DOCUMENT SUMMARY

This review article by Bannister and Kouzarides provides a comprehensive overview of the regulation of chromatin by histone modifications. It details the various types of modifications, including acetylation, phosphorylation, methylation, and others, and the enzymes that regulate them. The paper discusses the functional consequences of these modifications, particularly in transcription, and how they act by either directly perturbing chromatin structure or by recruiting effector proteins.

FILENAME

bannister_2011_research_report_chromatin_histone_modifications

METADATA

Category: RESEARCH

Type: report

Relevance: Core

Update Frequency: Static

Tags: #histone-modifications #chromatin #epigenetics #gene-regulation #transcription #acetylation #methylation #cancer-epigenetics #histone-code

Related Docs: N/A

Supersedes: N/A

FORMATTED CONTENT

Bannister_2011_Regulation_of_chromatin_by_histone_modifications

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Abstract

> **Chromatin** is not an inert structure, but rather an instructive DNA scaffold that can respond to external cues to regulate the many uses of DNA. A principle component of **chromatin** that plays a key role in this regulation is the modification of **histones**.

There is an ever-growing list of these modifications and the complexity of their action is only just beginning to be understood. However, it is clear that **histone modifications** play fundamental roles in most biological processes that are involved in the

manipulation and expression of DNA. Here, we describe the known **histone modifications**, define where they are found genomically and discuss some of their functional consequences, concentrating mostly on transcription where the majority of characterisation has taken place.

Keywords: histone; **modifications**; **chromatin**

Introduction

Ever since Vincent Allfrey's pioneering studies in the early 1960s, we have known that **histones** are post-translationally modified [1]. We now know that there are a large number of different **histone post-translational modifications (PTMs)**. An insight into how these modifications could affect **chromatin** structure came from solving the high-resolution X-ray structure of the nucleosome in 1997 [2]. The structure indicates that highly basic **histone** amino (N)-terminal tails can protrude from their own nucleosome and make contact with adjacent nucleosomes. It seemed likely at the time that modification of these tails would affect inter-nucleosomal interactions and thus affect the overall **chromatin** structure. We now know that this is indeed the case. Modifications not only regulate **chromatin** structure by merely being there, but they also recruit remodelling enzymes that utilize the energy derived from the hydrolysis of ATP to reposition nucleosomes. The recruitment of proteins and complexes with specific enzymatic activities is now an accepted dogma of how modifications mediate their function. As we will describe below, in this way modifications can influence transcription, but since **chromatin** is ubiquitous, modifications also affect many other DNA processes such as repair, replication and recombination.

Histone acetylation

Allfrey et al. [1] first reported **histone acetylation** in 1964. Since then, it has been shown that the **acetylation** of lysines is highly dynamic and regulated by the opposing action of two families of enzymes, **histone acetyltransferases (HATs)** and **histone deacetylases (HDACs)**; for review, see reference [3]). The **HATs** utilize acetyl CoA as cofactor and catalyse the transfer of an acetyl group to the e-amino group of lysine side chains. In doing so, they neutralize the lysine's positive charge and this action has the potential to weaken the interactions between **histones** and DNA (see below). There are two major classes of **HATs**: type-A and type-B. The type-B **HATs** are predominantly cytoplasmic, acetylating free **histones** but not those already deposited into **chromatin**. This class of **HATs** is highly conserved and all type-B **HATs** share sequence homology with scHat1, the founding member of this type of **HAT**. Type-B **HATs** acetylate newly synthesized histone H4 at K5 and K12 (as well as certain sites within H3), and this pattern of **acetylation** is important for deposition of the **histones**, after which the marks are removed [4].

The type-A **HATs** are a more diverse family of enzymes than the type-Bs. Nevertheless, they can be classified into at least three separate groups depending on amino-acid sequence homology and conformational structure: **GNAT**, **MYST** and **CBP/p300** families [5]. Broadly speaking, each of these enzymes modifies multiple sites within the **histone** N-terminal tails. Indeed, their ability to neutralize positive charges, thereby

disrupting the stabilizing influence of electrostatic interactions, correlates well with this class of enzyme functioning in numerous transcriptional coactivators [6]. However, it is not just the **histone** tails that are involved in this regulation, but there are additional sites of **acetylation** present within the globular **histone** core, such as **H3K56** that is acetylated in humans by hGCN5 [7]. The H3K56 side chain points towards the DNA major groove, suggesting that **acetylation** would affect **histone**/DNA interaction, a situation reminiscent of the proposed effects of acetylating the **histone** N-terminal tail lysines. Interestingly, knockdown of the p300 **HAT** has also been shown to be associated with the loss of **H3K56ac** [8], suggesting that p300 may also target this site. However, unlike GCN5 knockdown, p300 knockdown increases DNA damage, which may indirectly affect **H3K56ac** levels [7].

In common with many histone-modifying enzymes, the type-A **HATs** are often found associated in large multiprotein complexes [6]. The component proteins within these complexes play important roles in controlling enzyme recruitment, activity and substrate specificity. For instance, purified scGCN5 acetylates free **histones** but not those present within a nucleosome. In contrast, when scGCN5 is present within the so-called **SAGA complex**, it efficiently acetylates nucleosomal **histones** [9].

HDAC enzymes oppose the effects of **HATs** and reverse lysine **acetylation**, an action that restores the positive charge of the lysine. This potentially stabilizes the local **chromatin** architecture and is consistent with **HDACs** being predominantly transcriptional repressors. There are four classes of **HDAC** [6]: Classes I and II contain enzymes that are most closely related to yeast scRpd3 and scHda1, respectively, class IV has only a single member, HDAC11, while class III (referred to as **sirtuins**) are homologous to yeast scSir2. This latter class, in contrast to the other three classes, requires a specific cofactor for its activity, NAD^+.

In general, **HDACs** have relatively low substrate specificity by themselves, a single enzyme being capable of deacetylating multiple sites within **histones**. The issue of enzyme recruitment and specificity is further complicated by the fact that the enzymes are typically present in multiple distinct complexes, often with other **HDAC** family members. For instance, HDAC1 is found together with HDAC2 within the **NuRD**, **Sin3a** and **Co-REST** complexes [10]. Thus, it is difficult to determine which activity (specific **HDAC** and/or complex) is responsible for a specific effect. Nevertheless, in certain cases, it is possible to at least determine which enzyme is required for a given process. For example, it has been shown that HDAC1, but not HDAC2, controls embryonic stem cell differentiation [11].

Histone phosphorylation

Like **histone acetylation**, the **phosphorylation** of **histones** is highly dynamic. It takes place on serines, threonines and tyrosines, predominantly, but not exclusively, in the N-terminal **histone** tails [3]. The levels of the modification are controlled by **kinases** and **phosphatases** that add and remove the modification, respectively [12].

All of the identified **histone kinases** transfer a phosphate group from ATP to the hydroxyl group of the target amino-acid side chain. In doing so, the modification adds significant negative charge to the **histone** that undoubtedly influences the **chromatin** structure. For the majority of **kinases**, however, it is unclear how the enzyme is accurately recruited to its site of action on **chromatin**. In a few cases, exemplified by the mammalian MAPK1 enzyme, the **kinase** possesses an intrinsic DNA-binding domain with which it is tethered to the DNA [13]. This may be sufficient for specific recruitment, similar to bona fide DNA-binding transcription factors. Alternatively, its recruitment may require association with a **chromatin**-bound factor before it directly contacts DNA to stabilize the overall interaction.

The majority of **histone phosphorylation** sites lie within the N-terminal tails. However, sites within the core regions do exist. One such example is **phosphorylation** of **H3Y41**, which is deposited by the non-receptor tyrosine **kinase JAK2** (see below) [14].

Less is known regarding the roles of **histone phosphatases**. Certainly, given the extremely rapid turnover of specific **histone phosphorylations**, there must be high **phosphatase** activity within the nucleus. We do know, e.g., that the **PP1 phosphatase** works antagonistically to **Aurora B**, the **kinase** that lays down genome-wide **H3S10ph** and **H3S28ph** at mitosis [15, 16].

Histone methylation

Histone methylation mainly occurs on the side chains of lysines and arginines. Unlike **acetylation** and **phosphorylation**, however, **histone methylation** does not alter the charge of the **histone** protein. Furthermore, there is an added level of complexity to bear in mind when considering this modification; lysines may be **mono-**, **di-** or **tri-methylated**, whereas arginines may be **mono-**, **symmetrically** or **asymmetrically di-methylated** (for reviews see references [3, 17-19]).

Lysine methylation

The first **histone lysine methyltransferase (HKMT)** to be identified was **SUV39H1** that targets H3K9 [20]. Numerous **HKMTs** have since been identified, the vast majority of which methylate lysines within the N-terminal tails. Strikingly, all of the **HKMTs** that methylate N-terminal lysines contain a so-called **SET domain** that harbours the enzymatic activity. However, an exception is the **Dot1** enzyme that methylates **H3K79** within the **histone** globular core and does not contain a **SET domain**. Why this enzyme is structurally different than all of the others is not clear, but perhaps this reflects the relative inaccessibility of its substrate H3K79. In any case, all **HKMTs** catalyse the transfer of a methyl group from S-adenosylmethionine (SAM) to a lysine's ε-amino group.

HKMTs tend to be relatively specific enzymes. For instance, *Neurospora crassa* **DIM5** specifically methylates H3K9 whereas **SET7/9** targets H3K4. Furthermore, **HKMT** enzymes also modify the appropriate lysine to a specific degree (i.e., **mono-**, **di-** and/or **tri-methyl** state). Maintaining the same examples, **DIM5** can **tri-methylate** H3K9 [21] but **SET7/9** can only **mono-methylate** H3K4 [22]. These specific reaction products can

be generated using only the purified enzymes; so the ability to discriminate between different **histone** lysines and between different methylated states is an intrinsic property of the enzyme. It turns out from X-ray crystallographic studies that there is a key residue within the enzyme's catalytic domain that determines whether the enzyme activity proceeds past the **mono-methyl** product. In **DIM5**, there is a phenylalanine (F281) within the enzyme's lysine-binding pocket that can accommodate all the methylated forms of the lysine, thereby allowing the enzyme to generate the **tri-methylated** product [23]. In contrast, **SET7/9** has a tyrosine (Y305) in the corresponding position such that it can only accommodate the **mono-methyl** product [22]. Elegant mutagenesis studies have shown that mutagenesis of **DIM5** F281 to Y converts the enzyme to a **mono-methyltransferase**, whereas the reciprocal mutation in **SET7/9** (Y305 to F) creates an enzyme capable of **tri-methylating** its substrate [23]. More generally speaking, it seems that the aromatic determinant (Y or F) is a mechanism widely employed by **SET domain**-containing **HKMTs** to control the degree of **methylation** [24, 25].

Arginine methylation

There are two classes of **arginine methyltransferase**, the **type-I** and **type-II** enzymes. The **type-I** enzymes generate Rme1 and Rme2as, whereas the **type-II** enzymes generate Rme1 and Rme2s. Together, the two types of **arginine methyltransferases** form a relatively large protein family (11 members), the members of which are referred to as **PRMTs**. All of these enzymes transfer a methyl group from SAM to the w-guanidino group of arginine within a variety of substrates. With respect to **histone arginine methylation**, the most relevant enzymes are **PRMT1**, **4**, **5** and **6** (reviewed in [18, 26]).

Methyltransferases, for both arginine and lysine, have a distinctive extended catalytic active site that distinguishes this broad group of **methyltransferases** from other SAM-dependent enzymes [27]. Interestingly, the SAM-binding pocket is on one face of the enzyme, whereas the peptidyl acceptor channel is on the opposite face. This indicates that a molecule of SAM and the **histone** substrate come together from opposing sides of the enzyme [27]. Indeed, this way of entering the enzyme's active site may provide an opportunity to design selective drugs that are able to distinguish between **histone arginine/lysine methyltransferases** and other **methyltransferases** such as DNMTs.

Histone demethylases

For many years, **histone methylation** was considered a stable, static modification. Nevertheless, in 2002, a number of different reactions/pathways were suggested as potential **demethylation** mechanisms for both lysine and arginine [28], which were subsequently verified experimentally.

Initially, the conversion of arginine to citrulline via a **deimination** reaction was discovered as a way of reversing **arginine methylation** [29, 30]. Although this pathway is not a direct reversal of **methylation** (see **deimination** below), this mechanism reversed the dogma that **methylation** was irreversible. More recently, a reaction has been reported which reverses **arginine methylation**. The jumonji protein **JMJD6** was shown to be capable of performing a **demethylation** reaction on **histones H3R2** and

H4R3 [31]. However, these findings have yet to be recapitulated by other independent researchers.

In 2004, the first **lysine demethylase** was identified. It was found to utilize FAD as co-factor, and it was termed as **lysine-specific demethylase 1 (LSD1)** [32]. The **demethylation** reaction requires a protonated nitrogen and it is therefore only compatible with **mono-** and **di-methylated** lysine substrates. In vitro, purified **LSD1** catalyses the removal of methyl groups from **H3K4me1/2**, but it cannot demethylate the same site when presented within a nucleosomal context. However, when **LSD1** is complexed with the **Co-REST** repressor complex, it can demethylate nucleosomal **histones**. Thus, complex members confer nucleosomal recognition to **LSD1**. Furthermore, the precise complex association determines which lysine is to be demethylated by **LSD1**. As already mentioned, **LSD1** in the context of **Co-REST** demethylates **H3K4me1/2**, but when **LSD1** is complexed with the androgen receptor, it demethylates H3K9. This has the effect of switching the activity of **LSD1** from a repressor function to that of a coactivator (reviewed in [33]; see below).

In 2006, another class of **lysine demethylase** was discovered [34]. Importantly, certain enzymes in this class were capable of demethylating **tri-methylated** lysines [35]. They employ a distinct catalytic mechanism from that used by **LSD1**, using Fe(II) and a-ketoglutarate as co-factors, and a radical attack mechanism. The first enzyme identified as a **tri-methyl lysine demethylase** was **JMJD2** that demethylates **H3K9me3** and **H3K36me3** [35]. The enzymatic activity of **JMJD2** resides within a **JmjC jumonji domain**. Many **histone lysine demethylases** are now known and, except for **LSD1**, they all possess a catalytic **jumonji domain** [36]. As with the **lysine methyltransferases**, the **demethylases** possess a high level of substrate specificity with respect to their target lysine. They are also sensitive to the degree of lysine **methylation**; for instance, some of the enzymes are only capable of demethylating **mono-** and **di-methyl** substrates, whereas others can demethylate all three states of the **methylated** lysine.

Other modifications

Deimination

This reaction involves the conversion of an arginine to a citrulline. In mammalian cells, this reaction on **histones** is catalysed by the **peptidyl deiminase PADI4**, which converts peptidyl arginines to citrulline [29, 30]. One obvious effect of this reaction is that it effectively neutralizes the positive charge of the arginine since citrulline is neutral. There is also evidence that **PADI4** converts **mono-methyl arginine** to citrulline, thereby effectively functioning as an **arginine demethylase** [29, 30]. However, unlike a 'true' demethylase, the **PADI4** reaction does not regenerate an unmodified arginine.

B-N-acetylglucosamine

Many non-**histone** proteins are regulated via modification of their serine and threonine side chains with single **β-N-acetylglucosamine (O-GlcNAc)** sugar residues. Recently, **histones** were added to the long list of **O-GlcNAc**-modified proteins [37]. Interestingly,

in mammalian cells, there appears to be only a single enzyme, **O-GlcNAc transferase**, which catalyses the transfer of the sugar from the donor substrate, UDP-GlcNAc, to the target protein. Like most of the other **histone PTMs**, **O-GlcNAc** modification appears to be highly dynamic with high turnover rates and, as with the forward reaction, there appears to be only a single enzyme capable of removing the sugar, **β-N-acetylglucosaminidase (O-GlcNAcase)**. So far, **histones H2A**, **H2B** and **H4** have been shown to be modified by **O-GlcNAc** [37].

ADP ribosylation

Histones are known to be **mono-** and **poly-ADP ribosylated** on glutamate and arginine residues, but relatively little is known concerning the function of this modification [38]. What we do know is that once again the modification is reversible. For example, **poly-ADP-ribosylation** of **histones** is performed by the **poly-ADP-ribose polymerase (PARP)** family of enzymes and reversed by the **poly-ADP-ribose-glycohydrolase** family of enzymes. These enzymes function together to control the levels of **poly-ADP ribosylated histones** that have been correlated with a relatively relaxed **chromatin** state [38]. Presumably, this is a consequence, at least in part, of the negative charge that the modification confers to the **histone**. In addition, though, it has been reported that the activation of **PARP-1** leads to elevated levels of core **histone acetylation** [39]. Moreover, **PARP-1**-mediated **ribosylation** of the **H3K4me3 demethylase KDM5B** inhibits the demethylase and excludes it from **chromatin**, while simultaneously excluding H1, thereby making target promoters more accessible [40].

Histone mono-ADP-ribosylation is performed by the **mono-ADP-ribosyltransferases** and has been detected on all four core **histones**, as well as on the linker **histone H1**. Notably, these modifications significantly increase upon DNA damage implicating the pathway in the DNA damage response [38].

Ubiquitylation and sumoylation

All of the previously described **histone modifications** result in relatively small molecular changes to amino-acid side chains. In contrast, **ubiquitylation** results in a much larger covalent modification. **Ubiquitin** itself is a 76-amino acid polypeptide that is attached to **histone** lysines via the sequential action of three enzymes, **E1-activating**, **E2-conjugating** and **E3-ligating** enzymes [41]. The enzyme complexes determine both substrate specificity (i.e., which lysine is targeted) as well as the degree of **ubiquitylation** (i.e., either **mono-** or **poly-ubiquitylated**). For **histones**, **mono-ubiquitylation** seems most relevant although the exact modification sites remain largely elusive. However, two well-characterised sites lie within H2A and H2B. **H2AK119ub1** is involved in gene silencing [42], whereas **H2BK123ubl** plays an important role in transcriptional initiation and elongation [43, 44] (see below). Even though **ubiquitylation** is such a large modification, it is still a highly dynamic one. The modification is removed via the action of **isopeptidases** called **de-ubiquitin enzyme** and this activity is important for both gene activity and silencing [3 and references therein].

Sumoylation is a modification related to **ubiquitylation** [45], and involves the covalent attachment of small **ubiquitin**-like modifier molecules to **histone** lysines via the action of E1, E2 and E3 enzymes. **Sumoylation** has been detected on all four core **histones** and seems to function by antagonizing **acetylation** and **ubiquitylation** that might otherwise occur on the same lysine side chain [46, 47]. Consequently, it has mainly been associated with repressive functions, but more work is clearly needed to elucidate the molecular mechanism(s) through which **sumoylation** exerts its effect on **chromatin**.

Histone tail clipping

Perhaps the most radical way to remove **histone modifications** is to remove the **histone** N-terminal tail in which they reside, a process referred to as **tail clipping**. It was first identified in *Tetrahymena* in 1980 [48], where the first six amino acids of H3 are removed. However, it is now apparent that this type of activity also exists in yeast and mammals (mouse) where the first 21 amino acids of H3 are removed [49, 50]. In yeast, the proteolytic enzyme remains unknown, but the clipping process has been shown to be involved in regulating transcription [49]. The mouse enzyme was identified as **Cathepsin L**, which cleaves the N-terminus of H3 during ES cell differentiation [50].

Histone proline isomerization

The dihedral angle of a peptidyl proline's peptide bond naturally interconverts between the *cis* and *trans* conformations, the states differing by 180°. **Proline isomerases** facilitate this interconversion, which has the potential to stably affect peptide configuration. One **proline isomerase** shown to act on **histones** is the yeast enzyme **scFpr4**, which isomerizes H3P38 [51]. This activity is linked to **methylation** of H3K36, presumably by **scFpr4** affecting the recognition of K36 by the **scSet2 methyltransferase** and the **scJMJD2 demethylase** though the exact mechanism remains unclear [51, 52]. Nevertheless, this example highlights the fact that **proline isomerization** is an important modification of the **histone** tail. It is, however, not a true covalent modification since the enzyme merely 'flips' the peptide bond by 180°, thereby generating chemical isomers rather than covalently modified products.

Mode of action of histone modifications

Histone modifications exert their effects via two main mechanisms. The first involves the modification(s) directly influencing the overall structure of **chromatin**, either over short or long distances. The second involves the modification regulating (either positively or negatively) the binding of effector molecules. Our review has a transcriptional focus, simply reflecting the fact that most studies involving **histone modifications** have also had this focus. However, **histone modifications** are just as relevant in the regulation of other DNA processes such as repair, replication and recombination. Indeed, the principles described below are pertinent to any biological process involving DNA transactions.

Direct structural perturbation

Histone acetylation and **phosphorylation** effectively reduce the positive charge of **histones**, and this has the potential to disrupt electrostatic interactions between **histones** and DNA. This presumably leads to a less compact **chromatin** structure, thereby facilitating DNA access by protein machineries such as those involved in transcription. Notably, **acetylation** occurs on numerous **histone** tail lysines, including H3K9, H3K14, H3K18, H4K5, H4K8 and H4K12 [53]. This high number of potential sites provides an indication that in **hyper-acetylated** regions of the genome, the charge on the **histone** tails can be effectively neutralized, which would have profound effects on the **chromatin** structure. Evidence for this can be found at the β-globin locus where the genes reside within a **hyper-acetylated** and transcriptionally competent **chromatin** environment that displays DNase sensitivity, and therefore general accessibility [54]. Multiple **histone acetylations** are also enriched at enhancer elements and particularly in gene promoters, where they presumably again facilitate the transcription factor access [55]. However, multiple **histone acetylations** are not an absolute pre-requisite for inducing structural change - **histones** specifically acetylated at H4K16 have a significant negative effect on the formation of the 30 nm fibre, at least in vitro [56].

Histone phosphorylation tends to be very site-specific and there are far fewer sites compared with acetylated sites. As with H4K16ac, these single-site modifications can be associated with gross structural changes within **chromatin**. For instance, **phosphorylation** of H3S10 during mitosis occurs genome-wide and is associated with **chromatin** becoming more condensed [57]. This seems somewhat counterintuitive since the phosphate group adds negative charge to the **histone** tail that is in close proximity to the negatively charged DNA backbone. But it may be that displacement of **heterochromatin protein 1 (HP1)** from **heterochromatin** during metaphase by uniformly high levels of H3S10ph [58, 59] (see below) is required to promote the detachment of chromosomes from the interphase scaffolding. This would facilitate chromosomal remodeling that is essential for its attachment to the mitotic spindle.

Ubiquitylation adds an extremely large molecule to a **histone**. It seems highly likely that this will induce a change in the overall conformation of the nucleosome, which in turn will affect intra-nucleosomal interactions and/or interactions with other **chromatin**-bound complexes. **Histone tail clipping**, which results in the loss of the first 21 amino acids of H3 will have similar effects. In contrast, neutral modifications such as **histone methylation** are unlikely to directly perturb **chromatin** structure since these modifications are small and do not alter the charge of **histones**.

Regulating the binding of chromatin factors

Numerous **chromatin**-associated factors have been shown to specifically interact with modified **histones** via many distinct domains (Figure 1) [3]. There is an ever-increasing number of such proteins following the development and use of new proteomic approaches [60, 61]. These large data sets show that there are multivalent proteins and complexes that have specific domains within them that allow the simultaneous recognition of several modifications and other nucleosomal features.

> Notably, there are more distinct domain types recognizing **lysine methylation** than any other modification, perhaps reflecting the

modification's relative importance (Figure 1). These include **PHD fingers** and the so-called **Tudor 'royal' family** of domains, comprising **chromodomains**, **Tudor**, **PWWP** and **MBT domains** [62-64].

Within this group of **methyl-lysine** binders, numerous domains can recognize the same modified **histone** lysine. For instance, **H3K4me3** a mark associated with active transcription – is recognized by a **PHD finger** within the **ING** family of proteins (ING1-5) (reviewed in [62]). The **ING** proteins in turn recruit additional **chromatin** modifiers such as **HATs** and **HDACs**. For example, **ING2** tethers the repressive **mSin3a-HDAC1 HDAC** complex to active proliferation-specific genes following DNA damage [65]. **Tri-methylated H3K4** is also bound by the tandem **chromodomains** within **CHD1**, an ATP-dependent remodelling enzyme capable of repositioning nucleosomes [66], and by the tandem **Tudor domains** within **JMJD2A**, a **histone demethylase** [67]. In these cases, **H3K4me3** directly recruits the **chromatin**-modifying enzyme.

A further example of specific **methylated lysine** binding is provided by the **HP1** recognition of **H3K9me3** a mark associated with repressive **heterochromatin**. **HP1** binds to **H3K9me3** via its N-terminal **chromodomain** and this interaction is important for the overall structure of **heterochromatin** [68, 69]. **HP1** proteins dimerise via their C-terminal **chromoshadow domains** to form a bivalent **chromatin** binder. Interestingly, **HP1** also binds to **methylated H1.4K26** via its **chromodomain** [70]. Since H1.4 is also involved in **heterochromatin** architecture, it is tempting to speculate that **HP1** dimers integrate this positional information (**H3K9me** and **H1.4K26me**) in a manner that is important for **chromatin** compaction.

The **L3MBTL1** protein is another factor that integrates positional information. Like **HP1**, **L3MBTL1** dimerises thereby providing even more local contacts with the **chromatin**. It possesses three **MBT domains**, the first of which binds to **H4K20me1/2** and **H1bK26me1/2**. In doing so, **L3MBTL1** compacts nucleosomal arrays bearing the two **histone modifications** [71]. Importantly, **L3MBTL1** associates with **HP1**, and the **L3MBTL1/HP1** complex, with its multivalent **chromatin**-binding potential, binds **chromatin** with a higher affinity than that of either of the two individual proteins alone.

Histone acetylated lysines are bound by **bromodomains**, which are often found in **HATs** and **chromatin**-remodelling complexes [72]. For example, **Swi2/Snf2** contains a **bromodomain** that targets it to **acetylated histones**. In turn, this recruits the **SWI/SNF** remodelling complex, which functions to 'open' the **chromatin** [73]. Recently, it has also been shown that **PHD fingers** are capable of specifically recognizing **acetylated histones**. The **DPF3b** protein is a component of the **BAF chromatin-remodelling complex** and it contains tandem **PHD fingers** that are responsible for recruiting the **BAF complex** to **acetylated histones** [74].

Mitogen induction leads to a rapid activation of immediate early genes such as **c-jun**, which involves **phosphorylation** of **H3S10** within the gene's promoter [75]. This modification is recognized by the **14-3-3%** protein, a member of the **14-3-3 protein family** [76]. Furthermore, studies in *Drosophila melanogaster* have indicated that this

protein family is involved in recruiting components of the elongation complex to **chromatin** [77]. Another example of a protein that specifically binds to **phosphorylated histones** is **MDC1**, which is involved in the DNA-repair process and is recruited to sites of double-strand DNA breaks (DSB). **MDC1** contains tandem **BRCT domains** that bind to **yH2AX**, the DSB-induced **phosphorylated H2A** variant [78].

Histone modifications do not only function solely by providing dynamic binding platforms for various factors. They can also function to disrupt an interaction between the **histone** and a **chromatin** factor. For instance, **H3K4me3** can prevent the **NuRD complex** from binding to the H3 N-terminal tail [79, 80]. This simple mechanism seems to make sense because **NuRD** is a general transcriptional repressor and **H3K4me3** is a mark of active transcription. **H3K4 methylation** also disrupts the binding of **DNMT3L's PHD finger** to the H3 tail [81]. Indeed, this very N-terminal region of H3 seems to be important in regulating these types of interaction, though the regulation is not solely via modification of K4. For instance, **phosphorylation** of H3T3 prevents the **INHAT** transcriptional repressor complex from binding to the H3 tail [82].

Histone modification cross-talk

The large number of possible histone modifications provides scope for the tight control of chromatin structure. Nevertheless, an extra level of complexity exists due to cross-talk between different modifications, which presumably helps to fine-tune the overall control (Figure 2). This cross-talk can occur via multiple mechanisms [53].

(I) There may be competitive antagonism between modifications if more than one modification pathway is targeting the same site(s). This is particularly true for lysines that can be acetylated, methylated or ubiquitylated.

(II) One modification may be dependent upon another. A good example of this trans-regulation comes from the work in Saccharomyces cerevisiae; methylation of H3K4 by scCOMPASS and of H3K79 by scDot1 is totally dependent upon the ubiquitylation of H2BK123 by scRad6/Bre1 [43]. Importantly, this mechanism is conserved in mammals, including humans [44].

(III) The binding of a protein to a particular modification can be disrupted by an adjacent modification. For example, as discussed above, HP1 binds to H3K9me2/3, but during mitosis, the binding is disrupted due to phosphorylation of H3S10 [59]. This action has been described as a 'phospho switch'. In order to regulate binding in this way, the modified amino acids do not necessarily have to be directly adjacent to each other. For instance, in S. pombe, acetylation of H3K4 inhibits binding of spChp1 to H3K9me2/3 [83].

(IV) An enzyme's activity may be affected due to modification of its substrate. In yeast, the scFpr4 proline isomerase catalyses interconversion of the H3P38 peptide bond and this activity affects the ability of the scSet2 enzyme to methylate H3K36, which is linked to the effects on gene transcription [51].

(V) There may be cooperation between modifications in order to efficiently recruit specific factors. For example, PHF8 specifically binds to H3K4me3 via its PHD finger, and this interaction is stronger when H3K9 and H3K14 are also acetylated on the same tail of H3 [60]. However, this stabilization of binding may be due to additional factors in a complex with PHF8 rather than a direct effect on PHF8 itself.

There may also be cooperation between **histone modifications** and **DNA methylation**. For instance, the **UHRF1** protein binds to nucleosomes bearing **H3K9me3**, but this binding is significantly enhanced when the nucleosomal DNA is **CpG methylated** [61]. Conversely, **DNA methylation** can inhibit protein binding to specific **histone modifications**. A good example here is **KDM2A**, which only binds to nucleosomes bearing **H3K9me3** when the DNA is not **methylated** [61].

Genomic localization of histone modifications

From a **chromatin** point of view, eukaryotic genomes can generally be divided into two geographically distinct environments [3]. The first is a relatively relaxed environment, containing most of the active genes and undergoing cyclical changes during the cell cycle. These 'open' regions are referred to as **euchromatin**. In contrast, other genomic regions, such as centromeres and telomeres, are relatively compact structures containing mostly inactive genes and are refractive to cell-cycle cyclical changes. These more 'compact' regions are referred to as **heterochromatin**. This is clearly a simplistic view, as recent work in *D. melanogaster* has shown that there are five genomic domains of **chromatin** structure based on analysing the pattern of binding of many **chromatin** proteins [84]. However, given that most is known about the two simple domains described above, references below will be defined to these two types of genomic domains.

Both **heterochromatin** and **euchromatin** are enriched, and indeed also depleted, of certain characteristic **histone modifications**. However, there appears to be no simple rules governing the localization of such modifications, and there is a high degree of overlap between different **chromatin** regions. Nevertheless, there are regions of demarcation between **heterochromatin** and **euchromatin**. These **'boundary elements'** are bound by specific factors such as **CTCF** that play a role in maintaining the boundary between distinct **chromatin** 'types' [85]. Without such factors, **heterochromatin** would encroach into and silence the **euchromatic** regions of the genome. **Boundary elements** are enriched for certain modifications such as **H3K9me1** and are devoid of others such as **histone acetylation** [86]. Furthermore, a specific **histone** variant, **H2A.Z**, is highly enriched at these sites [86]. How all of these factors work together in order to maintain these boundaries is far from clear, but their importance is undeniable.

Heterochromatin

Although generally repressive and devoid of **histone acetylations**, over the last few years it has become evident that not all **heterochromatin** is the same. Indeed, in multicellular organisms, two distinct heterochromatic environments have been defined: (a) **facultative** and (b) **constitutive heterochromatin**.

(a) **Facultative heterochromatin** consists of genomic regions containing genes that are differentially expressed through development and/or differentiation and which then become silenced. A classic example of this type of **heterochromatin** is the inactive X-chromosome present within mammalian female cells, which is heavily marked by **H3K27me3** and the **Polycomb repressor complexes (PRCs)** [87]. This co-localization makes sense because the **H3K27 methyltransferase EZH2** resides within the trimeric **PRC2 complex**. Indeed, recent elegant work has shed light on how **H3K27me3** and **PRC2** are involved in positionally maintaining **facultative heterochromatin** through DNA replication [88]. Once established, it seems that **H3K27me3** recruits **PRC2** to sites of DNA replication, facilitating the maintenance of **H3K27me3** via the action of **EZH2**. In this way, the **histone** mark is 'replicated' onto the newly deposited **histones** and the **facultative heterochromatin** is maintained.

(b) **Constitutive heterochromatin** contains permanently silenced genes in genomic regions such as the centromeres and telomeres. It is characterised by relatively high levels of **H3K9me3** and **HP1α/β** [87]. As discussed above, **HP1** dimers bind to **H3K9me2/3** via their **chromodomains**, but importantly they also interact with **SUV39**, a major **H3K9 methyltransferase**. As DNA replication proceeds, there is a redistribution of the existing modified **histones** (bearing **H3K9me3**), as well as the deposition of newly synthesized **histones** into the replicated **chromatin**. Since **HP1** binds to **SUV39**, it is tempting to speculate that the proteins generate a feedback loop capable of maintaining **heterochromatin** positioning following DNA replication [68]. In other words, during DNA replication, **HP1** binds to nucleosomes bearing **H3K9me2/3**, thereby recruiting the **SUV39 methyltransferase**, which in turn methylates H3K9 in adjacent nucleosomes containing unmodified H3. Furthermore, this positive feedback mechanism helps to explain, at least in part, the highly dynamic nature of **heterochromatin**, not least its ability to encroach into **euchromatic** regions unless it is checked from doing so.

Euchromatin

In stark contrast to **heterochromatin**, **euchromatin** is a far more relaxed environment containing active genes. However, as with **heterochromatin**, not all **euchromatin** is the same. Certain regions are enriched with certain **histone modifications**, whereas other regions seem relatively devoid of modifications. In general, modification-rich 'islands' exist, which tend to be the regions that regulate transcription or are the sites of active transcription [86]. For instance, active transcriptional enhancers contain relatively high levels of **H3K4me1**, a reliable predictive feature [89]. However, active genes themselves possess a high enrichment of **H3K4me3**, which marks the transcriptional start site (TSS) [86, 90]. In addition, **H3K36me3** is highly enriched throughout the entire transcribed region [91]. The mechanisms by which **H3K4me1** is laid down at enhancers is unknown, but work in yeast has provided mechanistic detail into how the H3K4 and H3K36 **methyltransferases** are recruited to genes, which in turn helps to explain the distinct distribution patterns of these two modifications (Figure 3). The **scSet1 H3K4 methyltransferase** binds to the serine 5 phosphorylated CTD of RNAPII, the initiating form of polymerase situated at the TSS [92]. In contrast, the **scSet2 H3K36 methyltransferase** binds to the serine 2 phosphorylated CTD of RNAPII, the

transcriptional elongating form of polymerase [93]. Thus, the two enzymes are recruited to genes via interactions with distinct forms of RNAPII, and it is therefore the location of the different forms of RNAPII that defines where the modifications are laid down (reviewed in reference [3]).

Taken together, we are beginning to understand how some enzymes are recruited to specific locations, but our knowledge is far from complete. In addition, another question that needs to be considered relates to how different **histone modifications** integrate in order to regulate DNA processes such as transcription. Staying with **H3K4me3** in budding yeast as an example, it has been shown that **H3K4me3** recruits **scYng1**, which binds via its **PHD finger** [94]. This in turn stabilizes the interaction of the **scNuA3 HAT** leading to hyperacetylation of its substrate, H3K14 (Figure 3). Thus, **methylation** at H3K4 is intricately linked to **acetylation** at H3K14. In a similar manner, and again in yeast, **H3K36me3** has been shown to recruit the **scRpd3S HDAC** complex, which deacetylates **histones** behind the elongating RNAPII (Figure 3). This is important because it prevents cryptic initiation of transcription within coding regions [95, 96]. Together, these examples show how the recruitment of two opposing enzyme activities (**HATs** and **HDACs**) is important at active genes in yeast. However, it is not clear whether these mechanisms are completely conserved in mammals. There is evidence for **H3K4me3**-dependent **HAT** recruitment, [55] but no evidence exists for **H3K36me3**-dependent **HDAC** recruitment.

In mammals, regulatory mechanisms governing the activity of certain genes can involve specific components more commonly associated with heterochromatic events. For example, repression of the cell-cycle-dependent cyclin E gene by the retinoblastoma gene product **RB** involves recruitment of **HDACs**, H3K9 methylating activity and **HP1β** [97, 98]. Thus, the repressed cyclin E gene promoter appears to adopt a localized structure reminiscent of **constitutive heterochromatin**, i.e., presence of **H3K9me2/3** and **HP1**. However, unlike true **heterochromatin**, this is a transitory structure that is lost as the cell progresses from G1- into S-phase when the cyclin E gene is activated. Thus, components of **heterochromatin** are utilized in a **euchromatic** environment to regulate gene activity.

Histone modifications and cancer

Crudely speaking, full-blown **cancer** may be described as having progressed through two stages, initiation and progression. As we discuss below, changes in **'epigenetic modifications'** can be linked to both of these stages. However, before describing specific examples, we will consider the mechanisms by which aberrant **histone modification** profiles, or indeed the dysregulated activity of the associated enzymes, may actually give rise to **cancer**. Current evidence indicates that this can occur via at least two mechanisms; (i) by altering gene expression programmes, including the aberrant regulation of **oncogenes** and/or **tumour suppressors**, and (ii) on a more global level, **histone modifications** may affect genome integrity and/or chromosome segregation. Although it is beyond the scope of this review to fully discuss all of these possibilities, we will provide a few relevant examples highlighting these mechanisms.

Mouse models are invaluable tools for determining whether a particular factor is capable of inducing or initiating **tumourigenesis**. A good example is provided by the analysis of the **MOZ-TIF2** fusion that is associated with **acute myeloid leukaemia (AML)** [99, 100]. The **MOZ** protein is a **HAT** [101] and **TIF2** is a nuclear receptor coactivator that binds another **HAT**, **CBP** [102]. When the **MOZ-TIF2** fusion was transduced into normal committed murine haematopoietic progenitor cells, which lack self-renewal capacity, the fusion conferred the ability to self-renew in vitro and resulted in **AML** in vivo [103]. Thus, the fusion protein induces properties typical of leukaemic stem cells. Interestingly, the intrinsic **HAT** activity of **MOZ** is required for neither self-renewal nor leukaemic transformation, but its nucleosome-binding motif is essential for both [103, 104]. Importantly, the **CBP** interaction domain within **TIF2** is also essential for both processes [103, 104]. Thus, it seems that both self-renewal and leukaemic transformation involve aberrant recruitment of **CBP** to **MOZ** nucleosome-binding sites. Consequently, the transforming ability of **MOZ-TIF2** most likely involves an erroneous **histone acetylation** profile at **MOZ**-binding sites. These findings provide a clear indication that the dysregulated function of **histone** modifying enzymes can be linked to the initiation stage of **cancer** development.

An activating mutation within the non-receptor tyrosine **kinase JAK2** is believed to be a **cancer**-inducing event leading to the development of several different haematological malignancies, but there were few insights into how this could occur [105, 106]. Recently however, **JAK2** was identified as an H3 **kinase**, specifically phosphorylating H3Y41 in haematopoietic cells. **JAK2**-mediated **phosphorylation** of H3Y41 prevents **HP1a** from binding, via its **chromoshadow domain**, to this region of H3 and thereby relieves gene repression [14]. This antagonistic mechanism was shown to operate at the **Imo2** gene, a key haematopoietic **oncogene** [14, 107, 108].

In humans, extensive gene silencing caused by overexpression of **EZH2** has been linked to the progression of multiple solid malignancies, including those of breast, bladder and prostate [109-111]. This process almost certainly involves widespread elevated levels of **H3K27me3**, the mark laid down by **EZH2**. However, it has also recently been reported that **EZH2** is inactivated in numerous myeloid malignancies, suggesting that **EZH2** is a **tumour suppressor** protein [112, 113]. This is clearly at odds to the situation in solid tumours where elevated **EZH2** activity is consistent with an oncogenic function. One possible explanation for this apparent dichotomy is that the levels of **H3K27me3** need to be carefully regulated in order to sustain cellular homeostasis. In other words, aberrant perturbation of the equilibrium controlling **H3K27me3** (in either direction) may promote **cancer** development. In this regard, it is noteworthy that mutations in **UTX** (an **H3K27me3 demethylase**) have been identified in a variety of tumours [114], supporting the notion that **H3K27me3** levels are a critical parameter for determining cellular identity.

Finally, changes in **histone modifications** have been linked to genome instability, chromosome segregation defects and **cancer**. For example, homozygous null mutant embryos for the gene **PR-Set7** (an H4K20me1 HMT) display early lethality due to cell-cycle defects, massive DNA damage and improper mitotic chromosome condensation [115]. Moreover, mice deficient for the **SUV39 H3K9 methyltransferase** demonstrate

reduced levels of heterochromatic **H3K9me2/3** and they have impaired genomic stability and show an increased risk of developing **cancer** [116].

> It now seems clear that aberrant **histone modification** profiles are intimately linked to **cancer**. Crucially, however, unlike DNA mutations, changes in the **epigenome** associated with **cancer** are potentially reversible, which opens up the possibility that **'epigenetic drugs'** may have a powerful impact within the treatment regimes of various cancers.

Indeed, **HDAC inhibitors** have been found to be particularly effective in inhibiting tumour growth, promoting apoptosis and inducing differentiation (reviewed in [117]), at least in part via the reactivation of certain **tumour suppressor** genes. Moreover, the Food and Drug Administration has recently approved them for therapeutic use against specific types of **cancer**, such as T-cell cutaneous lymphoma, and other compounds are presently in phase II and III clinical trials [118].

Other **histone**-modifying enzyme inhibitors, such as **HMT inhibitors**, are presently in the developmental phase. But before we plunge head-first into a full discovery programme for other inhibitors, we should consider a number of important issues relevant to the development of such initiatives (see [118] for full discussion). First, we do not fully understand how **HDAC inhibitors** achieve their efficacy. Do they for instance exert their effects via modulating the **acetylation** of **histone** or non-**histone** substrates? Second, the majority of **HDAC inhibitors** are not enzyme-specific, that is, they inhibit a broad range of different **HDAC** enzymes. It is not known whether this promotes their efficacy or whether it would be therapeutically advantageous to develop inhibitors capable of targeting specific **HDACs**. Thus, when developing new inhibitors such as those targeting **HMTs**, we need to consider whether we should aim for enzyme-specific inhibitors, enzyme subfamily specific inhibitors, or similarly to the **HDAC inhibitors**, pan-inhibitors. Nevertheless, the fact that these drugs are safe and the fact that they work at all, given the broad target specificity, are extremely encouraging. So the truth is that even though there is still a lot to learn about **chromatin** as a target, **'epigenetic' drugs** clearly show great promise.

Future perspectives

We have identified many **histone modifications**, but their functions are just beginning to be uncovered. Certainly, there will be more modifications to discover and we will need to identify the many biological functions they regulate. Perhaps most importantly, there are three areas of sketchy knowledge that need to be embellished in the future.

The first is the delivery and control of **histone modifications** by **RNA**. There is an emerging model that short and long **RNAs** can regulate the precise positioning of modifications and they can do so by interacting with the enzyme complexes that lay down these marks [119-122]. Given the huge proportion of the genome that is converted into uncharacterised **RNAs** [123, 124], there is little doubt that this form of regulation is far more prevalent than is currently considered.

The second emerging area of interest follows the finding that **kinases** receiving signals from external cues in the cytoplasm can transverse into the nucleus and modify **histones** [14, 125]. This direct communication between the extracellular environment and the regulation of gene function may well be more widespread. It could involve many of the **kinases** that are currently thought to regulate gene expression indirectly via signalling cascades. Such direct signalling to **chromatin** may change many of our assumptions about **kinases**, as drug targets and may rationalise even more the use of **chromatin**-modifying enzymes as targets.

The third and perhaps the most ill-defined process that will be of interest is that of **epigenetic inheritance** and the influence of the environment on this process. We know of many biological phenomena that are inherited from mother to daughter cell, but the precise mechanism of how this happens is unclear [126]. Do **histone modifications** play an important role in this? The answer is yes, and as far as we know they are responsible for perpetuating these events. However, how does the **epigenetic** signal start off? Is the deposition of the modifications at the right place during replication enough to explain the process? Or is there a ‘memory molecule’, such an **RNA**, transmitted from mother to daughter cell [127], which can deliver **histone modifications** to the right place? These are fundamental questions at the heart of 'true' **epigenetic** research, and they will take us a while longer to answer.

Figures

Figure 1: Domains binding modified histones.

This figure illustrates examples of proteins with domains that specifically bind to modified histones. It shows the 'Royal family' of domains (Chromodomain, Tudor domain, MBT domain) and others like PHD finger, Bromodomain, and 14-3-3, and indicates which modified histone sites they recognize (e.g., H3K4me, H3K9me, H3K27me, H4K20me, H3K14ac, H3S10ph).

Figure 2: Histone modification cross-talk.

This figure depicts how histone modifications can positively (arrowhead) or negatively (flat head) affect other modifications on histones H3, H2B, H2A, and H4. It illustrates a complex web of interactions, such as H2BK123 ubiquitylation positively affecting H3K4 and H3K79 methylation.

Figure 3: Interplay of factors at an active gene in yeast.

This figure shows a model of the interplay between different factors at an active gene in yeast. It illustrates how the RNAPII CTD, in its different phosphorylation states (S5-ph at the TSS, S2-ph in the transcribed region), recruits different enzyme complexes (SET1, SET2, NuA3 HAT, Rpd3S HDAC) to lay down or remove specific histone modifications (H3K4me3, H3K36me3, H3K14ac) across the gene.

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