic modulation of immune synaptic-cytoskeletal networks potentiates $\gamma\delta$ T cell-mediated cytotoxicity in lung cancer. *Nat Commun* 2021;12(1):1–18.

- [25] Kim H, et al. Elevated mRNA levels of DNA methyltransferase-1 as an independent prognostic factor in primary nonsmall cell lung cancer. *Cancer* 2006;107(5):1042–9.
- [26] Sui C, et al. miR-148b reverses cisplatin-resistance in non-small cell cancer cells via negatively regulating DNA (cytosine-5)-methyltransferase 1(DNMT1) expression. *J Transl Med* 2015;13:132.
- [27] Tang H, et al. Upregulation of SPP1 is a marker for poor lung cancer prognosis and contributes to cancer progression and cisplatin resistance. *Front Cell Dev Biol* 2021;9:646390.
- [28] Zhao Z, Shilatifard A. Epigenetic modifications of histones in cancer. *Genome Biol* 2019;20(1):1–16.
- [29] Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res* 2011;21(3):381–95.
- [30] Neganova ME, et al. Histone modifications in epigenetic regulation of cancer: Perspectives and achieved progress. *Semin Cancer Biol* 2020.
- [31] Song JS, et al. Global histone modification pattern associated with recurrence and disease-free survival in non-small cell lung cancer patients. *Pathol Int* 2012;62(3):182–90.
- [32] Morgan MA, Shilatifard A. Reevaluating the roles of histone-modifying enzymes and their associated chromatin modifications in transcriptional regulation. *Nat Genet* 2020;52(12):1271–81.
- [33] Mohan M, Herz H-M, Shilatifard A. Snapshot: chromatin lysine methylase complexes: transcriptional regulation and epigenetics. *Cell* 2012;149(2):498.
- [34] Simon JA, Lange CA. Roles of the EZH2 histone methyltransferase in cancer epigenetics. *Mutat Res* 2008;647(1–2):21–9.
- [35] Matsubara T, et al. The association and prognostic impact of enhancer of zeste homologue 2 expression and epithelial-mesenchymal transition in resected lung adenocarcinoma. *PLoS One* 2019;14(5):e0215103.
- [36] Geng J, et al. EZH2 promotes tumor progression via regulating VEGF-A/AKT signaling in non-small cell lung cancer. *Cancer Lett* 2015;359(2):275–87.
- [37] Huqun, et al. Enhancer of zeste homolog 2 is a novel prognostic biomarker in nonsmall cell lung cancer. *Cancer* 2012;118(6):1599–606.
- [38] Nutt SL, et al. EZH2 function in immune cell development. *Biol Chem* 2020;401(8):933–43.
- [39] Yao Y, et al. Downregulation of enhancer of zeste homolog 2 (EZH2) is essential for the induction of autophagy and apoptosis in colorectal cancer cells. *Genes* 2016;7(10):83.
- [40] Ito T, et al. Regulation of cellular senescence by polycomb chromatin modifiers through distinct DNA damage-and histone methylation-dependent pathways. *Cell Rep* 2018;22(13):3480–92.
- [41] Batoor A, Jin C, Liu Y-X. Role of EZH2 in cell lineage determination and relative signaling pathways. *Front Biosci* 2019;24:947–60.
- [42] Hoy SM. Tazemetostat: first approval. *Drugs* 2020;80(5):513–21.
- [43] Ishikawa R, et al. Enhancer of zeste homolog 2 is a novel prognostic biomarker in nonsmall cell lung cancer. *Cancer* 2012;118(6):1599–606.
- [44] Gardner EE, et al. Chemosensitive relapse in small cell lung cancer proceeds through an EZH2-SLFN11 axis. *Cancer Cell* 2017;31(2):286–99.
- [45] Eich M-L, et al. EZH2-targeted therapies in cancer: hype or a reality. *Cancer Res* 2020;80(24):5449–58.
- [46] Wang D, et al. Targeting EZH2 reprograms intratumoral regulatory T cells to enhance cancer immunity. *Cell Rep* 2018;23(11):3262–74.
- [47] Goswami S, et al. Modulation of EZH2 expression in T cells improves efficacy of anti-CTLA-4 therapy. *J Clin Investig* 2018;128(9):3813–18.
- [48] Zhao Y, et al. EZH2 regulates PD-L1 expression via HIF-1 α in non-small cell lung cancer cells. *Biochem Biophys Res Commun* 2019;517(2):201–9.
- [49] Bledau AS, et al. The H3K4 methyltransferase Setd1a is first required at the epiblast stage, whereas Setd1b becomes essential after gastrulation. *Development* 2014;141(5):1022–35.

- [50] Clouaire T, et al. Cfp1 integrates both CpG content and gene activity for accurate H3K4me3 deposition in embryonic stem cells. *Genes Dev* 2012;26(15):1714–28.
- [51] Wu M, et al. Molecular regulation of H3K4 trimethylation by Wdr82, a component of human Set1/COMPASS. *Mol Cell Biol* 2008;28(24):7337–44.
- [52] Li Z, et al. The histone methyltransferase SETD1A regulates thrombomodulin transcription in vascular endothelial cells. *Biochim Biophys Acta Gene Regul Mech* 2018;1861(8):752–61.
- [53] Du M, et al. Histone methyltransferase SETD1A participates in lung cancer progression. *Thorac Cancer* 2021;12(16):2247–57.
- [54] Kang J-Y, et al. The H3K4 methyltransferase SETD1A is required for proliferation of non-small cell lung cancer cells by promoting S-phase progression. *Biochem Biophys Res Commun* 2021;561:120–7.
- [55] Wang R, et al. An SETD1A/Wnt/β-catenin feedback loop promotes NSCLC development. *J Exp Clin Cancer Res* 2021;40(1):318.
- [56] Bosgana P, et al. H3K4 methylation status and lysine specific methyltransferase KMT2C expression correlate with prognosis in lung adenocarcinoma. *Curr Mol Pharmacol* 2021;14(6):1028–36.
- [57] Mastoraki S, et al. KMT2C promoter methylation in plasma-circulating tumor DNA is a prognostic biomarker in non-small cell lung cancer. *Mol Oncol* 2021;15(9):2412–22.
- [58] Augert A, et al. Small cell lung cancer exhibits frequent inactivating mutations in the histone methyltransferase KMT2D/MLL2: CALGB 151111 (Alliance). *J Thorac Oncol* 2017;12(4):704–13.
- [59] Alam H, et al. KMT2D deficiency impairs super-enhancers to confer a glycolytic vulnerability in lung cancer. *Cancer Cell* 2020;37(4):599–617.e7.
- [60] Sun QY, et al. SETDB1 accelerates tumourigenesis by regulating the WNT signalling pathway. *J Pathol* 2015;235(4):559–70.
- [61] Lafuente-Sanchis A, et al. Prognostic value of ERCC1, RRM1, BRCA1 and SETDB1 in early stage of non-small cell lung cancer. *Clin Transl Oncol* 2016;18(8):798–804.
- [62] Mazur PK, et al. SMYD3 links lysine methylation of MAP3K2 to Ras-driven cancer. *Nature* 2014;510(7504):283–7.
- [63] Li J, et al. SMYD3 overexpression indicates poor prognosis and promotes cell proliferation, migration and invasion in non-small cell lung cancer. *Int J Oncol* 2020;57(3):756–66.
- [64] Huang T, et al. G9A promotes tumor cell growth and invasion by silencing CASP1 in non-small-cell lung cancer cells. *Cell Death Dis* 2017;8(4):e2726.
- [65] Liu S, Tian W, Li B. Integrative bioinformatics analysis the clinical value of KMT5A in different subtypes of lung cancer. *Comput Biol Chem* 2022;96:107603.
- [66] Piao L, et al. SETD8 is a prognostic biomarker that contributes to stem-like cell properties in non-small cell lung cancer. *Pathol Res Pract* 2020;216(12):153258.
- [67] Cao L, et al. Downregulation of SETD7 promotes migration and invasion of lung cancer cells via JAK2/STAT3 pathway. *Int J Mol Med* 2020;45(5):1616–26.
- [68] Yang X, et al. Methyltransferase SETD2 inhibits tumor growth and metastasis via STAT1-IL-8 signaling-mediated epithelial-mesenchymal transition in lung adenocarcinoma. *Cancer Sci* 2022;113(4):1195–207.
- [69] Hudlebusch HR, et al. The histone methyltransferase and putative oncoprotein MMSET is overexpressed in a large variety of human tumors. *Clin Cancer Res* 2011;17(9):2919–33.
- [70] Toyokawa G, et al. Histone lysine methyltransferase Wolf-Hirschhorn syndrome candidate 1 is involved in human carcinogenesis through regulation of the Wnt pathway. *Neoplasia* 2011;13(10):887.
- [71] Kang D, et al. The histone methyltransferase Wolf–Hirschhorn syndrome candidate 1-like 1 (WHSC1L1) is involved in human carcinogenesis. *Genes Chromosomes Cancer* 2013;52(2):126–39.
- [72] Mahmood SF, et al. PPAPDC1B and WHSC1L1 are common drivers of the 8p11–12 amplicon, not only in breast tumors but also in pancreatic adenocarcinomas and lung tumors. *Am J Pathol* 2013;183(5):1634–44.

- [73] Kuo C-H, et al. Lung tumor-associated dendritic cell-derived resistin promoted cancer progression by increasing Wolf–Hirschhorn syndrome candidate 1/Twist pathway. *Carcinogenesis* 2013;34(11):2600–9.
- [74] Galván-Femenía I, et al. Genomic profiling in advanced stage non-small-cell lung cancer patients with platinum-based chemotherapy identifies germline variants with prognostic value in SMYD2. *Cancer Treat Res Commun* 2018;15:21–31.
- [75] Wu L, et al. SMYD2 promotes tumorigenesis and metastasis of lung adenocarcinoma through RPS7. *Cell Death Dis* 2021;12(5):439.
- [76] Kim W, et al. Deficiency of H3K79 histone methyltransferase Dot1-like protein (DOT1L) inhibits cell proliferation. *J Biol Chem* 2012;287(8):5588–99.
- [77] Yoon K-A, et al. Novel polymorphisms in the SUV39H2 histone methyltransferase and the risk of lung cancer. *Carcinogenesis* 2006;27(11):2217–22.
- [78] Yoon K-A, et al. Genetic polymorphisms in the Rb-binding zinc finger gene RIZ and the risk of lung cancer. *Carcinogenesis* 2007;28(9):1971–7.
- [79] Lee J, et al. EHMT1 knockdown induces apoptosis and cell cycle arrest in lung cancer cells by increasing CDKN1A expression. *Mol Oncol* 2021;15(11):2989–3002.
- [80] Sengupta D, et al. NSD2 dimethylation at H3K36 promotes lung adenocarcinoma pathogenesis. *Mol Cell* 2021;81(21):4481–4492.e9.
- [81] Walter DM, et al. Systematic in vivo inactivation of chromatin-regulating enzymes identifies Setd2 as a potent tumor suppressor in lung adenocarcinoma. *Cancer Res* 2017;77(7):1719–29.
- [82] Zhou Y, et al. Histone methyltransferase SETD2 inhibits tumor growth via suppressing CXCL1-mediated activation of cell cycle in lung adenocarcinoma. *Aging (Albany NY)* 2020;12(24):25189–206.
- [83] Daks A, et al. Set7/9 controls proliferation and genotoxic drug resistance of NSCLC cells. *Biochem Biophys Res Commun* 2021;572:41–8.
- [84] Wagner KW, et al. KDM2A promotes lung tumorigenesis by epigenetically enhancing ERK1/2 signaling. *J Clin Investig* 2013;123(12):5231–46.
- [85] Teng Y-C, et al. Histone demethylase RBP2 promotes lung tumorigenesis and cancer metastasis. *Cancer Res* 2013;73(15):4711–21.
- [86] Wang S, et al. RBP2 induces epithelial-mesenchymal transition in non-small cell lung cancer. *PLoS One* 2013;8(12):e84735.
- [87] Lv T, et al. Over-expression of LSD1 promotes proliferation, migration and invasion in non-small cell lung cancer. *PLoS One* 2012;7(4):e35065.
- [88] Mallette FA, Richard S. JMJD2A promotes cellular transformation by blocking cellular senescence through transcriptional repression of the tumor suppressor CHD5. *Cell Rep* 2012;2(5):1233–43.
- [89] Hayami S, et al. Overexpression of the JmjC histone demethylase KDM5B in human carcinogenesis: involvement in the proliferation of cancer cells through the E2F/RB pathway. *Mol Cancer* 2010;9(1):1–14.
- [90] Hong Y, Li X, Zhu J. LSD1-mediated stabilization of SEPT6 protein activates the TGF- β 1 pathway and regulates non-small-cell lung cancer metastasis. *Cancer Gene Ther* 2022;29(2):189–201.
- [91] Liu Q, et al. TdIF1-LSD1 axis regulates epithelial—mesenchymal transition and metastasis via histone demethylation of E-cadherin promoter in lung cancer. *Int J Mol Sci* 2021;23(1):250.
- [92] Soini Y, Kosma V-M, Pirinen R. KDM4A, KDM4B and KDM4C in non-small cell lung cancer. *Int J Clin Exp Pathol* 2015;8(10):12922.
- [93] Jiang K, et al. miR-150 promotes the proliferation and migration of non-small cell lung cancer cells by regulating the SIRT2/JMJD2A signaling pathway. *Oncol Rep* 2018;40(2):943–51.
- [94] Hao F. Systemic profiling of KDM5 subfamily signature in non-small-cell lung cancer. *Int J Gen Med* 2021;14:7259.

- [95] Kuo K-T, et al. Histone demethylase JARID1B/KDM5B promotes aggressiveness of non-small cell lung cancer and serves as a good prognostic predictor. *Clin Epigenetics* 2018;10(1):1–12.
- [96] Wang F, Quan Q. The long non-coding RNA SNHG4/microRNA-let-7e/KDM3A/p21 pathway is involved in the development of non-small cell lung cancer. *Mol Ther Oncolytics* 2021;20:634–45.
- [97] Terashima M, et al. Epigenetic regulation of epithelial-mesenchymal transition by KDM6A histone demethylase in lung cancer cells. *Biochem Biophys Res Commun* 2017;490(4):1407–13.
- [98] Lee YM, et al. Epigenetic role of histone lysine methyltransferase and demethylase on the expression of transcription factors associated with the epithelial-to-mesenchymal transition of lung adenocarcinoma metastasis to the brain. *Cancers* 2020;12(12):3632.
- [99] Lachat C, et al. EZH2 and KDM6B expressions are associated with specific epigenetic signatures during EMT in non small cell lung carcinomas. *Cancers* 2020;12(12):3649.
- [100] Li S, et al. The prognostic significance of JMJD3 in primary sarcomatoid carcinoma of the lung, a rare subtype of lung cancer. *OncoTargets Ther* 2019;12:9385.
- [101] Ge T, Zhou Y, Lu H. The diagnostic performance of lysine (K)-specific demethylase 6B (KDM6B) in non-small cell lung cancer. *Artif Cells Nanomed Biotechnol* 2019;47(1):2155–60.
- [102] Xu L, Wu H, Hu X. Histone demethylase KDM5A enhances cell proliferation, induces EMT in lung adenocarcinoma cells, and have a strong causal association with paclitaxel resistance. *Acta Biochim Pol* 2021;68(4):593–602.
- [103] Zhang Q, et al. KDM5C expedites lung cancer growth and metastasis through epigenetic regulation of microRNA-133a. *OncoTargets Ther* 2021;14:1187.
- [104] Zhan M, et al. JMJD1A promotes tumorigenesis and forms a feedback loop with EZH2/let-7c in NSCLC cells. *Tumour Biol* 2016;37(8):11237–47.
- [105] Cao Y, et al. Lysine-specific demethylase 2 contributes to the proliferation of small cell lung cancer by regulating the expression of TFPI-2. *Mol Med Rep* 2018;18(1):733–40.
- [106] Wanna-Udom S, et al. KDM2B is involved in the epigenetic regulation of TGF- β -induced epithelial–mesenchymal transition in lung and pancreatic cancer cell lines. *J Biol Chem* 2021;296.
- [107] Hu Y, Mu H, Yang Y. Histone demethylase PHF8 promotes cell growth and metastasis of non-small-cell lung cancer through activating Wnt/ β -catenin signaling pathway. *Histol Histopathol* 2021;18349.
- [108] Li N, Jiang D. Jumonji domain containing 2C promotes cell migration and invasion through modulating CUL4A expression in lung cancer. *Biomed Pharmacother* 2017;89:305–15.
- [109] Wu X, et al. Histone demethylase KDM4C activates HIF1 α /VEGFA signaling through the costimulatory factor STAT3 in NSCLC. *Am J Cancer Res* 2020;10(2):491.
- [110] Toyokawa G, et al. The prognostic impact of Jumonji domain-containing 2B in patients with resected lung adenocarcinoma. *Anticancer Res* 2016;36(9):4841–6.
- [111] He L, et al. PRMT1 is critical to FEN1 expression and drug resistance in lung cancer cells. *DNA Repair (Amst)* 2020;95:102953.
- [112] Yoshimatsu M, et al. Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers. *Int J Cancer* 2011;128(3):562–73.
- [113] Gu Z, et al. Protein arginine methyltransferase 5 is essential for growth of lung cancer cells. *Biochem J* 2012;446(2):235–41.
- [114] Zhang S, et al. Targeting PRMT5/Akt signalling axis prevents human lung cancer cell growth. *J Cell Mol Med* 2019;23(2):1333–42.
- [115] Avasarala S, et al. PRMT6 promotes lung tumor progression via the alternate activation of tumor-associated macrophages. *Mol Cancer Res* 2020;18(1):166–78.
- [116] Tang J, et al. PRMT6 serves an oncogenic role in lung adenocarcinoma via regulating p18. *Mol Med Rep* 2020;22(4):3161–72.

- [117] Cheng D, et al. PRMT7 contributes to the metastasis phenotype in human non-small-cell lung cancer cells possibly through the interaction with HSPA5 and EEF2. *Oncotargets Ther* 2018;11:4869.
- [118] Wu D, et al. CARM1 promotes non-small cell lung cancer progression through upregulating CCNE2 expression. *Aging (Albany NY)* 2020;12(11):10578–93.
- [119] Litt M, Qiu Y, Huang S. Histone arginine methylations: their roles in chromatin dynamics and transcriptional regulation. *Biosci Rep* 2009;29(2):131–41.
- [120] Shi Y, et al. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 2004;119(7):941–53.
- [121] Fang R, et al. Human LSD2/KDM1b/AOF1 regulates gene transcription by modulating intragenic H3K4me2 methylation. *Mol Cell* 2010;39(2):222–33.
- [122] Metzger E, et al. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* 2005;437(7057):436–9.
- [123] Hayami S, et al. Overexpression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers. *Int J Cancer* 2011;128(3):574–86.
- [124] Lim S-Y, et al. LSD1 modulates the non-canonical integrin β 3 signaling pathway in non-small cell lung carcinoma cells. *Sci Rep* 2017;7(1):1–12.
- [125] Cloos PA, et al. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes Dev* 2008;22(9):1115–40.
- [126] Dhar SS, et al. Transcriptional repression of histone deacetylase 3 by the histone demethylase KDM2A is coupled to tumorigenicity of lung cancer cells. *J Biol Chem* 2014;289(11):7483–96.
- [127] Li J, et al. The molecular landscape of histone lysine methyltransferases and demethylases in non-small cell lung cancer. *Int J Med Sci* 2019;16(7):922.
- [128] Shen X, et al. JARID1B modulates lung cancer cell proliferation and invasion by regulating p53 expression. *Tumor Biol* 2015;36(9):7133–42.
- [129] Leng X, et al. Histone 3 lysine-27 demethylase KDM6A coordinates with KMT2B to play an oncogenic role in NSCLC by regulating H3K4me3. *Oncogene* 2020;39(41):6468–79.
- [130] Hu S, et al. MicroRNA-449a delays lung cancer development through inhibiting KDM3A/HIF-1 α axis. *J Transl Med* 2021;19(1):1–11.
- [131] Chan JC, Maze I. Nothing is yet set in (hi) stone: novel post-translational modifications regulating chromatin function. *Trends Biochem Sci* 2020;45(10):829–44.
- [132] Marmorstein R, Zhou M-M. Writers and readers of histone acetylation: structure, mechanism, and inhibition. *Cold Spring Harb Perspect Biol* 2014;6(7):a018762.
- [133] Bannister AJ, Kouzarides T. The CBP co-activator is a histone acetyltransferase. *Nature* 1996;384(6610):641–3.
- [134] Ogryzko VV, et al. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 1996;87(5):953–9.
- [135] Gao Y, et al. Expression of p300 and CBP is associated with poor prognosis in small cell lung cancer. *Int J Clin Exp Pathol* 2014;7(2):760.
- [136] Hou X, et al. p300 promotes proliferation, migration, and invasion via inducing epithelial-mesenchymal transition in non-small cell lung cancer cells. *BMC Cancer* 2018;18(1):641.
- [137] Li X, et al. GATA3 acetylation at K119 by CBP inhibits cell migration and invasion in lung adenocarcinoma. *Biochem Biophys Res Commun* 2018;497(2):633–8.
- [138] Gorgoulis VG, et al. Transcription factor E2F-1 acts as a growth-promoting factor and is associated with adverse prognosis in non-small cell lung carcinomas. *J Pathol* 2002;198(2):142–56.
- [139] Kishimoto M, et al. Mutations and deletions of the CBP gene in human lung cancer. *Clin Cancer Res* 2005;11(2):512–19.

- [140] Zhao L, et al. Histone acetyltransferase hMOF promotes S phase entry and tumorigenesis in lung cancer. *Cell Signal* 2013;25(8):1689–98.
- [141] Li N, et al. hMOF reduction enhances radiosensitivity through the homologous recombination pathway in non-small-cell lung cancer. *Onco Targets Ther* 2019;12:3065–75.
- [142] Van Den Broeck A, et al. Activation of a Tip60/E2F1/ERCC1 network in human lung adenocarcinoma cells exposed to cisplatin. *Carcinogenesis* 2012;33(2):320–5.
- [143] Liang Z, et al. Tip60-siRNA regulates ABCE1 acetylation to suppress lung cancer growth via activation of the apoptotic signaling pathway. *Exp Ther Med* 2019;17(4):3195–202.
- [144] Yang Y, et al. Tat-interactive protein-60KDA (TIP60) regulates the tumorigenesis of lung cancer in vitro. *J Cancer* 2017;8(12):2277–81.
- [145] Cai D, et al. Steroid receptor coactivator-3 expression in lung cancer and its role in the regulation of cancer cell survival and proliferation. *Cancer Res* 2010;70(16):6477–85.
- [146] Chen Y, et al. Elevated SRC3 expression predicts pemetrexed resistance in lung adenocarcinoma. *Biomed Pharmacother* 2020;125:109958.
- [147] Xiao Y, et al. Ku80 cooperates with CBP to promote COX-2 expression and tumor growth. *Oncotarget* 2015;6(10):8046–61.
- [148] Chen L, et al. Lysine acetyltransferase GCN5 potentiates the growth of non-small cell lung cancer via promotion of E2F1, cyclin D1, and cyclin E1 expression. *J Biol Chem* 2013;288(20):14510–21.
- [149] Li T, et al. DDT3 and KAT2A proteins regulate TNFRSF10A and TNFRSF10B expression in endoplasmic reticulum stress-mediated apoptosis in human lung cancer cells. *J Biol Chem* 2015;290(17):11108–18.
- [150] Simó-Riudalbas L, et al. KAT6B is a tumor suppressor histone H3 lysine 23 acetyltransferase undergoing genomic loss in small cell lung cancer. *Cancer Res* 2015;75(18):3936–45.
- [151] Sasaki H, et al. Histone deacetylase 1 mRNA expression in lung cancer. *Lung Cancer* 2004;46(2):171–8.
- [152] Zhang L, et al. HDAC1 knockdown inhibits invasion and induces apoptosis in non-small cell lung cancer cells. *Biol Chem* 2018;399(6):603–10.
- [153] Dong ZY, Zhou YR, Wang LX. HDAC1 is indirectly involved in the epigenetic regulation of p38 MAPK that drive the lung cancer progression. *Eur Rev Med Pharmacol Sci* 2018;22(18):5980–6.
- [154] Cai SX, et al. Roles of HDAC2, eIF5, and eIF6 in lung cancer tumorigenesis. *Curr Med Sci* 2021;41(4):764–9.
- [155] Minamiya Y, et al. Strong expression of HDAC3 correlates with a poor prognosis in patients with adenocarcinoma of the lung. *Tumor Biol* 2010;31(5):533–9.
- [156] Jin K, et al. MiR-520b restrains cell growth by targeting HDAC4 in lung cancer. *Thorac Cancer* 2018;9(10):1249–54.
- [157] Osada H, et al. Reduced expression of class II histone deacetylase genes is associated with poor prognosis in lung cancer patients. *Int J Cancer* 2004;112(1):26–32.
- [158] Lei Y, et al. Hdac7 promotes lung tumorigenesis by inhibiting Stat3 activation. *Mol Cancer* 2017;16(1):170.
- [159] Okudela K, et al. Expression of HDAC9 in lung cancer—potential role in lung carcinogenesis. *Int J Clin Exp Pathol* 2014;7(1):213.
- [160] Yang Y, et al. HDAC10 promotes lung cancer proliferation via AKT phosphorylation. *Oncotarget* 2016;7(37):59388–401.
- [161] Liu X, et al. HDAC10 is positively associated with PD-L1 expression and poor prognosis in patients with NSCLC. *Front Oncol* 2020;10:485.
- [162] Bora-Singhal N, et al. Novel HDAC11 inhibitors suppress lung adenocarcinoma stem cell self-renewal and overcome drug resistance by suppressing Sox2. *Sci Rep* 2020;10(1):1–20.

- [163] Grbesa I, et al. Expression of sirtuin 1 and 2 is associated with poor prognosis in non-small cell lung cancer patients. *PLoS One* 2015;10(4):e0124670.
- [164] Gong J, et al. Associations of sirtuins with clinicopathological parameters and prognosis in non-small cell lung cancer. *Cancer Manag Res* 2018;10:3341–56.
- [165] Liu L, et al. E3 ubiquitin ligase HRD1 promotes lung tumorigenesis by promoting sirtuin 2 ubiquitination and degradation. *Mol Cell Biol* 2020;40(7).
- [166] Cao K, et al. Sirt3 promoted DNA damage repair and radioresistance through ATM-Chk2 in non-small cell lung cancer cells. *J Cancer* 2021;12(18):5464–72.
- [167] Xiong Y, et al. SIRT3 is correlated with the malignancy of non-small cell lung cancer. *Int J Oncol* 2017;50(3):903–10.
- [168] Zhu B, et al. Downregulation of SIRT6 is associated with poor prognosis in patients with non-small cell lung cancer. *J Int Med Res* 2018;46(4):1517–27.
- [169] Krishnamoorthy V, Vilwanathan R. Silencing Sirtuin 6 induces cell cycle arrest and apoptosis in non-small cell lung cancer cell lines. *Genomics* 2020;112(5):3703–12.
- [170] Bai L, et al. Upregulation of SIRT6 predicts poor prognosis and promotes metastasis of non-small cell lung cancer via the ERK1/2/MMP9 pathway. *Oncotarget* 2016;7(26):40377–86.
- [171] Sang Y, et al. Histone deacetylase 7 inhibits plakoglobin expression to promote lung cancer cell growth and metastasis. *Int J Oncol* 2019;54(3):1112–22.
- [172] Yang X-J, Seto E. Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol Cell* 2008;31(4):449–61.
- [173] Wang L, et al. Depletion of HDAC6 enhances cisplatin-induced DNA damage and apoptosis in non-small cell lung cancer cells. *PLoS One* 2012.
- [174] Hu C, et al. The USP10-HDAC6 axis confers cisplatin resistance in non-small cell lung cancer lacking wild-type p53. *Cell Death Dis* 2020;11(5):1–18.
- [175] Zheng H, et al. HDAC inhibitors enhance T-cell chemokine expression and augment response to PD-1 immunotherapy in lung adenocarcinoma. *Clin Cancer Res* 2016;22(16):4119–32.
- [176] Adeegbe DO, et al. Synergistic immunostimulatory effects and therapeutic benefit of combined histone deacetylase and bromodomain inhibition in non–small cell lung cancer. *Cancer Discov* 2017;7(8):852–67.
- [177] Bag A, et al. Coupling the immunomodulatory properties of the HDAC6 inhibitor ACY241 with Oxaliplatin promotes robust anti-tumor response in non-small cell lung cancer. *Oncoimmunology* 2022;11(1):2042065.
- [178] Cao Y, et al. SIRT3 promotion reduces resistance to cisplatin in lung cancer by modulating the FOXO3/CDT1 axis. *Cancer Med* 2021;10(4):1394–404.
- [179] Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* 2010;79:351–79.
- [180] O'Brien J, et al. Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Front Endocrinol* 2018;9:402.
- [181] Hou Y, et al. MiR-101-3p regulates the viability of lung squamous carcinoma cells via targeting EZH2. *J Cell Biochem* 2017;118(10):3142–9.
- [182] Zhang G, Wu YJ, Yan F. MicroRNA-130-5p promotes invasion as well as migration of lung adenocarcinoma cells by targeting the EZH2 signaling pathway. *Eur Rev Med Pharmacol Sci* 2019;23(21):9480–8.
- [183] Wu J, et al. Decreased miR-124 contributes to the epithelial-mesenchymal transition phenotype formation of lung adenocarcinoma cells via targeting enhancer of zeste homolog 2. *Pathol Res Pract* 2020;216(6):152976.

- [184] Chen L, et al. MiR-92b inhibits proliferation and invasion of lung cancer by targeting EZH2. *Eur Rev Med Pharmacol Sci* 2020;24:3166–73.
- [185] Sun J, et al. MicroRNA-4465 suppresses tumor proliferation and metastasis in non-small cell lung cancer by directly targeting the oncogene EZH2. *Biomed Pharmacother* 2017;96:1358–62.
- [186] Chen J, et al. MiRNA-26a contributes to the acquisition of malignant behaviors of doxorubicin-resistant lung adenocarcinoma cells through targeting EZH2. *Cell Physiol Biochem* 2017;41(2):583–97.
- [187] Tan M, Wu J, Cai Y. Suppression of Wnt signaling by the miR-29 family is mediated by demethylation of WIF-1 in non-small-cell lung cancer. *Biochem biophys Res Commun* 2013;438(4):673–9.
- [188] Chen B, et al. A regulatory circuitry comprising TP53, miR-29 family, and SETDB1 in non-small cell lung cancer. *Biosci Rep* 2018;38(5).
- [189] Fabbri M, et al. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci U S A* 2007;104(40):15805–10.
- [190] Wu X, et al. MicroRNA-708-5p acts as a therapeutic agent against metastatic lung cancer. *Oncotarget* 2016;7(3):2417.
- [191] Liu T, et al. Downregulation of DNMT3A by miR-708-5p inhibits lung cancer stem cell-like phenotypes through repressing Wnt/β-catenin signaling. *Clin Cancer Res* 2018;24(7):1748–60.
- [192] Zhang X, et al. Oncogene LSD1 is epigenetically suppressed by miR-137 overexpression in human non-small cell lung cancer. *Biochimie* 2017;137:12–19.
- [193] Guan Y, Rao Z, Chen C. miR-30a suppresses lung cancer progression by targeting SIRT1. *Oncotarget* 2018;9(4):4924–34.
- [194] Sun C, et al. MicroRNA-125a-5p modulates radioresistance in LTED-a2 non-small cell lung cancer cells by targeting SIRT7. *Cancer Biomark* 2020;27(1):39–49.
- [195] Guan Y, et al. Long noncoding RNA CBR3 antisense RNA 1 promotes the aggressive phenotypes of non-small-cell lung cancer by sponging microRNA-509-3p and competitively upregulating HDAC9 expression. *Oncol Rep* 2020;44(4):1403–14.
- [196] Saleh R, et al. Role of epigenetic modifications in inhibitory immune checkpoints in cancer development and progression. *Front Immunol* 2020;11:1469.
- [197] Cortez MA, et al. PDL1 regulation by p53 via miR-34. *J Natl Cancer Inst* 2016;108(1).
- [198] Zhang ZW, et al. Long intergenic non-protein coding RNA 319 aggravates lung adenocarcinoma carcinogenesis by modulating miR-450b-5p/EZH2. *Gene* 2018;650:60–7.
- [199] Zhang L, Fang F, He X. Long noncoding RNA TP73-AS1 promotes non-small cell lung cancer progression by competitively sponging miR-449a/EZH2. *Biomed Pharmacother* 2018;104:705–11.
- [200] Liu JQ, et al. linc01088 promotes cell proliferation by scaffolding EZH2 and repressing p21 in human non-small cell lung cancer. *Life Sci* 2020;241:117134.
- [201] Wang X, et al. Long intergenic non-coding RNA 00467 promotes lung adenocarcinoma proliferation, migration and invasion by binding with EZH2 and repressing HTRA3 expression. *Mol Med Rep* 2019;20(1):640–54.
- [202] Yuan C, et al. Copy number amplification-activated long non-coding RNA LINC00662 epigenetically inhibits BIK by interacting with EZH2 to regulate tumorigenesis in non-small cell lung cancer. *J Cancer* 2022;13(5):1640–51.
- [203] Niu Y, et al. Long non-coding RNA TUG1 is involved in cell growth and chemoresistance of small cell lung cancer by regulating LIMK2b via EZH2. *Mol Cancer* 2017;16(1):5.
- [204] Kang X, et al. LncRNA MIR210HG promotes proliferation and invasion of non-small cell lung cancer by upregulating methylation of CACNA2D2 promoter via binding to DNMT1. *Onco Targets Ther* 2019;12:3779–90.
- [205] Zhang X, et al. Long noncoding RNA LINC00337 accelerates the non-small-cell lung cancer progression through inhibiting TIMP2 by recruiting DNMT1. *Am J Transl Res* 2019;11(9):6075–83.

- [206] Ma F, et al. LncRNA NEAT1 interacted with DNMT1 to regulate malignant phenotype of cancer cell and cytotoxic T cell infiltration via epigenetic inhibition of p53, cGAS, and STING in lung cancer. *Front Genet* 2020;11:250.
- [207] Wu J, et al. LncRNA APCDD1L-AS1 induces icotinib resistance by inhibition of EGFR autophagic degradation via the miR-1322/miR-1972/miR-324-3p-SIRT5 axis in lung adenocarcinoma. *Biomark Res* 2021;9(1):9.
- [208] Stepanov I, et al. Carcinogenic tobacco-specific N-nitrosamines in US cigarettes: three decades of remarkable neglect by the tobacco industry. *Tob Control* 2012;21(1):44–8.
- [209] Zong D, et al. The role of cigarette smoke-induced epigenetic alterations in inflammation. *Epigenetics Chromatin* 2019;12(1):1–25.
- [210] Adcock IM, et al. Epigenetic regulation of airway inflammation. *Curr Op Immunol* 2007;19(6):694–700.
- [211] Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010;31(1):27–36.
- [212] Joubert BR, et al. DNA methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. *Am J Hum Genet* 2016;98(4):680–96.
- [213] Lin R-K, et al. The tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation and tumor suppressor gene hypermethylation in mice and lung cancer patients. *J Clin Investig* 2010;120(2):521–32.
- [214] Seiler CL, et al. Inhalation exposure to cigarette smoke and inflammatory agents induces epigenetic changes in the lung. *Sci Rep* 2020;10(1):1–15.
- [215] Hudlikar RR, et al. Tobacco carcinogen 4-[methyl (nitroso) amino]-1-(3-pyridinyl)-1-butanone (NNK) drives metabolic rewiring and epigenetic reprogramming in A/J mice lung cancer model and prevention with diallyl sulphide (DAS). *Carcinogenesis* 2022;43(2):140–9.
- [216] Tessema M, et al. Genome-wide unmasking of epigenetically silenced genes in lung adenocarcinoma from smokers and never smokers. *Carcinogenesis* 2014;35(6):1248–57.
- [217] Tessema M, et al. Concomitant promoter methylation of multiple genes in lung adenocarcinomas from current, former and never smokers. *Carcinogenesis* 2009;30(7):1132–8.
- [218] Tan Q, et al. Epigenomic analysis of lung adenocarcinoma reveals novel DNA methylation patterns associated with smoking. *OncoTargets Ther* 2013;6:1471.
- [219] Henderson DW, Leigh J. The history of asbestos utilization and recognition of asbestos-induced diseases. In: Dodson RF, Hammar SP, editors. *Asbestos: risk assessment, epidemiology and health effects*. 2nd ed. Taylor Francis; 2011, p. 1–22.
- [220] Alberg AJ, et al. Epidemiology of lung cancer: diagnosis and management of lung cancer: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* 2013;143(5):e1S–e29S.
- [221] Ospina D, et al. Analyzing biological and molecular characteristics and genomic damage induced by exposure to asbestos. *Cancer Manag Res* 2019;11:4997.
- [222] Santarelli L, et al. Four-miRNA signature to identify asbestos-related lung malignancies. *Cancer Epidemiol Prev Biomarkers* 2019;28(1):119–26.
- [223] Nymark P, et al. Integrative analysis of microRNA, mRNA and aCGH data reveals asbestos-and histology-related changes in lung cancer. *Genes Chromosomes Cancer* 2011;50(8):585–97.
- [224] Pervez Hussain S, Harris CC. Inflammation and cancer: an ancient link with novel potentials. *Int J cancer* 2007;121(11):2373–80.
- [225] Kettunen E, et al. Asbestos-associated genome-wide DNA methylation changes in lung cancer. *Int J Cancer* 2017;141(10):2014–29.
- [226] Dammann R, et al. CpG island methylation and expression of tumour-associated genes in lung carcinoma. *Eur J cancer* 2005;41(8):1223–36.

- [227] Gu C, et al. Association between MGMT promoter methylation and non-small cell lung cancer: a meta-analysis. *PLoS One* 2013;8(9):e72633.
- [228] Krewski D, et al. Residential radon and risk of lung cancer: a combined analysis of 7 North American case-control studies. *Epidemiology* 2005;137–45.
- [229] Wu J, et al. Effects of radon on miR-34a-induced apoptosis in human bronchial epithelial BEAS-2B cells. *J Toxicol Environ Health A* 2019;82(16):913–19.
- [230] Bush ZM, et al. Temozolomide treatment for aggressive pituitary tumors: correlation of clinical outcome with O6-methylguanine methyltransferase (MGMT) promoter methylation and expression. *J Clin Endocrinol Metab* 2010;95(11):E280–90.
- [231] Bastide K, et al. Molecular analysis of the Ink4a/Rb1–Arf/Tp53 pathways in radon-induced rat lung tumors. *Lung Cancer* 2009;63(3):348–53.
- [232] Belinsky SA, et al. Plutonium targets the p16 gene for inactivation by promoter hypermethylation in human lung adenocarcinoma. *Carcinogenesis* 2004;25(6):1063–7.
- [233] Nie J, et al. Expression profiles of long non-coding RNA in mouse lung tissue exposed to radon. *J Toxicol Environ Health A* 2019;82(15):854–61.
- [234] Mok TS. Personalized medicine in lung cancer: what we need to know. *Nat Rev Clin Oncol* 2011;8(11):661–8.
- [235] Fukuoka M, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). *J Clin Oncol* 2011;29(21):2866–74.
- [236] Soria JC, et al. Osimertinib in untreated EGFR-mutated advanced non-small-cell lung cancer. *N Engl J Med* 2018;378(2):113–25.
- [237] Solomon BJ, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N Engl J Med* 2014;371(23):2167–77.
- [238] Peters S, et al. Alectinib versus crizotinib in untreated ALK-positive non-small-cell lung cancer. *N Engl J Med* 2017;377(9):829–38.
- [239] Quintas-Cardama A, Santos FP, Garcia-Manero G. Therapy with azanucleosides for myelodysplastic syndromes. *Nat Rev Clin Oncol* 2010;7(8):433–44.
- [240] O'Connor OA, et al. Belinostat in patients with relapsed or refractory peripheral T-cell lymphoma: results of the pivotal phase II BELIEF (CLN-19) study. *J Clin Oncol* 2015;33(23):2492–9.
- [241] Mann BS, et al. Vorinostat for treatment of cutaneous manifestations of advanced primary cutaneous T-cell lymphoma. *Clin Cancer Res* 2007;13(8):2318–22.
- [242] Whittaker SJ, et al. Final results from a multicenter, international, pivotal study of romidepsin in refractory cutaneous T-cell lymphoma. *J Clin Oncol* 2010;28(29):4485–91.
- [243] Stein EM, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. *Blood* 2017;130(6):722–31.
- [244] DiNardo CD, et al. Durable remissions with ivosidenib in IDH1-mutated relapsed or refractory AML. *N Engl J Med* 2018;378(25):2386–98.
- [245] Beckers T, et al. Distinct pharmacological properties of second generation HDAC inhibitors with the benzamide or hydroxamate head group. *Int J Cancer* 2007;121(5):1138–48.
- [246] Fournel M, et al. MGCD0103, a novel isotype-selective histone deacetylase inhibitor, has broad spectrum antitumor activity in vitro and in vivo. *Mol Cancer Ther* 2008;7(4):759–68.
- [247] Gray J, et al. Combination of HDAC and topoisomerase inhibitors in small cell lung cancer. *Cancer Biol Ther* 2012;13(8):614–22.
- [248] Siu LL, et al. Phase I study of MGCD0103 given as a three-times-per-week oral dose in patients with advanced solid tumors. *J Clin Oncol* 2008;26(12):1940–7.

- [249] Chan E, et al. Phase I/II study of mocetinostat in combination with gemcitabine for patients with advanced pancreatic cancer and other advanced solid tumors. *Cancer Chemother Pharmacol* 2018;81(2):355–64.
- [250] Ramalingam SS, et al. Phase I and pharmacokinetic study of vorinostat, a histone deacetylase inhibitor, in combination with carboplatin and paclitaxel for advanced solid malignancies. *Clin Cancer Res* 2007;13(12):3605–10.
- [251] Komatsu N, et al. SAHA, a HDAC inhibitor, has profound anti-growth activity against non-small cell lung cancer cells. *Oncol Rep* 2006;15(1):187–91.
- [252] Owonikoko TK, et al. Vorinostat increases carboplatin and paclitaxel activity in non-small-cell lung cancer cells. *Int J Cancer* 2010;126(3):743–55.
- [253] Schelman WR, et al. A phase I study of vorinostat in combination with bortezomib in patients with advanced malignancies. *Invest New Drugs* 2013;31(6):1539–46.
- [254] Hoang T, et al. Vorinostat and bortezomib as third-line therapy in patients with advanced non-small cell lung cancer: a Wisconsin Oncology Network Phase II study. *Invest New Drugs* 2014;32(1):195–9.
- [255] Ramalingam SS, et al. Carboplatin and Paclitaxel in combination with either vorinostat or placebo for first-line therapy of advanced non-small-cell lung cancer. *J Clin Oncol* 2010;28(1):56–62.
- [256] Traynor AM, et al. Vorinostat (NSC# 701852) in patients with relapsed non-small cell lung cancer: a Wisconsin Oncology Network phase II study. *J Thorac Oncol* 2009;4(4):522–6.
- [257] Lin CY, et al. Vorinostat combined with brigatinib overcomes acquired resistance in EGFR-C797S-mutated lung cancer. *Cancer Lett* 2021;508:76–91.
- [258] Takeuchi S, et al. Phase I study of vorinostat with gefitinib in BIM deletion polymorphism/epidermal growth factor receptor mutation double-positive lung cancer. *Cancer Sci* 2020;111(2):561–70.
- [259] Connolly RM, Rudek MA, Piekarz R. Entinostat: a promising treatment option for patients with advanced breast cancer. *Future Oncol* 2017;13(13):1137–48.
- [260] Connolly RM, et al. Combination epigenetic therapy in advanced breast cancer with 5-azacitidine and entinostat: a phase II National Cancer Institute/stand up to cancer study. *Clin Cancer Res* 2017;23(11):2691–701.
- [261] Ryan QC, et al. Phase I and pharmacokinetic study of MS-275, a histone deacetylase inhibitor, in patients with advanced and refractory solid tumors or lymphoma. *J Clin Oncol* 2005;23(17):3912–22.
- [262] Kummar S, et al. Phase I trial of MS-275, a histone deacetylase inhibitor, administered weekly in refractory solid tumors and lymphoid malignancies. *Clin Cancer Res* 2007;13(18 Pt 1):5411–17.
- [263] Gore L, et al. A phase I and pharmacokinetic study of the oral histone deacetylase inhibitor, MS-275, in patients with refractory solid tumors and lymphomas. *Clin Cancer Res* 2008;14(14):4517–25.
- [264] Witta SE, et al. Randomized phase II trial of erlotinib with and without entinostat in patients with advanced non-small-cell lung cancer who progressed on prior chemotherapy. *J Clin Oncol* 2012;30(18):2248–55.
- [265] Hellmann MD, et al. Entinostat plus pembrolizumab in patients with metastatic NSCLC previously treated with anti-PD-(L)1 therapy. *Clin Cancer Res* 2021;27(4):1019–28.
- [266] Topper MJ, et al. Epigenetic therapy ties MYC depletion to reversing immune evasion and treating lung cancer. *Cell* 2017;171(6):1284–1300.e21.
- [267] de Marinis F, et al. A phase II study of the histone deacetylase inhibitor panobinostat (LBH589) in pre-treated patients with small-cell lung cancer. *J Thorac Oncol* 2013;8(8):1091–4.
- [268] Bieliauskas AV, Pflum MK. Isoform-selective histone deacetylase inhibitors. *Chem Soc Rev* 2008;37(7):1402–13.
- [269] Luchenko VL, et al. Schedule-dependent synergy of histone deacetylase inhibitors with DNA damaging agents in small cell lung cancer. *Cell Cycle* 2011;10(18):3119–28.

- [270] Weiser TS, et al. Sequential 5-Aza-2 deoxycytidine-depsipeptide FR901228 treatment induces apoptosis preferentially in cancer cells and facilitates their recognition by cytolytic T lymphocytes specific for NY-ESO-1. *J Immunother* 2001;24(2):151–61.
- [271] Sandor V, et al. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. *Clin Cancer Res* 2002;8(3):718–28.
- [272] Gaillard SL, et al. A phase 1 trial of the oral DNA methyltransferase inhibitor CC-486 and the histone deacetylase inhibitor romidepsin in advanced solid tumors. *Cancer* 2019;125(16):2837–45.
- [273] Gerber DE, et al. Phase 1 study of romidepsin plus erlotinib in advanced non-small cell lung cancer. *Lung Cancer* 2015;90(3):534–41.
- [274] Schrump DS, et al. Clinical and molecular responses in lung cancer patients receiving Romidepsin. *Clin Cancer Res* 2008;14(1):188–98.
- [275] Otterson GA, et al. Phase II study of the histone deacetylase inhibitor Romidepsin in relapsed small cell lung cancer (Cancer and Leukemia Group B 30304). *J Thorac Oncol* 2010;5(10):1644–8.
- [276] Siu LL, et al. Activity of pivaloyloxymethyl butyrate, a novel anticancer agent, on primary human tumor colony-forming units. *Invest New Drugs* 1998;16(2):113–19.
- [277] Patnaik A, et al. A phase I study of pivaloyloxymethyl butyrate, a prodrug of the differentiating agent butyric acid, in patients with advanced solid malignancies. *Clin Cancer Res* 2002;8(7):2142–8.
- [278] Reid T, et al. Phase II trial of the histone deacetylase inhibitor pivaloyloxymethyl butyrate (Pivanex, AN-9) in advanced non-small cell lung cancer. *Lung Cancer* 2004;45(3):381–6.
- [279] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349(21):2042–54.
- [280] Brock MV, et al. DNA methylation markers and early recurrence in stage I lung cancer. *N Engl J Med* 2008;358(11):1118–28.
- [281] Sandoval J, et al. A prognostic DNA methylation signature for stage I non-small-cell lung cancer. *J Clin Oncol* 2013;31(32):4140–7.
- [282] Forde PM, Brahmer JR, Kelly RJ. New strategies in lung cancer: epigenetic therapy for non-small cell lung cancer. *Clin Cancer Res* 2014;20(9):2244–8.
- [283] Reed MD, et al. Aerosolised 5-azacytidine suppresses tumour growth and reprogrammes the epigenome in an orthotopic lung cancer model. *Br J Cancer* 2013;109(7):1775–81.
- [284] Mahesh S, et al. Intratracheally administered 5-azacytidine is effective against orthotopic human lung cancer xenograft models and devoid of important systemic toxicity. *Clin Lung Cancer* 2010;11(6):405–11.
- [285] Mateen S, et al. Silibinin synergizes with histone deacetylase and DNA methyltransferase inhibitors in upregulating E-cadherin expression together with inhibition of migration and invasion of human non-small cell lung cancer cells. *J Pharmacol Exp Ther* 2013;345(2):206–14.
- [286] Cowan LA, Talwar S, Yang AS. Will DNA methylation inhibitors work in solid tumors? A review of the clinical experience with azacitidine and decitabine in solid tumors. *Epigenomics* 2010;2(1):71–86.
- [287] Fu LL, et al. Inhibition of BET bromodomains as a therapeutic strategy for cancer drug discovery. *Oncotarget* 2015;6(8):5501–16.
- [288] Kato F, et al. MYCL is a target of a BET bromodomain inhibitor, JQ1, on growth suppression efficacy in small cell lung cancer cells. *Oncotarget* 2016;7(47):77378–88.
- [289] Lenhart R, et al. Sensitivity of small cell lung cancer to BET inhibition is mediated by regulation of ASCL1 gene expression. *Mol Cancer Ther* 2015;14(10):2167–74.
- [290] Meng Y, et al. Trichostatin A downregulates bromodomain and extra-terminal proteins to suppress osimertinib resistant non-small cell lung carcinoma. *Cancer Cell Int* 2021;21(1):216.
- [291] Liu S, et al. Targeting HER2 aberrations in non-small cell lung cancer with osimertinib. *Clin Cancer Res* 2018;24(11):2594–604.

- [292] Zhang H, et al. Oncogenic deregulation of EZH2 as an opportunity for targeted therapy in lung cancer. *Cancer Discov* 2016;6(9):1006–21.
- [293] Lim SY, et al. LSD1 modulates the non-canonical integrin beta3 signaling pathway in non-small cell lung carcinoma cells. *Sci Rep* 2017;7(1):10292.
- [294] Mohammad HP, et al. A DNA hypomethylation signature predicts antitumor activity of LSD1 inhibitors in SCLC. *Cancer Cell* 2015;28(1):57–69.
- [295] Macheleidt IF, et al. Preclinical studies reveal that LSD1 inhibition results in tumor growth arrest in lung adenocarcinoma independently of driver mutations. *Mol Oncol* 2018;12(11):1965–79.
- [296] Lu Z, et al. ORY-1001 suppresses cell growth and induces apoptosis in lung cancer through triggering HK2 mediated Warburg effect. *Front Pharmacol* 2018;9:1411.
- [297] Navarro A, et al. Final safety and efficacy data from CLEPSIDRA trial in 2L ED-SCLC. In: ESMO Congress 2020, Madrid; 2020.
- [298] Belinsky SA, et al. Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. *Cancer Res* 2011;71(2):454–62.
- [299] Lin J, et al. A phase I dose-finding study of 5-azacytidine in combination with sodium phenylbutyrate in patients with refractory solid tumors. *Clin Cancer Res* 2009;15(19):6241–9.
- [300] Braiteh F, et al. Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. *Clin Cancer Res* 2008;14(19):6296–301.
- [301] Juergens RA, et al. Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer Discov* 2011;1(7):598–607.
- [302] Chai G, et al. HDAC inhibitors act with 5-aza-2'-deoxycytidine to inhibit cell proliferation by suppressing removal of incorporated abases in lung cancer cells. *PLoS One* 2008;3(6):e2445.
- [303] Belinsky SA, et al. Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. *Cancer Res* 2003;63(21):7089–93.
- [304] Chu BF, et al. Phase I study of 5-aza-2'-deoxycytidine in combination with valproic acid in non-small-cell lung cancer. *Cancer Chemother Pharmacol* 2013;71(1):115–21.
- [305] Bedard PL, et al. Tumour heterogeneity in the clinic. *Nature* 2013;501(7467):355–64.
- [306] Almendro V, Marusyk A, Polyak K. Cellular heterogeneity and molecular evolution in cancer. *Annu Rev Pathol* 2013;8:277–302.
- [307] de Sousa VML, Carvalho L. Heterogeneity in lung cancer. *Pathobiology* 2018;85(1–2):96–107.
- [308] Terry S, Buart S, Chouaib S. Hypoxic stress-induced tumor and immune plasticity, suppression, and impact on tumor heterogeneity. *Front Immunol* 2017;8:1625.
- [309] Dominguez PM, Teater M, Shaknovich R. The new frontier of epigenetic heterogeneity in B-cell neoplasms. *Curr Opin Hematol* 2017;24(4):402–8.
- [310] Dawson MA. The cancer epigenome: concepts, challenges, and therapeutic opportunities. *Science* 2017;355(6330):1147–52.
- [311] Ryser MD, et al. Epigenetic heterogeneity in human colorectal tumors reveals preferential conservation and evidence of immune surveillance. *Sci Rep* 2018;8(1):17292.
- [312] Hua X, et al. Genetic and epigenetic intratumor heterogeneity impacts prognosis of lung adenocarcinoma. *Nat Commun* 2020;11(1):2459.
- [313] Martinez-Cardus A, et al. Epigenetic homogeneity within colorectal tumors predicts shorter relapse-free and overall survival times for patients with locoregional cancer. *Gastroenterology* 2016;151(5):961–72.
- [314] Gregorc V, et al. Intratumoral cellular heterogeneity: implications for drug resistance in patients with non-small cell lung cancer. *Cancers (Basel)* 2021;13(9).
- [315] Zhang B, et al. Effective targeting of quiescent chronic myelogenous leukemia stem cells by histone deacetylase inhibitors in combination with imatinib mesylate. *Cancer Cell* 2010;17(5):427–42.

- [316] Sharma SV, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010;141(1):69–80.
- [317] Issa ME, et al. Epigenetic strategies to reverse drug resistance in heterogeneous multiple myeloma. *Clin Epigenetics* 2017;9:17.
- [318] Pribluda A, de la Cruz CC, Jackson EL. Intratumoral heterogeneity: from diversity comes resistance. *Clin Cancer Res* 2015;21(13):2916–23.
- [319] Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002;21(35):5427–40.
- [320] Liu Z, et al. A new trend in cancer treatment: the combination of epigenetics and immunotherapy. *Front Immunol* 2022;13:809761.
- [321] Luo N, et al. DNA methyltransferase inhibition upregulates MHC-I to potentiate cytotoxic T lymphocyte responses in breast cancer. *Nat Commun* 2018;9(1):248.
- [322] Chiappinelli KB, et al. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. *Cell* 2015;162(5):974–86.
- [323] Ghoneim HE, et al. De novo epigenetic programs inhibit PD-1 blockade-mediated T cell rejuvenation. *Cell* 2017;170(1):142–157.e19.
- [324] Villanueva L, Alvarez-Errico D, Esteller M. The contribution of epigenetics to cancer immunotherapy. *Trends Immunol* 2020;41(8):676–91.
- [325] Dangaj D, et al. Cooperation between constitutive and inducible chemokines enables T cell engraftment and immune attack in solid tumors. *Cancer Cell* 2019;35(6):885–900.e10.
- [326] Wrangle J, et al. Alterations of immune response of non-small cell lung cancer with azacytidine. *Oncotarget* 2013;4(11):2067–79.
- [327] Covre A, et al. Antitumor activity of epigenetic immunomodulation combined with CTLA-4 blockade in syngeneic mouse models. *Oncobiology* 2015;4(8):e1019978.
- [328] Gray JE, et al. Phase I/Ib study of pembrolizumab plus vorinostat in advanced/metastatic non-small cell lung cancer. *Clin Cancer Res* 2019;25(22):6623–32.

Breast cancer epigenetics

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1 Introduction

Breast cancer tumorigenesis has been long acknowledged to relate to the accumulation of genetic mutations which may result in oncogene activation and tumor suppressor gene inactivation.

Reversible alterations in histone proteins and deoxyribonucleic acid (DNA) are now recognized to also facilitate tumorigenesis, and “nonmutational epigenetic reprogramming” has been recently described as an emerging hallmark of cancer after decades of research in this space [1,2].

Epigenetic regulation is critical for normal cell growth and development, but is also associated with pathological processes, including autoimmune diseases and cancer [3]. Epigenetics refers to alterations in gene expression that are distinct from changes in the primary nucleotide sequence of a gene, and may result in changes in chromatin structure which is comprised of DNA and histone proteins. Changes in chromatin structure can lead to a repressive state and silence both gene expression and transcription of DNA into ribonucleic acid (RNA). Epigenetic alterations include both histone hypoacetylation and abnormal methylation of DNA in the promoter region of important genes [4]. These alterations affect DNA accessibility and histone function which ultimately affect cellular functions, such as DNA replication, repair, and RNA transcription. Given reversal of genetic mutations is a challenging anticancer treatment strategy, modulation of epigenetic mechanisms is an attractive target for cancer therapeutics [5,6].

This chapter summarizes current knowledge regarding epigenetic modifications associated with breast cancer, including histone modification, DNA methylation, and microRNAs. The potential role of classes of drugs which aim to reverse these alterations, so called “epigenetic modifiers” is also discussed, with a particular focus on the intersection between the fields of epigenetics and immunology. Completed and ongoing clinical trials which incorporate epigenetic modifiers alone or as combination strategies in breast cancer are described, as well as the challenges in investigating these agents in patients with solid tumors.

2 Epigenetic alterations in breast cancer

2.1 Histone modification

Chromatin is chromosomal DNA coiled around structural proteins called histones which maintain the shape and structure of chromatin. Posttranslational alterations, such as acetylation, and methylation, take place at the amino acid tail of histones leading to conformational changes in chromatin which lead to changes in transcription of important genes, such as tumor suppressors. Acetylation and methylation are relatively stable and carried over through multiple cell cycles, in addition phosphorylation is a posttranslational modification which can be more transient [7].

Histone acetylation is controlled by histone acetyltransferases (HAT) which add an acetyl group to histones leading to uncoiling or “opening” of the chromatin, facilitating gene transcription. Another regulatory enzyme histone deacetylases (HDAC), remove acetyl groups from the histones leading to coiling or “closing” of chromatin which inhibits transcription [8]. HDACs are known to play an instrumental role in expression of genes important for cell survival, proliferation, differentiation, and apoptosis [9]. HDACs are also present as part of protein complexes responsible for recruitment of transcription factors to the promoter region of genes, including those of tumor suppressors, and regulation of acetylation status of specific cell cycle regulatory proteins [10]. Alterations of HDAC expression, such as increased HDAC expression, and decreased histone acetylation have been observed in cancer with associated transcriptional repression of genes and as such,

inhibition of HDACs via novel drug development has become an active area of investigation in cancer therapeutics [11].

Epigenetic alterations are prevalent in breast cancers, prompting much interest in their clinical significance and whether these can be manipulated. These alterations can include aberrant histone marks, which refer to specific differences in the number of modifications at specific sites, but can also include varied expression of histone modification enzymes HDACs and HATs which in turn control patterns of histone modifications [2]. One example of a histone modification present in many breast cancers is a reduction in trimethylation of H4K20me3 [12]. An example of aberrant HDAC activity associated with breast cancer include alterations of Class I and class II HDACs resulting in overexpression of HDACs that lead to modifications in cell cycle proliferation, apoptosis and estrogen receptor (ER) signaling in breast cancer [13,14]. Specifically, *HDAC 1* expression has been associated with advanced stage and aggressive histology in certain cancer types [15]. *HDAC 1* expression as identified in breast cancer biopsy samples has, however, been associated with estrogen receptor (ER) and progesterone receptor (PR) expression, earlier stage of disease at diagnosis, and improved survival outcomes [16]. Finally, *HDAC6* messenger RNA (mRNA) is more frequently expressed in patients with breast cancer less than 2 cm in size, low grade, and with ER and PR-positivity. Multivariate analyses have, however, failed to confirm that *HDAC6* expression was an independent prognostic factor for survival [17].

2.2 DNA methylation

Adenine, guanine, cytosine and thymine are the four bases which are the building blocks of our genetic make-up. DNA methyltransferase enzymes (DNMTs) add a methyl group ($-CH_3$) to the pyrimidine ring of cytosine to form methyl cytosine (DNA methylation) which can result in gene silencing when methylation occurs at the promoter region of genes and is a heritable epigenetic mechanism regulating gene expression [9]. Of the five DNMT proteins that have been discovered in mammals; DNMT1, DNMT3a, and DNMT3b have catalytic methyltransferase activity. DNMT1 targets hemi-methylated DNA as a substrate [18], whereas the DNMT3 enzymes are considered *de novo* methyltransferases, thus targeting unmethylated DNA [19].

This process of DNA methylation occurs where cytosines precede guanine in the DNA sequence; the CpG dinucleotide. These CpG dinucleotides are present throughout the genome and are usually heavily methylated (~70%) and thus prevent transcription of genes at those sites [20]. CpG islands have been defined as a number of dinucleotides at the promoter regions of genes. In normal tissue CpG islands are more commonly unmethylated, facilitating gene transcription. In cancer, the reverse can be seen with abnormal DNA methylation or hypermethylation of CpG islands that impede transcription of important genes, such as tumor suppressor genes [21]. In many cancers, breast cancer included, CpG methylation patterns can serve as prognostic biomarkers [22,23]. In addition, synthetic CpG islands can be used to elicit an immune response and are currently under investigation both in isolation and conjugated to other drugs and antibodies to promote an antitumor immune response [24]. Numerous preclinical investigations have led to clinical trials utilizing this as a strategy to improve response to immune therapies in breast cancer [25].

When considering aberrant DNA methylation in breast cancer, global DNA hypomethylation is more prevalent in breast cancer specimens compared to other tumor types [26]. This global hypomethylation has been associated with poor prognostic factors, including tumor size, stage, and

grade [27]. DNA hypomethylation can also affect individual breast cancer genes [28]. Breast cancer-related genes are more commonly hypermethylated and thus silenced, when compared to noncancerous tissue. There are a number of methylated tumor suppressor genes important for critical process, such as regulation of cell cycle (p14ARF, p16INK4a, p57KIP2 cyclin D2, $14 - 3 - 3\sigma$), growth (RAR β , SYK, RASSF1A, TGF β R-II, HIN1, SOCS1, NES1, SFRP1, WIF1), hormone receptors (ER α , ER β , PR), evasion of apoptosis (DAPK1, APC, HOXA5, HIC1, TWIST, TMS1), DNA repair (GSTP1, BRCA1, MGMT), invasion and metastasis (CDH1, CDH13, APC, prostasin, BCSG1, TIMP-3), as well as cell differentiation (e.g., *RARbeta*) [20,29]. Similar to alterations in enzymes that control histone modifications, alterations in enzymes that control DNA methylation are associated with breast carcinogenesis. For example, DNA methyltransferase 1 is overexpressed in preinvasive breast cancers [30]. These studies indicate that promoter methylation of specific genes or alterations in enzymes that control methylation, may be utilized as potential prognostic biomarkers in breast cancer patients if validated in future studies.

A genome-wide methylation array technology (methylome analysis) has been used to assess if methylation of specific breast cancer genes can predict clinical outcome [31]. The Illumina Infinium HumanMethylation27 array was used to analyze both primary invasive breast cancers ($n = 103$) and normal breast samples ($n = 21$). ER-positive tumors demonstrate a higher frequency of methylation versus ER-negative tumors (higher methylation at 5264 loci in ER-positive vs at 3112 loci in ER-negative tumors). Such analysis suggests that the hypermethylated loci in ER-negative tumors, which cluster closer to the transcriptional start sites are responsible for tighter control of transcriptional repression as compared to ER-positive tumors. The methylation patterns are also distinct with 27 (ER-positive) and 13 (ER-negative) loci showing the highest subtype specificity in individual tumor samples. These type of analyses are now being used to identify molecular pathways best targeted in the individual breast cancer subtypes and also as predictors associated with metastatic disease [32].

Development of an epigenomic signature to aid prediction of outcome in patients with breast cancer would be of great interest. For example, a 100 CpG loci signature has been identified that was significantly associated with disease progression in patients newly diagnosed with breast cancer treated with or without adjuvant therapy [32]. Approximately 20% of the loci in this signature were from homeobox-containing genes including *HOX* suggestive of a prominent role in tumor progression. Associations between aberrant DNA methylation and breast cancer outcomes have also been explored [33]. However, additional studies are required to more optimally delineate the role of gene methylation signatures as a prognostic and predictive biomarker in breast cancer. Methylation-specific polymerase chain reaction (MSP) can differentiate promoter hypermethylation at CpG islands [34]. Quantitative multiplex-methylation-specific PCR (QM-MSP) can be used to accurately assess promoter hypermethylation for many genes simultaneously in small samples, termed a “candidate marker approach” [35]. Thus advances in gene array technologies now also allow for a comprehensive whole-genome methylation array analysis (“methylome analysis”) in cancer samples will facilitate evaluation of these techniques in larger populations of patients to help determine their clinical utility [4]. Prominent expression of ER defines one of the most prevalent subtypes of breast cancer (~78%) and is the primary target of therapy for patients with this breast cancer subtype [36]. Development of ER-positive breast cancer has been linked to epigenetic dysregulation of the *ER* and as such, many of these epigenetic factors, including methylation of the ESR1 promotor and regulation of DNMT1 have been targeted for ER-directed therapy [37]. The

prevalence of *ER* methylation has specifically been examined after establishing that the *ER* indeed has a CpG island in its A and B promoters and first exon [38]. The *ER* gene is unmethylated at the *ER* CpG island in normal tissues and in several ER-positive human breast cancer cell lines. Only 36% of human breast cancers that express both the ER and PR proteins are methylated at the *ER* promoter, compared to 72% of tumors that are ER-positive but PR-negative, and 100% of tumors that are ER and PR-negative [38]. These observations prompted further investigation as to whether reversal of methylation at the *ER* promoter would sensitize the tumors to hormone treatment and is described below.

DNA methylation profiling has also identified breast cancer subtypes that are distinct from the “intrinsic subtypes” classified by gene-expression profiling (luminal A, luminal B, HER2 and basal) [39]. Methylome analysis performed on frozen primary breast cancer samples has highlighted six separate methylation clusters [40]. It was shown for the first time that DNA methylation profiles can reflect the cell-type composition of the tumor microenvironment, with a T lymphocyte infiltration of these tumors in particular in HER2-enriched and basal-like tumors. Interestingly, high expression of certain immune-related genes were found to be associated with improved relapse-free survival providing further insight into the importance of the immune system and tumor microenvironment in certain breast cancer subtypes.

Whether methylation of specific breast cancer genes can predict benefit from breast cancer therapies has also been studied. The poly (adenosine diphosphate)-ribose polymerase (PARP) inhibitors are approved for use in many BRCA1/BRCA2-associated cancers, and the *BRCA1* gene can be inactivated in sporadic breast and ovarian cancers through DNA methylation [41]. Interestingly, hypermethylation of the *BRCA1* gene in a breast cancer cell line was found to have equal sensitivity to PARP inhibitors as did the *BRCA1* mutation [42]. Treatment of this cell line with AZA restored expression of the gene. In addition, approximately 40% of “triple-negative” (ER/PR/HER2-negative, TNBC) breast tumors were found to exhibit *BRCA1* methylation, suggesting a possible future treatment option for this patient cohort. Patient with BRCA1/2 methylation are being considered eligible for ongoing clinical trials to investigate the clinical relevance of these findings (NCT03205761).

2.3 MicroRNAs

MicroRNAs (miRNAs) are small, noncoding RNA molecules that can also regulate gene expression and thus cellular activity. MiRNAs are downregulated in many tumor types including breast cancer, with aberrant miRNA expression profiles observed in cancer development and progression. These molecules are of great interest currently based on their potential to be used as biomarkers for early breast cancer detection or prognosis [43,44], predict response to breast cancer therapies, and support development of novel therapeutic approaches [45]. The clinical utility of miRNAs has yet to be determined, however, and may relate to a lack of a standardized approach to detection and analysis. That miRNAs can be detected in circulating blood, including encapsulated in exosomes which are nanovesicles that can be isolated from serum and other bodily fluids, is supportive of further investigation of this space [46]. Hypermethylation of miRNAs may also lead to their silencing and inability to function as tumor suppressors [47]. Whether miRNAs reflect a novel target in breast cancer requires further evaluation.

3 Targeted epigenetic therapies

3.1 HDAC inhibitors

Aberrant HDAC activity has been documented in a variety of tumor types and led to the development of HDAC inhibitors (HDACi) as anticancer therapeutics (Table 13.1). HDACi increase acetylation of cellular proteins by blocking HDAC activity and are classified either by their specificity for HDAC targets or by their chemical structure. Currently available HDACi target a variety of HDAC isoenzymes with class 1 (HDAC 1, 2, 3, and 8), class 2 (HDAC 4–7 and 9–10), and class 4 (HDAC 11) activity [11]. HDAC 1, 2, and 3 are the most highly conserved and are the “workhorses” of nuclear HDACs whereas HDAC 6 is the workhorse in the cytoplasm (Table 13.1). The other HDACs are summarized in Table 13.1 and have various substrates related to breast tumor cell functions. Agents, such as valproic acid and phenylbutyrate, are less potent than newer HDACi and were used in earlier investigations of this class of drug in advanced solid tumors or hematologic malignancies with modest clinical impact [48]. More potent HDACi include class-specific inhibitors (entinostat and romidepsin) and pan HDAC inhibitors (vorinostat, belinostat and panobinostat). Romidepsin and vorinostat have regulatory approval globally for treatment of T-cell lymphoma. Clinical investigation of HDACi in solid tumors are ongoing, with those relevant to breast cancer described later in this review.

Table 13.1 Classification of Histone Deacetylase Enzymes (HDACs).

HDAC	Class	Cellular Location	Function	Substrate in Breast cancer
1, 2, 3	1	Nuclear “workhorses”	Deacetylate histones and transcriptional regulators, highly conserved	Histones and transcriptional regulators. 1 and 2 = p53, E2F, ATM kinase, CAF1, DNMT1, LSD1. 3 = STAT3 and FoxP3
8	1	Nuclear	Fatty acid deacetylation	Histones, p53, SMC3, ERRα, ARID1a, longer chain acyllysine residues
11	4	Nuclear	Transcriptional regulator with an important role in immunomodulation	Longer chain acyllysine residues
6	2	Cytoplasmic “workhorses” with ability to be nuclear	Cytoskeleton and cell mobility and repair of protein misfolding	α -Tubulin and cortactin, Hsp90
4, 5, 7, 9	2	Nuclear and cytoplasmic	Scaffolding protein, catalysis as a means of eventual signal termination	Not well characterized
10	2	Nuclear and cytoplasmic	Transcriptional repressor, involved in processes, such as autophagy, immunoregulation, and DNA repair	Acetylpolyamines

3.2 Preclinical activity of the HDAC inhibitors

Laboratory research conducted to date supports the investigation of HDACi for the treatment of breast cancer. HDAC inhibitors are known for overall inhibition of tumor growth and via apoptosis of cancer cells. However, the exact mechanisms leading toward decreased growth and increased apoptosis depend not only on the specific effects on tumor cells but also cells in the surrounding tumor microenvironment (TME). Given that less than 10% of the genome is significantly altered after treatment with an HDACi, it is possible to narrow down some of the most significant effects of these drugs and in which cell types they are most important to promote an antitumor response [49].

HDACi exhibit an antitumor response *via their direct action on tumor cells*. Firstly, HDACi drives cancer cells toward nonproliferative fates involving a combination of differentiation, immunomodulation, chromatin instability, reduced DNA damage repair, reactive oxygen species production, cell cycle arrest, apoptosis, autophagy, and the reduction of angiogenesis and cell migration. In vitro studies demonstrated that vorinostat for example, inhibits clonogenic growth of both ER-positive and ER-negative breast cancer cell lines by inducing G1 and G2/M cell cycle arrest and subsequent apoptosis [50]. Exposure to low concentrations of vorinostat is also associated with accumulation of cells mainly in G1, while higher vorinostat concentrations cause cell cycle arrest predominantly in G2/M [51]. In other luminal models of breast cancer entinostat has been shown to downregulate Myc gene signatures leading to reduction in tumor growth [52].

Relief of transcriptional repression of hormone receptors is yet another mechanism by which HDACi affect sensitivity of breast tumor cells to antitumor therapies. The accumulation of acetylated H3 and H4 histone tails in conjunction with re-expression of a functional ER in ER-negative breast cancer cell lines has been observed with a novel HDAC inhibitor, scriptaid [53]. Treatment of ER-negative breast cancer cell lines with vorinostat is associated with reactivation of silenced *ER*, as well as downregulation of DNMT1 and EGFR protein expression [54]. The significance of an epigenetically reactivated *ER* was demonstrated when tamoxifen sensitivity was restored in the ER-negative MDA-MB-231 breast cancer cells following treatment with both HDAC (trichostatin A) and DNMT inhibitors (DAC) [55]. Entinostat has been shown to induce not only re-expression of *ER α* , but also the androgen receptor and the aromatase enzyme (CYP19) both *in vitro* and in triple-negative breast cancer xenografts [56]. Unfortunately, the strong preclinical rationale for use of entinostat to re-sensitize ER-positive breast cancer to endocrine therapy has not been successful to date in clinical trials as described below [57].

Entinostat has been shown to decrease seeding and growth of metastases in the more basal-like MDA-MB-231 model via reversal of the epithelial to mesenchymal transition by epigenetic repression of E-cadherin [58]. In erbB2-overexpressing breast cancer cells, entinostat induced cancer cell apoptosis via expression of miR-125a, miR-125b, and miR-205, which act in concert to downregulate erbB2/erbB3 in breast cancer cells [59]. Also, in erbB2 mouse models, entinostat has been shown to slow tumor growth and improve survival when combined with immunotherapies [60]. Furthermore, in erbB2-amplified breast cancer cell lines HDACi led to promotion of proteosomal degradation of HER2 and enhanced apoptosis induced by trastuzumab, docetaxel, epothilone B, and gemcitabine [61]. HDAC inhibitors also significantly enhance trastuzumab-induced growth inhibition in trastuzumab sensitive, *HER2*-overexpressing breast cancer cells, providing a strong rationale for clinical studies with this combination in patients with *HER2*-positive disease [62,63]. These represent only a fraction of the numerous different mechanisms in which HDACi's can affect

tumor cells leading to tumor elimination or improved survival. However, most of these preclinical studies point out that HDAC inhibitors affect numerous other cell types in addition to the tumor cells themselves in order to achieve these outcomes.

In addition to their effects on tumor cells, the *effect of HDACi on immune cells within the tumor microenvironment (TME) is under investigation* as a novel strategy to modulate immunotherapy; via transformation or priming of a pro-tumor/immune suppressed TME to that of an antitumor/immune permissive TME that is more responsive to immune checkpoint inhibition (ICI) [60,64,65]. For example, entinostat has been shown to affect myeloid derive suppressor cell (MDSC) phenotype and suppressive function in multiple TMEs, including breast, lung, kidney, and colon [60,64–66], and demonstrates a shift toward a less suppressive MDSC phenotype which promotes efficacy of adaptive immunity and antitumor response. Single cell RNA sequencing of entinostat treated murine breast tumors demonstrate significant shifts in other myeloid cell types, such as M1 and M2 macrophages, dendritic cells as well as T cells, most notably T-regulatory cells [64]. It has also been shown to increase tumor neoantigen burden in support of a robust antitumor immune response [67]. These preclinical studies point out the importance of understanding the effects of HDACi on numerous different immune cell types that contribute to the overall antitumor response. They also highlight that the most successful use of HDACi has thus far been their use in combination with other therapeutics, such as ICI and chemotherapy. There is much left to learn about the mechanism of HDACi in priming of different TMEs affecting both primary and metastatic growth of breast to fully take advantage of this drug class in the therapeutic setting.

Preclinical investigations have suggested histone acetylation occurs relatively quickly, within 30 minutes after exposure to HDACi, with the effect on chromatin remodeling in both tumor samples and peripheral blood mononuclear cells (PBMCs), occurring after more prolonged exposure to these agents (24–48 hours minimum) [68,69]. Thus analysis of PBMCs has been suggested as a potentially less invasive way to assess pharmacodynamic effect of HDAC inhibitors. However, given the more recent advances suggesting the extensive and important effects of HDACi on cells specific to the TME, mechanistic studies will still rely upon evaluation of HDACi on cells within tumors. These new insights may also explain why previous studies have failed to show a clear correlation between the level of hyperacetylation and response to therapy with HDAC inhibitors [68].

3.3 Clinical investigation of HDAC inhibitors

Based on a growing body of preclinical work, and regulatory approval of HDACi for hematologic malignancies [70,71], clinical trials have been developed and conducted in solid tumors, including breast cancer. These clinical trials now span phase 1–3 designs and have considered the use of HDACi as single agents or in combination with chemotherapy, targeted therapy ICIs (Table 13.2). Clinical trials continue to be developed in this space, with the ultimate goal of identifying a role for this class of epigenetic modifier in solid tumors.

3.3.1 Endocrine therapy combinations

The most promising areas of clinical investigation with HDACi in breast cancer has been in the setting of endocrine resistance in hormone receptor (HR)-positive disease. Vorinostat, a potent HDACi targeting class 1 and 2 HDACs, inhibited proliferation of ER-positive and ER-negative breast cancer cell lines and induced cell cycle arrest [51]. A presurgical or “window” biomarker

Table 13.2 Select Completed Trials of Epigenetic Therapies in Breast Cancer.

HDACi Backbone	Partnering Agent(s)	Rationale for HDACi	Breast Cancer Subtypes/ Treatment Setting	Phase I/II/ III	Select Reported Outcomes	Refs
Vorinostat	None	Modulation of breast cancer-associated gene expression	ER/PR + / neoadjuvant	I	N/A	[72]
Vorinostat	Tamoxifen	Re-sensitization of tumors to endocrine therapy	ER/PR + endocrine-resistant advanced breast cancer	II	ORR 19%, 40% CBR	[69]
Entinostat	Exemestane			II, ENCORE301	OS 26.94 versus 20.33 PFS 4.28 versus 2.27 months in the control group (exemestane alone)	[73]
Entinostat	Exemestane			III, E2112	No PFS/OS advantage	[74]
Vorinostat	Chemotherapy	Improve pathologic complete response	Primary operable breast cancer—TNBC and ER/PR +	I/II, TBCRC 008	27% across arms and subtypes, investigational arm not more promising	[75]
Entinostat	Nivolumab + ipilimumab	Improve response to immune checkpoint inhibition	TNBC and ER/PR + advanced breast cancer	Ib, ETCTN-9844	ORR 16% (overall cohort included other cancer types)	[76]
Entinostat	Atezolizumab		TNBC advanced breast cancer	II, ENCORE602	PFS, was not met and investigational arm associated with more toxicity	[77]
Vorinostat	Pembrolizumab + tamoxifen		ER/PR + advanced breast cancer	Ib/II, MORPHEUS	Limited clinical efficacy but observed encouraging changes to immune response	[78]

ER, estrogen receptor, ORR, objective response rate, OS, overall survival, PFS, progression free survival, PR, progesterone receptor.

study evaluated oral administration of 300 mg given twice daily for six doses to women awaiting definitive breast surgery. Potential biomarkers of response to this brief period of HDACi administration were investigated, and a reduction in proliferation-related gene expression by RT-PCR (reverse transcriptase polymerase chain reaction) using the Oncotype Dx assay was observed between baseline and surgery. Contrary to the hypotheses proposed, changes in candidate gene methylation or expression such as the ER were not observed. This suggested that while short term

administration of this oral HDACi can impact proliferation, modulation of breast cancer-associated gene expression may require a combination approach [72].

A phase 2 trial in patients with advanced breast cancer evaluated the combination of vorinostat with tamoxifen, a selective estrogen receptor modulator, based on supportive preclinical experiments [69]. Vorinostat 400 mg daily (21 days of a 28-day cycle) and tamoxifen 20 mg daily were administered orally to 43 patients. Tamoxifen had already been prescribed to a majority (60%) of patients in the early-stage setting (adjuvant), and over 50% had prior exposure to aromatase inhibitors (AIs); thus an endocrine-resistant population. Promising clinical efficacy was observed with a reported objective response rate (ORR) of 19% and a clinical benefit rate of 40%, and no concerning safety signals were observed. Exploratory correlative analyses suggested that histone hyperacetylation and high baseline HDAC2 levels were predictive of response. This hypothesis generating clinical trial supported further investigation of the combination.

Parallel preclinical and clinical evaluation of entinostat, a class 1 selective HDACi, in combination with AIs was also highly supportive of this approach. The ENCORE301 trial was designed to compare the clinical efficacy of entinostat plus exemestane (steroidal AI) versus exemestane plus placebo, in a randomized phase 2 study in the advanced breast cancer setting. Postmenopausal women who had experienced disease progression after taking a nonsteroidal AI were randomized to exemestane 25 mg daily plus entinostat 5 mg or placebo weekly [73]. A significant improvement in progression-free survival (PFS) was noted in the entinostat arm compared to placebo (median 4.28 vs 2.27 months, respectively); meeting the primary objective of the clinical trial. Unexpectedly, overall survival (OS), a secondary endpoint, was also significantly longer in the entinostat arm versus the placebo arm (26.94 versus 20.33 months, respectively), and led to FDA Breakthrough Designation of this combination [74]. A preplanned subset analysis examining early change (after 2 weeks of therapy) in protein lysine acetylation in PBMCs of patients receiving entinostat ($n = 27$) found that the median PFS was 8.5 months in those exhibiting protein lysine *hyperacetylation* versus 2.7 months for those who did not exhibit this change [75]. Based on these findings, a phase 3 study was developed to further delineate the role of entinostat in endocrine-resistant breast cancer.

The overarching aim of the phase 3 E2112 trial was to validate the preclinical and clinical findings supporting the role of HDACi in overcoming resistance to endocrine therapy in breast cancer. This international double-blind placebo-controlled registration trial conducted by the ECOG-ACRIN research group under the sponsorship of the National Cancer Institute (NCI) evaluated entinostat or placebo, in combination with exemestane, in men and women with locally advanced or metastatic HR-positive and HER2-negative breast cancer who had experienced disease progression after a nonsteroidal AI [57]. The design of E2112 followed as closely as possible that of ENCORE301, with the primary objective of E2112 assessing whether the addition of entinostat (5 mg weekly oral) to exemestane (25 mg daily oral) improved PFS by central review and OS. A sample size of 600 patients (300 per arm) was required to provide adequate power for the OS endpoint, and the trial was activated in early 2014. Stratification factors included the setting in which patient developed resistance to prior nonsteroidal AI (adjuvant/metastatic), presence of visceral disease, geographic region, and prior fulvestrant use. Accrual was completed in October 2018 ($n = 608$). Over 80% of patients had received prior AI in the advanced disease setting, suggesting indeed an endocrine resistant population, and 35% had also received prior cyclin-dependent kinase (CDK) inhibitor which became more widely available for use during study conduct. Disappointingly, the combination of exemestane and entinostat *did not* improve survival in AI

resistant advanced breast cancer despite strong rationale for performing the study. The median PFS observed across the study cohort was approximately 3 months and thus extremely short, suggesting that the use of single agent endocrine therapy in this endocrine-resistant setting is not an optimal approach in the majority of patients with much further work required to improve patient outcomes.

Interestingly, a second phase 3 trial incorporating a HDACi in endocrine resistant breast cancer in Asia has yielded a different outcome. The phase 3 randomized placebo-controlled ACE trial has led to regulatory approval of the combination of exemestane and another HDACi tucidinostat in China [79]. Postmenopausal patients who had previously received at least one prior endocrine therapy were randomized 2:1 to exemestane with tucidinostat or placebo (n = 365). The primary objective of that trial was met based on a 3.6 month advantage in investigator-assessed PFS observed between the arms. OS data, a secondary objective of the trial, is not yet available but the study was not powered to assess same. The apparent differences in outcome between the E2112 and ACE trials may be reflective of differing study designs and enrolled patient populations; with those in the ACE trial for example being less heavily pretreated with endocrine therapy in the advanced setting and thus perhaps having less endocrine resistant disease. Higher rates of adverse events were also observed in the ACE trial suggesting variable HDAC inhibition or off-target effects with the agents. The role of HDACi in endocrine resistant disease thus remains unclear, in particular in light of a number of new drug approvals in this space. Both CDK and phosphoinositide 3-kinase inhibitors are now approved in advanced HR-positive breast cancer. Combination strategies with epigenetic agents may be of interest if robust rationale present through careful preclinical investigation.

3.3.2 Chemotherapy combinations

HDACi have also been investigated in combination with standard cytotoxic agents, but have not suggested a clinical benefit with this approach despite promising preclinical data [80]. In the early-stage breast cancer setting, patients with primary operable breast cancer were randomized to 12 weeks of preoperative or neoadjuvant nab-paclitaxel and carboplatin plus vorinostat or placebo [75]. The primary objective was to determine pathologic complete response (pCR) rates to neoadjuvant chemotherapy with or without vorinostat, in patients with TNBC and HR-positive breast cancer. The pCR rate was 27% across arms and breast cancer subtypes, but the investigational arm did not appear more promising than the control arm. Vorinostat has also been combined with paclitaxel and bevacizumab in the 1st line advanced breast cancer setting, with an overall response rate of 55% observed consistent with studies evaluating the paclitaxel/bevacizumab combination alone [81]. Bevacizumab is no longer in routine use for managing advanced breast cancer and so the implications of this single arm study outcome remain unclear. A pilot study is investigating the combination of entinostat with capecitabine in both advanced breast cancer and high-risk early breast cancer after neoadjuvant therapy (NCT03473639).

3.3.3 Immunotherapy combinations

Harnessing the immune system to improve breast cancer outcomes has now reached the clinical, with recent regulatory approvals for use of ICIs in TNBC that has changed the standard of care internationally for this patient cohort. Clinical trials have demonstrated the value of adding the ICI pembrolizumab to a chemotherapy backbone in both high-risk, early-stage (KEYNOTE-522) [82] and metastatic TNBC (KEYNOTE-355) [83]. However, not all patients benefit from this approach with disease recurrence or progression a reality for a proportion of patients. Of increasing interest

in recent years is the evaluation of epigenetic agents *in combination with ICIs*, supported by a significant body of preclinical investigation as described above in this chapter.

The novel combination of entinostat and nivolumab with or without ipilimumab showed encouraging safety, tolerability, and antitumor activity in a phase 1 trial ($n = 33$) of patients with solid tumors, including advanced breast cancer [76]. Patients received an entinostat window or run-in period for 2 weeks, in order to evaluate biomarkers to HDAC inhibition alone, prior to the addition of the ICIs. Biospecimens (blood and tumor tissue samples) were collected at baseline, after the entinostat window, and after 8 weeks of combination therapy. The objective response rate in the overall cohort was 16% and included a complete response in a patient with triple-negative breast cancer. Biomarker evaluation also revealed that CD8/FoxP3 ratio increased in tumor biopsies following the addition of ICIs to entinostat, but not after the entinostat treatment alone. An additional cohort ($n = 24$) in HER2-negative advanced breast cancer treated with this combination has also yielded interesting results with an ORR of 25% (5/20 evaluable patients) [84], and a follow-up study is in the planning stages.

Other ICIs have also been investigated in combination with HDACi. The ENCORE602 trial ($n = 81$) evaluated the efficacy and safety of atezolizumab, a PDL1-inhibitor, plus entinostat versus atezolizumab plus placebo in advanced TNBC [77]. The primary endpoint of the study, PFS, was not met and indeed the investigational arm was associated with more toxicity. This combination is also being evaluated in the MROPHEUS Phase Ib/II trial in patients with HR-positive advanced breast cancer (NCT04802759). The combination of tamoxifen, pembrolizumab and vorinostat has also been evaluated in a randomized phase II trial, with limited clinical efficacy observed but a suggestion that T-cell exhaustion and treatment-induced depletion of regulatory T cells might identify those more likely to benefit from this approach [78].

Despite only one regulatory approval of a HDACi in China to date despite decades of clinical investigation of these agents in solid tumors, these orally available agents continue to be investigated in clinical trials. A more complete discussion is beyond the scope of this chapter. These completed and ongoing trials involve combination strategies with a variety of other anticancer agents, including HER2-targeted agents, and DNMT inhibitors as described below.

4 DNMT inhibitors

DNMT inhibitors (DNMTi), also referred to as demethylating agents, have been under preclinical and clinical investigation for over 30 years [85]. The two most widely studied are 5-azacitidine (AZA) and decitabine (DAC), both nucleoside cytidine analogs, that are incorporated into DNA after activation to a triphosphate moiety. The mechanism of action of these agents is well known and in short, after formation of an irreversible complex with DNMT1, degradation of the enzyme occurs preventing methylation of daughter DNA in CpG islands during DNA replication. AZA (but not DAC) is converted into a ribonucleoside moiety and is incorporated into RNA, interfering with protein translation [86]. The concentrations of these agents utilized in experiments lead to different functions; at low concentrations (e.g., 30 nM DAC, 300 nM AZA) these inhibitors are potent DNA hypomethylators, whereas at high concentrations ($\approx 3\text{--}10 \mu\text{M}$) they result in cytotoxicity [87]. Given that early clinical trials investigating AZA and DAC utilized cytotoxic doses, there was

excessive toxicity, and this possibly led to the lack of overall efficacy [88]. A better understanding of the DNA hypomethylating effects of AZA and DAC led to clinical trials in hematologic malignancies that have subsequently resulted in approval by the FDA for use in the treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [89–91]. Other DNMTi in an earlier phase of development include DAC analogs, such as SGI-110 (guadecitabine) and zebularine.

4.1 Preclinical activity of the DNMT inhibitors

DNMT1 is implicated in breast cancer tumorigenesis and more specifically via repression of ER, promotion of epithelial to mesenchymal transition, and induction of autophagy and promotion of breast cancer stem cells. Low nanomolar (nM) doses of AZA and DAC (e.g., 100 nM DAC and 500 nM AZA) resulted in the development of an antitumor “memory” response in xenograft models of breast cancer and demonstrate growth inhibition, including traditionally resistant stem-like cells, without evidence of cytotoxicity [92]. Sustained, genome-wide alterations in promoter methylation and gene expression which affected major cell signaling pathways were also observed. Of significant interest are alterations in methylation of the promoter of tumor suppressor genes, including *ER*, *BRCA-1*, *E-cadherin*, *PTEN*, and *MASPIN*, following treatment with DMNTi [93–95].

DNMTi have also been shown to sensitize breast cancer cell lines to the chemotherapeutic agent doxorubicin by inducing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [96]. These effects have been most notable in TNBC given they demonstrate higher levels of genome-wide hypomethylation compared with other breast cancer subtypes, but also affects other breast cancer subtypes [97]. Unfortunately, preclinical studies suggesting promise of AZA and DAC in breast cancer did not pan out in clinical trials [5]; however, second-generation DNMTi including SGI-110 suggest similar efficacy in promoting antitumor immune responses via its effects on cytotoxic T cells and MDSCs [98,99]. SGI-110 has also demonstrated promising responses in combination with ICIs, demonstrating similar mechanisms of action as those observed with HDACi, as well as with other targeted therapies approved in breast cancer, such as poly (ADP-ribose) polymerase inhibitors (PARPi) [97]. Ongoing studies aim to elucidate novel combination strategies utilizing traditional therapies, such as tamoxifen, aromatase inhibitors, trastuzumab, and cytotoxic agents, amongst others. In addition, novel DNMTi, such as antroquinonol D, kazinol Q, and isofistularin-3, are under development and show preliminary activity against growth and migration and EMT in breast cancer cell lines [100–102].

4.2 Clinical investigation of DNMT inhibitors

DNMTi have been investigated in patients with breast cancer for decades. Evidence of clinical activity in breast cancer was observed in all comers trials, with reported response rates as high as 18% in breast cancer [88]. However, high cytotoxic doses of DNMTi were used in these studies, rather than the more recent approach of utilizing doses likely to impact transcriptional repression. Clinical trials over the last decade have thus considered administration of DNMTi at a putative “optimal epigenetic dose” to evaluate the pharmacodynamic effects of these agents, alongside clinical efficacy.

4.2.1 Epigenetic combinations

As a combination of HDACi and DNMTi may result in greater re-expression of silenced genes and overcome endocrine resistance than either agent alone, patients with advanced TNBC and HR-positive breast cancer received a combination of low-dose AZA (40 mg/m^2) on days 1–5 and 8–10, and entinostat 7 mg on days 3 and 10 of a 28-day cycle [103]. In this multicenter phase II clinical trial, tumor biopsies prior to and after therapy were performed to assess the modulation of candidate gene methylation and expression. *ER* modulation was observed in approximately 50% of posttreatment biopsies in the HR-positive cohort. Combination epigenetic therapy was well tolerated, but ORR was low with this treatment strategy and thus the primary endpoint was not met. An optional continuation phase, after progression on the initial treatment combination, suggested that some women may benefit from reintroduction of endocrine therapy beyond progression.

4.2.2 Chemotherapy and immunotherapy combinations

There are limited clinical data supporting the combination of DNMTi and cytotoxic chemotherapy in breast cancer. AZA and carboplatin yielded an ORR of 22% in patients with platinum-resistant ovarian cancer (disease progression within 6 months of platinum, $n = 18$) prompting further evaluation of the combination [104]. However, a study evaluating decitabine and carboplatin in advanced TNBC was closed early due to slow patient accrual (NCT03295552). A phase 1/2 clinical trial of AZA and nab-paclitaxel in patients with advanced solid tumors included a small cohort with breast cancer. Patients received escalating AZA doses daily for 5 days, followed by nab-paclitaxel at the standard 100 mg/m^2 weekly dose for 3 weeks in 4-week cycles. A number of tumor responses were observed in this heavily pretreated population, including an unconfirmed response in a patient with breast cancer [105].

Additional investigation is warranted with this combination and other chemotherapy backbones to elucidate further the role of these agents in breast cancer. An open-label, multicenter, phase 2 study of a short course (window) of sequential decitabine followed by the ICI pembrolizumab administered prior to a standard neoadjuvant chemotherapy regimen for patients with locally advanced HER2-negative breast cancer is ongoing at this time (NCT02957968). The primary objective of the trial is to determine if the investigational combination increases the presence and percentage of tumor and/or stromal area of infiltrating lymphocytes prior to commencement of neoadjuvant chemotherapy.

Other DNMT inhibitors are under investigation in solid tumors including guadecitabine and an oral azacytidine formulation (CC-486) [3]. To our knowledge the only study investigating CC-486 in breast cancer, combined this oral DNMT inhibitor with fulvestrant in advanced endocrine-resistant breast cancer but was also terminated early (NCT02374099).

5 Newer epigenetic modifiers

A number of other epigenetic targets and related therapies are under investigation in breast cancer, but have been evaluated to a much lesser extent than the HDACi and DNMTi described above. Agents of interest include HDAC6 inhibitors, EZH2 methyltransferase inhibitors, LSD1 inhibitors and bromodomain and extraterminal (BET) inhibitors [3].

BET proteins are “epigenetic readers” that read acetyl groups on histone lysines, recruit complexes to chromatin, with resultant regulation of cancer-associated gene transcription [106,107]. The BET-family includes BRD2, BRD3, BRD4 and BRDT, with the associated adjacent bromodomains BD1 and BD2 being a target for many of the inhibitors in development. These inhibitors impact BET protein association with chromatin, and thus oncogene expression. Preclinical studies in breast cancer have shown promise in both TNBC and HR-positive/endocrine resistant cell line and mouse models; prompting early clinical development [108,109]. An interesting avenue may be the role of BET inhibitors, with or without fibroblast growth factor receptor 1 (FGFR1) inhibitors, in invasive lobular breast cancer; a rare breast cancer subtype [110]. Clinical trial data in this space is limited to date. A first-in-human study of an oral pan-inhibitor of BET proteins, mivebresib, has shown limited efficacy in a cohort of patients with advanced solid tumors ($n = 72$, 11% breast cancer) [111]. A phase 1 study has also evaluated alobresib in advanced cancers including a cohort with advanced HR-positive breast cancer alongside exemestane or fulvestrant (NCT02392611). The study has completed accrual but results are pending. Molibresib, another orally bioavailable BET protein inhibitor, has also been investigated in advanced cancers including breast cancer with limited activity in this cohort [112], as well as in combination with fulvestrant (NCT02964507). Additional studies are warranted to further delineate the role of these agents in breast cancer, and combination strategies will likely be preferred.

6 Current status and future directions

Epigenetic alterations are frequently detected in breast tumors, and as such agents that target these alterations have and continue to be of great interest to the research community. Despite significant promise in particular in the endocrine resistant space, however, clinical trial results have not shown a consistent benefit for these agents. Regulatory approval has been achieved in China for use of a HDACi in combination with endocrine therapy, but the improvement in PFS observed with this combination was modest and no OS advantage has been noted to date. More recently, epigenetic control of cell types that assist in either tumor growth, metastatic spread or immune response is also being studied as potential targets to aid in novel therapeutic development. Some promise has been observed clinically in studies in which HDACi are given prior to the addition of ICIs in the phase 1 setting [76]. Phase 2 trials will confirm or refute this success and preclinical studies will continue to investigate mechanisms of action. However, several challenges must be overcome to allow for optimal incorporation of epigenetic modifiers into the solid tumor and specifically breast cancer clinical treatment paradigm.

In regards the optimal dose and schedule of both HDACi and DNMTi inhibitors in breast cancer clinical trials, current data suggests that pretreatment with lower doses may be efficacious in particular if the goal is modulation of the TME. The clinical impact in breast cancer of using epigenetic modifiers at an optimally “epigenetic dose” rather than a cytotoxic dose remains unclear, however preclinical data suggests lower doses are likely to be more effective. Ultimately, however, investigation of combination strategies of epigenetic modifiers with other anticancer agents, such as standard chemotherapy, immunotherapy (ICIs specifically), anti-HER2 therapy, PARPi, and other small molecule inhibitors, may yield greater results than with either agent alone in the clinic. Enhancing the activity of chimeric antigen receptor (CAR) T cell therapy in solid tumors is also an avenue that has been explored *in vitro* and *ex vivo* with promising results [113]. The schedule of

administration of these agents is also likely to matter and should ideally be modeled preclinically prior to the initiation of clinical trials. For example, an attempt to enhance efficacy to ICIs or standard chemotherapy by “priming” cells with HDACi or DNMTi are of interest [64,114].

The design of clinical trials which incorporate epigenetic modifiers, especially in the context of immune checkpoint inhibition, requires careful consideration. In addition to various schedules of drug administration noted above, clinical trial endpoints should consider the experience in hematologic malignancy with these agents and drug mechanism of action. For example, in hematologic malignancies, a high response rate with AZA and DAC did not translate to a survival benefit; challenging use of ORR by RECIST criteria as a surrogate for longer term clinical benefit [115]. Consideration of modification of the standard response assessment criteria for trials investigating epigenetic agents is a matter for discussion, similar to the development of newer immune-related RECIST criteria for investigation of immune therapies as anticancer agents [116]. So called “window-of-opportunity” studies can also be considered when investigating novel agents and their pharmacodynamic impact on tumors [117]. Investigational agents can be administered during a short period (days to weeks) pre chemotherapy or pre surgery for breast cancer, and serial biopsies can be obtained for correlative analyses to elucidate mechanism of action of the drug. These study designs, however, require significant buy in from clinical teams and patients themselves to be successful. The ability of single agent AZA to induce expression of the *ER* and *PR* genes in patients with TNBC prior to primary breast cancer surgery, for example, was terminated early due to an inability to accrue patients (NCT01292083).

Finally, robust biomarkers of response and resistance to epigenetic modifiers in the breast cancer setting have not yet been defined. Alterations in lysine acetylation [68], or evaluation of histone hyperacetylation over time [69], have been evaluated in clinical trials however, lack of confirmed clinical benefit in confirmatory trials make these results hard to interpret. A focus on the changes made by HDACi and DNMTi to immune cells, such as changes in neoantigens, or changes in immune cell phenotype or function, suggest a novel approach to biomarker discovery for these classes of drugs. Prospective collection of serial biospecimens in well-designed clinical trials will be essential to optimally define who may benefit most from these therapies in the future; or indeed if they have a role in the solid tumor setting.

In conclusion, the field of epigenetics is constantly evolving, and the clinical relevance of HDACi and DNMTi for patients with breast cancer has yet to be determined. Significant challenges remain regarding the addition of these agents to the growing portfolio of therapeutic agents used to treat breast cancer, however their role in modulation of the TME to improve response to immune therapies is emerging. The rapid pace of new technologies coming to the market which can support biomarker evaluation has yet to fully elucidate mechanisms of response to these agents, which become more and more complex as our knowledge of their treatment effects grows. In order to continue to improve outcomes for patients with breast cancer, we must collaboratively and carefully design preclinical experiments and clinical trials which build on the extensive work performed in recent decades.

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References

- [1] Juergens RA, Wrangle J, Vendetti FP, Murphy SC, Zhao M, Coleman B, et al. Combination epigenetic therapy has efficacy in patients with refractory advanced non–small cell lung cancer. *Cancer Discov* 2011; Available from: <https://doi.org/10.1158/2159-8290.CD-11-0214>.
- [2] Hanahan D. Hallmarks of cancer: new dimensions. *Cancer Discov* 2022;12(1):31–46. Available from: <https://pubmed.ncbi.nlm.nih.gov/35022204/>.
- [3] Bates SE. Epigenetic therapies for cancer. *N Engl J Med* 2020;383(7):650–63. Available from: <https://pubmed.ncbi.nlm.nih.gov/32786190/>.
- [4] Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 2007;8(4):286–98. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17339880.
- [5] Connolly R, Stearns V. Epigenetics as a therapeutic target in breast cancer. *J Mammary Gland Biol Neoplasia* 2012;17(3–4):191–204. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22836913>.
- [6] Veeck J, Esteller M. Breast cancer epigenetics: from DNA methylation to microRNAs. *J Mammary Gland Biol Neoplasia* 2010;15(1):5–17. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20101446.
- [7] Munshi A, Shafi G, Aliya N, Jyothy A. Histone modifications dictate specific biological readouts. *J Genet Genomics* 2009;36(2):75–88. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19232306>.
- [8] Jenuwein T, Allis CD. Translating the histone code. *Science* 2001;293(5532):1074–80. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11498575>.
- [9] Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3(6):415–28. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12042769>.
- [10] Arts J, de Schepper S, Van Emelen K. Histone deacetylase inhibitors: from chromatin remodeling to experimental cancer therapeutics. *Curr Med Chem* 2003;10(22):2343–50. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14529477.
- [11] Prince HM, Bishton MJ, Harrison SJ. Clinical studies of histone deacetylase inhibitors. *Clin Cancer Res* 2009;15(12):3958–69. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19509172>.
- [12] Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet* 2005;37(4):391–400. Available from: <https://pubmed.ncbi.nlm.nih.gov/15765097/>.
- [13] Muller BM, Jana L, Kasajima A, Lehmann A, Prinzler J, Budczies J, et al. Differential expression of histone deacetylases HDAC1, 2 and 3 in human breast cancer—overexpression of HDAC2 and HDAC3 is associated with clinicopathological indicators of disease progression. *BMC Cancer* 2013;13:215. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23627572>.

- [14] Duong V, Bret C, Altucci L, Mai A, Duraffourd C, Loubersac J, et al. Specific activity of class II histone deacetylases in human breast cancer cells. *Mol Cancer Res* 2008;6(12):1908–19. Available from: <https://pubmed.ncbi.nlm.nih.gov/19074835/>.
- [15] Miyake K, Yoshizumi T, Imura S, Sugimoto K, Batmunkh E, Kanemura H, et al. Expression of hypoxia-inducible factor-1alpha, histone deacetylase 1, and metastasis-associated protein 1 in pancreatic carcinoma: correlation with poor prognosis with possible regulation. *Pancreas* 2008;36(3):e1–9. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18362831.
- [16] Krusche CA, Wulffing P, Kersting C, Vloet A, Bocker W, Kiesel L, et al. Histone deacetylase-1 and -3 protein expression in human breast cancer: a tissue microarray analysis. *Breast Cancer Res Treat* 2005;90(1):15–23. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15770522.
- [17] Zhang Z, Yamashita H, Toyama T, Sugiura H, Omoto Y, Ando Y, et al. HDAC6 expression is correlated with better survival in breast cancer. *Clin Cancer Res* 2004;10(20):6962–8. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15501975.
- [18] Pedrali-Noy G, Weissbach A. Mammalian DNA methyltransferases prefer poly(dI-dC) as substrate. *J Biol Chem* 1986;261(17):7600–2. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/3711099>.
- [19] Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999;99(3):247–57. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10555141>.
- [20] Widschwender M, Jones PA. DNA methylation and breast carcinogenesis. *Oncogene* 2002;21(35):5462–82. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12154408.
- [21] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349(21):2042–54. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14627790.
- [22] Du T, Liu B, Wang Z, Wan X, Wu Y. CpG methylation signature predicts prognosis in breast cancer. *Breast Cancer Res Treat* 2019;178(3):565–72. Available from: <https://pubmed.ncbi.nlm.nih.gov/31520283/>.
- [23] Jacot W, Lopez-Crapez E, Mollevi C, Boissière-Michot F, Simony-Lafontaine J, Ho-Pun-Cheung A, et al. BRCA1 promoter hypermethylation is associated with good prognosis and chemosensitivity in triple-negative breast cancer. *Cancers (Basel)* 2020;12(4). Available from: <https://pubmed.ncbi.nlm.nih.gov/32235500/>.
- [24] Li Z, Jang JK, Lechner MG, Hu P, Khawli L, Scannell CA, et al. Generation of tumor-targeted anti-body-CpG conjugates. *J Immunol Methods* 2013;389(1–2):45–51. Available from: <https://pubmed.ncbi.nlm.nih.gov/23279945/>.
- [25] Xia Y, Gupta GK, Castano AP, Mroz P, Avci P, Hamblin MR. CpG oligodeoxynucleotide as immune adjuvant enhances photodynamic therapy response in murine metastatic breast cancer. *J Biophotonics* 2014;7(11–12):897–905. Available from: <https://pubmed.ncbi.nlm.nih.gov/23922221/>.
- [26] Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G. Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. *BMC Genomics* 2010;11(1). Available from: <https://pubmed.ncbi.nlm.nih.gov/20181289/>.
- [27] Soares J, Pinto AE, Cunha CV, Andre S, Barao I, Sousa JM, et al. Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. *Cancer* 1999;85(1):112–18. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9921982.

- [28] Gupta A, Godwin AK, Vanderveer L, Lu A, Liu J. Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res* 2003;63(3):664–73. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12566312.
- [29] Brooks J, Cairns P, Zeleniuch-Jacquotte A. Promoter methylation and the detection of breast cancer. *Cancer Causes Control* 2009;20(9):1539–50. Available from: <https://pubmed.ncbi.nlm.nih.gov/19768562/>.
- [30] Xu X, Gammon MD, Hernandez-Vargas H, Herceg Z, Wetmur JG, Teitelbaum SL, et al. DNA methylation in peripheral blood measured by LUMA is associated with breast cancer in a population-based study. *FASEB J* 2012;26(6):2657–66. Available from: <https://pubmed.ncbi.nlm.nih.gov/22371529/>.
- [31] Fackler MJ, Umbricht CB, Williams D, Argani P, Cruz LA, Merino VF, et al. Genome-wide methylation analysis identifies genes specific to breast cancer hormone receptor status and risk of recurrence. *Cancer Res* 2011;71(19):6195–207. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21825015.
- [32] Legendre C, Gooden GC, Johnson K, Martinez RA, Liang WS, Salgia B. Whole-genome bisulfite sequencing of cell-free DNA identifies signature associated with metastatic breast cancer. *Clin Epigenetics* 2015;7(1). Available from: <https://pubmed.ncbi.nlm.nih.gov/26380585/>.
- [33] Hill VK, Ricketts C, Bieche I, Vacher S, Gentle D, Lewis C, et al. Genome-wide DNA methylation profiling of CpG islands in breast cancer identifies novel genes associated with tumorigenicity. *Cancer Res* 2011;71(8):2988–99. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21363912.
- [34] Licchesi JD, Herman JG. Methylation-specific PCR. *Methods Mol Biol* 2009;507:305–23. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18987823.
- [35] Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, et al. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in *in situ* and invasive lobular breast carcinoma. *Int J Cancer* 2003;107(6):970–5. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14601057.
- [36] Female Breast Cancer Subtypes—Cancer Stat Facts. <https://seer.cancer.gov/statfacts/html/breast-subtypes.html>.
- [37] Garcia-Martinez L, Zhang Y, Nakata Y, Chan HL, Morey L. Epigenetic mechanisms in breast cancer therapy and resistance. *Nat Commun* 2021;12(1). Available from: <https://pubmed.ncbi.nlm.nih.gov/33741974/>.
- [38] Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, et al. Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. *Clin Cancer Res* 1996;2(5):805–10. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9816234>.
- [39] Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature* 2000;406(6797):747–52. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10963602.
- [40] Dedeurwaerder S, Desmedt C, Calonne E, Singhal SK, Haibe-Kains B, Defrance M, et al. DNA methylation profiling reveals a predominant immune component in breast cancers. *EMBO Mol Med* 2011;3(12):726–41. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21910250.
- [41] Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med*

- 2000;343(19):1350–4. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11070098.
- [42] Veeck J, Ropero S, Setien F, Gonzalez-Suarez E, Osorio A, Benitez J, et al. BRCA1 CpG island hypermethylation predicts sensitivity to poly(adenosine diphosphate)-ribose polymerase inhibitors. *J Clin Oncol* 2010;28(29):e563–4 author reply e565–6. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20679605.
- [43] Schrauder MG, Strick R, Schulz-Wendtland R, Strissel PL, Kahmann L, Loehberg CR, et al. Circulating micro-RNAs as potential blood-based markers for early stage breast cancer detection. *PLoS One* 2011;7(1):e29770. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22242178.
- [44] Ferracin M, Querzoli P, Calin GA, Negrini M. MicroRNAs: toward the clinic for breast cancer patients. *Semin Oncol* 2011;38(6):764–75. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22082762.
- [45] Davey MG, Lowery AJ, Miller N, Kerin MJ. MicroRNA expression profiles and breast cancer chemotherapy. *Int J Mol Sci* 2021;22(19). Available from: <https://pubmed.ncbi.nlm.nih.gov/34639152/>.
- [46] Joyce DP, Kerin MJ, Dwyer RM. Exosome-encapsulated microRNAs as circulating biomarkers for breast cancer. *Int J Cancer* 2016;139(7):1443–8. Available from: <https://pubmed.ncbi.nlm.nih.gov/27170104/>.
- [47] Lujambio A, Esteller M. CpG island hypermethylation of tumor suppressor microRNAs in human cancer. *Cell Cycle* 2007;6(12):1455–9. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17581274.
- [48] Gore SD, Weng LJ, Zhai S, Figg WD, Donehower RC, Dover GJ, et al. Impact of the putative differentiating agent sodium phenylbutyrate on myelodysplastic syndromes and acute myeloid leukemia. *Clin Cancer Res* 2001;7(8):2330–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11489809>.
- [49] Li W, Sun Z. Mechanism of action for HDAC inhibitors—insights from omics approaches. *Int J Mol Sci* 2019;20(7). Available from: <https://pubmed.ncbi.nlm.nih.gov/30939743/>.
- [50] Huang L, Pardee AB. Suberoylanilide hydroxamic acid as a potential therapeutic agent for human breast cancer treatment. *Mol Med* 2000;6(10):849–66. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11126200>.
- [51] Munster PN, Troso-Sandoval T, Rosen N, Rifkind R, Marks PA, Richon VM. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. *Cancer Res* 2001;61(23):8492–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11731433>.
- [52] Tanioka M, Mott KR, Hollern DP, Fan C, Darr DB, Perou CM. Identification of Jun loss promotes resistance to histone deacetylase inhibitor entinostat through Myc signaling in luminal breast cancer. *Genome Med* 2018;10(1). Available from: <https://pubmed.ncbi.nlm.nih.gov/30497520/>.
- [53] Keen JC, Yan L, Mack KM, Pettit C, Smith D, Sharma D, et al. A novel histone deacetylase inhibitor, scriptaid, enhances expression of functional estrogen receptor alpha (ER) in ER negative human breast cancer cells in combination with 5-aza 2'-deoxycytidine. *Breast Cancer Res Treat* 2003;81(3):177–86. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/14620913>.
- [54] Zhou Q, Shaw PG, Davidson NE. Inhibition of histone deacetylase suppresses EGF signaling pathways by destabilizing EGFR mRNA in ER-negative human breast cancer cells. *Breast Cancer Res Treat* 2009;117(2):443–51. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18683042.
- [55] Sharma D, Saxena NK, Davidson NE, Vertino PM. Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes. *Cancer Res* 2006;66(12):6370–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16778215>.

- [56] Sabinis GJ, Goloubeva O, Chumsri S, Nguyen N, Sukumar S, Brodie AM. Functional activation of the estrogen receptor-alpha and aromatase by the HDAC inhibitor entinostat sensitizes ER-negative tumors to letrozole. *Cancer Res* 2011;71(5):1893–903. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21245100>.
- [57] Connolly RM, Zhao F, Miller KD, Lee MJ, Piekarz RL, Smith KL, et al. E2112: randomized phase III trial of endocrine therapy plus entinostat or placebo in hormone receptor-positive advanced breast cancer. A trial of the ECOG-ACRIN Cancer Research Group. *J Clin Oncol* 2021;39(28):3171–81. Available from: <https://pubmed.ncbi.nlm.nih.gov/34357781/>.
- [58] Schech A, Kazi A, Yu S, Shah P, Sabinis G. Histone deacetylase inhibitor entinostat inhibits tumor-initiating cells in triple-negative breast cancer cells. *Mol Cancer Ther* 2015;14(8):1848–57. Available from: <https://pubmed.ncbi.nlm.nih.gov/26037781/>.
- [59] Wang S, Huang J, Lyu H, Lee CK, Tan J, Wang J, et al. Functional cooperation of miR-125a, miR-125b, and miR-205 in entinostat-induced downregulation of erbB2/erbB3 and apoptosis in breast cancer cells. *Cell Death Dis* 2013;4:e556. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23519125>.
- [60] Christmas BJ, Rafie CI, Hopkins AC, Scott BA, Ma HS, Cruz KA, et al. Entinostat converts immune-resistant breast and pancreatic cancers into checkpoint-responsive tumors by reprogramming tumor-infiltrating MDSCs. *Cancer Immunol Res* 2018;6(12):1561–77.
- [61] Fuino L, Bali P, Wittmann S, Donapati S, Guo F, Yamaguchi H, et al. Histone deacetylase inhibitor LAQ824 down-regulates Her-2 and sensitizes human breast cancer cells to trastuzumab, taxotere, gemcitabine, and epothilone B. *Mol Cancer Ther* 2003;2(10):971–84. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/14578462>.
- [62] Huang X, Wang S, Lee CK, Yang X, Liu B. HDAC inhibitor SNDX-275 enhances efficacy of trastuzumab in erbB2-overexpressing breast cancer cells and exhibits potential to overcome trastuzumab resistance. *Cancer Lett* 2011;307(1):72–9. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21497990.
- [63] Bali P, Pranpat M, Swaby R, Fiskus W, Yamaguchi H, Balasis M, et al. Activity of suberoylanilide hydroxamic acid against human breast cancer cells with amplification of her-2. *Clin Cancer Res* 2005;11(17):6382–9. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16144943.
- [64] Sidiropoulos DN, Rafie CI, Jang JK, Castanon S, Baugh AG, Gonzalez E, et al. Entinostat decreases immune suppression to promote antitumor responses in a HER2+ breast tumor microenvironment. *Cancer Immunol Res* 2022;10(5):656–69. Available from: <https://pubmed.ncbi.nlm.nih.gov/35201318/>.
- [65] Kim K, Skora AD, Li Z, Liu Q, Tam AJ, Blosser RL, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci U S A* 2014;111(32):11774–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25071169>.
- [66] Lu Z, Zou J, Li S, Topper MJ, Tao Y, Zhang H, et al. Epigenetic therapy inhibits metastases by disrupting premetastatic niches. *Nature* 2020;579(7798):284–90. Available from: <https://pubmed.ncbi.nlm.nih.gov/32103175/>.
- [67] Truong AS, Zhou M, Krishnan B, Utsumi T, Manocha U, Stewart KG, et al. Entinostat induces antitumor immune responses through immune editing of tumor neoantigens. *J Clin Invest* 2021;131(16). Available from: <https://pubmed.ncbi.nlm.nih.gov/34396985/>.
- [68] Tomita Y, Lee MJ, Lee S, Tomita S, Chumsri S, Cruickshank S, et al. The interplay of epigenetic therapy and immunity in locally recurrent or metastatic estrogen receptor-positive breast cancer: correlative analysis of ENCORE 301, a randomized, placebo-controlled phase II trial of exemestane with or without entinostat. *Oncoimmunology* 2016;5(11):e1219008. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27999738>.

- [69] Munster PN, Thurn KT, Thomas S, Raha P, Lacevic M, Miller A, et al. A phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer. *Br J Cancer* 2011;104(12):1828–35. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21559012.
- [70] Olsen EA, Kim YH, Kuzel TM, Pacheco TR, Foss FM, Parker S, et al. Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. *J Clin Oncol* 2007;25(21):3109–15. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17577020.
- [71] Piekarz RL, Frye R, Prince HM, Kirschbaum MH, Zain J, Allen SL, et al. Phase II trial of romidepsin in patients with peripheral T-cell lymphoma. *Blood* 2010;. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21355097.
- [72] Stearns V, Jacobs LK, Tsangaris TN, Cheng Z, Slater S, Fackler MJ, et al. Association of vorinostat with decrease in gene expression of proliferation-related genes in tumors from women with newly diagnosed breast cancer. *J Clin Oncol* 2010;28(15s).
- [73] Yardley DA, Ismail-Khan RR, Melichar B, Lichinitser M, Munster PN, Klein PM, et al. Randomized phase II, double-blind, placebo-controlled study of exemestane with or without entinostat in postmenopausal women with locally recurrent or metastatic estrogen receptor-positive breast cancer progressing on treatment with a nonsteroidal aromatase inhibitor. *J Clin Oncol* 2013;31(17):2128–35. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23650416>.
- [74] Corrigan-Curay J, McKee AE, Stein P. Breakthrough-therapy designation—an FDA perspective. *N Engl J Med* 2018;378(15):1457–8. Available from: <https://pubmed.ncbi.nlm.nih.gov/29641963/>.
- [75] Connolly RM, Leal JP, Goetz MP, Zhang Z, Zhou XC, Jacobs LK, et al. TBCRC 008: early change in 18F-FDG uptake on PET predicts response to preoperative systemic therapy in human epidermal growth factor receptor 2-negative primary operable breast cancer. *J Nucl Med* 2015;56(1):31–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25476537>.
- [76] Roussos Torres ET, Rafie C, Wang C, Lim D, Brufsky A, LoRusso P, et al. Phase I study of entinostat and nivolumab with or without ipilimumab in advanced solid tumors (ETCTN-9844). *Clin Cancer Res* 2021;27(21):5828–37. Available from: <https://pubmed.ncbi.nlm.nih.gov/34135021/>.
- [77] O'Shaughnessy J, Moroese RL, Babu S, Baramidze K, Chan D, Leitner SP, et al. Results of ENCORE 602 (TRIO025), a phase II, randomized, placebo-controlled, double-blinded, multicenter study of atezolizumab with or without entinostat in patients with advanced triple-negative breast cancer (aTNBC). *J Clin Oncol* 2020;38(15_suppl):1014.
- [78] Terranova-Barberio M, Pawlowska N, Dhawan M, Moasser M, Chien AJ, Melisko ME, et al. Exhausted T cell signature predicts immunotherapy response in ER-positive breast cancer. *Nat Commun* 2020;11(1). Available from: <https://pubmed.ncbi.nlm.nih.gov/32681091/>.
- [79] Jiang Z, Li W, Hu X, Zhang Q, Sun T, Cui S, et al. Tucidinostat plus exemestane for postmenopausal patients with advanced, hormone receptor-positive breast cancer (ACE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2019;20(6):806–15. Available from: <https://pubmed.ncbi.nlm.nih.gov/31036468/>.
- [80] Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F. Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. *Cancer Res* 2003;63(21):7291–300. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/14612526>.
- [81] Ramaswamy B, Fiskus W, Cohen B, Pellegrino C, Hershman DL, Chuang E, et al. Phase I-II study of vorinostat plus paclitaxel and bevacizumab in metastatic breast cancer: evidence for vorinostat-induced tubulin acetylation and Hsp90 inhibition in vivo. *Breast Cancer Res Treat* 2011;. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22200869>.

- [82] Schmid P, Cortes J, Dent R, Pusztai L, McArthur H, Kümmel S, et al. Event-free survival with pembrolizumab in early triple-negative breast cancer. *N Engl J Med* 2022;386(6):556–67. Available from: <https://pubmed.ncbi.nlm.nih.gov/35139274/>.
- [83] Cortes J, Cescon DW, Rugo HS, Nowecki Z, Im SA, Yusof MM, et al. Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): a randomised, placebo-controlled, double-blind, phase 3 clinical trial. *Lancet (London, Engl)* 2020;396(10265):1817–28. Available from: <https://pubmed.ncbi.nlm.nih.gov/33278935/>.
- [84] Torres ETR, Leatherman J, Rafie C, Brufsky A, Lorusso P, Eder JP, et al. 964MO Entinostat, nivolumab and ipilimumab in advanced HER2-negative breast cancer (ETCTN-9844). *Ann Oncol* 2021;32:S833. Available from: <http://www.annalsofoncology.org/article/S092375342103578X/fulltext>.
- [85] Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 1980;20(1):85–93. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6156004.
- [86] Ghoshal K, Datta J, Majumder S, Bai S, Kutay H, Motiwala T, et al. 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Mol Cell Biol* 2005;25(11):4727–41. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15899874>.
- [87] Qin T, Youssef EM, Jelinek J, Chen R, Yang AS, Garcia-Manero G, et al. Effect of cytarabine and decitabine in combination in human leukemic cell lines. *Clin Cancer Res* 2007;13(14):4225–32. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17634552>.
- [88] Cowan LA, Talwar S, Yang AS. Will DNA methylation inhibitors work in solid tumors? A review of the clinical experience with azacitidine and decitabine in solid tumors. *Epigenomics* 2010;2(1):71–86. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22122748>.
- [89] Roboz GJ, Ravandi F, Wei AH, Dombret H, Thol F, Voso MT, et al. Oral azacitidine prolongs survival of patients with AML in remission independently of measurable residual disease status. *Blood* 2022;139(14):2145–55. Available from: <https://pubmed.ncbi.nlm.nih.gov/34995344/>.
- [90] Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol* 2009;10(3):223–32. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19230772>.
- [91] Steensma DP, Baer MR, Slack JL, Buckstein R, Godley LA, Garcia-Manero G, et al. Multicenter study of decitabine administered daily for 5 days every 4 weeks to adults with myelodysplastic syndromes: the alternative dosing for outpatient treatment (ADOPT) trial. *J Clin Oncol* 2009;27(23):3842–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19528372>.
- [92] Tsai HC, Li H, Van Neste L, Cai Y, Robert C, Rassool FV, et al. Transient low doses of DNA demethylating agents exert durable anti-tumor effects on hematological and epithelial tumor cells. *Cancer Cell* 2012;. Available from: <https://doi.org/10.1016/j.ccr.2011.12.029>.
- [93] Krawczyk B, Fabianowska-Majewska K. Alteration of DNA methylation status in K562 and MCF-7 cancer cell lines by nucleoside analogues. *Nucleosides Nucleotides Nucleic Acids* 2006;25(9–11):1029–32. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17065059.
- [94] Krawczyk B, Rudnicka K, Fabianowska-Majewska K. The effects of nucleoside analogues on promoter methylation of selected tumor suppressor genes in MCF-7 and MDA-MB-231 breast cancer cell lines. *Nucleosides Nucleotides Nucleic Acids* 2007;26(8–9):1043–6. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18058533.

- [95] Wozniak RJ, Klimecki WT, Lau SS, Feinstein Y, Futscher BW. 5-Aza-2'-deoxycytidine-mediated reductions in G9A histone methyltransferase and histone H3 K9 di-methylation levels are linked to tumor suppressor gene reactivation. *Oncogene* 2007;26(1):77–90. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16799634.
- [96] Xu J, Zhou JY, Tainsky MA, Wu GS. Evidence that tumor necrosis factor-related apoptosis-inducing ligand induction by 5-Aza-2'-deoxycytidine sensitizes human breast cancer cells to adriamycin. *Cancer Res* 2007;67(3):1203–11. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17283156.
- [97] Wong KK. DNMT1: a key drug target in triple-negative breast cancer. *Semin Cancer Biol* 2021;72:198–213. Available from: <https://pubmed.ncbi.nlm.nih.gov/32461152/>.
- [98] Ma G, Pan PY, Eisenstein S, Divino CM, Lowell CA, Takai T, et al. Paired immunoglobulin-like receptor-B regulates the suppressive function and fate of myeloid-derived suppressor cells. *Immunity* 2011;34(3):385–95. Available from: <http://www.cell.com/article/S1074761311000422/fulltext>.
- [99] Groth C, Hu X, Weber R, Fleming V, Altevogt P, Utikal J, et al. Immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) during tumour progression. *Br J Cancer* 2018;120(1):16–25. Available from: <https://www.nature.com/articles/s41416-018-0333-1>.
- [100] Wang SC, Lee TH, Hsu CH, Chang YJ, Chang MS, Wang YC, et al. Antroquinonol D, isolated from *Antrodia camphorata*, with DNA demethylation and anticancer potential. *J Agric Food Chem* 2014;62(24):5625–35. Available from: <https://pubs.acs.org/doi/abs/10.1021/jf4056924>.
- [101] Weng JR, Lai IL, Yang HC, Lin CN, Bai LY. Identification of Kazinol Q, a natural product from formosan plants, as an inhibitor of DNA methyltransferase. *Phyther Res* 2014;28(1):49–54. Available from: <https://onlinelibrary.wiley.com/doi/full/10.1002/ptr.4955>.
- [102] Florean C, Schnekenburger M, Lee J-Y, Kim KR, Mazumder A, Song S, et al. Discovery and characterization of Isofistularin-3, a marine brominated alkaloid, as a new DNA demethylating agent inducing cell cycle arrest and sensitization to TRAIL in cancer cells. *Oncotarget* 2016;7(17):24027–49. Available from: <https://www.oncotarget.com/article/8210/text/>.
- [103] Connolly RM, Jankowitz RC, Andreopoulou E, Allred J, Jeter SC, Zorzi J, et al. A phase 2 study investigating the safety, efficacy and surrogate biomarkers of response of 5-azacitidine (5-AZA) and entinostat (MS-275) in patients with advanced breast cancer. *SABCS 2011*:
- [104] Fu S, Hu W, Iyer R, Kavanagh JJ, Coleman RL, Levenback CF, et al. Phase 1b-2a study to reverse platinum resistance through use of a hypomethylating agent, azacitidine, in patients with platinum-resistant or platinum-refractory epithelial ovarian cancer. *Cancer* 2011;117(8):1661–9. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21472713.
- [105] Von Hoff DD, Rasco DW, Heath EI, Munster PN, Schellens JHM, Isambert N, et al. Phase I study of CC-486 alone and in combination with carboplatin or nab-paclitaxel in patients with relapsed or refractory solid tumors. *Clin Cancer Res* 2018;24(17):4072–80. Available from: <https://pubmed.ncbi.nlm.nih.gov/29764853/>.
- [106] Shorstova T, Foulkes WD, Witcher M. Achieving clinical success with BET inhibitors as anti-cancer agents. *Br J Cancer* 2021;124(9):1478–90. Available from: <https://pubmed.ncbi.nlm.nih.gov/33723398/>.
- [107] Andrikopoulou A, Lontos M, Koutsoukos K, Dimopoulos MA, Zagouri F. The emerging role of BET inhibitors in breast cancer. *Breast* 2020;53:152–63. Available from: <https://pubmed.ncbi.nlm.nih.gov/32827765/>.
- [108] Shu S, Lin CY, He HH, Witwicki RM, Tabassum DP, Roberts JM, et al. Response and resistance to BET bromodomain inhibitors in triple-negative breast cancer. *Nature* 2016;529(7586):413–17. Available from: <https://pubmed.ncbi.nlm.nih.gov/26735014/>.
- [109] Pérez-Salvia M, Simó-Riudalbas L, Llinàs-Arias P, Roa L, Setien F, Soler M, et al. Bromodomain inhibition shows antitumoral activity in mice and human luminal breast cancer. *Oncotarget* 2017;8(31):51621–9. Available from: <https://pubmed.ncbi.nlm.nih.gov/28881673/>.

- [110] Walsh L, Haley KE, Moran B, Mooney B, Tarrant F, Madden SF, et al. BET inhibition as a rational therapeutic strategy for invasive lobular breast cancer. *Clin Cancer Res* 2019;25(23):7139–50. Available from: <https://pubmed.ncbi.nlm.nih.gov/31409615/>.
- [111] Piha-Paul SA, Sachdev JC, Barve M, LoRusso P, Szmulewitz R, Patel SP, et al. First-in-human study of mivebresib (ABBV-075), an oral pan-inhibitor of bromodomain and extra terminal proteins, in patients with relapsed/refractory solid tumors. *Clin Cancer Res* 2019;25(21):6309–19. Available from: <https://pubmed.ncbi.nlm.nih.gov/31420359/>.
- [112] Cousin S, Blay JY, Garcia IB, de Bono JS, Le Tourneau C, Moreno V, et al. Safety, pharmacokinetic, pharmacodynamic and clinical activity of molibresib for the treatment of nuclear protein in testis carcinoma and other cancers: results of a phase I/II open-label, dose escalation study. *Int J Cancer* 2022;150(6):993–1006. Available from: <https://pubmed.ncbi.nlm.nih.gov/34724226/>.
- [113] Lei X, Ou Z, Yang Z, Zhong J, Zhu Y, Tian J, et al. A pan-histone deacetylase inhibitor enhances the antitumor activity of B7-H3-specific CAR T cells in solid tumors. *Clin Cancer Res* 2021;27(13):3757–71. Available from: <https://pubmed.ncbi.nlm.nih.gov/33811153/>.
- [114] Scandura JM, Roboz GJ, Moh M, Morawa E, Brenet F, Bose JR, et al. Phase 1 study of epigenetic priming with decitabine prior to standard induction chemotherapy for patients with AML. *Blood* 2011;118(6):1472–80. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21613261.
- [115] Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009;45(2):228–47. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19097774.
- [116] Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *Lancet Oncol* 2017;18(3):e143–52. Available from: <https://pubmed.ncbi.nlm.nih.gov/28271869/>.
- [117] Arnedos M, Rouleaux Dugage M, Perez-Garcia J, Cortes J. Window of Opportunity trials for biomarker discovery in breast cancer. *Curr Opin Oncol* 2019;31(6):486–92. Available from: <https://pubmed.ncbi.nlm.nih.gov/31464762/>.

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Therapeutic applications of the prostate cancer epigenome

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1 Introduction to prostate cancer

Prostate cancer (PCa) is the second most common cancer in men and the fifth leading cause of cancer-related deaths. An estimated 1.4 million men worldwide were diagnosed in 2020, accounting for 14.1% of male cancers and 6.8% of male cancer-related deaths. Significant global disparities exist in PCa incidence and mortality; incidence rates are threefold higher in developed countries over developing [1] and vary more than 13-fold worldwide. The rates are highest in Northern America, Australia/New Zealand, Southern Africa, and Northern and Western Europe and lowest in Asian populations, pointing toward environmental and genetic risk factors [1]. The marked variation in incidence can largely be attributed to the practice of prostate-specific antigen (PSA) testing or screening in certain geographical regions. Conversely, mortality rates are highest in developing regions [1].

PCa is a remarkably heterogeneous disease whose etiology is not well understood; most tumors have a slow natural trajectory and pose little likelihood of clinical manifestation, deemed indolent in nature [2]. Several large randomized controlled trials have assessed the benefit of PSA screening on PCa mortality. Overall, results show that PSA screening has little/no impact on reducing death from PCa (with 1000 men needing to be screened to save one death), but does significantly increase the detection of indolent disease and thus the risks of complications and side-effects from treatment [3,4]. However, a proportion of prostate tumors are aggressive, with the potential to metastasize and become castration resistant. Discerning indolent from aggressive disease is still challenging. Progression to metastatic castration-resistant prostate cancer (mCRPC) is commonly driven by persistent androgen receptor (AR) signaling.

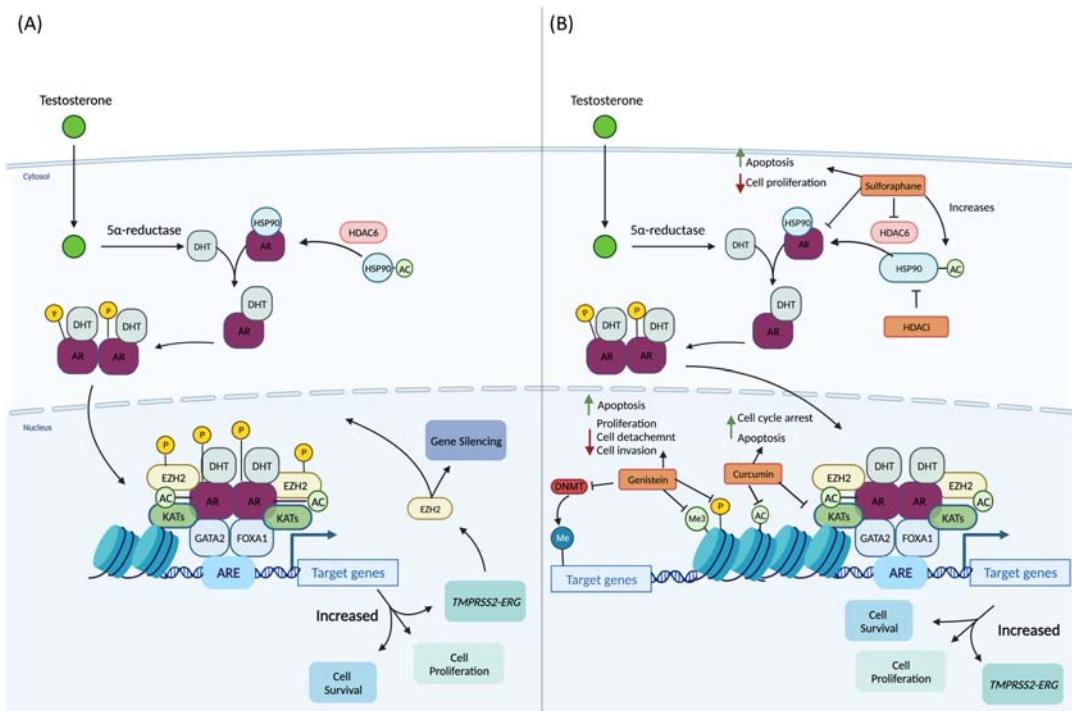
1.1 The androgen receptor signaling axis

The development, differentiation and growth of the prostate rely on androgens and their stimulation of the AR. The reliance on AR signaling is retained in PCa (Figure 14.1A). There are three sources of androgenic steroids: the testes, which synthesize testosterone from adrenal precursors; the adrenal glands, which secrete the weakly androgenic precursors androstenedione and dehydroepiandrosterone, and diet [5]. Within prostate cells, 5 α -reductase converts testosterone into the more potent dihydrotestosterone, which has a high affinity for the AR. Ligand activated AR functions as a transcription factor, by homodimerizing and translocating to the nucleus, where it promotes transcription of target genes (including *KLK3* (PSA) and *TMRPSS2*), leading to androgen-dependent cell growth.

1.2 Clinical management and treatment of prostate cancer

Localized disease can be successfully managed through radical prostatectomy, external beam radiotherapy, brachytherapy, and active surveillance for low/intermediate-risk disease; all of which offer close to 100% 10-year disease-specific survival rates [6,7].

Approximately 20%–25% of patients develop a recurrence following local therapy [8]. Androgen deprivation therapy is typically utilized for patients with a short PSA-doubling time (i.e., increased risk of lethal PCa), disease recurrence or metastasis [9]. Reducing circulating androgens

**FIGURE 14.1**

The androgen receptor signaling cascade in (A) a healthy prostate cell and (B) a cancerous prostate cell, including the effect epigenetic drugs and nutraceuticals have on this cascade. Free testosterone enters prostate cells and is converted to the more potent dihydrotestosterone (DHT) by the enzyme 5 α -reductase. Binding of DHT to the androgen receptor (AR) induces dissociation from heat-shock proteins (HSPs) and receptor phosphorylation. The AR dimerizes and translocate to the nucleus. The AR engages with a host of coactivators including lysine acetyltransferases (KAT3A (CBP) and KAT3B (p300)) and pioneer factors FOXA1 and GATA2 to bind to the androgen response element (ARE) and drive expression of its target genes. Activation of target genes leads to biological responses including proliferation and cell survival and the production of prostate-specific antigen (*KLK3*) and *TMPRSS2*.

(by luteinizing hormone-releasing hormone analogs) and/or antiandrogens (which block androgen signaling by targeting the ligand-binding domain of the AR), instigates a favorable response in most men, measured by a decline in serum PSA and radiographic imaging, and in improvement in disease-related symptoms. However, the positive response is short-lived, and the disease invariably progresses to the lethal castration-resistant phase [6,10].

Prostate tumors can escape AR inhibition through reactivation of AR signaling via a number of mechanisms (such as AR gene mutation or amplification, increased expression of the AR protein, promiscuous activation of the AR, aberrant behavior of AR coregulators, intracrine androgen production) or via lineage plasticity, involving transdifferentiation of adenocarcinoma to neuroendocrine disease

[11]. Another important mechanism of therapeutic resistance is the constitutively active AR splice variants (particularly AR-v7); truncated versions of the full receptor that lack the ligand-binding domain, making them invisible to current AR-directed therapies [12].

The last two decades have heralded a transformation in the treatment of advanced PCa, with survival rates significantly improving, due to advances made with second and third generation inhibitors of the AR pathway [13–15]. The androgen biosynthesis inhibitor Abiraterone targets the CYP17A1 enzyme inhibiting residual androgen synthesis [16]. Abiraterone in combination with prednisone improved survival of CRPC patients by 3.9 months in a phase III study [13,17]. The AR antagonist enzalutamide achieved significant serum PSA responses in >50% of chemotherapy-naïve CRPC patients in phase I/II studies [18,19]. A phase II study confirmed that enzalutamide in a safe and effective monotherapy for hormone -naïve mCRPC. Long-term treatment with enzalutamide gave a reduction in PSA levels (100% response rate at week 97) whilst maintaining safety and tolerability with limited side effects [20]. A 2012 study demonstrated that apalutamide, previously ARN-509, a second-generation antiandrogen similar in structure to enzalutamide, had characteristics which predicated a higher therapeutic index to already existing AR antagonists [21]. In 2018 the FDA-approved apalutamide as a therapeutic for nonmetastatic CRPC. This decision came from the double-blind multicenter trial consisting of over 1200 participants, which proved that apalutamide prolonged metastasis-free survival in high-risk patients [9].

Resistance to these “second-generation” AR antagonists can evolve. [22]. Nevertheless, the continued reliance of the cell on AR signaling warrants the development of further next-generation AR antagonists. The emergence however, of AR-independent clones (e.g., neuroendocrine, small cell morphology) at any stage during disease progression poses a major clinical challenge, as these cells do not respond to AR inhibition. This chapter will provide a synopsis of research activity into epigenetic therapies as viable alternatives or adjuncts to conventional therapeutics for CRPC.

2 A snapshot of the prostate cancer epigenome

2.1 The prostate cancer methylome

PCa has an unusually low mutation frequency [23]. By contrast, promoter hypermethylation of tumor suppressor genes and genes with important regulatory functions is widespread, affecting virtually all cellular pathways [24].

There is overwhelming evidence to support a role for promoter hypermethylation of individual genes, in particular the intracellular detoxification enzyme glutathione s-transferase pi (*GSTP1*), at the earliest stages of PCa initiation [25–27]. Promoter hypermethylation and silencing of *GSTP1* is a molecular hallmark of PCa, observed in more than 90% of tumors and in ~75% of preinvasive high-grade prostatic intraepithelial neoplastic (HGPIN) lesions, a finding that has been replicated by many independent groups. Building on this seminal work, quantitative assessments of multiple gene panels have demonstrated an “epigenetic catastrophe” during tumor initiation, with widespread hypermethylation and silencing of regulatory and tumor suppressor genes, such as *RARBeta* and *RASSF1A* [28]. There is also strong evidence to support clonal inheritance of DNA methylation patterns during metastatic progression [29]. Indeed, distinct subsets of genes become hypermethylated in late stage PCa, during which epigenetic changes become markedly more heterogeneous

[30,31]. While causes of widespread promoter hypermethylation in PCa are unclear, expression of the DNA demethylase enzyme ten-eleven translocation 1 (TET1) has been shown to be significantly reduced in tumors [32].

De novo losses in DNA methylation (or hypomethylation) are approximately four times more common than hypermethylated alterations in PCa metastases, and notably show no enrichment at promoters or with any particular gene ontology or cancer-related gene sets [33]. Extensive losses in 5mC are pronounced at genomic regions of low CpG density and tend to be accompanied by focal gains in DNA methylation at promoter CpG islands (CGIs) in PCa metastases [34]. There remains very limited evidence of *de novo* promoter hypomethylation and subsequent proto-oncogene activation in PCa [35–37]. Indeed, global analysis of DNA methylation in parallel with gene expression in PCa metastases showed that promoter hypomethylation has a negligible effect on gene expression [33]. Substantial differences in the timing and extent of DNA hypomethylation are evident between different cancer types, which could relate to the proliferative index of the tissue and the metabolism of one-carbon. This would explain why tumors with a protracted natural history (such as prostate) do not exhibit global losses in 5mC until more advanced stages of disease. Yegnasubramanian et al. also showed that DNA hypomethylation occurs in a very late stage of PCa progression, and only significantly at the metastatic stage [34]. Other studies have shown that PLAU (or uPA), a gene which is normally repressed by methylation and plays a role in tumor invasion and metastasis development is hypomethylated and leads to a higher expression than in benign prostate tissue [37].

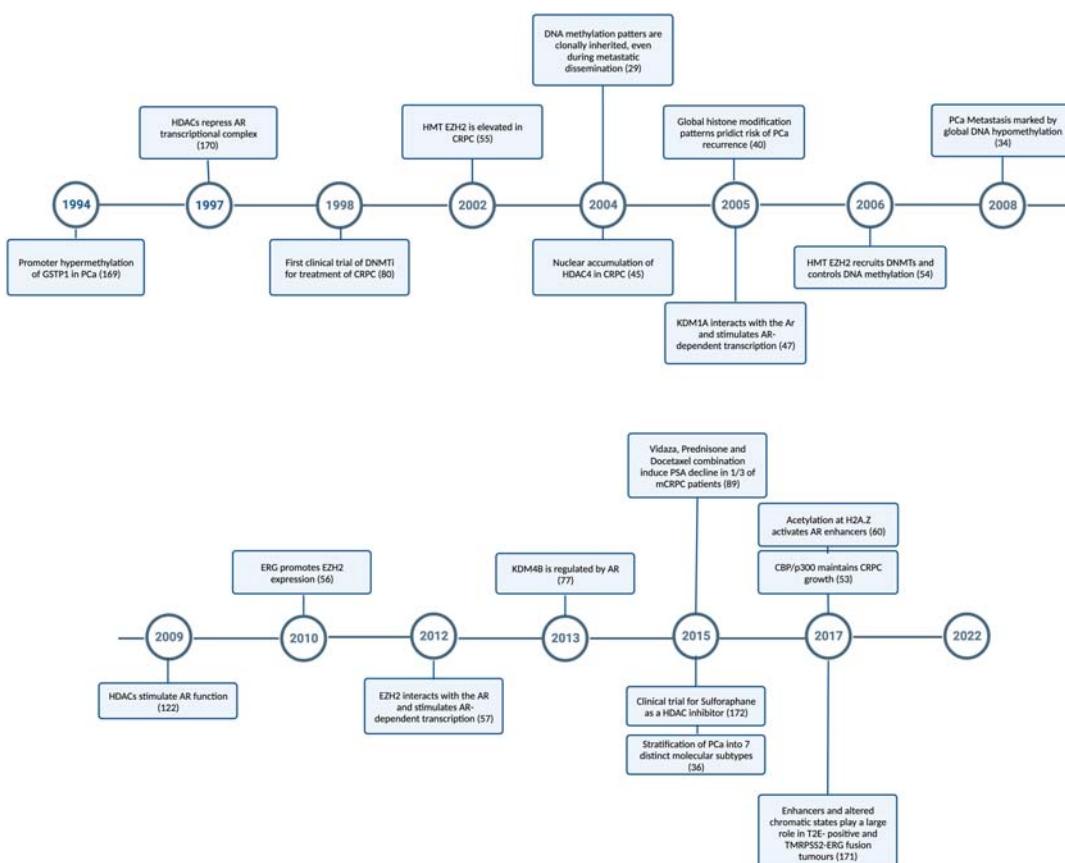
Since the first edition of this book, the advent of The Cancer Genome Atlas (TCGA) consortium has enabled powerful parallel omic assessments of many cancer types, including prostate (Figure 14.2) [38]. An integrative analysis of the genomes and epigenomes from 333 primary prostate tumors revealed a diverse DNA methylation landscape that mapped to seven molecularly distinct subsets of tumors. ERG-fusion positive tumors mostly displayed moderately elevated DNA methylation, with one-third of cases exhibiting an exclusive distinct hypermethylation pattern. ETV1 and ETV4 fusion-positive tumors showed more heterogeneous methylation. In contrast, SPOP and FOXA1-mutant tumors exhibited homogeneous epigenetic profiles. The low-frequency IDH1-mutant tumors were unique due to their highly elevated levels of genome-wide DNA hypermethylation, and represented a distinct subgroup of early-onset disease, with fewer DNA copy-number alterations or other canonical genomic lesions [38].

More recently the DNA methylation landscape of advanced PCa was characterized through a parallel deep-sequencing assessment of the genomes, epigenomes, and transcriptomes of one hundred castration-resistant metastases [53]. One-fifth of tumors demonstrated a novel epigenomic subtype, defined by hypermethylation and somatic mutations within the *BRAF*, *DNMT3B*, *IDH1*, and *TET2* genes. In addition to regulatory elements (such as promoters and enhancers), differential DNA methylation was found to colocalize with somatic mutational hotspots.

Collectively, these integrative omic studies provide strong indications for an interplay between distortion of the genome and epigenome in PCa, although the mechanisms involved need to be further teased out.

2.2 Histone modifications, variants, and epigenetic enzymes in prostate cancer

Various posttranslational modifications of the histone tails become altered in PCa. Notable examples include a global decrease in histone acetylation, with hyperacetylation at H3K18,

**FIGURE 14.2**

A snapshot of prostate cancer epigenomics research over the past 30 years [29,34,39–52].

and methylation at H3K4 and H3K9 and H3K27, all of which have been associated with higher tumor grade, disease recurrence, and castration resistance [39,54–56]. Profiling histone modifications (H3K27 acetylation and methylation) in neuroendocrine PCa identified approximately 15,000 regulatory elements that were dormant in prostate adenocarcinoma, a significant proportion of which were bound by FOXA1, implying a dependency on this pioneer transcription factor and the potential for therapeutic intervention [11].

In turn, the overexpression of histone modifying enzymes has also been linked with PCa progression, with many shown to be independent prognostic markers. For example, several classes of histone deacetylases (HDACs) are overexpressed in aggressive PCa and CRPC [40,57,58]. So too are the lysine methylases (KMTs; such as EZH2 and DOT1L) and demethylases (KDMs; such as KDM5C), which also offer prognostic value [41,59–62]. The HAT CBP/p300 which is responsible for the acetylation of both histone and nonhistone proteins involved in cell growth and tumorigenesis [63] plays an important role in maintaining CRPC growth [64].

Enhancer of zeste homolog 2 (EZH2), a subunit of Polycomb repressive complex 2 (PRC2), silences gene expression through its lysine methyltransferase activity on H3K27 (mostly tri-methylation). EZH2 also acts as a molecular scaffold, attracting DNMTs to gene promoters [42], thus bridging two distinct modes of epigenetic gene regulation: nucleosome remodeling and DNA methylation. EZH2 is frequently overexpressed in many cancers, including prostate, and especially CRPC; its overexpression represses tumor and metastases suppressor genes, is associated with elevated proliferation and increases tumor aggressiveness [43]. Elevated EZH2 is also linked with poor survival, making it a relevant therapeutic target. It was recently shown that expression of the *EZH2* gene is stimulated by the ERG transcription factor, which is overexpressed in approximately half of all prostate tumors, due to fusion with the androgen regulated *TMPRSS2* [44]. However, the oncogenic properties of EZH2 have in fact been attributed to a PRC2-independent transcriptional activating function of the enzyme [45].

A genome-wide systematic analysis of the functional significance of 615 epigenetic proteins in PCa cells revealed that subsets of enzymes influence different cancer cell phenotypes [65]. Several KDMs were highly expressed and found to mainly impact cell proliferation, whereas HDACs were primarily involved in regulating AR expression.

Another factor to consider in how epigenetic modifications impact chromatin accessibility is the histone variants, which have evolved to carry out functions distinct from those of the core histones. The core histone variant H2A.Z operates as both a transcriptional repressor and activator, influenced by posttranslational modifications (particularly acetylation), H2A.Z-H2B heterotypic dimers and multiple isoforms, namely H2A.Z1 and H2A.Z2 [66,67]. H2A.Z has been proposed to play a role in deregulation of gene expression and tumor progression in PCa with a global reorganization of its acetylation. H2A.Zac at the promoters of oncogenes leads to their activation, whilst conversely loss of H2A.Zac at the promoters of tumor suppressor genes is associated with their transcriptional silencing [67]. Indeed increased expression of the H2A.Z gene (*H2AFZ*) is reported in a CRPC xenograft model [68] as well as HGPin and primary tumors [69]. Sirtuin 1, a class III HDAC, reduces histone variant H2A.Z levels via proteasomal degradation; the two show reciprocal levels of gene expression in PCa [69]. Another noteworthy example is the centromeric H3 variant centromere protein A (CENPA), which modulates expression of critical proliferation, cell-cycle, and centromere/kinetochore genes and has been shown to be highly overexpressed in PCa correlating with disease stage [70].

3 Epigenetic modulation of androgen receptor signaling

3.1 AR acetylation

AR coregulators (activators or repressors) modify AR gene transactivation and prostate cell survival through acetylation in particular, but also via phosphorylation, ubiquitination, and sumoylation [71]. Lysine acetyltransferases (KATs), such as p300 (KAT3B), TIP60 (TAT interactive protein 60, KAT5) [72], and CBP (cAMP response element binding protein, KAT3A), behave as generic coactivators of many transcription factors, including the AR and p53. Other notable coactivators are PCAF (the p300-CBP-associated factor, KAT2B) and ARD1 [73]. The acetylases modify the AR at individual lysine residues and upregulation of these proteins is often associated with PCa

progression and aggression [74]. Acetylation is critical for the transcriptional activation activity of the AR; it also precludes its polyubiquitination and degradation [75]. For example, ARD1 acetylates the AR at lysine 618, inducing HSP90 dissociation and AR activation [74,76]. Expression of p300 is correlated with AKT phosphorylation (a surrogate for PTEN inactivation and a prevalent feature of metastatic PCa). Mechanistically, PTEN inactivation increases AR phosphorylation at serine 81, which promotes p300 binding and acetylation of the AR [75]. Thus p300 behaves as a *bona fide* oncogenic factor in PCa. Similarly, by promoting transcriptional activation activity of the AR, these coactivators promote CRPC [77]. CBP and p300 both serve as coactivators of AR, playing a critical role in the histone acetylation required for AR activity and expression of its target genes and are necessary to maintain cell growth in CRPC [64]. Inhibition of the CBP/P300 bromodomain (through small molecule inhibitor) impeded CA cell growth both *in vitro* and *in vivo* PDX models [64].

Transactivation by the AR is also dependent on access to its binding sites (androgen response elements (AREs)) at target gene enhancers or promoters, which is influenced by local chromatin structure. For example, a connection was made recently between H2A.Z acetylation and AR signaling. H2A.Zac plays an active role in AR enhancer function during hormone ablation and higher levels of global H2A.Zac correlate with poorer prognosis, reinforcing its pro-oncogenic role in PCa [78].

Following AR acetylation, KATs further facilitate AR transcriptional activity by nucleosome remodeling via histone acetylation and by recruiting the RNA polymerase II complex to AR-regulated promoters [79,80]. In addition, other factors (such as the androgen regulated AR coactivator ADAT2), modulate the transcriptional activity of the AR by promoting an open chromatin structure, which facilitates AR binding, creating a positive feedback loop [81,82]. This more open chromatin model is indicative of CRPC, with CRPC having unique open chromatin cites that are not found in androgen-sensitive PCa or in BPH, in addition to these sections of open chromatin being longer in CRPC [82].

3.2 AR methylation

Lysine (de)methylation contributes to AR transcriptional activation, at the level of histones, the AR protein itself and the interplay between the two. EZH2 (KMT6) methylates the AR, at lysine 630 and 632, which potentiates its transactivation function. Again, this has been mechanistically linked with AKT hyperactivity/PTEN loss, which mediates EZH2 phosphorylation at serine 21, stimulating AR methylation, through the SET domain of EZH2 [45]. The histone methyltransferase DOT1L has been linked to the progression of PCa (see Section 6) and increased AR activity. DOT1L additionally methylates the N-terminus of AR, recruiting PCa-associated lncRNAs, PRNCR1 and PCGEM1, enhancing AR target gene activation [83].

Conversely, lysine demethylase LSD1 (KDM1A) also interacts with the AR and promotes androgen-dependent transcription of target genes by demethylating repressive histone marks (mono- and dimethylation at H3K9) [41]. Several members of the jumonji domain containing family of KDMs (KDM4A, KDM4B, KDM4C, and KDM4D) also function as AR coactivators [46]. KDM1A and KDM4C colocalize and cooperatively stimulate AR-dependent gene transcription in the prostate microenvironment by removing methyl groups (me1, me2, and me3) from H3K9 [84]. KDM4B was identified as the first androgen-regulated KDM [46]. It influences AR transcriptional activation via its histone demethylation activity but also by directly modulating ubiquitination of

the AR. Knockdown of KDM4B almost completely depletes AR protein in the LNCaP cell line. Furthermore, KDM4B expression in clinical specimens positivity correlates with clinical grade, implying that KDM4B may be a viable therapeutic target for PCa.

4 Drugging the methylome for the treatment of castration-resistant prostate cancer

DNA methylation patterns are clonally maintained and can serve as driver events making them attractive therapeutic targets. Unlike genetic alterations, DNA methylation can be dynamic and reversible. Genes silenced by promoter hypermethylation are typically wild type in their genetic code, thus normal genetic function might be restored by removing the DNA methyl marks.

DNA methyltransferase inhibitors (DNMTi) can be classed as either nucleoside analogs, which include both 5-Aza-cytidine (5-Aza-CR; vidaza) and 5-Aza-2'-deoxycytidine (5-Aza-CdR; decitabine) or nonnucleoside inhibitors, which include naturally occurring compounds, new chemical entities, or the repurposing of existing drugs.

Preclinical studies of the nucleoside analogs have validated their use in restoring expression of numerous hypermethylated gene loci in PCa cell lines. Their application in PCa xenograft models has shown their potential to delay progression to CRPC [85]. As early as 1998, a phase II clinical trial investigated intravenous administration of decitabine for the treatment of CRPC in men following complete androgen blockade and flutamide withdrawal. Only 12 men were evaluable, of which 2 (16.67%) showed a time of progression of >10 weeks [47]. In 2011, a phase II trial of vidaza in a larger number of CRPC patients ($n = 36$) found that PSA-doubling time improved by ≥ 3 months in more than half (55.8%) of the cohort [86].

Over the last decade, research has focused on the potential of the DNMTi to amplifying the efficacy of conventional therapies (either chemotherapeutic agents or androgen withdrawal/blockade) in men with advanced PCa. Treatment of DU145 PCa cell lines with docetaxel and either 5-Aza-CdR or 5-Aza-CR enhanced docetaxel sensitivity through hypomethylation and reactivation of DNA damage response gene *GADD45A* [87]. Similarly, 5-Aza-CR pretreatment of DU145 cells resistant to docetaxel, enhanced their response to cabazitaxel [88]. Decitabine used in combination with either paclitaxel or cisplatin caused a synergistic growth suppression in prostate cell lines through enhanced induction of apoptosis and G₂/M cell cycle arrest [89,90]. In the TRAMP model of PCa, combined castration and decitabine significantly improved survival over either single treatment ($P < .05$) and reduced the presence of malignant disease ($P < .0001$) [91]. In preclinical cell line and xenograft models, vidaza has been shown to restore AR expression and enhance the apoptotic effects of antiandrogen bicalutamide [92]. 5-Azacytidine has also been demonstrated to enhance DU145 and PC3 sensitivity to Doxorubicin and Topotecan, potentially through demethylating and reactivating miR-34a expression [93]. Since the first edition of this book, a small number of early phase clinical trials assessing the efficacy of the canonical DNMTi for the treatment of recurrent or advanced PCa have commenced, mostly in combination with standard of care (Table 14.1). For example, the combination of vidaza, prednisone and docetaxel in mCRPC patients who progressed on or after docetaxel chemotherapy was well tolerated and elicited a PSA decline of 50% in half of the 19 evaluable patients treated in the trial [48]. It will be very interesting to

Table 14.1 DNMT Inhibitors in Clinical Trials for Prostate Cancer

Drug (DNMT inhibitor)	Clinical trial identifier	Study design	Phase	Enrollment	Status	Aim	Description	Primary outcomes measured	Results
Decitabine	NA		II	14	Completed	To find out the effects of decitabine on patients with CRPC	IV decitabine (75 mg/m ²) every 8 h for 3 disease, repeated every 5–8 weeks.	(1) To determine whether vidaza can restore hormone-responsiveness in CRPC	12/14 men enrolled were evaluable. 2/12 men had stable disease and delayed time to progression ≥ 10 weeks [47]
Decitabine in combination with All-Trans Retinoic Acid	NCT03572387	Prospective, open-label, randomized, cross-over	Pilot study	14	Active, not yet recruiting	A pilot study to assess the DPFS and safety of the combination of 5-AZA and All-trans Retinoic Acid (ATRA) for PCa with PSA-only recurrence after definitive local treatment	A 1:1 randomization to either the “5-AZA + ATRA” group or the “no therapy” group. Treatment on a 28-day cycle, in the absence of prohibitive toxicities, for 3 cycles. 5-Aza (40 mg/m ²) SC on days 1–5. ATRA (45 mg/m ²) orally on days 3–7 of each cycle, divided into two doses. Lupron (7.5 mg × 1)	(1) PSA RR after 12 weeks, defined by >30% decrease from baseline and (2) Percentage of adverse events by grade	NYA
Decitabine and Cedazuridine in combination with Enzalutamide	NCT05037500	Interventional, open label	Ib	19	Recruiting	To establish the safety of oral decitabine and cedazuridine with enzalutamide in patients with mCRPC	A dose-escalation study. Decitabine and cedazuridine PO QD on either days 1–3, 1–4, or 1–5 and enzalutamide PO QD on days 1–28. Treatments repeat every 28 days for up to 6 cycles in the absence of disease progression or unacceptable toxicity.	Incidence of AEs after 30 days posttreatment	NYA

Vidaza	NCT00384839	Open label	II	36	Completed	To determine if Vidaza can convert hormone-refractory prostate cancer to a hormone-responsive state	Injectable suspension of vidaza (75 mg/m^2) days 1–5 every 28-day cycle. Maximum 12 cycles	Percentage of patients With PSA DT ≥ 3 months	34/36 men enrolled were evaluable. 80.6% had metastatic disease. PSA-DT ≥ 3 months was attained in 19 patients (55.8%). Median PFS was 12.4 weeks ^a
Vidaza, Docetaxel and Prednisone	NCT00503984	Nonrandomized, open label	I/II	22	Terminated (withdrawal of funding)	To study the side effects and best dose of azacytidine and docetaxel when given together with prednisone and to see how well they work in treating patients with CRPC who did not respond to hormone therapy	Phase I: IV vidaza over 30 min days 1–5 of each 3-weekly cycle, docetaxel: IV over 1 h on day 6 of each 3-weekly cycle, prednisone (5 mg) twice a day from days 1–21 of each cycle. Phase II: as above without prednisone	Phase I: MTD of vidaza and docetaxel combination Phase II: Response, defined as PSA response or complete or partial response, by RECIST criteria	19/22 men enrolled were evaluable. 10/19 (52.63%) of men achieved a PSA response in accordance with Prostate Cancer Working Group 1 criteria ($\geq 50\%$ decline in PSA levels) [48]
Disulfiram	NCT01118741	Open label	Dose escalation	19	Completed	To determine the effect of disulfiram on DNA methylation in men with recurrent PCa	250 mg PO daily and 500 mg PO daily	Demethylation response	22% of participants in the 250 mg treatment arm and 30% of participants in the 500 mg treatment arm had a demethylation response [94]

(Continued)

Table 14.1 DNMT Inhibitors in Clinical Trials for Prostate Cancer *Continued*

Drug (DNMT inhibitor)	Clinical trial identifier	Study design	Phase	Enrollment	Status	Aim	Description	Primary outcomes measured	Results
Disulfiram in combination with copper	NCT02963051	Open-label, nonrandomized	Ib	9	Terminated (lack of efficacy)	To determine the safety and optimal dosing of IV copper chloride and disulfiram in mCRPC patients	Copper chloride (1–7 mg) IV with disulfiram (80 mg) PO three times daily and copper gluconate (1.5 mg) PO three times daily	Number of AEs	Bone and nodal metastases showed differential and heterogeneous copper uptake. No confirmed PSA declines or radiographic responses were observed. Common adverse events included fatigue and psychomotor depression; no Grade 4/5 AEs were observed [95]

^aSonpavde G, Aparicio AM, Zhan F, North B, Delaune R, Garbo LE, et al. Azacitidine favorably modulates PSA kinetics correlating with plasma DNA LINE-1 hypomethylation in men with chemonaive castration-resistant prostate cancer. *Urol Oncol* 2011;29(6):682–689 [86].

learn how trials investigating DNMTi in combination with second-generation AR antagonists (such as enzalutamide) progress.

4.1 New classes of DNMT inhibitors

The poor bioavailability, chemical instability and inherent toxicity of the nucleoside analog DNMTi has steered research toward compounds that target DNMTs more directly, rather than intercalating into the DNA double helix. One of the most promising of these agents is SGI-1027, a quinoline-based dinucleotide molecule. It is effective at a low micromolar concentration, inhibiting both DNMT1 and DNMT3A, arresting proliferation and reactivating tumor suppressor genes [96]. A novel analog of SGI-1027 is *N*-(3-(2-amin-o-6-methylpyrimidin-4-ylamino)phenyl)-3-(quinolinolin-4-ylamino)benzamide MC3343, which demonstrates higher potency and reduced toxicity than SGI-1027. It reduced viability and proliferation of PC3 PCa cells and impaired EMT by inducing E-cadherin and reducing MMP2 mRNA and protein levels [96].

RG108, another nonnucleoside inhibitor, reduced the viability of DU145, LNCaP, and 22Rv1 PCa cells by inducing apoptosis, in a time and dose dependent manner. RG108 was most effective in androgen-dependent LNCaP cells, reducing DNMT1 expression and activity causing global DNA hypomethylation accompanied by reduced promoter hypermethylation of cancer-related genes, including *GSTP1* [97].

Hydralazine, a potent arterial vasodilator approved by FDA for treatment of severe hypertension and heart failure, has demonstrated weak DNA demethylation activity and synergistic effects when used in combination with HDACi valproic acid, both *in vitro* and *in vivo*. Clinical trial results from other solid tumors look promising, in terms of both safety as well as efficacy in overcoming chemotherapeutic resistance. In PCa, preclinical research using cell line models demonstrated that hydralazine could reverse the PCa cell phenotype through inhibiting EGFR signaling, decrease DNMT expression, and restore expression of epigenetically silenced genes involved in prostate carcinogenesis [98]. More recently the combined treatments of hydralazine with HDACi (either panobinostat or valproic acid) or enzalutamide had synergistic growth inhibitory effects in the panel of PCa cell lines [99,100].

The final noteworthy example is disulfiram, a thiol-reactive compound used to treat alcoholism, which possesses intrinsic DNMTi properties. In PCa xenograft and cell line models, the drug globally reduced 5-mC levels, reactivated *RARβ2* and *APC* tumor suppressor genes and functionally reduced proliferation and induced apoptosis [101]. Subsequently, a clinical trial (NCT01118741) completed in 2012 showed that 22% of PCa patients treated with low-dose disulfiram (250 mg) and 30% of patients treated with high-dose disulfiram (500 mg) had a positive demethylation response ($\geq 10\%$ decrease in global 5mC levels compared to baseline) (Table 14.1) [94]. However, no changes in PSA kinetics were observed with either dose and the drug was poorly tolerated. Interestingly, the anticancer activity of disulfiram has been shown to be dependent on the presence of copper. The AR drives transcription of several genes involved in copper homeostasis leading to the accumulation of copper within prostate cells. However, a Phase 1b dose escalation study of copper and disulfiram (NCT02963051) in men with mCRPC again failed to show any biochemical or radiologic improvement [95].

While each of these individual compounds is potentially promising, progression beyond these early-stage preclinical studies has been limited due to their low potency and largely unknown

mechanisms of action. More work needs to be done and in more physiologically relevant models, such as organoids and explant systems, to better recapitulate the heterogeneity of the disease and overcome the challenges that this brings.

5 HDAC inhibitors for the treatment of castration-resistant prostate cancer

HDAC inhibitors (HDACi) are the other major class of epigenome modulating agents under investigation for the treatment of CRPC. HDAC inhibition causes histones to become hyperacetylated and chromatin to adopt an open conformation conducive to interaction with the transcriptional apparatus. Interestingly, it has been reported that <10% of genes are influenced directly by HDACi [102]. Of course HDACs interact with numerous nonhistone proteins, for example the AR, thus implying more pleiotropic mechanisms of HDACi activity (see Section 6). The strong attraction for developing HDACi is their selective action on tumor cells, inducing apoptosis, growth arrest and autophagy, amongst other phenotypic effects (reviewed elsewhere).

Multiple dose-dependent mechanisms of action have been observed for HDACi, both epigenetic and cytotoxic. There is a wealth of preclinical information showing that many HDACi exert effective antiproliferative and pro-apoptotic effects in PCa cell lines and xenograft models. For example, valproic acid (VPA, a short chain fatty acid inhibitor) inhibits growth of PCa cells *in vitro* and reduces tumor xenograft growth in athymic nude mice by modulating multiple pathways, including cell cycle arrest, apoptosis, angiogenesis, and senescence through its effects on HDACI1 acetylation [103,104]. Recently it was found that VPA suppresses EMT in PCa cells through increasing E-cadherin and the ubiquitination enzyme TIF1gamma and decreasing N-cadherin and vimentin [105].

The more potent hydroxamate HDACi inhibit HDAC activity at low micro/nanomolar ranges through binding to the catalytic pocket of the enzyme. Both TSA and SAHA (vorinostat) induce cell death and inhibit growth in PCa cell lines through inhibiting AR gene expression [106,107]. SAHA also suppresses tumor growth and volume with little toxicity in mice transplanted with CWR22 human prostate tumors in addition to enhancing T-cell mediated lysis of LNCaP PCa cells [108,109].

Micromolar concentrations of the benzamide HDACi Ms-275 (entinostat) cause growth arrest of PC3 and LNCaP cell lines and induce cell death in DU145 cells and PC3, derived *in vivo* subcutaneous xenografts. Molecular analysis shows that treatment increases histone H3 acetylation and CDKN1A (p21) expression in tumors. In the transgenic adenocarcinoma of mouse prostate (TRAMP) mouse model, long-term treatment with Ms-275 slowed tumor progression with significant reduction in cell proliferation [110].

De et al. demonstrated that MHY219 inhibited HDAC1 activity in a dose-dependent manner, although this varied between PCa cell type. It also inhibited migration of LNCaP (androgen-dependent) and DU145 (androgen-independent) cells by downregulating MMP-1 and MMP-2 and upregulating TIMP-1 [111].

A number of clinical trials with HDACi have been instigated in PCa patients; however, the response rate is generally poor (Table 14.2) [112–114]. A phase I/II study assessing the efficacy of HDACi panobinostat in combination with antiandrogen bicalutamide in patients who were resistant to second line antiandrogen therapy, established a synergistic antitumor effect with reduced AR

Table 14.2 HDAC inhibitors in clinical trials for prostate cancer.

Drug	Clinical trial identifier	Study design	Phase	Enrollment	Status	Aim	Description	Primary outcomes measured	Results
SB939	NCT01075308	Nonrandomized, open label	Phase II	32	Completed	To determine the efficacy and PFS of patients with recurrent or metastatic CRPC.	Oral SB939 once daily on days 1, 3, 5, 8, 10, 12, 15, 17, and 19. Treatment repeats every 4 weeks for up to 12 courses in the absence of disease progression or unacceptable toxicity.	(1) PSA-response and (2) PFS	SB939 was well tolerated. Two patients (6%) showed a PSA response lasting 3 and 21.6 months. SB939 did not show sufficient activity to warrant further study as a single agent in unselected patients with CRPC [116].
Panobinostat	NCT00667862	Open label	Phase II	35	Completed	To characterize the safety, tolerability, and efficacy of IV panobinostat as a single-agent treatment in patients with CRPC.	IV panobinostat (20 mg/m^2) on days 1 and 8 of a 21-day cycle.	(1) PFS at 24 weeks	Of the 35 patients enrolled, 4 (11.4%) were progression-free at 24 weeks; 0 exhibited a PSA decline $\geq 50\%$. IV panobinostat did not show sufficient level of clinical activity to pursue further activity as a single agent in CRPC [112].
Panobinostat	NCT00493766	Nonrandomized, open label	Phase I	16	Terminated (strategic decision)	To define the MTD, toxicity, activity, and pharmacokinetics of oral panobinostat, alone (Arm A) and in combination with docetaxel (Arm B) in patients with CRPC.	Arm A: Oral panobinostat (20 mg) on days 1, 3, and 5 for 2 consecutive weeks, followed by 1 week break. Arm B: Oral panobinostat (15 mg) administered on the same schedule in combination with docetaxel (75 mg/m^2) every 21 days.	(1) MTD and DLT of escalating doses of panobinostat. (2) MTD and DLT of escalating doses of panobinostat in combination with standard dose of docetaxel and daily prednisone.	16 patients were enrolled, 8 in each arm. Grade 3 toxicities were observed. Arm A: All patients developed progressive disease. Arm B: 5 patients had a PSA decline $\geq 50\%$. Docetaxel had no apparent effect on the pharmacokinetics of panobinostat. ^a

(Continued)

Table 14.2 HDAC inhibitors in clinical trials for prostate cancer. *Continued*

Drug	Clinical trial identifier	Study design	Phase	Enrollment	Status	Aim	Description	Primary outcomes measured	Results
Panobinostat combined with Bicalutamide (Casodex)	NCT00878436	Randomized, open label	Phase I (panobinostat) & Phase II (bicalutamide)	52	Completed	To investigate the safety, dosing schedule, and efficacy of the combination treatment of Panobinostat and hormone therapy for recurrent PCa.	Arm A: 21-day treatment cycle of bicalutamide 50 mg P.O. daily, continuously, with the addition of 40 mg panobinostat 3 times/ week (120 mg/week) for 2 consecutive weeks with one week rest. Arm B: 21-day treatment cycle of bicalutamide 50 mg P.O. daily, continuously, with the addition of 20 mg panobinostat 3 times/ week (60 mg/week) for 2 consecutive weeks with one week rest	The proportion of patients free of progression and without symptomatic deterioration by 9 months of therapy.	41/52 patients were evaluated. 42% of patients (Arm A) and 19% of patients (Arm B) were free of progression and without symptomatic deterioration at 6 months.
Romidepsin	NCT00106418	Open label, nonrandomized, single arm	Phase II	35	Completed	To evaluate the activity and tolerability of romidepsin in patients with metastatic PCa with a rising PSA while on hormonal therapy.	IV romidepsin infusion (13 mg/m ²) on days 1, 8, and 15 every 28-day cycle.	(1) No evidence of radiological progression at 6 months.	2/35 (5.71%) patients achieved a confirmed radiological partial response lasting \geq 6 months and a PSA decline \geq 50%; 11 were discontinued due to toxicities. At the selected dose and schedule, romidepsin demonstrated minimal antitumor activity in chemo-naïve patients with CRPC.

Vorinostat	NCT00330161	Open label, single arm	Phase II	29	Completed	To evaluate vorinostat in patients with advanced PCa who have progressed on 1 prior chemotherapy.	Oral vorinostat once daily on days 1–21. Treatment repeats every 21 days for at least 4 courses in the absence of disease progression or unacceptable toxicity.	Number of patients with PFS at 6 months.	27/29 patients were evaluable. All 27 were off therapy before the 6-month time point. 13 (48%) were removed due to progression, 11 (41%) to toxicity, and 3 (11%) for other reasons [113].
Vorinostat combined with Androgen deprivation therapy prior to radical prostatectomy	NCT00589472	Open-label, single-arm	Phase II	19	Completed	To determine the efficacy of neoadjuvant androgen deprivation therapy and vorinostat followed by radical prostatectomy for the treatment of localized PCa.	Bicalutamide PO QD for 1 month and leuprorelin acetate IM or goserelin acetate SC once a month until surgery, vorinostat PO QD beginning on the first day of androgen depletion therapy and continuing for up to 8 weeks or until the day of surgery (open or laparoscopic radical prostatectomy). Patients with positive surgical margins undergo immediate adjuvant external beam radiotherapy.	(1) Pathologic complete response at the time of surgery (12 weeks).	18/19 patients were evaluable. 0/18 (0%) demonstrated a complete pathologic response.
Tasquinimod	NCT02057666	Randomized, double-bind, placebo-controlled	Phase III	146	Terminated (Development of Tasquinimod in prostate cancer discontinued)	To confirm the effect of Tasquinimod in delaying disease progression or death as compared with placebo in chemo-naïve Asian men with mCRPC.	Drug: 0.25 mg/day, titrated through 0.5 mg/day (from day 15) to a maximum of 1 mg/day (from day 29).	(1) Radiological PFS (up to 3 years).	The median time for radiological progression for patients treated with Tasquinimod ($n = 96$) was 11 months versus 7.53 months for the placebo arm ($n = 50$), $P = .027$

(Continued)

Table 14.2 HDAC inhibitors in clinical trials for prostate cancer. *Continued*

Drug	Clinical trial identifier	Study design	Phase	Enrollment	Status	Aim	Description	Primary outcomes measured	Results
Tasquinimod	NCT01234311	Randomized, double-blind, placebo-controlled	Phase III	1245	Completed	To confirm the effect of Tasquinimod in delaying disease progression or death as compared with placebo in asymptomatic/mildly symptomatic men with mCRPC.	Arm A: Tasquinimod 0.25, 0.5, or 1 mg/day delivered orally; n = 832. Arm B: Placebo; n = 413.	(1) Radiological PFS (up to 3 years).	Estimated median radiological PFS was 7.0 months with Tasquinimod and 4.4 months with placebo, $P < .001$. The median OS was 21.3 months with Tasquinimod and 24.0 months with placebo $P = .25$. Grade ≥ 3 adverse events were more frequent with Tasquinimod (42.8% vs 33.6%), the most common being anemia, fatigue, and cancer pain. In chemotherapy-naïve men with mCRPC, Tasquinimod significantly improved radiological PFS, however, no OS benefit was observed. ^b
Tasquinimod	NCT01732549	Randomized, double-blind, placebo-controlled	Phase II	144	Terminated	Proof of concept study of maintenance therapy with Tasquinimod in patients with mCRPC who are not progressing after a first line Docetaxel based chemotherapy.	Drug: 0.25 mg/day, escalated to 0.5 or 1 mg/day until disease progression or toxicity.	(1) Radiological PFS (up to 3.5 years).	Radiological PFS was 31.7 weeks in the Tasquinimod arm and 22.7 weeks in the placebo arm, $P = .016$. Maintenance Tasquinimod therapy significantly reduced the risk of rPFS by 40%. ^c

JKI-802	NCT05268666	Multicenter, first in human, open-label, 2-part, dose escalation and expansion study	126	Recruiting	Phase I: to determine the MTD and recommended Phase 2 dose in patients with advanced solid tumors. Phase II: To evaluate the efficacy of JKI-802 in patients with solid tumors of neuroendocrine differentiation.	10 mg JKI-802 once daily as the starting dose with 4 days on/ 3 days off cycle.	(1) MTD and (2) objective response rate	NYA
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^aRathkopf D, Wong BY, Ross RW, Anand A, Tanaka E, Woo MM, et al. A phase I study of oral panobinostat alone and in combination with docetaxel in patients with castration-resistant prostate cancer. *Cancer Chemother Pharmacol* 2010;66(1):181–9.

^bSternberg C, Armstrong A, Pili R, Ng S, Huddart R, Agarwal N, et al. Randomized, double-blind, placebo-controlled phase III study of tasquinimod in men with metastatic castration-resistant prostate cancer. *J Clin Oncology* 2016;34(22):2636–43.

^cFizazi K, Ulys A, Sengelov L, Moe M, Ladoire S, Thiery-Vuillemin A, et al. A randomized, double-blind, placebo-controlled phase II study of maintenance therapy with tasquinimod in patients with metastatic castration-resistant prostate cancer responsive to or stabilized during first-line docetaxel chemotherapy. *Ann Oncol* 2017;28(11):2741–6.

activity. Patients receiving 40 mg of Panobinostat had a 47.5% chance of remaining radiological-progression-free survival (rPF) [115] (Table 14.2). A phase II study of the HDACi SB939 in patients with CRPC demonstrated a PSA response in only two out of thirty-two patients. Although SB939 was tolerable, the lack of PSA response did not warrant any further investigations as a stand-alone drug in CRPC patients [116]. A small phase I study showed an acceptable safety profile of entinostat with enzalutamide for the treatment of CRPC [117].

Overall, there is a large body of evidence supporting the positive effect of HDACi *in vitro*. These results, however, have largely failed to translate to clinical trials. This may be a result of the pan-activity of most HDACi, which results in activation of unwanted pro-survival genes. This could be counteracted by developing more selective inhibitors, for example, tasquinimod, which has been shown to double progression-free survival time in men with metastatic CRPC [118]. Tasquinimod mediates its anticancer effects, in part by specifically binding allosterically to and inactivating HDAC4. This subsequently prevents the formation of the HDAC4/N-CoR/HDAC3 complex, thus inhibiting deacetylation of histones and other “client” proteins (such as transcription factors), that are required for cancer cell survival and angiogenic response [119]. A number of phase II/III clinical trials assessing the efficacy of tasquinimod for the treatment of metastatic CRPC has shown that the drug improved radiological PFS compared to the placebo (Table 14.2). Thus providing evidence that more selective inhibitors may be beneficial for the treatment of CRPC.

5.1 Synergistic activity

HDACi have been used with demethylating agents to synergistically reactivate expression of methylated genes. For example, cotreatment of AR-negative cell line DU145 with 5-Aza-CR and TSA is more effective in restoring functional expression of the *AR* gene and its downstream targets, compared with either agent alone [120]. In LNCaP and 22RV1 cells, Ms-275 induced maspin-mediated AR-repression, promoting enzalutamide efficacy in addition to reducing PSA levels [121]. Other studies support a combination of HDACi with conventional chemotherapeutic agents or antiandrogens. Combined treatment of an HDACi (Depsipeptide, SAHA, Sodium butyrate or TSA) with TNF α -Related Apoptosis-Inducing Ligand (TRAIL) leads to enhanced cytotoxicity and synergistic apoptosis in PCa cell lines LNCaP and DU145 [122–124]. Combined treatment of PC3 cells with panobinostat and the dual PI3K-mTOR inhibitor, BEZ235 significantly attenuates DNA damage repair protein ATM and increases antitumor activity over either agent alone [125].

6 Targeting AR signaling by epigenetic drugs

The potential for epigenetic therapies in PCa cannot be considered without factoring in their influence on the pivotal AR signaling axis. Traditionally, HDACi have potentiated AR-driven transcription, by enabling the activity of KATs on the AR signaling axis. Antiandrogens, such as bicalutamide, recruit corepressors (i.e., NCoR or SMRT) that complex with HDACs and inhibit AR-transactivation. However, several lines of evidence support a negative effect of HDACi on AR signaling [49]. Certain HDACi result in hyperacetylation of HSP90, dissociation from the AR and subsequent AR degradation [126,127] (Figure 14.1B). In addition, HDACi (TSA, SAHA and

LBH589) block transcriptional activation of HDAC-dependent AR target genes, predominantly by interfering with the assembly of the RNA polymerase II complex, but also by inhibiting transcription of the *AR* gene [49,106]. Recent evidence suggests that Enzalutamide in conjunction with a HDACi (SAHA) could be a promising strategy. The hybrid molecule [2–45,53–82] targets both Hsp90 and AR, and successfully inhibits the growth of Enzalutamide-resistant PCa cells and xenograft models. Notably, it was found to be effective against both full-length AR and the NTD-truncated splice variant v7 and because it is retained in the cytoplasm, it inhibits HDAC6 without impacting nuclear HDACs, thus potentially overcoming resistance and toxicity associated with classical AR antagonists and HDACi [128]. A novel HDACi called CN133 (decreases HDAC 2 and 3 expression, inhibiting AR signaling pathway activity) reduced tumor size by 50% compared to placebo and was more effective than SAHA whilst also being more effective at reducing cell proliferation, invasion, and migration [129].

An exciting thought is the possibility of suppressing AR and AR coactivator activity simultaneously. This could be achieved by developing inhibitors of AR coactivators, in addition to conventional agents that deplete androgens or compete for binding with the AR. A small number of KAT inhibitors (KATi) have been reported. In LNCaP and PC3 PCa cell line models, a synthetic inhibitor of TIP60, namely NU9056, inhibited proliferation and induced apoptosis in a concentration- and time-dependent manner (IC₅₀: 2 μM). Reduced levels of the AR, PSA, and p53 were observed in response to treatment [130]. A novel inhibitor of the transcriptional coactivator CBP/p300 bromodomain (plays a vital role in AR activity and CRPC) GNE-049 prevents the acetylation of H3K27, thus inhibiting PCa growth both in vitro and in vivo [64].

The bromodomain and extraterminal (BET) family of chromatin readers modulate gene expression by binding to acetylated histone tails and subsequently activating RNA polymerase II-driven transcriptional elongation. Small molecule BET inhibitors block binding of BET proteins to acetylated chromatin marks, thus preventing transcriptional activation of BET target genes [131].

BRD4 plays an important role in transcription by RNA polymerase II. BRD4 is known to interact with several transcription factors, including the AR. It was recently shown that this interaction can be hindered in AR signaling competent CRPC cell lines, by JQ1, a small molecule BET-inhibitor [132]. Phenotypically, JQ1 causes cell cycle arrest and apoptosis. Like enzalutamide, JQ1 disrupts AR recruitment to target gene loci. JQ1 appears to function downstream of the AR, thus offering promise in the context of castration resistance, mediated by AR amplification or mutation. Recent evidence however suggests that JQ1 can also increase invasion and metastasis independent of BET proteins, for example, through FOXA1 inhibition; thus more research is vital [133]. ABBV-744, a recently developed selective inhibitor of BRD2 domain proteins of the BET family, is largely antiproliferative in cancer cell lines with full length AR and showed great activity in PCa xenografts. ABBV-744 additionally displaced BRD4 from AR-containing super enhancers and inhibited AR-dependent transcription [134].

Another potential target is DOT1L, which is overexpressed in CRPC tumors and is associated with poor outcomes [61]. Inhibition of DOT1L decreased the viability of AR-positive PCa cells and organoids and enzalutamide- and castration-resistant cells. It was concluded that the selectivity for AR-positive cells was due to a methylated K79 on a distal enhancer in the *MYC* gene (not present in AR-negative PCa cells) which is bound to DOT2L and AR. Overall, DOT1L inhibition resulted in reduced *MYC* expression in addition to increased *MYC* and AR degradation [61].

In essence, there are many ways in which the AR signaling cascade can be targeted using epigenetic mechanisms. The transactivation or expression of AR could be inhibited, in addition to an increase in its degradation. Although more research is needed, the targeting of AR splice variant also proves to be a promising therapeutic pathway.

7 Chemoprevention and neutraceutical therapies

The chronic nature of latent PCa makes it appealing to consider therapeutic and lifestyle interventions that could reduce the risk of disease progression into clinically apparent disease and/or that could prevent the formation of lethal metastases. Autopsy studies reveal the presence of precursor lesions and microscopic tumor foci in up to 29% of men aged in their 30s and 40s [135]. There is substantial evidence in support of an “epigenetic catastrophe” at the earliest stages of prostate carcinogenesis, positioning adverse epigenetic changes as seminal events in PCa initiation [136].

There is considerable epidemiological evidence to support a role for dietary factors as chemopreventative agents against PCa. Elucidating the molecular mechanisms by which the “active” compounds contained in these dietary constituents mediate their anticancer activity has lagged behind. This section attempts to summarize our current knowledge of how dietary agents may serve as therapeutic modalities by restoring epigenomics normality.

7.1 Isothiocyanates

A high intake of cruciferous vegetables is associated with a reduced risk of PCa in epidemiological studies [137]. There is preliminary preclinical evidence that sulforaphane, derived from glucoraphanin found in a number of cruciferous vegetables, may prevent and induce regression of PCa and other malignancies. Sulforaphane treatment of PCa cells *in vitro* inhibits HDAC activity and induces caspase-dependent apoptosis [138]. It can cause methylation H3K4me2 of and acetylation of H3K18, inhibiting human Telomerase Reverse Transcriptase (hTERT) expression and activity in PCa cell lines, where expression is associated with a high risk of PCa recurrence and poor prognosis [139]. Similarly, sulforaphane inhibits PCa progression and pulmonary metastasis in TRAMP mice, by reducing cell proliferation and augmenting natural killer cell lytic activity [140]. Sulforaphane inhibits HDAC6 enzymatic activity and enhances HSP90 acetylation and AR proteasomal degradation, ultimately reducing AR occupancy at target gene enhancers and expression of target genes (*KLK3* and *TMPRSS2*) [127]. Treatment of benign and malignant prostate cells in culture also inhibits DNMT expression and exerts broad changes on DNA methylation patterns [141], although the functional consequences of these observations remain to be elucidated. Sulforaphane altered the expression of over 100 lncRNAs in PCa, such as those involved in cell cycle and metabolism [142]. Furthermore, a clinical trial (NCT01950143) to investigate the chemoprotective effect of broccoli soup on low-risk PCa patients deduced an inverse association between broccoli/glucoraphanin consumption and PCa progression. The authors also saw that the broccoli soup treatments suppressed gene changes seen within the control arm in a dose-dependent manner (Table 14.3) [143].

Table 14.3 Nutraceutical epigenetic drugs in clinical trials for prostate cancer.

Drug	Clinical trial identifier	Study design	Phase	Status	Study objective	Description	Outcome measures	Results
Sulforaphane (SFN) glucosinolate	NCT01265953	Randomized, double-blind	Chemoprevention	Recruiting	To identify mechanisms by which dietary compounds, such as those found in cruciferous vegetables decrease PCa risk	Four weeks SFN glucosinolate capsules: 250 mg of broccoli seed extract (30 mg SFN glucosinolate), 8 capsules (4 capsules B.I.D.) daily in subjects at risk of PCa undergoing prostate biopsy	Primary: (1) Presence of SFN and its metabolites (SFN-Cys, SFN-NAC) (2) Expression of acetylated H3 and H4, and absolute histone levels Secondary: (1) Methylation of <i>GSTP1</i> , <i>AR</i> , sigma14–3-3, <i>P21</i> and global 5mC levels. (2) Cell proliferation (Ki-67 expression) and apoptosis (TUNEL assay) analyses will be carried out on peripheral blood plasma, urine and prostate biopsy cores following supplementation with SFN or placebo.	Presence of SFN metabolites in treatment group was significantly higher in the treatment group, but there was no significant difference in HDAC activity or prostate tissue biomarkers [144]
Sulforaphane (SFN) glucosinolate	NCT01950143	Randomized, double-blind	Chemoprevention	Recruiting (invite-only)	To determine whether a 12-month diet rich in SFN will prevent PCa progression in men diagnosed with low- and intermediate-risk prostate cancer on active surveillance.	Group A: 2 portions of standard broccoli soup/week for 12 months. Group B: 2 portions of glucoraphanin-enriched broccoli soup/week for 12 months. Group C: 2 portions of glucoraphanin-extra enriched broccoli soup/week for 12 months.	Primary: (1) Global transcriptome expression analysis (baseline and 12 months). Secondary: (1) metabolite concentration (baseline and 12 months)	SFN (consumed in the glucoraphanin-rich soup) altered gene expression in several hundred genes in oncogenic pathways in a dose-dependent manner. There was an inverse association between cancer progression and cruciferous vegetables [143]

(Continued)

Table 14.3 Nutraceutical epigenetic drugs in clinical trials for prostate cancer. *Continued*

Drug	Clinical trial identifier	Study design	Phase	Status	Study objective	Description	Outcome measures	Results
Sulforaphane (SFN) glucosinolate	NCT00946309	Randomized, double-blind	Phase II	Recruiting (invite-only)	To study the biological <i>in vivo</i> effects of SFN supplementation on normal prostate tissue.	Experimental: 100 µmol sulforaphane, every other day for 5 weeks. Placebo: 250 mg microcrystalline cellulose NF, every other day for 6 weeks	Primary: (1) Gene expression of Phase II enzymes. (2) DNA and lipid oxidation. (3) DHT levels. All assessed at baseline and 5 weeks	GPX4 had the largest fold change expression of -0.36 (0.26 in the placebo group) in the treatment group compared to the baseline
Sulforaphane (SFN) glucosinolate	NCT01228084	Open-label, single group assignment	Phase II	Completed	To study the effects of sulforaphane in patients with biochemical recurrence of PCa	Experimental: four 50µmol capsules Sulforaphane taken once daily from week 1 day 1 to week 20 day 7.	Primary: (1) Proportion of patients who achieve a 50% decline in PSA Secondary: (1) % change in PSA from baseline to end of study (20 weeks). (2) Toxicities. (3) Half-life of SFN in blood in relation to patient's GSTM1 genotype	5% of patients had a decline in PSA levels with sulforaphane
Curcumin	NCT03290417	Randomized, open-label, parallel group assignment	NA	Completed	To investigate if eating curcumin (turmeric), omega 3, and vitamin D slows the growth of PCa in patients on active surveillance	Experimental: Vitamin D: one 5000 IU/cap daily, Omega 3: one 720 mg/cap three times daily, turmeric: two 250 mg/cap four times daily	Primary: (1) Gene expression of very low and low risk PCa patients on AS up to 6 months. (2) Gene expression of very low and low risk PCa patients on AS up to 12 months. Secondary: (1) AS failure up to 6 months. (2) AS failure up to 12 months	NYA

7.2 Curcumin

The isoflavone curcumin, a component of turmeric, has numerous medicinal and anticancer properties (reviewed elsewhere). Curcumin can alter the expression of genes involved in multiple pathways, including inhibition of NF- κ B and Wnt signaling pathways, and upregulation of cell cycle arrest and apoptosis [145]. Curcumin has radiosensitizing properties in PCa cells *in vitro* [146] and in combination with phenethyl isothiocyanate (PEITC) significantly reduces the growth of PC3 xenografts in mice [147]. One study suggested that the radiosensitizing properties and radiation-induced autophagy could be due to the activation (through curcumin-induced hypomethylation) of miR-143 and miR-145 [148]. In addition, curcumin inhibited PCa growth and increased apoptosis by inhibiting the JNKs pathway, in part through repressing H3K9me3 [149]. Curcumin can also regulate PCa progression through inducing miR-30a-5p, a known tumor suppressor, in both PC3 and DU145 PCa cell lines [150]. Mechanistically, curcumin represses histone H4 acetylation at AREs and subsequent AR recruitment by impinging on p300 and CPB and GATA2 and FOXA1 occupancy at these enhancer elements. These effects were observed in xenograft models mimicking both androgen sensitive PCa and CRPC [77]. TSA and SAHA (HDACi) reversed the inhibitory effects of curcumin on cell survival, thereby implying that the ability of curcumin to attenuate AR activity is dependent on alteration of histone acetylation. The findings of this preclinical study are of strong clinical relevance because HAT coactivators and pioneer factors support activity of the AR in a castrate-resistant environment. Serum PSA levels of PCa patients were also reduced by curcumin in conjunction with isoflavones, in addition to reducing the severity of urinary symptoms [151]. Lastly, an analog of curcumin, ASC-J9 or dimethylcurcumin, which acts as an enhancer of AR-degradation, has also shown to reduce PCa cell viability in both AR + and AR - PCa cell lines through both AR dependent and independent mechanisms [152,153].

7.3 Phytoestrogens

Plasma and serum phytoestrogen levels of Japanese men are at least 10-fold higher than Caucasian men in the United Kingdom [154]. Soy isoflavones, for example, genistein, classified as phytoestrogens, act as both estrogen agonists and antagonists by differentially binding to the estrogen receptor alpha or beta and/or altering enzymes involved in hormone metabolism [155]. Epidemiological studies link an increased intake of dietary genistein with reduced rates of PCa metastasis and mortality [156]. Although this is also disputed by some studies [157–159], Genistein was found to induce apoptosis through increased caspase-3 enzymatic activity, gene expression, and protein levels [160]. Using both *in vitro* and *in vivo* PCa models, genistein impedes cell detachment [161] and inhibits cell invasion [162]. It inhibits proliferation through reduced p38 MAPK gene expression and decreased PC3 cell metastatic ability by decreasing MMP2 activity [160]. Subsequently, genistein was found to inhibit cell invasion by directly inhibiting the activity of p38 MAPK activator MEK4 at nanomolar concentrations *in vitro* [163]. Evidence on the effects of genistein on the epigenome is conflicting. A 2017 study utilizing clinical trial specimens saw a change in methylation and expression patterns in genes involved in developmental processes and proliferation between a Genistein treatment group and a placebo group (an increase in PTEN activity and a reduction in MYC activity) [164]. In androgen-dependent and -independent cell lines, genistein dose inhibits DNMT activity and reactivates methylation-associated silenced genes, for example,

GSTP1 and *RASSF1A* [165,166]. However, feeding studies with genistein increased DNA methylation *in vivo* [167]. Genistein reduced promoter methylation of the PCa tumor suppressor gene ER- β in androgen-dependent PCa cell lines, increasing its expression and reducing PCa cell proliferation [168]. Genistein-rich diets fed to mice, have also been shown to suppress the trimethylation of H3K9 and the phosphorylation of H3S10 and increase methylation of Wnt signaling related genes *SFRP2*, *SFRP5* and *WNT5A* genes, suppressing their expression and maintain normal levels of Wnt signaling [169]. Recent developments into nanocarriers and particles have also illustrated Genistein anticancer effect in PCa, overcoming the issue of its low bioavailability and allowing a more targeted administration [170,171].

7.4 Nutraceutical therapies in clinical trial

These studies significantly demonstrate the prevention of epigenetic aberrations and restoration to normal physiological states by common dietary constituents in parts of the world that experience some of the lowest incidence of PCa. Thus one may speculate that inhibiting hypermethylation-induced inactivation of key tumor suppressor genes or avoiding hyperstimulation of AR signaling by dietary molecules could afford a chemopreventive effect against PCa. It is possible that long-term consumption of polyphenols, isoflavones and isothiocyanates with dietary DNMT/HDACi, may have a cumulative effect over a man's lifetime, providing protection against PCa development [147,172]. Thus further research, in addition to clinical trials, is key. There are several ongoing clinical trials assessing the preventative properties of sulforaphane, curcumin, and genistein. In addition to investigate their interaction with the epigenome of PCa (Table 14.3). There are a number of completed and ongoing clinical trials which assess the efficacy of these three compounds in reducing PCa progression and other markers of prostate tumorigenesis. For example, a phase three clinical trial (NCT03769766) is currently underway to investigate the effect of curcumin on low-risk PCa progression in patients under active surveillance.

8 Conclusion

In the past, epigenetic research has concentrated around promoter CGIs because of their innate association with gene expression. Thus epigenetic cancer therapies have focused largely on reactivating tumor suppressor genes and restoring their functional products. However, the ever-growing surge in epigenome-wide capabilities coupled with Next-Gen sequencing presents the opportunity to explore epigenomic therapeutics beyond the promoter CGI. Elucidating the full range of molecular mechanisms involved in the therapeutic responsiveness to epigenetic drugs is needed before their successful clinical application for treatment of CRPC.

Data from clinical trial results do not support the use of conventional DNMTi and HDACi as effective single-agent CRPC therapies. However, some evidence may support a combinatorial approach in some patients, combining epigenetic therapies with existing agents, for example, the phase II clinical trial for assessing a low dose of panobinostat to enhance second-line hormonal therapy with bicalutamide (see Section 5) (Table 14.2). Due to geographical variation in disease incidence, dietary factors have been examined as therapeutic avenues, many of which have been shown to directly alter the epigenome and/or modify the behavior of epigenetic enzymes.

Other potential avenues are nonconventional approaches to perturb AR signaling. Many studies have investigated the plausibility of epigenetically targeting AR coactivators, and/or splice variants, in addition to other fundamentals of the AR signaling cascade. Many of these studies however are carried out *in vitro*; thus further investigations using more developed models, and subsequently, clinical trials are imperative.

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Abbreviations

AR	Androgen receptor
ARE	Androgen response element
CGI	CpG island
CRPC	Castration-resistant prostate cancer
mCRPC	Metastatic castration-resistant prostate cancer
DNMT	DNA methyltransferase
EZH2	Enhancer of zeste homolog 2
KAT	Lysine acetyl transferase
KDM	Lysine demethylase
HDAC	Histone deacetylase
HGPIN	High-grade prostatic intraepithelial neoplastic
KMT	Lysine methyltransferase
KDM	Lysine demethylase
PCa	Prostate cancer
PSA	Prostate-specific antigen
rPFS	Radiological progression-free survival
SAHA	Suberoylanilide hydroxamic acid
TRAMP	Transgenic adenocarcinoma of mouse prostate mouse model
TSA	Trichostatin A
5-Aza-CR	5-Azacytidine; 5-Aza-cytidine; vidaza
5-Aza-CdR	5-Aza-2'-deoxycytidine; decitabine

References

- [1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71(3):209–49.
- [2] Rawla P. Epidemiology of prostate cancer. World J Oncol 2019;10(2):63–89.
- [3] Martin RM, Donovan JL, Turner EL, Metcalfe C, Young GJ, Walsh EI, et al. Effect of a low-intensity PSA-based screening intervention on prostate cancer mortality: the CAP randomized clinical trial. JAMA 2018;319(9):883–95.

- [4] Ilic D, Djulbegovic M, Jung JH, Hwang EC, Zhou Q, Cleves A, et al. Prostate cancer screening with prostate-specific antigen (PSA) test: a systematic review and meta-analysis. *BMJ* 2018;362:k3519.
- [5] Dart DA, Brooke GN, Sita-Lumsden A, Waxman J, Bevan CL. Reducing prohibitin increases histone acetylation, and promotes androgen independence in prostate tumours by increasing androgen receptor activation by adrenal androgens. *Oncogene*. 2012;31(43):4588–98.
- [6] Teo MY, Rathkopf DE, Kantoff P. Treatment of advanced prostate cancer. *Annu Rev Med* 2019;70:479–99.
- [7] Siegel DA, O’Neil ME, Richards TB, Dowling NF, Weir HK. Prostate cancer incidence and survival, by stage and race/ethnicity—United States, 2001–2017. *MMWR Morb Mortal Wkly Rep* 2020;69(41):1473–80.
- [8] Simon NI, Parker C, Hope TA, Paller CJ. Best approaches and updates for prostate cancer biochemical recurrence. *Am Soc Clin Oncol Educ Book* 2022;42:1–8.
- [9] Chandler R, de Bono J. Second-generation antiandrogens in nonmetastatic CRPC. *Nat Rev Urol* 2018;15(6):342–4.
- [10] Desai K, McManus JM, Sharifi N. Hormonal therapy for prostate cancer. *Endocr Rev* 2021;42(3):354–73.
- [11] Baca SC, Takeda DY, Seo JH, Hwang J, Ku SY, Arafah R, et al. Reprogramming of the FOXA1 cistrome in treatment-emergent neuroendocrine prostate cancer. *Nat Commun* 2021;12(1):1979.
- [12] Paschalidis A, Sharp A, Welti JC, Neeb A, Raj GV, Luo J, et al. Alternative splicing in prostate cancer. *Nat Rev Clin Oncol* 2018;15(11):663–75.
- [13] de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011;364(21):1995–2005.
- [14] Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med* 2012;367(13):1187–97.
- [15] Fizazi K, Tran N, Fein L, Matsubara N, Rodriguez-Antolin A, Alekseev BY, et al. Abiraterone plus prednisone in metastatic, castration-sensitive prostate cancer. *N Engl J Med* 2017;377(4):352–60.
- [16] Reid AH, Attard G, Danila DC, Oommen NB, Olmos D, Fong PC, et al. Significant and sustained antitumor activity in post-docetaxel, castration-resistant prostate cancer with the CYP17 inhibitor abiraterone acetate. *J Clin Oncol* 2010;28(9):1489–95.
- [17] Danila DC, Morris MJ, de Bono JS, Ryan CJ, Denmeade SR, Smith MR, et al. Phase II multicenter study of abiraterone acetate plus prednisone therapy in patients with docetaxel-treated castration-resistant prostate cancer. *J Clin Oncol* 2010;28(9):1496–501.
- [18] Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, et al. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 2009;324(5928):787–90.
- [19] Scher HI, Beer TM, Higano CS, Anand A, Taplin ME, Efstathiou E, et al. Antitumour activity of MDV3100 in castration-resistant prostate cancer: a phase 1–2 study. *Lancet*. 2010;375(9724):1437–46.
- [20] Tombal B, Borre M, Rathenborg P, Werbrouck P, Van Poppel H, Heidenreich A, et al. Long-term efficacy and safety of enzalutamide monotherapy in hormone-naïve prostate cancer: 1- and 2-year open-label follow-up results. *Eur Urol* 2015;68(5):787–94.
- [21] Clegg NJ, Wongvipat J, Joseph JD, Tran C, Ouk S, Dilhas A, et al. ARN-509: a novel antiandrogen for prostate cancer treatment. *Cancer Res* 2012;72(6):1494–503.
- [22] Schmidt KT, Huitema ADR, Chau CH, Figg WD. Resistance to second-generation androgen receptor antagonists in prostate cancer. *Nat Rev Urol* 2021;18(4):209–26.
- [23] Kan Z, Jaiswal BS, Stinson J, Janakiraman V, Bhatt D, Stern HM, et al. Diverse somatic mutation patterns and pathway alterations in human cancers. *Nature*. 2010;466(7308):869–73.
- [24] Yegnasubramanian S, De Marzo AM, Nelson WG. Prostate cancer epigenetics: from basic mechanisms to clinical implications. *Cold Spring Harb Perspect Med* 2019;9(4).

- [25] Brooks JD, Weinstein M, Lin X, Sun Y, Pin SS, Bova GS, et al. CG island methylation changes near the *GSTP1* gene in prostatic intraepithelial neoplasia. *Cancer Epidemiol Biomarkers Prev* 1998;7(6):531–6.
- [26] Kang GH, Lee S, Lee HJ, Hwang KS. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. *J Pathol* 2004;202(2):233–40.
- [27] Perry AS, Loftus B, Moroosie R, Lynch TH, Hollywood D, Watson RW, et al. In silico mining identifies *IGFBP3* as a novel target of methylation in prostate cancer. *Br J Cancer* 2007;96(10):1587–94.
- [28] Kirby MK, Ramaker RC, Roberts BS, Lasseigne BN, Gunther DS, Burwell TC, et al. Genome-wide DNA methylation measurements in prostate tissues uncovers novel prostate cancer diagnostic biomarkers and transcription factor binding patterns. *BMC Cancer* 2017;17(1):273.
- [29] Yegnasubramanian SKJ, Goncalgo ML, Zahurak M, Piantadosi S, Walsh PC, Bova GS, et al. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res* 2004;64(6):1975–86.
- [30] Kim JH, Dhanasekaran SM, Prensner JR, Cao X, Robinson D, Kalyana-Sundaram S, et al. Deep sequencing reveals distinct patterns of DNA methylation in prostate cancer. *Genome Res* 2011;21(7):1028–41.
- [31] Mahapatra S, Klee EW, Young CY, Sun Z, Jimenez RE, Klee GG, et al. Global methylation profiling for risk prediction of prostate cancer. *Clin Cancer Res* 2012;18(10):2882–95.
- [32] Spans L, Van den Broeck T, Smeets E, Prekovic S, Thienpont B, Lambrechts D, et al. Genomic and epigenomic analysis of high-risk prostate cancer reveals changes in hydroxymethylation and TET1. *Oncotarget*. 2016;7(17):24326–38.
- [33] Aryee MJ, Liu W, Engelmann JC, Nuhn P, Gurel M, Haffner MC, et al. DNA methylation alterations exhibit intraindividual stability and interindividual heterogeneity in prostate cancer metastases. *Sci Transl Med* 2013;5(169):169ra10.
- [34] Yegnasubramanian S, Haffner MC, Zhang Y, Gurel B, Cornish TC, Wu Z, et al. DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. *Cancer Res* 2008;68(21):8954–67.
- [35] Ogishima T, Shiina H, Breault JE, Tabatabai L, Bassett WW, Enokida H, et al. Increased heparanase expression is caused by promoter hypomethylation and up-regulation of transcriptional factor early growth response-1 in human prostate cancer. *Clin Cancer Res* 2005;11(3):1028–36.
- [36] Wang Q, Williamson M, Bott S, Brookman-Amissah N, Freeman A, Nariculam J, et al. Hypomethylation of *WNT5A*, *CRIP1* and *S100P* in prostate cancer. *Oncogene*. 2007;26(45):6560–5.
- [37] Shukeir N, Pakneshan P, Chen G, Szyf M, Rabbani SA. Alteration of the methylation status of tumor-promoting genes decreases prostate cancer cell invasiveness and tumorigenesis in vitro and in vivo. *Cancer Res* 2006;66(18):9202–10.
- [38] TCGA. The molecular taxonomy of primary prostate cancer. *Cell*. 2015;163(4):1011–25.
- [39] Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, et al. Global histone modification patterns predict risk of prostate cancer recurrence. *Nature*. 2005;435(7046):1262–6.
- [40] Halkidou K, Cook S, Leung HY, Neal DE, Robson CN. Nuclear accumulation of histone deacetylase 4 (HDAC4) coincides with the loss of androgen sensitivity in hormone refractory cancer of the prostate. *Eur Urol* 2004;45(3):382–9 author reply 9.
- [41] Metzger E, Wissmann M, Yin N, Muller JM, Schneider R, Peters AH, et al. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature*. 2005;437(7057):436–9.
- [42] Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature*. 2006;439(7078):871–4.
- [43] Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature*. 2002;419(6907):624–9.

- [44] Yu J, Yu J, Mani RS, Cao Q, Brenner CJ, Cao X, et al. An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. *Cancer Cell* 2010;17(5):443–54.
- [45] Xu K, Wu ZJ, Groner AC, He HH, Cai C, Lis RT, et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is polycomb-independent. *Science*. 2012;338(6113):1465–9.
- [46] Coffey K, Rogerson L, Ryan-Munden C, Alkharaf D, Stockley J, Heer R, et al. The lysine demethylase, KDM4B, is a key molecule in androgen receptor signalling and turnover. *Nucleic Acids Res* 2013;41(8):4433–46.
- [47] Thibault A, Figg WD, Bergan RC, Lush RM, Myers CE, Tompkins A, et al. A phase II study of 5-aza-2’deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori*. 1998;84(1):87–9.
- [48] Singal R, Ramachandran K, Gordian E, Quintero C, Zhao W, Reis IM. Phase I/II study of azacitidine, docetaxel, and prednisone in patients with metastatic castration-resistant prostate cancer previously treated with docetaxel-based therapy. *Clin Genitourin Cancer* 2015;13(1):22–31.
- [49] Welsbie DS, Xu J, Chen Y, Borsu L, Scher HI, Rosen N, et al. Histone deacetylases are required for androgen receptor function in hormone-sensitive and castrate-resistant prostate cancer. *Cancer Res* 2009;69(3):958–66.
- [50] Lee WH, Morton RA, Epstein JI, Brooks JD, Campbell PA, Bova GS, et al. Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc Natl Acad Sci U S A* 1994;91(24):11733–7.
- [51] Halkidou K, Gnanapragasam VJ, Mehta PB, Logan IR, Brady ME, Cook S, et al. Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. *Oncogene*. 2003;22(16):2466–77.
- [52] Kron KJ, Murison A, Zhou S, Huang V, Yamaguchi TN, Shiah YJ, et al. TMPRSS2-ERG fusion co-opts master transcription factors and activates NOTCH signaling in primary prostate cancer. *Nat Genet* 2017;49(9):1336–45.
- [53] Zhao SG, Chen WS, Li H, Foye A, Zhang M, Sjöström M, et al. The DNA methylation landscape of advanced prostate cancer. *Nat Genet* 2020;52(8):778–89.
- [54] Bianco-Miotto T, Chiam K, Buchanan G, Jindal S, Day TK, Thomas M, et al. Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. *Cancer Epidemiol Biomarkers Prev* 2010;19(10):2611–22.
- [55] Ellinger J, Kahl P, von der Gathen J, Rogenhofer S, Heukamp LC, Gutgemann I, et al. Global levels of histone modifications predict prostate cancer recurrence. *Prostate*. 2010;70(1):61–9.
- [56] Ngollo M, Lebert A, Daures M, Judes G, Rifai K, Dubois L, et al. Global analysis of H3K27me3 as an epigenetic marker in prostate cancer progression. *BMC Cancer* 2017;17(1):261.
- [57] Weichert W, Roske A, Gekeler V, Beckers T, Stephan C, Jung K, et al. Histone deacetylases 1, 2 and 3 are highly expressed in prostate cancer and HDAC2 expression is associated with shorter PSA relapse time after radical prostatectomy. *Br J cancer* 2008;98(3):604–10.
- [58] Huffman DM, Grizzle WE, Bamman MM, Kim JS, Eltoum IA, Elgavish A, et al. SIRT1 is significantly elevated in mouse and human prostate cancer. *Cancer Res* 2007;67(14):6612–18.
- [59] Kahl P, Gullotti L, Heukamp LC, Wolf S, Friedrichs N, Vorreuther R, et al. Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. *Cancer Res* 2006;66(23):11341–7.
- [60] Yuan H, Han Y, Wang X, Li N, Liu Q, Yin Y, et al. SETD2 restricts prostate cancer metastasis by integrating EZH2 and AMPK signaling pathways. *Cancer Cell* 2020;38(3):350–65 e7.
- [61] Vatapalli R, Sagar V, Rodriguez Y, Zhao JC, Unno K, Pamarthi S, et al. Histone methyltransferase DOT1L coordinates AR and MYC stability in prostate cancer. *Nat Commun* 2020;11(1):4153.

- [62] Stein J, Majores M, Rohde M, Lim S, Schneider S, Krappe E, et al. KDM5C is overexpressed in prostate cancer and is a prognostic marker for prostate-specific antigen-relapse following radical prostatectomy. *Am J Pathol* 2014;184(9):2430–7.
- [63] Weinert BT, Narita T, Satpathy S, Srinivasan B, Hansen BK, Schölz C, et al. Time-resolved analysis reveals rapid dynamics and broad scope of the CBP/p300 acetylome. *Cell*. 2018;174(1):231–244.e12.
- [64] Jin L, Garcia J, Chan E, de la Cruz C, Segal E, Merchant M, et al. Therapeutic targeting of the CBP/p300 bromodomain blocks the growth of castration-resistant prostate cancer. *Cancer Res* 2017;77(20):5564–75.
- [65] Bjorkman M, Ostling P, Harma V, Virtanen J, Mpindi JP, Rantala J, et al. Systematic knockdown of epigenetic enzymes identifies a novel histone demethylase PHF8 overexpressed in prostate cancer with an impact on cell proliferation, migration and invasion. *Oncogene*. 2012;31(29):3444–56.
- [66] Dryhurst D, Ausio J. Histone H2A.Z deregulation in prostate cancer. Cause or effect? *Cancer metastasis reviews* 2014;33(2–3):429–39.
- [67] Valdes-Mora F, Song JZ, Statham AL, Strbenac D, Robinson MD, Nair SS, et al. Acetylation of H2A.Z is a key epigenetic modification associated with gene deregulation and epigenetic remodeling in cancer. *Genome Res* 2012;22(2):307–21.
- [68] Dryhurst D, McMullen B, Fazli L, Rennie PS, Ausio J. Histone H2A.Z prepares the prostate specific antigen (PSA) gene for androgen receptor-mediated transcription and is upregulated in a model of prostate cancer progression. *Cancer Lett* 2012;315(1):38–47.
- [69] Baptista T, Graca I, Sousa EJ, Oliveira AI, Costa NR, Costa-Pinheiro P, et al. Regulation of histone H2A.Z expression is mediated by sirtuin 1 in prostate cancer. *Oncotarget*. 2013;4(10):1673–85.
- [70] Saha AK, Contreras-Galindo R, Niknafs YS, Iyer M, Qin T, Padmanabhan K, et al. The role of the histone H3 variant CENPA in prostate cancer. *J Biol Chem* 2020;295(25):8537–49.
- [71] Samaržija I. Post-translational modifications that drive prostate cancer progression. *Biomolecules*. 2021;11(2).
- [72] Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. *J Biol Chem* 2002;277(29):25904–13.
- [73] Wang Z, Wang Z, Guo J, Li Y, Bavaria JH, Qian C, et al. Inactivation of androgen-induced regulator ARD1 inhibits androgen receptor acetylation and prostate tumorigenesis. *Proc Natl Acad Sci U S A* 2012;109(8):3053–8.
- [74] Kuhns KJ, Zhang G, Wang Z, Liu W. ARD1/NAA10 acetylation in prostate cancer. *Exp Mol Med* 2018;50(7):1–8.
- [75] Zhong J, Ding L, Bohrer LR, Pan Y, Liu P, Zhang J, et al. p300 acetyltransferase regulates androgen receptor degradation and PTEN-deficient prostate tumorigenesis. *Cancer Res* 2014;74(6):1870–80.
- [76] DePaolo JS, Wang Z, Guo J, Zhang G, Qian C, Zhang H, et al. Acetylation of androgen receptor by ARD1 promotes dissociation from HSP90 complex and prostate tumorigenesis. *Oncotarget*. 2016;7(44):71417–28.
- [77] Shah S, Prasad S, Knudsen KE. Targeting pioneering factor and hormone receptor cooperative pathways to suppress tumor progression. *Cancer Res* 2012;72(5):1248–59.
- [78] Valdés-Mora F, Gould CM, Colino-Sanguino Y, Qu W, Song JZ, Taylor KM, et al. Acetylated histone variant H2A.Z is involved in the activation of neo-enhancers in prostate cancer. *Nat Commun* 2017;8(1):1346.
- [79] Fu M, Rao M, Wang C, Sakamaki T, Wang J, Di Vizio D, et al. Acetylation of androgen receptor enhances coactivator binding and promotes prostate cancer cell growth. *Mol Cell Biol* 2003;23(23):8563–75.
- [80] Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Mol Cell* 2002;9(3):601–10.

- [81] Zou JX, Guo L, Revenko AS, Tepper CG, Gemo AT, Kung HJ, et al. Androgen-induced coactivator ANCCA mediates specific androgen receptor signaling in prostate cancer. *Cancer Res* 2009;69(8):3339–46.
- [82] Urbanucci A, Barfeld SJ, Kyölä V, Itkonen HM, Coleman IM, Vodák D, et al. Androgen receptor deregulation drives bromodomain-mediated chromatin alterations in prostate cancer. *Cell Rep* 2017;19(10):2045–59.
- [83] Yang L, Lin C, Jin C, Yang JC, Tanasa B, Li W, et al. lncRNA-dependent mechanisms of androgen receptor-regulated gene activation programs. *Nature*. 2013;500(7464):598–602.
- [84] Wissmann M, Yin N, Muller JM, Greschik H, Fodor BD, Jenuwein T, et al. Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. *Nat Cell Biol* 2007;9(3):347–53.
- [85] Wang X, Gao H, Ren L, Gu J, Zhang Y, Zhang Y. Demethylation of the miR-146a promoter by 5-Aza-2'-deoxycytidine correlates with delayed progression of castration-resistant prostate cancer. *BMC Cancer* 2014;14:308.
- [86] Sonpavde G, Aparicio AM, Zhan F, North B, Delaune R, Garbo LE, et al. Azacitidine favorably modulates PSA kinetics correlating with plasma DNA LINE-1 hypomethylation in men with chemonaïve castration-resistant prostate cancer. *Urol Oncol* 2011;29(6):682–9.
- [87] Ramachandran K, Gopisetty G, Gordian E, Navarro L, Hader C, Reis IM, et al. Methylation-mediated repression of GADD45alpha in prostate cancer and its role as a potential therapeutic target. *Cancer Res* 2009;69(4):1527–35.
- [88] Ramachandran K, Speer C, Nathanson L, Claros M, Singal R. Role of DNA Methylation in Cabazitaxel Resistance in Prostate Cancer. *Anticancer Res* 2016;36(1):161–8.
- [89] Shang D, Liu Y, Liu Q, Zhang F, Feng L, Lv W, et al. Synergy of 5-aza-2'-deoxycytidine (DAC) and paclitaxel in both androgen-dependent and -independent prostate cancer cell lines. *Cancer Lett* 2009;278(1):82–7.
- [90] Fang X, Zheng C, Liu Z, Ekman P, Xu D. Enhanced sensitivity of prostate cancer DU145 cells to cis-platinum by 5-aza-2'-deoxycytidine. *Oncol Rep* 2004;12(3):523–6.
- [91] Zorn CS, Wojno KJ, McCabe MT, Kuefer R, Gschwend JE, Day ML. 5-aza-2'-deoxycytidine delays androgen-independent disease and improves survival in the transgenic adenocarcinoma of the mouse prostate mouse model of prostate cancer. *Clin Cancer Res* 2007;13(7):2136–43.
- [92] Gravina GL, Marampon F, Di Staso M, Bonfili P, Vitturini A, Jannini EA, et al. 5-azacitidine restores and amplifies the bicalutamide response on preclinical models of androgen receptor expressing or deficient prostate tumors. *Prostate*. 2010;70(11):1166–78.
- [93] Liao H, Xiao Y, Hu Y, Xiao Y, Yin Z, Liu L, et al. Methylation-induced silencing of miR-34a enhances chemoresistance by directly upregulating ATG4B-induced autophagy through AMPK/mTOR pathway in prostate cancer. *Oncol Rep* 2016;35(1):64–72.
- [94] Schweizer MT, Lin J, Blackford A, Bardia A, King S, Armstrong AJ, et al. Pharmacodynamic study of disulfiram in men with non-metastatic recurrent prostate cancer. *Prostate Cancer Prostatic Dis* 2013;16(4):357–61.
- [95] Zhang T, Kephart J, Bronson E, Anand M, Daly C, Spasojevic I, et al. Prospective clinical trial of disulfiram plus copper in men with metastatic castration-resistant prostate cancer. *Prostate*. 2022;82(7):858–66.
- [96] Zwergel C, Schnekenburger M, Sarno F, Battistelli C, Manara MC, Stazi G, et al. Identification of a novel quinoline-based DNA demethylating compound highly potent in cancer cells. *Clin Epigenetics* 2019;11(1):68.

- [97] Graça I, Sousa EJ, Baptista T, Almeida M, Ramalho-Carvalho J, Palmeira C, et al. Anti-tumoral effect of the non-nucleoside DNMT inhibitor RG108 in human prostate cancer cells. *Curr Pharm Des* 2014;20(11):1803–11.
- [98] Graça I, Sousa EJ, Costa-Pinheiro P, Vieira FQ, Torres-Ferreira J, Martins MG, et al. Anti-neoplastic properties of hydralazine in prostate cancer. *Oncotarget*. 2014;5(15):5950–64.
- [99] Pacheco MB, Camilo V, Lopes N, Moreira-Silva F, Correia MP, Henrique R, et al. Hydralazine and panobinostat attenuate malignant properties of prostate cancer cell lines. *Pharmaceuticals (Basel)* 2021;14(7).
- [100] Lopes N, Pacheco MB, Soares-Fernandes D, Correia MP, Camilo V, Henrique R, et al. Hydralazine and enzalutamide: synergistic partners against prostate cancer. *Biomedicines*. 2021;9(8).
- [101] Lin J, Haffner MC, Zhang Y, Lee BH, Brennen WN, Britton J, et al. Disulfiram is a DNA demethylating agent and inhibits prostate cancer cell growth. *Prostate* 2011;71(4):333–43.
- [102] Peart MJ, Smyth GK, van Laar RK, Bowtell DD, Richon VM, Marks PA, et al. Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. *Proc Natl Acad Sci U S A* 2005;102(10):3697–702.
- [103] Shabbeer S, Sqk M, Kachhap S, Galloway N, Rodriguez R, Carducci MA. Multiple Molecular pathways explain the anti-proliferative effect of valproic acid on prostate cancer cells in vitro and in vivo. *Prostate*. 2007;67(10):1099–110.
- [104] Xia Q, Sung J, Chowdhury W, Chen CL, Hoti N, Shabbeer S, et al. Chronic administration of valproic acid inhibits prostate cancer cell growth in vitro and in vivo. *Cancer Res* 2006;66(14):7237–44.
- [105] Qi G, Lu G, Yu J, Zhao Y, Wang C, Zhang H, et al. Up-regulation of TIF1 γ by valproic acid inhibits the epithelial mesenchymal transition in prostate carcinoma through TGF- β /Smad signaling pathway. *Eur J Pharmacol* 2019;860:172551.
- [106] Rokhlin OW, Glover RB, Guseva NV, Taghiyev AF, Kohlgraf KG, Cohen MB. Mechanisms of cell death induced by histone deacetylase inhibitors in androgen receptor-positive prostate cancer cells. *Mol Cancer Res* 2006;4(2):113–23.
- [107] Marrocco DL, Tilley WD, Bianco-Miotto T, Evdokiou A, Scher HI, Rifkind RA, et al. Suberoylanilide hydroxamic acid (vorinostat) represses androgen receptor expression and acts synergistically with an androgen receptor antagonist to inhibit prostate cancer cell proliferation. *Mol Cancer Ther* 2007;6(1):51–60.
- [108] Gameiro SR, Malamas AS, Tsang KY, Ferrone S, Hodge JW. Inhibitors of histone deacetylase 1 reverse the immune evasion phenotype to enhance T-cell mediated lysis of prostate and breast carcinoma cells. *Oncotarget*. 2016;7(7):7390–402.
- [109] Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C, et al. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. *Cancer Res* 2000;60(18):5165–70.
- [110] Qian DZ, Wei YF, Wang X, Kato Y, Cheng L, Pili R. Antitumor activity of the histone deacetylase inhibitor MS-275 in prostate cancer models. *Prostate*. 2007;67(11):1182–93.
- [111] De U, Kundu S, Patra N, Ahn MY, Ahn JH, Son JY, et al. A new histone deacetylase inhibitor, MHY219, inhibits the migration of human prostate cancer cells via HDAC1. *Biomol Ther (Seoul)* 2015;23(5):434–41.
- [112] Rathkopf DE, Picus J, Hussain A, Ellard S, Chi KN, Nydam T, et al. A phase 2 study of intravenous panobinostat in patients with castration-resistant prostate cancer. *Cancer Chemother Pharmacol* 2013;72(3):537–44.
- [113] Molife LR, Attard G, Fong PC, Karavasilis V, Reid AH, Patterson S, et al. Phase II, two-stage, single-arm trial of the histone deacetylase inhibitor (HDACi) romidepsin in metastatic castration-resistant prostate cancer (CRPC). *Ann Oncol* 2010;21(1):109–13.

- [114] Bradley D, Rathkopf D, Dunn R, Stadler WM, Liu G, Smith DC, et al. Vorinostat in advanced prostate cancer patients progressing on prior chemotherapy (National Cancer Institute Trial 6862): trial results and interleukin-6 analysis: a study by the Department of Defense Prostate Cancer Clinical Trial Consortium and University of Chicago Phase 2 Consortium. *Cancer*. 2009;115(23):5541–9.
- [115] Ferrari AC, Alumkal JJ, Stein MN, Taplin ME, Babb J, Barnett ES, et al. Epigenetic therapy with panobinostat combined with bicalutamide rechallenge in castration-resistant prostate cancer. *Clin Cancer Res* 2019;25(1):52–63.
- [116] Eigl BJ, North S, Winquist E, Finch D, Wood L, Sridhar SS, et al. A phase II study of the HDAC inhibitor SB939 in patients with castration resistant prostate cancer: NCIC clinical trials group study IND195. *Invest N Drugs* 2015;33(4):969–76.
- [117] Lin J, Elkorn J, Ricart B, Palmer E, Zevallos-Delgado C, Noonepal S, et al. Phase I study of entinostat in combination with enzalutamide for treatment of patients with metastatic castration-resistant prostate cancer. *Oncologist*. 2021;26(12):e2136–42.
- [118] Pili R, Haggman M, Stadler WM, Gingrich JR, Assikis VJ, Bjork A, et al. Phase II randomized, double-blind, placebo-controlled study of tasquinimod in men with minimally symptomatic metastatic castrate-resistant prostate cancer. *J Clin Oncol* 2011;29(30):4022–8.
- [119] Isaacs JT, Antony L, Dalrymple SL, Brennen WN, Gerber S, Hammers H, et al. Tasquinimod is an allosteric modulator of HDAC4 survival signaling within the compromised cancer microenvironment. *Cancer Res* 2013;73(4):1386–99.
- [120] Nakayama TWM, Suzuki H, Toyota M, Sekita N, Hirokawa Y, Mizokami A, et al. Epigenetic regulation of androgen receptor gene expression in human prostate cancers. *Laboratory Investigation* 2000;80:1789–96.
- [121] Tang S, Lian X, Jiang J, Cheng H, Guo J, Huang C, et al. Tumor suppressive maspin-sensitized prostate cancer to drug treatment through negative regulating androgen receptor expression. *Front Cell Dev Biol* 2020;8:573820.
- [122] Lakshmikanthan V, Kaddour-Djebbar I, Lewis RW, Kumar MV. SAHA-sensitized prostate cancer cells to TNFalpha-related apoptosis-inducing ligand (TRAIL): mechanisms leading to synergistic apoptosis. *Int J Cancer* 2006;119(1):221–8.
- [123] VanOosten RL, Earel Jr JK, Griffith TS. Histone deacetylase inhibitors enhance Ad5-TRAIL killing of TRAIL-resistant prostate tumor cells through increased caspase-2 activity. *Apoptosis*. 2007;12(3):561–71.
- [124] VanOosten RL, Moore JM, Ludwig AT, Griffith TS. Depsipeptide (FR901228) enhances the cytotoxic activity of TRAIL by redistributing TRAIL receptor to membrane lipid rafts. *Mol Ther* 2005;11(4):542–52.
- [125] Ellis L, Ku SY, Ramakrishnan S, Lasorsa E, Azabdaftari G, Godoy A, et al. Combinatorial antitumor effect of HDAC and the PI3K-Akt-mTOR pathway inhibition in a Pten defecient model of prostate cancer. *Oncotarget*. 2013;4(12):2225–36.
- [126] Kovacs JJ, Murphy PJ, Gaillard S, Zhao X, Wu JT, Nicchitta CV, et al. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Mol Cell* 2005;18(5):601–7.
- [127] Gibbs A, Schwartzman J, Deng V, Alumkal J. Sulforaphane destabilizes the androgen receptor in prostate cancer cells by inactivating histone deacetylase 6. *Proc Natl Acad Sci U S A* 2009;106(39):16663–8.
- [128] Hu WY, Xu L, Chen B, Ou S, Mazzarelli KM, Hu DP, et al. Targeting prostate cancer cells with enzalutamide-HDAC inhibitor hybrid drug 2–75. *Prostate*. 2019;79(10):1166–79.
- [129] Chen Z, Wang X, Yang X, Xu Y, Yang Y, Wang H, et al. Imaging assisted evaluation of antitumor efficacy of a new histone deacetylase inhibitor in the castration-resistant prostate cancer. *Eur J Nucl Med Mol Imaging* 2021;48(1):53–66.

- [130] Coffey K, Blackburn TJ, Cook S, Golding BT, Griffin RJ, Hardcastle IR, et al. Characterisation of a Tip60 specific inhibitor, NU9056, in prostate cancer. *PLoS One* 2012;7(10):e45539.
- [131] Wyce A, Degenhardt Y, Bai Y, Le B, Korenchuk S, Crouthame MC, et al. Inhibition of BET bromodomain proteins as a therapeutic approach in prostate cancer. *Oncotarget*. 2013;4(12):2419–29.
- [132] Asangani IA, Dommeti VL, Wang X, Malik R, Cieslik M, Yang R, et al. Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. *Nature*. 2014;510(7504):278–82.
- [133] Wang L, Xu M, Kao CY, Tsai SY, Tsai MJ. Small molecule JQ1 promotes prostate cancer invasion via BET-independent inactivation of FOXA1. *J Clin Invest* 2020;130(4):1782–92.
- [134] Faivre EJ, McDaniel KF, Albert DH, Mantena SR, Plotnik JP, Wilcox D, et al. Selective inhibition of the BD2 bromodomain of BET proteins in prostate cancer. *Nature*. 2020;578(7794):306–10.
- [135] Sakr WAHG, Cassin BF, Pontes JE, Crissman JD. The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. *J Urol* 1993;150(2 Pt 1):379–85.
- [136] Ge R, Wang Z, Montironi R, Jiang Z, Cheng M, Santoni M, et al. Epigenetic modulations and lineage plasticity in advanced prostate cancer. *Ann Oncol* 2020;31(4):470–9.
- [137] Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. A prospective study of cruciferous vegetables and prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2003;12(12):1403–9.
- [138] Myzak MC, Hardin K, Wang R, Dashwood RH, Ho E. Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. *Carcinogenesis*. 2006;27(4):811–19.
- [139] Abbas A, Hall JA, Patterson 3rd WL, Ho E, Hsu A, Al-Mulla F, et al. Sulforaphane modulates telomerase activity via epigenetic regulation in prostate cancer cell lines. *Biochem Cell Biol* 2016;94(1):71–81.
- [140] Singh SV, Warin R, Xiao D, Powolny AA, Stan SD, Arlotti JA, et al. Sulforaphane inhibits prostate carcinogenesis and pulmonary metastasis in TRAMP mice in association with increased cytotoxicity of natural killer cells. *Cancer Res* 2009;69(5):2117–25.
- [141] Wong CP, Hsu A, Buchanan A, Palomera-Sanchez Z, Beaver LM, Houseman EA, et al. Effects of sulforaphane and 3,3'-diindolylmethane on genome-wide promoter methylation in normal prostate epithelial cells and prostate cancer cells. *PLoS One* 2014;9(1):e86787.
- [142] Beaver LM, Kuintzle R, Buchanan A, Wiley MW, Glasser ST, Wong CP, et al. Long noncoding RNAs and sulforaphane: a target for chemoprevention and suppression of prostate cancer. *J Nutr Biochem* 2017;42:72–83.
- [143] Traka MH, Melchini A, Coode-Bate J, Al Kadhi O, Saha S, Defernez M, et al. Transcriptional changes in prostate of men on active surveillance after a 12-mo glucoraphanin-rich broccoli intervention—results from the Effect of Sulforaphane on prostate CAncer PrEvention (ESCAPE) randomized controlled trial. *Am J Clin Nutr* 2019;109(4):1133–44.
- [144] Zhang Z, Garzotto M, Davis 2nd EW, Mori M, Stoller WA, Farris PE, et al. Sulforaphane bioavailability and chemopreventive activity in men presenting for biopsy of the prostate gland: a randomized controlled trial. *Nutr Cancer* 2020;72(1):74–87.
- [145] Katta S, Srivastava A, Thangapazham RL, Rosner IL, Cullen J, Li H, et al. Curcumin-gene expression response in hormone dependent and independent metastatic prostate cancer cells. *Int J Mol Sci* 2019;20(19).
- [146] Chendil D, Ranga RS, Meigooni D, Sathishkumar S, Ahmed MM. Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3. *Oncogene*. 2004;23(8):1599–607.
- [147] Khor TO, Keum YS, Lin W, Kim JH, Hu R, Shen G, et al. Combined inhibitory effects of curcumin and phenethyl isothiocyanate on the growth of human PC-3 prostate xenografts in immunodeficient mice. *Cancer Res* 2006;66(2):613–21.
- [148] Liu J, Li M, Wang Y, Luo J. Curcumin sensitizes prostate cancer cells to radiation partly via epigenetic activation of miR-143 and miR-143 mediated autophagy inhibition. *J Drug Target* 2017;25(7):645–52.

- [149] Zhao W, Zhou X, Qi G, Guo Y. Curcumin suppressed the prostate cancer by inhibiting JNK pathways via epigenetic regulation. *J Biochem Mol Toxicol* 2018;32(5):e22049.
- [150] Pan L, Sha J, Lin W, Wang Y, Bian T, Guo J. Curcumin inhibits prostate cancer progression by regulating the miR-30a-5p/PCLAF axis. *Exp Ther Med* 2021;22(3):969.
- [151] Mansouri K, Rasoulooor S, Daneshkhah A, Abolfathi S, Salari N, Mohammadi M, et al. Clinical effects of curcumin in enhancing cancer therapy: a systematic review. *BMC Cancer* 2020;20(1):791.
- [152] Cheng MA, Chou FJ, Wang K, Yang R, Ding J, Zhang Q, et al. Androgen receptor (AR) degradation enhancer ASC-J9® in an FDA-approved formulated solution suppresses castration resistant prostate cancer cell growth. *Cancer Lett* 2018;417:182–91.
- [153] Tian H, Chou FJ, Tian J, Zhang Y, You B, Huang CP, et al. ASC-J9® suppresses prostate cancer cell proliferation and invasion via altering the ATF3-PTK2 signaling. *J Exp Clin Cancer Res* 2021;40(1):3.
- [154] Morton MS, Arisaka O, Miyake N, Morgan LD, Evans BA. Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. *J Nutr* 2002;132(10):3168–71.
- [155] Ho E, Beaver LM, Williams DE, Dashwood RH. Dietary factors and epigenetic regulation for prostate cancer prevention. *Adv Nutr* 2011;2(6):497–510.
- [156] Severson RK, Nomura AM, Grove JS, Stemmermann GN. A prospective study of demographics, diet, and prostate cancer among men of Japanese ancestry in Hawaii. *Cancer Res* 1989;49(7):1857–60.
- [157] Travis RC, Allen NE, Appleby PN, Price A, Kaaks R, Chang-Claude J, et al. Prediagnostic concentrations of plasma genistein and prostate cancer risk in 1,605 men with prostate cancer and 1,697 matched control participants in EPIC. *Cancer Causes Control* 2012;23(7):1163–71.
- [158] Kurahashi N, Iwasaki M, Inoue M, Sasazuki S, Tsugane S. Plasma isoflavones and subsequent risk of prostate cancer in a nested case-control study: the Japan Public Health Center. *J Clin Oncol* 2008;26(36):5923–9.
- [159] Ward H, Chapelais G, Kuhnle GG, Luben R, Khaw KT, Bingham S. Lack of prospective associations between plasma and urinary phytoestrogens and risk of prostate or colorectal cancer in the European Prospective into Cancer-Norfolk study. *Cancer Epidemiol Biomarkers Prev* 2008;17(10):2891–4.
- [160] Shafiee G, Saidijam M, Tayebinia H, Khodadadi I. Beneficial effects of genistein in suppression of proliferation, inhibition of metastasis, and induction of apoptosis in PC3 prostate cancer cells. *Arch Physiol Biochem* 2022;128(3):694–702.
- [161] Lakshman M, Xu L, Ananthanarayanan V, Cooper J, Takimoto CH, Helenowski I, et al. Dietary genistein inhibits metastasis of human prostate cancer in mice. *Cancer Res* 2008;68(6):2024–32.
- [162] Huang X, Chen S, Xu L, Liu Y, Deb DK, Plataniias LC, et al. Genistein inhibits p38 map kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells. *Cancer Res* 2005;65(8):3470–8.
- [163] Xu L, Ding Y, Catalona WJ, Yang XJ, Anderson WF, Jovanovic B, et al. MEK4 function, genistein treatment, and invasion of human prostate cancer cells. *J Natl Cancer Inst* 2009;101(16):1141–55.
- [164] Bilir B, Sharma NV, Lee J, Hammarstrom B, Svindland A, Kucuk O, et al. Effects of genistein supplementation on genome-wide DNA methylation and gene expression in patients with localized prostate cancer. *Int J Oncol* 2017;51(1):223–34.
- [165] Fang MZ, Chen D, Sun Y, Jin Z, Christman JK, Yang CS. Reversal of hypermethylation and reactivation of p16INK4a, RARbeta, and MGMT genes by genistein and other isoflavones from soy. *Clin Cancer Res* 2005;11(19 Pt 1):7033–41.
- [166] Majid S, Dar AA, Shahryari V, Hirata H, Ahmad A, Saini S, et al. Genistein reverses hypermethylation and induces active histone modifications in tumor suppressor gene B-Cell translocation gene 3 in prostate cancer. *Cancer* 2010;116(1):66–76.
- [167] Day JK, Bauer AM, DesBordes C, Zhuang Y, Kim BE, Newton LG, et al. Genistein alters methylation patterns in mice. *J Nutr* 2002;132(8 Suppl):2419S–2423SS.

- [168] Mahmoud AM, Al-Alem U, Ali MM, Bosland MC. Genistein increases estrogen receptor beta expression in prostate cancer via reducing its promoter methylation. *J Steroid Biochem Mol Biol* 2015;152:62–75.
- [169] Zhang Y, Li Q, Chen H. DNA methylation and histone modifications of Wnt genes by genistein during colon cancer development. *Carcinogenesis*. 2013;34(8):1756–63.
- [170] Tian JY, Chi CL, Bian G, Xing D, Guo FJ, Wang XQ. PSMA conjugated combinatorial liposomal formulation encapsulating genistein and plumbagin to induce apoptosis in prostate cancer cells. *Colloids Surf B: Biointerfaces* 2021;203:111723.
- [171] Vodnik VV, Mojić M, Stamenović U, Otoničar M, Ajdžanović V, Maksimović-Ivančić D, et al. Development of genistein-loaded gold nanoparticles and their antitumor potential against prostate cancer cell lines. *Mater Sci Eng C: Mater Biol Appl* 2021;124:112078.
- [172] Fang M, Chen D, Yang CS. Dietary polyphenols may affect DNA methylation. *J Nutr* 2007;137(1 Suppl):223S–228SS.

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Neuroblastoma

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1 Neuroblastoma

Neuroblastoma is a malignant pediatric cancer, originating from immature cells of the sympathetic nervous system during embryonic development or early postnatal life. It is the most common solid extracranial malignancy of childhood and the most common malignant tumor in infants. The overall incidence of neuroblastoma is, on average, 10 cases per million and approximately 15% of all childhood cancer deaths can be attributed to the disease [1].

Neuroblastoma displays significant heterogeneity between patients both in disease progression and in clinical outcome. Some tumors will spontaneously regress or mature into a benign ganglioneuroma even without therapy, while others display a very aggressive, malignant behavior that is poorly responsive to current intensive, multimodal therapy. Others display a very aggressive, malignant behavior that is poorly responsive to current intensive, multimodal therapy. At the time of neuroblastoma diagnosis, more than 50% of patients will already have metastatic disease. Metastatic sites most commonly include bone and bone marrow, less frequently lymph nodes and liver, and rarely lung, brain, or skin.

The International Neuroblastoma Risk Group Staging System (INRGSS) is the universal system used to classify tumors and direct optimal treatment plans. Neuroblastoma tumors can be stratified into four groups (L1, L2, M, or Ms) according to risk factors which include age at diagnosis, tumor localization and size, histopathologic classification, DNA content, chromosomal abnormalities, and *MYCN* status (extensively reviewed by [1]). Of note, *MYCN* oncogene amplification occurs in approximately 20–30% of neuroblastomas and is the most reliable genetic marker of poor prognosis. High-risk neuroblastoma is identified as a child under 18 months presenting with metastatic disease or a patient at any age presenting with stage L2, M, or Ms with *MYCN* amplification. This marker is currently used in combination with clinical characteristics to stratify newly diagnosed patients into “risk groups” based on the likelihood of their developing an aggressive or less-severe form of neuroblastoma and predicts overall survival rate. The overall survival rates for patients with *MYCN* amplification is strikingly low at 20–30% [2].

Among the molecular features contributing to neuroblastoma heterogeneity and stratification are whole chromosome gains and a large number of recurrent large-scale chromosome imbalances, such as loss of heterozygosity at chromosome arms 1p, 3p, 14q, and 11q, unbalanced gain of 1q, 11p, and 17q and numerous mutations in key genes, such as *ALK*, *PHOX2B*, and *PTPRD* (reviewed in [3]).

Growing evidence indicates that features such as epigenetic changes and miRNA expression are highly correlated with clinical behavior, providing insight into the molecular basis of clinical heterogeneity and offering better defined prognostic signatures. These advances may eventually be included in risk assessment and stratification to improve treatment regimens and prediction of patient outcome [3].

Half of all neuroblastoma diagnoses are classified as high-risk and require immediate treatment. The 5-year survival rate for children with low-risk neuroblastoma is higher than 95% and for those with intermediate-risk neuroblastoma is 80–90%. Long-term survival for children with high-risk neuroblastoma is very poor, about 30–50%.

Standard of care regimens include chemotherapy, surgery, myeloablative radiation and in some cases followed by a restorative autologous stem cell transplant and/or immunotherapy. Unfortunately, at least 20% of high-risk neuroblastoma cases will be refractory (resistant) to one or the other type of therapy. After remission more than one-third of children will subsequently relapse. These are the cases with the highest risk. One of the greatest concerns in high-risk neuroblastoma

is the persistence of minimal residual disease (MRD) after chemotherapy or radiation which often results in recurrence.

In this chapter, we discuss the key epigenetic factors contributing to neuroblastoma, the significance of such in relation to improved understanding of neuroblastoma predisposition and development of new therapies.

2 Epigenetic changes

The hallmark of cancer is dysregulated gene expression. It is now widely accepted that both genetic and epigenetic factors impact gene expression and contribute to cancer development and progression. Epigenetic alterations are defined as those heritable changes in gene expression that do not result from direct changes in DNA sequence. Mechanisms of epigenetic regulation that impact gene expression most commonly include DNA methylation, modification of histones, and changes in microRNA (miRNA) expression.

2.1 DNA methylation

Altered DNA methylation patterns are widely observed in development and progression of various types of cancer including neuroblastoma. Methylation pattern detection has become an enormously important area of study (see Chapter 2 for more details). DNA methylation triggers the binding of DNA-specific binding proteins to CpG sites, attracting histone-modifying enzymes that, in turn, focally establish a silenced or active chromatin state. Aberrant hypermethylation of CpGs within gene promoters can lead to gene inactivation, while genome wide or global hypomethylation affects mostly repetitive DNA sequences. These alterations are the most frequent cancer-related epigenetic changes, while the epigenetic silencing is a common mechanism for loss of tumor suppressor gene function rather than mutation. Both single-gene methylation and methylation of multiple promoter CpG islands (known as CIMP) have been shown to have prognostic significance in neuroblastoma.

2.1.1 Aberrant hypermethylation of CpGs within gene promoters

The first DNA methylation studies in neuroblastoma discovered that epigenetic inactivation of caspase 8 (*CASP8*) and RAS-association domain family 1 isoform A (*RASSF1A*) are important in the development and progression of disease. With the addition of *DcR1/2*, these are the three most studied genes in the context of how methylation affects survival outcome in neuroblastoma. Other more recent examples include *RBI*, *TDGF1*, and *MEGF10*.

The promoter of tumor suppressor *RASSF1A*, mapped to 3p21.3 was *de novo* hypermethylated in 55–100% neuroblastoma tumors as reviewed by Decock [4]. *RASSF1A* inhibits accumulation of cyclin D1, preventing cell cycle progression and effectively inducing growth arrest. Epigenetic inactivation of this gene and its downstream processes has been shown to contribute to progression of various cancers [4]. *RASSF1A* methylation status correlates with poor prognosis in neuroblastoma, suggesting that the epigenetic silencing of this tumor suppressor gene could contribute to aberrations of RAS signaling pathways and progression of this disease [5]. Hypermethylated *RASSF1A* is also

shown to be a reliable circulating biomarker with potential to be used to monitor disease progression in patients [6].

Both CASP8 and DCR2 are involved in regulation of apoptosis and are both commonly methylated and inactivated in neuroblastoma. *CASP8* encodes a cysteine protease involved in the tumor necrosis factor-related apoptosis pathway and *DcR1/2* encodes one of the antiapoptotic decoy receptors in the same pathway. In neuroblastoma cell lines, inactivation of the *CASP8* gene has been shown to be caused by deletion or hypermethylation. However, methylation of its promoter region was the primary mechanism for its silencing in neuroblastoma tumor samples. Interestingly, complete inactivation of *CASP8* was observed predominantly in neuroblastomas with amplified *MYCN*, which potentially identifies it as an additional prognostic tool [7]. A consistent CpG island promoter methylation of *CASP8* has been detected in 14–91% neuroblastoma tumors. For example, in a cohort of 70 neuroblastoma tumor samples *CASP8* displayed 56% hypermethylation which was correlated to poor outcome [8]. Applying clustering of a limited number of hypermethylated genes, *CASP8* was found to be methylated in 77% of the neuroblastoma cell lines investigated, further supporting the importance of the methylation status of this gene *in vitro* [9]. Similarly, high levels of *DcR1/2* methylation have been observed in both cell lines and neuroblastoma patient samples contributing to loss of gene expression [10].

More recently Almutairi et al. carried out genome-wide methylation analysis identifying *GATA3*, which encodes for the regulator of sympathetic neural system development, to be hypomethylated in neuroblastoma tumor samples. This epigenetic regulation leads to persistent mRNA transcription and protein expression as well as poor disease outcome. Knockdown of *GATA3* inhibited proliferation and induced apoptosis in neuroblastoma cell lines [11]. Thus highlighting the importance of genome-wide analysis to identify other actionable epigenetic targets in cancer.

Advances in genome-wide methylation discrimination technologies have made it possible to identify nearly 80 different DNA methylation candidate genes associated with patient survival in neuroblastoma and risk factors, such as *MYCN* amplification, patient age, and tumor stage. Examples of such technologies include DNA methylation promoter assay after affinity-based capture, re-expression analysis after treatment with 5-aza-2'-deoxycytidine (DAC), methylation microarray after bisulfate treatment and next-generation sequencing. The genes identified are involved in fundamental biological processes, namely, tumor suppression cell cycle control, DNA damage response, cell migration, apoptosis, etc. More recently Decock et al. have identified genes associated with neural crest development and differentiation to be hypermethylated in the 4S subset of patients, the subset which has a generally good prognosis and often undergoes spontaneous regression. This study highlights the importance of this sequencing technology in identifying methylation signatures to identify specific genetic pathways involved in disease progression (or regression) as well as improving patient stratification [12].

CpG island methylator phenotype (CIMP) results in inactivation of several genes, namely tumor-suppressor genes, and is characterized by vast hypermethylation of promoter CpG island sites. The *PCDHB* family remains the most studied of such sites in neuroblastoma. In combination with other sites, high levels of methylation of *PCDHB*, as the consensus marker of CIMP, correlated with poor OS in two separate patient datasets and showed a close association with *MYCN* amplification [13,14]. This methylation signature could contribute to patient stratification and prediction of prognosis, however, further studies (currently underway), are required to validate the clinical utility.

Noticeably, a significant correlation between *RASSF1A* and *CASP8* methylation in neuroblastoma was demonstrated [9,15], suggesting that a subset of neuroblastoma may have a CpG island methylator phenotype (CIMP) as described in other cancers.

2.1.2 Genome-wide aberrant hypermethylation of CpGs islands assessment

The first genome-wide assessment of DNA methylation patterns in neuroblastoma, ganglioneuroma, and ganglioneuroblastoma tumors was carried out by Buckley et al. [16]. This study identified recurrent large-scale blocks of contiguously hypermethylated promoter/CpG island sites ($n = 70$) in the tumors. The highest number of large-scale DNA methylation blocks (eight blocks in total) was demonstrated for chromosome 19, which may be explained by the greater gene density of this chromosome. The study recognized a total of 63 methylation blocks that are potentially disease related. In contrast, DNA methylation analysis of three human chromosomes in normal tissue identified a significant correlation for methylation of regions only over distances 1000 bp, suggesting that larger methylated regions may be disease specific. Of note, a significant overrepresentation of methylated blocks toward telomeric ends (31% of the blocks occurring <2 Mb from telomere) was identified.

2.2 Histone modifications

Lysine-rich tails of core histones (H2A, H2B, H3, and H4) overhang from the nucleosome providing sites for reversible modifications that modify chromatin structure and modulate gene expression (see Chapter 3 for more details). The most characterized histone modifications in cancer are acetylation, deacetylation, methylation and demethylation.

2.2.1 Histone acetylation/deacetylation

Currently very little is known about the role of histone acetyltransferases (HATs) and acetylation in neuroblastoma. However, much research has been carried out on histone deacetylases (HDACs) giving insights into new methods of patient stratification as well interrogation of potential therapeutic targets.

HDACs are a family of protein deacetylating enzymes that remove acetyl groups from lysine residues of histone and nonhistone proteins (see Chapter 3 for more details). Their enzyme activity can be inhibited by small molecule compounds termed HDAC inhibitors (HDACi). HDACi are now recognized as a promising mode of anticancer treatment with clinical trials under way (Table 15.1).

The first study investigating expression of all HDAC family members at mRNA level in neuroblastoma was conducted on a large cohort of 251 tumors [21]. Increasing expression of HDAC8 mRNA showed significant correlation with disease progression, known clinical and molecular risk factors, and poor clinical outcome. Individual HDAC family members control different cellular pathways in neuroblastoma; summarized in Figure 15.1 and reviewed in [22]. This may explain the different biological effects observed with nonselective HDAC inhibitors on cancer cells.

2.2.2 Histone methylation/demethylation

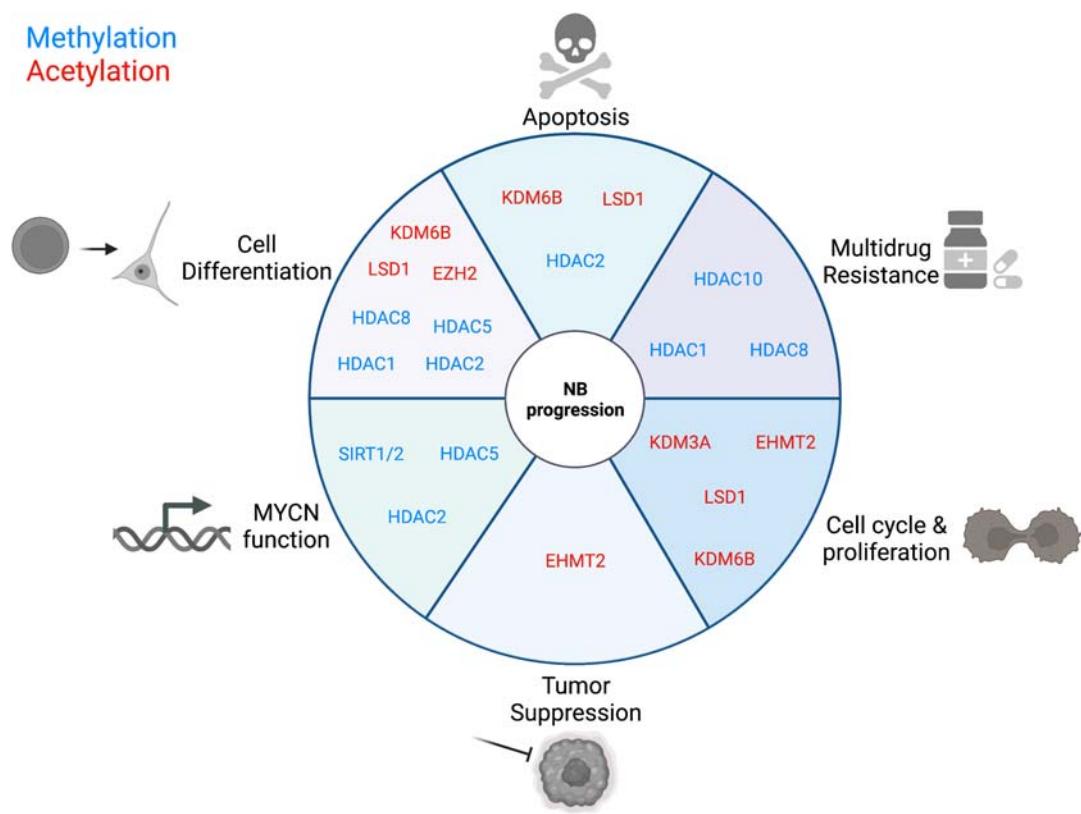
Histone methylation is a dynamic process and can result in both activation and repression of genes based on various factors.

The histone methyltransferase, *EZH2* (methylates H3K27) has been shown to be expressed at increased levels in neuroblastoma [23]. Multiple studies have shown an association with *MYCN*-amplified tumors with *MYCN* driving *EZH2* expression via promoter binding [24–26]. Increased expression of *EZH2* in

Table 15.1 Active Clinical Trials in Neuroblastoma with Epigenetic Targeting Therapeutics

Epigenetic Focus	NCT number	Title	Status	Results Posted	Conditions	Age Groups	Phase	Enrollment	References
N/A	NCT01857934	Therapy for Children with Advanced Stage Neuroblastoma	Active, not recruiting	Yes	Neuroblastoma	Child/adult	2	153	[17]
RA	NCT01701479	Long-Term Continuous Infusion Ch14.18/CHO Plus s.c. Aldesleukin (IL-2)	Active, not recruiting	No	Neuroblastoma	Child/adult	1/2	288	N/A
RA	NCT00026312	Isotretinoin with or without Monoclonal Antibody Ch14.18, Aldesleukin, and Sargramostim Following Stem Cell Transplant in Treating Patients with Neuroblastoma	Active, not recruiting	Yes	Neuroblastoma	Child/adult	3	1449	[18]
RA	NCT01711554	Lenalidomide and Dinutuximab With or Without Isotretinoin in Treating Younger Patients With Refractory or Recurrent Neuroblastoma	Active, not recruiting	Yes	Recurrent/refractory neuroblastoma	Child/adult	1	27	[19]
N/A	NCT03057626	Late Effects After Treatment in Patients With Previously Diagnosed High-Risk Neuroblastoma	Active, not recruiting	No	Recurrent neuroblastoma, neuroblastoma at different stages	Child/adult	Observational	400	N/A
RA	NCT03786783	Dinutuximab, Sargramostim, and Combination Chemotherapy in Treating Patients With Newly Diagnosed High-Risk Neuroblastoma Undergoing Stem Cell Transplant	Active, not recruiting	No	Ganglioneuroblastoma, high-risk neuroblastoma	Child/adult	2	45	N/A

RA	NCT03126916	Iobenguane I-131 or Crizotinib and Standard Therapy in Treating Younger Patients With Newly-Diagnosed High-Risk Neuroblastoma or Ganglioneuroblastoma	Active, recruiting	Yes	Ganglioneuroblastoma, neuroblastoma	Child/ adult	3	813	[20]	
HDAC inhibitors	NCT03332667	MIBG With Dinutuximab +/- Vorinostat	Active, recruiting	No	Neuroblastoma	Child/ adult	1	32	N/A	
HDAC inhibitors	NCT03561259	A Study of Therapeutic Iobenguane (131-I) and Vorinostat for Recurrent or Progressive High-Risk Neuroblastoma Subjects (OPTIMUM)	Active, recruiting	No	Neuroblastoma/ Neuroectodermal Tumors/neoplasms	Child/ adult/ senior	2	60	N/A	
HDAC inhibitors	NCT02559778	Pediatric Precision Laboratory Advanced Neuroblastoma Therapy (PEDS-PLAN)	Active, recruiting	No	Neuroblastoma	Child/ adult	2	500	N/A	
HDAC inhibitors	NCT04308330	Vorinostat in Combination With Chemotherapy in Relapsed/Refractory Solid Tumors and CNS Malignancies (NYMC195)	Active, recruiting	No	Ewing sarcoma/ rhabdomyosarcoma/ Wilms tumor/ neuroblastoma/ hepatoblastoma/germ cell tumor	Child/ adult	1	30	N/A	

**FIGURE 15.1**

Histone modifications contributing to neuroblastoma disease.

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neuroblastoma promotes an undifferentiated phenotype, is associated with poor outcome [27], and facilitates silencing of multiple gene signatures [28]. Inhibition of EHMT2 (methylates H3K9) reduced proliferation of neuroblastoma cell lines [29] and re-activated expression of tumor suppressor genes [30].

Similarly, inhibition of DOT1L (methylates H3K79) reduced growth in *MYCN*-amplified cell lines *in vitro* and attenuated tumor growth improving OS *in vivo* [31].

Three separate studies focused on DNA methylation status of *PTGER2* [32], *NSD1* [33], and *GATA3* [11] using chromatin immunoprecipitation (ChIP) assays have detected and discussed histone modifications in neuroblastoma.

The *PTGER2* gene, mapping to 14q22, encodes a receptor for prostaglandin E2, which has different biologic activities in a wide range of tissues. Dissection of *PTGER2* promoter region revealed segments of different susceptibility to methylation in neuroblastoma cells [32]. Hypermethylation patterns were concordant with loss of *PTGER2* protein expression. Subsequent experimental validation of histone modification status of promoter region of *PTGER2* by ChIP suggested that around its promoter region, histone H3 and H4 are deacetylated and histone H9 is

di- and tri-methylated in neuroblastoma cells lacking expression of this gene. The methylation status of *PTGER2* correlated with the aggressiveness of neuroblastoma tumors.

NSD1 gene mapped to 5q35 encodes a histone methyl transferase. CpG island hypermethylation of *NSD1* leads to reduced levels of NSD1 protein in neuroblastoma and glioma [33]. This epigenetic silencing was associated with global reduced levels of trimethylated histone H4 and H3 and poor survival.

Previously mentioned gene, *MEGF10*, has been shown to be silenced via DNA hypermethylation but also by repressive H3K27/K9 modifications in neuroblastoma cell lines when compared to the precursor, neural crest cells [34]. In cell lines with low levels of *MEGF10* methylation, this repressive histone modification could successfully silence gene expression, highlighting how histone modifications and DNA methylation are closely linked.

In contrast, much less is known regarding histone demethylation in neuroblastoma. The majority of research (reviewed in [35]) has focused on various subfamilies of lysine demethylases (KDMs). The effects of LSD1 (KDM1A), a histone demethylase, in various processes has been reported including poor cell differentiation, inhibition of proteins involved in cell cycle control, apoptosis and metastasis and autophagy [36–38]. KDM3A [39], KDM4B [40], KDM5B [41], and KDM6B [42] have also been implicated in neuroblastoma disease affecting various pathways, illustrated in Figure 15.1.

DNA methylation in neuroblastoma displays a complex pattern, yet to be fully elucidated. Integrated epigenetic events, such as DNA methylation and histone modification are involved in epigenetic gene silencing. Almost 80 differentially DNA methylated genes were validated using various detection techniques. Some of them have been shown to have the prognostic potential as epigenetic biomarkers.

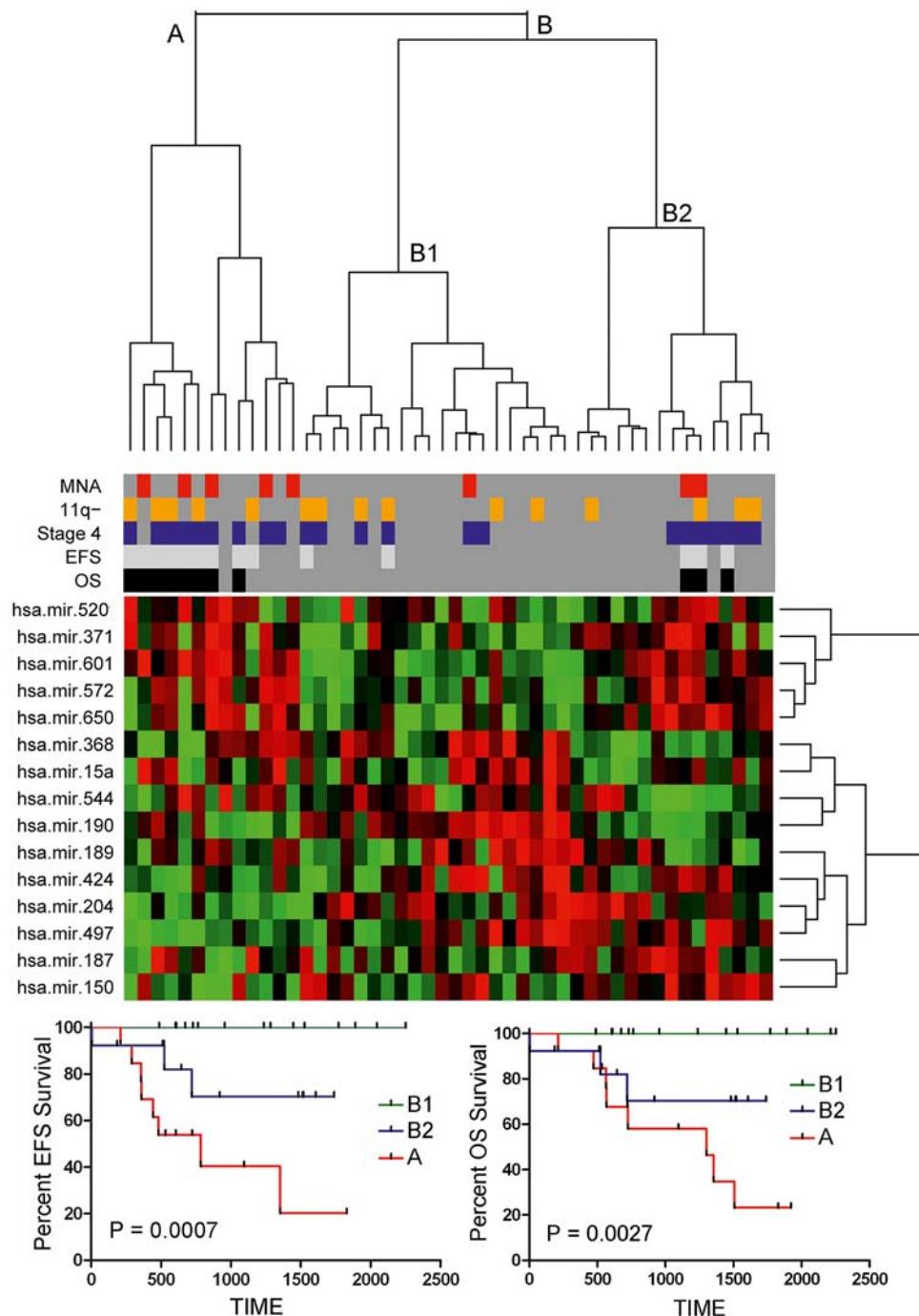
Importantly, epigenetic changes are reversible; hence DNA methylating agents may be able to restore functionality of epigenetically inactivated genes with potential tumor growth inhibitory effects.

2.3 miRNA

Epigenetic inactivation of miRNAs with tumor-suppressor activities is now recognized as a major hallmark of neuroblastoma tumors. miRNAs are a class of small, noncoding RNAs that function in gene expression regulation. MiRNAs are known to regulate oncogenes, tumor suppressor genes, genes involved in cell cycle control, cell migration, differentiation, development, apoptosis, and angiogenesis (reviewed in Chapter 5). MiRNA expression in tumors, and particularly in neuroblastoma has been observed to be up- or downregulated in high versus low-risk tumors, supporting their complex dual role as either “oncomirs” or tumor suppressors, respectively [43]. Remarkably, select miRNA signatures can classify multiple cancers more accurately than data from ~16,000 mRNAs [44]. Similarly, to protein encoding gene expression, the activity of miRNAs is also under epigenetic regulation. Therapeutic targeting of miRNA in neuroblastoma is currently being explored [45,46].

2.3.1 miRNA expression patterns

The first miRNA expression profiling study of primary neuroblastoma tumors was published by Chen and Stallings in 2007 [47], demonstrating that many miRNAs are differentially expressed in different genomic subtypes of neuroblastoma. Importantly, those miRNA profiles were correlated with the clinical outcome. Distinguished miRNA expression patterns were found between *MYCN* amplified and other tumor subtypes, leading to the hypothesis that overexpression of *MYCN* results in the downregulation of a large set of miRNAs that have antiproliferative or pro-apoptotic effects



and the upregulation of a smaller set of miRNAs that promote tumor growth. This observation has been validated further in the two independent miRNA expression profiling studies involving larger neuroblastoma tumor cohorts and miRNA loci. The hypothesis was confirmed by ChIP studies, which demonstrated that MYCN binds in close proximity to certain miRNA loci [48]. Further support for the concept that MYCN contributes to aggressive disease pathogenesis through the regulation of miRNAs came from the study by Beckers et al. [49]. These authors demonstrated that miRNAs that are upregulated by MYCN/MYC signaling directly downregulate a large set of protein-coding genes that are significantly associated with patient survival. Thus MYCN directly regulates expression of some miRNAs [48,49].

Other factors that significantly alter miRNA expression in neuroblastoma are the recurrent large-scale chromosomal imbalances, including loss of 1p, 3p, 11q, and 14q, along with gain of 1q and 17q [50]. Bray et al. used a machine learning algorithm to explore combinations of miRNAs that are either overexpressed or under expressed in unfavorable tumors. This study identified a 15-miRNA signature predictive of OS and EFS, as illustrated in [Figure 15.2](#), as reproduced from Bray et al. [50]. Notably, this signature is independent of MYCN copy number and the status of other prognostic markers thus can be used for advanced stratification of neuroblastomas.

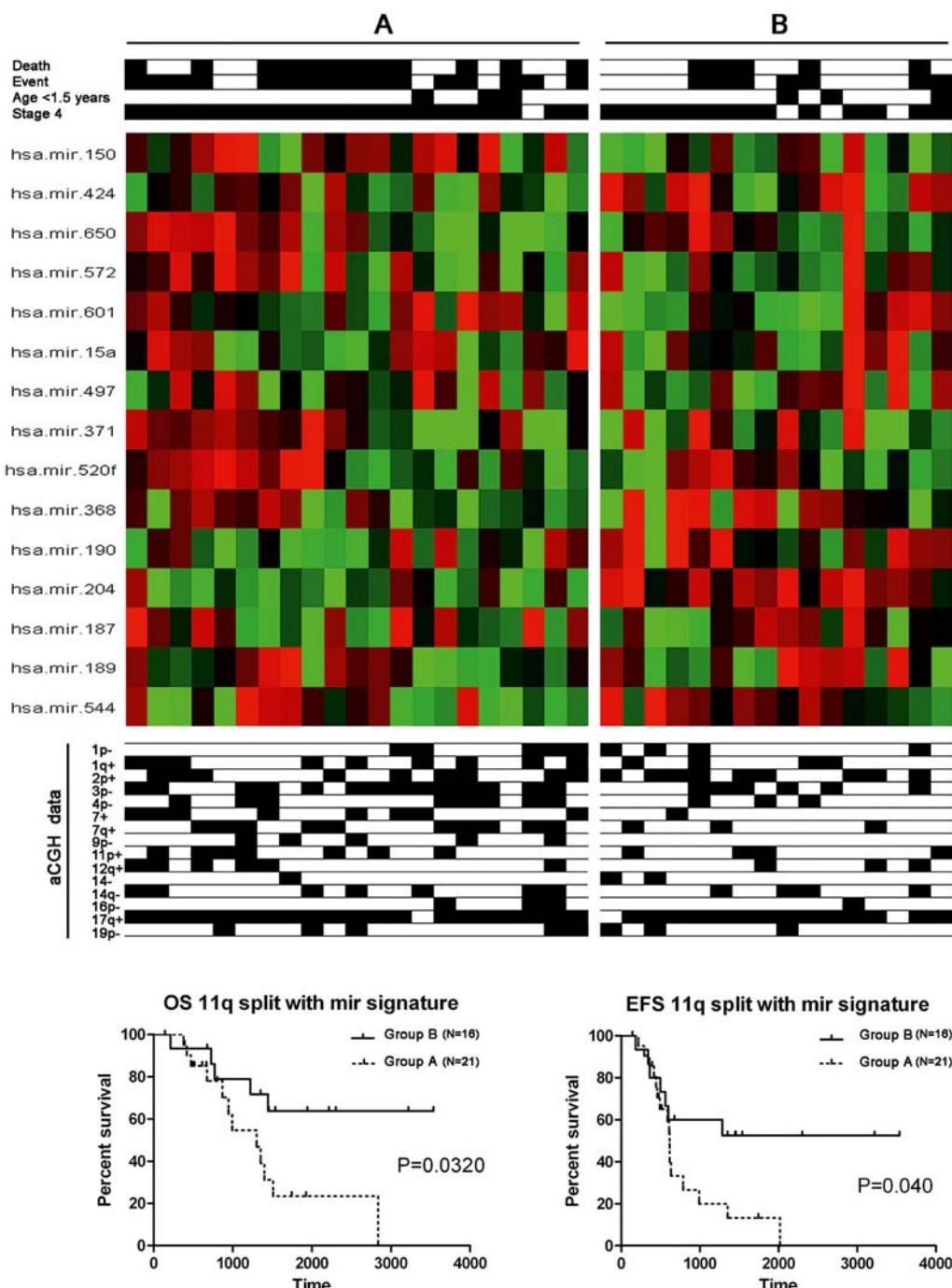
Buckley et al. [51] split tumors with 11q deletions into two distinct subtypes using the 15-miRNA signature originally developed by Bray [50]. These subtypes differed significantly in clinical outcome and the overall frequency of large-scale genomic imbalances, with the poor survival group having more imbalances. This is consistent with a study by Fisher et al. where 11q-tumors were subdivided based on mRNA expression profiling into two clinically distinct groups [52]. However, Buckley's study also discovered cases where miRNA expression was inversely related to the genomic imbalance. Some miRNAs were under-expressed despite mapping to a region of DNA copy number gain. This strongly suggests that alternative mechanisms can, in some instances, neutralize the effects of DNA dosage. Importantly, an miRNA expression signature predictive of clinical outcome for 11q-tumors was identified for neuroblastoma, emphasizing the potential for miRNA-mediated diagnostics and therapeutics ([Figure 15.3](#)).

The characteristic change in miRNA expression patterns associated with neuroblastoma progression, as well as their abundance in serum and plasma, places them as potential as biomarkers of tumor stage, metastasis presence, and drug resistance [53]. A differential abundance analysis, performed on the samples of patients with localized and metastatic disease, revealed tumor stage to have the most impact on the miRNA serum level, with the level of the miRNA proportional to the stage [54].

FIGURE 15.2

Hierarchical cluster analysis using a 15-miRNA expression signature divides neuroblastoma patients into two major groups, A and B, which significantly differ in survival. Patients with tumors in group A have significantly worse event-free and overall survival than patients from group B. The analysis represents an independent validation of the signature. Red indicates high miRNA expression and green low expression on the heat map.

This figure is the original figure published by Bray et al. [50] and is reproduced here with permission of the authors and their agreement with the publisher.



Differential abundance analysis, performed on the samples from the patients with metastatic and localized disease, revealed nine miRNA strongly associated with metastatic stage 4 disease, eight of which were specific for neuroblastoma (miR-873-3p, miR-149-5p, miR-124-3p, miR-218-5p, miR-490-5p, miR-323a-3p, miR-10b-3p, and miR-129-5p) [54]. In addition, those markers were predictive of OS in a cohort of patients including those from the low-, intermediate-, and high-risk groups but not associated with prognosis in the high-risk group alone.

In addition to staging, miRNA might be used to predict the response to the induction therapy in high-risk neuroblastoma patients. A SIOPEN study investigated the changes in exosomal miRNA expression in a cohort of high-risk neuroblastoma patients following induction chemotherapy and the correlation between selected exosomal miRNA levels and response to treatment [55]. The chemotherapy resulted in significant downregulation of 62 exosomal miRNAs in serum compared to levels pretreatment. Further analysis highlighted miR-29c, miR-342-3p, and let-7b to be significantly downregulated after treatment in minimal response patients, while remaining almost unchanged in very good partial response patients. While further verification is required in larger cohorts, this data demonstrates that circulating miRNA levels and patterns could contribute to improvements in patient and tumor stratification.

2.3.2 Individual miRNAs

Collectively, miRNAs can strongly influence tumorigenesis and tumor progression. However, certain individual miRNAs have been shown to be more influential than others in neuroblastoma pathogenesis. miRNAs regulate important genes involved in this process, playing a complex role as either “oncomirs” or tumor suppressors [56]. One of the tumor suppressor miRNAs in neuroblastoma is miR-34a [47,57,58]. Welch et al. first demonstrated that miR-34a, which maps to a region on distal chromosome 1p, is expressed at lower levels in tumors with 1p deletion, and that ectopic overexpression of this miRNA in neuroblastoma cell lines leads to the arrest of cell proliferation and the induction of a caspase-mediated apoptotic pathway [47]. miR-34a directly targets a number of genes in neuroblastoma, including the *E2F3* and *MYCN* transcription factors [59,60], as well as other genes involved in cell proliferation or apoptosis, such as *BCL2*, *CCND1*, *CDK6*, Notch1, survivin, *CD44*, *ATG5*, and many others [61].

Other miRNAs can also act as tumor suppressors in neuroblastoma, such as let-7, miR-101, miR-15a-5p, miR-15b-5p, and miR-16-5p, which directly regulate *MYCN* expression [62], pro-apoptotic miR-184 [63], the antiinvasive miR-335 [64], miR-542-5p [65], and several differentiation-related miRNA [66,67].

FIGURE 15.3

Analysis of neuroblastoma tumors possessing deletion of chromosome 11q using the same miRNA expression signature described in Figure 16.2. The 11q-tumors could be split by the unsupervised *k*-means partitional algorithm into two groups, A and B, on the basis of miRNA expression. Groups A and B differed significantly in event-free and overall survival, as well as in the frequency of segmental chromosome imbalances. The authors concluded that two distinct biological subtypes that differ in miRNA expression, clinical outcome, and frequency of segmental chromosomal imbalances.

This figure is the original figure published by Buckley et al. [51] and is reproduced here with permission of the authors and their agreement with the publisher.

Oncogenic potential of the miR-17-5p-92 polycistronic cluster (miR-17-5p, -18a, -19a, -20a, and -92) was provided by Fontana et al. [68]. This functional study in neuroblastoma reported that the cluster is directly upregulated by MYCN showing that some members of this cluster act in an oncogenic, growth promoting manner *in vitro* and *in vivo*. The upregulation of this cluster is correlated with its host gene, *MIRHG1*, which is significantly associated with neuroblastoma patient survival [69]. The experimentally validated targets of miR-17-5p are the p21 tumor suppressor gene (*CDKN1A*) responsible for enhanced cell proliferation and tumorigenesis; and pro-apoptotic gene, *BIM* [68]. The effects of the miR-17-5p-92 cluster appear to be quite extensive, as Chayka et al. [70] demonstrated direct targeting of the cluster (CLU) in gene mRNA by members of this polycistron. Another miRNA associated with tumor progression is miR-221, the overexpression of which increases MYCN expression and is associated with poorer prognosis [71].

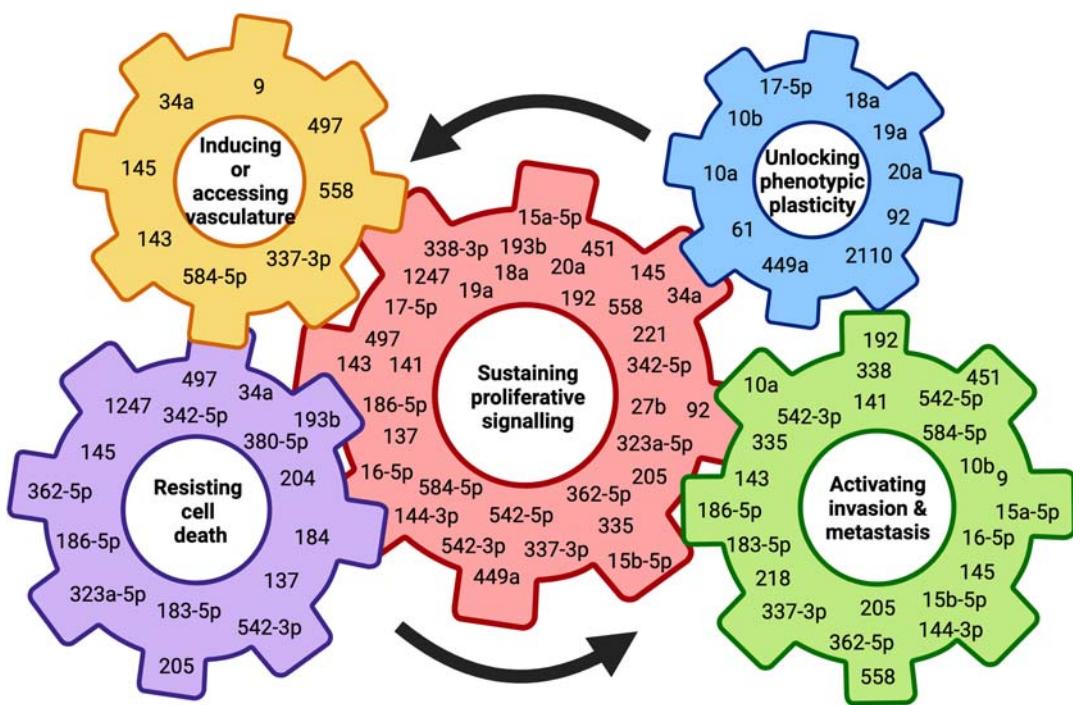
Other miRNAs can contribute to cisplatin sensitivity in neuroblastoma, such as miR-204 [72] and miR-21 [73] which have been shown to increase and decrease sensitivity respectively. 13q14.3 mono-allelic deletion and subsequent miRNA-15a/16-1 downregulation is associated with etoposide resistance in neuroblastoma cell model [74]. Several other miRNA, including -19b, -27b, -218, and 338 are also implicated in therapy responsiveness, as decreased levels are associated with poorer response [75].

Currently there are 12 accepted hallmarks of cancer (8 core and 4 perspective additions) [76] and in general miRNAs have associated functions with all 12 hallmarks. The list of specific miRNAs validated as contributors to neuroblastoma pathogenesis is continually expanding many of which have been shown to be involved in the processes underlying the hallmarks of cancer (illustrated in Figure 15.4, reviewed extensively by [45]). Specific to neuroblastoma and verified in patient/*in vivo* studies, at least 5 out of 12 hallmarks are impacted by specific miRNAs including (1) sustaining proliferative signaling, (2) resisting cell death, (3) inducing or accessing vasculature, (4) activating invasion and metastasis and the emerging hallmark, and (5) unlocking phenotypic plasticity. This is a nonexhaustive list, however, it highlights the importance of continued research into the complexity of miRNAs, both in neuroblastoma and other cancers.

2.3.3 Epigenetic control of miRNA expression

miRNA expression in neuroblastoma is under control of multiple mechanisms, including aberrations in DNA copy number, altered transcriptional activators/repressors, aberrant DNA methylation, or defects of the proteins involved in the miRNA biogenesis machinery and in the posttranscriptional regulation of miRNA expression. The deep understanding of the mechanisms involved in miRNA regulation is required not only to better understand the role miRNAs play in the development of the disease, but also may help us to identify new therapeutic targets.

Hypermethylation of CpG islands is associated with specific miRNAs. A recent study, reviewing the methylation data available from several different neoplasms, discovered that miRNAs displayed a higher magnitude of methylation in comparison to protein coding genes, with about 11.6% of all known miRNAs being methylated [77]. Therefore this epigenetic mechanism has been proposed as one of the mechanisms by which the miRNA is selectively downregulated in tumors. In cases where the miRNA is positioned in the coding region of a gene, methylation may simultaneously suppress expression of both the protein-coding gene and its embedded miRNA. However, very little is known in this area in relation to neuroblastoma disease. Das et al. demonstrated that DNA methylation could be responsible for the dysregulation of miRNA expression in

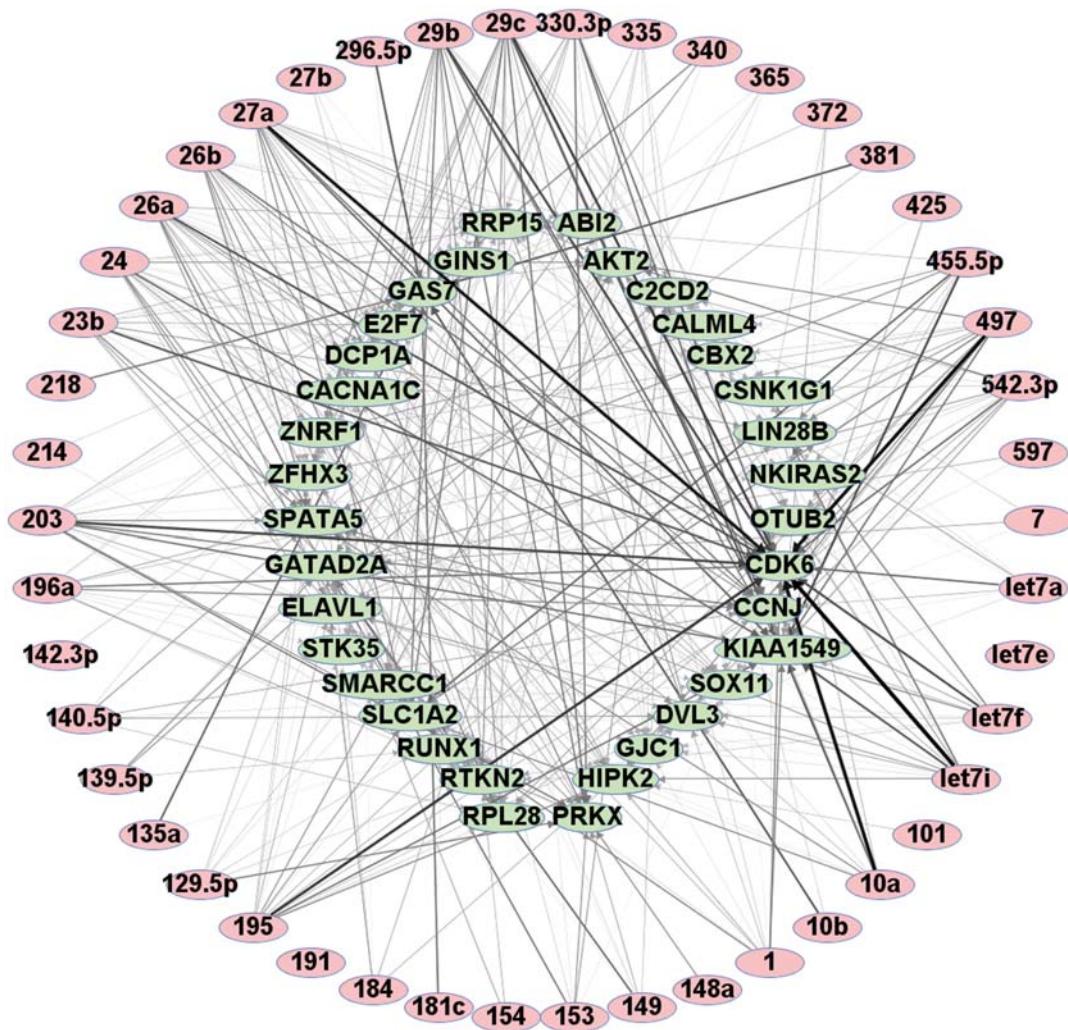
**FIGURE 15.4**

Individual miRNAs involved in neuroblastoma and their relationship to the hallmarks of cancer.

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neuroblastoma [78]. In-depth analysis of DNA methylation patterns together with miRNA and mRNA expression profiles in neuroblastoma samples resulted in the identification of a large set of epigenetically regulated miRNAs with significantly enriched target sites in the 3'-UTRs of genes overexpressed in unfavorable tumor subtypes. Remarkably, a high proportion of both the methylated miRNAs (42%) and their associated mRNA targets (56% of the highly redundantly targeted mRNAs) were highly associated with poor clinical outcome when under- and overexpressed in tumors, respectively. The list of potential epigenetically regulated miRNAs consisted of well-characterized tumor suppressor miRNAs in neuroblastoma, such as some of the let-7 miRNAs, miR-29c, miR-101, miR-335, and miR-184. Importantly, many of the genes targeted by this miRNA panel are known to play oncogenic roles in neuroblastoma, such as *AKT2*, *LIN28B*, and *CDK6*, suggesting that epigenetic silencing of miRNAs could contribute to the overexpression of oncogenes in neuroblastoma (Figure 15.5).

The epigenetically modulated miRNAs target genes involved in cell differentiation. 13-cis-retinoic acid is currently used as part of the treatment regimen for high-risk neuroblastoma patients [79] and induces some neuroblastoma cell lines to differentiate, leading to profound changes in mRNA and miRNA expression [66]. Thus the discovery and validation of specific genetic targets causing neuroblastoma cells to differentiate could be of potential therapeutic benefit. In a study by

**FIGURE 15.5**

A radial bipartite graph showing mRNAs (inner circle) that are most redundantly targeted by the epigenetically silenced miRNAs (outer circle) and which show significant correlations with poor patient survival when upregulated in tumors as identified in the recent study [78]. Evidence for cooperativity and redundant targeting of a large set of mRNAs by the miRNA panel was detected. The links between the miRNAs and the mRNA targets are displayed, with the thickness of each line being proportional to both inverse expression correlation between the miRNA and mRNA and the overrepresentation of predicted miRNA binding sites.

This figure is adapted from the original figure published by Das et al. [78] and is reproduced here with permission of the authors and their agreement with the publisher.

Das et al., DNA methylation changes were compared in SKNBE ATRA-treated versus untreated cells using methylated DNA immunoprecipitation applied to microarrays [80]. The authors identified a total of 402 gene promoters demethylated following all-trans retinoic acid (ATRA) treatment, while only 88 genes became hypermethylated. The demethylation events were explained in part by the downregulation of the methyltransferases DNMT1 and DNMT3 along with the upregulation of endogenous miRNAs targeting them, such as miR-152 and miR-26a/b.

Several reports have validated individual miRNAs that play a major role in neuroblastoma cell differentiation, a highly complex and poorly understood process (reviewed extensively by [45]). Le et al. reported that ectopic overexpression of both miR-124a and miR-125b induces neurite outgrowth in SH-SY5Y cells [67]. Importantly, miR-125b directly bound and repressed the expression of 10 genes with seed matches in their 3' UTRs. These genes are key contributors of neuronal differentiation. The targeting of these key genes by miR-125b launched a complex cascade of transcriptional alterations of downstream players for neuronal differentiation. Discovery of specific miRNA involved in differentiation is being recognized as a potential therapeutic effector of epigenetic therapeutics (Figure 15.6).

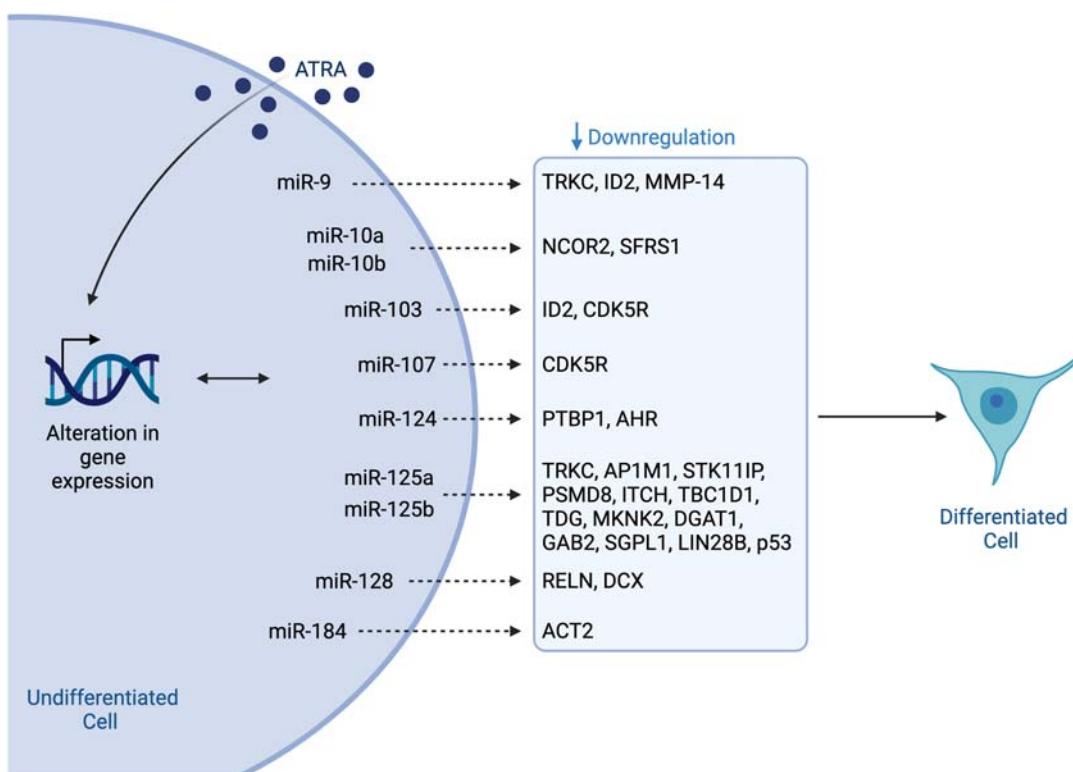


FIGURE 15.6

ATRA-regulated miRNA in neuroblastoma.

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2.4 Noncoding RNAs

2.4.1 Long noncoding RNAs

Long noncoding RNAs (lncRNAs) comprise another class of regulatory ncRNAs. lncRNAs are commonly defined as 200–100,000-nt-long mRNA-like transcripts lacking protein-coding features, such as open-reading frames. Functions of lncRNAs include modulation of chromatin function, regulation of the assembly and function of membrane-less nuclear bodies, alteration of the stability and translation of cytoplasmic mRNAs, and interfering with signaling pathways (extensively reviewed in [81]). lncRNAs have altered expression in human cancers, including neuroblastoma. Recently Baldini et al. have reviewed the most prominent lncRNAs associated with neuroblastoma [82] summarized in Table 15.2. Herein, we will focus only on three clinically relevant lncRNAs: *DLX6-AS1*, *FOXD3-AS1*, and *lncNB1*.

MYCN status is one of the most common means of neuroblastoma patient stratification. Overexpression of *lncNB1* is associated with *MYCN*-amplified neuroblastoma cell lines and knockdown of *lncNB1* lead to neuroblastoma tumor regression *in vivo* [85]. The mechanism by which this lncRNA acts yet to be fully elucidated but it is thought to exert effects through cell cycle alterations and increased apoptosis. The association with *MYCN*-amplification adds a separate layer by which patient stratification could be based on.

Similarly, reduced levels of *FOXD3-AS* have been shown to be associated advanced disease in various cancers, including neuroblastoma. One study has identified a causal relationship between this lncRNA and PARP1 protein, which is involved in various biological process namely DNA repair and gene regulation. Treatment with a *FOXD3-AS1* construct extended mouse survival in a NB xenograft model [84].

Conversely, knockdown of *DLX6-AS1* reduced tumor growth *in vivo* affecting various process *in vitro*, such as differentiation, cellular apoptosis, and invasion. This lncRNA is shown as upregulated in neuroblastoma tissues and cell lines and has been proposed as a useful marker for neuroblastoma stage and therefore prediction of outcome [83].

Table 15.2 lncRNAs and Their Relationship to Neuroblastoma Research

lncRNA	Expression in Neuroblastoma	Prognostic Marker	Involved in Differentiation	References
DLX6-AS1	Down	Y	Y	[83]
FOXD3-AS1	Down	Y	N	[84]
LncNB1	Up	Y	N	[85]
NDM29	Down	N	Y	[86]
SNHG1	Up	Y	N	[87,88]
SNHG16	Up	Y	N	[89,90]
NBAT1	Down	Y	N	[91]
CASC15	Down	Y	Y	[92]
LINC00839	Up	Y	N	[93]
FIRRE	Up	Y	N	[93]
LOC283177	Up	N	Y	[93]
LOC101928100	Up	N	Y	[93]

Research of lncRNA in relation to neuroblastoma is still in its infancy with no clinical trials currently active or recruiting. Discovery of lncRNAs associated with neuroblastoma, many not mentioned here, proceeds at a fast pace. However, more robust preclinical evaluation of lncRNAs *in vivo* and in patient samples is required to accelerate design, development, and ultimately, translation of these lncRNAs into actionable targets.

2.4.2 Circular RNAs

Recently a new type of ncRNAs, called circRNAs, was identified to participate in tumorigenesis. These stable closed loop structures were implicated in tumor cell proliferation, epithelial–mesenchymal transition, invasion, metastasis and chemoresistance in different types of cancer [94].

A recent circRNA of interest, *circDGKB*, promoted the proliferation, migration, invasion, and tumorigenesis of neuroblastoma cells and reduced cell apoptosis *in vitro* and *in vivo*; the ncRNA acted through inhibiting miR-873 and enhancing GLI1 expression [95]. In addition, *circCUX1* contributed to aerobic glycolysis, and the knockdown of this circRNA resulted in suppressed growth and aggressiveness of neuroblastoma cells; the overexpression of *circCUX1* was associated with poor clinical outcome [96].

3 Epigenetic targeting agents

Mechanisms of epigenetic regulation most commonly include DNA methylation, modification of histones, and changes in miRNA expression. Epigenetic changes are reversible; hence, the epigenetic status of the cancer genome can be restored to a nonmalignant state through epigenetic reprogramming through pharmacological manipulations resulting in tumor growth termination and elimination. Currently there are 11 active clinical trials in which epigenetic regulation in neuroblastoma is a focus, with 23 other such clinical trials completed from phase 1 to phase 3.

3.1 DNA methylation inhibitors

GSK-J4 is a small molecule inhibitor of lysine 27 of histone 3 demethylase and histone demethylase Jumonji D3. This compound induces neuroblastoma differentiation as well as upregulation of p53, modulator of apoptosis, which results in neuroblastoma cell death [97]. In animal models, this compound effectively inhibited growth of chemo-refractory patient-derived xenograft models of high-risk neuroblastoma; furthermore, it exhibited synergistic antitumor effect when combined with retinoic acid (RA) [97].

Decitabine (5-aza-2-deoxycytidine) is a cytosine analog that inhibits DNA methyltransferases, reverses methylation, and can reactivate silenced genes. It has shown therapeutic activity in patients with different cancers [98].

A phase I clinical trial was conducted through The Children's Oncology Group (COG) in which decitabine (5-aza-2 = -deoxycytidine), was given, together with doxorubicin and cyclophosphamide, to children with relapsed/refractory solid tumors [99]. Low-dose decitabine in combination with doxorubicin/cyclophosphamide displayed tolerable toxicity in children. Unfortunately, doses of decitabine capable of producing therapeutic effects were not well tolerated with this combination.

Alternative combinatory strategies of demethylating agents with noncytotoxic, biologically targeted agents, such as HDAC inhibitors, should attract more attention. At present, decitabine is being tested in clinical trials for neuroblastoma followed by cancer-antigen vaccination [100].

3.2 Inhibitors of histone modification enzymes

Histones provide a compact structure of DNA. These proteins together with the DNA form the major components of chromatin. Changes in histones can affect chromatin structure. They can be modified by methylation and acetylation, mediated by histone acetyl transferases (HATs) and deacetylases (HDACs), and histone methyltransferases (HMTs). Each of these processes alters histone function, which, in turn alters the structure of chromatin and therefore the accessibility of DNA to transcription factors.

Various inhibitors of HDACs (HDACi) have been developed and tested (reviewed by [22]) showing success in preclinical models with a handful of therapeutics translating to the clinic. Currently there are eight ongoing neuroblastoma clinical trials involving HDACi, summarized in Table 15.1.

Vorinostat (suberoylanilide hydroxamic acid or SAHA) inhibits deacetylation of HDACs through binding to the active site and leads to an accumulation of hyperacetylated histones and transcription factors. This results in the activation of expression of cyclin-dependent kinase p21, followed by G1 cell-cycle arrest and/or apoptosis. In addition, hyperacetylation of tumor suppressor p53, alpha tubulin, and heat shock protein 90 produces antiproliferative effects. Vorinostat was FDA-approved for treatment of cutaneous T-cell lymphoma in 2006 and several clinical trials have been completed or are ongoing in neuroblastoma both alone or in combination with other drugs, such as isotretinoin, MIBG therapy, etoposide, and the immunotherapeutic dinutuximab (Table 15.1).

A preclinical study suggests that vorinostat can also function as a radiosensitizer in neuroblastoma demonstrating that a combination of radiation and vorinostat significantly increased γ -H2AX expression, resulting in decreased tumor size compared to single modality alone in a murine metastatic neuroblastoma model [101]. Further assessment in a phase I study has identified tolerable doses of vorinostat combined with MIBG treatment with a phase II trial ongoing comparing single-agent treatments [102]. Thus vorinostat potentiates antineoplastic effects of radiation in neuroblastoma possibly due to inhibitory effects on DNA repair enzymes.

Evidently, HDACi have a great potential to work synergistically with other drugs. Several HDACi, such as trichostatin A, sodium butyrate, suberoylanilide hydroxamic acid, and TH34, were shown to decrease neuroblastoma cell viability and proliferation *in vitro*; their action was shown to be potentiated by ATRA addition [103,104]. This is not surprising given the involvement of HDACs in ATRA mechanism of action, typically inducing cell differentiation. While ATRA treatment alone has proved ineffective in neuroblastoma, combination therapies with HDACi are under investigation with each agent potentiating the effects of the other. As such, combination administration of low-dose ATRA with HDACi (100 mg/kg) resulted in growth inhibition comparable with that achieved with 200 mg/kg CBHA on human neuroblastoma xenografts of the SMS-KCN-69n cell line in mice. Synergy between ATRA and HDACi was confirmed by combining noninhibitory doses of CBHA (50 mg/kg) and ATRA (2.5 mg/kg) and achieving a statistically significant 52% reduction in final tumor size compared with control [105]. Similarly, coadministration of HDACi

PCI-48012 and 13-*cis* RA resulted in more prominent tumor volume reduction in BE(2)-C xenografted NMRI nude mice compared to the single drug treatments [106].

A recent RNAi screen has identified coexpression of *HDAC8* and *ALK* to be associated with poor prognosis in two large neuroblastoma datasets. Subsequently, combination inhibition of both *ALK* and *HDAC8* decreased cell viability and efficiently killed neuroblastoma cells *in vitro* while reducing tumor growth *in vivo*.

Aside from HDAC inhibitors various therapies targeting other histone-modifying enzymes are becoming increasingly more important. Two therapeutics to target HATs, PU139 and PU140, have proved successful in neuroblastoma preclinical mouse models by reducing tumor growth. PU139 also showed synergism when combined with the chemotherapeutic agent doxorubicin [107]. BIX-01294 inhibits EHMT2, a histone lysine methyltransferase, and in doing so has been shown to reduce neuroblastoma tumor growth *in vivo* [108] as well as reducing neuroblastoma cell mobility, proliferation and invasiveness *in vitro* [29].

3.3 Differentiation therapeutics

Epigenetic changes trigger the differentiation process during embryo development. Some cancer cells undergo differentiation under such stimuli like ATRA. Differentiation agents re-program cancer cells toward a nonmalignant phenotype, and consequently may suppress tumor growth and development. The process of differentiation is particularly important in neuroblastoma tumors, as patients with more differentiated tumors have a better clinical outcome [109].

RA is an active metabolite of vitamin A under the family retinoid. Previous functional studies identified that ATRA and 13-*cis*-RA, isoforms of RA, inhibit the proliferation and induces markers of apoptosis and differentiation in neuroblastoma (reviewed by [110]). RA isoforms have been shown to induce differentiation and have anticancer activities in several other types of cancer cells *in vitro* and in *ex vivo* [111].

13-*cis*-RA is a recognized component of the treatment of high-risk neuroblastoma, regardless of discouraging results of early phase II trials conducted with low-dose 13-*cis* RA. This trial showed limited clinical benefit in patients with recurrent disease [112,113]. However, more recent randomized trials demonstrated improved survival in patients treated with high dose, pulsed 13-*cis* RA after myeloablative chemotherapy [114,115]. Other strategies are currently under development to achieve pharmacological efficacy of retinoid therapy. While ATRA and IFN α 2a cotreatment failed to benefit neuroblastoma patients in phase II clinical trial, retinoid therapy may be more effective in combination with other pharmacologic options, such as HDAC inhibitors as discussed above.

Synthetic atypical retinoids are an attractive alternative, as their mechanism of action is different from the classic retinoids and, might be effective in treating RA-resistant neuroblastomas [116,117]. As such, phase II clinical trial is currently investigating the efficacy of powdered lipid formulation of fenretinide, a pro-apoptotic retinoid derivative that had a promising pharmacological profile [116].

Neuroblastoma patients, however, are not universally responsive to RA treatment due to various reasons. To achieve pharmacologically efficacious 13-*cis* RA levels, it is important to administrate adequate dose and optimal schedules due to different individual metabolisms or/and acquired resistance mechanisms [118].

Tumor genotype can critically contribute to sensitivity to ATRA. Mutations in the neurofibromatosis type 1 (NF1) tumor suppressor gene were determined in neuroblastoma cell lines [119] and in primary tumors [120,121] and affect response on ATRA treatment in neuroblastoma patients [122]. Loss of NF1 activates signaling in the RAS–MEK pathway, resulting in the downregulation of ZNF423, which is a critical transcriptional coactivator of the RA receptors [122,123]. Inhibition of MEK signaling downstream of NF1 restores sensitivity to RA. This may represent an alternative approach for the treatment protocol to overcome RA resistance in NF1-deficient neuroblastomas. Another mechanism of resistance to 13-cis RA is c-MYC overexpression caused by the overactivation of MK2 and OCT4 transcription factors. The increase in those markers was associated with progressive disease in neuroblastoma. Conversely, shRNA-mediated knockdown of OCT4 restored RA-resistant neuroblastoma sensitivity to 13-cis RA [124].

Recent studies discovered several novel pathways that induce neuroblastoma differentiation. Among them is a Hedgehog signaling cascade, a developmental pathway involved in neural crest cell maturation. As such, Hedgehog signaling and its transcription factor GLI1 were shown to exert tumor-suppressive actions in neuroblastoma, while isoxazole, a small molecule activator of GLI1, potently inhibited neuroblastoma cell proliferation [125].

There is a remarkable potential in a combinational therapy, as it can reduce toxicity of single-agent therapy. Activation of epigenetically silenced miRNA through demethylation or differentiation agents may provide increased therapeutic efficiency in the management of neuroblastoma. Importantly, these agents efficiently modulate miRNA re-expression at low doses [78], and as such make them less toxic and easily tolerated while achieving therapeutic efficacy. This advantage makes this area extremely important in neuroblastoma treatment development, and management.

4 miRNA-based therapeutics

The widespread involvement of miRNAs in various human pathogenic diseases opens a new avenue for the study and development of new therapeutic strategies. Most small molecule inhibitor therapeutics target a single oncogene resulting in a modest induction of therapeutic response. In contrast, a single miRNA can repress many oncogenes simultaneously at the mRNA level across various cell pathways, hence providing a strong rationale for developing miRNA-based cancer therapeutics. Notably, miRNA exhibits its function in cytoplasm without damaging DNA, and thus leading to fewer or completely reversible side effects when compared to current treatments.

The principles for developing miRNA-based therapies follow the same strategy as for other targeted therapies that take the path from drug target to drug. Target identification and validation are key steps in the selection of potential miRNAs. Since the discovery of miRNAs, several candidates have already progressed into product and clinical development. Currently there are several companies developing miRNA therapeutics for different human pathological conditions, including cancer (extensively reviewed in [126]). There are two tactics to developing miRNA-based therapeutics: miRNA antagonists and miRNA mimics; in addition, an miRNA could be used as diagnostic tool. miRNA antagonists aim to inhibit endogenous miRNAs showing a gain of function in diseased tissues; while miRNA mimics aim to restore a loss of function observed in healthy cells. The first approach is also known as “miRNA knockdown therapy,” the second—“miRNA replacement

therapy.” miRNAs under investigation are those that not only yield satisfactory efficacy in various disease models, but also sufficient experimental data that allow a precise placement of the miRNA into disease-related pathways. Because the miRNA mimic therapeutics is in the pipeline for anti-cancer therapy rather than anti-miRNA based, we will mainly focus on miRNA mimics and briefly outline prospective anti-miRNA candidates in neuroblastoma.

4.1 miRNA replacement therapy

The reintroduction of miRNA mimics is expected to restore pathways required for normal cellular function and blocks those that drive the disease. miRNA mimics are ideally to be well tolerated in normal tissues. miRNA mimics are designed and synthesized to have the same sequence as the naturally occurring equivalent, so are expected to target the same set of genes. Since most normal cells already express the miRNA with tumor suppressor function, administration of miRNA mimics to normal tissue is unlikely to induce adverse events as the cellular pathways affected by the mimic are already activated or inactivated by the endogenous miRNA. A subset of the miRNAs which have shown therapeutic promise in neuroblastoma is outlined below and illustrated in [Figure 15.4](#).

To date, most targeted cancer therapeutics tackle a gain-of-function, so miRNA replacement therapy provides an exciting new opportunity to exploit tumor suppressors. Proof of concept for miRNA replacement therapy for neuroblastoma has been demonstrated by mimics of tumor suppressor miRNAs that stimulate antioncogenic pathways, apoptosis and eventually lead to an abolition of tumor cells ([Table 15.3](#)).

Table 15.3 miRNA-Mediated Experimental Therapeutics for Neuroblastoma

miRNA	Murine Models	Neuroblastoma Cell Lines	Mode of Delivery	Targeted	References
34a	Orthotopic	NB1691Luc, SK-N-ASLuc	Silica nanoparticles	Anti-GD2	[127]
34a	Pseudo metastatic (tail vein) and orthotopic	LAN-5, SH-SY5Y, HTLA-230	Liposomes	Anti-GD2	[128]
145	Subcutaneous	SH-SY5Y	Lentiviral stable transfections	N	[129]
184a	Orthotopic	NB1691Luc, SK-N-ASLuc	miRNA pretransfected cells	N	[130]
186	Orthotopic	CHLA-136-Fluc	Anionic lipopolyplex nanoparticle	Anti-GD2	[131]
323a-5p	Subcutaneous	SK-N-AS, SK-N-BE(2)	miRNA pretransfected cells	N	[132]
342-5p	Subcutaneous	SK-N-AS, SK-N-BE(2)	miRNA pretransfected cells	N	[132]
497-5p	Subcutaneous	SK-N-BE(2)Fluc	An inducible miR-497 expression system	N	[133]
Let-7a	Pseudo metastatic and orthotopic	LAN-5, SH-SY5Y, HTLA-230	Liposomes	Anti-GD2	[128]

A strategic target downregulated by miR-34a in neuroblastoma is MYCN, an oncogene that is amplified in 25% of neuroblastomas with proven difficulty in therapeutic targeting [134,135]. As transcription factor, MYCN regulates the expression of a countless number of genes participating in cell cycle, proliferation, and metastasis, among others. miR-34a functions within the p53 pathway and inhibits cancer cell growth by repressing MYC/MYCN, MET, BCL2, and other oncogenes. The tumor suppressor function of miR-34a, which is lost or expressed at reduced levels in most solid and hematologic malignancies, has been validated by numerous *in vitro* and *in vivo* studies [136]. Therapeutic delivery of a miR-34 mimic blocked tumor growth in murine models of lung [137], prostate cancer, and neuroblastoma [127,128] miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44 [138]. The therapeutic efficacy is antiproliferative and pro-apoptotic in lung tumor cells with a specific repression of CDK4, MET, and BCL2 [137]. In addition, therapeutic delivery of miR-34 did not induce an elevation of cytokines or liver and kidney enzymes in serum, suggesting that treatment is well tolerated, and the antioncogenic effects are mediated by a specific mechanism of miR-34. The tumor suppressor potential of miR-34a was successfully explored in a mouse orthotopic model of neuroblastoma by two independent groups [127,128].

Both groups encapsulated miR-34a mimics in synthetic nanoparticles conjugated to a disialo-ganglioside GD2 (GD2) antibody. Neuroblastomas express high levels of the cell surface antigen GD2, providing a target for tumor-specific delivery. Thus targeted delivery is to provide a higher concentration of miR-34a to the tumor site, potentially increasing the efficacy of this mode of treatment. Encapsulated miRNA was administered to a well characterized murine orthotopic xenograft disease model. The targeted delivery of tumor suppressor miR-34a significantly inhibited tumor growth (Figure 15.7). Immunohistochemical staining of tumors treated with miR-34a revealed that multiple mechanisms, including increased apoptosis, and decreased angiogenesis, were responsible for the antitumorigenic effects of this miRNA [127]. Both studies demonstrated the viability of a targeted delivery miRNA-based therapy for neuroblastoma.

Commercial MRX34, a liposomal miR-34a mimic, is one of the first clinical miRNA replacement therapeutics [139]. The therapeutic activity of MRX34 has been tested in a survival study using an orthotopic mouse model of hepatocellular carcinoma. This study demonstrated that all animals treated with MRX34 stayed alive in comparison with negative controls. Importantly, the histopathologic and molecular analyses of these animals did not find any evidence of remaining tumor cells. In autumn 2013, MRX34 therapeutic was transferred to the Phase I clinical trials for patients with primary liver cancer or those with liver metastasis from other cancers (NCT01829971). Unfortunately, this trial was terminated and phase II in melanoma patients was withdrawn (NCT02862145) due to the five serious immunological adverse events resulting in four patients' death [140]. Nevertheless, this first clinical trial of miRNA-based therapeutics provided invaluable lessons for future development pipeline of this class of drugs. Both nonspecific target delivery and liposomal formulation of delivery vesicles could be the potential reasons behind the prematurely terminated trial.

4.2 miRNA knockdown therapy

miRNA antagonists are synthetic molecules that inhibit endogenous miRNAs with a gain of function in diseased tissues. This concept is like other inhibitory therapeutics that target a single-gene

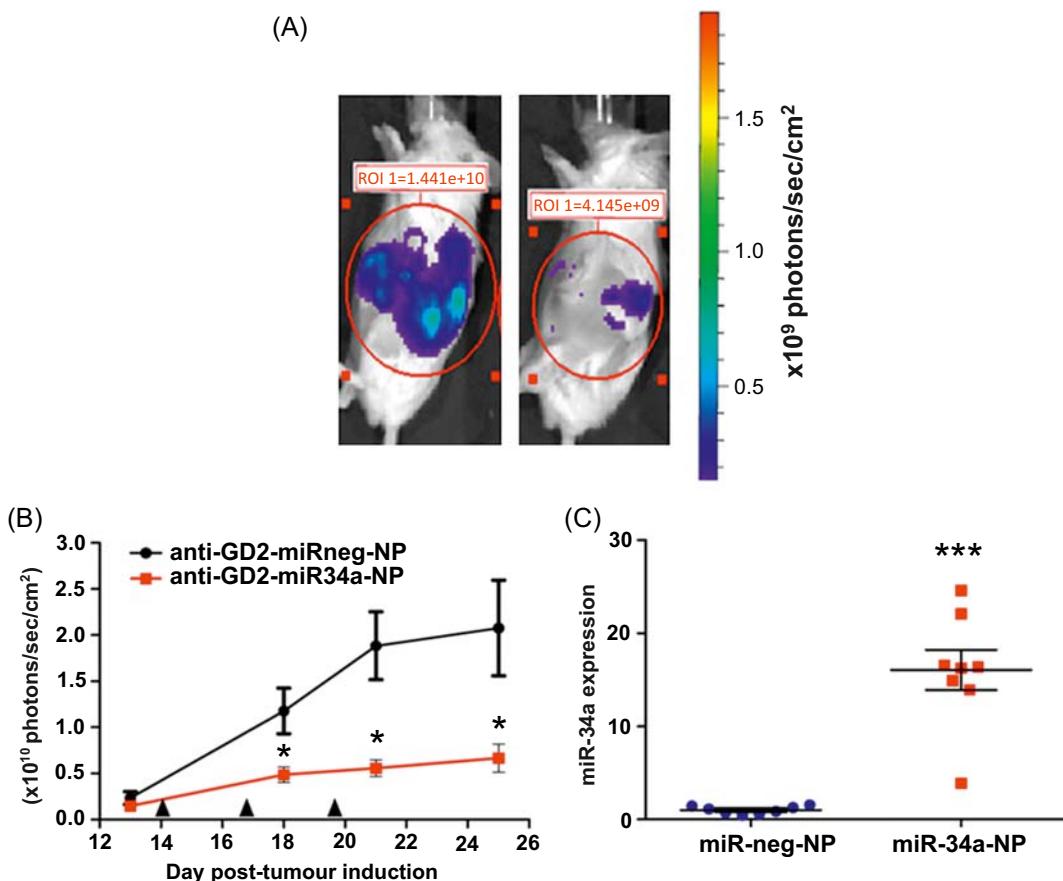


FIGURE 15.7

Antineuroblastoma effect of anti-GD2 conjugated nanoparticles bearing miR-34a *in vivo*. (A) Bioluminescent images representative of mice bearing NB1691luc tumors treated with anti-GD2-miRneg-NP (left) or anti-GD2-miR34a-NP (right). (B) Tumor growth curves from mice bearing NB1691luc tumors treated with anti-GD2-miRneg-NP (black line) or anti-GD2-miR34a-NP (red line). Time points for systemic administration of nanoparticles are indicated by the symbol ▲. Differences in tumor growth between mice injected with anti-GD2-miR34a-NP versus anti-GD2-miRneg-NP were statistically significant for both models. (C) Mature miR-34a transcript levels were significantly higher in anti-GD2-miR34a-NP-treated tumors relative to anti-GD2-miRneg-NP-treated control tumors in both.

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product, such as small-molecule inhibitors and short-interfering RNAs (siRNAs). The inhibitory synthetic molecule is a chemically modified miRNA passenger strand (anti-miR or antagomir) that binds with high affinity to the active miRNA strand. This binding is irreversible, so the new miRNA duplex is unable to be processed by RISC and/or degraded. As a main concern, the

antagonist can potentially bind nonspecifically to other RNAs, which could result in unwanted side effects. To date, there is no miRNA antagonist-based therapeutic in clinical trials for cancer conditions, while reviewed for other human pathological conditions elsewhere (reviewed in [141]). Some miRNAs of the miR-17-5p-92 polycistronic cluster are the perspective candidates for miRNA knockdown/antagonist in various types of cancer [142], including neuroblastoma [68]. Most impressively, antagonization of miR-17-5p significantly inhibits tumor growth in a mouse xenograft model of the disease, illustrating the potential for miRNA antagonist mediated therapy of neuroblastoma [68,70].

miRNA functional studies suggest the enormous potential of miRNA-based therapy. Through in-depth validation of a single miRNA targets, it is feasible to select candidates that simultaneously restore nonmalignant cell function through direct targeting of oncogenes. Advancing delivery of these miRNA-based therapeutics in animal models will facilitate clinical trials for miRNA therapeutics for treatment of neuroblastoma in the near future.

References

- [1] Chung C, et al. Neuroblastoma. *Pediatric Blood Cancer* 2021;68(S2):e28473.
- [2] Otte J, et al. MYCN function in neuroblastoma development. *Front Oncol* 2021;10.
- [3] Takita J. Molecular basis and clinical features of neuroblastoma. *JMA J* 2021;4(4):321–31.
- [4] Decock A, et al. Neuroblastoma epigenetics: from candidate gene approaches to genome-wide screenings. *Epigenetics* 2011;6(8):962–70.
- [5] Yang Q, et al. Association of epigenetic inactivation of RASSF1A with poor outcome in human neuroblastoma. *Clin Cancer Res* 2004;10(24):8493–500.
- [6] van Zogchel LMJ, et al. Hypermethylated RASSF1A as circulating tumor DNA marker for disease monitoring in neuroblastoma. *JCO Precis Oncol* 2020;4.
- [7] Teitz T, et al. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nat Med* 2000;6(5):529–35.
- [8] Yang Q, et al. Methylation of CASP8, DCR2, and HIN-1 in neuroblastoma is associated with poor outcome. *Clin Cancer Res* 2007;13(11):3191–7.
- [9] van Noesel MM, et al. Clustering of hypermethylated genes in neuroblastoma. *Genes Chromosomes Cancer* 2003;38(3):226–33.
- [10] van Noesel MM, et al. Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation. *Cancer Res* 2002;62(7):2157–61.
- [11] Almutairi B, et al. Epigenetic deregulation of GATA3 in neuroblastoma is associated with increased GATA3 protein expression and with poor outcomes. *Sci Rep* 2019;9(1):18934.
- [12] Decock A, et al. Stage 4S neuroblastoma tumors show a characteristic DNA methylation portrait. *Epigenetics* 2016;11(10):761–71.
- [13] Asada K, Abe M, Ushijima T. Clinical application of the CpG island methylator phenotype to prognostic diagnosis in neuroblastomas. *J Hum Genet* 2013;58(7):428–33.
- [14] Abe M, et al. CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res* 2005;65(3):828–34.
- [15] Lázcoz P, et al. Frequent promoter hypermethylation of RASSF1A and CASP8 in neuroblastoma. *BMC Cancer* 2006;6:254.

- [16] Buckley PG, et al. Genome-wide DNA methylation analysis of neuroblastic tumors reveals clinically relevant epigenetic events and large-scale epigenomic alterations localized to telomeric regions. *Int J Cancer* 2011;128(10):2296–305.
- [17] Furman WL, et al. Improved outcome in children with newly diagnosed high-risk neuroblastoma treated with chemoimmunotherapy: updated results of a phase II study using hu14.18K322A. *J Clin Oncol* 2022;40(4):335–44.
- [18] Yu AL, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med* 2010;363(14):1324–34.
- [19] Marachelian A, et al. A phase I NANT study of lenalidomide with ch14.18 and isotretinoin (RA) in patients with refractory/recurrent neuroblastoma (RR-NB). *J Clin Oncol* 2018;36(15_suppl):10522.
- [20] Weiss BD, et al. A safety and feasibility trial of (131) I-MIBG in newly diagnosed high-risk neuroblastoma: a Children’s Oncology Group study. *Pediatr Blood Cancer* 2021;68(10):e29117.
- [21] Oehme I, et al. Histone deacetylase 10 promotes autophagy-mediated cell survival. *Proc Natl Acad Sci U S A* 2013;110(28):E2592–601.
- [22] Phimmacanh M, et al. Histone deacetylases and histone deacetylase inhibitors in neuroblastoma. *Front Cell Dev Biol* 2020;8:578770.
- [23] Cohen AL, et al. Genomic pathway analysis reveals that EZH2 and HDAC4 represent mutually exclusive epigenetic pathways across human cancers. *BMC Med Genomics* 2013;6(1):35.
- [24] Chen L, et al. CRISPR-Cas9 screen reveals a MYCN-amplified neuroblastoma dependency on EZH2. *J Clin Invest* 2018;128(1):446–62.
- [25] Tsubota S, et al. PRC2-mediated transcriptomic alterations at the embryonic stage govern tumorigenesis and clinical outcome in MYCN-driven neuroblastoma. *Cancer Res* 2017;77(19):5259–71.
- [26] Corvetta D, et al. Physical interaction between MYCN oncogene and polycomb repressive complex 2 (PRC2) in neuroblastoma: functional and therapeutic implications. *J Biol Chem* 2013;288(12):8332–41.
- [27] Li Z, et al. EZH2 regulates neuroblastoma cell differentiation via NTRK1 promoter epigenetic modifications. *Oncogene* 2018;37(20):2714–27.
- [28] Henrich KO, et al. Integrative genome-scale analysis identifies epigenetic mechanisms of transcriptional deregulation in unfavorable neuroblastomas. *Cancer Res* 2016;76(18):5523–37.
- [29] Lu Z, et al. Histone-lysine methyltransferase EHMT2 is involved in proliferation, apoptosis, cell invasion, and DNA methylation of human neuroblastoma cells. *Anticancer Drugs* 2013;24(5):484–93.
- [30] Bellamy J, et al. Increased efficacy of histone methyltransferase G9a inhibitors against MYCN-amplified neuroblastoma. *Front Oncol* 2020;10:818.
- [31] Wong M, et al. The histone methyltransferase DOT1L promotes neuroblastoma by regulating gene transcription. *Cancer Res* 2017;77(9):2522–33.
- [32] Sugino Y, et al. Epigenetic silencing of prostaglandin E receptor 2 (PTGER2) is associated with progression of neuroblastomas. *Oncogene* 2007;26(53):7401–13.
- [33] Berdasco M, et al. Epigenetic inactivation of the Sotos overgrowth syndrome gene histone methyltransferase NSD1 in human neuroblastoma and glioma. *Proc Natl Acad Sci U S A* 2009;106(51):21830–5.
- [34] Charlet J, et al. Genome-wide DNA methylation analysis identifies MEGF10 as a novel epigenetically repressed candidate tumor suppressor gene in neuroblastoma. *Mol Carcinog* 2017;56(4):1290–301.
- [35] Jubierre L, et al. Targeting of epigenetic regulators in neuroblastoma. *Exp & Mol Med* 2018;50(4):1–12.
- [36] Ambrosio S, et al. LSD1 mediates MYCN control of epithelial-mesenchymal transition through silencing of metastatic suppressor NDRG1 gene. *Oncotarget* 2017;8(3):3854–69.
- [37] Ambrosio S, et al. Lysine-specific demethylase LSD1 regulates autophagy in neuroblastoma through SESN2-dependent pathway. *Oncogene* 2017;36(48):6701–11.
- [38] Amente S, et al. Lysine-specific demethylase (LSD1/KDM1A) and MYCN cooperatively repress tumor suppressor genes in neuroblastoma. *Oncotarget* 2015;6(16):14572–83.

- [39] Tee AE, et al. The histone demethylase JMJD1A induces cell migration and invasion by up-regulating the expression of the long noncoding RNA MALAT1. *Oncotarget* 2014;5(7):1793–804.
- [40] Yang J, et al. The role of histone demethylase KDM4B in Myc signaling in neuroblastoma. *J Natl Cancer Inst* 2015;107(6):djv080.
- [41] Kuo YT, et al. JARID1B expression plays a critical role in chemoresistance and stem cell-like phenotype of neuroblastoma cells. *PLoS One* 2015;10(5):e0125343.
- [42] Yang L, et al. Histone demethylase KDM6B has an anti-tumorigenic function in neuroblastoma by promoting differentiation. *Oncogenesis* 2019;8(1):3.
- [43] Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med* 2012;4(3):143–59.
- [44] Wiemer EA. The role of microRNAs in cancer: no small matter. *Eur J Cancer* 2007;43(10):1529–44.
- [45] Aravindan N, et al. MicroRNAs in neuroblastoma tumorigenesis, therapy resistance, and disease evolution. *Cancer Drug Resist* 2019;2:1086–105.
- [46] Misiak D, et al. The microRNA landscape of MYCN-amplified neuroblastoma. *Front Oncol* 2021;11.
- [47] Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* 2007;26(34):5017–22.
- [48] Murphy DM, et al. Global MYCN transcription factor binding analysis in neuroblastoma reveals association with distinct E-box motifs and regions of DNA hypermethylation. *PLoS One* 2009;4(12):e8154.
- [49] Beckers A, et al. MYCN-targeting miRNAs are predominantly downregulated during MYCN-driven neuroblastoma tumor formation. *Oncotarget* 2015;6(7):5204–16.
- [50] Bray I, et al. Widespread dysregulation of MiRNAs by MYCN amplification and chromosomal imbalances in neuroblastoma: association of miRNA expression with survival. *PLoS One* 2009;4(11):e7850.
- [51] Buckley PG, et al. Chromosomal and microRNA expression patterns reveal biologically distinct subgroups of 11q- neuroblastoma. *Clin Cancer Res* 2010;16(11):2971–8.
- [52] Fischer M, et al. Differential expression of neuronal genes defines subtypes of disseminated neuroblastoma with favorable and unfavorable outcome. *Clin Cancer Res* 2006;12(17):5118–28.
- [53] Blondal T, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. *Methods* 2013;59(1):S1–6.
- [54] Zeka F, et al. Circulating microRNA biomarkers for metastatic disease in neuroblastoma patients. *JCI Insight* 2018;3(23).
- [55] Morini M, et al. Exosomal microRNAs from longitudinal liquid biopsies for the prediction of response to induction chemotherapy in high-risk neuroblastoma patients: a proof of concept SIOPEN study. *Cancers (Basel)* 2019;11(10).
- [56] Lu J, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435(7043):834–8.
- [57] Cheng X, et al. miR-34a inhibits progression of neuroblastoma by targeting autophagy-related gene 5. *Eur J Pharmacol* 2019;850:53–63.
- [58] De Antonellis P, et al. Early targets of miR-34a in neuroblastoma. *Mol Cell Proteom* 2014;13(8):2114–31.
- [59] Cole KA, et al. A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene. *Mol Cancer Res* 2008;6(5):735–42.
- [60] Wei JS, et al. The MYCN oncogene is a direct target of miR-34a. *Oncogene* 2008;27(39):5204–13.
- [61] Buechner J, et al. Tumour-suppressor microRNAs let-7 and mir-101 target the proto-oncogene MYCN and inhibit cell proliferation in MYCN-amplified neuroblastoma. *Br J Cancer* 2011;105(2):296–303.
- [62] Chava S, et al. miR-15a-5p, miR-15b-5p, and miR-16-5p inhibit tumor progression by directly targeting MYCN in neuroblastoma. *Mol Oncol* 2020;14(1):180–96.
- [63] Foley NH, et al. MicroRNA-184 inhibits neuroblastoma cell survival through targeting the serine/threonine kinase AKT2. *Mol Cancer* 2010;9:83.

- [64] Lynch J, et al. MiRNA-335 suppresses neuroblastoma cell invasiveness by direct targeting of multiple genes from the non-canonical TGF- β signalling pathway. *Carcinogenesis* 2012;33(5):976–85.
- [65] Bray I, et al. MicroRNA-542-5p as a novel tumor suppressor in neuroblastoma. *Cancer Lett* 2011;303(1):56–64.
- [66] Foley NH, et al. MicroRNAs 10a and 10b are potent inducers of neuroblastoma cell differentiation through targeting of nuclear receptor corepressor 2. *Cell Death Differ* 2011;18(7):1089–98.
- [67] Le MT, et al. MicroRNA-125b promotes neuronal differentiation in human cells by repressing multiple targets. *Mol Cell Biol* 2009;29(19):5290–305.
- [68] Fontana L, et al. Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS One* 2008;3(5):e2236.
- [69] Wei JS, et al. microRNA profiling identifies cancer-specific and prognostic signatures in pediatric malignancies. *Clin Cancer Res* 2009;15(17):5560–8.
- [70] Chayka O, et al. Clusterin, a haploinsufficient tumor suppressor gene in neuroblastomas. *J Natl Cancer Inst* 2009;101(9):663–77.
- [71] He XY, et al. microRNA-221 enhances MYCN via targeting nemo-like kinase and functions as an oncogene related to poor prognosis in neuroblastoma. *Clin Cancer Res* 2017;23(11):2905–18.
- [72] Ryan J, et al. MicroRNA-204 increases sensitivity of neuroblastoma cells to cisplatin and is associated with a favourable clinical outcome. *Br J Cancer* 2012;107(6):967–76.
- [73] Schulte JH, et al. Deep sequencing reveals differential expression of microRNAs in favorable versus unfavorable neuroblastoma. *Nucleic Acids Res* 2010;38(17):5919–28.
- [74] Marengo B, et al. Etoposide-resistance in a neuroblastoma model cell line is associated with 13q14.3 mono-allelic deletion and miRNA-15a/16-1 down-regulation. *Sci Rep* 2018;8(1):13762.
- [75] Marengo B, et al. Potential role of miRNAs in the acquisition of chemoresistance in neuroblastoma. *J Personalized Med* 2021;11(2):107.
- [76] Hanahan D. Hallmarks of cancer: new dimensions. *Cancer Discov* 2022;12(1):31–46.
- [77] Liu X, et al. Regulation of microRNAs by epigenetics and their interplay involved in cancer. *J Exp Clin Cancer Res* 2013;32(1):96.
- [78] Das S, et al. Modulation of neuroblastoma disease pathogenesis by an extensive network of epigenetically regulated microRNAs. *Oncogene* 2013;32(24):2927–36.
- [79] Peinemann F, et al. Retinoic acid postconsolidation therapy for high-risk neuroblastoma patients treated with autologous haematopoietic stem cell transplantation. *Cochrane Database Syst Rev* 2017;8(8):Cd010685.
- [80] Das S, et al. MicroRNA mediates DNA demethylation events triggered by retinoic acid during neuroblastoma cell differentiation. *Cancer Res* 2010;70(20):7874–81.
- [81] Statello L, et al. Gene regulation by long non-coding RNAs and its biological functions. *Nat Rev Mol Cell Biol* 2021;22(2):96–118.
- [82] Baldini F, et al. An overview of long non-coding (lnc)RNAs in neuroblastoma. *Int J Mol Sci* 2021;22(8).
- [83] Zhang HY, et al. Long noncoding RNA DLX6-AS1 promotes neuroblastoma progression by regulating miR-107/BDNF pathway. *Cancer Cell Int* 2019;19:313.
- [84] Zhao X, et al. Risk-associated long noncoding RNA FOXD3-AS1 inhibits neuroblastoma progression by repressing PARP1-mediated activation of CTCF. *Mol Ther* 2018;26(3):755–73.
- [85] Liu PY, et al. The long noncoding RNA lncNB1 promotes tumorigenesis by interacting with ribosomal protein RPL35. *Nat Commun* 2019;10(1):5026.
- [86] Alloisio S, et al. Generation of a functional human neural network by NDM29 overexpression in neuroblastoma cancer cells. *Mol Neurobiol* 2017;54(8):6097–106.
- [87] Zhang N, et al. LncRNA SNHG1 contributes to tumorigenesis and mechanism by targeting miR-338-3p to regulate PLK4 in human neuroblastoma. *Eur Rev Med Pharmacol Sci* 2019;23(20):8971–83.

- [88] Sahu D, et al. Co-expression analysis identifies long noncoding RNA SNHG1 as a novel predictor for event-free survival in neuroblastoma. *Oncotarget* 2016;7(36):58022–37.
- [89] Xu Z, et al. SNHG16 promotes tumorigenesis and cisplatin resistance by regulating miR-338-3p/PLK4 pathway in neuroblastoma cells. *Cancer Cell Int* 2020;20:236.
- [90] Yu Y, et al. lncRNA SNHG16 is associated with proliferation and poor prognosis of pediatric neuroblastoma. *Int J Oncol* 2019;55(1):93–102.
- [91] Pandey GK, et al. The risk-associated long noncoding RNA NBAT-1 controls neuroblastoma progression by regulating cell proliferation and neuronal differentiation. *Cancer Cell* 2014;26(5):722–37.
- [92] Mondal T, et al. Sense-antisense lncRNA pair encoded by locus 6p22.3 determines neuroblastoma susceptibility via the USP36-CHD7-SOX9 regulatory axis. *Cancer Cell* 2018;33(3):417–434.e7.
- [93] Meng X, et al. Identification of prognostic long noncoding RNAs associated with spontaneous regression of neuroblastoma. *Cancer Med* 2020;9(11):3800–15.
- [94] Fontemaggi G, et al. New molecular mechanisms and clinical impact of circRNAs in human cancer. *Cancers (Basel)* 2021;13(13).
- [95] Yang J, et al. Circular RNA DGKB promotes the progression of neuroblastoma by targeting miR-873/GLI1 axis. *Front Oncol* 2020;10:1104.
- [96] Li H, et al. Therapeutic targeting of circ-CUX1/EWSR1/MAZ axis inhibits glycolysis and neuroblastoma progression. *EMBO Mol Med* 2019;11(12):e10835.
- [97] Lochmann TL, et al. Targeted inhibition of histone H3K27 demethylation is effective in high-risk neuroblastoma. *Sci Transl Med* 2018;10(441).
- [98] Derissen EJ, Beijnen JH, Schellens JH. Concise drug review: azacitidine and decitabine. *Oncologist* 2013;18(5):619–24.
- [99] George RE, et al. Phase I study of decitabine with doxorubicin and cyclophosphamide in children with neuroblastoma and other solid tumors: a Children's Oncology Group study. *Pediatr Blood Cancer* 2010;55(4):629–38.
- [100] Krishnadas DK, Shapiro T, Lucas K. Complete remission following decitabine/dendritic cell vaccine for relapsed neuroblastoma. *Pediatrics* 2013;131(1):e336–41.
- [101] Mueller S, et al. Cooperation of the HDAC inhibitor vorinostat and radiation in metastatic neuroblastoma: efficacy and underlying mechanisms. *Cancer Lett* 2011;306(2):223–9.
- [102] DuBois SG, et al. Phase I study of vorinostat as a radiation sensitizer with 131I-metaiodobenzylguanidine (131I-MIBG) for patients with relapsed or refractory neuroblastoma. *Clin Cancer Res* 2015;21(12):2715–21.
- [103] Kolbinger FR, et al. The HDAC6/8/10 inhibitor TH34 induces DNA damage-mediated cell death in human high-grade neuroblastoma cell lines. *Arch Toxicol* 2018;92(8):2649–64.
- [104] De los Santos M, et al. Histone deacetylase inhibitors regulate retinoic acid receptor beta expression in neuroblastoma cells by both transcriptional and posttranscriptional mechanisms. *Mol Endocrinol* 2007;21(10):2416–26.
- [105] Coffey DC, et al. The histone deacetylase inhibitor, CBHA, inhibits growth of human neuroblastoma xenografts in vivo, alone and synergistically with all-trans retinoic acid. *Cancer Res* 2001;61(9):3591–4.
- [106] Rettig I, et al. Selective inhibition of HDAC8 decreases neuroblastoma growth in vitro and in vivo and enhances retinoic acid-mediated differentiation. *Cell Death Dis* 2015;6(2):e1657.
- [107] Gajer JM, et al. Histone acetyltransferase inhibitors block neuroblastoma cell growth in vivo. *Oncogenesis* 2015;4(2):e137.
- [108] Ke XX, et al. Inhibition of H3K9 methyltransferase G9a repressed cell proliferation and induced autophagy in neuroblastoma cells. *PLoS One* 2014;9(9):e106962.

- [109] Goto S, et al. Histopathology (international neuroblastoma pathology classification) and MYCN status in patients with peripheral neuroblastic tumors: a report from the Children's Cancer Group. *Cancer* 2001;92(10):2699–708.
- [110] Bayeva N, Coll E, Piskareva O. Differentiating neuroblastoma: a systematic review of the retinoic acid, its derivatives, and synergistic interactions. *J Pers Med* 2021;11(3).
- [111] Masetti R, et al. Retinoids in pediatric onco-hematology: the model of acute promyelocytic leukemia and neuroblastoma. *Adv Ther* 2012;29(9):747–62.
- [112] Adamson PC, et al. A phase 2 trial of all-trans-retinoic acid in combination with interferon-alpha2a in children with recurrent neuroblastoma or Wilms tumor: a pediatric oncology branch, NCI and Children's Oncology Group Study. *Pediatr Blood Cancer* 2007;49(5):661–5.
- [113] Finklestein JZ, et al. 13-cis-retinoic acid (NSC 122758) in the treatment of children with metastatic neuroblastoma unresponsive to conventional chemotherapy: report from the Childrens Cancer Study Group. *Med Pediatr Oncol* 1992;20(4):307–11.
- [114] Matthay KK, et al. Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: a children's oncology group study. *J Clin Oncol* 2009;27(7):1007–13.
- [115] Park JR, et al. Outcome of high-risk stage 3 neuroblastoma with myeloablative therapy and 13-cis-retinoic acid: a report from the Children's Oncology Group. *Pediatr Blood Cancer* 2009;52(1):44–50.
- [116] Maurer BJ, et al. Phase I trial of fenretinide delivered orally in a novel organized lipid complex in patients with relapsed/refractory neuroblastoma: a report from the New Approaches to Neuroblastoma Therapy (NANT) consortium. *Pediatr Blood Cancer* 2013;60(11):1801–8.
- [117] Villablanca JG, et al. Phase I trial of oral fenretinide in children with high-risk solid tumors: a report from the Children's Oncology Group (CCG 09709). *J Clin Oncol* 2006;24(21):3423–30.
- [118] Veal GJ, et al. Pharmacokinetics and metabolism of 13-cis-retinoic acid (isotretinoin) in children with high-risk neuroblastoma—a study of the United Kingdom Children's Cancer Study Group. *Br J Cancer* 2007;96(3):424–31.
- [119] The I, et al. Neurofibromatosis type 1 gene mutations in neuroblastoma. *Nat Genet* 1993;3(1):62–6.
- [120] Origone P, et al. Homozygous inactivation of NF1 gene in a patient with familial NF1 and disseminated neuroblastoma. *Am J Med Genet A* 2003;118a(4):309–13.
- [121] Serra E, et al. Confirmation of a double-hit model for the NF1 gene in benign neurofibromas. *Am J Hum Genet* 1997;61(3):512–19.
- [122] Hölszel M, et al. NF1 is a tumor suppressor in neuroblastoma that determines retinoic acid response and disease outcome. *Cell* 2010;142(2):218–29.
- [123] Huang S, et al. ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome. *Cancer Cell* 2009;15(4):328–40.
- [124] Wei S-J, et al. MYC transcription activation mediated by OCT4 as a mechanism of resistance to 13-cisRA-mediated differentiation in neuroblastoma. *Cell Death Dis* 2020;11(5):368.
- [125] Koeniger A, et al. Activation of cilia-independent Hedgehog/GLI1 signaling as a novel concept for neuroblastoma therapy. *Cancers (Basel)* 2021;13(8).
- [126] Kara G, Calin GA, Ozpolat B. RNAi-based therapeutics and tumor targeted delivery in cancer. *Adv Drug Deliv Rev* 2022;182:114113.
- [127] Tivnan A, et al. Inhibition of neuroblastoma tumor growth by targeted delivery of microRNA-34a using anti-disialoganglioside GD2 coated nanoparticles. *PLoS One* 2012;7(5):e38129.
- [128] Di Paolo D, et al. Combined replenishment of miR-34a and let-7b by targeted nanoparticles inhibits tumor growth in neuroblastoma preclinical models. *Small* 2020;16(20):1906426.
- [129] Zhao J, et al. MicroRNA-145 overexpression inhibits neuroblastoma tumorigenesis in vitro and in vivo. *Bioengineered* 2020;11(1):219–28.

- [130] Tivnan A, et al. MicroRNA-184-mediated inhibition of tumour growth in an orthotopic murine model of neuroblastoma. *Anticancer Res* 2010;30(11):4391–5.
- [131] Pilkington EH, et al. From influenza to COVID-19: lipid nanoparticle mRNA vaccines at the frontiers of infectious diseases. *Acta Biomater* 2021;131:16–40.
- [132] Soriano A, et al. Functional high-throughput screening reveals miR-323a-5p and miR-342-5p as new tumor-suppressive microRNA for neuroblastoma. *Cell Mol Life Sci* 2019;76(11):2231–43.
- [133] Soriano A, et al. MicroRNA-497 impairs the growth of chemoresistant neuroblastoma cells by targeting cell cycle, survival and vascular permeability genes. *Oncotarget* 2016;7(8):9271–87.
- [134] Llombart V, Mansour MR. Therapeutic targeting of “undruggable” MYC. *eBioMedicine* 2022;75:103756.
- [135] Liu Z, et al. Targeting MYCN in pediatric and adult cancers. *Front Oncol* 2020;10:623679.
- [136] Li WJ, et al. MicroRNA-34a: potent tumor suppressor, cancer stem cell inhibitor, and potential anticancer therapeutic. *Front Cell Dev Biol* 2021;9:640587.
- [137] Wiggins JF, et al. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. *Cancer Res* 2010;70(14):5923–30.
- [138] Liu C, et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nat Med* 2011;17(2):211–15.
- [139] Bader AG. miR-34—a microRNA replacement therapy is headed to the clinic. *Front Genet* 2012;3:120.
- [140] Hong DS, et al. Phase 1 study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours. *Br J Cancer* 2020;122(11):1630–7.
- [141] Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* 2017;16(3):203–22.
- [142] Li M-H, Fu S-B, Xiao H-s. Genome-wide analysis of microRNA and mRNA expression signatures in cancer. *Acta Pharmacol Sin* 2015;36(10):1200–11.

PART

Targeting aberrant
epigenetics

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Epigenetic therapies—update on lysine methyltransferase/PRC complex inhibitors

16

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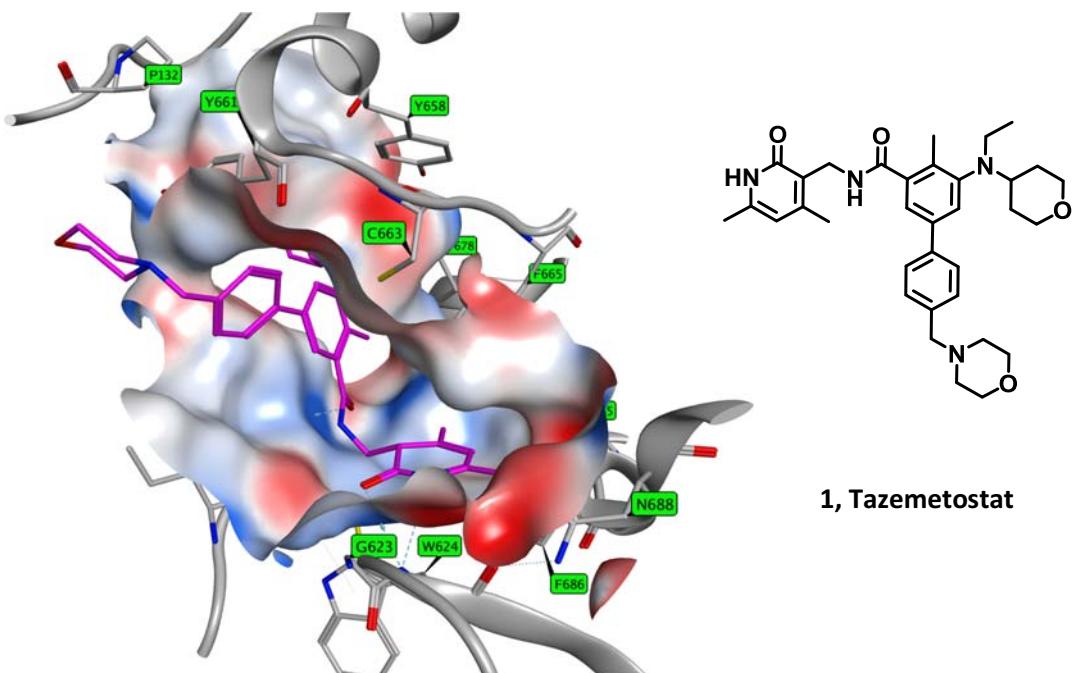
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1 Introduction

Since publication of the previous review article [1] on the emerging role of trithorax and polycomb group (PcG) proteins in the development of cancer, the most significant advances in polycomb repressive complex 2 (PRC2) inhibitors have focused on the clinical development of catalytic inhibitors of enhancer of zeste homolog 2 (EZH2), a component of PRC2. The US Food and Drug Administration (FDA) approval of Tazverik (tazemetostat, Epizyme; Figure 16.1) for the treatment of epithelial sarcoma and follicular lymphoma (FL) in 2020 marked the culmination of a decade-long effort to develop a new epigenetic therapy based on a molecular understanding of the role of aberrant histone methylation in transcriptional regulation. The fundamental role and enzymology of EZH2, together with the

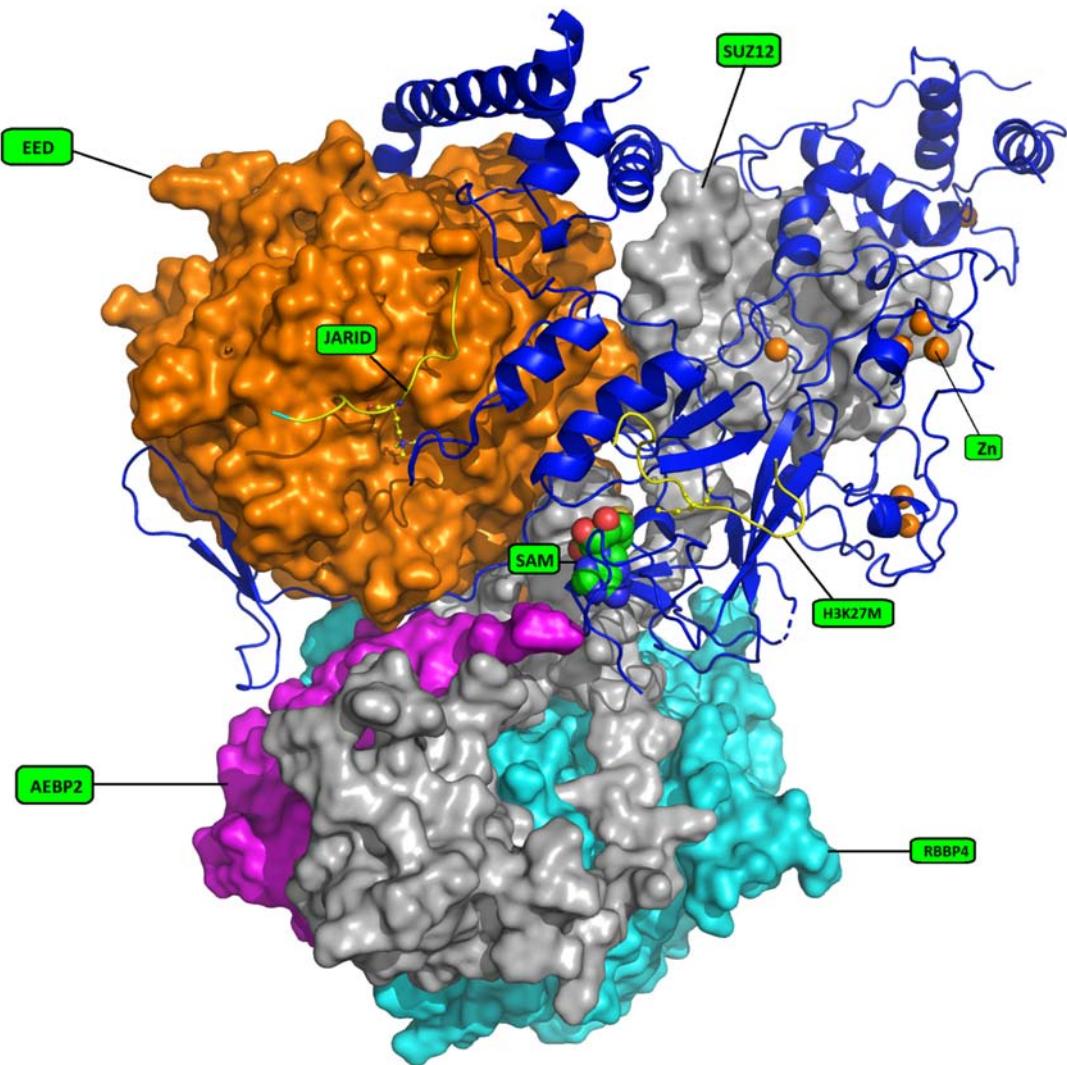
**FIGURE 16.1**

Computationally modeled view of tazemetostat binding to EZH2.

normal cellular function of PRC2, has been reviewed recently [2–4] and will not be covered extensively in this chapter. Nonetheless, different strategies to affect PRC2 activity beyond EZH2 catalytic inhibition are emerging and promising novel agents are primed to enter clinical development soon. This updated review will highlight these newer agents, summarize current interesting studies of the effects of disrupting PRC2 function in disease indications beyond lymphomas, and provide an overview of the emerging clinical experience with existing PRC2 modulators.

2 PRC enzyme mechanisms

The (PcG) is composed of a set of genes that silence gene transcription, and was originally identified in Drosophila for their roles to repress the bithorax homeobox (Hox) gene cluster, where inactivating mutations led to improper body segmentation [4]. PcG consists of two protein groups, polycomb repressor complexes 1 and 2 (PRC1 and PRC2), and their main function is to silence gene transcription by posttranscriptional modification of histone 3 via dimethylation and trimethylation of lysine 27 (H3K27me2/3) [5]. PRC2 is composed of four core proteins: EZH1/2, embryonic ectoderm development (EED), suppressor of zeste 12 (SUZ12), and retinoblastoma-binding protein 7/4 (RBBP7/4) (Figure 16.2).

**FIGURE 16.2**

Multicomponent PRC2 complex showing EZH2 (ribbon), EED, SUZ12, and RbAp. AEBP is shown with SAM depicted in solid spheres.

EZH1/2 represent the catalytic subunits of PRC2 that execute the transfer of a methyl group from cofactor *S*-adenosyl methionine (SAM) to H3K27 in a successive manner to reach a stable trimethylated form that keeps the chromatin in a repressed state for gene transcription [6]. EZH2 exhibits higher enzymatic activity compared with EZH1, and PRC2/EZH2 establishes H3K27me2/3 levels, whereas PRC2/EZH1 restores methylation that has been lost owing to demethylase activity [7]. Reflective of these

functional differences, EZH2 expression is higher in proliferating cells, whereas EZH1 is predominantly detected in differentiated cells. In addition, AEBP2, Pcls, and Jarid2 are three accessory proteins that interact with the PRC2 complex to regulate its enzymatic activity. Although these proteins are not required for PRC2 activity *in vitro*, they are proposed to enhance PRC2 enzymatic activity in certain cellular contexts [4]. H3K27me3, once fully formed by PRC2, recruits PRC1 to execute mono-ubiquitylation of lysine 119 of histone H2A (H3AK119ub), which further modifies the chromatin structure to maintain full repression of gene transcription [5]. The core subunits of PRC1 include Bmi, Ring1a, Ring1b, CBX, PH1, PH2, NSPC1, and MEL18, where Ring1a and Ring1b represent the ligases that ubiquitylate the lysine [8].

3 PRC2 in cancer

Data from human genome sequencing efforts have shown that a wide variety of human cancers harbor mutations in the genes encoding histone-modifying chromatin regulators [2]. Numerous independent investigations have generated a large amount of evidence to highlight a dichotomous link between EZH2 and cancer, where alterations in enzyme activity can either promote or block oncogenesis depending on the cellular context and underlying genetic backgrounds [9]. EZH2 overexpression was first identified in prostate cancer as one of the gene products associated with poor clinical outcomes in this tumor setting [10]. Since then, EZH2 overexpression has been reported in multiple tumor types, including breast cancer, bladder cancer, B-cell lymphoma, endometrial cancer, and melanoma; and elevated levels of EZH2 are correlated with worse outcomes in these tumors [11–13].

Heterozygous mutations in the C-terminus catalytic SET domain of EZH2, including a point mutation at tyrosine 641 (Y641), have been detected in 22% of germinal center diffuse large B-cell lymphomas (DLBCL) and in 7% to 12% of FLs as gain-of-function mutations [14,15]. The mutations at Y641 (Y641F, Y641N, T641S, Y641C, and Y641H) enhance the methyltransferase activity of the enzyme for dimethylated H3K27 histones, leading to elevated levels of trimethylated H3K27. Other EZH2 gain-of-function mutations, such as those at A677 and A687, have also been identified in non-Hodgkin lymphoma [16,17]. In addition to EZH2 mutations, loss-of-function mutations in proteins that antagonize EZH2 activity can lead to hypermethylated H3K27me3. For example, ubiquitously transcribed tetratricopeptide repeat gene on X-chromosome (UTX or KDM6A) is a histone lysine demethylase that removes methyl groups from H3K27, and inactivating mutations have been reported in medulloblastoma, bladder, and prostate cancers [18–20]. Nearly all of these mutations result in the loss of the Jumonji C (JmjC) domain of UTX, which is required for demethylation activity. The absence of JmjC activity keeps the H3K27me3 in a hypermethylated state, which is therefore functionally equivalent to gain-of-function mutations in EZH2. The main consequence of these events is the repression of critical tumor suppressor genes that safeguard cells from unchecked proliferation, including INK4a/Arf and p21, the inactivation of which promotes malignant growth and eventual tumorigenesis [2]. Taken together, the evidence suggests that aberrant EZH2 activity favors the development of a neoplastic phenotype, especially in cancers in which EZH2 is overexpressed or that harbor gain-of-function EZH2 mutations. The evidence also suggests that blocking EZH2 activity may be a potential therapeutic strategy for cancer.

However, as mentioned earlier, EZH2 has a dichotomous role in oncogenesis; in certain cancers such as those from the myeloid lineage, EZH2 has been shown to act as a tumor suppressor, where its inhibition can lead to the opposite outcome and promotion of tumor development.

Myeloid malignancies are clonal hematopoietic disorders of myeloid lineage that include cancers classified as myeloproliferative neoplasms (MPN) and myelodysplastic syndrome (MDS) [21]. Inactivating mutations of EZH2 have been found in 10% to 13% of MPN/MDS and 13% of myelofibrosis (MF) [22,23]. These mutations are largely missense, frameshift, and nonsense mutations. Patients with MPN/MDS who harbor homozygous mutations show shorter survival time and worse prognosis than those with heterozygous mutations, suggesting that EZH2 acts as a tumor suppressor in these cancers. Various mechanisms have been proposed to explain the relationship between loss of EZH2 activity and the development of myeloid malignancies, the majority of which suggest an epigenetic dysfunction in the presence of specific cooccurring mutations. In cancers lacking Additional Sex Combs-Like 1 (ASXL1), loss of EZH2 reduces global levels of H3K27me3 and activates oncogenes such as *HoxA9* to promote cell transformation and leukemogenesis [21]. Additional major mutations, such as in *TET2*, *RUNX1*, and *SF3B1*, have also been shown to cooperate with EZH2 loss to promote malignant cell growth. In cancers harboring Janus kinase 2 (JAK2) V617F mutation, loss of EZH2 activates genes known to be prooncogenic, including *HMGA2* (High Mobility Group AT-Hook 2) [24]. Treating JAK2/V617F transgenic mice with EZH2 inhibitor further accelerates tumor growth and results in a shorter survival, demonstrating that EZH2 inhibition enhances JAK2-V617F oncogenic activity. Taken together, these data demonstrate that, contrary to observations in prostate cancer or B-cell lymphomas, inhibition of EZH2 in myeloid cancer cells results in activation of oncogenes that exacerbate the tumorigenic phenotype. This again speaks to the polypharmacology of EZH2, where its activity can promote tumor growth or inhibition, based on the genes affected and the mechanisms unique to different cell types or lineages.

Although histone modification is one of the most intensely studied areas of EZH2 biology, newly emerging biological insights have shown that EZH2 participates in a number of noncanonical pathways independent of PRC2 to promote tumorigenesis. These include its role as a transcriptional activator/coactivator in endocrine tissue cancers, as a modifier of nonhistone proteins, and as an RNA binding protein [25]. In the estrogen receptor (ER)-positive breast cancer cell line, MCF-7, EZH2 forms a ternary complex with estrogen receptor α (ER α) and β -catenin to activate *MYC* and *cyclin D1* transcription [26]. In ER-negative basal-like breast cancer cells, EZH2 binds to RelA and RelB to activate the transcription of NF- κ B target genes, such as *interleukin (IL)-6* and *tumor necrosis factor (TNF)* [27]. In addition to acting as a coactivator, EZH2 can function as a sole transcription factor by binding directly to a gene promoter. Kim et al. showed that EZH2 can bind directly to and activate the androgen receptor (AR) promoter, leading to increased expression of prostate-specific antigen (PSA), TMPRSS2, and FKBP5, which are known markers for prostate cancer development [28]. EZH2 is also overexpressed in invasive and hormone-refractory metastatic prostate cancers [10]. Nonhistone proteins that have been shown to be methylated by EZH2 include GATA4, ROR α , and STAT3 [25]. ROR α methylation is recognized by the CUL4 ubiquitin ligase complex, leading to its degradation [29]. In oral cell squamous cell carcinoma and glioblastoma, EZH2 enhances the activity of phosphorylated STAT3, to promote neoplastic cell growth [30,31]. In summary, EZH2 biology is highly complex and can lead to tumor growth or tumor suppression. For therapies directed toward blocking PRC2/EZH2 activity, selecting the appropriate patients with cancer based on a clear understanding of the biological and mechanistic dependency is essential for successful clinical development.

4 Synthetic lethality

The mammalian SWItch/Sucrose NonFermentable complex (SWI/SNF) is a multisubunit chromatin remodeler composed of 12–15 proteins, which functions mainly to alter the position of nucleosomes, so as to control the promoter access for epigenetic regulators, including polycomb proteins [32]. Inactivating mutations of the SWI/SNF complex are found in 20% of human cancers, and it is a major tumor suppressor whose prevalence is similar to that of p53 [32]. SWI/SNF and polycomb proteins have opposing functions, and the cancers that harbor mutant SWI/SNF are highly dependent on EZH2, where EZH2 inhibition results in cell growth arrest and senescence or apoptosis. The earliest evidence for this synthetic lethality was observed in pediatric malignant rhabdoid tumors and T-cell lymphomas lacking SNF5/INI1 (also known as BAF47), in which genetic or pharmacologic inhibition of EZH2 led to complete tumor growth inhibition in multiple cell lines and mouse models [33]. Studies to understand this mechanism demonstrated that the absence of SNF5/INI1 resulted in the recruitment of EZH2 to the promoters of major tumor suppressors, including INK4A/p16, resulting in silencing of their expression via increasing H3K27me3 levels. Reintroducing SNF5/INI1 then replacing EZH2 at the p16 locus and derepressing the gene, results in growth arrest. Using an engineered reporter system leveraging the Oct4 locus, Kadoc and colleagues demonstrated that SWI/SNF evicted polycomb proteins occupying the promoter in an ATP-dependent manner, providing direct evidence for their antagonism [34]. ARID1A, which is the DNA-binding component of the SWI/SNF complex, is the most frequently mutated subunit, and is altered in 50% of ovarian cancers, 20% of gastric cancers, 20% of bladder cancers, 14% of hepatocellular cancers, 12% of melanomas, and 10% of colorectal cancers [9]. Genetic and pharmacologic inhibition of EZH2 in ovarian cancer cells harboring *ARID1A* mutation has been shown to restore *PIK3IP1* expression, resulting in inhibition of PI3K/AKT signaling and cell growth arrest [35]. Taken together, these data demonstrate the antagonistic/synthetic lethal relationship between SWI/SNF and polycomb proteins, where inactivation of the former would lead to a dependency on the latter. EZH2 inhibition could therefore be an effective therapeutic strategy against SWI/SNF mutant cancers.

As described previously, the histone lysine demethylase, UTX/KDM6A can also directly antagonize PRC2/EZH2 by removing the methyl marks on H3K27 [18–20]. In cancers harboring the inactivating mutations of *KDM6A* (equivalent to gain-of-function mutations in EZH2), inhibiting EZH2 activity leads to growth inhibition. In multiple myeloma cells lacking *KDM6A*, disruption of EZH2 inhibition results in robust growth inhibition, where the inhibitor had no effect in the wild-type cells [36]. Restoring KDM6A activity alters EZH2 sensitivity, confirming the synthetic lethal relationship between the two proteins. Synthetic lethality for EZH2 inhibitor was also observed in mesothelioma cell lines lacking the H2aK119Ub deubiquitinase BAP1 and BRAC1 deficient breast cancers, where the latter also required coinhibition of ATM [37,38].

5 Tumor immunity

In addition to oncogenes and tumor suppressors, the PRC2 complex regulates a diverse set of immune-related genes in both tumor and immune cells. Similar to what was described previously

for effects on oncogenesis and tumor growth, PRC2 may act to either suppress or promote tumor immunity, depending on the mechanism and cell type. Multiple reports have shown that in tumor cell models EZH2 represses the transcription of cytokines that promote T-cell infiltration. Using an ovarian cancer cell model, Peng et al. have shown that EZH2 repressed the Th1 chemokines, CXCL9 and CXCL10, resulting in decreased trafficking of effector CD8⁺ T cells and a lack of response to antiprogrammed cell death ligand 1 (PD-L1) therapy [39]. The repression of CXCL9/10 transcription not only involved elevated H3K27me3 mark on histones by EZH2 but also increased DNA methylation by DNA methyltransferase (DNMT). Treatment with EZH2 and DNMT inhibitors then restored CXCL9/10 expression, increased T-cell trafficking, and sensitized the tumors to a PD-L1 antibody, where the triple combination of EZH2 and DNMT inhibitors and PD-L1 antibody led to a robust antitumor activity. The same laboratory also reported similar findings in colon cancer models, where EZH2 directly silenced cytokine transcription in DLD-1 and SW480 cells [40]. Genetic knockdown of EZH2 or SUZ12 then removed the H3K27me3 mark on the CXCL10 promoter, resulting in increased T-cell trafficking and enhanced antitumor immunity of the checkpoint blockage agent. PRC2 also represses major histocompatibility complex (MHC)-I/II transcription in tumor cells, leading to decreased antigen presentation at the cell surface and minimizing engagement of the effector T cells. Burr et al. performed a CRISPR screen in MHC-I negative K-562 cells and have found that EED and SUZ12 were the most strongly associated genes associated with MHC-I suppression [41]. Depletion of EED and SUZ12 reduced H3K27me3 at the MHC-I APP gene, which restored MHC-I expression and interferon (IFN)- γ response. Pharmacologic inhibition of EZH2 or EED resulted in enhanced tumor cell killing by OT-I T cells, suggesting that EZH2 treatment can re-sensitize tumors that initially lack MHC-I expression [41]. In addition, EZH2 has also been shown to silence MHC2TA transcription. In uveal melanoma Holling et al. observed high H3K27me3 levels at the MHC2TA promoter IV (CIITA-PIV) bound by EZH2, with depletion of EZH2 activity resulting in enhanced CIITA transcription upon IFN- γ induction [42]. Finally, EZH2 also suppresses the dsRNA-STING-IFN response pathway, which plays a critical role in checkpoint inhibitor sensitivity. Using a prostate cancer model, Morel et al. have demonstrated that EZH2 negatively regulates type I/II IFN-stimulated genes (ISGs), with tumors that show <5% PD-L1 positivity showing high level of H3K27me3 mark and low dsRNA staining [43]. EZH2 inhibitor treatment derepressed endogenous dsRNA expression, which in turn activated ISGs in a STING-dependent manner. EZH2/PD1 inhibitor combination led to robust antitumor activity in this model [43]. In summary, EZH2 represses numerous genes associated with tumor immunity, including MHC1/2 and STING-IFN pathway genes, where the outcome often leads to lack of response to PD-L1 antibodies. Taken together, the findings suggest that administration of an EZH2 inhibitor in combination with checkpoint inhibitor therapy may be an effective strategy to promote tumor immunity.

EZH2 function has been investigated in all major types of immune cells, including CD4⁺/CD8⁺ T cells, T helper cells, T regulatory (Treg) cells, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages. The data from these studies are consistent with those described previously, where EZH2-mediated mechanisms can lead to opposite outcomes depending on the cell type and context. In CD8⁺ T cells, EZH2 suppresses CDKN2A and CDK2C in activated, naive T cells, and its activity is required for CD8⁺ T cells to differentiate to central memory precursor T cells upon antigen stimulation [44,45]. When EZH2 knockout CD8⁺ T cells were transferred to a mouse tumor model, they were unable to proliferate and form memory cells as seen in the EZH2

wild-type, resulting in a reduced and less durable antitumor response [44]. Despite data suggesting EZH2 inhibition may reduce tumor immunity, studies on Tregs demonstrate an opposite effect, where EZH2 disruption of Treg function promotes the acquisition of proinflammatory functions in tumor-infiltrating Tregs (TI-Tregs). Thus blocking EZH2 activity in Tregs was shown to promote the remodeling of the tumor microenvironment and increased in CD4⁺ and CD8⁺ effector T-cell recruitment [46]. In this study, EZH2 depletion induced a robust antitumor activity in immunocompetent MC38 mouse colorectal cancer model, while showing no effects in the T-cell-deficient Rag-1^{-/-} model, suggesting the antitumor activity was mainly driven by a tumor extrinsic mechanism. EZH2 deficiency reduced FOXP3 expression in Tregs and increased the levels of proinflammatory cytokines, including TNF- α , IFN- γ , and IL-2. Further characterization of these Tregs demonstrated that EZH2 inhibition did not affect the proliferation; rather, immunosuppressive functions were impaired, suggesting that EZH2 disruption can enhance anticancer immunity without significant risk of autoimmunity.

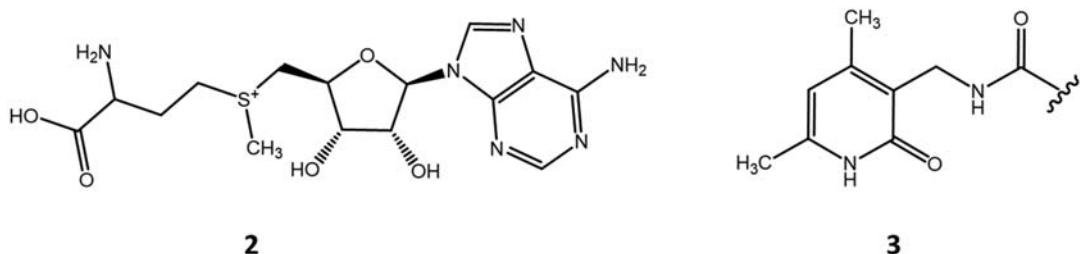
MDSCs are immature cells of myeloid lineage that have immunosuppressive activity and are known to be elevated in patients with cancer [47]. MDSCs utilize multiple mechanisms to suppress adaptive and innate immunity, including regulation of indoleamine-pyrrole 2,3-dioxygenase (IDO), arginase, inducible nitric oxide synthase (iNOS), TGF- β , and IL-10. EZH2 prevents the formation of MDSC, and the inhibition of EZH2 can therefore promote the production of MDSCs, leading to enhanced tumor growth. Studies by Huang et al. demonstrated that pharmacologic inhibition of EZH2 with GSK126 increased MDSC formation, while reducing CD4⁺ and CD8⁺ T cells in the tumor microenvironment [48]. This disruption of EZH2 activity increased the CD11⁺ Gr-1 MDSC population, which could then be reversed by the treatment with Gr-1 neutralizing antibody. The combination treatment of an EZH2 inhibitor and Gr-1 antibody restored the antitumor activity of GSK126 by removing the cell population that opposed the inhibitor activity on cancer cells.

In summary, similar to the cases observed with oncogenes and tumor suppressors in cancer cells, the role of EZH2 in immune cells is multifaceted. Owing to this biological complexity, the development of PRC2/EZH2 therapies in the clinic can therefore be quite challenging, despite the encouraging activity observed in numerous preclinical models.

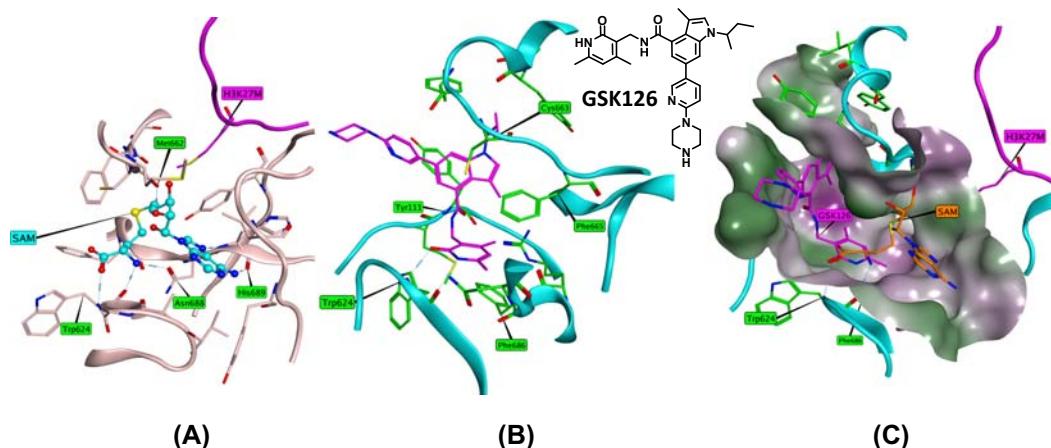
6 First-generation EZH2 inhibitors

As discussed in the previous section, the multicomponent PRC2 complex is composed of four core subunits: EZH2, SUZ12, EED, and RbAp46 (Figure 16.2, PDB ID: 6C23) and is regulated by cofactors including JARID2/4 and AEBP2 [49]. The discovery of S-adenosyl-L-methionine (Figure 16.3, compound 2, SAM)-competitive inhibitors of the catalytic activity of EZH2 has been described extensively in previous reviews [50,51]. Notably, high-throughput screening of multiple diverse, small molecule compound libraries identified the 6-methylpyridone fragment 3 as a common pharmacophore [16,52,53] for accessing the SAM cofactor binding pocket within the EZH2 catalytic domain.

Subsequently, X-ray cocrystal structures of various 6-methylpyridone-containing EZH2 inhibitors (Figure 16.4, PDB IDs: 5LS6, 5WG6) have revealed the key interactions of this moiety and have provided a structural understanding of the importance of this critical recognition motif [54,55].

**FIGURE 16.3**

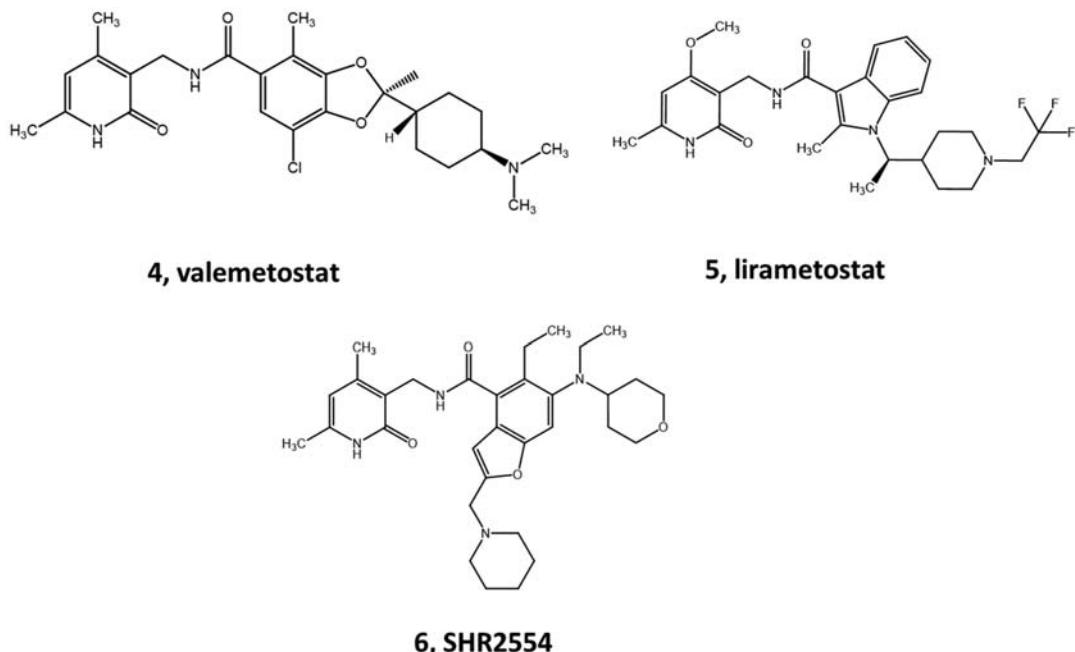
Compound 2 (SAM) and compound 3.

**FIGURE 16.4**

View of PRC2 complex highlighting interactions of SAM (A), GSK126 (B) and an overlay of the two showing the critical interactions of the pyridone group (C).

Insights derived from the crystal structures have provided the impetus for the design and development of additional SAM site directed EZH2 inhibitors with enhanced potency, selectivity, or more favorable pharmaceutical properties. Among these analogs, valemestostat (DS-3201, Daiichi-Sankyo [56]), lirametostat (CPI-1205, MorphoSys [54]), and SHR2554 (Jiangsu Hengrui [57]) have advanced to clinical development (Figure 16.5).

Other first-generation compounds of note are EI1 (compound 7, Novartis [53]) and compound 8 (Shanghai Haihe Biopharma, WO2019170063 [58]), compound 9 (Hefei Institute, WO2019170063 [59]), and HH2853 (compound 10, Hefei Institute, WO2018045971 [60]) (Figure 16.6) from the patent literature. However, none of these appear to currently be in clinical development.

**FIGURE 16.5**

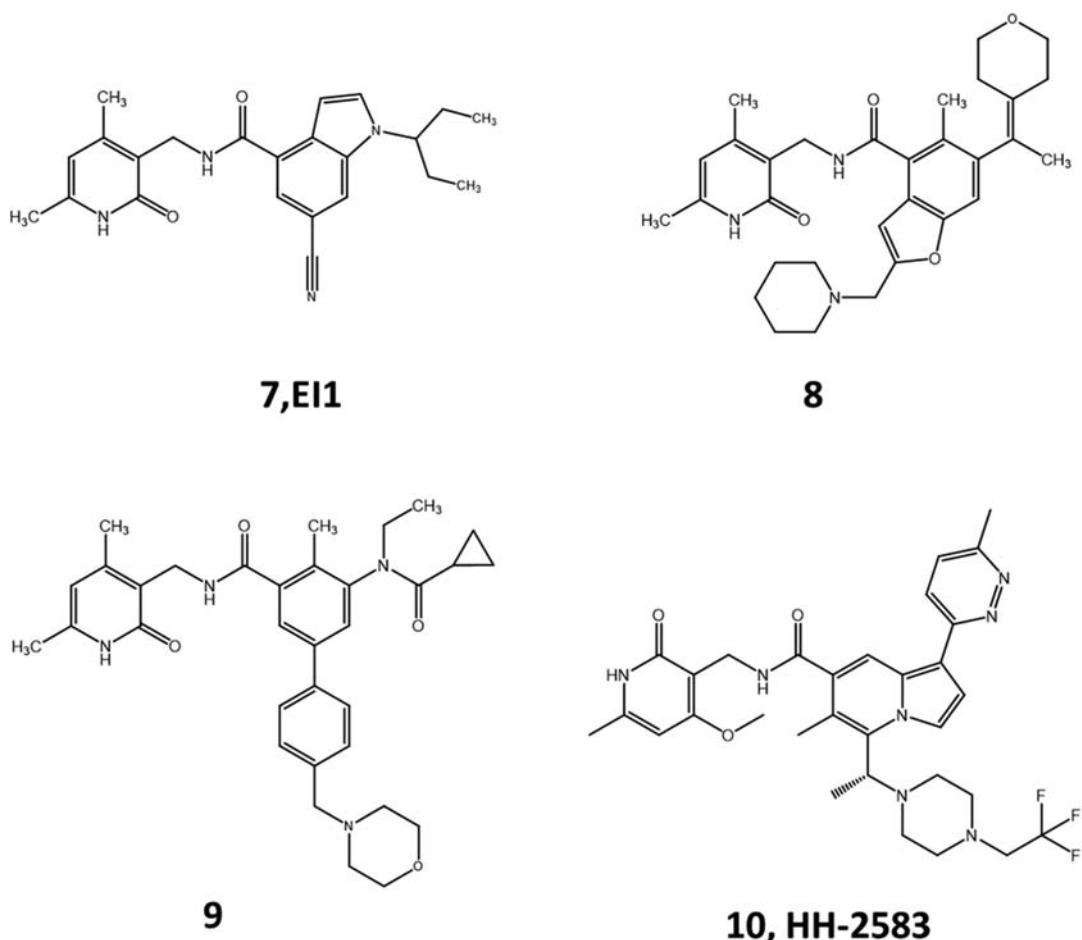
Clinical stage first-generation EZH2 inhibitors.

7 Conformationally constrained EZH2 inhibitors

Beyond the first-generation compounds, attention has turned toward identifying alternate scaffolds as a way to improve solubility and *in vivo* pharmacokinetics, resulting in the development of structurally distinct inhibitors. Conformational constraint by cyclization of the pyridone and amide linker in compound **11** allows for optimal orientation of the pyridone and pendant phenyl ring, leading to the dihydroisoquinolone scaffold that was further optimized to clinical candidate, PF-06821497 (compound **12**, Pfizer [59]). A related compound, HM97662 (compound **13**, Hanmi [61]) is poised to enter first-in-human studies in 2022 (Figure 16.7).

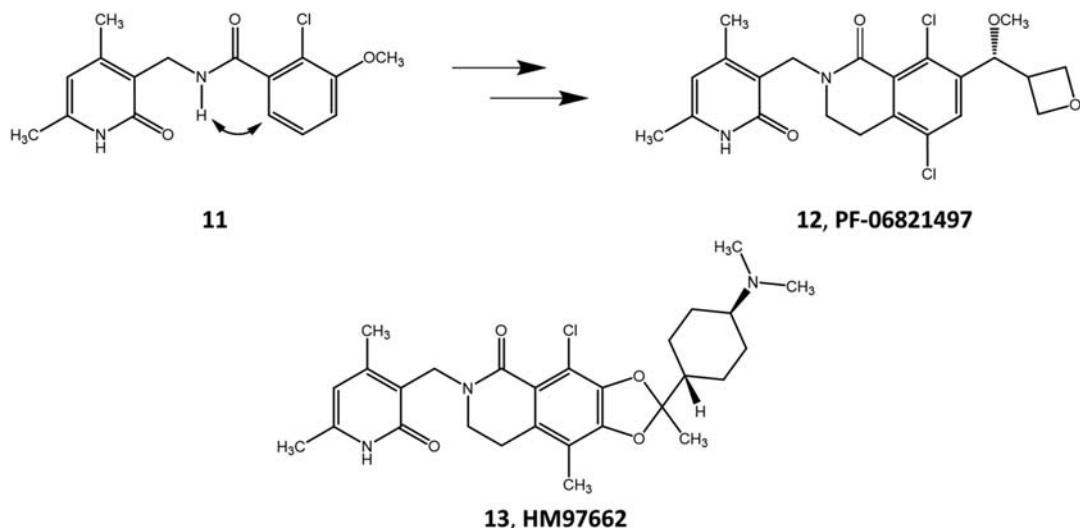
Inhibition of EZH2 in the brain may be beneficial in the treatment of central nervous system (CNS) malignancies, especially in pediatric patients [62]. Since the amide cyclization strategy reduces H-bond donor count, it is predicted to reduce P-gp transporter-mediated efflux, potentially enhancing CNS permeability. *N*-methylation of the pyridine moiety further assists with this strategy; indeed, this design has led to the discovery of novel and potent brain-penetrant EZH2 inhibitors, including TDI-6118 (Figure 16.8, compound **14**, Tri-Institutional Therapeutics Discovery Institute [63]).

In a remarkable finding, consideration of pyridone tautomerization in the context of EZH2 binding led to identification of a 4-thiomethyl pyridone substitution as being a highly favorable, potency-enhancing element [64]. Incorporation of this type of substitution has led to some of the

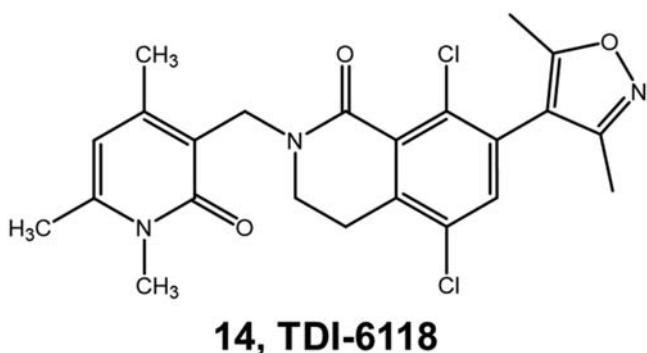
**FIGURE 16.6**

Preclinical first-generation EZH2 inhibitors.

most potent EZH2 inhibitors reported, with K_i values in the femtomolar range. K_i values in this range for noncovalent or nonmechanism-based inhibitors are extraordinary and, in this case, were proposed to be a result of favorable sulfur- π interactions between the 4-thiomethyl group and F665 and F686 in the pyridone-binding locus [64]. Careful and extensive biochemical analysis of the binding kinetics of various pyridone-containing inhibitors has also revealed details of the molecular basis of the increased potency of the thioalkyl analogs. Despite similar association rates, the single atom change of oxygen to sulfur (i.e., methoxy to thiomethyl) was reported to dramatically reduce the dissociation rate of compounds from the activated PRC2 complex. As a result, S-alkyl analogs have a very long enzyme residence time, which translates to superior activity in cellular and *in vivo* mouse xenograft models [64–66]. Further chemical modification in the scaffold provided

**FIGURE 16.7**

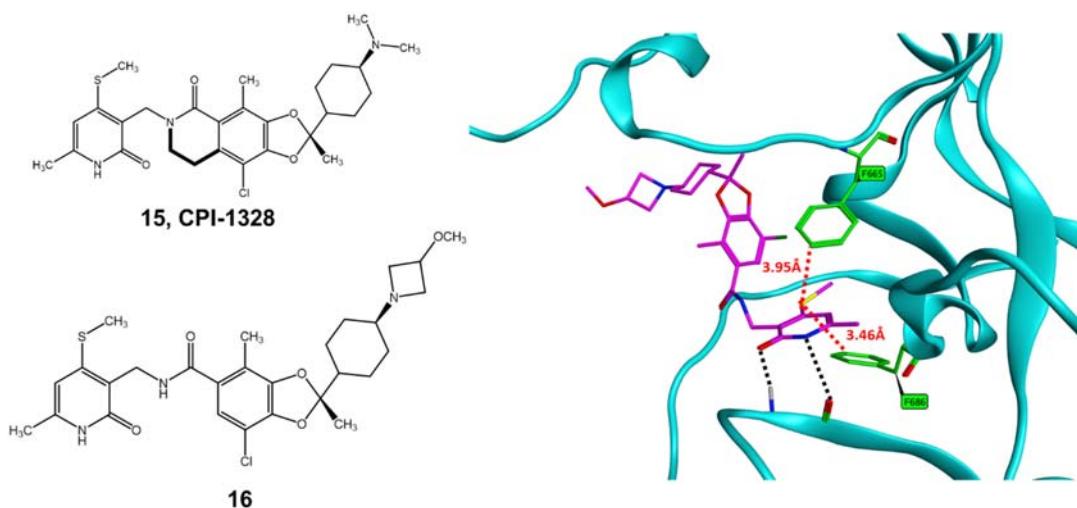
Design of conformation constrained clinical stage EZH2 inhibitors.

**14, TDI-6118****FIGURE 16.8**

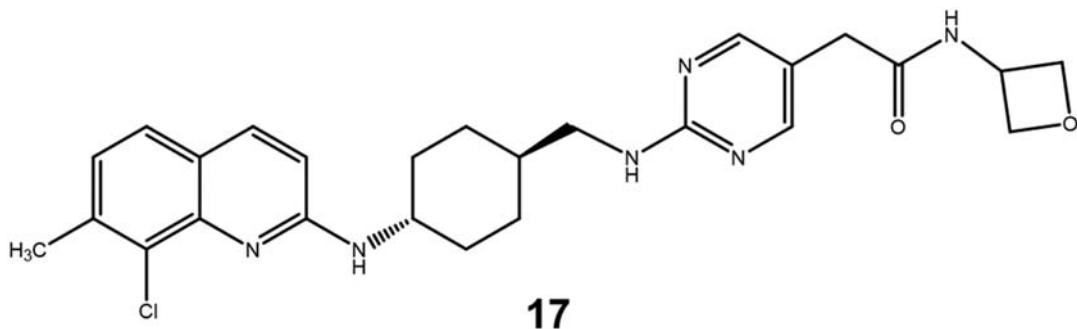
Structure of TDI-6118.

compounds, such as CPI-1328 (compound **15**, WO2019226491 [67], MorphoSys) and compound **16** (WO2021016409 [68], MorphoSys) (Figure 16.9), which are likely related to a clinical candidate, CPI-0209 (structure not disclosed).

More recently, azaquinolines, such as compound **17** (Figure 16.10, WO2022033492 [69], Novartis) representing a novel, nonpyridone-containing template have been disclosed in the patent literature as potent EZH2 inhibitors. The mechanism of action or the mode of binding of this chemotype has not yet been reported.

**FIGURE 16.9**

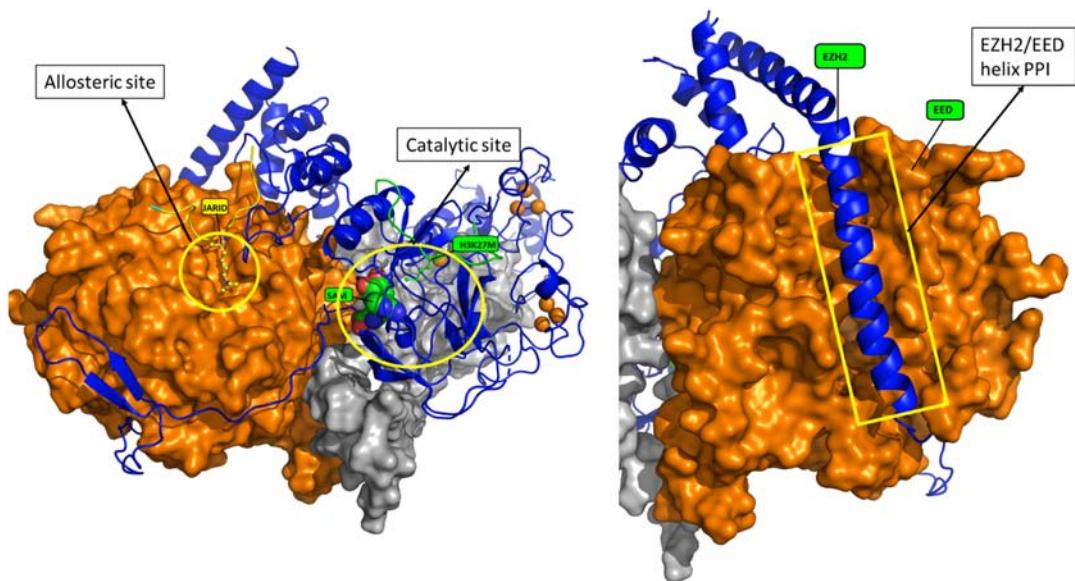
4-Thiomethyl pyridone analogs, CPI-1328 and compound **16**. Compound **16** is modeled in the EZH2 binding site (PDB ID: 5HYN) showing the sulfur π interactions with F665 and F686 proposed to lead to longer enzyme residence time [64].

**FIGURE 16.10**

Representative example of azaquinoline-containing EZH2 inhibitors.

8 EED-targeted PRC2 modulators

In contrast to direct, SAM-competitive EZH2 inhibitors, several groups have explored targeting the EED protein as a way to indirectly inhibit EZH2-mediated H3K27 trimethylation. EED is a required component of the multiprotein PRC2 complex that interacts with EZH2 via an extended EZH2 N-terminus α-helix. EED also binds to a distinct pocket in the cofactor JARID4 and further activates the catalytic EZH2 domain to methylate adjacent nucleosomes (Figure 16.11, PDB ID: 5HYN) [49].

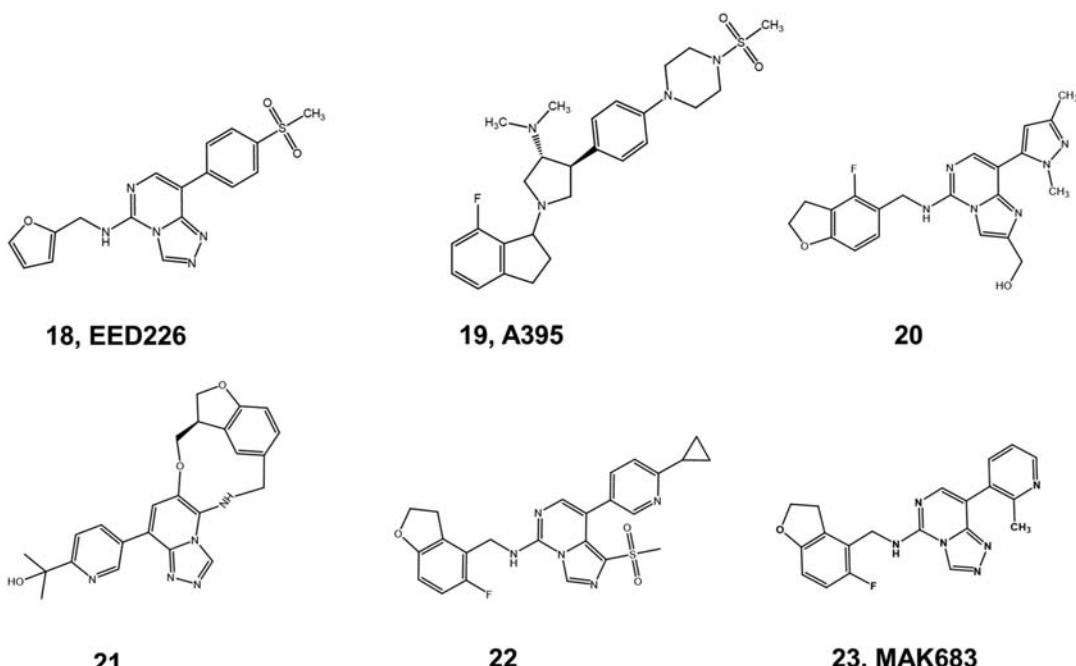
**FIGURE 16.11**

View of PRC2 complex showing EED, EZH2 (ribbon), and SUZ12 interactions.

Since EED is critical in stabilizing PRC2 [70–73], disruption of the EED:EZH2 interaction can result in a functional loss of the complex. The advantages of targeting EED binders over EZH2 catalytic inhibitors include the potential to abrogate any scaffolding function of PRC2 and to increase selectivity over EZH1 inhibition. In practice, drug-like small molecules able to alter histone lysine binding, such as EED226 (Figure 16.12, compound **18**, Novartis [74,75]), behave as competitive H3K27 inhibitors [76] and effectively inhibit trimethylation without PRC2 destabilization [75]. In cellular and *in vivo* assays, EED226 acts synergistically with SAM-competitive EZH2 inhibitors, suggesting that the stability of the PRC2 complex is retained.

EED226 and A395 (Figure 16.12, compound **19**) have also been reported to be active against cell lines resistant to the catalytic site inhibitors EI1 and GSK126 [74,75], indicating a unique, differentiated biological mechanism for such compounds with potential clinical utility in patients who develop resistance to SAM-competitive EZH2 inhibitors. Following the discovery of EED226, other structurally related derivatives (Figure 16.12) including compound **20** (WO2020247475 [77], Mirati Therapeutics), compound **21** (WO2020190754 [78], Fulcrum Therapeutics), and the exceptionally potent compound **22** [79,80] have been reported. Amongst these analogs, MAK683 (compound **23** [81], Novartis) has advanced to clinical development (NCT02900651 [82]).

Targeting PRC2 function by interfering with the extended α -helical domain protein-protein interaction has also been explored [83] and described in more detail elsewhere [84]. Although promising tractable and selective leads have been identified [85–87], sufficiently potent, drug-like compounds have not yet been developed.

**FIGURE 16.12**

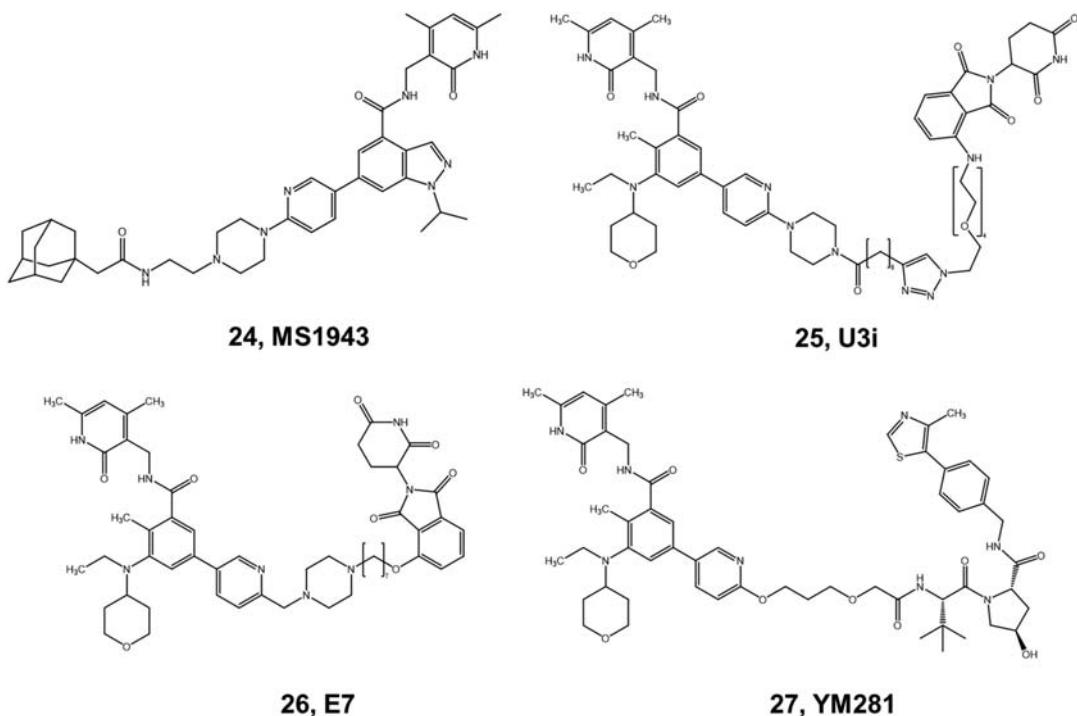
EED binders related to EED226.

9 EZH2 and EED degraders

The discovery of the ubiquitin-mediated proteasomal degradation pathway [88–90], followed by the pioneering development of proteolysis targeting chimeras (PROTACs [91]) represents a major paradigm shift in small molecule anticancer drug design and development. Moreover, the selective elimination of intracellular proteins of interest (POIs) by degradation allows for all functions of the POI (catalytic, scaffolding, etc.) to be abolished, often leading to new and more profound effects on cellular phenotypes and tissue function. A detailed description of targeted protein degradation is beyond the scope of this review, but has been extensively reviewed elsewhere [92–94]. The availability of potent and selective EZH2 and EED small molecule binders has allowed for development of multiple PRC2 degraders, based on both hydrophobic tagging [95] and PROTAC approaches [96–100]. These agents have been used to remove either of these critical PRC2 components.

Following some elegant work [101], Ms1943 (compound 24; Figure 16.13), a first-in-class EZH2 degrader, was reported to selectively reduce intracellular EZH2 and SUZ12 levels without affecting levels of EED or EZH1 [95].

In contrast, cereblon and VHL-engaging EZH2 PROTAC degraders U3i (compound 25 [96]), E7 (compound 26 [97]), and YM281 (compound 27 [98]; Figure 16.13) degraded EZH2, EED, and SUZ12, respectively, while still sparing EZH1. This indicates potential differences in the ability of targeted degraders to influence the ultimate fate of POIs, depending on the mode of the induced degradation.

**FIGURE 16.13**

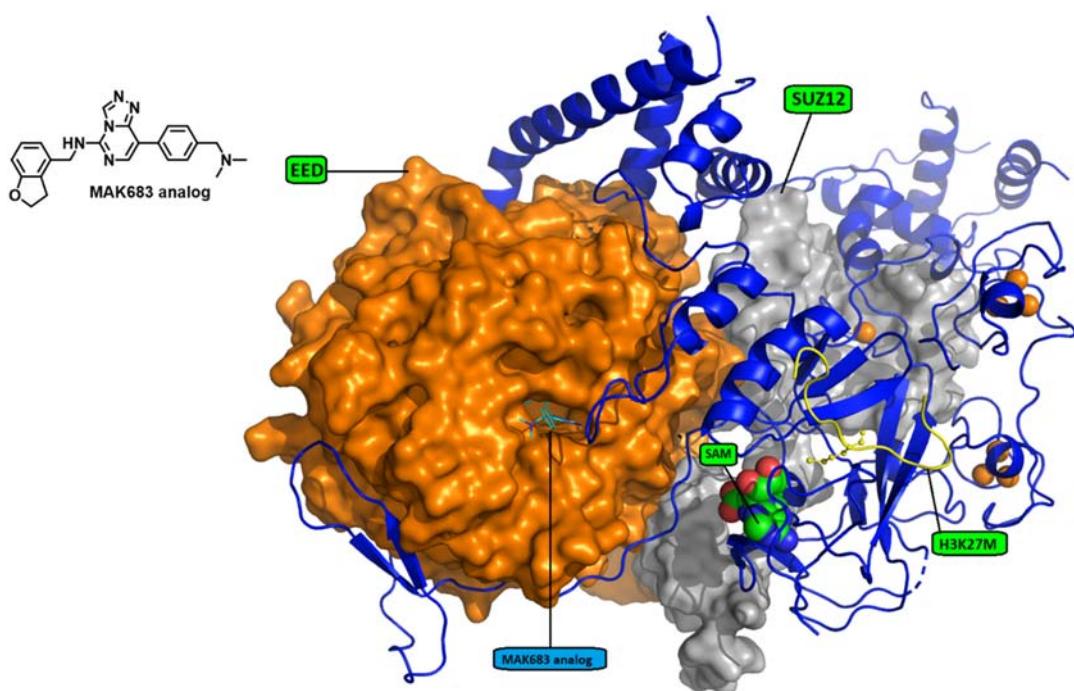
EZH2 degraders.

By analogy to EZH2 degraders, various EED-binding warheads have been coupled with VHL-engaging ligands [102]. In the design of these bifunctional compounds, the optimal vector for linker attachment of the ligase binding moiety is critical, and its identification can be greatly enabled with structural information on the binding mode of the POI-targeting molecule [102,103]. The cocrystal structure of a MAK683 analog ([81], Figure 16.14) clearly reveals the vector for such functionalization and rationally leads to the PROTACS, UNC6852 (Figure 16.15, compound 28 [99]) and compound 29 [100], which have been characterized for their ability to remove intracellular EED and associated PRC2 components.

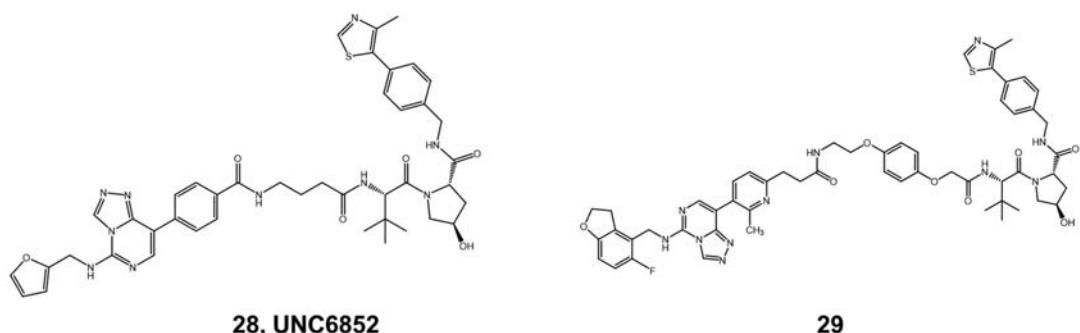
Consistent with the requirement for protein re-synthesis to generate a functional complex, the PRC2 degraders show superior activity in cellular and mouse models, including prolonging suppression of H2K27me3 levels relative to nondegrading controls [100].

10 Covalent EZH2 inhibitors

The clinical success of diverse covalent kinase inhibitors, such as sotorasib [104], ibrutinib [105], and osimertinib [106], targeting KRAS, BTK, and EGFR, respectively, has spurred interest in designing selective, covalent, drug-like EZH2 inhibitors. Indeed, some weakly active, natural product-related compounds were

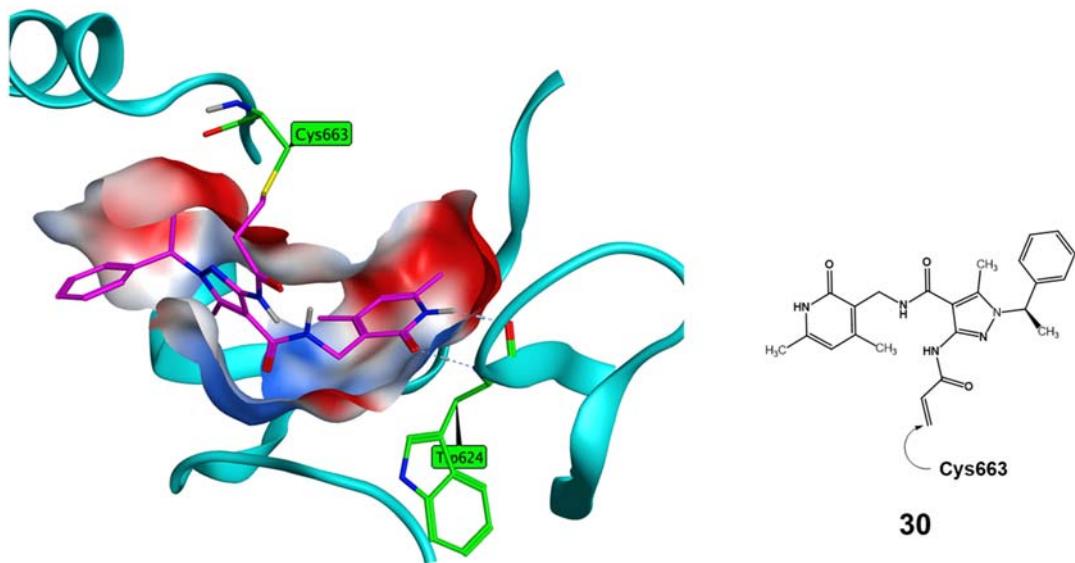
**FIGURE 16.14**

MAK683 analog binding to EED showing vector available for design of bifunctional degraders.

**FIGURE 16.15**

EED PROTACs.

reported to covalently label EZH2 [107]; although recently synthetic small molecule and peptide-based approaches have been reported [108–110]. SKLB-0335 (compound **30** [109]) and the structurally related analog SKLB-03176 [110] target the conserved Cys663 in EZH2 (Figure 16.16) and form an irreversible covalent bond with the residue via the acrylamide warhead of the inhibitor.

**FIGURE 16.16**

Covalent EZH2 inhibition by targeting Cys663 with pyridone-based acrylamides.

The presence of the pyridone is consistent with these compounds being SAM competitive in their binding mode, but the irreversible nature of the interaction distinguishes these molecules from earlier generation EZH2 inhibitors, including CPI-0209. Although the reported cellular potency of compound **30** was inferior to reversible EZH2 inhibitors, further optimization of the compound may allow for improved cellular potency and *in vivo* pharmacology.

11 Clinical activity of EZH2 inhibitors

Nine compounds inhibiting EZH2 have been tested in clinical trials since 2016 (Table 16.1), including the aforementioned tazemetostat (compound **1**), GSK126 (Figure 16.4), valemestostat (compound **4**), liramerestostat (CPI-1205, compound **5**), CPI-0209, PF-06821497 (compound **12**), SH2554 (compound **6**), MAK683 (compound **23**), and HH2853 (compound **10**). The most clinically advanced of these, tazemetostat, was approved in 2020 to treat metastatic or locally advanced epithelioid sarcoma and relapsed/refractory (R/R) lymphoma [111–113]. It is currently being studied in multiple tumor types, including mesothelioma, nerve sheath tumors, and rhabdoid tumors (Table 16.2).

11.1 Tazemetostat: approved indications

As previously discussed (see Section 4), INI1, encoded by SMARCB1, is a subunit of the SWI/SNF chromatin-remodeling complex that opposes the enzymatic function of EZH2, and loss of

TABLE 16.1 EZH2 inhibitors in clinical development.

Compound	Developer	MOA	Status
Tazemetostat (Tazverik)	Epizyme	Inhibits mutant and wild-type EZH2	<ul style="list-style-type: none"> Approved: epithelioid sarcoma Approved: relapsed/refractory follicular lymphoma Under investigation: solid tumors and hematologic malignancies
GSK126	GSK	Inhibits mutant and wild-type EZH2	Terminated; results available
Valemetostat	Daiichi-Sankyo	Inhibits EZH1 and EZH2	Ongoing; results available
Liramestat (CPI-1205)	Constellation/ MorphoSys	Inhibits mutant and wild-type EZH2	Terminated; results available
CPI-0209	Constellation/ MorphoSys	Inhibits EZH1 and EZH2	Ongoing; results available
PF-06821497	Pfizer	EZH2	Ongoing
SH2554	Shire	EZH2	Ongoing; results available
MAK683	Novartis	EED inhibitor	Ongoing; results available
HH2853	Haihe Biopharma	Inhibits mutant and wild-type EZH1 and EZH2	Ongoing

INI1 expression in tumors leads to unopposed, constitutive activation and oncogenic dependence on EZH2 [114]. Epithelioid sarcoma is a rare and aggressive tumor that is characterized by the loss of INI1 expression and/or *SMARCB1* activation, leading to dysregulation of EZH2 activity and promoting tumorigenesis [114].

Tazemetostat is an orally available inhibitor of wild-type (wt) and mutated (mut) EZH2, displaying nanomolar potency and 35-fold selectivity over EZH1. Tazemetostat was granted accelerated approval in epithelioid sarcoma in January 2020 based on the results from a single-arm (cohort 5, $n = 61$) of the phase 2 EZH-202 study in patients (16 years or older) with documented loss of INI1 expression or *SMARCB1* alterations or R/R synovial sarcoma [115]. Efficacy was measured as overall response rate (ORR) and duration of response (DOR). Objective responses were observed in nine patients (ORR 15%), with one patient having a complete response. Median DOR was not reached; however, 67% of responding patients had responses lasting 6 months or longer [115]. Median progression-free survival (PFS) and overall survival (OS) were 5.5 months and 10 months, respectively, with better responses observed in the treatment-naïve population [115]. The majority of adverse events (AEs) occurring during tazemetostat treatment were of grade 1 or 2 severity [115]. The most common grade ≥ 3 treatment-related AEs included anemia (6%) and weight loss (3%) [115]. Serious treatment-related AEs occurred in 3% of patients [115]. A phase 3 study of tazemetostat in combination with doxorubicin as first-line treatment for advanced epithelioid sarcoma is currently ongoing at the time of preparing this review (NCT04204941).

EZH2 mutations are present in approximately 20% of DLBCL and 12% of FL [116,117]. As previously described (see Section 3), EZH2 is required to maintain the developmental integrity of B cells, and activating mutations in, or overexpression of EZH2 that result in the maintenance of the germinal center may act as an oncolytic driver in a subset of patients [116,117]. Preclinical

TABLE 16.2 Ongoing clinical studies of tazemetostat (*excludes investigator-sponsored research*).

NCT number	Sponsor/collaborators	Title	Conditions	Enrollment	Phases	Status
NCT02875548	Epizyme, Inc.	Tazemetostat Rollover Study (TRuST): An Open-Label, Rollover Study	Lymphoma, sarcoma mesothelioma, advanced solid tumors	100	Phase 1/2	Active, not recruiting
NCT05228158	Eisai Co.	A Study of Tazemetostat on Safety in Participants With Relapsed or Refractory Follicular Lymphoma With Enhancer of Zeste Homolog 2 (EZH2) Gene Mutation in Japan	Lymphoma, follicular	145		Recruiting
NCT04537715	Epizyme, Inc.	A Two-part Study to Characterize Drug-Drug Interaction Effects on Steady-State Pharmacokinetics of Oral Tazemetostat	All malignancies	42	Phase 1	Active, not recruiting
NCT05467943	Hutchison MediPharma Limited	Tazemetostat for the Treatment of Relapsed/Refractory Follicular Lymphoma	Relapsed/refractory follicular lymphoma with EZH2	39	Phase 2	Not yet recruiting
NCT04241835	Epizyme, Inc.	A Study of Oral Tazemetostat in Subjects With Moderate and Severe Hepatic Impairment With Advanced Malignancies	Hepatic impairment, advanced malignant solid tumor	24	Phase 1	Recruiting
NCT02601950	Epizyme, Inc.	A Phase II, Multicenter Study of the EZH2 Inhibitor Tazemetostat in Adult Subjects With INI1-Negative Tumors or Relapsed/Refractory Synovial Sarcoma	Malignant rhabdoid tumors, synovial sarcoma, INI1-negative tumors	250	Phase 2	Active, not recruiting
NCT04204941	Epizyme, Inc.	Tazemetostat in Combination With Doxorubicin as Frontline Therapy for Advanced Epithelioid Sarcoma	Advanced soft tissue sarcoma, advanced epithelioid sarcoma	164	Phase 3	Recruiting
NCT04762160	Epizyme, Inc. Swedish Cancer Institute	SYMPHONY-2, A Trial to Examine Combination of Tazemetostat With Rituximab in Subjects With Relapsed/Refractory Follicular Lymphoma	Follicular lymphoma	59	Phase 2	Recruiting
NCT05205252	Epizyme, Inc.	Multi Cohort Study of Tazemetostat in Combination With Various Treatments For R/R Hematologic Malignancies	Relapsed/refractory hematological malignancies	156	Phase 1/2	Recruiting

TABLE 16.2 Ongoing clinical studies of tazemetostat (*excludes investigator-sponsored research*).
Continued

NCT number	Sponsor/collaborators	Title	Conditions	Enrollment	Phases	Status
NCT04179864	Epizyme, Inc.	CELLO-1, Study of Tazemetostat With Enzalutamide or Abiraterone/Prednisone in Subjects With Castration-Resistant Prostate Cancer Who Have Not Received Chemotherapy	Metastatic prostate cancer	104	Phase 1/2	Recruiting
NCT04224493	Epizyme, Inc.	Study in Subjects With Relapsed/Refractory Follicular Lymphoma	Relapsed/refractory follicular lymphoma	540	Phase 3	Recruiting

studies have demonstrated that FL with either wild-type or mutant EZH2 are dependent on EZH2 activity [118,119]; as a result, EZH2 inhibitors represent a promising target in the treatment of FL.

In June 2020 the FDA granted tazemetostat accelerated approval for the third-line or later treatment of EZH2 mutation-positive adults with R/R FL and adult patients with R/R FL who have no satisfactory alternative treatment options, representing the first epigenetic therapy approved for FL [112] [REF]. The FDA also approved a companion diagnostic (Roche Molecular Systems) in conjunction with the tazemetostat approval [112] [REF]. The approval was based on two open-label, single-arm cohorts from the phase 1/2 E7438-G000-101 study in patients with histologically confirmed FL after at least two prior systemic therapies (NCT01897571); the first cohort enrolled patients with mutEZH2 FL (cohort 4) and the second with wtEZH2 FL (cohort 5) [117]. Efficacy was generally better in the mutEZH2 patients, with an observed ORR of 69% and DOR of 10.9 months (cohort 4) compared with an ORR of 35% and DOR of 13.0 months in the wtEZH2 cohort (cohort 5) [117]. Median PFS in the mutant and wild-type populations were 13.8 months and 11.1 months, respectively [117]. Treatment-related AEs occurred in 81% of patients, with the most common being thrombocytopenia (3%), neutropenia (3%), and anemia (2%). Four percent of patients experienced serious treatment-related AEs events [117].

In 2018 the FDA halted enrollment in trials of tazemetostat for 5 months after a pediatric patient enrolled in the phase 1 pediatric study (NCT02601937) developed a secondary T-cell lymphoma [120]. Long-term inhibition of EZH2 resulting in loss of function can be pathogenic for MDS or MPNs, and low expression of EZH2 in patients with MDS is associated with decreased survival and increased acute myeloid leukemia (AML) transformation, although the mechanism(s) of these effects is not clear [121–123]. Additional evidence that the development of secondary T-cell lymphoma may be a class effect of EZH2 inhibitors comes from 13-week repeat-dose toxicity studies in rats receiving tazemetostat or two structurally similar, but chemically distinct EZH2 inhibitors [124]. In these experiments, all three inhibitors were associated with multiple cases of T-cell lymphoblastic lymphoma (T-LBL) [124]. Further analysis of all patients treated with tazemetostat ($n = 725$ at the time of the ODAC) found that six (0.8%) developed secondary malignancies. Because of the clinical and preclinical observations, tazemetostat carries a boxed warning for secondary malignancies, including T-LBL and T-cell acute lymphoblastic leukemia, as well as MDS and AML.

11.2 Tazemetostat: ongoing trials

Preliminary results of combination studies of tazemetostat with atezolizumab or R-CHOP (combination of rituximab, cyclophosphamide, hydroxydaunorubicin hydrochloride, vincristine, and prednisone) in R/R DLBCL and lenalidomide in R/R FL have been reported.

An ongoing phase 1b/3 study of tazemetostat in combination with R² (rituximab + lenalidomide) in pretreated patients with R/R FL (SYMPHONY-1; EZH-301; NCT04224493) has yielded impressive preliminary results [125,126]. In 44 patients treated with tazemetostat + R² (400 mg [$n = 4$], 600 mg [$n = 19$], or 800 mg [$n = 21$]), 36/38 evaluable patients responded to therapy with an ORR of 95%, including a 50% complete response rate [125]. Patients with mutEZH2 ($n = 30$) and wtEZH2 ($n = 5$) had ORRs of 100% and 94%, respectively, and complete responses of 47% and 60%, respectively [125,126]. Notably, rituximab-refractory patients ($n = 13$, 36%) and progression of disease (POD) patients ($n = 10$) demonstrated an ORR of 100%, with complete responses observed in 46% and 40% of patients, respectively [125,126]. These preliminary efficacy results build on preclinical data which suggest a synergistic effect of tazemetostat and lenalidomide [127] and are especially noteworthy given the responses in historically difficult to treat rituximab-refractory and patients with POD within 24 months of initial treatment. Safety results were consistent with those reported in the prescribing information for both tazemetostat and R², respectively, with grade 3/4 treatment-emergent AEs (TEAEs) observed in 25 patients (57%) [125,126].

In contrast to FL, only modest antitumor activity has been observed with tazemetostat in patients with DLBCL. Although an exact reason for this reduced antitumor activity is unclear, it is worth noting that whereas approximately 12%–22% of FL and DLBCL cases have activating mutations in EZH2, only 8% of DLBCL cases have a gain at the *EZH2* locus on chromosome 7, compared with 24% of FL cases (and 39% of transformed FL cases) [116,128]. Tumors harboring a chromosomal gain in *EZH2* have been shown to possess significantly higher levels of EZH2 protein [128]. Although the consequence of chromosomal gains at the *EZH2* locus and increased level of EZH2 protein is not yet fully understood, it is possible that this might explain the higher efficacy observed with EZH2 inhibitors in FL.

A phase 2 study of tazemetostat as monotherapy or in combination with prednisolone in patients with DLBCL (NCT01897571) was halted in 2018 after an interim analysis concluded that the clinical activity was not sufficient to warrant further development [129]. For tazemetostat monotherapy, an ORR of 17% was observed in both mutEZH2 ($n = 36$; 1 complete response and 5 partial responses) and wtEZH2 ($n = 121$; 11 complete responses and 9 partial responses) [125]. For the combination of tazemetostat with prednisolone, the ORR was 9% ($n = 69$; 1 complete response, 5 partial responses) [125]. Adverse events were consistent with the label, with 27% of patients reporting grade ≥ 3 treatment-related adverse events.

In another phase 1b study (NCT02220842), R/R DLBCL patients ($n = 43$) were treated with a combination of tazemetostat (800 mg) and atezolizumab (1200 mg IV every 3 weeks) [130]. In a heavily pretreated population (median 3 prior lines of therapy), the ORR was 16%, PFS was 1.9 months, and OS was 13 months [130]. An analysis of EZH2 mutation status in 28 patients revealed a correlation between higher responses and EZH2 mutation, with a 75% response rate in patients with mutEZH2 and no observed responders in patients with wtEZH2 [130]. Adverse events were consistent with the label, with 95% of patients reporting ≥ 1 AE, 49% reporting grade ≥ 3 AEs, and 40% reporting serious AEs [130]. Reduced effectiveness of Tregs resulting from altered

expression of FOXP3 has been associated with EZH2 inhibition [131]. However, Treg reduction and association of FOXP3 expression with tumor shrinkage was not observed [130].

Increased expression of EZH2 in association with loss of BAP1 has been identified in malignant pleural mesothelioma, and BAP1 loss of function is known to sensitize tumor cells to EZH2 inhibition [37]. As discussed previously (see Section 2), Pcg proteins function as tumor suppressors by maintaining gene silencing and preserving cell-fate determination and stem cell pluripotency [37]. BAP1 is a member of the Pcg and loss of BAP1 function leads to increases in H3K27 trimethylation and EZH2-dependent transformation [37]. Unfortunately, despite positive preclinical results, tazemetostat has demonstrated minimal activity in malignant pleural mesothelioma patients.

In an open-label, single-arm phase 2 study (NCT02860286), 61 patients with R/R malignant pleural mesothelioma who had received treatment with 1 pemetrexed-containing regimen received oral tazemetostat [132]. Responses were unimpressive, with an ORR of 5% (no complete responses; two partial responses), disease control rate of 54% (primary endpoint), and PFS and OS of 18 weeks and 36 weeks, respectively [132]. TEAEs occurred in 99% of patients and were consistent with those reported in the prescribing information for tazemetostat [132]. Forty-nine percent of patients experienced grade ≥ 3 TEAEs and SAEs occurred in 34% of patients [132].

In addition to epithelioid sarcomas, loss of INI1 expression is a defining characteristic of malignant rhabdoid tumors and poorly differentiated chordomas [133]. In a phase 1 study of 109 patients of 16 years of age and older with R/R INI1-negative tumors treated with tazemetostat (1200 mg/m^2 twice daily), an ORR of 14% was observed in dose expansion, with higher responses reported in poorly differentiated chordomas (33%), atypical teratoid rhabdoid tumor (24%), and epithelioid sarcoma (23%) [133]. Of note, neutrophil counts at cycle 1 day 1 were correlated with response to tazemetostat therapy, with 100% of responders having less than 125,000 total neutrophils at cycle 1 day 1 versus 42% of nonresponders [133]. Grade ≥ 3 AEs were observed in 22% of patients in the dose expansion and were consistent with the label [133].

11.3 Other EZH2 inhibitors

In addition to tazemetostat, eight other EZH2 inhibitors have entered the clinic and preliminary results for six of these have been published (Table 16.1).

As discussed previously (see Section 3), EZH2 overexpression has been identified in a wide variety of solid tumors, including breast, prostate, and lung cancers. Notably, EZH2 overexpression correlates with increased tumor aggressiveness and poor prognosis [134]. Additionally, EZH2 expression in the tumor microenvironment can indirectly promote cancer progression by stimulating tumor-associated fibroblasts to produce proangiogenic factors [134]. Unfortunately, with the exception of certain sarcomas, EZH2 inhibitors have not demonstrated appreciable responses in solid tumors.

GSK126 (see Figure 16.4) is a potent and selective inhibitor of both mutEZH2 and wtEZH2 with a 150-fold selectivity over EZH1 [135]. GSK126 is administered intravenously twice a week in a 3-week-on/1-week-off, 28-day cycle. A 2-part phase 1 study (NCT02082977) consisting of dose-escalation and dose-expansion phases was initially planned; however, the study was terminated early because of dosing challenges and lack of a therapeutic window; consequently, the dose-expansion phase was not initiated [135]. Twenty-one patients with stage III or IV solid tumors and 20 patients with lymphomas were enrolled. In patients with solid tumors, no partial or complete

responses were observed and 38% had stable disease [135]. In patients with lymphoma, one patient achieved a partial response and 30% had stable disease. All patients experienced an AE on study, with 32% experiencing SAEs [135]. The most frequent treatment-related AEs were fatigue (49%) and nausea (39%) [135]. Despite promising preclinical results, GSK126 was terminated because of the limitation of twice-weekly intravenous dosing and ineffective biological exposure at tolerated doses encountered in this trial [135].

Similar to the function in B cells, EZH2 catalyzes H3K27 trimethylation in T cells to function as an epigenetic repressor by protecting downstream gene expression [116]. Of note, EZH2 depletion in T lymphocytes has been shown to disrupt genomic transcriptome and gene bivalent states, resulting in cell death via apoptosis [116].

Valemetostat (compound **4**) [56] is an oral, potent, and selective inhibitor of EZH1 and EZH2. A pivotal phase 2 study (VALENTINE-PTCL01; NCT04703192) in 25 Japanese patients with R/R adult T-cell leukemia/lymphoma revealed a disease control rate of 88% and ORR of 48%, with 5 patients achieving a complete response and 7 achieving partial responses [136]. The median DOR was not reached (median follow-up 6.5 months). Sixty percent of patients experienced grade ≥ 3 TEAEs, the most common of which were platelet count decrease (80%), anemia (48%), alopecia (40%), and dysgeusia (36%) [136]. Based on these results, a new drug application for valemetostat was submitted in Japan in December 2021 [136].

EZH2 has been shown to act as a transcriptional activator in castration-resistant prostate cancer (CRPC) [137]. Lirametostat (CPI-1205; compound **5**) is an oral, potent, reversible, cofactor-competitive, small molecule inhibitor of EZH2 and is the first EZH2 inhibitor to report clinical results in advanced, metastatic CRPC patients enrolled in the phase 1b/2 ProSTAR trial (NCT03480646) [138]. In the phase 1b portion, CPI-1205 was tested in combination with abiraterone ($n = 20$) or enzalutamide ($n = 16$) in heavily pretreated patients [138]. Patients treated with CPI-1205 in combination with abiraterone achieved a disease control rate of 75% (six patients with stable disease, no complete or partial responders), and patients treated with CPI-1205 in combination with enzalutamide achieved a disease control rate of 60% (one partial response, two stable disease, no complete responses) [138]. Twenty-four percent of patients achieved a $\geq 80\%$ reduction in PSA level (20% in the abiraterone arm and 27% in the enzalutamide arm) [138], all of which were observed in AR splice variant 7 (AR-V7)-negative patients. This is consistent with clinical data showing that AR-V7 patients typically have better responses to AR inhibitors than those with AR-V7 isoforms. Owing to the lack of definitive activity in metastatic CRPC, development of lirametostat was discontinued in 2021 [139].

CPI-0209 (compound **5**) [54] is an oral, second-generation, selective inhibitor of EZH2 that exhibits more potent preclinical activity compared with first-generation inhibitors [140]. As a second-generation inhibitor, CPI-0209 was designed to possess increased potency, longer resistance time on target, and lack self-induced metabolism [140]. Preliminary results for heavily pretreated patients have been reported from a phase 1/2 study in advanced solid tumors ($n = 40$; NCT04104776) [140]. Efficacy was evaluated in four patients with BAP1 loss mesothelioma. The disease control rate was 100% (one partial response, three stable disease) [140]. Among all 40 patients enrolled, 43% experienced grade ≥ 3 TEAEs and 28% of patients had ≥ 1 SAE [140]. The most common TEAEs included thrombocytopenia (reversible and dose dependent), diarrhea, asthenic conditions, nausea, anemia, dysgeusia, abdominal pain, and alopecia [140].

SHR2554 (compound **6**) [57] is a potent and selective oral inhibitor of both mutEZH2 and wtEZH2, with a 22-fold selectivity over EZH1 [141]. In a phase 1 study of 113 Chinese patients with R/R, mature lymphoid neoplasms (NCT03603951), 43% of patients achieved an overall response to therapy, with an estimated DOR of 9.3 months [141]. Notably, of the patients with FL, 59% of patients achieved an overall response, with an estimated DOR of 9.3 months [141]. Subanalysis looking at the EZH2 mutation status of patients with FL revealed slightly higher responses for patients with an EZH2 mutation (63% vs 55% for wtEZH2) and a slightly lower duration in the mutEZH2 population (5.6 months vs 11.2 for wtEZH2) [141]. Thirty-four percent of patients experienced grade ≥ 3 treatment-related AEs, the most common of which were decreased platelet count (18%), decreased neutrophil count (9%), decreased white blood cell count (8%), and anemia (7%) [141]. Sixteen percent of patients experienced serious treatment-related AEs, the most common of which were decreased platelet count (5%) and pneumonia (3%) [141]. Two percent ($n = 2$) patients had treatment-related deaths, including from skin infection and toxic epidermal necrolysis and respiratory failure [141].

As described previously (see Section 2), EED is a catalytic subunit of PRC2 that, like EZH2, acts as a histone methyltransferase for H3K27 [81]. Currently, one EED inhibitor, MAK683 (compound **23**), has entered clinical development. MAK683 is a potent, oral inhibitor of EED that displays inhibitory effects on the methyltransferase activity of EZH1 and both mutEZH2 and wtEZH2 [81]. Preliminary results for 31 heavily pretreated DLBCL patients in a phase 1/2 study (NCT02900651) demonstrated a ORR of 16% (2 complete responses, 3 partial responses) [142]. Sixty-eight percent of patients experienced treatment-related AEs, most commonly thrombocytopenia (29%) and anemia (23%) [142]. Forty-five percent of patients experienced grade ≥ 3 treatment-related AEs, most commonly being thrombocytopenia (19%), neutropenia (16%), and decreased neutrophil count (16%) [142].

12 Resistance mechanisms to EZH2 inhibition

Two primary resistance mechanisms for EZH2 inhibitors have been reported: (1) acquired mutations to mutEZH2 and wtEZH2 that interfere with drug-target binding and (2) activation of survival pathways, including those mediated by PI3K, MEK, and IGF1R [143–145]. Preclinical studies have demonstrated that the specific mutations and pathways involved in EZH2 inhibitor resistance can vary based on experimental conditions, including the choice of cell line and inhibitor [145]. Additional studies are necessary to determine the conditions under which these resistance mechanisms arise in patients and the best strategies to combat EZH2 inhibitor resistance in liquid and solid tumors.

13 Conclusions

Owing to its role in silencing gene transcription, PcG has emerged as a desirable target in oncology. EZH2 is a catalytic subunit of PRC2, and catalytic inhibitors of PRC2/EZH2 activity represent the most significant progress toward inhibition of PcG. Overexpression of EZH2 has been reported in a variety of solid and liquid tumors and is typically correlated with a worse prognosis. However,

inhibition of EZH2 is complicated by a dichotomous role, with alterations in EZH2 activity acting to promote or block oncogenesis depending on the cellular context. Current EZH2 inhibitors have shown limited clinical activity outside FL and certain sarcomas. Nonetheless, following the approval of tazemetostat in 2020, different strategies to affect PRC2 activity beyond EZH2 catalytic inhibition have emerged. In particular, promising preclinical activity has been observed with second-generation inhibitors beyond lymphoma and in combination with immune modulators. Despite the challenging complexity of targeting EZH2, this next class of inhibitors promises to extend on the current success of tazemetostat, potentially bringing a new class of therapy to patients with solid and liquid tumors with high unmet medical needs.

References

- [1] Pande V, Pocalyko DJ, Dhanak D. Chapter 19 – Emerging epigenetic therapies—lysine methyltransferase/PRC complex inhibitors. In: Gray SG, editor. *Epigenetic cancer therapy*. Boston: Academic Press; 2015, p. 427–37.
- [2] Kim KH, Roberts CWM. Targeting EZH2 in cancer. *Nat Med* 2016;22:128–34.
- [3] Holoch D, Margueron R. Mechanisms regulating PRC2 recruitment and enzymatic activity. *Trends Biochem Sci* 2017;42:531–42.
- [4] Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. *Nature* 2011;469:343–9.
- [5] Simon JA, Kingston RE. Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat Rev Mol Cell Biol* 2009;10:697–708.
- [6] Zee BM, Levin RS, Xu B, LeRoy G, Wingreen NS, Garcia BA. In vivo residue-specific histone methylation dynamics. *J Biol Chem* 2010;285:3341–50.
- [7] Margueron R, Li G, Sarma K, Blais A, Zavadil J, Woodcock CL, et al. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. *Mol Cell* 2008;32:503–18.
- [8] Vidal M, Starowicz K. Polycomb complexes PRC1 and their function in hematopoiesis. *Exp Hematol* 2017;48:12–31.
- [9] Eich ML, Athar M, Ferguson 3rd JE, Varambally S. EZH2-targeted therapies in cancer: hype or a reality. *Cancer Res* 2020;80:5449–58.
- [10] Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 2002;419:624–9.
- [11] Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. *J Clin Oncol* 2006;24:268–73.
- [12] Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J* 2003;22:5323–35.
- [13] Sauvageau M, Sauvageau G. Polycomb group proteins: multi-faceted regulators of somatic stem cells and cancer. *Cell Stem Cell* 2010;7:299–313.
- [14] Bödör C, O’Riain C, Wrench D, Matthews J, Iyengar S, Tayyib H, et al. EZH2 Y641 mutations in follicular lymphoma. *Leukemia* 2011;25:726–9.
- [15] Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet* 2010;42:181–5.
- [16] McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature* 2012;492:108–12.

- [17] Majer CR, Jin L, Scott MP, Knutson SK, Kuntz KW, Keilhack H, et al. A687V EZH2 is a gain-of-function mutation found in lymphoma patients. *FEBS Lett* 2012;586:3448–51.
- [18] Gui Y, Guo G, Huang Y, Hu X, Tang A, Gao S, et al. Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder. *Nat Genet* 2011;43:875–8.
- [19] Pugh TJ, Weeraratne SD, Archer TC, Pomeranz Krummel DA, Auclair D, Bochicchio J, et al. Medulloblastoma exome sequencing uncovers subtype-specific somatic mutations. *Nature* 2012;488:106–10.
- [20] Waddell N, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, et al. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 2015;518:495–501.
- [21] Rinke J, Chase A, Cross NCP, Hochhaus A, Ernst T. EZH2 in myeloid malignancies. *Cells* 2020;9:1639.
- [22] Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet* 2010;42:722–6.
- [23] Nikoloski G, Langemeijer SMC, Kuiper RP, Knops R, Massop M, Tonnissen ER, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet* 2010;42:665–7.
- [24] Shimizu T, Kubovcakova L, Nienhold R, Zmajkovic J, Meyer SC, Hao-Shen H, et al. Loss of Ezh2 synergizes with JAK2-V617F in initiating myeloproliferative neoplasms and promoting myelofibrosis. *J Exp Med* 2016;213:1479–96.
- [25] Huang J, Gou H, Yao J, Yi K, Jin Z, Matsuoka M, et al. The noncanonical role of EZH2 in cancer. *Cancer Sci* 2021;112:1376–82.
- [26] Shi B, Liang J, Yang X, Wang Y, Zhao Y, Wu H, et al. Integration of estrogen and Wnt signaling circuits by the polycomb group protein EZH2 in breast cancer cells. *Mol Cell Biol* 2007;27:5105–19.
- [27] Lee ST, Li Z, Wu Z, Aau M, Guan P, Karuturi RK, et al. Context-specific regulation of NF- κ B target gene expression by EZH2 in breast cancers. *Mol Cell* 2011;43:798–810.
- [28] Kim J, Lee Y, Lu X, Song B, Fong KW, Cao Q, et al. Polycomb- and methylation-independent roles of EZH2 as a transcription activator. *Cell Rep* 2018;25(2808–2820):e2804.
- [29] Lee JM, Lee JS, Kim H, Kim K, Park H, Kim JY, et al. EZH2 generates a methyl degron that is recognized by the DCAF1/DDB1/CUL4 E3 ubiquitin ligase complex. *Mol Cell* 2012;48:572–86.
- [30] Kim E, Kim M, Woo DH, Shin Y, Shin J, Chang N, et al. Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. *Cancer Cell* 2013;23:839–52.
- [31] Zheng M, Cao MX, Luo XJ, Li L, Wang K, Wang SS, et al. EZH2 promotes invasion and tumour glycolysis by regulating STAT3 and FoxO1 signalling in human OSCC cells. *J Cell Mol Med* 2019;23:6942–54.
- [32] Kadoc C, Hargreaves DC, Hodges C, Elias L, Ho L, Ranish J, et al. Proteomic and bioinformatic analysis of mammalian SWI/SNF complexes identifies extensive roles in human malignancy. *Nat Genet* 2013;45:592–601.
- [33] Wilson BG, Wang X, Shen X, McKenna ES, Lemieux ME, Cho YJ, et al. Epigenetic antagonism between polycomb and SWI/SNF complexes during oncogenic transformation. *Cancer Cell* 2010;18:316–28.
- [34] Kadoc C, Williams RT, Calarco JP, Miller EL, Weber CM, Braun SM, et al. Dynamics of BAF-Polycomb complex opposition on heterochromatin in normal and oncogenic states. *Nat Genet* 2017;49:213–22.
- [35] Bitler BG, Aird KM, Garipov A, Li H, Amatangelo M, Kossenkov AV, et al. Synthetic lethality by targeting EZH2 methyltransferase activity in ARID1A-mutated cancers. *Nat Med* 2015;21:231–8.
- [36] Ezponda T, Dupere-Richer D, Will CM, Small EC, Varghese N, Patel T, et al. UTX/KDM6A loss enhances the malignant phenotype of multiple myeloma and sensitizes cells to EZH2 inhibition. *Cell Rep* 2017;21:628–40.
- [37] LaFave LM, Beguelin W, Koche R, Teater M, Spitzer B, Chramiec A, et al. Loss of BAP1 function leads to EZH2-dependent transformation. *Nat Med* 2015;21:1344–9.
- [38] Ratz L, Brambillasca C, Bartke L, Huetzen MA, Goergens J, Leidecker O, et al. Combined inhibition of EZH2 and ATM is synthetic lethal in BRCA1-deficient breast cancer. *Breast Cancer Res* 2022;24:41.

- [39] Peng D, Kryczek I, Nagarsheth N, Zhao L, Wei S, Wang W, et al. Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy. *Nature* 2015;527:249–53.
- [40] Nagarsheth N, Peng D, Kryczek I, Wu K, Li W, Zhao E, et al. PRC2 epigenetically silences Th1-type chemokines to suppress effector T-cell trafficking in colon cancer. *Cancer Res* 2016;76:275–82.
- [41] Burr ML, Sparbier CE, Chan KL, Chan YC, Kersbergen A, Lam EYN, et al. An evolutionarily conserved function of polycomb silences the MHC Class I antigen presentation pathway and enables immune evasion in cancer. *Cancer Cell* 2019;36(385–401):e388.
- [42] Holling TM, Bergevoet MW, Wilson L, Van Eggemon MC, Schooten E, Steenbergen RD, et al. A role for EZH2 in silencing of IFN-gamma inducible MHC2TA transcription in uveal melanoma. *J Immunol* 2007;179:5317–25.
- [43] Morel KL, Sheahan AV, Burkhardt DL, Baca SC, Boufaied N, Liu Y, et al. EZH2 inhibition activates a dsRNA-STING-interferon stress axis that potentiates response to PD-1 checkpoint blockade in prostate cancer. *Nat Cancer* 2021;2:444–56.
- [44] Chen G, Subedi K, Chakraborty S, Sharov A, Lu J, Kim J, et al. Ezh2 regulates activation-induced CD8 (+) T cell cycle progression via repressing Cdkn2a and Cdkn1c expression. *Front Immunol* 2018;9:549.
- [45] Qi W, Zhao K, Gu J, Huang Y, Wang Y, Zhang H, et al. An allosteric PRC2 inhibitor targeting the H3K27me3 binding pocket of EED. *Nat Chem Biol* 2017;13:381–8.
- [46] Wang D, Quiros J, Mahuron K, Pai CC, Ranzani V, Young A, et al. Targeting EZH2 reprograms intratumoral regulatory T cells to enhance cancer immunity. *Cell Rep* 2018;23:3262–74.
- [47] Gabrilovich DI, Bronte V, Chen SH, Colombo MP, Ochoa A, Ostrand-Rosenberg S, et al. The terminology issue for myeloid-derived suppressor cells. *Cancer Res* 2007;67:425 author reply 426.
- [48] Huang S, Wang Z, Zhou J, Huang J, Zhou L, Luo J, et al. EZH2 inhibitor GSK126 suppresses antitumor immunity by driving production of myeloid-derived suppressor cells. *Cancer Res* 2019;79:2009–20.
- [49] Kasinath V, Faini M, Poepsel S, Reif D, Feng XA, Stjepanovic G, et al. Structures of human PRC2 with its cofactors AEBP2 and JARID2. *Science* 2018;359:940–4.
- [50] Fioravanti R, Stazi G, Zwergel C, Valente S, Mai A. Six years (2012–2018) of researches on catalytic EZH2 inhibitors: the boom of the 2-pyridone compounds. *Chem Rec* 2018;18:1818–32.
- [51] Stazi G, Zwergel C, Mai A, Valente S. EZH2 inhibitors: a patent review (2014–2016). *Expert Opin Therapeutic Pat* 2017;27:797–813.
- [52] Knutson SK, Wigle TJ, Warholic NM, Sneeringer CJ, Allain CJ, Klaus CR, et al. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. *Nat Chem Biol* 2012;8:890–6.
- [53] Qi W, Chan H, Teng L, Li L, Chuai S, Zhang R, et al. Selective inhibition of Ezh2 by a small molecule inhibitor blocks tumor cells proliferation. *Proc Natl Acad Sci U S A* 2012;109:21360–5.
- [54] Vaswani RG, Gehling VS, Dakin LA, Cook AS, Nasveschuk CG, Duplessis M, et al. Identification of (R)-N-((4-Methoxy-6-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-2-methyl-1-(1-(2,2,2-trifluoroethyl)piperidin-4-yl)ethyl-1H-indole-3-carboxamide (CPI-1205), a potent and selective inhibitor of histone methyltransferase EZH2, suitable for phase I clinical trials for B-cell lymphomas. *J Med Chem* 2016;59:9928–41.
- [55] Bratkowski M, Yang X, Liu X. An evolutionarily conserved structural platform for PRC2 inhibition by a class of Ezh2 inhibitors. *Sci Rep* 2018;8:9092.
- [56] Yamagishi M, Hori M, Fujikawa D, Ohsugi T, Honma D, Adachi N, et al. Targeting excessive EZH1 and EZH2 activities for abnormal histone methylation and transcription network in malignant lymphomas. *Cell Rep* 2019;29(2321–2337):e2327.
- [57] Song Y, Liu Y, Li ZM, Li L, Su H, Jin Z, et al. SHR2554, an EZH2 inhibitor, in relapsed or refractory mature lymphoid neoplasms: a first-in-human, dose-escalation, dose-expansion, and clinical expansion phase 1 trial. *Lancet Haematol* 2022;9:e493–503.
- [58] Chen X, Chen Y, Huang Y, Geng M, Zhang Q, Ding J, et al. Indolizine compounds, preparation method and use thereof. Shanghai Haihe Pharmaceutical Co Ltd; Shanghai Inst Materia Medica, Chinese Academy of Sciences; 2019.

- [59] Kung PP, Bingham P, Brooun A, Collins M, Deng YL, Dinh D, et al. Optimization of orally bioavailable enhancer of zeste homolog 2 (EZH2) inhibitors using ligand and property-based design strategies: identification of development candidate (R)-5,8-dichloro-7-(methoxy(oxetan-3-yl)methyl)-2-((4-methoxy-6-methyl-2-oxo-1,2- dihydropyridin-3-yl)methyl)-3,4-dihydroisoquinolin-1(2H)-one (PF-06821497). *J Med Chem* 2018;61:650–65.
- [60] Chen X, Geng M, Jiang L, Chen Y, Cao J, Jiang Q, et al. Pyrido five-element aromatic ring compound, preparation method therefor and use thereof. Shanghai Haihe Pharmaceutical Co Ltd; Shanghai Inst Materia Medica, Chinese Academy of Sciences; 2018.
- [61] Jung SH, Hong D, Hwang J, Park S, Byun J, Lee M, et al. Abstract 1142: a novel and potent EZH1/2 dual inhibitor, HM97662, demonstrates antitumor activity in malignant tumors. *Cancer Res* 2021;81:1142.
- [62] Paskeh MDA, Mehrabi A, Gholami MH, Zabolian A, Ranjbar E, Saleki H, et al. EZH2 as a new therapeutic target in brain tumors: molecular landscape, therapeutic targeting and future prospects. *Biomed Pharmacother* 2022;146:112532.
- [63] Liang R, Tomita D, Sasaki Y, Ginn J, Michino M, Huggins DJ, et al. A chemical strategy toward novel brain-penetrant EZH2 inhibitors. *ACS Med Chem Lett* 2022;13:377–87.
- [64] Khanna A, Cote A, Arora S, Moine L, Gehling VS, Brenneman J, et al. Design, synthesis, and pharmacological evaluation of second generation EZH2 inhibitors with long residence time. *ACS Med Chem Lett* 2020;11:1205–12.
- [65] Van Aller GS, Pappalardi MB, Ott HM, Diaz E, Brandt M, Schwartz BJ, et al. Long residence time inhibition of EZH2 in activated polycomb repressive complex 2. *ACS Chem Biol* 2014;9:622–9.
- [66] Stuckey JI, Cantone NR, Cote A, Arora S, Vivat V, Ramakrishnan A, et al. Identification and characterization of second-generation EZH2 inhibitors with extended residence times and improved biological activity. *J Biol Chem* 2021;296:100349.
- [67] Côté A, Khanna A, Moine L. Modulators of methyl modifying enzymes, compositions and uses thereof. Constellation Pharmaceuticals, Inc; 2019.
- [68] Bradley W. Ezh2 inhibition in combination therapies for the treatment of cancers. Constellation Pharmaceuticals, Inc; 2021.
- [69] Dai X, Dore M, Gu X-J, Li L, Liu K, Mak SY, et al. Quinoline compounds and compositions for inhibiting Ezh2. Dai, Xuan: Novartis AG; 2022.
- [70] Brooun A, Gajiwala KS, Deng YL, Liu W, Bolanos B, Bingham P, et al. Polycomb repressive complex 2 structure with inhibitor reveals a mechanism of activation and drug resistance. *Nat Commun* 2016;7:11384.
- [71] Justin N, Zhang Y, Tarricone C, Martin SR, Chen S, Underwood E, et al. Structural basis of oncogenic histone H3K27M inhibition of human polycomb repressive complex 2. *Nat Commun* 2016;7:11316.
- [72] Sanulli S, Justin N, Teissandier A, Ancelin K, Portoso M, Caron M, et al. Jarid2 methylation via the PRC2 complex regulates H3K27me3 deposition during cell differentiation. *Mol Cell* 2015;57:769–83.
- [73] Margueron R, Justin N, Ohno K, Sharpe ML, Son J, Drury 3rd WJ, et al. Role of the polycomb protein EED in the propagation of repressive histone marks. *Nature* 2009;461:762–7.
- [74] He Y, Selvaraju S, Curtin ML, Jakob CG, Zhu H, Comess KM, et al. Erratum: the EED protein-protein interaction inhibitor A-395 inactivates the PRC2 complex. *Nat Chem Biol* 2017;13:922.
- [75] He Y, Selvaraju S, Curtin ML, Jakob CG, Zhu H, Comess KM, et al. The EED protein-protein interaction inhibitor A-395 inactivates the PRC2 complex. *Nat Chem Biol* 2017;13:389–95.
- [76] Huang Y, Zhang J, Yu Z, Zhang H, Wang Y, Lingel A, et al. Discovery of first-in-class, potent, and orally bioavailable embryonic ectoderm development (EED) Inhibitor with robust anticancer efficacy. *J Med Chem* 2017;60:2215–26.
- [77] Marx M, Ketcham J, Burns A. Imidazo[1,2-C]pyrimidine derivatives as Prc2 inhibitors for treating cancer. Mirati Therapeutics, Inc; 2020.

- [78] Efremov IV, Kazmirski S, Li Q, Thompson I, Wallace OB, Johnstone SD, et al. Macrocyclic azolopyridine derivatives as Eed and Prc2 modulators. Fulcrum Therapeutics, Inc; 2020.
- [79] Rej RK, Wang C, Lu J, Wang M, Petrunak E, Zawacki KP, et al. EEDI-5285: an exceptionally potent, efficacious, and orally active small-molecule inhibitor of embryonic ectoderm development. *J Med Chem* 2020;63:7252–67.
- [80] Wang S, Rej R, Wang C, Wang M, Lu J, Yang C-Y, et al. Preparation of imidazopyrimidines as EED inhibitors and the use thereof. The Regents of the University of Michigan; 2021.
- [81] Huang Y, Sendzik M, Zhang J, Gao Z, Sun Y, Wang L, et al. Discovery of the clinical candidate MAK683: an EED-directed, allosteric, and selective PRC2 inhibitor for the treatment of advanced malignancies. *J Med Chem* 2022;65:5317–33.
- [82] Novartis Pharmaceuticals. A phase I/II, multicenter, open-label study of MAK683 in adult patients with advanced malignancies. <[clinicaltrials.gov](#)>; 2022.
- [83] Kim W, Bird GH, Neff T, Guo G, Kerenyi MA, Walensky LD, et al. Targeted disruption of the EZH2-EED complex inhibits EZH2-dependent cancer. *Nat Chem Biol* 2013;9:643–50.
- [84] Tomassi S, Romanelli A, Zwergerl C, Valente S, Mai A. Polycomb repressive complex 2 modulation through the development of EZH2-EED interaction inhibitors and EED binders. *J Med Chem* 2021;64:11774–97.
- [85] Kong X, Chen L, Jiao L, Jiang X, Lian F, Lu J, et al. Astemizole arrests the proliferation of cancer cells by disrupting the EZH2-EED interaction of polycomb repressive complex 2. *J Med Chem* 2014;57:9512–21.
- [86] Chen H, Gao S, Li J, Liu D, Sheng C, Yao C, et al. Wedelolactone disrupts the interaction of EZH2-EED complex and inhibits PRC2-dependent cancer. *Oncotarget* 2015;6:13049–59.
- [87] Du D, Xu D, Zhu L, Stazi G, Zwergerl C, Liu Y, et al. Structure-guided development of small-molecule PRC2 inhibitors targeting EZH2-EED interaction. *J Med Chem* 2021;64:8194–207.
- [88] Ciechanover A, Elias S, Heller H, Ferber S, Hershko A. Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. *J Biol Chem* 1980;255:7525–8.
- [89] Hershko A, Eytan E, Ciechanover A, Haas AL. Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. Relationship to the breakdown of abnormal proteins. *J Biol Chem* 1982;257:13964–70.
- [90] Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* 1983;258:8206–14.
- [91] Sakamoto KM, Kim KB, Kumagai A, Mercurio F, Crews CM, Deshaies RJ. Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *Proc Natl Acad Sci U S A* 2001;98:8554–9.
- [92] Burslem GM, Crews CM. Small-molecule modulation of protein homeostasis. *Chem Rev* 2017;117:11269–301.
- [93] Pettersson M, Crews CM. PROteolysis TArgeting Chimeras (PROTACs)—past, present and future. *Drug Discov Today Technol* 2019;31:15–27.
- [94] Schapira M, Calabrese MF, Bullock AN, Crews CM. Targeted protein degradation: expanding the toolbox. *Nat Rev Drug Discov* 2019;18:949–63.
- [95] Ma A, Stratikopoulos E, Park KS, Wei J, Martin TC, Yang X, et al. Discovery of a first-in-class EZH2 selective degrader. *Nat Chem Biol* 2020;16:214–22.
- [96] Wang C, Chen X, Liu X, Lu D, Li S, Qu L, et al. Discovery of precision targeting EZH2 degraders for triple-negative breast cancer. *Eur J Med Chem* 2022;238:114462.
- [97] Liu Z, Hu X, Wang Q, Wu X, Zhang Q, Wei W, et al. Design and synthesis of EZH2-based PROTACs to degrade the PRC2 complex for targeting the noncatalytic activity of EZH2. *J Med Chem* 2021;64:2829–48.

- [98] Tu Y, Sun Y, Qiao S, Luo Y, Liu P, Jiang ZX, et al. Design, synthesis, and evaluation of VHL-based EZH2 degraders to enhance therapeutic activity against lymphoma. *J Med Chem* 2021;64:10167–84.
- [99] Potjewyd F, Turner AW, Beri J, Rectenwald JM, Norris-Drouin JL, Cholensky SH, et al. Degradation of polycomb repressive complex 2 with an EED-targeted bivalent chemical degrader. *Cell Chem Biol* 2020;27(47–56):e15.
- [100] Hsu JH, Rasmusson T, Robinson J, Pachl F, Read J, Kawatkar S, et al. EED-targeted PROTACs degrade EED, EZH2, and SUZ12 in the PRC2 complex. *Cell Chem Biol* 2020;27(41–46):e17.
- [101] Yang X, Li F, Konze KD, Meslamani J, Ma A, Brown PJ, et al. Structure-activity relationship studies for enhancer of zeste homologue 2 (EZH2) and enhancer of zeste homologue 1 (EZH1) inhibitors. *J Med Chem* 2016;59:7617–33.
- [102] Bricelj A, Steinebach C, Kuchta R, Güttschow M, Sosić I. E3 ligase ligands in successful PROTACs: an overview of syntheses and linker attachment points. *Front Chem* 2021;9:707317.
- [103] Bricelj A, Dora Ng, YL, Ferber D, Kuchta R, Müller S, Monschke M, et al. Influence of linker attachment points on the stability and neosubstrate degradation of cereblon ligands. *ACS Med Chem Lett* 2021;12:1733–8.
- [104] Lanman BA, Allen JR, Allen JG, Amegadzie AK, Ashton KS, Booker SK, et al. Discovery of a covalent inhibitor of KRAS(G12C) (AMG 510) for the treatment of solid tumors. *J Med Chem* 2020;63:52–65.
- [105] Honigberg LA, Smith AM, Sirisawad M, Verner E, Loury D, Chang B, et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc Natl Acad Sci U S A* 2010;107:13075–80.
- [106] Cross DA, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, et al. AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer. *Cancer Discov* 2014;4:1046–61.
- [107] Wang X, Cao W, Zhang J, Yan M, Xu Q, Wu X, et al. A covalently bound inhibitor triggers EZH2 degradation through CHIP-mediated ubiquitination. *EMBO J* 2017;36:1243–60.
- [108] Wang Y, Huang X, Geng M, Li B, Yang H, Shi Q. Preparation of N-[(oxopyridinyl)methyl]benzamide/indole carboxamide derivatives as EZH2 covalent irreversible inhibitor and used for the prevention and/or treatment of tumors. Sun Yat-Sen University, Shanghai Institute of Materia Medica, Chinese Academy of Sciences; 2021.
- [109] Zhang Q, Hu X, Li L, Zhang L, Wan G, Feng Q, et al. The discovery of SKLB-0335 as a paralog-selective EZH2 covalent inhibitor. *Chem Commun (Cambridge, Engl)* 2021;57:3006–9.
- [110] Zhang Q, Chen X, Hu X, Duan X, Wan G, Li L, et al. Covalent inhibitors of EZH2: design, synthesis and evaluation. *Biomed Pharmacother* 2022;147:112617.
- [111] US FDA. FDA approves tazemetostat for advanced epithelioid sarcoma; 2020.
- [112] US FDA. FDA granted accelerated approval to tazemetostat for follicular lymphoma; 2020.
- [113] Epizyme Inc. TAZVERIK (tazemetostat) tablets [prescribing information]; 2020.
- [114] Tansir G, Rastogi S, Shamim SA, Barwad A. Early clinical and metabolic response to tazemetostat in advanced relapsed INI1 negative epithelioid sarcoma. *Future Sci OA* 2021;7 FSO675.
- [115] Gounder M, Schoffski P, Jones RL, Agulnik M, Cote GM, Villalobos VM, et al. Tazemetostat in advanced epithelioid sarcoma with loss of INI1/SMARCB1: an international, open-label, phase 2 basket study. *Lancet Oncol* 2020;21:1423–32.
- [116] Li B, Chng WJ. EZH2 abnormalities in lymphoid malignancies: underlying mechanisms and therapeutic implications. *J Hematol Oncol* 2019;12:118.
- [117] Morschhauser F, Tilly H, Chaidos A, McKay P, Phillips T, Assouline S, et al. Tazemetostat for patients with relapsed or refractory follicular lymphoma: an open-label, single-arm, multicentre, phase 2 trial. *Lancet Oncol* 2020;21:1433–42.

- [118] Beguelin W, Popovic R, Teater M, Jiang Y, Bunting KL, Rosen M, et al. EZH2 is required for germinal center formation and somatic EZH2 mutations promote lymphoid transformation. *Cancer Cell* 2013;23:677–92.
- [119] Huet S, Sujobert P, Salles G. From genetics to the clinic: a translational perspective on follicular lymphoma. *Nat Rev Cancer* 2018;18:224–39.
- [120] Epizyme Inc. Epizyme provides update regarding tazemetostat clinical program; 2018.
- [121] Xu F, Liu L, Chang CK, He Q, Wu LY, Zhang Z, et al. Genomic loss of EZH2 leads to epigenetic modifications and overexpression of the HOX gene clusters in myelodysplastic syndrome. *Oncotarget* 2016;7:8119–30.
- [122] Sakhdari A, Class C, Montalban-Bravo G, Sasaki K, Bueso-Ramos CE, Patel KP, et al. Immunohistochemical loss of enhancer of Zeste Homolog 2 (EZH2) protein expression correlates with EZH2 alterations and portends a worse outcome in myelodysplastic syndromes. *Mod Pathol* 2022.
- [123] Zheng Z, Li L, Li G, Zhang Y, Dong C, Ren F, et al. EZH2/EHMT2 histone methyltransferases inhibit the transcription of DLX5 and promote the transformation of myelodysplastic syndrome to acute myeloid leukemia. *Front Cell Developmental Biol* 2021;9:619795.
- [124] NDA 211723 tazemetostat applicant: epizyme, in Oncologic Drugs Advisory Committee, FDA briefing document; 2019.
- [125] Ribrag V, Morschhauser F, McKay P, Salles GA, Batlevi CL, Schmitt A, et al. Interim results from an ongoing phase 2 multicenter study of tazemetostat, an EZH2 inhibitor, in patients with relapsed or refractory (R/R) diffuse large B-cell lymphoma (DLBCL). *Blood* 2018;132:4196.
- [126] Epizyme Inc. Epizyme presents updates from SYMPHONY-1 Tazemetostat + R² combination study in relapsed/refractory follicular lymphoma at the 2022 ASCO annual meeting; 2022.
- [127] Tong KI, Yoon S, Isaev K, Bakhtiari M, Lackraj T, He MY, et al. Combined EZH2 inhibition and IKAROS degradation leads to enhanced antitumor activity in diffuse large B-cell lymphoma. *Clin Cancer Res* 2021;27:5401–14.
- [128] Huet S, Xerri L, Tesson B, Mareschal S, Taix S, Mescam-Mancini L, et al. EZH2 alterations in follicular lymphoma: biological and clinical correlations. *Blood Cancer J* 2017;7:e555.
- [129] Epizyme Inc. Epizyme reports second quarter 2018 financial results and provides business updates; 2018.
- [130] Palomba ML, Cartron G, Popplewell L, Ribrag V, Westin J, Huw LY, et al. Combination of atezolizumab and tazemetostat in patients with relapsed/refractory diffuse large B-cell lymphoma: results from a phase Ib study. *Clin Lymphoma Myeloma Leuk* 2022;22:504–12.
- [131] Yang XP, Jiang K, Hirahara K, Vahedi G, Afzali B, Sciume G, et al. EZH2 is crucial for both differentiation of regulatory T cells and T effector cell expansion. *Sci Rep* 2015;5:10643.
- [132] Zauderer MG, Szlosarek P, Le Moulec S, Popat S, Taylor P, Planchard D, et al. Phase 2, multicenter study of the EZH2 inhibitor tazemetostat as monotherapy in adults with relapsed or refractory (R/R) malignant mesothelioma (MM) with BAP1 inactivation. *J Clin Oncol* 2018;36:8515.
- [133] Chi SN, Bourdeaut F, Casanova M, Kilburn LB, Hargrave DR, McCowage GB, et al. Update on phase 1 study of tazemetostat, an enhancer of zeste homolog 2 inhibitor, in pediatric patients with relapsed or refractory integrase interactor 1-negative tumors. *J Clin Oncol* 2022;40:10040.
- [134] Kang N, Eccleston M, Clermont PL, Latarani M, Male DK, Wang Y, et al. EZH2 inhibition: a promising strategy to prevent cancer immune editing. *Epigenomics* 2020;12:1457–76.
- [135] Yap TA, Winter JN, Giulino-Roth L, Longley J, Lopez J, Michot JM, et al. Phase I study of the novel enhancer of zeste homolog 2 (EZH2) inhibitor GSK2816126 in patients with advanced hematologic and solid tumors. *Clin Cancer Res* 2019;25:7331–9.
- [136] Daiichi-Sankyo. Valemetostat pivotal data shows promising response rates in patients with adult T-cell leukemia/lymphoma. Press Release; 2021.

- [137] Xu K, Wu ZJ, Groner AC, He HH, Cai C, Lis RT, et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. *Science* 2012;338:1465–9.
- [138] Taplin M-E, Hussain A, Shah S, Shore ND, Edenfield WJ, Sartor OA, et al. Abstract CT094: Phase Ib results of ProSTAR: CPI-1205, EZH2 inhibitor, combined with enzalutamide (E) or abiraterone/prednisone (A/P) in patients with metastatic castration-resistant prostate cancer (mCRPC). *Cancer Res* 2019;79:CT094.
- [139] Constellation Pharmaceuticals Inc. Constellation Pharmaceuticals provides updates of MANIFEST study for CPI-0610 and EZH2 franchise; 2020.
- [140] Lakhani NJ, Gutierrez M, Duska LR, Do KT, Sharma M, Gandhi L, et al. Phase 1/2 first-in-human (FIH) study of CPI-0209, a novel small molecule inhibitor of enhancer of zeste homolog 2 (EZH2) in patients with advanced tumors. *J Clin Oncol* 2021;39:3104.
- [141] Song Y, Liu Y, Li Z-M, Li L, Su H, Jin Z, et al. SHR2554, an enhancer of zeste homolog 2 (EZH2) inhibitor, in relapsed or refractory (r/r) mature lymphoid neoplasms: a first-in-human phase 1 study. *J Clin Oncol* 2022;40:7525.
- [142] Ribrag V, Michot J-M, Igleias L, Tan D, Ma B, Duca M, et al. Phase I/II study of MAK683 in patients with advanced malignancies, including diffuse large B-cell lymphoma. *Blood* 2021;138:1422.
- [143] Bisserier M, Wajapeyee N. Mechanisms of resistance to EZH2 inhibitors in diffuse large B-cell lymphomas. *Blood* 2018;131:2125–37.
- [144] Gibaja V, Shen F, Harari J, Korn J, Ruddy D, Saenz-Vash V, et al. Development of secondary mutations in wild-type and mutant EZH2 alleles cooperates to confer resistance to EZH2 inhibitors. *Oncogene* 2016;35:558–66.
- [145] Preston SEJ, Emond A, Pettersson F, Dupere-Richer D, Abraham MJ, Riva A, et al. Acquired resistance to EZH2 inhibitor GSK343 promotes the differentiation of human DLBCL cell lines toward an ABC-like phenotype. *Mol Cancer Ther* 2022;21:511–21.

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Inhibitors of Jumonji-C domain-containing histone demethylases

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1 Jumonji-C domain-containing family of writers

Histone methylation represents an epigenetic modification that plays important roles not only for the transcriptional regulation but also for the preservation of genomic stability in eukaryotes. The methylation status of specific lysines is kept in balance by the competitive roles of two enzyme families, that is, the histone methyltransferases (HMTs) [1,2] and the histone demethylases (KDMs) [3–7]. A variety of diseases, including inflammation, leukemia, and breast and prostate cancers, are linked to alterations from its normal status of this epigenetic modification [8,9].

Nine groups of human $N\epsilon$ -methyl-lysine demethylases (KDM1–9) have been identified that catalyze the demethylation of N -methyl-lysine residues in histones through oxidative mechanisms acting on different $N\epsilon$ -methylation states and specific protein sequences [3]. KDMs are grouped into two families: the flavin-dependent lysine-specific demethylases (LSDs/KDM1s) and the 2-oxoglutarate (2OG)-, ferrous iron-, and oxygen-dependent demethylases [Jumonji-C (JmjC) KDMs], the latter being the largest family of KDMs. More than 100 members of the JmJC domain-containing proteins have been reported, including KDMs [10].

The KDM2–9 or JmjC domain-containing subfamily of demethylase enzymes (JHDMS) [11–13], which were called after the “Jumonji” protein in which this domain was first described [14], comprise a conserved class of enzymes that catalyze demethylation of all three $N\epsilon$ -methylated lysine residues on the histone tails, in particular the *N*-terminal tail of histone H3 (H3K4, H3K9, H3K27, and H3K36) [10,15–17]. However, the activity of these enzymes does not involve demethylation [18,19]. Instead, these enzymes promote the oxidative decarboxylation of 2OG to generate a reactive iron(IV)-oxo species [20], which is the reagent required for hydroxylation of the N -methyl groups [18,19]. Lysine methyl hydroxylation precedes the hydrolysis of the resultant hemiaminal intermediate, releasing formaldehyde and demethylated product (see Section 3). Since the substrate does not require a lone pair on nitrogen, they could catalyze the demethylation of $N\epsilon$ -trimethyl-lysine substrates (except in some reported cases for steric reasons) [3].

The demethylases are conserved from yeast to humans. Analysis of the public protein-domain databases, JmjC domain evolution and domain architectures, led to first propose of seven groups (subfamilies) for classification of the 20 human Jmj-KDMs family enzyme members [10,21]. More recently, based on sequence analysis of their catalytic domain, JmjC KDMs were grouped into six subfamilies: KDM2, KDM3, KDM4, KDM5, KDM6, and KDM7 [6,18], although KDM2 and KDM7 are recognized as a combined subfamily (KDM2/7) because their catalytic domains showed

a close sequence identity [22]. Most of them possess lysine demethylase (KDM) activity, and a subset of the reported JmjC-containing oxygenases were further identified as protein hydroxylases [23,24] and arginine demethylases (KDM3A, KDM4A, KDM5C, and KDM6B) promoting arginine demethylation of histone and nonhistone fragments [25].

In addition to the JmjC catalytic domain for histone demethylation, KDM4 contains the JmjN domain in a different relative localization within the protein, which is considered to play a pivotal role in the enzyme's structural stability. The KDM4 (JMJD2) subfamily requires both JmjN and JmjC domains for catalytic activity. Likewise, KDM5/JARID1 subfamily members also contain and atypical split catalytic Jumonji domain and the insertion of DNA binding AT-rich interaction domain (ARID) and histone interacting PHD1 domain in sections JmjN and JmjC [26,27]. However, it has been found that the ARID and PHD1 domains are crucial for *in vitro* enzymatic activity of KDM5 family members, whereas only the Zn-binding domain, which is next to C-terminal to JmjC is essential for enzymatic activity [26], and therefore the constructed linked JmjN-JmjC domain from KDM5A preserves structural integrity of the cofactor (metal ion and α-KG) binding as in other Jumonji-domain-containing demethylases [26].

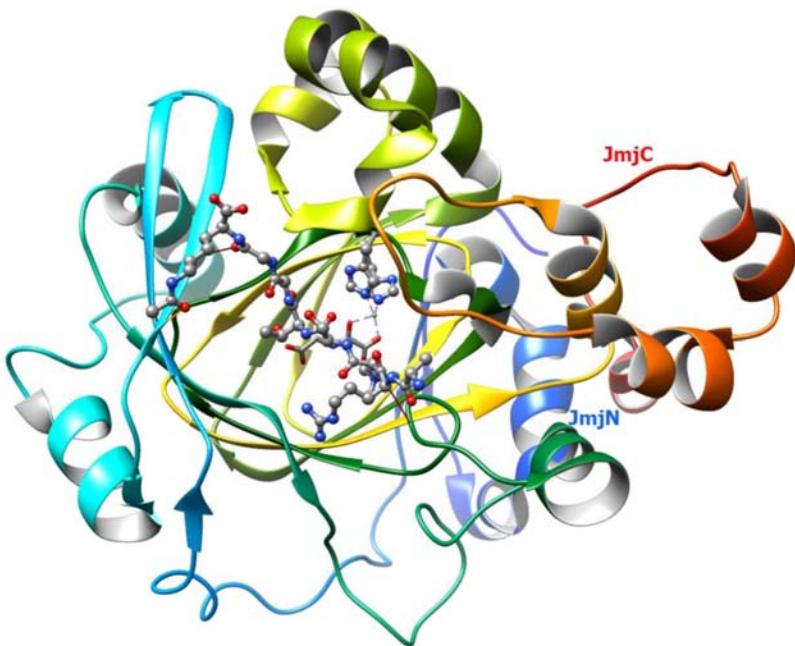
A very large number of crystal structures of unbound and bound α-ketoglutarate-KDMs/JHDMs complexes deposited in the RCSB PDB (more than 700 as of June 2022) have increased our knowledge about their structure and mechanism [6,28,29]. Crystal structures of complexes including peptides have also generates models for the interactions between the enzyme and substrate mimics [30].

JmjC KDMs have a conserved active site to bind 2-OG and Fe(II) with a structure of barrel-like double-stranded β-helix (DSBH) fold, with eight antiparallel strands as catalytic core to accommodate Fe(II) and 2-OG together with the substrate. KDM4/5 contain a JmjN domain with a Zn-binding site located close to the active site entrance at the N-terminus of the JmjC domain, promoting the interaction with vicinal domains and structural integrity [31]. This jellyroll fold of α-helices and β-strands binds the iron ion by chelation with three strictly conserved residues (one Asp/Glu and two His residues) adopting a octahedral binding geometry with the rest of the sites occupied by water molecules [32]. The JmjN domain, with three helices placed between two β-strands, is positioned opposite the catalytic center and interacts largely with JmjC. In addition to the JmjC/N domains, the KDM2/7, KDM4, KDM5, and KDM7 subfamilies contain one or more PHD domain, which recognizes the H3K4me3 mark therefore serving as a nucleosome-binding domain (Figure 17.1).

Given their close relationship to KDM2, KDM7 and Plant Homeodomain Finger (PHF) are considered part of the same subfamily (KDM2/7) [6,22]. Besides JmjC domain, the KDM2 subfamily has a CxxC zinc finger domain, a PHD domain, and an F-box domain. Whereas KDM2A demethylates H3K36me2/1, KDM2B demethylates H3K4me3 and H3K36me2. Since both H3K36 and H3K4 methylation activate gene transcription, the KDM2 subfamily promotes gene repression. The KDM7 subfamily consists of KDM7A-C, and demethylates H3K9me2/1, H3K27me2/1, and H4K20me1. Although the catalytic domains are similar, the PHF proteins recognize H3K4me3 through their PHD domain and demethylate H3K9me2/me1, H3K27me2/me1, and nucleosomal H4K20me1. Contrasting substrate specificities of PHF8 and KDM7A have been noted [6].

The KDM3 family consists of three members, KDM3A-C. KDM3A and KDM3B are specific for removing H3K9me2/1 [6].

The KDM4 (Jmj-D2) subfamily of histone KDMs includes five members, KDM4A-E, and a likely pseudogene KDM4F, and they prefer tri- and di-*N*^ε-methylated H3K9 substrates. KDM4A-C

**FIGURE 17.1**

X-ray structure of KDM4A (PDB ID: 2OQ6) [33].

promotes the demethylation of H3K9me3/2 and H3K36me3/2, KDM4D only demethylates H3K9me3/2 and KDM4E demethylates both H3K9me3 and H3K56me3. KDM4A, 4B, and 4C share more than 50% sequence homology, comprising JmjN, JmjC, two PHD, and two Tudor domains [6,34]. In addition, all KDM4 subfamily members are able to demethylate H1.4K26me2/3 [35]. The KDM4 enzymes are overexpressed in several tumors as well as cardiovascular diseases and mental retardation [36]. Furthermore, they have a role in regulating heterochromatin, replication timing and chromosomal copy numbers [37]. KDM4A-E are all crucial in tumorigenesis with the exception of KDM4E, whose function is still unknown [38]. KDM4s therefore act as transcriptional activators, and their overexpression can activate gene transcription in cancer [39]. KDM4s are implicated in several biological processes, such as cell cycle regulation, DNA damage, and transcriptional regulation [35].

The KDM5 subfamily [19] [also known as the Jumonji/AT-rich interacting domain-containing protein 1 (JARID1) subfamily], consists of four members (A–D) with an high sequence homology and domain organization [6,26,40], but different tissue distributions and cellular localizations, and therefore cell functions. The KDM5/JARID1 subfamily catalyzes the demethylation of H3K4me3/2, a transcription activating mark [41]. Being associated to oncogenic functions, KDM5 enzymes are considered promising drug targets for cancer therapy [42].

The KDM6 subfamily of histone demethylases includes KDM6B (JMJD3), KDM6A (UTX) and KDM6C (UTY). They catalyze the demethylation of both the trimethylated and demethylated

lysine 27 on histone H3 (H3K27) [6,43,44]. KDM6s are members of multiprotein complexes, such as MLL2, MLL3, and MLL4, involved in the regulation of Polycomb family proteins. Although UTX and UTY share >96% sequence similarity in their catalytic JmjC domains, KDM6B only shares 86%. KDM6 enzymes have an effect on cancer development and progression by inhibiting or activating gene transcription [45]. KDM6B (JMJD3) is considered a therapeutic target both in inflammatory diseases and in cancer.

2 Role of JmjC proteins in human cancer

JmjC domain-containing histone demethylases (JmjC-KDMs) act as important regulators of the methylation state of chromatin and gene expression by removing methyl groups on specific lysines. They contain a JmjC catalytic domain and an additional JmjN domain involved in the structural integrity and activation of enzymatic function, as well as noncatalytic domains, such as PHD and Tudor domains, that determine substrate specificity and control enzymatic activity [46]. JmjC-KDMs are also involved in the modification of nonhistone substrates, regulating their abundance, stability, or activity, and in RNA modifications, such as m⁶A and 5-mc, affecting RNA stability and mRNA translation [7].

JmjC-KDM expression is also regulated by somatic mutations, posttranslational acetylation and phosphorylation, and the metabolic state of cells. These modulators contribute to physiological processes, and their altered activity and/or expression is associated with cell plasticity, the development of several cancers, and chemoresistance.

Histone methylation plays a pivotal role in the activation or repression of gene transcription by controlling the access of TFs to regulatory regions and by regulating DNA replication and repair, chromatin status, and cell cycle progression. Generally, H3K4 and H3K36 methylation are active histone marks associated with transcriptional activation, whereas H3K9, H3K27, and H4K20 methylation are repressive histone marks related to transcriptional repression [47].

In this section, we summarize the recent and growing body of evidence highlighting the intriguing role of JmjC-KDMs in tumorigenesis and describe their potential use as new drug targets and as an alternative approach in targeted cancer therapies [48].

2.1 JmjC proteins in solid cancers

Aberrant KDM expression is associated with several solid cancers, such as GMB, NSCLC, GC, BC, CC, PCa, and CRC.

2.1.1 KDM2A and KDM2B

KDM2A is involved in heterochromatin formation, and its deletion increases genomic instability. This enzyme is able to bind CpG islands and although mainly demethylates H3K36me2, it can also bind nonhistone substrates, such as NF-κB, modulating intracellular signals. It is involved in regulating cell proliferation and senescence via modulation of p15 and p53 expression [49]. KDM2A exerts an oncogenic role in several types of cancer. KDM2A upregulation is found in NSCLC, where it causes transcriptional repression of DUSP3, which dephosphorylates ERK1/2, leading to

its deactivation and thus in turn activating ERK1/2 and their signaling pathways. Activation of ERK1/2 signaling induces tumorigenesis, cell proliferation, and invasiveness. However, *in vitro* and *in vivo* models of KDM2A KD were found to inhibit proliferation, tumor growth, and metastatization, and high levels of KDM2 were associated with poor prognosis in two distinct NSCLC patient cohorts [50]. Furthermore, *HDAC3* is a target gene of KDM2A, which, by demethylating H3K36me2 at the *HDAC3* promoter region, causes its transcriptional repression. Consequently, loss of HDAC3 results in the activation of HDAC3 target genes, such as those associated with cell cycle (*CDK6* and *NEK7*) and invasion (*NANOS1* and *RAPHI*), activating tumorigenesis and invasion in NSCLC cells overexpressing KDM2A [51]. In addition, an integrative genomic approach combining CRISPR/Cas9 screening identified KDM2A as a target gene in AD and SCLC cell lines [52].

The role of KDM2A in GC is poorly understood. RNA and protein levels of the enzyme result elevated in GC tissue compared to adjacent nontumor tissues. KDM2A upregulation promotes the growth and progression of GC cells via expression of PCNA, phosphorylated ERK, and cyclin D1, and downregulation of PDCD4. Furthermore, KDM2A KD abolishes the metastatic capability of GC cells *in vivo* [53].

Recent studies show a correlation between KDM2A and lncRNAs in GC. Specifically, the highly expressed LINC00460 and LINC00511 may exert their oncogenic activity by downregulating miR-342-3p and miR-29-b, respectively, which in turn leads to upregulation of KDM2A [54,55].

In BC, KDM2A upregulation is associated with poor prognosis. The oncogenic activity of KDM2A results from its ability to aberrantly activate NOTCH signaling pathway. Specifically, KDM2A binds to the promoter region of JAG1, a ligand of NOTCH1, inducing its expression and driving tumor proliferation and angiogenesis. Tumor angiogenesis is also supported by KDM2A-mediated PDGFA activation. In addition, JAG1/NOTCH signaling pathway induces expression of SOX2, a stemness promoter detected at the early stage of tumor development. In BC cells, KDM2A KD suppresses these cancer-promoting effects, which can be rescued by JAG1 overexpression, highlighting the involvement of JAG1 in KDM2A-mediated cancer regulation [56].

Another KDM2A target in BC is the DNA demethylase TET2. KDM2A binds to the promoter region of TET2, repressing its gene transcription. TET2 KD suppresses the expression of two TET2 target genes, E-cadherin and EpCAM, causing tumor progression, while KDM2 depletion reduces invasion and migration by reactivating the two genes. In TNBC tissues, TET2 and KDM2A levels are also negatively correlated, and high TET2 levels predict a better survival [57]. KDM2A is also overexpressed in CAFs and is linked to advanced tumor stage and poor prognosis in BC. BC cell-secreted cytokines activate expression of KDM2A. Stromal KDM2A induces senescence and PD-L1 in CAFs, contributing to BC promotion [49]. In EOC KDM2A has a crucial role in cancer development and induction of EMT. KDM2A KD in EOC cells reduces cell proliferation and colony formation and decreases cell migration through increased levels of E-cadherin and downregulation of vimentin and N-cadherin, which control tumor progression [58]. In GBM, the function of KDM2A has not been fully elucidated; the only available findings suggest that KDM2A is a miR-3666 target and that its silencing, or miR-3666 overexpression, suppresses proliferation and invasion [59]. In CRC tissue, high levels of KDM2A also contribute to cancer growth by positively regulating cyclin D1 expression [60].

KDM2B, the second member of the KDM2 subfamily, is also overexpressed in numerous cancers, promoting cell migration by binding to migration-associated genes and inhibiting cell

senescence by repressing senescence-associated genes [61]. In LC and PC cell lines, it has a crucial role in EMT activation mediated by TGF- β . KDM2B is a member of PRC1, mediating H2A monoubiquitination on lysine 119 (H2AK119) on the regulatory regions of epithelial marker genes, such as *CDH1*, *miR-200a*, and *CGN*, thereby activating enhancer of EZH2 recruitment and histone H3K27 methylation, repressing gene expression. KDM2B was found to influence expression of the mesenchymal marker genes *ZEB1* and *ZEB2* through interaction with miR-200a; other markers were not impacted by KDM2B levels [62]. In CRC and PCa, KDM2B is able to affect migration by deregulating normal cytoskeleton organization and function. It regulates EMT through repression of E-cadherin and ZO-1 and induction of N-cadherin and is also involved in actin cytoskeleton reorganization via control of RhoA/B GTPases [63,64]. In CRC, KDM2B overexpression positively correlates with clinical and TNM stage. Downregulation of KDM2B inhibits cell proliferation by activating p21 and p27 and decreasing cyclin D1, and also affects cell progression, cell growth, cell renewal capacity, and stemness, thus suppressing sphere formation. Specifically, KDM2B decreases expression of EZH2, which activates PI3K/AKT signaling, maintaining CSCs *in vitro* [65]. In CC, KDM2B exerts its oncogenic role through interaction with CDC25A, a CDC25 phosphatase involved in several biological processes and CC development. The TF ALX3 engages KDM2B at CDC25A promoter region, enhancing its transcription through H3K4me3 demethylation, thereby causing cell cycle progression and proliferation. Consequently, PI3K/AKT pathway activation increases cancer cell motility [66]. In glioma cells, downregulation of KDM2B inhibits cell proliferation and migration, through activation of p21 and a reduction in cyclin D1 and EZH2 [67].

2.1.2 KDM3

KDM3A is upregulated in several cancers and is linked to poor prognosis. Downregulation of KDM3A inhibits tumor progression, EMT, invasion, metastasis, and xenograft tumor formation [68]. In PC, KDM3A levels are higher than in normal tissue, and the enzyme is involved in tumor growth through regulation of cell cycle-associated genes, such as *CDK6*, via an H3K9-dependent pathway [69]. In CRC, KDM3A promotes cancer development by activating Wnt/ β -catenin pathway. KDM3A can directly associate with β -catenin on Wnt gene promoter, modifying histone H3K9 methylation status [70], or can exert its oncogenic function indirectly by recruiting MLL1, a methyltransferase of H3K4, which leads to recruitment on chromatin of BCL9/PYGO, two key coactivators of Wnt/ β -catenin signaling pathway [71]. In PCa, high levels of KDM3A are associated with cell proliferation, migration, and invasion both *in vitro* and *in vivo*. The oncogenic role of the enzyme may be involved in regulation of AR and c-Myc, as well as in activation of Snail, an important regulator of EMT, by affecting its promoter methylation state [72]. In CC, downregulation of KDM3A inhibits expression of Bcl-2, N-cadherin, and vimentin, and increases expression of E-cadherin and proapoptotic genes, such as *Bax*, highlighting the role of the enzyme in regulating tumor progression and growth. KDM3A is also tightly connected to expression of the oncogene *ETS1* [73]. However, KDM3A can regulate tumor formation through methylation of a nonhistone target, PGC-1 α . The enzyme regulates PGC-1 α depending on oxygen availability; hypoxic conditions inhibit KDM3A activity, increasing PGC-1 α K224me, thus resulting in a decrease in mitochondrial biogenesis [74].

The role of KDM3B in cancer is controversial; it may have tumor-suppressive or tumor-promoting activities depending on specific protein interactions. KDM3B exerts an oncogenic role

via regulation of the autophagy process, which is dysregulated in tumors and enhances cancer cell survival, by increasing transcription of autophagy-related genes. In nutrient-deprivation conditions, KDM3B levels increase, leading to activation of autophagy-inducing genes due to demethylation of H3K9me2 [75]. KDM3B is involved in regulating cell cycle progression in HCC, and its depletion leads to a reduction in cyclin D1, proliferation factor CDC123, and HepG2 proliferation [76].

The oncogenic role of KDM3C is mainly exerted in CRC and PCa. In CRC, the interaction between KDM3C and ATF2, a TF involved in cancer development, leads to tumor progression, lymph node metastasis, and poor clinical outcome. Both *in vitro* and *in vivo*, KDM3C KD reduces metastasis formation, partially decreased by ATF2 overexpression. KDM3C binds to the ATF2 promoter region, demethylating H3K9me2 regulating its expression [77]. KDM3C plays an important role in AR-negative PCa, suggesting its potential use as a new therapeutic target. KDM3C and AR may cooperate in the suppression of TNF α downstream genes and NF- κ B pathway [78].

2.1.3 KDM4

KDM4A is overexpressed in 20% of cancers and is associated with poor prognosis. It acts as a transcriptional activator or repressor depending on its role as a histone demethylase or as a recruiter of chromatin factors to methyl marks on chromatin. KDM4A affects processes involved in genome stability, such as cell cycle progression, apoptosis, and DNA damage response and repair. Thus aberrant expression of KDM4A leads to chromatin instability and cancer [35]. In PCa, the catalytic domain or C-terminus of KDM4A binds to AR and stimulates AR-dependent transcription of proliferative and survival genes, including c-Myc, contributing to malignant transformation [79]. High levels of KDM4A contribute to PCa initiation through interaction with ETV1 and activation of YAP1 [80]. In BC, KDM4A forms a complex with ER α , promoting the transcription of ER α target genes [81]. In CRC, high levels of KDM4A promote tumorigenesis by hampering 53BP1, a tumor suppressor, recruitment to DNA damage sites and consequent DNA repair [82].

Levels of KDM4B and its catalytic activity are negatively influenced by hypoxic conditions, suggesting that HIF-1 α is a promoter of its expression [35]. However, KDM4B is found overexpressed in several cancers. In EOC, KDM4B is overexpressed in both primary and metastatic tumors. Hypoxic conditions promote expression of KDM4B, which, by demethylating H3K9me3 at target regulatory regions, induces the expression of genes involved in peritoneal dissemination of EOC cells [83]. In PTEN-deficient TNBC, inhibition of KDM4B activates UPR pathway, causing apoptosis through interaction with eIF2 α , a component of UPR signaling [84]. In CRC, KDM4B is fundamental for cancer cell proliferation as it activates glucose metabolism. KDM4B interacts with the ubiquitin E3 ligase TRAF6, required for AKT activation, leading to GLUT1 overexpression and thus to glucose uptake [85]. It also interacts with Wnt/ β -catenin pathway, inducing oncogene expression, and with p53, inhibiting p53-related genes upon DNA damage [86]. In PCa, KDM4B binds to the C-terminus of AR and promotes expression of AR-target genes, indicating that KDM4B and KDM4A are important AR coactivators [79].

KDM4C acts as an oncogene in several cancers. In EOC, KDM4C plays an important role in CSC maintenance by binding to the promoter region of OCT-4 and regulating its expression. KDM4C depletion reduces CSC properties, invasion, and migration [87]. In LC, KDM4C plays a key role in antitumor immunity. High KDM4C levels lead to a decrease in H3K36me3 at CXCL10

promoter region and therefore to its transcriptional repression. This causes a decrease in CD8⁺ T cells infiltration and activation, making LC resistant to radio- and immunotherapy. Pharmacological KDM4C inhibition represents a promising strategy in LC treatment [88]. In PCa, high levels of KDM4C induce overexpression of AKT signaling proteins and c-Myc, promoting cell proliferation [89]. In CRC, KDM4C expression has an important function in sphere formation, where it is expressed downstream of β-catenin, the major TF of Wnt signaling pathway. KDM4C recruits β-catenin to JAG1 promoter, leading to its expression, which is pivotal for colonosphere formation [90]. In BC, KDM4C promotes HIF-1α-related gene expression by removing K3K9me3, inducing tumor growth and metastasis [35].

In RCC, KDM4D induces tumor progression and angiogenesis through interaction with JAG1 and upregulation of VEGFR-3 [91]. In PCa, KDM4D exerts its oncogenic function in binding AR and stimulating AR-dependent transcription [81]. In CRC, KDM4D can drive cell proliferation and survival, suggesting it as a promising target in anticancer therapy. KDM4D induces tumorigenesis through Wnt/β-catenin signaling pathway activation, leading to transcriptional activation of c-Myc, cyclin D1, MMP2, and MMP9, and tumor formation in mice [92]. In HCC, KDM4D may counteract the tumor suppressor activity of p53 by inhibiting its recruitment to p21 and PUMA promoter regions and by activating oncogenic pathways [93].

2.1.4 KDM5

KDM5A, also known as RBP2 due to its binding with pRb, is highly expressed in GC, BC, EOC, promoting angiogenesis, drug resistance, EMT, metastasis, invasiveness, proliferation, and cell motility. Its expression is correlated with poor clinical outcome. In EOC, KDM5A overexpression stimulates EMT, metastasis, and cell cycle progression and mediates drug resistance in paclitaxel-resistant cells [94]. In GC, KDM5A may be recruited by TGF-β through phospho-SMAD3, both of which are important regulators of EMT, to the E-cadherin promoter region, inhibiting its expression and inducing metastasis [95]. In tamoxifen-resistant BC cells, KDM5A overexpression leads to tamoxifen resistance *in vitro* and *in vivo*, while its depletion confers tamoxifen sensitivity. These effects occur through KDM5A-mediated enhancement of ER-IGF1R-ErbB signaling cascade [96]. Furthermore, KDM5A-B activity is associated with cellular transcriptomic heterogeneity, causing an increase in drug resistance and poor prognosis [97].

KDM5B is also reported overexpressed in several cancers and is identified as a potential oncogene [38]. In PCa, mRNA levels of KDM5B are higher than in normal prostate tissue. KDM5B inhibition leads to upregulation of tumor suppressor genes, causing cancer growth arrest and apoptosis and highlighting KDM5B role as a repressor of tumor suppressor genes [98]. Furthermore, KDM5B regulates PI3K/AKT signaling *in vitro* and *in vivo* [85]. In BC, overexpression of KDM5B is associated with EMT and cancer growth via regulation of lipid metabolic reprogramming [99]. In HCC, KDM5B upregulation is linked to poor prognosis, promoting cell proliferation, EMT, and migration [100]. KDM5C is overexpressed in several tumors and is involved in regulating tumor-related gene expression. It inhibits *p53*, *PCNA*, and *p21*, thus promoting cell proliferation. KDM5C is involved in X-linked pathologies and also in human papillomavirus-associated tumorigenesis [36]. In CRC, KDM5C downregulates transcription of the tumor suppressor FBXW7, reducing the proto-oncogene c-Jun degradation [101]. In LC, KDM5C regulates miR-133a promoter methylation state, inhibiting expression of miR-133a and promoting cancer growth and metastasis [102]. In BC, KDM5C exerts a dual role, either activating or repressing gene expression. It interacts

with ER α , regulating the expression of ER α -target genes, and suppresses type I interferons, promoting cancer growth and metastasis [103].

KDM5D also has an oncosuppressive effect in PCa, where its downregulation is involved in tumor initiation and tumor/grade stage [104], and in GC, where its expression induces CUL4A promoter demethylation and EMT inhibition [105].

2.1.5 KDM6

The role of KDM6A (also known as UTX) as a suppressor or activator remains unclear, as it is recruited by different TFs and chromatin modifiers. It is a member of the H3K4 methylation protein complex, which includes MLL3/4, inducing MLL4/p300 recruitment and H3K27 acetylation. KDM6A also associates with CBP and SMARCA4, the enzymatic subunit of the SWI/SNF chromatin-remodeling complex. KDM6A can also exert its function through interaction with MLL3/4, without involving its enzymatic activity. These interactions promote chromatin opening and gene transcription activation [106]. Although many studies describe the role of KDM6A as a tumor suppressor, some findings indicate its oncogenic effect in several types of cancer. KDM6A promotes cell proliferation, invasion, and migration in MCF-7 cells, a BC cell line that expresses ER α , through colocalization with ER α and expression of gene targets, such as *GREB1*, *TFI1*, *MYB*, and *CXCR4*, all of which are involved in BC development [107]. However, KDM6A is also recruited by the TF GATA3, forming the GATA3/UTX complex, which activates KDM6A itself and the tumor suppressor Dicer, thus inhibiting tumor progression [108].

KDM6B is inducible by several cytokines, growth factors, and lipopolysaccharides during inflammation and is directly regulated by NF- κ B. KDM6B is activated via NF- κ B binding to its promotor region by TNF α and in turn upregulates MAPK pathway, thus promoting cancer cell growth and survival in a catalytically independent manner [45]. KDM6B may exert its function independently of its demethylase activity, interacting with TFs involved in chromatin remodeling. It is also known to be involved in cancer, but its role as an oncogene or tumor suppressor is unclear [109]. In GBM, inhibition of KDM6B leads to the maintenance of a stable methylation state on H3K27 where a mutation in *H3F3A* gene reduces H3K27 methylation; it also exerts its oncogenic activity by activating expression of EMT markers, such as SNAI1, which increases N-cadherin and CXCL12 levels. However, KDM6B acts as tumor suppressor in GBM by regulating p53, and via its regulation by STAT3, whose overexpression reduces KDM6B levels and induces neurosphere formation, proliferation, and metastasis [109]. In PC, overexpression of KDM6B and EZH2 is dependent on cancer type. The role of KDM6B is also controversial in CRC. As a tumor suppressor, its activation limits the expression of EMT markers, promotes chromatin access to genes implicated in cell adhesion, and induces tumor immunity by enhancing transcription of the chemokines CXCL9 and CXCL10, apoptosis, and expression of the tumor suppressor p15. In contrast, KDM6B has an oncogenic role in EpCAM genes, and its interaction with NOTCH leads to tumor formation and tumor growth. In BC, KDM6B can induce expression of Bcl-2 in genomic manner in nonresistant BC, while in resistant-BC Bcl-2 levels increase through EZH2-KDM6B interaction. In NSCLC, KDM6B impacts cell proliferation by affecting cell cycle and may influence cell migration through regulation of EMT [45].

KDM6C is only present on Y-chromosome, regulating male fertility, development, and differentiation of male-specific organs. In PCa, KDM6C was shown to be a downstream mediator of *NKX3.1*,

a homeobox gene that regulates cellular differentiation *in vitro* and tumor growth *in vivo*. To induce prostate differentiation NKX3.1 requires the methyltransferase G9a, which binds to the promoter of *UTY*. Further investigations are necessary to enhance our knowledge in *UTY* role in cancer [110].

2.1.6 KDM7

KDM7A mostly exerts its oncogenic role in PCa by inducing AR-dependent cell growth, and its suppression inhibits tumor development in xenograft models;[111] in BCA, KDM7A mediates tumor growth through interaction with AR, especially under drug resistance;[82] in BC, its overexpression is associated with tumor sphere formation, promotion of BC stem cells through interaction with c-Myc and KLF4, and apoptosis suppression by enhancing phosphorylated levels of Bcl-2 and BAD [112].

In CRC, overexpression of KDM7B was shown to be associated with TNM stage predicting poor survival, tumor progression, and migration through its regulation mediated by the tumor suppressor miR-488 [113]. KDM7B also acts as an oncogene in HER2⁺ BC cells, leading to tumor development, EMT, and resistance to anticancer therapy [114]. Furthermore, the proto-oncogene c-Myc seems to regulate KDM7B expression in GC through miR-22-3p suppression, enhancing KDM7B-mediated proliferation, migration, and invasion [115]. KDM7B also exerts its oncogenic role in PCa, NSCLC, and LC [116].

KDM7C has tumor suppressor effects in several cancers. In CRC and GC, it acts as a tumor suppressor by interacting with p53 and enhancing p53 expression by demethylating H3K9me2 at its promoter region [117]. In HCC, KDM7C is targeted by miR-221, leading to downregulation of the enzyme, cell migration, tumor development, and poor overall survival [118]. In BC, transcriptional stimulation of KDM7C by FOXP2 inhibits EMT and metastasis, conferring the epithelial phenotype on cancer cells [119].

2.2 JmjC proteins in hematological cancers

2.2.1 KDM2

In myeloid malignancies, KDM2A has an antileukemic role and acts as an eraser, counteracting the action of ASH1L, a methyltransferase of H3K36 that maintains H3K36me2, read by LEDGF, recruiting and stabilizing MLL on its promoter region, inducing transcription of MLL genes (*HOX*, *MEIS1*). In this way, KDM2A demethylates histone H3K36me2 at MLL target genes and promotes chromatin dissociation of MLL [120].

Unlike KDM2A, the role of KDM2B seems to be controversial and context dependent. In AML, it was shown to exert its oncogenic activity through inhibition of p15 (Ink4b), leading to cell cycle progression and AML transformation by Hoxa9/Meis1 in mice [121,122]. In transgenic mice, KDM2B overexpression in HSCs promotes myeloid or B-lymphoid leukemia by enhancing levels of Nsg2 and OXPHOS, involved in HSC differentiation and metabolic activation, respectively [123]. By contrast, KDM2B acts as a tumor suppressor in myelodysplastic syndrome cell lines through inhibition of EZH2 expression by the miRNA let-7b; overexpression of let-7b induces KDM2B and EZH2 downregulation, a reduction in H3K27me3 levels, inhibition of cyclin D1, and an increase in G0/G1 cell cycle phase [124]. In AML, KDM2B overexpression also leads to inhibition of Hox10 and Smarca4/Brg1 and to activation of interferon program-associated genes [125].

2.2.2 KDM3

In the MLL-AF9-driven murine model of AML, KDM3A has a proleukemic role and is recruited by Oct1, a TF involved in malignant transformation, at the CDX2 promoter, removing repressive H3K9me2 and overexpressing CDX2 [126].

The role of KDM3B in leukemia is controversial. In AML, deletion of *KDM3B* gene loci inhibits colony formation [127]. By contrast, in acute lymphocytic leukemia (ALL), KDM3B is recruited along with CBP to the promoter region of *Imo2*, a leukemic oncogene that regulates erythropoiesis and inhibits erythroid differentiation, reducing H3K9me2/1, thus promoting its transcription and *Imo2*-mediated leukemogenesis [128]. However, the noncatalytic activity of KDM3B is exerted by its interaction with Hoxa9, leading to the activation of Hoxa9-related genes.

The oncogenic role of KDM3C is mediated by its catalytic and noncatalytic activities. KDM3C is recruited by AE to target genes involved in the proliferation and survival of multiple AML cells, inducing transcription [129]. In addition, loss of KDM3C upregulates expression of IL3 receptor and enhances RAS/MAPK and JAK/STAT pathways [130].

2.2.3 KDM4

KDM4A-D demethylases have a proleukemic role, while the function of KDM4E remains unclear in myeloid malignancies.

In AML, KDM4A exerts an oncogenic role through interaction with PAF1, involved in transcriptional elongation and mediation of the oncogenic program, inducing a decrease in H3K9me3 levels leading to leukemogenesis [131]. Furthermore, in MLL-AF9-driven leukemia, depletion of KDM4A enhances apoptosis.

KDM4B is also overexpressed in MLL-AF9 leukemia, where the enzyme is involved in the activation of S100A8/9, and in Hodgkin's lymphoma, where it leads to an aggressive phenotype and radioresistance [132].

KDM4C is upregulated in AML cells and activates MALAT1, a lncRNA associated with increased proliferation, causing an increase in cyclin D2. In addition, KDM4C removes H3K9me3 at the promoter of the m6A demethylase ALKBH5, enhancing the stability of AXL and thus PI3K/AKT/mTOR signaling [133]. KDM4C also interacts with the oncogenic dual-phosphatase PRL2, which facilitates the ligand of KDM4C to the promoter region of *Leo1*, and mediates oncogenic activity in AML [134].

In AML, high levels of KDM4D correlate with poor prognosis, causing an increase in proliferation and expression of antiapoptotic genes, such as *MCL1*, through removal of H3K9me3 [135].

2.2.4 KDM5

Both KDM5A and KDM5B have a proleukemic role.

KDM5A shows aberrantly high expression in AML. KDM5A can interact with *NUP98* and create a fusion gene, NUP98-KDM5A, which activates *Hoxa* genes, regulates hematopoietic cell proliferation, and affects myeloerythropoietic differentiation by demethylating H3K4me2/3 [136]. In CML, in the CML-BP KDM5A is underexpressed, while in CML-CP KDM5A levels are higher, determining inhibition of *miR-21*, an oncogene that stimulates leukemia cell proliferation and block of apoptosis. KDM5A binds to the proximal promoter of *miR-21* by demethylating H3K4me3, inhibiting its expression and activity, thus activating PDCD4 [137].

KDM5B is overexpressed in normal CD34⁺ HSPCs in AML and CML and is required for HSC self-renewal in mice [138]. In CML-BP, KDM5B expression levels are increased in patients compared to healthy controls. In B-ALL, KDM5B is repressed by the TF Ikaros, which exerts a tumor suppressor activity in leukemia. Ikaros interacts with HDAC1, which binds KDM5B upstream regulatory element, resulting in cellular growth arrest [139].

2.2.5 KDM6

KDM6A is highly expressed in HSPC lineages. It is a frequent target of loss-of-function mutations in leukemia and may exert tumor-suppressor action in AML cells, where loss of KDM6A leads to drug resistance. In TAL1-positive cells, a molecular subtype of T-ALL, the oncogene *TAL1* leads to expression of specific genes involved in cell growth and leukemia maintenance through interaction with KDM6A both *in vitro* and *in vivo*. KDM6A interacts with genomic sites normally silenced and removes histone H3K27 methylation, activating their transcription. By contrast, in TAL1-negative T-ALL, KDM6A exerts a tumor suppressor action, inhibiting tumor growth and development [140]. KDM6A is upregulated in CML cells and cooperates with the TF YY1 for transcriptional activation of the NGF receptor TRKA in a demethylase-independent manner. The NGF-TRKA axis is constitutively active and induces imatinib tolerance. KDM6A and TRKA were found upregulated in patient-derived CML cells, particularly in imatinib-resistant cells, and loss of KDM6A activity repressed NGF-induced resistance [141].

KDM6B exerts both anticancer and carcinogenic roles by directing different signaling pathways. It is overexpressed in bone marrow HSPCs of patients with MDS and CMML, where it can interact with Set1/MLL in H3K4 methylation, inducing dysregulation of innate immune genes [142]. In transgenic mice, overexpression of KDM6B induces leukopenia and dysplasia and impairs the repopulation capacity of HSPCs, which can be reversed after treatment with GSK-J4 in both mice and human patients [143]. Furthermore, the oncogenic role of KDM6B in T-ALL is associated with NF-κB-NOTCH interaction, whose activation induces an inflammatory state and proliferation of T cells; here, KDM6B activates expression of T-cell-specific oncogenic target genes, such as *Hes1*, through modulation of H3K27me3 at the promoter region [144]. In AML, KDM6B plays an onco-suppressor role in two AML subtypes, M1 and M2, by directing modulation of H3K4 and H3K27 methylation states, activating expression of myelopoietic regulators. KDM6B/C/EBP β interaction is in fact fundamental for AML cells to undergo myeloid differentiation and lose their capability to re-establish leukemia *in vivo* [145].

Most KDMs function as oncogenes or as tumor suppressors and are strongly disease stage and cell lineage dependent (Table 17.1).

3 Mechanism of inhibition

As indicated above, KDMs are mononuclear Fe(II)-dependent dioxygenases that oxidatively demethylate specific lysines by using α -KG and oxygen as cosubstrates. The protein family contains as structural motif a 2-histidine-1-carboxylate facial triad coordinated to a nonheme Fe(II) at the catalytic center [20].

Table 17.1 KDM enzymes: targets and roles in cancer.

Enzyme	Histone Target	Biological Properties	Potential Role	Type of Cancer	Target	Effects
KDM2A FBXL11 JHDM1A	H3K36me2	Epigenetic repression	Oncogenic	NSCLC	↓DUSP3	Activation of ERK1/2, tumorigenesis, cell proliferation, invasiveness
	H3K36me2	Transcriptional repression	Oncogenic	NSCLC	↓HDAC3	Activation of HDAC3-targeted genes involved in cell proliferation and invasion
	H3K36me2	Transcriptional regulation	Oncogenic	GC	↓PDCD4 ↑PCNA, cyclin D1	Tumor growth and cell proliferation
	H3K36me2/1 H3K4	Transcriptional regulation	Oncogenic	BC	↑JAG1 ↑JAG1, PDGFA ↑JAG1, SOX2	NOTCH activation, chemoresistanceAngiogenesisStemness,
	H3K36me2	Transcriptional repression	Oncogenic	BC	↓TET2	Cell migration and invasion, high tumor grade
	H3K36me2	Transcriptional regulation	Oncogenic	EOC	↓E-cadherin ↑N-cadherin, vimentin	Cell proliferation, colony formation, EMT
	H3K36me2	Transcriptional regulation	Oncogenic	CRC	↑cyclin D1	Cancer development
	H3K36me2	Transcriptional repression	Oncosuppressor	AML	↓MLL-target genes	MLL-chromatin dissociation
	H2AK119	Transcriptional repressor	Oncogenic	LC, PC	↓miR-200a, CGN, CDH1	EMT, EZH2 recruitment
	H3K36me2	Transcriptional regulation	Oncogenic	CRC, PCa	↓E/cadherin, ZO-1 ↑N/cadherin	EMT
KDM2B FBXL10 JHDM1B	H3K36me2	Transcriptional regulation	Oncogenic	CRC	↓p21, p27 ↑cyclin D1 ↑EZH2	Cell proliferation, cell growth, stemness CSCs
	H3K4me3	Transcriptional activation	Oncogenic	CC	↑CDC25A	Activation of PI3K/Akt pathway, cell motility
	H3K36me2	Transcriptional regulation	Oncogenic	GBM	↓p21 ↑cyclin D1, EZH2	Cell proliferation, migration,
	H3K36me2	Transcriptional regulation	Oncogenic	AML	↓p15↑Hoxa9/Meis1	Cell cycle progression
	H3K36me2	Transcriptional regulation	Oncogenic	AML	↑Nsg2, OXPHOS	HSC differentiation
	H3K36me2	Transcriptional regulation	Oncosuppressor	AML	↓Hox10, Smarca4/Brg1	Activation of interferon program-associated genes

KDM3A JHDM2A JMJD1A JMJD1	H3K9me2/1	Transcriptional activation	Oncogenic	PC	↑CDK6, cell cycle-associated genes ↑Wnt-related genes	Tumor growth
	H3K9me2	Transcriptional activation	Oncogenic	CRC	↑AR, c-Myc, Snail	Tumorigenesis, CSCs, MLL1 recruitment
	H3K9me2/1	Transcriptional activation	Oncogenic	PCa	↑AR, c-Myc, Snail	EMT, proliferation, invasion
	H3K9me2	Transcriptional regulation	Oncogenic	CC	↓E-cadherin, Bax ↑N-cadherin, Bcl-2, vimentin ↑ETS1 ↑CDX2	Tumor progression and growth Tumor growth
	H3K9me2	Transcriptional activation	Oncogenic	AML	↑Autophagy-related genes	Tumor progression
KDM3B JHDM2B JMJD1B	H3K9me2	Transcriptional activation	Oncogenic	CRC	↑Autophagy-related genes	Autophagy
	H3K9me2	Gene expression	Oncogenic	HCC	↑Cyclin D1, CDC123	Cell proliferation
	H3K9me2/1	Transcriptional activation	Oncogenic	ALL	↑Imo2	Leukemogenesis
KDM3C JHDM2C	H3K9me2	Transcriptional activation	Oncogenic	CRC	↑ATF2	Tumor progression, poor clinical outcome
	H3K9me2	Transcriptional activation	Oncogenic	AR-negative PCa	↑AR-downstream genes	Suppression of TNFα downstream genes and NF-κB pathway
KDM4A JMJD2 JMJD2A JHDM3A TDRD14B KIAA0677	H3K9me2	Transcriptional activation	Oncogenic	AML	↑AE-related genes	Proliferation, survival
	H3K9me2	Transcriptional activation	Oncogenic	PCa	↑AR-related genes ↑ETV1	Neoplastic formation YAP1 activation
	H3K9me3	Transcriptional activation	Oncogenic	BC	↑E α target genes	Cell growth, tumor progression
	H3K9me3	Repression	Oncogenic	CRC	↓53BP1	Tumorigenesis
	H3K9me3	Transcriptional activation	Oncogenic	AML	↑PAF1	Leukemogenesis

(Continued)

Table 17.1 KDM enzymes: targets and roles in cancer. *Continued*

Enzyme	Histone Target	Biological Properties	Potential Role	Type of Cancer	Target	Effects
KDM4B JMJD2B JHDM3B KIAA0876	H3K9me3	Transcriptional activation	Oncogenic	TNBC	↓ UPR pathway	Tumor progression
	H3K36me3	Transcriptional activation	Oncogenic	CRC	↑ GLUT1	Glucose uptake, ATP production
	H3K9me3	Transcriptional activation	Oncogenic	CRC	↓ p53-related genes ↑ β -catenin/TCF4 target	Tumor progression
	H3K9me3	Transcriptional activation	Oncogenic	PCa	↑ AR-related genes	Tumorigenesis
	H3K9me3	Transcriptional activation	Oncogenic	MLL-AF9 Hodgkin's lymphoma	↑ S100A8/9	Aggressive phenotype, radioresistance
KDM4C JMJD2C JHDM3C GASC1 KIAA0780	H3K9me3/2	Transcriptional activation	Oncogenic	EOC	↑ OCT-4	CSCs, migration, invasion
	H3K36me3	Transcriptional repression	Oncogenic	LC	↓ CXCL10	Resistance to radio- and immunotherapy
	H3K9me3	Transcriptional activation	Oncogenic	PCa	↑ c-Myc, AKT	Proliferation
	H3K9me3	Transcriptional activation	Oncogenic	CRC	↑ JAG1	Sphere formation
	H3K9me3	Transcriptional activation	Oncogenic	BC	↑ HIF-1 α related genes	Metastasis, tumor growth
	H3K9me3	Transcriptional activation	Oncogenic	AML	↑ MALAT1, CCND2 ↑ Leo1	Proliferation, tumor progression
	H3K9me3	Transcriptional activation	Oncogenic	RCC	↑ JAG1, VEGFR-3	Tumor progression, angiogenesis
KDM4D JMJD2D	H3K9me3	Transcriptional activation	Oncogenic	PCa	↑ AR-related genes	Tumor development and progression
	H3K9me3	Transcriptional activation	Oncogenic	CRC	↑ Wnt/ β -catenin signaling pathway	Transcriptional activation of c-Myc, cyclin D1, MMP2, MMP9
	H3K9me3	Inhibition Transcriptional activation	Oncogenic	HCC	↓ p53 ↑ Wnt/ β -catenin	Cancer progression and development
	H3K9me3	Transcriptional activation	Oncogenic	AML	↑ Antiapoptotic genes	Poor prognosis, proliferation

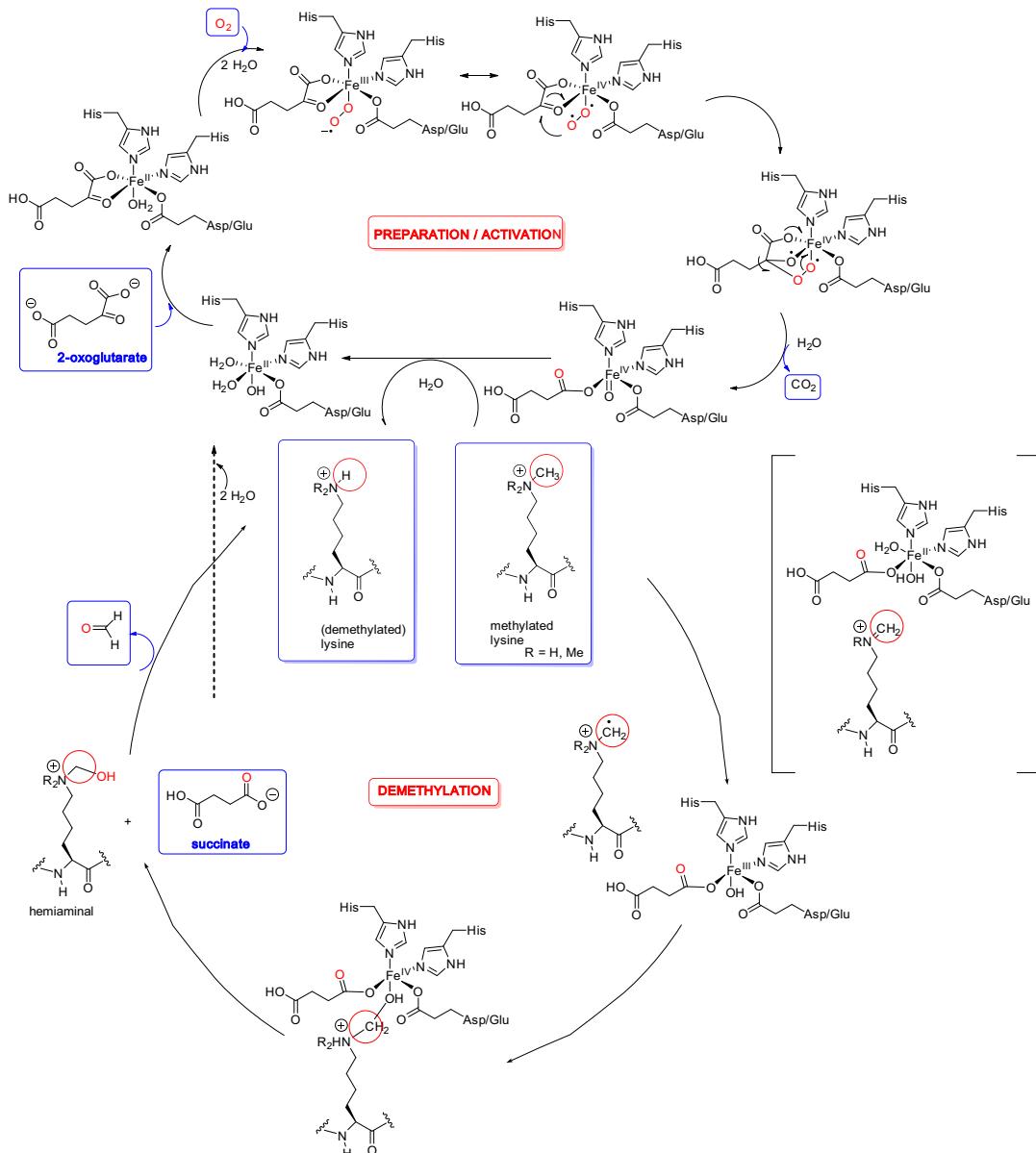
KDM5A RBBP2 JARID1A	H3K4me3	Transcriptional regulation	Oncogenic	EOC	↓ Epithelial markers ↑ Mesenchymal markers ↓ E-cadherin	EMT, metastasis, drug resistance
	H3K4me3	Transcriptional repression	Oncogenic	GC	↓ E-cadherin	EMT
	H3K4me3	Transcriptional activation	Oncogenic	BC	↑ ER/IGF1R/ErbB	Tamoxifen-resistance
	H3K4me3	Transcriptional activation	Oncogenic	AML	↑ Hoxa-genes	Cell proliferation, myeloerythropoietic differentiation
KDM5B JARID1B	H3K4me3	Transcriptional regulation	Oncosuppressor	CML	↓ miR-21 ↑ PDCD4	Apoptosis
	H3K4me3	Transcriptional repression	Oncogenic	PCa	↓ Tumor suppressors	Tumor growth
	H3K4me3	Transcriptional repression	Oncogenic	BC	↓ Tumor suppressor	EMT, cancer growth
	H3K4me3	Transcriptional repression	Oncogenic	HCC	↓ Tumor suppressor	EMT, poor prognosis
KDM5C SMCX JARID1C MRX13	H3K4me3	Transcriptional repression	Oncogenic	Cancer	↓ p53, PCNA, p21	Cell proliferation
	H3K4me3	Transcriptional repression	Oncogenic	CRC	↓ FBXW7	Reducing c-Jun degradation
	H3K4me3	Transcriptional repression	Oncogenic	LC	↓ miR-133a	Cancer growth, metastasis
	H3K4me3	Repriming/activating gene expression	Oncogenic	BC	↓ IFNs ↑ ER α	Cancer growth, metastasis
KDM5D JARID1D	H3K4me3	Transcriptional regulation	Oncosuppressor	PCa	↓ Invasion-associated genes	Tumor inhibition
	H3K4me3	Transcriptional activation	Oncosuppressor	GC	↑ CUL4A	EMT inhibition
KDM6A UTX	H3K27me2	Transcriptional activation	Oncogenic	BC	↑ Er α -targeted genes	Tumor development, invasion, migration
	H3K27me2	Transcriptional activation	Oncosuppressor	BC	↑ Dicer	Inhibition of tumor progression
	H3K27me2	Transcriptional activation	Oncogenic	CML	↑ TRKA	Imatinib-tolerance

(Continued)

Table 17.1 KDM enzymes: targets and roles in cancer. *Continued*

Enzyme	Histone Target	Biological Properties	Potential Role	Type of Cancer	Target	Effects
KDM6B JMJD3	H3K27me2	Transcriptional activation	Oncogenic	Cancer	↑ MAPK pathway	Cell growth
	H3K27me2	Transcriptional activation	Oncogenic	GBM	↑ SNAI1, N-cadherin, CXCL12	EMT, metastasis
	Direct interaction with p53	Transcriptional activation	Tumor suppressor	GBM	↑ p53	Antiproliferative effects
	H3K27me2	Transcriptional regulation	Tumor suppressor	CRC	↓ EMT-markers ↑ CXCL9, CXCL10, p15	Cell adhesion, apoptosis
	H3K27me2	Transcriptional activation	Oncogenic	CRC	↑ Notch	Tumor formation and growth
	H3K27me2	Transcriptional activation	Oncogenic	BC	↑ Bcl-2	Tumor progression
	H3K27me2	Transcriptional activation	Oncogenic	NSCLC	↓ p21 ↑ EMT-related genes	Cell cycle, EMT, proliferation
	H3K4	Transcriptional activation	Oncogenic	MDS CMML	Set1/MLL	Dysregulation of innate immune genes
	H3K27me3	Transcriptional activation	Oncogenic	T-ALL	↑ T-cell-specific oncogenic target	Inflammatory state, T cells proliferation
	H3K4/27	Transcriptional activation	Oncosuppressor	AML	↑ Myelopoietic regulators	Cell death, differentiation
KDM6C UTY	H3K4/27	Transcriptional activation	Oncogenic	PCa	↑ NKX3.1	Cell differentiation
KDM7A JHDM1D	H2K27me2	Transcriptional activation	Oncogenic	PCa, bladder	↑ AR-related genes	Cell growth and proliferation
	H2K27me2	Transcriptional activation	Oncogenic	BC	↑ c-Myc, KLF4 ↑ Bcl-2, BAD	BCSc Apoptosis inhibition
KDM7B PHF8	H3K9me2/3	Transcriptional activation	Oncogenic	CRC	↑ Tumor-related genes	Tumor progression, TNM-stage
	H3K9me2/3	Transcriptional activation	Oncogenic	BC HER2 +	↑ HER2-signaling	EMT, drug resistance
	H3K9me2/3	Transcriptional activation	Oncogenic	GC	↑ EMT-related genes	Migration, invasion
KDM7C PHF2	H3K9me2	Enhance expression	Tumor suppressor	CRC, GC	↑ p53	Tumor suppressor

Mechanistic proposals on the JmjC KDM catalytic cycle (Scheme 17.1) are considered to follow the principles established for 2-OG-dependent oxygenases by spectroscopic [20], kinetic [32], and computational studies [146]. Thus the oxidative demethylation reaction catalyzed by the JmjC



SCHEME 17.1

Mechanism of *N*-methyl-lysine demethylation by the KDM/JHD enzymes.

family is analogous to those operated by dioxygenases using Fe(II) in their catalytic domain as shown in [Scheme 17.1](#) [20,33,147,148].

In the resting enzyme, Fe(II) is octahedrally coordinated by three amino acids and two to three water molecules. Bidentate coordination of the 2-OG (via 1-carboxylate and oxo groups) and displacement of two water molecules occur after the binding of ferrous iron [Fe(II)] in the active site [19]. The coordination of the 2-oxo fragment selects the trans orientation relative to the metal-coordinating carboxylate of aspartic/glutamic acid. Sequential binding of the methylated lysine substrate weakens the binding of the water molecule to the metal and activates dioxygen binding ([Scheme 17.1](#)). Crystal structures are consistent with the proposal [19,20]. The key oxidative decarboxylation process occurs in the ternary complex incorporating succinate, the Fe(IV)-oxo complex generated upon oxygen binding to the Fe(II), and the methylated lysine. First, the α -ketoglutarate complexed Fe(II) transfers an electron to the coordinated oxygen, resulting in a peroxide anion (superoxide radical) and Fe(III). Nucleophilic attack of the anion to the carbonyl group (C2) of α -ketoglutarate generates an Fe(IV) bicyclic peroxyhemiketal and the intermediate is decarboxylated to produce succinate. The oxoferryl group of the highly unstable Fe(IV)-oxo intermediate thus produced removes a hydrogen atom from the methyl group of *N*-methylated lysine, releasing a Fe(III) hydroxide. Next, the radical recombination gives rise to a carbinolamine that losses formaldehyde and provides the demethylated lysine-containing peptide. Release of succinate and coordination of water molecules regenerates Fe(II) and completes the catalytic cycle ([Scheme 17.1](#)).

4 Chemical biology tools for the discovery of JmjC inhibitors

Since there are \sim 60 human 2-OG-dependent oxygenases [149], it is of paramount importance to develop new types of selective KDM inhibitors. The majority of KDM inhibitors are active site ion chelators containing Fe^{2+} -chelation ligands designed to compete with the 2-OG cofactor since they mimic at least one of the carboxylate groups of the 2-OG cosubstrate in the active site and coordinate forming one or two metal-ligand bonds. Being 2-OG analogs these compounds are pan-oxygenase inhibitors and chelate Fe(II) by using in general carboxylate and amide groups [150–152]. Among the functional groups involved in chelation of Fe(II) ion, catechols, hydroxamic acids, pyridyl and bipyridyl carboxylates, 8-hydroxyquinolines, pyrimidine carboxylates, and hydroxamic acids are common scaffolds found in the structure of KDM inhibitors. Given the fact that other unrelated enzymes are also dependent on Fe(II)/2-OG, selective inhibitors for the KDM subfamilies, and more importantly, within subfamilies, are still highly challenging [100,153].

The chemoproteomics approach based on affinity pulldowns, which need derivatization of the test molecules with linkers and additional functional groups serving as pulldown tags, has recently allowed the purification of dioxygenases [17]. Incubation of the beads with cell extracts in the absence or presence of unselective active-site drug-like inhibitor compounds, as well as cellular cofactors, followed by mass spectrometry-based quantification of bead enzymes, enabled the affinity capturing of around 40 different dioxygenase enzymes from human cells. Affinities for the cosubstrate 2-OG and for oncometabolites, such as 2-hydroxyglutarate, were determined by mass spectrometry-based quantification of bead-bound enzymes using a free-ligand competition binding format [17]. The comprehensive set of data comprising cofactor, metabolite, and inhibitor binding

properties for the 2-OG-dependent dioxygenase target class indicated a good correlation between measured binding affinities and enzymatic inhibition for small compounds. However, the correlation is not so reliable for metabolites, since increased concentrations of metabolites were found to exert complex effects in disease progression via deregulation of epigenetic mechanisms, DNA repair programs, and other dioxygenase-controlled cellular functions.

An alternative approach is focused on the development of acyclic and cyclic peptides targeting the substrate binding site [154–156], but the section will not be covered in this chapter.

5 Development of selective inhibitors

5.1 2-OG competitive inhibitors

Reviews on active site iron chelators and selective modulators for KDM5A [153] and KDM5B [100,157,158] have been recently published. Therefore the revision will focus on the period 2017–2021.

5.1.1 KDM2/7 inhibitors

A first-in-class inhibitor of histone substrate instead of the 2-OG cosubstrate, namely (S,S)-1 (Figure 17.2A), was identified after screening of a library of known binders to methyl lysine reader domain and histone methyltransferases against a panel of KDMs. Enantiopure (S,S)-1 [159] was shown to induce a dose-dependent increase of H3K36me2 levels with $IC_{50} = 0.16 \mu\text{M}$ using immunofluorescence assays in HeLa cells ectopically expressing catalytically active KDM2A. Cytotoxicity to HeLa and HAP1 cells was noticed at higher concentrations. Remarkable selectivity towards KDM2A (> 100-fold) was demonstrated when using a panel of KDMs and other epigenetic enzymes, with the

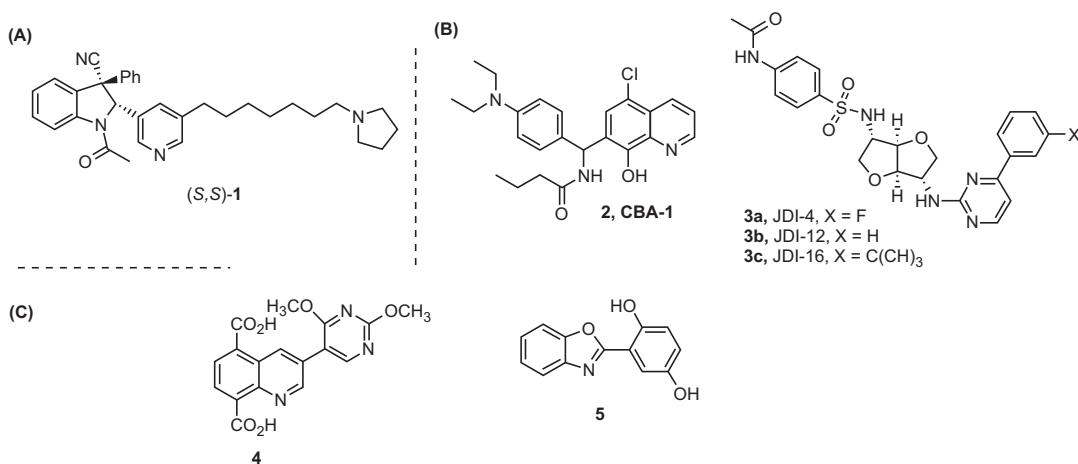


FIGURE 17.2

(A) KDM2, (B) KDM3, and (C) KDM6 inhibitors.

exception of KDM7A [159]. Tandem MS analysis confirmed the 1:1 ratio for binding of ligand (S,S)-**1** to KDM2A, and kinetic analysis revealed that the compound did not display competitive inhibition with respect to either 2-OG or the peptide substrate. Further experiments suggested the presence of an alternative (allosteric) binding site specific to KDM2A/7A [159].

5.1.2 KDM3 inhibitors

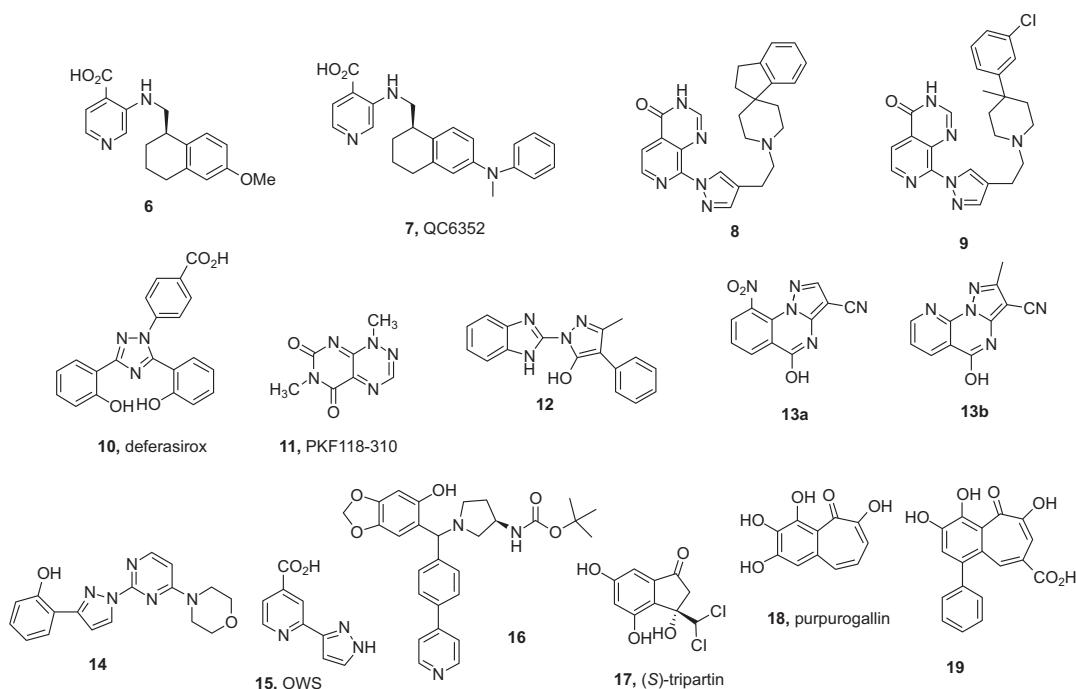
Carboxamide-substituted benzhydrol amines (CBAs) as members of a compound library were shown to inhibit Wnt signaling induced by either LiCl or Wnt protein in the stable HEK293T cell line. In particular, CBA-1 (**2**) (Figure 17.2B) inhibited Wnt signaling at 500 nM concentrations downstream of β -catenin, most likely by blocking β -catenin activity in the nucleus. It was also found to significantly inhibit colon cancer organoids development, indicating that it could inhibit colon cancer cells with APC and K-ras mutations [160]. CBA-1 (**2**) increased the levels of H3K9Me2 on the promoter of Wnt target genes, c-Myc and Cyclin B1. The use of a biotinylated analog of CBA-1 (**2**) confirmed that the compound bound endogenous KDM3A and inhibited its activity *in vitro* at low micromolar levels. CBA-1 (**2**) exhibited also inhibitory activity against KDM3B, but the activity was weaker than with KDM3A. *In vivo* studies in larvae of a Wnt/ β -catenin zebrafish reporter line indicated that CBA-1 (**2**) inhibited Wnt signaling in the tail and rescued Bio-induced eye defects in a dose-dependent manner with no obvious toxicity or embryo deaths with up to 20 μ M concentration. Moreover, the inhibition of KDM3A by the ligand was the crucial event in the observed Wnt inhibition [160].

A high-throughput virtual ligand screening of 149,519 natural products and 33,765 Chinese medicine components against JMJD1C led to the discovery of JDI-4 (**3a**) and JDI-12 (**3b**) (Figure 17.2B) as the most potent of the 15 active compounds [161]. Surface plasmon resonance analysis indicated modest binding affinity of JDI-4 (**3a**) and JDI-12 (**3b**) for KDM3 JMJD1C ($IC_{50} = 2.67 \times 10^{-5}$ M; $IC_{50} = 2.7 \times 10^{-6}$ M, respectively) and also for KDM3B ($IC_{50} = 1.63 \times 10^{-5}$ M; $IC_{50} = 1.36 \times 10^{-7}$ M, respectively). *In vitro* demethylation assays confirmed that increasing concentrations of the compounds induce H3K9me1 levels in MV4–11 cells. The two ligands precisely inhibited the proliferation of several *MLLr* AL and other hematopoietic malignancies-derived cell lines except KG-1, and also colony formation on these cells [161].

Among the analogs assayed after computer docking studies and SPR analysis, JDI-16 **3c** was found to be the most active ($IC_{50} = 8.06 \times 10^{-6}$ M for KDM3 JMJD1C; $IC_{50} = 7.79 \times 10^{-6}$ M for KDM3B) and showed the most significant repression on colony formation in MV4–11, MOLM-13, and SEM cell lines, and increase of the H3K9me1 levels in leukemia cell line SEM and primary leukemia samples when compared to analogs JDI-4 **3a** and JDI-12 **3b**. Significant cell apoptosis of MV4–11 and SEM cells and also on primary AML cells, as well as induction of genes and related metabolites involved in cell metabolism were determined for JDI-16 **3c**. Synergistic effect with differentiation-inducing agent all-*trans*-retinoic acid and demethylation agent decitabine were observed to arrest growth of some *MLLr* AML cell lines but no cytotoxic effect were seen on leukemia cells resistant to JMJD1 depletion or cord blood cells [161].

5.1.3 KDM6 inhibitors

In addition to GSK-J1 (**48**, see Figure 17.3) [162,163], ligands of KDM6 based on quinoline-5,8-dicarboxylic acid and on benzoxazole-2-yl scaffolds have been discovered using a fragment-based approach [164,165].

**FIGURE 17.3**

Selection of KDM inhibitors.

KDM6B inhibitors with quinoline-5,8-dicarboxylic acid substructures were designed after *in silico* screening of a fragment library of metal chelators as putative metalloprotein inhibitors, followed by molecular modeling of the presumed interactions established on the available crystal structures of KDM6B with charges at iron and coordinating amino acids refined by DFT computations and molecular docking [164]. Incorporation of a second carboxylic acid at the initial quinoline-8 carboxylic acid led to improved interactions with additional amino acids without affecting the global conformation. Additional stabilizing interactions were assigned to ligands with H-bond donors/acceptors functional groups and hydrophobic substituents. The most potent and selective of the series of compounds was **4** (Figure 17.2C), which showed an $IC_{50} = 1.52 + 0.50 \mu\text{M}$ for KDM6B (90% inhibitory activity) and, when used at $10 \mu\text{M}$, no significant inhibition of KDM6A (UTX), KDM4C and KDM4D, but weak inhibition of KDM3A (12%) and KDM2A (15%) and relatively lower activity on KDM5A (33%) and KDM5B (26%) [164].

Compound **5** (Figure 17.2C), containing a 2-benzoxazol-2-yl-phenol scaffold [165], was derived from previous work [164] that carried out *in silico* screening of a metal chelator fragment common to metalloprotein inhibitors. Whereas both 2-(benzo[*d*]oxazol-2-yl)phenol and 2-(benzo[*d*]thiazol-2-yl)phenol fragments were considered to properly get locked into the α -KG cavity that hosts the methylated lysine, only the former proved to be enzymatically active using the AlphaScreen protocol. Structural modifications near the phenol moiety to optimize the $\pi - \pi$ interactions with the

surrounding residues of KDM6B led to the selection of the benzoxazole derivatives [162,163]. Lead compound **5** showed an IC₅₀ value of 1.22 + 0.22 μM against JMJD3 by AlphaScreen assay and 25.7 + 2.3 μM against KDM6A (UTX) [165]. Ligand **5** showed an acceptable apparent permeability value (Papp) of $9.12 \times 10^{-7} + 1.67 \times 10^{-8}$ cm/s using the parallel artificial membrane permeability assay technique (PAMPA). Upon treating A375 melanoma cells at different concentrations of **5**, cell proliferation in a concentration-dependent manner was noted at 20 μM, and cells were arrested at the S phase at 100 μM. The IC₅₀ value was determined as 24.7 + 1.1 μM. In addition, a slight decrease of the H3K27me2 levels was detected, which was ascribed to the low inhibitor activity of the compound against KDM6A [165].

5.1.4 KDM4 inhibitors

Compound QC6352 (**7**, Figure 17.4), structurally related to pyridine-2,4-dicarboxylic acids that showed inhibition of KDM4C [166], was designed through structural modifications of the 3-(methylamino)isonicotinic acid scaffold [167], which itself showed notable enzymatic potency. Since the *R* enantiomers of these analogs with tetrahydronaphthalene fragments were more potent and selective inhibitors than the *S* counterparts, further studies focused on these enantiomers, and structural modifications were suggested by the space available at the methoxy-substituted tetrahydronaphthalene ring in the crystal structure. Lead compound **6** (Figure 17.4) with an IC₅₀ value of 6 + 2 nM for KDM4C, was further optimized to QC6352 (**7**), with higher cellular potency (KYSE-150 EC₅₀ = 3.5 + 1 nM;

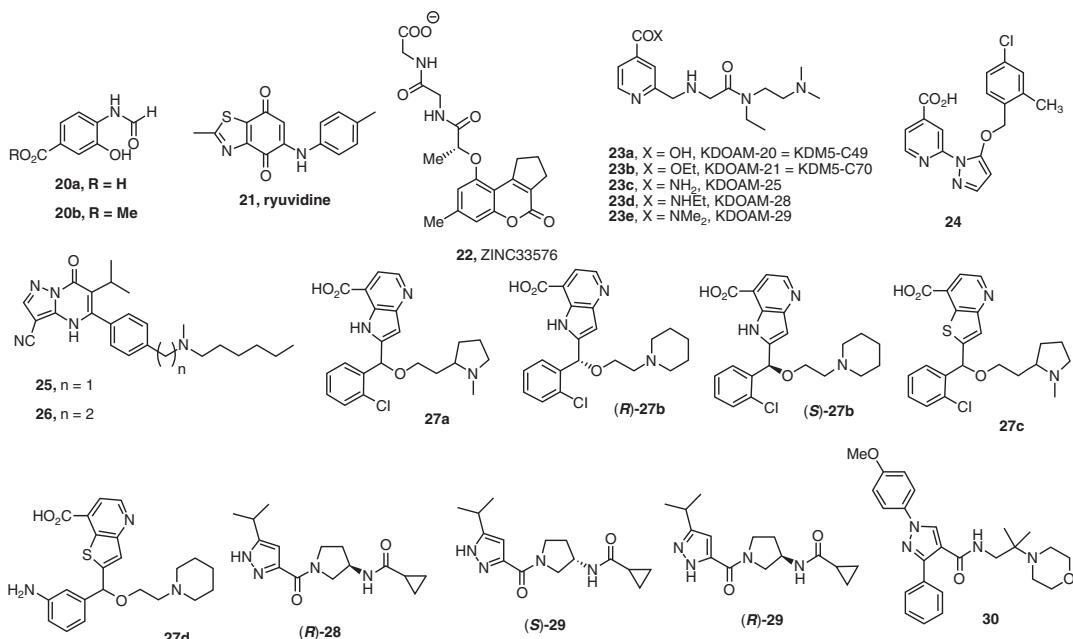


FIGURE 17.4

KDM4 inhibitors, including natural products and natural products-inspired KDM4 inhibitors.

H3K36 MOA = $1.3 + 0.3$ nM), and inactive in IMR-90, a normal fibroblast cell line [166]. Compound QC6352 (**7**) showed greater potency against the KDM4 family (KDM4A, $IC_{50} = 104 + 10$ nM; KDM4B, $IC_{50} = 56 + 6$ nM; KDM4C, $IC_{50} = 35 + 8$ nM; KDM4D, $IC_{50} = 104 + 18$ nM), and higher selectivity against other family members, except KDM5B ($IC_{50} = 750 + 170$ nM) [166]. The compound was also active to inhibit colony formation and cell viability in BR0869f, a HER-2-positive patient-derived BC organoid model ($IC_{50} = 5$ nM after a 5-day incubation) and in SU60, a patient-derived CRC organoid model ($IC_{50} = 13$ nM) [166]. Administered intravenously (dose of 5 mg/kg) to female CD-1 mice, QC6352 (**7**) exhibited low systemic clearance and volume of distribution, 6.9 mL/min/kg and 675 mL/kg, respectively, and it was readily absorbed following oral administration (at a dose of 10 mg/kg) with AUC of 10,400 ng/mL·h and oral bioavailability of 30% [166]. *In vivo* efficacy using the BR0869f PDX model in mice was also studied, founding that the tumor growth rate diminished in a dose-dependent manner.

A series of C8-pyrido[3,4-*d*]-pyrimidin-4(3*H*)-one derivatives [168] were tested in the InCELL Hunter™ assay for KDM4B and KDM5B cellular target engagement, and several ligands (Figure 17.4) showed EC₅₀ values close to 1 μM [169]. Among them, **8** (KDM4A $IC_{50} = 0.100 + 0.041$ μM; KDM4B $IC_{50} = 0.043 + 0.021$ μM; KDM5B $IC_{50} = 0.038$ μM; KDM5C $IC_{50} = 0.123$ μM; Caco-2: 11.64×10^{-6} cm/s) and **9** (KDM4A $IC_{50} = 0.107 + 0.020$ μM; KDM4B $IC_{50} = 0.029 + 0.006$ μM; KDM5B $IC_{50} = 0.014$ μM; KDM5C not tested; Caco-2: 13.33×10^{-6} cm/s) were more promising. In the cellular IF assay, analogs **8** and **9** inhibited KDM4A demethylase activity in cells (EC₅₀ = 4.7 and 5.9 μM, respectively) and KDM5B with lower potency (EC₅₀ = 13.4 and 20.8 μM, respectively). The cellular KDM4A and KDM4B EC₅₀ values determined for **8** were like the corresponding biochemical IC₅₀ values (IC₅₀ = 2.21 and 0.79 μM, respectively) at physiological 2OG concentrations (approximately 1 mM). Notable reduction from biochemical to cell-based activity was noted for these analogs upon cellular profiling in two orthogonal target engagement assays (InCELL Hunter™ and an inmunofluorescence assay), which appeared consistent with 2OG competition. Thus subnanomolar biochemical potency was proposed for these analogs to achieve submicromolar target inhibition in cells [169].

Deferasirox (**10**, Figure 17.4) is a clinically used drug (as well as deferoxamine and deferiprone, not shown) for the treatment of patients with excess levels of ions in their blood (hemochromatosis). Deferasirox (**10**) showed potent inhibition of demethylation of H3K9me3 peptides by KDM4, with IC₅₀ values between 3 and 17 μM [170]. Enzyme kinetics analysis revealed that only inhibition by deferasirox (**10**) (but not deferoxamine or deferiprone) was, at least in part, competitive with the cosubstrate 2-OG. Using the LANCEUltra assay system, potent and unselective inhibition of KDM5A and KDM6B was demonstrated by using the corresponding substrates, namely H3K4me3 and H3K27me3 [170]. Spectroscopic analysis using pseudomodulated field-sweep echo electron paramagnetic resonance (EPR) spectra of KDM4A with bound Fe³⁺ (in place of the catalytically active Fe²⁺) showed EPR signature between 400 and 650 mT typical of nitrogen-complexed Fe³⁺. Further experiments, including pulsed ESEEM experiments and NMR spectroscopy using recombinant protein, also confirmed the formation of the Fe³⁺ complex with the ligand within the active site of KDM4A [170]. Molecular Docking experiments suggested binding of the ligand (**10**) to the active site of KDM4A, KDM5A, and KDM6B, by chelation of the Fe²⁺ ion in a tridentate manner via its triazole *N* atom and both phenol groups in KDM4A, in a bidentate manner in KDM6B and also in a bidentate manner via its triazole *N* atom and one phenol O atom in KDM5A [170].

Deferasirox (**10**) showed potent antiproliferative effects in KYSE-150 esophageal cancer cells and HL-60 leukemia cells in an MTS assay with GI_{50} values of $3.27 + 0.62$ and $5.5 + 0.4 \mu\text{M}$, respectively [170]. Potent growth inhibition of a panel of esophageal cancer cell lines was observed for the methyl ester of deferasirox. The effects on a panel of 18 lung adenocarcinoma cell lines, and three nontransformed lung-derived cell lines (BEAS-2B, IRM-90, and WI-38), in comparison with other epigenetic ligands (including KDM inhibitor JIB-04 **41**, Figure 17.3, and HDAC inhibitors KK16 and vorinostat) further supported that the both deferasirox (**10**) and its methyl ester act as *bona fide* JmjC KDM inhibitors within cells, which was consistent with the overall increase in the level of histone H3 methylation and methylation-specific actions of deferasirox (**10**) [170].

The levels of H3K9 trimethylation were likewise increased upon treatment of KYSE-150 cancer cells with deferasirox (**10**) (the same effect was found for some of its chemical derivatives), as observed by immunofluorescence microscopy, with IC_{50} value of $40.5 \mu\text{M}$ (cf. $3.3 \mu\text{M}$ for the methyl ester). Likewise, the levels of H3K9me3 staining in U2OS cells ectopically expressing KDM4A were increased with deferasirox (**10**) in a dose-dependent manner, thus suggesting direct inhibition of demethylation by KDM4A in cells [170]. Lastly, deferasirox (**10**) was also observed to inhibit human 2-OG-dependent hypoxia-inducible factor prolyl hydroxylase activity, which was considered to account for some of the complex effects observed in the cellular studies.

Toxoflavin (PKF118-310, **11**, Figure 17.4), an antagonist of transcription factor 4 (TCF4)/ β -catenin signaling, was identified after 3D-structural based virtual ligand screening, and shown to inhibit KDM4A in a dose-dependent manner, with IC_{50} of $10 \mu\text{M}$ *in vitro* [171]. Direct interaction with KDM4A was demonstrated through its stabilization at 44°C upon stimulation with the ligand using cellular thermal shift assay experiments. Anticancer activity was demonstrated on liquid and solid tumor cells, namely wild-type HCT-116, HCT-116 p53^{-/-}, and U937 leukemia cells, and immortalized cell line MePR2B, in a dose-dependent manner. Greater impact was observed on the cell cycle upon treatment of the U937 leukemia cell line with toxoflavin (PKF118-310 [11]).

Benzimidazole pyrazolones were identified on the ChemBioNet (CBN) library (consisting of 32,032 small molecules) as scaffolds for recombinant human KDM4E inhibition after a formaldehyde dehydrogenase (FDH) coupled enzyme assay [34]. The most potent of the series preserving the pyrazole hydroxyl moiety, analog **12** (Figure 17.4), showed an IC_{50} value of $13 \mu\text{M}$ using the ELISA-based method [34]. No inhibition of FDH was found, whereas the α -KG dependent K_m value of $22 \mu\text{M}$ was measured for KDM4E, which increased to $69 \mu\text{M}$ in the presence of the JmjC-KDM competitive inhibitor pyridine 2,4-dicarboxylic acid (2,4-PDCA). A noncompetitive mechanism of inhibition was suggested since increasing concentrations of the compound decreased V_{max} values with no effect on the substrate's K_m . The stabilization of a KDM4E: α -KG complex was consistent with the decreasing K_m and V_{max} values [172]. Further experiments confirmed that the benzimidazole pyrazole inhibitor completely removed the active-site metal ions and inactivated the enzyme. Thus a complex mechanism of inhibition was suggested by competition with the enzyme for binding the active-site Fe^{2+} and by populating a distal site on the enzyme surface, as deduced from X-ray analysis of some analogs [172].

Compound **12** inhibited the growth of all PCa cell lines tested, including DU145 cells and PC3 cells, plus a nondisease control cell line (HuPrEC), with similar efficacies and GI_{50} values between 8 and $26 \mu\text{M}$. The cytotoxic effect was mediated in part through inhibition of intracellular KDMs. Treatment of LnCaP cells with the inhibitor at $25 \mu\text{M}$ decreased the PSA AR-dependent expression, which diminished when using a mixture of the compound and DHT. Reduced expression of

androgen-receptor-associated genes in PCa cells, together with the lower levels of the H3K9me3 epigenetic mark in untreated cells, confirmed the KDM4 activity inhibition in cell lines expressing these targets [172].

Molecular docking-based virtual screening against several chemical libraries (including Specs, ChemDiv and the in-house library) based on the crystal structure of the JmjC domain of KDM4D (PDB entry: SFP4) led to the discovery of compounds **13a** and **13b** (Figure 17.4), with inhibition rates of more than 50% against KDM4D when used at 10 µM. Although they also inhibited KDM2D, KDM3B, and KDM5A, only compound **13a** showed a better selectivity against KDM4D [173]. Structural modifications led to analog **14**, with IC₅₀ value of 0.41 + 0.03 µM for KDM4D and almost no activity (> 10 µM) against KDM2B, KDM3B and KDM5A. Another analog, named OWS (**15**), was also discovered, which showed an IC₅₀ value of 4.28 µM for KDM4D [174].

AlphaLisa-based screening of an in-house chemical library of about 4000 compounds, structural optimization and SAR studies, led the discovery of a new class of KDM4D inhibitors based on the 2-(aryl(pyrrolidine-1-yl)methyl)phenol scaffold. The most potent of the series, compound **16** (Figure 17.4) showed an IC₅₀ = 0.023 + 0.004 µM [175], with an equilibrium dissociation constant (Kd) of 0.036 µM determined using isothermal titration calorimetry (ITC) assay. A differential scanning fluorimetry (DSF) assay indicated a dose-dependent negative thermal shift (ΔTm), contrary to other 2-OG competitive inhibitors, which suggested a different mechanism of inhibition. Binding to the 2-OG binding pocket was discarded after kinetic analysis indicating that the compound showed no competitive inhibition kinetics towards 2-OG with a Ki_{app} value of 0.293 µM. Based on Molecular Docking studies, it was proposed that the ligand instead occupies the H3K9me3 peptide-binding site [175]. Since compound **16** displayed high selectivity (SI > 1500) not only against other JMJD family members (KDM7B, KDM3B, KDM4A, KDM5A, and KDM6A) but also within the KDM4 subfamily, it was therefore considered the first KDM4D-selective inhibitor [175].

In an MTT assay, dose-dependent antiviability effects against HCT116, SW620, and SW480 cell lines were determined for **16** with IC₅₀ values of 10.73, 19.20, and 10.70 µM, respectively. Although the ligand inhibited colony formation in a dose-dependent manner, its potency was weaker with additional tumor cell lines. Ligand **16** could also blocks the migration of HCT116 cells in a dose- and time-dependent manner [175]. The compound was found to downregulate Gli1, β-catenin, and c-Myc in HCT116 cells in a dose-dependent manner, and, probably, also, KDM4D, suggesting that its anticancer properties are due to its ability to inhibit both activity and expression of KDM4D enzyme [175].

Few natural inhibitors of 2-OG-dependent oxygenases, including the KDMs, have been described [19,176] (S)-Tripartin (**17**, Figure 17.4), with the unusual dichloromethylcarbinol dihydroxyindanedione structural motif, was isolated from a culture of *Streptomyces* sp. associated with a larva of the dung beetle *Copris tripartitus* Waterhouse, and found to inhibit KDM4 enzymes in cells [177]. Both synthetic tripartin enantiomers caused moderate increase in the H3K9me3 and H3K9me2 levels. However, none of the tripartin enantiomers were shown to significantly modify the H3K9me1 level. Further re-evaluation showed that it significantly increased H3K9me3 levels in HCT-116 cells by Western blot analysis, but no or very slight inhibition was detected when tested at 100 µM against isolated KDM4A, B, C, D, or E under standard assay conditions [178]. Thus the mechanism of action of tripartin is undefined, and the formation of further metabolites as active KDM inhibitors, or the indirect effect on the H3K9 methylation status, could instead justify the biological activities of the natural product [178].

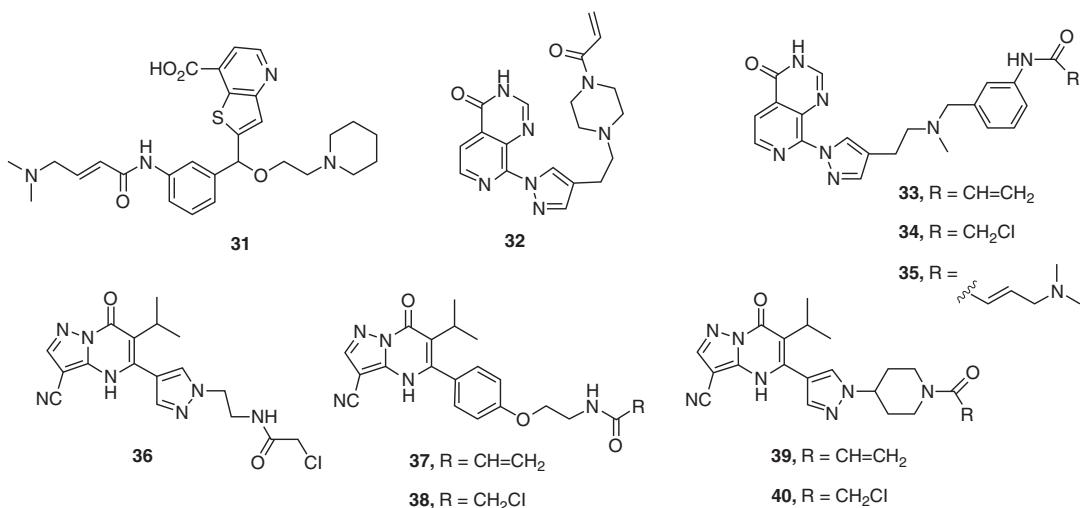
By using high-throughput screening techniques, we have also identified purpurogallin (**18**, Figure 17.4), a natural product isolated from nutgalls and oak bark and used as component of edible oils, as Jumonji-C (JmjC) inhibitor [179]. Compound **19** (Figure 17.4) showed the highest potency among those synthesized that were inspired in the structure and biogenesis of purpurogallin (**18**). Analog **19** showed 70% inhibitory activity of HCT-116 CRC cells with IC₅₀ of 26.5 μM. Furthermore, when treated with **19** at 10 μM for 24 h, 48 h, or after double administration for 24 h, an increase of the signals corresponding to two KDM4A targets, namely H3K9me2 and H3K36me3, both after 24 h and after double stepwise administration, was observed by Western blot analysis. As expected, **19** did not modulate the activity of other targets with lysine demethylases activities, such as H3K27me2, H3K4me1, H3K4me2, and H3K4me3 [179], which is in agreement with being a nonspecific substrate for this enzyme [180].

The anticancer activity of **19** relative to well-known epigenetic modulators was also examined through cell cycle analyses on HCT-116 CRC, NB4 APL, and U937 AML cells. Significant cell death induction by **19**, in a time- and dose-dependent manner was demonstrated [179]. In addition, compound **19** induced a robust cell death in all cancer cell lines tested, including A549 (lung), MiaPaCa (pancreas) HT-29 (colon), Hep-G2 (human hepatocyte carcinoma), and MCF7 (breast), with IC₅₀ values ranging from 7.04 to 4.04 μM after 72 h of treatment [179].

5.1.5 KDM5 inhibitors

Ligands containing *o*-hydroxyanilide functional groups, such as **20a** and **20b**, which were proposed to also chelate iron and behave as a novel pharmacophore, were discovered using the inhibitory-based fragment merging strategy after association of the *ortho*-substituted aniline substructure to HDAC inhibition [181]. From the *o*-substituted-anilides screened against KDM5A in the AlphaScreen assay, only those analogs with the hydroxy-substituted formanilides bearing 3-carboxylate group showed pronounced KDM5A inhibitory activity and greater potency than parent CPI-455 (see 47, Figure 17.3) [181]. IC₅₀ values of 46 + 11 μM for KDM5A were determined for **20a**, with only 20% and 38% inhibition of KDM4A and KDM6B, respectively, whereas HDAC1 inhibitory activity required a much higher (100 μM) concentration. Since the KDM5A inhibitory activity of compound **20a** at 200 μM decreased from 97% to 44% as the concentration of α-KG increased from 50 to 1000 μM in the FDH-coupled assay, it was concluded that the inhibitory effect might be due to the interaction with the α-KG binding site of KDM5A. Although in A549 human LG cells, in which KDM5A is overexpressed, compound **20a** did not affect H3K4me3 levels, its methyl ester **20b** as putative prodrug did increase the H3K4me3/H3 ratio by ca. 1.7–2.2 times at 100–250 μM. None of these compounds showed significant toxicity against A549 cells at 250 μM [181].

A collection of 3865 small molecules with known effects on biological processes from several drug libraries (Microsource International Drug, MicroSource US Drug, Prestwick, and Tocris) was evaluated using the AlphaScreen method in the search for inhibitors of recombinant KDM5A after His-tagged KDM5A1–797 produced in Sf9 insect cells and purified using the His tag. A group of 60 compounds that showed inhibition rates higher than 70% at 5 μM with a Fe(II) concentration of either 3 or 50 μM to discard inhibitory effects through chelation to Fe(II) or competition with Fe (II) were selected. In cell reporter assays using the human TFPI-2 promoter responsive to KDM5A inhibitors, 10 compounds were found to increase luciferase activity by more than twofold at 10 μM. MALDI-TOF/M studies confirmed their activity as KDM5A inhibitors. After discarding

**FIGURE 17.5**

KDM5 inhibitors.

those that appear to compete with 2-OG and other that did not show clear repression of reduction of H3K4me3 levels, and further studies in the effects of the inhibitors on the growth of gefitinib-tolerant human SCLC PC9 cells and drug-tolerant expanded persisters (DTEPs), ryuvidine (**21**, Figure 17.5) a derivative of benzothiazoledione, was found to inhibit the growth of DTEPs at concentrations at which parental PC9 cells were mostly unaffected [182]. Ryuvidine (**21**) inhibited the family members KDM5A, B, and C with different efficiencies (IC_{50} values of $0.57 + 0.1 \mu\text{M}$ for KDM5A, $0.026 + 0.01 \mu\text{M}$ for KDM5B, and $8.73 + 1.8 \mu\text{M}$ for KDM5C) with KDM5B being the most sensitive and KDM5C the more resistant to the compound [182].

The cyclopenta[*c*]chromene derivative ZINC33576 (**22**, Figure 17.5) was classified as the principal candidate after performing hierarchical *in silico* virtual ligand screening (using the internal coordinate mechanics ICM method) towards the KDM5A catalytic pocket of a chemical library containing 90,000 natural products or natural product-related structures from the ZINC compound library, followed by biochemical characterization [183]. To this end, the 17 compounds selected by the *in silico* analysis were screened against KDM5A using an *in vitro* chemiluminescence assay. Out of the four hits showing greater than 50% inhibitory activity on KDM5A in MDA-MB-231 BC cells, ZINC33576 (**22**) showed the greatest potency both *in vitro* and *in cellulo*. Incubation of MDA-MB-231 cells with ZINC33576 (**22**) significantly increased H3K4me3 and H3K4me2 levels. The ligand was shown to target KDM5A *in cellulo* using the cellular thermal shift assay (CETSA) after incubation at $3 \mu\text{M}$ with MDA-MB-231 lysates for 30 minutes and detection by Western blotting. ZINC33576 (**20**) selectively and dose-dependently inhibited KDM5A demethylase activity (IC_{50} 23.8 nM) over that of KDM4 (IC_{50} 100 μM) using an *in vitro* chemiluminescence assay. Moreover, it showed a more selective inhibition of the members of the KDM5 family than CPI-455 (**47**, Figure 17.3) after comparison of the IC_{50} values *in vitro* and the transcriptional genes specifically regulated by KDM5A (*Bak-1*), KDM4A (*p21*), KDM5B (*CAVI*) and KDM5C (*SCNA*) *in*

cellulo. *In silico* docking studies of ZINC33576 (**22**) with KDM5A suggested that the molecule occupies the 2-OG binding domain, likely through a competitive mode of action.

Using the MTT assay, ZINC33576 (**22**) showed strong cytotoxicity against MDA-MB-231, MDA-MB-489, and MCF-7 BC cell lines, and relatively low toxicity against MCF-10A and LO2 cells. A colony formation assay using the MDA-MB-231 cell line confirmed the inhibition of colony formation by the KDM5A inhibitor in a dose-dependent manner. *In cellulo* studies in MDA-MB-231 cells indicated that ZINC33576 (**22**) promoted an increase on the amplification of the promoters of *p16* and *p27* genes, which suggested disruption of the recruitment of KDM5A to H3K4me3, increasing the trimethylation of H3K4 at the promoters of *p16* and *p27*, and thus raising their transcription. G1 arrest and senescence induction via transcriptional regulation of *p16* and *p27* levels was assigned to the blocking of the KDM5A-mediated H3K4-demethylation activity by ZINC33576 (**22**) [183].

Given the potent inhibition of KDM5B *in vitro* by the ester prodrug KDOAM-21 (**23b**) [184], derivatives of the carboxylic acid with less labile amide groups were considered to retain KDM5 potency and selectivity. KDOAM-25 (**23c**, Figure 17.5) was the most potent KDM5B inhibitor of the functionalized amide derivatives, with biochemical half maximal inhibitory concentration values of <100 nM *in vitro*, with an IC₅₀ of 19 nM against KDM5B (IC₅₀ = 71, 69 and 69 nM for KDM5A, 5C and 5D, respectively) and improved selectivity over KDM4C. Analogs KDOAM-28 **23d** and KDOAM-29 **23e** proved to be less potent (IC₅₀ = 98 nM and 270 nM, respectively) [185]. Primary amide KDOAM-25 **23c** was characterized as a selective inhibitor of the KDM5 subfamily since no inhibition was observed on other 2-OG oxygenases at concentrations below 4.8 μM. Moreover, it showed no inhibition when profiled in the CEREP express on a panel of 55 common off-targets and therefore was proposed to act as a partial competitor of the cosubstrate 2-OG. Antiproliferative effects of KDOAM-25 **23c** in the MM1S multiple myeloma cell line using a fluorescent cell viability assay showed little effect after three days, but considerable reduction after a delay of 5–7 days with an IC₅₀ of 30 μM. In addition, using the ChIP-Rx strategy for quantification, an increase in the level of global H3K4me3 was observed. No significant cytotoxicity to A549 cells at 250 μM was noted for the series of compounds [185].

An analog with a pyrazole pyridine core **24** (Figure 17.5) was designed with the purpose of replacing the pyridine ring of KDM4E inhibitors [186] with a less basic and weaker metal chelator pyrazole ring, under the assumption that it should show lower toxicity [187]. Modeling studies suggested that the extension of a pendant aryl ring into the peptide binding region would allow to improve the selectivity for the KDM5 family. Additional derivatives were prioritized, mainly those with bidentate metal chelation by both nitrogen atoms on the pyridine and pyrazole rings as well as stabilization by π-stacking interactions. Optimization of the designed analogs led to **24**, a potent and selective inhibitor of KDM5A and KDM5B (IC₅₀ = 0.013 μM and IC₅₀ = 0.002 μM, respectively) as shown by the induction of H3K4me3 levels up to 10-fold in the ZR-75-1 BC cell line with EC₅₀ = 0.09 μM. It was also highly selective against other KDM family members except KDM4C (IC₅₀ = 0.041 μM) [187].

Pharmacokinetic parameters in female CD-1 mice after intravenous dose administration of **24** (5 mg/kg) revealed a low systemic clearance of 2.1 mL/min/kg and a low volume of distribution of 0.16 L/kg. It was readily absorbed after oral administration (10 mg/kg) with an AUC of 44,000 ng/mL·h and oral bioavailability of 55%. *In vivo* PK/PD studies on the MCF-7 human BC xenograft model using female nude mice indicated the induction of H3K4me3 by **24** at 75 mg/kg, which was sustained until the 24 h time point [187].

Using an inhibitor-based fragment merging strategy, further analogs of 3-cyanopyrazolo[1,5-*a*]pyrimidin-7-one [188,189] differing in the size on the methylene linker were designed (Figure 17.5). Compound **25** efficiently ($97.9 + 0.5\%$ at $1 \mu\text{M}$) inhibited KDM5A *in vitro* and showed IC_{50} value of $22.7 + 3.2 \text{ nM}$, which is 22 times stronger than CPI-455 (**47**, see Figure 17.3) [190]. Homologous tertiary amine **26** with an ethylene linker showed a 5-times more activity on KDM5A inhibition than **25**. However, AlphaLISA screen assay confirmed that compound **25** did not show selectivity among the subfamily members, since it provided similar IC_{50} values for inhibition of KDM5B and KDM5C (IC_{50} values of $51.1 + 5.3 \text{ nM}$ and $5.84 + 0.65 \text{ nM}$, respectively) than for KDM5A. IC_{50} values for homolog **26** were also similar for the three subtypes (IC_{50} values of $4.37 + 0.19 \text{ nM}$; $1.34 + 0.16 \text{ nM}$; $6.24 + 0.70 \text{ nM}$, for KDM5A, KDM5B, and KDM5C, respectively) [190]. Selectivity against other KDM family proteins (KDM2A, KDM3A, KDM4A, KDM6B, and KDM7B) was nevertheless demonstrated, with estimated IC_{50} values of **25** for KDM4A and **26** for KDM3A that vary between 25 and $50 \mu\text{M}$, and higher values for the other KDMs tested.

Cellular assays using the human lung cancer cell line A549 indicated inhibition of KDM5s by **25** and **26** when used at $25 \mu\text{M}$ concentration, with increased levels of H3K4me3 by Western blot analysis without affecting the methylation levels of H3K9me3 and H3K27me3. The growth of A549 cells was also inhibited by $50 \mu\text{M}$ concentration of **25** and **26** in a dose-dependent manner, with GI_{50} values of $40.0 \mu\text{M}$ and $29.6 \mu\text{M}$, respectively [190].

In efforts to understand the differences in biological activities of the individual enantiomers of chiral epigenetic inhibitors, selected ligands were evaluated as racemates and as enantiopure compounds [191]. In the case of **27** (Figure 17.5) the racemates were first evaluated using the purified minimal catalytic domain KDM5A(1–588) Δ AP, and their dissociation constants determined by isothermal titration calorimetry (ITC). It was concluded that the identity of the basic substituent at the terminus of the alkyl ether and the length of the linker had a small impact on binding affinity and therefore the core was tolerant to structural modifications [191]. AlphaLISA-based determination of the potency of these molecules on enzyme inhibition using an antibody against methylated H3K4me3 yielded inhibitory activities that correlated reasonably well with the relative binding affinities for the KDM5A linked Jumonji domain constructs, except in the case of the two enantiomers [191]. The biological evaluation of enantiopure analogs showed enantiodiscrimination. The most potents of the series were the (*R*)- and (*S*)-(27) and (*R*)- and (*S*)-(28). *In vitro*, (*S*)-**27** and (*R*)-**28** (Figure 17.5) exhibited greater binding and more potent inhibition of KDM5A [191]. For the former, a $K_D = 60 \text{ nM}$, ca. fourfold better than the *R* enantiomer was determined. For the latter, the difference in binding affinity between the two enantiomers associated with the relative inhibitory activity (IC_{50} values of $22 + 3 \mu\text{M}$ for (*R*)-**28** and $140 + 30 \mu\text{M}$ for (*S*)-**28**) in α -KG-competition binding experiments ($1 \text{ mM } \alpha\text{-KG}$). (*R*)-**28** showed enhanced accumulation of H3K4me3 and greater growth inhibition (about 10-fold) than its enantiomer when tested with different subtypes of BC cells, including BT474 and SKBR3 (HER2^+), MCF7 (ER^+), MDA-MB-231 (triple negative for ER, PR, and HER2) cells at concentrations ranging from 0.1 to $10 \mu\text{M}$. Selectivity was confirmed by the minimal effect of these enantiomers on other histone methylation marks, including H3K9me3 and H3K27me3, substrates for KDM4 and KDM6 subfamilies, respectively.

More potent inhibitors were obtained upon replacement of the pyrrole ring NH group with a sulfur atom transforming the pyrrolo[3,2-*b*]pyridine to a thieno[3,2-*b*]pyridine moiety (Figure 17.5). Binding affinity was improved by twofold (**27a**: $K_D = 0.15 + 0.01 \mu\text{M}$; **27c**: $K_D = 0.26 + 0.03 \mu\text{M}$) when compared to the K_D values obtained by isothermal titration calorimetry. Furthermore, the

replacement of the 2-chlorophenyl by a 3-aminophenyl group, and the substitution of piperidine by pyrrolidine led to **27d** with binding affinity of $K_D = 0.49 \mu\text{M}$ in KDM5B(1–755)DAP (AlphaLISA assay; $[\text{Fe}^{2+}] = 50 \mu\text{M}$; $\alpha\text{-KG}, 25 \mu\text{M}$), which are 8.6-fold greater for KDM5B relative to KDM4A. The structure was the basis for the design and development of irreversible inhibitors (see below).

High-throughput screening of the Genentech/Roche library [189,192] identified pyrazole derivatives as potent KDM5 inhibitors. Further SAR studies through modifications of the skeletal fragments identified cyclobutyl and cyclopropyl amide fragments as those with the highest biochemical potency ($\text{IC}_{50} = 80$ and 65 nM , respectively). In particular, the more active cyclopropyl enantiomer (*R*)-**29** (45 vs 90 nM) showed EC_{50} of 960 nM , reduced lipophilicity ($\text{Log}D = 1.3$ at pH 7.4), improved LE (0.49) and LLE (5.9) [192]. (*R*)-**29** was characterized as a pan-KDM5 inhibitor (KDM5A $\text{IC}_{50} = 0.045 \mu\text{M}$; KDM5B $\text{IC}_{50} = 0.056 \mu\text{M}$; KDM5C $\text{IC}_{50} = 0.055 \mu\text{M}$). It was significantly less potent against other KDM enzymes (1A, 32B, 3B, 4C, 6A, 7B), being strongest with KDM4A ($\text{IC}_{50} = 4.1 \mu\text{M}$) and displayed 91-fold selectivity for KDM4C versus KDM5A. (*R*)-**29** showed improved *in vitro* metabolic stability across mice species. *In vivo* testing indicated moderate clearance (28 mL/min/kg) in mice with good oral bioavailability (F% 34), low plasma protein binding in mice (40%), with an unbound C_{\max} observed as being about twice of its cell EC_{50} value (960 nM) following a 5 mg/kg oral dose [192]. When tested at $10 \mu\text{M}$ against Invitrogen kinase (300 kinases) and Cerep (40 enzymes/receptor/ion channels) panels, (*R*)-**29** did not show $>50\%$ inhibition against any target.

Using structure-based virtual screening of a 2 million (Enamine library) and an in house (500 compounds) library against the crystal structure (PDB: 5FYZ) of KDM5B, biochemical screening, identification of pyrazole derivatives as KDM5B-selective inhibitors, and further optimization led to ligand **30**, which showed an $\text{IC}_{50} = 0.0244 \mu\text{M}$, and inhibited 95.78% KDM5B at a concentration of $10 \mu\text{M}$ [193]. Compound **30** could stabilize KDM5B levels in MKN45 GC cells (which was previously shown to be the cell line with the highest expression of KDM5B) in a dose-dependent manner. Significant accumulation of the KDM5B substrate H3K4me2/3 was induced after 5 days treatment of MKN45 cells with the compound at concentrations from 0.5 to $20 \mu\text{M}$, and the amount of H3K4me1 was kept constant. The compound failed to induce the expression of substrates of KDM4 and KDM6 subfamilies, including H3K9me2, H3K9me3 and H3K27me2, and induced the accumulation of H3K4me2/3 *in situ* in a dose-dependent manner when applied to MNK45 cells for 5 days [193]. To analyze the role of KDM5B as contributor to GC cell proliferation and metastasis, compound **30** was tested in GC cells. It was found to inhibit MKN45 cell proliferation (after 4 days, $\text{IC}_{50} = 26.72 + 2.28 \mu\text{M}$) but not normal human gastric epithelial GES-1 cells, as well as wound healing and migration in a time and dose-dependent manner [193].

5.1.6 Covalent inhibitors

Since the Cys481 residue of KDM5A is invariant among the KDM5 subfamily (corresponding to Cys497 in KDM5B, Cys502 in KDM5C, and Cys512 in KDM5D) but is not present in other Jmj-KDM subfamilies, irreversible KDM5 inhibitors have been designed based on the structure of GSK-J1 (**48**, see Figure 17.3) through incorporation of an electron acceptor to nucleophile Cys481 [191]. In fact, cocrystal structure of GSK-J1 (**48**) bound to KDM5A and Mn(II) (PDB 6DQ4) [191] revealed that the primary amine was located at a close distance (4.2 \AA) to Cys481. The potential reactivity of the latter nucleophile was exploited by attaching a dimethylamino-2-butenamide moiety harboring a reactive acrylamide (the dimethylamino-2-butenamide) group as in compound **31**.

Crystal structure analysis showed the formation of a covalent bond between the butenamide moiety and the sulfur atom of Cys481 by Michael addition.

Kinetic studies also showed that the inhibitor was noncompetitive with the 2-OG cofactor. ALPHALISA-based assay against the activities of KDM5A, KDM5B, KDM4A, and KDM6A confirmed the 24-fold selectivity of **31** for KDM5B over KDM4A when compared to noncovalent inhibitors **27c** and **27d**. (Figure 17.5). Derivative **31** (Figure 17.6), generated by replacement of the 2-chlorophenyl by a 3-aminophenyl group, showed improved binding affinity (24-fold for KDM5B against KDM4A) and selectivity ($K_D = 0.22 + 0.05 \mu\text{M}$) in the KDM5B(1–755)DAP (AlphaLISA assay; $[\text{Fe}^{2+}] = 50 \mu\text{M}$; $\alpha\text{KG} 25 \mu\text{M}$) [191].

Although the isopropyl ester derivative of **31** showed greater permeability and some growth inhibitory and cytotoxic activity against BT474 (HER2^+) and MCF7 (ER^+) BC cells, unfortunately had no impact on cellular H3K4me3 methylation levels at doses up to $5 \mu\text{M}$ [194].

KDM5 covalent inhibitors based on cyanopyrazole CPI-455 (**47**, see Figure 17.3) [168] were likewise developed in efforts to target cysteine residues that are only present in this subfamily, namely

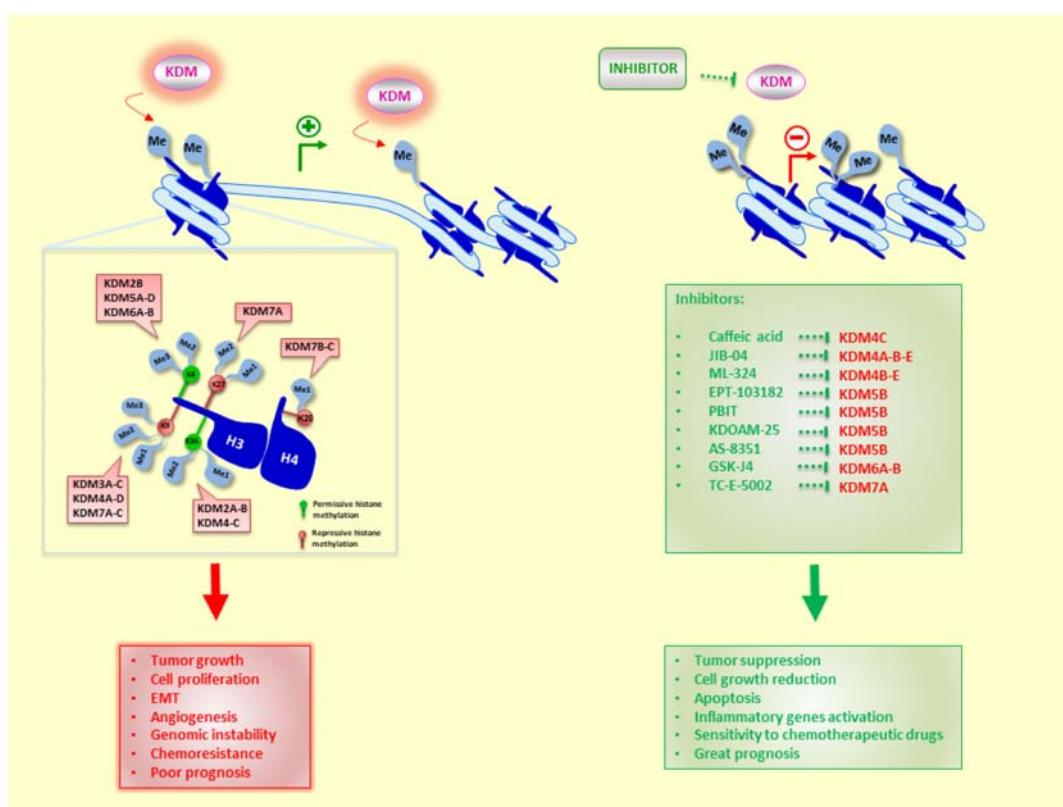


FIGURE 17.6

KDM5 covalent inhibitors.

C497, which is a noncatalytic residue located close to the binding site of the KDM5 members, and a unique Cys480 residue in KDM5B relative to other family members (KDM5A: Ser464; KDM5C: Ser485; KDM5D: Ser495) [195]. By connecting the 2-chloroacetamide moiety into the pyrazolyl fragment of CPI-455 (**47**, see Figure 17.3) conveniently modified through an ethylamine linker, the acrylamide ligand **32** was generated [195]. Cocrystal structure of **32** with KDM5B confirmed the formation of a covalent bond of the acrylamide β -carbon with C480 sulfur with the ligand binding through bidentate metal coordination and salt bridging/hydrogen bonding with K517/Y425.

Chloroacetamide **34** was the most potent of a related series of covalent inhibitors and showed the highest k_{inact}/K_i values ($40 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). Although acrylamide **33** was less active than **34** ($k_{\text{inact}}/K_i = 7.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) it showed a drop in IC₅₀ of 12-fold after an hour incubation. Dimethylamine-substituted crotonamide **35** was less potent ($k_{\text{inact}}/K_i = 6.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) than acrylamide **33**, which itself was fivefold more potent than the corresponding piperazine acrylamide **32** ($k_{\text{inact}}/K_i = 1.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) [195]. Moderate selectivity for KDM5B over KDM5C (38-fold) was demonstrated for the series, but analog **32** strongly inhibited KDM5A and KDM5D with IC₅₀ values of 30 and 83 nM, respectively. The covalent inhibition of KDM5B, but not of KDM4B or KDM5C, was confirmed by 2-OG competition assays and by MS-labeling experiments. In contrast to the noncovalent analog [168], which was characterized as a dual KDM4 and KDM5 inhibitor, covalent inhibitors showed greater selectivity (50-fold) for KDM5B over KDM4A/B/C [195].

Additional covalent inhibitors **36–40** (Figure 17.6) inspired by CPI-455 (**47**, see Figure 17.3) were moreover designed to target residue C497 of KDM5B. However, although X-Ray structures of **36** and **39** showed the proximity of the reactive fragments (chloroacetyl and acrylamide functional groups) to C497 sulfur nucleophile, no covalent bond was seen. IC₅₀ values of the inhibitors measured using an AlphaScreen assay provided k_{inact} and K_i values. Their comparative analysis ($k_{\text{inact}}/K_i = 25 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for **36**; $20 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for **39**; $19 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for **40**; $6.9 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for **37**; $5.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for **38**) confirmed that both series of compounds possessed time-dependent inhibition (potent nanomolar IC₅₀ values) through covalent binding to KDM5B [195]. MS-labeling experiments likewise confirmed the covalent binding of the inhibitors to KDM5B, and a protein-inhibitor adduct was also detected by MS upon incubation of **36** with KDM5B. LC–MS/MS analysis after digestion of the adducts suggested the covalent modification of C497 of KDM5B [195].

Compounds of the cyanopyrazole series (**36–40**) showed high activity with IC₅₀ in the low nanomolar range against most KDM5 family members, with almost equipotency against KDM5A/B/D, but 30- to 100-fold selective over KDM5C. Compound **36** (KDM5A/B IC₅₀ 10 nM), showed the best selectivity profile of the series. Chloroacetamide **40** showed the highest selectivity for KDM5B with more than 100-fold over KDM5C. Both series showed the greatest selectivity over KDM2A and 3A, from 200- to 1500-fold, and over the KDM4 series.

A 2-OG competition assay confirmed that compound **36** was 37-times more potent than the non-covalent precursor [189] at 100 μM 2-OG, indicating the reduced competition with the cofactor [195]. Compound **34** showed similar potency for KDM5A, 5B, and 5D, with IC₅₀ values of 10, 7, and 38 nM (determined using an AlphaScreen assay), respectively, but it was 81-fold less active for KDM5C. More than 500-fold selectivity for KDM5B over KDM4A/B and of 253-fold selectivity over KDM4C was reported. Greater selectivity (> 800 -fold) was also measured over KDM3A, whereas no inhibition was found for KDM2A and KDM6A even at 100 μM [195].

Cellular target engagement was assayed using a PZ-based cell-permeable fluorescent tracer assay for KDM target fused to NanoLuciferase containing the fluorescence dye NanoBRET₅₉₀®. The selectivity of the tracer was confirmed [189,196] with all the KDM5 family members in a dose-dependent manner [195]. For the series, the former analogs **37** and **38** showed the best activities (IC_{50} 0.53 and 0.30 μ M, respectively), whereas **39** was the most potent of its group (IC_{50} 10.6 μ M). These compounds were noncytotoxic to HEK293 cells, except for **34**. Using ChIP-seq experiments, compounds **33**, **36**, and **38** showed significant increases of H3K4m32 levels around TSS in HEK293 cells.

6 JmjC inhibitors in cancer therapy

Deregulation of KDMs in cancer and their association with the progression of various cancer types make them potential therapeutic targets for cancer treatment (Figure 17.7). Downregulation or pharmacological inhibition of KDMs could thus be a valid approach in cancer therapy. Epidrugs are small molecules that modulate chromatin-remodeling enzymes, and some have already been approved by the US FDA for different cancer therapies [36]. Discovering selective inhibitors is challenging due to the chemical similarities between KDMs. Peptide inhibitors (not covered in this work) mimic the histone substrate or bind to KDMs allosterically, but their use is limited by their low cellular permeability [159].

Numerous promising inhibitors of KDMs have been described, and we have just described those reported in the period 2017–2021. The most important KDM4 inhibitor is caffeic acid (**42**, Figure 17.3), a natural molecule that mainly targets KDM4C and displays a strong anticancer activity against esophageal cancer *in vitro* and *in vivo*. The most advanced preclinical inhibitor is JIB-04 (**41**, Figure 17.3), which is cell permeable and targets KDM4A/B/E, exhibiting selective anticancer properties *in vitro* and *in vivo*. Notably, JIB-04 (**41**) lowered histone demethylase activity in

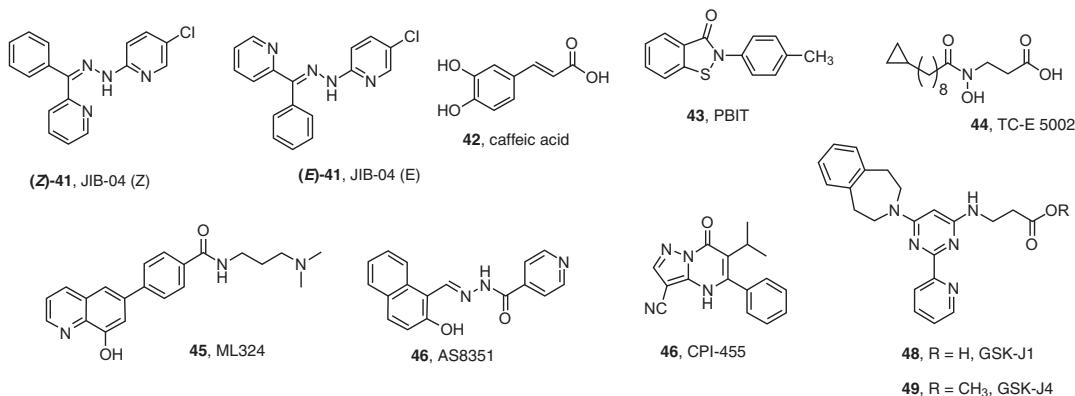


FIGURE 17.7

The role of KDM overexpression and inhibition in cancer development.

tumors, reduced tumor burden, and prolonged cancer survival *in vivo* in mice [197]. JIB-04 (41) treatment was able to block colony formation, growth, and migration *in vitro* and also tumorigenic activity in a CRC model *in vivo*. The mechanism was associated to capability to downregulate genes that are involved in Wnt signaling pathway and thus crucial in cancerogenesis. However, it remains to be tested in clinical trials, still [198].

Another important KDM inhibitor is ML324 (45, Figure 17.3), which targets KDM4B-E, and showed promise for the treatment of TNBC and PCa by inhibiting cellular proliferation *in vitro* and *in vivo* [34,84]. Specifically, in PTEN-deficient TNBC, which occurs in up to 35% of TNBCs, KDM4B inhibition promoted apoptosis in combination with PI3K inhibitors, exerting a strong effect in tumor suppression both *in vitro* and *in vivo* [84]. To date, ML324 (45) has not been tested in clinical trials.

EPT-103182 (structure not disclosed) is the most promising KDM5 inhibitor for the treatment of hematological and solid cancers, while PBIT (43, Figure 17.3) and KDOAM-25 (23c, Figure 17.5) showed encouraging results in the treatment of BC; however, all three molecules are still to be tested in clinical trials [38]. The inhibitor AS-8351 (46, Figure 17.3) targets KDM5B in BC, reversing EMT and inhibiting cell proliferation [99].

Another promising inhibitor is GSK-J4 (49, Figure 17.3), which targets KDM6A/B and displayed efficacy in numerous preclinical trials [38]. Initial studies investigated KDM6A/B inhibition in NK cell cytokine regulation, which was found to lead to an increase in H3K27 methylation around the transcriptional start sites of *IFNG*, *TNF*, and other inflammatory genes, thus underscoring the antiinflammatory effects of KDM6A/B inhibition [199]. GSK-J4 (49) could also be used for the treatment of AML due to its effect on endoplasmic reticulum stress, an important biological reaction that affects cell cycle progression, cell growth, and apoptosis regulation. In AML KG-1a cells, GSK-J4 (49) induced endoplasmic reticulum stress-related proteins, such as GRP78, ATF4, and caspase-12, inducing cell cycle arrest in S phase and upregulation of proapoptotic proteins. GSK-J4 (49) could also downregulates PKC- α , which has an antiapoptotic role and induces chemoresistance in leukemia through interaction with Bcl-2 [200]. Furthermore, GSK-J4 (49) exerted antiproliferative effects in AML cell lines by inhibiting expression of important genes involved in DNA replication and cell cycle phase transition (such as *FEN-1*, *PCNA*, *MCM3*, *MCM4*, and *HSP90AA1*) and *HOX* genes by increasing H3K27me3 levels in their transcriptional start sites, while *in vivo* GSK-J4 (49) reduced tumor burden in a xenograft mouse model [201]. Other studies describe the role of GSK-J4 (49) in inhibiting leukemia cell growth. The role of GSK-J4 (49) was investigated in downregulation of CREB, a TF upregulated in AML, leading cell growth and survival, and blocking apoptosis. GSK-J4 (49) led to proteasome downregulation of CREB at protein level, without affecting mRNA levels, and impacted the accessibility of CREB via an increase in H3K27me2/3 [202]. In TAL1-positive T-ALL, GSK-J4 (49) promoted apoptosis by blocking TAL1-mediated leukemic gene activation mediated by KDM6A.

GSK-J4 (49) may also exert its antitumor effect in LUAD by inhibiting KDM6B activity and reducing glutamate, which induces metabolic and oxidative stress and affects KDM activity through a reduction in α -KG [203]. Another study highlights the importance of GSK-J4 (49) treatment in glioma cells, where levels of KDM6 are higher and levels of H3K27me2/3 are lower than in endothelial cells. Thus the molecule inhibited cell proliferation and migration and promoted apoptosis in glioma cells but not in nonglioma cells, indicating that the compound had no adverse side effects [204].

7 JmjC-based combinatorial approaches and drug resistance

Overexpression of KDM enzymes leads to drug resistance in several types of cancer.

GSK-J4 (49) may be used in combination with decitabine, a strong inhibitor of DNA methyltransferase, in the treatment of AML. This combinatorial treatment increased apoptosis compared to treatment with GSK-J4 (49) or decitabine alone, especially in patients resistant to decitabine. GSK-J4 (49) was also used in combination with venetoclax (a Bcl-2 inhibitor) in the treatment of neuroblastoma, but further studies are required [200]. GSK-J4 (49) displayed a synergistic effect when used in combination with the antileukemic agent cytosine arabinoside on the inhibition of cell proliferation and colony formation, sensitizing leukemic cells to chemotherapeutic drugs [201]. In combination with retinoic acids, GSK-J4 (49) was able to reduce tumor formation and cell viability in neuroblastoma. In CRC, KDM6B overexpression induced oxaliplatin resistance, which can be made reversible through treatment with GSK-J4 (49) [205].

KDM5A mediates drug resistance to Wee1 inhibition in AML and in EOC resistant to paclitaxel. Findings show that the chemoresistant AML cell lines Molm13 and Jurkat could be re-sensitized to AZD1775, an inhibitor of Wee1-kinase, treatment via KD of KDM5A either directly by using the KDM5 inhibitor CPI-455 (47, Figure 17.3) or indirectly by inhibiting HDACs that negatively regulate KDM5A activity. In BC, KDM5A/B inhibition increased sensitivity to fulvestrant in endocrine-resistant cells [97]. PI3K inhibitors and KDM4B inhibitors may exert a synergic role in PTEN-TNBCs [84]. KDM4C is involved in cytarabine resistance in AML, thus corroborating the role of KDM4C as a promising target in AML.

In PCa, the KDM7A inhibitor TC-E 5002 (44, Figure 17.3), in cotreatment with the AR inhibitor enzalutamide, synergistically induced cell death [82], while KDM7B induced chemoresistance to trastuzumab in BC.

8 Clinical trials

Currently, the only KDM4C (GASC1) inhibitor, caffeoic acid (42, Figure 17.3), is being tested in clinical trials for Chinese advanced esophageal squamous cell cancer (NCT04648917). The study is now in phase 3 and is currently recruiting participants. Another clinical trial is active but not recruiting for the treatment of esophageal cancer (NCT03070262). KDM5C is being evaluated as a potential prognostic biomarker in T1-stage RCC (NCT03694912) and the study is currently recruiting patients. One clinical trial evaluated a KDM6A mutation in pancreaticobiliary tumors and is completed (NCT02893085), while another is investigating KDM6A genomic alterations after treatment with apalutamide in metastatic castration-sensitive PCa (NCT04601441). This latter study is in phase 4 and is recruiting participants.

9 Current perspectives and future directions

The KDM family of enzymes plays an important role in the development of several solid and hematological cancers by targeting genomic and nongenomic substrates. Although their

physiological role is not yet fully understood, they may serve as new therapeutic targets, especially in cancers that have no druggable site or that have stopped responding to pharmacological treatment. Some inhibitors have shown good selectivity over KDM2/3/6, but selectivity over the KDM4 and KDM5 subfamilies is quite challenging since the primary binding residues of the catalytic domain are conserved in KDM4 and KDM5. A small number of drugs have already been tested in preclinical studies and have produced encouraging results. Further studies are essential to identify selective inhibitors that directly target specifically KDM, thus avoiding adverse side effects or lack of selectivity.

Conflict of interest

The authors declare no conflict of interest.

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Abbreviations

2OG	2-Oxoglutarate
5-mc	5-Methylcytosine
53BP1	p53-binding protein 1
m⁶A	N6-methyladenosine
AD	Adenocarcinoma
AE	ML1-ETO
AR	Androgen receptor
ARID	AT-rich interaction domain
ATF2	Cyclic AMP-dependent transcription factor
AUC	Area under the curve
AXL	Receptor tyrosine kinase
BAD	BCL2-associated agonist of cell death
BC	Breast cancer
BCA	Bladder cancer
CAF	Cancer-associated fibroblast
CBA	Carboxamide-substituted benzhydryl amine
CBP	CREB-binding protein
CC	Cervical cancer

CDC25A	Cell division cycle 25A
CETSA	Cellular thermal shift assay
CML	Chronic myeloid leukemia
CML-BP	CML blastic phase
CML-CP	CML chronic phase
CMM	Chronic myelomonocytic leukemia
CRC	Colorectal cancers
CSC	Cancer stem cell
CXCL10	C-X-C motif chemokine ligand 10
DFT	Density functional theory
DTEP	Drug-tolerant expanded persisters
DUSP3	Dual specificity phosphatase 3
EMT	Epithelial–mesenchymal transition
EOC	Epithelial ovarian cancer
EpCAM	Epithelial cell adhesion molecule
EPR	Electron paramagnetic resonance
ERα	Estrogen receptor alpha
ETV1	ETS variant transcription factor 1
EZH2	Enhancer of zeste homolog 2
FDH	Formaldehyde dehydrogenase
FOXP2	Forkhead box P2
GBM	Glioblastoma
GC	Gastric cancer
GLUT1	Glucose transporter 1
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor-1 alpha
HMT	Histone methyltransferase
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
ITC	Isothermal titration calorimetry
KD	Knockdown
KDM	Histone demethylases
α - KG	Alpha-ketoglutarate
KLF4	Kruppel-like factor 4
LC	Lung cancer
lncRNA	Long noncoding RNAs
LE	Ligand efficiency
LEDGF	Lens epithelium-derived growth factor
LLE	Ligand-lipophilicity efficiency
LSDs/KDM1	Lysine-specific demethylase
LUAD	Lung adenocarcinoma
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
MLL1	Mixed lineage leukemia 1
MMP	Matrix metalloproteinase

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NK	Natural killer
NMR	Nuclear magnetic resonance
NSCLC	Non–small-cell lung cancer
NSG2	Neuronal vesicle trafficking-associated protein 2
Oct-4	Octamer-binding transcription factor 4
OXPHOS	Mitochondrial oxidative phosphorylation system
PAF1	Polymerase-associated factor 1
Papp	Apparent permeability
PAMPA	Parallel artificial membrane permeability assay technique
PC	Pancreatic cancer
PCa	Prostate cancer
PCNA	Proliferation cell nuclear antigen
PD	Pharmacodynamics
PDB	Protein data bank
PD-L1	Programmed death-ligand 1
PDCD4	Programmed cell death 4
PDGFA	Platelet-derived growth factor alpha
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
PHD	Plant homeodomain
PHF	Plant homeodomain finger
PK	Pharmacokinetic
pRb	Retinoblastoma protein
PRC1	Polycomb-repressive complex 1
PTEN	Phosphatase and tensin homolog
RCC	Renal cell carcinoma
SCLC	Small-cell lung cancer
SMARCA4	WI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4
SPR	Surface plasmon resonance
TCF4	Transcription factor 4
TET2	Tet methylcytosine dioxygenase 2
TF	Transcription factors
TGF-β3	Transforming growth factor-beta
TNBC	Triple-negative breast cancer
TNFα	Tumor necrosis factor-alpha
TNM	Tumor/node/metastasis
TRAF6	TNF receptor-associated factor 6
TRKA	Receptor tyrosine kinase 1 .
UPR	Unfolded protein response
UTX	Ubiquitously transcribed tetratricopeptide repeat X-chromosome
UTY	Ubiquitously transcribed tetratricopeptide repeat Y-chromosome
VEGFR-3	Vascular endothelial growth factor receptor 3
YAP1	Yes-associated protein 1
YY1	Yin yang 1

References

- [1] Hú K, Konze KD, Jin J. Selective inhibitors of protein methyltransferases. *J Med Chem* 2015; 58(4):1596–629.
- [2] Schapira M. Chemical inhibition of protein methyltransferases. *Cell Chem Biol*. 2016;23(9):1067–76.
- [3] Kooistra SM, Helin K. Molecular mechanisms and potential functions of histone demethylases. *Nat Rev Mol Cell Biol* 2012;13(5):297–311.
- [4] Hojfeldt JW, Agger K, Helin K. Histone lysine demethylases as targets for anticancer therapy. *Nat Rev Drug Discov* 2013;12:917–30.
- [5] Thinnnes CC, England KS, Kawamura A, Chowdhury R, Schofield CJ, Hopkinson RJ. Targeting histone lysine demethylases—progress, challenges, and the future. *Biochim Biophys Acta (BBA) Gene Regul Mech* 2014;1839(12):1416–32.
- [6] Kaniskan HÜ, Martini ML, Jin J. Inhibitors of protein methyltransferases and demethylases. *Chem Rev* 2018;118(3):989–1068.
- [7] Arifuzzaman S, Khatun MR, Khatun R. Emerging of lysine demethylases (KDMs): From pathophysiological insights to novel therapeutic opportunities. *Biomedicine & Pharmacotherapy* 2020;129:110392.
- [8] Black JC, Van Rechem C, Whetstine JR. Histone lysine methylation dynamics: establishment, regulation, and biological impact. *Mol Cell* 2012;48:491–507.
- [9] Morera L, Lübbert M, Jung M. Targeting histone methyltransferases and demethylases in clinical trials for cancer therapy. *Clin Epigen*. 2016;8:57.
- [10] Klose RJ, Kallin EM, Zhang Y. JmjC-domain-containing proteins and histone demethylation. *Nat Rev Genetics*. 2006;7:715–27.
- [11] Tsukada YI, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, et al. Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 2006;439(7078):811–16.
- [12] Yamane K, Toumazou C, Tsukada Y-I, Erdjument-Bromage H, Tempst P, Wong J, et al. JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell*. 2006;125(3):483–95.
- [13] Whetstine JR, Nottke A, Lan F, Huarte M, Smolikov S, Chen Z, et al. Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. *Cell*. 2006;125(3):467–81.
- [14] Takeuchi T, Yamazaki Y, Katoh-Fukui Y, Tsuchiya R, Kondo S, Motoyama J, et al. Gene trap capture of a novel mouse gene, jumonji, required for neural tube formation. *Genes Dev* 1995;9(10):1211–22.
- [15] Anand R, Marmorstein R. Structure and mechanism of lysine-specific demethylase enzymes. *J Biol Chem* 2007;282:35425–9.
- [16] Loenarz C, Schofield CJ. Expanding chemical biology of 2-oxoglutarate oxygenases. *Nat Chem Biol* 2008;4:152–6.
- [17] Joberty G, Boesche M, Brown JA, Eberhard D, Garton NS, Humphreys PG, et al. Interrogating the druggability of the 2-oxoglutarate-dependent dioxygenase target class by chemical proteomics. *ACS Chem Biol* 2016;11(7):2002–10.
- [18] Markolovic S, Leissing TM, Chowdhury R, Wilkins SE, Lu X, Schofield CJ. Structure–function relationships of human JmjC oxygenases—demethylases versus hydroxylases. *Curr Opin Struct Biol* 2016;41:62–72.
- [19] Rose NR, McDonough MA, King ONF, Kawamura A, Schofield CJ. Inhibition of 2-oxoglutarate dependent oxygenases. *Chem Soc Rev* 2011;40(8):4364–97.
- [20] Martinez S, Hausinger RP. Catalytic mechanisms of Fe(II)- and 2-oxoglutarate-dependent oxygenases. *J Biol Chem* 2015;290(34):20702–11.
- [21] Klose RJ, Zhang Y. Regulation of histone methylation by demethylimation and demethylation. *Nat Rev Mol Cell Biol* 2007;8(4):307–18.

- [22] Rose NR, Woon ECY, Tumber A, Walport LJ, Chowdhury R, Li XS, et al. Plant growth regulator daminozide is a selective inhibitor of human KDM2/7 histone demethylases. *J Med Chem* 2012; 55(14):6639–43.
- [23] Unoki M, Masuda A, Dohmae N, Arita K, Yoshimatsu M, Iwai Y, et al. Lysyl 5-hydroxylation, a novel histone modification, by jumonji domain containing 6 (JMD6). *J Biol Chem* 2013;288:6053–62.
- [24] Markovic S, Zhuang Q, Wilkins SE, Eaton CD, Abboud MI, Katz MJ, et al. The Jumonji-C oxygenase JMJD7 catalyzes (3 S)-lysyl hydroxylation of TRAFAC GTPases. *Nat Chem Biol* 2018;14(7):688–95.
- [25] Walport LJ, Hopkinson RJ, Chowdhury R, Schiller R, Ge W, Kawamura A, et al. Arginine demethylation is catalysed by a subset of JmjC histone lysine demethylases. *Nat Commun*. 2016;7.
- [26] Horton John R, Liu X, Gale M, Wu L, Shanks John R, Zhang X, et al. Structural basis for KDM5A histone lysine demethylase inhibition by diverse compounds. *Cell Chem Biol*. 2016;23(7):769–81.
- [27] Pilka ES, James T, Lisztwan JH. Structural definitions of Jumonji family demethylase selectivity. *Drug Disc Today*. 2015;20(6):743–9.
- [28] Hou H, Yu H. Structural insights into histone lysine demethylation. *Curr Opin Struct Biol* 2010; 20(6):739–48.
- [29] Aik W, McDonough MA, Thalhammer A, Chowdhury R, Schofield CJ. Role of the jelly-roll fold in substrate binding by 2-oxoglutarate oxygenases. *Curr Op Struct Biol*. 2012;22(6):691–700.
- [30] Romier C, Wurtz J-M, Renaud J-P, Cavarelli J. Structural biology of epigenetic targets. In: Sippl W, Jung M, editors. Epigenetics targets in drug discovery. Methods and principles in medicinal chemistry. Weinheim: Wiley-VCH; 2009. p. 23–56.
- [31] Clifton II, McDonough MA, Ehrismann D, Kershaw NJ, Granatino N, Schofield CJ. Structural studies on 2-oxoglutarate oxygenases and related double-stranded β -helix fold proteins. *J Inorg Biochem* 2006;100(4):644–69.
- [32] Costas M, Mehn MP, Jensen MP, Que L. Dioxygen activation at mononuclear nonheme iron active sites: enzymes, models, and intermediates. *Chem Rev* 2004;104(2):939–86.
- [33] Ng SS, Kavanagh KL, McDonough MA, Butler D, Pilka ES, Lienard BMR, et al. Crystal structures of histone demethylase JMD2A reveal basis for substrate specificity. *Nature* 2007;448:87–91.
- [34] Carter DM, Specker E, Przygoda J, Neuenschwander M, von Kries JP, Heinemann U, et al. Identification of a novel benzimidazole pyrazolone scaffold that inhibits KDM4 lysine demethylases and reduces proliferation of prostate cancer cells. *Slas Discov*. 2017;22(7):801–12.
- [35] Lee DH, Kim GW, Jeon YH, Yoo J, Lee SW, Kwon SH. Advances in histone demethylase KDM4 as cancer therapeutic targets. *FASEB J* 2020;34(3):3461–84.
- [36] Franci G, Ciotta A, Altucci L. The Jumonji family: past, present and future of histone demethylases in cancer. *Biomol Concepts*. 2014;5:209–24.
- [37] Black JC, Atabakhsh E, Kim J, Biette KM, Van Rechem C, Ladd B, et al. Hypoxia drives transient site-specific copy gain and drug-resistant gene expression. *Genes Dev* 2015;29(10):1018–31.
- [38] Punnia-Moorthy G, Hersey P, Al Emran A, Tiffen J. Lysine demethylases: promising drug targets in melanoma and other cancers. *Front Gen*. 2021;12:680633.
- [39] Young NL, Dere R. Mechanistic insights into KDM4A driven genomic instability. *Biochem Soc Trans* 2021;49(1):93–105.
- [40] Horton JR, Engstrom A, Zoeller EL, Liu X, Shanks JR, Zhang X, et al. Characterization of a linked Jumonji domain of the KDM5/JARID1 family of histone H3 lysine 4 demethylases. *J Biol Chem* 2016;291:2631–46.
- [41] Christensen J, Agger K, Cloos PAC, Pasini D, Rose S, Sennels L, et al. RBP2 belongs to a family of demethylases, specific for tri- and dimethylated lysine 4 on histone 3. *Cell*. 2007;128(6):1063–76.
- [42] Harmeyer KM, Facompre ND, Herlyn M, Basu D. JARID1 histone demethylases: emerging targets in cancer. *Trends Cancer*. 2017;3(10):713–25.

- [43] Agger K, Cloos PAC, Christensen J, Pasini D, Rose S, Rappoport J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature* 2007;449(7163):731–4.
- [44] Walport LJ, Hopkinson RJ, Vollmar M, Madden SK, Gileadi C, Oppermann U, et al. Human UTY (KDM6C) is a male-specific No μ -methyl lysyl demethylase. *J Biol Chem* 2014;289(26):18302–13.
- [45] Hua CY, Chen JQ, Li ST, Zhou JA, Fu JH, Sun WJ, et al. KDM6 demethylases and their roles in human cancers. *Front Oncol.* 2021;11:779918.
- [46] Sterling J, Menezes SV, Abbassi RH, Munoz L. Histone lysine demethylases and their functions in cancer. *Int J Cancer* 2021;148(10):2375–88.
- [47] Hyun K, Jeon J, Park K, Kim J. Writing, erasing and reading histone lysine methylations. *Exp Mol Med* 2017;49:e324.
- [48] D’Oto A, Tian QW, Davidoff AM, Yang J. Histone demethylases and their roles in cancer epigenetics. *J Med Oncol Ther.* 2016;1(2):7.
- [49] Chen JY, Li CF, Lai YS, Hung WC. Lysine demethylase 2A expression in cancer-associated fibroblasts promotes breast tumour growth. *Br J Cancer* 2021;124(2):484–93.
- [50] Wagner KW, Alam H, Dhar SS, Giri U, Li N, Wei YK, et al. KDM2A promotes lung tumorigenesis by epigenetically enhancing ERK1/2 signaling. *J Clin Invest* 2013;123(12):5231–46.
- [51] Dhar SS, Alam H, Li N, Wagner KW, Chung JY, Ahn YW, et al. Transcriptional repression of histone deacetylase 3 by the histone demethylase KDM2A is coupled to tumorigenicity of lung cancer cells. *J Biol Chem* 2014;289(11):7483–96.
- [52] Sauta E, Reggiani F, Torricelli F, Zanetti E, Tagliavini E, Santandrea G, et al. CSNK1A1, KDM2A, and LTB4R2 are new druggable vulnerabilities in lung cancer. *Cancers.* 2021;13(14):3477.
- [53] Huang YF, Liu YQ, Yu LJ, Chen J, Hou J, Cui LH, et al. Histone demethylase KDM2A promotes tumor cell growth and migration in gastric cancer. *Tumor Biol.* 2015;36(1):271–8.
- [54] Wang F, Liang S, Liu XHL, Wang J QD. LINC00460 modulates KDM2A to promote cell proliferation and migration by targeting miR-342-3p in gastric cancer. *Oncotargets Ther.* 2018;11:12.
- [55] Zhao YL, Chen XB, Jiang J, Wan XC, Wang YF, Xu P. Epigallocatechin gallate reverses gastric cancer by regulating the long noncoding RNA LINC00511/miR-29b/KDM2A axis. *Biochim Biophys Acta Mol Bas Dis.* 2020;1866(10):165856.
- [56] Chen JY, Li CF, Chu PY, Lai YS, Chen CH, Jiang SS, et al. Lysine demethylase 2A promotes stemness and angiogenesis of breast cancer by upregulating Jagged1. *Oncotarget.* 2016;7(19):27689–710.
- [57] Chen JY, Luo CW, Lai YS, Wu CC, Hung WC. Lysine demethylase KDM2A inhibits TET2 to promote DNA methylation and silencing of tumor suppressor genes in breast cancer. *Oncogenesis.* 2017;6:e369.
- [58] Lu DH, Yang J, Gao LK, Min J, Tang JM, Hu M, et al. Lysine demethylase 2A promotes the progression of ovarian cancer by regulating the PI3K pathway and reversing epithelial-mesenchymal transition. *Oncol Rep* 2019;41(2):917–27.
- [59] Shou TT, Yang HY, Lv J, Liu D, Sun XY. MicroRNA-3666 suppresses the growth and migration of glioblastoma cells by targeting KDM2A. *Mol Med Rep.* 2019;19(2):1049–55.
- [60] Cao LL, Du CZ, Liu HQ, Pei L, Qin L, Jia M, et al. Lysine-specific demethylase 2A expression is associated with cell growth and cyclin D1 expression in colorectal adenocarcinoma. *Int J Biol Markers* 2018;33(4):407–14.
- [61] Yan MN, Yang XX, Wang H, Shao QX. The critical role of histone lysine demethylase KDM2B in cancer. *Am J Transl Res.* 2018;10(8):2222–33.
- [62] Wanna-Udom S, Terashima M, Suphakpong K, Ishimura A, Takino T TS. KDM2B is involved in the epigenetic regulation of TGF- β -induced epithelial-mesenchymal transition in lung and pancreatic cancer cell lines. *J Biol Chem* 2021;296:100213.
- [63] Zacharopoulou N, Tsapara A, Kallergi G, Schmid E, Alkahtani S, Alarifi S, et al. The epigenetic factor KDM2B regulates EMT and small GTPases in colon tumor cells. *Cell Physiol Biochem.* 2018;47(1):368–77.

- [64] Zacharopoulou N, Tsapara A, Kallergi G, Schmid E, Tsichlis PN, Kampranis SC, et al. The epigenetic factor KDM2B regulates cell adhesion, small rho GTPases, actin cytoskeleton and migration in prostate cancer cells. *Biochim Biophys Acta Mol Cell Res* 2018;1865(4):587–97.
- [65] Sanches JGP, Song B, Zhang QQ, Cui XY, Yabasin IB, Ntim M, et al. The role of KDM2B and EZH2 in regulating the stemness in colorectal cancer through the PI3K/AKT pathway. *Front Oncol*. 2021;11:637298.
- [66] Qi JH, Zhou L, Li DQ, Yang JY, Wang H, Cao HF, et al. Oncogenic role of ALX3 in cervical cancer cells through KDM2B-mediated histone demethylation of CDC25A. *BMC Cancer* 2021;21(1):819.
- [67] Wang YW, Zang J, Zhang DY, Sun ZX, Qiu B, Wang XJ. KDM2B overexpression correlates with poor prognosis and regulates glioma cell growth. *Oncotarg Ther*. 2018;11:201–9.
- [68] Yoo J, Jeon YH, Cho HY, Lee SW, Kim GW, Lee DH, et al. Advances in histone demethylase KDM3A as a cancer therapeutic target. *Cancers*. 2020;12(5):1098.
- [69] Hou XY, Li QG, Yang LP, Yang ZL, He J, Li QL, et al. KDM1A and KDM3A promote tumor growth by upregulating cell cycle-associated genes in pancreatic cancer. *Exp Biol Med* 2021;246(17):1869–83.
- [70] Hoyle RG, Wang HQ, Cen YN, Zhang Y, Li J. IOX1 suppresses Wnt target gene transcription and colorectal cancer tumorigenesis through inhibition of KDM3 histone demethylases. *Mol Cancer Ther* 2021;20(1):191–202.
- [71] Li Jiong, Yu Bo, Deng Peng, Cheng Yingduan, Yu Yongxin, Kevork Kareena, et al. KDM3 epigenetically controls tumorigenic potentials of human colorectal cancer stem cells through Wnt/β-catenin signalling. *Nat Commun*. 2017;8:15146.
- [72] Tang DE, Dai Y, Fan LL, Geng XY, Fu DX, Jiang HW. Histone demethylase JMJD1A promotes tumor progression via activating snail in prostate cancer. *Mol Cancer Res* 2022;20(2):333.
- [73] Liu JY, Li DQ, Zhang X, Li YY, Ou J. Histone demethylase KDM3A promotes cervical cancer malignancy through the ETS1/KIF14/Hedgehog axis. *Oncotargets Ther*. 2020;13:11957–73.
- [74] Qian X, Li XJ, Shi ZM, Bai XM, Xia Y, Zheng YH, et al. KDM3A senses oxygen availability to regulate PGC-1 alpha-mediated mitochondrial biogenesis. *Mol Cell* 2019;76(6):885–895.e7.
- [75] Jung H, Seo SB. Histone lysine demethylase 3B (KDM3B) regulates the propagation of autophagy via transcriptional activation of autophagy-related genes. *PLoS One* 2020;15(7):e0236403.
- [76] An MJ, Kim DH, Kim CH, Kim M, Rhee S, Seo SB, et al. Histone demethylase KDM3B regulates the transcriptional network of cell-cycle genes in hepatocarcinoma HepG2 cells. *Biochem Biophys Res Commun* 2019;508(2):576–82.
- [77] Chen C, Aihemaiti M, Zhang X, Qu H, Sun QL, He QS, et al. Downregulation of histone demethylase JMJD1C inhibits colorectal cancer metastasis through targeting ATF2. *Am J Cancer Res* 2018;8(5):852–65.
- [78] Yoshihama Y, LaBella KA, Kim E, Bertolet L, Colic M, Li JX, et al. AR-negative prostate cancer is vulnerable to loss of JMJD1C demethylase. *Proc Natl Acad Sci U S A* 2021;118(36) e2026324118.
- [79] Shin S, Janknecht R. Activation of androgen receptor by histone demethylases JMJD2A and JMJD2D. *Biochem Biophys Res Commun* 2007;359(3):742–6.
- [80] Kim TD, Jin F, Shin S, Oh S, Lightfoot SA, Grande JP, et al. Histone demethylase JMJD2A drives prostate tumorigenesis through transcription factor ETV1. *J Clin Invest* 2016;126(2):706–20.
- [81] Varghese B, Del Gaudio N, Cobellis G, Altucci L, Nebbioso A. KDM4 involvement in breast cancer and possible therapeutic approaches. *Front Oncol* 2021;11:750315.
- [82] Lee KH, Kim BC, Jeong SH, Jeong CW, Ku JH, Kim HH, et al. Histone Demethylase KDM7A regulates androgen receptor activity, and its chemical inhibitor TC-E 5002 overcomes cisplatin-resistance in bladder cancer cells. *Int J Mol Sci* 2020;21(16).
- [83] Wilson C, Qiu L, Hong Y, Karnik T, Tadros G, Mau B, et al. The histone demethylase KDM4B regulates peritoneal seeding of ovarian cancer. *Oncogene* 2017;36(18):2565–76.

- [84] Wang WY, Oguz G, Lee PL, Bao Y, Wang PP, Terp MG, et al. KDM4B-regulated unfolded protein response as a therapeutic vulnerability in PTEN-deficient breast cancer. *J Exp Med* 2018; 215(11):2833–49.
- [85] Li GL, Kanagasabai T, Lu WF, Zou MR, Zhang SM, Celada SI, et al. KDM5B is essential for the hyperactivation of PI3K/AKT signaling in prostate tumorigenesis. *Cancer Res* 2020;80(21):4633–43.
- [86] Wang ZZ, Cai HR, Zhao ER, Cui HJ. The diverse roles of histone demethylase KDM4B in normal and cancer development and progression. *Front Cell Dev Biol* 2022;9:790129.
- [87] Chen GQ, Ye P, Ling RS, Zeng F, Zhu XS, Chen L, et al. Histone demethylase KDM4C is required for ovarian cancer stem cell maintenance. *Stem Cells Int* 2020;2020:8860185.
- [88] Jie XH, Chen YS, Zhao Y, Yang XJ, Xu YZ, Wang J, et al. Targeting KDM4C enhances CD8(+) T cell mediated antitumor immunity by activating chemokine CXCL10 transcription in lung cancer. *J Immunother Cancer* 2022;10(2):e003716.
- [89] Lin CY, Wang BJ, Chen BC, Tseng JC, Jiang SS, Tsai KK, et al. Histone demethylase KDM4C stimulates the proliferation of prostate cancer cells via activation of AKT and c-Myc. *Cancers* 2019; 11(11):1785.
- [90] Yamamoto S, Tateishi K, Kudo Y, Yamamoto K, Isagawa T, Nagae G, et al. Histone demethylase KDM4C regulates sphere formation by mediating the cross talk between Wnt and Notch pathways in colonic cancer cells. *Carcinogenesis* 2013;34(10):9.
- [91] Yan H, Zhu LS, Zhang J, Lin ZM. Histone demethylase KDM4D inhibition suppresses renal cancer progression and angiogenesis through JAG1 signaling. *Cell Death Disc* 2021;7(1):284.
- [92] Peng K, Kou L, Yu L, Bai C, Li M, Mo P, et al. Histone demethylase JMJD2D interacts With β -catenin to induce transcription and activate colorectal cancer cell proliferation and tumor growth in mice. *Gastroenterology* 2019;156(4):1112.
- [93] Li M, Deng Y, Zhuo MH, Zhou H, Kong X, Xia XG, et al. Demethylase-independent function of JMJD2D as a novel antagonist of p53 to promote Liver Cancer initiation and progression. *Theranostics* 2020;10(19):8863–79.
- [94] Feng TF, Wang Y, Lang Y, Zhang YZ. KDM5A promotes proliferation and EMT in ovarian cancer and closely correlates with PTX resistance. *Mol Med Rep* 2017;16(3):3573–80.
- [95] Liang XM, Zeng JP, Wang LX, Shen L, Ma XP, Li SY, et al. Histone demethylase RBP2 promotes malignant progression of gastric cancer through TGF-beta 1-(p-Smad3)-RBP2-E-cadherin-Smad3 feed-back circuit. *Oncotarget* 2015;6(19):17661–74.
- [96] Choi HJ, Joo HS, Won HY, Min KW, Kim HY, Son T, et al. Role of RBP2-induced ER and IGF1R-ErbB signaling in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 2018;110(4):400–10.
- [97] Hinohara K, Wu HJ, Vigneau S, McDonald TO, Igarashi KJ, Yamamoto KN, et al. KDM5 histone demethylase activity links cellular transcriptomic heterogeneity to therapeutic resistance. *Cancer Cell* 2019;35(2):330–2.
- [98] Liu BKR, Chao HP, Mehmood R, Ji Y, Tracz A, et al. Evidence for context-dependent functions of KDM5B in prostate development and prostate cancer. *Oncotarget* 2020;11(46):4243–51.
- [99] Zhang ZG, Zhang HS, Sun HL, Liu HY, Liu MY, Zhou Z. KDM5B promotes breast cancer cell proliferation and migration via AMPK-mediated lipid metabolism reprogramming. *Exp Cell Res* 2019; 379(2):182–90.
- [100] Fu Y-D, Huang M-J, Guo J-W, You Y-Z, Liu H-M, Huang L-H, et al. Targeting histone demethylase KDM5B for cancer treatment. *Eur J Med Chem* 2020;208:112760.
- [101] Lin HS, Ma NN, Zhao L, Yang GW, Cao BW. KDM5c promotes colon cancer cell proliferation through the FBXW7-c-Jun regulatory axis. *Front Oncol* 2020;10:535449.
- [102] Zhang Q, Xu L, Wang JJ, Zhu XM, Ma ZH, Yang JF, et al. KDM5C expedites lung cancer growth and metastasis through epigenetic regulation of microRNA-133a. *Oncotargets Therapy* 2021;14:1187–204.

- [103] Shen HF, Zhang WJ, Huang Y, He YH, Hu GS, Wang L, et al. The dual function of KDM5C in both gene transcriptional activation and repression promotes breast cancer cell growth and tumorigenesis. *Adv Sci* 2021;8(9):2004635.
- [104] Crea F, Sun L, Mai A, Chiang YT, Farrar WL, Danesi R, et al. The emerging role of histone lysine demethylases in prostate cancer. *Mol Cancer* 2012;11:52.
- [105] Shen XD, Hu KW, Cheng GL, Xu LM, Chen ZR, Du P, et al. KDM5D inhibit epithelial-mesenchymal transition of gastric cancer through demethylation in the promoter of Cul4A in male. *J Cell Biochem* 2019;120(8):12247–58.
- [106] Tran N, Braun A, Ge K. Lysine demethylase KDM6A in differentiation, development, and cancer. *Mol Cell Biol* 2020;40(20) e00341-20.
- [107] Xie G, Liu X, Zhang Y, Li W, Liu S, Chen Z, et al. UTX promotes hormonally responsive breast carcinogenesis through feed-forward transcription regulation with estrogen receptor. *Oncogene* 2017;36(39):5497–511.
- [108] Yu WQ, Huang W, Yang Y, Qiu RF, Zeng Y, Hou YQ, et al. GATA3 recruits UTX for gene transcriptional activation to suppress metastasis of breast cancer. *Cell Death Dis* 2019;10:832.
- [109] Sanchez A, Khoufaf FZH, Idrissou M, Penault-Llorca F, Bignon YJ, Guy L, et al. The functions of the demethylase JMJD3 in cancer. *Int J Mol Sci* 2021;22(2):968.
- [110] Dutta A, Le Magnen C, Mitrofanova A, Ouyang XS, Califano A, Abate-Shen C. Identification of an NKX3.1-G9a-UTX transcriptional regulatory network that controls prostate differentiation. *Science* 2016;352(6293):1576–80.
- [111] Lee KH, Hong S, Kang M, Jeong CW, Ku JH, Kim HH, et al. Histone demethylase KDM7A controls androgen receptor activity and tumor growth in prostate cancer. *Int J Cancer* 2018;143(11):2849–61.
- [112] Meng ZZ, Liu Y, Wang J, Fan HJ, Fang H, Li S, et al. Histone demethylase KDM7A is required for stem cell maintenance and apoptosis inhibition in breast cancer. *J Cell Physiol* 2020;235(2):932–43.
- [113] Lv Y, Shi Y, Han QL, Dai GH. Histone demethylase PHF8 accelerates the progression of colorectal cancer and can be regulated by miR-488 in vitro. *Mol Med Rep* 2017;16(4):4437–44.
- [114] Liu Q, Borcherding NC, Shao P, Maina PK, Zhang WZ, Qi HH. Contribution of synergism between PHF8 and HER2 signalling to breast cancer development and drug resistance. *Ebiomedicine* 2020;51:102612.
- [115] Cai MZ, Wen SY, Wang XJ, Liu Y, Liang H. MYC regulates PHF8, which promotes the progression of gastric cancer by suppressing miR-22-3p. *Technol Cancer Res Treat* 2020;19 1533033820967472.
- [116] Park SY, Park JW, Chun YS. Jumonji histone demethylases as emerging therapeutic targets. *Pharmacol Res* 2016;105:146–51.
- [117] Lee KH, Park JW, Sung HS, Choi YJ, Kim WH, Lee HS, et al. PHF2 histone demethylase acts as a tumor suppressor in association with p53 in cancer. *Oncogene* 2015;34(22):2897–909.
- [118] Fu Y, Liu MY, Li FX, Qian L, Zhang P, Lv FW, et al. MiR-221 promotes hepatocellular carcinoma cells migration via targeting PHF2. *Biomed Res Int* 2019;2019:4371405.
- [119] Liu YX, Chen TL, Guo MY, Li Y, Zhang Q, Tan GX, et al. FOXA2-interacting FOXP2 prevents epithelial-mesenchymal transition of breast cancer cells by stimulating E-cadherin and PHF2 transcription. *Front Oncol* 2021;11:605025.
- [120] Zhu L, Li Q, Wong SHK, Huang M, Klein BJ, Shen JF, et al. ASH1L links histone H3 lysine 36 dimethylation to MLL leukemia. *Cancer Discov* 2016;6(7):770–83.
- [121] Nakamura S, Tan L, Nagata Y, Takemura T, Asahina A, Yokota D, et al. JmjC-domain containing histone demethylase 1B-mediated p15Ink4b suppression promotes the proliferation of leukemic progenitor cells through modulation of cell cycle progression in acute myeloid leukemia. *Mol Carcinogen* 2013;52(1):57–69.
- [122] He J, Anh TN, Zhang Y. KDM2b/JHDM1b, an H3K36me2-specific demethylase, is required for initiation and maintenance of acute myeloid leukemia. *Blood* 2011;117(14):3869–80.

- [123] Ueda T, Nagamachi A, Takubo K, Yamasaki N, Matsui H, Kanai A, et al. Fbxl10 overexpression in murine hematopoietic stem cells induces leukemia involving metabolic activation and upregulation of Nsg2. *Blood* 2015;125(22):3437–46.
- [124] Karoopongse E, Yeung C, Byon J, Ramakrishnan A, Holman ZJ, Jiang PYZ, et al. The KDM2B-Let-7b-EZH2 axis in myelodysplastic syndromes as a target for combined epigenetic therapy. *PLoS One* 2014;9(9).
- [125] Andricovich J, Kai Y, Peng WQ, Foudi A, Tzatsos A. Histone demethylase KDM2B regulates lineage commitment in normal and malignant hematopoiesis. *J Clin Inv* 2016;126(3):905–20.
- [126] Jafek JL, Shakya A, Tai PY, Ibarra A, Kim H, Maddox J, et al. Transcription factor Oct1 protects against hematopoietic stress and promotes acute myeloid leukemia. *Exp Hematol* 2019;76:38–48.
- [127] Xu X, Nagel S, Quentmeier H, Wang ZJ, Pommerenke C, Dirks WG, et al. KDM3B shows tumor-suppressive activity and transcriptionally regulates HOXA1 through retinoic acid response elements in acute myeloid leukemia. *Leuk Lymphoma* 2018;59(1):204–13.
- [128] Kim JY, Kim KB, Eom GH, Choe N, Kee HJ, Son HJ, et al. KDM3B is the H3K9 demethylase involved in transcriptional activation of lmo2 in leukemia. *Mol Cell Biol* 2012;32(14):2917–33.
- [129] Chen M, Zhu N, Liu XC, Laurent B, Tang ZY, Eng R, et al. JMJD1C is required for the survival of acute myeloid leukemia by functioning as a coactivator for key transcription factors. *Gene Dev* 2015;29(20):2123–39.
- [130] Izaguirre-Carbonell J, Christiansen L, Burns R, Schmitz J, Li CX, Mokry RL, et al. Critical role of Jumonji domain of JMJD1C in MLL-rearranged leukemia. *Blood Adv* 2019;3(9):1499–511.
- [131] Massett ME, Monaghan L, Patterson S, Mannion N, Bunschoten RP, Hoose A, et al. A KDM4A-PAF1-mediated epigenomic network is essential for acute myeloid leukemia cell self-renewal and survival. *Cell Death Dis* 2021;12(6):573.
- [132] Milan T, Celton M, Lagace K, Roques E, Safa-Tahar-Henni S, Bresson E, et al. Epigenetic changes in human model KMT2A leukemias highlight early events during leukemogenesis. *Haematologica* 2022;107(1):86–99.
- [133] Wang JZ, Li YC, Wang PP, Han GQ, Zhang TT, Chang JW, et al. Leukemogenic chromatin alterations promote AML leukemia stem cells via a KDM4C-ALKB5-AXL signaling axis. *Cell Stem Cell* 2020;27(1):81.
- [134] Chong PSY, Zhou JB, Cheong LL, Liu SC, Qian JR, Guo TN, et al. LEO1 is regulated by PRL-3 and mediates its oncogenic properties in acute myelogenous leukemia. *Cancer Res* 2014;74(11):3043–53.
- [135] Wu W, Cao XNA, Mo LX. Overexpression of KDM4D promotes acute myeloid leukemia cell development by activating MCL-1. *Am J Trans Res* 2021;13(4):2308.
- [136] Cardin S, Bilodeau M, Roussy M, Aubert L, Milan T, Jouan L, et al. Human models of NUP98-KDM5A megakaryocytic leukemia in mice contribute to uncovering new biomarkers and therapeutic vulnerabilities. *Blood Adv* 2019;3(21):3307–21.
- [137] Zhou MR, Zeng JP, Wang XM, Wang XY, Huang T, Fu Y, et al. Histone demethylase RBP2 decreases miR-21 in blast crisis of chronic myeloid leukemia. *Oncotarget* 2015;6(2):1249–61.
- [138] Stewart MH, Albert M, Srocynska P, Cruckshank VA, Guo YP, Rossi DJ, et al. The histone demethylase Jarid1b is required for hematopoietic stem cell self-renewal in mice. *Blood* 2015;125(13):2075–8.
- [139] Wang HJ, Song CH, Ding YL, Pan XK, Ge Z, Tan BH, et al. Transcriptional regulation of JARID1B/KDM5B histone demethylase by Ikaros, histone deacetylase 1 (HDAC1), and Casein Kinase 2 (CK2) in B-cell acute lymphoblastic leukemia. *J Biol Chem* 2016;291(8):4004–18.
- [140] Benyoucef A, Palii CG, Wang CC, Porter CJ, Chu A, Dai FT, et al. UTX inhibition as selective epigenetic therapy against TAL1-driven T-cell acute lymphoblastic leukemia. *Gene Dev* 2016;30(5):508–21.
- [141] Zhang CW, Shen L, Zhu YF, Xu R, Deng ZK, Liu XN, et al. KDM6A promotes imatinib resistance through YY1-mediated transcriptional upregulation of TRKA independently of its demethylase activity in chronic myelogenous leukemia. *Theranostics* 2021;11(6):2691–705.

- [142] Wei Y, Chen R, Dimicoli S, Bueso-Ramos C, Neuberg D, Pierce S, et al. Global H3K4me3 genome mapping reveals alterations of innate immunity signaling and overexpression of JMJD3 in human myelodysplastic syndrome CD34+ cells. *Leukemia* 2013;27(11):2177–86.
- [143] Wei Y, Zheng H, Bao NR, Jiang S, Bueso-Ramos CE, Khouri J, et al. KDM6B overexpression activates innate immune signaling and impairs hematopoiesis in mice. *Blood Adv* 2018; 2(19):2491–504.
- [144] Ntziachristos P, Tsirigos A, Welstead GG, Trimarchi T, Bakogianni S, Xu L, et al. Contrasting roles of histone 3 lysine 27 demethylases in acute lymphoblastic leukaemia. *Nature* 2014;514(7523):513–17.
- [145] Yu SH, Zhu KY, Chen J, Liu XZ, Xu PF, Zhang W, et al. JMJD3 facilitates C/EBP beta-centered transcriptional program to exert oncorepressor activity in AML. *Nat Commun* 2018;9:3369.
- [146] Blomberg MRA, Borowski T, Himo F, Liao R-Z, Siegbahn PEM. Quantum chemical studies of mechanisms for metalloenzymes. *Chem Rev* 2014;114(7):3601–58.
- [147] Couture J-F, Collazo E, Ortiz-Tello PA, Brunzelle JS, Trievel RC. Specificity and mechanism of JMJD2A, a trimethyllysine-specific histone demethylase. *Nat Struct Mol Biol* 2007;14:689–95.
- [148] Chen Z, Zang J, JKappler J, Hong X, Crawford F, Wang Q, et al. Structural basis of the recognition of a methylated histone tail by JMJD2A. *Proc Natl Acad Sci U S A* 2007;104:10818–23.
- [149] Loenarz C, Schofield CJ. Physiological and biochemical aspects of hydroxylations and demethylations catalyzed by human 2-oxoglutarate oxygenases. *Trends Biochem Sci* 2011;36(1):7–18.
- [150] Hamada S, Kim T-D, Suzuki T, Itoh Y, Tsumoto H, Nakagawa H, et al. Synthesis and activity of N-oxalylglycine and its derivatives as Jumonji C-domain-containing histone lysine demethylase inhibitors. *Bioorg Med Chem Lett* 2009;19:2852–5.
- [151] Hamada S, Suzuki T, Mino K, Koseki K, Oehme F, Flamme I, et al. Design, synthesis, enzyme-inhibitory activity, and effect on human cancer cells of a novel series of Jumonji domain-containing protein 2 histone demethylase inhibitors. *J Med Chem* 2010;53(15):5629–38.
- [152] Rose NR, Woon ECY, Kingham GL, King ONF, Mecinovic J, Clifton II, et al. Selective inhibitors of the JMJD2 histone demethylases: combined nondenaturing mass spectrometric screening and crystallographic approaches. *J Med Chem* 2010;53:1810–18.
- [153] Yang G-J, Wu J, Miao L, Zhu M-H, Zhou Q-J, Lu X-J, et al. Pharmacological inhibition of KDM5A for cancer treatment. *Eur J Med Chem* 2021;226:113855.
- [154] Lohse B, Nielsen AL, Kristensen JBL, Helgstrand C, Cloos PAC, Olsen L, et al. Targeting histone lysine demethylases by truncating the histone 3 tail to obtain selective substrate-based inhibitors. *Angew Chem Int Ed* 2011;50(39):9100–3.
- [155] Woon ECY, Tumber A, Kawamura A, Hillringhaus L, Ge W, Rose NR, et al. Linking of 2-oxoglutarate and substrate binding sites enables potent and highly selective inhibition of JmjC histone demethylases. *Angew Chem Int Ed* 2012;51(7):1631–4.
- [156] Kawamura A, Münz M, Kojima T, Yapp C, Bhushan B, Goto Y, et al. Highly selective inhibition of histone demethylases by de novo macrocyclic peptides. *Nat Commun* 2017;8:14773.
- [157] Zheng Y-C, Chang J, Wang L-C, Ren H-M, Pang J-R, Liu H-M. Lysine demethylase 5B (KDM5B): a potential anti-cancer drug target. *Eur J Med Chem* 2019;161:131–40.
- [158] Jose A, Shenoy GG, Sunil Rodrigues G, Kumar NAN, Munisamy M, Thomas L, et al. Histone demethylase KDM5B as a therapeutic target for cancer therapy. *Cancers* 2020;12(8):2121.
- [159] Gerken PA, Wolstenholme JR, Tumber A, Hatch SB, Zhang Y, Müller S, et al. Discovery of a highly selective cell-active inhibitor of the histone lysine demethylases KDM2/7. *Angew Chem Int Ed* 2017;56(49):15555–9.
- [160] Zhang W, Sviripa VM, Xie Y, Yu T, Haney MG, Blackburn JS, et al. Epigenetic regulation of wnt signaling by carboxamide-substituted benzhydryl amines that function as histone demethylase inhibitors. *iScience* 2020;23(12):101795.

- [161] Xu X, Wang L, Hu L, Dirks WG, Zhao Y, Wei Z, et al. Small molecular modulators of JMJD1C preferentially inhibit growth of leukemia cells. *Int J Cancer* 2020;146(2):400–12.
- [162] Kruidenier L, Chung C-W, Cheng Z, Liddle J, Che K, Joberty G, et al. A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. *Nature* 2012;488(7411):404–8.
- [163] Kruidenier L, Chung C-W, Cheng Z, Liddle J, Che K, Joberty G, et al. Kruidenier et al. reply. *Nature* 2014;514(7520):E2–E.
- [164] Giordano A, del Gaudio F, Johansson C, Riccio R, Oppermann U, Di Micco S. virtual fragment screening identification of a quinoline-5,8-dicarboxylic acid derivative as a selective JMJD3 inhibitor. *ChemMedChem* 2018;13(12):1160–4.
- [165] Giordano A, Forte G, Terracciano S, Russo A, Sala M, Scala MC, et al. Identification of the 2-benzoxazol-2-yl-phenol scaffold as new hit for JMJD3 inhibition. *ACS Med Chem Lett* 2019;10(4):601–5.
- [166] Chen YK, Bonaldi T, Cuomo A, Del Rosario JR, Hosfield DJ, Kanouni T, et al. Design of KDM4 inhibitors with antiproliferative effects in cancer models. *ACS Med Chem Lett* 2017;8(8):869–74.
- [167] Westaway SM, Preston AGS, Barker MD, Brown F, Brown JA, Campbell M, et al. Cell penetrant inhibitors of the KDM4 and KDM5 families of histone lysine demethylases. 1. 3-Amino-4-pyridine carboxylate derivatives. *J Med Chem* 2016;59(4):1357–69.
- [168] Bavetsias V, Lanigan RM, Ruda GF, Atrash B, McLaughlin MG, Tumber A, et al. 8-Substituted pyrido[3,4-d]pyrimidin-4(3 H)-one derivatives as potent, cell permeable, KDM4 (JMJD2) and KDM5 (JARID1) histone lysine demethylase inhibitors. *J Med Chem* 2016;59(4):1388–409.
- [169] Le Bihan Y-V, Lanigan RM, Atrash B, McLaughlin MG, Velupillai S, Malcolm AG, et al. C8-substituted pyrido[3,4-d]pyrimidin-4(3 H)-ones: studies towards the identification of potent, cell penetrant Jumonji C domain containing histone lysine demethylase 4 subfamily (KDM4) inhibitors, compound profiling in cell-based target engagement assays. *Eur J Med Chem* 2019;177:316–37.
- [170] Roatsch M, Hoffmann I, Abboud MI, Hancock RL, Tarhonskaya H, Hsu K-F, et al. The clinically used iron chelator deferasirox is an inhibitor of epigenetic JumonjiC domain-containing histone demethylases. *ACS Chem Biol* 2019;14(8):1737–50.
- [171] Sarno F, Nebbioso A, Altucci L. Identification and characterization of PKF118-310 as a KDM4A inhibitor. *Epigenetics* 2017;12(3):198–205.
- [172] Carter DM, Specker E, Małekci PH, Przygoda J, Dudaniec K, Weiss MS, et al. Enhanced properties of a benzimidazole benzylpyrazole lysine demethylase inhibitor: mechanism-of-action, binding site analysis, and activity in cellular models of prostate cancer. *J Med Chem* 2021;64(19):14266–82.
- [173] Fang Z, Wang T-Q, Li H, Zhang G, Wu X-A, Yang L, et al. Discovery of pyrazolo[1,5-a]pyrimidine-3-carbonitrile derivatives as a new class of histone lysine demethylase 4D (KDM4D) inhibitors. *Bioorg Med Chem Lett* 2017;27(14):3201–4.
- [174] Wang T, Liu Y, Zhang H, Fang Z, Zhang R, Zhang W, et al. Crystal structures of two inhibitors in complex with histone lysine demethylase 4D (KDM4D) provide new insights for rational drug design. *Biochem Biophys Res Commun* 2021;554:71–5.
- [175] Fang L, Hu Z, Yang Y, Chen P, Zhou J, Zhang H. Discovery of 3,5-dimethylisoxazole derivatives as novel, potent inhibitors for bromodomain and extraterminal domain (BET) family. *Bioorg Med Chem* 2021;39:116133.
- [176] McAllister TE, England KS, Hopkinson RJ, Brennan PE, Kawamura A, Schofield CJ. Recent progress in histone demethylase inhibitors. *J Med Chem* 2016;59(4):1308–29.
- [177] Kim S-H, Kwon SH, Park S-H, Lee JK, Bang H-S, Nam S-J, et al. Tripartin, a histone demethylase inhibitor from a bacterium associated with a dung beetle larva. *Org Lett* 2013;15:1834–7.
- [178] Guillade L, Sarno F, Tarhonskaya H, Nebbioso A, Alvarez S, Kawamura A, et al. Synthesis and biological evaluation of tripartin, a putative KDM4 natural product inhibitor, and 1-dichloromethylinden-1-ol analogues. *ChemMedChem* 2018;13(18):1949–56.

- [179] Souto JA, Sarno F, Nebbioso A, Papulino C, Álvarez R, Lombino J, et al. A new family of Jumonji C domain-containing KDM inhibitors inspired by natural product purpurogallin. *Front Chem* 2020;8(312).
- [180] Berry WL, Janknecht R. KDM4/JMJD2 histone demethylases: epigenetic regulators in cancer cells. *Cancer Res* 2013;73:2936–42.
- [181] Jaikhan P, Buranrat B, Itoh Y, Chotitumnavee J, Kurohara T, Suzuki T. Identification of ortho-hydroxy anilide as a novel scaffold for lysine demethylase 5 inhibitors. *Bioorg Med Chem Lett* 2019; 29(10):1173–6.
- [182] Mitsui E, Yoshida S, Shinoda Y, Matsumori Y, Tsujii H, Tsuchida M, et al. Identification of ryuvidine as a KDM5A inhibitor. *Sci Rep* 2019;9(1):9952.
- [183] Yang G-J, Ko C-N, Zhong H-J, Leung C-H, Ma D-L. Structure-based discovery of a selective KDM5A inhibitor that exhibits anti-cancer activity via inducing cell cycle arrest and senescence in breast cancer cell lines. *Cancers* 2019;11(1):92.
- [184] Labelle M., Boesen T., Mehrotra M., Khan Q., Ullah F. Inventors inhibitors of histone demethylases. International Patent 2014; 2014.
- [185] Tumber A, Nuzzi A, Hookway ES, Hatch SB, Velupillai S, Johansson C, et al. Potent and selective KDM5 inhibitor stops cellular demethylation of H3K4me3 at transcription start sites and proliferation of MM1S myeloma cells. *Cell Chem Biol*. 2017;24(3):371–80.
- [186] Chang K-H, King ONF, Tumber A, Woon ECY, Heightman TD, McDonough MA, et al. Inhibition of histone demethylases by 4-carboxy-2,2'-bipyridyl compounds. *ChemMedChem* 2011;6(5):759–64.
- [187] Nie Z, Shi L, Lai C, O'Connell SM, Xu J, Stansfield RK, et al. Structure-based design and discovery of potent and selective KDM5 inhibitors. *Bioorg Med Chem Lett* 2018;28(9):1490–4.
- [188] Gehling VS, Bellon SF, Harmange J-C, LeBlanc Y, Poy F, Odate S, et al. Identification of potent, selective KDM5 inhibitors. *Bioorg Med Chem Lett* 2016;26(17):4350–4.
- [189] Liang J, Zhang B, Labadie S, Ortwinne DF, Vinogradova M, Kiefer JR, et al. Lead optimization of a pyrazolo[1,5-a]pyrimidin-7(4 H)-one scaffold to identify potent, selective and orally bioavailable KDM5 inhibitors suitable for in vivo biological studies. *Bioorg Med Chem Lett* 2016;26:4036–41.
- [190] Miyake Y, Itoh Y, Hatanaka A, Suzuma Y, Suzuki M, Kodama H, et al. Identification of novel lysine demethylase 5-selective inhibitors by inhibitor-based fragment merging strategy. *Bioorg Med Chem* 2019;27(6):1119–29.
- [191] Horton JR, Liu X, Wu L, Zhang K, Shanks J, Zhang X, et al. Insights into the action of inhibitor enantiomers against histone lysine demethylase 5 A. *J Med Chem* 2018;61(7):3193–208.
- [192] Liang J, Labadie S, Zhang B, Ortwinne DF, Patel S, Vinogradova M, et al. From a novel HTS hit to potent, selective, and orally bioavailable KDM5 inhibitors. *Bioorg Med Chem Lett* 2017;27(13):2974–81.
- [193] Zhao B, Liang Q, Ren H, Zhang X, Wu Y, Zhang K, et al. Discovery of pyrazole derivatives as cellular active inhibitors of histone lysine specific demethylase 5B (KDM5B/JARID1B). *Eur J Med Chem* 2020;192:112161.
- [194] Horton JR, Woodcock CB, Chen Q, Liu X, Zhang X, Shanks J, et al. Structure-based engineering of irreversible inhibitors against histone lysine demethylase KDM5A. *J Med Chem* 2018; 61(23):10588–601.
- [195] Vazquez-Rodriguez S, Wright M, Rogers CM, Cribbs AP, Velupillai S, Philpott M, et al. Design, synthesis and characterization of covalent KDM5 inhibitors. *Angew Chem Int Ed* 2019;58(2):515–19.
- [196] Hatch SB, Yapp C, Montenegro RC, Savitsky P, Gamble V, Tumber A, et al. Assessing histone demethylase inhibitors in cells: lessons learned. *Epigen Chrom* 2017;10(1):9.
- [197] Wang L, Chang JJ, Varghese D, Dellinger M, Kumar S, Best AM, et al. A small molecule modulates Jumonji histone demethylase activity and selectively inhibits cancer growth. *Nat Commun* 2013;4.
- [198] Kim MS, Cho HI, JungYoon H, Ann YH, Park EJ, Jin YH, et al. JIB-04, a small molecule histone demethylase inhibitor, selectively targets colorectal cancer stem cells by inhibiting the Wnt/beta-catenin signaling pathway. *Sci Rep* 2018;8.

- [199] Cribbs A, Hookway ES, Wells G, Lindow M, Obad S, Oerum H, et al. Inhibition of histone H3K27 demethylases selectively modulates inflammatory phenotypes of natural killer cells. *J Biol Chem* 2018;293(7):2422–37.
- [200] Chu X, Zhong L, Yu LH, Xiong L, Li J, Dan WR, et al. GSK-J4 induces cell cycle arrest and apoptosis via ER stress and the synergism between GSK-J4 and decitabine in acute myeloid leukemia KG-1a cells. *Cancer Cell Int* 2020;20(1).
- [201] Li YN, Zhang MY, Sheng MY, Zhang P, Chen ZZ, Xing W, et al. Therapeutic potential of GSK-J4, a histone demethylase KDM6B/JMJD3 inhibitor, for acute myeloid leukemia. *J Cancer Res Clin* 2018;144(6):1065–77.
- [202] Illiano M, Conte M, Salzillo A, Ragone A, Spina A, Nebbioso A, et al. The KDM inhibitor GSKJ4 triggers CREB downregulation via a protein kinase A and proteasome-dependent mechanism in human acute myeloid leukemia cells. *Front Oncol* 2020;10.
- [203] Hong BJ, Park WY, Kim HR, Moon JW, Lee HY, Park JH, et al. Oncogenic KRAS sensitizes lung adenocarcinoma to GSK-J4-induced metabolic and oxidative stress. *Cancer Res* 2019;79(22):5849–59.
- [204] Sui AX, Xu YB, Li YT, Hu QL, Wang ZY, Zhang HT, et al. The pharmacological role of histone demethylase JMJD3 inhibitor GSK-J4 on glioma cells. *Oncotarget* 2017;8(40):68591–8.
- [205] Wang Q, Chen X, Jiang YH, Liu SH, Liu HS, Sun XH, et al. Elevating H3K27me3 level sensitizes colorectal cancer to oxaliplatin. *J Mol Cell Biol* 2020;12(2):125–37.

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Emerging epigenetic therapies—lysine acetyltransferase inhibitors

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1 Introduction

Environmental cues are processed into cellular responses through the regulation of differential gene expression networks. Histones as well as nonhistone proteins undergo an extensive repertoire of covalent modifications that alter their functions under various stimuli. These posttranslational modifications (PTMs) dictate critical cellular processes which are indispensable for the maintenance of cell homeostasis.

The reversible lysine acetylation is one such PTM that has a crucial role in cellular events, such as transcription, replication, DNA damage response, signaling cascades, and metabolic pathways. Owing to its immense importance in cell function, aberrant lysine acetylation is often associated with the onset and progression of pathological conditions, such as neurodegenerative disorders, metabolic diseases, and malignancies.

Acetylation of the lysine residue comprises of the transfer of the acetyl moiety from acetyl coenzyme A (acetyl-CoA) to the ϵ -nitrogen on the lysine side chain. Lysine acetylation dynamics are modulated by the concerted action of the writers, lysine acetyltransferases (KATs), and the erasers lysine deacetylases (KDACs). Reversible lysine acetylation is a mark of openly accessible chromatin which facilitates the binding of protein effectors machinery involved in transcription, replication, or DNA damage. Lysine acetylation has been associated with various cellular functions including chromatin architecture, DNA repair, protein-protein interaction, and protein stability. Proteomic studies have revealed the ubiquitous presence of lysine acetylation modification across different subcellular compartments regulating cellular homeostasis [1].

Even though acetylation is the most well studied lysine acylation, the lysine residues can undergo other acylations as well, namely formylation, propionylation, butyrylation, crotonylation, succinylation, malonylation, and β -hydroxybutyrylation. With improved sensitivity of detection techniques, the repertoire of acylation modifications has been increasing. Emerging studies have implicated the role of lysine acylations in different chromatin functions and differentiation and development programs, such as spermatogenesis [2], neural stem cell fate decision [3], muscle differentiation [4], adipogenesis [5], and mesoendodermal commitment in human embryonic stem cells [6].

1.1 Lysine acetylation/acylation and its consequences in cancer

Alterations in the lysine acetylation pattern of histones and nonhistone substrates have been extensively linked to cancer [1,7]. Among histones, these alterations can occur across whole genome (globally) [8] or onto specific gene loci. Impairment in the acetylation patterns or levels leads to deregulation in the expression of genes governing vital cellular processes thereby favoring tumorigenic process. Hypoacetylation on tumor suppressor genes (due to inactivating mutations, haploinsufficiency or low expression of KATs), hyperacetylation on oncogenes (due to overexpression and aberrant recruitment of KATs) and genetic aberrations in the reader proteins are among the prominent scenarios that contribute to the oncogenic reprogramming of the genome thereby promoting cancer phenotype [9,10].

Several of the acetylated histone marks are biomarkers for specific cancers and can serve as a prognostic tool for cancer prediction (Figure 18.1). Hypoacetylation of H4K16 has been so extensively observed in various human cancers that it is considered as a hallmark for malignant

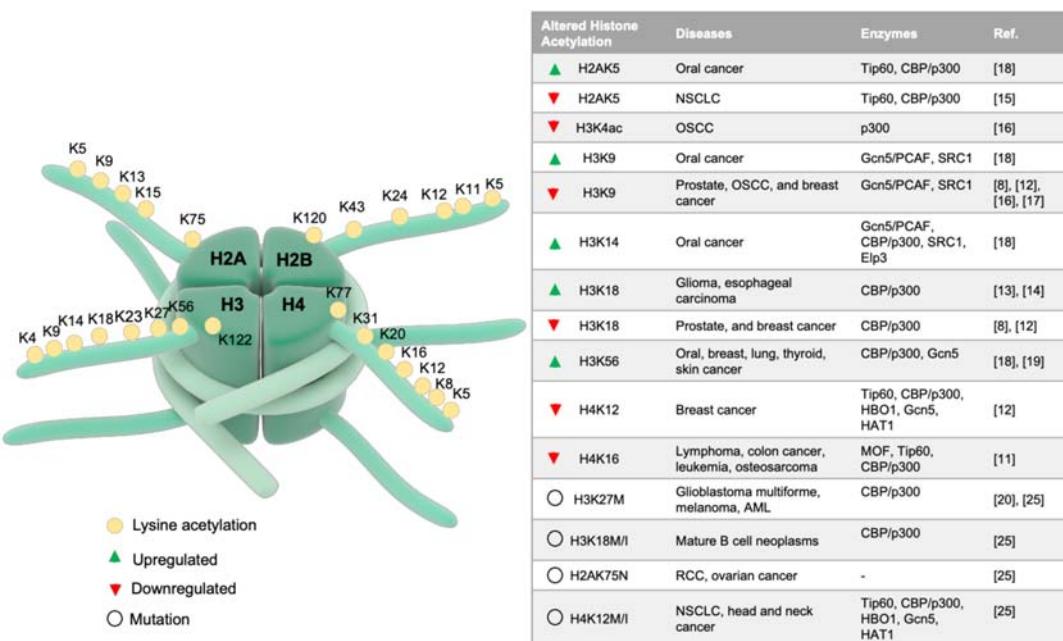
**FIGURE 18.1**

Illustration of a nucleosome depicting the positions of histone acetylation marks on the N-terminal tails of the four core histones, H2A, H2B, H3, and H4. The table alongside summarizes the altered histone acetylation in different cancers.

transformation [11]. In breast carcinomas, moderate-to-low levels of H3K9ac, H3K18ac and H4K12ac correlated with poor prognosis [12]. In glioblastoma and esophageal carcinoma, lower levels of H3K18ac were shown to be predictive of better patient survival [13,14]. In nonsmall-cell lung carcinoma (NSCLC), hypoacetylation of H2AK5 is associated with poor survival, whereas hypoacetylation of H3K4ac correlated with better survival of the patients [15]. In oral squamous cell carcinoma (OSCC), reduced levels of H3K9ac and H3K4ac was observed to be associated with poor prognosis and lower survival rates in patients [16,17], while our group observed hyperacetylation at several residues of histone H3 (K9, K14, K56) and at H2AK5 in tumor tissues of OSCC patients [18]. In prostate cancer, higher levels of H3K9ac and H3K18ac correlated with better prognosis and disease-free survival. This study demonstrated that patterns of histone modifications are better indicator of tumor recurrence and can be availed as a prognostic biomarker [8]. Elevated levels of H3K56ac has been observed to be proportional to tumor grade in many cancer tissues and positively correlated with dedifferentiated cancer cells and undifferentiated cells [19]. Though histone acetylation marks do serve as predictive prognostic markers, these altered histone acetylation marks may only be a snippet of a much larger epigenetic landscape that we are yet to comprehend in its full capacity. However, the use of single-cell analytical tools has paved the way to better understand tumor heterogeneity for precision medicine designed towards patient-specific epigenetic signatures.

To further add to the complexity of histone codes, the emerging role of “oncohistone” mutational landscape has also been extensively studied in oncogenesis. Notably, many of the mutations occurring in histones were found at or around prevalent histone modification sites, for example, H3.3K27M was identified in glioblastoma multiforme [20], H3.3K36M in 95% of chondroblastomas [21], histone H1 oncohistones in diffuse large B-cell lymphomas [22], H3K36M in head and neck cancers [23], and mutations on H2A/H2B in ovarian and uterine carcinosarcomas [24]. Nacev et al. reported additional histone oncomutations occurring on the tail and globular regions on histones including several lysine residue mutations including H3K4M/I, H3K18M/I, H4K12M/I, H2AK74N, and H2AK75N, in addition to the known lysine histone mutations [25]. It is known that the combinatorial effects of histone modifications play an important role in dictating cellular processes, thus these oncohistone mutations can effectively disrupt epigenetic signals thereby facilitating tumorigenesis.

1.2 Rewiring of acetyl-CoA metabolism in cancer

Acetyl-CoA is used as an essential cofactor by KATs for histone and nonhistone posttranslational modifications. Metabolic reprogramming is one of the hallmarks of cancer. Metabolic rewiring in cancer cells leads to an altered cellular concentration of acetyl-CoA which influences the activity of KATs and hence the protein acetylation patterns. The altered expression of metabolic enzymes is one of the facets of metabolic rewiring. ATP-citrate lyase (ACLY) and cytosolic acetyl-CoA synthetase (ACSS2) are the two key enzymes that produce acetyl-CoA from citrate and acetate respectively. Both ACLY and ACSS2 get upregulated or activated in several cancers. In human lung carcinoma, acetylated ACLY was observed to be upregulated. PCAF mediated acetylation of ACLY under high glucose condition was shown to increase its stability and promoted *de novo* lipid synthesis and tumor growth [26]. In hepatocellular carcinoma (HCC), the expression of acetyl-CoA synthetases (ACSS1/2) was shown to be high. In these cells, ACSS2 activity sustains acetyl-CoA production by utilizing acetate as an alternative carbon source. Mechanistically, under hypoxic conditions, acetate induced hyperacetylation of H3 at K9, K27, and K56 residues at the promoter regions of lipogenic genes (ACACA, FASN) increases their expression [27,28]. Interestingly, acetate, in spite of its low concentration has emerged as a primary alternative source of acetyl-CoA when the contribution of glucose for acetyl-CoA production drops under hypoxic conditions [29]. Both ACLY and ACSS2 are directly regulated by various signaling pathways. PI3K-AKT signaling pathway, commonly activated in various cancers, regulates histone acetylation through an increase in the phosphorylation of ACLY that leads to an increase in its activity [30,31]. In mutant-KRAS driven pancreatic carcinogenesis, enhanced phosphorylation and activity of AKT and ACLY was observed which led to the increase in abundance of acetyl-CoA that eventually resulted in elevated histone acetylation prior to pancreatic carcinogenesis [32]. AMPK, a cellular stress sensor, phosphorylates ACSS2. AMPK mediated phosphorylation of ACSS2 under glucose deprivation conditions leads to its nuclear translocation where it binds to transcription factor EB and locally produces acetyl-CoA for H3 acetylation at the promoter regions of genes involved in lysosomal biogenesis, autophagy, cell survival, and brain tumorigenesis [33].

In the subsequent sections, we will highlight the role of a few KATs in cancer and the current modalities being developed to inhibit the functions of deregulated KATs in tumorigenesis.

2 Deregulation of KATs in cancer

In this section we will discuss the involvement of KATs in the initiation, progression, and manifestation of cancer.

2.1 KATs and cancer

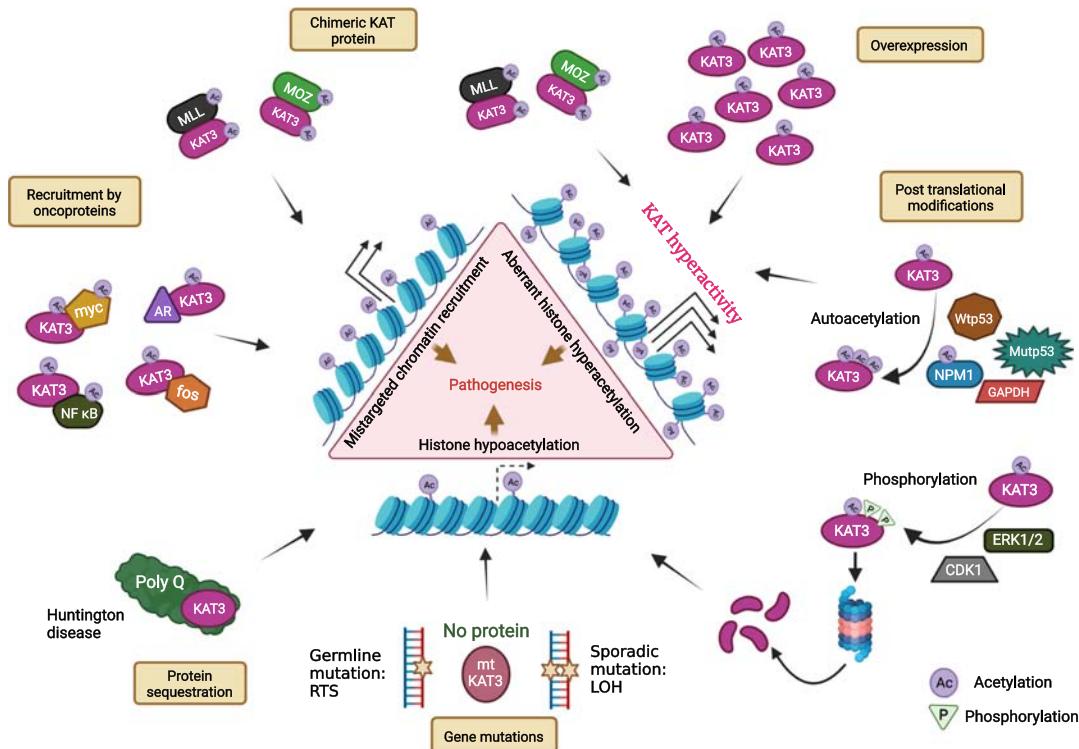
Lysine acetyltransferases (KATs) are classified into three major families based on their structural homology and sequence similarity. They are: GNAT (Gcn5 related N-acetyltransferase) family, MYST (MOZ, Ybf2, Sas2, TIP60) family, and the orphan (p300/CBP and nuclear receptors) family [34]. The role of KATs in the context of cancer can be either tumor suppressors or oncogenes. The fact that various somatic mutations identified in KATs in multiple cancers and their necessity for the functions of several tumor suppressors proteins render KATs tumor suppressive in nature. On the other hand, as oncogenes, hyperactivity or misregulated activity of KATs can contribute to tumorigenesis.

2.1.1 p300/CBP family

p300 (KAT3B) and CBP (KAT3A), the two paralog proteins, are transcriptional coactivators with intrinsic acetyltransferase activity [35]. They share high sequence identity in several of their structured regions, such as KAT domain (90% homology), bromodomain (93% homology), acetyl-lysine binding domain, and an overall sequence identity of 63% [36]. As p300/CBP is involved in several basic biological processes, such as transcription, cell cycle progression, DNA damage response, cellular proliferation, and apoptosis, perturbations of any kind in their functionality can lead to severe pathological conditions. The role of KAT3 proteins in cancer can be of a tumor suppressor or an oncogene which depends on various parameters.

Several somatic heterozygous mutations have been identified in p300/CBP gene in multiple cancers and heterozygous germline mutations in CBP (majorly) and EP300 gene occurs in Rubinstein-Taybi syndrome (a developmental disorder characterized by intellectual disability, physical defects, and increased risk of developing tumors) [37]. A typical characteristic of any tumor suppressor gene is the loss of heterozygosity (LOH) and same is the case with mutations in EP300 and CBP genes, identified in cell lines and primary tumors [38]. CBP and p300 are essential for maintaining hematopoietic stem cell self-renewal and hematopoietic differentiation respectively. Though, p300 and CBP have distinct roles in maintaining hematopoiesis, the absence of either of them in cells injected to generate chimeric mice resulted in the development of hematological cancers [39]. The ability of p300/CBP to interact and acetylate several tumor suppressor proteins, such as p53, E2F, BRCA1, FOXO-1, and SMAD, renders it essential for suppression of tumorigenesis [39] (Figure 18.2).

On the contrary, the reports of p300/CBP being involved in oncogenic transformation are not scarce. Chromosomal translocations of acetyltransferases lead to the formation of gain-of-function fusion proteins or chimeric oncoproteins which causes aberrant acetylation of histones at the genomic targets of its fusion partner and a higher overall gene expression level. Examples include – a fusion between MOZ and CBP or p300, and between MLL and CBP or p300 in various hematological malignancies [40]. In addition, p300/CBP interacts with several oncoproteins, such as c-jun, c-fos, c-myc, and c-myb, to further the process of cell proliferation and tumor formation [41–44] (Figure 18.2). Recent findings have shown that methyltransferase EZH2 (enhancer of zeste homolog 2) interacts noncanonically with

**FIGURE 18.2**

Deregulation of p300/CBP in diseases. Figure illustrating the deregulation of KAT3 proteins (p300/CBP) in three major ways that contributes to pathogenesis. Firstly, mistargeted recruitment of p300/CBP on chromatin by oncoproteins or gain-of-function fusion proteins; second, aberrant histone hyperacetylation caused by hyperactive p300/CBP resulting from chimeric proteins (MOZ-p300/CBP, MLL-p300/CBP), overexpression of p300/CBP or autoacetylation induction by GAPDH, NPM1, mutant p53; third, histone hypoacetylation due to reduced or loss of KAT activity due to ERK1/2 and CDK1 mediated phosphorylation and degradation of KAT3 proteins, germline or sporadic mutations leading to loss of heterozygosity (LOH) in various malignancies or their sequestration by poly (Q) proteins in Huntington disease. (Created with BioRender.com).

c-myc and p300 and thereby mediates gene activation and oncogenesis in acute leukemia [45]. p300 but not CBP controls enhancer acetylation through its interaction with a transcription factor TFAP2 β and regulates oncogenic transcription in high-risk neuroblastoma [46]. Interestingly, H3.3 was shown to act as a nucleosomal cofactor for p300. Upon phosphorylation, H3.3 stimulated p300 HAT activity in trans and thereby established new enhancers during differentiation [47]. Gain-of-function (GOF) mutations in *CREBBP/EP300* gene imparted radiation resistance in head and neck squamous cell carcinoma (HNSCC) whereas inhibition of their HAT activity sensitizes *CREBBP/EP300*-mutated tumors to radiation [48]. Overexpression of p300 has been widely reported in several cancers, such as prostate cancer, breast cancer, HCC, NSCLC, and the elevated levels of p300 is associated with poor survival of these

cancer patients [49–52]. Depletion of p300/CBP or targeting the interaction between p300/CBP and their oncogenic interacting partners, such as β -catenin, HIF1- α , NF- κ B, and AR, led to inhibition in cancer cell proliferation, invasion and tumor progression [53,54]. p300 and CBP are also required for androgen regulated expression of specific set of genes in AR (androgen receptor) positive prostate cancer cells [55]. Targeting p300/CBP has been shown to attenuate HCC progression via decrease in H3K18ac and H3K27ac levels at the promoter regions of amino acid metabolism and nucleotide synthesis enzyme genes [56].

The activity of p300/CBP can be modulated by their posttranslational modifications. p300 was shown to get phosphorylated by cyclin dependent kinase 1 and ERK1/2 on Ser1038 and Ser2039 which led to its degradation and promoted lung cancer progression [57]. Autoacetylation of p300/CBP on the unstructured, lysine rich regulatory loop present at the active site leads to their hyperactivation (Figure 18.2) (discussed in detail in the Section 2.2).

2.1.2 GNAT family

GCN5 (KAT2A) and PCAF (KAT2B) are homologous transcription coactivators of GNAT family. They function as a part of large multiprotein 2 MDa complexes, such as human STAGA (SPT3-TAF9-GCN5 acetyltransferase), TFTC (TATA binding protein (TBP)-free-TAF complex), PCAF complexes, and 700-kDa ATAC complex. Along with the locus-specific coactivator functions, these complexes carry out acetylation of histone and nonhistone substrates, making them indispensable for normal cellular functions [58].

PCAF acts as a coactivator for p53-directed transcription of p21 resulting in cell cycle arrest [59]. PCAF acetylates p53 at residue K320 which is necessary for its transactivation functions following DNA damage [60]. PCAF also has an intrinsic E3 ligase activity and controls the stability of Hdm2 thus contributing to p53 stabilization upon DNA damage [61]. PCAF locus (3p24) was found to be frequently deleted in esophageal squamous cell carcinoma (ESCC) and was identified to be downregulated in primary ESCC tumors and cell lines. PCAF could suppress the tumorigenicity of ESCC cells, mechanistically through upregulation of p21, Smad4, Rb and downregulation of CDK2 [62]. PCAF was downregulated in several HCC cell lines and its overexpression induced apoptosis and growth arrest [63]. PCAF expression was significantly downregulated in intestinal type gastric cancer and correlated with the poor clinical outcome [64]. These findings are strongly indicative of PCAF being a tumor suppressor. PCAF overexpression promoted autophagy mediated degradation of δ -catenin through acetylation which suppressed prostate cancer cell growth and motility [65].

On the other hand, PCAF is known to impart drug resistance to cells either through enhanced E2F1 expression [66] or by Twist1 mediated Y-box binding protein-1 (Yb-1) expression dependent manner [67]. PCAF/GCN5 mediated elevated H3K9ac levels at MDR1 (Multidrug Resistant Protein-1) gene promoter conferred multidrug resistance to breast cancer cells, whereas RNAi mediated knockdown of PCAF or GCN5 resulted in reduced MDR1 expression thus sensitizing cancer cells to drugs [68]. GCN5 and PCAF when associates with oncprotein c-Myc, gets recruited to c-Myc-target promoters in chromatin [69]. N-terminal truncated form of c-Myc protein could not interact with STAGA complex and was deficient in promoting malignant transformation due to its reduced transactivation potential [70]. High expression of GCN5 has been reported in NSCLC which correlated with the tumor size. Elevated levels of GCN5 promoted cell growth and G1/S transition in several lung cancer cell lines via upregulation of E2F1, cyclin D1 and cyclin E1

expression [71]. Fascin gets acetylated by PCAF which reduces its actin bundling activity and filopodium formation thereby suppressing esophageal cancer cell migration and tumor metastasis [72]. The oncogenic signaling axis PCAF-PAX3–FOXO1 promotes alveolar rhabdomyosarcoma (ARMS) which is abrogated through PCAF inhibition [73].

2.1.3 MYST family

KATs of the MYST family are evolutionarily highly conserved sharing a conserved MYST domain that contains zinc finger domain and an acetyl-CoA binding domain. In mammals, MYST family comprises of five acetyltransferases – TIP60 (KAT5), MOZ (KAT6A), MORF (KAT6B), MOF (KAT8) and HBO1 (KAT7). These KATs are involved in critical cellular functions, such as gene regulation, DNA damage repair, cell cycle, stem cell development and self-renewal, replication, and heterochromatin formation, and hence are implicated in several pathophysiological conditions like cancer [74,75].

Tip60 (HIV-1 Tat interacting protein) is intricately involved in DNA repair pathway. Upon DNA damage, Tip60 acetylates and activates ATM kinase, which further phosphorylates its downstream targets, such as p53, chk2, and histone variant H2AX (γ -H2AX) [76,77]. Tip60 acetylates p53 at K120 which was found to be critical for p53-mediated apoptosis upon prolonged DNA damage [78]. Tip60 has been found to be downregulated in multiple cancers, such as colon and lung cancers, which compromises the activity of p53 [79]. Downregulation of Tip60 in gastric cancer significantly correlated with patient age, tumor invasion and metastasis [80]. Tip60 is particularly downregulated in breast cancer and its low level correlates with p53 mutations. Heterozygous deletion of Tip60 promoted mammary tumorigenesis due to reduced or defective DNA repair [81]. Low levels of Tip60 leads to reduction in H3K4ac and development of ER-negative tumors [82]. Hence, Tip60 acts as a haplo-insufficient tumor suppressor whose critical levels are required to mount a DNA damage response [83]. Tip60 expression was significantly reduced in metastatic melanoma and associated with poor disease-specific survival in melanoma patients. Also, Tip60 overexpression inhibited melanoma cell migration and enhanced chemosensitivity [84]. HPV E6 utilizes the function of E3 ligase EDD1 to degrade Tip60 in cervical cancer, thereby contributing to HPV E6 mediated transformation [85]. All these observations establish Tip60 as a putative tumor suppressor.

Tip60 is also has potentially oncogenic roles. Tip60 can function as a coactivator for oncoprotein c-Myc, and along with GCN5, causes acetylation mediated stabilization of c-Myc [86]. It has been shown that Tip60-Myc interaction is required for Myc-associated transforming activities in adult T-cell leukemogenesis [87]. Tip60 is involved in the development of androgen independent prostate cancer progression. Tip60 is an AR coactivator protein. Androgen withdrawal led to upregulation and nuclear accumulation of Tip60 [88] and promoted Tip60 mediated nuclear localization of AR during prostate cancer progression. Furthermore, Tip60 silencing reduced the growth of castration-resistant prostate cancer cells [89]. Tip60 was observed to be overexpressed in cisplatin resistant human lung cancer cells and is shown to be involved in the repair of cisplatin-induced DNA damage through upregulation of ERCC1 protein expression [90,91].

hMOF is the human ortholog of the Drosophila MOF (males absent on the first), a key component of MSL (Male specific lethal) complex involved in dosage compensation in Drosophila [92]. hMOF associates with two distinct multisubunit human histone acetyltransferase complexes hMSL and hNSL (nonspecific lethal) with distinct substrate specificities. While hMSL is responsible for majority of H4K16 acetylation in human cells, hNSL has a relaxed specificity and can acetylate

histone H4 on K5 and K8 in addition to K16 [93,94]. Hypoacetylation of H4K16 is considered as a hallmark of cancer and downregulation of hMOF is also observed in several cancers, such as gastric cancer, colorectal cancer, renal cell carcinoma, and ovarian cancer [95,96]. Upon DNA damage, hMOF acetylates p53 on K120 which is required for p53 mediated activation of proapoptotic target genes [97].

Contrastingly, hMOF level was observed to be elevated in oral tongue squamous cell carcinoma (OTSCC) and high hMOF expression correlated with poor overall and disease-free survival [98]. hMOF was found to be overexpressed in NSCLC, contributes to drug resistance and tumor growth and is associated with tumor size, metastasis and poor prognosis [99].

MOZ (Monocytic Leukemia Zinc Finger Protein) and MORF (MOZ-related factor) are homologous transcriptional coactivators with acetyltransferase activity [100,101]. MOZ was first identified as a chimeric protein fused with CBP due to chromosomal translocation in AML patients. MOZ-CBP GOF fusion protein caused aberrant chromatin acetylation during leukemogenesis [102]. Similarly, MOZ-p300 fusion protein is also observed in AML [103]. Another fusion partner for MOZ in AML is transcription intermediary factor 2 (TIF2), a nuclear receptor transcriptional coactivator [104]. MOZ-TIF2 fusion protein recruits CBP through TIF2 CBP interaction domain (CID) to form a hyperactive chimera that causes deregulated acetylation and may exert a dominant negative effect over tumor suppressive functions of CBP. The recruitment of CBP by TIF2 was found to be critical for MOZ-TIF2 mediated transformation and leukemogenesis [105]. GOF fusion protein has also been reported for MORF with CBP. MORF-CBP, like MOZ-CBP, leads to aberrant acetylation and gene expression programs [106]. p53 GOF mutants were shown to regulate the chromatin through upregulation of MLL and MOZ expression which elevated the levels of active histone modifications H3K4me3 and H3K9ac globally and contributed to GOF effects of mutant p53 [107]. This study for the first time revealed the role of epigenetic-based therapy in combating GOF mutant p53 driven cancers.

HBO1 (HAT bound to ORC1), was identified as histone acetyltransferase that interacts with ORC1 protein, the largest subunit of origin recognition complex [108]. HBO1 is involved in prereplicative complex assembly and replication initiation [109]. HBO1 is the catalytic subunit of tumor suppressive protein complexes, such as JADE (JADE1/2/3) and Ing (Ing4/5) [110,111]. p53 interacts with HBO1 and negatively regulates its HAT activity to stall replication during physiological stress [112]. HBO1 was found to be abundantly expressed in human primary tumors of breast, stomach, bladder, testes, ovary as compared to adjacent normal tissues [113]. HBO1 gets phosphorylated by Cyclin E/Cdk2 and thereby promotes enrichment of cancer stem-like cells (CSC) [114]. Phosphorylation of HBO1 by Polo-like kinase 1 (Plk1) leads to drug resistance in pancreatic cells. HBO1 phosphorylation transcriptionally increased c-Fos expression which in turn elevated the expression of one of its target gene MDR1 that conferred gemcitabine resistance [115].

2.2 Autoacetylation in cancer progression

Similar to kinases, acetyltransferases are also subjected to autoacetylation. Almost all KATs are known to be autoacetylated. The autoacetylation of KATs enhances their catalytic activity. p300/CBP undergo transautoacetylation process that can regulate their catalytic activity [116]. A lysine-rich autoinhibitory loop was identified in the HAT domain of p300 which upon getting autoacetylated at several of its key lysine sites, increases the acetyltransferase activity of p300 to several folds

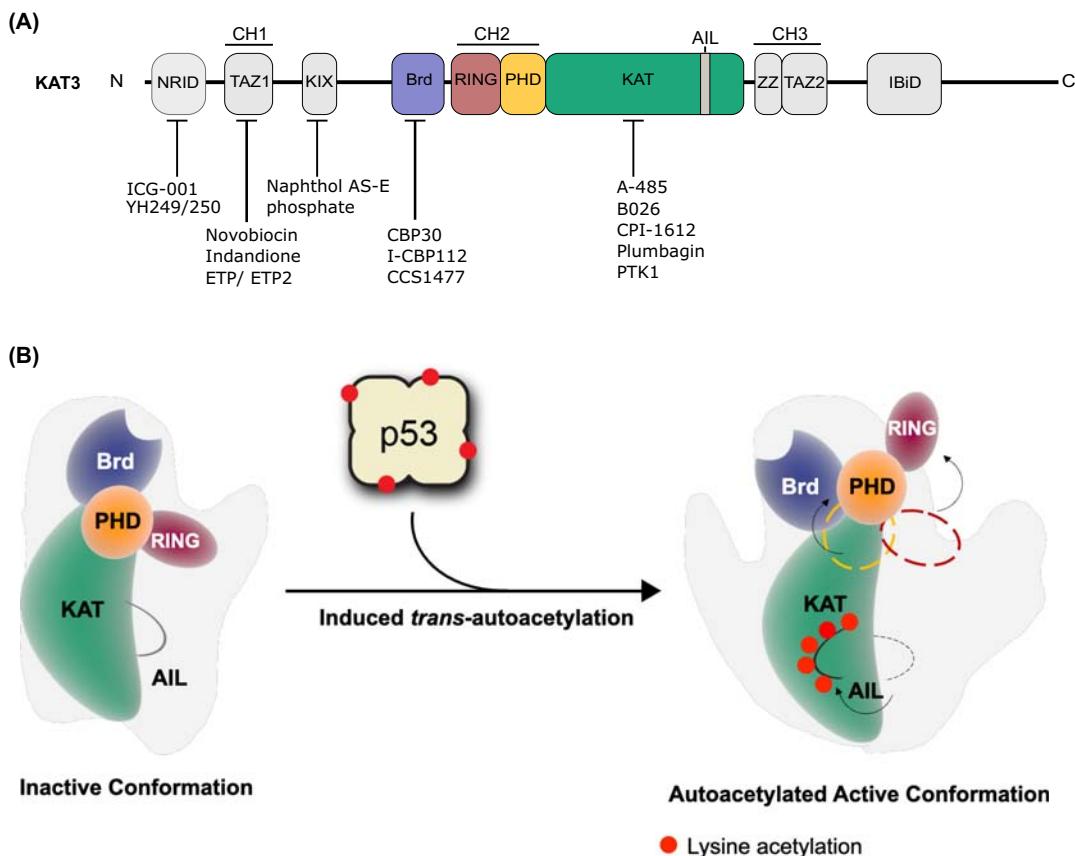
(Figure 18.3) [117]. Autoacetylation of p300 was identified to be a highly cooperative and intermolecular process by kinetic and mass spectrometric analysis [118]. Also, surface-enhance Raman spectroscopy revealed the structural alterations induced by autoacetylation in the p300 HAT domain [119]. Several inducers of p300 autoacetylation have been reported—two subunits (APC5/7) of anaphase promoting complex/Cyclosome (APC/C) [120], transcription factor MAML1 [121], GAPDH [122], NPM1 [18,123], wild-type p53, and several GOF mutants of p53 [124]. NPM1 and GAPDH gets overexpressed in several cancers including oral cancer. Our group has reported nitric oxide (NO) signal-dependent upregulation of NPM1 and GAPDH in tumor tissues of OSCC patients that contributed to increase in the levels of autoacetylated p300 (acp300) and hence hyperacetylation of histones [18]. Upon exploring the mechanism behind NPM1 mediated p300 autoacetylation induction, it was observed that NPM1 oligomerization along with its reversible binding (chaperoning) to p300 is required for p300 autoacetylation induction [123]. Interestingly, p300 acetylates NPM1 and enhances its activity. Both NPM1 and acetylated NPM1 (AcNPM1) get overexpressed in oral cancer. Recent findings have confirmed the role of AcNPM1 as a coactivator during RNA Pol II-driven transcription of genes involved in oral tumorigenesis [125]. Master tumor suppressor protein p53 has also been shown to be a bona fide inducer of p300 autoacetylation which causes redistribution of acp300 and its preferential chromatin enrichment that assists in p53-targeted gene expression [124,126] (Figure 18.3). Surprisingly, several GOF hotspot mutants of p53 (R273H, R249S, R248W, V143A) were also identified as potent inducers of p300 autoacetylation [124]. Further studies that involves non-hotspot GOF p53 mutants, such as P152Lp53 [127], along with detailed mechanistic insights would establish mutant p53-induced p300 autoacetylation induction as one of the GOF mechanism employed by p53 mutants to execute their oncogenic functions.

3 Lysine acetyltransferase: a potential target for therapeutics

Cancer is caused by the interplay of genetic and epigenetic abnormalities. The reversible nature of lysine acetylation has made lysine acetyltransferases a potential therapeutic target in cancers. Lysine acetylation is a crucial factor in the maintenance of cellular homeostasis and several cancers are known to be driven by aberrant protein lysine acetylation, therefore the need for specific small-molecule inhibitors of KAT is of utmost importance [1,7].

3.1 Bisubstrate inhibitors

KATs are bisubstrate enzymes that bind to acetyl-CoA and lysine containing peptides. The earliest known inhibitor of acetyltransferase activity was a multisubstrate analog formed by an acetic acid linkage between acetyl-CoA and spermidine known as N-[2-(S-coenzyme A) acetyl] spermidine amide [128]. Bisubstrate inhibitors are acetyl-CoA molecules conjugated to a lysine substrate mimic that act as highly selective, potent, dead-end inhibitors [129]; Lys-CoA a specific inhibitor for p300 and H3-CoA-20 for PCAF with IC₅₀ values $\leq 0.5 \mu\text{M}$ [130]. Based on the same rationale, substrate-based inhibitor, H4K16CoA, was synthesized targeting Esa1 and Tip60 [131]. However, the major setback in developing bisubstrate inhibitors into potential therapeutics was the lack of cell permeability and poor metabolic stability. The cell permeability issue was resolved by linking

**FIGURE 18.3**

Regulation of KAT3 catalytic regulation. (A) Domain architecture of KAT3 proteins [NRID (nuclear receptor interaction domain), TAZ1 (transcriptional adapter zinc-finger domain 1), KIX (kinase-inducible domain interacting domain), Brd (bromodomain), RING (really interesting new gene), PHD (plant homeodomain), KAT (Lysine acetyltransferase domain), ZZ (ZZ-type zinc finger domain), TAZ2 (transcriptional-adapter zinc-finger domain 2), IBID (IRF3-binding domain), CH1/2/3 (cysteine-histidine-rich (CH) regions), AIL (Autoinhibitory loop)] and examples of small molecules targeting specific domains. (B) p53-mediated enhancement of p300 (KAT3B) autoacetylation and activation. Tetrameric p53 binds to p300 leading to a rearrangement of the core catalytic domains (Brd-RING-PHD-KAT) from an inactive conformation to an activated open conformation. This spatial rearrangement of p300 domains facilitates *trans*-autoacetylation and activation of p300 catalytic activity [124,126].

a cell permeabilizing peptide, tat, to the inhibitors [132]. However, the linkage to tat peptide reduced the potency and selectivity of Lys-CoA. To further optimize the activity of the conjugate, the cell permeabilizing peptide was reversibly linked to Lys-CoA derivative via a disulfide bond, which could be reduced inside the cell thereby releasing the inhibitor from the permeabilizing

peptide [133]. Spermidine linked via a thioglycolic acid bond to the S-terminus of Co-enzymeA forming Spd-CoA forms a nontoxic, histone acetylation inhibitor. Spd-CoA sensitized cells to chemotherapeutic drugs and UV-radiation by blocking cell cycle [134]. Recently bisubstrate inhibitors, H4K5CoA and H4K12CoA, targeting cytoplasmic HAT1 were reported with a K_i in the nanomolar range [135].

3.2 Natural KAT inhibitors and derivatives

There have been many challenges to create a KATi which is cell-permeable, potent, and selective. Natural compounds have proven to be a great source of complex molecules harboring biologically relevant activity. Compounds extracted from natural sources have served as a great starting point for potent small molecule KATi.

3.2.1 Anacardic acid and derivatives

Anacardic acid (AA, 6-pentadecylsalicylic acid), extracted from *Anacardium occidentale* (cashew nut) shell liquid, was the first natural compound shown to possess KAT inhibitory activity [136]. AA has been shown to sensitize tumors to radiation through the inhibition of Tip60-mediated DNA damage repair to ionizing radiation [137]. Even though AA lacks specificity and can inhibit p300, PCAF, and Tip60, it has proven to be an invaluable scaffold for series of potent KAT modulators with improved selectivity, cell permeability, and potency. AA derivative optimized to a binding model of AA for PCAF exhibited specificity towards PCAF. The inhibitors were derived by substituting the 6-alkyl chain for other functional groups. The salicylate derivative displayed a twofold improved inhibitory activity against histone acetylation in HepG2 cells [138]. MG149, a derivative with a phenethyl moiety replacement at the 6-position of AA exhibited selective inhibition of recombinant Tip60 ($IC_{50} = 74 \mu\text{M}$) and MOF ($IC_{50} = 47 \mu\text{M}$) over p300 and PCAF. Enzyme kinetics revealed that the inhibition of Tip60 was competitive to acetyl-CoA, and noncompetitive to the histone substrate [139].

N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (CTPB) an amide derivative of AA curiously activated p300 catalytic activity without affecting PCAF activity [136]. Benzamide derivative of CTPB with shorter chains demonstrated p300 inhibition. The derivative with the shortest alkyl chain effectively induced apoptosis in leukemia cells [140]. The anti-inflammatory drugs salicylate and acetylsalicylic acid have shown to possess inhibitory activity against CBP/p300 through direct competition with acetyl-CoA binding at the active site. Based on chemical structure similarity, difunisal, another anti-inflammatory drug, was discovered as a p300 inhibitor, capable of blocking p300-dependent cell growth in leukemia cell lines expression AML1-ETO [141].

3.2.2 Polyphenols

Garcinol, polyisoprenylated benzophenone, extracted from *Garcinia indica*, is a potent nonselective KAT inhibitor with IC_{50} values of $5 \mu\text{M}$ for p300 and $7 \mu\text{M}$ for PCAF, which can alter global gene expression and induce apoptosis in HeLa cells [142]. Garcinol is a potential cancer chemopreventive agent. Garcinol can alter histone and nonhistone protein PTMs to modulate the expression of key epigenetic enzymes, thereby inhibiting cell growth in MCF7 cells [143]. Garcinol can exert its antitumor effect through the suppression of proinflammatory STAT3 and NF-κB pathways in head

and neck squamous cell carcinoma [143]. In HCC, garcinol was shown to prevent the activation of inflammatory pathways by impairing the activation, dimerization, and acetylation of STAT3 [144]. Garcinol mediated antitumor effects in HNSCC and HCC xenograft models through the inhibition of inflammatory pathways [143,144]. Garcinol is a good lead molecule for further optimization into nontoxic, potent, and selective KAT inhibitors. Intramolecular cyclization of garcinol formed iso-garcinol (IG), which is a scaffold for the nontoxic LTK compound series. The LTK series was synthesized by the controlled modification of IG. Monosubstitution at the 14-position generated 14-isopropoxy IG (LTK-13) and 14-methoxy IG (LTK-14) while disubstitution of IG lead to 13,14-disulfoxyl IG (LTK-19). Among other derivatives in the series, LTK-13, LTK-14, and LTK19 exhibited p300-specific inhibition while displaying no effect on PCAF KAT activity. The IC₅₀ values of these molecules ranged between 5 and 7 μM. The mode of inhibition differed from garcinol which is competitive for histones and mixed type for acetyl-CoA, while LTK14 appeared to exhibit mixed type kinetics for both histones and acetyl-CoA, suggesting that the molecule maybe binding to a pocket on the enzyme other than the active site, thereby specifically modulating p300 KAT activity [145]. Isogarcinol derivative LTK14A (13,14 dimethoxy isogarcinol) has been shown to inhibit p300-mediated butyrylation at a concentration of 25 μM. LTK14A reduced the expression of pro-adipogenic genes and attenuated weight gain in obesity mouse models [5]. Garcinol derivatization through molecular pruning led to the benzylidenebarbituric acid analog, EML425, which is more cell permeable than the parent compound and a specific, competitive inhibitor of p300/CBP. Human leukemia cells treated with EML425 resulted in cell cycle arrest in the G0/G1 phase and marked reduction in H3K9 and H4K5 acetylation [146].

Epigallocatechin-3-gallate (EGCG) is a polyphenol present in green tea. EGCG has been shown to have anti-inflammatory activity in a variety of malignancies. The anti-KAT activity of EGCG results in the abrogation of RelA activation through p300-mediated acetylation and the subsequent downstream NF-κB proinflammatory signal transduction [147].

The p300-specific inhibitor curcumin, isolated from *Curcuma longa* (turmeric) rhizome, is a polyphenol shown to possess antioxidant, anti-inflammatory, antiproliferative, and antitumor properties [148]. Even though curcumin has poor bioavailability due its sparse water solubility, curcumin derivatives hydrazinobenzoyl curcumin (HBC) and its water-soluble salt, CTK7A, has shown immense antitumor activity in castration-resistant prostate cancer and oral cancer xenografts respectively [18,149]. The antimalignant potency of CTK7A may be attributed to the inhibition of p300 autoacetylation [18]. CTK7A-mediated inhibition of p300 autoacetylation also leads to the loss of interaction between p300 and hypoxia-inducible factor-1 (HIF-1) resulting in its reduced accumulation and activity in gastric cancer cells [150].

The polyphenol delphinidin (DP), extracted from pomegranate (*Punica granatum*), is a p300-specific KAT inhibitor. DP, at 100 μM, inhibited p300/CBP activity by 70% *in vitro*. DP exerts its anti-inflammatory activity by preventing p300-mediated acetylation and activation of RelA (NF-κB) [151].

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is a phytochemical which is anticancerous and anti-inflammatory. GA is an uncompetitive inhibitor for p300/CBP. In A549 lung cancer cells, GA inhibits NF-κB signaling by the abrogation of p300-mediated RelA acetylation. *In vivo*, GA reduces the levels of LPS-induced interleukin-6 [152].

Procyanidin B3 is a p300-specific inhibitor that abolishes p300-mediated AR acetylation and activation and suppresses prostate cancer cell proliferation [153].

Quercetin is a dietary polyphenolic bioflavonoid which possesses a wide range of biological activities including antitumor and antiinflammatory properties. Quercetin is an inhibitor of p300 KAT activity and blocks the binding of p300 to transactivators NF-κB, c-Jun, CREB2, and C/EBP β to the COX-2 promoter, a protein involved in cancer progression [154].

The natural polyphenol, carnosol, found in edible plants, such as oregano, sage, and rosemary, promotes ROS-dependent proteasomal degradation of p300 and PCAF proteins with no effect on Gcn5 and MOF levels. Moreover, carnosol showed p300-specific KAT inhibitory activity *in vitro* but did not inhibit recombinant PCAF or Gcn5 catalytic activity [155]. Carnosol suppresses the proliferation, migration, and invasion and promotes autophagy in highly invasive breast cancer cells [156,157].

3.2.3 Quinone

Plumbagin is a hydroxynaphthoquinone isolated from the roots of *Plumbago rosea*, a medicinal herb. Plumbagin is selective to p300 over PCAF and inhibits p300 *in vitro* as well as in cells, through a noncompetitive mechanism. Docking studies revealed that a single hydroxyl group on plumbagin forms a hydrogen bond with p300 catalytic domain at the residue K1358 [158]. Plumbagin has demonstrated anticancer activity against a wide range of cancer cell lines, including melanoma, OSCC, lung cancer, brain tumor, and breast cancer, and in addition possesses antioxidant, antiinflammatory, and antidiabetic properties [159]. PTK1, a 1,4-naphthoquinone derivative, has an improved toxicity profile compared to plumbagin while retaining the p300 KAT inhibitory activity [160].

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) extracted from *Embelia ribes* is a cell permeable, proapoptotic, and antiinflammatory PCAF-specific inhibitor [161,162]. Embelin was shown to inhibit PCAF-mediated muscle differentiation [161] and PCAF-PAX3–FOXO1 oncogenic signaling axis in ARMS [57]. Embelin treatment inhibited tumorigenesis in rhabdomyosarcoma xenograft model [73].

3.2.4 Alkaloids

Sanguinarine is a benzophenanthridine alkaloid DNA intercalator extracted from the rhizomes of *Sanguinaria canadensis* and *Argemone mexicana* and is a known anti-inflammatory and antiproliferative agent that promotes health span and innate immunity [163,164]. Interestingly, sanguinarine can modulate the epigenetic landscape and gene regulatory networks by inhibiting p300/CBP and methyltransferases G9a and CARM1 [165].

Customized structure-based virtual screen from natural product libraries led to the discovery of alkaloids NP-2 (spinosine, $IC_{50} = 0.69 \mu M$), NP-3 (palmatine, $IC_{50} = 1.05 \mu M$), NP-9 (venenatine, $IC_{50} = 0.58 \mu M$), and NP-15 (taxodionine, $IC_{50} = 4.85 \mu M$) as potent p300 inhibitors. These molecules also displayed moderate inhibitory activity against PCAF (IC_{50} values of NP-2, NP-3, NP-9, and NP-15 were 14.13, 10.0, 27.1, and 7.16 μM , respectively) [166].

3.2.5 Peptides

Microorganism broth library screen to find novel p300 KAT inhibitors identified two secondary metabolites of *Penicillium* species, NK13650A and NK13650B with IC_{50} values of 11 and 22 nM, respectively. These compounds possess unique pseudopeptide structures containing a citrate. These peptide-based molecules showed inhibitory activity AR-dependent transcriptional activation and NK13650A treatment abrogated prostate cancer cell proliferation [167].

3.3 Synthetic KAT inhibitors

Though natural compounds have great value as KATi, their biological use is restricted by their cytotoxicity, poor bioavailability, and pleiotropic activity. Therefore high-throughput screens and structure–function-based optimizations have been the go-to modality to identify potent and selective KATi. Search for novel scaffolds with minimal off-target effects is of pressing priority.

MB-3 (α -methylene- γ -butyrolactone) was the first synthetic cell-permeable, small molecule inhibitor of Gcn5 with an IC_{50} value of 100 μ M [168]. A phenotypic screen in *Saccharomyces cerevisiae* was performed to identify modulators of Gcn5p, leading to the discovery of CPTH2 (cyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl]hydrazone), a novel molecule derived from thiazole [169]. (Thiazol-2-yl)hydrazones derivatives were shown to be potent p300 inhibitors and could effectively induce apoptosis and cytodifferentiation in human leukemia cells U937 and colon cancer cell HCT116 [170]. The efficacy of CPTH2 has been demonstrated against the progression of clear cell renal carcinoma (ccRCC) through the inhibition of p300 activity and reduction of H3K18 acetylation [171].

The isothiazolone-based HAT inhibitors, CCT077791 and CCT077792 were identified through a high-throughput screen as antagonist of p300 and PCAF catalytic activity. These compounds were shown to decrease global acetylation in a time-dependent and concentration-dependent manner in HCT116 and HT28 colon carcinoma cell lines [172]. 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides exhibited growth inhibition of HepG2 HCC cells through the inhibition of PCAF [173]. However, most isothiazolones are thiol-reactive, leading to nonspecificity and limited biological use [174,175]. The thiazole-based synthetic compound 1-(4-(4-chlorophenyl)thiazol-2-yl)-2-(propan-2-ylidene)hydrazine (BF1) demonstrated inhibitory activity against recombinant Gcn5 and p300 and was tested in aggressive neuroblastoma and glioblastoma cell lines. BF1 treatment resulted in global reduction in histone H3 acetylation [176].

NU9056 (1,2-bis(isothiazol-5-yl)disulfane) was identified as an inhibitor of Tip60 (IC_{50} = 2 μ M) through a high-throughput virtual screen of structurally diverse compound library. NU9056 exhibited efficacy against the growth of prostate cancer cells [177]. Drug design based on Tip60 structure led to the discovery of a novel inhibitor TH1834 [178]. TH1834 treatment effectively inhibited Tip60-mediated breast cancer progression [179].

High-throughput screening of vast chemical libraries has led to the identification of several potent KAT inhibitors, notably, the discovery of C646, a potent, cell-permeable, p300-specific inhibitor with a K_i of 400 nM. Computational modeling supported by site-directed mutagenesis study revealed that C646 interacts with T1411, W1466, R1410, and Y1467 of p300 via hydrogen bonds [180]. C646 sensitizes NSCLC cells to ionizing radiation, but not normal human lung fibroblasts, through G2 checkpoint abrogation and mitotic catastrophe [181]. C646 was also reported to inhibit tumor growth in pancreatic xenograft mouse model by suppressing the transcription of G3/M cell cycle proteins cyclin B1 and CDK1 [182]. However, it was shown that C646 exhibited thiol-reactivity, thus greatly limiting its biological use [183,184]. Through another high-throughput virtual screen of 800,000 molecules and a further 1300 compound screen through radioactivity-based HAT assay, A-485 was identified as a potent inhibitor of p300 in the nanomolar range (IC_{50} value of 60 nM). A-485 was shown to be highly specific to p300/CBP over other KAT family proteins, BET domain proteins and over 150 nonepigenic proteins. A-485 displayed antiproliferative properties against 10 different types of solid tumors and hematological cancers and was efficacious

against castration resistant prostate cancer xenograft model [185]. Using an AI-assisted drug discovery pipeline to discover KAT3 inhibitors with desirable drug-like properties, the orally efficacious compound B026 ($IC_{50} = 1.8$ nM) was discovered with a 30-fold higher potency against p300 than A-485, cancer cell growth inhibition in the nanomolar range, effective tumor growth inhibition and good pharmacokinetics profile [186]. Recent reports of orally bioavailable p300 inhibitors with nanomolar inhibitory activity have shown promising advances in the field of KATi therapeutics [187,188].

3.4 KAT bromodomain inhibitors

Targeting conserved bromodomains has been more effective than targeting the catalytic activity of KATs in the development of anticancer therapeutics. Many KAT proteins possess acetylated lysine recognition domains. CBP/p300 bromodomain inhibitor (Brdi) has witnessed great success as anti-tumor drugs. The potent CBP/p300 Brdi, CBP30 exhibits selectivity towards p300 ($K_D = 32$ nM) and CBP ($K_D = 26$ nM) over BRD4 ($K_d = 885$ nM) [189]. CBP/p300 Brdi I-CBP112 ($K_d = 151 \pm 6$ nmol/L for CBP and $K_D = 167 \pm 8$ nmol/L for p300) inhibited leukemia-initiating cells synergistically in combination with doxorubicin [190]. The potent CBP/p300 Brdi CCS1477 binds CBP ($K_d = 1.7$ nM) and p300 ($K_d = 1.3$ nM) with high affinity and selectivity (BRD4 $K_d = 222$ nM). CCS1477 inhibited AML and multiple myeloma (MM) cell lines, and oral doses of the drug in AML and MM xenograft models led to tumor regression [191]. CCS1477 exhibited synergistic effects with JQ1 in prostate cancer cells [192]. Triazolophthalazine-based L-45 is a potent inhibitor of PCAF bromodomain. L-45 is nontoxic and has shown good cell permeability and metabolic stability [193]. 3-(2-Nitrophenoxyl) propan-1-amine derivatives are potent PCAF Brdi with strong inhibitory activity against HIV replication [194].

3.5 Inhibitors of KATs and effector protein interactions

Disruption of the interaction between KATs and oncogenic effectors has proved to be an effective modality to treat cancers. Since KATs are integral to cancer signaling networks, specific interaction inhibitors may prove to be invaluable as anticancer therapeutics.

Survival under hypoxic conditions is a hallmark of solid tumors. This specialized adaptation of tumor cells is mediated through the activation of the hypoxia-induced transcription factor, HIF1 α , which regulates hypoxic-gene expression programs leading to increased angiogenesis, altered metabolism, and enhanced tumorigenesis. p300 is a coactivator for HIF1 α -target genes and the interaction between HIF1 α -p300 is a potential target for anticancer therapeutics. Novobiocin, an aminocoumarin antibiotic, can directly block the interaction between HIF1 α C-terminal activation domain (CTAD) and the p300/CBP cysteine-histidine rich (CH1) domain, thereby inhibiting hypoxic signaling in tumors. Novobiocin demonstrated inhibitory effects on breast cancer cells MCF7 proliferation and colony formation [195]. Natural product high throughput screen identified indandione and benzoquinone derivatives as inhibitors of HIF1 α and CH1 domain of p300. Ninhedrin, the active species of indandione, and naphthoquinones induced the ejection of Zn(II) from p300 and KDM4 catalytic domain [196]. Natural compounds, such as the marine alkaloid eudistidine A ($IC_{50} = 75$ μ M), and epidithiodiketopiperazine (ETP) family molecules can disrupt HIF-1 α -p300 protein-protein interaction [197,198]. ETPs have been shown to inhibit angiogenesis and tumor

growth in prostate xenograft mouse model [198]. Dimeric ETP (ETP2) can selectively disrupt HIF-1 α -p300 interaction and suppress hypoxic gene expression programs. ETP2 was shown to be efficacious against hypoxia-induced resistance and tumor growth in breast cancer model [199].

The Wnt/ β -catenin signaling cascade regulates cell fate decisions associated with pluripotency, self-renewal, and differentiation. Dysregulation of the Wnt/ β -catenin pathway promotes dedifferentiation, cancer stem cell self-renewal, and malignancy [200]. Wnt/ β -catenin associates with either CBP or p300 to regulate distinct subsets of genes. The small-molecule antagonist, ICG-001, selectively binds to CBP thereby disrupting the β -catenin/CBP without affecting the p300/ β -catenin signaling cascade. ICG-001 was observed to induce apoptosis and inhibit growth of colon cancer cells but not in normal colon cells and was efficacious in colon cancer xenograft mouse models [201]. ICG-001 can sensitize CSC to radiation and combinatorial therapies in colon cancer [202], colorectal cancer [203], HCC [204], gastric cancer [205], pancreatic ductal adenocarcinoma [206], ovarian carcinoma [207], leukemia [208], and melanoma [209]. The p300/ β -catenin signaling pathway promotes differentiation. p300/ β -catenin antagonists, IQ-1 [210] and YH249/250 [211] can specifically disrupt the interaction between p300 and β -catenin. Inhibition of p300/ β -catenin pathway in ESCs by YH249/250 results in the maintenance of pluripotency [211]. The inhibition of the p300/ β -catenin axis by IQ-1 prevents differentiation in lung progenitor cells alveolar epithelial type II (AT2) cells, tracheal epithelial cells, and C2C12 myoblasts [210].

The transcription factor, c-myb regulates the gene expression networks related to hematopoietic lineage specification, cell proliferation, and differentiation. Dereulation of c-myb has been implicated in the development of leukemia. c-Myb-mediated transcription programs are dependent on its association with p300; therefore the p300/c-myb interaction is a viable target for c-myb inhibitors. The small molecule, Naphthol AS-E phosphate can specifically bind to the KIX domain of p300 and CBP with IC₅₀ values of 30 and 43 μ M, respectively, thereby inhibiting the interaction between the coactivators and c-myb. Naphthol AS-E phosphate was shown to induce apoptosis in leukemic cells [212].

Autoacetylation of KATs is another layer of complexity in the fine-tuning of KAT catalytic function. In the case of KAT3 proteins, several protein and nonprotein modulators of autoacetylation are known, both negative and positive, that tweak KAT3 acetyltransferase activity depending on environmental cues. The multimeric protein NPM1, a known histone chaperone and transcription coactivator, acts as a modulator of p300 autoacetylation and catalytic function through its molecular chaperone activity. Interestingly, disruption of NPM1 oligomers using the small molecule inhibitor NSC348884 [213] can prevent the induction of NPM1-mediated intermolecular autoacetylation of p300 [123]. The ability to prevent NPM1-driven autoacetylation and hyperacetylation may be a viable target in cancers, such as OSCC [18]. The tumor suppressor p53 is another potent inducer of p300 autoacetylation and intriguingly oncogenic mutant p53 protein retain the ability to enhance the catalytic activity of p300, possibly hijacking the process to induce tumorigenic transformation. Inhibiting the interaction between mutant p53 and p300 using phosphomimic N-terminal p53 peptides has been shown to be an effective mode of inhibiting cancer cell migration in cell lines expressing GOF R273H p53 mutant protein [124].

3.6 Targeted degradation of KATs: PROTACs

Another effective strategy of inhibiting KAT-mediated aberrant acetylation is through the targeted degradation of KATs using PROTACs (proteolysis-targeting chimeras) [214,215]. CBP/p300 are

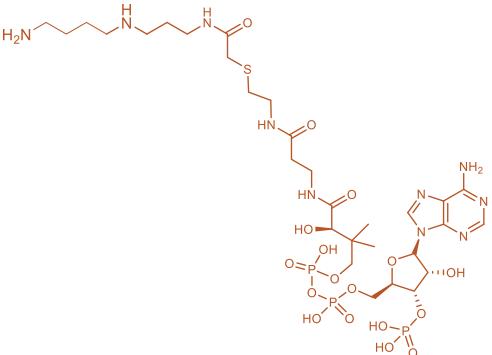
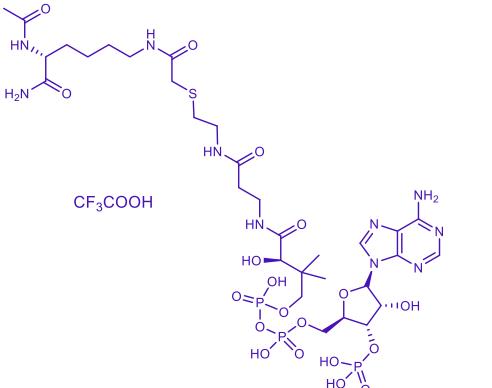
multidomain transcription coactivators and are known to interact with over 400 proteins [216]. CBP/p300 are integral members of the enhanceosome complex, which facilitates cooperative activation of target genes [217]. Complete depletion of CBP/p300 is achieved by the chemical degrader dCBP-1 which engages the CBP/p300 bromodomain to the E3 ligase cereblon. dCBP-1-mediated degradation of CBP/p300 results in abolishment of enhancer-driven expression of myc oncogene and causes cell death in multiple myeloma cells [218].

4 Conclusion

Lysine acetylation is one of the key posttranslational modifications in the maintenance of cellular equilibrium. Deregulation in the fine-tuning of acetylation-driven cellular programs often lead to the onset and manifestation of cancer. The involvement of lysine acetylation in malignant transformation has led to the development of therapeutics targeting the modification. Aberrant activity of KATs has been associated with a wide range of diseases, in particular cancer. KDACi have exhibited efficacy as potent anticancer therapeutics. Vorinostat, belinostat, romidepsin, and panobinostat are FDA-approved drugs in use against hematological cancers. Currently KDACis are being extensively tested in clinical trials against solid tumors. The lysine acetylation readers are also being tested as potential antineoplastic therapeutics, especially molecules targeting pan-bromodomains and BET domains. Recent clinical trial of BETi molibresib (GSK525762) has shown partial response in nuclear protein in testis (NUT) carcinoma and other solid tumor patients [219,220]. However, cancer cells can be sensitized to BETi resistance by the combinatorial inhibition of CBP/p300 [190,192,221].

Development of KATi has been plagued by the lack of specificity, clinically relevant potency, and bioavailability. However, KATi scaffolds have served as potential lead molecules for the derivatization of more efficacious inhibitors. Nonspecific KATi, curcumin and ECGC have been tested against a wide range of diseases in clinical trials, however their efficacy cannot be attributed to KAT inhibition alone due to their pleiotropic effects. Currently there are no KAT catalytic inhibitors in clinical trials; however, molecules targeting conserved domains, such as the NRID and bromodomain of KAT3 proteins, have shown immense potential for clinical use. The small molecule antagonist of CBP/β-catenin interaction, PRI-724 (ICG-001 isomer) has been tested in phase I/II clinical trials against advanced solid tumors (NCT01302405), acute and chronic myeloid leukemia (NCT01606579), and advanced and metastatic adenocarcinoma in combination with the chemotherapeutic agent gemcitabine (NCT01764477). The CBP/p300 bromodomain inhibitor CCS1477 is presently in clinical trials against hematological malignancies (NCT04068597), and metastatic castration resistant prostate cancer (mCRPC) and advanced solid tumors (NCT03568656). FT-7051, an oral, potent, p300/CBP bromodomain selective inhibitor, that has exhibited promising preclinical potency against enzalutamide-resistant prostate cancer models [222], is currently being examined in Phase I trials against mCRPC (NCT04575766) (<https://clinicaltrials.gov/>). With the advancement of inhibitor screening technology with the integration of AI, it is hoped that more potent, selective KAT inhibitors can be developed as therapeutics (Tables 18.1 and 18.2).

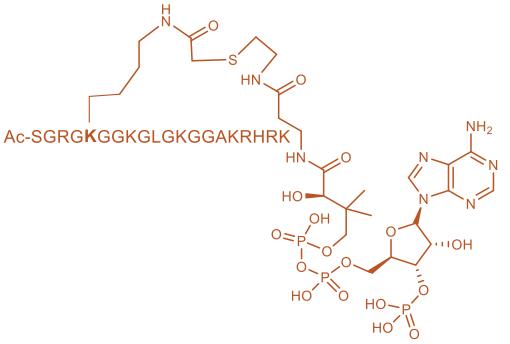
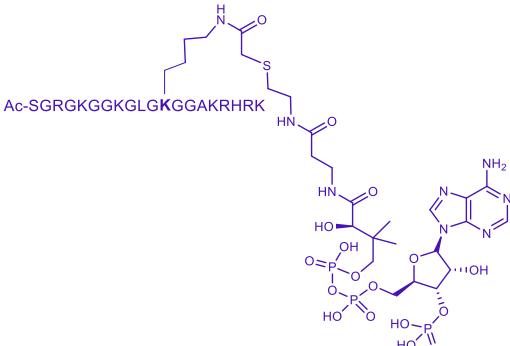
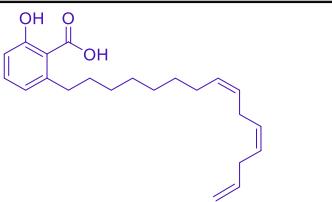
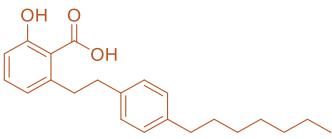
Table 18.1 KAT catalytic activity inhibitors.

Name	Structure	Target enzyme	IC ₅₀	Cellular effect	References
Bisubstrate inhibitors					
Spd-CoA		—	Ki < 10 nM	—	[128]
Lys-CoA		p300	0.5 μM	—	[130]

(Continued)

Table 18.1 KAT catalytic activity inhibitors. *Continued*

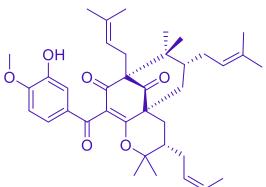
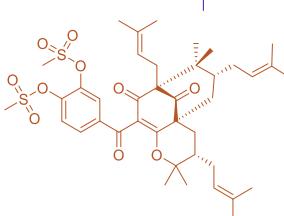
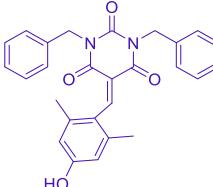
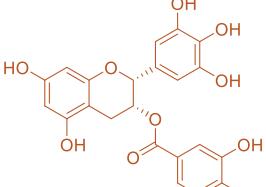
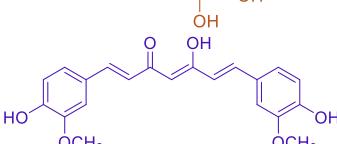
Name	Structure	Target enzyme	IC ₅₀	Cellular effect	References
H3-CoA-20		PCAF	0.5 μM	—	[130]
H4K16CoA		Tip60 Esa1	17.59 μM (Tip60) 5.51 mM (Esa1)	—	[131]

H4K5CoA		HAT1	0.083 μM	—	[135]
H4K12CoA		HAT1	0.023 μM	—	[135]
Natural compound inhibitors					
Anacardic acid		p300 PCAF	8.5 μM (p300) 5 μM (PCAF)	Sensitize tumors to radiotherapy, antiproliferative, anti-inflammatory	[136,137]
MG149		Tip60 MOF	74 μM (Tip60) 47 μM (MOF)	Anti-inflammatory, induces apoptosis in malignant pleural mesothelioma	[139,223,224]

(Continued)

Table 18.1 KAT catalytic activity inhibitors. *Continued*

Name	Structure	Target enzyme	IC ₅₀	Cellular effect	References
Benzamide derivative of CTPB		p300	50 μM	Induces apoptosis in leukemia cells	[140]
Garcinol		p300 PCAF	7 μM (p300) 5 μM (PCAF)	Antiproliferative, anti-inflammatory, antitumor	[142]
Isogarcinol		p300	7 μM	Induces apoptosis in human promyelocytic leukemia	[145,225]
LTK-13		p300	5–7 μM	Nontoxic, reduces histone acetylation	[145]

LTK-14		p300	5–7 μM	Nontoxic, inhibits HIV multiplication, reduces histone and p53 acetylation.	[145]
LTK-19		p300	5–7 μM	Nontoxic, reduces histone acetylation	[145]
EML425		p300	2.9 μM	G0/G1 cell cycle arrest, reduces H3K9 and H4K5 acetylation	[140]
Epigallocatechin-3-gallate		p300, CBP, PCAF, Tip60	30 μM/L (p300), 50 μM/L (CBP), 60 μM/L (PCAF), 70 μM/L (Tip60)	Inhibits EBV-induced B lymphocyte transformation	[147]
Curcumin		p300/ CBP	25 μM	Antioxidant, anti-inflammatory, antiproliferative, and antitumor properties	[148]

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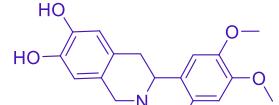
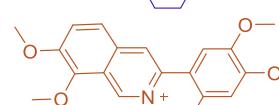
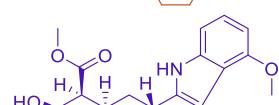
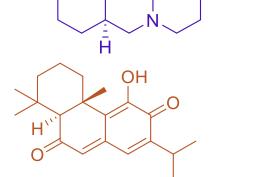
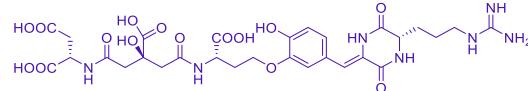
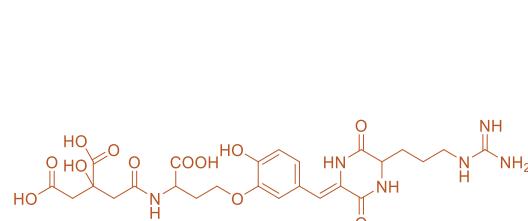
Table 18.1 KAT catalytic activity inhibitors. *Continued*

Name	Structure	Target enzyme	IC ₅₀	Cellular effect	References
Hydrazinobenzoyl curcumin		p300	80 μM	Antitumor activity in castration-resistant prostate cancer xenograft model	[149]
CTK7A		p300, PCAF	100 μM	Inhibits oral tumor cell growth in nude mice	[18]
Delphinidin		p300/CBP	100 μM	Anti-inflammatory	[151]
Gallic acid		p300, CBP, PCAF, Tip60	14 μM/L (p300), 24 μM/L (CBP), 34 μM/L (PCAF), 25 μM/L Tip60	Inhibits inflammatory cytokine production in human cancer cells	[152]
Procyanidin B3		p300		Suppresses prostate cancer cell proliferation	[153]

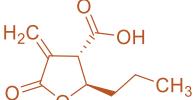
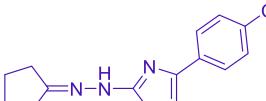
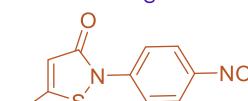
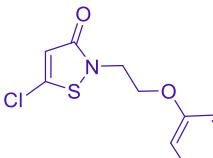
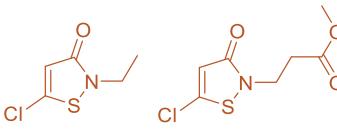
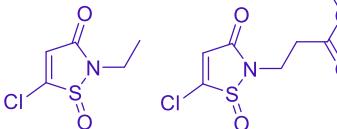
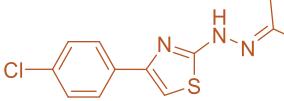
Quercetin		p300	200 μM	Antitumor and anti-inflammatory properties	[154]
Carnosol		p300	50 μM	Suppresses the proliferation, migration, invasion, and promotes autophagy in highly invasive breast cancer cells	[155]
Plumbagin		p300	20–25 μM	Proapoptotic, antiangiogenic and antimetastatic	[158]
PTK1		p300	100 μM	Nontoxic, reduced histone acetylation in cells	[160]
Embelin		PCAF	20 μM	Inhibits PCAF-mediated muscle differentiation and tumorigenesis in alveolar rhabdomyosarcoma xenograft model	[73,161]
Sanguinarine		p300	5–20 μM	Antitumor and anti-inflammatory	[165]

(Continued)

Table 18.1 KAT catalytic activity inhibitors. *Continued*

Name	Structure	Target enzyme	IC ₅₀	Cellular effect	References
NP-2		p300, PCAF	0.69 μM (p300) 14.13 μM (PCAF)	–	[166]
NP-3		p300, PCAF	1.05 μM (p300) 10.0 μM (PCAF)	–	[166]
NP-9		p300, PCAF	0.58 μM (p300) 27.1 μM (PCAF)	–	[166]
NP-15		p300, PCAF	4.85 μM (p300) 7.16 μM (PCAF)	–	[166]
NK13650A		p300	11 nM	Inhibitory activity against AR-dependent transcriptional activation, abrogation of prostate cancer cell proliferation	[167]
NK13650B		p300	22 nM	Inhibitory activity against AR-dependent transcriptional activation	[167]

Synthetic inhibitors

MB-3		Gcn5	100 μM	–	[168]
CPTH2		Gcn5p, p300	0.8 mM (Gcn5)	Inhibition of clear cell renal carcinoma	[169]
CCT077791		PCAF	7.3 μM	Decrease global acetylation in HCT116 and HT28 colon carcinoma cell lines	[172]
CCT077792		PCAF	15.0 μM	Decrease global acetylation in HCT116 and HT28 colon carcinoma cell lines	[172]
5-Chloroisothiazolones		PCAF	2–3 μM	Inhibits proliferation of HepG2 cells	[173]
5-Chloroisothiazolone-1-oxides		PCAF	10 μM, 5.6 μM	Inhibits proliferation of HepG2 cells	[173]
BF1		p300	0.6 mM	Global reduction in histone H3 acetylation	[176]

(Continued)

Table 18.1 KAT catalytic activity inhibitors. *Continued*

Name	Structure	Target enzyme	IC ₅₀	Cellular effect	References
C646		p300	Ki = 400 nM	Sensitizes NSCLC cells to ionizing radiation, inhibit tumor growth in pancreatic xenograft mouse model	[180–182]
NU9056		Tip60	2 μM	Inhibits growth of prostate cancer cells	[177]
TH1834		Tip60	500 μM	Inhibits Tip60-mediated breast cancer progression	[178]
A-485		p300	0.060 μM	Antiproliferative properties against 10 different types of solid tumors and hematological cancers and was efficacious against castration resistant prostate cancer xenograft model	[185]

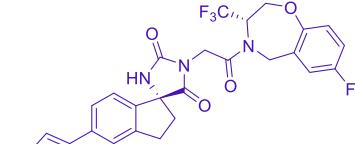
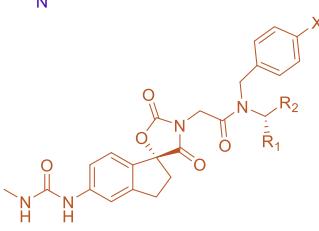
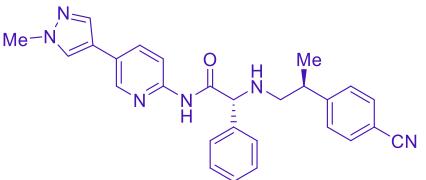
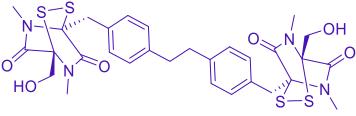
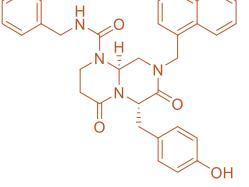
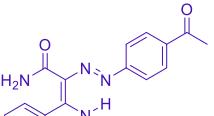
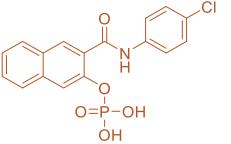
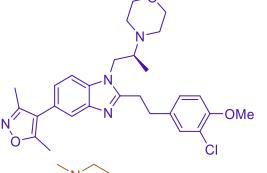
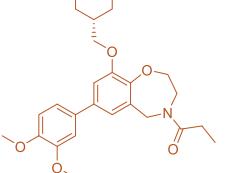
B026		p300	1.8 nM	Cancer cell growth inhibition in the nanomolar range, effective tumor growth inhibition	[186]
Spirooxazolidinediones	 Compd R ₁ R ₂ X 22 Me cyclopropyl H 23 CF ₃ cyclopropyl F 24 Me CF ₃ F	p300	22 (p300 0.025 μM), 23 (p300 0.032 μM), 24 (p300 0.060 μM)	Improved oral bioavailability and pharmacokinetics	[187]
CPI-1612		p300/CBP	< 0.5 nM (p300) 2.9 nM (CBP)	Orally bioavailable, biologically relevant efficacious dose in JEKO-1 mantle cell lymphoma mouse xenograft	[188]

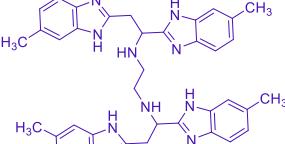
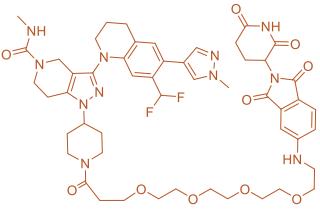
Table 18.2 KAT interaction inhibitors and KAT3 PROTAC.

Name	Structure	Target	IC ₅₀	Cellular effect	References
Novobiocin		p HIF-1α-p300/CBP interaction	100–400 μM	Disrupts the interaction between HPC4-HIF1α and endogenous p300	[195]
Indandione		p HIF-1α-p300/CBP interaction	4 (53.9 μM) 5 (7.0 μM) 6 (15.7 μM)	—	[196]
Eudistidine A		HIF-1α-p300 interaction	75 μM	—	[197]
Epidithiodiketopiperazine (ETPs)		HIF-1α-p300 interaction	Gliotoxin (250 nM), Chaetocin (25 nm), and chetomin (25 nM)	ETPs suppresses tumor angiogenesis and prostate cancer progression	[198]

ETP2		HIF1α-p300/CBP	0.6 μM	Suppresses hypoxic gene expression programs	[199]
ICG-001		CBP/β-catenin interaction	100 μM	Sensitize cancer stem-like cells to radiation and combinatorial therapies in colon cancer, colorectal cancer, hepatocellular carcinoma, gastric cancer, pancreatic ductal adenocarcinoma, ovarian carcinoma, leukemia 108, and melanoma	[201–206,209]
IQ-1		p300/β-catenin interaction	5 μM	Prevents differentiation of primary alveolar epithelial type II (AT2) cells, tracheal epithelial cells, and C2C12 myoblasts	[210]
Naphthal AS-E phosphate		p300/CBP KIX domain	30 μM (p300) 43 μM (CBP)	Induces apoptosis in leukemic cells	[212]
CBP30		CBP/p300 Brdi	2 μM	Selective inhibition of the CBP/p300 bromodomain with CBP30 strongly reduces secretion of IL-17A	[189]
I-CBP112		CBP/p300 Brdi	600 ± 50 nmol	Increases the cytotoxic activity of BET bromodomain inhibitor JQ1 as well as doxorubicin	[190]

(Continued)

Table 18.2 KAT interaction inhibitors and KAT3 PROTAC. *Continued*

Name	Structure	Target	IC ₅₀	Cellular effect	References
NSC348884		NPM1/ p300 interaction	5 μM	Prevents p300 NPM1 interaction and reduces p300 autoacetylation	[123,213]
dCBP-1		p300/ CBP- specific PROTAC	—	Abolishes enhancer-driven expression of myc oncogene and causes cell death in multiple myeloma cells	[218]

Abbreviations

AA	anacardic acid
ACACA	acetyl-CoA carboxylase alpha
ACLY	ATP citrate lyase
ACSS	acetyl-CoA synthetase
AI	artificial intelligence
AML	acute myeloid leukemia
AMPK	5' AMP-activated protein kinase
APC	anaphase-promoting complex
AR	androgen receptor
ARMS	alveolar rhabdomyosarcoma
BETi	bromo- and extra-terminal domain inhibitor
BRCA1	breast cancer type 1 susceptibility protein
Brd	bromodomain
CARM1	coactivator associated arginine methyltransferase 1
CBP	CREB-binding protein
ccRCC	clear cell renal cell carcinoma
CDK1	cyclin dependent kinase1
CH1	cysteine-histidine rich
CPTH2	cyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl]hydrazone
CSC	cancer stem-like cells
CTAD	C-terminal activation domain
CTPB	N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide
DP	delphinidin
EDD1	E3 identified by differential display
EGCG	epigallocatechin gallate
ERCC1	Excision Repair Cross-Complementation Group 1
ERK1/2	extracellular signal-regulated protein kinase
ESCC	esophageal squamous cell carcinoma
ETP	epidithiodiketopiperazine
EZH2	enhancer of zeste homolog 2
FASN	fatty acid synthase
FOXO-1	Forkhead Box O1
GA	gallic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCN5	general control nondepressible 5
GNAT	Gcn5-related N-acetyltransferase
HBO1	HAT bound to ORC1
HCC	hepatocellular carcinoma
Hdm2	double minute 2
HIF-1α	hypoxia-inducible factor 1-alpha
HIV	human immunodeficiency virus
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
IG	isogarcinol
Ing	inhibitor of growth
KAT	lysine acetyltransferase

KAT1	lysine acetyltransferase inhibitor
KDACi	lysine deacetylase inhibitor
LOH	loss of heterozygosity
LPS	lipopolysaccharide
MAML1	mastermind like transcriptional coactivator 1
MB-3	α -methylene- γ -butyrolactone
mCRPC	metastatic castration resistant prostate cancer
MDR-1	multidrug resistant protein-1
MLL	mixed-lineage leukemia
MM	multiple myeloma
MOF	males-absent on the first
MORF	MOZ-related factor
MOZ	monocytic leukemia zinc finger protein
MSL	male specific lethal
MYST	MOZ, Ybf2, Sas2, TIP60
NF-κB	nuclear factor kappa B
NPM1	nucleophosmin 1
NSCLC	nonsmall-cell lung carcinoma
NSL	nonspecific lethal
NUT	nuclear protein in testis
ORC1	Origin Recognition Complex Subunit 1
OSCC	oral squamous cell carcinoma
OTSCC	oral tongue squamous cell carcinoma
p300	adenoviral E1A-binding protein of 300 kDa
PCAF	P300/CBP-associated factor
Plk1	Polo-like kinase 1
PROTAC	proteolysis-targeting chimeras
PTM	posttranslational modification
SMAD	Suppressor of mothers against decapentaplegic
STAGA	SPT3-TAF9-GCN5 acetyltransferase
TFTC	Tata binding protein (TBP)-free TAF complex
TIF2	transcription intermediary factor2
Tip60	tat interactive protein 60 kDa
Yb-1	Y-box binding protein

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References

- [1] Kaypee S, Sudarshan D, Shanmugam MK, Mukherjee D, Sethi G, Kundu TK. Aberrant lysine acetylation in tumorigenesis: Implications in the development of therapeutics. Pharmacol Ther 2016;162:98–119. Available from: <https://doi.org/10.1016/J.PHARMATHERA.2016.01.011>.

- [2] Goudarzi A, et al. Dynamic competing histone H4 K5K8 acetylation and butyrylation are hallmarks of highly active gene promoters. *Mol Cell* 2016;62(2):169–80. Available from: <https://doi.org/10.1016/J.MOLCEL.2016.03.014>.
- [3] Dai S-K, et al. Histone crotonylation regulates neural stem cell fate decisions by activating bivalent promoters. *EMBO Rep* 2021;22(10):e52023. Available from: <https://doi.org/10.1525/EMBR.202052023>.
- [4] Lagerwaard B, et al. Propionate hampers differentiation and modifies histone propionylation and acetylation in skeletal muscle cells. *Mech Ageing Dev* 2021;196. Available from: <https://doi.org/10.1016/J.MAD.2021.111495>.
- [5] Bhattacharya A, et al. EP300 (p300) mediated histone butyrylation is critical for adipogenesis. *bioRxiv* 2022;. Available from: <https://doi.org/10.1101/2021.08.01.454641> 2021.08.01.454641.
- [6] Fang Y, et al. Histone crotonylation promotes mesoendodermal commitment of human embryonic stem cells. *Cell Stem Cell* 2021;28(4):748–763.e7. Available from: <https://doi.org/10.1016/J.STEM.2020.12.009>.
- [7] Kaypee S, Mandal S, Chatterjee S, Kundu TK. Emerging Epigenetic Therapies: Lysine Acetyltransferase Inhibitors. *Epigenetic Cancer Ther* 2015;471–94. Available from: <https://doi.org/10.1016/B978-0-12-800206-3.00021-5>.
- [8] Seligson DB, et al. Global levels of histone modifications predict prognosis in different cancers. *Am J Pathol* 2009;174(5):1619–28. Available from: <https://doi.org/10.2353/AJPATH.2009.080874>.
- [9] di Cerbo V, Schneider R. Cancers with wrong HATs: the impact of acetylation. *Brief Funct Genomics* 2013;12(3):231–43. Available from: <https://doi.org/10.1093/BFGP/ELS065>.
- [10] Miyoshi I, et al. BRD4 bromodomain gene rearrangement in aggressive carcinoma with translocation t(15;19). *Am J Pathol* 2001;159(6):1987–92. Available from: [https://doi.org/10.1016/S0002-9440\(10\)63049-0](https://doi.org/10.1016/S0002-9440(10)63049-0).
- [11] Fraga MF, et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet* 2005;37(4):391–400. Available from: <https://doi.org/10.1038/NG1531>.
- [12] Elsheikh SE, et al. Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome. *Cancer Res* May 2009;69(9):3802–9. Available from: <https://doi.org/10.1158/0008-5472.CAN-08-3907>.
- [13] Tzao C, et al. Prognostic significance of global histone modifications in resected squamous cell carcinoma of the esophagus. *Mod Pathol* 2009;22(2):252–60. Available from: <https://doi.org/10.1038/MODPATHOL.2008.172>.
- [14] Liu BL, et al. Global histone modification patterns as prognostic markers to classify glioma patients. *Cancer Epidemiol Biomarkers Prev* 2010;19(11):2888–96. Available from: <https://doi.org/10.1158/1055-9965.EPI-10-0454>.
- [15] Barlési F, et al. Global histone modifications predict prognosis of resected non small-cell lung cancer. *J Clin Oncol* 2007;25(28):4358–64. Available from: <https://doi.org/10.1200/JCO.2007.11.2599>.
- [16] Chen YW, Kao SY, Wang HJ, Yang MH. Histone modification patterns correlate with patient outcome in oral squamous cell carcinoma. *Cancer* 2013;119(24):4259–67. Available from: <https://doi.org/10.1002/CNCR.28356>.
- [17] Webber LP, et al. Hypoacetylation of acetyl-histone H3 (H3K9ac) as marker of poor prognosis in oral cancer. *Histopathology* 2017;71(2):278–86. Available from: <https://doi.org/10.1111/HIS.13218>.
- [18] Arif M, et al. Nitric oxide-mediated histone hyperacetylation in oral cancer: target for a water-soluble HAT inhibitor, CTK7A. *Chem Biol* 2010;17(8):903–13. Available from: <https://doi.org/10.1016/J.CHEMBIOL.2010.06.014>.
- [19] Das C, Lucia MS, Hansen KC, Tyler JK. CBP/p300-mediated acetylation of histone H3 on lysine 56. *Nature* May 2009;459(7243):113–17. Available from: <https://doi.org/10.1038/NATURE07861>.
- [20] Schwartzentruber J, et al. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature* 2012;482(7384):226–31. Available from: <https://doi.org/10.1038/nature10833>.

- [21] Behjati S, et al. Distinct H3F3A and H3F3B driver mutations define chondroblastoma and giant cell tumor of bone. *Nat Genet* 2013;45(12):1479–82. Available from: <https://doi.org/10.1038/ng.2814>.
- [22] Lohr JG, et al. Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. *Proc Natl Acad Sci U S A* 2012;109(10):3879–84. Available from: https://doi.org/10.1073/PNAS.1121343109/SUPPL_FILE/SD05.XLS.
- [23] Papillon-Cavanagh S, et al. Impaired H3K36 methylation defines a subset of head and neck squamous cell carcinomas. *Nat Genet* 2017;49(2):180–5. Available from: <https://doi.org/10.1038/ng.3757>.
- [24] Zhao S, et al. Mutational landscape of uterine and ovarian carcinosarcomas implicates histone genes in epithelial-mesenchymal transition. *Proc Natl Acad Sci U S A* 2016;113(43):12238–43. Available from: https://doi.org/10.1073/PNAS.1614120113/SUPPL_FILE/PNAS.1614120113.SAPP.PDF.
- [25] Nacev BA, et al. The expanding landscape of ‘oncohistone’ mutations in human cancers. *Nature* 2019;567(7749):473–8. Available from: <https://doi.org/10.1038/s41586-019-1038-1>.
- [26] Lin R, et al. Acetylation stabilizes ATP-citrate lyase to promote lipid biosynthesis and tumor growth. *Mol Cell* 2013;51(4):506–18. Available from: <https://doi.org/10.1016/J.MOLCEL.2013.07.002>.
- [27] Gao X, et al. Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia. *Nat Commun* 2016;7. Available from: <https://doi.org/10.1038/NCOMMS11960>.
- [28] Comerford SA, et al. Acetate dependence of tumors. *Cell* 2014;159(7):1591–602. Available from: <https://doi.org/10.1016/J.CELL.2014.11.020>.
- [29] Kamphorst JJ, Chung MK, Fan J, Rabinowitz JD. Quantitative analysis of acetyl-CoA production in hypoxic cancer cells reveals substantial contribution from acetate. *Cancer Metab* 2014;2(1). Available from: <https://doi.org/10.1186/2049-3002-2-23>.
- [30] Potapova IA, El-Maghrabi MR, Doronin Sv, Benjamin WB. Phosphorylation of recombinant human ATP:citrate lyase by cAMP-dependent protein kinase abolishes homotropic allosteric regulation of the enzyme by citrate and increases the enzyme activity. Allosteric activation of ATP:citrate lyase by phosphorylated sugars. *Bio* 2000;39(5):1169–79. Available from: <https://doi.org/10.1021/BI992159Y>.
- [31] Berwick DC, Hers I, Heesom KJ, Kelly Moule S, Tavaré JM. The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. *J Biol Chem* 2002;277(37):33895–900. Available from: <https://doi.org/10.1074/JBC.M204681200>.
- [32] Carrer A, et al. Acetyl-CoA Metabolism Supports Multistep Pancreatic Tumorigenesis. *Cancer Discov* 2019;9(3):416–35. Available from: <https://doi.org/10.1158/2159-8290.CD-18-0567>.
- [33] Li X, et al. Nucleus-translocated ACSS2 promotes gene transcription for lysosomal biogenesis and autophagy. *Mol Cell* 2017;66(5):684–697.e9. Available from: <https://doi.org/10.1016/J.MOLCEL.2017.04.026>.
- [34] Marmorstein R, Zhou MM. Writers and readers of histone acetylation: structure, mechanism, and inhibition. *Cold Spring Harb Perspect Biol* 2014;6(7). Available from: <https://doi.org/10.1101/CSHPERSPECT.A018762>.
- [35] Ogryzko Vv, Schiltz RL, Russianova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 1996;87(5):953–9. Available from: [https://doi.org/10.1016/S0092-8674\(00\)82001-2](https://doi.org/10.1016/S0092-8674(00)82001-2).
- [36] Kalkhoven E. CBP and p300: HATs for different occasions. *Biochem Pharmacol* 2004;68(6):1145–55. Available from: <https://doi.org/10.1016/J.BCP.2004.03.045>.
- [37] Roelfsema JH, et al. Genetic heterogeneity in Rubinstein-Taybi syndrome: mutations in both the CBP and EP300 genes cause disease. *Am J Hum Genet* 2005;76(4):572–80. Available from: <https://doi.org/10.1086/429130>.
- [38] Iyer NG, Özdag H, Caldas C. p300/CBP and cancer. *Oncogene* May 2004;23(24):4225–31. Available from: <https://doi.org/10.1038/SJ.ONC.1207118>.
- [39] Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. *Genes Dev* 2000;14(13):1553–77. Available from: <https://doi.org/10.1101/GAD.14.13.1553>.
- [40] Krivtsov Av, Armstrong SA. MLL translocations, histone modifications and leukaemia stem-cell development. *Nat Rev Cancer* 2007;7(11):823–33. Available from: <https://doi.org/10.1038/NRC2253>.

- [41] Bannister AJ, Kouzarides T. CBP-induced stimulation of c-Fos activity is abrogated by E1A. *EMBO J* 1995;14(19):4758–62. Available from: <https://doi.org/10.1002/J.1460-2075.1995.TB00157.X>.
- [42] Dai P, et al. CBP as a transcriptional coactivator of c-Myb. *Genes Dev* 1996;10(5):528–40. Available from: <https://doi.org/10.1101/GAD.10.5.528>.
- [43] Bannister AJ, Oehler T, Wilhelm D, Angel P, Kouzarides T. Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro. *Oncogene* 1995;11(12):2509–14. Available from: <https://europepmc.org/article/med/8545107> [accessed 22.07. 22].
- [44] Vervoorts J, et al. Stimulation of c-MYC transcriptional activity and acetylation by recruitment of the cofactor CBP. *EMBO Rep* May 2003;4(5):484–90. Available from: <https://doi.org/10.1038/SJ.EMBOR.EMBOR821>.
- [45] Wang J, et al. EZH2 noncanonically binds cMyc and p300 through a cryptic transactivation domain to mediate gene activation and promote oncogenesis. *Nat Cell Biol* 2022;24(3):384–99. Available from: <https://doi.org/10.1038/S41556-022-00850-X>.
- [46] Durbin AD, et al. EP300 selectively controls the enhancer landscape of MYCN-amplified neuroblastoma. *Cancer Discov* 2022;12(3):730–51. Available from: <https://doi.org/10.1158/2159-8290.CD-21-0385>.
- [47] Martire S, et al. Phosphorylation of histone H3.3 at serine 31 promotes p300 activity and enhancer acetylation. *Nat Genet* 2019;51(6):941–6. Available from: <https://doi.org/10.1038/S41588-019-0428-5>.
- [48] Kumar M, et al. Inhibition of histone acetyltransferase function radiosensitizes CREBBP/EP300 mutants via repression of homologous recombination, potentially targeting a gain of function. *Nat Commun* 2021;12(1). Available from: <https://doi.org/10.1038/S41467-021-26570-8>.
- [49] Yokomizo C, et al. High expression of p300 in HCC predicts shortened overall survival in association with enhanced epithelial mesenchymal transition of HCC cells. *Cancer Lett* 2011;310(2):140–7. Available from: <https://doi.org/10.1016/J.CANLET.2011.06.030>.
- [50] Hou X, et al. High expression of the transcriptional co-activator p300 predicts poor survival in resectable non-small cell lung cancers. *Eur J Surg Oncol* 2012;38(6):523–30. Available from: <https://doi.org/10.1016/J.EJSO.2012.02.180>.
- [51] Xiao XS, et al. High expression of p300 in human breast cancer correlates with tumor recurrence and predicts adverse prognosis. *Chin J Cancer Res* 2011;23(3):201–7. Available from: <https://doi.org/10.1007/S11670-011-0201-5>.
- [52] Debes JD, Sebo TJ, Lohse CM, Murphy LM, Anna Haugen DL, Tindall DJ. Advances in brief p300 in prostate cancer proliferation and progression. *CANCER Res* 2003;63:7638–40. Available from: <http://aacrjournals.org/cancerres/article-pdf/63/22/7638/2509284/zch02203007638.pdf> [accessed 22.07. 22].
- [53] Santer FR, et al. Inhibition of the acetyltransferases p300 and CBP reveals a targetable function for p300 in the survival and invasion pathways of prostate cancer cell lines. *Mol Cancer Ther* 2011;10(9):1644–55. Available from: <https://doi.org/10.1158/1535-7163.MCT-11-0182>.
- [54] Yoshida T, et al. Transcriptional upregulation of HIF-1 α by NF- κ B/p65 and its associations with β -catenin/p300 complexes in endometrial carcinoma cells. *Lab Invest* 2013;93(11):1184–93. Available from: <https://doi.org/10.1038/LABINVEST.2013.111>.
- [55] Ianculescu I, Wu DY, Siegmund KD, Stallcup MR. Selective roles for cAMP response element-binding protein binding protein and p300 protein as coregulators for androgen-regulated gene expression in advanced prostate cancer cells. *J Biol Chem* 2012;287(6):4000–13. Available from: <https://doi.org/10.1074/JBC.M111.300194>.
- [56] Cai LY, et al. Targeting p300/CBP attenuates hepatocellular carcinoma progression through epigenetic regulation of metabolism. *Cancer Res* 2021;81(4):860–72. Available from: <https://doi.org/10.1158/0008-5472.CAN-20-1323>.
- [57] Wang SA, Hung CY, Chuang JY, Chang WC, Hsu TI, Hung JJ. Phosphorylation of p300 increases its protein degradation to enhance the lung cancer progression. *Biochim Biophys Acta* 2014;1843(6):1135–49. Available from: <https://doi.org/10.1016/J.BBAMCR.2014.02.001>.

- [58] Nagy Z, Tora L. Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. *Oncogene* 2007;26(37):5341–57. Available from: <https://doi.org/10.1038/SJ.ONC.1210604>.
- [59] Love IM, Sekaric P, Shi D, Grossman SR, Androphy EJ. The histone acetyltransferase PCAF regulates p21 transcription through stress-induced acetylation of histone H3. *Cell Cycle* 2012;11(13):2458–66. Available from: <https://doi.org/10.4161/CC.20864>.
- [60] Sakaguchi K, et al. DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev* 1998;12(18):2831–41. Available from: <https://doi.org/10.1101/GAD.12.18.2831>.
- [61] Linares LK, et al. Intrinsic ubiquitination activity of PCAF controls the stability of the oncoprotein Hdm2. *Nat Cell Biol* 2007;9(3):331–8. Available from: <https://doi.org/10.1038/NCB1545>.
- [62] Zhu C, et al. Characterization of tumor suppressive function of P300/CBP-associated factor at frequently deleted region 3p24 in esophageal squamous cell carcinoma. *Oncogene* 2009;28(31):2821–8. Available from: <https://doi.org/10.1038/ONC.2009.137>.
- [63] Zheng X, et al. Histone acetyltransferase PCAF up-regulated cell apoptosis in hepatocellular carcinoma via acetylating histone H4 and inactivating AKT signaling. *Mol Cancer* 2013;12(1). Available from: <https://doi.org/10.1186/1476-4598-12-96>.
- [64] Ying MZ, et al. The p300/CBP associated factor is frequently downregulated in intestinal-type gastric carcinoma and constitutes a biomarker for clinical outcome. *Cancer Biol Ther* 2010;9(4):312–20. Available from: <https://doi.org/10.4161/CBT.9.4.10748>.
- [65] Zhou R, et al. p300/CBP-associated factor promotes autophagic degradation of δ-catenin through acetylation and decreases prostate cancer tumorigenicity. *Sci Rep* 2019;9(1). Available from: <https://doi.org/10.1038/S41598-019-40238-W>.
- [66] Hirano G, et al. Enhanced expression of PCAF endows apoptosis resistance in cisplatin-resistant cells. *Mol Cancer Res* 2010;8(6):864–72. Available from: <https://doi.org/10.1158/1541-7786.MCR-09-0458>.
- [67] Shiota M, et al. P300/CBP-associated factor regulates Y-box binding protein-1 expression and promotes cancer cell growth, cancer invasion and drug resistance. *Cancer Sci* 2010;101(8):1797–806. Available from: <https://doi.org/10.1111/J.1349-7006.2010.01598.X>.
- [68] Toth M, Boros IM, Balint E. Elevated level of lysine 9-acetylated histone H3 at the MDR1 promoter in multidrug-resistant cells. *Cancer Sci* 2012;103(4):659–69. Available from: <https://doi.org/10.1111/J.1349-7006.2012.02215.X>.
- [69] Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B. Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes Dev* 2001;15(16):2069–82. Available from: <https://doi.org/10.1101/GAD.906601>.
- [70] Liu X, Tesfai J, Evrard YA, Dent SYR, Martinez E. c-Myc transformation domain recruits the human STAGA complex and requires TRRAP and GCN5 acetylase activity for transcription activation. *J Biol Chem* May 2003;278(22):20405–12. Available from: <https://doi.org/10.1074/JBC.M211795200>.
- [71] Chen L, et al. Lysine acetyltransferase GCN5 potentiates the growth of non-small cell lung cancer via promotion of E2F1, cyclin D1, and cyclin E1 expression. *J Biol Chem* May 2013;288(20):14510–21. Available from: <https://doi.org/10.1074/JBC.M113.458737>.
- [72] Cheng YW, et al. P300/CBP-associated factor (PCAF)-mediated acetylation of Fascin at lysine 471 inhibits its actin-bundling activity and tumor metastasis in esophageal cancer. *Cancer Commun (Lond)* 2021;41(12):1398–416. Available from: <https://doi.org/10.1002/CAC2.12221>.
- [73] Bharathy N, et al. P/CAF mediates PAX3-FOXO1-dependent oncogenesis in alveolar rhabdomyosarcoma. *J Pathol* 2016;240(3):269–81. Available from: <https://doi.org/10.1002/PATH.4773>.
- [74] Sapountzi V, Côté J. MYST-family histone acetyltransferases: beyond chromatin. *Cell Mol Life Sci* 2011;68(7):1147–56. Available from: <https://doi.org/10.1007/S00018-010-0599-9>.

- [75] Avvakumov N, Côté J. The MYST family of histone acetyltransferases and their intimate links to cancer. *Oncogene* 2007;26(37):5395–407. Available from: <https://doi.org/10.1038/SJ.ONC.1210608>.
- [76] Shroff R, et al. Distribution and dynamics of chromatin modification induced by a defined DNA double-strand break. *Curr Biol* 2004;14(19):1703–11. Available from: <https://doi.org/10.1016/J.CUB.2004.09.047>.
- [77] Sun Y, Jiang X, Chen S, Fernandes N, Price BD. A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. *Proc Natl Acad Sci U S A* 2005;102(37):13182–7. Available from: <https://doi.org/10.1073/PNAS.0504211102>.
- [78] Tang Y, Luo J, Zhang W, Gu W. Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol Cell* 2006;24(6):827–39. Available from: <https://doi.org/10.1016/J.MOLCEL.2006.11.021>.
- [79] LLeonart ME, et al. New p53 related genes in human tumors: significant downregulation in colon and lung carcinomas. *Oncol Rep* 2006;16(3):603–8. Available from: <https://doi.org/10.3892/OR.16.3.603>.
- [80] Sakuraba K, et al. TIP60 as a potential marker for the malignancy of gastric cancer. *Anticancer Res* 2011;31(1):77–9.
- [81] Bassi C, et al. The acetyltransferase Tip60 contributes to mammary tumorigenesis by modulating DNA repair. *Cell Death Differ* 2016;23(7):1198–208. Available from: <https://doi.org/10.1038/CDD.2015.173>.
- [82] Judes G, et al. TIP60: an actor in acetylation of H3K4 and tumor development in breast cancer. *Epigenomics* 2018;10(11):1415–30. Available from: <https://doi.org/10.2217/EPI-2018-0004>.
- [83] Gorrini C, et al. Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. *Nature* 2007;448(7157):1063–7. Available from: <https://doi.org/10.1038/NATURE06055>.
- [84] Chen G, Cheng Y, Tang Y, Martinka M, Li G. Role of Tip60 in human melanoma cell migration, metastasis, and patient survival. *J Invest Dermatol* 2012;132(11):2632–41. Available from: <https://doi.org/10.1038/JID.2012.193>.
- [85] Subbaiah VK, et al. E3 ligase EDD1/UBR5 is utilized by the HPV E6 oncogene to destabilize tumor suppressor TIP60. *Oncogene* 2016;35(16):2062–74. Available from: <https://doi.org/10.1038/ONC.2015.268>.
- [86] Patel JH, et al. The c-MYC oncoprotein is a substrate of the acetyltransferases hGCN5/PCAF and TIP60. *Mol Cell Biol* 2004;24(24):10826–34. Available from: <https://doi.org/10.1128/MCB.24.24.10826-10834.2004>.
- [87] Awasthi S, et al. A human T-cell lymphotropic virus type 1 enhancer of Myc transforming potential stabilizes Myc-TIP60 transcriptional interactions. *Mol Cell Biol* 2005;25(14):6178–98. Available from: <https://doi.org/10.1128/MCB.25.14.6178-6198.2005>.
- [88] Halkidou K, et al. Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. *Oncogene* 2003;22(16):2466–77. Available from: <https://doi.org/10.1038/SJ.ONC.1206342>.
- [89] Shiota M, et al. Tip60 promotes prostate cancer cell proliferation by translocation of androgen receptor into the nucleus. *Prostate* 2010;70(5):540–54. Available from: <https://doi.org/10.1002/PROS.21088>.
- [90] van den Broeck A, Nissou D, Brambilla E, Eymin B, Gazzeri S. Activation of a Tip60/E2F1/ERCC1 network in human lung adenocarcinoma cells exposed to cisplatin. *Carcinogenesis* 2012;33(2):320–5. Available from: <https://doi.org/10.1093/CARCIN/BGR292>.
- [91] Miyamoto N, et al. Tip60 is regulated by circadian transcription factor clock and is involved in cisplatin resistance. *J Biol Chem* 2008;283(26):18218–26. Available from: <https://doi.org/10.1074/JBC.M802332200>.
- [92] Rea S, Xouri G, Akhtar A. Males absent on the first (MOF): from flies to humans. *Oncogene* 2007;26(37):5385–94. Available from: <https://doi.org/10.1038/SJ.ONC.1210607>.
- [93] Cai Y, et al. Subunit composition and substrate specificity of a MOF-containing histone acetyltransferase distinct from the male-specific lethal (MSL) complex. *J Biol Chem* 2010;285(7):4268–72. Available from: <https://doi.org/10.1074/JBC.C109.087981>.
- [94] Smith ER, Cayrou C, Huang R, Lane WS, Côté J, Lucchesi JC. A human protein complex homologous to the Drosophila MSL complex is responsible for the majority of histone H4 acetylation at lysine 16. *Mol Cell Biol* 2005;25(21):9175–88. Available from: <https://doi.org/10.1128/MCB.25.21.9175-9188.2005>.

- [95] Cao L, et al. Correlation of low expression of hMOF with clinicopathological features of colorectal carcinoma, gastric cancer and renal cell carcinoma. *Int J Oncol* 2014;44(4):1207–14. Available from: <https://doi.org/10.3892/IJO.2014.2266>.
- [96] Liu N, et al. A potential diagnostic marker for ovarian cancer: Involvement of the histone acetyltransferase, human males absent on the first. *Oncol Lett* 2013;6(2):393–400. Available from: <https://doi.org/10.3892/OL.2013.1380>.
- [97] Sykes SM, et al. Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell* 2006;24(6):841–51. Available from: <https://doi.org/10.1016/J.MOLCEL.2006.11.026>.
- [98] Li Q, Sun H, Shu Y, Zou X, Zhao Y, Ge C. hMOF (human males absent on the first), an oncogenic protein of human oral tongue squamous cell carcinoma, targeting EZH2 (enhancer of zeste homolog 2). *Cell Prolif* 2015;48(4):436–42. Available from: <https://doi.org/10.1111/CPR.12177>.
- [99] Chen Z, et al. The histone acetyltransferase hMOF acetylates Nrf2 and regulates anti-drug responses in human non-small cell lung cancer. *Br J Pharmacol* 2014;171(13):3196–211. Available from: <https://doi.org/10.1111/BPH.12661>.
- [100] Champagne N, et al. Identification of a human histone acetyltransferase related to monocytic leukemia zinc finger protein. *J Biol Chem* 1999;274(40):28528–36. Available from: <https://doi.org/10.1074/JBC.274.40.28528>.
- [101] Champagne N, Pelletier N, Yang XJ. The monocytic leukemia zinc finger protein MOZ is a histone acetyltransferase. *Oncogene* 2001;20(3):404–9. Available from: <https://doi.org/10.1038/SJ.ONC.1204114>.
- [102] Borrow J, et al. The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. *Nat Genet* 1996;14(1):33–41. Available from: <https://doi.org/10.1038/NG0996-33>.
- [103] Kitabayashi I, et al. Fusion of MOZ and p300 histone acetyltransferases in acute monocytic leukemia with a t(8;22)(p11;q13) chromosome translocation. *Leukemia* 2001;15(1):89–94. Available from: <https://doi.org/10.1038/SJ.LEU.2401983>.
- [104] Carapeti M, Aguiar RCT, Goldman JM, Cross NCP. A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. *Blood* May 1998;91(9):3127–33. Available from: <https://doi.org/10.1182/BLOOD.V91.9.3127>.
- [105] Deguchi K, et al. MOZ-TIF2-induced acute myeloid leukemia requires the MOZ nucleosome binding motif and TIF2-mediated recruitment of CBP. *Cancer Cell* 2003;3(3):259–71. Available from: [https://doi.org/10.1016/S1535-6108\(03\)00051-5](https://doi.org/10.1016/S1535-6108(03)00051-5).
- [106] Panagopoulos I, et al. Fusion of the MORF and CBP genes in acute myeloid leukemia with the t(10;16)(q22; p13). *Hum Mol Genet* 2001;10(4):395–404. Available from: <https://doi.org/10.1093/HMG/10.4.395>.
- [107] Zhu J, et al. Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth. *Nature* 2015;525(7568):206–11. Available from: <https://doi.org/10.1038/NATURE15251>.
- [108] Iizuka M, Stillman B. Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. *J Biol Chem* 1999;274(33):23027–34. Available from: <https://doi.org/10.1074/JBC.274.33.23027>.
- [109] Iizuka M, Matsui T, Takisawa H, Smith MM. Regulation of replication licensing by acetyltransferase Hbo1. *Mol Cell Biol* 2006;26(3):1098–108. Available from: <https://doi.org/10.1128/MCB.26.3.1098-1108.2006>.
- [110] Saksouk N, et al. HBO1 HAT complexes target chromatin throughout gene coding regions via multiple PHD finger interactions with histone H3 tail. *Mol Cell* 2009;33(2):257–65. Available from: <https://doi.org/10.1016/J.MOLCEL.2009.01.007>.
- [111] Doyon Y, et al. ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Mol Cell* 2006;21(1):51–64. Available from: <https://doi.org/10.1016/J.MOLCEL.2005.12.007>.
- [112] Iizuka M, Sarmento OF, Sekiya T, Scoble H, Allis CD, Smith MM. Hbo1 Links p53-dependent stress signaling to DNA replication licensing. *Mol Cell Biol* 2008;28(1):140–53. Available from: <https://doi.org/10.1128/MCB.00662-07>.

- [113] Iizuka M, et al. Histone acetyltransferase Hbo1: catalytic activity, cellular abundance, and links to primary cancers. *Gene* 2009;436(1–2):108–14. Available from: <https://doi.org/10.1016/J.GENE.2009.01.020>.
- [114] Duong MLT, et al. Hbo1 is a cyclin E/CDK2 substrate that enriches breast cancer stem-like cells. *Cancer Res* 2013;73(17):5556–68. Available from: <https://doi.org/10.1158/0008-5472.CAN-13-0013>.
- [115] Song B, et al. Plk1 phosphorylation of orc2 and hbo1 contributes to gemcitabine resistance in pancreatic cancer. *Mol Cancer Ther* 2013;12(1):58–68. Available from: <https://doi.org/10.1158/1535-7163.MCT-12-0632>.
- [116] Hamamori Y, et al. Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. *Cell* 1999;96(3):405–13. Available from: [https://doi.org/10.1016/S0092-8674\(00\)80553-X](https://doi.org/10.1016/S0092-8674(00)80553-X).
- [117] Thompson PR, et al. Regulation of the p300 HAT domain via a novel activation loop. *Nat Struct Mol Biol* 2004;11(4):308–15. Available from: <https://doi.org/10.1038/NSMB740>.
- [118] Karanam B, Jiang L, Wang L, Kelleher NL, Cole PA. Kinetic and mass spectrometric analysis of p300 histone acetyltransferase domain autoacetylation. *J Biol Chem* 2006;281(52):40292–301. Available from: <https://doi.org/10.1074/JBC.M608813200>.
- [119] Arif M, Kumar GVP, Narayana C, Kundu TK. Autoacetylation induced specific structural changes in histone acetyltransferase domain of p300: probed by surface enhanced Raman spectroscopy. *J Phys Chem B* 2007;111(41):11877–9. Available from: <https://doi.org/10.1021/JP0762931>.
- [120] Turnell AS, et al. The APC/C and CBP/p300 cooperate to regulate transcription and cell-cycle progression. *Nature* 2005;438(7068):690–5. Available from: <https://doi.org/10.1038/NATURE04151>.
- [121] Hansson ML, et al. The transcriptional coactivator MAML1 regulates p300 autoacetylation and HAT activity. *Nucleic Acids Res* 2009;37(9):2996–3006. Available from: <https://doi.org/10.1093/NAR/GKP163>.
- [122] Sen N, et al. Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. *Nat Cell Biol* 2008;10(7):866–73. Available from: <https://doi.org/10.1038/NCB1747>.
- [123] Kaypee S, et al. Oligomers of human histone chaperone NPM1 alter p300/KAT3B folding to induce autoacetylation. *Biochim Biophys Acta Gen Subj* 2018;1862(8):1729–41. Available from: <https://doi.org/10.1016/J.BBAGEN.2018.05.003>.
- [124] Kaypee S, et al. Mutant and wild-type tumor suppressor p53 induces p300 autoacetylation. *iScience* 2018;4:260–72. Available from: <https://doi.org/10.1016/J.ISCI.2018.06.002/ATTACHMENT/CE59C335-90CE-4FFC-AE9D-5A60522B548B/MMC1.PDF>.
- [125] Senapati P, et al. Histone chaperone nucleophosmin regulates transcription of key genes involved in oral tumorigenesis. *Mol Cell Biol* 2022;42(2). Available from: <https://doi.org/10.1128/MCB.00669-20>.
- [126] Ghosh R, Kaypee S, Shasmal M, Kundu TK, Roy S, Sengupta J. Tumor suppressor p53-mediated structural reorganization of the transcriptional coactivator p300. *Biochemistry* 2019;58(32):3434–43. Available from: https://doi.org/10.1021/ACS.BIOCHEM.9B00333/SUPPL_FILE/B19B00333_SI_002.AVI.
- [127] Singh S, et al. The cancer-associated, gain-of-function TP53 variant P152Lp53 activates multiple signaling pathways implicated in tumorigenesis. *J Biol Chem* 2019;294(38):14081–95. Available from: <https://doi.org/10.1074/JBC.RA118.007265>.
- [128] Cullis PM, Wolfenden R, Cousens LS, Alberts BM. Inhibition of histone acetylation by N-[2-(S-coenzyme A)acetyl] spermidine amide, a multisubstrate analog. *J Biol Chem* 1982;257(20):12165–9.
- [129] Lau OD, et al. p300/CBP-associated factor histone acetyltransferase processing of a peptide substrate. Kinetic analysis of the catalytic mechanism. *J Biol Chem* 2000;275(29):21953–9. Available from: <https://doi.org/10.1074/JBC.M003219200>.
- [130] Lau OD, et al. HATs off: selective synthetic inhibitors of the histone acetyltransferases p300 and PCAF. *Mol Cell* 2000;5(3):589–95. Available from: [https://doi.org/10.1016/S1097-2765\(00\)80452-9](https://doi.org/10.1016/S1097-2765(00)80452-9).
- [131] Wu J, Xie N, Wu Z, Zhang Y, Zheng YG. Bisubstrate inhibitors of the MYST HATs Esa1 and Tip60. *Bioorg Med Chem* 2009;17(3):1381–6. Available from: <https://doi.org/10.1016/J.BMCC.2008.12.014>.

- [132] Guidez F, et al. Histone acetyltransferase activity of p300 is required for transcriptional repression by the promyelocytic leukemia zinc finger protein. *Mol Cell Biol* 2005;25(13):5552–66. Available from: <https://doi.org/10.1128/MCB.25.13.5552-5566.2005/ASSET/DBBA1FAA-9BA5-4283-8F94-F14B576658F7/ASSETS/GRAPHIC/ZMB0130551070010.jpeg>.
- [133] Zheng Y, et al. Synthesis and evaluation of a potent and selective cell-permeable p300 histone acetyltransferase inhibitor. *J Am Chem Soc* 2005;127(49):17182–3. Available from: https://doi.org/10.1021/JA0558544SUPPL_FILE/JA0558544SI20051031_041715.PDF.
- [134] Bandyopadhyay K, Banères JL, Martin A, Blonski C, Parello J, Gjerset RA. Spermidinyl-CoA-based HAT inhibitors block DNA repair and provide cancer-specific chemo- and radiosensitization. *Cell Cycle* 2009;8(17):2779–88. Available from: <https://doi.org/10.4161/CC.8.17.9416>.
- [135] Ngo L, Brown T, Zheng YG. Bisubstrate inhibitors to target histone acetyltransferase 1 (HAT1). *Chem Biol Drug Des* May 2019;93(5):865. Available from: <https://doi.org/10.1111/CBDD.13476>.
- [136] Balasubramanyam K, Swaminathan V, Ranganathan A, Kundu TK. Small molecule modulators of histone acetyltransferase p300. *J Biol Chem* May 2003;278(21):19134–40. Available from: <https://doi.org/10.1074/JBC.M301580200>.
- [137] Sun Y, Jiang X, Chen S, Price BD. Inhibition of histone acetyltransferase activity by anacardic acid sensitizes tumor cells to ionizing radiation. *FEBS Lett* 2006;580(18):4353–6. Available from: <https://doi.org/10.1016/J.FEBSLET.2006.06.092>.
- [138] Ghizzoni M, Boltjes A, de Graaf C, Haisma HJ, Dekker FJ. Improved inhibition of the histone acetyltransferase PCAF by an anacardic acid derivative. *Bioorg Med Chem* 2010;18(16):5826–34. Available from: <https://doi.org/10.1016/J.BMC.2010.06.089>.
- [139] Ghizzoni M, Wu J, Gao T, Haisma HJ, Dekker FJ, George Zheng Y. 6-alkylsalicylates are selective Tip60 inhibitors and target the acetyl-CoA binding site. *Eur J Med Chem* 2012;47(1):337–44. Available from: <https://doi.org/10.1016/J.EJMECH.2011.11.001>.
- [140] Souto JA, et al. Synthesis of benzamides related to anacardic acid and their histone acetyltransferase (HAT) inhibitory activities. *ChemMedChem* 2008;3(9):1435–42. Available from: <https://doi.org/10.1002/CMDC.200800096>.
- [141] Shirakawa K, et al. Salicylate, diflunisal and their metabolites inhibit CBP/p300 and exhibit anticancer activity. *eLife* 2016;5. Available from: <https://doi.org/10.7554/ELIFE.11156>.
- [142] Balasubramanyam K, et al. Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *J Biol Chem* 2004;279(32):33716–26. Available from: <https://doi.org/10.1074/JBC.M402839200>.
- [143] Collins HM, et al. Differential effects of garcinol and curcumin on histone and p53 modifications in tumour cells. *BMC Cancer* 2013;13. Available from: <https://doi.org/10.1186/1471-2407-13-37>.
- [144] Sethi G, et al. Inhibition of STAT3 dimerization and acetylation by garcinol suppresses the growth of human hepatocellular carcinoma in vitro and in vivo. *Mol Cancer* 2014;13(1). Available from: <https://doi.org/10.1186/1476-4598-13-66>.
- [145] Mantelingu K, et al. Specific inhibition of p300-HAT alters global gene expression and represses HIV replication. *Chem Biol* 2007;14(6):645–57. Available from: <https://doi.org/10.1016/J.CHEMBIOL.2007.04.011>.
- [146] Milite C, et al. A novel cell-permeable, selective, and noncompetitive inhibitor of KAT3 histone acetyltransferases from a combined molecular pruning/classical isosterism approach. *J Med Chem* 2015;58(6):2779–98. Available from: <https://doi.org/10.1021/JM5019687>.
- [147] Choi KC, et al. Epigallocatechin-3-gallate, a histone acetyltransferase inhibitor, inhibits EBV-induced B lymphocyte transformation via suppression of RelA acetylation. *Cancer Res* 2009;69(2):583–92. Available from: <https://doi.org/10.1158/0008-5472.CAN-08-2442>.
- [148] Balasubramanyam K, et al. Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone/nonhistone proteins and histone acetyltransferase-

- dependent chromatin transcription. *J Biol Chem* 2004;279(49):51163–71. Available from: <https://doi.org/10.1074/JBC.M409024200>.
- [149] Wu M, et al. Hydrazinobenzoylcucumin inhibits androgen receptor activity and growth of castration-resistant prostate cancer in mice. *Oncotarget* 2015;6(8):6136–50. Available from: <https://doi.org/10.18632/ONCOTARGET.3346>.
- [150] Rath S, Jena AB, Bhattacharyya A, Dandapat J. CTK7A, a curcumin derivative, can be a potential candidate for targeting HIF-1 α /p300 complex: Evidences from in vitro and computational studies. *Biophys Chem* 2022;287:106828. Available from: <https://doi.org/10.1016/J.BPC.2022.106828>.
- [151] Seong AR, et al. Delphinidin, a specific inhibitor of histone acetyltransferase, suppresses inflammatory signaling via prevention of NF- κ B acetylation in fibroblast-like synoviocyte MH7A cells. *Biochem Biophys Res Commun* 2011;410(3):581–6. Available from: <https://doi.org/10.1016/J.JBRC.2011.06.029>.
- [152] Choi KC, et al. Gallic acid suppresses lipopolysaccharide-induced nuclear factor-kappaB signaling by preventing RelA acetylation in A549 lung cancer cells. *Mol Cancer Res* 2009;7(12):2011–21. Available from: <https://doi.org/10.1158/1541-7786.MCR-09-0239>.
- [153] Choi KC, et al. Procyanidin B3, an inhibitor of histone acetyltransferase, enhances the action of antagonist for prostate cancer cells via inhibition of p300-dependent acetylation of androgen receptor. *Biochem J* 2011;433(1):235–44. Available from: <https://doi.org/10.1042/BJ20100980>.
- [154] Xiao X, et al. Quercetin suppresses cyclooxygenase-2 expression and angiogenesis through inactivation of P300 signaling. *PLoS One* 2011;6(8):e22934. Available from: <https://doi.org/10.1371/JOURNAL.PONE.0022934>.
- [155] Alsamri H, et al. Carnosol is a novel inhibitor of p300 acetyltransferase in breast cancer. *Front Oncol* 2021;11. Available from: <https://doi.org/10.3389/FONC.2021.664403>.
- [156] al Dhaheri Y, et al. Carnosol induces ROS-mediated beclin1-independent autophagy and apoptosis in triple negative breast cancer. *PLoS One* 2014;9:10. Available from: <https://doi.org/10.1371/journal.pone.0109630>.
- [157] Alsamri H, el Hasasna H, al Dhaheri Y, Eid AH, Attoub S, Iratni R. Carnosol, a natural polyphenol, inhibits migration, metastasis, and tumor growth of breast cancer via a ROS-dependent proteasome degradation of STAT3. *Front Oncol* 2019;9(AUG):743. Available from: <https://doi.org/10.3389/fonc.2019.00743>.
- [158] Ravindra KC, et al. Inhibition of lysine acetyltransferase KAT3B/p300 activity by a naturally occurring hydroxynaphthoquinone, plumbagin. *J Biol Chem* 2009;284(36):24453–64. Available from: <https://doi.org/10.1074/JBC.M109.023861>.
- [159] Yin Z, et al. Anticancer effects and mechanisms of action of plumbagin: review of research advances. *BioMed Res Int* 2020;2020. Available from: <https://doi.org/10.1155/2020/6940953>.
- [160] Vasudevarao MD, et al. Naphthoquinone-mediated inhibition of lysine acetyltransferase KAT3B/p300, basis for non-toxic inhibitor synthesis. *J Biol Chem* 2014;289(11):7702–17. Available from: <https://doi.org/10.1074/JBC.M113.486522>.
- [161] Modak R, et al. Probing p300/CBP associated factor (PCAF)-dependent pathways with a small molecule inhibitor. *ACS Chem Biol* 2013;8(6):1311–23. Available from: https://doi.org/10.1021/CB4000597/SUPPL_FILE/CB4000597_SI_003.XLSX.
- [162] Ko JH, et al. The application of embelin for cancer prevention and therapy. *Molecules* 2018;23(3). Available from: <https://doi.org/10.3390/MOLECULES23030621>.
- [163] Fu C, Guan G, Wang H. The anticancer effect of sanguinarine: a review. *Curr Pharm Des* 2018;24(24):2760–4. Available from: <https://doi.org/10.2174/138161282466180829100601>.
- [164] Liu F, et al. Sanguinarine promotes healthspan and innate immunity through a conserved mechanism of ROS-mediated PMK-1/SKN-1 activation. *iScience* 2022;25(3):103874. Available from: <https://doi.org/10.1016/J.ISCI.2022.103874>.
- [165] Selvi B R, et al. Sanguinarine interacts with chromatin, modulates epigenetic modifications, and transcription in the context of chromatin. *Chem Biol* 2009;16(2):203–16. Available from: <https://doi.org/10.1016/J.CHEMBIOL.2008.12.006>.

- [166] Li GB, Huang LY, Li H, Ji S, Li LL, Yang SY. Identification of new p300 histone acetyltransferase inhibitors from natural products by a customized virtual screening method. *RSC Adv* 2016;6 (66):61137–40. Available from: <https://doi.org/10.1039/C6RA11240D>.
- [167] Tohyama S, et al. Discovery and characterization of NK13650s, naturally occurring p300-selective histone acetyltransferase inhibitors. *J Org Chem* 2012;77(20):9044–52. Available from: https://doi.org/10.1021/JO301534B/SUPPL_FILE/JO301534B_SI_001.PDF.
- [168] Biel M, Kretsovali A, Karatzali E, Papamatheakis J, Giannis A. Design, synthesis, and biological evaluation of a small-molecule inhibitor of the histone acetyltransferase Gcn5. *Angew Chem Int Ed Engl* 2004;43(30):3974–6. Available from: <https://doi.org/10.1002/ANIE.200453879>.
- [169] Chimenti F, et al. A novel histone acetyltransferase inhibitor modulating Gen5 network: cyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl]hydrazone. *J Med Chem* 2009;52(2):530–6. Available from: https://doi.org/10.1021/JM800885D/SUPPL_FILE/JM800885D_SI_001.PDF.
- [170] Carradori S, et al. Evaluation of a large library of (thiazol-2-yl)hydrazones and analogues as histone acetyltransferase inhibitors: enzyme and cellular studies. *Eur J Med Chem* 2014;80:569–78. Available from: <https://doi.org/10.1016/J.EJMECH.2014.04.042>.
- [171] Cocco E, et al. KAT3B-p300 and H3AcK18/H3AcK14 levels are prognostic markers for kidney ccRCC tumor aggressiveness and target of KAT inhibitor CPTH2. *Clin Epigenetics* 2018;10(1). Available from: <https://doi.org/10.1186/S13148-018-0473-4>.
- [172] Stimson L, et al. Isothiazolones as inhibitors of PCAF and p300 histone acetyltransferase activity. *Mol Cancer Ther* 2005;4(10):1521–32. Available from: <https://doi.org/10.1158/1535-7163.MCT-05-0135>.
- [173] Ghizzoni M, Haisma HJ, Dekker FJ. Reactivity of isothiazolones and isothiazolone-1-oxides in the inhibition of the PCAF histone acetyltransferase. *Eur J Med Chem* 2009;44(12):4855–61. Available from: <https://doi.org/10.1016/J.EJMECH.2009.07.025>.
- [174] Wapenaar H, Dekker FJ. Histone acetyltransferases: challenges in targeting bi-substrate enzymes. *Clin Epigenetics* May 2016;8:1. Available from: <https://doi.org/10.1186/S13148-016-0225-2>.
- [175] Wisastra R, Ghizzoni M, Maarsingh H, Minnaard AJ, Haisma HJ, Dekker FJ. Isothiazolones; thiol-reactive inhibitors of cysteine protease cathepsin B and histone acetyltransferase PCAF. *Org Biomol Chem* 2011;9(6):1817–22. Available from: <https://doi.org/10.1039/C0OB00464B>.
- [176] Secci D, et al. Synthesis of a novel series of thiazole-based histone acetyltransferase inhibitors. *Bioorg Med Chem* 2014;22(5):1680–9. Available from: <https://doi.org/10.1016/J.BMC.2014.01.022>.
- [177] Coffey K, et al. Characterisation of a Tip60 specific inhibitor, NU9056, in prostate cancer. *PLoS One* 2012;7(10). Available from: <https://doi.org/10.1371/JOURNAL.PONE.0045539>.
- [178] Gao C, et al. Rational design and validation of a Tip60 histone acetyltransferase inhibitor. *Sci Rep* 2014;4. Available from: <https://doi.org/10.1038/SREP05372>.
- [179] Idrissou M, et al. TIP60 inhibitor TH1834 reduces breast cancer progression in xenografts in mice. *OMICS* 2019;23(9):457–9. Available from: <https://doi.org/10.1089/OMI.2019.0126>.
- [180] Bowers EM, et al. Virtual ligand screening of the p300/CBP histone acetyltransferase: identification of a selective small molecule inhibitor. *Chem Biol* 2010;17(5):471. Available from: <https://doi.org/10.1016/J.CHEMBIOL.2010.03.006>.
- [181] Oike T, et al. C646, a selective small molecule inhibitor of histone acetyltransferase p300, radiosensitizes lung cancer cells by enhancing mitotic catastrophe. *Radiother Oncol* 2014;111(2):222–7. Available from: <https://doi.org/10.1016/J.RADONC.2014.03.015>.
- [182] Ono H, et al. C646 inhibits G2/M cell cycle-related proteins and potentiates anti-tumor effects in pancreatic cancer. *Sci Rep* 2021;11(1). Available from: <https://doi.org/10.1038/S41598-021-89530-8>.
- [183] Shrimp JH, Sorum AW, Garlick JM, Guasch L, Nicklaus MC, Meier JL. Characterizing the covalent targets of a small molecule inhibitor of the lysine acetyltransferase P300. *ACS Med Chem Lett* 2016;7(2):151–5. Available from: https://doi.org/10.1021/ACSMEDCHEMLETT.5B00385/SUPPL_FILE/ML5B00385_SI_001.PDF.

- [184] Dahlin JL, et al. Assay interference and off-target liabilities of reported histone acetyltransferase inhibitors. *Nat Commun* 2017;8(1). Available from: <https://doi.org/10.1038/S41467-017-01657-3>.
- [185] Lasko LM, et al. Discovery of a selective catalytic p300/CBP inhibitor that targets lineage-specific tumours. *Nature* 2017;550(7674):128–32. Available from: <https://doi.org/10.1038/nature24028>.
- [186] Yang Y, et al. Discovery of highly potent, selective, and orally efficacious p300/CBP histone acetyltransferases inhibitors. *J Med Chem* 2020;63(3):1337–60. Available from: <https://doi.org/10.1021/ACS.JMEDCHEM.9B01721>.
- [187] Michaelides MR, et al. Discovery of spiro oxazolidinediones as selective, orally bioavailable inhibitors of p300/CBP histone acetyltransferases. *ACS Med Chem Lett* 2017;9(1):28–33. Available from: <https://doi.org/10.1021/ACSMEDCHEMLETT.7B00395>.
- [188] Wilson JE, et al. Discovery of CPI-1612: a potent, selective, and orally bioavailable EP300/CBP histone acetyltransferase inhibitor. *ACS Med Chem Lett* 2020;11(6):1324–9. Available from: <https://doi.org/10.1021/ACSMEDCHEMLETT.0C00155>.
- [189] Hammitzsch A, et al. CBP30, a selective CBP/p300 bromodomain inhibitor, suppresses human Th17 responses. *Proc Natl Acad Sci U S A* 2015;112(34):10768–73. Available from: https://doi.org/10.1073/PNAS.1501956112/SUPPL_FILE/PNAS.1501956112.SD01.XLS.
- [190] Picaud S, et al. Generation of a selective small molecule inhibitor of the CBP/p300 bromodomain for leukemia therapy. *Cancer Res* 2015;75(23):5106–19. Available from: <https://doi.org/10.1158/0008-5472.CAN-15-0236>.
- [191] Brooks N, Raja M, Young BW, Spencer GJ, Somervaille TC, Pegg NA. CCS1477: a novel small molecule inhibitor of p300/CBP bromodomain for the treatment of acute myeloid leukaemia and multiple myeloma. *Blood* 2019;134(Supplement_1):2560. Available from: <https://doi.org/10.1182/BLOOD-2019-124707>.
- [192] Brooks N, Prosser A, Young B, Gaughan L, Elvin P, Pegg N. Abstract 3826: CCS1477, a potent and selective p300/CBP bromodomain inhibitor, is targeted & differentiated from BET inhibitors in prostate cancer cell lines in vitro. *Cancer Res* 2019;79(13_Supplement):3826. Available from: <https://doi.org/10.1158/1538-7445.AM2019-3826>.
- [193] Moustakim M, et al. Discovery of a PCAF bromodomain chemical probe. *Angew Chem Int Ed Engl* 2017;56(3):827–31. Available from: <https://doi.org/10.1002/ANIE.201610816>.
- [194] Wang Q, Wang R, Zhang B, Zhang S, Zheng Y, Wang Z. Small organic molecules targeting PCAF bromodomain as potent inhibitors of HIV-1 replication. *Medchemcomm* 2013;4(4):737–40. Available from: <https://doi.org/10.1039/C3MD20376J>.
- [195] Wu D, Zhang R, Zhao R, Chen G, Cai Y, Jin J. A novel function of novobiocin: disrupting the interaction of HIF 1 α and p300/CBP through direct binding to the HIF1 α C-terminal activation domain. *PLoS One* 2013;8(5):e62014. Available from: <https://doi.org/10.1371/JOURNAL.PONE.0062014>.
- [196] Jayatunga MKP, et al. Inhibition of the HIF1 α -p300 interaction by quinone- and indandione-mediated ejection of structural Zn(II). *Eur J Med Chem* 2015;94:509–16. Available from: <https://doi.org/10.1016/J.EJMECH.2014.06.006>.
- [197] Chan STS, et al. Structural elucidation and synthesis of eudistidine a: an unusual polycyclic marine alkaloid that blocks interaction of the protein binding domains of p300 and HIF-1 α . *J Am Chem Soc* 2015;137(16):5569–75. Available from: <https://doi.org/10.1021/JACS.5B02156>.
- [198] Reece KM, et al. Epidithiodiketopiperazines (ETPs) exhibit in vitro antiangiogenic and in vivo antitumor activity by disrupting the HIF-1 α /p300 complex in a preclinical model of prostate cancer. *Mol Cancer* 2014;13(1). Available from: <https://doi.org/10.1186/1476-4598-13-91>.
- [199] Dubey R, et al. Suppression of tumor growth by designed dimeric epidithiodiketopiperazine targeting hypoxia-inducible transcription factor complex. *J Am Chem Soc* 2013;135(11):4537–49. Available from: <https://doi.org/10.1021/JA400805B>.
- [200] Zhang Y, Wang X. Targeting the Wnt/ β -catenin signaling pathway in cancer. *J Hematol Oncol* 2020;13(1). Available from: <https://doi.org/10.1186/S13045-020-00990-3>.

- [201] Emami KH, et al. A small molecule inhibitor of β -catenin/cyclic AMP response element-binding protein transcription. *Proc Natl Acad Sci U S A* 2004;101(34):12682–7. Available from: <https://doi.org/10.1073/PNAS.0404875101/ASSET/624BD5A5-7D70-463A-BD68-B2AD85887C85/ASSETS/GRAPHIC/ZPQ0350458490005.jpeg>.
- [202] Z L, et al. Combination of auranofin and ICG-001 suppress the proliferation and metastasis of colon cancer. *Front Oncol* 2021;11. Available from: <https://doi.org/10.3389/FONC.2021.738085>.
- [203] Choi JH, et al. The small-molecule Wnt inhibitor ICG-001 efficiently inhibits colorectal cancer stemness and metastasis by suppressing MEIS1 expression. *Int J Mol Sci* 2021;22(24). Available from: <https://doi.org/10.3390/IJMS222413413>.
- [204] Huang Y, et al. Wnt/ β -catenin inhibitor ICG-001 enhances the antitumor efficacy of radiotherapy by increasing radiation-induced DNA damage and improving tumor immune microenvironment in hepatocellular carcinoma. *Radiother Oncol* 2021;162:34–44. Available from: <https://doi.org/10.1016/J.RADONC.2021.06.034>.
- [205] Liu Y, et al. ICG-001 suppresses growth of gastric cancer cells and reduces chemoresistance of cancer stem cell-like population. *J Exp Clin Cancer Res* 2017;36(1). Available from: <https://doi.org/10.1186/S13046-017-0595-0>.
- [206] Arensman MD, et al. The CREB-binding protein inhibitor ICG-001 suppresses pancreatic cancer growth. *Mol Cancer Ther* 2014;13(10):2303–14. Available from: <https://doi.org/10.1158/1535-7163.MCT-13-1005>.
- [207] Nagaraj AB, et al. Critical role of Wnt/ β -catenin signaling in driving epithelial ovarian cancer platinum resistance. *Oncotarget* 2015;6(27):23720–34. Available from: <https://doi.org/10.18632/ONCOTARGET.4690>.
- [208] Zhao Y, et al. CBP/catenin antagonist safely eliminates drug-resistant leukemia-initiating cells. *Oncogene* 2016;35(28):3705–17. Available from: <https://doi.org/10.1038/ONC.2015.438>.
- [209] Kaochar S, et al. ICG-001 exerts potent anticancer activity against uveal melanoma cells. *Invest Ophthalmol Vis Sci* 2018;59(1):132–43. Available from: <https://doi.org/10.1167/IOVS.17-22454>.
- [210] Rieger ME, et al. p300/ β -catenin interactions regulate adult progenitor cell differentiation downstream of WNT5a/protein kinase C (PKC). *J Biol Chem* 2016;291(12):6569–82. Available from: <https://doi.org/10.1074/JBC.M115.706416>.
- [211] Higuchi Y, Nguyen C, Yasuda S-Y, McMillan M, Hasegawa K, Kahn M. Specific direct small molecule p300/ β -catenin antagonists maintain stem cell potency. *Curr Mol Pharmacol* 2016;9(3):272–9. Available from: <https://doi.org/10.2174/187446720866150526155146>.
- [212] Uttarkar S, Dukare S, Bopp B, Goblirsch M, Jose J, Klempnauer KH. Naphthol AS-E phosphate inhibits the activity of the transcription factor Myb by blocking the interaction with the KIX domain of the coactivator p300. *Mol Cancer Ther* 2015;14(6):1276–85. Available from: <https://doi.org/10.1158/1535-7163.MCT-14-0662>.
- [213] Qi W, et al. NSC348884, a nucleophosmin inhibitor disrupts oligomer formation and induces apoptosis in human cancer cells. *Oncogene* 2008;27(30):4210–20. Available from: <https://doi.org/10.1038/onc.2008.54>.
- [214] Nalawansha DA, Crews CM. PROTACs: an emerging therapeutic modality in precision medicine. *Cell Chem Biol* 2020;27(8):998–1014. Available from: <https://doi.org/10.1016/J.CHEMBIOL.2020.07.020>.
- [215] Nabet B, et al. The dTAG system for immediate and target-specific protein degradation. *Nat Chem Biol* May 2018;14(5):431–41. Available from: <https://doi.org/10.1038/S41589-018-0021-8>.
- [216] Dancy BM, Cole PA. Protein lysine acetylation by p300/CBP. *Chem Rev* 2015;115(6):2419–52. Available from: <https://doi.org/10.1021/CR500452K>.
- [217] Merika M, Williams AJ, Chen G, Collins T, Thanos D. Recruitment of CBP/p300 by the IFN beta enhanceosome is required for synergistic activation of transcription. *Mol Cell* 1998;1(2):277–87. Available from: [https://doi.org/10.1016/S1097-2765\(00\)80028-3](https://doi.org/10.1016/S1097-2765(00)80028-3).
- [218] Vannam R, et al. Targeted degradation of the enhancer lysine acetyltransferases CBP and p300. *Cell Chem Biol* 2021;28(4):503–514.e12. Available from: <https://doi.org/10.1016/J.CHEMBIOL.2020.12.004>.

- [219] Cousin S, et al. Safety, pharmacokinetic, pharmacodynamic and clinical activity of molibresib for the treatment of nuclear protein in testis carcinoma and other cancers: Results of a Phase I/II open-label, dose escalation study. *Int J Cancer* 2022;150(6):993–1006. Available from: <https://doi.org/10.1002/IJC.33861>.
- [220] Piha-Paul SA, et al. Phase 1 study of molibresib (GSK525762), a bromodomain and extra-terminal domain protein inhibitor, in NUT carcinoma and other solid tumors. *JNCI Cancer Spectr* 2020;4(2). Available from: <https://doi.org/10.1093/JNCICS/PKZ093>.
- [221] Tontsch-Grunt U, et al. Therapeutic impact of BET inhibitor BI 894999 treatment: backtranslation from the clinic. *Br J Cancer* 2022;. Available from: <https://doi.org/10.1038/S41416-022-01815-5>.
- [222] Armstrong AJ, et al. Abstract P202: Initial findings from an ongoing first-in-human phase 1 study of the CBP/p300 inhibitor FT-7051 in men with metastatic castration-resistant prostate cancer. *Mol Cancer Therapeutics* 2021;20(12_Supplement):P202. Available from: <https://doi.org/10.1158/1535-7163.TARG-21-P202>.
- [223] Liu Y, et al. MG149 inhibits histone acetyltransferase KAT8-mediated IL-33 acetylation to alleviate allergic airway inflammation and airway hyperresponsiveness. *Signal Transduct Target Ther* 2021;6(1). Available from: <https://doi.org/10.1038/S41392-021-00667-4>.
- [224] Cregan S, et al. KAT5 (Tip60) is a potential therapeutic target in malignant pleural mesothelioma. *Int J Oncol* 2016;48(3):1290–6. Available from: <https://doi.org/10.3892/IJO.2016.3335>.
- [225] Pieme CA, Ambassa P, Yankep E, Saxena AK. Epigarcinol and isogarcinol isolated from the root of *Garcinia ovalifolia* induce apoptosis of human promyelocytic leukemia (HL-60 cells). *BMC Res Notes* 2015;8(1). Available from: <https://doi.org/10.1186/S13104-015-1596-8>.

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Epigenetic therapies: histone deacetylases

19

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1 Introduction

The deregulation of gene expression is generally responsible for the development of diseases. DNA mutations are one of the most described mechanisms involved in this process. Epigenetics, which affects chromatin through heritable and labile modifications that do not change the DNA sequence and also controls gene expression, has been identified as being involved in many diseases when it is dysregulated. DNA methylations on cytosine residues and histone modifications, mainly on their

N-terminal tails consisting on acetylation, methylation, and phosphorylation among others, are the most studied epigenetic modifications. The combination of these epigenetic modifications alters the structure of chromatin, the accessibility of DNA to transcription factors, and then the expression of genes. Three different families of enzymes classified as writers, erasers, and readers and regulate the epigenetic “code.” Various pathologies can result from dysregulation of epigenetic pathways. Malignant diseases belong to the category of diseases with many epigenetic alterations, including changes in DNA methylation and histone acetylation. DNA methylation is catalyzed by DNA methyl transferase (DNMT) and consists of the addition of a methyl group to cytosine residues. Three DNMTs were described, the maintenance methyl transferase DNMT1 and the *de novo* methyl transferases DNMT 3A and 3B. Histone acetyl transferases (HATs) and histone deacetylases (HDACs) control histone acetylation by antagonistic actions. Addition of acetyl groups by HATs to the N-terminal tails of histones leads to chromatin relaxation and is associated with increased gene expression, whereas deacetylation of histones by HDACs leads to chromatin compaction and represses gene expression. It has been generally described that hypermethylation of tumor suppressor gene (TSG) promoters and histone hypoacetylation linked to HDAC dysregulation in cancer cells lead to TSG repression. Thus DNMTs and HDACs, in particular, have emerged as potentially interesting therapeutic targets. This chapter will focus mainly on HDACs and their inhibitors (HDACi), which have been the focus of intensive research for several decades, with the aim of producing molecules with significant therapeutic potential, particularly for the treatment of cancers. Indeed, the promising results obtained in *in vitro* and *in vivo* models support the clinical potential of this family of molecules.

2 Histone deacetylases

Eighteen histone deacetylases have been identified in the previous work. On the basis of homologies with yeast deacetylases, their function, localization, and substrates (Table 19.1), HDACs are classified into four categories.

Class I, II, and IV HDACs, the group of “classical” HDACs comprising 11 metalloenzymes, require the presence of a zinc cation (Zn^{2+}) in their active site to be functional. Class I HDACs (HDACs 1, 2, 3, and 8) are located in the nucleus of cells and widely expressed in human tissues. Class IIa HDACs (HDACs 4, 5, 7, and 9) are expressed in a tissue specific manner, unlike other HDACs. They can translocate in the cytoplasm following posttranslational modifications, such as multiple phosphorylation on serine residues [1], and present the particularity to contain a histidine to replace the key tyrosine in the

TABLE 19.1 Classification of histone deacetylase inhibitor.

Class	HDACs	Localization	Zn^{2+}	Expression
I	1, 2, 3, 8	Nucleus	Yes	Ubiquitous
IIa	4, 5, 7, 9	Nucleus, cytoplasm	Yes	Tissue specific
IIb	6, 10	Cytoplasm	Yes	Tissue specific
III	Sirtuins 1–7	Nucleus, cytoplasm, mitochondria	No	Variable
IV	11	Nucleus, cytoplasm	Yes	Ubiquitous

active site leading to a weak catalytic activity [2]. The N-terminal domain has no similarities with HDACs from the others classes and bind to MEF2 family of transcriptional repressors [3]. Enzymes from class IIb (HDACs 6 and 10) are found in nucleus and cytoplasm. HDAC 6 is composed of two catalytic domains organized in tandem, CD1 and CD2, and exerts its lysine deacetylation activity mainly in the cytoplasm on well-known substrates, such as α -tubulin and cortactin [4], involved in cytoskeleton and cell mobility, and the chaperone Hsp90 [4]. HDAC 10, whereas in the same subgroup than HDAC6, is quite different. Indeed, recent work has identified an acetyl polyamine hydrolase activity, due to the presence of a glutamate residue in the active site, which may be superior to the hydrolytic activity of acetyl lysine [5,6]. Class III HDACs constituted of seven enzymes, called sirtuins (SIRTs), do not require Zn^{2+} in their active site but use nicotinamide adenine dinucleotide (NAD^+) as a cofactor. Sirtuins are located in nucleus, cytoplasm, or mitochondria depending on the isoform [7]. HDAC11 is the only member of class IV enzymes. This HDAC is located in the nucleus of cells and, as class I HDACs, it is widely expressed in human tissues. This is also the smallest HDACs with the catalytic domain covering 80% of the protein sequence.

The function of HDAC is mainly to remove the acetyl group on the N - ϵ -lysine side chain present in the N-terminal tail of histones. This catalytic activity increases the positive charge of the histones, and leads to stabilization of DNA–histone complexes by electrostatic interactions resulting in chromatin compaction and transcription repression. However, the cytoplasmic HDACs can deacetylate nonhistone proteins, such as transcription factors [8–14], DNA repair enzyme, heat shock protein (Hsp90) [15], signaling pathway proteins (STAT3) [16], and α -tubulin [17]. The impact of the acetylation status of the nonhistone proteins can modify their activity or their stability and then, deeply impact the cellular machinery.

The function of HDACs was initially studied through the development of knockout (KO) mouse models due to the absence of isoform-specific inhibitors [18,19]. Indeed, *HDAC1* KO leads to early embryonic death due to generalized growth defects. In *HDAC2* KO, cardiac defects are responsible for the death of mice shortly after birth. Embryonic lethality is observed following disruption of *HDAC3* gene probably due to defect in cell cycle regulation, particularly in S-phase control, and DNA repair mechanisms [20]. Lethality in the first weeks of life is observed in *HDAC4* KO mice. This phenotype is explained by the presence of bone formation defects. *HDAC7* is specifically expressed in endothelial cells during embryogenesis. Thus mice deleted for *HDAC7* die early in embryonic development due to vascular defects leading to hemorrhage [21]. Additional functions of *HDAC7* were described as an implication in immunity [22–24] and osteoclastogenesis [25,26]. On the contrary of the majority of HDACs, *HDAC5* and *HDAC9* KO mice are not lethal. However, cardiac abnormalities are observed when these two HDACs are inhibited suggesting redundant functions. According to an important activity in the cytoplasm on nonhistone proteins, *HDAC6* KO mice present high acetylation of tubulin in multiple organs. However, these mice are viable with no evident phenotype [27].

The use of KO mouse models has highlighted the major functions of HDACs during development, in particular for class I HDACs whose KOs lead to early death in mice.

3 HDACs and cancer

Cancer cells are generally characterized by the presence of epigenetic alterations. In a large number of cancers, mutations in HDACs, changes in HDAC expression and changes in histone acetylation

was observed [19]. Changes in HDAC expression was described in several cancers [28–33]. However, only a small number of HDACs were generally associated with prognosis, a particular subtype or resistance to treatment. Although an association between overexpression of HDACs and shorter patient survival was described in the majority of published studies, in some cases an association with better prognosis was found and thus the contribution of these enzymes to disease outcome is unclear [34–36].

The involvement of HDACs in the development of cancer could occur throughout the course of the disease, particularly during the initiation or progression phases [37]. During cancer initiation, gene repression, usually of tumor suppressor genes, leading to uncontrolled cell proliferation, differentiation and decreased apoptosis has been described and linked to decreased histone acetylation by HDACs. During cancer progression, histone deacetylation due to increased HDAC activity induces loss of cell adhesion, participating in cell migration, invasion and angiogenesis. Therefore the modification of histone acetylation related to the deregulation of HDACs in cancer and its consequences on tumor cells have led to the identification of these enzymes as promising therapeutic targets.

4 HDACi

Initially, HDACi were identified from natural sources. The first efficient HDACi, trichostatin A, was identified there is 30 years by Yoshida and collaborators from *Streptomyces* [38]. The basic description of the structure of this molecule, constituted of a zinc binding group, a linker and a cap involved in isoform selectivity, was then largely used to develop a high number of derivatives [39] (Figure 19.1). Thus new molecules were developed with the objective to improve their activity and their specificity. The inhibitory activity of compounds targeting zinc-dependent HDACs is related to their strong zinc chelating properties. To date a high number of compounds are available and evaluated in preclinical or clinical studies. According to their chemical structure, HDACi are classified into four classes [40].

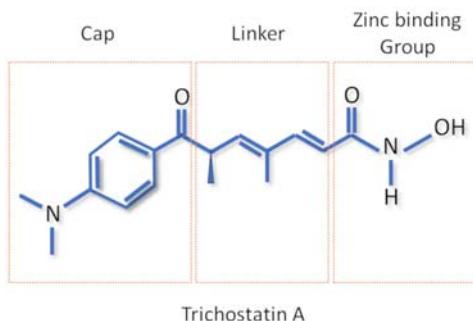


FIGURE 19.1

Structure of trichostatin A. Rectangle indicates the different parts of the molecule that constitute the general scaffold of HDACi.

Hydroxamates are the larger family. These compounds are usually unselective HDAC inhibitors with an activity in the range of micromolar to nanomolar concentrations. Three hydroxamates are particularly well-known, vorinostat (SAHA), belinostat (PDX101), and panobinostat (LBH589), and approved by the US food and drug administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL) [41], of patients with relapse or refractory peripheral T-cell lymphoma (PTCL) [42] or of multiple myeloma (MM) [43], respectively. Although it exhibited anticancer activity at nanomolar concentrations on malignant cells *in vitro*, trichostatin A (TSA) has been excluded from clinical use due to its high toxicity [44]. However, hydroxamates constitute the main family of compounds under clinical evaluation in the field of cancers. Molecules involved are abexinostat (PCI-24781), pracinostat (SB239), resminostat (RAS2410), givinostat (ITF2357), and quisinostat (JNJ-26481585) [45].

Cyclic peptides and benzamides are mainly class I HDAC inhibitors. Romidepsin (FK2208), a depsipeptide isolated from bacteria, and entinostat (MS-275) are the prototypes of these families, respectively. Romidepsin was approved by FDA for the treatment of CTCL [46] and PTCL [47]. Since, additional natural molecules have been isolated, such as spiruchostatine and largazol [48]. These compounds are characterized by the presence of common free thiol constituting the zinc binding group following reduction (Figure 19.2). Concerning benzamide molecules, entinostat and

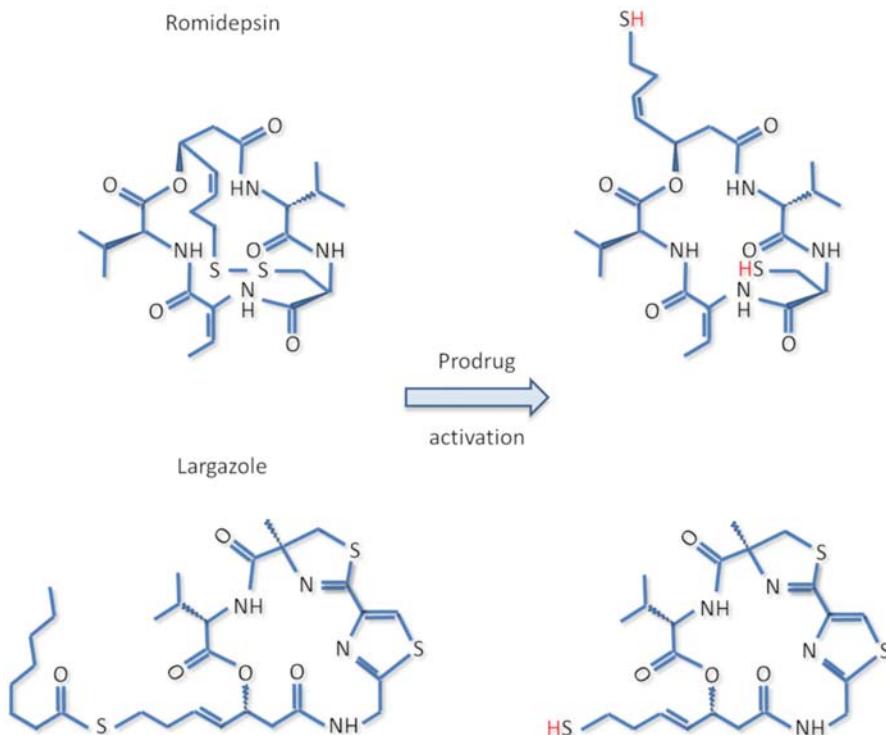


FIGURE 19.2

Structure and activation of natural HDACi prodrugs.

other members of this family, such as mocetinostat (MGCD0103) and tucidinostat (Chidamide), are under clinical investigation, the latter being approved in China for the treatment of PTCL [49].

Short chain fatty acids, valproic acid (VPA) and sodium butyrate, inhibit class I and class IIa HDACs, however, at millimolar concentrations. The inhibitory activity of these molecules relies on the presence of a carboxylic function that binds Zn^{2+} . VPA (Depakine) is well-known for its anti-epileptic and mood stabilizing properties.

5 HDACi effects on tumor cells

From microarray experiments, it appears that less than 10% of the transcriptomic profile of cells is impacted after a HDACi treatment [50]. However, in a cancer cell, it was shown that HDACi can induce cell cycle arrest, sensitivity to apoptosis, changes in DNA damage repair machinery, reduction of angiogenesis, immunomodulation, and modification of epithelial-to-mesenchymal transition (EMT) status (Figure 19.3) [51].

The induction of CDK inhibitor (CDKi) expression is the main mechanism of cell cycle control by HDACi. It was shown in several studies that the expression of the p21^{WAF1/CIP1} gene was induced following HDACi treatment [52–55]. Thus depending on the cancer cell line and on the HDACi used, a cell cycle arrest in G0/G1, G1/S or G2/M phase was observed [45]. HDACi-induced cell cycle arrest appears to be mainly mediated by induction of p21 expression, although other CDKi genes are induced by these molecules [56]. Expression of p21 was demonstrated to be under the control of P53 through binding to its promotor [57]. However, while some studies described that HDACi can induce p53 activation [58,59], the increase in p21 gene expression following HDAC inhibition appears to be independent of the status of p53 in cells [53,60,61]. Dephosphorylation of retinoblastoma protein (Rb) [62–64], inhibition of the expression of genes coding cyclin D and cyclin A, regulating the activities of CDK2 and CDK4 kinases, respectively

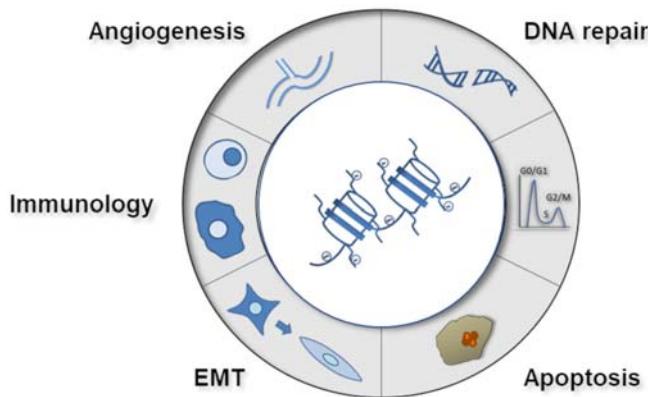


FIGURE 19.3

Processes impacted by modification of histone acetylation.

[65,66], and decrease of E2F transcriptional activity [67] were described as additional mechanisms contributing to regulation of cell cycle by HDACi.

Resistance to death is a usual property of cancer cells. Induction of apoptosis by HDACi usually involves the regulation of genes encoding proapoptotic or antiapoptotic proteins. HDACi also have the ability to modulate the expression of genes in the intrinsic and extrinsic pathways of apoptosis. Apoptosis induced by extrinsic pathway involves the interaction of tumor necrosis factor (TNF)-super family ligands (TRAIL, TNF α , Fas-L) with death receptors (Dr4, Dr5). In cancer cells, HDACi induce Dr4 and Dr5 receptor expressions [67–70]. In addition, some studies demonstrated that HDACi can regulate the expression of intracellular adaptor proteins, such as FLICE-inhibitory protein (c-FLIP) [68,69,71], an inhibitor of apoptosis, or by acting on the interaction between the Fas-associated death domain (FADD) interaction and the death-inducing signaling complex (DISC) [68,72]. Stress signals induced by chemotherapeutic agents, for example, by induction of ROS or DNA damage, can trigger the intrinsic apoptotic pathway and lead to mitochondrial permeability and thus caspase activation following the release of proapoptotic proteins. Intrinsic apoptosis is regulated in cells by the expression of proapoptotic BCL-2 proteins (Bak and Bax) and antiapoptotic BCL-2 proteins (BCL-2, BCL-XL, MCL-1). BH3 proteins only (Bad, Bik, Bid, Bim, Puma, Noxa) are another family of apoptosis regulators that help fine-tune the level of cell death. HDAC inhibition has been shown to result in increased expression of proapoptotic BCL-2 and BH3 proteins only [73]. However, the genes regulated depend on the HDACi and the cancer cells used. These interesting properties of HDACi have led to the consideration of these molecules in combination with other classical chemotherapeutic agents to act as apoptosis sensitizers.

In the development of tumors, angiogenesis plays a major role in providing nutrients to malignant tumors but also in promoting their dissemination in the body. Many therapies have been developed to limit this process in order to reduce tumor growth and metastasis. Several studies have shown that angiogenesis pathways can be targeted using HDACi. Indeed, HDACi can directly decrease angiogenic capacity of endothelial cells [74–78]. At the mechanistic level, several actions were described such as a downregulation of the expression of VEGF expression and hypoxia-inducible factor-1 α (HIF1 α) in numerous model of cancers [79–83]. In addition to their action on the expression of HIF1 α , HDACi induce its degradation through the induction of the expression of the tumor suppressor gene von Hippel Lindau (VHL) and altering the chaperone function of Hsp90 by modifying its acetylation [84,85]. Induction of the expression of antiangiogenic factors, such as activin-A and thrombospondin-1, has also been reported with VPA [86].

The sensitivity of cancer cells to chemotherapy or radiation therapy may be conditioned by DNA damage repair (DDR) pathways. HDACi can interfere with these mechanisms and lead to improve treatment efficiency. Indeed, the combination of HDACi with radiotherapy increases DNA damages by decreasing the capacity of cells to repair double-strand break (DSB) [87–90]. A possible mechanistic explanation is the downregulation of the expression of proteins, such as Rad50, Ku70, and Ku80, involved in DDR, observed after HDACi treatment [89]. It was also observed that the treatment with VPA, NaBu, vorinostat, and MS-275 increased the duration of γ H2AX foci, associated with the presence of DNA damages, following irradiation of cancer cells [90]. HDACi, such as TSA, vorinostat, and abexinostat, were shown to inhibit the homologous recombination and of the nonhomologous recombination end through the downregulation of breast cancer 1 (BRCA1) and RAD51 and thus DDR [91,92]. Interestingly, in normal cells, DDR was not altered following HDACi treatment [90,93–95]. HDACi can also directly impact the function of proteins by

modifying their acetylation. As with apoptosis, the impact of HDACi on DDR mechanisms suggests that a combination with radiotherapy may be effective in inducing cancer cell death after induction of DNA damages.

Induction of DNA damages by HDACi has also been shown to be associated with increased production of reactive oxygen species (ROS) in cancer cells [96,97]. The use of free radical scavenger molecules, such as *N*-acetyl-cysteine (NAC), have confirmed the involvement of ROS in the cellular cytotoxicity induced by HDACi [96,98,99]. Several mechanisms were described to explain this effect of HDACi. Mainly, an impact on the expression of proteins involved in the regulation of redox state in cells was described. Notably, a reduction of the expression of thioredoxin (TRX), an ROS scavenger, and an induction of the expression of the thioredoxin-binding protein-2 (TBP-2), that binds and inhibits TRX, were described [100]. The induction of another negative regulator of TRX by HDACi, the thioredoxin-interacting protein (TXNIP), was also observed [101,102].

In cancer cells, changes in the epithelial-mesenchymal transition (EMT) are generally associated with changes in migration/invasion properties and subsequently in metastatic capacity [103]. Epigenetics plays an important role in this process by controlling the expression of key transcription factors through DNA methylation, histone acetylation, and histone methylation [104]. Usually, HDACi lead to the induction of epithelial markers, such as E-cadherin, and to the downregulation of mesenchymal transcription factors, such as Zeb1, Snail, and Slug [78,105–111]. However, conflicting results were obtained using the same cell models and molecules, suggesting that experimental conditions, such as culture conditions, duration, and repetition of treatments, might interfere with the action of HDACi on the EMT state of cells [112–115].

The immunogenicity of cancer cells is crucial for the induction of an antitumor immune response. Reduced expression of key factors of the antigen processing machinery (APM) is responsible for the loss of HLA class I molecules on the surface of cancer cells, which contributes to immune escape. Recent studies have demonstrated that treatment with HDACi, particularly with vorinostat, induces expression of APM proteins, TAP1 and TAP2, and of HLA class I molecules on the surface of merkel carcinoma cells [116]. HDACi were also described to induce the expression of program cell death ligand-1 (PD-L1) interacting with program death-1 (PD-1) to inhibit T cells [117–119]. This suggests that combination with anti-PD-1 or anti-PD-L1 therapies may be effective in enhancing the antitumor effects of HDACi. However, HDACi implication in antitumor response is complex, given that these molecules also act on several immune cells of the tumor microenvironment, such as T lymphocytes, regulatory T cells, and myeloid cells, and need to better understand to anticipate unexpected effects [120].

6 FDA-approved HDACi

6.1 Vorinostat

Vorinostat or suberoylanilide hydroxamic acid (SAHA) is a hydroxamate HDACi structurally and functionally related to trichostatin A (Table 19.2). This molecule has a broad-spectrum of action (class I, II, and IV HDACi) and is probably the HDACi the most studied and evaluated in clinic. Vorinostat was the first HDACi approved by US FDA for the treatment of cutaneous T-cell lymphoma (CTCL) in 2006. The half-life of vorinostat in plasma is ranging from 1.5 to 2 hours, when

TABLE 19.2 Structure and applications of the FDA-approved HDACi.

Names	Structure	FDA approved	Application
Suberoylanilide hydroxamic acid (Vorinostat)		2006	Cutaneous T-cell lymphoma
Romidepsin (Istodax)		2009 2011	Cutaneous T-cell lymphoma Peripheral T-cell lymphoma
Belinostat (Beleodaq)		2014	Peripheral T-cell lymphoma
Panobinostat (Farydak)		2015	Multiple myeloma
Chidamide (Epidaza)		2014	Peripheral T-cell lymphoma (China)

administered orally [121,122]. The fast metabolism is the main mechanism for drug elimination [122]. Vorinostat is well tolerated at a dose of 400 mg/day. However, several adverse events have been described, mainly fatigue, thrombocytopenia, diarrhea and anorexia [123]. Other abnormalities, hematological, biochemical (hyperglycemia, proteinuria) and serum electrolyte parameters, have been observed, generally of grade 1–2. Unfortunately, a poor efficacy was observed on solid tumors when used as a single agent [124]. Therefore combination strategies with other chemotherapeutic agents were and are evaluated (approximately 83 phase II clinical trials and 6 phase III

clinical trials, ClinicalTrials.gov). Vorinostat is currently evaluated in phase III clinical trials in combination with proteasome inhibitor (bortezomib), alkylating agents (temozolomide, carboplatin, thalidomide, lenalidomide), antimicrotubular taxane (paclitaxel), anthracyclines (daunorubicin, idarubicin) and/or antimetabolite (cytarabine) (Table 19.3).

TABLE 19.3 Phase III clinical trials including HDACi in combination with chemotherapeutic agents.

HDACi	Study number	Title
Vorinostat	NCT00473889	A Clinical Trial of Vorinostat (MK-0683, SAHA) in Combination With FDA Approved Cancer Drugs in Patients With Advanced Non-Small Cell Lung Cancer (NSCLC) (0683-056)
	NCT00773747	Study of Vorinostat (MK-0683) or Placebo, in Combination With Bortezomib in Participants With Multiple Myeloma (MK-0683-088 AMN)
	NCT01236560	Vorinostat, Temozolomide, or Bevacizumab in Combination With Radiation Therapy Followed by Bevacizumab and Temozolomide in Young Patients With Newly Diagnosed High-Grade Glioma
	NCT01554852	Use of Thalidomide, Lenalidomide, Carfilzomib, Bortezomib and Vorinostat in the Initial Treatment of Newly Diagnosed Multiple Myeloma Patients
	NCT01802333	Cytarabine and Daunorubicin Hydrochloride or Idarubicin and Cytarabine With or Without Vorinostat in Treating Younger Patients With Previously Untreated Acute Myeloid Leukemia
	NCT01386398	Vorinostat With or Without Bortezomib in Treating Patients With Refractory or Recurrent Stage IIB, Stage III, or Stage IV Cutaneous T-Cell Lymphoma
Romidepsin	NCT03355768	Romidepsin Versus Combination of Romidepsin Plus Pralatrexate in PTCL
Panobinostat	NCT01023308	Panobinostat or Placebo With Bortezomib and Dexamethasone in Patients With Relapsed Multiple Myeloma
Chidamide	NCT02482753	Trial of Chidamide in Combination With Exemestane in Patients With Advanced Breast Cancer
	NCT05191914	Clinical Study of Fulvestrant Combined With Chidamide in the Treatment of Hormone Receptor-positive Advanced Breast Cancer Resistant to CDK4/6 Inhibitors
	NCT04668690	Clinical Study of Mitoxantrone Hydrochloride Liposome Injection vs. Chidamide in Patients With Relapsed/Refractory PTCL
	NCT05075460	Tucidinostat, Azacitidine Combined With CHOP Versus CHOP in Patients With Untreated Peripheral T-cell Lymphoma
	NCT04231448	Phase III Study of Tucidinostat in Combination With R-CHOP in Patients With Newly Diagnosed Double-Expressor DLBCL
	NCT05253066	Chidamide Combined With Exemestane (+/- Goserelin) Versus Neoadjuvant Chemotherapy in Patients of Stage II-III HR-positive/HER2-negative Breast Cancer
	NCT04490590	A Clinical Trial of Chidamide Combined With Etoposide in Relapsed or Refractory NK/T-cell Lymphoma

6.2 Romidepsin

Romidepsin is a natural bicyclic peptide isolated from *Chromobacterium violaceum* [125,126] (Table 19.2). This molecule inhibits mainly class I HDACs. Romidepsin is a prodrug that requires reduction of the disulfide bond in its structure to interact with the zinc in the HDAC catalytic site. After systemic administration, the plasma half-life is ranging from 1.9 to 8.1 hours [127–129]. This molecule was approved by FDA in 2009 for the treatment of patients with CTCL who have received at least one prior systemic therapy after a large phase II study showing an overall response rate of 34% [130]. Romidepsin also showed an interesting significant activity in patients with PTCL [131]. Therefore FDA approved romidepsin for the treatment of patients with PTCL who have failed or who were refractory to at least one prior systemic therapy in 2011. The route of administration of Romidepsin is intravenously. Nausea, fatigue and hematologic disorders, which can be serious in some cases, constitute the main adverse effects described. Electrocardiography and serum electrolytes abnormalities have also been reported [46].

As for vorinostat, the limited activity of romidepsin in solid tumors as a single agent has led to the evaluation of combination strategies in clinical trials. (21 phase II clinical trials and 1 phase III clinical trials, ClinicalTrials.gov) (Table 19.3).

6.3 Belinostat

Belinostat is a class I and class II HDACi belonging to the hydroxamate family (Table 19.2). In 2014 belinostat was approved by the FDA for the treatment of patients with refractory or relapsed PTCL after prior therapy [42]. After intravenous injection, this HDACi has a plasma half-life ranging from 0.7 to 4 hours [132–134]. Results from a phase II clinical trial in patients with relapsed or refractory PTCL were the basis for approval [135]. Belinostat was administered at a dose of 1000 mg/m² intravenously on days 1–5 of a 21-day cycle. In this study, the overall response rate was 25.8%. Side effects were limited, showing that this molecule was well tolerated. These results were confirmed in a second phase II clinical trial and demonstrated greater activity of belinostat on PTCL than on CTCL (objective response rate of 25% and 14%, respectively) [136]. This molecule has also been evaluated using an oral formulation. As with intravenous administration, patients received 1000 mg/m² of oral belinostat. This formulation did not alter the toxicity profile and led to an AUC and plasma half-life similar to those obtained after intravenous injection [137]. Thus oral rather than intravenous administration of belinostat is supported by these results.

As with other HDACi, the low activity of belinostat in solid tumors [138] has led to the evaluation of this molecule in combination with current chemotherapeutic agents (26 phase II clinical trials, ClinicalTrials.gov), mainly alkylating agents (cisplatin, carboplatin and paclitaxel).

6.4 Panobinostat

Panobinostat is a hydroxamate HDACi inhibiting class I, II, and IV HDACs (Table 19.2). The first route of administration of this HDACi evaluated was intravenous. Depending on the dose administrated, the plasmatic half-life is ranging from 7.8 to 36.9 hours (from 4.8 to 20 mg/m²) [139,140]. An oral formulation was developed and evaluated in clinic (single oral dose of 30 mg). In this case, the plasmatic half-life is approximately 30 h [141,142]. With these two routes of administration, a good tolerance

profile was observed in these phase I clinical trials. The first interest of panobinostat in clinic was observed in a phase II study in patients with relapsed or refractory Hodgkin's lymphoma after autologous stem cell transplantation. Indeed, in 74% of the patients, a tumor reduction was observed with an objective response rate of 27% [143]. Panobinostat was administrated orally, in this study, at a dose of 40 mg three times per week in a 21 days cycle. However, numerous adverse events were observed (79% grade 3–4 thrombocytopenia).

Recently, panobinostat was approved by FDA, in combination with bortezomib and dexamethasone, for the treatment of patients with multiple myeloma who have received at least two prior regimens, including bortezomib and immunomodulatory agent. A phase III clinical trials, enrolling 768 patients separated in two arms and named PANORAMA1, is at the origin of the approval [144]. In the panobinostat group, a significant increase of the median progression-free survival was observed compared with the placebo group (11.99 months and 8.08 months respectively). Moreover, in the panobinostat group, an increase of the proportion of patients with a complete or near complete response was also observed compared with placebo group (27·6% and 15·7%, respectively).

Panobinostat was and is currently evaluated in clinical trials in combination with chemotherapeutic agents (40 phase II clinical trials and 2 phase III clinical trials, ClinicalTrials.gov) (Table 19.3). Regarding the interesting results obtained, a high number of phase II or III clinical trials are ongoing to evaluate the efficacy of panobinostat alone or in combination for the treatment of a large panel of cancer.

6.5 Chidamide

Chidamidine is the first benzamide to be approved in clinic for the treatment of relapsed and refractory PTCL in China in 2014 [49] (Table 19.2). This molecule was administrated orally. The molecule was relatively well-tolerated and toxicity was dependent of the doses (32.5–50 mg) and of the schedule of administration (2 or 3 times per week for 4 consecutive weeks every 6 weeks) [145]. The half-life in blood ranged from 16 to 18 hours depending on the dose (25–50 mg). The maximal plasma concentration was rapidly reached within 0.5–2 hours and return to baseline after 48 hours. A phase II clinical trial was performed on 79 patients with PTCL treated orally with 30 mg twice per week [146]. The overall response rate was 28% (22 of 79). A particular activity was obtained in angioimmunoblastic T-cell lymphoma subtype. The main grade 3 adverse effects observed were thrombocytopenia (22%), leucopenia (13%) and neutropenia (11%).

Several clinical trials using chidamidine in combination with other chemotherapeutic agents have been completed or are ongoing, all in China (84 phase II clinical trials and 7 phase III clinical trials, ClinicalTrials.gov) (Table 19.3).

Currently a large number of molecules are under development. The molecules under clinical evaluation are mainly new generation hydroxamates, such as entinostat, abexinostat, pracinostat, and quisinostat, but also ricolinostat, a selective HDAC6 inhibitor [147]. Many other compounds with promising properties on cancer cells are currently being studied, such as TMP195, a selective class IIa inhibitor, and citarinostat, a selective second-generation HDAC6 inhibitor, among others.

7 Why HDACi failed in clinic for the treatment of solid tumors?

The main clinical application of HDACi is currently in the treatment of hematological malignancies, as mentioned above. In solid tumors, encouraging results have been obtained in phase I

clinical trials with several HDACi as monotherapies [148]. Unfortunately, in phase II clinical trials, results have been disappointing, with a small number of patients achieving a complete or partial response [148]. The explanation for this difference of activity is not fully understood. However, several characteristics of these molecules may be responsible for these failures, such as poor pharmacokinetics that prevent therapeutic concentrations from being reached.

8 Short half-life

HDACi, and particularly hydroxamates, present a fast clearance from blood. For examples, when administrated intravenously vorinostat, romidepsin, belinostat, and panobinostat have a half-life of 1.5–2 hours [121,122], 1.9–8 hours [127–129], 0.7–4 hours [132–134], and 7.8–36.9 hours [139,140], respectively. These values are dependent of the dose injected. The administration route can also affect the elimination rate of HDACi such as for panobinostat that displays a plasmatic half-life of approximately 30 hours when administrated orally [141,142]. Thus the rapid clearance of these molecules and the limited concentrations obtained in the blood lead to a too low proportion of HDACi reaching the malignant tissue to obtain a major antitumor effect. Indeed, in the case of hematological malignancies, HDACi are in direct contact with circulating tumor cells and can thus exert their antitumor activity. To overcome these problems, an increase in the amount of molecule administered could be considered. Unfortunately, systemic injection of HDACi is generally associated with adverse effects, some of which are quite serious.

9 Toxicity

The induction of adverse effects is one of the major limitations in the use of HDACi in the clinics. The route of administration, usually systemic, induces serious hematological toxicity (\geq grade 3) that leads to thrombocytopenia, neutropenia, leucopenia and anemia depending on the molecule used. Additional adverse effects were described with a consequence on the general state of the patients, such as asthenia, nausea, vomiting, cardiovascular disorders (atrial fibrillation, tachycardia, thrombosis, hyperglycemia, and hypertension), and ionogram anomalies (hyponatremia and hypophosphatemia) [148]. Some of these side effects normalize after treatment is stopped. The similar toxicity profile of HDACi, regardless of the nature of the molecule, suggests that they are more dependent on effects on the target than on nonspecific activity.

10 Metabolism

The rapid metabolism of HDACi is a major concern that contributes to their limited antitumor effect observed in the clinic. Depending on their nature, different mechanisms leading to the inactivation of HDACi are involved. The main class of HDACi, the hydroxamates, including vorinostat, belinostat, and panobinostat, is generally oxidized and O-glucuronylated by UDP-glucuronosyltransferases [149,150]. Some molecules, mainly derived from natural sources, such as

romidepsin, largazole, and psammaplin A, require metabolic activation by reduction of a disulfide bond which releases the zinc-binding group [151]. A glutathione conjugate, metabolized by cytochrome P450, was described and contribute to inaction of romidepsin *in vivo* [152,153]. Phenyl butyrate (PB), a HDACi from short chain fatty acid, is β -oxidized to phenylacetate and rapidly eliminated *in vivo* upon glutamine addition [154].

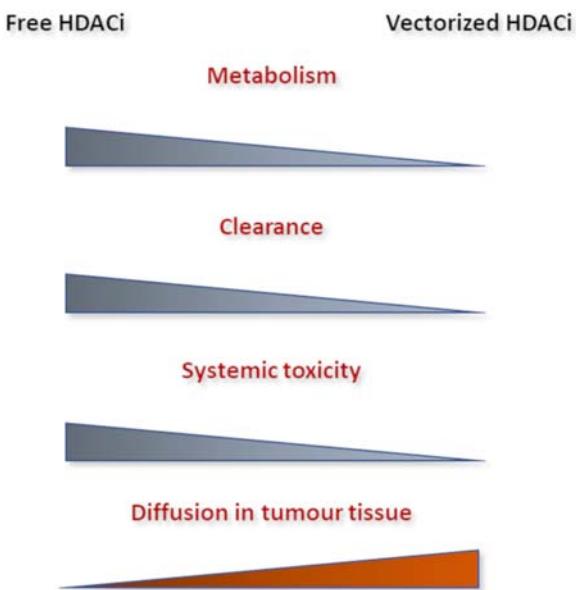
11 New perspectives

11.1 Vectorization

As mentioned above, HDACi are rapidly cleared from the blood circulation due to short half-life in plasma and fast metabolism [40]. These defaults probably contribute to the limited efficacy of these molecules on solid tumors by limiting the fraction of active molecules reaching malignant tissues. Unfortunately, the toxicities of HDACi demonstrated in clinical trials prevent the increase of the doses administered to improve the antitumor effect.

The vectorization of HDACi, to improve their antitumor activity *in vivo* and also to decrease the adverse effects, appears to be a promising strategy. Indeed, this field of research is rapidly expanding and has led to interesting studies suggesting that this approach could be appropriate for this class of epigenetic modulators. The objectives of vectorization are mainly to improve half-life of molecules in plasma, to decrease hematological toxicity by protecting active molecules during blood circulation, to allow the use of poorly soluble molecules, and to increase the quantity of molecules reaching the tumor tissues (Figure 19.4). The latter objective is based on the description of the enhanced permeability and retention effect (EPR), demonstrated by Sato et al. in tumor blood vessels [155]. The efficacy of this strategy has already been proven with doxorubicin. A formulation of this chemotherapeutic agent encapsulated in pegylated liposomes, called Doxil, was approved by FDA for the treatment of advanced metastatic ovarian cancer, Kaposi's sarcoma and for use in combination with bortezomib in patients with multiple myeloma. The main improvement observed with this vectorized doxorubicin is a better distribution of the molecule *in vivo*, which leads to a decrease in adverse effects such as cardiac toxicity in human clinical trials [156–158].

Several studies have applied this strategy to different HDACi and mainly to vorinostat. Vectorized vorinostat was evaluated *in vivo* in rats after iv or oral administration using solid lipid nanoparticles or poly(ethylene glycol)-b-poly (DL-lactic acid) (PEG-b-PLA) micelles [159,160]. An improvement of the solubility, half-life in blood and clearance were observed. The antitumor effect of vorinostat-loaded poly(DL-lactide-co-glycolide) (PLGA) nanoparticles was demonstrated *in vitro* on lung cancer and glioblastoma cells compared to free molecules [161,162]. This formulation was shown to be biocompatible and to lead to a wide distribution of vorinostat *in vivo* [163]. Unfortunately, the *in vivo* antitumor activity of PLGA-vorinostat was not evaluated in these studies. However, using xenograft models of colorectal and prostate carcinomas, it was demonstrated that combination of PLGA-vorinostat with chemoradiotherapy improves the antitumor effect compared to the same combination using free vorinostat [164]. The same benefice was obtained with redox-sensitive iodinated polymersomes loaded with vorinostat in a breast cancer model in mice [165]. As for vorinostat, belinostat-loaded PLGA nanoparticles, modified with a penetrating polymer, induced a sustained hyperacetylation and an increased toxicity in bladder cancer cells compared to

**FIGURE 19.4**

Improvement of HDACi properties using vectorization.

the free drug. *In vivo*, an increase of the intratumoral acetylation of histone H4 associated with a strong decrease of the tumor volume was obtained confirming the promising results observed *in vitro* [166]. A pH sensitive PLGA-vorinostat, surrounded with hybrid membrane derived from red blood cells, also showed an improve activity on lung cancer metastasis in mice [167]. On breast cancer and lung cancer cell lines, the cytotoxicity of vorinostat was improved when loaded in solid lipid nanoparticles or nanostructured lipid carriers [160,168]. In a study using pH-sensitive vorinostat prodrug loaded on norbornenyl-poly(ethylene oxide) nanoparticles, in a model of asbestos related cancer called mesothelioma, the authors observed an intratumoral induction of histone acetylation following iv injection compared to free drug without hematological or systemic toxicities. However, despite the demonstration of vorinostat activity into the tumor tissue, no decrease in tumor mass was observed [169]. The same group applied this strategy to vectorize CI-994 (tacidenaline), a HDACi belonging to the benzamide family [170]. To improve the benefit of their approach *in vivo*, the authors used a new HDACi analog of trichostatin A, called NODH, active at nanomolar concentrations [171]. In a syngeneic mice model of intraperitoneal mesothelioma, they observed a strong reduction of the tumor mass (80%) with the vectorized NODH whereas the free molecule has no effect [172]. This demonstrates the interest of vectorization strategy for HDACi to improve their delivery in tumor tissues and then, their efficacy [173]. Solid mesoporous silica nanoparticles were also used to deliver vorinostat into cancer cells. Improved solubility, permeability, and anticancer activity were achieved *in vitro* [174]. *In vivo*, this approach was evaluated with silica nanoparticles coloaded with c-FLIP siRNA and shown a safe profile [175]. A study using β -cyclodextrin-poly (β -Amino Ester) nanoparticles loaded with panobinostat showed an interesting

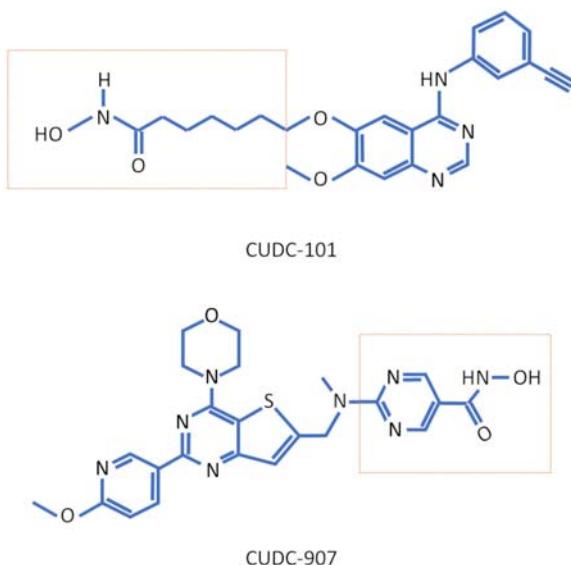
antitumor effect after intracranial administration in a model of glioma in mice. This formulation allowed for a slow release of the drugs, which could help improve their action [176]. With the objective to target CD44 expressing cancer cells, solid lipid nanoparticles loaded with vorinostat coated with hyaluronic acid (HA) were produced [160]. This active targeting strategy was also efficient *in vivo*, in a model of lung cancer, with hyaluronan-based copolymer encapsulating gefitinib and vorinostat. Indeed, a targeting of tumors was obtained after iv injection as well as a reduced toxicity compared to free drugs. A stronger decrease of tumor growth was also obtained after intrapulmonary administration compared to free drugs [177]. This combination of vorinostat with other chemotherapeutic loaded in a nanoparticle (Wnt- β catenin antagonist [178], tamoxifen [179], erlotinib [180]) nanogels (with etoposide [181], doxorubicin [182] or supramolecular self-assemblies (cisplatin [183]) or doxorubicin [184]) was evaluated *in vitro* and *in vivo* and showed a benefit in the antitumor activity and toxicity compared to free drugs. This strategy was also applied to benzamide-based HDACi. Indeed, the antitumor activity of chidamide was enhanced when loaded into nanoparticles with targeting properties based on the use of magnetism or folate-based functionalization *in vivo* [185,186]. Short-chain fatty acid HDACi were also vectorized to evaluate the benefit of this approach on this class of molecules. Cholesteryl butyrate solid lipid has shown a better activity than free butyrate on breast and promyelocytic leukemia cancer cell lines [187]. Recently VPA was loaded on cellulose or dextran-based nanoparticles through a cleaved linkage inside the cells to achieve intracellular delivery of the molecule [188–190]. Codelivery systems comprising VPA and chemotherapeutic agents, cisplatin or doxorubicin, using metal/organic or polymeric prodrug micelles were also developed and showed an improved activity in cells and *in vivo* [191,192].

In conclusion, vectorization strategies could be a promising alternative to reduce side effects and allow the use of HDACi for the treatment of a broad spectrum of cancers. However, these promising results must now be confirmed by clinical trials in order to confirm the interest of these approaches.

12 Dual inhibitor compounds

Recent developments in HDACi therapy include combinations with conventional chemotherapeutic agents [193]. These strategies appear to work in part by “sensitization” of cancer cells by HDAC inhibitors to other antitumor agents in a synergistic or additive manner. To improve antitumor efficacy and also to facilitate drug administration, bifunctional molecules including HDACi have therefore been designed. This formulation also provides new properties such as reversing drug resistance by the modification of metabolism and/or escape to multidrug resistance.

In dual inhibitors, the zinc binding group is preserved to maintain HDAC inhibitory activity and the cap region, not involved in critical interactions with HDAC active site, can be used to link another molecule. Currently the main dual inhibitors are constituted of HDACi with kinase inhibitors. In CUDC-101 molecule, erlotinib is coupled to SAHA (Figure 19.5). This molecule exhibits strong HDACi activity as well as strong inhibition of RTKs, including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and the human epidermal receptor 2 (HER2), at nanomolar concentrations [194]. Several anticancer activities, such as

**FIGURE 19.5**

Structure of bifunctional molecules evaluated in clinic. Rectangle indicates the HDACi part of the molecule.

apoptosis induction and reduction of proliferation were observed on cell lines from different cancers, such as nonsmall-cell lung cancer (NSCLC) and thyroid cancer, *in vitro* and *in vivo* [194]. CUDC-101 was evaluated in phase I clinical trials and showed a good tolerability when used alone or combined with cisplatin and radiotherapy [195,196]. However, additional studies are necessary to conclude on the safety and the potential of this molecule in the clinic. A second molecule combining HDAC and phosphatidyl inositol-3 kinase (PI3K) inhibition, obtained with the PI3K inhibitor pictilisib, was developed with nanomolar activity on both targets (Figure 19.5). PI3K is involved in the regulation of several oncogenic properties, such as proliferation, migration, and resistance to apoptosis among others. This molecule, called fimepinostat or CUDC-907, has shown an interesting activity on diffuse large B-cell lymphoma (DLBCL), characterized by an activation of PI3K, as well as on chronic lymphocytic leukemia (CLL) by suppressing STAT3 and RAF/MEK/ERK signaling leading to a decrease in the expression of antiapoptotic proteins of the BCL2-family MCL-1, BCL-XL, and BCL-2. In preclinical models of hematologic and solid cancers, fimepinostat was more effective in reducing tumor growth compared with molecules used as single agents. In particular, this dual inhibitor repressed the expression of *MYC* gene, in different *in vivo* models of *MYC*-amplified cancers including PDX models [197]. These results suggest that CUDC-907 may be of interest for the treatment of *MYC*-dependent cancers. In a recent phase I clinical trial, CUDC-907 was evaluated, alone or in combination with rituximab, in patients with DLBCL including *MYC* altered cases. The objective response rate was 64% in *MYC*-altered patients while it was 29% in *MYC* unaltered, and 17% in patients with unknown *MYC* status. The median of duration of response was 13.6 months in *MYC*-altered patients, 6.0 months in *MYC*-unaltered, and 7.8 months in patients with unknown *MYC* status [198]. CUDC-907 is in advanced stage of clinical trials.

Recently, a molecule with selective HDAC6 and JAK2 inhibition at nanomolar concentrations was produced. This molecule showed an antiproliferative effect on a large panel of hematologic cells as well as an inhibition of colony formation and an induction of apoptosis [199]. The same group synthetized a second series of vorinostat/ruxolitinib derivatives with a less selective activity on specific HDAC or JAK family members with always an anticancer effect on cell lines [200].

Several derivatives of vorinostat coupled with other chemotherapeutic agents, such as topoisomerase II inhibitors [201], tamoxifen or raloxifene [202], were also produced and showed anticancer activity. The synthesis of dual inhibitors is a vast area of research. The number and the diversity of molecules increases regularly as well as the proteins cotargeted. For example, dual molecules with inhibition of HDAC and tubulin, fibroblast growth factor receptor (FGFR), cyclin-dependent kinases (CDK) or vascular endothelial growth factor receptor (VEGFR) were described (For review: Ref. [203]).

13 Combination with immunotherapies

Whereas the antitumor effect of HDACi on solid tumors is disappointing, numerous studies demonstrated the potential of these molecules in combination with classical chemotherapeutic agents (For reviews see Ref. [193,204]). Immunotherapy, mainly immune check point inhibitors (ICI), has recently revolutionized the therapeutic management of cancers due to the spectacular antitumor effect observed in some patients. However, depending on the origin of the malignant tumors, the efficacy is not equivalent and in too many cases, patients do not benefit from these therapies due to resistances, which are currently being investigated. In particular, the tumor microenvironment and the poor immunogenicity of tumor cells are involved in the limited efficacy of immunotherapies. Interestingly, several studies have shown that HDACi can act on these two aspects to increase anti-tumor immune responses. Thus this part will focus on the potential of HDACi in combination with immunotherapies.

HDACi have been described to increase tumor immunogenicity and immunotherapy efficacy by different mechanisms. We have previously mentioned that HDACi lead to an increase in HLA class I molecules on the surface of cancer cells that improves their recognition by immune cells. However, in several cancer cells, HDACi increase PD-L1 expression [117,205,206], which interacts with PD-1 to inhibit immune responses. This may explain the limited efficacy of HDACi used alone in solid tumors. Interestingly, HDACi, including vorinostat, romidepsin, and entinostat, has also been shown to enhance anti-PD-1 therapies in melanoma and lung cancer models *in vivo* compared to these therapies used alone [205,207,208]. This suggests that this combination should be a strategy to improve HDACi antitumor activity *in vivo*. A study performed with belinostat, in a model of hepatocellular carcinoma, suggests that depending on the tumor sensitivity to ICI, combination with anti-PD-1 or anti-CTLA-4 will be more efficient [209]. Thus a good selection of the patients could significantly improve the clinical benefit of these combinations.

Inhibitors of DNA methyl transferases (DNMTi) were demonstrated to be immunogenic by increasing immunogenicity of tumor cells. Different mechanisms were described including activation of interferon response through reactivation of endogenous retroviruses (ERVs) [210,211] and induction/increase expression of cancer testis antigens, such as NY-ESO-1 and MAGE-A3

[212,213]. The combination of HDACi, vorinostat or VPA, with DNMTi, in particular decitabine, was shown to improve decitabine-induced immunogenicity in different model of cancers *in vitro* and *in vivo* [214–216] by acting on methylation and acetylation of gene promotors as described for *CTAG1B* gene encoding for NY-ESO-1 [217,218]. Association with an anti-PD-1 and anti-CTLA-4 improve the antitumor effect of decitabine/HDACi combination in mice models of colon and breast cancers [219].

These results obtained in preclinical cancer models have led to the evaluation of HDACi in combination with immunotherapies in the clinic [120] (Table 19.4). Several phase I and II clinical

TABLE 19.4 Clinical trials including HDACi in combination with immunotherapy agents.

HDACi	Immunotherapy	Study number	Title
Vorinostat	Pembrolizumab	NCT04357873	Efficacy of Immunotherapy Plus a Drug in Patients With Progressive Advanced Mucosal Cancer of Different Locations
	Pembrolizumab	NCT04190056	Pembrolizumab and Tamoxifen With or Without Vorinostat for the Treatment of Estrogen Receptor Positive Breast Cancer
	Pembrolizumab	NCT02395627	Reversing Therapy Resistance With Epigenetic-Immune Modification. (Breast neoplasms)
	Pembrolizumab	NCT03426891	Pembrolizumab and Vorinostat Combined With Temozolamide for Newly Diagnosed Glioblastoma
	Pembrolizumab	NCT03150329	Pembrolizumab and Vorinostat in Treating Patients With Relapsed or Refractory Diffuse Large B-Cell Lymphoma, Follicular Lymphoma, or Hodgkin Lymphoma
	Pembrolizumab	NCT02619253	Phase I/Ib Study of Pembrolizumab With Vorinostat for Patients With Advanced Renal or Urothelial Cell Carcinoma
Romidepsin	Pembrolizumab	NCT03278782	Study of Pembrolizumab (MK-3475) in Combination With Romidepsin
	Durvalumab	NCT04257448	Safety and Tolerance of Epigenetic and Immunomodulating Drugs Combined With Chemotherapeutics in Patients Suffering From Advanced Pancreatic Cancer
	Durvalumab	NCT03161223	Durvalumab in Different Combinations With Pralatrexate, Romidepsin and Oral 5-Azacitidine for Lymphoma
	Nivolumab	NCT02393794	Cisplatin Plus Romidepsin & Nivolumab in Locally Recurrent or Metastatic Triple Negative Breast Cancer (TNBC)
Belinostat	Durvalumab, tremelimumab	NCT05154994	Tremelimumab, Durvalumab, and Belinostat for the Treatment of ARID1A Mutated Metastatic or Unresectable, Locally Advanced Urothelial Carcinoma (RESOLVE)
	Nivolumab, ipilimumab	NCT04315155	Evaluating Safety & Efficacy Belinostat Combo w Nivo Alone & w Ipi in Patients w Treated Metastatic/Advanced Carcinomas w ARID1A Lof Mutation

(Continued)

TABLE 19.4 Clinical trials including HDACi in combination with immunotherapy agents.
Continued

HDACi	Immunotherapy	Study number	Title
Panobinostat	Ipilimumab	NCT02032810	Phase I of Histone Deacetylase (HDAC) Inhibitor Panobinostat With Ipilimumab With Unresectable III/IV Melanoma
Chidamide	Nivolumab	NCT02718066	Study of HBI-8000 With Nivolumab in Melanoma, Renal Cell Carcinoma and Non-Small Cell Lung Cancer
	Pembrolizumab	NCT05141357	A Study of HBI-8000 (Tucidinostat) With Pembrolizumab in Non-Small Cell Lung Cancer

clinicaltrials.gov.

trials with HDACi and ICI were conducted using vorinostat, entinostat, mocetinostat, domatinostat or romidepsin in different solid tumors. No major adverse events were observed. Entinostat was evaluated in combination with an anti-PD-1 in a phase II clinical trial on NSCLC and melanoma. Whereas no significant effect was observed in NSCLC (ORR: 9%) [220], an ORR of 19% was obtained in melanoma patients [120]. In this study, patients have progressed after ICI therapy before to receive the combination. This situation, a resistance profile to immunotherapy, could not be the appropriate population of patients to evaluate this combination. In another phase II study in triple negative breast cancer (TNBC) patients evaluating entinostat combined with anti-PD-1 and anti-CTLA-4, an ORR 30% was obtained that needs now to be confirmed. Indeed, only 20 patients were evaluable in this study [221]. Combination of entinostat with anti-PD-L1 was also evaluated in TNBC in a phase II clinical trial. However, no clinical benefit and an increase of severe adverse events were observed [222]. This observation raises the question of the interest of the combination HDACi with anti-PD-1 rather than with anti-PD-L1. Further studies are needed to answer this question. Two class I HDACi, mocetinostat and domatinostat, are under clinical evaluation in combination with ICI in solid tumors. A good safety profile was observed as well as an interesting antitumor response associated with changes in the tumor microenvironment such as an increase in T-cell infiltration.

Combinations including HDACi and decitabine were mainly evaluated in phase I or II clinical trials in acute myeloid leukemia (AML) or myelodysplastic syndrome (MS). The association with VPA did not improve the effect of decitabine and resulted in toxicities [223–225]. This combination has also been evaluated in patients with NSCLC. While activity was demonstrated by re-expression of hypermethylated genes, toxicity was observed. The induction of adverse effects led to consideration of other HDACi in combination with decitabine [226]. The combination decitabine with vorinostat has been studied in different settings. In patients with solid tumors, non-Hodgkin's lymphomas, AML or MS, the combination was tolerable and showed promising activity [227–229]. To enhance the benefits of these combinations, the addition of chemotherapies has been used. Depending on the therapeutic agents combined with decitabine and vorinostat, toxicities have been observed in pediatric patients with relapsed or refractory B-cell acute lymphoblastic leukemia (ALL) [230]. However, in a study of relapsed ALL patients and in a recent phase I/II clinical

trial in relapsed/refractory pediatric AML, interesting efficacy was observed particularly in patients with epigenetic alterations [231,232]. In two recent clinical studies using chidamide in combination with decitabine and cytarabine, aclarubicin or idarubicin, and G-CSF in patients with relapsed/refractory AML, good tolerability was achieved with interesting efficacy. All these results suggest that combination therapies including HDACi and decitabine could improve the antitumor effects of conventional chemotherapeutic agents. With the recent development of immunotherapies and the preclinical data on the immunogenicity of the decitabine/HDACi combination, the combination of these molecules could be interesting to induce antitumor immune responses.

14 Conclusion

HDACi have demonstrated their potential for the treatment of several malignant diseases. Unfortunately, some of their properties limit their use in solid tumors and raise the question of their interest in this context. However, recent works open new perspectives to improve the efficacy of these epigenetic modulators such as the modification of their formulation and the combination with immunotherapies. The effects observed *in vivo* are not only the result of HDACi activity on tumor cells. Indeed, it has been shown that HDACi can modify the tumor microenvironment by acting on different immune cells, directly or not, showing the complexity of the actions of epigenetic modulators *in vivo*. All the new therapeutic approaches and a better understanding of the impact of HDACi on tumor microenvironment should provide a better overview of the real potential of HDACi for the treatment of cancer in the coming years.

References

- [1] Yang XJ, Gregoire S. Class II histone deacetylases: from sequence to function, regulation, and clinical implication. *Mol Cell Biol* 2005;25:2873–84.
- [2] Ho TCS, Chan AHY, Ganesan A. Thirty years of HDAC inhibitors: 2020 insight and hindsight. *J Med Chem* 2020;63.
- [3] Asfaha Y, et al. Recent advances in class IIa histone deacetylases research. *Bioorg Med Chem* 2019;27.
- [4] Valenzuela-Fernandez A, Cabrero JR, Serrador JM, Sanchez-Madrid F. HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions. *Trends Cell Biol* 2008;18.
- [5] Hai Y, Shinsky SA, Porter NJ, Christianson DW. Histone deacetylase 10 structure and molecular function as a polyamine deacetylase. *Nat Commun* 2017;8:15368.
- [6] Shinsky SA, Christianson DW. Polyamine deacetylase structure and catalysis: prokaryotic acetylpolyamine amidohydrolase and eukaryotic HDAC10. *Biochemistry* 2018;57:3105–14.
- [7] Frye RA. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* 2000;273:793–8.
- [8] Chen L, Fischle W, Verdin E, Greene WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science* 2001;293:1653–7.
- [9] Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. *J Biol Chem* 2002;277:25904–13.

- [10] Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 1997;90:595–606.
- [11] Jeong JW, et al. Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. *Cell* 2002;111:709–20.
- [12] Martinez-Balbas MA, Bauer UM, Nielsen SJ, Brehm A, Kouzarides T. Regulation of E2F1 activity by acetylation. *EMBO J* 2000;19:662–71.
- [13] Patel JH, et al. The c-MYC oncprotein is a substrate of the acetyltransferases hGCN5/PCAF and TIP60. *Mol Cell Biol* 2004;24:10826–34.
- [14] Wang C, et al. Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. *J Biol Chem* 2001;276:18375–83.
- [15] Kovacs JJ, et al. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Mol Cell* 2005;18:601–7.
- [16] Yuan ZL, Guan YJ, Chatterjee D, Chin YE. Stat3 dimerization regulated by reversible acetylation of a single lysine residue. *Science* 2005;307:269–73.
- [17] Zhang Y, et al. HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. *EMBO J* 2003;22:1168–79.
- [18] Morris MJ, Karra AS, Monteggia LM. Histone deacetylases govern cellular mechanisms underlying behavioral and synaptic plasticity in the developing and adult brain. *Behav Pharmacol* 2010;21:409–19.
- [19] Witt O, Deubzer HE, Milde T, Oehme I. HDAC family: what are the cancer relevant targets? *Cancer Lett* 2009;277:8–21.
- [20] Bhaskara S, et al. Deletion of histone deacetylase 3 reveals critical roles in S phase progression and DNA damage control. *Mol Cell* 2008;30:61–72.
- [21] Chang S, et al. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. *Cell* 2006;126:321–34.
- [22] Kasler HG, et al. Histone deacetylase 7 regulates cell survival and TCR signaling in CD4/CD8 double-positive thymocytes. *J Immunol* 2011;186:4782–93.
- [23] Kasler HG, Verdin E. Histone deacetylase 7 functions as a key regulator of genes involved in both positive and negative selection of thymocytes. *Mol Cell Biol* 2007;27:5184–200.
- [24] Navarro MN, Goebel J, Feijoo-Carnero C, Morrice N, Cantrell DA. Phosphoproteomic analysis reveals an intrinsic pathway for the regulation of histone deacetylase 7 that controls the function of cytotoxic T lymphocytes. *Nat Immunol* 2011;12:352–61.
- [25] Jin Z, Wei W, Dechow PC, Wan Y. HDAC7 inhibits osteoclastogenesis by reversing RANKL-triggered beta-catenin switch. *Mol Endocrinol* 2013;27:325–35.
- [26] Pham L, et al. HDAC3 and HDAC7 have opposite effects on osteoclast differentiation. *J Biol Chem* 2011;286:12056–65.
- [27] Yan B, et al. HDAC6 regulates IL-17 expression in T lymphocytes: implications for HDAC6-targeted therapies. *Theranostics* 2017;7:1002–9.
- [28] Giaginis C, et al. Clinical significance of histone deacetylase (HDAC)-1, HDAC-2, HDAC-4, and HDAC-6 expression in human malignant and benign thyroid lesions. *Tumour Biol J Int Soc Oncodev Biol Med* 2014;35:61–71.
- [29] Gueugnon F, et al. New histone deacetylase inhibitors improve cisplatin antitumor properties against thoracic cancer cells. *Oncotarget* 2014;5.
- [30] Lee J-S, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatol* 2004;40:667–76.
- [31] Mithraprabhu S, Kalff A, Chow A, Khong T, Spencer A. Dysregulated Class I histone deacetylases are indicators of poor prognosis in multiple myeloma. *Epigenetics* 2014;9:1511–20.
- [32] Weichert W. HDAC expression and clinical prognosis in human malignancies. *Cancer Lett* 2009;280:168–76.

- [33] Yang H, et al. Analysis of class I and II histone deacetylase gene expression in human leukemia. *Leuk Lymphoma* 2015;1–8. Available from: <https://doi.org/10.3109/10428194.2015.1034705>.
- [34] Adams H, Fritzsche FR, Dirnhofer S, Kristiansen G, Tzankov A. Class I histone deacetylases 1, 2 and 3 are highly expressed in classical Hodgkin's lymphoma. *Expert Opin Ther Targets* 2010;14:577–84.
- [35] Seo J, et al. Expression of histone deacetylases HDAC1, HDAC2, HDAC3, and HDAC6 in invasive ductal carcinomas of the breast. *J Breast Cancer* 2014;17:323–31.
- [36] Wilmott JS, et al. Expression of the class 1 histone deacetylases HDAC8 and 3 are associated with improved survival of patients with metastatic melanoma. *Mod Pathol* 2015;28:884–94.
- [37] Parbin S, et al. Histone deacetylases: a saga of perturbed acetylation homeostasis in cancer. *J Histochem Cytochem* 2014;62:11–33.
- [38] Yoshida M, Hoshikawa Y, Koseki K, Mori K, Beppu T. Structural specificity for biological activity of trichostatin A, a specific inhibitor of mammalian cell cycle with potent differentiation-inducing activity in Friend leukemia cells. *J Antibiot (Tokyo)* 1990;43:1101–6.
- [39] Jung M, et al. Amide analogues of trichostatin A as inhibitors of histone deacetylase and inducers of terminal cell differentiation. *J Med Chem* 1999;42:4669–79.
- [40] Bertrand P, Martinet N. Interpreting clinical assays for histone deacetylase inhibitors. *Cancer Manag Res* 2011;117. Available from: <https://doi.org/10.2147/CMR.S9661>.
- [41] Marks PA. Discovery and development of SAHA as an anticancer agent. *Oncogene* 2007;26:1351–6.
- [42] Poole RM. Belinostat: first global approval. *Drugs* 2014;74:1543–54.
- [43] Richardson PG, et al. Panobinostat: a novel pan-deacetylase inhibitor for the treatment of relapsed or relapsed and refractory multiple myeloma. *Expert Rev Anticancer Ther* 2015;15:737–48.
- [44] Vanhaecke T, Papeleu P, Elaut G, Rogiers V. Trichostatin A-like hydroxamate histone deacetylase inhibitors as therapeutic agents: toxicological point of view. *Curr Med Chem* 2004;11:1629–43.
- [45] Mottamal M, Zheng S, Huang TL, Wang G. Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents. *Molecules* 2015;20:3898–941.
- [46] Grant C, et al. Romidepsin: a new therapy for cutaneous T-cell lymphoma and a potential therapy for solid tumors. *Expert Rev Anticancer Ther* 2010;10:997–1008.
- [47] Iyer SP, Foss FF. Romidepsin for the treatment of peripheral T-cell lymphoma. *Oncologist* 2015;20:1084–91.
- [48] Ganesan A. Romidepsin and the zinc-binding thiol family of natural product HDAC Inhibitors. *Success Drug Discovery* 2016;2:13–24.
- [49] Lu X, Ning Z, Li Z, Cao H, Wang X. Development of chidamide for peripheral T-cell lymphoma, the first orphan drug approved in China. *Intractable Rare Dis Res* 2016;5:185–91.
- [50] Li W, Sun Z. Mechanism of action for HDAC inhibitors—insights from omics approaches. *Int J Mol Sci* 2019;20:E1616.
- [51] Eckschlager T, Plch J, Stiborova M, Hrabetá J. Histone deacetylase inhibitors as anticancer drugs. *Int J Mol Sci* 2017;18:E1414.
- [52] Eickhoff B, et al. Trichostatin A modulates expression of p21waf1/cip1, Bcl-xL, ID1, ID2, ID3, CRAB2, GATA-2, hsp86 and TFIID/TAFII31 mRNA in human lung adenocarcinoma cells. *Biol Chem* 2000;381:107–12.
- [53] Saito A, et al. A synthetic inhibitor of histone deacetylase, MS-27–275, with marked in vivo antitumor activity against human tumors. *Proc Natl Acad Sci U S A* 1999;96:4592–7.
- [54] Shin JY, Kim HS, Park J, Park JB, Lee JY. Mechanism for inactivation of the KIP family cyclin-dependent kinase inhibitor genes in gastric cancer cells. *Cancer Res* 2000;60:262–5.
- [55] Vrana JA, et al. Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. *Oncogene* 1999;18:7016–25.

- [56] Archer SY, Meng S, Shei A, Hodin RA. p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc Natl Acad Sci U S A* 1998;95:6791–6.
- [57] el-Deiry WS, et al. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817–25.
- [58] Condorelli F, Gnemmi I, Vallario A, Genazzani AA, Canonico PL. Inhibitors of histone deacetylase (HDAC) restore the p53 pathway in neuroblastoma cells. *Br J Pharmacol* 2008;153:657–68.
- [59] Zhao Y, et al. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21(Waf1/Cip1). *Mol Cell Biol* 2006;26:2782–90.
- [60] Gui CY, Ngo L, Xu WS, Richon VM, Marks PA. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. *Proc Natl Acad Sci USA* 2004;101:1241–6.
- [61] Sowa Y, et al. Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor. *Cancer Res* 1999;59:4266–70.
- [62] Florenes VA, Skrede M, Jorgensen K, Nesland JM. Deacetylase inhibition in malignant melanomas: impact on cell cycle regulation and survival. *Melanoma Res* 2004;14:173–81.
- [63] Greenberg VL, Williams JM, Cogswell JP, Mendenhall M, Zimmer SG. Histone deacetylase inhibitors promote apoptosis and differential cell cycle arrest in anaplastic thyroid cancer cells. *Thyroid* 2001;11:315–25.
- [64] Strait KA, et al. Cell cycle blockade and differentiation of ovarian cancer cells by the histone deacetylase inhibitor trichostatin A are associated with changes in p21, Rb, and Id proteins. *Mol Cancer Ther* 2002;1:1181–90.
- [65] Qiu L, et al. Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. *Mol Biol Cell* 2000;11:2069–83.
- [66] Sandor V, et al. P21-dependent g1arrest with downregulation of cyclin D1 and upregulation of cyclin E by the histone deacetylase inhibitor FR901228. *Br J Cancer* 2000;83:817–25.
- [67] Fandy TE, Shankar S, Ross DD, Sausville E, Srivastava RK. Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and expressions of cell cycle regulatory genes in multiple myeloma. *Neoplasia* 2005;7:646–57.
- [68] Guo F, et al. Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death inducing signaling complex activity and apoptosis of human acute leukemia cells. *Cancer Res* 2004;64:2580–9.
- [69] Shankar S, et al. Interactive effects of histone deacetylase inhibitors and TRAIL on apoptosis in human leukemia cells: involvement of both death receptor and mitochondrial pathways. *Int J Mol Med* 2005;16:1125–38.
- [70] Singh TR, Shankar S, Srivastava RK. HDAC inhibitors enhance the apoptosis-inducing potential of TRAIL in breast carcinoma. *Oncogene* 2005;24:4609–23.
- [71] Iacomino G, Medici MC, Russo GL. Valproic acid sensitizes K562 erythroleukemia cells to TRAIL/Apo2L-induced apoptosis. *Anticancer Res* 2008;28:855–64.
- [72] Inoue S, Harper N, Walewska R, Dyer MJ, Cohen GM. Enhanced Fas-associated death domain recruitment by histone deacetylase inhibitors is critical for the sensitization of chronic lymphocytic leukemia cells to TRAIL-induced apoptosis. *Mol Cancer Ther* 2009;8:3088–97.
- [73] Matthews GM, Newbold A, Johnstone RW. Intrinsic and extrinsic apoptotic pathway signaling as determinants of histone deacetylase inhibitor antitumor activity. *Adv Cancer Res* 2012;116:165–97.
- [74] Cheng HT, Hung WC. Inhibition of proliferation, sprouting, tube formation and Tie2 signaling of lymphatic endothelial cells by the histone deacetylase inhibitor SAHA. *Oncol Rep* 2013;30:961–7.
- [75] Hellebrekers DM, et al. Identification of epigenetically silenced genes in tumor endothelial cells. *Cancer Res* 2007;67:4138–48.
- [76] Hellebrekers DM, et al. Epigenetic regulation of tumor endothelial cell anergy: silencing of intercellular adhesion molecule-1 by histone modifications. *Cancer Res* 2006;66:10770–7.

- [77] Kwon HJ, Kim MS, Kim MJ, Nakajima H, Kim KW. Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. *Int J Cancer* 2002;97:290–6.
- [78] Srivastava RK, Kurzrock R, Shankar S. MS-275 sensitizes TRAIL-resistant breast cancer cells, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition in vivo. *Mol Cancer Ther* 2010;9:3254–66.
- [79] Heider U, et al. Histone deacetylase inhibitors reduce VEGF production and induce growth suppression and apoptosis in human mantle cell lymphoma. *Eur J Haematol* 2006;76:42–50.
- [80] Mie Lee Y, et al. Inhibition of hypoxia-induced angiogenesis by FK228, a specific histone deacetylase inhibitor, via suppression of HIF-1alpha activity. *Biochem Biophys Res Commun* 2003;300:241–6.
- [81] Sasakawa Y, et al. Antitumor efficacy of FK228, a novel histone deacetylase inhibitor, depends on the effect on expression of angiogenesis factors. *Biochem Pharmacol* 2003;66:897–906.
- [82] Sawa H, et al. Histone deacetylase inhibitors such as sodium butyrate and trichostatin A inhibit vascular endothelial growth factor (VEGF) secretion from human glioblastoma cells. *Brain Tumor Pathol* 2002;19:77–81.
- [83] Zgouras D, Becker U, Loitsch S, Stein J. Modulation of angiogenesis-related protein synthesis by valproic acid. *Biochem Biophys Res Commun* 2004;316:693–7.
- [84] Kim MS, et al. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med* 2001;7:437–43.
- [85] Qian DZ, et al. Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha. *Cancer Res* 2006;66:8814–21.
- [86] Cinatl J, et al. Induction of differentiation and suppression of malignant phenotype of human neuroblastoma BE(2)-C cells by valproic acid: enhancement by combination with interferon-alpha. *Int J Oncol* 2002;20:97–106.
- [87] Camphausen K, et al. Enhancement of in vitro and in vivo tumor cell radiosensitivity by valproic acid. *Int J Cancer* 2005;114:380–6.
- [88] Camphausen K, et al. Enhanced radiation-induced cell killing and prolongation of gammaH2AX foci expression by the histone deacetylase inhibitor MS-275. *Cancer Res* 2004;64:316–21.
- [89] Munshi A, et al. Vorinostat, a histone deacetylase inhibitor, enhances the response of human tumor cells to ionizing radiation through prolongation of gamma-H2AX foci. *Mol Cancer Ther* 2006;5:1967–74.
- [90] Munshi A, et al. Histone deacetylase inhibitors radiosensitize human melanoma cells by suppressing DNA repair activity. *Clin Cancer Res* 2005;11:4912–22.
- [91] Adimoolam S, et al. HDAC inhibitor PCI-24781 decreases RAD51 expression and inhibits homologous recombination. *Proc Natl Acad Sci U S A* 2007;104:19482–7.
- [92] Zhang Y, et al. Attenuated DNA damage repair by trichostatin A through BRCA1 suppression. *Radiat Res* 2007;168:115–24.
- [93] Banuelos CA, et al. Radiosensitization by the histone deacetylase inhibitor PCI-24781. *Clin Cancer Res* 2007;13:6816–26.
- [94] Gaymes TJ, et al. Histone deacetylase inhibitors (HDI) cause DNA damage in leukemia cells: a mechanism for leukemia-specific HDI-dependent apoptosis? *Mol Cancer Res* 2006;4:563–73.
- [95] Lee JH, Choy ML, Ngo L, Foster SS, Marks PA. Histone deacetylase inhibitor induces DNA damage, which normal but not transformed cells can repair. *Proc Natl Acad Sci U S A* 2010;107:14639–44.
- [96] Rosato RR, Almenara JA, Grant S. The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21CIP1/WAF1 1. *Cancer Res* 2003;63:3637–45.
- [97] Ruefli AA, et al. Suberoylanilide hydroxamic acid (SAHA) overcomes multidrug resistance and induces cell death in P-glycoprotein-expressing cells. *Int J Cancer* 2002;99:292–8.
- [98] Hubaux R, et al. Valproic acid improves second-line regimen of small cell lung carcinoma in preclinical models. *ERJ Open Res* 2015;1:00028–2015.

- [99] Yu C, et al. Induction of apoptosis in BCR/ABL + cells by histone deacetylase inhibitors involves reciprocal effects on the RAF/MEK/ERK and JNK pathways. *Cancer Biol Ther* 2003;2:544–51.
- [100] Butler LM, et al. The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. *Proc Natl Acad Sci U S A* 2002;99:11700–5.
- [101] Lee JH, et al. Inhibition of histone deacetylase 10 induces thioredoxin-interacting protein and causes accumulation of reactive oxygen species in SNU-620 human gastric cancer cells. *Mol Cell* 2010;30:107–12.
- [102] Ungerstedt J, Du Y, Zhang H, Nair D, Holmgren A. In vivo redox state of human thioredoxin and redox shift by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). *Free Radic Biol Med* 2012;53:2002–7.
- [103] Mitra A, Mishra L, Li S. EMT, CTCs and CSCs in tumor relapse and drug-resistance. *Oncotarget* 2015;6:10697–711.
- [104] Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat Med* 2013;19:1438–49.
- [105] Buzzese F, et al. HDAC inhibitor vorinostat enhances the antitumor effect of gefitinib in squamous cell carcinoma of head and neck by modulating ErbB receptor expression and reverting EMT. *J Cell Physiol* 2011;226:2378–90.
- [106] Mateen S, Raina K, Agarwal C, Chan D, Agarwal R. Silibinin synergizes with histone deacetylase and DNA methyltransferase inhibitors in upregulating E-cadherin expression together with inhibition of migration and invasion of human non-small cell lung cancer cells. *J Pharmacol Exp Ther* 2013;345:206–14.
- [107] Meng F, Sun G, Zhong M, Yu Y, Brewer MA. Anticancer efficacy of cisplatin and trichostatin A or 5-aza-2'-deoxycytidine on ovarian cancer. *Br J Cancer* 2013;108:579–86.
- [108] Nalls D, Tang SN, Rodova M, Srivastava RK, Shankar S. Targeting epigenetic regulation of miR-34a for treatment of pancreatic cancer by inhibition of pancreatic cancer stem cells. *PLoS One* 2011;6:e24099.
- [109] Rhodes LV, et al. Suppression of triple-negative breast cancer metastasis by pan-DAC inhibitor panobostat via inhibition of ZEB family of EMT master regulators. *Breast Cancer Res Treat* 2014;145:593–604.
- [110] Shah P, Gau Y, Sabnis G. Histone deacetylase inhibitor entinostat reverses epithelial to mesenchymal transition of breast cancer cells by reversing the repression of E-cadherin. *Breast Cancer Res Treat* 2014;143:99–111.
- [111] Wang X, et al. Trichostatin A, a histone deacetylase inhibitor, reverses epithelial-mesenchymal transition in colorectal cancer SW480 and prostate cancer PC3 cells. *Biochem Biophys Res Commun* 2015;456:320–6.
- [112] Giudice FS, Pinto Jr. DS, Nor JE, Squarize CH, Castilho RM. Inhibition of histone deacetylase impacts cancer stem cells and induces epithelial-mesenchyme transition of head and neck cancer. *PLoS One* 2013;8:e58672.
- [113] Han RF, Li K, Yang ZS, Chen ZG, Yang WC. Trichostatin A induces mesenchymal-like morphological change and gene expression but inhibits migration and colony formation in human cancer cells. *Mol Med Rep* 2014;10:3211–16.
- [114] Ji M, et al. HDAC inhibitors induce epithelial-mesenchymal transition in colon carcinoma cells. *Oncol Rep* 2015;33:2299–308.
- [115] Jiang GM, et al. Histone deacetylase inhibitor induction of epithelial-mesenchymal transitions via up-regulation of Snail facilitates cancer progression. *Biochim Biophys Acta* 1833;663–71:2013.
- [116] Ritter C, et al. Epigenetic priming restores the HLA class-I antigen processing machinery expression in Merkel cell carcinoma. *Sci Rep* 2017;7:2290.

- [117] Bensaid D, et al. Assessment of new HDAC inhibitors for immunotherapy of malignant pleural mesothelioma. *Clin Epigenetics* 2018;10:79.
- [118] Sah VR, et al. Epigenetic therapy to enhance therapeutic effects of PD-1 inhibition in therapy-resistant melanoma. *Melanoma Res* 2021. Available from: <http://doi.org/10.1097/CMR.0000000000000791>.
- [119] Shi Y, et al. Romidepsin (FK228) regulates the expression of the immune checkpoint ligand PD-L1 and suppresses cellular immune functions in colon cancer. *Cancer Immunol Immunother* 2021;70:61–73.
- [120] Borcoman E, et al. HDAC inhibition to prime immune checkpoint inhibitors. *Cancers* 2022;14:66.
- [121] Kelly WK, et al. Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. *J Clin Oncol* 2005;23:3923–31.
- [122] Rubin EH, et al. A study to determine the effects of food and multiple dosing on the pharmacokinetics of vorinostat given orally to patients with advanced cancer. *Clin Cancer Res* 2006;12:7039–45.
- [123] Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. *Oncologist* 2007;12:1247–52.
- [124] Krug LM, et al. Vorinostat in patients with advanced malignant pleural mesothelioma who have progressed on previous chemotherapy (VANTAGE-014): a phase 3, double-blind, randomised, placebo-controlled trial. *Lancet Oncol* 2015;16:447–56.
- [125] Nakajima H, Kim YB, Terano H, Yoshida M, Horinouchi S. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. *Exp Cell Res* 1998;241:126–33.
- [126] Ueda H, Nakajima H, Hori Y, Goto T, Okuhara M. Action of FR901228, a novel antitumor bicyclic depsipeptide produced by Chromobacterium violaceum no. 968, on Ha-ras transformed NIH3T3 cells. *Biosci Biotechnol Biochem* 1994;58:1579–83.
- [127] Graham C, et al. Evaluation of the antitumor efficacy, pharmacokinetics, and pharmacodynamics of the histone deacetylase inhibitor depsipeptide in childhood cancer models *in vivo*. *Clin Cancer Res* 2006;12:223–34.
- [128] Sandor V, et al. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. *Clin Cancer Res* 2002;8:718–28.
- [129] Schrump DS, et al. Clinical and molecular responses in lung cancer patients receiving Romidepsin. *Clin Cancer Res* 2008;14:188–98.
- [130] Piekarz RL, et al. Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. *J Clin Oncol* 2009;27:5410–17.
- [131] Coiffier B, et al. Results from a pivotal, open-label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J Clin Oncol* 2012;30:631–6.
- [132] Lassen U, et al. A phase I study of the safety and pharmacokinetics of the histone deacetylase inhibitor belinostat administered in combination with carboplatin and/or paclitaxel in patients with solid tumours. *Br J Cancer* 2010;103:12–17.
- [133] Steele NL, et al. A phase 1 pharmacokinetic and pharmacodynamic study of the histone deacetylase inhibitor belinostat in patients with advanced solid tumors. *Clin Cancer Res* 2008;14:804–10.
- [134] Yeo W, et al. Epigenetic therapy using belinostat for patients with unresectable hepatocellular carcinoma: a multicenter phase I/II study with biomarker and pharmacokinetic analysis of tumors from patients in the Mayo Phase II Consortium and the Cancer Therapeutics Research Group. *J Clin Oncol* 2012;30:3361–7.
- [135] O'Connor OA, et al. Belinostat in patients with relapsed or refractory peripheral T-cell lymphoma: results of the pivotal phase II BELIEF (CLN-19) study. *J Clin Oncol* 2015;33:2492–9.
- [136] Foss F, et al. A phase II trial of belinostat (PXD101) in patients with relapsed or refractory peripheral or cutaneous T-cell lymphoma. *Br J Haematol* 2015;168:811–19.
- [137] Steele NL, et al. Pharmacokinetic and pharmacodynamic properties of an oral formulation of the histone deacetylase inhibitor belinostat (PXD101). *Cancer Chemother Pharmacol* 2011;67:1273–9.

- [138] Ramalingam SS, et al. Phase II study of belinostat (PXD101), a histone deacetylase inhibitor, for second line therapy of advanced malignant pleural mesothelioma. *J Thorac Oncol* 2009;4:97–101.
- [139] Giles F, et al. A phase I study of intravenous LBH589, a novel cinnamic hydroxamic acid analogue histone deacetylase inhibitor, in patients with refractory hematologic malignancies. *Clin Cancer Res* 2006;12:4628–35.
- [140] Morita S, et al. Phase I dose-escalating study of panobinostat (LBH589) administered intravenously to Japanese patients with advanced solid tumors. *Invest New Drugs* 2012;30:1950–7.
- [141] Sharma S, et al. A phase I, open-label, multicenter study to evaluate the pharmacokinetics and safety of oral panobinostat in patients with advanced solid tumors and varying degrees of renal function. *Cancer Chemother Pharmacol* 2015;75:87–95.
- [142] Slingerland M, et al. A phase I, open-label, multicenter study to evaluate the pharmacokinetics and safety of oral panobinostat in patients with advanced solid tumors and various degrees of hepatic function. *Cancer Chemother Pharmacol* 2014;74:1089–98.
- [143] Younes A, et al. Panobinostat in patients with relapsed/refractory Hodgkin's lymphoma after autologous stem-cell transplantation: results of a phase II study. *J Clin Oncol* 2012;30:2197–203.
- [144] San-Miguel JF, et al. Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, double-blind phase 3 trial. *Lancet Oncol* 2014;15:1195–206.
- [145] Dong M, et al. Phase I study of chidamide (CS055/HBI-8000), a new histone deacetylase inhibitor, in patients with advanced solid tumors and lymphomas. *Cancer Chemother Pharmacol* 2012;69:1413–22.
- [146] Chan TS, Tse E, Kwong Y-L. Chidamide in the treatment of peripheral T-cell lymphoma. *OncoTargets Ther* 2017;10:347–52.
- [147] Blanquart C, et al. Epigenetic metalloenzymes. *Curr Med Chem* 2018. Available from: <https://doi.org/10.2174/0929867325666180706105903>.
- [148] McClure JJ, Li X, Chou CJ. Chapter Six – Advances and challenges of HDAC inhibitors in cancer therapeutics. In: Tew KD, Fisher PB, editors. *Advances in cancer research*, vol. 138. Academic Press; 2018.
- [149] Du L, Musson DG, Wang AQ. Stability studies of vorinostat and its two metabolites in human plasma, serum and urine. *J Pharm Biomed Anal* 2006;42:556–64.
- [150] Parise RA, Holleran JL, Beumer JH, Ramalingam S, Egorin MJ. A liquid chromatography–electrospray ionization tandem mass spectrometric assay for quantitation of the histone deacetylase inhibitor, vorinostat (suberoylanilide hydroxamic acid, SAHA), and its metabolites in human serum. *J Chromatogr B* 2006;840:108–15.
- [151] Furumai R, et al. FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. *Cancer Res* 2002;62:4916–21.
- [152] Shiraga T, Tozuka Z, Ishimura R, Kawamura A, Kagayama A. Identification of cytochrome P450 enzymes involved in the metabolism of FK228, a potent histone deacetylase inhibitor, in human liver microsomes. *Biol Pharm Bull* 2005;28:124–9.
- [153] Xiao JJ, Byrd J, Marcucci G, Grever M, Chan KK. Identification of thiols and glutathione conjugates of depsipeptide FK228 (FR901228), a novel histone protein deacetylase inhibitor, in the blood. *Rapid Commun Mass Spectrom* 2003;17:757–66.
- [154] Ebbel EN, et al. Identification of phenylbutyrate-generated metabolites in Huntington disease patients using parallel liquid chromatography/electrochemical array/mass spectrometry and off-line tandem mass spectrometry. *Anal Biochem* 2010;399:152–61.
- [155] Sato NL, Niimura S, Fujisawa N, Maeda Y. Characterization of vascular permeability-increasing component isolated from solid tumors and the effect of highly polymerized dextran sulfate on its activity. *Jpn J Pharmacol* 1986;41:163–71.

- [156] Allen TM, Martin FJ. Advantages of liposomal delivery systems for anthracyclines. *Semin Oncol* 2004;31:5–15.
- [157] Gabizon A, Shmeeda H, Barenholz Y. Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies. *Clin Pharmacokinet* 2003;42:419–36.
- [158] Gabizon A, Martin F. Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale use solid tumours. *Drugs* 1997;54(Suppl. 4):15–21.
- [159] Mohamed EA, et al. Vorinostat with sustained exposure and high solubility in poly(ethylene glycol)-b-poly(DL-lactic acid) micelle nanocarriers: characterization and effects on pharmacokinetics in rat serum and urine. *J Pharm Sci* 2012;101:3787–98.
- [160] Tran TH, et al. Development of vorinostat-loaded solid lipid nanoparticles to enhance pharmacokinetics and efficacy against multidrug-resistant cancer cells. *Pharm Res* 2014;31:1978–88.
- [161] Kaur J, et al. Ultrasonic atomizer-driven development of biocompatible and biodegradable poly(d,l-lactide-co-glycolide) nanocarrier-encapsulated suberoylanilide hydroxamic acid to combat brain cancer. *ACS Appl Bio Mater* 2021;4:5627–37.
- [162] Sankar R, et al. Nanostructured delivery system for Suberoylanilide hydroxamic acid against lung cancer cells. *Mater Sci Eng C Mater Biol Appl* 2015;51:362–8.
- [163] Sankar R, Ravikumar V. Biocompatibility and biodistribution of suberoylanilide hydroxamic acid loaded poly (DL-lactide-co-glycolide) nanoparticles for targeted drug delivery in cancer. *Biomed Pharmacother* 2014;68:865–71.
- [164] Wang EC, et al. Nanoparticle formulations of histone deacetylase inhibitors for effective chemoradiotherapy in solid tumors. *Biomaterials* 2015;51:208–15.
- [165] Zhu Z, et al. Redox-sensitive iodinated polymersomes carrying histone deacetylase inhibitor as a dual-functional nano-radiosensitizer for enhanced radiotherapy of breast cancer. *Drug Deliv* 2021;28:2301–9.
- [166] Martin DT, et al. Nanoparticles for urothelium penetration and delivery of the histone deacetylase inhibitor belinostat for treatment of bladder cancer. *Nanomed.* 2013;9:1124–34.
- [167] Peng Q, et al. Hybrid artificial cell-mediated epigenetic inhibition in metastatic lung cancer. *J Colloid Interface Sci* 2021;603:319–32.
- [168] Tran TH, et al. Development of lipid nanoparticles for a histone deacetylases inhibitor as a promising anti-cancer therapeutic. *Drug Deliv* 2015;1–9. Available from: <https://doi.org/10.3109/10717544.2014.991432>.
- [169] Denis I, et al. Vorinostat-polymer conjugate nanoparticles for Acid-responsive delivery and passive tumor targeting. *Biomacromolecules* 2014;15:4534–43.
- [170] Denis I, et al. Histone deacetylase inhibitor-polymer conjugate nanoparticles for acid-responsive drug delivery. *Eur J Med Chem* 2015;95:369–76.
- [171] Charrier C, et al. Synthesis and modeling of new benzofuranone histone deacetylase inhibitors that stimulate tumor suppressor gene expression. *J Med Chem* 2009;52:3112–15.
- [172] El Bahhaj F, et al. Histone deacetylase inhibitors delivery using nanoparticles with intrinsic passive tumor targeting properties for tumor therapy. *Theranostics* 2016;6:795–807.
- [173] Baylin SB, Jones PA. A decade of exploring the cancer epigenome—biological and translational implications. *Nat Rev Cancer* 2011;11:726–34.
- [174] Meka AK, et al. Enhanced solubility, permeability and anticancer activity of vorinostat using tailored mesoporous silica nanoparticles. *Pharmaceutics* 2018;10:E283.
- [175] Phung CD, et al. Pre- and post-transcriptional regulation of cFLIP for effective cancer therapy using pH-ultrasensitive nanoparticles. *ACS Appl Mater Interfaces* 2021;13:5999–6010.
- [176] Chaudhuri S, et al. β -Cyclodextrin-poly (β -amino ester) nanoparticles are a generalizable strategy for high loading and sustained release of HDAC inhibitors. *ACS Appl Mater Interfaces* 2021;13:20960–73.

- [177] Jeannot V, et al. Anti-tumor efficacy of hyaluronan-based nanoparticles for the co-delivery of drugs in lung cancer. *J Control Rel J Control Rel Soc* 2018;275:117–28.
- [178] Shamsian A, et al. Targeting tumorigenicity of breast cancer stem cells using SAHA/Wnt-b catenin antagonist loaded onto protein corona of gold nanoparticles. *Int J Nanomed* 2020;15:4063–78.
- [179] Ma W, et al. Sensitizing triple negative breast cancer to tamoxifen chemotherapy via a redox-responsive vorinostat-containing polymeric prodrug nanocarrier. *Theranostics* 2020;10:2463–78.
- [180] Abdel-Ghany S, et al. Vorinostat-loaded titanium oxide nanoparticles (anatase) induce G2/M cell cycle arrest in breast cancer cells via PALB2 upregulation. *3 Biotech* 2020;10:407.
- [181] Kumar P, Wasim L, Chopra M, Chhikara A. Co-delivery of vorinostat and etoposide via disulfide cross-linked biodegradable polymeric nanogels: synthesis, characterization, biodegradation, and anti-cancer activity. *AAPS PharmSciTech* 2018;19:634–47.
- [182] Vijayaraghavalu S, Labhsetwar V. Nanogel-mediated delivery of a cocktail of epigenetic drugs plus doxorubicin overcomes drug resistance in breast cancer cells. *Drug Deliv Transl Res* 2018;8:1289–99.
- [183] Xu S, Zhu X, Huang W, Zhou Y, Yan D. Supramolecular cisplatin-vorinostat nanodrug for overcoming drug resistance in cancer synergistic therapy. *J Controlled Rel* 2017;266:36–46.
- [184] Wu C, et al. Supramolecularly self-assembled nano-twin drug for reversing multidrug resistance. *Biomater Sci* 2018;6:2261–9.
- [185] Ma Z, et al. Folate-mediated and pH-responsive chidamide-bound micelles encapsulating photosensitizers for tumor-targeting photodynamic therapy. *Int J Nanomed* 2019;14:5527–40.
- [186] Wang S, et al. Chidamide stacked in magnetic polypyrrole nano-composites counter thermotolerance and metastasis for visualized cancer photothermal therapy. *Drug Deliv* 2022;29:1312–25.
- [187] Foglietta F, et al. Modulation of butyrate anticancer activity by solid lipid nanoparticle delivery: an in vitro investigation on human breast cancer and leukemia cell lines. *J Pharm Pharm Sci* 2014;17:231–47.
- [188] Kühne M, et al. Biocompatible valproic acid-coupled nanoparticles attenuate lipopolysaccharide-induced inflammation. *Int J Pharm* 2021;601:120567.
- [189] Kühne M, et al. Biocompatible sulfated valproic acid-coupled polysaccharide-based nanocarriers with HDAC inhibitory activity. *J Control Rel J Control Rel Soc* 2021;329:717–30.
- [190] Lindemann H, et al. Polysaccharide nanoparticles bearing HDAC inhibitor as nontoxic nanocarrier for drug delivery. *Macromol Biosci* 2020;20:e2000039.
- [191] Guo W, et al. l-Cysteine decorated nanoscale metal-organic frameworks delivering valproic acid/cis-platin for drug-resistant lung cancer therapy. *Chem Commun Camb Engl* 2020;56:3919–22.
- [192] Senevirathne SA, et al. HDAC inhibitor conjugated polymeric prodrug micelles for doxorubicin delivery. *J Mater Chem B* 2017;5:2106–14.
- [193] Zhou M, et al. Combining histone deacetylase inhibitors (HDACis) with other therapies for cancer therapy. *Eur J Med Chem* 2021;226:113825.
- [194] Lai C-J, et al. CUDC-101, a multitargeted inhibitor of histone deacetylase, epidermal growth factor receptor, and human epidermal growth factor receptor 2, exerts potent anticancer activity. *Cancer Res* 2010;70:3647–56.
- [195] Galloway TJ, et al. A phase I study of CUDC-101, a multitarget inhibitor of HDACs, EGFR, and HER2, in combination with chemoradiation in patients with head and neck squamous cell carcinoma. *Clin Cancer Res J Am Assoc Cancer Res* 2015;21:1566–73.
- [196] Shimizu T, et al. Phase I first-in-human study of CUDC-101, a multitargeted inhibitor of HDACs, EGFR, and HER2 in patients with advanced solid tumors. *Clin Cancer Res J Am Assoc Cancer Res* 2014;20:5032–40.
- [197] Sun K, et al. Dual HDAC and PI3K inhibitor CUDC-907 downregulates MYC and suppresses growth of MYC-dependent cancers. *Mol Cancer Ther* 2017;16:285–99.

- [198] Oki Y, et al. CUDC-907 in relapsed/refractory diffuse large B-cell lymphoma, including patients with MYC-alterations: results from an expanded phase I trial. *Haematologica* 2017;102:1923–30.
- [199] Yang EG, et al. Design and synthesis of janus kinase 2 (JAK2) and histone deacetylase (HDAC) bispecific inhibitors based on pacritinib and evidence of dual pathway inhibition in hematological cell lines. *J Med Chem* 2016;59:8233–62.
- [200] Yao L, et al. Design and synthesis of ligand efficient dual inhibitors of janus kinase (JAK) and histone deacetylase (HDAC) based on ruxolitinib and vorinostat. *J Med Chem* 2017;60:8336–57.
- [201] Guerrant W, Patil V, Canzoneri JC, Oyelere AK. Dual targeting of histone deacetylase and topoisomerase II with novel bifunctional inhibitors. *J Med Chem* 2012;55:1465–77.
- [202] Ning CQ, et al. Macroyclic compounds as anti-cancer agents: design and synthesis of multi-acting inhibitors against HDAC, FLT3 and JAK2. *Eur J Med Chem* 2015;95:104–15.
- [203] Tomaselli D, Lucidi A, Rotili D, Mai A. Epigenetic polypharmacology: a new frontier for epi-drug discovery. *Med Res Rev* 2020;40:190–244.
- [204] Ruzic D, et al. Targeting histone deacetylases: opportunities for cancer treatment and chemoprevention. *Pharmaceutics* 2022;14:209.
- [205] Woods DM, et al. HDAC inhibition upregulates PD-1 ligands in melanoma and augments immunotherapy with PD-1 blockade. *Cancer Immunol Res* 2015;3:1375–85.
- [206] Hicks KC, et al. Epigenetic priming of both tumor and NK cells augments antibody-dependent cellular cytotoxicity elicited by the anti-PD-L1 antibody avelumab against multiple carcinoma cell types. *Oncoimmunology* 2018;7:e1466018.
- [207] Zheng H, et al. HDAC inhibitors enhance T-cell chemokine expression and augment response to PD-1 immunotherapy in lung adenocarcinoma. *Clin Cancer Res J Am Assoc Cancer Res* 2016;22:4119–32.
- [208] Wu R, et al. SOX2 promotes resistance of melanoma with PD-L1 high expression to T-cell-mediated cytotoxicity that can be reversed by SAHA. *J Immunother Cancer* 2020;8:e001037.
- [209] Llopiz D, et al. Enhanced anti-tumor efficacy of checkpoint inhibitors in combination with the histone deacetylase inhibitor belinostat in a murine hepatocellular carcinoma model. *Cancer Immunol Immunother CII* 2019;68:379–93.
- [210] Roulois D, et al. DNA-demethylating agents target colorectal cancer cells by inducing viral mimicry by endogenous transcripts. *Cell* 2015;162:961–73.
- [211] Chiappinelli KB, et al. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. *Cell* 2015;162:974–86.
- [212] Coral S, et al. 5-aza-2'-deoxycytidine-induced expression of functional cancer testis antigens in human renal cell carcinoma: immunotherapeutic implications. *Clin Cancer Res J Am Assoc Cancer Res* 2002;8:2690–5.
- [213] Woloszynska-Read A, Mhawech-Fauceglia P, Yu J, Odunsi K, Karpf AR. Intertumor and intratumor NY-ESO-1 expression heterogeneity is associated with promoter-specific and global DNA methylation status in ovarian cancer. *Clin Cancer Res J Am Assoc Cancer Res* 2008;14:3283–90.
- [214] Stone ML, et al. Epigenetic therapy activates type I interferon signaling in murine ovarian cancer to reduce immunosuppression and tumor burden. *Proc Natl Acad Sci U S A* 2017;114:E10981–90.
- [215] Oi S, et al. Synergistic induction of NY-ESO-1 antigen expression by a novel histone deacetylase inhibitor, valproic acid, with 5-aza-2'-deoxycytidine in glioma cells. *J Neurooncol* 2009;92:15–22.
- [216] Leclercq S, et al. A 5-aza-2'-deoxycytidine/valproate combination induces cytotoxic T-cell response against mesothelioma. *Eur Respir J* 2011;38:1105–16.
- [217] Wischnewski F, Pantel K, Schwarzenbach H. Promoter demethylation and histone acetylation mediate gene expression of MAGE-A1, -A2, -A3, and -A12 in human cancer cells. *Mol Cancer Res MCR* 2006;4:339–49.
- [218] Carton P-F, Blanquart C, Hervouet E, Gregoire M, Vallette FM. HDAC1-mSin3a-NCOR1, Dnmt3b-HDAC1-Egr1 and Dnmt1-PCNA-UHRF1-G9a regulate the NY-ESO1 gene expression. *Mol Oncol* 2013;7:452–63.

- [219] Kim K, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci U S A* 2014;111:11774–9.
- [220] Hellmann MD, et al. Entinostat plus pembrolizumab in patients with metastatic NSCLC previously treated with anti-PD-(L)1 therapy. *Clin Cancer Res J Am Assoc Cancer Res* 2021;27:1019–28.
- [221] Roussos Torres ET, et al. 964MO entinostat, nivolumab and ipilimumab in advanced HER2-negative breast cancer (ETCTN-9844). *Ann Oncol* 2021;32:S833.
- [222] O'Shaughnessy J, et al. Results of ENCORE 602 (TRIO025), a phase II, randomized, placebo-controlled, double-blinded, multicenter study of atezolizumab with or without entinostat in patients with advanced triple-negative breast cancer (aTNBC). *J Clin Oncol* 2020;38:1014.
- [223] Issa J-P, et al. Results of phase 2 randomized study of low-dose decitabine with or without valproic acid in patients with myelodysplastic syndrome and acute myelogenous leukemia. *Cancer* 2015;121:556–61.
- [224] Blum W, et al. Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. *J Clin Oncol J Am Soc Clin Oncol* 2007;25:3884–91.
- [225] Lübbert M, et al. Valproate and retinoic acid in combination with decitabine in elderly nonfit patients with acute myeloid leukemia: results of a multicenter, randomized, 2 × 2, phase II trial. *J Clin Oncol J Am Soc Clin Oncol* 2020;38:257–70.
- [226] Chu BF, et al. Phase I study of 5-aza-2'-deoxycytidine in combination with valproic acid in non-small-cell lung cancer. *Cancer Chemother Pharmacol* 2013;71:115–21.
- [227] Stathis A, et al. Phase I study of decitabine in combination with vorinostat in patients with advanced solid tumors and non-Hodgkin's lymphomas. *Clin Cancer Res J Am Assoc Cancer Res* 2011;17:1582–90.
- [228] Kirschbaum M, et al. A phase 1 clinical trial of vorinostat in combination with decitabine in patients with acute myeloid leukaemia or myelodysplastic syndrome. *Br J Haematol* 2014;167:185–93.
- [229] How J, et al. A phase I trial of two sequence-specific schedules of decitabine and vorinostat in patients with acute myeloid leukemia. *Leuk Lymphoma* 2015;56:2793–802.
- [230] Burke MJ, et al. Decitabine and vorinostat with chemotherapy in relapsed pediatric acute lymphoblastic leukemia: a TACL pilot study. *Clin Cancer Res J Am Assoc Cancer Res* 2020;26:2297–307.
- [231] Burke MJ, et al. A therapeutic trial of decitabine and vorinostat in combination with chemotherapy for relapsed/refractory acute lymphoblastic leukemia. *Am J Hematol* 2014;89:889–95.
- [232] Pommert L, et al. Decitabine and vorinostat with FLAG chemotherapy in pediatric relapsed/refractory AML: report from the therapeutic advances in childhood leukemia and lymphoma (TACL) consortium. *Am J Hematol* 2022;97:613–22.

PART

Issues to overcome/
areas of concern

4

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Epigenetic intratumoral heterogeneity 20

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Chapter outline

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1 Introduction

The epigenetic modifications are found to interlink the environmental risk factors and many disease [1–4]. It is also highly believed that epigenetics changes are directly related to the origin of human cancer [5]. Among the epigenetics markers, DNA methylation (DNAm) is stable enough to be measured accurately given a small amount of DNA content [6,7]. With the advancement of array technology, the cost of whole-genome DNAm quantifications is relatively affordable. Among those, the most widely used platforms to measure the human whole-genome DNAm are Illumina Infinium 450k beadchip [8] and 850k/EPIC beadchip [9], in addition to whole-genome bisulfite sequencing (WGBS). The high coverage and low cost of DNAm profile make it a potential promising biomarker on which researchers can rely to identify the cause of many diseases and for prediction purposes. In the last decade, many Epigenome-Wide Association Studies (EWAS) have been performed, using DNAm measured in bulk tissues of individuals from the control and disease groups. Although the technologies to measure DNAm profile at single-cell level have emerged [10], the considerable cost and the sparsity nature of the data prevent us from generating such data on a large scale. Therefore researchers still exploit the abundant bulk tissue DNAm data when performing EWAS. Of note, the well-known The Cancer Genome Atlas (TCGA) provides bulk tissue DNAm data using a 450k beadchip for all cancer types [11].

Most EWAS tries to identify differentially methylated CpGs (DMCs) between the disease and control groups. The endeavor, however, is hindered by the fact that the DNAm profile is tissue and cell-type-specific, and the baseline DNAm level varies between tissues/cell types [12–14].

Coupling with the difference in cell-type heterogeneity in individuals, the resulting EWAS can sometimes give us unexpected and wrong inferences about DMC. For example, Jaffe et al. reported that the fractions of blood cell subtypes (neutrophils, eosinophils, basophils, monocytes, CD⁴⁺ T cells, CD⁸⁺ T cells, B cells, and NK cells) confound with age-related variability; the difference of DNAm levels along ages is likely to be driven by the difference of fractions of blood cell subtypes but not actual DNAm changes [15]. Later, Liu et al. also found that without considering the cell-type heterogeneity, they would end up with a long list of false positive DMCs when comparing 354 rheumatoid arthritis (RA) cases with 337 controls in an EWAS performed in whole blood [16]. In these two examples, the cell-type heterogeneity would cause false positive inference.

Moreover, the cell-type heterogeneity could also drive false negative inference in EWAS. To better visualize the issue, let us suppose we have measured bulk tissue DNAm in control versus case setting (Figure 20.1). In the bulk tissue, we have three different cell types, among which only

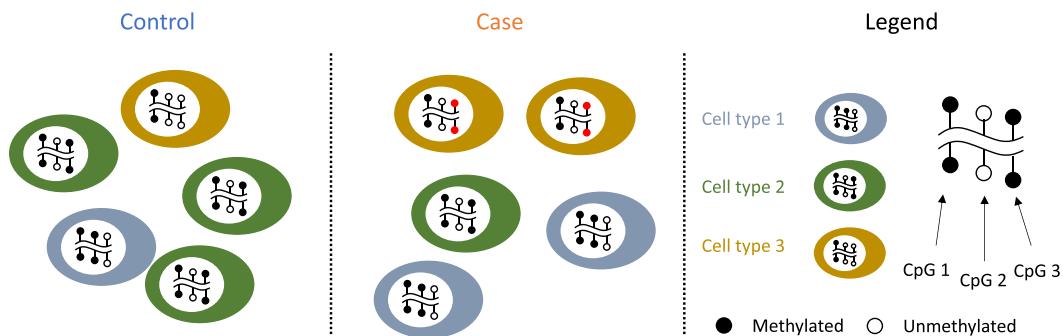


FIGURE 20.1

Demonstration of false positive and false negative calls in a control-case EWAS. On the top, we consider two groups of individuals—control individuals and case individuals. Different colors represent different cell types. Here, we only consider three CpGs labeled as CpG 1, CpG 2, and CpG 3 from left to right. A hollow circle denotes the unmethylated CpG, while the solid circle for methylated CpG. Among 3 CpGs, only CpG 3 is truly differentially methylated between case and control, and such differential methylation only occurs in cell-type 3 (denoted as the solid red circles). At the bottom, we display the beta value reading for each CpG, the difference of beta value readings, whether the CpG is truly differentially methylated between case and control, and the type of DMC inference.

CpG 3 of the cell-type 3 is truly differentially methylated. When we use the beta value to represent the DNAm level of a CpG, it tells us the percentage of methylated counts over all counts for a single CpG. Due to the difference in cell-type fraction between case and control, CpG 2 will be wrongly reported as a DMC. In the meantime, CpG 3 will not be correctly reported as a DMC. This simple example depicts how the cell-type heterogeneity could affect EWAS inference and highlights the necessity of considering cell-type heterogeneity in all EWAS [15,17], including cancer research.

In the last couple of years, many methods/software have been developed to tackle cell-type heterogeneity in EWAS [18,19]. Those methods could be separated into two main categories: statistical methods to identify differentially methylated CpGs and statistical methods to identify differentially methylated cell types. We can think of category B methods as more advanced, as they not only infer whether a CpG is differentially methylated but also tell you in which cell type(s) the CpG is differentially methylated. However, there are still cases where we only want to infer DMCs considering cell-type heterogeneity. Among the statistical methods to identify differentially methylated CpGs, there are two main types: reference-based and reference-free (Table 20.1). Reference-based methods require *a priori* constructed reference DNAm matrix consisting of the cell types of interest, while reference-free methods do not. We will then discuss these two types in detail in the following sections.

Table 20.1 Statistical Methods to Identify Differentially Methylated CpGs.

Statistical Method	Type	Output	Developed Specifically for Cell-Type Heterogeneity in EWAS	References
Houseman's CP	Reference based	Cell-type fractions	Yes	[20]
CIBERSORT	Reference based	Cell-type fractions	Yes	[21,22]
RPC (EpiDISH)	Reference based	Cell-type fractions	Yes	[23,24]
MethylResolver	Reference based	Cell-type fractions	Yes	[25]
EWASher	Reference free	Latent components	Yes	[26]
RefFreeEWAS	Reference free	DMCs	Yes	[27]
RefFreeCellMix	Supervised			
	Reference free	Putative cell-type fractions and putative reference matrix	Yes	[28]
MeDeCom	Reference free	Putative cell-type fractions and putative reference matrix	Yes	[29]
ReFACTOr	Reference free	Latent components	Yes	[30]
SVA	Reference free	DMCs	No	[31]
ISVA	Supervised			
	Reference free	DMCs	No	[32]
RUVm	Supervised			
	Reference free	DMCs	No	[33]
	Supervised			

CP, Constrained projection; DMCs, differentially methylated CpGs; ISVA, independent surrogate variable analysis; RPC, robust partial correlation; SVA, surrogate variable analysis.

2 Using reference-based statistical methods to identify differentially methylated CpGs

All reference-based methods in [Table 20.1](#) assume the same underlying statistical model:

$$X = R \cdot F + \varepsilon$$

where $X \in R^{m \times n}$ is the subset DNAm beta value matrix for m cell-type-specific CpGs and n samples, $R \in R^{m \times k}$ is the reference matrix for m cell-type-specific CpGs and k different cell types, $F \in R^{k \times n}$ is the cell-type fraction matrix, and $\varepsilon \in R^{m \times n}$ is the error matrix. Note that when we estimate the cell-type fractions using these reference-based methods, we omit the DNAm difference associated with the phenotype of interest with the assumption that most cell-type-specific CpGs in the reference matrix are not affected by the phenotype of interest.

The predefined reference matrix is usually constructed using DNAm profiles of sorted cells or purified cell lines to maximize the distinguishing power between different cell types. With a predefined reference matrix, estimating the cell-type fraction matrix becomes a deconvolution problem. With the assumption of the DNAm profile being a weighted linear combination of different cell types, one could quickly think of using a least-square regression model to solve the unknown fractions/weights matrix. However, the elements of a fraction matrix need to satisfy two requirements to make it interpretable: first, each element should be greater than or equal to 0 and less than or equal to 1; second, all fraction values of each cell should add to 1. (Although one can alternatively require the sums of fractions is less than or equal to 1 to allow inference with some cell types missing from the reference matrix, the alternative has not been systematically applied and validated.) The most used reference-based method, Houseman's constrained projection [\[20\]](#), imposes these two requirements during the model fitting with quadratic programming. It has been shown that Houseman's CP works exceptionally well in some tissue types, such as whole blood [\[16,34\]](#). Though, it might not be the best method among reference-based methods when applied to other data. Unlike Houseman's CP, which imposes the requirements during the inference, the other three reference-based methods in [Table 20.1](#) impose requirements as *a posteriori*. For those three methods, the direct output (preliminary fraction values) from the regression model fitting could be negative or greater than one and may not add to 1; the direct output is further coerced and normalized to satisfy the above two requirements. The nonconstrained method CIBERSORT, originally developed and validated in the context of gene expression data, uses support vector regression to search for a solution in a penalized multivariate regression fashion [\[22,35\]](#). Studies have shown that CIBERSORT also works well for DNAm data [\[21,23\]](#). A similar nonconstrained method, robust partial correlation (RPC), was also proven to work well for DNAm data [\[23\]](#). Interestingly, in a study where the authors compared Houseman's CP, CIBERSORT, and RPC in the context of DNAm data, the two nonconstrained methods, CIBERSORT and RPC, outperformed Houseman's CP when the noise was relatively high [\[23\]](#). This finding is consistent with the fact that CIBERSORT was developed to be robust concerning noise and exceeded CP in gene expression data [\[22,35\]](#), which inherently exhibits higher noise than DNAm data. More recently, another nonconstrained method—the Least Trimmed Squares regression-based method MethylResolver was proposed and validated to infer fractions of blood cell subtypes from methylation profiles of tumor admixtures [\[25\]](#). The rationale behind MethylResolver is that some CpGs might be outliers if the origin tissue to construct the reference does not match the tissue of interest to infer the cell-type

fractions. For example, we could use a whole blood reference matrix to estimate relative fractions of blood cell subtypes in tumor tissue. However, we do not have any estimation for the nonblood cell subtypes in the tumor tissue. MethylResolver would filter the cell-type-specific CpGs in the reference matrix to minimize the effect of outliers before fitting the regression model [25]. While some results indicate that one of the methods may outperform other methods in some tissue types and at some noise levels, there is still a lack of systematic comparison between all reference-based methods to infer (relative) cell-type fractions in the context of DNA methylation data. As the performance difference between methods seems marginal, we believe all reference-based methods would work reasonably well when there is no need for high-resolution and highly accurate estimation of cell-type fractions from bulk tissue DNA methylation data.

As one can see, one of the most critical ingredients for any reference-based method is a reference DNA methylation (DNAm) matrix. Researchers have built and validated DNAm references for the whole blood [20,23,25,36,37], cord blood [38–40], prefrontal cortex [41], generic epithelial tissues [42] (which could be used on tumor samples), and head and neck squamous cell carcinoma tissue [21]. Koestler et al. [43] have shown that the accuracy of fraction estimation depends on the quality of the reference DNAm profiles, which subsequently impacts the downstream analysis. We may pay attention to two related aspects of any DNAm reference matrix, the number of cell-type-specific CpGs and collinearity between cell types. We could exploit several strategies when constructing a DNAm reference to improve its quality. First, we could balance the number of exclusive cell-type-specific CpGs between cell types. In a supervised selection of cell-type-specific CpGs, the CpGs exhibit a significant DNAm difference between one cell type to all other cell types tend to be highly ranked. You may end up with a DNAm reference with most CpGs specific to one of the cell types among all cell types. Optimizing the conditioning number of features for each cell-type results in a DNAm reference matrix more stable to the accidental loss of observations of CpGs [22]. Second, we should include as many samples of each sorted cell type with a variety of demographic. Moreover, we may use prior biological knowledge to help select the cell-type-specific CpGs. For example, one can use the cell-type-specific DNase hypersensitive site [44] to filter CpGs that are likely to be true cell-type-specific CpGs in all tissues [23]. The collinearity between cell types could be tricky sometimes. For example, in the context of whole blood, the DNAm profiles of CD⁴⁺ T cells and CD⁸⁺ T cells could be similar. Another challenging situation is when we need to consider different hierarchies, such as in tumor tissues, where we have epithelial cells, fibroblasts, and immune cells. Since the difference between epithelial and immune cells is so big, and the difference between immune cell subtypes is relatively much smaller, we might not be able to get an excellent DNAm reference, which circumvents the collinearity for epithelial cells, fibroblasts, and all immune cell subtypes. Therefore we might need to go an alternative route by inferring the cell-type fractions in a two-stage or hierarchical way [42].

We end up with a cell-type fraction matrix for all reference-based methods. Such a fraction matrix could be included as a covariate matrix in almost any supervised model of EWAS. Moreover, the fraction matrix, in principle, could be used to infer alterations in cell-type fractions. Studies have shown that the ratios of fractions of two blood cell subtypes in tumor samples and the level of immune cell infiltration (absolute fractions of the immune cell of tumor samples) may have prognostic and diagnostic power [21,25,35,45–57]. On the other hand, reference-based methods are limited by the presence of a predefined reference matrix. To construct such a DNAm reference for tissue of interest, DNAm profiles of sorted cells from the tissue are usually required.

Due to the high cost and lack of cell-type-specific antibodies of high specificity, it is unrealistic for us to get a reference for all tissue types. There are cases/tissues where we cannot apply a reference-based method. Another disadvantage of reference-based methods over reference-free methods is that reference-based methods cannot account for cell-to-cell interactions due to the relatively simple model assumption.

3 Using reference-free statistical methods to identify differentially methylated CpGs

Unlike reference-based methods, the underlying model of reference-free methods varies across methods. While some methods, such as RefFreeEWAS [27], were explicitly designed for adjusting cell-type heterogeneity, other methods, including surrogate variable analysis (SVA) [31], independent surrogate variable analysis (ISVA) [32], and RUVm [33] are more general methods that also adjust for unknown confounders other than cell-type heterogeneity when performing EWAS. SVA is a supervised method designed for expression data initially, requiring an input of phenotype of interest (POI) [31,58]. It assumes that the underlying components associated with confounders are orthogonal to that associated with POI. After regressing the effect of POI with a linear model, SVA uses components from principal component analysis (PCA) orthogonal to POI as surrogate variables, which will be used as covariates in a linear model with POI. ISVA, one of the variations of SVA, uses independent component analysis (ICA) [59] instead of PCA to factorize the residual variation matrix [32]. Both SVA and ISVA were designed for expression, but they also work for DNAm data since they are relatively assumption free. RUVm is one of the Removing Unwanted Variation (RUV) family methods [33] and was developed specifically for Illumina HumanMethylation450 [8] array DNAm data. RUVm relies on the unique negative control probes on Illumina HumanMethylation450 array, which should not associate with biological effect, to get empirical controls. RUV-inverse, a generalized least square regression model, will subsequently use the empirical controls to refit the model and find the “true effect” associated with POI. Since the Illumina HumanMethylation850 [9] array has a similar design, including negative control probes, RUVm also works for Illumina HumanMethylation850 array DNAm data. For other types of DNAm data, such as WGBS data, another member of the RUV family method—RUV2 might be an alternative [60]. Note that for those three supervised methods, the nonspoken assumption is that the POI is not confounded with cell-type composition, which is violated [35] in some cases. We should refrain from using the supervised methods if there is *prior* knowledge informing us of the confounding between POI and cell-type composition. RefFreeEWAS could be considered a variant of SVA, but it explicitly assumes the cell-type mixtures model [27]. EWASher and ReFACTOr are kind of similar in terms of model assumption. Both assume the top principal components of variation of the whole DNAm matrix are driven by cell-type composition [26,30]. As both do not use POI when inferring the cell-type heterogeneity components, the POI effect will not be protected from the inferred latent components. Although the assumption of EWASher and ReFACTOr might be valid in some blood EWAS where the DNAm changes caused by POI are not captured by top principal components [30], it is not valid in an EWAS where the DNAm changes caused by POI are so significant, such as in some cancer EWAS. One example is the application of ReFACTOr on

the TCGA breast cancer data would remove a lot of true DMCs, as cancer samples usually show genome-wide DNAm changes [61]. RefFreeCellMix [28] and MeDeCom [29] assume an explicit mixture model and use nonnegative matrix factorization (NMF) to obtain a cell-type fraction matrix and a cell-type-specific average DNAm matrix. As both are reference-free methods, the cell types inferred by them are putative cell types, which need to be *posteriorly* mapped to real cell types. The difference between RefFreeCellMix and MeDeCom is that RefFreeCellMix requires it to be less than or equal to 1. At the same time, MeDeCom imposes a constraint requiring the sums of cell-type fractions to be precisely 1, making its fraction matrix more interpretable.

There has been limited objective benchmarking studies for reference-free methods. McGregor et al. [62] compared Houseman's CP, EWASher, RefFreeEWAS, SVA, ISVA, and RUV using both simulated and real data. They concluded there is no single method that always works best. The performance fluctuates across scenarios for all methods, although SVA seems the most robust method. Taking reference-based and reference-free methods into consideration, some researchers believe the reference-based methods work better than all reference-free methods when there is a good reference available for the tissue type of interest [63,64]. In summary, the advantages of reference-free methods include no need for a knowledge of cell types and reference matrix; applicable to any tissue type; and adjusting for other unknown factors. On the other hand, the performance of the reference-free methods relies significantly on the model assumption valid on specific data. Moreover, they do not provide a fully interpretable cell-type fraction matrix.

4 Statistical methods that identify differentially methylated cell types

As we have pointed out, all the above statistical methods could identify differentially methylated CpGs by including the estimated cell-type fractions or surrogate variables as covariates in the linear model or obtaining the inference results directly from the methods. However, as we have illustrated in Figure 20.1, sometimes the DNAm changes only occur in one or some cell types but not all of them. Therefore novel statistical methods seeking to identify the cell type in which the differential methylation at the CpG level occurs (DMCTs) have been proposed, such as CellDMC [65], TCA [66], TOAST [67], and HIRE [68]. Generally, these methods use the following model (all underlying models are based on this general model):

$$\beta_{ij} = \sum_k \left(f_{jk} \mu_{ik} + f_{jk} \xrightarrow{p_j} \cdot \xrightarrow{\beta_{ik}} \right) + \varepsilon_{ij}$$

where $\vec{p}_j = [p_{1j}, p_{2j}, \dots, p_{rj}]$ and $\vec{\beta}_{ik} = [\beta_{ik1}, \beta_{ik2}, \dots, \beta_{ikr}]$. β_{ij} represents the observed DNAm level of CpG i for sample j ; f_{jk} represents for the cell-type fraction of cell-type k for sample j with sums of all cell types equal to 1; μ_{ik} represents the baseline DNAm level of CpG i for cell-type k ; \vec{p}_j and $\vec{\beta}_{ik}$ are vectors representing phenotypes of sample j and phenotype-specific effect for cell-type k (the cell-type-specific DNAm changes); ε_{ij} is the error term. All methods make statistical inference on the phenotype effect parameter β_{ik} for a given CpG i with the null hypothesis $\beta_{ik} = 0$.

The required inputs for all methods are a DNAm matrix, a phenotype of interest, and a matrix of cell-type fractions. CellDMC considers one phenotype at a time and allows for the inclusion of other covariates, such as batches. At the same time, TOAST uses the above general model and allows for

inference of difference between cell types and multiple phenotypes. Both TCA and HIRE optimize the input cell-type fractions during the inference, while TCA differs from HIRE by using tensor composition analysis (TCA) for deconvolution. Although optimizing the fraction matrix could result in better inference, it could also lead to over-fitting. A comprehensive benchmarking study on these methods is needed for the researcher to conclude which method is the best under certain circumstances.

Despite the lack of comparisons between methods, there have been some successful applications of these methods in the context of cancer EWAS. For example, with DMCT inference, researchers identified a novel endothelial-to-mesenchymal transition (EndoMT) signature in the case of lung squamous cell carcinoma [69]. In endometrial cancer, CellDMC confirmed that the *HAND2*, a transcription factor that mediates the tumor-suppressive effects of progesterone, is hypermethylated in epithelial cells, fibroblasts, and immune cells [65].

5 Conclusion and future perspectives

In summary, the cell-type heterogeneity hampers the inference of differentially methylated CpGs in EWAS. When performing EWAS using bulk DNAm data, we should adjust for cell-type heterogeneity using an appropriate statistical method. However, the optimal choice of a method depends on the characteristic of the data and the underlying assumption of a statistical method. To further delineate the cell type in which DNAm change occurs, one should use one method that identifies DMCTs. The correct inference of differentially methylated CpGs and the differentially methylated cell type(s) help us better understand the relationship between cancer and DNAm and its biological mechanism.

Note that in this chapter, we have only discussed the epigenetic heterogeneity caused by different cell types. The epigenetic heterogeneity is also observed within specific cell types [70] and cancer clones [71–74]. Such a topic is not discussed here.

Looking into the future, the cell-type heterogeneity will persist in being an issue in EWAS until the technologies for single-cell methylomics become more accessible and reliable. As there is evidence that reference-based methods might work better with a DNAm reference of high quality and the cell-type fraction matrix is helpful as a biomarker, it is tempting for the community to construct more DNAm reference profiles of different tissue types. The recent EpiSCORE algorithm uses high-resolution single-cell RNA sequencing atlas data to impute DNAm reference [69]. Such an effort avoids high-cost DNAm profiling of sorted cells and results in a publicly available pan-tissue DNAm atlas [75].

References

- [1] Petronis A. Epigenetics as a unifying principle in the aetiology of complex traits and diseases. *Nature* 2010;465:721–7.
- [2] van Veldhoven K, et al. Epigenome-wide association study reveals decreased average methylation levels years before breast cancer diagnosis. *Clin Epigenetics* 2015;7:67.
- [3] Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. *Nat Rev Genet* 2011;12:529–41.
- [4] Lappalainen T, Greally JM. Associating cellular epigenetic models with human phenotypes. *Nat Rev Genet* 2017;18:441–51.

- [5] Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006;7:21–33.
- [6] Beck S. Taking the measure of the methylome. *Nat Biotechnol* 2010;28:1026–8.
- [7] Daugaard I, Kjeldsen TE, Hager H, Hansen LL, Wojdacz TK. The influence of DNA degradation in formalin-fixed, paraffin-embedded (FFPE) tissue on locus-specific methylation assessment by MS-HRM. *Exp Mol Pathol* 2015;99:632–40.
- [8] Sandoval J, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics* 2011;6:692–702.
- [9] Moran S, Arribas C, Esteller M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. *Epigenomics* 2016;8:389–99.
- [10] Karemaker ID, Vermeulen M. Single-cell DNA methylation profiling: technologies and biological applications. *Trends Biotechnol* 2018;36:952–65.
- [11] Chang K, et al. The Cancer Genome Atlas Pan-Cancer analysis project. *Nat Genet* 2013;45:1113–20.
- [12] Ziller MJ, et al. Charting a dynamic DNA methylation landscape of the human genome. *Nature* 2013;500:477–81.
- [13] Kundaje A, et al. Integrative analysis of 111 reference human epigenomes. *Nature* 2015;518:317–30.
- [14] Baron U, et al. DNA methylation analysis as a tool for cell typing. *Epigenetics* 2006;1:55–60.
- [15] Jaffe AE, Irizarry RA. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biol* 2014;15:R31.
- [16] Liu Y, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. *Nat Biotechnol* 2013;31:142–7.
- [17] Qi L, Teschendorff AE. Cell-type heterogeneity: why we should adjust for it in epigenome and biomarker studies. *Clin Epigenetics* 2022;14:31.
- [18] Teschendorff AE, Relton CL. Statistical and integrative system-level analysis of DNA methylation data. *Nat Rev Genet* 2018;19:129–47.
- [19] Teschendorff AE, Zheng SC. Cell-type deconvolution in epigenome-wide association studies: a review and recommendations. *Epigenomics-uk* 2017;9:757–68.
- [20] Houseman EA, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. *Bmc Bioinforma* 2012;13:86.
- [21] Chakravarthy A, et al. Pan-cancer deconvolution of tumour composition using DNA methylation. *Nat Commun* 2018;9:3220.
- [22] Newman AM, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* 2015;12:453–7.
- [23] Teschendorff AE, Breeze CE, Zheng SC, Beck S. A comparison of reference-based algorithms for correcting cell-type heterogeneity in Epigenome-Wide Association Studies. *Bmc Bioinforma* 2017;18:105.
- [24] Zheng SC, et al. EpiDISH web server: Epigenetic Dissection of Intra-Sample-Heterogeneity with online GUI. *Bioinformatics* 2019;36:1950–1.
- [25] Arneson D, Yang X, Wang K. MethylResolver—a method for deconvoluting bulk DNA methylation profiles into known and unknown cell contents. *Commun Biol* 2020;3:422.
- [26] Zou J, Lippert C, Heckerman D, Aryee M, Listgarten J. Epigenome-wide association studies without the need for cell-type composition. *Nat Methods* 2014;11:309–11.
- [27] Houseman EA, Molitor J, Marsit CJ. Reference-free cell mixture adjustments in analysis of DNA methylation data. *Bioinformatics* 2014;30:1431–9.
- [28] Houseman EA, et al. Reference-free deconvolution of DNA methylation data and mediation by cell composition effects. *Bmc Bioinforma* 2016;17:259.
- [29] Lutsik P, et al. MeDeCom: discovery and quantification of latent components of heterogeneous methylomes. *Genome Biol* 2017;18:55.

- [30] Rahmani E, et al. Sparse PCA corrects for cell-type heterogeneity in epigenome-wide association studies. *Nat Methods* 2016;13:443–5.
- [31] Leek JT, Storey JD. Capturing heterogeneity in gene expression studies by surrogate variable analysis. *PLoS Genet* 2007;3:e161.
- [32] Teschendorff AE, Zhuang J, Widschwender M. Independent surrogate variable analysis to deconvolve confounding factors in large-scale microarray profiling studies. *Bioinformatics* 2011;27:1496–505.
- [33] Maksimovic J, Gagnon-Bartsch JA, Speed TP, Oshlack A. Removing unwanted variation in a differential methylation analysis of Illumina HumanMethylation450 array data. *Nucleic Acids Res* 2015;43:e106.
- [34] Accomando WP, Wiencke JK, Houseman EA, Nelson HH, Kelsey KT. Quantitative reconstruction of leukocyte subsets using DNA methylation. *Genome Biol* 2014;15:R50.
- [35] Gentles AJ, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med* 2015;21:938–45.
- [36] Reinius LE, et al. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. *PLoS One* 2012;7:e41361.
- [37] Salas LA, et al. An optimized library for reference-based deconvolution of whole-blood biospecimens assayed using the Illumina HumanMethylationEPIC BeadArray. *Genome Biol* 2018;19:64.
- [38] Gervin K, et al. Cell-type specific DNA methylation in cord blood: a 450K-reference data set and cell count-based validation of estimated cell-type composition. *Epigenetics* 2016;11:690–8.
- [39] Cardenas A, et al. Validation of a DNA methylation reference panel for the estimation of nucleated cells types in cord blood. *Epigenetics* 2016;11:773–9.
- [40] Bakulski KM, et al. DNA methylation of cord blood cell-types: applications for mixed cell birth studies. *Epigenetics* 2016;11:354–62.
- [41] Quintinvano J, Aryee MJ, Kaminsky ZA. A cell epigenotype specific model for the correction of brain cellular heterogeneity bias and its application to age, brain region and major depression. *Epigenetics* 2013;8:290–302.
- [42] Zheng SC, et al. A novel cell-type deconvolution algorithm reveals substantial contamination by immune cells in saliva, buccal and cervix. *Epigenomics-uk* 2018;10:925–40.
- [43] Koestler DC, et al. Improving cell mixture deconvolution by identifying optimal DNA methylation libraries (IDOL). *BMC Bioinforma* 2016;17:120.
- [44] Thurman RE, et al. The accessible chromatin landscape of the human genome. *Nature* 2012;489:75–82.
- [45] Koestler DC, et al. DNA methylation-derived neutrophil-to-lymphocyte ratio: an epigenetic tool to explore cancer inflammation and outcomes. *Cancer Epidemiol Prev Biomarkers* 2017;26:328–38.
- [46] Titus AJ, Gallimore RM, Salas LA, Christensen BC. Cell-type deconvolution from DNA methylation: a review of recent applications. *Hum Mol Genet* 2017;26:R216–24.
- [47] Li B, et al. Comprehensive analyses of tumor immunity: implications for cancer immunotherapy. *Genome Biol* 2016;17:174.
- [48] Newman AM, Gentles AJ, Liu CL, Diehn M, Alizadeh AA. Data normalization considerations for digital tumor dissection. *Genome Biol* 2017;18:128.
- [49] Strouml;bel P, et al. Paraneoplastic myasthenia gravis correlates with generation of mature naive CD4 + T cells in thymomas. *Blood* 2002;100:159–66.
- [50] Ottensmeier CH, et al. Upregulated glucose metabolism correlates inversely with CD8 + T-cell infiltration and survival in squamous cell carcinoma. *Cancer Res* 2016;76:4136–48.
- [51] Bethwaite PB, Holloway LJ, Yeong ML, Thornton A. Effect of tumour associated tissue eosinophilia on survival of women with stage IB carcinoma of the uterine cervix. *J Clin Pathol* 1993;46:1016.
- [52] Sjöberg E, et al. A minority-group of renal cell cancer patients with high infiltration of CD20 + B-cells is associated with poor prognosis. *Br J Cancer* 2018;119:840–6.
- [53] Cai C, et al. Interleukin 10-expressing B cells inhibit tumor-infiltrating T cell function and correlate with T cell Tim-3 expression in renal cell carcinoma. *Tumor Biol* 2016;37:8209–18.

- [54] Tosolini M, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, Th2, Treg, Th17) in patients with colorectal cancer. *Cancer Res* 2011;71:1263–71.
- [55] Chakravarthy A, et al. Human papillomavirus drives tumor development throughout the head and neck: improved prognosis is associated with an immune response largely restricted to the oropharynx. *J Clin Oncol* 2016;34:4132–41.
- [56] Ward MJ, et al. Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. *Brit J Cancer* 2014;110:489–500.
- [57] Kim S, Eliot M, Koestler DC, Wu W-C, Kelsey KT. Association of neutrophil-to-lymphocyte ratio with mortality and cardiovascular disease in the Jackson heart study and modification by the duffy antigen variant. *JAMA Cardiol* 2018;3:455.
- [58] Leek JT, Storey JD. A general framework for multiple testing dependence. *Proc Natl Acad Sci U S A* 2008;105:18718–23.
- [59] Hyvärinen A, Oja E. Independent component analysis: algorithms and applications. *Neural Netw* 2000;13:411–30.
- [60] Gagnon-Bartsch JA, Speed TP. Using control genes to correct for unwanted variation in microarray data. *Biostatistics* 2012;13:539–52.
- [61] Zheng SC, et al. Correcting for cell-type heterogeneity in epigenome-wide association studies: revisiting previous analyses. *Nat Methods* 2017;14:216–17.
- [62] McGregor K, et al. An evaluation of methods correcting for cell-type heterogeneity in DNA methylation studies. *Genome Biol* 2016;17:84.
- [63] Hattab MW, et al. Correcting for cell-type effects in DNA methylation studies: reference-based method outperforms latent variable approaches in empirical studies. *Genome Biol* 2017;18:24.
- [64] Kaushal A, et al. Comparison of different cell-type correction methods for genome-scale epigenetics studies. *BMC Bioinforma* 2017;18:216.
- [65] Zheng SC, Breeze CE, Beck S, Teschendorff AE. Identification of differentially methylated cell-types in epigenome-wide association studies. *Nat Methods* 2018;15:1059–66.
- [66] Rahmani E, et al. Cell-type-specific resolution epigenetics without the need for cell sorting or single-cell biology. *Nat Commun* 2019;10:3417.
- [67] Li Z, Wu Z, Jin P, Wu H. Dissecting differential signals in high-throughput data from complex tissues. *Bioinformatics* 2019;35:3898–905.
- [68] Luo X, Yang C, Wei Y. Detection of cell-type-specific risk-CpG sites in epigenome-wide association studies. *Nat Commun* 2019;10:3113.
- [69] Teschendorff AE, Zhu T, Breeze CE, Beck S. EPISCORE: cell-type deconvolution of bulk tissue DNA methylomes from single-cell RNA-Seq data. *Genome Biol* 2020;21:221.
- [70] Landan G, et al. Epigenetic polymorphism and the stochastic formation of differentially methylated regions in normal and cancerous tissues. *Nat Genet* 2012;44:1207–14.
- [71] Landau DA, et al. Locally disordered methylation forms the basis of intratumor methylome variation in chronic lymphocytic leukemia. *Cancer Cell* 2014;26:813–25.
- [72] Li S, et al. Distinct evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia. *Nat Med* 2016;22:792–9.
- [73] Teschendorff AE, et al. DNA methylation outliers in normal breast tissue identify field defects that are enriched in cancer. *Nat Commun* 2016;7:10478.
- [74] Teschendorff AE, et al. Epigenetic variability in cells of normal cytology is associated with the risk of future morphological transformation. *Genome Med* 2012;4:24.
- [75] Zhu T, et al. A pan-tissue DNA methylation atlas enables in silico decomposition of human tissue methylomes at cell-type resolution. *Nat Methods* 2022;19:296–306.

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Challenges for single-cell epigenetic analysis **21**

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1 Why single-cell epigenetics?

Cell-to-cell heterogeneity is a fundamental property of multicellular organisms. Although the cells in the body have the same DNA, different cell types carry out different functions by eliciting distinct gene expression programs. The epigenome, the set of nucleo-protein interactions and chemical modifications regulating chromatin structure and DNA accessibility, is the fundamental determinant of this diversity of gene expression. The epigenomic landscape is defined biochemically by DNA methylation and histone modifications, DNA accessibility, and chromatin architecture [1]. The composition and post-translational modifications (PTMs) of nucleosomes regulate chromatin accessibility by affecting nucleosome affinity for chromatin remodelers and transcription factor (TF) binding via steric hindrance. The accessibility continuum, which varies from closed to highly dynamic and permissive chromatin, reflects, to some degree, the regulatory potential of a given genomic locus and provides insight into cell type and cell states (Figure 21.1).

In contrast to bulk epigenomic measurements, where the signal is averaged across cells, single-cell epigenomic measurements enable the identification of cellular subpopulations defined by similarities or differences in their epigenomic landscape. The measurement of this epigenomic landscape helps to (1) identify cis- and trans-regulatory factors that define diverse subpopulations and (2) define the complex regulatory networks that lead to their development.

In the context of cancer, single-cell epigenomics can reveal the gene-regulatory diversity within cell types in the tumor microenvironment, such as cancer, immune, and stromal cells, as well as the diversity of cell states across tumorigenesis, tumor progression, and drug treatment. For example, while mechanisms of drug resistance have traditionally been proposed to be genetic due to the acquisition of mutations, recent work has demonstrated that particular cells may be epigenetically

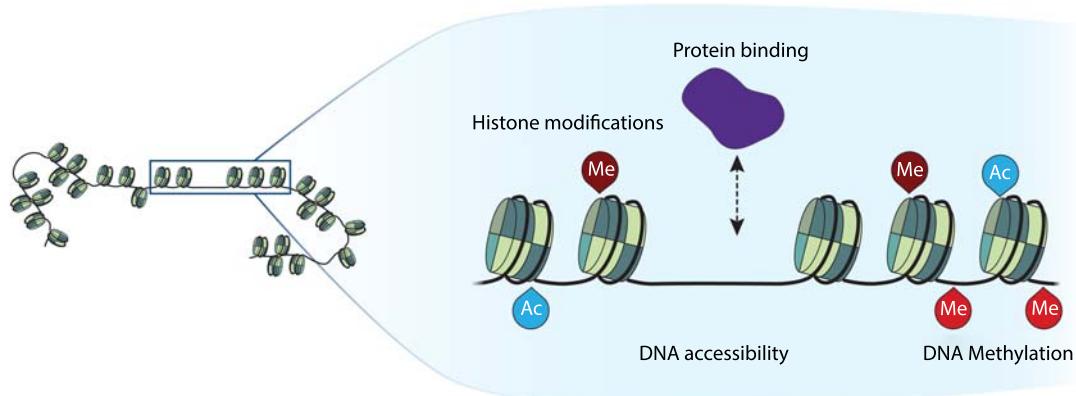


FIGURE 21.1

Single-cell epigenomic modalities. Diverse processes contribute to the epigenome landscape profile, including DNA protein binding (such as TFs and related co-factors), histone modifications (such as H3K4me and H3K27ac), and DNA methylation. These dynamic processes control the underlying gene expression by modulating regulatory elements, such as enhancer accessibility and repressive histone modifications.

primed to develop drug resistance [2]. In melanoma, vemurafenib resistance is an epigenetic phenomenon, wherein cells transition between pre-resistant and nonresistant cell states [3] that are then “locked in” upon drug treatment. Using CRISPR-Cas9 genetic screens, Torre et al. [4] discovered that pushing cells towards a more differentiated epigenetic state increased resistance to vemurafenib, suggesting that modulating epigenetic cellular plasticity could be used to treat drug resistance in cancer. This chapter will provide an overview of current single-cell epigenetic assays in the field, discuss applications of single-cell epigenomics in cancer, describe general principles and technical challenges of single-cell epigenomic assays and analysis, and outline the remaining challenges in using these technologies to study cancer.

2 Current single-cell epigenetic assays and associated challenges

By far the most widely used single-cell technology is single-cell RNA sequencing (scRNA-seq). While scRNA-seq quantifies transcripts in individual cells, gene expression levels arise from complex gene regulatory networks that drive expression through the binding of TFs to promoters and distal regulatory elements. However, TFs are often lowly expressed and may only be expressed in specific critical periods. TFs bind multiple loci in the genome, sometimes simultaneously regulating the expression of multiple genes. Hence, it is often challenging to correlate the TF expression level with the expression of the potential regulatory targets of this TF, complicating the interpretation of the underlying regulatory network. In contrast, single-cell epigenetic assays directly measure the chromatin landscape features that modulate gene expression, including chromatin accessibility, various DNA and histone modifications, as well as the DNA sequence motifs present at these biochemical features. This section will describe the key epigenetic assays, their strengths and limitations, and how they complement each other.

2.1 Single-cell ATAC-seq surveys the epigenome for accessible regions

The assay of transposase-accessible chromatin using sequencing (ATAC-seq) has been widely adopted since its development [5] as an attractive method to survey the epigenomic landscape. Chromatin accessibility, which broadly measures the degree to which nuclear macromolecules physically contact chromatinized DNA, is determined by the occupancy and topology of nucleosomes and other chromatin-binding factors [1]—highly accessible chromatin implies that trans-acting factors such as TFs have relatively unfettered access to DNA, allowing them to bind and exert their gene regulatory activities. In ATAC-seq, a prokaryotic Tn5 transposase loaded with sequencing adapters is used to simultaneously fragment double-stranded DNA and ligate sequencing adapters. Tn5 can only bind in regions of accessible chromatin, enabling the identification of accessible chromatin regions by mapping sequenced reads and identifying regions of increased density of these reads (“peaks” of accessibility as observed by pileups of sequencing reads in a genomic track browser). Analyzing the DNA sequences enriched in these accessible peaks nominates specific TF-binding motifs as relevant regulatory elements.

Single-cell ATAC libraries are generated by using array-, droplet-, or split-pool-based approaches. In the array-based approach, cells are isolated into individual chambers using dedicated

equipment, such as Fluidigm C1 [6] or ICELL8 [7], followed by fragmentation and pooling the library for sequencing. The main challenge in this technology, besides the unique equipment necessary, is the relatively low throughput of these approaches, generally allowing 96–1800 cells to be processed at a time. Droplet-based technologies, such as the commercially available 10x Genomic [8] and BioRad kits [9], represent perhaps the most commonly used single-cell epigenetic analysis platforms. These approaches encapsulate single nuclei with barcoded beads into subnanoliter droplets, where single-cell barcoding occurs. Droplets are then pooled together, where they undergo the sequential DNA sequencing library preparation steps of the scATAC-seq protocol. These approaches offer relatively high-quality data with relatively high throughput, allowing the processing of 100s of thousands of cells or more in approximately one day. Despite the popularity and availability of products using this methodology in the scientific community, these kits are relatively expensive, and these approaches require encapsulation equipment that may not be available to all labs. Stepwise combinatorial cell indexing [10,11] represents an exciting alternative single-cell approach that eschews complex microfluidics or droplet methodologies, and even circumvents the need to isolate and process individual cells. In this approach, nuclei populations are split and barcoded in 96 wells containing different cell barcodes, then pooled, diluted, and redistributed in wells containing a second set of barcodes. Repeating this split-pool process multiple times gives each nucleus a barcoding combination that is likely distinct from other cells. Finally, all cells are pooled, processed, and sequenced. This methodology omits the need for a specialized microfluidic device, can be carried out in any modern laboratory, and drastically reduces the cost of processing. However, despite its low cost, this methodology requires expertise as the protocols contain many steps, sample-specific optimizations, and “homemade” reagents.

In the last few years, scATAC-seq has been paired with other molecular modalities to integrate the chromatin regulatory landscape with other molecular phenotypes of the cell. These efforts include combining scATAC-seq data generation with scRNA-seq [12–15], with scDNAm-seq [16,17], with both scRNA-seq and protein abundance [18,19], with TCR sequencing [20], or with guide capture for single-cell CRISPR-based perturbations [21].

Although scATAC-seq has been widely adopted in single-cell epigenetic research, the method has its associated challenges. Human diploid cells have exactly two DNA copies compared to numerous transcribed mRNA molecules, making the DNA molecule capture process comparatively more challenging per accessible locus, resulting in a relatively sparse dataset [22]. Second, Tn5 displays some sequence preference in its insertion sites with a binding motif that generally favors GC-rich sequences [23–26]. This bias effect is most visible at single base pair resolution, for instance, in the core motif region of aggregate footprinting plots, where nearly identical aligned sequences display strong Tn5 bias between neighboring base pairs. Some methods seek to correct this bias by accounting for the observed native sequence preference of Tn5 [27]. Tn5 bias highly correlates to GC content when aggregating insertions at the peak level, thus some computational methods use per-peak GC content as a covariate during analysis [28]. Lastly, ATAC-seq does not directly identify which proteins are bound to DNA, but rather measures which motifs in the genome are accessible. Accessibility measurements from scATAC-seq can be supplemented with knowledge about specific proteins’ binding motifs (e.g., JASPAR [29]) or by using methods to detect where specific proteins are bound. Furthermore, the correlation between chromatin accessibility at TF motifs and gene expression of the TF proteins themselves also provides further evidence of which TFs might be driving the observed chromatin accessibility patterns.

2.2 Single-cell CUT&Tag surveys histone modification and TF-binding sites

Cleavage Under Targets and Tagmentation (CUT&Tag) [30] identifies the genomic localization of proteins or histone modifications using a Tn5 tethered to protein A (pA-Tn5), which binds to antibodies, thereby enabling targeted tagmentation at specific sites with chromatin interacting proteins. Due to the specificity of antibody-bound Tn5, CUT&Tag offers an alternative to traditional ChIP-seq and presents a far better signal-to-noise ratio and very low background signal while avoiding the bias introduced by the ChIP-seq cross-linking step [31,32]. For example, for an abundant target, such as H3K27me3, which is associated with gene repression, an extremely low number of cells is sufficient to generate a significant signal (the original bulk assay assayed as few as 60 cells). The high sensitivity and specificity of CUT&Tag enable the application of the method to primary and rare cells, allowing the identification of epigenetic signatures within heterogeneous cancer samples (scCUT&Tag) [32,33]. Bartosovic et al. [33] examined histone modifications characteristic of active promoters, enhancers, and gene bodies (H3K4me3, H3K27ac, and H3K36me3) and inactive regions (H3K27me3) in single cells and by identifying the location of specific histone modifications in the genome with scCUT&Tag, they identified evidence of gene regulatory mechanisms, such as promoter bivalency, and spreading of H3K4me3.

Despite its elegant design and demonstrated advantages, scCUT&Tag has some experimental challenges due to its reliance on specific antibodies and Tn5 tagmentation. First, the assay depends on highly efficient and independently validated antibodies, such as the rigorously optimized antibodies targeting diverse histone PTMs and antibodies for a few other abundant and well-validated targets. For low-signal targets, such as low expressed TFs or targets with indirect binding to DNA, the antibodies might not efficiently detect their targets. Furthermore, transiently binding proteins could be destabilized by the stringent high salt wash conditions necessary to minimize the nonspecific binding of pA-Tn5 to accessible DNA. Second, because Tn5 tagments open-chromatin, antibody binding to nonaccessible or heterochromatin regions may suffer from inferior tagmentation efficiency and thus become “invisible” to downstream steps. Some recent efforts have aimed to enhance scCUT&Tag by combining it with surface protein levels (scCUT&Tag-pro [34]) or with scRNA-seq [35,36].

2.3 Single-cell DNA methylation assays survey accessible CpG islands in the genome

DNA methylation is a heritable epigenetic mark associated with silenced genomic regions [37] deposited by methyltransferase enzymes that preferentially couple methyl group (CH3) to deoxyribonucleosides at accessible DNA. The most common methylated DNA base is 5-methylcytosine (5meC) [38] which tends to cluster in CpG islands (dense DNA regions with CpG enrichment) generally associated with promoters [39]. Abnormal DNA methylation is implicated in disease processes, such as fragile X syndrome [40] and, most notably, in cancer [41,42].

Measurement of base-resolved methylation signals from single cells is achieved by bisulfite conversion and enzymatic conversion methods. Single-cell bisulfite methods rely on the chemical conversion of 5meC to uracil by sodium bisulfite, which is read out as thymidine in the sequencer. Although chemical conversion achieves very high efficiency (> 99%) in commercial kits, it also induces DNA degradation [43]. DNA content losses due to degradation during the conversion limit the coverage of data obtained for downstream applications. To minimize DNA loss in library preparation steps, Miura et al. [44] developed the postbisulfite adaptor tagging methodology, which was then implemented by many single-cell

DNA methylation protocols. Many single-cell methylation assays rely on bisulfite conversion-based technology [45,46], and they can be further divided into RRBS (reduced representation bisulfite sequencing) and WGBS (whole-genome bisulfite sequencing) adaptations: RRBS-based assays are focused on a subset of CpG-rich regions only, and therefore decrease downstream costs by assaying only a fraction of the genome, whereas WGBS-based assays survey the entire methylome, and have simpler protocols but require more sequencing per cell to uncover the genomic methylation patterns.

A fundamental challenge in assaying epigenetic states using WGBS approaches is the relatively low genome coverage that stems from limited CpG islands sites. This partial genome coverage makes the study of distal regulatory elements, intergenic regions, and allele- or strand-specific difficult to decipher [47–50]. To improve detected coverage, enzymatic methods have been developed to detect unmethylated Cs, aiming to avoid the limitations imposed by the bisulfite conversion methods by, for example, using methylation-sensitive restriction enzymes to probe for the presence of methylated Cs [51–53]. However, this type of semi-targeted assay suffers from reduced coverage that is dependent on the restriction enzymes' site abundance.

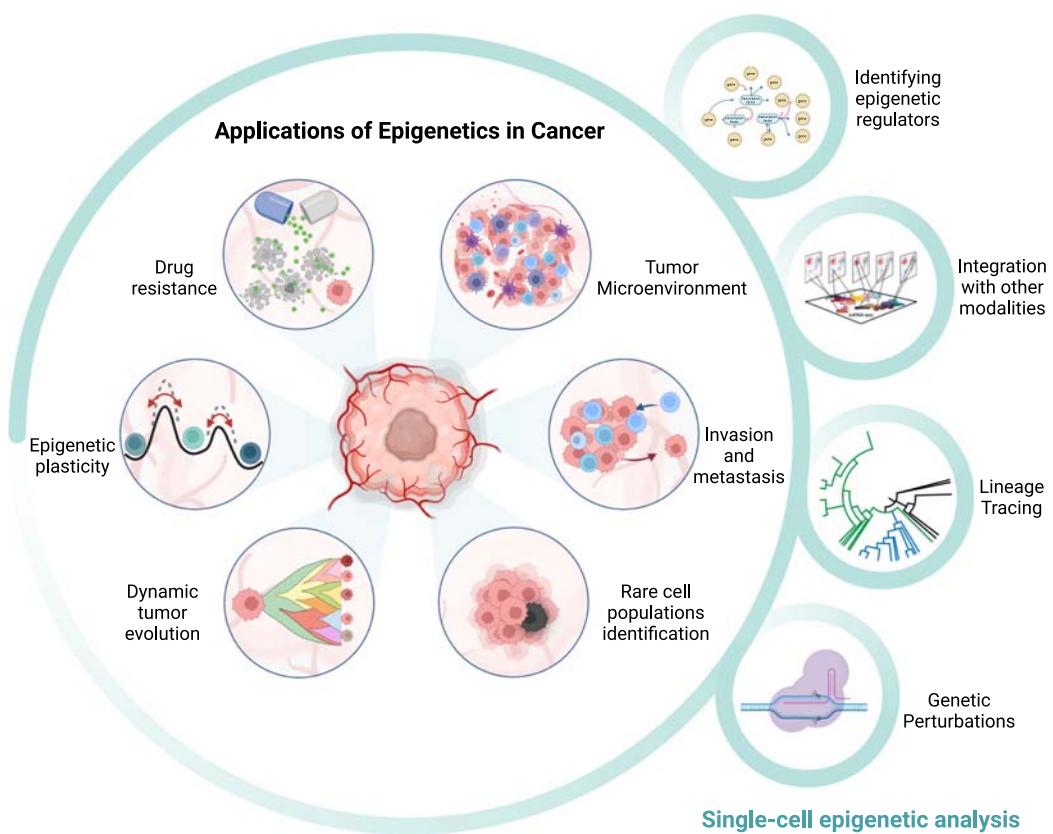
Although generally associated with gene repression, DNA methylation can also mark active gene expression [54–56]. In cancer, the role of DNA methylation is more ambiguous due to altered regulatory networks, resulting in more challenging understanding of the methylation pattern in regard to gene activity [57–59]. Given these complexities, many groups have aimed to combine methylation-based assays with other molecular readouts in single cells, including the combination of WGBS-based data with single-cell transcriptomics [16,60], accessibility [61,62], or all three of the modalities together [63].

3 Applications of single-cell epigenetics in cancer

Tumors are complex environments consisting of malignant transformed cancer cells, normal stromal cells, and infiltrating immune cells. Each of these underlying cell types can also occupy a continuum of cell states, which can be probed using single-cell epigenomics. For example, malignant cancer cells can span different stages of differentiation from a more stem cell-like state to a more differentiated state [64], and tumor-infiltrating lymphocytes can be activated to elicit an immune response against cancer cells or exhausted from chronic inflammation [65]. In cancer, studying the epigenomes of single cells allows the mapping of inter- and intra-tumor heterogeneity, thereby isolating the cell type-specific chromatin regulatory processes that may contribute to tumorigenesis and tumor progression. In addition, coupling single-cell epigenomic assays such as scATAC-seq with single-cell transcriptomics (scRNA-seq) enables both the identification of epigenetic regulators driving these diverse cell states, and the effects of these regulators on gene expression. Identifying the epigenetic drivers of dysfunctional cell states in cancer and immune cells can also be exploited therapeutically to reactivate infiltrating immune cells and overcome drug resistance. In this section, we will briefly review a few of the applications of single-cell epigenomics in cancer (Figure 21.2).

3.1 Defining epigenetic changes underlying malignant transformation and oncogenesis

Epigenetic changes have recently been demonstrated to be central to malignant transformation. In animal models of multiple myeloma and diffuse large B-cell lymphoma, transient induction of

**FIGURE 21.2**

Applications of single-cell epigenetics in cancer. Epigenetic measurements and analysis of tumors are used as comprehensive tools to understand the complex and ever-changing tumor environment.

oncogene expression (*MafB* and *Bcl6*, respectively) was sufficient to cause cancer initiation [66,67]. Across these models, cancer cells contained either hyper- or hypomethylated CpG islands and promoters, which were inherited throughout B-cell development, suggesting that the temporal expression of *Bcl6* is sufficient to epigenetically prime cells for cancer initiation [66]. By coupling temporal genetic perturbations and scATAC-seq in a mouse model of pancreatitis, Alonso-Curbelo et al. [68] demonstrated that following pancreatic injury, a permissive cancer-associated epigenetic state emerges. In the presence of an oncogenic *KRAS* genetic mutation, a unique chromatin remodeling program diverts BRD4-mediated transcription from normal lineage-specifying loci to cancer-defining loci, facilitating the pancreatic metaplasia to neoplasia transition [68]. While *KRAS* mutations alone are insufficient to initiate cancer, this work demonstrates the role of permissive epigenetic states in acquiring genetic mutations to initiate cancer.

Although it is more challenging to study oncogenesis in human models due to the difficulty in obtaining samples across transformation, the findings observed in animal models have also been seen in the limited existing human data. Becker et al. [69] defined cell state and composition

changes that occur along a malignant transformation trajectory from normal tissue to polyps to colorectal cancer by profiling single-cell transcriptomes and epigenomes in patients with familial adenomatous polyposis. Polyps and colorectal cancers contain more stem-like cells than unaffected tissues, and these cells exhibit dysfunctional epigenetic and gene expression programs distinct from normal stem cells. This analysis identified TFs associated with the normal colon to carcinoma transformation, including increased accessibility at TCF and LEF TF motifs and decreased accessibility at KLF motifs. Thus, in both animal and human models, single-cell epigenomics have revealed the increased plasticity and permissive epigenetic states that can underlie tumor initiation.

3.2 Characterizing epigenetics underpinnings of intratumoral heterogeneity

Single-cell epigenomic studies have defined cell state transitions underlying cancer progression and intratumoral heterogeneity. Gliomas have long been known to exhibit intratumoral heterogeneity in gross pathology, but recent single-cell technologies have allowed for the characterization of distinct transcriptomic and epigenomic profiles underlying this heterogeneity [70]. Single-cell RNA sequencing of primary human glioblastomas (GBMs) identified a continuum of four cellular states (neural progenitor-like, oligodendrocyte progenitor-like, astrocyte-like, and mesenchymal-like) [71]. More recently, the integration of single-cell transcriptomics with single-cell epigenomics, including chromatin accessibility and DNA methylation, has revealed the epigenomic underpinnings of the heterogeneity and plasticity of GBMs [72,73]. Using scATAC-seq in GBM stem cells, the population of self-renewing cells driving cancer progression, three subsets of reactive, constructive, and invasive cells were identified [73]. Each of these glioblastoma stem cell (GSC) states is defined by a unique gene regulatory program driven by distinct TFs, and the invasive GSC epigenomic signature was shown to be predictive of low patient survival [73]. Because DNA methylation state is largely inherited in cell division, it can be used as a “molecular flight recorder” to infer the lineage of individual cells in a tumor. By integrating the single-cell transcriptomic profiles with inferred lineage from single-cell DNA methylation, Chaligne et al. [72] demonstrated that while *IDH*-wild type gliomas exhibit greater plasticity and reversibly switch between stem-like and differentiated-like states, *IDH*-mutant gliomas exhibit a hierarchical differentiation from stem-like cells to glial-like cells [72,74].

3.3 Identifying the gene-regulatory mechanisms of metastasis

Bulk epigenomic studies have profiled the changes in chromatin accessibility that occur across metastasis and identified the driving epigenetic regulators. In a genetically engineered mouse model of small cell lung cancer, the comparison of genome-wide chromatin accessibility profiles of primary tumors to metastases revealed an immense increase in accessible distal regulatory elements in metastases. In particular, differentially accessible sites were highly enriched for Nfib motifs. Nfib promotes pro-metastatic neuronal gene expression programs, which drive metastasis [75]. While bulk epigenomic assays have identified changes between primary tumors and metastases, single-cell epigenomics can also be used to identify the continuum of chromatin state transitions across the progression towards metastasis. For example, single-cell transcriptomics and epigenomics revealed coaccessible regulatory programs that occurred in a murine model of lung

adenocarcinoma. They demonstrated that activation of RUNX TFs can drive a premetastatic transition and is predictive of survival in lung adenocarcinoma patients [76].

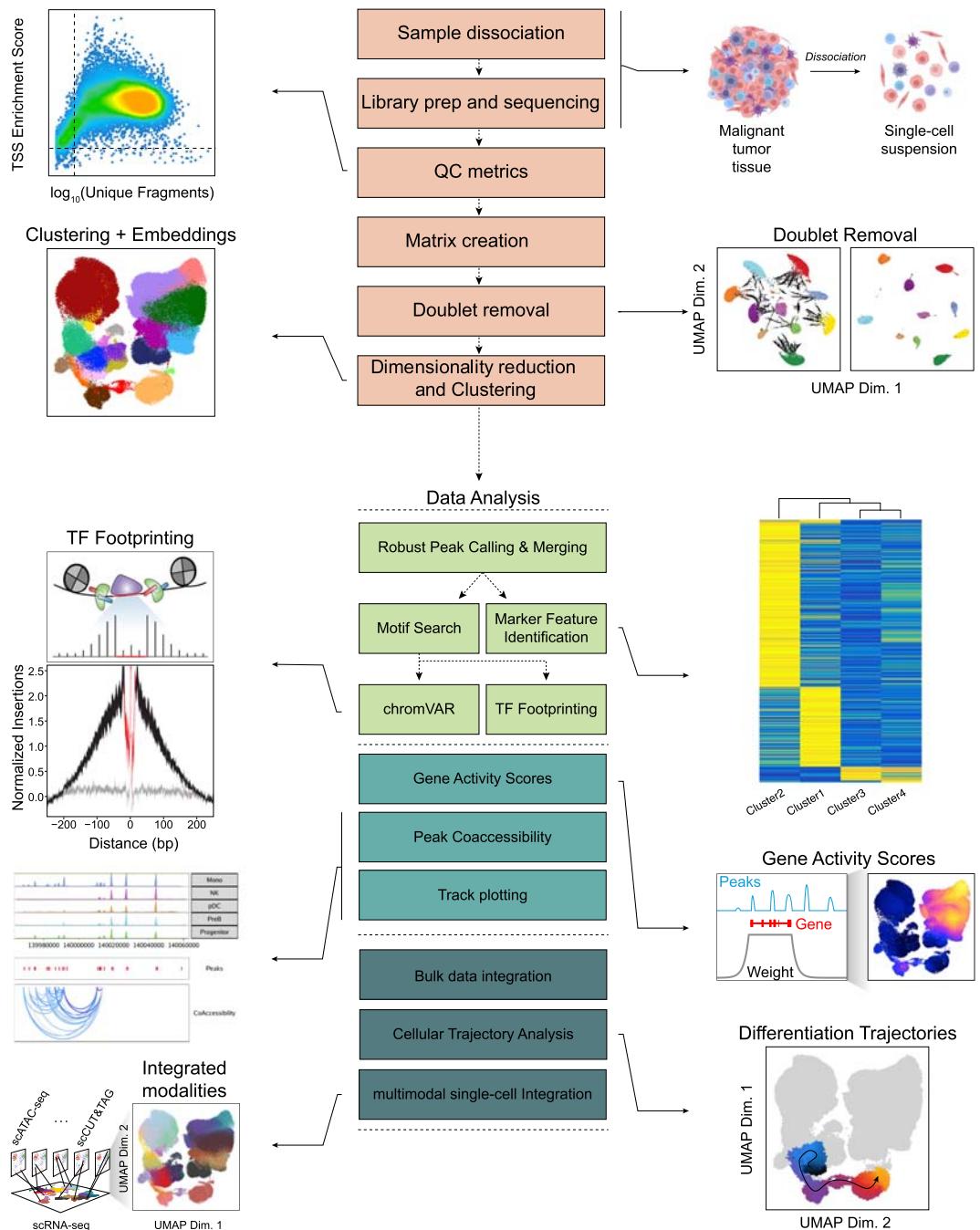
Pseudo-time trajectories can be constructed from single-cell epigenomics datasets, enabling the inference of the progression of cell states across cancer progression. However, the sole use of pseudo-time models to infer cancer progression may obscure the complexities of reversible paths of dedifferentiation and dead ends that do not promote cancer progression, such as senescence. Recently, Ludwig et al. [77] demonstrated the use of somatic mitochondrial DNA (mtDNA) *in vitro* and *in vivo* as natural genetic barcodes. Somatic mutations in mtDNA can be detected with single-cell ATAC and single-cell RNA sequencing, allowing for the coupling of cell lineage and cell states in cancer.

3.4 Defining epigenetic signatures of the tumor microenvironment

Recently, the tumor microenvironment (TME) has become a central focus for understanding cancer pathogenesis. In addition to the genetic and epigenetic state of cancer cells, the ecosystem of cancer, immune, and stromal cells in the TME informs cancer progression and response to therapy. For example, tumor-infiltrating cytotoxic T cells can recognize and attack cancer cells. However, the epigenetic state of tumor-infiltrating T cells influences their efficacy in immunosurveillance. Upon chronic activation during tumorigenesis, infiltrating T cells can enter an exhausted state, characterized by the expression of inhibitory receptors, such as PD-1 and CTLA-4, that prevent their cytotoxic function [78]. The expression of PD-1 and CTLA-4 can also act as an inhibitory signal to neighboring immune cells, dampening the immune response against cancer cells. Exhausted cytotoxic T cells exhibit distinct chromatin accessibility profiles compared to either functional or memory T cells [79,80]. While only ~10% of genes are differentially expressed between functional and exhausted T cells, ~50% of chromatin regions are differentially accessible [81].

In addition to adaptive immune cells, innate immune cells such as macrophages exhibit epigenetic plasticity in the TME. Tumor-associated macrophages (TAMs) can promote an immune response against tumors by presenting antigens to adaptive immune cells and secreting pro-inflammatory cytokines, or they can promote tumor progression by producing proteolytic enzymes, secreting growth factors, and promoting the epithelial-mesenchymal transition [82,83]. The pro-inflammatory and anti-inflammatory states of TAMs are characterized by distinct chromatin accessibility profiles driven by the expression of TFs, such as Stat6 [84]. Single-cell RNA sequencing data has shown that beyond the traditional *M1* (pro-inflammatory macrophages) and *M2* (anti-inflammatory macrophages) states, there is a continuum of macrophage expression programs [85,86]. Defining dysfunctional epigenetic states of immune cells in the TME and potential paths towards “correcting” these pathological states can lead to the development of therapeutics that modulate the epigenetic state to reprogram exhausted T cells and TAMs [87].

Single-cell RNA- and ATAC-seq enable the identification and epigenetic profiling of diverse cell types in the tumor microenvironment. These techniques have been used to uncover how T cells become exhausted and dysfunctional in cancer, how tumor-associated macrophages can promote cancer stemness, and how immune cells undergo distinct metabolic reprogramming in cancer. However, challenges remain in applying single-cell epigenomics to the tumor microenvironment. Technical limitations, such as the different susceptibility of immune cell types to depletion in single-cell preparations, can skew relative immune cell abundances and TME landscapes.



(Continued)

4 Practical considerations for single-cell epigenetic analysis (and associated challenges)

From sample processing to data analysis, we will describe the general principles and challenges of single-cell epigenetic analysis, following the scATAC-seq pipeline as an analysis framework (Figure 21.3).

4.1 Dissociation

As with all single-cell-based methods, high-quality single-cell suspension preparation is critical for data quality. Apoptotic cells or chromatin debris may not only increase background noise, but may also reduce the sensitivity of the assay by interfering with enzymatic reactions. Here, we will describe key considerations in designing a dissociation strategy.

For freshly collected samples, time to dissociation and time to assay are critical for optimal preservation of cell state. Prolonged tissue exposure to room temperature, saline buffers, or other storage methods can induce artifacts, such as the upregulation of stress response genes [88]. Therefore minimizing and recording the time to dissociation and time to assay can help to reduce these confounding responses that may present as batch effects in downstream analyses. Viability stains or dyes are common and effective ways to assess if fresh tissue dissociation was sufficient to dissociate the cells without leading to cell death.

In the case of preserved samples (i.e., fresh-frozen or fixed samples), the details of the preservation method may lead to disruption or depletion of specific cell types, or changes to the cell state. Evaluating the impact of preservation on cell type distributions through flow cytometry or immunofluorescence using markers for cell type of interest is often a straightforward and effective pilot experiment to confirm if preservation approaches are appropriate for a specific experimental question.

The second parameter to consider is the method of dissociation. Fresh samples can be used to generate dissociated live cells or prepared as nuclei. However, if samples have been preserved before analysis, then direct nuclei preparation is likely the only available option. For fresh enzymatic dissociation, often a careful review of the literature and pilot testing to dissociate the tissue that maximizes viability while minimizing doublets is usually required. Oftentimes, a dissociation method will need to be optimized for the tissue of interest, in which case there are two axes to

◀ FIGURE 21.3

scATAC-seq analysis workflow. General scATAC-seq preprocessing and analysis fundamental steps allow a thorough investigation of cancer samples. Preanalysis steps (top six) are necessary to obtain high-quality data as this will underlie the subsequent analysis steps. scATAC-seq data facilitate complex analysis, such as marker feature identification, TF footprinting, cellular trajectory analysis, integration with other modalities, and inference on gene activity.

Adapted from Granja J.M., et al. ArchR is a scalable software package for integrative single-cell chromatin accessibility analysis. Nat Genet 2021;53:403–411.

focus on for optimization: (1) the mechanical force applied to the tissue and (2) the type and extent of enzymatic digestion. To optimize the first axis, multiple commercial protocols and devices are available for mechanical disruption of tissues, such as the gentleMACS system. Coupled with enzymatic components, these commercial protocols provide an accessible starting place for additional optimizations if needed. Throughout these optimizations, cell viability measurements and doublets/multiplets quantifications can be used to identify the best protocol for further processing.

Unlike relatively unique, tissue-specific strategies for fresh enzymatic dissociations, multiple methods for nuclei preparation from preserved tissues have worked robustly across different tissue/tumor types [89,90]. These methods generally combine mechanical homogenization and detergent-based lysis to create a nuclei suspension.

Finally, once a satisfactory means of generating single-cell suspension is worked out, specific cell types might need to be enriched or depleted to appropriately capture the diversity of cell types in a given sample. For enrichment/depletion, both flow or magnetic separation of antibody-labeled cells/nuclei have been broadly used across tissues or tumors [91]. Further enrichment for intact whole cells and nuclei by Fluorescence-Activated Cell/Nuclei Sorting (FACS or FANS, respectively) can improve assay quality by minimizing free floating chromatin that may increase background noise.

4.2 Single-cell epigenomic platforms

Following sample dissociation, an appropriate single-cell platform should be chosen based on sensitivity, throughput, and cell type of interest for the experiment. Here, we describe major categories of single cell platforms and their strengths and weaknesses.

All single-cell methods rely on linking an identifying, unique cellular barcode to relevant molecules of interest within an individual cell. This cell-to-barcode linkage can be accomplished either by physical isolation of cells followed by a separate reaction that links barcodes to molecules (plate-based or droplet-based) or by dynamic barcoding by split-pool, which combinatorially builds up unique barcodes for each individual cell.

Historically, initial single-cell RNA-seq applications relied on physically isolating individual cells into a plate, then processing RNA-seq data from each well, generating high-quality data—an approach that remains perhaps the gold standard for single-cell RNA-seq data generation [92]. Methods for physical isolation have also been used for epigenomic profiling to perform highly sensitive measurements of chromatin accessibility (scATAC-seq) and protein-DNA interactions (scCUT&Tag) [32]. While these methods allow for higher sensitivity, they have significant limitations on throughput. Because individual cells must be sorted into unique wells and prepared into libraries in parallel, these methods are limited to a few thousand cells without requiring extensive automation.

Another class of single-cell platforms relies on physically isolating each cell into a droplet created as a water-in-oil emulsion. These advances in droplet-based isolation of single cells by commercial providers such as 10x and Bio-Rad have enabled the broad adoption of single-cell epigenomic profiling. These methods simultaneously separate and immediately label each cell in a microfluidics system, enabling higher throughput while maintaining assay sensitivity. The higher throughput of droplet-based methods has enabled high-quality epigenomic profiling of 10s to 100s

of thousands of cells [79]. The major drawback for these commercial products is the steep cost of the reagents and devices necessary to perform these experiments.

Finally, split-pool-based methods forego the physical isolation of cells for dynamic combinatorial barcoding to enable higher throughput at lower cost. Conceptually, split-pool-based methods rely on splitting and pooling cells through multiple rounds of barcoding to dynamically create a unique barcode combination for each cell. For example, cells can be split up into 96 unique barcodes and then pooled back together to be split up again into 96 unique barcodes [93]. Because the probability that two cells travel together through the same barcodes over multiple rounds of splitting and pooling decreases exponentially with increasing number of barcodes and rounds, split-pool methods can dynamically label a large number of single cells in parallel. Therefore, the strength of this approach is in its scalability. Because split-pool combinatorial indexing approaches do not require physical isolation of single cells, a large number of cells can be labeled in parallel across an arbitrarily scalable number of unique barcodes for each round, which significantly reduces the cost while increasing throughput [10,93]. However, split-pool-based methods require prolonged sample processing times, which can lead to reduced sensitivity of the assay as well as cell type-specific biases that may exacerbate downstream analyses.

Lastly, an important consideration beyond cost and throughput is cell-type specific capture biases that may be unique for each single cell platform. For example, droplet-based isolation methods often encourage removing granulocytes from peripheral blood monocyte preparations due to their propensity to degranulate. However, certain plate-based single-cell platforms have been able to capture them effectively due to the more gentle mechanical forces involved in their isolation methods [94]. Therefore choosing the most appropriate platform for the sample type and experimental question or leveraging a combination of platforms [95] will be essential for capturing the relevant cell types for the experimental question.

4.3 Analysis: quality metrics

Initial analysis of single-cell epigenomic data begins with per-cell quality metric assessment and creating a consensus data structure for further analysis. Here we will describe key considerations for quality metrics in single-cell epigenomics datasets.

Quantification of assay sensitivity often provides figures of merit that report the number of molecules detected for each cell, such as fragments per cell or the number of fragments expected at “infinite” sequencing depth (i.e., absolute complexity). Filtering low-quality cells based on this metric will remove low-quality cells or under-sequenced cells from the analysis. Metrics of specificity generally assess how accurately the data generated from a single cell represents the underlying epigenetic cell state. For example, in single-cell ATAC assays, the TSS ratio of a given cell, (i.e., the fraction of fragments originating from the annotated transcription start sites divided by fragments originating from flanking regions) can be used to filter low-quality nuclei with naked chromatin or background nonnuclear chromatin debris. Furthermore, in assays such as sc-Cut&Tag where fragments are not necessarily coming from well-defined sites of the genome, assessing the per cell number of reads in peaks can be used as a metric for specificity. To quantify reads in peaks, fragments from cells in a given cluster are treated as a bulk sample (or pseudobulked) to perform peak calling. This “bulkification” of single-cell data is necessary because single-cell epigenomic data can only capture either 0, 1, or 2 fragments from a given site in the genome,

necessitating pseudobulked data to perform peak calling based on pile up of fragments. Quantifying the number of fragments in peaks per cell on pseudobulked clusters from preliminary clustering can be used to identify low-quality cells in an assay agnostic manner if the epigenetic marker of interest is not enriched in the TSS.

4.4 Analysis: single-cell epigenomic data matrix preparation

While single-cell transcriptomic measurements are limited to the set of all genes in the human genome (~25,000) per cell, single-cell epigenomic measurements generate genomic fragments that can align anywhere on the 3 billion base pairs of the human genome. Therefore binning the fragments detected in single-cell epigenomic assays into computationally tractable and biologically meaningful bins is the first step in creating a data structure for downstream analysis. These data structures are common in the format of matrices with cells as rows and genomic loci as columns. Two approaches are often used to bin the fragment counts in single-cell epigenomic data: fixed-width tiles and variable-width peaks. By starting with fixed-width tiles, all standard dimensionality reduction, clustering, and cluster identification methods can be performed without relying on a biologically specific set of genome regions. This approach thus allows for unbiased cluster generation and can allow additional samples to be added to the dataset without further analysis. Alternatively, the peaks that are called on a per sample or per cluster level can be used as elements in the data matrix. However, most recent tools perform dimensionality reduction and clustering using the tile matrix for cluster generation and identification followed by peak calling on a per-cluster level to preserve the cell-type-specific peaks for downstream analysis [96].

4.5 Analysis: clustering, annotation, and doublet removal

After preprocessing, single cells within a data set are typically organized by “clustering”—that is, organizing cells with similar molecular signatures together. Clustering then allows for pseudo-bulk analysis (i.e., aggregation of data from similar cells), which increases the power to measure differential epigenetic signatures, and reduces the total number of such comparisons to a more manageable number. Diverse computational tools can carry out this clustering analysis, given a data matrix, and while computational details vary, all methods strive to identify dimensions of salient variable features in the data set and group cells that cluster together in a subspace of this data manifold [96–98].

However, while single-cell computational tools can easily generate clusters at different levels of resolution, these clusters must be annotated based on prior biological knowledge to produce collections of cells that comprise a relevant biological function at the relevant level of cellular resolution. To achieve this annotation, iterative subclustering, or the clustering of cells into finer collections until the additional clusters that have been identified do not have meaningful differential biological information, is often useful. To enable this iterative subclustering, the separation of broad subsets of cell type (e.g., epithelial, stromal, endothelial, and immune compartments) before performing iterative subclustering can focus the variable feature selection to cell types within a given compartment and away from the large differences between compartments which can be irrelevant to identifying these more subtle biological differences in the subclusters.

Existing annotations from large consortium datasets [99,100] can also be used by unsupervised cluster identification methods that link signatures from one data set to the signatures from a “gold standard” consortium data set, transferring consortium annotations from the nearest cell in that data set to each of the cells in the provided data set [101]. While such methods are widely used in scRNA-seq datasets, epigenomic reference datasets are currently fairly limited. To plug this gap in data sets, methods for cross-modality label transfer have been developed [96]. These methods rely on using Gene Scores (a proxy for gene expression derived from accessibility about a promoter) to identify clusters using reference scRNA-seq datasets. Recent developments in multiomic methods allow datasets that capture multiple modalities to function as a “bridge” between two datasets that were generated independently. This enables cluster annotation of epigenomic datasets when transcriptomic datasets have not been generated for it [102].

Cluster identification can also enable doublet removal—droplets containing two cells will likely map to regions “between” biological bone fide clusters. Often, these doublets form “connecting bridges” linking cell types with no biological or developmental relationship. For these technical doublets, computational methods that create synthetic doublets from distinct bone fide biological cell types can then nominate cells in the dataset that could be candidate doublets and cluster with these synthetic doublets [96]. However, doublet removal sometimes does not identify a significant number of cells that influence a given cluster to alter the downstream analysis and therefore should be tested before being applied to the dataset [103].

4.6 Analysis: feature-based analysis

Following cluster identification and annotation, a cluster-specific feature identification will then define epigenomic features of interest for downstream analysis. Oftentimes, performing per cluster feature identification after fixed-width tile-based dimensionality reduction enables better resolution of biologically relevant features (i.e., peaks) for downstream analysis. In most cases, this starts with performing peak calling on a pseudobulked profile of each cluster to identify cluster-specific peaks. A challenge with peak calling is creating a unified peak set for all cells in a given dataset. Although many approaches have been developed, the iterative peak collapsing for scATAC-seq data described in ArchR represents one approach to capture variable peak sizes and peaks that are present in a limited number of cells [96]. Depending on the assay type, additional bias corrections (i.e., Tn5 bias [34]) or the creation of fixed-width peaks may be necessary for creating the peak matrix. After creating a feature matrix, subsequent differential feature analysis can be performed to highlight regions of the genome that may be variable across different cell types or samples. Furthermore, enrichment of *trans* factor (such as TFs or remodelers) colocalization based on footprinting or integration with bulk datasets can shed mechanistic insights into key gene expression signatures or regulatory regions of interest.

4.7 Mechanistic insights from single-cell epigenomic analysis

4.7.1 Peak-to-gene linkages

A common way to leverage single-cell epigenomic datasets is to integrate with a single-cell gene expression dataset to identify regions of the genome with an epigenetic signal that is

correlated with expression of a given gene. This peak-to-gene linkage identifies potential regulatory regions of the genome in a cell type-specific manner, which can better focus on critical regulatory regions in specific pathways. Many tools, such as ArchR, SnapATAC, Signac, FigR, and others, allow for this style of analysis [96,97]. Furthermore, regulatory regions that have the most peak-to-gene linkages are enriched for super-enhancers that have been implicated in common developmental pathways [13]. Identifying key regulatory regions in a specific cell type or process can also be used to link the likely transcriptional effects of causal genomic mutations in regulatory regions.

4.7.2 Pseudotime trajectory analysis/velocity and potential

Trajectory analysis identifies and orders cells along a continuous path intended to trace out a biological state change. Specifically, in the context of cancer, these trajectories might span the process of malignant transformation [69,104,105] or cellular responses to treatment [79]. Pseudotime alignment methods have been applied to scATAC-seq data to link centers of clusters in a supervised manner to trace the best fit line across a set of cells (ArchR, Signac, SnapATAC). These methods often require significant prior knowledge to define the start or end clusters. Recent efforts used multiomic measurements (RNA and ATAC) to incorporate both the changes in accessibility of a given locus as well as the subsequent gene expression changes in the genes linked to those peaks [13,103]. These trajectories can then be used to identify key regulatory regions and genes that correlate with the continuum of the cellular process. For example, Becker et al. [69] demonstrated anticorrelation between DNA methylation and accessibility across a continuum of colorectal cancer cell transformation at the single-cell resolution, identifying key regulatory regions impacted by an aberrant gain of methylation.

4.7.3 Inferring lineage from single-cell epigenomic data

While genetic lineage tracing is a powerful tool for studying primary tumor and tumor-infiltrating lymphocyte heterogeneity and metastases in model organisms [106,107], these powerful tools cannot be applied to primary human samples where genetic manipulations are not feasible. Approaches that have used somatic mutations are often error-prone or are unable to identify cells beyond the first generation [108,109]. Recent work leveraging the higher frequency and stable transmission of mitochondrial mutations has demonstrated that lineage tracing in human samples was possible across multiple single-cell methods [110]. Specifically, by leveraging the high read coverage of mtDNA in scATAC-seq that are often filtered out in preprocessing, the authors were able to identify lineages within the population as well as simultaneously capture the accessibility landscape [110]. Lineage information, as supplied by this strategy of mitochondrial lineage tracing, linked to the epigenomic landscape of cancer cells can provide insights into drivers of metastases, treatment-induced lineage selection, and tumor evolution.

4.7.4 CNV detection

Genome instability leads to copy number variation (CNV), which subsequently drives cancer development and evolution. Identifying CNVs often requires whole genome sequencing to quantify regions of the genome that have undergone amplification or loss. However, because epigenomic assays capture and sequence DNA associated with an epigenetic feature, these methods can also infer the copy number of genomic regions. Specifically, ATAC-seq has been used to quantify

increased copy numbers of a genomic region with a TF implicated in the metastatic potential of a mouse model of lung cancer [75,89]. With sufficient coverage, similar approaches will be viable in single-cell epigenomic assays.

5 Challenges and unanswered questions in epigenetic research in cancer

5.1 Open questions in cancer epigenomics

Despite the growing number of single-cell epigenomic studies in cancer, many unanswered questions still remain. While single-cell epigenomics can resolve the diverse cell types and cell states present in the tumor microenvironment [111], integrating cell states with spatial information to understand cell-cell contacts, immune cell infiltration, and clonality will likely uncover additional regulatory mechanisms of cancer and immune cells. Spatial transcriptomic studies have already revealed the existence of tumor-specific immune cell niches [111], and spatial-multimodal analysis would identify cis- and trans-regulatory factors defining these states.

While the dysfunctional epigenetic state of exhausted tumor-infiltrating lymphocytes has been well characterized, the aberrant cell states of stromal and innate immune cells in the TME are less understood. For example, cancer-associated fibroblasts (CAFs) remodel the extracellular matrix and mediate crosstalk with cancer cells and infiltrating immune cells. CAFs are characterized by heterogeneity in function [112], and a recent scRNA-seq of genetically engineered mouse model of breast cancer revealed three CAF subpopulations demonstrating distinct functions and spatial locations within the tumor [113]. While CAF subsets have been defined transcriptionally, single-cell transcriptomics are ripe for integration with scATAC-seq to identify epigenetic drivers of the CAF subpopulations.

Drug resistance, traditionally attributed to the acquisition of genetic mutations, is increasingly being understood as an epigenetic phenomenon [4]. Future work in identifying the epigenetic drivers of drug resistance, understanding the persistence and heritability of epigenetic states, and in exploiting epigenetic states therapeutically to overcome drug resistance are exciting avenues of investigation. Beyond characterizing cell states in the TME, modulating and “correcting” the epigenetic states of cancer and immune cells to prevent metastasis and harness the immune system against cancer cells remain open areas of potential therapeutic intervention.

5.2 Identifying cancer cell types and cell states

Identifying a cell’s state and clustering similar cells together is a fundamental step in any single-cell analysis assay, providing structured data for downstream analysis steps. Assigning cell type/state can be done in a supervised clustering, where the cells are being compared to known and curated datasets [114,115] or in an unsupervised clustering, where clusters of similar cells are annotated manually by examining the cluster’s features. For noncancerous samples, a compendium of reference datasets and atlases exist, allowing relatively fast cell-type and state identification, especially for transcriptomic data. User-friendly web interfaces have also been developed in order to facilitate easier data access (e.g. CELLxGENE [116]), eliminating the need for advanced computational expertise. In contrast, there are inherent challenges in cancer-related single-cell research.

First, tumors are known to contain a mixture of malignant and normal cells, as well as infiltrating cells from the tissue-of-origin. Each of these cells can harbor a different epigenetic profile, which complicates the cell-state clustering, either in a supervised or an unsupervised manner. Further, the specific single-cell epigenetic profiles can be unique in a way that makes supervised comparison to a reference less accurate. For example, Corces et al. [117] surveyed chromatin accessibility profiles from AML patient-derived primary cells and compared them to normal hematopoietic cell types. They showed the degree of patient-to-patient variability of AML cells' epigenomic states, clustering as normal monocytes, as cancer cells, or even as distinct from any normal cell type. The study also suggested that leukemia is derived from a dysregulated stem cell pool, which necessitates a more complex unsupervised analysis when comparing cancer samples to other datasets. This example illustrates the intra- and inter-patient cancer epigenomic variability, making the identification and subsequent clustering of the cells a fundamental challenge in cancer research.

5.3 Identifying tissue- and cell-of-origin

During normal differentiation, the epigenome changes in a regulated manner. In stem cell-like states, the epigenome is often considered to be more permissive, with DNA methylation, histone PTMs and other repressive marks in low abundance. During differentiation, cells exhibit a gradual increase of repressive markers, such as DNA methylation, and a concomitant loss of chromatin accessibility [118]. Other differentiation programs are more step-wise and might have distinctive phases of epigenetic changes [119]. Comparing epigenetic alterations during tumor progression allows identification of similarities and distinctions between cancer and normal differentiation states [69,71]. These comparisons may reveal that some cancers appear to exhibit impaired differentiation or dedifferentiation, revealing key developmental regulators responsible for these aberrant signals and, thus potential therapeutic targets. Although many dynamic processes, such as keratino-genesis [120], adipogenesis [119], and B cell maturation [121] have been characterized epigenetically, many other differentiation programs still lack comprehensive epigenetic profiling. Thus acquiring more comprehensive epigenetic profiles from normal and cancer oncogenesis processes will allow us to decipher the common and different epigenetic states and mechanisms that lead to malignancies.

5.4 Handling data sparsity

Despite dramatic increases in the number of cells that can be profiled in a single-cell experiment, current technologies still only detect a fraction from the total epigenomic landscape in any given individual cell. For example, when surveying DNA, a molecule that is present in only two copies in a diploid cell, the low copy number results in an inherent data sparsity measurement. This limited sensitivity primarily originates from inefficiencies in target molecule capture. Further fragment losses occur during sample processing, i.e., loss from liquid handling and DNA degradation during bisulfate conversions [43]. These characteristics render the single-cell data very sparse—1%–10% of accessible regions in scATAC-seq [6,22] and GpC site coverage of 15% using scNMT-seq [63]. This data sparsity poses challenges in identifying cell-specific regions of differential signal and is likely to affect downstream analysis, such as clustering and detection of regulatory features [27,34]. One way to overcome this challenge is to use a multimodal assay (e.g., capturing accessibility,

methylation and transcription from the same cell [63]) where the different modalities complement each other's specific deficiencies and result in higher quality single-cell characterization.

6 Concluding remarks

Single-cell epigenomic assays and analyses hold much promise in studying clinically relevant aspects of cancer biology. In the next decade, we foresee rapid technological advances in epigenomic measurements that will facilitate better single-cell profiling in terms of coverage and quality and will further allow novel multimodality measurements from the same cell. These technologies and the massive efforts by various groups will surely provide much-needed research toward meaningful benefit to patients.¹

References

- [1] Klemm SL, Shipony Z, Greenleaf WJ. Chromatin accessibility and the regulatory epigenome. *Nat Rev Genet* 2019;20:207–20.
- [2] Taavitsainen S, et al. Single-cell ATAC and RNA sequencing reveal pre-existing and persistent cells associated with prostate cancer relapse. *Nat Commun* 2021;12:5307.
- [3] Shaffer SM, et al. Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. *Nature* 2017;546:431–5.
- [4] Torre EA, et al. Genetic screening for single-cell variability modulators driving therapy resistance. *Nat Genet* 2021;53:76–85.
- [5] Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* 2013;10:1213–18.
- [6] Buenrostro JD, et al. Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature* 2015;523:486–90.
- [7] Mezger A, et al. High-throughput chromatin accessibility profiling at single-cell resolution. *Nat Commun* 2018;9:3647.
- [8] 10x Genomics. Single Cell ATAC—Official 10x Genomics Support. <https://www.10xgenomics.com/support/single-cell-atac>. Accessed 12.01.2002.
- [9] BD Biosciences. Single-Cell Multiomics Systems. BD Biosciences. <https://www.bd biosciences.com/en-us/products/instruments/single-cell-multiomics-systems>. Accessed 12.01.2002.
- [10] Cusanovich DA, et al. Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science* 2015;348:910–14.
- [11] Domcke S, et al. A human cell atlas of fetal chromatin accessibility. *Science* 2020;370:eaba7612.
- [12] Cao J, et al. Joint profiling of chromatin accessibility and gene expression in thousands of single cells. *Science* 2018;361:1380–5.
- [13] Ma S, et al. Chromatin potential identified by shared single-cell profiling RNA chromatin. *Cell* 2020;183:1103–1116.e20.
- [14] Zhu C, et al. An ultra high-throughput method for single-cell joint analysis of open chromatin and transcriptome. *Nat Struct Mol Biol* 2019;26:1063–70.

¹Some illustrations were created with BioRender.com

- [15] Chen S, Lake BB, Zhang K. High-throughput sequencing of the transcriptome and chromatin accessibility in the same cell. *Nat Biotechnol* 2019;37:1452–7.
- [16] Angermueller C, et al. Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. *Nat Methods* 2016;13:229–32.
- [17] Hu Y, et al. Simultaneous profiling of transcriptome and DNA methylome from a single cell. *Genome Biol* 2016;17:88.
- [18] Chen AF, et al. NEAT-seq: simultaneous profiling of intra-nuclear proteins, chromatin accessibility, and gene expression in single cells. *BioRxiv* 2021;. Available from: <https://doi.org/10.1101/2021.07.29.454078>.
- [19] Chen X, et al. Joint single-cell DNA accessibility and protein epitope profiling reveals environmental regulation of epigenomic heterogeneity. *Nat Commun* 2018;9:4590.
- [20] Satpathy AT, et al. Transcript-indexed ATAC-seq for precision immune profiling. *Nat Med* 2018;24:580–90.
- [21] Pierce SE, Granja JM, Greenleaf WJ. High-throughput single-cell chromatin accessibility CRISPR screens enable unbiased identification of regulatory networks in cancer. *Nat Commun* 2021;12:2969.
- [22] Chen H, et al. Assessment of computational methods for the analysis of single-cell ATAC-seq data. *Genome Biol* 2019;20:241.
- [23] Subramaniyam S, et al. Statistical analysis of variability in TnSeq data across conditions using zero-inflated negative binomial regression. *BMC Bioinforma* 2019;20:603.
- [24] Miravet-Verde S, Burgos R, Delgado J, Lluch-Senar M, Serrano L. FASTQINS and ANUBIS: two bioinformatic tools to explore facts and artifacts in transposon sequencing and essentiality studies. *Nucleic Acids Res* 2020;48:e102.
- [25] Herron PR, Hughes G, Chandra G, Fielding S, Dyson PJ. Transposon Express, a software application to report the identity of insertions obtained by comprehensive transposon mutagenesis of sequenced genomes: analysis of the preference for in vitro Tn5 transposition into GC-rich DNA. *Nucleic Acids Res* 2004;32:e113.
- [26] Green B, Bouchier C, Fairhead C, Craig NL, Cormack BP. Insertion site preference of Mu, Tn5, and Tn7 transposons. *Mob DNA* 2012;3:3.
- [27] Bentsen M, et al. ATAC-seq footprinting unravels kinetics of transcription factor binding during zygotic genome activation. *Nat Commun* 2020;11:4267.
- [28] Schep AN, Wu B, Buenrostro JD, Greenleaf W. J. chromVAR: inferring transcription-factor-associated accessibility from single-cell epigenomic data. *Nat Methods* 2017;14:975–8.
- [29] Fornes O, et al. JASPAR 2020: update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res* 2020;48:D87–92.
- [30] Kaya-Okur HS, et al. CUT&Tag for efficient epigenomic profiling of small samples and single cells. *BioRxiv* 2019;. Available from: <https://doi.org/10.1101/568915>.
- [31] Baranello L, Kouzine F, Sanford S, Levens D. ChIP bias as a function of cross-linking time. *Chromosome Res* 2016;24:175–81.
- [32] Kaya-Okur HS, et al. CUT&Tag for efficient epigenomic profiling of small samples and single cells. *Nat Commun* 2019;10:1930.
- [33] Bartosovic M, Kabbe M, Castelo-Branco G. Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues. *Nat Biotechnol* 2021;39:825–35.
- [34] Zhang H, et al. Comprehensive understanding of Tn5 insertion preference improves transcription regulatory element identification. *NAR Genom Bioinform* 2021;3:lqab094.
- [35] Zhu C, et al. Joint profiling of histone modifications and transcriptome in single cells from mouse brain. *Nat Methods* 2021;18:283–92.
- [36] Xiong H, Luo Y, Wang Q, Yu X, He A. Single-cell joint detection of chromatin occupancy and transcriptome enables higher-dimensional epigenomic reconstructions. *Nat Methods* 2021;18:652–60.

- [37] Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012;13:484–92.
- [38] Culp LA, Dore E, Brown GM. Methylated bases in DNA of animal origin. *Arch Biochem Biophys* 1970;136:73–9.
- [39] Deaton AM, Bird A. CpG islands and the regulation of transcription. *Genes Dev* 2011;25:1010–22.
- [40] Kraan CM, Godler DE, Amor DJ. Epigenetics of fragile X syndrome and fragile X-related disorders. *Dev Med Child Neurol* 2019;61:121–7.
- [41] Robertson KD. DNA methylation and human disease. *Nat Rev Genet* 2005;6:597–610.
- [42] Koch A, et al. Analysis of DNA methylation in cancer: location revisited. *Nat Rev Clin Oncol* 2018;15:459–66.
- [43] Hong SR, Shin K-J. Bisulfite-converted DNA quantity evaluation: a multiplex quantitative real-time PCR system for evaluation of bisulfite conversion. *Front Genet* 2021;12:618955.
- [44] Miura F, Enomoto Y, Dairiki R, Ito T. Amplification-free whole-genome bisulfite sequencing by post-bisulfite adaptor tagging. *Nucleic Acids Res* 2012;40:e136.
- [45] Ahn J, Heo S, Lee J, Bang D. Introduction to single-cell DNA methylation profiling methods. *Biomolecules* 2021;11.
- [46] Karemaker ID, Vermeulen M. Single-cell DNA methylation profiling: technologies and biological applications. *Trends Biotechnol* 2018;36:952–65.
- [47] Yu B, et al. Genome-wide, single-cell DNA methylomics reveals increased non-CpG methylation during human oocyte maturation. *Stem Cell Rep* 2017;9:397–407.
- [48] Yong W-S, Hsu F-M, Chen P-Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin* 2016;9:26.
- [49] Clark SJ, et al. Genome-wide base-resolution mapping of DNA methylation in single cells using single-cell bisulfite sequencing (scBS-seq). *Nat Protoc* 2017;12:534–47.
- [50] Rauluseviciute I, Drablos F, Rye MB. DNA methylation data by sequencing: experimental approaches and recommendations for tools and pipelines for data analysis. *Clin Epigenetics* 2019;11:193.
- [51] Kantlehner M, et al. A high-throughput DNA methylation analysis of a single cell. *Nucleic Acids Res* 2011;39:e44.
- [52] Han L, et al. Bisulfite-independent analysis of CpG island methylation enables genome-scale stratification of single cells. *Nucleic Acids Res* 2017;45:e77.
- [53] Cheow LF, Quake SR, Burkholder WF, Messerschmidt DM. Multiplexed locus-specific analysis of DNA methylation in single cells. *Nat Protoc* 2015;10:619–31.
- [54] Moore LD, Le T, Fan G. DNA methylation and its basic function. *Neuropsychopharmacology* 2013;38:23–38.
- [55] Dhar GA, Saha S, Mitra P, Nag Chaudhuri R. DNA methylation and regulation of gene expression: Guardian of our health. *Nucl (Calcutta)* 2021;64:259–70.
- [56] Kim M, Costello J. DNA methylation: an epigenetic mark of cellular memory. *Exp Mol Med* 2017;49: e322.
- [57] Kim MS, Lee J, Sidransky D. DNA methylation markers in colorectal cancer. *Cancer Metastasis Rev* 2010;29:181–206.
- [58] Chen J, et al. High-resolution bisulfite-sequencing of peripheral blood DNA methylation in early-onset and familial risk breast cancer patients. *Clin Cancer Res* 2019;25:5301–14.
- [59] Zhan Y-X, Luo G-H. DNA methylation detection methods used in colorectal cancer. *World J Clin Cases* 2019;7:2916–29.
- [60] Bian S, et al. Single-cell multiomics sequencing and analyses of human colorectal cancer. *Science* 2018;362:1060–3.
- [61] Gu C, Liu S, Wu Q, Zhang L, Guo F. Integrative single-cell analysis of transcriptome, DNA methylome and chromatin accessibility in mouse oocytes. *Cell Res* 2019;29:110–23.

- [62] Pott S. Simultaneous measurement of chromatin accessibility, DNA methylation, and nucleosome phasing in single cells. *eLife* 2017;6.
- [63] Clark SJ, et al. scNMT-seq enables joint profiling of chromatin accessibility DNA methylation and transcription in single cells. *Nat Commun* 2018;9:781.
- [64] Wainwright EN, Scaffidi P. Epigenetics and cancer stem cells: unleashing, hijacking, and restricting cellular plasticity. *Trends Cancer* 2017;3:372–86.
- [65] Thommen DS, Schumacher TN. T cell dysfunction in cancer. *Cancer Cell* 2018;33:547–62.
- [66] Green MR, et al. Transient expression of Bcl6 is sufficient for oncogenic function and induction of mature B-cell lymphoma. *Nat Commun* 2014;5:3904.
- [67] Vicente-Dueñas C, et al. A novel molecular mechanism involved in multiple myeloma development revealed by targeting MafB to haematopoietic progenitors. *EMBO J* 2012;31:3704–17.
- [68] Alonso-Curbelo D, et al. A gene-environment-induced epigenetic program initiates tumorigenesis. *Nature* 2021;590:642–8.
- [69] Becker WR, et al. Single-cell analyses define a continuum of cell state and composition changes in the malignant transformation of polyps to colorectal cancer. *Nat Genet* 2022;54:985–95.
- [70] Mathur R, Costello JF. Epigenomic contributions to tumor cell heterogeneity and plasticity. *Nat Genet* 2021;53:1403–4.
- [71] Neftel C, et al. An integrative model of cellular states, plasticity, and genetics for glioblastoma. *Cell* 2019;178:835–849.e21.
- [72] Chaligne R, et al. Epigenetic encoding, heritability and plasticity of glioma transcriptional cell states. *Nat Genet* 2021;53:1469–79.
- [73] Guilhamon P, et al. Single-cell chromatin accessibility profiling of glioblastoma identifies an invasive cancer stem cell population associated with lower survival. *eLife* 2021;10.
- [74] Johnson KC, et al. Single-cell multimodal glioma analyses identify epigenetic regulators of cellular plasticity and environmental stress response. *Nat Genet* 2021;53:1456–68.
- [75] Denny SK, et al. Nfib promotes metastasis through a widespread increase in chromatin accessibility. *Cell* 2016;166:328–42.
- [76] Marjanovic ND, et al. Emergence of a high-plasticity cell state during lung cancer evolution. *Cancer Cell* 2020;38:229–246.e13.
- [77] Ludwig LS, et al. Transcriptional states and chromatin accessibility underlying human erythropoiesis. *Cell Rep* 2019;27:3228–3240.e7.
- [78] Day CL, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006;443:350–4.
- [79] Satpathy AT, et al. Massively parallel single-cell chromatin landscapes of human immune cell development and intratumoral T cell exhaustion. *Nat Biotechnol* 2019;37:925–36.
- [80] Gennert DG, et al. Dynamic chromatin regulatory landscape of human CAR T cell exhaustion. *Proc Natl Acad Sci U S A* 2021;118.
- [81] Sen DR, et al. The epigenetic landscape of T cell exhaustion. *Science* 2016;354:1165–9.
- [82] Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* 2014;41:49–61.
- [83] Lin Y, Xu J, Lan H. Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *J Hematol Oncol* 2019;12:76.
- [84] Yu T, et al. Modulation of M2 macrophage polarization by the crosstalk between Stat6 and Trim24. *Nat Commun* 2019;10:4353.
- [85] Azizi E, et al. Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. *Cell* 2018;174:1293–1308.e36.

- [86] Chung W, et al. Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. *Nat Commun* 2017;8:15081.
- [87] Sarode P, et al. Reprogramming of tumor-associated macrophages by targeting β -catenin/FOSL2/ARID5A signaling: A potential treatment of lung cancer. *Sci Adv* 2020;6:eaaz6105.
- [88] Denisenko E, et al. Systematic assessment of tissue dissociation and storage biases in single-cell and single-nucleus RNA-seq workflows. *Genome Biol* 2020;21:130.
- [89] Corces MR, et al. The chromatin accessibility landscape of primary human cancers. *Science* 2018;362.
- [90] Grandi FC, Modi H, Kampman L, Corces MR. Chromatin accessibility profiling by ATAC-seq. *Nat Protoc* 2022;17:1518–52.
- [91] Nguyen QH, Pervolarakis N, Nee K, Kessenbrock K. Experimental considerations for single-cell RNA sequencing approaches. *Front Cell Dev Biol* 2018;6:108.
- [92] Picelli S, et al. Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* 2014;9:171–81.
- [93] Cao J, et al. Comprehensive single-cell transcriptional profiling of a multicellular organism. *Science* 2017;357:661–7.
- [94] Wilk AJ, et al. A single-cell atlas of the peripheral immune response in patients with severe COVID-19. *Nat Med* 2020;26:1070–6.
- [95] Travaglini KJ, et al. A molecular cell atlas of the human lung from single-cell RNA sequencing. *Nature* 2020;587:619–25.
- [96] Granja JM, et al. ArchR is a scalable software package for integrative single-cell chromatin accessibility analysis. *Nat Genet* 2021;53:403–11.
- [97] Stuart T, Srivastava A, Madad S, Lareau CA, Satija R. Single-cell chromatin state analysis with Signac. *Nat Methods* 2021;18:1333–41.
- [98] Stuart T, et al. Comprehensive integration of single-cell data. *Cell* 2019;177:1888–1902.e21.
- [99] Zhang K, et al. A single-cell atlas of chromatin accessibility in the human genome. *Cell* 2021;184:5985–6001.e19.
- [100] Cusanovich DA, et al. A single-cell atlas of in vivo mammalian chromatin accessibility. *Cell* 2018;174:1309–1324.e18.
- [101] Hao Y, et al. Integrated analysis of multimodal single-cell data. *Cell* 2021;184:3573–3587.e29.
- [102] Hao Y, et al. Dictionary learning for integrative, multimodal, and scalable single-cell analysis. *BioRxiv* 2022;. Available from: <https://doi.org/10.1101/2022.02.24.481684>.
- [103] Trevino AE, et al. Chromatin and gene-regulatory dynamics of the developing human cerebral cortex at single-cell resolution. *Cell* 2021;184:5053–5069.e23.
- [104] LaFave LM, et al. Epigenomic state transitions characterize tumor progression in mouse lung adenocarcinoma. *Cancer Cell* 2020;38:212–228.e13.
- [105] Granja JM, et al. Single-cell multiomic analysis identifies regulatory programs in mixed-phenotype acute leukemia. *Nat Biotechnol* 2019;37:1458–65.
- [106] Quinn JJ, et al. Single-cell lineages reveal the rates, routes, and drivers of metastasis in cancer xenografts. *Science* 2021;371.
- [107] Yang D, et al. Lineage tracing reveals the phylodynamics, plasticity, and paths of tumor evolution. *Cell* 2022;185:1905–1923.e25.
- [108] Ju YS, et al. Somatic mutations reveal asymmetric cellular dynamics in the early human embryo. *Nature* 2017;543:714–18.
- [109] Lodato MA, et al. Somatic mutation in single human neurons tracks developmental and transcriptional history. *Science* 2015;350:94–8.
- [110] Ludwig LS, et al. Lineage tracing in humans enabled by mitochondrial mutations and single-cell genomics. *Cell* 2019;176:1325–1339.e22.

- [111] Jerby-Arnon L, et al. A cancer cell program promotes T cell exclusion and resistance to checkpoint blockade. *Cell* 2018;175:984–997.e24.
- [112] Sahai E, et al. A framework for advancing our understanding of cancer-associated fibroblasts. *Nat Rev Cancer* 2020;20:174–86.
- [113] Bartoschek M, et al. Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing. *Nat Commun* 2018;9:5150.
- [114] Bujold D, et al. The international human epigenome consortium data portal. *Cell Syst* 2016;3:496–499.e2.
- [115] Davis CA, et al. The Encyclopedia of DNA elements (ENCODE): data portal update. *Nucleic Acids Res* 2018;46:D794–801.
- [116] Tabula Sapiens Consortium, et al. The Tabula Sapiens: a multiple-organ, single-cell transcriptomic atlas of humans. *Science* 2022;376:eabl4896.
- [117] Corces MR, et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet* 2016;48:1193–203.
- [118] Gulati GS, et al. Single-cell transcriptional diversity is a hallmark of developmental potential. *Science* 2020;367:405–11.
- [119] Rauch A, et al. Osteogenesis depends on commissioning of a network of stem cell transcription factors that act as repressors of adipogenesis. *Nat Genet* 2019;51:716–27.
- [120] Kim DS, et al. The dynamic, combinatorial cis-regulatory lexicon of epidermal differentiation. *Nat Genet* 2021;53:1564–76.
- [121] Oakes CC, et al. DNA methylation dynamics during B cell maturation underlie a continuum of disease phenotypes in chronic lymphocytic leukemia. *Nat Genet* 2016;48:253–64.

Epigenetics of cisplatin resistance

22

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1 Introduction

Currently, the standard of care for many cancers involves treatment regimens containing a platinum agent. Many patients, however, gain no benefit from this treatment, due to innate intrinsic tumor resistance. For example, the first-line treatment for all patients with malignant pleural mesothelioma (MPM) involves treatment with either cisplatin/pemetrexed or cisplatin/raltitrexed. However, only 41% will demonstrate an initial response to this therapy. This means that a large proportion of MPM tumors’ have intrinsic resistance to platinum-based therapy.

Another common issue that emerges when patients are treated with cisplatin is that while there may be an initial response to treatment, over time this response fails. In other words, the tumor develops acquired resistance to the platinum agent, such as cisplatin.

Epigenetics is currently defined as a specialized form of gene regulation whereby stable and heritable changes are effected on gene expression, which are not due to changes in the primary DNA sequence. The current established epigenetic mechanisms identified to date involve: DNA CpG methylation, histone posttranslational modifications (PTMs), histone variants, and noncoding RNA (ncRNA).

In the following chapter using primarily nonsmall-cell lung cancer (NSCLC) and ovarian cancer as examples, epigenetics, and the cellular machinery involved with epigenetic regulation will be discussed in the context of the development of both innate and acquired resistance to cisplatin. An overview is provided in [Figure 22.1](#).

2 DNA methylation

In a previous chapter, the general issue of DNA CpG methylation and hydroxy-methylation in cancer has been discussed. It is now well established that aberrant DNA CpG methylation is a common element in the pathogenesis of cancer including ovarian, testicular germ cell tumors (TGCTs), and NSCLC [1–4].

2.1 DNA methylation changes associated with cisplatin resistance

Early large-scale genome-wide epigenetic analyses have identified many differentially methylated regions (DMRs) and loci associated with the development of resistance to cisplatin in both ovarian

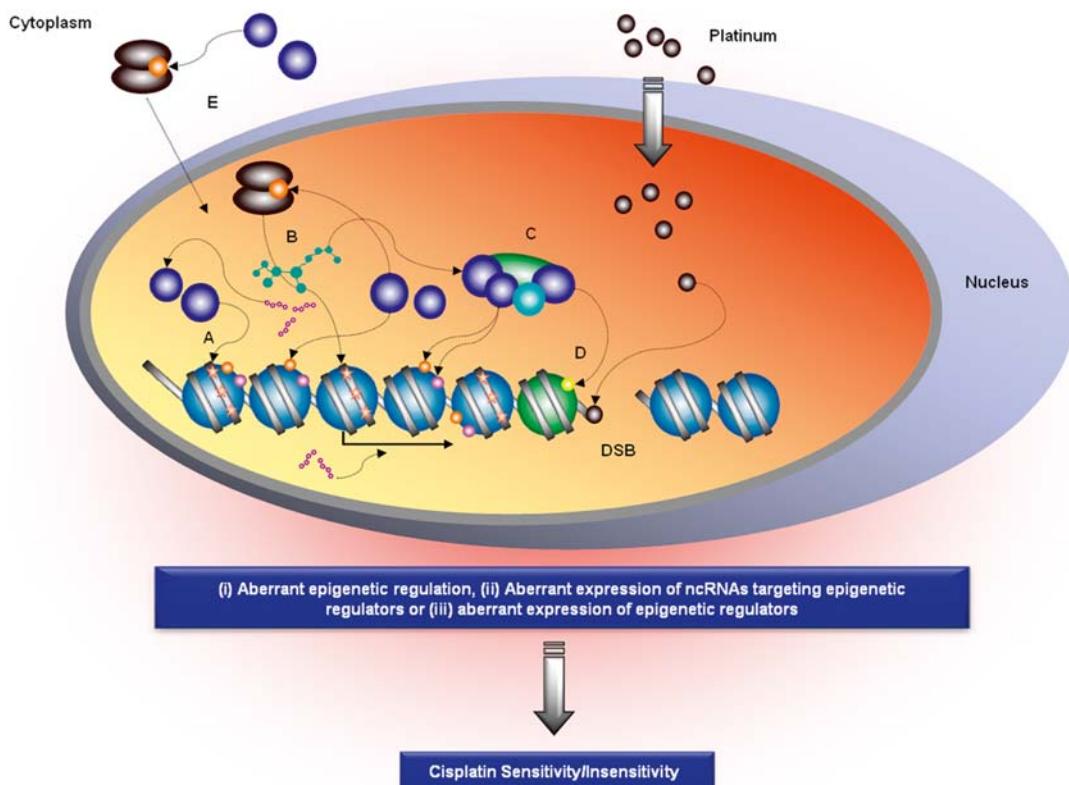


FIGURE 22.1

Epigenetics underpinning cisplatin resistance. Diagram summarizing the available evidence linking the various forms of epigenetic regulation to how cancer cells respond to cisplatin based therapies. (A) DNA methylation; (B) noncoding RNA (including miRNAs and lncRNAs); (C) Epigenetic readers, writers and erasers (including multisubunit complexes); (D) Histone variants; and (E) nonhistone PTMs.

and NSCLC cell lines [5–7]. However, methylation in cancer cell lines may not truly reflect the methylation pattern in the primary tumor as elegantly shown by Jones and colleagues [8].

More recently studies on primary patient material from both surgically resected primary tumors and patient-derived xenografts (PDXs) have elucidated numerous DMRs in ovarian cancer linked to both cisplatin resistance and response to demethylating agents (discussed later in more detail) [9–12]. However, it must be noted that epigenetic intratumoral heterogeneity (epITH) a common feature in NSCLC [13], and discussed in greater detail in a separate chapter of this edition, may also play confounding roles in the identification of DMRs and affected genes truly associated with cisplatin resistance in these settings. In this regard a recent study in NSCLC [14], links epITH to patient prognosis in lung adenocarcinomas, and while no links with cisplatin resistance were identified in this analysis, epITH may still play a defining role in individual tumor responses to therapy.

3 Epigenetic readers, writers, and erasers and associated links with cisplatin response

Cisplatin is often considered to impair normal DNA functions by generating intra- and interstrand DNA crosslinks, as well as monoadducts, interfering with normal cellular functions [15,16]. It has also been shown to irreversibly link chromatin itself through cisplatin-nucleosome adducts that impair the nucleosome remodeling that is vital for normal biological functions [17].

The epigenetic regulatory mechanism for chromosomal remodeling involves PTM of the nucleosomal histone proteins. This involves the addition and removal of modifications, such as acetylation and methylation, in defined patterns that represent a dynamic “code” used by the cellular machinery to regulate transcription. To utilize this code, various families of proteins have emerged with different functions based on their ability to either “read,” “write,” or “erase” these modifications [18]. Evidence is now emerging that many of these proteins may also play roles in cisplatin resistance.

3.1 Lysine acetyltransferases

Of the many epigenetic “writers” lysine acetyltransferases are emerging as major elements in responses to cisplatin and the development of resistance to this agent.

For instance, the lysine acetyltransferases KAT13D (Clock) [19], KAT5 (Tip60) [20,21], KAT2B (PCAF) [22], KAT3A [23], and SRC-3 [24] have all been linked to resistance to cisplatin in lung cancer, while KAT6A has recently been found to be associated with cisplatin sensitivity in ovarian cancer [25].

3.2 Tip60/Kat5

Methylation at Histone H3 links DNA damage detection at DNA double-strand breaks (DSBs) to activation of Tip60 (KAT5) [26].

An early study linked Tip60 (KAT5) and HDAC6 as important regulators of lung cancer cell responses to cisplatin [20,27]. The acetyltransferase Tip60 acetylates an important splicing factor SRSF2 on its lysine 52 residue promoting its proteasomal degradation, while HDAC6 abrogates this. In response to cisplatin an acetylation/phosphorylation signaling network regulates both the accumulation of SRSF2 and splicing of caspase-8 pre-mRNA and determines whether cells undergo apoptosis or G(2)/M cell cycle arrest [27].

Since this initial observation, other mechanisms through which Tip60 (KAT5) exerts effects on cisplatin resistance have been identified. For example, alternative splicing resulting in multiple isoforms of key proteins is an important element in drug resistance [28]. In breast cancer, one such gene is serine-arginine protein kinase 1 (SRPK1), and Tip60 (KAT5) has now been shown to be key to this process, whereby Tip60 (KAT5)-mediated acetylation of SRPK1 is closely associated with chemotherapy sensitivity. Acetylation of SRPK1 is normally induced by cisplatin. However, in resistant cells, the level of acetylation is reduced, leading to a switch in alternative splicing of key apoptotic proteins from proapoptotic to antiapoptotic forms. Moreover, by enhancing SRPK1 acetylation cisplatin-resistant cells could be resensitized [29,30].

Another mechanism identified has found that Tip60 (KAT5) functionally associates with BMI1 to regulate expression of the multidrug resistance protein 1 (MDR1). In the presence of cisplatin, a functional interaction between TIP60 (KAT5) and BMI-1 occurs at a cluster of E-box elements in the MDR1 promoter. This results in chromatin remodeling of the promoter via acetylation of histone H2A and H3 by Tip60 (KAT5) and results in the upregulation of MDR1 expression [21].

3.3 KMTs

Histone methyltransferases fall in to two large subfamilies, those that modify lysine residues or lysine methyltransferases (KMTs) [31], and those that modify arginine residues or arginine methyltransferases (also known as protein arginine or PRMTs) [32], with many roles in carcinogenesis [33].

As such unsurprisingly, links between histone methylation and cisplatin resistance have emerged.

One of the best known histone methyltransferases is EZH2 [34]. One of the first indicators that the lysine methyltransferase EZH2 could be involved with cisplatin resistance came from a study in ovarian cancer, which found that EZH2 was overexpressed in cisplatin-resistant ovarian cancer cells compared with cisplatin-sensitive cells [35,36]. Targeting EZH2 resulted in enhanced sensitivity to platinum-based therapy in a cisplatin resistant cell line both *in vitro* and *in vivo* [35,37,38]. This observation was also seen in NSCLC lung adenocarcinoma, with overexpression noted in primary tissues and cisplatin resistant cell lines [39,40].

The critical role of EZH2 in the development of resistance to platinum agents has recently been highlighted in small-cell lung cancer (SCLC) [41]. This is an aggressive poorly differentiated neuroendocrine tumor accounting for 13% of all lung cancer cases [42,43], and with significant aberrant epigenetics [44]. SCLC is highly responsive to cytotoxic chemotherapy and is traditionally treated using with a platinum-based agent (cisplatin or carboplatin) in combination with etoposide [45,46]. However, the vast majority of patients have only a short response to therapy, quickly developing acquired resistance to therapy and 5-year survival rates for this cancer remain at less than 7% [47,48]. One potential biomarker for predicting response to cisplatin therapy in SCLC is SLFN11 [49]. Moreover, treatments with cisplatin result in the downregulation of SLFN11 suggesting a mechanism for the rapid development of therapeutic resistance in SCLC [49]. Using DNA methylation analysis on PDXs Poirier et al., identified EZH2 as amongst the most significantly overexpressed genes in SCLC, expressed >12-fold higher than in normal lung tissue [50]. In a follow-up study using paired SCLC chemo-naive and chemoresistant PDX samples it was found that in resistant tumors, EZH2 epigenetically downregulated SLFN11 [51]. Combining standard cytotoxic therapies with an EZH2 inhibitor was shown to prevent the emergence of acquired resistance and to augment chemotherapeutic efficacy in both the chemo-sensitive and chemo-resistant models of SCLC [51]. Another cancer for which EZH2 has shown potential as a biomarker and as a candidate therapeutic option to induce sensitivity to cisplatin is breast cancer [52]. In contrast a recent study in testicular cancer found that inhibition of EZH2 led to the acquisition of resistance to cisplatin, while a KDM inhibitor resensitized cisplatin resistant cells to platinum-based therapy [53].

Another KMT, SETD2, has been found to be commonly mutated in NSCLC [54], and a particular mutation in SETD2 (p.T1171K), has been shown to confer resistance to cisplatin in NSCLC cell lines [55].

Other lysine methyltransferases associated with resistance to cisplatin include DOT1L in ovarian cancer [56] and SMYD3 in breast cancer and NSCLC [57,58].

3.4 PRMTs

Protein arginine methyltransferases (PRMTs) are a diverse family of proteins with the ability to methylate diverse arginine residues on histones (and other proteins), with known roles in the DNA damage response [32]. One member of this family, PRMT5, has been shown to have altered expression in NSCLC [59,60], and ovarian cancer [59,60]. In this regard, given the functional role of PRMT5 in DNA damage responses a selective inhibitor of PRMT5 was recently shown to enhance the efficacy of cisplatin in NSCLC cell lines [61].

Another member of the PRMTs, PRMT1, has been shown to have elevated expression in many cancers [62], including ovarian cancer cell lines [63] and NSCLC cell lines and tumors [64]. Critically, PRMT1 was identified as a regulator of the DNA damage response [65], and in ovarian cancer cell lines treated with cisplatin, PRMT5 becomes phosphorylated, inducing its recruitment to chromatin at proinflammatory gene promoters to induce a senescence-associated secretory phenotype [66]. A recent study has now shown that at least for primary ovarian cancer, levels of PRMT1 can predict sensitivity of cisplatin therapy [67]. In this regard no current evidence has been shown for a role of PRMT1 in NSCLC, but evidence has shown that it may function in the regulation of epithelial-mesenchymal transition (EMT) [68] in NSCLC, which has been linked to the development of resistance to receptor tyrosine kinase inhibitors [69]. Finally, chromatin target of protein arginine methyltransferase (CHTOP) has been identified as a key factor in the development of resistance to cisplatin in ovarian cancer [70,71].

3.5 HDACs

The initial evidence linking histone deacetylases (HDACs) to cisplatin resistance found that diverse members of these proteins and associated HDAC containing complexes are linked to cisplatin resistance [72–75]. More recent analyses have now confirmed strong links between HDACs and HDAC complexes with resistance to cisplatin [76–83], and the potential to target these important epigenetic “erasers” is discussed in more detail in a later section of this chapter.

3.6 KDMs

There are a large number of histone lysine demethylases (KDMs), which can be classified into two families based on the catalytic mechanisms [84].

The first are flavin adenine dinucleotide (FAD)-dependent amine oxidases and include LSD1 (KDM1A) and LSD2 (KDM1B) [84]. The second family are 2-oxoglutarate (2-OG) and Fe(II)-dependent hydroxylases, and comprise the Jumonji (JmjC-domain) containing demethylases and include the families of KDM2-KDM8 [84]. Aberrant expression of all of these histone lysine demethylases have been observed in many cancers (including NSCLC and ovarian cancer) [85–88], and indeed additional links between their expression and cisplatin are also emerging.

LSD1/KDM1A is a lysine demethylase, which acts on both histones and nonhistone proteins to regulate not only gene expression, but also multiple pathways in various cellular processes [89].

Aberrant expression of LSD1 has been established for many cancers [90], including NSCLC [91] and ovarian cancer [92,93]. Links between LSD1 and sensitivity/resistance to cisplatin are emerging [94,95], and so it is conceivable that such an approach may warrant further investigation in the clinical setting. The other member of this family, LSD2/KDM1B, has also been linked to cisplatin resistance in ovarian cancer [96].

KDM5B/JARID1B is another lysine demethylase found to be overexpressed in NSCLC [97,98]. In NSCLC cell lines, cisplatin treatment results in upregulation of Jarid1b expression, resulting in altered sensitivity to cisplatin therapy [99].

JARID2 is also a member of the PRC2 complex, and while it is classed as a lysine demethylase, it contains amino acid substitutions that are thought to abolish its catalytic activity [100]. A recent publication found that JARID2 expression is upregulated in cisplatin resistant lung cancer cells, and in patients, high expression is further associated with advanced TNM stage, shorter overall survival, and poor chemotherapeutic response [101].

KDM4/JMJD2 proteins are a family consisting of six members: KDM4A–F, of which KDM4E and KDM4F have been reported as protein noncoding pseudogenes [84]. In NSCLC cell lines over-expression of KDM4B (JMJD2B) has been identified as an important element in resistance to cisplatin [102].

3.7 Epigenetic readers

Epigenetic readers can be thought of as effector proteins that contain domains, which recognize and are recruited to specific marks on histones or nucleotides. It must be noted that proteins, which “write” or “erase” epigenetic marks may contain such reader domains, thus leading coordinated “reading/writing” and “reading/erasing” modifications for the regulation or setting of epigenetic events. Proteins that contain these reader domains can loosely be classified into four groups: chromatin architectural proteins, chromatin remodeling enzymes, chromatin modifiers, and adaptor proteins that function to recruit/assemble the protein complexes involved in gene expression. Some of the best recognized reader domains are the chromo-domains, WD repeat domains, Tudor Domains, plant homeodomain (PHD) domains and Bromodomains (BrD) [103], and evidence is emerging to link several of these domain containing proteins with cisplatin resistance.

For instance the WD repeat protein, WDR82, which recognises histone H2B ubiquitination-dependent manner [104] and is a regulatory component of the Setd1a and Setd1b histone H3K4 methyltransferase complexes [105], has been found to be associated in a Tox4 protein complex, which recognises DNA adducts generated by cisplatin [106]. Likewise another WD repeat containing protein F-box/WD repeat-containing protein 7 (FBW7), has been shown to be involved with sensitivity to cisplatin [107].

BRD4 is a member of the Bromodomain and extra-terminal domain (BET) family of reader proteins that recognizes histone lysine acetylation, and plays multiple roles in the regulation of gene transcription [108]. A large body of evidence now links aberrant BRD4 expression to both NSCLC [109], and ovarian cancer [110], and also to response to cisplatin therapy [111,112]. While normally associated with immune checkpoint inhibitor therapy, PD-L1 has also been shown to have an intrinsic oncogenic function via a role in regulating the DNA damage response that facilitates resistance to chemotherapies. DNA damaging agents, such as cisplatin, have indeed been shown to induce PD-L1 [113,114], but a recent article suggests that in lung cancer, BRD4 inhibitor treatment

inhibits chemoradiation-induced PD-L1 expression [115], and moreover, synergizes *in vivo* with chemoradiotherapy and PD-1 blockade to elicit strong antitumoral responses without any significant toxicity [115].

3.8 BRCA1 complexes containing epigenetic readers/writers and erasers as a critical element in cisplatin resistance

Epigenetic readers, writers and erasers are often found in large multiprotein complexes. Complexes involving BRCA1 and the epigenetic regulatory machinery are now emerging as playing important roles in cisplatin sensitivity and resistance.

3.8.1 BRCA1 complexes, the epigenetic machinery, and DNA damage response

The Breast Cancer 1 Gene (BRCA1) has two important functions namely: (1) the regulation of gene transcription and (2) the regulation of the cellular response to DNA damage (DNA Repair) [116]. In this regard, BRCA1 acts mainly as a tumor suppressor through transcriptionally regulating genes involved with DNA repair [117].

Loss of BRCA1 expression is a frequent event in NSCLC [118,119], and loss of expression due to epigenetic inactivation via DNA CpG methylation is a factor in 18%–30% of tumors [118,120]. Likewise, BRCA1 is also commonly lost in ovarian cancer, and methylation at the BRCA1 promoter is a causative event in a proportion of patients [121]. Following the functional isolation of the first HDACs in 1996, it was soon shown that BRCA1 was able to associate with HDACs, indicating that chromatin remodeling may be an integral element in BRCA1 regulation of genome integrity [122]. In response to DNA damage, the BRCA1 protein forms several complexes, containing epigenetic readers, writers and erasers to both execute and coordinate various aspects of the DNA damage response [123]. In this regard, it has recently been shown that the lysine demethylase, KDM5B, is required for efficient DSB repair and for the recruitment of Ku70 and BRCA1 [124]. Another critical component of DSB is the Mediator of DNA-damage checkpoint 1 (MDC1) protein, and its function is regulated by posttranslational phosphorylation, ubiquitylation and sumoylation [125]. It has also recently been shown that the lysine demethylase, JMJD1C, plays a crucial role in this process [126]. In response to DSBs, JMJD1C demethylates MDC1 to regulate BRCA1-mediated chromatin responses to DNA breaks [126].

With respect to cisplatin, in response to DNA damage, BRCA1 forms a heterodimer with the protein BRCA1-associated RING domain-1 (BARD1) generating a complex with ubiquitin E3 ligase activity [127], that ubiquitylates both H2A and H2B histones [128], effecting chromatin remodeling [129]. Cisplatin has been shown to directly bind to BRCA1 and its transcriptional trans-activation activity is dramatically diminished in the presence of multiple cisplatin-damaged DNA sites [130]. Furthermore, when complexed with BARD1, cisplatin treatment results in a significantly reduced E3 ligase activity [131].

BRCA1 is also associated with the Mi-2/NuRD (nucleosome remodeling and deacetylase NuRD) complex. This complex has been shown to assemble on DSBs in breast cancer cells treated with cisplatin. Critically, phosphorylation of HDAC2 would appear to be essential for the formation of the NuRD complex at the DSB [132]. Other histone PTMs would appear to play essential roles in the repair of DSBs by NuRD. Upon induction of DSBs, the catalytic subunit of NuRD, CHD4

stimulates the accrual of RFN168 and RFN8, two proteins which together mediate ubiquitylation of histone H2A, resulting in a chromatin environment at the DSB that is permissive for the assembly of checkpoint and repair proteins including BRCA1 [133–135].

The effects of BRCA1 mutation in human and mouse cells are mitigated by concomitant deletion of the protein p53BP1. This protein binds histone H4 di-methylated at position lysine 20 (H4K20me2) to promote nonhomologous end joining, suggesting that a balance between BRCA1 and 53BP1 is responsible for regulating DSB repair mechanism choice. Greenberg and colleagues have shown that the activities of KATs and HDACs are essential to this balance. In particular, hypoacetylation due to loss of KAT5 (TIP60) acetyltransferase deficiency mimicked BRCA1 mutation, resulting in reduced BRCA1 and Rad51 DSB localization, and enhanced radial chromosome formation associated with increases in 53BP1, whereas HDAC inhibition yielded the opposite effect [136]. The data suggests that acetylation of H4K16 plays a central role in determining the balance of BRCA1 and 53BP1 at DSB chromatin by reducing, albeit not eliminating 53BP1 Tudor domain's binding affinity for H4K20me2 when present on the same H4 tail [136]. Critically, loss or impairment of KAT5 (TIP60) activity reduced homologous recombination and conferred sensitivity to PARP inhibition (PARPi), suggesting that inhibitors directed at Tip60 (KAT5) may be useful in this context. Indeed, other combinations of epigenetic therapies combined with PARPi are emerging as an important potential therapeutic avenue in treating cisplatin resistance [137].

3.8.2 BRCA1 is linked with sensitivity to cisplatin

The critical role that BRCA1 plays in determining sensitivity to cisplatin originally came from murine studies of breast cancer. In these studies, cells that were deficient for BRCA1 were found to be cisplatin sensitive, whereas restoring BRCA1 in these cells caused increased resistance. Xenograft studies confirmed these initial observations [138–140].

Such results have been confirmed in ovarian cancer. In 115 primary sporadic ovarian carcinomas, 39 (34%) had low BRCA1 protein while 49 (42%) had low BRCA2 expression. Restoration of BRCA1 and BRCA2 mediated resistance to platinum chemotherapy in recurrent BRCA1 and BRCA2 mutated hereditary ovarian carcinomas [141]. Inactivation of BRCA1 by DNA CpG methylation predicts enhanced sensitivity to platinum-derived drugs to the same extent as BRCA1 mutations in breast and ovarian cancer. Most importantly, BRCA1 hypermethylation proved to be a predictor of longer time to relapse and improved overall survival in ovarian cancer patients undergoing chemotherapy with cisplatin [142].

Perhaps the first indicator within the clinical setting that levels of BRCA1 could predict response to cisplatin in NSCLC came from a study of Gemcitabine/Cisplatin in the neoadjuvant setting. This study found that patients whose tumors had low levels of BRCA1 mRNA had a better outcome than those whose tumors had high levels of BRCA1 mRNA [119,143]. Further evidence for this came from a study within the second-line setting, where low BRCA1 expression levels were significantly correlated with higher response rates, longer progression free survival and median overall survival [144]. Finally, in more advanced metastatic disease Baorui Liu and colleagues confirmed that BRCA1 mRNA expression levels measured in pleural effusions from patients with metastatic NSCLC were inversely correlated with sensitivity to cisplatin [145].

To test the possibility that BRCA1 levels could be used to customize therapy of NSCLC, Rosell and colleagues conducted a phase II clinical trial where patients were segregated and treated based on Epidermal Growth Factor Receptor (EGFR) mutation status and BRCA1 expression. Those with

tumors harboring an EGFR mutation received erlotinib, whereas those with EGFR wild-type tumors received chemotherapy with or without cisplatin based on their BRCA1 mRNA expression as follows: low, cisplatin plus gemcitabine; intermediate, cisplatin plus docetaxel; high, docetaxel alone. To determine if any BRCA1 interacting partners could provide additional prognostic value, levels of interacting partner proteins (RAP80 and Abraxas) were also examined. It was found that patients with both low BRCA1 and RAP80 levels, had a median survival exceeding 26 months compared to 11 months for patients with low BRCA1 alone. RAP80 was a significant factor for survival in patients treated according to BRCA1 levels (hazard ratio, 1.3 [95% CI, 1–1.7]; $P = .05$) [146]. Supporting evidence for the role of BRCA1 expression in predicting sensitivity to cisplatin in NSCLC has come from additional studies [147–151]. Furthermore, BRCA1 expression also has predictive value for patients with metastatic NSCLC undergoing second-line cisplatin-based chemotherapy [144]. For those patients who have high expression of BRCA1, antitubulin-containing regimens are emerging as a potential therapeutic intervention [117], NSCLC patients with high BRCA1 mRNA expression were found to benefit more from this type of treatment (8.7 vs 13.0 months) [152].

Despite the strong links between BRCA1 and RAP80 expression and sensitivity to cisplatin, two follow-up biomarker driven randomized trials in both European and Chinese patients were conducted in NSCLC. Both however, failed to show any clinical benefit in the experimental over the control arm [153,154], and as such further work will be required to determine the true potential of BRCA as a biomarker for stratifying patients for cisplatin therapy.

3.8.3 The link between BRCA1, K-methyltransferases, and acquired cisplatin resistance

KDMs are also linked to BRCA1 and response to cisplatin. The first indicators for this came from studies in murine BRCA1-deficient mammary tumor cells, which were found to be selectively sensitive to EZH2 (KMT6) inhibitors [155]. This KMT added methyl groups to lysine-27 of histone H3, and loss of trimethyl lysine 27 (H3K27me3) is prognostic for poor outcome in ovarian cancer [156]. In a similar manner, high levels of H3K27me3 were found to have better prognosis in NSCLC [157]. Indeed, across all pathological stages of NSCLC, patients with high levels of EZH2 have significantly poorer prognosis than patients with low EZH2 [158].

Ovarian or NSCLC patients with either high BRCA1 or EZH2 levels might potentially benefit from treatments with EZH2 inhibitors, such as DZNep. Indeed, targeting EZH2 in NSCLC cell lines with DZNep is found to sensitize lung cancer cells to cisplatin [40]. As mentioned in a previous section, loss or downregulation of BRCA1 is common in ovarian cancer, and indeed it has now been shown that overexpression of EZH2 is linked to reduced expression of BRCA1 in manner similar to that seen in lung cancer [37].

4 Noncoding RNAs (ncRNAs)

Noncoding RNAs have been explored in detail in earlier chapters of this edition. Two types of non-coding RNAs, microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), play important roles in epigenetic regulation. Furthermore, there is increasing evidence that both of these forms of ncRNA also play functional roles in cisplatin sensitivity/resistance [159,160].

4.1 miRNAs associated with cisplatin resistance/sensitivity

Intriguingly, various miRNAs associated with resistance to cisplatin have been found to be downregulated by DNA CpG methylation [161]. For instance, in ovarian cancer the let7e miRNA was found to be significantly reduced in a cisplatin-resistant human epithelial ovarian cancer (EOC) cell line A2780/CP compared with the parental A2780 cell [162]. When A2780, SKOV3 and ES2 ovarian cancer cells were treated with cisplatin, the levels of this miRNA decreased in a concentration-dependent manner. Overexpression of let-7e by transfection of agomir could resensitize A2780/CP and reduce the expression of cisplatin-resistant-related proteins EZH2 and cyclin D1 (CCND1), whereas let-7e inhibitors increased resistance to cisplatin in parental A2780 cells [162]. Quantitative methylation-specific PCR analysis showed hypermethylation of the CpG island adjacent to let-7e in A2780/CP cells, and demethylation treatment with 5-aza-CdR or transfection of pYr-let-7e-shRNA plasmid containing unmethylated let-7e DNA sequence could restore let-7e expression and partly reduce the chemoresistance. In addition, cisplatin combined with let-7e agomirs inhibited the growth of A2780/CP xenograft more effectively than cisplatin alone [162]. These reduced levels of let-7e were also confirmed in chemoresistant ovarian cancer tissues, which may in part be due to its effect on BRCA1 and Rad51 expression [163]. In ovarian SKOV3 cells, Xiao et al. also found that let-7e sensitized cells to cisplatin through its targeting of PARP1 [163]. Likewise, levels of this miRNA have been shown to be reduced in NSCLC and associated with poorer overall survival [164].

Let-7e can also be bound by the lncRNA, SNHG4, which through a SNHG4/let-7e/KDM3A/p21 axis is involved in the carcinogenesis of NSCLC [165]. In an additional microarray-based cisplatin resistant NSCLC study, a number of miRNA were altered following A549/CDDP treatment with 5-Aza-dc, with this drug resensitizing resistant cells to cisplatin, with miR-320a thought to play a role [166]. In ovarian cancer cell lines (A2780/CDDP), expression of miR-143 enhanced sensitivity to cisplatin through the inhibition of DNMT3A [167].

Overexpression of EZH2 has been shown to contribute to the development of acquired cisplatin resistance in ovarian cancer cells *in vitro* and *in vivo* [35]. The miRNA, miR-101, is a critical regulator of EZH2 and loss or depletion of this miRNA results in elevated levels of EZH2 [168]. In both NSCLC and ovarian cancer, levels of miR-101 are downregulated [169,170], indicating that this miRNA may be an effective target for potentially sensitizing/resensitizing NSCLC and ovarian cancer to platinum-based therapy. It has also been shown that FBP (also known as FUBP1), a single-strand DNA and RNA-binding protein, can bind to EZH2 in an ovarian cancer model [171]. Knockdown of FBP1 resulted in improved sensitivity to cisplatin with the reduction of EZH2 and the trimethylation of H3 at K27 [171]. FUBP1 can also regulate c-Myc expression. In ovarian cancer cells, high expression of c-Myc has been identified in cisplatin resistant cells, where it amplifies the expression of EZH2, which in turns suppresses miR-137 [172]. Inhibition of this pathway increased sensitivity to cisplatin in resistant cell lines, and furthermore ovarian tissue samples taken from patients with resistance to platinum drugs/recurrent disease demonstrated an activated c-Myc-miR-137-EZH2 pathway [172].

There are many other examples of miRNAs associated with cisplatin resistance in NSCLC and ovarian cancer through their activities on particular genes. However, miRNAs often have multiple potential RNA targets, which has led to the formulation of the contentious “competitive endogenous RNA” or ceRNA hypothesis [173,174]. If ceRNA networks are elements of cisplatin

resistance perhaps miRNA signatures may be useful to identify such key networks [175]. Several such signatures have recently been identified in many models of cisplatin resistance including those that predict patient responses in NSCLC [176–178] but validation of these signatures is problematic [179].

4.2 epi-miRNAs and cisplatin sensitivity

A subset of miRNAs have been shown to regulate the expression of epigenetic effector proteins, which has led to them being described as epi-miRNAs [180–182]. The importance of an epi-miRNA network in cisplatin resistance was demonstrated by Edward Ratvitski and colleagues who have identified a network of miRNAs and the Tumor Protein p63 as a major element in the development of chemoresistance to cisplatin in laryngeal squamous cell carcinoma. Many of these include miRNAs that directly target the epigenetic machinery including miR-297 (DNMT3A), miR-92b-3p (HDAC9), and miR-485-5p (KDM4C) [183], while in a response to cisplatin a network of miRNA promoters was found to be regulated by p63 in complexes with other transcriptional and chromatin-associated factors, including HDACs, KATs, KMTs, and KDMs [184]. Clearly in the future the functional roles between miRNAs and the epigenetic machinery that influence cellular responses to cisplatin will become better defined.

4.3 lncRNAs associated with resistance/sensitivity

The role of lncRNAs in epigenetic regulation has been discussed in depth in an earlier chapter, and the reader is also directed to overviews in the following reviews [185,186]. lncRNAs are also emerging as epigenetic elements involved with resistance to cisplatin [187]. However, in recent years the subject area has become muddied by the emergence of a high number of fabricated publications associated with paper mills [188–191]. As such, overall, while the data in the literature suggest that lncRNAs may be important elements modulating the epigenetic landscape in the cellular response to cisplatin, a certain degree of caution must currently be observed in ascribing any functionality, and the reader is advised to proceed with caution.

5 Cancer stem cells and cisplatin resistance

The cancer stem cell (CSC) hypothesis suggests that there is a small subset of cancer cells that are responsible for tumor initiation and growth, possessing properties, such as indefinite self-renewal, slow replication, intrinsic resistance to chemotherapy and radiotherapy, and an ability to give rise to differentiated progeny [192]. Critically, CSCs or side-populations may be an emerging central element in cisplatin resistance. We and others have shown that cisplatin can induce/increase CSC/side-populations as cells develop resistance to cisplatin [193,194]. Moreover, targeting such side-populations has been shown to re-sensitize resistant cells to cisplatin therapy [195,196], and this notion of epigenetic plasticity in maintaining permissive chromatin states and allowing for the development of drug-tolerant persisting cells with stem like features is gaining traction [197,198].

In this regard, it is well established that in the regulation of embryonic stem cell (ESC) identity, KAT5 (Tip60) plays critical roles through an associated Tip60 (KAT5)-p400 complex [199]. However, it has now been shown that HDAC6 regulates Tip60 (KAT5)-p400 function in stem cells [200]. In contrast to differentiated cells, where HDAC6 is mainly cytoplasmic, HDAC6 is largely nuclear in ESCs, neural stem cells (NSCs), and some cancer cell lines, and interacts with Tip60 (KAT5)-p400 in each. Given that in a previous section the evidence linking resistance to cisplatin to both Tip60 (KAT5) and HDAC6 was discussed [27], it is tempting to therefore speculate that this may link cisplatin resistance with the subpopulations of CSCs present within a tumor. Indeed, pharmacologically targeting HDAC6 in isogenic oral squamous cell carcinoma cell line models of cisplatin resistance results in both a reduction of the cancer stemness phenotype associated with the resistance, and a corresponding increased sensitivity to platinum [201].

In a previous section, we discussed how JARID2 was shown to be associated with the development of resistance to cisplatin. The mechanism for this has now been linked to enhanced stem-like features caused by the upregulation of Notch1 by JARID2 in NSCLC cells [101]. Another important epigenetic regulatory protein that has now been shown to link drug resistance to cancer “stemness” is PRMT5, which has been shown to be a critical regulator of stem cell function in breast cancer [202]. Given the strong links between CSCs and cisplatin resistance in NSCLC, coupled with the development of drugs capable of targeting PRMT5 (discussed in a later section), direct links between PRMT5 and the CSC niche in cisplatin resistant lung cancer will no doubt be identified in the future.

6 The epitranscriptome and cisplatin?

A controversial area in epigenetics, the epitranscriptome describes what are essentially modifications to RNA. In this regard, these modifications can be considered to be similar to those observed on DNA/chromatin in that various families of readers, writers, and erasers of these modifications have been described [203,204]. A large body of evidence is emerging that the epitranscriptome plays important roles in many biological processes with potential roles in cancer drug resistance [205], for example in TGCT [206,207]. The current literature linking epitranscriptomics and resistance to cisplatin is sparse but increasing, and includes links with NSCLC [208], but more data will be required to truly link the epitranscriptome to cisplatin resistance.

7 Targeting cisplatin resistance epigenetically?

In the previous sections the data linking epigenetics as a key mechanism underpinning cisplatin resistance has been set out. It would seem that potentially targeting the enzymes involved could be used as a therapeutic approach to enhance cisplatin activity and/or resensitize resistant tumors’ to cisplatin-based chemotherapy regimens. In the following sections data showing that targeting the epigenetic machinery may have clinical potential will be discussed.

7.1 Targeting HDACs

A significant number of early studies have shown that histone deacetylase inhibitors (HDACi) have the ability to either synergize with cisplatin or resensitize resistant NSCLC [209–214] or ovarian cancer cell lines to cisplatin [81,215,216].

In other developments the HDACi, belinostat, was found to act synergistically with cisplatin in cisplatin-resistant NSCLC cancer cell lines either when given combined or if given sequentially (belinostat followed by cisplatin), and demonstrated that belinostat circumvented cisplatin resistance through combined downregulation of ABCC2 (an efflux transporter) and a critical DNA repair-mediated gene, ERCC1 [214]. Earlier reports demonstrated that the HDACis, vorinostat and valproic acid, can sensitize SCLC to cisplatin treatment [44] (and references therein). The possibility of combining a HDACi with another agent to resensitize tumors to cisplatin therapy is a rapidly developing area of research exemplified by recent studies such as that by Dallavalle et al., with DNA polymerase α and HDACi [217].

7.2 Targeting KDMs

The development of lysine demethylase inhibitors has been extensively covered in a separate chapter of this volume and in a following section a small sample of the current available data with respect to studies pertaining to cisplatin resistance are described.

The family of Lysine Demethylases (KDMs) are known to be FE + dependent. As such many of the early approaches used tended to target these members of the epigenetic machinery indirectly, using iron chelators, such as deferoxamine (DFO). One of the first reports to target these enzymes and enhance cisplatin sensitivity came from a study in breast cancer by Beland and colleagues [218], and while numerous studies have shown that DFO treatment can enhance sensitivity to cisplatin in both NSCLC [219] and ovarian cancer [220] no direct links to the involvement of inhibition of chromatin remodeling enzymes were investigated. In another recent development, the use of an iron chelator KDMi (VLX600) was found to synergize with platinum compounds and PARPi in ovarian cancer cells, and the authors raise the notion that such an approach could be used to induce homologous recombination (HR) defects in ovarian cancers and correspondingly sensitize them to platinum agents and PARPi [221]. However, it must be noted that a phase I trial of this compound (NCT02222363) in solid tumors was terminated due to (1) lack of accrual and (2) lack of efficacy despite the completion of several dose steps.

Other lysine demethylases inhibitors, such as JIB-04 and ML324, have been shown to overcome cisplatin resistance *in vitro* and *in vivo* through a mechanism involving degradation of key DNA repair proteins [222]. Of the lysine demethylases, focus on KDM4 has been intensive and there are various inhibitors in preclinical development [84]. One of these is TACH101, and recent studies of this agent in diffuse large B-cell lymphoma have shown potent *in vitro* (cell line) and *in vivo* (PDX) activity [223]. In the solid tumor setting, TACH101 has recently opened for recruiting a phase I (NCT05076552) trial in gastrointestinal cancers and metastatic colorectal cancer, and it will be interesting to see the outcome of this trial.

Another potential key target in ovarian cancer is the lysine demethylase Lysine-specific demethylase 1 (LSD1), which was first shown to be a candidate target to sensitize ovarian cancer to cisplatin by the use of a pLKO-LSD1-short hairpin RNA [94]. A significant number

of LSD1 inhibitors are currently in clinical development [90,224,225], and it will be interesting to see if such inhibitors can enhance responses to cisplatin-based therapies in the future.

7.3 Targeting KMTs

A significant amount of activity has been undertaken in the pharmaceutical sector to target EZH2 [226]. The potential for targeting this protein to enhance sensitivity to cisplatin in NSCLC has been demonstrated in cell lines using the inhibitor EPZ-6438 [38], and in SCLC using DZNep [41]. In ovarian cancer, while high EZH2 expression has been associated as a prognostic marker for cisplatin resistance [227,228], and potentially actionable [36], other studies suggest that at least in a subset of patient's loss of EZH2 drives resistance to carboplatin [229]. NCT03348631 is a phase II clinical trial of an EZH2 inhibitor (tazemetostat) in patients with recurrent ovarian or endometrial cancer but is currently suspended to assess enrollments. More data will be required to truly ascertain if KMTi have a role to play in targeting cisplatin resistance.

7.4 Targeting bet proteins and BrD containing epigenetic readers

As discussed in an earlier section, Bromodomain and extra-terminal domain (BET) family proteins play important roles in both NSCLC and ovarian cancer. A number of BET and BrD inhibitors are currently in clinical development [230,231], and a large body of evidence links BET proteins with the DNA Damage Response (DDR) pathway [232], which suggests that agents targeting BET proteins may be attractive targets for use with cisplatin-based therapies, and indeed in preclinical studies BETi have been shown to impair DDR pathways in NSCLC cell lines [233], and to also synergize with cisplatin with associated enhanced inhibition of cell proliferation [111]. Studies on the potential use of BETi in ovarian cancer have also demonstrated potential [234,235]. However, on a note of caution, it must be noted that in a PDX study of pediatric brain tumors, EZH2i and cisplatin did not show any superiority to cisplatin alone [236].

7.5 Targeting PRMTs

Given the important roles identified for PRMTs in cisplatin resistance, the development of inhibitors to these epigenetic writers has garnered much interest particularly for PRMT5. Several of the inhibitors developed are currently undergoing phase I clinical trials (including patients with NSCLC) [237,238]. It is interesting to note that in a case of potential drug repurposing, tadalafil was identified as a PRMT5 inhibitor [239,240] which have been shown to enhance sensitivity to cisplatin at least in breast cancer models [239].

7.6 Natural bioactives

Natural bioactives that target the epigenetic machinery have been discussed in detail in a separate chapter in this book. Evidence continues to be published suggesting that such bioactives could potentially be able to sensitize to cisplatin. For example, sulforaphane (a polyphenol histone deacetylase inhibitor found in cruciferous vegetables) has been shown to sensitize

ovarian cancer cell lines to cisplatin [241], and while the data are encouraging significant issues remain with bioactives particularly with respect to their delivery in meaningful amounts to elicit appropriate responses [242].

8 Clinical trials

Many clinical trials involving epigenetic therapies and cisplatin have been conducted. Initially, the results of such trials were discouraging. For example, a phase I trial of cisplatin plus decitabine, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable NSCLC, came to the conclusion that the cisplatin plus decitabine combination did not exhibit significant antitumor activity in patients with NSCLC at the dose and schedule applied to justify its further evaluation [243].

New trials are being initiated with second generation DNMTi, such as guadecitabine (a novel prodrug of decitabine), which are beginning to show some promise. The results of a phase I trial of guadecitabine plus cisplatin in cisplatin-resistant germ cell tumors (NCT02429466) has recently been reported, with an ORR of 23%, and a combined clinical benefit of 46% [244]. Likewise, a phase I trial of guadecitabine plus carboplatin in platinum resistant recurrent ovarian cancer (NCT01696032) also reported similar efficacy with an ORR of 15% and a clinical benefit of 45% [245]. These results warrant the expansion of guadecitabine into the phase II setting.

Despite the data suggesting that HDACi can affect responses to cisplatin therapy, the results of very few clinical trials have been reported. The results of a phase I trial (NCT00926640) of belinostat in combination with Etoposide/Cisplatin has been reported for patients with SCLC and neuroendocrine cancer [246]. In this trial, “objective responses were observed in 11 (39%) of 28 patients and seven (47%) of 15 patients with neuroendocrine tumors (including SCLC)” [246]. In contrast to this, a two-stage phase II study of another HDACi, chidamide, given in combination with cisplatin in patients with metastatic triple-negative breast cancer failed to reach its primary endpoint and was halted at the end of its first stage prior to expansion [247].

Other trials have attempted to combine various epigenetic therapy inhibitors with cisplatin. One such combination is hydralazine/valproate (valproic acid). Hydralazine acts as a DNMT inhibitor [248], while valproic acid functions to inhibit HDACs [249]. A phase II clinical trial using Hydralazine/Valproic acid was tested in combination with various chemotherapies in refractory solid tumors to see if this combination could overcome chemotherapy resistance including cisplatin [250]. The results of this small clinical trial were encouraging and found a clinical benefit in 12 (80%) patients. Particularly noteworthy from this study is that of seven ovarian cancer patients included in the trial all achieved either partial responses ($n = 3$) or disease stabilization ($n = 4$) [250].

In a separate phase I clinical trial, the effect of azacytidine and valproate in combination with carboplatin was examined on solid tumors. Minor responses or stable disease lasting ≥ 4 months were achieved by six patients (18.8%), including three with platinum-resistant or platinum-refractory ovarian cancer, suggesting that regimens including epigenetic targeting agents may have a role to play in the management of platinum refractory ovarian cancer [251]. However, it must be noted that the clinicaltrial.gov identifier linked to this study is NCT00529022, which was officially titled “Phase I Trial of Sequential Azacitidine and Valproic Acid Plus Carboplatin in the Treatment

of Patients With Platinum Resistant Epithelial Ovarian Cancer,” and so a certain degree of caution in the interpretation of these results is warranted as only 10 out of 32 patients enrolled actually had ovarian cancer.

Moving away from HDACs, several lysine demethylase inhibitors are being examined in the clinical setting. For example, numerous inhibitors of LSD1 are in current clinical development [90,224] and recent trials, such as NCT02875223 (CC-90011), demonstrate favorable tolerability profiles, alongside clinical activity and durable responses [252,253].

LSD1 inhibitors, such as T-3775440 or ORY-1001 (iadademstat), have been identified as a potential therapeutic possibility in SCLC [254,255], and based partially on the data from the CLEPSIDRA (EudraCT no 2018-000469-35) trial [256], in June 2022, iademstat recently received Orphan Drug Status by the FDA for treating SCLC [257]. However, it must be noted that a phase I trial of GSK2879552 (an LSD1 inhibitor) in SCLC (NCT02034123) was “*terminated, as the risk-benefit profile did not favor continuation*” [258]. In this regard, a recent analysis suggests that a limited number of SCLC patients with a neuroendocrine differentiation state may be the target population for such LSDi [259], and a new trial (NCT05420636) is currently about to launch to investigate “Iademstat in Combination With Paclitaxel in Relapsed/Refractory SCLC” and extra-pulmonary high grade neuroendocrine carcinomas.

As other drugs targeting KDMs have recently completed phase I trials [260–262], it will be interesting to see whether any of the clinical trials can be used to enhance cisplatin therapy.

Might targeting EZH2 or lysine methyltransferases have any efficacy? Several inhibitors of EZH2 are currently under development, and one tazemetostat is the first epigenetic targeting agent to receive FDA approval for a solid tumor [263] and has also been approved for relapsed or refractory follicular lymphoma [264].

Perhaps we could therefore target epigenetic readers? Several BET inhibitors have completed initial clinical trials, but as yet none have received regulatory approval [232,265,266]. Some of the limitations with the current inhibitors involve severe dose-limiting toxicities (DLTs), and the rapid development of multiple mechanisms of resistance in solid tumors, including triple-negative breast cancer (TNBC), castration resistant prostate cancer (CRPC), lung cancer, and ovarian cancer [232,267].

8.1 Circadian clocks/metronomic scheduling

Circadian clock proteins, such as Clock, are involved with many cellular functions, such as the cell cycle and cell division, and as such the appropriate timing of chemotherapy may therefore increase the efficacy of chemotherapeutic drugs and ameliorate their side effects. In this regard, Tip60 (KAT5) is regulated by Clock and required for cisplatin resistance in NSCLC [20] and Ovarian cancer [268]. In a wide ranging review of this field, Aziz Sancar, David Hsu and colleagues explore this issue [269], and concluded that that “in general, circadian rhythmicity is lost” and conclude that “*the efficacy of anticancer drugs, would be expected to be circadian time-dependent; however, research so far has not shown this to be the case*” [269]. However, despite this, in NSCLC phase II trial of metronomic scheduling or chronochemotherapy of vinorelbine in combination with cisplatin alongside concomitant radiotherapy ClinicalTrials.gov (NCT02709720) has completed [270]. The results of this trial were broadly positive, with an overall response rate (ORR) of 66.2%, and a median overall survival (OS) of 35.6 months. The main conclusion from this study was that this

regimen of “Metronomic oral vinorelbine and cisplatin obtains similar efficacy results with significantly lower toxicity than the same chemotherapy at standard doses” [270].

One of the common issues with the use of epigenetic therapies in the clinical setting centers on how we can functionally measure epigenetic effects in real time to estimate efficacy. Many of the current failures with respect to epigenetic therapy in clinical trials of solid tumors often reflect the fact that in many cases the epigenetic targeting agent fails to penetrate the tumors [271,272]. How to resolve this is often an issue, but recently a methodology has been utilized to develop a PET/CT imaging approach to monitor HDACi activity *in vivo* [273], which may help to resolve some of these outstanding issues. Other long-term issues that remain to be resolved include issues of solubility, adverse side effects, selectivity profiles, and pharmacokinetic interactions [274].

8.2 Low-dose therapies as “epigenetic priming” events

Given the failure of epigenetic targeting agents particularly in the solid tumor setting in trials, such as VANTAGE-014 [275] and ENCORE-601 [276], this raises important questions regarding how we move these agent’s forwards in the clinic as recently highlighted by Ganesan and colleagues [277]. Data is emerging to show that low-dose treatments with epigenetic targeting agents may prove to be a breakthrough in this area. Essentially, by using clinically relevant noncytotoxic doses that target the epigenetic machinery but do not cause any cytotoxicity, cancers may become “epigenetically primed” for standard chemotherapy or targeted therapy. In probably the most important development with regard to this hypothesis, transient treatments with clinically relevant nanomolar doses of DNA-demethylating agents were found to exert durable antitumor effects on hematological and epithelial tumor cells. Treatments generated an antitumor “memory” response, including inhibition of subpopulations of cancer stem-like cells, accompanied by sustained decreases in genome wide promoter DNA methylation, gene reexpression, and antitumor changes in key cellular regulatory pathways [278]. This has obvious implications for targeting tumors, which have become resistant to cisplatin.

The most persuasive evidence for epigenetic priming has come from a phase I/II trial (NCT01004991) in patients newly diagnosed with diffuse large B-cell lymphoma (DLBCL), which demonstrated very strong responses. Eleven of 12 patients achieved a complete response and 10 remained in remission with a median follow-up of 13 months (range 5–28 months) at the time of publication [279].

In NSCLC, a nontoxic dose of the HDACi, chidamide, was shown to synergistically enhance platinum-induced responses and cell death [280]. Subsequently a phase I trial (ChiCTR-ONC-12002283) of chidamide combined with paclitaxel and carboplatin in patients with NSCLC was completed with some clinical benefit shown, and a further phase II trial (NCT01836679) completed in 2015, but no data from this trial have yet been reported. It must be noted that a separate phase II trial of chidamide in combination with cisplatin has been reported for patients with metastatic triple-negative breast cancer [247], with disappointing results and did not meet its primary endpoint.

In another disappointment, a dosing regimen involving low-dose DNMTi (gaudecitatine) plus carboplatin in ovarian cancer (NCT01696032) failed to meet its primary objective [281]. Intriguingly, a downstream analysis of this trial demonstrated that “Lower DNMT1 and 3B protein levels in pretreatment tumors were associated with improved progression-free survival” [10].

Nevertheless, despite this failure, “epigenetic priming” with HDACi appears to sensitize ovarian cancer cells to cisplatin, and to reverse resistance to cisplatin in already resistant cells [282].

A more recent phase II trial (NCT03985007) of a double epigenetic priming strategy using chidamide and decitabine prior to the salvage treatment of relapsed/refractory acute myeloid leukemia using cytarabine, idarubicin, and granulocyte-colony stimulating factor was found to have a completed remission rate (CRR) of 42.9%. The median OS time was 11.7 months, and when stratified responders (median OS of 18.4 months) versus nonresponders (median OS of 7.4 months) [283].

The potential for epigenetic priming for enhancing response to immune checkpoint inhibitors (ICIs) is also emerging as a potential treatment postdevelopment of cisplatin resistance. In a separate trial (NCT02901899) regarding epigenetic priming of platinum-resistant ovarian cancer patients with guadecitabine prior to therapy with pembrolizumab resulted in 8.6% of patients showing partial responses, stable disease in 22.9% and an overall clinical benefit rate of 31.4% [284]. Similar results were observed in a phase I trial in advanced solid tumors (HyPeR—NCT02998567), in which an ORR of 7% was observed, with 37% of patients having a PFS of ≥ 24 weeks [285]. Of interest in this trial, were the data pertaining to patients with NSCLC. Notably of these 10 patients (83%) had previously progressed on anti-PD-1/PD-L1 therapy, and following treatment on this trial 5 patients (42%) experienced disease control for ≥ 24 weeks [285].

Likewise, the METADUR phase I trial (NCT02811497) also examined the efficacy of a different DNMTi (CC-486) plus pembrolizumab in various solid tumors [286]. This trial also resulted in a similar disease control rate of 7.1%. However, perhaps the most important results to have come from these two trials are the following: (1) that CC-486 did not penetrate the tumors and (2) these responses were not durable, and as such these combined DNMTi plus immune checkpoint blockade regimens were not considered strong enough to move forward with this therapy [272]. In this regard, a similar pattern emerged for lung cancer with the failure of entinostat (HDACi) combined with pembrolizumab in the phase I/II Encore 601 study [276] which was potentially flagged as an issue in early preclinical studies which concluded “Taken together, these data do not support broadly applicable epigenetic priming in NSCLC” [287].

Clearly, while the notion of low-dose epigenetic priming may become important, currently our knowledge is suboptimal with respect to identifying those patients who might best respond to such an approach.

9 Conclusions

It is clear that epigenetics plays important roles in the cellular response to cisplatin, and strong evidence exists linking epigenetic mechanisms to both the development and maintenance of resistance to platinum-based chemotherapy regimens.

The emerging clinical data for combinatorial low-dose epigenetic therapies is compelling and future work will determine if such therapies will revolutionize the way chemotherapy resistant tumors are treated. One concern, however, remains the issue of intratumoral heterogeneity [288], and if this may affect how epigenetic therapies (both standard and epigenetic priming) will affect the long-term outcome of patients treated to resensitize to cisplatin-based regimens.

Glossary

Epigenetic Priming The use of epigenetic therapy to prime a patient for subsequent other interventions

Epigenetic Machinery A generic term loosely grouping epigenetic readers, writers and erasers into one large subgroup.

Epi-miRNA A subset of miRNAs which have been shown to directly target and regulate other members of the epigenetic regulatory machinery

List of abbreviations

5-aza-dC	5-Aza-2-deoxycytidine
ARIE	Acute radiation-induced esophagitis
AzaC	5'-Azacytidine
BARD	BRCA1-associated RING domain-1
BER	Base excision repair
BRCA1	Breast Cancer 1 gene
CSC	Cancer stem cell
DFO	Desferrioxamine
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSB	Double-strand breaks
DZNep	3-Deazaneplanocin A
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
EOC	Epithelial ovarian cancer
ERCC1	Excision repair cross-complementation group 1
EZH2	Enhancer of zeste homolog 2
FBW7	F-box/WD repeat-containing protein 7
Foxo	Forkhead O
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
KAT	Lysine acetyltransferase
KDM	Lysine demethylase
KMT	Lysine methyltransferase
LCL	Lymphoblastic cell line
lncRNA	Long noncoding RNA
MBD2	Methyl-CpG-binding domain protein 2
MDC1	Mediator of DNA-damage checkpoint 1
miRNA	MicroRNA
MPM	Malignant pleural mesothelioma
ncRNA	Noncoding RNA
NER	Nucleotide excision repair
NFkB	Nuclear Factor kappa Beta

NuRD	Nucleosome remodeling and deacetylase
NSCLC	Nonsmall-cell lung cancer
OS	Overall survival
PD	Progressive disease
PFS	Progression-free survival
PHD	Plant homeodomain
PR	Partial response
PRC	Polycomb repressive complex
PTM	Posttranslational modification
QTL	Quantitative trait loci
RGS	Regulator of G protein signaling
SCLC	Small-cell lung cancer
SD	Stable disease
shRNA	Short hairpin RNA
SNP	Single-nucleotide polymorphism
TSA	Trichostatin A
XPG	Xeroderma pigmentosum complementation group G

References

- [1] Wang Y, Huang Z, Li B, Liu L, Huang C. The emerging roles and therapeutic implications of epigenetic modifications in ovarian cancer. *Front Endocrinol (Lausanne)* 2022;13:863541.
- [2] Reid BM, Fridley BL. DNA methylation in ovarian cancer susceptibility. *Cancers (Basel)* 2020;13(1).
- [3] Duan J, Zhong B, Fan Z, et al. DNA methylation in pulmonary fibrosis and lung cancer. *Expert Rev Respir Med* 2022;16(5):519–28.
- [4] Singh R, Fazal Z, Bikorimana E, et al. Reciprocal epigenetic remodeling controls testicular cancer hypersensitivity to hypomethylating agents and chemotherapy. *Mol Oncol* 2022;16(3):683–98.
- [5] Yu W, Jin C, Lou X, et al. Global analysis of DNA methylation by Methyl-Capture sequencing reveals epigenetic control of cisplatin resistance in ovarian cancer cell. *PLoS One* 2011;6(12):e29450.
- [6] Zeller C, Dai W, Steele NL, et al. Candidate DNA methylation drivers of acquired cisplatin resistance in ovarian cancer identified by methylome and expression profiling. *Oncogene* 2012;31(42):4567–76.
- [7] Zhang YW, Zheng Y, Wang JZ, et al. Integrated analysis of DNA methylation and mRNA expression profiling reveals candidate genes associated with cisplatin resistance in non-small cell lung cancer. *Epigenetics* 2014;9(6).
- [8] De Carvalho DD, Sharma S, You JS, et al. DNA methylation screening identifies driver epigenetic events of cancer cell survival. *Cancer Cell* 2012;21(5):655–67.
- [9] Chan DW, Lam WY, Chen F, et al. Genome-wide DNA methylome analysis identifies methylation signatures associated with survival and drug resistance of ovarian cancers. *Clin Epigenetics* 2021;13(1):142.
- [10] Cardenas H, Fang F, Jiang G, et al. Methylomic signatures of high grade serous ovarian cancer. *Epigenetics* 2021;16(11):1201–16.
- [11] Lund RJ, Huhtinen K, Salmi J, et al. DNA methylation and transcriptome changes associated with cisplatin resistance in ovarian cancer. *Sci Rep* 2017;7(1):1469.
- [12] Tomar T, de Jong S, Alkema NG, et al. Genome-wide methylation profiling of ovarian cancer patient-derived xenografts treated with the demethylating agent decitabine identifies novel epigenetically regulated genes and pathways. *Genome Med* 2016;8(1):107.
- [13] Quek K, Li J, Estecio M, et al. DNA methylation intratumor heterogeneity in localized lung adenocarcinomas. *Oncotarget* 2017;8(13):21994–2002.

- [14] Hua X, Zhao W, Pesatori AC, et al. Genetic and epigenetic intratumor heterogeneity impacts prognosis of lung adenocarcinoma. *Nat Commun* 2020;11(1):2459.
- [15] Woźniak K, Błasiak J. Recognition and repair of DNA-cisplatin adducts. *Acta Biochim Pol* 2002;49(3):583–96.
- [16] Rottenberg S, Disler C, Perego P. The rediscovery of platinum-based cancer therapy. *Nat Rev Cancer* 2021;21(1):37–50.
- [17] Moon HM, Park JS, Lee IB, et al. Cisplatin fastens chromatin irreversibly even at a high chloride concentration. *Nucleic Acids Res* 2021;49(21):12035–47.
- [18] Shah MA, Denton EL, Liu L, Schapira M. ChromoHub V2: cancer genomics. *Bioinformatics* 2014;30(4):590–2.
- [19] Igarashi T, Izumi H, Uchiumi T, et al. Clock and ATF4 transcription system regulates drug resistance in human cancer cell lines. *Oncogene* 2007;26(33):4749–60.
- [20] Miyamoto N, Izumi H, Noguchi T, et al. Tip60 is regulated by circadian transcription factor clock and is involved in cisplatin resistance. *J Biol Chem* 2008;283(26):18218–26.
- [21] Banerjee Mustafi S, Chakraborty PK, Naz S, et al. MDR1 mediated chemoresistance: BMI1 and TIP60 in action. *Biochim Biophys Acta* 2016;1859(8):983–93.
- [22] Hirano G, Izumi H, Kidani A, et al. Enhanced expression of PCAF endows apoptosis resistance in cisplatin-resistant cells. *Mol Cancer Res* 2010;8(6):864–72.
- [23] Wu ZZ, Sun NK, Chao CC. Knockdown of CITED2 using short-hairpin RNA sensitizes cancer cells to cisplatin through stabilization of p53 and enhancement of p53-dependent apoptosis. *J Cell Physiol* 2011;226(9):2415–28.
- [24] Cai D, Shames DS, Raso MG, et al. Steroid receptor coactivator-3 expression in lung cancer and its role in the regulation of cancer cell survival and proliferation. *Cancer Res* 2010;70(16):6477–85.
- [25] Liu W, Zhan Z, Zhang M, et al. KAT6A, a novel regulator of β -catenin, promotes tumorigenicity and chemoresistance in ovarian cancer by acetylating COP1. *Theranostics* 2021;11(13):6278–92.
- [26] Sun Y, Jiang X, Xu Y, et al. Histone H3 methylation links DNA damage detection to activation of the tumour suppressor Tip60. *Nat Cell Biol* 2009;11(11):1376–82.
- [27] Edmond V, Moysan E, Khochbin S, et al. Acetylation and phosphorylation of SRSF2 control cell fate decision in response to cisplatin. *EMBO J* 2011;30(3):510–23.
- [28] Dehm SM. mRNA splicing variants: exploiting modularity to outwit cancer therapy. *Cancer Res* 2013;73(17):5309–14.
- [29] Wang C, Zhou Z, Subbaramanyam CS, et al. SRPK1 acetylation modulates alternative splicing to regulate cisplatin resistance in breast cancer cells. *Commun Biol* 2020;3(1):268.
- [30] Bassi C, Li YT, Khu K, et al. The acetyltransferase Tip60 contributes to mammary tumorigenesis by modulating DNA repair. *Cell Death Differ* 2016;23(7):1198–208.
- [31] Hyun K, Jeon J, Park K, Kim J. Writing, erasing and reading histone lysine methylations. *Exp Mol Med* 2017;49(4):e324.
- [32] Guccione E, Richard S. The regulation, functions and clinical relevance of arginine methylation. *Nat Rev Mol Cell Biol* 2019;20(10):642–57.
- [33] Zhao S, Allis CD, Wang GG. The language of chromatin modification in human cancers. *Nat Rev Cancer* 2021;21(7):413–30.
- [34] Gan L, Yang Y, Li Q, Feng Y, Liu T, Guo W. Epigenetic regulation of cancer progression by EZH2: from biological insights to therapeutic potential. *Biomark Res* 2018;6:10.
- [35] Hu S, Yu L, Li Z, et al. Overexpression of EZH2 contributes to acquired cisplatin resistance in ovarian cancer cells in vitro and in vivo. *Cancer Biol Ther* 2010;10(8):788–95.
- [36] Sun S, Zhao S, Yang Q, et al. Enhancer of zeste homolog 2 promotes cisplatin resistance by reducing cellular platinum accumulation. *Cancer Sci* 2018;109(6):1853–64.

- [37] Li T, Cai J, Ding H, Xu L, Yang Q, Wang Z. EZH2 participates in malignant biological behavior of epithelial ovarian cancer through regulating the expression of BRCA1. *Cancer Biol Ther* 2014;15(3):271–8.
- [38] Cao Z, Wu W, Wei H, Zhang W, Huang Y, Dong Z. Downregulation of histone-lysine N-methyltransferase EZH2 inhibits cell viability and enhances chemosensitivity in lung cancer cells. *Oncol Lett* 2021;21(1):26.
- [39] Lv Y, Yuan C, Xiao X, et al. The expression and significance of the enhancer of zeste homolog 2 in lung adenocarcinoma. *Oncol Rep* 2012;28(1):147–54.
- [40] Riquelme E, Suraokar M, Behrens C, et al. VEGF/VEGFR-2 upregulates EZH2 expression in lung adenocarcinoma cells and EZH2 depletion enhances the response to platinum-based and VEGFR-2-targeted therapy. *Clin Cancer Res* 2014;20(14):3849–61.
- [41] Koyen AE, Madden MZ, Park D, et al. EZH2 has a non-catalytic and PRC2-independent role in stabilizing DDB2 to promote nucleotide excision repair. *Oncogene* 2020;39(25):4798–813.
- [42] Gazdar AF, Bunn PA, Minna JD. Small-cell lung cancer: what we know, what we need to know and the path forward. *Nat Rev Cancer* 2017;17(12):725–37.
- [43] Rudin CM, Poirier JT, Byers LA, et al. Molecular subtypes of small cell lung cancer: a synthesis of human and mouse model data. *Nat Rev Cancer* 2019;19(5):289–97.
- [44] Khan P, Siddiqui JA, Maurya SK, et al. Epigenetic landscape of small cell lung cancer: small image of a giant recalcitrant disease. *Semin Cancer Biol* 2022;83:57–76.
- [45] Basumallik N, Agarwal M. Small cell lung cancer. Treasure Island (FL): StatPearls Publishing; 2022.
- [46] Sui JSY, Martin P, Gray SG. Pre-clinical models of small cell lung cancer and the validation of therapeutic targets. *Expert Opin Ther Targets* 2020;24(3):187–204.
- [47] Rudin CM, Brambilla E, Faivre-Finn C, Sage J. Small-cell lung cancer. *Nat Rev Dis Prim* 2021;7(1):3.
- [48] Byers LA, Rudin CM. Small cell lung cancer: where do we go from here? *Cancer* 2015;121(5):664–72.
- [49] Allison Stewart C, Tong P, Cardnell RJ, et al. Dynamic variations in epithelial-to-mesenchymal transition (EMT), ATM, and SLFN11 govern response to PARP inhibitors and cisplatin in small cell lung cancer. *Oncotarget* 2017;8(17):28575–87.
- [50] Poirier JT, Gardner EE, Connis N, et al. DNA methylation in small cell lung cancer defines distinct disease subtypes and correlates with high expression of EZH2. *Oncogene* 2015;34(48):5869–78.
- [51] Gardner EE, Lok BH, Schneeberger VE, et al. Chemosensitive relapse in small cell lung cancer proceeds through an EZH2-SLFN11 axis. *Cancer Cell* 2017;31(2):286–99.
- [52] Puppe J, Opdam M, Schouten PC, et al. EZH2 is overexpressed in BRCA1-like breast tumors and predictive for sensitivity to high-dose platinum-based chemotherapy. *Clin Cancer Res* 2019;25(14):4351–62.
- [53] Singh R, Fazal Z, Corbet AK, et al. Epigenetic remodeling through downregulation of polycomb repressive complex 2 mediates chemotherapy resistance in testicular germ cell tumors. *Cancers (Basel)* 2019;11(6).
- [54] Tessema M, Rossi MR, Picchi MA, et al. Common cancer-driver mutations and their association with abnormally methylated genes in lung adenocarcinoma from never-smokers. *Lung Cancer* 2018;123:99–106.
- [55] Kim IK, McCutcheon JN, Rao G, et al. Acquired SETD2 mutation and impaired CREB1 activation confer cisplatin resistance in metastatic non-small cell lung cancer. *Oncogene* 2019;38(2):180–93.
- [56] Liu D, Zhang XX, Li MC, et al. C/EBP β enhances platinum resistance of ovarian cancer cells by reprogramming H3K79 methylation. *Nat Commun* 2018;9(1):1739.
- [57] Wang L, Xu ML, Wang C, et al. SET and MYND domain-containing protein 3 inhibits tumor cell sensitivity to cisplatin. *Oncol Lett* 2020;19(5):3469–76.
- [58] Li J, Zhao L, Pan Y, et al. SMYD3 overexpression indicates poor prognosis and promotes cell proliferation, migration and invasion in non-small cell lung cancer. *Int J Oncol* 2020;57(3):756–66.

- [59] Ibrahim R, Matsubara D, Osman W, et al. Expression of PRMT5 in lung adenocarcinoma and its significance in epithelial-mesenchymal transition. *Hum Pathol* 2014;45(7):1397–405.
- [60] Shilo K, Wu X, Sharma S, et al. Cellular localization of protein arginine methyltransferase-5 correlates with grade of lung tumors. *Diagn Pathol* 2013;8:201.
- [61] Bajbouj K, Ramakrishnan RK, Saber-Ayad M, et al. PRMT5 selective inhibitor enhances therapeutic efficacy of cisplatin in lung cancer cells. *Int J Mol Sci* 2021;22(11).
- [62] Yoshimatsu M, Toyokawa G, Hayami S, et al. Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers. *Int J Cancer* 2011;128(3):562–73.
- [63] Akter KA, Mansour MA, Hyodo T, Ito S, Hamaguchi M, Senga T. FAM98A is a novel substrate of PRMT1 required for tumor cell migration, invasion, and colony formation. *Tumour Biol* 2016;37(4):4531–9.
- [64] Elakoum R, Gauchotte G, Oussalah A, et al. CARM1 and PRMT1 are dysregulated in lung cancer without hierarchical features. *Biochimie* 2014;97:210–18.
- [65] Boisvert FM, Déry U, Masson JY, Richard S. Arginine methylation of MRE11 by PRMT1 is required for DNA damage checkpoint control. *Genes Dev* 2005;19(6):671–6.
- [66] Musiani D, Giambruno R, Massignani E, et al. PRMT1 is recruited via DNA-PK to chromatin where it sustains the senescence-associated secretory phenotype in response to cisplatin. *Cell Rep* 2020;30(4):1208–22 e9.
- [67] Matsubara H, Fukuda T, Awazu Y, et al. PRMT1 expression predicts sensitivity to platinum-based chemotherapy in patients with ovarian serous carcinoma. *Oncol Lett* 2021;21(2):162.
- [68] Avasarala S, Van Scoyk M, Karuppusamy Rathinam MK, et al. PRMT1 is a novel regulator of epithelial-mesenchymal-transition in non-small cell lung cancer. *J Biol Chem* 2015;290(21):13479–89.
- [69] Iderzorig T, Kellen J, Osude C, et al. Comparison of EMT mediated tyrosine kinase inhibitor resistance in NSCLC. *Biochem Biophys Res Commun* 2018;496(2):770–7.
- [70] Feng X, Li L, Wang L, Luo S, Bai X. Chromatin target of protein arginine methyltransferase regulates invasion, chemoresistance, and stemness in epithelial ovarian cancer. *Biosci Rep* 2019;39(4).
- [71] Feng X, Bai X, Ni J, et al. CHTOP in chemoresistant epithelial ovarian cancer: a novel and potential therapeutic target. *Front Oncol* 2019;9:557.
- [72] Liang XJ, Finkel T, Shen DW, Yin JJ, Aszalos A, Gottesman MM. SIRT1 contributes in part to cisplatin resistance in cancer cells by altering mitochondrial metabolism. *Mol Cancer Res* 2008;6(9):1499–506.
- [73] Kim MG, Pak JH, Choi WH, Park JY, Nam JH, Kim JH. The relationship between cisplatin resistance and histone deacetylase isoform overexpression in epithelial ovarian cancer cell lines. *J Gynecol Oncol* 2012;23(3):182–9.
- [74] Toiber D, Erdel F, Bouazoune K, et al. SIRT6 recruits SNF2H to DNA break sites, preventing genomic instability through chromatin remodeling. *Mol Cell* 2013;51(4):454–68.
- [75] Kothandapani A, Gopalakrishnan K, Kahali B, Reisman D, Patrick SM. Downregulation of SWI/SNF chromatin remodeling factor subunits modulates cisplatin cytotoxicity. *Exp Cell Res* 2012;318(16):1973–86.
- [76] Yang Y, Li M, Zhou X, et al. PHF5A contributes to the maintenance of the cancer stem-like phenotype in non-small cell lung cancer by regulating histone deacetylase 8. *Ann Clin Lab Sci* 2022;52(3):439–51.
- [77] Fan Q, Li L, Wang TL, Emerson RE, Xu Y. A novel ZIP4-HDAC4-VEGFA axis in high-grade serous ovarian cancer. *Cancers (Basel)* 2021;13(15).
- [78] Xiao Y, Lin FT, Lin WC. ACTL6A promotes repair of cisplatin-induced DNA damage, a new mechanism of platinum resistance in cancer. *Proc Natl Acad Sci U S A* 2021;118(3).
- [79] Wang W, Zhao M, Cui L, et al. Characterization of a novel HDAC/RXR/HtrA1 signaling axis as a novel target to overcome cisplatin resistance in human non-small cell lung cancer. *Mol Cancer* 2020;19(1):134.

- [80] Taylan E, Zayou F, Murali R, et al. Dual targeting of GSK3B and HDACs reduces tumor growth and improves survival in an ovarian cancer mouse model. *Gynecol Oncol* 2020;159(1):277–84.
- [81] Bandolik JJ, Hamacher A, Schrenk C, Weishaupt R, Kassack MU. Class I-histone deacetylase (HDAC) inhibition is superior to pan-HDAC inhibition in modulating cisplatin potency in high grade serous ovarian cancer cell lines. *Int J Mol Sci* 2019;20(12).
- [82] Luo S, Ma K, Zhu H, et al. Molecular, biological characterization and drug sensitivity of chidamide-resistant non-small cell lung cancer cells. *Oncol Lett* 2017;14(6):6869–75.
- [83] Sobczak M, Strachowska M, Gronkowska K, Robaszkiewicz A. Activation of ABCC genes by cisplatin depends on the CoREST occurrence at their promoters in A549 and MDA-MB-231 cell lines. *Cancers (Basel)* 2022;14(4).
- [84] Wu Q, Young B, Wang Y, Davidoff AM, Rankovic Z, Yang J. Recent advances with KDM4 inhibitors and potential applications. *J Med Chem* 2022;65(14):9564–79.
- [85] Berry WL, Janknecht R. KDM4/JMJD2 histone demethylases: epigenetic regulators in cancer cells. *Cancer Res* 2013;73(10):2936–42.
- [86] Pavlenko E, Ruengeler T, Engel P, Poepsel S. Functions and interactions of mammalian KDM5 demethylases. *Front Genet* 2022;13:906662.
- [87] Liang X, Zhang H, Wang Z, et al. JMJD8 is an M2 macrophage biomarker, and it associates with DNA damage repair to facilitate stemness maintenance, chemoresistance, and immunosuppression in pan-cancer. *Front Immunol* 2022;13:875786.
- [88] Liu H, Lin J, Zhou W, et al. KDM5A inhibits antitumor immune responses through downregulation of the antigen-presentation pathway in ovarian cancer. *Cancer Immunol Res* 2022;10(8):1028–38.
- [89] Perillo B, Tramontano A, Pezone A, Migliaccio A. LSD1: more than demethylation of histone lysine residues. *Exp Mol Med* 2020;52(12):1936–47.
- [90] Dong J, Pervaiz W, Tayyab B, et al. A comprehensive comparative study on LSD1 in different cancers and tumor specific LSD1 inhibitors. *Eur J Med Chem* 2022;240:114564.
- [91] Lv T, Yuan D, Miao X, et al. Over-expression of LSD1 promotes proliferation, migration and invasion in non-small cell lung cancer. *PLoS One* 2012;7(4):e35065.
- [92] Chen C, Ge J, Lu Q, Ping G, Yang C, Fang X. Expression of lysine-specific demethylase 1 in human epithelial ovarian cancer. *J Ovarian Res* 2015;8:28.
- [93] Konovalov S, Garcia-Bassets I. Analysis of the levels of lysine-specific demethylase 1 (LSD1) mRNA in human ovarian tumors and the effects of chemical LSD1 inhibitors in ovarian cancer cell lines. *J Ovarian Res* 2013;6(1):75.
- [94] Shao G, Wan X, Lai W, et al. Inhibition of lysine-specific demethylase 1 prevents proliferation and mediates cisplatin sensitivity in ovarian cancer cells. *Oncol Lett* 2018;15(6):9025–32.
- [95] Chen ZY, Chen H, Qiu T, et al. Effects of cisplatin on the LSD1-mediated invasion and metastasis of prostate cancer cells. *Mol Med Rep* 2016;14(3):2511–17.
- [96] Lee YK, Lim J, Yoon SY, Joo JC, Park SJ, Park YJ. Promotion of cell death in cisplatin-resistant ovarian cancer cells through KDM1B-DCLRE1B modulation. *Int J Mol Sci* 2019;20(10).
- [97] Kuo KT, Huang WC, Bamodu OA, et al. Histone demethylase JARID1B/KDM5B promotes aggressiveness of non-small cell lung cancer and serves as a good prognostic predictor. *Clin Epigenetics* 2018;10(1):107.
- [98] Shen X, Zhuang Z, Zhang Y, et al. JARID1B modulates lung cancer cell proliferation and invasion by regulating p53 expression. *Tumour Biol* 2015;36(9):7133–42.
- [99] Tortelli TC, Tamura RE, de Souza Junqueira M, et al. Metformin-induced chemosensitization to cisplatin depends on P53 status and is inhibited by Jarid1b overexpression in non-small cell lung cancer cells. *Aging (Albany NY)* 2021;13(18):21914–40.

- [100] Landeira D, Fisher AG. Inactive yet indispensable: the tale of Jarid2. *Trends Cell Biol* 2011;21(2):74–80.
- [101] Wang Q, Wu J, Wei H, et al. JARID2 promotes stemness and cisplatin resistance in non-small cell lung cancer via upregulation of Notch1. *Int J Biochem Cell Biol* 2021;138:106040.
- [102] Duan L, Perez RE, Chastain 2nd PD, Mathew MT, Bijukumar DR, Maki CG. JMJD2 promotes acquired cisplatin resistance in non-small cell lung carcinoma cells. *Oncogene* 2019;38(28):5643–57.
- [103] Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell* 2012;150(1):12–27.
- [104] Wu M, Wang PF, Lee JS, et al. Molecular regulation of H3K4 trimethylation by Wdr82, a component of human Set1/COMPASS. *Mol Cell Biol* 2008;28(24):7337–44.
- [105] Lee JH, You J, Dobrota E, Skalnik DG. Identification and characterization of a novel human PP1 phosphatase complex. *J Biol Chem* 2010;285(32):24466–76.
- [106] Bounaix Morand du Puch C, Barbier E, Kraut A, et al. TOX4 and its binding partners recognize DNA adducts generated by platinum anticancer drugs. *Arch Biochem Biophys* 2011;507(2):296–303.
- [107] Yu HG, Wei W, Xia LH, et al. FBW7 upregulation enhances cisplatin cytotoxicity in non-small cell lung cancer cells. *Asian Pac J Cancer Prev* 2013;14(11):6321–6.
- [108] Cheung KL, Kim C, Zhou MM. The functions of BET proteins in gene transcription of biology and diseases. *Front Mol Biosci* 2021;8:728777.
- [109] Liao YF, Wu YB, Long X, et al. High level of BRD4 promotes non-small cell lung cancer progression. *Oncotarget* 2016;7(8):9491–500.
- [110] Drumond-Bock AL, Bieniasz M. The role of distinct BRD4 isoforms and their contribution to high-grade serous ovarian carcinoma pathogenesis. *Mol Cancer* 2021;20(1):145.
- [111] Zhou X, Sun T, Meng Y, et al. BET inhibitors combined with chemotherapy synergistically inhibit the growth of NSCLC cells. *Oncol Rep* 2021;45(5).
- [112] Andrikopoulou A, Liotatos M, Koutsoukos K, Dimopoulos MA, Zagouri F. Clinical perspectives of BET inhibition in ovarian cancer. *Cell Oncol (Dordr)* 2021;44(2):237–49.
- [113] Grabosch S, Bulatovic M, Zeng F, et al. Cisplatin-induced immune modulation in ovarian cancer mouse models with distinct inflammation profiles. *Oncogene* 2019;38(13):2380–93.
- [114] Mesnage SJL, Auguste A, Genestie C, et al. Neoadjuvant chemotherapy (NACT) increases immune infiltration and programmed death-ligand 1 (PD-L1) expression in epithelial ovarian cancer (EOC). *Ann Oncol* 2017;28(3):651–7.
- [115] Wang J, Xu Y, Rao X, et al. BRD4-IRF1 axis regulates chemoradiotherapy-induced PD-L1 expression and immune evasion in non-small cell lung cancer. *Clin Transl Med* 2022;12(1):e718.
- [116] Foulkes WD, Shuen AY. In brief: BRCA1 and BRCA2. *J Pathol* 2013;230(4):347–9.
- [117] Price M, Monteiro AN. Fine tuning chemotherapy to match BRCA1 status. *Biochem Pharmacol* 2010;80(5):647–53.
- [118] Lee MN, Tseng RC, Hsu HS, et al. Epigenetic inactivation of the chromosomal stability control genes BRCA1, BRCA2, and XRCC5 in non-small cell lung cancer. *Clin Cancer Res* 2007;13(3):832–8.
- [119] Rosell R, Skrzypski M, Jassem E, et al. BRCA1: a novel prognostic factor in resected non-small-cell lung cancer. *PLoS One* 2007;2(11):e1129.
- [120] Wang Y, Zhang D, Zheng W, Luo J, Bai Y, Lu Z. Multiple gene methylation of nonsmall cell lung cancers evaluated with 3-dimensional microarray. *Cancer* 2008;112(6):1325–36.
- [121] Catteau A, Harris WH, Xu CF, Solomon E. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. *Oncogene* 1999;18(11):1957–65.
- [122] Yarden RI, Brody LC. BRCA1 interacts with components of the histone deacetylase complex. *Proc Natl Acad Sci U S A* 1999;96(9):4983–8.
- [123] Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer* 2012;12(1):68–78.

- [124] Li X, Liu L, Yang S, et al. Histone demethylase KDM5B is a key regulator of genome stability. *Proc Natl Acad Sci U S A* 2014;111(19):7096–101.
- [125] Lu J, Matunis MJ. A mediator methylation mystery: JMJD1C demethylates MDC1 to regulate DNA repair. *Nat Struct Mol Biol* 2013;20(12):1346–8.
- [126] Watanabe S, Watanabe K, Akimov V, et al. JMJD1C demethylates MDC1 to regulate the RNF8 and BRCA1-mediated chromatin response to DNA breaks. *Nat Struct Mol Biol* 2013;20(12):1425–33.
- [127] Baer R, Ludwig T. The BRCA1/BARD1 heterodimer, a tumor suppressor complex with ubiquitin E3 ligase activity. *Curr Opin Genet Dev* 2002;12(1):86–91.
- [128] Thakar A, Parvin J, Zlatanova J. BRCA1/BARD1 E3 ubiquitin ligase can modify histones H2A and H2B in the nucleosome particle. *J Biomol Struct Dyn* 2010;27(4):399–406.
- [129] Zhao Y, Brickner JR, Majid MC, Mosammaparast N. Crosstalk between ubiquitin and other post-translational modifications on chromatin during double-strand break repair. *Trends Cell Biol* 2014;24(7):426–34.
- [130] Ratanaphan A, Wasiksiri S, Canyuk B, Prasertsan P. Cisplatin-damaged BRCA1 exhibits altered thermostability and transcriptional transactivation. *Cancer Biol Ther* 2009;8(10):890–8.
- [131] Atipairin A, Canyuk B, Ratanaphan A. The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by the platinum-based anticancer drugs. *Breast Cancer Res Treat* 2010;.
- [132] Sun JM, Chen HY, Davie JR. Differential distribution of unmodified and phosphorylated histone deacetylase 2 in chromatin. *J Biol Chem* 2007;282(45):33227–36.
- [133] Larsen DH, Poinsignon C, Gudjonsson T, et al. The chromatin-remodeling factor CHD4 coordinates signaling and repair after DNA damage. *J Cell Biol* 2010;190(5):731–40.
- [134] Luijsterburg MS, Acs K, Ackermann L, et al. A new non-catalytic role for ubiquitin ligase RNF8 in unfolding higher-order chromatin structure. *EMBO J* 2012;31(11):2511–27.
- [135] Smeenk G, Wiegant WW, Vrolijk H, Solari AP, Pastink A, van Attikum H. The NuRD chromatin-remodeling complex regulates signaling and repair of DNA damage. *J Cell Biol* 2010;190(5):741–9.
- [136] Tang J, Cho NW, Cui G, et al. Acetylation limits 53BP1 association with damaged chromatin to promote homologous recombination. *Nat Struct Mol Biol* 2013;20(3):317–25.
- [137] Dominici C, Sgarioto N, Yu Z, et al. Synergistic effects of type I PRMT and PARP inhibitors against non-small cell lung cancer cells. *Clin Epigenetics* 2021;13(1):54.
- [138] Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J Biol Chem* 2000;275(31):23899–903.
- [139] Tassone P, Tagliaferri P, Perricelli A, et al. BRCA1 expression modulates chemosensitivity of BRCA1-defective HCC1937 human breast cancer cells. *Br J Cancer* 2003;88(8):1285–91.
- [140] Tassone P, Di Martino MT, Ventura M, et al. Loss of BRCA1 function increases the antitumor activity of cisplatin against human breast cancer xenografts *in vivo*. *Cancer Biol Ther* 2009;8(7):648–53.
- [141] Swisher EM, Gonzalez RM, Taniguchi T, et al. Methylation and protein expression of DNA repair genes: association with chemotherapy exposure and survival in sporadic ovarian and peritoneal carcinomas. *Mol Cancer* 2009;8:48.
- [142] Stefansson OA, Villanueva A, Vidal A, Martí L, Esteller M. BRCA1 epigenetic inactivation predicts sensitivity to platinum-based chemotherapy in breast and ovarian cancer. *Epigenetics* 2012;7(11):1225–9.
- [143] Taron M, Rosell R, Felip E, et al. BRCA1 mRNA expression levels as an indicator of chemoresistance in lung cancer. *Hum Mol Genet* 2004;13(20):2443–9.
- [144] Papadaki C, Sfakianaki M, Ioannidis G, et al. ERCC1 and BRCA1 mRNA expression levels in the primary tumor could predict the effectiveness of the second-line cisplatin-based chemotherapy in pre-treated patients with metastatic non-small cell lung cancer. *J Thorac Oncol* 2012;7(4):663–71.

- [145] Wang L, Wei J, Qian X, et al. ERCC1 and BRCA1 mRNA expression levels in metastatic malignant effusions is associated with chemosensitivity to cisplatin and/or docetaxel. *BMC Cancer* 2008;8:97.
- [146] Rosell R, Perez-Roca L, Sanchez JJ, et al. Customized treatment in non-small-cell lung cancer based on EGFR mutations and BRCA1 mRNA expression. *PLoS One* 2009;4(5):e5133.
- [147] Bonanno L, Costa C, Majem M, et al. The predictive value of 53BP1 and BRCA1 mRNA expression in advanced non-small-cell lung cancer patients treated with first-line platinum-based chemotherapy. *Oncotarget* 2013;4(10):1572–81.
- [148] Li Z, Qing Y, Guan W, et al. Predictive value of APE1, BRCA1, ERCC1 and TUBB3 expression in patients with advanced non-small cell lung cancer (NSCLC) receiving first-line platinum-paclitaxel chemotherapy. *Cancer Chemother Pharmacol* 2014;.
- [149] Qin X, Yao W, Li W, et al. ERCC1 and BRCA1 mRNA expressions are associated with clinical outcome of non-small cell lung cancer treated with platinum-based chemotherapy. *Tumour Biol* 2014;35(5):4697–704.
- [150] Wang TB, Zhang NL, Wang SH, Li HY, Chen SW, Zheng YG. Expression of ERCC1 and BRCA1 predict the clinical outcome of non-small cell lung cancer in patients receiving platinum-based chemotherapy. *Genet Mol Res* 2014;13(2):3704–10.
- [151] Xian-Jun F, Xiu-Guang Q, Li Z, et al. ERCC1 and BRCA1 mRNA expression predicts the clinical outcome of non-small cell lung cancer receiving platinum-based chemotherapy. *Pak J Med Sci* 2014;30(3):488–92.
- [152] Su C, Zhou S, Zhang L, et al. ERCC1, RRM1 and BRCA1 mRNA expression levels and clinical outcome of advanced non-small cell lung cancer. *Med Oncol* 2011;28(4):1411–17.
- [153] Moran T, Wei J, Cobo M, et al. Two biomarker-directed randomized trials in European and Chinese patients with nonsmall-cell lung cancer: the BRCA1-RAP80 Expression Customization (BREC) studies. *Ann Oncol* 2014;25(11):2147–55.
- [154] Wei J, Qian XP, Zou ZY, et al. Chinese multicenter randomized trial of customized chemotherapy based on BRCA1 (breast cancer susceptibility gene 1)-RAP80 (receptor-associated protein 80) mRNA expression in advanced non-small cell lung cancer (NSCLC) patients. *Zhonghua Zhong Liu Za Zhi* 2016;38(11):868–73.
- [155] Puppe J, Drost R, Liu X, et al. BRCA1-deficient mammary tumor cells are dependent on EZH2 expression and sensitive to Polycomb Repressive Complex 2-inhibitor 3-deazaneplanocin A. *Breast Cancer Res* 2009;11(4):R63.
- [156] Wei Y, Xia W, Zhang Z, et al. Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. *Mol Carcinog* 2008;47(9):701–6.
- [157] Chen X, Song N, Matsumoto K, et al. High expression of trimethylated histone H3 at lysine 27 predicts better prognosis in non-small cell lung cancer. *Int J Oncol* 2013;43(5):1467–80.
- [158] Kikuchi J, Kinoshita I, Shimizu Y, et al. Distinctive expression of the polycomb group proteins Bmi1 polycomb ring finger oncogene and enhancer of zeste homolog 2 in nonsmall cell lung cancers and their clinical and clinicopathologic significance. *Cancer* 2010;116(12):3015–24.
- [159] Loren P, Saavedra N, Saavedra K, et al. Contribution of microRNAs in chemoresistance to cisplatin in the top five deadliest cancer: an updated review. *Front Pharmacol* 2022;13:831099.
- [160] Ashrafizadeh M, Zarrabi A, Hushmandi K, et al. Lung cancer cells and their sensitivity/resistance to cisplatin chemotherapy: role of microRNAs and upstream mediators. *Cell Signal* 2021;78:109871.
- [161] Drayton RM. The role of microRNA in the response to cisplatin treatment. *Biochem Soc Trans* 2012;40(4):821–5.
- [162] Cai J, Yang C, Yang Q, et al. Dereulation of let-7e in epithelial ovarian cancer promotes the development of resistance to cisplatin. *Oncogenesis* 2013;2:e75.

- [163] Xiao M, Cai J, Cai L, et al. Let-7e sensitizes epithelial ovarian cancer to cisplatin through repressing DNA double strand break repair. *J Ovarian Res* 2017;10(1):24.
- [164] Zhu WY, Luo B, An JY, et al. Differential expression of miR-125a-5p and let-7e predicts the progression and prognosis of non-small cell lung cancer. *Cancer Invest* 2014;.
- [165] Wang F, Quan Q. The long non-coding RNA SNHG4/microRNA-let-7e/KDM3A/p21 pathway is involved in the development of non-small cell lung cancer. *Mol Ther Oncolytics* 2021;20:634–45.
- [166] Lu M, Hu C, Wu F, et al. MiR-320a is associated with cisplatin resistance in lung adenocarcinoma and its clinical value in non-small cell lung cancer: a comprehensive analysis based on microarray data. *Lung Cancer* 2020;147:193–7.
- [167] Han X, Liu D, Zhou Y, et al. The negative feedback between miR-143 and DNMT3A regulates cisplatin resistance in ovarian cancer. *Cell Biol Int* 2021;45(1):227–37.
- [168] Varambally S, Cao Q, Mani RS, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science* 2008;322(5908):1695–9.
- [169] Semaan A, Qazi AM, Seward S, et al. MicroRNA-101 inhibits growth of epithelial ovarian cancer by relieving chromatin-mediated transcriptional repression of p21(waf1/cip1). *Pharm Res* 2011;28(12):3079–90.
- [170] Yang Y, Li X, Yang Q, et al. The role of microRNA in human lung squamous cell carcinoma. *Cancer Genet Cytogenet* 2010;200(2):127–33.
- [171] Xiong X, Lai X, Zhang J, et al. FBP1 knockdown decreases ovarian cancer formation and cisplatin resistance through EZH2-mediated H3K27me3. *Biosci Rep* 2022;.
- [172] Sun J, Cai X, Yung MM, et al. miR-137 mediates the functional link between c-Myc and EZH2 that regulates cisplatin resistance in ovarian cancer. *Oncogene* 2019;38(4):564–80.
- [173] Karreth FA, Pandolfi PP. ceRNA cross-talk in cancer: when ce-bling rivalries go awry. *Cancer Discov* 2013;3(10):1113–21.
- [174] Thomson DW, Dinger ME. Endogenous microRNA sponges: evidence and controversy. *Nat Rev Genet* 2016;17(5):272–83.
- [175] Ding Z, Zu S, Gu J. Evaluating the molecule-based prediction of clinical drug responses in cancer. *Bioinformatics* 2016;32(19):2891–5.
- [176] Xu X, Yu S, Sun W, et al. MiRNA signature predicts the response of patients with advanced lung adenocarcinoma to platinum-based treatment. *J Cancer Res Clin Oncol* 2018;144(3):431–8.
- [177] MacDonagh L, Gallagher MF, Ffrench B, et al. MicroRNA expression profiling and biomarker validation in treatment-naïve and drug resistant non-small cell lung cancer. *Transl Lung Cancer Res* 2021;10(4):1773–91.
- [178] Fekete JT, Welker Á, Győrffy B. miRNA expression signatures of therapy response in squamous cell carcinomas. *Cancers (Basel)* 2020;13(1).
- [179] Berghmans T, Ameye L, Lafitte JJ, et al. Prospective validation obtained in a similar group of patients and with similar high throughput biological tests failed to confirm signatures for prediction of response to chemotherapy and survival in advanced NSCLC: a prospective study from the european lung cancer working party. *Front Oncol* 2014;4:386.
- [180] Mortazavi D, Sohrabi B, Mosallaei M, et al. Epi-miRNAs: regulators of the histone modification machinery in human cancer. *J Oncol* 2022;2022:4889807.
- [181] Papadimitriou MA, Panoutsopoulou K, Pilala KM, Scorilas A, Avgeris M. Epi-miRNAs: modern mediators of methylation status in human cancers. *Wiley Interdiscip Rev RNA* 2022;e1735.
- [182] Karimzadeh MR, Pourdavoud P, Ehtesham N, et al. Regulation of DNA methylation machinery by epi-miRNAs in human cancer: emerging new targets in cancer therapy. *Cancer Gene Ther* 2021;28(3–4):157–74.

- [183] Ratovitski EA. Phospho-DeltaNp63alpha/microRNA network modulates epigenetic regulatory enzymes in squamous cell carcinomas. *Cell Cycle* 2014;13(5):749–61.
- [184] Huang Y, Kesselman D, Kizub D, Guerrero-Preston R, Ratovitski EA. Phospho-DeltaNp63alpha/microRNA feedback regulation in squamous carcinoma cells upon cisplatin exposure. *Cell Cycle* 2013;12(4):684–97.
- [185] Wanowska E, Samorowska K, Szcześniak MW. Emerging roles of long noncoding RNAs in breast cancer epigenetics and epitranscriptomics. *Front Cell Dev Biol* 2022;10:922351.
- [186] Hu Q, Ma H, Chen H, Zhang Z, Xue Q. LncRNA in tumorigenesis of non-small-cell lung cancer: From bench to bedside. *Cell Death Discov* 2022;8(1):359.
- [187] Song H, Liu D, Dong S, et al. Epitranscriptomics and epiproteomics in cancer drug resistance: therapeutic implications. *Signal Transduct Target Ther* 2020;5(1):193.
- [188] Bik E. The Tadpole Paper Mill. <https://scienceintegritydigestcom/2020/02/21/the-tadpole-paper-mill/>; 2020.
- [189] Christopher J. The raw truth about paper mills. *FEBS Lett* 2021;595(13):1751–7.
- [190] Else H, Van Noorden R. The fight against fake-paper factories that churn out sham science. *Nature* 2021;591(7851):516–19.
- [191] Schneider L. Editors and Other Real Papermill Heroes. <https://forbettersciencecom/2021/11/10/editors-and-other-real-papermill-heroes/>; 2021.
- [192] O'Flaherty JD, Barr M, Fennell D, et al. The cancer stem-cell hypothesis: its emerging role in lung cancer biology and its relevance for future therapy. *J Thorac Oncol* 2012;7(12):1880–90.
- [193] Barr MP, Gray SG, Hoffmann AC, et al. Generation and characterisation of cisplatin-resistant non-small cell lung cancer cell lines displaying a stem-like signature. *PLoS One* 2013;8(1):e54193.
- [194] Wang L, Liu X, Ren Y, et al. Cisplatin-enriching cancer stem cells confer multidrug resistance in non-small cell lung cancer via enhancing TRIB1/HDAC activity. *Cell Death Dis* 2017;8(4):e2746.
- [195] MacDonagh L, Gray SG, Breen E, et al. BBI608 inhibits cancer stemness and reverses cisplatin resistance in NSCLC. *Cancer Lett* 2018;428:117–26.
- [196] MacDonagh L, Gallagher MF, Ffrench B, et al. Targeting the cancer stem cell marker, aldehyde dehydrogenase 1, to circumvent cisplatin resistance in NSCLC. *Oncotarget* 2017;8(42):72544–63.
- [197] Flavahan WA, Gaskell E, Bernstein BE. Epigenetic plasticity and the hallmarks of cancer. *Science* 2017;357(6348).
- [198] Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010;141(1):69–80.
- [199] Fazzio TG, Huff JT, Panning B. An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. *Cell* 2008;134(1):162–74.
- [200] Chen PB, Hung JH, Hickman TL, et al. Hdac6 regulates Tip60-p400 function in stem cells. *Elife* 2013;2:e01557.
- [201] Tavares MO, Milan TM, Bighetti-Trevisan RL, Leopoldino AM, de Almeida LO. Pharmacological inhibition of HDAC6 overcomes cisplatin chemoresistance by targeting cancer stem cells in oral squamous cell carcinoma. *J Oral Pathol Med* 2022;51(6):529–37.
- [202] Chiang K, Zielinska AE, Shaaban AM, et al. PRMT5 is a critical regulator of breast cancer stem cell function via histone methylation and FOXP1 expression. *Cell Rep* 2017;21(12):3498–513.
- [203] Primac I, Penning A, Fuks F. Cancer epitranscriptomics in a nutshell. *Curr Opin Genet Dev* 2022;75:101924.
- [204] Berdasco M, Esteller M. Towards a druggable epitranscriptome: compounds that target RNA modifications in cancer. *Br J Pharmacol* 2022;179(12):2868–89.
- [205] Huang H, Weng H, Chen J. m(6)A modification in coding and non-coding RNAs: roles and therapeutic implications in cancer. *Cancer Cell* 2020;37(3):270–88.

- [206] Lobo J, Costa AL, Cantante M, et al. m(6)A RNA modification and its writer/reader VIRMA/YTHDF3 in testicular germ cell tumors: a role in seminoma phenotype maintenance. *J Transl Med* 2019;17(1):79.
- [207] Miranda-Gonçalves V, Lobo J, Guimarães-Teixeira C, et al. The component of the m(6)A writer complex VIRMA is implicated in aggressive tumor phenotype, DNA damage response and cisplatin resistance in germ cell tumors. *J Exp Clin Cancer Res* 2021;40(1):268.
- [208] Shi L, Gong Y, Zhuo L, Wang S, Chen S, Ke B. Methyltransferase-like 3 upregulation is involved in the chemoresistance of non-small cell lung cancer. *Ann Transl Med* 2022;10(3):139.
- [209] Kiweler N, Wünsch D, Wirth M, et al. Histone deacetylase inhibitors dysregulate DNA repair proteins and antagonize metastasis-associated processes. *J Cancer Res Clin Oncol* 2020;146(2):343–56.
- [210] He Y, Chen D, Yi Y, et al. Histone deacetylase inhibitor sensitizes ERCC1-high non-small-cell lung cancer cells to cisplatin via regulating miR-149. *Mol Ther Oncolytics* 2020;17:448–59.
- [211] Al-Keilani MS, Alzoubi KH, Jaradat SA. The effect of combined treatment with sodium phenylbutyrate and cisplatin, erlotinib, or gefitinib on resistant NSCLC cells. *Clin Pharmacol* 2018;10:135–40.
- [212] Huang WJ, Tang YA, Chen MY, et al. A histone deacetylase inhibitor YCW1 with antitumor and anti-metastasis properties enhances cisplatin activity against non-small cell lung cancer in preclinical studies. *Cancer Lett* 2014;346(1):84–93.
- [213] Gueugnon F, Cartron PF, Charrier C, et al. New histone deacetylase inhibitors improve cisplatin antitumor properties against thoracic cancer cells. *Oncotarget* 2014;5(12):4504–15.
- [214] To KK, Tong WS, Fu LW. Reversal of platinum drug resistance by the histone deacetylase inhibitor belinostat. *Lung Cancer* 2017;103:58–65.
- [215] Cacan E, Ali MW, Boyd NH, Hooks SB, Greer SF. Inhibition of HDAC1 and DNMT1 modulate RGS10 expression and decrease ovarian cancer chemoresistance. *PLoS One* 2014;9(1):e87455.
- [216] Meng F, Sun G, Zhong M, Yu Y, Brewer MA. Anticancer efficacy of cisplatin and trichostatin A or 5-aza-2'-deoxycytidine on ovarian cancer. *Br J Cancer* 2013;108(3):579–86.
- [217] Dallavalle S, Musso L, Cincinelli R, et al. Antitumor activity of novel POLA1-HDAC11 dual inhibitors. *Eur J Med Chem* 2022;228:113971.
- [218] Pogribny IP, Tryndyak VP, Pogribna M, et al. Modulation of intracellular iron metabolism by iron chelation affects chromatin remodeling proteins and corresponding epigenetic modifications in breast cancer cells and increases their sensitivity to chemotherapeutic agents. *Int J Oncol* 2013;42(5):1822–32.
- [219] Liu WJ, Pan PY, Sun Y, et al. Deferoxamine counteracts cisplatin resistance in A549 lung adenocarcinoma cells by increasing vulnerability to glutamine deprivation-induced cell death. *Front Oncol* 2021;11:794735.
- [220] Wang L, Li X, Mu Y, et al. The iron chelator desferrioxamine synergizes with chemotherapy for cancer treatment. *J Trace Elem Med Biol* 2019;56:131–8.
- [221] Ekstrom TL, Pathoulas NM, Huehls AM, Kanakkanthara A, Karnitz LM. VLX600 disrupts homologous recombination and synergizes with PARP inhibitors and cisplatin by inhibiting histone lysine demethylases. *Mol Cancer Ther* 2021;20(9):1561–71.
- [222] Duan L, Perez RE, Calhoun S, Maki CG. Inhibitors of Jumonji C domain-containing histone lysine demethylases overcome cisplatin and paclitaxel resistance in non-small cell lung cancer through APC/Cdh1-dependent degradation of CtIP and PAF15. *Cancer Biol Ther* 2022;23(1):65–75.
- [223] Perabo F, Chandhasin C, Yoo S, et al. Abstract 3720: TACH101, a first-in-class inhibitor of KDM4 histone lysine demethylase for the treatment of diffuse large B-cell lymphoma. *Cancer Res* 2022;82(12_Supplement):3720.
- [224] Lv YX, Tian S, Zhang ZD, Feng T, Li HQ. LSD1 inhibitors for anticancer therapy: a patent review (2017-present). *Expert Opin Ther Pat* 2022;

- [225] Sacilotto N, Dessanti P, Lufino MMP, et al. Comprehensive in vitro characterization of the LSD1 small molecule inhibitor class in oncology. *ACS Pharmacol Transl Sci* 2021;4(6):1818–34.
- [226] Zeng J, Zhang J, Sun Y, et al. Targeting EZH2 for cancer therapy: from current progress to novel strategies. *Eur J Med Chem* 2022;238:114419.
- [227] Reid BM, Vyas S, Chen Z, et al. Morphologic and molecular correlates of EZH2 as a predictor of platinum resistance in high-grade ovarian serous carcinoma. *BMC Cancer* 2021;21(1):714.
- [228] Sun S, Yang Q, Cai E, et al. EZH2/H3K27Me3 and phosphorylated EZH2 predict chemotherapy response and prognosis in ovarian cancer. *PeerJ* 2020;8:e9052.
- [229] Naskou J, Beiter Y, van Rensburg R, et al. EZH2 loss drives resistance to carboplatin and paclitaxel in serous ovarian cancers expressing ATM. *Mol Cancer Res* 2020;18(2):278–86.
- [230] Schwalm MP, Knapp S. BET bromodomain inhibitors. *Curr Opin Chem Biol* 2022;68:102148.
- [231] Cochran AG, Conery AR, Sims 3rd RJ. Bromodomains: a new target class for drug development. *Nat Rev Drug Discov* 2019;18(8):609–28.
- [232] Sarnik J, Popławski T, Tokarz P. BET proteins as attractive targets for cancer therapeutics. *Int J Mol Sci* 2021;22(20).
- [233] Takashima Y, Kikuchi E, Kikuchi J, et al. Bromodomain and extraterminal domain inhibition synergizes with WEE1-inhibitor AZD1775 effect by impairing nonhomologous end joining and enhancing DNA damage in nonsmall cell lung cancer. *Int J Cancer* 2020;146(4):1114–24.
- [234] Momeny M, Eyyvani H, Barghi F, et al. Inhibition of bromodomain and extraterminal domain reduces growth and invasive characteristics of chemoresistant ovarian carcinoma cells. *Anticancer Drugs* 2018;29(10):1011–20.
- [235] Bagratuni T, Mavrianou N, Gavalas NG, et al. JQ1 inhibits tumour growth in combination with cisplatin and suppresses JAK/STAT signalling pathway in ovarian cancer. *Eur J Cancer* 2020;126:125–35.
- [236] Qi L, Lindsay H, Kogiso M, et al. Evaluation of an EZH2 inhibitor in patient-derived orthotopic xenograft models of pediatric brain tumors alone and in combination with chemo- and radiation therapies. *Lab Invest* 2022;102(2):185–93.
- [237] Chen Y, Shao X, Zhao X, et al. Targeting protein arginine methyltransferase 5 in cancers: roles, inhibitors and mechanisms. *Biomed Pharmacother* 2021;144:112252.
- [238] Yuan Y, Nie H. Protein arginine methyltransferase 5: a potential cancer therapeutic target. *Cell Oncol (Dordr)* 2021;44(1):33–44.
- [239] Yang Z, Xiao T, Li Z, Zhang J, Chen S. Novel chemicals derived from tadalafil exhibit PRMT5 inhibition and promising activities against breast cancer. *Int J Mol Sci* 2022;23(9).
- [240] Wu Y, Wang Z, Han L, et al. PRMT5 regulates RNA m6A demethylation for doxorubicin sensitivity in breast cancer. *Mol Ther* 2022;30(7):2603–17.
- [241] Gong TT, Liu XD, Zhan ZP, Wu QJ. Sulforaphane enhances the cisplatin sensitivity through regulating DNA repair and accumulation of intracellular cisplatin in ovarian cancer cells. *Exp Cell Res* 2020;393(2):112061.
- [242] Kurek M, Benaida-Debbache N, Elez Garofulić I, et al. Antioxidants and bioactive compounds in food: critical review of issues and prospects. *Antioxid (Basel)* 2022;11(4).
- [243] Schwartzmann G, Schunemann H, Gorini CN, et al. A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer. *Invest N Drugs* 2000;18(1):83–91.
- [244] Albany C, Fazal Z, Singh R, et al. A phase 1 study of combined guadecitabine and cisplatin in platinum refractory germ cell cancer. *Cancer Med* 2021;10(1):156–63.
- [245] Matei D, Ghamande S, Roman L, et al. A phase I clinical trial of guadecitabine and carboplatin in platinum-resistant, recurrent ovarian cancer: clinical, pharmacokinetic, and pharmacodynamic analyses. *Clin Cancer Res* 2018;24(10):2285–93.

- [246] Balasubramaniam S, Redon CE, Peer CJ, et al. Phase I trial of belinostat with cisplatin and etoposide in advanced solid tumors, with a focus on neuroendocrine and small cell cancers of the lung. *Anticancer Drugs* 2018;29(5):457–65.
- [247] Meng Y, Jin J, Gong C, et al. Phase II study of chidamide in combination with cisplatin in patients with metastatic triple-negative breast cancer. *Ann Palliat Med* 2021;10(11):11255–64.
- [248] Cornacchia E, Golbus J, Maybaum J, Strahler J, Hanash S, Richardson B. Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. *J Immunol* 1988;140(7):2197–200.
- [249] Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem* 2001;276(39):36734–41.
- [250] Candelaria M, Gallardo-Rincon D, Arce C, et al. A phase II study of epigenetic therapy with hydralazine and magnesium valproate to overcome chemotherapy resistance in refractory solid tumors. *Ann Oncol* 2007;18(9):1529–38.
- [251] Falchook GS, Fu S, Naing A, et al. Methylation and histone deacetylase inhibition in combination with platinum treatment in patients with advanced malignancies. *Invest N Drugs* 2013;31(5):1192–200.
- [252] Hollebecque A, Salvagni S, Plummer R, et al. Clinical activity of CC-90011, an oral, potent, and reversible LSD1 inhibitor, in advanced malignancies. *Cancer* 2022;.
- [253] Moreno V, Sepulveda JM, Vieito M, et al. Phase I study of CC-90010, a reversible, oral BET inhibitor in patients with advanced solid tumors and relapsed/refractory non-Hodgkin's lymphoma. *Ann Oncol* 2020;31(6):780–8.
- [254] Takagi S, Ishikawa Y, Mizutani A, et al. LSD1 inhibitor T-3775440 inhibits SCLC cell proliferation by disrupting LSD1 interactions with SNAG domain proteins INSM1 and GFI1B. *Cancer Res* 2017;77(17):4652–62.
- [255] Augert A, Eastwood E, Ibrahim AH, et al. Targeting NOTCH activation in small cell lung cancer through LSD1 inhibition. *Sci Signal* 2019;12(567).
- [256] Navarro Mendivil AF, Gutierrez S, Maes T, Bullock R, Ropacki M, Buesa C. 1751TiP—CLEPSIDRA trial: a pilot, biomarker-guided study to assess safety, tolerability, dose finding and efficacy of iadademstat in combination with platinum-etoposide in patients with relapsed, extensive-stage small cell lung cancer. *Ann Oncol* 2019;30:v717.
- [257] Faller D., Buesa C., ORYZON GENOMICS S.A. ORYZON Announces FDA Orphan Drug Designation Granted to Iademstat for Treatment of Small Cell Lung Cancer. [https://wwworyzoncom/en/news-events/news/oryzon-announces-fda-orphan-drug-designation-granted-iademstat-treatment-small/](https://wwworyzoncom/en/news-events/news/oryzon-announces-fda-orphan-drug-designation-granted-iademstat-treatment-small;); 2022.
- [258] Bauer TM, Besse B, Martinez-Martí A, et al. Phase I, open-label, dose-escalation study of the safety, pharmacokinetics, pharmacodynamics, and efficacy of GSK2879552 in relapsed/refractory SCLC. *J Thorac Oncol* 2019;14(10):1828–38.
- [259] Yan W, Chung CY, Xie T, et al. Intrinsic and acquired drug resistance to LSD1 inhibitors in small cell lung cancer occurs through a TEAD4-driven transcriptional state. *Mol Oncol* 2022;16(6):1309–28.
- [260] Salamero O, Montesinos P, Willekens C, et al. First-in-human phase I study of iademstat (ORY-1001): a first-in-class lysine-specific histone demethylase 1A inhibitor, in relapsed or refractory acute myeloid leukemia. *J Clin Oncol* 2020;38(36):4260–73.
- [261] Roboz GJ, Yee K, Verma A, et al. Phase I trials of the lysine-specific demethylase 1 inhibitor, GSK2879552, as mono- and combination-therapy in relapsed/refractory acute myeloid leukemia or high-risk myelodysplastic syndromes. *Leuk Lymphoma* 2022;63(2):463–7.
- [262] Wass M, Göllner S, Besenbeck B, et al. A proof of concept phase I/II pilot trial of LSD1 inhibition by tranylcypromine combined with ATRA in refractory/relapsed AML patients not eligible for intensive therapy. *Leukemia* 2021;35(3):701–11.

- [263] Rothbart SB, Baylin SB. Epigenetic therapy for epithelioid sarcoma. *Cell* 2020;181(2):211.
- [264] Straining R, Eighmy W. Tazemetostat: EZH2 inhibitor. *J Adv Pract Oncol* 2022;13(2):158–63.
- [265] Ameratunga M, Braña I, Bono P, et al. First-in-human Phase 1 open label study of the BET inhibitor ODM-207 in patients with selected solid tumours. *Br J Cancer* 2020;123(12):1730–6.
- [266] Aggarwal RR, Schweizer MT, Nanus DM, et al. A phase Ib/Ia study of the Pan-BET inhibitor ZEN-3694 in combination with enzalutamide in patients with metastatic castration-resistant prostate cancer. *Clin Cancer Res* 2020;26(20):5338–47.
- [267] Sun Y, Zhang Z, Zhang K, et al. Epigenetic heterogeneity promotes acquired resistance to BET bromodomain inhibition in ovarian cancer. *Am J Cancer Res* 2021;11(6):3021–38.
- [268] Sun Y, Jin L, Sui YX, Han LL, Liu JH. Circadian gene CLOCK affects drug-resistant gene expression and cell proliferation in ovarian cancer SKOV3/DDP cell lines through autophagy. *Cancer Biother Radiopharm* 2017;32(4):139–46.
- [269] Yang Y, Lindsey-Boltz LA, Vaughn CM, et al. Circadian clock, carcinogenesis, chronochemotherapy connections. *J Biol Chem* 2021;297(3):101068.
- [270] Provencio M, Majem M, Guirado M, et al. Phase II clinical trial with metronomic oral vinorelbine and tri-weekly cisplatin as induction therapy, subsequently concomitant with radiotherapy (RT) in patients with locally advanced, unresectable, non-small cell lung cancer (NSCLC). Analysis of survival and value of ctDNA for patient selection. *Lung Cancer* 2021;153:25–34.
- [271] Juo YY, Gong XJ, Mishra A, et al. Epigenetic therapy for solid tumors: from bench science to clinical trials. *Epigenomics* 2015;7(2):215–35.
- [272] Chiappinelli KB, Baylin SB. Inhibiting DNA methylation improves antitumor immunity in ovarian cancer. *J Clin Invest* 2022;132(14).
- [273] Yeh SH, Lin MH, Leo Garcia II F, et al. In vivo evaluation of the combined anticancer effects of cisplatin and SAHA in nonsmall cell lung carcinoma using [(18)F]FAHA and [(18)F]FDG PET/CT imaging. *Mol Imaging* 2021;2021:6660358.
- [274] Vaidya GN, Rana P, Venkatesh A, et al. Paradigm shift of “classical” HDAC inhibitors to “hybrid” HDAC inhibitors in therapeutic interventions. *Eur J Med Chem* 2021;209:112844.
- [275] Krug LM, Kindler H, Calvert H, et al. VANTAGE 014: vorinostat (V) in patients with advanced malignant pleural mesothelioma (MPM) who have failed prior pemetrexed and either cisplatin or carboplatin therapy: a phase III, randomized, double-blind, placebo-controlled trial. *Eur J Cancer* 2011;47 (Supplement 2):2–3.
- [276] Hellmann MD, Jänne PA, Opyrchal M, et al. Entinostat plus pembrolizumab in patients with metastatic NSCLC previously treated with anti-PD-(L)1 therapy. *Clin Cancer Res* 2021;27(4):1019–28.
- [277] Ganesan A, Arimondo PB, Rots MG, Jeronimo C, Berdasco M. The timeline of epigenetic drug discovery: from reality to dreams. *Clin Epigenetics* 2019;11(1):174.
- [278] Tsai HC, Li H, Van Neste L, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer Cell* 2012;21(3):430–46.
- [279] Clozel T, Yang S, Elstrom RL, et al. Mechanism-based epigenetic chemosensitization therapy of diffuse large B-cell lymphoma. *Cancer Discov* 2013;3(9):1002–19.
- [280] Zhou Y, Pan DS, Shan S, et al. Non-toxic dose chidamide synergistically enhances platinum-induced DNA damage responses and apoptosis in non-small-cell lung cancer cells. *Biomed Pharmacother* 2014;68 (4):483–91.
- [281] Oza AM, Matulonis UA, Alvarez Secord A, et al. A randomized phase II trial of epigenetic priming with guadecitabine and carboplatin in platinum-resistant, recurrent ovarian cancer. *Clin Cancer Res* 2020;26(5):1009–16.

- [282] Rodrigues Moita AJ, Bandolik JJ, Hansen FK, Kurz T, Hamacher A, Kassack MU. Priming with HDAC inhibitors sensitizes ovarian cancer cells to treatment with cisplatin and HSP90 inhibitors. *Int J Mol Sci* 2020;21(21).
- [283] Yin J, Wan CL, Zhang L, et al. A phase II trial of the double epigenetic priming regimen including chidamide and decitabine for relapsed/refractory acute myeloid leukemia. *Front Oncol* 2021;11:726926.
- [284] Chen S, Xie P, Cowan M, et al. Epigenetic priming enhances antitumor immunity in platinum-resistant ovarian cancer. *J Clin Invest* 2022;132(14).
- [285] Papadatos-Pastos D, Yuan W, Pal A, et al. Phase 1, dose-escalation study of guadecitabine (SGI-110) in combination with pembrolizumab in patients with solid tumors. *J Immunother Cancer* 2022;10(6).
- [286] Taylor K, Loo Yau H, Chakravarthy A, et al. An open-label, phase II multicohort study of an oral hypomethylating agent CC-486 and durvalumab in advanced solid tumors. *J Immunother Cancer* 2020;8(2).
- [287] Vendetti FP, Topper M, Huang P, et al. Evaluation of azacitidine and entinostat as sensitization agents to cytotoxic chemotherapy in preclinical models of non-small cell lung cancer. *Oncotarget* 2015;6 (1):56–70.
- [288] Fan XX, Wu Q. Decoding lung cancer at single-cell level. *Front Immunol* 2022;13:883758.

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Emerging epigenetic therapies: protein arginine methyltransferase inhibitors

23

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1 Introduction

Protein arginine methyltransferase (PRMT) proteins are ancient biological molecules found nearly ubiquitously in eukaryotes [1]. It is thus unsurprising that these proteins play essential roles in cellular physiology. This is highlighted by the detrimental phenotypes associated with a loss of any PRMT protein and embryonic lethality observed with knockout of either PRMT1 or PRMT5—the two most widely active family members [2,3]. PRMT proteins serve to create a unique posttranslational modification (PTM): the methylation of arginine residues, specifically at nitrogen atoms

within guanidinium side chains. Whilst other PTMs have hundreds of effectors, arginine methylation is only known to be carried out by PRMT protein family members, of which there are just 9. This restriction could explain why PRMT protein dysfunction evokes such deleterious effects and is known to be associated with multiple diseases, including those of the cardiovascular, respiratory, renal, and nervous systems [4]. Most heavily studied, however, is the part that these proteins play in carcinogenesis. Widely overexpressed in cancer and often correlated with poor prognosis [5], PRMT overactivity has, surprisingly, only recently come to the forefront of research. Despite this, rapid and exciting progress has been made in the past few years, with the first therapeutics now entering into clinical trials.

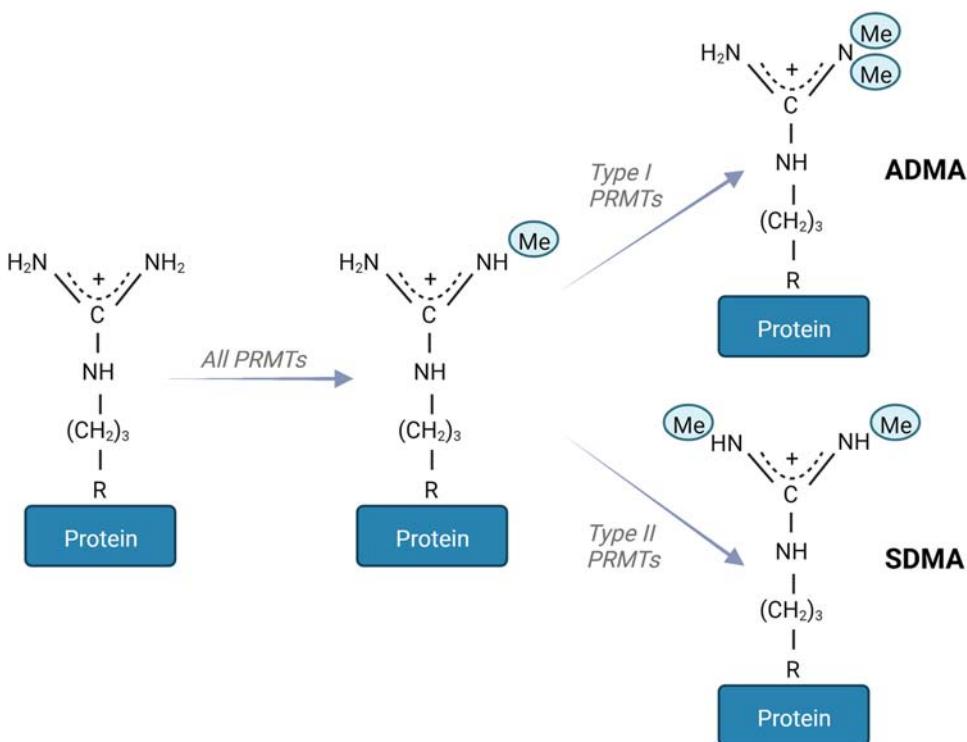
2 PRMT protein summary

There are 9 known PRMT proteins in humans, each of which possesses the ability to transfer a methyl group from *S*-adenosylmethionine (SAM) to an arginine residue. This is achieved through the presence of a conserved catalytic core of 300 amino acids which contains a SAM binding site, dimerization arm, and beta-barrel for substrate binding [5]. While most PRMTs are active as dimers, PRMT7 and PRMT9 are able to function as monomers due to their duplicated methyltransferase domains [5].

PRMT proteins can be classified as either type I, type II, or type III based on the nature of their methyltransferase activity (Figure 23.1). PRMT1 is the major type I enzyme and accounts for around 85% of type I activity in mammals [6], while the major type II enzyme is PRMT5. All PRMT proteins initially monomethylate arginine residues, with class I and II enzymes then being able to proceed to asymmetrical dimethylation and symmetrical dimethylation, respectively. PRMT7 is the only class III enzyme and is classified separately as its activity is limited solely to monomethylation. This occurs due to the narrow active site of PRMT7, which limits its methylation ability [7].

The addition of methyl groups to arginine residues as described above does not alter charge, in contrast to processes, such as lysine methylation but instead serves to enhance hydrophobicity and reduce hydrogen bonding capacity. Consequently, the ability of PRMT substrates to interact with other proteins or nucleic acids is impaired, thus altering their function [3,5,7,8]. Moreover, the addition of a methyl group enables pi stacking interactions with aromatic cages of Tudor domains, enabling “reading” of methyl-arginine by Tudor domain containing proteins and consequent downstream effects [7]. In particular, survival of motor neurone (SMN), splicing factor 30 (SPF30), and Tudor domain containing proteins (TDRDs) 1/2/3/6/9/11 are known to interact with methyl-arginine residues [7].

The vast number of PRMT targets enables every PRMT family member to enact a plethora of cellular roles (Table 23.1), including effects on transcription, RNA processing, translation, the DNA damage response, and signal transduction. This huge range of effects enables PRMT proteins to potently modulate cellular behavior, which appears to be particularly important in cancer cells. Interestingly, each PRMT protein seems to play a unique role within cells, with many PRMT proteins exhibiting specificity in their subcellular location. PRMT3 and PRMT5 reside mainly in the cytoplasm, while PRMT8 is membrane bound and PRMTs 4 and 6 are located mainly within the nucleus [1]. Interestingly, expression of PRMT8 is restricted to the nervous system, suggesting that it plays a unique physiological role compared to other, more widely expressed, family members [9].

**FIGURE 23.1**

An overview of PRMT methylation activity. All PRMT proteins are able generate monomethyl arginine. This is the final product for PRMT7 (type III), which is unable to proceed to dimethylation, but the first reaction step for type I and type II PRMTs. Following monomethylation, type I PRMT proteins (PRMT1–4, PRMT6, and PRMT8) add an additional methyl group to the same nitrogen atom previously targeted, producing asymmetric dimethyl arginine (ADMA). Comparatively, type II PRMT proteins (PRMT5 and PRMT9) add a second methyl group to an alternative nitrogen atom, generating symmetric dimethyl arginine (SDMA).

3 The role of PRMT proteins in cancer

PRMT proteins are widely overexpressed in both hematological and solid cancers, in which their excess is correlated with poor patient prognosis [5]. This pervasive overactivity, even in such a diverse range of cancer types, points to an important function for PRMT proteins in carcinogenesis and this has been heavily researched in recent years. PRMT1 and PRMT5 are at the forefront of such work, although newer evidence indicates that other PRMTs also play key roles. Excitingly, positive preclinical results have been demonstrated in a range of cancers following PRMT inhibition [2,3,5,7]. In this chapter, the focus will be on the epigenetic roles of PRMT proteins, which are many and varied. Perhaps it is their ability to regulate gene expression not just at the level of transcription, but additionally through modulation of splicing and translation, which accounts for the potent effects observed following loss of activity.

TABLE 23.1 A summary of PRMT proteins.

Protein	Class	Histone Targets	Notable nonhistone targets	Primary known epigenetic function(s)	Links to cancer
PRMT1	Type I	H4 (AR3)	Several RBPs, RBM15, FOXO transcription factors, p53, DNA pol beta, RUNX1, BRCA1, c-Myc, GLI1	Transcriptional coactivator, regulator of RNA splicing	Overexpressed in multiple cancers including breast, colorectal, ovarian, and lung cancer Associated with poor prognosis in colorectal cancer Vital for MLL transcription Hormone receptor coactivator
PRMT2	Type I	H3 (R8)	ER-alpha, AR SmB/B', snRNPs, splicing regulators (hnRNPs), SAM68	Transcriptional coactivator, regulator of RNA splicing	Overexpressed in breast cancer, glioblastoma multiforme, and hepatocellular carcinoma Androgen receptor coactivator
PRMT3	Type I		rpS2, GADPH, hnRNPA1 ^a	Regulator of ribosomal homeostasis	Overexpressed in breast cancer Upregulated and associated with poor survival in pancreatic cancer ^a Contributes to chemoresistance in pancreatic cancer ^a
PRMT4	Type I	H3 (R17, R26, and R42)	RBPs, splicing factors (SmB, SAP49, U1-C), CBP/p300, MED12, KMT2Dm RUNX1, NEAT1	Transcriptional coactivator, regulator of RNA splicing	Overexpressed in multiple cancers, including breast, prostate, ovarian, colorectal, leukemia, and melanoma Reduced expression in liver and pancreatic cancers Estrogen linked cell cycle progression in breast cancer
PRMT5	Type II	H2 (AR3), H4 (R3), H3 (R8)	Sm proteins, SRF1, FOXO1, p53, DNA pol beta	Transcriptional repressor (and activator), regulator of RNA splicing and fidelity	Overexpressed in multiple cancers including pancreatic cancer, glioblastoma, lung adenocarcinoma and squamous cell carcinoma Downregulates tumor suppressor genes in fibroblasts

TABLE 23.1 A summary of PRMT proteins. *Continued*

Protein	Class	Histone Targets	Notable nonhistone targets	Primary known epigenetic function(s)	Links to cancer
PRMT6	Type I	H3 (R2), H2 (AR29, AR3), H4 (R3)	FOXO1, p53, DNA pol beta, NF- κ B, HMGA1a, SIRT7	Transcriptional repressor and activator	Key for MYC-mediated splicing regulation Promotes lymphomagenesis via cooperation with cyclin D1 oncogenic mutant Represses p53 targets via p53 methylation Cofactor for androgen receptor and regulates androgen receptor expression—role in prostate cancer Overexpressed in breast, prostate, bladder, lung, colon, gastric, and endometrial cancers Associated with poor prognosis in glioblastoma ^b
PRMT7	Type III	H2 (AR29, R31), H4 (R17, R19)	EIF2S1, G3BP2, HNRNPA1, DVL3	Transcriptional repressor, regulator of RNA splicing and gene imprinting	Role in breast cancer metastasis
PRMT8	Type I	H2A, H4 (<i>in vitro</i>)	EWS, NIFK, Nav, G3BP1	Unknown (thought to be localized to cell membrane)	Mutated in ovarian, skin, and colon cancers Under-expressed in glioblastoma
PRMT9	Type II		SF3B2/SAP145, SF3B4/SAP49	Regulator of RNA splicing	Promotes invasion and metastasis in hepatocellular carcinoma, ^c in which it is associated with poor survival Under-expressed in osteosarcoma ^d

Information compiled from Spannhoff et al. [3], Jarrold et al. [5], Rakow et al. [10], Cura et al. [11], Gupta et al. [12], Halabelian et al. [13], Thiebaut et al. [14], Suresh et al. [15], and Dong et al. [9].

^aParticular reference: [16].

^bParticular reference: [17].

^cParticular reference: [18].

^dParticular reference: [19].

3.1 Transcription

PRMT proteins play a diverse array of roles in transcription, being involved in both gene activation and repression, with some family members able to carry out both of these roles.

PRMT1 and PRMT4 are both known to function mainly as activators of gene transcription, which is largely accounted for by the histone methylation marks they generate (Table 23.1). These are associated with active gene promoters and thought to function through TDRD3 recruitment. This enables topoisomerase TOP3B binding and R loop resolution to allow gene transcription [20]. The PRMT1 mark H4AR3me2a is also known to recruit histone acetyltransferases such as p300 to promote lysine acetylation on histones H3 and H4, with gene activation ensuing [21]. The additional role of PRMT1 in methylating and stabilizing multiple transcription factors also contributes to its function in elevating gene expression [14].

The activating role of PRMT1 is highly associated with carcinogenesis. For example, this function is critical for oncogenic transformation in acute myeloid leukemia (AML), in which PRMT1 is recruited to key leukemia genes (such as *Hoxa9*) by MLL fusion proteins and MOZ-TIF2 to generate the H4R3me2a activating mark and remove the H3K9me3 repressive mark [22]. Furthermore, the generation of H4R3me2a at the ZEB1 promoter is able to enhance epithelial-to-mesenchymal transition (EMT) [23], a key step cancer cells must undergo to allow invasion and metastasis. This mark is also known to recruit SMARCA4, a subunit of the SWI/SNF chromatin remodeling complex, to increase epithelial growth factor receptor (EGFR) expression in colorectal carcinoma and thus enhance proliferation [24]. The ability of PRMT1 to interact with transcription factors also contributes to its role in carcinogenesis. For example, methylation of Twist1 leads to inhibition of E-cadherin expression to evoke epithelial to mesenchymal transition and enhanced metastasis in nonsmall-cell lung cancer [25].

PRMT4 is able to asymmetrically dimethylate H3R17, H3R26, and H3R42 to enhance gene expression (Table 23.1). In breast cancer cell lines, the generation of the H3R17me2a mark by PRMT4 has been shown to upregulate the key cell cycle transcriptional regulator E2F1 to evoke cell growth in response to estrogen stimulation [26]. This same mark at the Notch2 promoter in gastric cancer cells also contributes to carcinogenesis through enhanced cancer progression [27]. In addition, in nonsmall-cell lung cancer PRMT4 generates H3R17me2a and H3R26me2a within the core promoter of the CCNE2 gene, activating its expression to augment proliferation and colony formation [28]. Transcriptional activation by PRMT4 additionally contributes to such properties in colorectal cancer, in which it regulates CUL4A/4B levels [29]. These results highlight PRMT4-mediated gene activation as a key process contributing to cancer cell growth and spread. However, it is important to note that the ability of PRMT4 to methylate nonhistone proteins enables it to play a more nuanced role in transcriptional regulator. For example, PRMT4 is able to act alongside p160 family members to promote gene activation as well as block CRE-CREB binding interactions to evoke gene repression [8]. As such, a complete transcriptional signature of PRMT4 needs to be determined to fully understand its contribution to the cancer cell phenotype.

In contrast to PRMT1 and PRMT4, the family members PRMT5, PRMT6, and PRMT7 serve mainly as transcriptional repressors. These proteins are known to generate inhibitory methyl-arginine marks at gene promoters (Table 23.1). However, both PRMT5 and PRMT6 also able to serve as transcriptional coactivators in a context dependent manner. For example, PRMT6 is able to act as a coactivator for the transcription factor NF- κ B to enhance expression of its target genes [30].

H3R2me2a is generated by PRMT6 and is vital in mediating its repressive function. This mark is found in the promoters of repressed genes and impedes histone readers from binding to H3 tails marked with activating PTMs [31]. In particular, this is known to prevent WDR5, an MLL complex component, from binding to such marks [31]. Interestingly, the symmetrical arginine dimethylation mark generated by PRMT5 on the same residue has the opposite effect, promoting WDR5 recruitment [32]. Subsequent H3K4me3 deposition leads to the activation of multiple genes including those involved in the genotoxic stress response and growth hormone mediated proliferation. This mechanism is known to be particularly important in breast cancer stem cells to enable activation of the FOXP1 promoter, aiding in self-renewal and proliferation [33]. In addition, this pathway is able to induce expression of SHARPIN, which is associated with cancer cell proliferation and invasion [34]. Although this would appear to put the activity of PRMT5 and PRMT6 in direct opposition of each other, this is not the case as H3K4me3 deposition (which occurs following H3R2me2s arginine methylation by PRMT5) serves to inhibit PRMT6 activity [35]. As such, PRMT6 does not silence the same genes which PRMT5 activates. Working together, these two proteins could enhance the cancer cell phenotype, with PRMT5 activating beneficial genes and PRMT6 silencing detrimental ones (such as tumor suppressors and angiogenesis inhibitors [12]).

H4R3me2s is a key repressive mark generated by PRMT5, which can be recognized by DNMT3a to promote DNA methylation and gene silencing [36]. This mark is known to silence epithelial junction genes to enhance cancer cell invasion, repress keratinocyte and osteoblast differentiation genes (which could be important in cancer cell stemness), and decrease expression of key tumor suppressor and CDK inhibitor genes [7]. H4R3me2s has also been demonstrated to be recognized by PHF1, leading to CUL4B-ring E3 ligase complex recruitment and downstream silencing of E-cadherin and FBXW7 to promote cell growth and migration [37]. In AML, the generation of H4R3me2s leads to decreased expression of the micro-RNA miR-29b [38]. As miR-29b usually serves to promote degradation of the Sp1 transcription factor, Sp1 levels rise following miR-29b repression, activating FLT3 expression to promote leukemia initiation. Furthermore, in breast cancer cells, PRMT5-mediated silencing of DKK1 and DKK3 leads to enhanced expression of C-MYC, CYCLIN D1, and SURVIVIN to promote cell growth and proliferation [39].

Transcriptional repression mediated by PRMT7 is also important in cancer biology, with elevated H4R3me2s levels associated with increased PRMT7 expression evoking loss of E-Cadherin and epithelial to mesenchymal transition in breast cancer [40]. Interestingly, PRMT7 overexpression has also been shown to upregulate matrix metalloproteinase 9 (MMP9) in breast cancer cells, again enhancing migration and invasiveness, despite its major role as a transcriptional repressor [41]. The precise mechanism of this activity still needs to be determined to assess whether this upregulation is achieved as a secondary effect of E-cadherin downregulation or whether PRMT7 is also able to directly upregulate gene expression, thus exhibiting both repressive and activating functions as seen with other PRMTs.

3.2 Splicing

Various roles exist for PRMT proteins in the regulation of splicing. This is likely to occur due to the glycine–arginine-rich motifs and Tudor domains commonly present in splicing factors and RNA-binding proteins, both of which are known to be favored targets of the PRMT family.

PRMT5 is the family member most strongly associated with splicing regulation and has repeatedly been shown to be vital in splicing fidelity. Critically, symmetrical dimethylation of Sm proteins SmD1 and SmD3 enables binding with snRNAs, recognition by SMN, and subsequent assembly into mature snRNP molecules [42]. A failure of this function, as occurs with PRMT5 inhibition or

depletion, impedes spliceosome assembly and leads to the generation of hundreds of aberrant transcripts due to exon skipping and intron retention [43–45]. Notably, in multiple cancers, PRMT5 depletion has been shown to promote a shift in MDM4 expression toward a short isoform which undergoes nonsense mediated decay, thus reducing its expression [44,45]. This subsequently removes repression of the p53 pathway to enhance cancer cell survival. In addition, PRMT5 inhibition in B-cell lymphomas leads to the mis-splicing of MYC transcripts to evoke cancer cell apoptosis [46], while in hematopoietic progenitor cells it causes alternative splicing of TIP60 to impair homologous recombination [47]. These results highlight a key role for PRMT5 in splicing and suggest that this vulnerability could be exploited for therapeutic benefit in cancer. Interestingly, the oncogene Myc is known to elevate the expression of both Sm proteins and PRMT5 [46]. It is thought that PRMT5 is an essential effector of Myc function, needed to maintain splicing fidelity in the face of elevated transcription levels evoked by Myc which are necessary for rapid cancer cell proliferation.

Evidence also exists for roles of other PRMT proteins in splicing, but these have not yet been as clearly elucidated. For example, PRMT1 and PRMT4 are each able to methylate several RNA-binding proteins (RBPs) and are thus thought to be important in splicing regulation [8]. PRMT4 is also known to asymmetrically dimethylate other proteins involved in splicing, including CA150 (a transcription elongation repressor), SAP49 and snRNP C (splicing factors), and Sm proteins [8]. In particular, PRMT1 has been shown to methylate the RBP RBM15 in acute megakaryoblastic leukemia (AMKL) [48], a cancer in which PRMT1 is both overexpressed and correlated with poor survival. RBM15 methylation results in targeting by the E3 ligase CNOT4 and subsequent degradation. The role of RBM15 in RNA export and splicing means that this reduced expression disrupts gene product isoform ratios and prevents differentiation (a defining feature of AMKL) [48]. PRMT2 is also known to be involved in splicing regulation through its ability to target SmB/B', snRNP components and hnRNPs. It has also been demonstrated that PRMT2 can interact with SAM68 (Scr-associated in mitosis 68-kDa protein) which regulates the alternative splicing of Bcl-X, an apoptosis regulator [49]. Furthermore, PRMT9, the least well characterized PRMT to date, is also thought to be involved in splicing fidelity, due to its ability to act on spliceosome associated protein 145 [50] (part of the U2 snRNP) and splicing factor SF3B2 to enhance SMN binding. Importantly, upon PRMT9 depletion widespread disruption to splicing transcripts is observed, including skipped exons and retained introns [50].

Together, this evidence points toward key roles for PRMT proteins in splicing regulation and maintenance which could be targeted for therapeutic benefit in various cancers. It also suggests that certain cancers, namely those with splicing defects, may be more likely to respond to anti-PRMT agents due to their diminished ability to tolerate further splicing disruption. This has already been demonstrated in glioblastoma, in which cells with reduced splicing associated protein expression were demonstrated to be more sensitive to PRMT5 inhibition [43]. In addition, in AML type I PRMT inhibition preferentially killed cells with splicing factor mutations, such as SRSF2 [51]. However, further work to confirm this still needs to be carried out in other cancers. A total of 45%–85% of myelodysplastic syndrome patients, for example, exhibit mutations in splicing machinery genes [52] and could serve to benefit greatly for PRMT inhibition. It is also worth investigating whether known immunogenic cancers could be a second group with enhanced sensitivity to PRMT inhibition. Splicing disruption through PRMT inhibition is theorized to result in the production of multiple neoepitopes, which could aid in inducing anticancer immunity. This also suggests

that PRMT targeting agents could be combined with immunotherapies to enhance efficacy and allow targeting of a wider range of cancer subtypes.

3.3 mRNA regulation and translation

Gene expression is further influenced by PRMT proteins at the level of translation (Table 23.2), mainly through the methylation of RNA binding proteins at RGG/RG motifs. These functions have been shown to be important in multiple cancers. For example, PRMT1 methylation of Aven and subsequent recruitment of DHX36 helicase is needed to allow translation of MLL fusion proteins vital for oncogenic transformation in acute lymphoblastic leukemia (ALL) [53].

TABLE 23.2 Roles of PRMT proteins in translation.

PRMT family member	Target	Effect(s)
PRMT1	Aven	Recruits DHX36 helicase to promote translation ^a
	eIF4G1	Contributes to eIF4G1 stability and translation initiation complex assembly ^b
	Ribosomal protein S3	Required for transport of rpS3 into the nucleolus and subsequent ribosome assembly ^c
	DNA topoisomerase 3B	Localization to stress granules and possible mRNA silencing ^d
PRMT3	Ribosomal protein S2	Enables binding of 40S ribosomal subunit to enable ribosomal assembly and enhance translation ^e
	hnRNPA1	Stabilizes ABCG2 mRNA, enhancing its expression and hence gemcitabine resistance in pancreatic cancer ^f
PRMT4	NEAT1	Prevents paraspeckle formation to enhance export of IRAlus containing mRNAs ^g
PRMT5	Nuclear riboprotein A1	Allows interaction with mRNAs containing internal ribosome entry sites (including MEP50, MYC, and CDKN1B) ^h
	Ribosomal protein S10	Enables S10 entry into ribosome complexes to regulate overall levels of translation ⁱ
PRMT7	eIF2S1	Allows eIF2α-mediated stress granule formation ^j
PRMT8	eIF4E, eIF4G1, eIF4H	Upregulated by PRMT8 at neuronal synapses (although mRNA levels not altered), contributing to translation initiation complex formation ^k

^aReference: [53].

^bReference: [54].

^cReference: [55].

^dReference: [56].

^eReference: [57].

^fReference: [58].

^gReference: [59].

^hReference: [60].

ⁱReference: [61].

^jReference: [62].

^kReference: [63].

Furthermore, PRMT4 is able to regulate the formation and function of paraspeckles [15], nuclear structures which modulate gene expression through the sequestration of certain mRNA molecules. Notably, paraspeckles target mRNAs containing inverted repeated Alu elements (IRAlus). PRMT4 is able to repress transcription of the long noncoding RNA NEAT1 (nuclear paraspeckle assembly transcript 1), which is central to paraspeckle formation, by binding to its promoter [59]. This disrupts paraspeckle formation, thus decreasing mRNA sequestration and allowing export of these mRNAs to the cytoplasm for translation to occur. Interestingly, however, PRMT4 inhibition also appears to have deleterious effects on paraspeckle assembly [64], suggesting that PRMT4 is a key regulator of this process. Given that paraspeckles have been shown to be essential for developmental progression, this function of PRMT4 could be highly important for generation of the cancer cell phenotype and heterogeneity.

3.4 PRMTs and cancer stem cells

The link between the PRMT family of proteins and stemness is becoming increasingly apparent not just in embryogenesis, but also in cancer (Figure 23.2). Cancer stem cells (CSCs) represent a small proportion of cancer cells and are characterized by their stem-like properties, namely their ability to proliferate indefinitely and produce differentiated progeny. As a result of this, CSCs are thought to be vital in initiating tumorigenesis, promoting metastasis, and enabling cancer recurrence following treatment. Furthermore, CSCs often demonstrate enhanced chemoresistance compared to the rest of the tumor bulk [65], making them difficult to ablate with our current therapy regimes. These properties make CSCs a particularly appealing target in cancer, which could perhaps be achieved through PRMT inhibition.

In normal development, PRMT5 has been shown to be essential for embryonic stem cell (ESC) proliferation (although not for maintenance of pluripotency) [66], with PRMT5 knockout being lethal in early embryogenesis [67]. This is likely to be linked to the ability of PRMT5 to upregulate Oct4 and Nanog in ESCs [67]. In adults, PRMT5 is involved in neural stemness and stem cell expansion following injury, while also being critical for the maintenance of hematopoietic stem cells [2]. Additionally, PRMT5 can act as a reprogramming factor alongside other Yamanaka factors to enhance induced pluripotent stem cell production [68]. Thus it is clear that PRMT5 is highly linked to stemness. This is also true in cancer, where PRMT5 is found to be overexpressed in chronic myeloid leukemia (CML) [69] and breast cancer stem cells [33]. Notably, PRMT5 depletion in human breast CSCs as well as PRMT5 inhibition in leukemic CSCs leads to impaired self-renewal. Furthermore, in xenograft models of breast cancer, breast CSC numbers could be significantly diminished through PRMT5 depletion [33]. In glioblastoma (GBM), PRMT5 inhibition has been shown to impair growth of cells in stem cell cultures and prolong survival in xenograft mouse models [70]. This function was linked to extensive disruption to RNA splicing, which seemed to particularly affect proteins involved in the cell cycle. These results highlight PRMT5 as a promising target in CSCs, inhibitors of which could be combined with other chemotherapy agents to ablate both bulk tumor and CSC populations.

PRMT5 is not the only family member linked to stemness, however, with PRMT1 needed for maintenance of embryonic stem cell pluripotency [71] and PRMT4, PRMT7, and PRMT8 able to upregulate drivers of stemness, such as Oct4, Sox2, Nanog, and c-Myc [5]. PRMT7, in particular, has been shown to modulate glycine metabolism through inducing glycine decarboxylase in CML

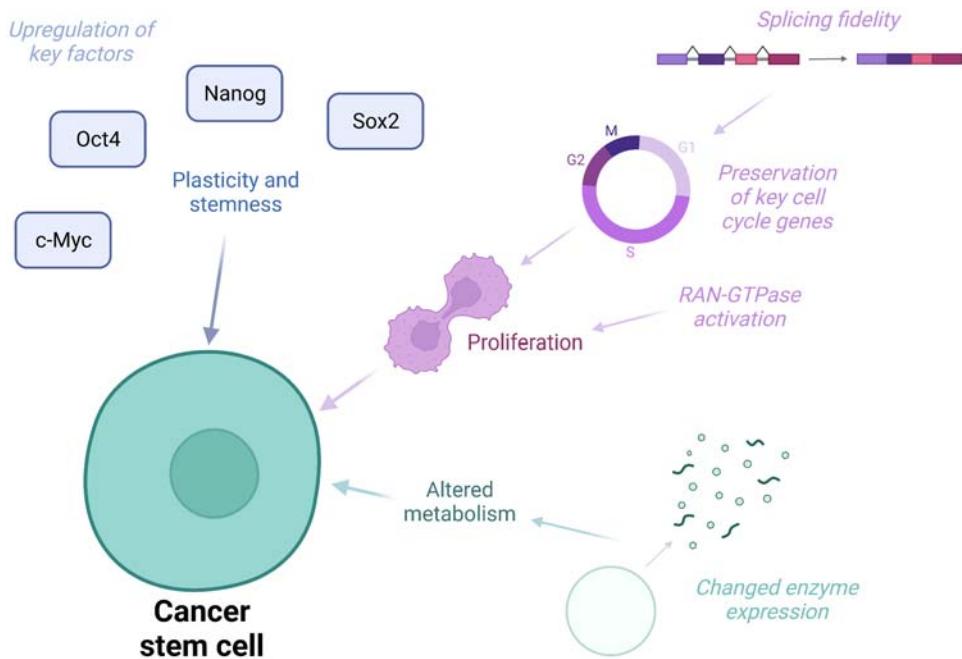


FIGURE 23.2

The roles of PRMT proteins in cancer stem cells. PRMT proteins have been increasingly linked to plasticity and stemness, both within the normal stem cell population and in cancer stem cells (CSCs). This is achieved through multiple mechanisms, namely epigenetic modulation to alter the transcriptional signature of cells. Multiple PRMT proteins (including PRMT4, PRMT5, PRMT7, and PRMT8) have been shown to upregulate key "stemness" factors, such as Oct4, Nanog, Sox2, and c-Myc. In addition, PRMT5 maintains splicing fidelity in a manner which is beneficial to CSCs, enabling proliferation, while PRMT6 methylates RCC1 to allow an increase in RAN-GTPase activation and PRMT7 induces glycine decarboxylase expression to alter glycine metabolism in CSCs.

stem cells, with pharmacological inhibition or depletion of PRMT7 impairing CSC renewal in murine models [72]. PRMT7 is also able to replace SOX2 as a reprogramming factor to induce formation of murine pluripotent stem cells [73]. Furthermore, PRMT6 has recently been linked to stemness in glioblastoma, in which its expression was shown to be elevated in tumorspheres and its depletion impeded sphere forming ability [17]. This function was linked to the ability of PRMT6 to methylate regulator of chromatin condensation 1 (RCC1) which enables chromatin binding, RAN-GTPase activation, and cell proliferation. Taken together, these results suggest that pan-PRMT inhibitors or inhibitors of PRMT proteins other than PRMT5 could also be effective in CSC ablation. Notably, high throughput screening in colon cancer cells revealed the type I PRMT inhibitor Ms023 to be a potent inducer of differentiation, later confirmed by proteomics and genomics, thus supporting this approach [74].

3.5 Important nonepigenetic roles

3.5.1 DNA damage response

Cells depleted of PRMT1 and PRMT5 demonstrate increased sensitivity to DNA damage and genome instability [47,75,76], indicative of an important role for PRMT proteins in the DNA damage response. PRMT1 is known to be involved in double stranded DNA (dsDNA) break repair, via its ability to methylate MRE11 [77], a member of the MRN complex. This facilitates anchoring of MRE11 to dsDNA breaks to aid in nuclease activity and repair. PRMT5 also participates in the repair of dsDNA breaks through regulation of homologous recombination[5]. In particular, PRMT5 methylates RUVBL1, a member of the TIP60 complex, which is needed to mobilize 53BP1 away from dsDNA breaks and promote HR over the alternative dsDNA break repair pathway nonhomologous end joining (NHEJ) [76]. PRMT5 also plays a secondary role in this process through maintaining splicing fidelity of DNA repair proteins, such as Tip60 [47]. In addition, both PRMT4 and PRMT5 are known to be involved in the suppression of R loop formation [8], which helps to prevent the formation of dsDNA breaks and subsequent genome instability.

PRMT proteins also play a role in the repair of single-stranded DNA breaks. For example, PRMT6 is able to interact with DNA polymerase beta, which is vital in base excision repair (BER) [78]. PRMT1 also plays a role in BER through its ability to methylate DNA polymerase beta and Flap endonuclease 1 (FEN1) [14], both of which are key enzymes in this process.

3.5.2 Signal transduction

PRMT1 and PRMT5 seem to be highly involved in growth factor signaling and interact with multiple pathways highly associated with cancer cells. This includes BMP, TGF- β , EGFR, WNT, and PDGR- α signaling pathways [14]. PRMT1 has also been shown to interact directly with estrogen receptor alpha (to enhance transcription) and acts a potent coactivator of the androgen receptor (AR) [11]. PRMT6 is another family member able to bind to the AR and serves to inhibit its phosphorylation, thereby promoting hormone dependent transactivation and contributing to signaling [79]. Furthermore, PRMT3 has been shown to stabilize c-MYC in colorectal cancer to evoke protumorigenic effects [80]. Also in colorectal cancer, it has recently been demonstrated that PRMT3 is able to methylate and stabilize HIF1 α , contributing to the HIF1/VEGFA signaling pathway in order to stimulate angiogenesis [81]. Interestingly, PRMT9 is reported to evoke HIF1 α instability in osteosarcoma, a cancer in which its expression is suppressed [19].

4 Discovery and efficacy of PRMT inhibitors

Given an increasing body of evidence highlighting the role of PRMT proteins in various medical conditions, including cancer, much work has been carried to develop small molecule inhibitors. Excitingly, multiple of these inhibitors are currently being tested in clinical trials (Table 23.3), with more set to enter trials soon. It is thus feasible that PRMT inhibitors could soon be a subset of epigenetic targeting agents utilized to treat cancer in the clinic.

Initial research aiming to identify PRMT inhibitors focused on the use of SAM analogs, such as methylthioadenosine, S-adenosylhomocysteine, and sinefungin, to compete for PRMT binding [3].

TABLE 23.3 PRMT inhibitors participating in clinical trials.

Protein	Inhibitor	Phase	Cancers included	Identifier
PRMT5	GSK3326595 (+ pembrolizumab in Part 3)	I	Non-Hodgkin's lymphoma, advanced solid tumors	NCT02783300
	GSK3326595	I	Relapsed or refractory myelodysplastic syndrome, chronic myelomonocytic leukemia, and acute myeloid leukemia	NCT03614728
	GSK3326595	II	Early stage breast cancer	NCT04676516
	JNJ-64619178	I	Relapsed or refractory B-cell non-Hodgkin lymphoma, advanced solid tumors, lower risk myelodysplastic syndromes	NCT03573310
	AMG 193 (+ docetaxel in phase II)	I/II	Metastatic or locally advanced MTAP-null nonsmall-cell lung cancer, previously treated with chemotherapy or checkpoint inhibition	NCT05094336
	PF-06939999 + docetaxel in later stages	I	Previously treated advanced or metastatic cancer (including nonsmall-cell lung cancer, head and neck squamous cell carcinoma, esophageal cancer, endometrial cancer, cervical cancer, and bladder cancer)	NCT03854227
	PRT811	I	High grade gliomas, CNS lymphomas, and advanced cancers	NCT04089449
	TNG908	I/II	Advanced or metastatic solid tumors with known MTAP deletion.	NCT05275478
	PRT543	I	Advanced solid tumors, diffuse large B cell lymphomas, myelodysplasias, myelofibrosis, mantle cell lymphomas, acute myeloid leukemias, and chronic myelomonocytic leukemias which are relapsed or refractory. Also adenoid cystic carcinoma.	NCT03886831
	MRTX1719	I/II	Advanced, unresectable, or metastatic malignancies with a homozygous deletion in the MTAP gene. Includes: mesotheliomas, nonsmall-cell lung cancers, malignant peripheral nerve sheath tumors, pancreatic adenocarcinomas, and other solid tumors.	NCT05245500
PRMT1	GSK3368715	I	Diffuse large B cell lymphomas and solid tumors with MTAP deficiencies (including pancreatic, bladder, and nonsmall-cell lung cancer)	NCT03666988

However, these proved to lack selectivity for PRMT proteins (also targeting DNA methyltransferases and other enzymes) and thus were unlikely to be used clinically. It was not until large scale screening approaches were employed that the first selective PRMT inhibitors were discovered. In 2004 Bedford et al. utilized virtual screening on a compound library of >1900 compounds to identify the AMI series of compounds [82], with subsequent screening experiments also identifying activity of diamidine

compounds against PRMT1 [4]. In subsequent years, multiple screening experiments have taken place and these, alongside a better understanding of PRMT structure and multiple rounds of drug optimization, have allowed highly specific inhibitors to be generated.

4.1 TYPE I PRMT inhibitors

Type I PRMT inhibitors were initially isolated via high throughput screening and act by binding to and masking recognition of PRMT1 substrates. Ms023, in particular, has been shown to potently inhibit type I PRMT proteins and has particular activity against PRMT1 [83]. GSK3368715 is another powerful type I PRMT inhibitor, which entered phase I trials in 2018 involving some assessment of activity in patients with hematological malignancies and MTAP depleted solid tumors (Table 23.3).

More selective inhibitors targeting type I PRMT proteins were subsequently developed, including several for PRMT1, PRMT3, PRMT6, and PRMT4. Currently PRMT3 and PRMT6 inhibitors have limited clinical indications [8], although further research should soon elucidate these. Comparatively, PRMT4 inhibitors have demonstrated significant anticancer effects in mouse models of hematological malignancies (including multiple myeloma [84] and AML [85]) as well as breast cancer [86]. Two PRMT1 specific inhibitors have also been reported: TC-R-5003 [87] and C7280948 [88], which have demonstrated antiproliferative effects in lung cancer and colorectal cancer, respectively. If positive results continue to be demonstrated with these drugs, perhaps we will see them entering clinical trials soon.

4.2 TYPE II PRMT inhibitors

Several type II PRMT inhibitors have also been developed, mainly targeting PRMT5 (the most active enzyme within this group). EPZ015666, GSK3326595 (pemrometastat), and GSK591 all act similarly by binding to the substrate binding pocket of PRMT5 and additionally making contact with the SAM binding pocket in order to diminish enzyme activity [8]. These drugs have proven to be effective in a number of cancer cell lines and seem to be particularly active against hematological tumors in mice [44,89]. Promisingly, GSK3326595 has already taken part in a dose-escalation study in solid tumors and non-Hodgkin lymphoma (Table 23.3), which demonstrated safety of the drug and response in patients with multiple tumor types. Additionally, a trial investigating safety and activity of GSK3326595 in myelodysplastic syndrome and AML (NCT03614728) has been recently completed (although results not yet reported) and it is also set to be used in a Phase II window of opportunity trial in early stage breast cancer (NCT04676516).

SAM competitive PRMT5 inhibitors, including LLY-283 and JNJ-64619178 [8], have also been isolated and have shown promising preclinical results. JNJ-64619178 is currently in clinical trials, being tested in those with lung cancer and hematological malignancies (Table 23.3). It has demonstrated encouraging preclinical results, including potent inhibition of proliferation in various cell lines [90]. Pancreatic, hematological, breast, colon, lung, and ovarian cancers were among the most sensitive to PRMT5 inhibition with this agent, with high proportions of cell lines demonstrating a GI_{50} of under 10^{-8} mol/L. This inhibitor also performed well in lung cancer xenograft models, in which it was able to induce growth inhibition and tumor regression [90].

The results highlighting the tolerability of PRMT5 inhibitors are especially promising, given prior concerns about this aspect of PRMT5 inhibition. Particular concerns existed surrounding the possibility of PRMT5 inhibition ablating the hematopoietic stem cell population and evoking severe pancytopenia, as was seen in conditional depletion of Prmt5 in mice [2]. In addition, PRMT5 appears to be involved in the promotion of hepatic gluconeogenesis during fasting [91], which implied potential for dangerous hypoglycemia to occur with PRMT5 inhibition. However, further work must still be carried out to confirm no long-term side effects arise from such therapy. For example, PRMT5 repression by amyloid beta has been linked to neuronal cell death seen in Alzheimer's disease [92] and this would suggest that PRMT5 inhibitors could have similar downstream effects, should penetration of the blood brain barrier be achieved (as was recently demonstrated to occur with the PRMT5 inhibitor LLY-283 [70]). Furthermore, arginine methylation could play a role in aging, with loss of PRMTs in transgenic mice being associated with cell death and premature aging [2]. As such, patients should be monitored following use of PRMT inhibitors to ensure that these drugs are not risk factors for conditions arising with increasing age.

4.3 TYPE III PRMT inhibitors

SGC3027 has proven to be a potent and selective PRMT7 inhibitor [93]. It enters cells as a pro-drug, where it is then converted to SGC8158 and competes with SAM for binding to PRMT7. Importantly, such inhibition has been demonstrated to sensitize cells to heat shock and proteasome inhibitors [94], the latter of which could prove important in cancer therapy. Furthermore, application of SCG3027 in melanoma cells led to upregulation of endogenous retroviral elements and enhanced antitumor immunity [94]. This highlights SCG3027 as an agent which could potentiate immunotherapy responses to enhance therapeutic efficacy in cancer.

4.4 Targeted use of PRMT inhibitors

It is possible that certain subsets of patients may exhibit increased sensitivity to PRMT inhibition and would serve to gain the most therapeutic benefit from this treatment avenue. In particular, cancer with splicing defects (such as those with change of function mutations in splicing factors SF3B1, U2AF1, SRSF2) demonstrate increased sensitivity to type I and PRMT5 inhibitors [7]. In addition, MTAP loss has been shown to similarly increase response to type I and PRMT5 targeting agents [7]. This avenue of targeted treatment is currently being explored in multiple clinical trials (Table 23.3). Furthermore, recent work has elucidated a subset of pancreatic cancer cells containing activating aberrations in the MYC pathway which exhibited increased responsiveness to PRMT5 inhibitors [95].

5 Conclusions and future directions

The pace of research into PRMT proteins has been rapid in recent years. These previously largely unknown proteins have been widely characterized, with a particular focus on their role in cancer, and (importantly) can now be targeted with small molecule inhibitors. Preclinical results have shown significant promise for PRMT inhibitors and, with the first clinical trial results now coming

out, these compounds could soon be revolutionizing treatments in clinical settings. Particularly interesting is the role that PRMT proteins appear to play in CSCs, in which they are essential for stemness. As stem-like properties are thought to arise through epigenetic modulation, which can evoke de-differentiation, an epigenetic targeting agent is likely to be required to reverse these effects. Perhaps we have found our answer in PRMT inhibitors.

Despite this, there are still key gaps in our knowledge that need to be filled. Work has focused most heavily on PRMT1, PRMT4, and PRMT5, with other PRMT proteins still needing to be better characterized. PRMT9, especially, is under-researched and needs to be investigated further. Given that research suggests that this is the only family member which has a distinct evolutionary origin [1], it will be interesting to see whether its unique derivation allows it to play cellular roles not carried out by other PRMTs. In addition, little is currently known about the protein interactions of PRMT2 and PRMT3, with even less being known about how such interactions impact on cellular physiology.

Furthermore, we need to better understand the interactions of PRMT proteins with each other within cells. “Substrate scavenging” following inhibition or depletion of a single PRMT protein has been documented [96], but what effect this will have on therapeutic efficacy within patients is as of yet unknown. It also begs the question of whether resistance to selective PRMT inhibition could be rapidly achieved through the upregulation of alternative family members and whether more broadly effective (e.g., type I) inhibitors could be a better approach. Immune interactions of PRMT inhibitors, and the impacts this may have on cancer cells, also need to be better characterized. PRMT5, in particular, is known to play an important role within peripheral immune cells and it is possible that its inhibition could have detrimental effects on antitumor immunity. Notably, one study found that PRMT5 knockdown or application of PRMT5 inhibitor GSK591 in mouse models of lung cancer led to rapid upregulation of PD-L1 to reduce immune infiltration, although this could be rectified through application of anti-PD-L1 checkpoint inhibitors [97].

Another question yet unanswered in the field is whether a true arginine demethylase exists. It is possible that lysine demethylases may also function to remove methyl groups from arginine too, which suggests that the JmjC proteins could perform this function. JmjD6 was reported as a putative arginine demethylase, but was later shown to be shown to be only a lysine hydroxylase [98]. Lysine demethylases KDM3A, KDM4E, and KDM5C have all shown arginine demethylase activity *in vitro* [99], but whether this translates *in vivo* is unknown. In addition, their preference methyl-lysine over methyl-arginine suggests that *in vivo* activity is likely to be limited. Protein arginine deaminases, which act to convert arginine to citrulline, have been proposed as an alternative modality for removal of methyl marks, but strongly favor unmodified arginine residues and so are unlikely to be physiologically relevant [100]. It will be important in future to determine whether the reversal of arginine methyl marks is possible, or whether this mark is a long-lasting PTM lost only with protein degradation. If an arginine demethylase is identified, its role in cancer will be interesting to investigate.

In summary, a great deal of progress has been made in understanding this previously understudied family of proteins and has highlighted their pivotal role in cancer epigenetics. Preclinical results of inhibitors are encouraging, highlighting the promise that modulating the cancer epigenome holds. Such manipulation of cancer cell phenotype appears not only to be a viable approach in theory, but, with clinical trial results of PRMT inhibitors soon to be revealed, could soon become much more widespread in clinical practice.

List of abbreviations

ADMA	Asymmetric dimethyl arginine
AML	Acute myeloid leukemia
AMKL	Acute megakaryoblastic leukemia
BER	Base excision repair
CML	Chronic myeloid leukemia
CSC	cancer stem cell
PRMT	protein arginine methyltransferase
SAM	<i>S</i> -Adenosylmethionine
SDMA	Symmetric dimethyl arginine
SMN	Survival of motor neurone
SPF30L	Splicing factor 30
TDRD	Tudor domain containing protein

References

- [1] Krause CD, Yang ZH, Kim YS, Lee JH, Cook JR, Pestka S. Protein arginine methyltransferases: evolution and assessment of their pharmacological and therapeutic potential. *Pharmacol Ther* 2007;113(1):50–87. Available from: <https://doi.org/10.1016/j.pharmthera.2006.06.007>.
- [2] Blanc RS, Richard S. Arginine methylation: the coming of age. *Mol Cell* 2017;65(1):8–24. Available from: <https://doi.org/10.1016/j.molcel.2016.11.003>.
- [3] Spannhoff A, Sippel W, Jung M. Cancer treatment of the future: Inhibitors of histone methyltransferases. *Int J Biochem Cell Biol* 2009;41(1):4–11. Available from: <https://doi.org/10.1016/j.biocel.2008.07.024>.
- [4] Hu H, Qian K, Ho MC, Zheng YG. Small molecule inhibitors of protein arginine methyltransferases. *Expert Opin Investigational Drugs* 2016;25(3):335–58. Available from: <https://doi.org/10.1517/13543784.2016.1144747>.
- [5] Jarrold J, Davies CC. PRMTs and arginine methylation: cancer's best-kept secret? *Trends Mol Med* 2019;25(11):993–1009. Available from: <https://doi.org/10.1016/j.TMOLMED.2019.05.007>.
- [6] Tang J, et al. PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. *J Biol Chem* 2000;275(11):7723–30. Available from: <https://doi.org/10.1074/jbc.275.11.7723>.
- [7] Xu J, Richard S. Cellular pathways influenced by protein arginine methylation: Implications for cancer. *Mol Cell* 2021;81(21):4357–68. Available from: <https://doi.org/10.1016/j.molcel.2021.09.011>.
- [8] Guccione E, Richard S. The regulation, functions and clinical relevance of arginine methylation. *Nat Rev Mol Cell Biol* 2019;20(10):642–57. Available from: <https://doi.org/10.1038/s41580-019-0155-x>.
- [9] Dong R, Li X, Lai KO. Activity and function of the prmt8 protein arginine methyltransferase in neurons. *Life* 2021;11(11). Available from: <https://doi.org/10.3390/life1111132>.
- [10] Rakow S, Pullamsetti SS, Bauer UM, Bouchard C. Assaying epigenome functions of PRMTs and their substrates. *Methods* 2020;175:53–65. Available from: <https://doi.org/10.1016/j.ymeth.2019.09.014>.
- [11] Cura V, Cavarelli J. Structure, activity and function of the prmt2 protein arginine methyltransferase. *Life* 2021;11(11):1263. Available from: <https://doi.org/10.3390/life1111263>.
- [12] Gupta S, Kadumuri RV, Singh AK, Chavali S, Dhayalan A. Structure, activity and function of the protein arginine methyltransferase 6. *Life* 2021;11(9):951. Available from: <https://doi.org/10.3390/life11090951>.
- [13] Halabelian L, Barsyte-Lovejoy D. Structure and function of protein arginine methyltransferase prmt7. *Life* 2021;11(8):768. Available from: <https://doi.org/10.3390/life11080768>.
- [14] Thiebaut C, Eve L, Poulard C, Le Romancer M. Structure, activity, and function of prmt1. *Life* 2021;11(11):1147. Available from: <https://doi.org/10.3390/life1111147>.

- [15] Suresh S, Huard S, Dubois T. CARM1/PRMT4: making its mark beyond its function as a transcriptional coactivator. *Trends Cell Biol* 2021;31(5):402–17. Available from: <https://doi.org/10.1016/j.tcb.2020.12.010>.
- [16] Hsu MC, et al. Protein arginine methyltransferase 3-induced metabolic reprogramming is a vulnerable target of pancreatic cancer. *J Hematol Oncol* 2019;12(1). Available from: <https://doi.org/10.1186/s13045-019-0769-7>.
- [17] Huang T, et al. PRMT6 methylation of RCC1 regulates mitosis, tumorigenicity, and radiation response of glioblastoma stem cells. *Mol Cell* 2021;81(6):1276–1291.e9. Available from: <https://doi.org/10.1016/j.molcel.2021.01.015>.
- [18] Jiang H, et al. PRMT9 promotes hepatocellular carcinoma invasion and metastasis via activating PI3K/Akt/GSK-3 β /Snail signaling. *Cancer Sci* 2018;109(5):1414–27. Available from: <https://doi.org/10.1111/cas.13598>.
- [19] Zhang H, et al. MiRNA-543 promotes osteosarcoma cell proliferation and glycolysis by partially suppressing PRMT9 and stabilizing HIF-1 α protein. *Oncotarget* 2017;8(2):2342–55. Available from: <https://doi.org/10.18632/oncotarget.13672>.
- [20] Yang Y, McBride KM, Hensley S, Lu Y, Chedin F, Bedford MT. Arginine methylation facilitates the recruitment of TOP3B to chromatin to prevent R loop accumulation. *Mol Cell* 2014;53(3):484–97. Available from: <https://doi.org/10.1016/j.molcel.2014.01.011>.
- [21] Wang H, et al. Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Sci (80-)* 2001;293(5531):853–7. Available from: <https://doi.org/10.1126/science.1060781>.
- [22] Cheung N, et al. Targeting aberrant epigenetic networks mediated by PRMT1 and KDM4C in acute myeloid leukemia. *Cancer Cell* 2016;29(1):32–48. Available from: <https://doi.org/10.1016/j.ccr.2015.12.007>.
- [23] Gao Y, et al. The dual function of PRMT1 in modulating epithelial-mesenchymal transition and cellular senescence in breast cancer cells through regulation of ZEB1. *Sci Rep* 2016;6. Available from: <https://doi.org/10.1038/srep19874>.
- [24] Yao B, et al. PRMT1-mediated H4R3me2a recruits SMARCA4 to promote colorectal cancer progression by enhancing EGFR signaling. *Genome Med* 2021;13(1):1–21. Available from: <https://doi.org/10.1186/s13073-021-00871-5>.
- [25] Avasarala S, et al. PRMT1 Is a novel regulator of epithelial-mesenchymal-transition in non-small cell lung cancer. *J Biol Chem* 2015;290(21):13479–89. Available from: <https://doi.org/10.1074/jbc.M114.636050>.
- [26] Fretze S, Lupien M, Silver PA, Brown M. CARM1 regulates estrogen-stimulated breast cancer growth through up-regulation of E2F1. *Cancer Res* 2008;68(1):301–6. Available from: <https://doi.org/10.1158/0008-5472.CAN-07-1983>.
- [27] Wang F, et al. Nup54-induced CARM1 nuclear importation promotes gastric cancer cell proliferation and tumorigenesis through transcriptional activation and methylation of Notch2. *Oncogene* 2022;41(2):246–59. Available from: <https://doi.org/10.1038/s41388-021-02078-9>.
- [28] Wu D, et al. CARM1 promotes non-small cell lung cancer progression through upregulating CCNE2 expression. *Aging (Albany NY)* 2020;12(11):10578–93. Available from: <https://doi.org/10.18632/aging.103280>.
- [29] Lu W, Yang C, He H, Liu H. The CARM1-p300-c-Myc-Max (CPCM) transcriptional complex regulates the expression of CUL4A/4B and affects the stability of CRL4 E3 ligases in colorectal cancer. *Int J Biol Sci* 2020;16(6):1071–85. Available from: <https://doi.org/10.7150/ijbs.41230>.
- [30] Di Lorenzo A, Yang Y, Macaluso M, Bedford MT. A gain-of-function mouse model identifies PRMT6 as a NF- κ B coactivator. *Nucleic Acids Res* 2014;42(13):8297–309. Available from: <https://doi.org/10.1093/nar/gku530>.
- [31] Gayatri S, Bedford MT. Readers of histone methylarginine marks. *Biochim Biophys Acta Gene Regul Mech* 2014;1839(8):702–10. Available from: <https://doi.org/10.1016/j.bbagr.2014.02.015>.

- [32] Migliori V, et al. Symmetric dimethylation of H3R2 is a newly identified histone mark that supports euchromatin maintenance. *Nat Struct Mol Biol* 2012;19(2):136–45. Available from: <https://doi.org/10.1038/nsmb.2209>.
- [33] Chiang K, et al. PRMT5 is a critical regulator of breast cancer stem cell function via histone methylation and FOXP1 expression. *Cell Rep* 2017;21(12):3498–513. Available from: <https://doi.org/10.1016/j.celrep.2017.11.096>.
- [34] Tamiya H, et al. SHARPIN-mediated regulation of protein arginine methyltransferase 5 controls melanoma growth. *J Clin Invest* 2018;128(1):517–30. Available from: <https://doi.org/10.1172/JCI95410>.
- [35] Guccione E, et al. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. *Nature* 2007;449(7164):933–7. Available from: <https://doi.org/10.1038/nature06166>.
- [36] Zhao Q, et al. PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. *Nat Struct Mol Biol* 2009;16(3):304–11. Available from: <https://doi.org/10.1038/nsmb.1568>.
- [37] Liu R, et al. PHD finger protein 1 (PHF1) is a novel reader for histone H4R3 symmetric dimethylation and coordinates with PRMT5-WDR77/CRL4B complex to promote tumorigenesis. *Nucleic Acids Res* 2018;46(13):6608–26. Available from: <https://doi.org/10.1093/nar/gky461>.
- [38] Tarighat SS, et al. The dual epigenetic role of PRMT5 in acute myeloid leukemia: Gene activation and repression via histone arginine methylation. *Leukemia* 2016;30(4):789–99. Available from: <https://doi.org/10.1038/leu.2015.308>.
- [39] Shailesh H, Siveen KS, Sif S. Protein arginine methyltransferase 5 (PRMT5) activates WNT/β-catenin signalling in breast cancer cells via epigenetic silencing of DKK1 and DKK3. *J Cell Mol Med* 2021;25 (3):1583–600. Available from: <https://doi.org/10.1111/jcmm.16260>.
- [40] Yao R, et al. PRMT7 induces epithelial-to-mesenchymal transition and promotes metastasis in breast cancer. *Cancer Res* 2014;74(19):5656–67. Available from: <https://doi.org/10.1158/0008-5472.CAN-14-0800>.
- [41] Mitchell Baldwin R, et al. Protein arginine methyltransferase 7 promotes breast cancer cell invasion through the induction of MMP9 expression. *Oncotarget* 2015;6(5):3013–32. Available from: <https://doi.org/10.18632/oncotarget.3072>.
- [42] Meister G, Fischer U. Assisted RNP assembly: SMN and PRMT5 complexes cooperate in the formation of spliceosomal UsnRNPs. *EMBO J* 2002;21(21):5853–63. Available from: <https://doi.org/10.1093/emboj/cdf585>.
- [43] Braun CJ, et al. Coordinated splicing of regulatory detained introns within oncogenic transcripts creates an exploitable vulnerability in malignant glioma. *Cancer Cell* 2017;32(4):411–26. Available from: <https://doi.org/10.1016/j.ccr.2017.08.018>.
- [44] Gerhart SV, et al. Activation of the p53-MDM4 regulatory axis defines the anti-tumour response to PRMT5 inhibition through its role in regulating cellular splicing. *Sci Rep* 2018;8. Available from: <https://doi.org/10.1038/s41598-018-28002-y>.
- [45] Bezzi M, et al. Regulation of constitutive and alternative splicing by PRMT5 reveals a role for Mdm4 pre-mRNA in sensing defects in the spliceosomal machinery. *Genes Dev* 2013;27(17):1903–16. Available from: <https://doi.org/10.1101/gad.219899.113>.
- [46] Koh CM, et al. MYC regulates the core pre-mRNA splicing machinery as an essential step in lymphomagenesis. *Nature* 2015;523:96–100. Available from: <https://doi.org/10.1038/nature14351>.
- [47] Hamard PJ, et al. PRMT5 regulates DNA repair by controlling the alternative splicing of histone-modifying enzymes. *Cell Rep* 2018;24(10):2643–57. Available from: <https://doi.org/10.1016/j.celrep.2018.08.002>.
- [48] Zhang L, et al. Cross-talk between PRMT1-mediated methylation and ubiquitylation on RBM15 controls RNA splicing. *eLife* 2015;4. Available from: <https://doi.org/10.7554/elife.07938>.

- [49] Vhuiyan MI, et al. PRMT2 interacts with splicing factors and regulates the alternative splicing of BCL-X. *J Biochem* 2017;162(1):17–25. Available from: <https://doi.org/10.1093/jb/mvw102>.
- [50] Yang Y, et al. PRMT9 is a Type II methyltransferase that methylates the splicing factor SAP145. *Nat Commun* 2015;6(1):1–12. Available from: <https://doi.org/10.1038/ncomms7428>.
- [51] Fong JY, et al. Therapeutic targeting of RNA splicing catalysis through inhibition of protein arginine methylation. *Cancer Cell* 2019;36(2):194–209.e9. Available from: <https://doi.org/10.1016/j.ccr.2019.07.003>.
- [52] Yoshida K, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature* 2011;478:64–9. Available from: <https://doi.org/10.1038/nature10496>.
- [53] Thandapani P, et al. Aven recognition of RNA G-quadruplexes regulates translation of the mixed lineage leukemia protooncogenes. *eLife* 2015;4. Available from: <https://doi.org/10.7554/eLife.06234>.
- [54] Hsu JHR, et al. PRMT1-mediated translation regulation is a crucial vulnerability of cancer. *Cancer Res* 2017;77(17):4613–25. Available from: <https://doi.org/10.1158/0008-5472.CAN-17-0216>.
- [55] Shin HS, Jang CY, Kim HD, Kim TS, Kim S, Kim J. Arginine methylation of ribosomal protein S3 affects ribosome assembly. *Biochem Biophys Res Commun* 2009;385(2):273–8. Available from: <https://doi.org/10.1016/j.bbrc.2009.05.055>.
- [56] Huang L, Wang Z, Narayanan N, Yang Y. Arginine methylation of the C-terminus RGG motif promotes TOP3B topoisomerase activity and stress granule localization. *Nucleic Acids Res* 2018;46:3061–74. Available from: <https://doi.org/10.1093/nar/gky103>.
- [57] Swiercz R, Cheng D, Kim D, Bedford MT. Ribosomal protein rpS2 is hypomethylated in PRMT3-deficient mice. *J Biol Chem* 2007;282(23):16917–23. Available from: <https://doi.org/10.1074/jbc.M609778200>.
- [58] Hsu MC, et al. Protein arginine methyltransferase 3 enhances chemoresistance in pancreatic cancer by methylating hnRNPA1 to increase ABCG2 expression. *Cancers (Basel)* 2019;11(1):8. Available from: <https://doi.org/10.3390/cancers11010008>.
- [59] Bin Hu S, et al. Protein arginine methyltransferase CARM1 attenuates the paraspecklemediated nuclear retention of mRNAs containing IRAlus. *Genes Dev* 2015;29(6):630–45. Available from: <https://doi.org/10.1101/gad.257048.114>.
- [60] Gao Y, Teschendorff AE. Epigenetic and genetic deregulation in cancer target distinct signaling pathway domains. *Nucleic Acids Res* 2017;45(2):583–96. Available from: <https://doi.org/10.1093/nar/gkw1100>.
- [61] Ren J, Wang Y, Liang Y, Zhang Y, Bao S, Xu Z. Methylation of ribosomal protein S10 by protein-arginine methyltransferase 5 regulates ribosome biogenesis. *J Biol Chem* 2010;285(17):12695–705. Available from: <https://doi.org/10.1074/jbc.M110.103911>.
- [62] Haghish N, et al. PRMT7 methylates eukaryotic translation initiation factor 2 α and regulates its role in stress granule formation. *Mol Biol Cell* 2019;30(6):778–93. Available from: <https://doi.org/10.1091/mbc.E18-05-0330>.
- [63] Penney J, et al. Loss of protein arginine methyltransferase 8 alters synapse composition and function, resulting in behavioral defects. *J Neurosci* 2017;37(36):8655–66. Available from: <https://doi.org/10.1523/JNEUROSCI.0591-17.2017>.
- [64] Hupalowska A, Jedrusik A, Zhu M, Bedford MT, Glover DM, Zernicka-Goetz M. CARM1 and para-speckles regulate pre-implantation mouse embryo development. *Cell* 2018;175(7):1902–1916.e13. Available from: <https://doi.org/10.1016/j.cell.2018.11.027>.
- [65] Prieto-Vila M, Takahashi RU, Usuba W, Kohama I, Ochiya T. Drug resistance driven by cancer stem cells and their niche. *Int J Mol Sci* 2017;18(12):2574. Available from: <https://doi.org/10.3390/ijms18122574>.
- [66] Gkountela S, Li Z, Chin CJ, Lee SA, Clark AT. PRMT5 is required for human embryonic stem cell proliferation but not pluripotency. *Stem Cell Rev Rep* 2014;10(2):230–9. Available from: <https://doi.org/10.1007/s12015-013-9490-z>.

- [67] Tee WW, et al. Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency. *Genes Dev* 2010;24:2772–7. Available from: <https://doi.org/10.1101/gad.606110>.
- [68] Nagamatsu G, et al. A germ cell-specific gene, Prmt5, works in somatic cell reprogramming. *J Biol Chem* 2011;286(12):10641–8. Available from: <https://doi.org/10.1074/jbc.M110.216390>.
- [69] Jin Y, et al. Targeting methyltransferase PRMT5 eliminates leukemia stem cells in chronic myelogenous leukemia. *J Clin Invest* 2016;126(10):3961–80. Available from: <https://doi.org/10.1172/JCI85239>.
- [70] Sachamitr P, et al. PRMT5 inhibition disrupts splicing and stemness in glioblastoma. *Nat Commun* 2021;12(1):1–17. Available from: <https://doi.org/10.1038/s41467-021-21204-5>.
- [71] Bao X, et al. CSNK1a1 regulates PRMT1 to maintain the progenitor state in self-renewing somatic tissue. *Dev Cell* 2017;43(2):227–239.e5. Available from: <https://doi.org/10.1016/j.devcel.2017.08.021>.
- [72] Liu C, et al. Loss of PRMT7 reprograms glycine metabolism to selectively eradicate leukemia stem cells in CML. *Cell Metab* 2022;34(6):818–835.e7. Available from: <https://doi.org/10.1016/j.cmet.2022.04.004>.
- [73] Wang B, Pfeiffer MJ, Drexler HCA, Fuellen G, Boiani M. Proteomic analysis of mouse oocytes identifies PRMT7 as a reprogramming factor that replaces SOX2 in the induction of pluripotent stem cells. *J Proteome Res* 2016;15(8):2407–21. Available from: <https://doi.org/10.1021/acs.jproteome.5b01083>.
- [74] Plotnikov A, et al. PRMT1 inhibition induces differentiation of colon cancer cells. *Sci Rep* 2020;10(1):1–15. Available from: <https://doi.org/10.1038/s41598-020-77028-8>.
- [75] Yu Z, Chen T, Hébert J, Li E, Richard S. A mouse PRMT1 null allele defines an essential role for arginine methylation in genome maintenance and cell proliferation. *Mol Cell Biol* 2009;29(11):2982–96. Available from: <https://doi.org/10.1128/mcb.00042-09>.
- [76] Clarke TL, et al. PRMT5-dependent methylation of the TIP60 coactivator RUVBL1 is a key regulator of homologous recombination. *Mol Cell* 2017;65(5):900–916.e7. Available from: <https://doi.org/10.1016/j.molcel.2017.01.019>.
- [77] Boisvert FM, Déry U, Masson JY, Richard S. Arginine methylation of MRE11 by PRMT1 is required for DNA damage checkpoint control. *Genes Dev* 2005;19:671–6. Available from: <https://doi.org/10.1101/gad.1279805>.
- [78] El-Andaloussi N, et al. Arginine methylation regulates DNA polymerase β . *Mol Cell* 2006;22(1):51–62. Available from: <https://doi.org/10.1016/j.molcel.2006.02.013>.
- [79] Scaramuzzino C, et al. Protein arginine methyltransferase 6 enhances polyglutamine-expanded androgen receptor function and toxicity in spinal and bulbar muscular atrophy. *Neuron* 2015;85(1):88–100. Available from: <https://doi.org/10.1016/j.neuron.2014.12.031>.
- [80] Hu Y, Su Y, He Y, Liu W, Xiao B. Arginine methyltransferase PRMT3 promote tumorigenesis through regulating c-MYC stabilization in colorectal cancer. *Gene* 2021;791. Available from: <https://doi.org/10.1016/j.gene.2021.145718>.
- [81] Zhang X, et al. PRMT3 promotes tumorigenesis by methylating and stabilizing HIF1 α in colorectal cancer. *Cell Death Dis* 2021;12(11). Available from: <https://doi.org/10.1038/s41419-021-04352-w>.
- [82] Cheng D, Yadav N, King RW, Swanson MS, Weinstein EJ, Bedford MT. Small molecule regulators of protein arginine methyltransferases. *J Biol Chem* 2004;279(23):23892–9. Available from: <https://doi.org/10.1074/jbc.M401853200>.
- [83] Eram MS, et al. A potent, selective, and cell-active inhibitor of human type I protein arginine methyltransferases. *ACS Chem Biol* 2016;11(3):772–81. Available from: <https://doi.org/10.1021/acscchembio.5b00839>.
- [84] Drew AE, et al. Identification of a CARM1 inhibitor with potent in vitro and in vivo activity in preclinical models of multiple myeloma. *Sci Rep* 2017;7(1):1–13. Available from: <https://doi.org/10.1038/s41598-017-18446-z>.
- [85] Greenblatt SM, et al. CARM1 is essential for myeloid leukemogenesis but dispensable for normal hematopoiesis. *Cancer Cell* 2018;33(6):1111–1127.e5. Available from: <https://doi.org/10.1016/j.ccr.2018.05.007>.

- [86] Wang L, et al. CARM1 methylates chromatin remodeling factor BAF155 to enhance tumor progression and metastasis. *Cancer Cell* 2014;25(1):21–36. Available from: <https://doi.org/10.1016/j.ccr.2013.12.007>.
- [87] Zhang P, Tao H, Yu L, Zhou L, Zhu C. Developing protein arginine methyltransferase 1 (PRMT1) inhibitor TC-E-5003 as an antitumor drug using INEI drug delivery systems. *Drug Deliv* 2020;27(1):491–501. Available from: <https://doi.org/10.1080/10717544.2020.1745327>.
- [88] Yin XK, et al. PRMT1 enhances oncogenic arginine methylation of NONO in colorectal cancer. *Oncogene* 2021;40(7):1375–89. Available from: <https://doi.org/10.1038/s41388-020-01617-0>.
- [89] Chan-Penebre E, et al. A selective inhibitor of PRMT5 with in vivo and in vitro potency in MCL models. *Nat Chem Biol* 2015;11(6):432–7. Available from: <https://doi.org/10.1038/nchembio.1810>.
- [90] Brehmer D, et al. Discovery and pharmacological characterization of JNJ-64619178, a novel small-molecule inhibitor of PRMT5 with potent antitumor activity. *Mol Cancer Ther* 2021;20(12):2317–28. Available from: <https://doi.org/10.1158/1535-7163.MCT-21-0367>.
- [91] Tsai WW, Niessen S, Goebel N, Yates JR, Guccione E, Montminy M. PRMT5 modulates the metabolic response to fasting signals. *Proc Natl Acad Sci U S A* 2013;110(22):8870–5. Available from: <https://doi.org/10.1073/pnas.1304602110>.
- [92] Quan X, et al. The protein arginine methyltransferase PRMT5 regulates A β -induced toxicity in human cells and *Caenorhabditis elegans* models of Alzheimer’s disease. *J Neurochem* 2015;134(5):969–77. Available from: <https://doi.org/10.1111/jnc.13191>.
- [93] Szewczyk MM, et al. Pharmacological inhibition of PRMT7 links arginine monomethylation to the cellular stress response. *Nat Commun* 2020;11(1). Available from: <https://doi.org/10.1038/S41467-020-16271-Z>.
- [94] Srour N, et al. PRMT7 ablation stimulates anti-tumor immunity and sensitizes melanoma to immune checkpoint blockade. *Cell Rep* 2022;38(13). Available from: <https://doi.org/10.1016/J.CELREP.2022.110582>.
- [95] Orben F, et al. Epigenetic drug screening defines a PRMT5 inhibitor–sensitive pancreatic cancer subtype. *JCI Insight* 2022;7(10). Available from: <https://doi.org/10.1172/jci.insight.151353>.
- [96] Dhar S, et al. Loss of the major type I arginine methyltransferase PRMT1 causes substrate scavenging by other PRMTs. *Sci Rep* 2013;. Available from: <https://doi.org/10.1038/srep01311>.
- [97] Hu R, et al. PRMT5 inhibition promotes PD-L1 expression and immuno-resistance in lung cancer. *Front Immunol* 2022;12:5877. Available from: <https://doi.org/10.3389/fimmu.2021.722188>.
- [98] Webby CJ, et al. Jmjd6 catalyses lysyl-hydroxylation of U2AF65, a protein associated with RNA splicing. *Sci (80-)* 2009;325:90–3. Available from: <https://doi.org/10.1126/science.1175865>.
- [99] Walport LJ, et al. Arginine demethylation is catalysed by a subset of JmjC histone lysine demethylases. *Nat Commun* 2016;7. Available from: <https://doi.org/10.1038/ncomms11974>.
- [100] Thompson PR, Fast W. Histone citrullination by protein arginine deiminase: is arginine methylation a green light or a roadblock? *ACS Chem Biol* 2006;1(7):433–41. Available from: <https://doi.org/10.1021/CB6002306/ASSET/IMAGES/MEDIUM/CB-2006-002306-TGR1.JPG>.

PART

Future directions:
translation to the clinic **5**

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Personalized epigenetic therapy—chemosensitivity testing

24

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1 Introduction

Diffuse large B-cell lymphomas (DLBCL) are common non-Hodgkin lymphoid (NHL) malignancies that for most patients follow an aggressive clinical course. Contrasting with most other “solid” tumors, DLBCL are potentially chemotherapy-curable diseases. Chemotherapy is therefore the cornerstone of DLBCL treatment. It has been known for more than forty years that some patients with NHL can be cured using chemotherapy in combination [1]. Since then, a great deal of effort has gone to identify the best combinatorial treatment for DLBCL patients. The best cost-effective regimen, now part of the standard in most countries, resulted to be CHOP, a combination of four chemotherapy agents (cyclophosphamide, doxorubicin, vincristine, and prednisolone) [2,3].

Most of these drugs target the DNA and/or mitosis, common strategies in the 1960s and 1970s to kill highly proliferating tumor cells. Cyclophosphamide, a nitrogen mustard, mainly acts as a DNA-crosslinker attaching into the nitrogen 7 of the guanine base and interfering with DNA replication by forming intrastrand and interstrand DNA crosslinks [4]. Vincristine is a semisynthetic

natural product with a well-characterized action mechanism that involves the disruption of the microtubules and the subsequent cell death by apoptosis [5]. The anthracycline doxorubicin intercalates into DNA and stabilizes the DNA topoisomerase II complex resulting in replication stopping [6]. For three decades there were no further substantial advances on the treatment of DLBCL until rituximab (an anti-CD20 monoclonal antibody) was adopted as part of the standard treatment (R-CHOP) [7]. As a result, the overall response rate for patients with advanced disease increased to 65–70% from about 50–55% with CHOP. Despite these improvements, approximately 25% of the patients will succumb to the disease either due to primary refractory DLBCL or because of a relapsing lymphoma.

Aggressive NHLs on the T-cell counterpart are represented by the heterogeneous group of peripheral T-cell lymphoma (PTCL) that typically associate with poor prognosis with conventional chemotherapy. Despite divergent cells of origin and mechanisms of lymphomagenesis, PTCL therapy has historically followed the treatment framework for DLBCL, in part due to the lack of dedicated prospective studies tailored to subtypes and biomarkers. Thus CHOP is the most prescribed initial treatment for PTCL. Except for anaplastic large cell lymphoma (ALCL) subtype of PTCL, CHOP delivers complete responses in 30%–40% of patients, and long-term survival (measured by 5-year overall survival) in the range of 20%–30% in non-ALCL subtypes [8–10] and relapses remain common [9,11].

2 Chemoresistance in lymphomas

The most consistent finding from a series of randomized controlled clinical trials for NHL patients is the superiority of an anthracycline-containing regimen over regimens that do not contain an anthracycline. Therefore anthracycline resistance, in particular to doxorubicin, remains the primary obstacle for improving the curability rate of aggressive lymphomas with chemotherapy. Chemoresistance may be intrinsic or acquired (a.k.a. adaptive) and may occur through multiple mechanisms. These mechanisms may be classified in two major groups. The first group is decreased drug accumulation and/or increased drug inactivation, for example via upregulation of the multidrug resistance genes. Although this mechanism has been characterized in cellular and animal models [12], its relevance to clinical resistance remains marginal [13].

A second mechanism involves biological mechanisms that prevent cells from dying after chemotherapy-induced damage and/or modify the capacity of cancer cells to repair DNA. The ability of most cancers to resist the cytotoxic effects of chemotherapy is more closely connected to biological abnormalities that affect critical cell cycle checkpoints and cell death pathways than to specific mechanisms of resistance unique to each drug. One approach to improve treatments results in lymphoma would be the addition of adjuvant drugs to “chemosensitize” the tumor cells by interfering with aberrant biological pathways that make cells to resist death from DNA damage. Thus and in the light of many decades of successful curative chemotherapy in aggressive lymphomas, chemosensitization emerges as an attractive strategy to defeat relapsed and refractory disease, and epigenetic therapy promises to be a valid approach to achieve it (Figure 24.1).

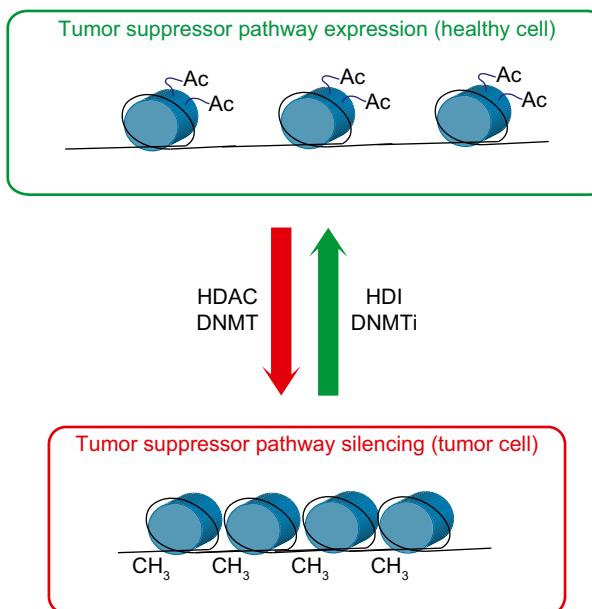


FIGURE 24.1

In cancer cells, the epigenetic balance is tilted to the silencing of tumor suppressor pathways by DNA hypermethylation of genes in the pathway through the action of the DNA methyltransferase and histone deacetylases. Histone deacetylase inhibitors and/or DNMT inhibitors can reverse the balance to reexpression of tumor suppressor pathways. DNMT, DNA methyltransferase.

3 Epigenetically encoded chemoresistance

Gene expression is coordinated by transcription factors (that can activate or repress genes) and by the regulatory state of the chromatin, both at the DNA and histone components. This regulatory state is orchestrated by chromatin modifications, such as DNA cytosine methylation and histone acetylation. Several enzymes, brought to regulatory transcriptional complexes by transcription factors and cofactors, impose these chemical modifications in the DNA and histones. Mutations in these enzymes or alteration in substrates and cofactors may induce aberrant DNA methylation patterns and histone modifications, a common feature in cancer. DNA methylation in mammalian cells occurs at the cytosines and, less frequently, at adenines. Cytosine methylation is catalyzed by maintenance DNA methyltransferase 1 (DNMT1) and de novo methyltransferases DNMT3A and DNMT3B. Demethylation can occur passively as a failure of methylation after replication (a process that increases in presence of DNA damage) or actively through the involvement of TET enzymes. Recurrent mutations in genes involved in the epigenetic machinery are relatively common in aggressive B- and T-cell lymphomas [14–16]. Inherent to the oncogenic state, cancer cells, compared to their cell of origin, exhibit an aberrant pattern of DNA methylation that is globally

characterized by increased DNA hypomethylation of repetitive genomic regions and DNA hypermethylation of promoter and other regulatory regions of, mostly, tumor suppressor genes. Decrease expression of tumor suppressor genes and checkpoints is one the most common mechanisms of lymphomagenesis. This can be achieved, among others mechanisms, by genetic lesions (e.g., mutations in TP53) as well as by epigenetic repression of genes (gene silencing) [17]. From the therapeutic point of view, epigenetic tumor suppressor silencing through DNA hypermethylation offers the opportunity of reversibility.

This aberrant epigenetic patterning is used by malignant cells not only to alter the expression of specific genes that will allow them to sustain the oncogenic state but also to survive extrinsic changes, such as those imposed by chemotherapy agents. Thus pharmacological target of enzymes imposing gene silencing allows the restoring of nonfunctional pathways in tumor cells that may increase their susceptibility to cancer chemotherapy. For example, hypermethylation of DNA damage response genes that are involved in cell death responses, such as MLH1 in solid tumors, are associated with chemoresistance [18,19]. In consequence, demethylation of MLH1 could sensitize cells to chemotherapy [20]. However, DNA methylation can also target and silence a gene that, although favoring tumor progression, increases the tumor's susceptibility to DNA damage. For example, decreased expression of the DNA repair gene encoding *O*-6-methylguanine-DNA methyltransferase MGMT due to promoter hypermethylation is associated with increased sensitivity to alkylating agents [21]. Similarly, DNA hypermethylation of BRCA1 that leads to homologous recombination (HR) deficiency may increase the susceptibility to platinum-based chemotherapy and PARP inhibitors [22]. These examples raise clinical challenges in using DNA hypomethylating agents to sensitize tumors to DNA-damaging agents, including cytotoxic chemotherapy and targeted agents.

4 The evolving epigenetic landscape of lymphomas: treatment and aging

During carcinogenesis, two mutually nonexclusive mechanisms have been described: in one of them the epigenetic alterations are an early step that precede mutations (e.g., when the aberrant methylome of the cancer cells is established at the premalignant state [23]) and, in the other, changes in the genome and epigenome are mutually dependent and occur in parallel (for example when mutations target epigenetic regulators) [24]. The resulting tumor DNA methylation pattern is therefore a composite of the cell-of-origin plus the tumor-specific alterations. In addition, epigenetic alterations in nonmalignant tumor microenvironmental cells are critical contributors in this patterning. These scenarios are also frequently at play during cancer evolution [24,25]. Therefore shaped by microenvironment factors (that include treatment, hypoxia, metabolism, etc.), relapsed and treatment refractory tumors exhibit unique epigenetic alterations [26]. Since epigenetic changes serves to adapt to the treatment pressure, it could also create new therapeutic vulnerabilities for epigenetic agents that can be exploited as combinatorial treatments with chemotherapy in relapsed and refractory tumors [27].

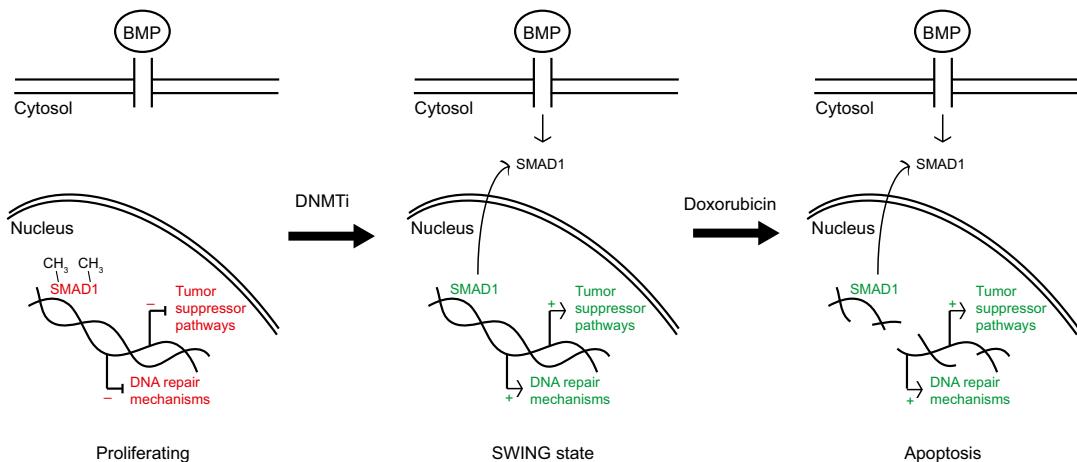
During the aging process, cellular DNA methylation and chromatin landscapes also undergo significant changes [28]. DNA methylation signatures parallel some changes seen in cancer. For

example, aging is also accompanied by an epigenetic drift, characterized by DNA hypomethylation in constitutive heterochromatin repeat intergenic regions (e.g., transposable elements) and DNA hypermethylation of promoters' CpG islands affecting tumor suppressors [28,29]. Moreover, age-associated changes in enhancer DNA methylation leads to chromatin disorganization into large loops, which are normally maintained by binding of boundary factors, such as CTCF and cohesin [28,30]. In addition, aging directly affects relative abundance of histone variants and histone post-translational modifications present in functional regions of the genome, such as enhancers and promoters. These changes occur in all cells to varying degrees ultimately manifesting as age-related epigenetic signatures that can serve as an estimator of the epigenetic age of cells and tissues. Since these epigenetic changes affect the function of malignant and nonmalignant cells, they provide distinct biological features to tumors arising at older age [31]. For example, DLBCL in older people tend to exhibit an epigenetic drift and have an inflammatory microenvironment with activation of NF- κ B [31]. DLBCL in older patients are more likely chemorefractory [31] that together with methylome alterations provides the rational for conducting epigenetic-primed chemosensitization trials in this population [32].

5 Implementing epigenetic therapy to chemosensitize lymphoma

In the current clinical setting several epigenetic-based chemosensitization strategies are being tested. Most therapeutic approaches work by either modifying the chromatin or inducing DNA hypomethylation. Histones and DNA form nucleosomes, the fundamental unit of the chromatin. Each of the core histones contains a few terminal amino acids called the histone tail that protrudes from the nucleosome. Amino acids in these tails can be covalently modified including acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation creating the "histone code" [33]. Addition and removal of these modifications are carried out by enzymes that are targeted pharmacologically. Examples of chromatin modifiers that have been used to chemosensitize tumors include histone deacetylases (HDIs), that decrease histone acetylation. However, since these compounds remove acetyl groups from acetyl-lysines contained in nonhistone proteins, the chemosensitization mechanism is not always primarily epigenetic [34].

DNA hypermethylation can be targeted with DNMT inhibitors (DNMTIs) that can reverse gene silencing, restoring the expression of pathways ultimately affecting lymphoma survival (Figure 24.2) [35]. Most extended DNMT inhibitors, or DNA hypomethylating agents, are 5-aza-2'-deoxycytidine (decitabine) and azacitidine. Both molecules are cytidine nucleosides analogues that incorporate into DNA inactivating irreversibly DNMTs by forming a covalent bond between their 5-azacytosine ring and the DNMT-enzyme. Thus DNMTs are not able to incorporate methyl groups in newly synthesized DNA, resulting in the reduction of DNA methylation. Novel chemical formulations allow for the oral administration of these compounds in combination with chemotherapy. These oral route compounds include the azacitidine analog CC-486 [36] and ASTX727 that is a combination of decitabine with the cytidine deaminase inhibitor cedazurine. In addition, there also direct enzymatic DNMT inhibitors with selectively toward DNMT1 versus DNMT3a/b that are in early stages of clinical development [37].

**FIGURE 24.2**

A depiction of the model proposed for the mechanism-based epigenetic chemosensitization of aggressive lymphomas upon low-dose DNMT inhibitor treatment. DNMT, DNA methyltransferase.

6 What antitumoral effect to expect from epigenetic drugs?

There are basically two main cellular effects from the administration of DNA hypomethylating agents, cell death or more likely, cellular reprogramming. Since DNMTIs are incorporated into DNA, they may induce dose-dependent DNA damage. When this DNA damage exceeds a critical threshold, it may cause apoptosis or any other form of cell death, but does not result in DNA hypomethylation, an effect requiring surviving cells. B- and, more frequently, T-cell lymphoma patients with loss-of-function mutations in the methylcytosine dioxygenase TET2 or isocitrate dehydrogenase 2 (IDH2) show extensive alterations on epigenetic regulation [38,39]. For example, in PTCL recurrently hypermethylated genes involved T-cell receptor signaling and T-cell differentiation genes that likely contribute to lymphomagenesis, providing a strong rationale for clinical application of hypomethylating agents [40,41]. In myelodysplastic syndrome, acute myeloid leukemia patients and preliminary data from PTCL patients indicate that the presence of TET2 mutations may increase the likelihood of response to hypomethylating agents. In this specific molecular background, it is possible to see induction of cell death upon the administration of hypomethylating agents; however, the most likely effect is the induction of cellular reprogramming toward cell differentiation or senescence. Thus although in patients harboring these mutations it is possible to see a decrease in lymphoma burden relatively fast (as with chemotherapy agents), this will not be the most typical effect for majority of the patients. Therefore prospective trials should focus on deliver an amount of drug necessary to maximally achieve a biological effect rather than in tumor burden decrease.

Compared to other cytosine analogs, such as cytarabine, DNMTIs are poor inducers of DNA damage and may create adaptive resistance to other DNA damage agents. Therefore DNMTIs must

be used at doses that induce DNA hypomethylation while minimizing the DNA damaging effect when aimed to restore lymphoma chemosensitivity to chemotherapeutic drugs. Recent data suggest that doses required to induce pathway reactivation and phenotypic changes are significantly lower than maximum tolerated doses for DNMTIs [42]. Epigenetically reprogramming affects cancer and microenvironment cells, that could increase the susceptibility to chemotherapy and other anti-neoplastic agents by a combination of cancer cell-intrinsic and -extrinsic factors. This newly acquired vulnerabilities are being actively exploited in several ongoing clinical trials of DNA hypomethylating agents in combinations with targeted agents.

7 Selecting the right drug for the right patient and vice versa

Although the clinical prognostic factors can stratify populations of patients with DLBCL into broad groups with respect to disease progression, deeper understanding of the molecular disease heterogeneity, both between patients and within lymphomas, is required to a personalized therapeutic approach. In this regard, molecular profiling of lymphomas and their microenvironments can serve to select candidates for epigenetic chemosensitizing therapies [43]. Patient selection and mechanism of epigenetic sensitization are sides of the same coin. There are at least two complimentary approaches toward tumor-selective personalized epigenetic chemosensitization: cellular reprogramming and synthetic lethality.

7.1 Cellular reprogramming

During carcinogenesis certain apoptotic and differentiation pathways are epigenetically inactivated to assure tumor cell survival, being many of these pathways also required for the antineoplastic effect of most chemotherapeutic agents. By reversing epigenetic changes, epigenetic therapy can result in cell reprogramming that, in turn, may trigger cell differentiation, cell death and/or senescence among other possible phenotypes. Cellular reprogramming may also restate pathways that lead to increased chemotherapy sensitivity.

For example, it has been reported that sequential treatment of drug-resistant breast cancer cells first with decitabine followed by doxorubicin, reverses this resistance in more than 80% of treated cells [44]. Decitabine pretreatment induces a depletion in the DNMT1 protein levels that relieves transcriptional repression of *p21* (which is responsible for cell cycle regulation), thereby increasing its expression [44,45]. This DNMT1 reduction also plays an indirect role in *p21* induction via reexpression of methylation-inactivated transcription factors, such as *EGR1*, *SMAD3*, and *HES6*, which interact with *p21* promoter and increase its expression in tumor cells [45–47]. Induction of *p21* causes cells to undergo G2/M arrest that might result in accumulation of Topo II. Since stabilization of Topo II is also an effect of doxorubicin, this pathway reactivation results in increasing doxorubicin tumor cytotoxic effects [48,49].

It is worth noting that reactivation of tumor cell-specific pathways may be more therapeutically crucial than reaching global epigenetic changes (i.e., increase in histone acetylation or DNA hypomethylation). In DLBCL, this is the case for the transforming growth factor β (TGF β) pathway, specifically for its intracellular transducer SMAD1. TGF β belongs to the superfamily of

growth factors involves in the control of cell growth, proliferation, differentiation, apoptosis, and homeostasis of normal B cells. These functions are mediated through proteins from the SMAD family that transduce the extracellular signals from the TGF β ligands to the nucleus where they activate target genes [50]. SMAD1 is epigenetically silenced by DNA hypermethylation in malignant B cells, allowing these cells to sustain the oncogenic phenotype [42,51,52]. Moreover, DNA methylation-mediated silencing of SMAD1 results in chemoresistance in DLBCL [42,53]. Treatment of DLBCL patients with the DNMTi azacitidine hypomethylates and reactivates SMAD1 expression. This makes the TGF β pathway to become functional again, similarly to what is seen in B cells [52,54]. Lymphoma cells exhibiting this gained functionality are now responsive to growth-inhibitory signals through the TGF β pathway, and more importantly, to chemotherapeutic drugs [42]. Therefore cellular epigenetic reprogramming restores a “normal” tumor suppressor pathway in lymphoma cells that causes higher vulnerability to chemotherapy agents. In preclinical DLBCL models, upregulation of TET activity with ascorbic acid-induced SMAD1 upregulation and chemosensitization (Figure 24.2) [55]. Although speculative, patients with genetic lesions affecting TGF β and/or SMAD1 could less likely benefit from this approach and will not be selected for such therapy.

7.2 Synthetic lethality

Originally described in the fruit fly [56] and later introduced to cancer biology [57], the concept of synthetic lethality represents a promising approach to be considered in the field of epigenetic therapy. Synthetic lethality originally refers to the relationship between two genes where loss or inhibition of either gene is compatible with cell viability, but loss or inhibition of both genes ends in cell death. This concept expands to synthetic lethal relationships among pathways that ultimately regulate essential survival functions.

In the context of epigenetics, mutations generated in the cancer cell can result in *de novo* dependences on the activity of certain gene products that in normal cells are not essential. Then, activation or inactivation of these new-essential genes by epigenetic drugs could lead to cell death on cancer cells, leaving normal cells largely unaffected [58]. In this line, we can speculate the following scenario leading to synthetic lethality: genes A and B form a synthetic lethal pair in normal cells. In turn, gene B expression is negatively regulated by a third gene C, normally hypermethylated. A DNMTI will hypomethylate and reactive C that in turn will inhibit B. This situation will be lethal only for cells lacking the synthetic lethal pair A, which may occur exclusively in cancer cells by mutations or any other mechanism. Patients bearing inactivating mutations in gene A will be eligible for a therapy with DNMTI's. Thus epigenetic therapy personalization in lymphoma must necessarily address the identification of the mutations present in the patient that confer synthetic lethality (Figure 24.3). Currently, the only clinical application of synthetic lethality is the use of poly (ADP-ribose) polymerase (PARP) inhibitors in patients bearing tumors with BRCA1 or BRCA2 mutations [59]. PARP detects single-strand DNA breaks and orchestrates its reparation [60]. When PARP activity is inhibited, an alternative form of DNA-repair, HR, buffers the PARP-lack of effect [61]. HR is reliant on functional BRCA1 and BRCA2 genes, and when these genes are mutated this form of DNA-repair cannot occur and cells die when exposed to PARP inhibitors.

	Genotype			Phenotype
No drug	A	+	B	C-CH ₃ Silenced Cell survival
	A	+	B	C-CH ₃ Silenced Cell survival
	A	+	B	C-CH ₃ Silenced Cell survival
	A	+	B	C-CH ₃ Silenced Cell death
DNMTi	A	+	B	C Expressed Cell survival
	A _{mut}	+	B	C Expressed Synthetic lethality

FIGURE 24.3

Genes A and B form a synthetic lethal pair. Loss or inhibition of either gene is compatible with cell viability, but loss or inhibition of both genes results in cell death. Gene C inhibits gene B. Gene C is normally hypermethylated and not expressed. Treatment with a DNA methyltransferase inhibitor restores gene C expression that will inhibit gene B. This approach will be lethal only to cell lacking gene A, for example, by a mutation (A_{mut}).

8 Conclusions

Pharmacologic regulation of epigenetic pathways represents a promising therapeutic strategy in DLBCL. In light of the preclinical and early clinical data, several epigenetic agents such as DNMTi's and chromatin modifiers have demonstrated encouraging pharmacodynamic effects. Importantly, epigenetic chemosensitization is emerging as a key mechanism of action underlying the effect of these drugs. This could probably be the most likely clinical scenario for the translation of pleiotropic epigenetic agents, such as hypomethylating agents.

Mutations in chromatin regulators are not rare events in lymphoma and other tumors. Gain-of-function mutations may lead to specific inhibitors, such as in cases with EZH2 and IDH1/2 mutations. However, mutations affecting HDAC could decrease the effect of HDI as emerging preclinical is showing [62] and in T-cell lymphomas mutations in DNMT3A may decrease the effect of hypomethylating agents. Other resistance mechanisms to epigenetic drugs are likely to arise, since perturbation of epigenetic patterning are frequent events in B-cell lymphomas [63].

The exploitation of cancer-specific vulnerabilities through the identification of synthetic lethal pairs will remain a key issue for the development of personalized epigenetic therapies. It will be crucial to identify and characterize epigenetic lesions present in each patient to select the best strategy to activate or inactivate the lethal counterpart. More importantly, the concept of biologically driven treatments should be included from the early beginning when designing clinical trials of epigenetic drugs. Research aimed to characterize tumor genetic, epigenetic and microenvironmental features, as well as to identify biomarkers that account for the molecular effect of these drugs will be necessary to successfully translate epigenetic therapies to patients.

Abbreviations

CHOP	cyclophosphamide, doxorubicin, vincristine, and prednisolone
DLBCL	diffuse large B-cell lymphomas
DNMT	DNA methyltransferases
DNMTi	DNA methyltransferase inhibitor
HAT	histone acetyltransferases
HDAC	histone deacetylases
HDI	histone deacetylase inhibitor
MDR/TAP	multidrug resistance/antigen peptide transporter

References

- [1] Levitt M, Marsh JC, DeConti RC, et al. Combination sequential chemotherapy in advanced reticulum cell sarcoma. *Cancer* 1972;29(3):630–6.
- [2] Gottlieb JA, Guterman JU, McCredie KB, Rodriguez V, Frei 3rd. E. Chemotherapy of malignant lymphoma with adriamycin. *Cancer Res* 1973;33(11):3024–8.
- [3] McKelvey EM, Gottlieb JA, Wilson HE, et al. Hydroxyldaunomycin (Adriamycin) combination chemotherapy in malignant lymphoma. *Cancer* 1976;38(4):1484–93.
- [4] Dong Q, Barsky D, Colvin ME, et al. A structural basis for a phosphoramido mustard-induced DNA interstrand cross-link at 5'-d(GAC). *Proc Natl Acad Sci U S A* 1995;92(26):12170–4.
- [5] Gidding CE, Kellie SJ, Kamps WA, de Graaf SS. Vincristine revisited. *Crit Rev Oncol Hematol* 1999;29(3):267–87.
- [6] Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol* 1999;57(7):727–41.
- [7] Coiffier B, Haioun C, Ketterer N, et al. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter phase II study. *Blood* 1998;92(6):1927–32.
- [8] Mourad N, Mounier N, Brière J, et al. Clinical, biologic, and pathologic features in 157 patients with angioimmunoblastic T-cell lymphoma treated within the Groupe d'Etude des Lymphomes de l'Adulte (GELA) trials. *Blood* 2008;111(9):4463–70.
- [9] Reimer P, Rüdiger T, Geissinger E, et al. Autologous stem-cell transplantation as first-line therapy in peripheral T-cell lymphomas: results of a prospective multicenter study. *J Clin Oncol* 2009;27(1):106–13.
- [10] Simon A, Peoch M, Casassus P, et al. Upfront VIP-reinforced-ABVD (VIP-rABVD) is not superior to CHOP/21 in newly diagnosed peripheral T cell lymphoma. Results of the randomized phase III trial GOELAMS-LTP95. *Br J Haematol* 2010;151(2):159–66.
- [11] d'Amore F, Relander T, Lauritsen GF, et al. Up-front autologous stem-cell transplantation in peripheral T-cell lymphoma: NLG-T-01. *J Clin Oncol* 2012;30(25):3093–9.
- [12] Binkhathlan Z, Lavasanifar A. P-glycoprotein inhibition as a therapeutic approach for overcoming multidrug resistance in cancer: current status and future perspectives. *Curr Cancer Drug Targets* 2013;13(3):326–46.
- [13] Libby E, Hromas R. Dismounting the MDR horse. *Blood* 2010;116(20):4037–8.
- [14] Jiang Y, Melnick A. The epigenetic basis of diffuse large B-cell lymphoma. *Semin Hematol* 2015;52(2):86–96.
- [15] Pasqualucci L, Dalla-Favera R. Genetics of diffuse large B-cell lymphoma. *Blood* 2018;131(21):2307–19.

- [16] Liu J, Li JN, Wu H, Liu P. The status and prospects of epigenetics in the treatment of lymphoma. *Front Oncol* 2022;12:874645.
- [17] Isshiki Y, Melnick A. Epigenetic mechanisms of therapy resistance in diffuse large B cell lymphoma (DLBCL). *Curr Cancer Drug Targets* 2021;21(4):274–82.
- [18] Zeller C, Dai W, Steele NL, et al. Candidate DNA methylation drivers of acquired cisplatin resistance in ovarian cancer identified by methylome and expression profiling. *Oncogene* 2012;31(42):4567–76.
- [19] Gallito M, Cheng He R, Inocencio JF, et al. Epigenetic preconditioning with decitabine sensitizes glioblastoma to temozolomide via induction of MLH1. *J Neurooncol* 2020;147(3):557–66.
- [20] Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. *Cancer Res* 2000;60(21):6039–44.
- [21] Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 2005;352(10):997–1003.
- [22] Garner IM, Brown R. Is there a role for epigenetic therapies in modulating DNA damage repair pathways to enhance chemotherapy and overcome drug resistance? *Cancers (Basel)* 2022;14(6).
- [23] Kretzmer H, Biran A, Purroy N, et al. Preneoplastic alterations define CLL DNA methylome and persist through disease progression and therapy. *Blood Cancer Discov* 2021;2(1):54–69.
- [24] Flavahan WA, Gaskell E, Bernstein BE. Epigenetic plasticity and the hallmarks of cancer. *Science* 2017;357(6348).
- [25] Tsagiropoulou M, Papakonstantinou N, Moysiadis T, et al. DNA methylation profiles in chronic lymphocytic leukemia patients treated with chemoimmunotherapy. *Clin Epigenetics* 2019;11(1):177.
- [26] Pan H, Jiang Y, Boi M, et al. Epigenomic evolution in diffuse large B-cell lymphomas. *Nat Commun* 2015;6:6921.
- [27] Hess BT, Cerchietti L, Hendrickson L, et al. Phase I study of oral azacitidine (CC-486) plus salvage chemotherapy in relapsed/refractory diffuse large B-cell lymphoma. *Blood* 2021;138(Supplement 1):3567.
- [28] Michalak EM, Burr ML, Bannister AJ, Dawson MA. The roles of DNA, RNA and histone methylation in ageing and cancer. *Nat Rev Mol Cell Biol* 2019;20(10):573–89.
- [29] Li Y, Tollefsbol TO. Age-related epigenetic drift and phenotypic plasticity loss: implications in prevention of age-related human diseases. *Epigenomics* 2016;8(12):1637–51.
- [30] Stadler MB, Murr R, Burger L, et al. DNA-binding factors shape the mouse methylome at distal regulatory regions. *Nature* 2011;480(7378):490–5.
- [31] Marullo R, Revuelta MV, Béguelin W, Lara-García J, Cerchietti L. Aging-associated epigenetic reprogramming alters the germinal center reaction and targets pathways related to lymphomagenesis. *Blood* 2021;138:676.
- [32] Brem EA, Li H, Beaven AW, et al. SWOG 1918: a phase II/III randomized study of R-miniCHOP with or without oral azacitidine (CC-486) in participants age 75 years or older with newly diagnosed aggressive non-Hodgkin lymphomas—aiming to improve therapy, outcomes, and validate a prospective frailty tool. *J Geriatr Oncol* 2022;13(2):258–64.
- [33] Strahl BD, Allis CD. The language of covalent histone modifications. *Nature* 2000;403(6765):41–5.
- [34] Ha K, Fiskus W, Choi DS, et al. Histone deacetylase inhibitor treatment induces 'BRCAness' and synergistic lethality with PARP inhibitor and cisplatin against human triple negative breast cancer cells. *Oncotarget* 2014;5(14):5637–50.
- [35] Li Y, Nagai H, Ohno T, et al. Aberrant DNA methylation of p57(KIP2) gene in the promoter region in lymphoid malignancies of B-cell phenotype. *Blood* 2002;100(7):2572–7.
- [36] Martin P, Bartlett NL, Chavez JC, et al. Phase 1 study of oral azacitidine (CC-486) plus R-CHOP in previously untreated intermediate- to high-risk DLBCL. *Blood* 2022;139(8):1147–59.
- [37] Xie T, Yu J, Fu W, et al. Insight into the selective binding mechanism of DNMT1 and DNMT3A inhibitors: a molecular simulation study. *Phys Chem Chem Phys* 2019;21(24):12931–47.

- [38] Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* 2016;127(20):2375–90.
- [39] de Leval L, Rickman DS, Thielen C, et al. The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (TFH) cells. *Blood* 2007;109(11):4952–63.
- [40] Wang C, McKeithan TW, Gong Q, et al. *IDH2^{R172}* mutations define a unique subgroup of patients with angioimmunoblastic T-cell lymphoma. *Blood* 2015;126(15):1741–52.
- [41] Odejide O, Weigert O, Lane AA, et al. A targeted mutational landscape of angioimmunoblastic T-cell lymphoma. *Blood* 2014;123(9):1293–6.
- [42] Clozel T, Yang S, Elstrom RL, et al. Mechanism-based epigenetic chemosensitization therapy of diffuse large B-cell lymphoma. *Cancer Discov* 2013;3(9):1002–19.
- [43] Kotlov N, Bagaev A, Revuelta MV, et al. Clinical and biological subtypes of B-cell lymphoma revealed by microenvironmental signatures. *Cancer Discov* 2021;11(6):1468–89.
- [44] Vijayaraghavalu S, Dermawan JK, Cheriyath V, Labhasetwar V. Highly synergistic effect of sequential treatment with epigenetic and anticancer drugs to overcome drug resistance in breast cancer cells is mediated via activation of p21 gene expression leading to G2/M cycle arrest. *Mol Pharm* 2013;10(1):337–52.
- [45] Young JI, Sedivy JM, Smith JR. Telomerase expression in normal human fibroblasts stabilizes DNA 5-methylcytosine transferase I. *J Biol Chem* 2003;278(22):19904–8.
- [46] Drenzek JG, Seiler NL, Jaskula-Sztul R, Rausch MM, Rose SL. Xanthohumol decreases Notch1 expression and cell growth by cell cycle arrest and induction of apoptosis in epithelial ovarian cancer cell lines. *Gynecol Oncol* 2011;122(2):396–401.
- [47] Ijichi H, Otsuka M, Tateishi K, et al. Smad4-independent regulation of p21/WAF1 by transforming growth factor-beta. *Oncogene* 2004;23(5):1043–51.
- [48] Walker JV, Nitiss JL. DNA topoisomerase II as a target for cancer chemotherapy. *Cancer Investig* 2002;20(4):570–89.
- [49] Quan ZW, Yue JN, Li JY, Qin YY, Guo RS, Li SG. Somatostatin elevates topoisomerase II alpha and enhances the cytotoxic effect of doxorubicin on gallbladder cancer cells. *Chemotherapy* 2008;54(6):431–7.
- [50] Massague J. TGFbeta in cancer. *Cell* 2008;134(2):215–30.
- [51] Gerlach MM, Stelling-Germani A, Ting Wu C, et al. SMAD1 promoter hypermethylation and lack of SMAD1 expression in Hodgkin lymphoma: a potential target for hypomethylating drug therapy. *Haematologica* 2021;106(2):619–21.
- [52] Stelling A, Wu CT, Bertram K, et al. Pharmacological DNA demethylation restores SMAD1 expression and tumor suppressive signaling in diffuse large B-cell lymphoma. *Blood Adv* 2019;3(20):3020–32.
- [53] Leshchenko VV, Kuo PY, Jiang Z, Thirukonda VK, Parekh S. Integrative genomic analysis of temozolomide resistance in diffuse large B-cell lymphoma. *Clin Cancer Res* 2014;20(2):382–92.
- [54] Stelling A, Hashwah H, Bertram K, Manz MG, Tzankov A, Muller A. The tumor suppressive TGF-beta/SMAD1/S1PR2 signaling axis is recurrently inactivated in diffuse large B-cell lymphoma. *Blood* 2018;131(20):2235–46.
- [55] Shenoy N, Bhagat T, Nieves E, et al. Upregulation of TET activity with ascorbic acid induces epigenetic modulation of lymphoma cells. *Blood Cancer J* 2017;7(7):e587.
- [56] Lucchesi JC. Synthetic lethality and semi-lethality among functionally related mutants of *Drosophila melanogaster*. *Genetics* 1968;59(1):37–44.
- [57] Hartwell LH, Szankasi P, Roberts CJ, Murray AW, Friend SH. Integrating genetic approaches into the discovery of anticancer drugs. *Science* 1997;278(5340):1064–8.
- [58] Mair B, Kubicek S, Nijman SM. Exploiting epigenetic vulnerabilities for cancer therapeutics. *Trends Pharmacol Sci* 2014;35(3):136–45.

- [59] Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* 2009;361(2):123–34.
- [60] Ame JC, Spenlehauer C, de Murcia G. The PARP superfamily. *Bioessays* 2004;26(8):882–93.
- [61] Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005;434(7035):917–21.
- [62] Cerchietti LC, Hatzi K, Caldas-Lopes E, et al. BCL6 repression of EP300 in human diffuse large B cell lymphoma cells provides a basis for rational combinatorial therapy. *J Clin Invest* 2010;120(12):4569–82.
- [63] Shaknovich R, Melnick A. Epigenetics and B-cell lymphoma. *Curr OpHematol* 2011;18(4):293–9.

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Epigenetic profiling in cancer: triage, prognosis, and precision oncology

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1 Introduction

Over the past decade, notable progress has been made in the field of cancer diagnosis and treatment. However, it is forecasted that cancer will become the leading cause of premature death over the course of this century. In 2020 World Health Organisation estimated 10 million cancer-related deaths [1], resulting in significant negative effects on patients, their families, and society at large,

as well as incurring a significant economic cost of \$167 billion. Due to demographic shifts towards an aging population, the global cancer burden is expected to double in the next 50 years [2]. A major contributor to these losses is the frequent diagnosis of cancer at advanced stages, coupled with inadequate treatment response. Research suggests that up to 50% of cancer cases could be prevented through the implementation of biomarkers with strong diagnostic, prognostic, and treatment-response predictive capabilities [2].

Epigenetic alterations, including DNA methylation, histone modification, and microRNA (miRNA) regulation, have been proposed as possible biomarker candidates as they play a role in every stage of tumor formation and progression. Distinct patterns of these modifications have been reproducibly linked to specific types of cancer. This chapter aims to provide an overview of the multifaceted involvement of these signatures in carcinogenesis and to evaluate the feasibility of clinical implementation of epigenetic-based tests through examination of selected case studies.

1.1 DNA methylation

The most studied epigenetic mark is DNA methylation, which involves the addition of methyl groups to position 5 of a cytosine residue. This reaction is catalyzed by DNA methyl transferases (DNMTs), resulting in 5-methylcytosine (5-mC). DNA methylation usually occurs on CpG dinucleotide sequence. Around 70%–80% of CpGs are methylated by default in somatic cells; however, CpG-rich sequences (CpG islands) are interspersed in the genome and often unmethylated in germ and somatic cells. CpG islands are characterized as regions with a methylated/unmethylated CpG ratio >0.6 [3]. The alteration of canonical DNA methylation patterns is a hallmark of human cancers, and it is typically associated with loss of global genomic DNA methylation (hypomethylation) accompanied by site-specific hypermethylation. Global DNA hypomethylation may disrupt chromosome condensation and stability, while site-specific DNA hypermethylation occurs on CpG islands present on regulatory sites. Specific examples will be discussed at length in the subsequent sections.

DNA methylation profiles change with age and so does the risk of cancer. Aging brings about global DNA hypermethylation and cancer might accelerate this event. As a matter of fact, recent data indicate that age-related methylation changes in blood accelerate during acute myeloid leukemia (AML) [4]. Steve Horvath was the first to use DNA methylation signatures that are correlated to chronological age to build an epigenetic clock for age prediction [5,6]. Like most epigenetic changes, DNA methylation is highly sensitive to the environment, with diet, exercise, medication, or smoking influencing levels at specific CpG sites. Therefore, the clock may predict aging deviations caused by lifestyle or the presence of disease. In fact, the epigenetic clock has been applied to cancer cohorts and the ages of glioblastoma tissues were found to be accelerated [7] and so were the ones of pancreatic cancers [8]. These data are of particular importance as it pertains to deviations of the norm, highlighting the potential of epigenetic aging as a biomarker for cancer outcomes.

It is well-established that epigenetic changes play a critical role in the cancer development and progression. Whilst the majority of such changes are of a somatic nature, instances of inherited constitutional epigenetic alterations, or epimutations, have also been reported [9]. These epimutations are characterized by the promoter methylation and transcription silencing of a single allele, in the absence of any genetic mutations. One example is hypermethylation of the *MLH1* promoter, which is commonly observed in colorectal cancer to disrupt the mismatch repair (MMR) pathway. This particular epimutation was observed in 20% of families with Lynch syndrome, a condition

characterized by susceptibility to the development of colorectal and additional cancers involving microsatellite instability as a consequence of an impaired MMR pathway [10]. Other examples of constitutional epimutations are present in familial breast and ovarian cancer syndrome with *BRCA1* and *RAD51C* showing promoter hypermethylation, accompanied by associated gene silencing events [11]. It is also worth noting that identifying epimutations can be a cost-effective method to select and refer patients with a strong familial history of cancer for germline analysis.

1.2 Histone modifications

Covalent modifications at specific amino acids on histones can greatly impact DNA-related processes such as transcription, repair, recombination, and packaging. Each histone core protein possesses a characteristic tail which is rich in lysine and arginine residues. Histone modifications, such as acetylation and methylation, can result from genetic mutations in histone modifiers, such as histone deacetylases (HDACs), acetyltransferases (HATs), methyltransferases (HMTs), or methyl demethylases (HDMs). These modifications neutralize the positive charge on histone tails, thereby weakening the electrostatic interaction between the DNA and histones, and subsequently influencing the compaction state of chromatin. Furthermore, such modifications create docking sites within chromatin that are recognizable by transcriptional complexes. One example of this can be observed in prostate cancer, where the transcription of androgen receptor (AR) target genes is affected. Agonists of AR promote the recruitment of coactivators with HAT activity at transcription starting sites, leading to histone acetylation and active transcription [12]. Inversely, antagonists of AR (i.e., bicalutamide) activate HDACs to limit expression [13].

1.3 miRNAs

Aberrant miRNA expression and is an attractive biomarker for diagnostic and/or prognostic predictors in many cancers. Generally, miRNAs effects are mediated through binding primarily to the 3'-untranslated region of the target mRNA(s), resulting in mRNA degradation and/or translational repression. One example of miRNA involvement in cancer is in colorectal cancer, where it was found that miRNAs can impact the Wnt/β-catenin pathway. This pathway is known to be one of the earliest events driving the development of colorectal cancer. When activated, the pathway promotes cell division and growth and is controlled by a protein called β-catenin. Constitutively active β-catenin upregulates the expression of Wnt target genes to promote division of cancer cells. To limit its movement into the nucleus, β-catenin is found in the cytoplasm, attached to the degradation complex. When Wnt is active, β-catenin is released and migrates to the nucleus where it stimulates Wnt signaling [14]. miR-224 was shown to enhance the Wnt/β-catenin signaling by downregulating the expression of degradation complex components, leading to β-catenin presence in the nucleus. Consequently, knockdown of miR-224 was found to restore transcription and inhibit Wnt/β-catenin-mediated cell metastasis and proliferation [14]. Many other miRNA species have been reported as being involved in carcinogenesis. In gastric cancer, six miRNA species (miR-601, miR-107, miR-18a, miR-370, miR-300, and miR-96) showed increased expression in cancer compared to normal or adenoma samples making them potential biomarkers for screening [15]. Similarly, in breast cancer, high miR-21, miR-155, miR-27a, miR-205, miR-145, and miR-320a have been associated with treatment resistance [16].

1.4 Feasibility for clinical implementation

Effective implementation of epigenetic profiling in clinical settings has its barriers. Notably, selection of the appropriate biomarkers and analytical platforms, the results turnaround time, the tools available to interpret the results as well as financial constraints are the most pressing challenges. We are generating a large number of biomarkers, when in reality, only a few can be integrated in clinical settings. A major goal of biomedical research in epigenetics is to identify feasible biomarkers from the high number of candidates generated from procedures like Whole Genome Bisulfite Sequencing (WGBS), Reduced Representation Bisulfite Sequencing (RRBS), Illumina microarray technology, Chromatin Immunoprecipitation Sequencing (ChIP-Seq), small RNA-seq long-noncoding RNA-seq among others. However, the extraction of relevant data is time consuming and requires the utilization of bio-informatic and computational procedures to analyze very large data sets. An integrated approach combining data generation, data selection, and AI analysis is needed to address this issue. Beyond identifying the right biomarkers, clinical implementation requires the new tests to be simple, validated in different settings, and performed in analytical methods already present in the clinics, like RT-qPCR and microarrays for both DNA methylation and miRNA analysis. Next-generation sequencing is also being introduced into clinical routines, partly due to the reduction in prices for high throughput analysis in the recent years. Adoption of new technologies in clinical settings joined by the efforts of the medical community, pharmaceutical industry, and medical device manufacturers will feasibly contribute to clinical implementation of epigenetic profiling.

Compared to proteins or RNA, DNA methylation, miRNA, and other post-translational modifications show high stability in biofluids or and in samples of moderate quality, such as formalin-fixed paraffin embedded (FFPE). To date, DNA methylation assays have been thoroughly validated, with reports of commercially available in vitro diagnostic (IVD) tools approved by the FDA ([Table 25.1](#)). As minimal invasiveness is preferred, DNA methylation testing extended beyond tissue analysis to circulating cell-free DNA (cfDNA), circulating tumor cells (CTCs) and exosomes to allow collection of information from sampling methods such as feces, urine, and liquid biopsies. For instance, the Epi proLung® originally received CE-IVD certification for the analysis of *SHOX2* methylation from bronchial lavage specimens. From 2011, the test shifted toward the analysis of cfDNA from blood plasma. In 2017 the Epi proLung® liquid biopsy test, looking at *SHOX2* and *PTGER4* methylation, received CE-IVD certification.

The analysis of DNA methylation, a relatively stable epigenetic mark, can be performed through methods based on four principles: (1) digestion with methylation-sensitive restriction enzymes, (2) use of anti-methylcytosine antibodies, (3) bisulfite or enzymatic treatment to convert unmethylated cytosines into uracils, (4) library preparation for third-generation sequencing, that is, nanopore technologies [[17](#)]. While many DNA methylation assays are reliable and can be implemented in a clinical setting, bisulfite pyrosequencing and amplicon bisulfite sequencing are considered the most cost-efficient and scalable options [[18](#)]. However, with the reduction in cost of sequencing, many more opportunities can be unlocked, such as the use of portable Oxford Nanopore devices for affordable point-of-care testing in low- and middle-income countries [[19](#)]. In addition, Galleri, a multi-cancer early detection test which utilizes custom Illumina arrays, is currently being trialed to evaluate feasibility of implementation in National Health Service (NHS) screening programs. This test provides insight into the potential of DNA methylation-based tests for population-scale cancer screening.

TABLE 25.1 Epigenetic assays for cancer triage, prognosis, and response prediction.

Cancer type	Epigenetic mark	Technology platform	Biospecimen	Commercial tool	Application
Cervical	DNA methylation on ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671	qMSP	Epithelial cells from cervical brush	GynTect®	Triage of hrHPV positive women to colposcopy
	DNA methylation on FAM19A4 and mir124-2	qMSP	Epithelial cells from cervical brush, urine	QIAsure Methylation Test	Triage of hrHPV positive women to colposcopy
	DNA methylation on ZNF582	qMSP	Epithelial cells from cervical brush	Cervi-M® assay	Early-stage cervical cancer detection
	DNA methylation on EPB41L3 and viral late genes of HPV16, HPV18, HPV31, and HPV33	Pyrosequencing	Epithelial cells from cervical brush, urine	n/a	Triage of hrHPV positive women to colposcopy
	DNA methylation on NDRG4 and BMP3	qMSP	Stool	Cologuard®	Early-stage colorectal cancer detection
Colorectal	DNA methylation on SEPT9 and ACTB	qMSP	cfDNA from blood plasma	Epi proColon® 2.0	Early-stage colorectal cancer detection
	DNA methylation on SDC2	qMSP	cfDNA from blood plasma	EarlyTect® CRC assay	Early-stage colorectal cancer detection
	DNA methylation on PDX1, EN2, and MSX1	qMSP	Fresh tissue	n/a	Prognosis of colorectal cancer severity
	miR-31-3p	RT-qPCR	FFPE	miRPreDX-31-3p	Response prediction to anti-EGFR therapy
Lung	DNA methylation on SHOX2, PTGER4	qMSP	cfDNA from blood plasma	Epi proLung®	Triage of patients at high-risk of lung cancer development
Prostate	DNA methylation on GSTP1, RASSF1, and APC	qMSP	FFPE	ConfirmMDx	Prognosis of prostate cancer, Supplement of traditional diagnosis to indicate cancer-free histopathology
	DNA methylation on HOXD3 and GSTP1	qMSP	cfDNA from urine	ProCUrE	Prognosis and detection of prostate cancer
	DNA methylation on GSTP1 and APC	qMSP	cfDNA from urine	EpiCaPture	Prognosis and detection of prostate cancer

(Continued)

TABLE 25.1 Epigenetic assays for cancer triage, prognosis, and response prediction. *Continued*

Cancer type	Epigenetic mark	Technology platform	Biospecimen	Commercial tool	Application
AML	DNA methylation on CD34, RHOC, SSCRN1, F2RL1, FAM92A1, MIR155HG, and VWA8	Pyrosequencing	cfDNA from blood	n/a	Prognosis and identification of novel AML subsets for treatment guidance
	DNA methylation on TET2	Pyrosequencing	cfDNA from blood	n/a	Response prediction to cytarabine-based chemotherapy
Breast	DNA methylation on PITX2	RT-qPCR	FFPE, DNA from blood	Therascreen PITX2 RGQ PCR Kit	Response prediction to anthracyclines chemotherapy
Glioblastoma	DNA methylation on MGMT	Pyrosequencing	FFPE, DNA from blood	Therascreen MGMT Pyro Kit	Response prediction to the alkylating agent temozolomide
Pancreatic	DNA hydroximethylation on GATA4, GATA6, PROX1, ONECUT1, MEIS2, YAP1, TEAD1, PROX1, IGF1	DNA sequencing on NextSeq550	cfDNA from blood plasma	n/a	Triage of pancreatic ductal adenocarcinoma based on stage
Unknown origin	Analysis of 450K CpG sites	Human methylation Beadchip 450K (Illumina)	FFPE	EPICUP	Triage of cancers with unknown origin based on primary origin site

2 Epigenetics testing in cancer screening and triage

Clinical investigations of common cancer types, such as cervical, lung, colorectal, or breast, proved that screening does save lives. One of the best examples of the power of early detection and reduction of cancer burden is for cervical cancer. Since the implementation of the first morphological based test (Pap smear) in the 1950s, mortality has been reduced by more than 70% [20]. However, for breast cancer, mammography screening in women aged 50–59, show that more than 1300 women need to be evaluated to save one single life [21]. When the biomarkers used lack the necessary accuracy, screening can potentially be harmful, leading to unnecessary or invasive follow-up tests as well as the anxiety derived from false-positive results. Prostate-specific antigen (PSA)-based screening for prostate cancer highlights the potential consequences of high false positives and overdiagnosis. Despite the PSA test benefiting some men, in 2018, the US Preventive Services Task Force concluded that the false positivity issue brings both physiological and physical complications to up to 50% men with positive PSA but no disease after biopsy [22]. In the need for performance biomarkers, epigenetics has been investigated for triaging high-risk individuals for adequate treatment.

2.1 Triaging HPV-positive women

The implementation of systematic cytology screening contributed to a reduction in cervical cancer-associated deaths. Yet, cervical cancer is still the fourth most common cancer in women worldwide with 604,000 cases in 2020, accounting for 7.5% of all female cancer deaths [1]. Persistent infection with high-risk HPV (hrHPV) is a necessary, but not sufficient factor for development of cervical cancer and its precursor, cervical intraepithelial neoplasia (CIN). However, most hrHPV infections are transient, persistent beyond 2 years and occur in <10% of women. As more than 90% of cases are caused by hrHPV, screening shifted toward hr-HPV testing with triage of HPV positive women to allow a further reduction in the incidence of cervical cancer. Triage generally relies on cytology as an option for a secondary triage test. Despite establishing HPV vaccination program since 2008, women born before 1991 are not covered, as a significant proportion of the population had not been immunized against HPV prior to becoming sexually active [20]. With the youngest women in their late 20 s, means that cervical screening will remain a dominant part of cervical cancer prevention for at least 30 years.

Cytology triage is subjective. Examining cells under the microscope relies on human judgment to interpret what is seen. Consequently, many more women may be considered morphologically abnormal when they are known to be positive for hrHPV. Although showing a higher specificity in identifying CIN2+ than HPV testing, cytology-triaging might not have the capacity to rule out hrHPV positive women without evidence of disease leading to a higher number of histology referrals (colposcopy) [23]. Notably, all hrHPV-positive women with abnormal cytology results are referred for colposcopy to receive the appropriate CIN diagnostics. Women can also be misdiagnosed at this stage if the histology sample does not include the diseased tissue. All CIN2+ women are treated by removal of the abnormal tissue, however CIN2 lesions might regress, which is difficult to predict, so surgical treatment is the default option. Moreover, some women are likely to be overtreated, young women, especially being at great risk of having their fertility impaired due to invasive surgery [23]. Overall, cytology analysis requires skilled workforce and constant financial

investment to assure systematic screening, being mostly available to countries with the relevant infrastructure.

Compared to the current morphologically based triage methodologies, epigenetic testing offers the advantages of potential automatization and objectivity. Epigenetic testing can be performed on the same clinician-collected or self-collected specimens used for HPV-testing. Unlike HPV genotyping, methylation biomarkers have the potential to distinguish between persistent and transient HPV infections.

Following persistent infection, HPV-induced methylation of host DNA was established to play an important role in viral immune evasion. The HPV oncoproteins E6 and E7 alter the DNA methylation in host cells by modulating DNMTs activity. HPV-E7 indirectly increases *DNMT1* expression by forming the E7-pRb complex which allows the released transcription factor E2F to activate the promoter of *DNMT1*. E7 was also demonstrated to directly bind to *DNMT1*, inducing conformational changes, which enhance its methyltransferase activity, promoting host DNA hypermethylation [24].

Approximately 10 human genes have been consistently identified to be differentially methylated in patients with cervical disease versus healthy controls. These are *CADM1*, *EPB41L3*, *FAM19A4*, *MAL*, *PAX1*, *SOX1*, *TERT*, *PRDM14*, *JAM3*, *C13ORF18*, and miR-124 [25]. The functional relevance of these markers has been shown through *in vitro* studies, which revealed that the onset of methylation might differ between genes. Verlaat et al. argued that DNA methylation occurs at the precancer stage and reaches the highest levels after hrHPV-induced carcinogenesis. This might be due to the different susceptibility of specific promoters to DNA methylation by the E6/E7 activated DNMTs. When analyzed in cervical exfoliated cells covering an array of histology types (no disease, CIN2, CIN3, and cancer), all genes analyzed except *C13ORF18*, showed a gradual increase in methylation with disease progression. In all cases there was a constant increase in methylation levels with disease progression and age [26]. These were considered promising biomarkers to identify hrHPV-positive women and/or women with abnormal cytology at risk for cervical cancer.

For methylation testing to be introduced in the current screening algorithms, its performance must be better than cytology. A recent meta-analysis described the performance of DNA methylation assays in early detection of CIN2+ and cancer [27]. All genes were pooled and a set specificity of 70.0% which is required for improving hrHPV positive women triage to colposcopy. As a triage test, DNA methylation had higher specificity than cytology. The reported sensitivity was 69% for CIN2+ and 71% for CIN3+ detection [27]. Although the sensitivity was lower than cytology for CIN2+, the set specificity was higher, meaning that the methylation markers, can help decrease the number of unnecessary biopsies and follow-up procedures at the expense of some false-negative results.

Although methylation assays have reached commercialization, real-world validation is required for adoption into screening algorithms. GynTect® and QIAsure Methylation Test are two examples of commercialized cervical cancer detection assays. The GynTect® assay investigates the methylation of *ASTN1*, *DLXI*, *ITGA4*, *RXFP3*, *SOX17*, and *ZNF671*. In a study including more than 300 women with abnormal colposcopy, the GynTect® assay showed a 68% sensitivity for CIN3+ detection, with all invasive cancers being correctly identified by the test. When a triage screening setting was modeled, with GynTect replacing cytology, the overall sensitivity declined, but specificity was 89% [28]. Having the possibility to triage hrHPV-positive women from the same self-collected specimen brings many advantages including a reduction in logistics associated with systematic screening and eliminating objectivity. However, the test did not perform as well in self-sampling specimens compared to physician collected specimens [29].

The QIAsure Methylation Test Kit looks at the promoter hypermethylation of *FAM19A4* and mir124-2. Using liquid-based cytology samples, Vink et al. reported that the CIN3 + sensitivity of *FAM19A4*/miR124-2 methylation analysis was comparable to that of cytology (71% vs 76%). Combining *FAM19A4*/miR124-2 methylation analysis with cytology resulted in an increased sensitivity of 84.6% at a set specificity of 70% [30]. QIAsure showed a good clinical performance as an alternative or addition to cytology triage in an EU-multicenter, retrospective study, with sensitivity and specificity being 77% and 78%, respectively. The likelihood of an individual not to have the disease prior to both hrHPV positive and methylation negative tests was 99.9% for cervical cancer and 93% for \geq CIN2 [31]. In addition, a study led by De Strooper et al., showed that the QIAsure methylation test can be performed in the same self-collected samples used for primary hrHPV testing. The performance of QIAsure to detect CIN3 + was 69% sensitivity and 76% specificity. In combination with HPV16/18 genotyping on the same samples, the sensitivity increased to 85% while specificity decreased to 55%. These data refer to the *FAM19A4*/miR124-2 methylation test for testing in a real-life pilot for alternative/addition to cytology triage [31].

Another test, the S5 methylation classifier, utilizes a combination of host (*EPB41L3*) and viral methylation targets (L1 and L2) of HPV16, HPV18, HPV31 and HPV33. Data shows good separation between women with CIN2 + from those with CIN1 or less (cytology negative). At a cutoff value of 0.8, sensitivity was 74%, specificity was 65% [32]. Additionally, the S5-classifier demonstrated improved triage performance compared to HPV genotyping, cytology or the combination thereof and has been validated in an HPV-positive cohort of women as part of three clinical trials [33]. Taking into account the prevalence of HPV infections as well as the difference in screening capacity and performance of populations can affect disease prevalence, the test has been designed to be “setting specific”, with an adjustable cutoff value [33]. A recent study shows that an increased cutoff with a lower number of false positives rate would maximize the detection of cancer, given the lack of resources in low and medium resource countries.

A fundamental aspect of molecular triage in cervical cancer screening is its ability to prioritize women for treatment. DNA-methylation based tests could help decision making by providing healthcare professionals with tools to assess disease risk. So far, none of these tests have been adopted in the screening algorithms, validation data on real-life settings being required. As cervical cancer incidence rates are disproportionately high in low and middle income countries compared to developed countries (18.8 vs 11.3 cases per 100,000) [1], methylation testing should be adapted according to any clinical setting. In a low- and middle-income setting, an increased threshold with a lower number of false-positives would maximize detection of cancer and save resources [33].

2.2 Non-invasive screening alternatives for colorectal cancer

Colorectal cancer (CRC) is the third most aggressive cancer worldwide with a high mortality rate due to the lack of robust early detection biomarkers. The five-year survival rates range from more than 90% for stage I to less than 10% for stage IV cancer, however 90% of deaths could be prevented if precancerous lesions are identified early. Despite latest guidelines recommending screening to begin from the age of 45, current programs are mostly available to those aged over 55. The two main problems in CRC screening are: invasiveness of accurate tests and low adherence rates of participation for screening as people scarcely comply with currently available methods [34]. Despite being the gold standard method for CRC screening and diagnosis, colposcopy is

labor-intensive, invasive to the patient and cannot be applied to everyone. Thus there is a great need for cost-effective and noninvasive CRC screening tests to improve the screening accuracy and acceptability.

The current screening guidelines include noninvasive tests alternatives based on stool, blood, or radiologic tests. The stool-based tests currently available are the guaiac-based fecal occult blood test (gFOBT), fecal immunochemical test (FIT) and from 2014, the Cologuard® stool DNA methylation test. The FIT for hemoglobin detection in stools is the most widely used test, but its sensitivity is relatively low in detecting early stage I CRC (53%) and advanced adenomas (27%) [35]. FIT is still limited by the hemoglobin degradation and the intermittent bleeding patterns, so that one in four CRC cases are still diagnosed in a late stage, leading to poor prognosis [36]. However, the use of circulating and/or fecal-based DNA or miRNAs could be the next generation CRC screening biomarkers.

The Cologuard® test is an example of a noninvasive, multitarget stool screening test (mt-sDNA) that involves a single random stool sample, collected by patients at home, without any preparation, or change in medications or diet [37]. As colonocytes consistently exfoliate and shed into the feces, DNA methylation could be easily interrogated, making it good material for alternative CRC screening. Cologuard® involves the quantitative analysis of KRAS mutations, aberrant DNA methylation levels of *NDRG4* and *BMP3*, combined with a human hemoglobin immunoassay for accurate detection of colon neoplasia [37]. It uses a composite score algorithm with the normalizing gene beta-actin to generate a single negative/positive result [38].

After promising results from clinical trials, Cologuard® was the first FDA-approved DNA methylation assay for general CRC screening for average-risk adults older than 50 years. Comparing Cologuard® to FIT in a target population of asymptomatic subjects considered at risk of developing colorectal cancer, Cologuard® demonstrated a better performance over FIT, optimizing for the minimum false-negative rate while keeping the overall performance [39]. Subsequent clinical studies followed the FDA approval, emphasizing on the need of correctly identifying the disease rather than reducing the number of unnecessary colonoscopies in the screening programme [40]. Applied in frozen samples for extended comparisons with FIT, sensitivity and specificity were 49% and 89%, respectively highlighting a better accuracy than FIT (25% and 96%, respectively) [38]. Although it is performing better than FIT, Cologuard® detected fewer than half of advanced adenomas, limiting its preventive role in early cancer screening.

Despite the clinical advantages, wide implementation of Cologuard® into clinics may prove difficult due to the high cost and slow turnaround of approximately two weeks from receiving the stool sample to healthcare provider. For the mt-sDNA test to be cost-effective, substantially higher screening participation rates are required [41]. Nonetheless, both the FDA and the US Preventive Services Task Force (USPSTF) approved the use of mt-sDNA tests in their screening exam recommendations [42]. Current guidelines recommend a regular fecal-based noninvasive examination, such as FIT (every year) and mt-sDNA (every 3 years) for patients aged 50–75 [38].

The Epi proColon® is another valuable example of an alternative screening test for CRC. In addition to feces, DNA methylation can be detected in liquid biopsies, allowing investigation of the *SEPT9* and *ACTB* methylation status from cfDNA [43]. The blood test was designed to address the invasive nature of sampling and maximize adherence to screening. The test was first implemented in Europe in 2008 and approved by the FDA in 2016 as the first blood test intended for early CRC detection.

In the initial retrospective case–control study, Epi proColon® had a high sensitivity of 81% and a specificity of 92%, indicating potential suitability for early disease detection [44]. Other studies indicate that the accuracy of the test increases exponentially with severity of lesion of CRC stage, although a negative result does not infer absence of disease [45]. While the overall sensitivity for CRC detection of Epi proColon® may be superior to gFOBT, it is inferior to that of FIT. In fact, a recent meta-analysis study found that the *SEPT9* assay is only superior to FIT in the symptomatic population [46]. Moreover, relative to Cologuard®, the Epi proColon® test is less sensitive to CRC and advanced adenoma detection [47].

However, subsequent investigations showed that Epi proColon® was not CRC specific. Methylation of the same region in *SEPT9* has been linked to other cancers (i.e., breast, prostate, and lung), the test being positive in 42% patients with other cancers with other cancers and it is currently being trialed for hepatocellular carcinoma detection among cirrhotic patients [48].

Despite all efforts in bringing new methylation biomarkers into the screening program, colonoscopy is still the universal gold standard method for CRC diagnostics. Although in Europe and most parts of Asia, only gFOBT or quantitative FIT are indicated for noninvasive screening, Chinese guidelines have recently recommended using Epi proColon® as a complement to other diagnostic approaches (gFOBT). In the United States, Epi proColon® is not intended to replace the CRC screening tests recommended by clinical guidelines, but the test was FDA-approved for screening of patients unwilling or unable to participate in recommended screening methods following guidelines [34].

3 Epigenetics profiling for cancer prognosis

Prognosis, or the estimation of the likely course and outcome of a disease, is of paramount importance in patient management. In the context of cancer, prognosis is often used to determine the chances of successful treatment and recovery. The identification and characterization of prognostic biomarkers play a crucial role in this assessment by providing insight into the aggressiveness of the disease, informing treatment strategies and ultimately impacting patient outcomes. By identifying patients at risk for recurrence or progression, physicians can tailor treatment plans accordingly. This is particularly important for those with indolent or less aggressive diseases, as overtreatment can result in unnecessary side effects and complications.

3.1 Improving treatment for acute myeloid leukemia by identifying prognosis biomarkers

Treatment response is a major issue for most adults diagnosed with AML. An estimated 40% of younger (age < 60 years) and 10% of older (age ≥ 60 years) patients achieve long-term survival [49]. Therefore novel strategies and targets for treatment are needed to improve outcomes. To date, classification and prognostication for patients have been largely based on cytogenetic and genetic testing, with little focus on other factors, including epigenetic changes [50].

Compared to other malignancies, genetic changes are infrequent in AML and often found at epigenetic regulator sites (*TET2*, *TPMT*, *DNMT3A*, *IDH1*, and *IDH2*), suggesting that an altered epigenome may underlie AML biology and disease prognosis. Aberrant methylation at CpG

islands has been repeatedly observed in subsets of primary origin cancers and has been named CGI methylator phenotype (CIMP) [51]. The CIMP phenotype was often associated with better outcomes for the patient. In line with CIMP, Marcucci et al. reported methylation-induced alterations of gene expression [52]. Each reported gene (*CD34*, *RHOC*, *SCRNI*, *F2RL1*, *FAM92A1*, *MIR155HG*, and *VWA8*) had high DMRs and low expression, associated with better outcome. Results were translated in a weighted summary expression score. When validated in a population of young and old patients, low expression of all seven genes had the best outcomes (complete remission rate: 94% and 87%, respectively; 3-year overall survival: 80% and 42%, respectively), irrespective of age.

Mutations of *IDH1/2* were associated with the CIMP phenotype in leukemia (I-CIMP). For instance, alterations of *IDH2* can cause enzymatic overproduction of 2-hydroxyglutarate, an inhibitor TET-mediated DNA demethylation, leading to aberrant hypermethylation [53,54]. CIMP can also be *IDH1/2* mutation independent (A-CIMP). DNA methylation patterns, genetic backgrounds and clinical characteristics are distinct between I-CIMP and A-CIMP. A pilot study looking at bone marrow samples from AML patients who have been treated with chemotherapy described better survival and benefits to the patient with the A-CIMP [55]. Subsequent studies brought similar results, highlighting CIMP analysis as an independent risk factor for prognosis in AML [56].

In 2020, Jiang et al. conducted a study investigating the relationship between whole genome methylation patterns and leukemia prognosis. The study analyzed blood samples from hundreds of acute myeloid leukemia (AML) patients, acute lymphoblastic leukemia (ALL) patients, and healthy individuals. It is not surprising when results revealed DMRs were identified in genes involved in the methylation machinery are mutated in leukemia. As a result, the authors proposed a methylation-based survival classifier for both ALL and AML that could successfully divide patients into high-risk and low-risk groups, with significant differences in clinical outcome in each leukemia type. The classifier, which is based on the methylation status of specific CpG sites, showed a high degree of sensitivity and specificity, with approximate values of 95% [57]. It is noteworthy that the classifier can be introduced in clinical settings as a PCR-based assay, which is easier to implement than other methylation analysis methods like the sequencing based-CIMP analysis. Although the preliminary data looks promising, further validation in a larger cohort of patients is needed to support the potential clinical utility of these signatures in predicting prognosis and outcomes of different leukemia types.

3.2 Stratification of aggressive prostate cancer

The age-standardized rate incidence of prostate cancer is 31 per 100,000 (lifetime cumulative risk: 3.9%), making this the most diagnosed cancer in men. Prostate cancer also contributed to over 375,000 deaths in 2020 [58]. Current screening methods for prostate cancer do not affect overall mortality [22]. Diagnosis is confirmed by prostate biopsy which involves the collection of 12 needle biopsy cores. Initial biopsies detect 65%–77% cancers and there is a requirement for subsequent, potentially unnecessary, procedures to be performed. However, clinically recognized prognostic markers lack sensitivity and specificity in distinguishing aggressive from indolent disease, particularly in patients with localized, intermediate-grade prostate cancer [22]. Thus there is a need for reliable prognostic biomarkers to characterize aggressive cases and recommend adequate treatment.

Generally, genes involved in cellular pathways including invasion, metastasis, cell cycle, apoptosis, DNA repair, and hormonal response were found hypermethylated in prostate tumors.

Hypermethylation of *RASSF1*, *APC*, *MGMT*, and *CDKN2A* was identified as early events in prostate carcinogenesis [59–61]. Somatic hypermethylation of glutathione-S transferase (*GSTP1*) is also associated with prostate carcinogenesis. Among other roles, the product of *GSTP1* is involved in defense against reactive oxygen species; *GSTP1* may be implicated in resistance toward chemotherapy agents. Hypermethylation is associated with loss of *GSTP1* expression and has been observed in 75% preinvasive high-grade prostatic intraepithelial neoplasms as well as 90% of tumors [62].

This knowledge has been translated into commercially available tests. ConfirmMDx is DNA-methylation assay for the management of patients considered for repeat prostate biopsy. Based on quantitative methylation specific PCR (qMSP), the test looks at the methylation levels of *GSTP1*, *RASSF1* and *APC* with a goal to distinguish true negative prostate biopsies from cancer. Although not FDA-approved, the test is already used in clinical practice as a supplement to traditional diagnostics [63]. Rightly so, it had a significant prognostic impact in the MATLOC trial, where a total of 500 men with histology negative prostate biopsies were investigated. The negative predictive value was 90% and a positive test result was highly associated with repeat biopsy outcome at 30 months after initial biopsies [64]. The assay was independently validated in a population of 350 PSA-screened men. The test showed an odds ratio of 2.69 and a negative predictive value of 88% for disease detection in repeat biopsy [65]. Together, these results indicate the potential prognostic power of the test as well as its ability to decrease unnecessary biopsies [64]. In fact, in 2018 the National Comprehensive Cancer Network (CNCC) recognized the utility of ConfirmMDx to demonstrate cancer-free histopathology [63].

ProCUrE investigates methylation status of *HOXD3* and *GSTP1* from urine. Initially, the test was based on a larger panel of methylation biomarkers (*APC*, *GSTP1*, *HOXD3*, *KLK10*, *TBX15*, and *TGFβ2*). Comparing histology negative to radical prostatectomy tumor samples, higher methylation on *HOXD3* and *GSTP1* was shown to be associated with clinical prognosis [66–68]. Therefore the two most optimal genes were selected to be part of the classifier model. The performance of the test was investigated in a cohort of 408 patients with ranging in risk of prostate cancer development from benign to high risk. The positive predictive value of the ProCUrE test was higher (59.4%–78%) than the one of PSA (38.2%–72.1%) [69]. To measure prognostic ability for aggressiveness, the test was compared with previously used *GSTP1* and *APC* methylation (EpiCaPture test) [70]. Furthermore, the false-positive rate of ProCUrE was almost twofold lower, compared to *GSTP1* and *APC* methylation, making the test an interesting prognostic candidate [69].

4 Epigenetic signatures predict response to treatment

Epigenetic alterations present a novel opportunity for the field of oncology as they allow for the development of personalized treatment strategies based on the specific signatures of a patient's malignancy. This approach, known as precision oncology, aims to optimize treatment outcomes by matching patients to the therapies most likely to be effective. To date, DNA methylation and miRNAs have been shown to be of particular clinical utility in this regard, due to their stability and ease of testing.

4.1 miRNAs in precision treatment of colorectal cancer patients

An estimated 25% of patients with colorectal cancer will present with metastatic disease (mCRC) at the time of diagnosis and up to an additional 50% of patients developing mCRC postinitial diagnosis. Survival of mCRC patients has significantly improved over the last decades and now reaches 30–40 months. However, few therapeutic options are available after failure of the first-line treatment [71]. Evidence suggests that previously third-line therapy with antiepidermal growth factor receptor (EGFR) monoclonal antibodies (mAb) therapy could be clinically beneficial as first-line therapy in combination with chemotherapy and lead to prolonged survival. Anti-EGFR mAb drugs like cetuximab and panitumumab competitively bind to EGFR with the natural ligands such as EGF to inhibit phosphorylation of EGFR tyrosine kinase and block a series of reactions target of rapamycin (mTOR) and the RAS/RAF/mitogen-activated protein kinase (MAPK) pathways [71]. The acquired cell resistance mechanism is a challenge, limiting the efficiency of anti-EGFR therapy. Approximately 30%–40% of patients with baseline RAS wild-type (WT) tumors displayed mutations in *KRAS* or *NRAS* oncogenes after treatment with cetuximab or panitumumab. In these patients, survival improved significantly when an antivascular endothelial growth factor (VEGF) agent (bevacizumab) was prescribed after anti-EGFR therapy. *RAS/KRAS* mutation was not predictive for bevacizumab therapy [72]. Therefore predicting which patients lose sensitivity to first-line anti-EGFR therapy would improve targeted treatment and survival.

The use of miRNAs as biomarkers for predicting treatment response in patients with mCRC has been the subject of much investigation. In a meta-analysis of a blood-based miRNAs, the overall sensitivity and specificity of colorectal cancer prediction were above 70% [73]. However, one limitation of using blood-based miRNA analysis for colorectal cancer is the lack of specificity, as miRNAs may also be elevated in patients with other types of cancer. Fecal-based miRNA detection has also been investigated as an alternative method for CRC diagnosis. miR-21 has been identified as a reliable candidate biomarker. The major bottleneck in this approach is the extraction of RNA from feces, which is abundant in proteins and microbe DNA. This can lead to low yield and make analysis difficult [74].

Another example, mir-31, which has both oncogenic and tumor suppressive roles is frequently deregulated in a variety of cancers. In CRC, mir-31 is commonly overexpressed with high levels correlating to advanced disease. Functional studies link mir-31 to pleiotropic activity which promotes CRC progression through enhanced cell migration and invasion [75,76]. One of the mature forms of mir-31 is miR-31-3p, which has been reported to be associated with outcomes for patients treated with anti-EGFR therapy. In a recent study patients with low miR-31-3p expression were associated with KRAS WT and were not harmed by the addition of cetuximab [77].

The predictive potential of miR-31-3p expression was studied in a randomized phase 3 interventional clinical study. Patients with RAF/KRAS WT showed low miR-31-3p expression in tumors treated with cetuximab. Patients with low miR-31-3p levels were also reported to have better outcomes when treated with cetuximab compared with bevacizumab, validating the use of anti-EGFR before anti-VEGF therapy.

These data led to the development of the first theragnostic test for mCRC, miRPreDX-31-3p, looking at miR-31-3p levels from total RNA extracted from FFPE samples of primary tumors in mCRC patients. The threshold of expression is 1.36, results being delivered in the form of high or low expression [78]. Therefore, a result below the threshold would indicate better outcomes with a

first-line treatment with anti-EGFR in combination with chemotherapy. A predictive tool for both overall survival and response, miRPreDX-31-3p aims to identify patients that could benefit from anti-EGFR therapy and was recently CE-IVD marked for commercial distribution.

Further validation of miRpredX-31-3p was performed by comparing first-line anti-EGFR to anti-VEGF therapy for multiple patient outcomes [79]. Low miR-31-3p expression in affected tissue was associated with a 12-month survival advantage and a 40% reduced risk of death when using anti-EGFR versus anti-VEGF therapy in patients with mCRC. In opposition, high miR-31-3p levels were associated with inferior outcomes and displayed no differences when treatment with either anti-EGFR or anti-VEGF [80]. Overall, patients whose expression level was below the predefined threshold, had a one year longer median overall survival and a 40% reduction in mortality risk when receiving the appropriate treatment.

The miRpredX-31-3p is a good example of an epigenetics precision oncology tool. Based on RT-qPCR, the assay has good feasibility for being easily implemented into clinical diagnostic laboratories. miRpredX-31-3p can analyze up to 12 samples and provide the results in one day. A limitation for the test is the quality of the FFPE sample it is performed in. As the integrity of FFPE material varies in quality, good total RNA recovery is essential for miR-31-3p quantification [80].

4.2 Precision treatment based on DNA methylation testing for breast cancer

Breast cancer is a highly heterogeneous disease resulting in diverse clinical behaviors and therapeutic responses. Most cases, 75%–80%, are hormone receptor positive, meaning that tumor cells express either the estrogen receptor (ER) and/or the progesterone receptor (PR) [81]. Disease recurrence and/or metastasis after curative treatment can occur in approximately 10% of patients with hormone receptor-positive breast cancer within 5 years and continues to be a risk with an annual rate of up to 2.2% over more than 20 years [81,82]. While systemic nonplatinum chemotherapy diminishes this risk, not all patients respond well, and some of the patients suffer from the harsh side-effects without any benefit. In fact, a recent phase 3 clinical study concluded that 46% of women with breast cancer who are at high clinical risk may receive little to no benefit from chemotherapy [83]. Until now biomarkers to predict outcome to nonplatinum chemotherapy are of high unmet medical need.

In breast cancer, DNA-methylation of *PITX2* has received increasing attention as a response prediction biomarker for triage to therapy. *PITX2* is a transcription factor, which is involved in pituitary-specific gene regulation and left-right patterning during embryonic and organogenic development. DNA-methylation of the *PITX2* promoter gene has been shown to predict risk of distant metastasis in node-negative, hormone receptor-positive breast cancer [84]. Additional evidence indicates that *PITX2* methylation predicts response outcome of ER positive, HER2 negative breast cancer patients to adjuvant anthracycline-based chemotherapy, having potential to support the clinicians to decide on the most effective therapy option. Hypermethylation of *PITX2* correlates to poor survival and resistance to treatment [85].

Translating research into clinical application, Qiagen supported the development of the Therascreen PITX2 RGQ PCR Kit. This involves a simple qPCR-based approach, it investigates *PITX2* methylation in DNA of FFPE samples derived from breast biopsies [86]. The test has been recently CE-IVD marked in 2018 and commercially available to help clinicians choose the best treatment course and estimate high-risk breast cancer patient's likelihood to respond to anthracyclines. Although the original application is for metastatic breast cancer, it has been shown that the

selective determination of the *PITX2* methylation status can serve as a cancer biomarker to predict responses to anthracycline-based adjuvant chemotherapy in patients with nonmetastatic triple negative breast cancer [87].

Further, it is well known that women carrying germline mutations in *BRCA* (gBRCAm) or *ATM* have an elevated risk for developing breast cancer in their lifetime. Methylation microarray analyses of peripheral blood DNA across several genes, indicated that body hypermethylation of *ATM* was associated with an estimated threefold increased risk of breast cancer [88]. Another study showed that *BRCA1* promoter methylation was associated with an estimated fivefold increased risk of breast cancer diagnosed before the age of 40 years [89]. These epimutations can mimic germline mutations in their effect on carcinogenesis and may be used as prognosis or response biomarkers.

The product of *BRCA1* gene is a protein involved in double strand DNA break repair. Deficiencies in the *BRCA1* protein are often seen in breast cancer and lead to a faulty activity of the homologous recombination pathway, making cells unable to repair double strand breaks in DNA. This creates synthetic lethality with inhibition of selected DNA repair pathways and this is exploited therapeutically by using poly(ADP-ribose) polymerase (PARP) inhibitors such as olaparib or talazoparib [90].

So far, only the two mentioned PARP inhibitors have been approved for treatment of gBRCAm carriers with metastatic HER2-negative breast cancer. Two phase 3 clinical trials compared single agent olaparib or talazoparib to nonplatinum single agent chemotherapy in gBRCAm carriers with metastatic disease. Both studies concluded that PARP inhibitors contributed to significant progression-free survival, however, the response rate for both PARP inhibitors were 60% with a median duration of approximately 6 months.

Apart from inactivating mutations, hypermethylation of the *BRCA1* promoter CpG islands can cause protein downregulation, and subsequent sensitivity to PARP inhibition [91]. Research showed that *BRCA1* promoter methylation in the wild-type UACC3199 breast cancer cell line was equally sensitive to PARP inhibition as the *BRCA1* mutant MDA-MB-436 line [89]. In addition, patient-derived xenograft models confirmed the presence of *BRCA1* methylation in a subset of tumors and that loss of methylation was associated with PARP inhibitor resistance. These preliminary results infer an epigenetic role in determining likely sensitivity to PARPi that could be used in treating patients with breast and/or ovarian cancer [89].

4.3 Enhancing treatment success for metastatic castration-resistant prostate cancer using epigenetic markers

Metastatic castration-resistant prostate cancer (mCRPC) represents a prostate disease state that develops after tumors progress after initial androgen deprivation therapy (ADT). Even with therapy advances, the median survival rate is approximately 3 years after mCRPC diagnosis. ADT agents aim to downregulate concentrations of circulating androgen by blocking transcriptional activation of the AR. Consequently, a decrease in PSA levels after primary ADT may result from tumor cell death or decreased expression of AR-stimulated PSA. Alternative molecular biomarkers in mCRPC focused on genomic alterations that predict response to systemic therapies or identifying mechanisms of resistance [92]. As most treatment resistant prostate cancers are still driven by AR signaling, epigenetic alterations are investigated as prognostic and predictive biomarkers for mCRPC.

Recent clinical trial data showed undetectable methylation on *GSTP1* in 55% of mCRPC patients treated with two rounds of ADT. This correlated with a longer overall survival and time to PSA progression. The mCRPC patients who maintained this methylation status after ADT were shown to have a longer time to clinical progression [92]. Therefore cfDNA methylation biomarkers might serve as predictive markers of response to different therapies allowing for more precision in choosing the right drug combinations.

While past studies only investigated DNA methylation in small regions of the genome predominantly focusing on CpG islands at promoter regions, whole-genome approaches have been used to better characterize mCRPC. Therefore Shuang et al. described a novel epigenomic subtype for mCRPC. Deep whole-genome bisulfite sequencing data coupled RNA-sequencing was performed on hundreds of mCRPC metastases tissue. Results showed specific hypermethylation alterations in *TET2*, *DNMT3B*, *IDH1*, *BRAF* and the oncogenic driver genes *AR*, *MYC* and *ERG* as well as in other DMRs in somatic mutational hotspots [93]. Another study points toward hypermethylation of tumor-suppressor genes, androgen receptor and estrogen receptor (*ESR1*), cell adhesion genes (*CD44*, *CDH1*) and cell-cycle genes (*CCND2*, *CDKN1B*, *SFN*) as mCRPC specific [94].

Apart from DNA methylation, other epigenetic modifications like covalent modifications at amino acids on histones are altered in prostate carcinogenesis. The prostate epigenome was investigated across various clinical states: from normal prostate epithelium to localized prostate cancer to CRPC. The epigenetically reprogrammed AR sites were identified based on acetylation of the lysine 27 position on histone H3 (H3K27ac) that did not arise *de novo*. During the evolution from localized to CRPC, there was reactivation of latent regulatory elements that were active during fetal prostate organogenesis [95]. Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of polycomb repressive complex 2 (PRC2) and is involved in transcriptional silencing through trimethylation of histone H3 lysine 27 (H3K27me3). EZH2 is overexpressed in many cancers, including advanced prostate cancer [96]. EZH2 can act as a coactivator of transcription factors such as the AR. Thus EZH2 is explored as a potential therapeutic target for mCRPC.

4.4 DNA methylation theragnostic may increase treatment success rate of glioblastoma treatment response

Glioblastomas develop from glial cells in the brain and spinal cord. Their highly invasive nature prevents complete resection of the tumor, causing significant neurologic morbidity and mortality. Despite the availability of aggressive treatments, the survival rate after a glioblastoma diagnosis is no more than 15 months. Approximately 40% glioblastoma patients show methylation at the *MGMT* promoter, as indicated by a recent meta-analysis including 3000 data points. *MGMT* methylation has, therefore, been investigated as a biomarker of response to the alkylating agent, temozolomide (TMZ), a front-line chemotherapy drug used for glioblastoma treatment. The methylation signature is associated with better progression-free survival as well as overall survival, independent of therapeutic programs. For the other 60%, therapy is focused on inhibition of MGMT combined with TMZ analogs [97]. The protein product of *MGMT* removes alkyl adducts from the O⁶-position of guanine. As alkylated guanines promotes double strand breaks, apoptosis and cell death, high MGMT activity is counteracting the alkylating activity of TMZ. Promoter methylation of is associated with genes silencing and sensitivity to alkylating agents [98]. Considering these data, NCCN guidelines have recently included analysis of the methylation status of the *MGMT* promoter in the glioblastoma treatment assessment.

5 Conclusion and further directions

The future of cancer management and treatment is likely to involve an increased use of epigenetic profiling in clinical settings. Epigenetic-based triaging and early detection already proved to be beneficial for managing high-risk populations. In the next years, the national screening programs may include tissue-specific methylation-based tests for triaging hrHPV-positive women or to decrease the number of unnecessary biopsies in the diagnosis of melanoma, pending encouraging results in well-designed population-wide trials. In fact, some tests are already commercially available, with the NCCN guidelines even recommending the use of Cologuard® in colorectal cancer screening programs. Although validation in real-life settings is required for many epigenetics tests, the future looks promising, even diving into population-scale early cancer screening. The use of liquid biopsies combined with advancements in sequencing technologies will push blood-based test closer to into clinics. For instance, the Galleri test by GRAIL aims to overcome the limitations of organ-specific screening using a pan-cancer approach. The array-based test examines methylation patterns on tumor-specific cfDNA in the blood, with the ability to detect difficult-to-diagnose cancers like pancreatic and ovarian [99]. Despite having to impove on test sensitivity, GRAIL has already begun clinical trials in the United Kingdom and United States to gather real-world data from population-scale early cancer screenings. However, the high cost of the test is currently the major limitation for its clinical implementation, but this may change as technology advances. GRAIL has received breakthrough device designation from the FDA and aims to gain full approval in 2023 [99]. It is expected that epigenetic profiling will go beyond triaging patients into monitoring the response to therapy, detecting recurrences, and making predictions about the course of the disease. Prognostic hypermethylation of *GSTP1*, *RASSF1*, and *APC* may identify indolent from aggressive prostate cancer and recommend adequate treatment. By matching therapies to subgroups of patients who are most likely to benefit from it, epigenetic profiling may facilitate precision oncology. For instance, the predictive potential of miR-31-3p expression improves survival of metastatic colorectal cancer through targeted treatments. Similarly, *PITX2* and *BRCA1* methylation may help clinicians choose the best treatment course for high-risk breast cancer patients. While there are still some limitations for implementation, the efforts of validating accurate biomarkers coupled with adoption of new technologies in clinical settings may help pave the way for widespread adoption of epigenetic profiling in the not so distant future.

List of abbreviations

5-mC	5-Methylcytosine
ADT	Androgen deprivation therapy
AML	Acute myeloid leukemia
AR	Androgen receptor
cfDNA	Cell-free DNA
CIMP	CpG island methylator phenotype
CIN	Cervical intraepithelial neoplasia

CNNC	National Comprehensive Cancer Network
CRC	Colorectal cancer
CTC	Circulating tumor cell
DMR	Differentially methylated region
DNMT	DNA methyltransferase
DRE	Digital rectal examination
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FFPE	Formalin-fixed paraffin embedded
FIT	Fecal immunochemical test
gBRCAm	Germline BRCA mutation
gFOBT	Guaiac-based fecal occult blood test
HAT	Histone acetyltransferases or methyl demethylases
HDAC	Histone deacetylase
HDM	Histone methyl demethylases
HMT	Histone methyl transferases
HPV	Human papilloma virus
hrHPV	High-risk human papilloma virus
IVD	<i>In vitro</i> diagnostic
mAb	Monoclonal antibodies
mCRC	Metastatic colorectal cancer
mCRPC	Metastatic castration resistant prostate cancer
MMR	Mismatch repair pathway
mt-sDNA	Multitarget stool screening test
PARP	Poly(ADP-ribose) polymerase
PR	Progesterone receptor
PSA	Prostate-specific antigen
qMSP	Methylation-specific PCR
TMZ	Temozolomide
TNBC	Triple-negative breast cancer
VEGF	Vascular endothelial growth factor
WT	Wild type

References

- [1] Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71:209–49.
- [2] Soerjomataram I, Bray F. Planning for tomorrow: global cancer incidence and the role of prevention 2020–2070. Nat Rev Clin Oncol 2021;18:663–72.
- [3] Strichman-Almashanu LZ, Lee RS, Onyango PO, et al. A genome-wide screen for normally methylated human CpG islands that can identify novel imprinted genes. Genome Res 2002;12:543–54.
- [4] Maegawa S, Gough SM, Watanabe-Okochi N, et al. Age-related epigenetic drift in the pathogenesis of MDS and AML. Genome Res 2014;24:580–91.
- [5] Horvath S, Raj K. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. Nat Rev Genet 2018;19:371–84.
- [6] Levine ME, Lu AT, Quach A, et al. An epigenetic biomarker of aging for lifespan and healthspan. Aging 2018;10:573–91.

- [7] Liao P, Ostrom QT, Stetson L, Barnholtz-Sloan JS. Models of epigenetic age capture patterns of DNA methylation in glioma associated with molecular subtype, survival, and recurrence. *Neuro-Oncol* 2018;20:942–53.
- [8] Chung M, Ruan M, Zhao N, et al. DNA methylation ageing clocks and pancreatic cancer risk: pooled analysis of three prospective nested case-control studies. *Epigenetics* 2021;16:1306–16.
- [9] Hitchins MP. Constitutional epimutation as a mechanism for cancer causality and heritability? *Nat Rev Cancer* 2015;15:625–34.
- [10] Hitchins MP, Wong JJL, Suthers G, et al. Inheritance of a cancer-associated MLH1 germ-line epimutation. *N Engl J Med* 2007;356:697–705.
- [11] Hansmann T, Pliushch G, Leubner M, et al. Constitutive promoter methylation of BRCA1 and RAD51C in patients with familial ovarian cancer and early-onset sporadic breast cancer. *Hum Mol Genet* 2012;21:4669–79.
- [12] Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Mol Cell* 2002;9:601–10.
- [13] Nagy L, Kao H-Y, Chakravarti D, et al. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* 1997;89:373–80.
- [14] Li T, Lai Q, Wang S, et al. MicroRNA-224 sustains Wnt/β-catenin signaling and promotes aggressive phenotype of colorectal cancer. *J Exp Clin Cancer Res* 2016;35:21.
- [15] Hwang J, Min B-H, Jang J, et al. MicroRNA expression profiles in gastric carcinogenesis. *Sci Rep* 2018;8:14393.
- [16] Teoh SL, Das S. The role of microRNAs in diagnosis, prognosis, metastasis and resistant cases in breast cancer. *Curr Pharm Des* 2017;23:1845–59.
- [17] Lee E-J, Luo J, Wilson JM, Shi H. Analyzing the cancer methylome through targeted bisulfite sequencing. *Cancer Lett* 2013;340:171–8.
- [18] Bock C, Halbritter F, Carmona FJ, et al. Quantitative comparison of DNA methylation assays for biomarker development and clinical applications. *Nat Biotechnol* 2016;34:726–37.
- [19] Euskirchen P, Bielle F, Labreche K, et al. Same-day genomic and epigenomic diagnosis of brain tumors using real-time nanopore sequencing. *Acta Neuropathol (Berl)* 2017;134:691–703.
- [20] Lei J, Ploner A, Lehtinen M, Sparén P, Dillner J, Elfström KM. Impact of HPV vaccination on cervical screening performance: a population-based cohort study. *Br J Cancer* 2020;123:155–60.
- [21] de Ruijter TC, van der Heide F, Smits KM, Aarts MJ, van Engeland M, Heijnen VCG. Prognostic DNA methylation markers for hormone receptor breast cancer: a systematic review. *Breast Cancer Res* 2020;22:13.
- [22] Ilic D, Djulbegovic M, Jung JH, et al. Prostate cancer screening with prostate-specific antigen (PSA) test: a systematic review and meta-analysis. *BMJ* 2018;362:k3519.
- [23] Ronco G, Zappa M, Franceschi S, et al. Impact of variations in triage cytology interpretation on human papillomavirus-based cervical screening and implications for screening algorithms. *Eur J Cancer* 2016;68:148–55.
- [24] Sen P, Ganguly P, Ganguly N. Modulation of DNA methylation by human papillomavirus E6 and E7 oncoproteins in cervical cancer. *Oncol Lett* 2018;15:11–22.
- [25] Cuschieri K, Ronco G, Lorincz A, et al. Eurogin roadmap 2017: triage strategies for the management of HPV-positive women in cervical screening programs. *Int J Cancer* 2018;143:735–45.
- [26] Verlaat W, Van Leeuwen RW, Novianti PW, et al. Host-cell DNA methylation patterns during high-risk HPV-induced carcinogenesis reveal a heterogeneous nature of cervical pre-cancer. *Epigenetics* 2018;13:769–78.
- [27] Kelly H, Benavente Y, Pavon MA, De Sanjose S, Mayaud P, Lorincz AT. Performance of DNA methylation assays for detection of high-grade cervical intraepithelial neoplasia (CIN2+): a systematic review and meta-analysis. *Br J Cancer* 2019;121:954–65.

- [28] Schmitz M, Wunsch K, Hoyer H, et al. Performance of a methylation specific real-time PCR assay as a triage test for HPV-positive women. *Clin Epigenetics* 2017;9:118.
- [29] Klischke L, von Ehr J, Kohls F, et al. Performance of a six-methylation-marker assay on self-collected cervical samples—a feasibility study. *J Virol Methods* 2021;295:114219.
- [30] Vink FJ, Lissenberg-Witte BI, Meijer CJLM, et al. FAM19A4/miR124-2 methylation analysis as a triage test for HPV-positive women: cross-sectional and longitudinal data from a Dutch screening cohort. *Clin Microbiol Infect* 2021;27:125.e1–6.
- [31] Bonde J, Floore A, Ejegod D, et al. Methylation markers FAM19A4 and miR124-2 as triage strategy for primary human papillomavirus screen positive women: a large European multicenter study. *Int J Cancer* 2021;148:396–405.
- [32] Lorincz AT, Brentnall AR, Scibior-Bentkowska D, et al. Validation of a DNA methylation HPV triage classifier in a screening sample. *Int J Cancer* 2016;138:2745–51.
- [33] Banila C, Lorincz AT, Scibior-Bentkowska D, et al. Clinical performance of methylation as a biomarker for cervical carcinoma in situ and cancer diagnosis: a worldwide study. *Int J Cancer* 2022;150:290–302.
- [34] Issa IA, Noureddine M. Colorectal cancer screening: an updated review of the available options. *World J Gastroenterol* 2017;23:5086–96.
- [35] Morikawa T, Kato J, Yamaji Y, Wada R, Mitsushima T, Shiratori Y. A comparison of the immunochemical fecal occult blood test and total colonoscopy in the asymptomatic population. *Gastroenterology* 2005;129:422–8.
- [36] Tepus M, Yau TO. Non-invasive colorectal cancer screening: an overview. *Gastrointest Tumors* 2020;7:62–73.
- [37] Imperiale TF, Ransohoff DF, Itzkowitz SH, et al. Multitarget stool DNA testing for colorectal-cancer screening. *N Engl J Med* 2014;370:1287–97.
- [38] Berger BM, Levin B, Hilsden RJ. Multitarget stool DNA for colorectal cancer screening: A review and commentary on the United States Preventive Services Draft Guidelines. *World J Gastrointest Oncol* 2016;8:450–8.
- [39] van Lanschot MCJ, Carvalho B, Coupé VMH, van Engeland M, Dekker E, Meijer GA. Molecular stool testing as an alternative for surveillance colonoscopy: a cross-sectional cohort study. *BMC Cancer* 2017;17:116.
- [40] Redwood DG, Asay ED, Blake ID, et al. Stool DNA testing for screening detection of colorectal neoplasia in alaska native people. *Mayo Clin Proc* 2016;91:61–70.
- [41] Ladabaum U, Mammalithara A. Comparative effectiveness and cost effectiveness of a multitarget stool DNA test to screen for colorectal neoplasia. *Gastroenterology* 2016;151(427–439):e6.
- [42] Lin JS, Piper MA, Perdue LA, et al. Screening for colorectal cancer: updated evidence report and systematic review for the US Preventive Services Task Force. *JAMA* 2016;315:2576–94.
- [43] deVos T, Tetzner R, Model F, et al. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. *Clin Chem* 2009;55:1337–46.
- [44] Potter NT, Hurban P, White MN, et al. Validation of a real-time PCR-based qualitative assay for the detection of methylated SEPT9 DNA in human plasma. *Clin Chem* 2014;60:1183–91.
- [45] Song L, Wang J, Wang H, et al. The quantitative profiling of blood mSEPT9 determines the detection performance on colorectal tumors. *Epigenomics* 2018;10:1569–83.
- [46] Song L, Jia J, Peng X, Xiao W, Li Y. The performance of the SEPT9 gene methylation assay and a comparison with other CRC screening tests: a meta-analysis. *Sci Rep* 2017;7:3032.
- [47] Pickhardt PJ. Emerging stool-based and blood-based non-invasive DNA tests for colorectal cancer screening: the importance of cancer prevention in addition to cancer detection. *Abdom Radiol N Y* 2016;41:1441–4.

- [48] Chen S, Zhou C, Liu W, et al. Methylated septin 9 gene for noninvasive diagnosis and therapy monitoring of breast cancer. *Transl Cancer Res* 2018;7:587–99.
- [49] Estey E. AML in older patients: are we making progress? *Best Pract Res Clin Haematol* 2009;22:529–36.
- [50] Dombret H. Gene mutation and AML pathogenesis. *Blood* 2011;118:5366–7.
- [51] Issa J-P. CpG island methylator phenotype in cancer. *Nat Rev Cancer* 2004;4:988–93.
- [52] Marcucci G, Yan P, Maharry K, et al. Epigenetics meets genetics in acute myeloid leukemia: clinical impact of a novel seven-gene score. *J Clin Oncol J Am Soc Clin Oncol* 2014;32:548–56.
- [53] Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* 2009;462:739–44.
- [54] Xu W, Yang H, Liu Y, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α -ketoglutarate-dependent dioxygenases. *Cancer Cell* 2011;19:17–30.
- [55] Kelly AD, Kroeger H, Yamazaki J, et al. A CpG island methylator phenotype in acute myeloid leukemia independent of IDH mutations and associated with a favorable outcome. *Leukemia* 2017;31:2011–19.
- [56] Fu H, Wu D, Zhou H, Shen J. CpG island methylator phenotype and its relationship with prognosis in adult acute leukemia patients. *Hematology* 2014;19:329–37.
- [57] Jiang H, Ou Z, He Y, et al. DNA methylation markers in the diagnosis and prognosis of common leukemias. *Signal Transduct Target Ther* 2020;5:1–10.
- [58] Gandaglia G, Leni R, Bray F, et al. Epidemiology and prevention of prostate cancer. *Eur Urol Oncol* 2021;4:877–92.
- [59] Feinberg AP, Koldobskiy MA, Göndör A. Epigenetic modulators, modifiers and mediators in cancer aetiology and progression. *Nat Rev Genet* 2016;17:284–99.
- [60] Yamanaka M, Watanabe M, Yamada Y, et al. Altered methylation of multiple genes in carcinogenesis of the prostate. *Int J Cancer* 2003;106:382–7.
- [61] Padar A, Sathyaranayana UG, Suzuki M, et al. Inactivation of cyclin D2 gene in prostate cancers by aberrant promoter methylation. *Clin Cancer Res J Am Assoc Cancer Res* 2003;9:4730–4.
- [62] Gurioli G, Martignano F, Salvi S, Costantini M, Gunelli R, Casadio V. GSTP1 methylation in cancer: a liquid biopsy biomarker? *Clin Chem Lab Med CCLM* 2018;56:702–17.
- [63] Paul Y, Sandra S, Justin C, et al. Clinical utility study of confirms mdx for prostate cancer in a community urology practice. *J Clin Oncol*. Available from: https://ascopubs.org/doi/abs/10.1200/JCO.2019.37.7_suppl.94 (accessed 22.05.22).
- [64] Stewart GD, Van NL, Delvenne P, et al. Clinical utility of an epigenetic assay to detect occult prostate cancer in histopathologically negative biopsies: results of the MATLOC study. *J Urol* 2013;189:1110–16.
- [65] Partin AW, Van Neste L, Klein EA, et al. Clinical validation of an epigenetic assay to predict negative histopathological results in repeat prostate biopsies. *J Urol* 2014;192:1081–7.
- [66] Liu L, Kron KJ, Pethe VV, et al. Association of tissue promoter methylation levels of APC, TGF β 2, HOXD3 and RASSF1A with prostate cancer progression. *Int J Cancer* 2011;129:2454–62.
- [67] Kron K, Liu L, Trudel D, et al. Correlation of ERG expression and DNA methylation biomarkers with adverse clinicopathologic features of prostate cancer. *Clin Cancer Res* 2012;18:2896–904.
- [68] Olkhov-Mitsel E, Van der Kwast T, Kron KJ, et al. Quantitative DNA methylation analysis of genes coding for kallikrein-related peptidases 6 and 10 as biomarkers for prostate cancer. *Epigenetics* 2012;7:1037–45.
- [69] Zhao F, Olkhov-Mitsel E, Kamdar S, et al. A urine-based DNA methylation assay, ProCUrE, to identify clinically significant prostate cancer. *Clin Epigenetics* 2018;10:147.
- [70] O'Reilly E, Tuzova AV, Walsh AL, et al. epiCaPture: a urine DNA methylation test for early detection of aggressive prostate cancer. *JCO Precis Oncol* 2019;1–18.

- [71] Ciardiello D, Martini G, Famiglietti V, et al. Biomarker-guided anti-EGFR rechallenge therapy in metastatic colorectal cancer. *Cancers* 2021;13:1941.
- [72] Kim ST, Park KH, Shin SW, Kim YH. Dose KRAS mutation status affect on the effect of VEGF therapy in metastatic colon cancer patients? *Cancer Res Treat J Korean Cancer Assoc* 2014;46:48–54.
- [73] Carter JV, Galbraith NJ, Yang D, Burton JF, Walker SP, Galandiuk S. Blood-based microRNAs as biomarkers for the diagnosis of colorectal cancer: a systematic review and meta-analysis. *Br J Cancer* 2017;116:762–74.
- [74] Yau TO, Tang C-M, Harriss EK, Dickins B, Polytarchou C. Faecal microRNAs as a non-invasive tool in the diagnosis of colonic adenomas and colorectal cancer: a meta-analysis. *Sci Rep* 2019;9:9491.
- [75] Wang C-J, Stratmann J, Zhou Z-G, Sun X-F. Suppression of microRNA-31 increases sensitivity to 5-FU at an early stage, and affects cell migration and invasion in HCT-116 colon cancer cells. *BMC Cancer* 2010;10:616.
- [76] Liu C-J, Tsai M-M, Hung P-S, et al. miR-31 ablates expression of the HIF regulatory factor FIH to activate the HIF pathway in head and neck carcinoma. *Cancer Res* 2010;70:1635–44.
- [77] Pugh S, Thiébaut R, Bridgewater J, et al. Association between miR-31-3p expression and cetuximab efficacy in patients with KRAS wild-type metastatic colorectal cancer: a post-hoc analysis of the New EPOC trial. *Oncotarget* 2017;8:93856–66.
- [78] Ramon L, David C, Fontaine K, et al. Technical validation of a reverse-transcription quantitative polymerase chain reaction in vitro diagnostic test for the determination of MiR-31-3p expression levels in formalin-fixed paraffin-embedded metastatic colorectal cancer tumor specimens. *Biomark Insights* 2018;13:1177271918763357.
- [79] Laurent-Puig P, Paget-Bailly S, Vernerey D, et al. Evaluation of miR 31 3p as a biomarker of prognosis and panitumumab benefit in RAS-wt advanced colorectal cancer (aCRC): Analysis of patients (pts) from the PICCOLO trial. *J Clin Oncol* 2015;33:3547.
- [80] Laurent-Puig P, Grisoni M-L, Heinemann V, et al. MiR 31 3p as a predictive biomarker of cetuximab efficacy effect in metastatic colorectal cancer (mCRC) patients enrolled in FIRE-3 study. *J Clin Oncol* 2016;34:3516.
- [81] Pan H, Gray R, Braybrooke J, et al. 20-Year risks of breast-cancer recurrence after stopping endocrine therapy at 5 years. *N Engl J Med* 2017;377:1836–46.
- [82] Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials. *Lancet Lond Engl* 2015;386:1341–52.
- [83] Arpino G, Generali D, Sapino A, et al. Gene expression profiling in breast cancer: a clinical perspective. *Breast Edinb Scotl* 2013;22:109–20.
- [84] Nimmrich I, Sieuwerts AM, Meijer-van Gelder ME, et al. DNA hypermethylation of PITX2 is a marker of poor prognosis in untreated lymph node-negative hormone receptor-positive breast cancer patients. *Breast Cancer Res Treat* 2008;111:429–37.
- [85] Hartmann O, Spyros F, Harbeck N, et al. DNA methylation markers predict outcome in node-positive, estrogen receptor-positive breast cancer with adjuvant anthracycline-based chemotherapy. *Clin Cancer Res J Am Assoc Cancer Res* 2009;15:315–23.
- [86] Schricker G, Napieralski R, Noske A, et al. Clinical performance of an analytically validated assay in comparison to microarray technology to assess PITX2 DNA-methylation in breast cancer. *Sci Rep* 2018;8:16861.
- [87] Absmaier M, Napieralski R, Schuster T, et al. PITX2 DNA-methylation predicts response to anthracycline-based adjuvant chemotherapy in triple-negative breast cancer patients. *Int J Oncol* 2018;52:755–67.
- [88] Flanagan JM, Cocciardi S, Waddell N, et al. DNA methylome of familial breast cancer identifies distinct profiles defined by mutation status. *Am J Hum Genet* 2010;86:420–33.

- [89] Wong EM, Southey MC, Fox SB, et al. Constitutional methylation of the BRCA1 promoter is specifically associated with BRCA1 mutation-associated pathology in early-onset breast cancer. *Cancer Prev Res (Phila Pa)* 2011;4:23–33.
- [90] Litton JK, Rugo HS, Ettl J, et al. Talazoparib in patients with advanced breast cancer and a germline BRCA mutation. *N Engl J Med* 2018;379:753–63.
- [91] Kondrashova O, Topp M, Nesic K, et al. Methylation of all BRCA1 copies predicts response to the PARP inhibitor rucaparib in ovarian carcinoma. *Nat Commun* 2018;9:3970.
- [92] Peter MR, Bilenyk M, Isserlin R, et al. Dynamics of the cell-free DNA methylome of metastatic prostate cancer during androgen-targeting treatment. *Epigenomics* 2020;12:1317–32.
- [93] Zhao SG, Chen WS, Li H, et al. DNA methylation landscapes in advanced prostate cancer. *Nat Genet* 2020;52:778–89.
- [94] Graça I, Pereira-Silva E, Henrique R, Packham G, Crabb SJ, Jerónimo C. Epigenetic modulators as therapeutic targets in prostate cancer. *Clin Epigenetics* 2016;8:98.
- [95] Pomerantz MM, Qiu X, Zhu Y, et al. Prostate cancer reactivates developmental epigenomic programs during metastatic progression. *Nat Genet* 2020;52:790–9.
- [96] Varambally S, Dhanasekaran SM, Zhou M, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 2002;419:624–9.
- [97] Ramirez YP, Mladek AC, Phillips RM, et al. Evaluation of novel imidazotetrazine analogues designed to overcome temozolomide resistance and glioblastoma regrowth. *Mol Cancer Ther* 2015;14:111–19.
- [98] Zhang K, Wang X, Zhou B, Zhang L. The prognostic value of MGMT promoter methylation in glioblastoma multiforme: a meta-analysis. *Fam Cancer* 2013;12:449–58.
- [99] GRAIL and the quest for earlier multi-cancer detection. Available from: <https://www.nature.com/articles/d42473-020-00079-y> (accessed 23.05.22).

Epigenetic priming—fact or falacy? 26

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1 Introduction

Epigenetic aberrations, which may be directly caused by somatic mutations resulting in gain or loss of function of epigenetic modulators, contribute to the onset and progression of cancer. This process often includes the epigenetically driven suppression of cancer immunity. Epigenetic modifications in the DNA methylation pattern and/or chromatin landscape (particularly on histone posttranslational modifications) result in alterations in gene expression leading to phenotypic changes. The regulatory proteins that modulate the epigenome can be classified into functional subtypes based on broad, simplistic functions as “epigenetic writers” that introduce epigenetic marks on DNA or histones; these marks are removed by “epigenetic erasers” and recognized by “epigenetic readers.” Several compounds, many clinically approved, have been developed to modify the epigenome by acting, with higher or lower specificity, on these regulatory proteins. In this chapter, we will discuss the potential use of these compounds to increase the cancer immunity. We will particularly focus on drugs that have demonstrated or are under clinical evaluation for this effect including hypomethylating agents, inhibitors of histone methyltransferase EZH2, inhibitors of histone deacetylases (HDAC), and inhibitors of histone arginine methyltransferases.

The immune system plays a critical role in cancer development and progression by eliminating cancer cells. In turn, cancer cells escape from the immune system by expressing suppressive phenotypes and decreasing immunogenicity (i.e., losing target antigens and neoantigens). Cancer cell

“extrinsic” mechanisms include poor immune cell infiltration, effector cell dysfunction and exhaustion, and mechanisms leading to an immunosuppressive microenvironment. In some tumors, these early and late development stages are intercalated with an intermediate “equilibrium” phase in which cancer cells persist, but the adaptive immune system impedes frank tumor’s growth. Cancer immunity is regulated by a dynamic interplay of costimulatory and inhibitory (e.g., PD1, CTLA-4, PDL1, and PDL2) signals that balance the immune response and self-tolerance. Cancer immunotherapy includes drugs and biological agents that aim to improve cancer immunity. These strategies include immune activators (e.g., lenalidomide), immune checkpoint inhibitors (e.g., anti-PD1, PDL1, and CTLA-4), bi-specific antibodies (e.g., anti-CD20xCD3), antibodies (e.g., anti-CD30 and anti-CD47) and cellular therapies (e.g., CAR-T cells). Some of these modalities are currently FDA approved and others are in clinical phase of development, and almost all of them have shown cancer cell intrinsic or extrinsic mechanisms of resistance.

2 Epigenetic mechanisms of immune escape

Epigenetic dysregulation, more often than genetic alterations, is among the several mechanisms that cancer cells employ to avoid immune recognition and suppress cancer immunity [1]. Cancer cells display an array for epigenetic regulated-immune escape mechanisms targeting the expression of tumor-associated and oncogenic viral-associated antigens, the antigen processing and presentation machinery, and immune checkpoint and costimulatory molecules. Some of these pathways can be dysregulated during the very early stages of carcinogenesis while others are suppressed at later stages, even after the administration of immune therapeutic approaches that modulate the emergency of epigenetically driven resistance [2]. Most of the described epigenetic aberrations target the molecules involved in the interaction, also referred as immune synapse, between cancer and effector T cells. In solid tumor and hematological malignancies, the epigenetic targeting of both HLA and immune checkpoint and costimulatory ligands in cancer cells may decrease tumor immunity as well as immunotherapy responses [3–5]. A pattern of aberrant methylation of immune synapse genes quite consistent across tumor types is characterized by hypermethylation (and downregulation) of costimulatory molecules and/or hypomethylation (and upregulation) of immune checkpoints [2].

3 Immune “cold” and “hot” tumors

Tumor immunophenotypes can be broadly categorized as “hot,” which are characterized by (mostly) T-cell infiltration and subsequent inflammation; immune-excluded, with minimal and inefficient infiltration; or immune-desert, that present no signs of immune attraction. The latter two representing a “cold” phenotype. There are several biological reasons underlying these phenotypes including epigenetic alterations in cancer cells. For example, epigenetic dysregulation that causes downregulation of immune synapse genes can promote a “cold” tolerogenic immune landscape in B-cell lymphomas [3,4]. Similarly, hypermethylation of costimulatory genes is associated with decrease tumor immunogenicity and recruitment of effector T cells in solid tumors [2,6]. Promoter

hypermethylation of genes that contain neoantigenic mutations represent a mechanism of immunoeediting and, accordingly, sparsely infiltrated nonsmall-cell lung cancers exhibit mechanisms of neoantigen presentation dysfunction [7]. Similarly, in B-cell lymphomas, an immune-depleted TME was associated with increased genome-wide DNA methylation in cancer cells [4].

4 Epigenetic therapy to warm up tumors

Epigenetic alterations in cancer cells can shape the immune microenvironment, which, in many cases, offer an opportunity for therapeutic reversion (Figure 26.1). Several groups have recently reported that anticancer agents that elicit an “antiviral-like” (AVR) response in tumor models potentiate the effect of immune checkpoint inhibitors and other forms of immunotherapy in solid tumors. A common mechanism triggering AVR in cancer cells is the induction of DNA hypomethylation, mostly consequence of DNMT downregulation, with the subsequent expression of retrotransposon-like elements including double-stranded (ds)RNAs [8]. This is in addition to inducing the re-expression of molecules that attract and/or activate immune cells. Therefore

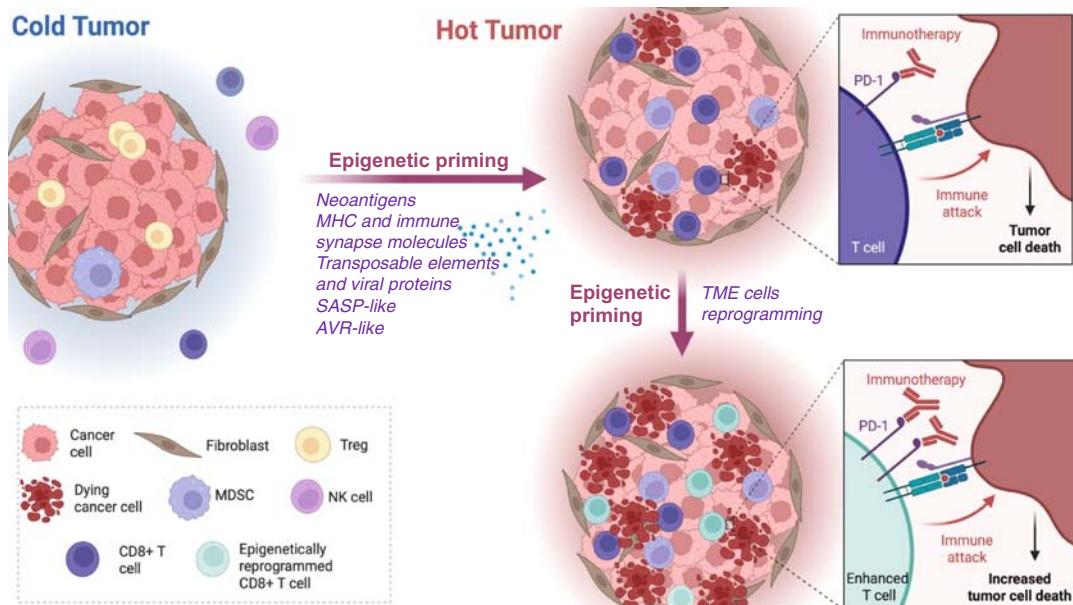


FIGURE 26.1

Therapeutic epigenetic priming. Hypomethylating and chromatin modifying agents can activate several mechanisms in cancer cells ultimately resulting in increased immunogenicity and microenvironment infiltration of cytotoxic T cells that favor the activity of immunotherapy agents. In addition, in “hot” tumors, epigenetic agents can reprogram tumor microenvironment cells to decrease their immune suppressive effects and/or enhance their cytotoxicity resulting in increased cancer cell killing upon immunotherapy.

immunologically “cold” tumors could become “hot” due to the activity of epigenetic agents. Preclinical B-cell lymphoma models and cells treated with hypomethylating agents develop a senescence-like phenotype characterized by reduced proliferation rate and reduced tolerance to DNA damage [9]. Molecularly, these lymphoma cells upregulate the expression of genes involved in cell cycle control (e.g., p21), TGF signaling (e.g., SMAD1), extracellular signaling (e.g., VAV3), and interferon response (e.g., IRF4), among others. A hallmark of senescent cells is the secretion of a variety of inflammatory cytokines and chemokines, immune modulators, and proteases, which collectively are defined as senescence-associated secretory phenotype (SASP). Recent data indicates that activation of the nucleic acid sensing pathway cGAS-STING is the molecular basis of the SASP [10,11]. In this scenario, the molecular recognition of double-strand DNA or RNA fragments triggers the production of interferon gamma and other cytokines through the activation of interferon responsive elements [12]. Therefore it is possible that hypomethylation-induced senescence is associated with a SASP that could in turn improve the cancer immune response. In a murine B-cell lymphoma model harboring a “cold” immune microenvironment, administration of low-dose azacytidine induced hypomethylation and changes in gene expression, including the upregulation of class I and II MHC genes [4]. This process was followed by increased tumor infiltration of T cells promoting a “hot” tumor microenvironment phenotype [4].

A similar gene upregulation was reported with the use of EZH2 inhibitors in lymphomas harboring EZH2 activating mutations [3]. This overall mechanism is supported by clinical data as shown in a recent clinical trial of oral azacytidine priming for 7 days in patients with B-cell lymphoma [13]. In this trial, the investigators described the upregulation of a transposable elements belonging to the SINE class (short interspersed nuclear elements). Although most mobilized SINEs have no described biological roles, they can activate common pathways resulting in AVR that, in these patients, elicited an interferon lambda systemic response [12,13]. Like interferon alpha and beta, interferon lambda exhibits a potent antiviral (and antitumor) activity, but its receptors are restricted to cells of epithelial origin [14,15]. Interestingly, the expression of interferon lambda receptor (i.e., *IFNLRI*) in cancer cells is epigenetically regulated and can be re-expressed by class I HDAC inhibitors in cellular models of glioblastoma [14]. A potential class of tumors benefiting from this mechanism is constituted by viral-driven cancers. For example, a subgroup of Epstein-Barr virus (EBV)-infected B-cell lymphomas epigenetically suppress immunogenic EBV antigens to, in part, evade immune responses as part of their oncogenic process [16]. In these tumors, hypomethylating agents can reactivate immunogenic EBV antigens sensitizing cells to lysis by EBV-specific cytotoxic T cells [17], proving a rationale for testing epigenetic priming followed by immunotherapy in this setting.

5 Epigenetic therapy to boost cancer immunity

Tumor infiltrating immune cells can be subjected to epigenetic reprogramming leading to cancer “tolerogenic” phenotypes and eventually arising as cancer cell-supporting entities in a process of “co-education.” Accordingly, epigenetic alterations have been described in several tumor microenvironment immune cells. Tumor infiltrating CD8⁺ T cells can differentiate into cytotoxic effector T cells and eliminate tumor cells. Although, as expected, the methylome pattern differs in naïve and reactive tumor infiltrating CD8⁺ T cells, most tumor-reactive T cells eventually exhibit an T

cell exhausted DNA methylation signature [18–21]. In addition, mounting evidence suggest that generation of memory T cells could be also epigenetically driven [22], potentially compromising the generation of long-lived cancer immunity memory. Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells characterized by their immunosuppressive function favoring cancer immune evasion. HDAC2 and HDAC11 have been implicated in the function of myeloid cells and expansion of MDSCs in the tumor microenvironment [23,24]. Tumor associated macrophages (TAMs) are an abundant component of the microenvironment of solid tumors and lymphomas. Although are generally associated with inflammation and poor prognosis, TAMs can show pro- and antitumoral properties that may depend on reversible epigenetic reprogramming. Although little is known on epigenetic TAM reprogramming, some studies have involved several epigenetic regulators in the phenotype and activation of macrophages subsets [25]. These include DNMT3B [26], HDAC3 [27], HDAC4 [28], HDAC9 [29], SIRT2 [30], SMYD3 [25], and KDM6B [31], among others. Thus it is possible to speculate that macrophage reprogramming into TAM subpopulations involve extensive epigenetic remodeling.

Although therapeutic reversing T-cell exhaustion can reinvigorate cancer immunity, there is a proportion of patients with low durable responses to these strategies that can be explained by the stable dysfunctional state of T cells epigenetically shaped. Increasing evidence indicate that the epigenetic reprogramming and thus the functional status of these cells can be, at least at early stages, therapeutically targeted [32,33] (Figure 26.1). For example, experimental studies in preclinical models indicated that HDAC inhibition increases the activity of Th1 cytokines that promote expression of cytotoxic T cells and generate other immunomodulatory effects on systemic immunity including a reduction of MDSC subsets. Similarly, in a murine model of ovarian cancer, the administration of low dose of the hypomethylating agent decitabine increases the expression of chemokines that recruit and activate natural killer (NK) and effector T cells [34]. Some epigenetic states can be susceptible for therapeutic epigenetic reprogramming. Preclinical cancer models indicate that dysfunctional T cells are more likely reprogrammable when they exhibit a plastic chromatin state [33], thus identification of such state in humans will be paramount for established successful combinatorial therapies. The likelihood of immune microenvironment epigenetic reprogramming may be lower at advanced stages or in immunotherapy relapsed tumors, which also questions whether the epigenetic effects in peripheral circulating cells faithfully represents changes in TME cells. A recent clinical trial examined the response to the pan-HDAC inhibitor entinostat with the PD1 inhibitor pembrolizumab in patients with PD1 axis inhibitor-resistant nonsmall-cell lung cancer [35]. This trial showed that the concurrent combination did not achieve the primary response rate endpoint but provided clinical benefit with objective response in 9% of the patients [35]. Increased lysine acetylation in circulating B cells, but not in T cells or monocytes, was associated with improved progression-free survival and there was evidence of T-cell “reinvigoration” upon entinostat that correlated with favorable duration of treatment [35]. However, the extent of the TME reprogramming was not evaluated in these patients and it is unlikely that most patients had an epigenetically driven mechanism underlying the resistance to PD1 axis inhibition. In another trial exploring the combination of epigenetic priming with the hypomethylating agent guadecitabine followed by pembrolizumab in platinum-resistant ovarian cancer demonstrated transcriptional evidence of activation of antitumor immunity in after two cycles of treatment [36]. In this trial, that achieved 31% clinical benefit, analysis of posttreatment TME in the available patients showed differences in the distribution of infiltrating T and B cells in relation to cancer cells [36].

6 Challenges to epigenetic priming

Patient selection and predictive biomarkers are cornerstones for implementing successful epigenetic therapeutic approaches to improve immunotherapy, while the so called “agnostic” approaches are likely to fail [37]. Although limited data suggest that baseline characteristics of the immune TME and/or peripheral blood immune subsets (like monocytes) may help to predict benefit from epigenetic-immunotherapy regimens [35,36], the composition and characteristics of the TME is highly dynamic. Thus ideally, these putative biomarkers must incorporate the dynamic changes TME and cancer cells experience during disease progression allowing for the identification of “windows of opportunity” for the combinatorial approach [6]. Tumors must be fully reprogrammed with epigenetic agents to a state in which the activity of the immunotherapeutic agents is optimized; this will not only potentiate efficacy but also it could decrease the likelihood of side effects. When the epigenetic therapy fails to induce biological effects leading to cell reprogramming, the clinical efficacy of an immunotherapy combinatorial approach is expected to be poor [38]. In an early trial exploring the combination of guadecitabine with CTLA-4 inhibitor ipilimumab in unresectable melanoma, the analysis of serial tumors samples at baseline and 4 and 12 weeks after treatment demonstrated immunomodulatory effects; however, for patients with improvements at 4 weeks the analysis of 12-week biopsies showed not further benefit [39]. In B-cell lymphoma patients, an epigenetic priming phase of oral azacytidine for 7 days was enough to activate innate immune responses (SINEs upregulation in tumors and increased interferon levels in plasma) [13], similarly to effects shown in preclinical models [4,12]. In ovarian cancer patients, decreased levels of LINE-1 methylation in peripheral blood cells has been detected with 4 days of the hypomethylating agent guadecitabine; however, there were not significant changes in the peripheral blood immune profiling in this time frame [36]. Similar designed studies have detected reduction in LINE-1 methylation and peripheral blood immune profiling with potentially corresponding changes in the TMEs of the tumors [40], but sample size, patient cohort heterogeneity and unavoidable sampling bias affected the robustness of these effects. Activation of transposable elements had been proposed as a general pharmacodynamic biomarker of hypomethylating agents in relation to potential immune effects. However, the type and extent to what transposable elements are methylated differ among tumor types, but even in peripheral blood cells the dynamic of methylation and demethylation of these elements is different from gene promoters. In contrast to large CpG islands associated with gene promoters, small CpG islands (200–500 base pairs) that are frequently associated with SINEs and other elements, are potentially more susceptible to the effect of demethylating agents. This could help to explain common discrepancies between epigenetic, transcriptional, and phenotypic (cellular) effects on evaluating the optimal duration of epigenetic priming in patients, that still need to be clearly established.

Although limited clinical data are available on dosing and treatment duration of immune checkpoints inhibitors and other immunotherapies [41], recent data suggest that dose intensity may impact on tumor response. In a phase 2 trial in nonsmall-lung cancer in which patients were randomized to pembrolizumab plus oral azacytidine or placebo, patients on the experimental arm, due to gastrointestinal toxicity, had potentially decreased exposure to pembrolizumab, leading to no significant differences in survival between the two arms [37]. It is also possible that epigenetic agents may have upregulated immune checkpoints or molecules related

with immune suppressive TMEs. This theoretical scenario is supported by data demonstrating baseline hypermethylation and downregulation of immune checkpoints in a subset of cancer patients [2] and the effect of hypomethylating agents in the upregulation of immune checkpoints and ligands [42,43] and/or molecules that negatively affect cancer immunity like IDO1 [13,36]. Overall, this data suggests that the epigenetic status of immune synapse genes at the prepriming phase together with tumor changes at the postpriming phase may be utilized for agent selection in combinatorial regimens.

Another critical point when implementing these therapies is the potential deleterious effect of the compound on the immune microenvironment, particularly on the cytotoxic effector T and NK cells [32,42–44]. On this regard careful consideration of the specific epigenetic regulators in different immune cell subsets as well as the selectivity of the compounds are paramount for selecting epigenetic primers [21]. In best case scenario, these drugs should improve the activity of effector cells, but at least they should not suppress cancer immunity. Preclinical data indicates that, for example, relatively unspecific HDAC inhibitors can enhance the expansion of immunosuppressive cells including myeloid-derived suppressive cells (MDSC) and T-cell regulatory (Tregs) as well as decrease the activity of effector T and NK cells [44–47]. Others have reported opposite effects of pan-HDAC inhibitors and differentially modulating MDSC subpopulations [48,49]. More specific inhibitors can limit the epigenetic reprogramming to the desired subset of cells. An example is provided by inactivating mutations of CREBBP that promote the development of HDAC3-dependent B-cell lymphomas and, accordingly, selective HDAC3 inhibition shows antitumoral effect by increasing antitumor immunity through indirect activation of cytotoxic T cells in preclinical setting [50,51].

One of the most attractive targets to improve immunotherapy is EZH2 [52]. Several inhibitors from preclinical to FDA-approved stages are available that prompted the development of combinatorial clinical trials. In cancer models of solid tumors and hematological malignancies, EZH2 inhibition has shown to convert an immunosuppressive TME into an immune promoting TME [53]. With variations according to the model and the tumors, the effect of EZH2 inhibitors has shown to decrease the immune evading mechanism in cancer cells as well as to directly enhance T-cell infiltration and activity of T and NK cells in the TME [6,54].

7 Conclusion

Epigenetic alterations in cancer and TME cells play a major role in establishment an immunosuppressive and immune evasive TME, which may decrease the efficacy of cancer immunotherapy. Since some of these epigenetic alterations can be pharmacologically reversed, it provides the rationale to modify the immunosuppressive TME as well as increase the effect of immunotherapy. However, successful implementation of such approaches will require deepening our knowledge on the specific epigenetic mechanisms and molecular players in the acquisition of aberrant epigenetic phenotypes by immune cells. The epigenetic drug toolbox has increased in the last few years. Parallel efforts should be implementing in the development of robust biomarkers to select candidates for these treatments as well as to measure the impact of epigenetic drugs in immune cells during the treatment.

References

- [1] Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou Yang TH, et al. The immune landscape of cancer. *Immunity* 2018;48:812–830.e814. Available from: <https://doi.org/10.1016/j.immuni.2018.03.023>.
- [2] Berglund A, Mills M, Putney RM, Hamaidi I, Mule J, Kim S. Methylation of immune synapse genes modulates tumor immunogenicity. *J Clin Invest* 2020;130:974–80. Available from: <https://doi.org/10.1172/JCI131234>.
- [3] Beguelin W, Teater M, Meydan C, Hoehn KB, Phillip JM, Soshnev AA, et al. Mutant EZH2 induces a pre-malignant lymphoma niche by reprogramming the immune response. *Cancer Cell* 2020;37:655–673.e611. Available from: <https://doi.org/10.1016/j.ccr.2020.04.004>.
- [4] Kotlov N, Bagaev A, Revuelta MV, Phillip JM, Cacciapuoti MT, Antysheva Z, et al. Clinical and biological subtypes of B-cell lymphoma revealed by microenvironmental signatures. *Cancer Discov* 2021;11:1468–89. Available from: <https://doi.org/10.1158/2159-8290.CD-20-0839>.
- [5] Sade-Feldman M, Jiao YJ, Chen JH, Rooney MS, Barzily-Rokni M, Eliane JP, et al. Resistance to checkpoint blockade therapy through inactivation of antigen presentation. *Nat Commun* 2017;8:1136. Available from: <https://doi.org/10.1038/s41467-017-01062-w>.
- [6] Zingg D, Arenas-Ramirez N, Sahin D, Rosalia RA, Antunes AT, Haeusel J, et al. The histone methyltransferase Ezh2 controls mechanisms of adaptive resistance to tumor immunotherapy. *Cell Rep* 2017;20:854–67. Available from: <https://doi.org/10.1016/j.celrep.2017.07.007>.
- [7] Rosenthal R, Cadieux EL, Salgado R, Bakir MA, Moore DA, Hiley CT, et al. Neoantigen-directed immune escape in lung cancer evolution. *Nature* 2019;567:479–85. Available from: <https://doi.org/10.1038/s41586-019-1032-7>.
- [8] Chiappinelli KB, Strissel PL, Desrichard A, Li H, Henke C, Akman B, et al. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. *Cell* 2015;162:974–86. Available from: <https://doi.org/10.1016/j.cell.2015.07.011>.
- [9] Clozel T, Yang S, Elstrom RL, Tam W, Martin P, Kormaksson M, et al. Mechanism-based epigenetic chemosensitization therapy of diffuse large B-cell lymphoma. *Cancer Discov* 2013;3:1002–19. Available from: <https://doi.org/10.1158/2159-8290.CD-13-0117>.
- [10] Gluck S, Guey B, Gulen MF, Wolter K, Kang TW, Schmacke NA, et al. Innate immune sensing of cytosolic chromatin fragments through cGAS promotes senescence. *Nat Cell Biol* 2017;19:1061–70. Available from: <https://doi.org/10.1038/ncb3586>.
- [11] Yang H, Wang H, Ren J, Chen Q, Chen ZJ. cGAS is essential for cellular senescence. *Proc Natl Acad Sci U S A* 2017;114:E4612–20. Available from: <https://doi.org/10.1073/pnas.1705499114>.
- [12] Leonova KI, Brodsky L, Lipchick B, Pal M, Novototskaya L, Chenchik AA, et al. p53 cooperates with DNA methylation and a suicidal interferon response to maintain epigenetic silencing of repeats and non-coding RNAs. *Proc Natl Acad Sci U S A* 2013;110:E89–98. Available from: <https://doi.org/10.1073/pnas.1216922110>.
- [13] Martin P, Bartlett NL, Chavez JC, Reagan JL, Smith SM, LaCasce AS, et al. Phase 1 study of oral azacitidine (CC-486) plus R-CHOP in previously untreated intermediate- to high-risk DLBCL. *Blood* 2022;139:1147–59. Available from: <https://doi.org/10.1182/blood.2021011679>.
- [14] Ding S, Khouri-Hanold W, Iwasaki A, Robek MD. Epigenetic reprogramming of the type III interferon response potentiates antiviral activity and suppresses tumor growth. *PLoS Biol* 2014;12:e1001758. Available from: <https://doi.org/10.1371/journal.pbio.1001758>.
- [15] Dumoutier L, Tounsi A, Michiels T, Sommereyns C, Kotenko SV, Renaud JC. Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-lambda 1: similarities with type I interferon signaling. *J Biol Chem* 2004;279:32269–74. Available from: <https://doi.org/10.1074/jbc.M404789200>.

- [16] Guo R, Zhang Y, Teng M, Jiang C, Schineller M, Zhao B, et al. DNA methylation enzymes and PRC1 restrict B-cell Epstein-Barr virus oncprotein expression. *Nat Microbiol* 2020;5:1051–63. Available from: <https://doi.org/10.1038/s41564-020-0724-y>.
- [17] Dalton T, Doubrovina E, Pankov D, Reynolds R, Scholze H, Selvakumar A, et al. Epigenetic reprogramming sensitizes immunologically silent EBV + lymphomas to virus-directed immunotherapy. *Blood* 2020;135:1870–81. Available from: <https://doi.org/10.1182/blood.2019004126>.
- [18] Ghoneim HE, Fan Y, Moustaki A, Abdelsamed HA, Dash P, Dogra P, et al. De novo epigenetic programs inhibit PD-1 blockade-mediated T cell rejuvenation. *Cell* 2017;170(142–157):e119. Available from: <https://doi.org/10.1016/j.cell.2017.06.007>.
- [19] Stephen TL, Payne KK, Chaurio RA, Allegrezza MJ, Zhu H, Perez-Sanz J, et al. SATB1 expression governs epigenetic repression of PD-1 in tumor-reactive T cells. *Immunity* 2017;46:51–64. Available from: <https://doi.org/10.1016/j.jimmuni.2016.12.015>.
- [20] Yang R, Cheng S, Luo N, Gao R, Yu K, Kang B, et al. Distinct epigenetic features of tumor-reactive CD8 + T cells in colorectal cancer patients revealed by genome-wide DNA methylation analysis. *Genome Biol* 2019;21:2. Available from: <https://doi.org/10.1186/s13059-019-1921-y>.
- [21] Zou Q, Wang X, Ren D, Hu B, Tang G, Zhang Y, et al. DNA methylation-based signature of CD8 + tumor-infiltrating lymphocytes enables evaluation of immune response and prognosis in colorectal cancer. *J Immunother Cancer* 2021;9. Available from: <https://doi.org/10.1136/jitc-2021-002671>.
- [22] Dogra P, Ghoneim HE, Abdelsamed HA, Youngblood B. Generating long-lived CD8(+) T-cell memory: insights from epigenetic programs. *Eur J Immunol* 2016;46:1548–62. Available from: <https://doi.org/10.1002/eji.201545550>.
- [23] Sahakian E, Powers JJ, Chen J, Deng SL, Cheng F, Distler A, et al. Histone deacetylase 11: a novel epigenetic regulator of myeloid derived suppressor cell expansion and function. *Mol Immunol* 2015;63:579–85. Available from: <https://doi.org/10.1016/j.molimm.2014.08.002>.
- [24] Youn JI, Kumar V, Collazo M, Nefedova Y, Condamine T, Cheng P, et al. Epigenetic silencing of retinoblastoma gene regulates pathologic differentiation of myeloid cells in cancer. *Nat Immunol* 2013;14:211–20. Available from: <https://doi.org/10.1038/ni.2526>.
- [25] Kittan NA, Allen RM, Dhaliwal A, Cavassani KA, Schaller M, Gallagher KA, et al. Cytokine induced phenotypic and epigenetic signatures are key to establishing specific macrophage phenotypes. *PLoS One* 2013;8:e78045. Available from: <https://doi.org/10.1371/journal.pone.0078045>.
- [26] Yang X, Wang X, Liu D, Yu L, Xue B, Shi H. Epigenetic regulation of macrophage polarization by DNA methyltransferase 3b. *Mol Endocrinol* 2014;28:565–74. Available from: <https://doi.org/10.1210/me.2013-1293>.
- [27] Chen X, Barozzi I, Termanini A, Prosperini E, Recchiti A, Dalli J, et al. Requirement for the histone deacetylase Hdac3 for the inflammatory gene expression program in macrophages. *Proc Natl Acad Sci U S A* 2012;109:E2865–74. Available from: <https://doi.org/10.1073/pnas.1121131109>.
- [28] Yang Q, Wei J, Zhong L, Shi M, Zhou P, Zuo S, et al. Cross talk between histone deacetylase 4 and STAT6 in the transcriptional regulation of arginase 1 during mouse dendritic cell differentiation. *Mol Cell Biol* 2015;35:63–75. Available from: <https://doi.org/10.1128/MCB.00805-14>.
- [29] Cao Q, Rong S, Repa JJ, St Clair R, Parks JS, Mishra N. Histone deacetylase 9 represses cholesterol efflux and alternatively activated macrophages in atherosclerosis development. *Arterioscler Thromb Vasc Biol* 2014;34:1871–9. Available from: <https://doi.org/10.1161/ATVBAHA.114.303393>.
- [30] Lo Sasso G, Menzies KJ, Mottis A, Piersigilli A, Perino A, Yamamoto H, et al. SIRT2 deficiency modulates macrophage polarization and susceptibility to experimental colitis. *PLoS One* 2014;9:e103573. Available from: <https://doi.org/10.1371/journal.pone.0103573>.
- [31] Satoh T, Takeuchi O, Vandenberg A, Yasuda K, Tanaka Y, Kumagai Y, et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol* 2010;11:936–44. Available from: <https://doi.org/10.1038/ni.1920>.

- [32] Lodewijk I, Nunes SP, Henrique R, Jeronimo C, Duenas M, Paramio JM. Tackling tumor microenvironment through epigenetic tools to improve cancer immunotherapy. *Clin Epigenetics* 2021;13:63. Available from: <https://doi.org/10.1186/s13148-021-01046-0>.
- [33] Philip M, Fairchild L, Sun L, Horste EL, Camara S, Shakiba M, et al. Chromatin states define tumour-specific T cell dysfunction and reprogramming. *Nature* 2017;545:452–6. Available from: <https://doi.org/10.1038/nature22367>.
- [34] Wang L, Amoozgar Z, Huang J, Saleh MH, Xing D, Orsulic S, et al. Decitabine enhances lymphocyte migration and function and synergizes with CTLA-4 blockade in a murine ovarian cancer model. *Cancer Immunol Res* 2015;3:1030–41. Available from: <https://doi.org/10.1158/2326-6066.CIR-15-0073>.
- [35] Hellmann MD, Janne PA, Opyrchal M, Hafez N, Raez LE, Gabrilovich DI, et al. Entinostat plus pembrolizumab in patients with metastatic NSCLC previously treated with anti-PD-(L)1 therapy. *Clin Cancer Res* 2021;27:1019–28. Available from: <https://doi.org/10.1158/1078-0432.CCR-20-3305>.
- [36] Chen S, Xie P, Cowan M, Huang H, Cardenas H, Keathley R, et al. Epigenetic priming enhances antitumor immunity in platinum-resistant ovarian cancer. *J Clin Invest* 2022;132. Available from: <https://doi.org/10.1172/JCI158800>.
- [37] Levy BP, Giaccone G, Besse B, Felip E, Garassino MC, Domine Gomez M, et al. Randomised phase 2 study of pembrolizumab plus CC-486 versus pembrolizumab plus placebo in patients with previously treated advanced non-small cell lung cancer. *Eur J Cancer* 2019;108:120–8. Available from: <https://doi.org/10.1016/j.ejca.2018.11.028>.
- [38] Taylor K, Loo Yau H, Chakravarthy A, Wang B, Shen SY, Ettayebi I, et al. An open-label, phase II multicohort study of an oral hypomethylating agent CC-486 and durvalumab in advanced solid tumors. *J Immunother Cancer* 2020;8. Available from: <https://doi.org/10.1136/jitc-2020-000883>.
- [39] Di Giacomo AM, Covre A, Finotello F, Rieder D, Danielli R, Sigalotti L, et al. Guadecitabine plus ipilimumab in unresectable melanoma: the NIBIT-M4 clinical trial. *Clin Cancer Res* 2019;25:7351–62. Available from: <https://doi.org/10.1158/1078-0432.CCR-19-1335>.
- [40] Papadatos-Pastos D, Yuan W, Pal A, Crespo M, Ferreira A, Gurel B, et al. Phase 1, dose-escalation study of guadecitabine (SGI-110) in combination with pembrolizumab in patients with solid tumors. *J Immunother Cancer* 2022;10. Available from: <https://doi.org/10.1136/jitc-2022-004495>.
- [41] Patnaik A, Kang SP, Rasco D, Papadopoulos KP, Ellassai-Schaap J, Beeram M, et al. Phase I study of pembrolizumab (MK-3475; anti-PD-1 monoclonal antibody) in patients with advanced solid tumors. *Clin Cancer Res* 2015;21:4286–93. Available from: <https://doi.org/10.1158/1078-0432.CCR-14-2607>.
- [42] Daver N, Boddu P, Garcia-Manero G, Yadav SS, Sharma P, Allison J, et al. Hypomethylating agents in combination with immune checkpoint inhibitors in acute myeloid leukemia and myelodysplastic syndromes. *Leukemia* 2018;32:1094–105. Available from: <https://doi.org/10.1038/s41375-018-0070-8>.
- [43] Wrangle J, Wang W, Koch A, Easwaran H, Mohammad HP, Vendetti F, et al. Alterations of immune response of non-small cell lung cancer with azacytidine. *Oncotarget* 2013;4:2067–79. Available from: <https://doi.org/10.18632/oncotarget.1542>.
- [44] Ogbomo H, Michaelis M, Kreuter J, Doerr HW, Cinatl Jr. J. Histone deacetylase inhibitors suppress natural killer cell cytolytic activity. *FEBS Lett* 2007;581:1317–22. Available from: <https://doi.org/10.1016/j.febslet.2007.02.045>.
- [45] Akimova T, Ge G, Golovina T, Mikheeva T, Wang L, Riley JL, et al. Histone/protein deacetylase inhibitors increase suppressive functions of human FOXP3+ Tregs. *Clin Immunol* 2010;136:348–63. Available from: <https://doi.org/10.1016/j.clim.2010.04.018>.
- [46] Rosborough BR, Castellaneta A, Natarajan S, Thomson AW, Turnquist HR. Histone deacetylase inhibition facilitates GM-CSF-mediated expansion of myeloid-derived suppressor cells in vitro and in vivo. *J Leukoc Biol* 2012;91:701–9. Available from: <https://doi.org/10.1189/jlb.0311119>.

- [47] Shi Y, Fu Y, Zhang X, Zhao G, Yao Y, Guo Y, et al. Romidepsin (FK228) regulates the expression of the immune checkpoint ligand PD-L1 and suppresses cellular immune functions in colon cancer. *Cancer Immunol Immunother* 2021;70:61–73. Available from: <https://doi.org/10.1007/s00262-020-02653-1>.
- [48] Hashimoto A, Fukumoto T, Zhang R, Gabrilovich D. Selective targeting of different populations of myeloid-derived suppressor cells by histone deacetylase inhibitors. *Cancer Immunol Immunother* 2020;69:1929–36. Available from: <https://doi.org/10.1007/s00262-020-02588-7>.
- [49] Cui Y, Cai J, Wang W, Wang S. Regulatory effects of histone deacetylase inhibitors on myeloid-derived suppressor cells. *Front Immunol* 2021;12:690207. Available from: <https://doi.org/10.3389/fimmu.2021.690207>.
- [50] Jiang Y, Ortega-Molina A, Geng H, Ying HY, Hatzi K, Parsa S, et al. CREBBP inactivation promotes the development of HDAC3-dependent lymphomas. *Cancer Discov* 2017;7:38–53. Available from: <https://doi.org/10.1158/2159-8290.CD-16-0975>.
- [51] Mondello P, Tadros S, Teater M, Fontan L, Chang AY, Jain N, et al. Selective inhibition of HDAC3 targets synthetic vulnerabilities and activates immune surveillance in lymphoma. *Cancer Discov* 2020;10:440–59. Available from: <https://doi.org/10.1158/2159-8290.CD-19-0116>.
- [52] Qiu J, Sharma S, Rollins RA, Paul TA. The complex role of EZH2 in the tumor microenvironment: opportunities and challenges for immunotherapy combinations. *Future Med Chem* 2020;12:1415–30. Available from: <https://doi.org/10.4155/fmc-2020-0072>.
- [53] Goswami S, Apostolou I, Zhang J, Skepner J, Anandhan S, Zhang X, et al. Modulation of EZH2 expression in T cells improves efficacy of anti-CTLA-4 therapy. *J Clin Invest* 2018;128:3813–18. Available from: <https://doi.org/10.1172/JCI99760>.
- [54] Wang D, Quiros J, Mahuron K, Pai CC, Ranzani V, Young A, et al. Targeting EZH2 reprograms intratumoral regulatory T cells to enhance cancer immunity. *Cell Rep* 2018;23:3262–74. Available from: <https://doi.org/10.1016/j.celrep.2018.05.050>.

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CRISPR, epigenetics, and cancer 27

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1 Introduction

Control of gene expression plays a key role in a wide variety of biological processes. The temporal and spatial control of gene expression is critical during development to ensure proper lineage commitment, cell fate determination, and organogenesis [1,2]. This tight regulation persists through all life stages of an organism. Processes, such as cellular stress responses, tissue homeostasis, and immunity, rely on precise amounts of proteins or noncoding RNA produced at exactly the right time and in the appropriate cell/tissue. Reflective of its importance the regulatory mechanisms controlling eukaryotic gene expression are extremely complex. Genetic regulation of gene expression is dependent on the

underlying DNA sequence around gene regulatory regions. By contrast, epigenetic regulation controls expression by regulating the chromatin structure, which in turn modulates the frequency, rate, or extent of transcription [3–5]. The epigenetic machinery is composed principally of four interconnected components that ultimately define active or inactive states of chromatin: DNA methylation, histone posttranslational modifications, chromatin three-dimensional conformation, and noncoding RNAs. In keeping with its importance, alterations of the epigenome contribute to the pathogenesis of various inherited or somatically acquired human diseases. In particular, many studies have shown that epigenetic changes are closely associated with each of the steps involved in cancer development and progression, including the acquisition of additional features essential for cancer metastasis [6–13].

Unlike most genetic defects, however, due to the dynamic nature of the epigenome, epigenetic defects are reversible. Such reversibility of epigenetic alterations opens a new avenue to targeted cancer therapeutics often termed as epigenetic editing. Several epigenetics based therapeutics have been developed, such as small molecule inhibitors preventing a DNA or histone modification from being deposited [14–16]. However, the major drawback associated with such inhibitors is that they lack gene specificity, and global chromatin changes are often induced resulting in important side effects [17–20]. On the other hand, the newly developed CRISPR-mediated epigenome editing technologies provide the excellent alternative to small molecule inhibitors [21]. The engineering of programmable enzymes with DNA-binding domains has enabled genome locus-specific targeting of epigenetic modifiers to alter specific local epigenetic modifications directly. In particular, the CRISPR system has become the most widely used epigenome-editing tool due to its high efficiency, versatility, specificity, and ease of use. The type II CRISPR system utilized by *Streptococcus pyogenes* is the best characterized system for genome and epigenome editing, consisting of the Cas9 nuclease, a CRISPR RNA (crRNA), and *trans*-activating CRISPR RNA (tracrRNA). The crRNA hybridizes with the tracrRNA, recruits Cas9 and binds to specific protospacer elements [22]. To simplify the application of this system, the two RNAs can be fused together forming a chimeric, single-guide RNA (sgRNA) [23] (Figure 27.1). Cas9 can be directed to almost any target through modification of this sgRNA molecule by alteration of the 20-bp guide sequence in the spacer.

For epigenome editing, cleavage of the DNA sequence is not required. As such, the Cas9 nuclease is deactivated to remove its catalytic activity. The basic requirement for CRISPR epigenome editing consists of three essential parts: a DNA-binding targeting protein, an effector protein and a unique gRNA sequence (Figure 27.1). With regard to epigenomic perturbation, a variety of epigenetic modulators have been linked to a nuclease-deficient Cas9 (dCas9) without impacting its genome interacting ability [24,25]. Utilizing the RNA-based genome targeting of dCas9, this combination thus allows for genetically defined epigenetic modifications.

In this chapter, we outline relations between epigenetics and cancer and the recent efforts in making the CRISPR technology an efficient molecular tool for targeted epigenomic editing and a promising therapeutic tool against cancer.

2 DNA methylation/demethylation

2.1 DNA methylation/demethylation processes and cancer

DNA methylation is one of the major mechanisms of epigenetic regulation, playing a crucial role in many regulatory processes, such as gene expression, genomic imprinting, and genomic stability.

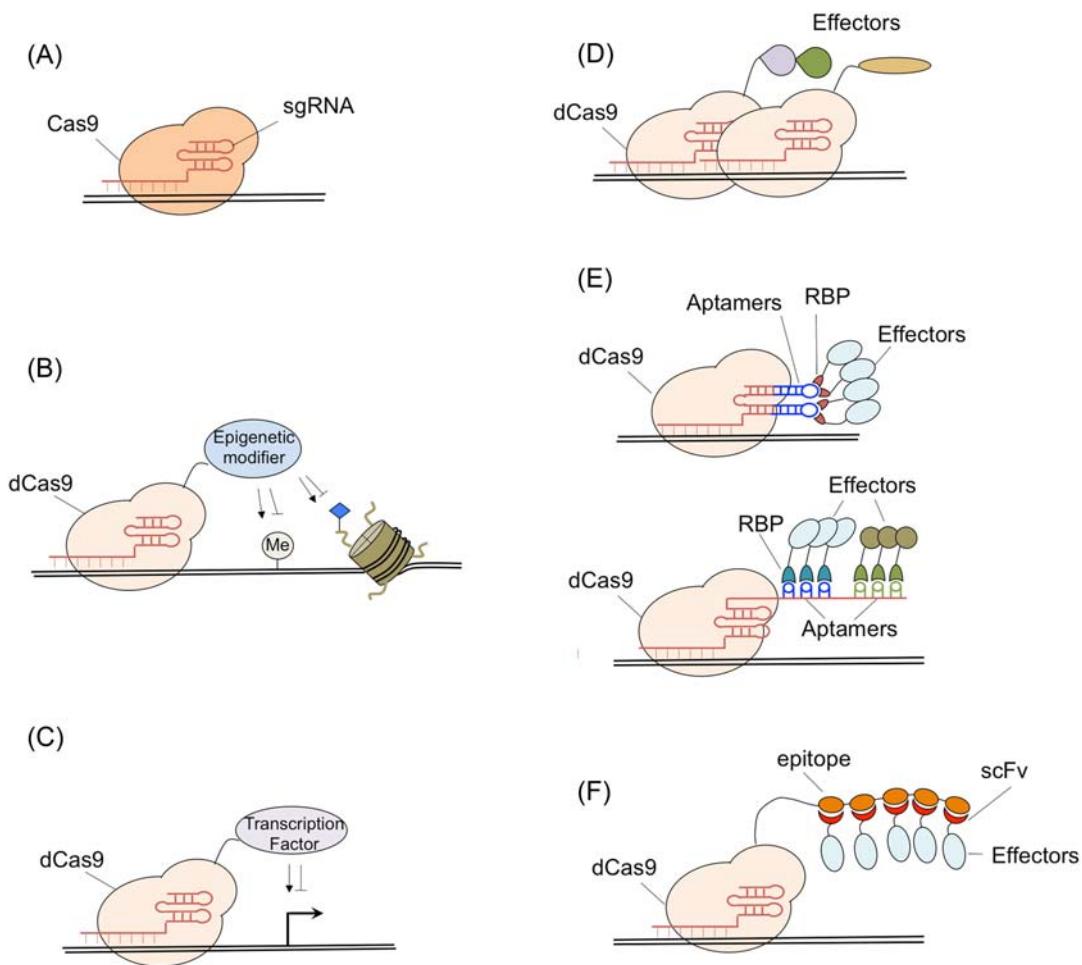


FIGURE 27.1 CRISPR tools for targeted epigenome editing.

(A) CRISPR-Cas9 induces double strand break at target genomic sites in mammalian cells and results in indel formation. (B) Catalytically dead Cas9 (dCas9) variant was engineered to prevent cleavage of double strand DNA and is tethered to an epigenetic modifier to edit epigenetic marks. (C) Transcriptional factor domains tethered to dCas9 to either repress or activate targeted genes. Various sgRNA/dCas9-effector design strategies have been developed to amplify the effector efficiency and to edit different epigenetic marks simultaneously. (D) Multiple effector domains fused to dCas9 system and/or recruitment of different dCas9-effectors fusion proteins. (E) dCas9-scRNA system provides the RNA-binding protein for attachment of effector domain. (F) dCas9-SunTag system which composed of antibody provides the attachment of effector domains.

As a consequence, aberrant methylation plays a role in the pathogenesis of many diseases, including cancers [26–29]. In mammals, CpG (cytosine-guanine dinucleotide) islands are commonly found within the promoter regions of regulatory genes or exons. DNA methylation occurs almost exclusively within the context of CpG islands via covalent addition of a methyl group to the fifth

carbon position of cytosine residues to generate a 5-methylcytosine (5mC). The methylation of cytosine at 5(C) is catalyzed by DNA methyltransferases (DNMTs) in a postreplication reaction, and the donor of the methyl group is the S-adenosyl methionine. DNMTs can both introduce methylation marks (de novo methylation) and maintain them after the genome is replicated (maintenance methylation). Within the mammalian DNMT family, DNMT1 is considered the maintenance methyltransferase, as it propagates the methylation pattern from parental to daughter strands during cell replication [30]. Methyltransferases DNMT3A, DNMT3B, and DNMT3L (a cofactor DNA methyltransferase without enzymatic activity) are instead crucial for de novo DNA methylation [31,32].

Demethylation, which leads to the removal of the methyl group from 5mC, can occur through either passive or active pathways [33]. Passive demethylation is replication dependent and occurs during DNA replication when maintenance methylation of a new DNA strand is inhibited [34]. Active demethylation is a replication-independent process and involves the conversion of methylcytosine to cytosine via either enzymatic oxidation or deamination followed by base excision repair. The DNA methylation enzymes are dioxygenases belonging to the ten-eleven translocation (TET) family (TET1, TET2, and TET3). TET enzymes catalyze the oxidation of methyl groups and produce intermediates that are then recognized and excised by thymine DNA glycosylase (TDG) [35].

DNA methylation is generally linked with transcriptional repression and abnormal DNA hypermethylation has been observed in almost all types of cancer cells, usually in the promoter regions of tumor suppressor genes [36]. Cancer-specific hypermethylated silenced genes are often involved in transcriptional regulation, apoptosis, oxidoreductase activity, and cellular metabolism and are associated with drug resistance. Aberrant hypomethylation is also a feature of several cancers and it often leads to expression of normally silenced genes promoting cell proliferation, chromatin reorganization and chromosomal instability [37,38]. More generally, cancer-associated aberrant DNA methylation entails global hypomethylation with region-specific hypermethylation [39].

3 CRISPR-mediated DNA methylation

To generate CRISPR-based tool capable of modulating DNA methylation at a specific genomic target, various effectors have been fused with the dCas9. In one of the designs, DNMT3A is fused with dCas9 resulting in the dCas9-DNMT3A fusion protein, which causes the suppression of gene by increasing methylation in the promoter region through the enzymatic action of DNMT3A. Higher methylation efficiency and specificity can be achieved combining the synergistic action of different effectors. For example, Stepper et al. used a combination of two DNA methyltransferases, DNMT3a–DNMT3L, fused to dCas9, achieving a approximately fivefold higher than the methylation realized through the targeting of DNMT3a alone. Recruitment of multiple copies of effector domains to the target locus enhances the activity for long-range methylation editing. This enhancement can be achieved by fusing multiple copies of effectors directly to dCas9 or relying on alternative recruitment strategies (Figure 27.1D–F). An example of this is the SUperNova TAGging (SunTag) system developed by Tanenbaum et al. [40]. The SunTag system employs a dCas9 fused to an array of peptide sequences with the capacity to recruit multiple copies of an scFv-fusion

effector domain at a target locus (Figure 27.1F). Huang et al. developed a dCas9-SunTag-DNMT3A system that was able to recruit multiple copies of DNMT3A to the HOXA locus in HEK293 cells obtaining higher methylation efficiency compared to the dCas9-DNMT3A fusion [41].

The transfection-based methylation gain obtained with the systems described above is transient, the CpG methylation achieved is not stable and is gradually lost. However, recent findings have shown that it is possible for epigenome editing to write a stable epigenetic mark that is remembered and propagated by cells without constitutive expression of the programmable epigenetic modulators. One approach is to combine the action of different effectors. For instance, Amabile et al. observed that the transient coexpression of dCas9-KRAB and dCas9-DNMT3A induced methylation and long-term repression of the target genes that was further improved by cotransfection with dCas9-DNMT3L [42]. The possible explanation is that KRAB is recruiting protein complexes that establish a chromatin environment conducive to *de novo* DNA methylation imposed by DNMT3A–DNMT3AL, resulting in compaction of chromatin and long-term memory of gene silencing.

The system we just described relies on the inefficient codelivery of three fusion proteins. More recent works focused on improving the system with alternative designs in which all the three different effectors are fused to a single dCas9 condensing all necessary domains into a single construct. Nakamura and colleagues showed that dCas9-KAL (KRAB, DNMT3a, and DNM3AL) was capable of inducing high levels of stable gene repression. They also highlighted the dependence of the silencing activity on the relative configuration of effector domains fused to dCas9 [43]. Same design was employed in the recently described CRISPR-off system [44]. The DNA methylation deposited by CRISPR-off was maintained for more than 450 cell divisions resulting in a form of gene silencing that is stable and heritable. Furthermore, CRISPR-off epigenetic memories can be reversed using a multipartite epigenetic editor they named CRISPRon, which removes DNA methylation and recruits the transcriptional machinery [44]. Engineering a desired and persistent epigenomic state is a complex and challenging effort and will likely require the use of multiplexed epigenetic modifiers.

Bacterial-derived DNMTs are not expected to recruit endogenous mammalian epigenetic modifiers, which may help to limit off-target effects. Lei et al. adapted the bacterial MQ1, for site-directed promoter methylation in human cells and mouse embryos [45]. To further reduce off-target events, a split version of the M.SssI was shown to generate efficient and targeted DNA methylation [46].

4 CRISPR-mediated DNA demethylation

Based on the same concept of using the catalytic domain instead of the whole enzyme, and in doing so avoiding issues linked with the enzyme (covalent/allosteric) regulation, direct fusion of the TET1 catalytic domain to dCas9 is the first reported strategy for targeted demethylation of 5mC marks [47–49]. dCas9-TET is used to target promoters of endogenous genes or regulatory regions outside of the promoter, such as enhancers.

To improve efficiency, researchers have developed a two-part RNA aptamer-derived system to recruit multiple copies of TET1 [50,51] (Figure 27.1E). This system, named synergistic activation

mediator (SAM), makes use of a scaffold RNA in which protein-binding RNA aptamers are fused with the sgRNA. The RNA aptamer recruits RNA-binding proteins (RBPs) to form RNA aptamer–RBP pairs. The RBPs, in turn, are fused to a TET catalytic domain. Recruitment of multiple copies of TET1 and higher demethylation levels can also be achieved using the SunTag multimerization system we already described for targeted methylation [52].

Targeted DNA demethylation usually erases repressive chromatin markers resulting in upregulation of the target gene; however, the activation level is often gene dependent and affected by the presence of other epigenetic marks. Higher and persistent target gene activation can be achieved with the corecruitment of TET1 and transcription factors domains [53–55]. Alternative demethylation strategies not based exclusively on TET activity have also been developed. For instance, Gregory et al. showed targeted DNA demethylation using TDG, a DNA glycosylase enzymes [56]. An RNA aptamer-derived system, similar to SAM, was utilized to corecruit TET1 and DNA glycosylases involved in the initial step of removing oxidized cytosines produced by TET1, facilitating DNA demethylation [57]. A plant-derived DNA glycosylase (ROS1) can remove 5-meC directly without prior modifications, avoiding formation of 5-mec derivatives [58], which are themselves considered as independent epigenetic marks responsible of unwanted regulatory functions [59,60].

Other strategies for targeted DNA demethylation are based on interference with the endogenous methylation processes. For instance, Lu et al. employed a dCas9-R2 modified gRNA capable of recruiting and sequestering endogenous DNMT1, thus inhibiting DNMT1 enzyme activity at the specific target site and preventing DNA methylation maintenance during replication [61]. In another recent report, researchers demonstrate that dCas9 alone can be used to physically block DNA methylation at specific targets [62]. When endogenous DNMT1 methylates the genome during DNA replication, it does not methylate sites blocked by dCas9.

5 Applications of CRISPR-mediated targeted methylation/demethylation in cancer

Several studies reported the reactivation of tumor suppressor genes through CRISPR-mediated targeted DNA demethylation. The majority of these studies, however, are *ex vivo*, that is, performed in cancerous cell lines that were afterwards transplanted into immune compromised mice. For instance, Choudhury et al. obtained demethylation of the BRCA1 gene delivering dCas9-TET1 in Hela and MCF7 cells. Upregulation of the BRCA1 gene and consequently, inhibition of cell growth was observed [49]. Morita et al. used the dCas9-SunTag-TET1 system for targeted demethylation of RHOXF2B, CARD9, SH3BP2, and CNKSR1 genes in human cells, with upregulation of targeted genes [52]. The dCas9-SunTag system was also recently employed to edit the DNA methylation of EBF3, a putative epigenetic driver of melanoma metastasis, in human melanoma cells [63]. Same CRISPR system was used to target SARI (suppressor of activator protein 1 regulated by IFN) a tumor suppressor gene that is inactivated in various cancers. Epigenetic demethylation of the SARI promoter region re-activates the silenced gene resulting in antitumor effects on tumor proliferation, apoptosis, and angiogenesis [64].

There are also examples of CRISPR-mediated DNA methylation to precisely methylate and silence the overactivation of oncogenic genes or genes involved in oncogenic signaling pathway.

For instance, Wang et al. achieved targeted methylation and stable repression of GNR. GNR encodes for Granulin protein, a secreted glycosylated peptide associated with oncogenesis. Epigenetic silencing of GRN decreases invasion and tumorsphere formation in Hep3B tumor cells thus demonstrating the potential utility of using dCas9 epi-suppressors in the development of epigenetic targeting against tumors [65]. Other studies on targeted DNA methylation are focused on probing the effects of methylation on genes implicated in oncogenesis to identify new epigenetic targets for cancer therapy. Wu and colleagues used the dCas9-DNMT3a to induce DNA methylation and silencing of SMARCA2 to understand the epigenetic regulation of the gene in lung cancer [66]. Targeted demethylation of CDKN2A with dCas9-DNMT3A-3L demonstrates the linkage of CDKN2A gene suppression and progression of breast cancer [67]. In a more recent report, Vizoso and colleagues focused their studies on IGFBP2, a gene regulated by DNA methylation, which has been reported to function both as a tumor-promoting and -suppressing gene. dCas9-DNMT3A targeting IGFBP2 induces DNA methylation, gene silencing and a phenotypic switch in cell migration and cell morphology [68].

Overall, these studies show how CRISPR-mediated DNA methylation/demethylation can potentially represent a new therapeutic tool against cancer and be employed to investigate the role of DNA methylation in cancer development.

6 Histone modification

6.1 Histone posttranslational modifications and cancer

Several posttranslational modifications of histones have been reported, including acetylation, methylation, citrullination, ubiquitination, SUMOylation, ADP-ribosylation, proline isomerization, and phosphorylation, occur on histones [69]. These modifications represent a major mechanism by which chromatin structure and function are regulated. The pattern of modifications has been suggested to act as an information code, the so-called histone code [70,71]. Distinct histone marks function sequentially or in combination to initiate distinct downstream changes that collectively influence chromatin packaging and gene regulation. Furthermore, histone modifications can interact with DNA methylation and together give rise to an epigenetic code associated with high or low levels of gene expression [72]. Histone modification are imposed, removed, or recognized by specific proteins.

The most studied histone modifications are histone methylation and acetylation [69]. The lysine residues on histone tails can be mono-, di-, or tri-methylated by a set of proteins, generically referred to as histone methyltransferases (HMT) and removed by histone demethylase (HDM). The transcriptional influence of lysine methylation is strongly context dependent. However, methylation of lysine 4 on histone 3 (H3K4) and lysine 36 (H3K36) are often associated with gene activation [73–75]. In contrast, modifications, such as methylation of lysine 9 on histone 3 (H3K9), lysine 27 (H3K27), or lysine 20 (H3K20), are often linked to silenced genes [73,76,77]. Lysine residues, such as lysine 36 (H3K36) and lysine 64 (H3K64), on histone 3 can be acetylated, which typically results in a less condensed chromatin state coupled with an increase in transcriptional activity [78–80]. Histone acetyl transferases (HATs) are the enzymes that deposit acetylation marks, and histone deacetylases (HDACs) are the enzymes that remove these epigenetic marks. Acetylated

histones are associated with active chromatin and actively transcribed genes, whereas gene repression and tight chromatin structure are often due to the deacetylation of histones [81].

It has been observed that, the loss of histone acetylation and methylation of core histone proteins H3 and H4 are linked with carcinogenesis [82,83]. Different histone methylation marks have been identified in all major types of cancer [84]. Recent pan-cancer analysis of over 15 cancer types demonstrated the reduction of H3K9me2, H3K9me3, H3K36me2, and H3K36me3 to be an oncogenic feature [85]. HATs and HDACs have also been identified in the initiation, progression, and metastasis of cancers [86,87].

Although less studied, other histone epigenetic marks, such as phosphorylation, ubiquitination, poly-ADP ribosylation, sumoylation, and glycosylation, have also been implicated in transcription regulation and in the development of cancer [88].

7 CRISPR-mediated histone methylation/demethylation

Targeted histone methylation can be achieved by direct fusion of the catalytic domain of specific HMTs to dCas9. Cano-Rodriguez et al. fused dCas9 to the catalytic domains of HMTs PRDM9 and DOT1L. The H3K79me mark, introduced by dCas9–DOT1L is required for the stability and maintenance of the H3K4me3 modification introduced by dCas9-PRDM9, and the effects this editing has on gene activation depends on the methylation level of the targeted DNA, an evident demonstration of the interplay between different histone marks and the preexisting chromatin microenvironment [89]. In another report, deposition of the repressive H3K9me3 mark and subsequent repression of the target gene HER2 was obtained using dCas9 fused to G9A [90]. Targeted histone modification with the addition of H3K27me3, another epigenetic mark often associated with gene silencing, is obtained targeting endogenous genes with dCas9 fused with the catalytic domain of EZH2, a subunit of the Polycomb Repressive Complex 2 (PCR2) [91]. To improve histone methylation levels, Chen et al., employed a more efficient sgRNA aptamer-based system with EZH2 fused to PP7 coat protein (PCP) resulting in the EZH2-PCP component. The dCas9/gRNA complex recruits EZH2-PCP on the target site to efficiently modify histone proteins adding the H3K27me3 marks [92]. H3K27me3 is a repressive marker, however, the effect on gene expression depends on the target DNA methylation status [91] and the presence of other histone modifications [93], highlighting once more the complex cooperation between different epigenetic marks and the preexisting chromatin context in modulating gene expression.

CRISPR-mediated histone demethylation is obtained using dCas9 in combination with the catalytic domain of LSD1, an HDM that catalyzes the removal of the methyl groups from H3K4me1/2 and H3K9me2, which are associated with active and repressive chromatin, respectively [94–98]. Recently the combinatorial action of LSD1 and KRAB in repressing enhancer activity was tested using an aptamer-based strategy combining dCas9-LSD1 together with Ms2-sgRNA to recruit MCP–KRAB repressor domains [98]. Alternative methods for targeted histone demethylation have been described. An interesting approach is based on a competitive binding approach to indirectly inhibit HMT activity. In this work, dCas9 is fused to a computationally designed protein that specifically binds and locally sequesters EED, a component of the PCR2 complex. The HMT EZH2 is part of PCR2 and the EED binder (EB) competes with EZH2 in binding EED. dCas9-EB allows for PRC2 inhibition at a precise locus resulting in targeted H3K27me3 reduction [99].

8 CRISPR-mediated histone acetylation/deacetylation

The most commonly used HAT family in the field of epigenetic studies is the p300/CBP family of HATs. The p300 catalytic domain fused to dCas9 enables acetylation of H3K27 in a locus-specific manner. Recruitment of dCas9-p300 to either promoters or enhancers induces target gene activation concomitant with the deposition of H3K27ac marker [100,101]. dCas9-p300 has been applied in different studies, on its own to identify transcriptional regulatory elements [102] or in combination with other epigenetic modifiers to evaluate the combinatorial action of different epigenetic modifiers in a locus-specific manner [53,98,103].

HDACs are the eraser enzymes that remove lysine acetylation, often resulting in transcriptional repression. Full-length HDAC3 and HDAC8 enzymes have been integrated in CRISPR systems for targeted histone deacetylation. dCas9-HDAC3 and dCas9-HDAC8 fusion protein were employed to target the promoter of endogenous genes and repress gene transcription following deacetylation of H3K27ac [103,104].

9 Applications of CRISPR-mediated histone modifications in cancer

CRISPR/dCas9 systems linked to histone modifiers have been used to modulate oncogenes and tumor suppressor genes. However, they are often used in combination with alternative CRISPR systems. In fact, for targeted gene activation/repression, several studies make use of more efficient CRISPR-based synthetic transcription factors that recruit transcriptional complexes, which in turn deposit epigenetic marks, rather than using CRISPR-based histone modifiers for direct histone modification. These systems are briefly described in the next paragraph.

Nonetheless, waking up of dormant tumor suppressor genes can be obtained using dCas9-p300. Transiently transfected dCas9-p300 systems were able to reactivate tumor suppressor genes epigenetically silenced in cancer cells [105]. CRISPR-mediated p300 and LSD1 recruitment has been employed to activate/repress oncogenic super-enhancer resulting in modulation of oncogenes expression and cancer progression [98,106]. These gain-of-function approaches facilitate the identification of gene-distal *cis*-regulatory elements that are necessary and/or sufficient for oncogene expression. The combination of multisuppressors, that is, dCas9-DNMT3a, dCas9-EZH2, and dCas9-KRAB, serves as an efficient epigenetic approach to inhibit the oncogenes for liver cancer therapy [64,107].

10 CRISPR-dCas9-based synthetic transcription factors

The CRISPR systems described above allow the fine-tuning of individual epigenetic perturbations to better understand the impact of single epigenetic modifications on cancer development and progression. From a therapeutic point of view these tools offer the advantage of precision and can potentially be employed for correction of specific disease-associated epigenetic aberrations. However, such systems may not maximize up- or downregulation of the target gene, as the impact of a specific epigenetic mark on gene expression depends from the chromatin context and the

presence of other epigenetic marks. CRISPR epigenetic modifiers might not be the best choice for targeted modulation of onco-suppressor genes.

Another set of CRISPR tools have been optimized for this purpose. CRISPR-dCas9-based synthetic transcription factors (dCas9-sTFs) consists in dCas9 combined with activation/repression domains of several transcription factors and they efficiently modulate the expression of target genes [108]. Herpes simplex viral protein 16 (VP16), and its multiple copies VP48, VP64, VP120, and transactivator domain of nuclear factor kappa B (p65) are the examples of transcription activating factors utilized in CRISPR activation (CRISPRa) systems [109,110]. On the other hand, transcription repressive factors, such as KRAB and MeCP2, are combined in CRISPR interference (CRISPRi) systems to repress target genes [111,112]. These domains promote the concomitant recruitment of chromatin-modifying proteins, additional transcription factors, and DNA-binding proteins, causing accumulation of epigenetic markers, chromatin de-condensation/ condensation, and ultimately transcriptional perturbation [113,114].

There are several published data on different oncogenes and tumor suppressor genes targeted with dCas9-sTFs. The majority of genes were targeted with sgRNAs against promoter proximal regions. For example, Moses and colleagues showed how dCas9 fused to the transactivator VP64-p65-Rta (VPR) can be directed to the PTEN proximal promoter in cancer cells that exhibited low levels of PTEN expression. The dCas9-VPR system increased PTEN expression in melanoma and TNBC cell lines [115]. Similar results were obtained in another study where dCas9-VPR was targeted to the promoter region of REPRIMO and MASPIN, tumor suppressor genes often silenced in cancer cells. Targeted gene activation led to a concomitant cell proliferation inhibition and apoptosis induction [105]. CRISPRa can also be used to express target antigenic peptide in cancer cells to enhance the elimination of tumor cells through the immune response generated by the peptide [116].

There are also examples of CRISPRi for oncogene repression. For instance, dCas9-KRAB was able to selectively repress a specific oncogenic isoform of TP63 without affecting alternative transcripts, making it a much safer alternative to RNAi, which is more prone to off-target events [117]. Targeted repression of the isoform Δ Np63 effectively decreased cell proliferation and induced apoptosis in lung and esophageal SCC cells *in vitro* and significantly inhibited tumor growth in a mouse lung SCC xenograft model *in vivo* [117].

CRISPRi can also be used to silence or inactivate multidrug resistance genes. For instance, the long noncoding RNA KCNQ1OT1 is overexpressed in squamous cell carcinoma tissues and lung cancer that are resistant to cisplatin. KCNQ1OT1 was targeted with dCas9-KRAB and its expression inhibited in CAL27-res and SCC9-res cells improving the chemosensitivity to cisplatin [118].

dCas9-sTFs have represents also efficient tools to study cancer progression and therapeutic resistance models. For instance, Chen et al. employed the SAM system (dCas9-VP64/sgRNA-Ms2-P65-HSF1) to induce the overexpression of methyltransferase-like 3 (METTL3) in human hepatocellular carcinoma MHCC97L cells. Upon subcutaneous injection of METTL3-overexpressing MHCC97L cells into BABL/cAnN-nude mice, they observed a marked increase in tumor growth and enhanced cellular migration [119]. In another report, dCas9-VP64-mediated transcriptional activation of O6-methylguanine–DNA methyltransferase (Mgmt) in B-cell lymphoma cells leads to resistance to temozolomide treatment after xenografting into immunocompetent C57BL6/J mice [120].

These examples show how dCas9-sTFs can be employed to activate tumor suppressor genes, silence oncogenes and remove tumor resistance mechanisms for targeted therapy. Future research should evaluate the possibility to combine dCas9-sTFs with drugs that have not been effective in treating cancer to investigate potential advantages to reduce cancer mortality in a cost-effective manner and with more efficient results.

11 Limitation and challenges

CRISPR-based epigenetic tools hold great promise for cancer treatment. However, there are still several limitations that must be addressed to fulfill the great potential of CRISPR-based therapies.

12 *In vivo* delivery of CRISPR systems

The efficient delivery of CRISPR systems is still one of the main challenging aspects and significant research is underway for the development of safe and effective *in vivo* delivery of CRISPR therapeutics [121]. In an ideal situation, an optimal delivery system should introduce CRISPR components specifically to the tissue of interest without inducing immunological reactions or other toxic events. One major constraint specific for dCas9-effector fusion proteins is their large size. Smaller Cas9 variants or the use of split-dCas9 systems could provide a solution to the size issue [122–124].

Delivery systems are mainly divided into physical process, viral delivery, and nonviral delivery. Physical methods include electroporation [125], microinjection [126], and hydrodynamic delivery [127]. The main concern associated with physical methods is their potential toxicity and the lack of tissue specific delivery in a natural anatomic location. The viral vectors employed for *in vivo* delivery are lentivirus and AAV viral vectors [128]. Benefits of using lentivirus for *in vivo* gene therapy include its 10 kb carrying capacity and prolonged transgene expression. Disadvantages of *in vivo* utilization include the potential for oncogenicity, insertional mutagenesis, and immunogenic responses. On the other hand, AAVs lack the immunogenic and tumorigenesis, however, a significant drawback is the packaging limitation of 4.2 kb in size. Nonviral vectors, such as liposomes, lipid nanoparticles (LNPs), polymeric nanoparticles, peptide nanoparticles, inorganic nanoparticles, and extracellular vesicles have the advantages of safety, minimal immunogenicity, low cost in large scale production, and better loading capacity [129,130]. Main disadvantages include endosomal degradation, phagocytic clearance, insufficient distribution at the target site, and instability under physiological conditions.

13 Off-target effects

Specificity of *in vivo* genome editing is critical to safe applications of CRISPR-Cas9 therapeutics in the clinic. CRISPR/Cas9 is used for precise gene editing; however, CRISPR systems suffer from off-target effects. Gene editing at off-target tissues and at the incorrect sites may lead to undesired effects. This is also relevant for CRISPR-based epigenetic tools, where the optimal design of

sgRNAs on the target gene must ensure that the deposited epigenetic marks does not spread on the neighboring genes. Several efforts have been made to reduce the off-target effects, including engineering Cas9 nucleases, such as high-fidelity Cas9 [131], hyperaccurate Cas9 [132], Cas9 variants with expanded PAM compatibilities [133], and Cas9 fused with artificial inhibitory domains [134]. Another concern is that overexpression of an epigenetic catalytic domain alone might be enough to induce genome-wide aspecific epigenetic alterations [135]. Transient exposure of the cellular genome to CRISPR-epigenetic modifiers limits the residence time of these proteins, also decreasing the likelihood of off-target activity. Development of epigenetic effector with reduced endogenous DNA binding affinity can also improve specificity [136]. Some other reported strategies are based on the more sophisticated control of effector recruitment [137]. Tissue-specific delivery of the CRISPR system is also important and innovative targeted delivery vectors play vital roles in reducing the delivery-mediated off-target effects. Smart systems, such as stimuli-responsive nonviral delivery systems, are promising to enable spatiotemporal genome editing by releasing or activating CRISPR-Cas9 components in target cells only when endogenous or exogenous signals trigger the release [138,139].

14 Future perspectives

CRISPR technology is rapidly evolving generating editing tools with high clinical potential. Several clinical trials with CRISPR for gene editing are ongoing with CRISPR/Cas9 against multiple cancers (ClinicalTrials.gov, <https://clinicaltrials.gov/ct2/home>). These clinical trials encourage the development of CRISPR tools for epigenetic editing as a therapeutic strategy against cancer. The potential to selectively silence a mutated gene or activate the healthy gene copy with long duration effects has vast potential in cancer treatment that could be achieved as CRISPR epigenome editing technology advances. CRISPRi, CRISPRa and CRISPR epigenome editors have mostly been tested *in vitro* to date. For an *in vivo* clinical application, several challenges still remain to be addressed. There is still uncertainty regarding the *in vivo* efficacy and safety of CRISPR-based epigenetic modifiers. More studies on genome-wide off-target effects are needed. The recent efforts on development of smaller Cas9 variants, with broader PAM compatibility, superior effector domains with high on-target specificity, and more efficient gRNA designs, are laying the foundations for the new generation CRISPR tools capable of higher on target editing, enhanced genome-wide targeting accuracy, and better delivery. We can for instance envision the rational design of more advanced dCas9-effector proteins capable of displaying the epigenetic catalytic domains only upon binding of the target sites further reducing off-target effects. Protein engineering for Cas9 nucleases can also aim to reduce immunogenicity, improve cellular entry, endosomal escaping, and nucleus entry. Epigenetic mechanisms are interlinked and oncogenesis is the collective response of several epigenome alterations. Although challenging it is of extreme interest the development of compact and efficient CRISPR systems capable of editing different epigenetic marks simultaneously. The multiplexing capability of CRISPR technology is indeed another attractive feature for treating an epigenetically complex disease like cancer and should be explored further. Tissue-specific systemic delivery is another important aspect that requires the development of novel more efficient viral and nonviral systems as well as novel vector designs.

15 Conclusions

Cancer is a complex disease associated with accumulative genetic mutations and epigenetic alterations. The presence of global epigenetic abnormalities in all human cancers suggests the necessity of developing therapeutic strategies to specifically target and reverse these pathogenic aberrations. CRISPR/Cas9 holds several advantages over other techniques including its simple design, easy operation, good specificity, and high efficiency. Personalized and targeted therapy based on CRISPR/Cas9 will possibly shape the development of tumor therapy in the future. CRISPR/Cas9 still has many unresolved problems that must be tackle, such as immunogenicity, *in vivo* delivery and targeting specificity. Rigorous clinical tests, including efficacy, safety, and specificity tests are required. Nonetheless, CRISPR/Cas9 holds great potentials for the treatment of human diseases, such as cancer.

References

- [1] Boland MJ, Nazor KL, Loring JF. Epigenetic regulation of pluripotency and differentiation. *Circ Res* 2014;115:311–24. Available from: <https://doi.org/10.1161/CIRCRESAHA.115.301517>.
- [2] Praggastis SA, Thummel CS. Right time, right place: the temporal regulation of developmental gene expression. *Genes Dev* 2017;31:847–8. Available from: <https://doi.org/10.1101/gad.301002.117>.
- [3] Lelli KM, Slattery M, Mann RS. Disentangling the many layers of eukaryotic transcriptional regulation. *Annu Rev Genet* 2012;46:43–68. Available from: <https://doi.org/10.1146/annurev-genet-110711-155437>.
- [4] Schneider R, Grosschedl R. Dynamics and interplay of nuclear architecture, genome organization, and gene expression. *Genes Dev* 2007;21:3027–43. Available from: <https://doi.org/10.1101/gad.1604607>.
- [5] Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. *Cell* 2007;128:669–81. Available from: <https://doi.org/10.1016/j.cell.2007.01.033>.
- [6] Lund AH, van Lohuizen M. Epigenetics and cancer. *Genes Dev* 2004;18:2315–35. Available from: <https://doi.org/10.1101/gad.1232504>.
- [7] Kanwal R, Gupta S. Epigenetics and cancer. *J Appl Physiol* 2010;109:598–605. Available from: <https://doi.org/10.1152/japplphysiol.00066.2010>.
- [8] Alzrigat M, Párraga AA, Jernberg-Wiklund H. Epigenetics in multiple myeloma: from mechanisms to therapy. *Semin Cancer Biol* 2018;51:101–15. Available from: <https://doi.org/10.1016/j.semcan.2017.09.007>.
- [9] Duruisseaux M, Esteller M. Lung cancer epigenetics: from knowledge to applications. *Semin Cancer Biol* 2018;51:116–28. Available from: <https://doi.org/10.1016/j.semcan.2017.09.005>.
- [10] Pasculli B, Barbano R, Parrella P. Epigenetics of breast cancer: biology and clinical implication in the era of precision medicine. *Semin Cancer Biol* 2018;51:22–35. Available from: <https://doi.org/10.1016/j.semcan.2018.01.007>.
- [11] Moran B, Silva R, Perry AS, Gallagher WM. Epigenetics of malignant melanoma. *Semin Cancer Biol* 2018;51:80–8. Available from: <https://doi.org/10.1016/j.semcan.2017.10.006>.
- [12] Timp W, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. *Nat Rev Cancer* 2013;13:497–510. Available from: <https://doi.org/10.1038/nrc3486>.
- [13] Chatterjee A, Rodger EJ, Eccles MR. Epigenetic drivers of tumourigenesis and cancer metastasis. *Semin Cancer Biol* 2018;51:149–59. Available from: <https://doi.org/10.1016/j.semcan.2017.08.004>.

- [14] James LI, Frye SV. Targeting chromatin readers. *Clin Pharmacol Ther* 2013;93:312–14. Available from: <https://doi.org/10.1038/clpt.2013.6>.
- [15] Mohammad HP, Barbash O, Creasy CL. Targeting epigenetic modifications in cancer therapy: erasing the roadmap to cancer. *Nat Med* 2019;25:403–18. Available from: <https://doi.org/10.1038/s41591-019-0376-8>.
- [16] Herold JM, Wigle TJ, Norris JL, Lam R, Korboukh VK, Gao C, et al. Small-molecule ligands of methyl-lysine binding proteins. *J Med Chem* 2011;54:2504–11. Available from: <https://doi.org/10.1021/jm200045v>.
- [17] Cossío FP, Esteller M, Berdasco M. Towards a more precise therapy in cancer: exploring epigenetic complexity. *Curr Opin Chem Biol* 2020;57:41–9. Available from: <https://doi.org/10.1016/j.cbpa.2020.04.008>.
- [18] el Bahhaj F, Dekker FJ, Martinet N, Bertrand P. Delivery of epidrugs. *Drug Discov Today* 2014;19:1337–52. Available from: <https://doi.org/10.1016/j.drudis.2014.03.017>.
- [19] Miyamoto K, Ushijima T. Diagnostic and therapeutic applications of epigenetics. *Jpn J Clin Oncol* 2005;35:293–301. Available from: <https://doi.org/10.1093/jjco/hyi088>.
- [20] Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 2004;429:457–63. Available from: <https://doi.org/10.1038/nature02625>.
- [21] Brezgin S, Kostyusheva A, Kostyushev D, Chulanov V. Dead Cas systems: types, principles, and applications. *Int J Mol Sci* 2019;20:6041. Available from: <https://doi.org/10.3390/ijms20236041>.
- [22] Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 2013;8:2281–308. Available from: <https://doi.org/10.1038/nprot.2013.143>.
- [23] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012;337:816–21. Available from: <https://doi.org/10.1126/science.1225829>.
- [24] Martella A, Fisher DI. Regulation of gene expression and the elucidative role of CRISPR-based epigenetic modifiers and CRISPR-induced chromosome conformational changes. *CRISPR J* 2021;4:43–57. Available from: <https://doi.org/10.1089/crispr.2020.0108>.
- [25] Gjaltema RAF, Rots MG. Advances of epigenetic editing. *Curr Opin Chem Biol* 2020;57:75–81. Available from: <https://doi.org/10.1016/j.cbpa.2020.04.020>.
- [26] Hollister JD, Gaut BS. Epigenetic silencing of transposable elements: a trade-off between reduced transposition and deleterious effects on neighboring gene expression. *Genome Res* 2009;19:1419–28. Available from: <https://doi.org/10.1101/gr.091678.109>.
- [27] Inbar-Feigenberg M, Choufani S, Butcher DT, Roifman M, Weksberg R. Basic concepts of epigenetics. *Fertil Steril* 2013;99:607–15. Available from: <https://doi.org/10.1016/j.fertnstert.2013.01.117>.
- [28] Sharp AJ, Stathaki E, Migliavacca E, Brahmachary M, Montgomery SB, Dupre Y, et al. DNA methylation profiles of human active and inactive X chromosomes. *Genome Res* 2011;21:1592–600. Available from: <https://doi.org/10.1101/gr.112680.110>.
- [29] Tucci V, Isles AR, Kelsey G, Ferguson-Smith AC, Tucci V, Bartolomei MS, et al. Genomic imprinting and physiological processes in mammals. *Cell* 2019;176:952–65. Available from: <https://doi.org/10.1016/j.cell.2019.01.043>.
- [30] Kar S, Deb M, Sengupta D, Shilpi A, Parbin S, Torrisani J, et al. An insight into the various regulatory mechanisms modulating human DNA methyltransferase 1 stability and function. *Epigenetics* 2012;7:994–1007. Available from: <https://doi.org/10.4161/epi.21568>.
- [31] Plasschaert RN, Bartolomei MS. Genomic imprinting in development, growth, behavior and stem cells. *Development* 2014;141:1805–13. Available from: <https://doi.org/10.1242/dev.101428>.
- [32] Veland N, Lu Y, Hardikar S, Gaddis S, Zeng Y, Liu B, et al. DNMT3L facilitates DNA methylation partly by maintaining DNMT3A stability in mouse embryonic stem cells. *Nucleic Acids Res* 2019;47:152–67. Available from: <https://doi.org/10.1093/nar/gky947>.

- [33] Cortellino S, Xu J, Sannai M, Moore R, Caretti E, Cigliano A, et al. Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. *Cell* 2011;146:67–79. Available from: <https://doi.org/10.1016/j.cell.2011.06.020>.
- [34] Hashimoto H, Liu Y, Upadhyay AK, Chang Y, Howerton SB, Vertino PM, et al. Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation. *Nucleic Acids Res* 2012;40:4841–9. Available from: <https://doi.org/10.1093/nar/gks155>.
- [35] Wu X, Zhang Y. TET-mediated active DNA demethylation: mechanism, function and beyond. *Nat Rev Genet* 2017;18:517–34. Available from: <https://doi.org/10.1038/nrg.2017.33>.
- [36] Kulis M, Esteller M. DNA methylation and cancer. *Adv Genet* 2010;27–56. Available from: <https://doi.org/10.1016/B978-0-12-380866-0.60002-2>.
- [37] Saghafinia S, Mina M, Riggi N, Hanahan D, Ciriello G. Pan-cancer landscape of aberrant DNA methylation across human tumors. *Cell Rep* 2018;25:1066–1080.e8. Available from: <https://doi.org/10.1016/j.celrep.2018.09.082>.
- [38] Wilson AS, Power BE, Molloy PL. DNA hypomethylation and human diseases. *Biochim Biophys Acta Rev Cancer* 2007;1775:138–62. Available from: <https://doi.org/10.1016/j.bbcan.2006.08.007>.
- [39] Klutstein M, Nejman D, Greenfield R, Cedar H. DNA methylation in cancer and aging. *Cancer Res* 2016;76:3446–50. Available from: <https://doi.org/10.1158/0008-5472.CAN-15-3278>.
- [40] Tanenbaum ME, Gilbert LA, Qi LS, Weissman JS, Vale RD. A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell* 2014;159:635–46. Available from: <https://doi.org/10.1016/j.cell.2014.09.039>.
- [41] Huang Y-H, Su J, Lei Y, Brunetti L, Gundry MC, Zhang X, et al. DNA epigenome editing using CRISPR-Cas SunTag-directed DNMT3A. *Genome Biol* 2017;18:176. Available from: <https://doi.org/10.1186/s13059-017-1306-z>.
- [42] Amabile A, Migliara A, Capasso P, Biffi M, Cittaro D, Naldini L, et al. Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. *Cell* 2016;167:219–232.e14. Available from: <https://doi.org/10.1016/j.cell.2016.09.006>.
- [43] Nakamura M, Ivec AE, Gao Y, Qi LS. Durable CRISPR-based epigenetic silencing. *BioDesign Res* 2021;2021:1–8. Available from: <https://doi.org/10.34133/2021/9815820>.
- [44] Nuñez JK, Chen J, Pommier GC, Cogan JZ, Replogle JM, Adriaens C, et al. Genome-wide programmable transcriptional memory by CRISPR-based epigenome editing. *Cell* 2021;184:2503–2519.e17. Available from: <https://doi.org/10.1016/j.cell.2021.03.025>.
- [45] Lei Y, Zhang X, Su J, Jeong M, Gundry MC, Huang Y-H, et al. Targeted DNA methylation *in vivo* using an engineered dCas9-MQ1 fusion protein. *Nat Commun* 2017;8:16026. Available from: <https://doi.org/10.1038/ncomms16026>.
- [46] Xiong T, Meister GE, Workman RE, Kato NC, Spellberg MJ, Turker F, et al. Targeted DNA methylation in human cells using engineered dCas9-methyltransferases. *Sci Rep* 2017;7:6732. Available from: <https://doi.org/10.1038/s41598-017-06757-0>.
- [47] Liu XS, Wu H, Ji X, Stelzer Y, Wu X, Czauderna S, et al. Editing DNA methylation in the mammalian genome. *Cell* 2016;167:233–247.e17. Available from: <https://doi.org/10.1016/j.cell.2016.08.056>.
- [48] Liu XS, Wu H, Krzisch M, Wu X, Graef J, Muffat J, et al. Rescue of fragile X syndrome neurons by DNA methylation editing of the FMR1 gene. *Cell* 2018;172:979–992.e6. Available from: <https://doi.org/10.1016/j.cell.2018.01.012>.
- [49] Choudhury SR, Cui Y, Lubecka K, Stefanska B, Irudayaraj J. CRISPR-dCas9 mediated TET1 targeting for selective DNA demethylation at *BRCA1* promoter. *Oncotarget* 2016;7:46545–56. Available from: <https://doi.org/10.18632/oncotarget.10234>.

- [50] Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 2015;517:583–8. Available from: <https://doi.org/10.1038/nature14136>.
- [51] Xu X, Tao Y, Gao X, Zhang L, Li X, Zou W, et al. A CRISPR-based approach for targeted DNA demethylation. *Cell Discov* 2016;2:16009. Available from: <https://doi.org/10.1038/celldisc.2016.9>.
- [52] Morita S, Noguchi H, Horii T, Nakabayashi K, Kimura M, Okamura K, et al. Targeted DNA demethylation in vivo using dCas9–peptide repeat and scFv–TET1 catalytic domain fusions. *Nat Biotechnol* 2016;34:1060–5. Available from: <https://doi.org/10.1038/nbt.3658>.
- [53] Morita S, Horii T, Kimura M, Hatada I. Synergistic upregulation of target genes by TET1 and VP64 in the dCas9–SunTag platform. *Int J Mol Sci* 2020;21:1574. Available from: <https://doi.org/10.3390/ijms21051574>.
- [54] Josipović G, Tadić V, Klasić M, Zanki V, Bečeheli I, Chung F, et al. Antagonistic and synergistic epigenetic modulation using orthologous CRISPR/dCas9-based modular system. *Nucleic Acids Res* 2019;47:9637–57. Available from: <https://doi.org/10.1093/nar/gkz709>.
- [55] Halmai JANM, Deng P, Gonzalez CE, Coggins NB, Cameron D, Carter JL, et al. Artificial escape from XCI by DNA methylation editing of the CDKL5 gene. *Nucleic Acids Res* 2020;48:2372–87. Available from: <https://doi.org/10.1093/nar/gkz1214>.
- [56] Gregory DJ, Mikhaylova L, Fedulov AV. Selective DNA demethylation by fusion of TDG with a sequence-specific DNA-binding domain. *Epigenetics* 2012;7:344–9. Available from: <https://doi.org/10.4161/epi.19509>.
- [57] Taghbalout A, Du M, Jillette N, Rosikiewicz W, Rath A, Heinen CD, et al. Enhanced CRISPR-based DNA demethylation by Casilio-ME-mediated RNA-guided coupling of methylcytosine oxidation and DNA repair pathways. *Nat Commun* 2019;10:4296. Available from: <https://doi.org/10.1038/s41467-019-12339-7>.
- [58] Devesa-Guerra I, Morales-Ruiz T, Pérez-Roldán J, Parrilla-Doblas JT, Dorado-León M, García-Ortiz MV, et al. DNA methylation editing by CRISPR-guided excision of 5-methylcytosine. *J Mol Biol* 2020;432:2204–16. Available from: <https://doi.org/10.1016/j.jmb.2020.02.007>.
- [59] Bachman M, Uribe-Lewis S, Yang X, Burgess HE, Iurlaro M, Reik W, et al. 5-Formylcytosine can be a stable DNA modification in mammals. *Nat Chem Biol* 2015;11:555–7. Available from: <https://doi.org/10.1038/nchembio.1848>.
- [60] Kohwi M, Lupton JR, Lai S-L, Miller MR, Doe CQ. Developmentally regulated subnuclear genome reorganization restricts neural progenitor competence in *Drosophila*. *Cell* 2013;152:97–108. Available from: <https://doi.org/10.1016/j.cell.2012.11.049>.
- [61] Lu A, Wang J, Sun W, Huang W, Cai Z, Zhao G, et al. Reprogrammable CRISPR/dCas9-based recruitment of DNMT1 for site-specific DNA demethylation and gene regulation. *Cell Discov* 2019;5:22. Available from: <https://doi.org/10.1038/s41421-019-0090-1>.
- [62] Sapozhnikov DM, Szyf M. Unraveling the functional role of DNA demethylation at specific promoters by targeted steric blockage of DNA methyltransferase with CRISPR/dCas9. *Nat Commun* 2021;12:5711. Available from: <https://doi.org/10.1038/s41467-021-25991-9>.
- [63] Smith J, Banerjee R, Waly R, Urbano A, Gimenez G, Day R, et al. Locus-specific DNA methylation editing in melanoma cell lines using a CRISPR-based system. *Cancers* 2021;13:5433. Available from: <https://doi.org/10.3390/cancers13215433>.
- [64] Wang Q, Dai L, Wang Y, Deng J, Lin Y, Wang Q, et al. Targeted demethylation of the SARI promotor impairs colon tumour growth. *Cancer Lett* 2019;448:132–43. Available from: <https://doi.org/10.1016/j.canlet.2019.01.040>.
- [65] Wang H, Guo R, Du Z, Bai L, Li L, Cui J, et al. Epigenetic targeting of granulin in hepatoma cells by synthetic CRISPR dCas9 epi-suppressors. *Mol Ther – Nucleic Acids* 2018;11:23–33. Available from: <https://doi.org/10.1016/j.omtn.2018.01.002>.

- [66] Wu J, He K, Zhang Y, Song J, Shi Z, Chen W, et al. Inactivation of SMARCA2 by promoter hypermethylation drives lung cancer development. *Gene* 2019;687:193–9. Available from: <https://doi.org/10.1016/j.gene.2018.11.032>.
- [67] Saunderson EA, Stepper P, Gomm JJ, Hoa L, Morgan A, Allen MD, et al. Hit-and-run epigenetic editing prevents senescence entry in primary breast cells from healthy donors. *Nat Commun* 2017;8:1450. Available from: <https://doi.org/10.1038/s41467-017-01078-2>.
- [68] Vizoso M, van Rheenen J. Diverse transcriptional regulation and functional effects revealed by CRISPR/Cas9-directed epigenetic editing. *Oncotarget* 2021;12:1651–62. Available from: <https://doi.org/10.18632/oncotarget.28037>.
- [69] Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res* 2011;21:381–95. Available from: <https://doi.org/10.1038/cr.2011.22>.
- [70] Gardner KE, Allis CD, Strahl BD. OPERating ON chromatin, a colorful language where context matters. *J Mol Biol* 2011;409:36–46. Available from: <https://doi.org/10.1016/j.jmb.2011.01.040>.
- [71] Prakash K, Fournier D. Evidence for the implication of the histone code in building the genome structure. *Biosystems* 2018;164:49–59. Available from: <https://doi.org/10.1016/j.biosystems.2017.11.005>.
- [72] Kondo Y. Epigenetic cross-talk between DNA methylation and histone modifications in human cancers. *Yonsei Med J* 2009;50:455. Available from: <https://doi.org/10.3349/ymj.2009.50.4.455>.
- [73] Vermeulen M, Eberl HC, Matarese F, Marks H, Denissov S, Butter F, et al. Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers. *Cell* 2010;142:967–80. Available from: <https://doi.org/10.1016/j.cell.2010.08.020>.
- [74] Greer EL, Shi Y. Histone methylation: a dynamic mark in health, disease and inheritance. *Nat Rev Genet* 2012;13:343–57. Available from: <https://doi.org/10.1038/nrg3173>.
- [75] Huang C, Zhu B. Roles of H3K36-specific histone methyltransferases in transcription: antagonizing silencing and safeguarding transcription fidelity. *Biophys Rep* 2018;4:170–7. Available from: <https://doi.org/10.1007/s41048-018-0063-1>.
- [76] Laugesen A, Höjfeldt JW, Helin K. Molecular mechanisms directing PRC2 recruitment and H3K27 methylation. *Mol Cell* 2019;74:8–18. Available from: <https://doi.org/10.1016/j.molcel.2019.03.011>.
- [77] Ninova M, Fejes Tóth K, Aravin AA. The control of gene expression and cell identity by H3K9 trimethylation. *Development* 2019;146:dev181180. Available from: <https://doi.org/10.1242/dev.181180>.
- [78] Di Cerbo V, Mohn F, Ryan DP, Montellier E, Kacem S, Tropberger P, et al. Acetylation of histone H3 at lysine 64 regulates nucleosome dynamics and facilitates transcription. *eLife* 2014;3:e01632. Available from: <https://doi.org/10.7554/eLife.01632>.
- [79] Joshi AA, Struhl K. Eaf3 chromodomain interaction with methylated H3-K36 links histone deacetylation to Pol II elongation. *Mol Cell* 2005;20:971–8. Available from: <https://doi.org/10.1016/j.molcel.2005.11.021>.
- [80] Marmorstein R, Zhou M-M. Writers and readers of histone acetylation: structure, mechanism, and inhibition. *Cold Spring Harb Perspect Biol* 2014;6:a018762. Available from: <https://doi.org/10.1101/cshperspect.a018762>.
- [81] Verdone L, Caserta M, Mauro ED. Role of histone acetylation in the control of gene expression. *Biochem Cell Biol* 2005;83:344–53. Available from: <https://doi.org/10.1139/o05-041>.
- [82] Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010;31:27–36. Available from: <https://doi.org/10.1093/carcin/bgp220>.
- [83] Zhao Z, Shilatifard A. Epigenetic modifications of histones in cancer. *Genome Biol* 2019;20:245. Available from: <https://doi.org/10.1186/s13059-019-1870-5>.
- [84] Baylin SB, Jones PA. Epigenetic determinants of cancer. *Cold Spring Harb Perspect Biol* 2016;8:a019505. Available from: <https://doi.org/10.1101/cshperspect.a019505>.

- [85] Shah MA, Denton EL, Arrowsmith CH, Lupien M, Schapira M. A global assessment of cancer genomic alterations in epigenetic mechanisms. *Epigenetics Chromatin* 2014;7:29. Available from: <https://doi.org/10.1186/1756-8935-7-29>.
- [86] Hai R, He L, Shu G, Yin G. Characterization of histone deacetylase mechanisms in cancer development. *Front Oncol* 2021;11:700947. Available from: <https://doi.org/10.3389/fonc.2021.700947>.
- [87] Wapenaar H, Dekker FJ. Histone acetyltransferases: challenges in targeting bi-substrate enzymes. *Clin Epigenetics* 2016;8:59. Available from: <https://doi.org/10.1186/s13148-016-0225-2>.
- [88] Shanmugam MK, Arfuso F, Arumugam S, Chinnathambi A, Jinsong B, Warrier S, et al. Role of novel histone modifications in cancer. *Oncotarget* 2018;9:11414–26. Available from: <https://doi.org/10.18632/oncotarget.23356>.
- [89] Cano-Rodriguez D, Gjaltema RAF, Jilderda LJ, Jellema P, Dokter-Fokkens J, Ruiters MHJ, et al. Writing of H3K4Me3 overcomes epigenetic silencing in a sustained but context-dependent manner. *Nat Commun* 2016;7:12284. Available from: <https://doi.org/10.1038/ncomms12284>.
- [90] O'Geen H, Ren C, Nicolet CM, Perez AA, Halmai J, Le VM, et al. dCas9-based epigenome editing suggests acquisition of histone methylation is not sufficient for target gene repression. *Nucleic Acids Res* 2017;45:9901–16. Available from: <https://doi.org/10.1093/nar/gkx578>.
- [91] Fukushima HS, Takeda H, Nakamura R. Targeted *in vivo* epigenome editing of H3K27me3. *Epigenetics Chromatin* 2019;12:17. Available from: <https://doi.org/10.1186/s13072-019-0263-z>.
- [92] Chen X, Wei M, Liu X, Song S, Wang L, Yang X, et al. Construction and validation of the CRISPR/dCas9-EZH2 system for targeted H3K27Me3 modification. *Biochem Biophys Res Commun* 2019;511:246–52. Available from: <https://doi.org/10.1016/j.bbrc.2019.02.011>.
- [93] O'Geen H, Bates SL, Carter SS, Nisson KA, Halmai J, Fink KD, et al. Ezh2-dCas9 and KRAB-dCas9 enable engineering of epigenetic memory in a context-dependent manner. *Epigenetics Chromatin* 2019;12:26. Available from: <https://doi.org/10.1186/s13072-019-0275-8>.
- [94] Shi Y, Lan F, Matson C, Mulligan P, Whetstone JR, Cole PA, et al. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 2004;119:941–53. Available from: <https://doi.org/10.1016/j.cell.2004.12.012>.
- [95] Mendenhall EM, Williamson KE, Reyont D, Zou JY, Ram O, Joung JK, et al. Locus-specific editing of histone modifications at endogenous enhancers. *Nat Biotechnol* 2013;31:1133–6. Available from: <https://doi.org/10.1038/nbt.2701>.
- [96] Kearns NA, Pham H, Tabak B, Genga RM, Silverstein NJ, Garber M, et al. Functional annotation of native enhancers with a Cas9–histone demethylase fusion. *Nat Methods* 2015;12:401–3. Available from: <https://doi.org/10.1038/nmeth.3325>.
- [97] Williams RM, Senanayake U, Artibani M, Taylor G, Wells D, Ahmed AA, et al. Genome and epigenome engineering CRISPR toolkit for *in vivo* modulation of *cis*-regulatory interactions and gene expression in the chicken embryo. *Development* 2018;dev.160333. Available from: <https://doi.org/10.1242/dev.160333>.
- [98] Li K, Liu Y, Cao H, Zhang Y, Gu Z, Liu X, et al. Interrogation of enhancer function by enhancer-targeting CRISPR epigenetic editing. *Nat Commun* 2020;11:485. Available from: <https://doi.org/10.1038/s41467-020-14362-5>.
- [99] Levy S, Somasundaram L, Raj IX, Ic-Mex D, Phal A, Schmidt S, et al. dCas9 fusion to computer-designed PRC2 inhibitor reveals functional TATA box in distal promoter region. *Cell Rep* 2022;38:110457. Available from: <https://doi.org/10.1016/j.celrep.2022.110457>.
- [100] Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, et al. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol* 2015;33:510–17. Available from: <https://doi.org/10.1038/nbt.3199>.

- [101] Okada M, Kanamori M, Someya K, Nakatsukasa H, Yoshimura A. Stabilization of Foxp3 expression by CRISPR-dCas9-based epigenome editing in mouse primary T cells. *Epigenetics Chromatin* 2017;10:24. Available from: <https://doi.org/10.1186/s13072-017-0129-1>.
- [102] Klann TS, Black JB, Chellappan M, Safi A, Song L, Hilton IB, et al. CRISPR–Cas9 epigenome editing enables high-throughput screening for functional regulatory elements in the human genome. *Nat Biotechnol* 2017;35:561–8. Available from: <https://doi.org/10.1038/nbt.3853>.
- [103] Chen L-F, Lin YT, Gallegos DA, Hazlett MF, Gómez-Schiavon M, Yang MG, et al. Enhancer histone acetylation modulates transcriptional bursting dynamics of neuronal activity-inducible genes. *Cell Rep* 2019;26:1174–1188.e5. Available from: <https://doi.org/10.1016/j.celrep.2019.01.032>.
- [104] Kwon DY, Zhao Y-T, Lamonica JM, Zhou Z. Locus-specific histone deacetylation using a synthetic CRISPR-Cas9-based HDAC. *Nat Commun* 2017;8:15315. Available from: <https://doi.org/10.1038/ncomms15315>.
- [105] Garcia-Bloj B, Moses C, Sgro A, Plani-Lam J, Arooj M, Duffy C, et al. Waking up dormant tumor suppressor genes with zinc fingers, TALEs and the CRISPR/dCas9 system. *Oncotarget* 2016;7:60535–54. Available from: <https://doi.org/10.18632/oncotarget.11142>.
- [106] Ginley-Hidinger M, Carleton JB, Rodriguez AC, Berrett KC, Gertz J. Sufficiency analysis of estrogen responsive enhancers using synthetic activators. *Life Sci Alliance* 2019;2:e201900497. Available from: <https://doi.org/10.26508/lsa.201900497>.
- [107] Moroi K, Sato T. Comparison between procaine and isocarboxazid metabolism in vitro by a liver microsomal amidase-esterase. *Biochem Pharmacol* 1975;24:1517–21. Available from: [https://doi.org/10.1016/0006-2952\(75\)90029-5](https://doi.org/10.1016/0006-2952(75)90029-5).
- [108] Dominguez AA, Lim WA, Qi LS. Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation. *Nat Rev Mol Cell Biol* 2016;17:5–15. Available from: <https://doi.org/10.1038/nrm.2015.2>.
- [109] Zhou H, Liu J, Zhou C, Gao N, Rao Z, Li H, et al. In vivo simultaneous transcriptional activation of multiple genes in the brain using CRISPR–dCas9-activator transgenic mice. *Nat Neurosci* 2018;21:440–6. Available from: <https://doi.org/10.1038/s41593-017-0060-6>.
- [110] Xu X, Qi LS. A CRISPR–dCas toolbox for genetic engineering and synthetic biology. *J Mol Biol* 2019;431:34–47. Available from: <https://doi.org/10.1016/j.jmb.2018.06.037>.
- [111] Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013;154:442–51. Available from: <https://doi.org/10.1016/j.cell.2013.06.044>.
- [112] Yeo NC, Chavez A, Lance-Byrne A, Chan Y, Menn D, Milanova D, et al. An enhanced CRISPR repressor for targeted mammalian gene regulation. *Nat Methods* 2018;15:611–16. Available from: <https://doi.org/10.1038/s41592-018-0048-5>.
- [113] Martella A, Firth M, Taylor BJM, Göppert A, Cuomo EM, Roth RG, et al. Systematic evaluation of CRISPRa and CRISPRi modalities enables development of a multiplexed, orthogonal gene activation and repression system. *ACS Synth Biol* 2019;8:1998–2006. Available from: <https://doi.org/10.1021/acssynbio.8b00527>.
- [114] Guo LY, Bian J, Davis AE, Liu P, Kempton HR, Zhang X, et al. Multiplexed genome regulation in vivo with hyper-efficient Cas12a. *Nat Cell Biol* 2022;24:590–600. Available from: <https://doi.org/10.1038/s41556-022-00870-7>.
- [115] Moses C, Nugent F, Waryah CB, Garcia-Bloj B, Harvey AR, Blancafort P. Activating PTEN tumor suppressor expression with the CRISPR/dCas9 system. *Mol Ther Nucleic Acids* 2019;14:287–300. Available from: <https://doi.org/10.1016/j.omtn.2018.12.003>.
- [116] Wang G, Chow RD, Bai Z, Zhu L, Errami Y, Dai X, et al. Multiplexed activation of endogenous genes by CRISPRa elicits potent antitumor immunity. *Nat Immunol* 2019;20:1494–505. Available from: <https://doi.org/10.1038/s41590-019-0500-4>.

- [117] Yoshida M, Yokota E, Sakuma T, Yamatsuji T, Takigawa N, Ushijima T, et al. Development of an integrated CRISPRi targeting Δ Np63 for treatment of squamous cell carcinoma. *Oncotarget* 2018;9:29220–32. Available from: <https://doi.org/10.18632/oncotarget.25678>.
- [118] Zhang S, Ma H, Zhang D, Xie S, Wang W, Li Q, et al. LncRNA KCNQ1OT1 regulates proliferation and cisplatin resistance in tongue cancer via miR-211-5p mediated Ezrin/Fak/Src signaling. *Cell Death Dis* 2018;9:742. Available from: <https://doi.org/10.1038/s41419-018-0793-5>.
- [119] Chen M, Wei L, Law C-T, Tsang FH-C, Shen J, Cheng CL-H, et al. RNA N6-methyladenosine methyltransferase-like 3 promotes liver cancer progression through YTHDF2-dependent posttranscriptional silencing of SOCS2. *Hepatology* 2018;67:2254–70. Available from: <https://doi.org/10.1002/hep.29683>.
- [120] Braun CJ, Bruno PM, Horlbeck MA, Gilbert LA, Weissman JS, Hemann MT. Versatile *in vivo* regulation of tumor phenotypes by dCas9-mediated transcriptional perturbation. *Proc Natl Acad Sci U S A* 2016;113. Available from: <https://doi.org/10.1073/pnas.1600582113>.
- [121] Song X, Liu C, Wang N, Huang H, He S, Gong C, et al. Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy. *Adv Drug Deliv Rev* 2021;168:158–80. Available from: <https://doi.org/10.1016/j.addr.2020.04.010>.
- [122] Truong D-JJ, Kühner K, Kühn R, Werfel S, Engelhardt S, Wurst W, et al. Development of an intein-mediated split–Cas9 system for gene therapy. *Nucleic Acids Res* 2015;43:6450–8. Available from: <https://doi.org/10.1093/nar/gkv601>.
- [123] Zhang X, Lv S, Luo Z, Hu Y, Peng X, Lv J, et al. MiniCAFE, a CRISPR/Cas9-based compact and potent transcriptional activator, elicits gene expression *in vivo*. *Nucleic Acids Res* 2021;49:4171–85. Available from: <https://doi.org/10.1093/nar/gkab174>.
- [124] Xu X, Chempathy A, Zeng L, Kempton HR, Shang S, Nakamura M, et al. Engineered miniature CRISPR–Cas system for mammalian genome regulation and editing. *Mol Cell* 2021;81:4333–4345.e4. Available from: <https://doi.org/10.1016/j.molcel.2021.08.008>.
- [125] Song B, Kang CY, Han JH, Kano M, Konnerth A, Bae S. *In vivo* genome editing in single mammalian brain neurons through CRISPR–Cas9 and cytosine base editors. *Comput Struct Biotechnol J* 2021;19:2477–85. Available from: <https://doi.org/10.1016/j.csbj.2021.04.051>.
- [126] Xu W. Microinjection and micromanipulation: a historical perspective. In: Liu C, Du Y, editors. *Microinjection*. New York: Springer; 2019. p. 1–16. Available from: https://doi.org/10.1007/978-1-4939-8831-0_1.
- [127] Suda T, Liu D. Hydrodynamic gene delivery: its principles and applications. *Mol Ther* 2007;15:2063–9. Available from: <https://doi.org/10.1038/sj.mt.6300314>.
- [128] Xu CL, Ruan MZC, Mahajan VB, Tsang SH. Viral delivery systems for CRISPR. *Viruses* 2019;11:28. Available from: <https://doi.org/10.3390/v11010028>.
- [129] Li L, Hu S, Chen X. Non-viral delivery systems for CRISPR/Cas9-based genome editing: challenges and opportunities. *Biomaterials* 2018;171:207–18. Available from: <https://doi.org/10.1016/j.biomaterials.2018.04.031>.
- [130] Banskota S, Raguram A, Suh S, Du SW, Davis JR, Choi EH, et al. Engineered virus-like particles for efficient *in vivo* delivery of therapeutic proteins. *Cell* 2022;185:250–265.e16. Available from: <https://doi.org/10.1016/j.cell.2021.12.021>.
- [131] Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, et al. High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 2016;529:490–5. Available from: <https://doi.org/10.1038/nature16526>.
- [132] Chen JS, Dagdas YS, Kleinstiver BP, Welch MM, Sousa AA, Harrington LB, et al. Enhanced proof-reading governs CRISPR–Cas9 targeting accuracy. *Nature* 2017;550:407–10. Available from: <https://doi.org/10.1038/nature24268>.

- [133] Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, et al. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* 2018;556:57–63. Available from: <https://doi.org/10.1038/nature26155>.
- [134] Aschenbrenner S, Kallenberger SM, Hoffmann MD, Huck A, Eils R, Niopek D. Coupling Cas9 to artificial inhibitory domains enhances CRISPR-Cas9 target specificity. *Sci Adv* 2020;6:eaay0187. Available from: <https://doi.org/10.1126/sciadv.aay0187>.
- [135] Galonska C, Charlton J, Mattei AL, Donaghey J, Clement K, Gu H, et al. Genome-wide tracking of dCas9-methyltransferase footprints. *Nat Commun* 2018;9:597. Available from: <https://doi.org/10.1038/s41467-017-02708-5>.
- [136] Hofacker D, Broche J, Laistner L, Adam S, Bashtrykov P, Jeltsch A. Engineering of effector domains for targeted DNA methylation with reduced off-target effects. *Int J Mol Sci* 2020;21:502. Available from: <https://doi.org/10.3390/ijms21020502>.
- [137] Chiarella AM, Butler KV, Gryder BE, Lu D, Wang TA, Yu X, et al. Dose-dependent activation of gene expression is achieved using CRISPR and small molecules that recruit endogenous chromatin machinery. *Nat Biotechnol* 2020;38:50–5. Available from: <https://doi.org/10.1038/s41587-019-0296-7>.
- [138] Mathew A, Cho K-H, Uthaman S, Cho C-S, Park I-K. Stimuli-regulated smart polymeric systems for gene therapy. *Polymers* 2017;9:152. Available from: <https://doi.org/10.3390/polym9040152>.
- [139] Yu C, Li L, Hu P, Yang Y, Wei W, Deng X, et al. Recent advances in stimulus-responsive nanocarriers for gene therapy. *Adv Sci* 2021;8:2100540. Available from: <https://doi.org/10.1002/advs.202100540>.

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Edited by Steven G. Gray

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