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5-Methylcytosine DNA Demethylation: More Than Losing a Methyl Group

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Abstract

Demethylation of 5-methylcytosine in DNA is integral to the maintenance of an intact epigenome. The balance between the presence or absence of 5-methylcytosine determines many physiological aspects of cell metabolism, with a turnover that can be measured in minutes to years. Biochemically, addition of the methyl group is shared among all living kingdoms and has been well characterized, whereas the removal or reversion of this mark seems diverse and much less understood. Here, we present a summary of how DNA demethylation can be initiated directly, utilizing the ten-eleven translocation (TET) family of proteins, activation-induced deaminase (AID), or other DNA modifying enzymes, or indirectly, via transcription, RNA metabolism, or DNA repair; how intermediates in those pathways are substrates of the DNA repair machinery; and how demethylation pathways are linked and possibly balanced, avoiding mutations.

INTRODUCTION

If mammalian CpG methylation (5-methyl modification on both cytosines of a double-stranded dimer) provides a means for local and heritable control of gene expression, then it must be reversible. This notion has fostered the search for mechanisms that lead to DNA demethylation. Biochemically, DNA methylation serves a multitude of purposes (**Figure 1*a***): (*a*) It alters van der Waals radii around C5 of cytosine by the addition of a bulky methyl group; (*b*) it can help foster the formation of non-B DNA (107); (*c*) it alters the base stacking potential of cytosine, possibly influencing compactness and winding around a nucleosome (1); (*d*) it inhibits the binding of some DNA binding proteins (63, 127, 137); and (*e*) it can enhance the binding of methyl-CpG binding proteins (MBPs) (148). Within the nucleus, DNA methylation serves to (**Figure 1*b***) (*a*) change the compaction of chromatin (108); (*b*) change the subnuclear localization of gene loci (74, 108); (*c*) change the activation potential of genes (136); (*d*) repress transposons and other selfish DNA (11); (*e*) alter DNA replication efficacy (3, 33, 106); and (*f*) influence the rate of RNA processing (126). Such diverse functionality can have profound effects on cellular proliferation and differentiation, pluripotency, development, imprinting, and oncogenesis (**Figure 1*c***).

Demethylation is subject to temporal and cellular regulation, raising the likelihood that several types of effectors are involved at any given time. In mammals, numerous examples of active DNA demethylation have been described. The extent of active demethylation is

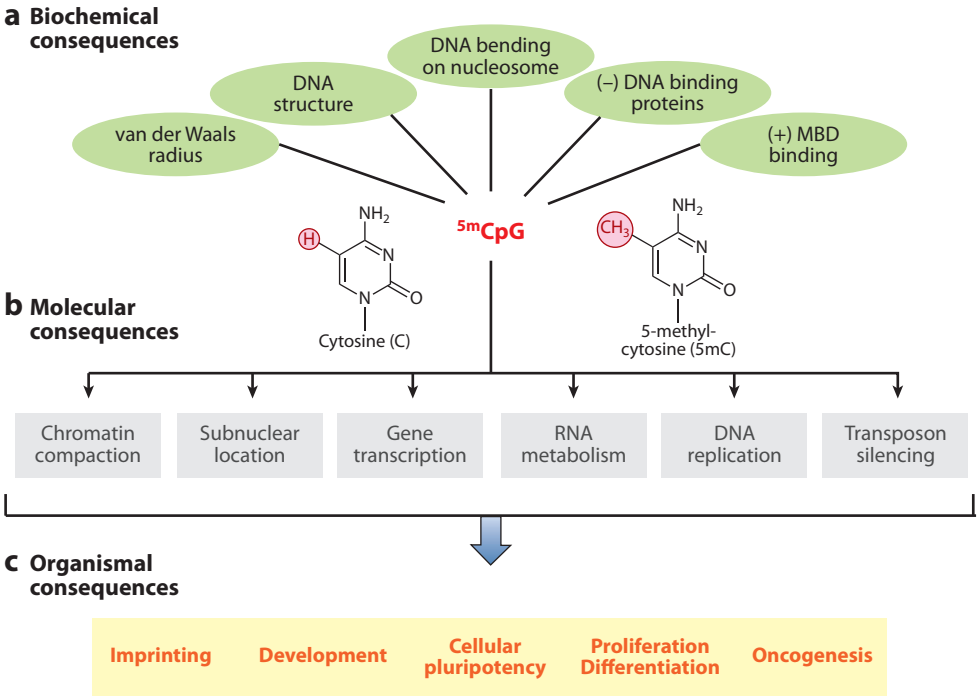

Figure 1

Overview of how methylation of cytosines influences biological processes. (*a*) The methyl group on cytosine can exert biochemical changes to DNA, which are either direct (van der Waals radius, DNA structure, DNA bending on nucleosomes) or indirect (enabling/disabling the association of protein binding). (*b*) The biochemical modifications serve as molecular signals for chromosome function via various means. (*c*) The combination of these molecular signals determines development, physiology, and pathology of an organism.

variable, ranging from global (i.e., genome-wide) to local gene-specific demethylation, possibly even at just a single cytosine (142).

Among the most active tissues for DNA demethylation are those carrying out very early steps in development, which are characterized by genome-wide loss of 5-methylcytosine (5mC). First, in the zygote, the paternal genome undergoes a massive wave of loss of 5mC marks right after fertilization (75, 105). Second, global demethylation can be observed during cellular migration of primordial germ cells (PGCs) to the genital ridge between embryonic day 10.5 and 12.5 (37, 61).

Aside from global DNA reprogramming events, evidence for active DNA demethylation in somatic cells has been reported. In mature neurons (72), lymphocytes (16, 58, 88), and breast cancer cell lines (118b) (56, 78) local DNA demethylation that leads to specific gene activation has been detected following cellular stimulation.

The wealth of data on global methylation status and local CpG demethylation derived from studies of physiological development, cancer, tissue differentiation, and stem cell survival cannot be explained by a simple single static pathway of DNA demethylation. As CpG methylation provides a mark for numerous molecular functions, its presence or absence will be detected over a broad dynamic scale, ranging from fluctuations in minutes to near perfect stability over generations. At times, demethylation may be cyclic and directly followed by remethylation for bursts of promoter activation. Demethylation may also persist only until a developmental path has been concluded, at which time genes and cells return to a more quiescent state, whereas some persistent demethylation is required in terminally differentiated cells.

This review highlights the pathways currently known to lead to DNA demethylation, focusing mostly on mammals. The importance of epigenetic reprogramming in health and disease is increasingly appreciated, and we also hope to provide the nonexpert with enough of an overview to allow independent exploration of this exciting field.

DNA Demethylation as a Loss of 5-Methylcytosine Recognition

The progress of science is dependent on available technologies. For many years, the techniques most commonly used to identify DNA methylation included restriction analysis with methylation-sensitive enzymes, staining with antibodies that recognize 5mC, and chemical modification (bisulfite treatment) followed by sequencing. Each approach has strengths and limitations, particularly because they may be sensitive to 5mC modifications. Either a 5mC or a modified 5mC may alter the pattern of restriction digests; antibody recognition may be prevented not only by loss of the methyl group but also by modifications of the methyl group; and a modified 5mC may have a different chemical property during bisulfite treatment than a 5mC.

The chemical differences that affect detection of 5mC in vitro are also important for molecular recognition of 5mC in vivo. Reversibility of methylation can therefore be understood in terms of function as well as chemistry. The chemical and biological functions of 5mC described above can be modulated not only by modification or removal of the methyl group but also by shielding or hiding it. Hiding is a potentially important source of regulation that has not been discussed or systematically tested experimentally. Hiding could, in principle, be achieved chemically via tautomerization of 5mC. As with most pyrimidines, 5mC is usually an *amino* tautomer, and this form is recognized by methyl-CpG-binding domains (MBDs) of MBPs. Local acidic pH changes (stimulated, for example, by the loss of basic protamines that buffer the DNA phosphodiester backbone) could induce transition to the *imino* form, possibly preventing MBD recognition. Return to a neutral pH would enable MBD recognition. Local changes in pH are commonly used and are precise means by which to influence biological activity, as

seen by the subtle pH shifts that promote catalysis by histidine-containing enzymes.

Nucleosomes can inhibit recognition of CpG by DNA nucleotide methyltransferase (DNMT) (28), thereby leading to a demethylated phenotype. Although, MBDs can recognize 5mC within chromatin (18, 64), the positioning of the 5mC with respect to the face of the core nucleosome can influence MBD binding, with linker DNA-associated 5mCs even more accessible. This suggests that a 5mC could be hidden by nucleosomes depending on the position of the nucleosome over the methylated region.

Modification of the 5-methyl moiety is another means to prevent the recognition of 5mC. This can induce changes in the epigenetic status of a locus [see discussion on ten-eleven translocation (TET) family proteins below]. If reversible, this allows for a transient and dynamic loss of methylation in response to developmental or environmental stimuli.

Base removal is the most stable mechanism of DNA demethylation but brings with it the highest price of mutability. Nonetheless, the discovery that several DNA repair pathways are involved in DNA demethylation (see following sections below for details) seems to indicate that this risk is evolutionary acceptable.

Absentee: A DNA Demethylase

A covalent modification such as protein phosphorylation can alter the 3D structure of a protein as well as its interaction with other proteins. The bond energy of protein phosphorylation allows for rapid addition and removal, a biological advantage that has been fully exploited, as seen by the large number of kinases and phosphatases participating in every aspect of biology. However, the chemistry of cytosine methylation (119) seems to forbid comparable transience. Methylation at the C5 position of cytosine is via a stable carbon-carbon bond that requires substantial energy to break. Electrons are shared throughout the aromatic cytosine ring, so input energy sufficient to break the carbon-carbon bond at the C5 position would dissipate and induce bond breakage at N1, N3, and the N-carbon bond on C4. This may preclude direct demethylation at the C5 position and explain why activities that directly demethylate 5mC DNA have not been identified. Instead, cytosine DNA demethylation appears to require a cascade of enzymes leading to the production of cytosine. As the term DNA demethylase suggests a single activity, it is not used in this review when discussing 5mC to avoid possible confusion.

However, DNA demethylases have been described that act directly to remove methylation by cleaving an N-carbon bond (6). AlkB dioxygenases, which are found in prokaryotes and eukaryotes, carry out oxidative demethylation to remove alkylation from DNA during DNA repair (4). These enzymes use oxygen, Fe(II), and 2-oxoglutarate as cofactors, and release methyl groups from 1-methyl adenine or 3-methyl cytosine as formaldehyde (27).

DEMETHYLATION PATHWAYS ACTING AT OR NEAR 5-METHYLCYTOSINE

There has been a recent explosion in the numbers of factors implicated in DNA demethylation of 5mC, and this may have led to a confusing and misleading picture. We attempt to simplify this (**Figure 2**) by separating those pathways into two groups: those in which a specific factor acts at or near 5mCpG [e.g., thymine DNA glycosylase (TDG), MBD4, TET, AID, DNMT1] and those in which such links have not been identified or are likely to be indirect (e.g., PARP, GADD45, hormones). The direct interacting proteins can be divided into those that are directly mutagenic [targeted lesion-induced demethylation (TLD)] and those that are indirectly or nonmutagenic [targeted modification-induced demethylation (TMD)]. (see sidebar, Triggering Active DNA Demethylation). These demethylation pathways may involve significant overlap among DNA repair proteins and pathways used.

Passive DNA Demethylation

Most DNA methylation is symmetrical in that the C5 carbon is methylated on base-paired CpG dinucleotides on both DNA strands. As replicative DNA polymerases cannot incorporate 5mC, a hemimethylated DNA intermediate is formed during DNA replication. In prokaryotes, repression of methyltransferase activities during replication leads to the dilution of methylated bases, with some daughter DNA molecules lacking any methylation.

DNMTs are essential for the establishment and maintenance of the methylation profile (32, 66, 91). During vertebrate DNA replication, a number of proteins associate with the replicative DNA polymerase, including processivity factors, nucleosome remodelers, and DNA repair proteins. The DNA polymerase clamp loader PCNA can interact with the maintenance methyltransferase DNMT1. Reducing DNMT1 expression (30, 51) or inhibiting the interaction of DNMT1 with PCNA (45) leads to genome-wide loss of CpG methylation and genome instability. DNMT1 possesses an autoinhibitory mechanism that ensures it targets only hemimethylated CpGs (112a), and this prevents DNMT1 from acting on DNA following demethylation of both DNA strands. Furthermore, inhibition of DNMT1 recruitment also leads to passive DNA demethylation. In the presence of UHFR1, bound to hemimethylated DNA with its SRA domain, DNMT1 is tethered to the locus. Accordingly, depletion of UHFR1 leads to a loss of genome-wide methylation marks (14, 116). Other DNMTs, including DNMT2, DNMT3A/B, and DNMT3L (32), can also act on hemimethylated CpGs; however, they are usually considered de novo methylating enzymes and their role in DNA demethylation is more indirect (see below).

The extent and dynamics of 5mC in the genome of mammals suggest that passive DNA demethylation alone cannot account for all changes. Unlike prokaryotes that can replicate their genome in minutes and even have multiple copies available during growth, mammalian methylation dynamics has to cover all occurrences from minutes to years, and some even without DNA replication.

TRIGGERING ACTIVE DNA DEMETHYLATION

Proteins that induce active 5mC demethylation can be divided into two types, those that proceed via a mutagenic intermediate and those that do not.

Targeted Lesion-Induced Demethylation (TLD). The first group includes those proteins that are able to induce DNA demethylation by introducing a lesion at or near the 5mC. This would include TDG and MBD4, as their product is an AP site, as well as DNA deaminases such as AID, which produce a T:G or U:G mismatch. Importantly, these pathways require a direct and efficient activation of the DNA repair machinery.

Targeted Modification-Induced Demethylation (TMD). The second category involves proteins able to modify the 5mC, which does not lead to any change in dsDNA base pairing. TET-like modifications can serve to hide the 5mC recognition or become a target for TLD. Importantly, these proteins do not have to be directly linked to DNA repair, allowing for demethylation to proceed via different kinetics than TLD.

DNA Glycosylases and Base Excision Repair

Mammalian genomes experience 20,000–30,000 DNA-damaging events a day (68), which are efficiently repaired via numerous DNA repair pathways. DNA glycosylases play a pivotal role in repair by removing damaged DNA bases from the genome (6). The glycosylases implicated in base excision repair (BER) are (2) (*a*) the uracil DNA glycosylases, mitochondrial UNG1 and nuclear UNG2; (*b*) TDG; (*c*) the single-strand selective monofunctional uracil DNA glycosylase SMUG1; (*d*) the MBP MBD4; and (*e*) oxidized base-specific glycosylases NTH1, OGG1, NEIL1, and NEIL2. Two of these DNA glycosylases, TDG and MBD4, have been reported to act on 5mC:G in vitro (145, 146). The BER pathway could thus be implicated in active DNA demethylation (147), with DNA glycosylases TDG and MBD4 removing the

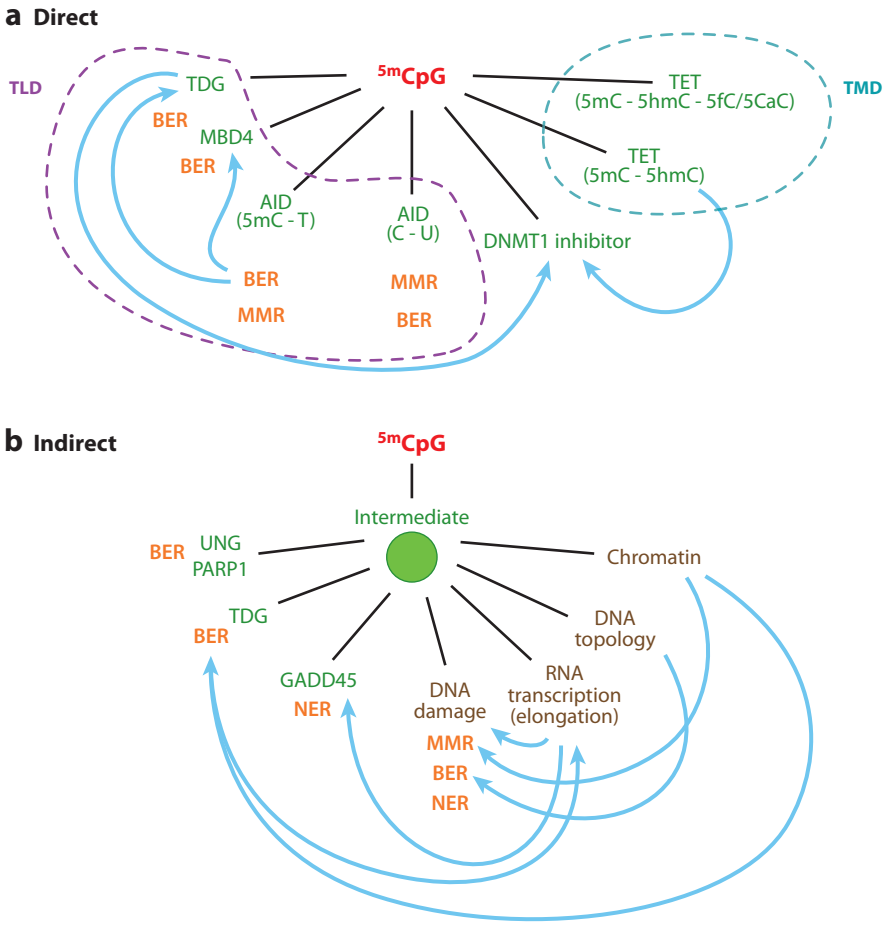

Figure 2

Active DNA demethylation machinery. (*a*) Proteins (*green*) and pathways (*orange*) that act at or near 5mCpG. Some proteins are classified as TLD (targeted lesion-induced demethylation) and some as TMD (targeted modification-induced demethylation) (see sidebar, Triggering Active DNA Demethylation). Currently, TLD proteins include TDG, MBD4, and AID, whereas TMD includes the TET protein family. (*b*) Proteins and pathways that may act through an unknown and possibly shared intermediate. Arrows indicate linked pathways.

5mC to generate an abasic (AP) site. The AP site then becomes a substrate for AP-endonuclease (APE), which in turn cleaves one strand of the DNA backbone and removes the deoxyribose-phosphate. The resulting single nucleotide gap is filled in by DNA polymerase β, which will incorporate C opposite G, and the backbone is ligated (52). Thus, this scenario results in replacement of 5mC with C (**Figure 3**).

A glycosylase-dependent DNA demethylation pathway has already been described for plants (46) and employs the DNA glycosylases ROS1 and DEMETER. These are bifunctional enzymes that initiate demethylation by first removing the 5mC base and then cleaving the resulting AP site. Demethylation in plants is not discussed in further detail, but the reader is encouraged to look at recent reviews (46, 147).

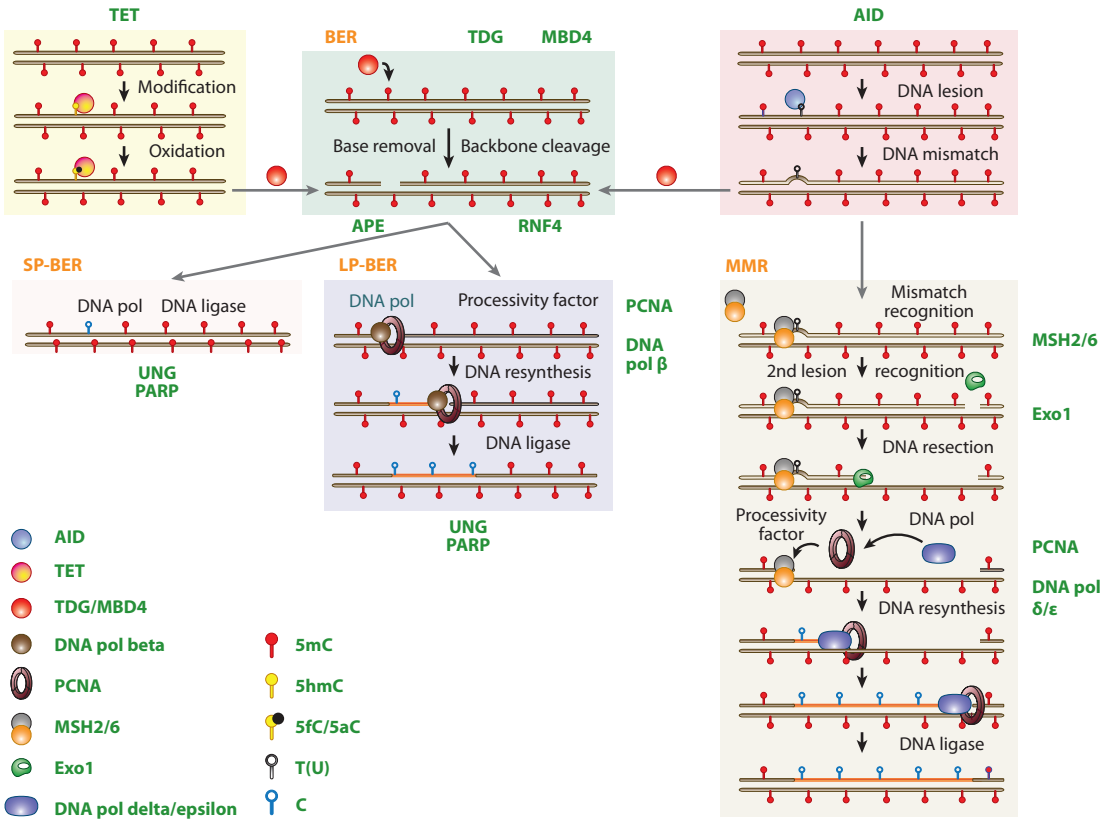

Figure 3

Mechanisms for active DNA demethylation. The current model for active DNA demethylation involves DNA repair pathways (*in orange*), including both short patch (SP) and long patch (LP) base excision repair (BER), and mismatch repair (MMR). TET-induced targeted modification-induced demethylation (TMD) pathway is shown on the top left. Activation-induced deaminase (AID)-induced targeted lesion-induced demethylation (TLD) pathway is shown on the top right. 5mC glycosylases-induced TLD is shown on the top center. Only key proteins are shown in the schematic. Intermediates from one pathway may be resolved via another pathway. DNA lesions not targeting CpGs may enter the repair pathway at various stages leading to demethylation. SP-BER leads to single base demethylation, whereas the activity of processive DNA polymerases in LP-BER and MMR may enable a single lesion to initiate extensive demethylation. Proteins are indicated in green.

Both TDG and MBD4 can act on 5mC:G pairs analogous to the plant enzymes. However, enzyme kinetics and in vivo genetics seem to preclude a direct function that would be analogous to the first step of ROS1 and DEMETER in plants.

Thymine DNA Glycosylase on 5-Methylcytosine

Glycosylase-dependent DNA demethylation was first proposed when TDG activity was described in chicken embryo extracts (55). Its function in coordination with BER explained an earlier observation that 5mC was replaced by cytosine rather than changed back to cytosine (104). The preferred substrate of TDG is thymine (or uracil) mispaired with guanine, with mismatches in a CpG context enhancing enzymatic activity (118). Although one report showed that TDG can directly excise 5mC from a guanine pair, this activity was reduced by a factor of 30 relative to a thymine:guanine pair (146). Subsequently, other laboratories

were unable to reproduce this finding (e.g., 39). The importance of TDG during epigenetic reprogramming was most aptly shown (21) when it was demonstrated that TDG-deficient mice exhibit reduced global methylation and embryonic lethality. The authors speculated that the observed phenotype was not only due to a global impairment in DNA methylation but possibly also due to a broader epigenetic regulatory function, as TDG interacts with transcription factors, histone acetyltransferases, and DNMT3 (21) (see below). A second TDG knockout confirmed its function in DNA demethylation (22), where authors speculated on a trimeric complex between TDG, AID, and GADD45, and proposed BER to drive global demethylation.

MBD4 on 5-Methylcytosine

As with TDG, the DNA glycosylase MBD4 removes thymine and uracil from mismatches and can initiate demethylation of 5mC in conjunction with the BER machinery. Similar to TDG, MBD4 activity is enhanced when the mismatches are in a CpG context and activity on a 5mC:G pair is reduced 30–40-fold (145). However, unlike the TDG knockout, mice deficient for MBD4 are viable and showed an increased C-to-T transition mutation rate at CpG and enhanced tumorigenesis (79), but active demethylation in the paternal pronucleus in zygotes was unaffected (109). This suggested that MBD4 is not essential for this global DNA demethylation event.

MBD4 has two functional domains, the N-terminal MBD and a highly conserved C-terminal glycosylase domain. MBD4 belongs to the family of MBD proteins that bind to symmetrically methylated cytosine exclusively in the context of CpG within double-stranded DNA (dsDNA) (76). Although MBD1, MBD2, and MECP2 play substantial roles in de novo silencing and the maintenance of the silent state of their target genes, the MBD of MBD4 binds preferentially to 5mCpG:TpG mismatches, which is the primary product of deamination of a single 5mC in a CpG context.

5-Methylcytosine Base Modifications

To circumvent the absence of direct deglycosylation of 5mC in mammals, there are other means to initiate DNA demethylation via oxidation, deamination, and radical S-adenosylmethionine (SAM)-based demethylation (84). All three result in modifications of 5mC that could lead to loss of DNA methylation recognition and/or loss of the methyl group (**Figure 2**).

TET Pathway

5-Hydroxymethylcytosine (5hmC) in DNA was discovered more than 60 years ago in bacteriophage T4 (143), which expresses glucosyltransferases that convert the bulk of the genomic cytosines to 5hmC. This modification protects the bacteriophage DNA against restriction by the host and serves as a mark for self-recognition. Despite the identification of 5hmC in the mammalian genome 20 years later (97), only the recent detection of 5hmC in neuronal cells and embryonic stem (ES) cells (60, 125) has provided a resurgence of interest in this modified base.

In mammals, the hydroxymethylation reaction is catalyzed by members of the TET protein family, which are 2-oxoglutarate and Fe(II)-dependent dioxygenases that are able to convert 5mC into 5hmC (50, 125) (**Figure 3**). TET proteins are expressed in tissues in which active DNA demethylation takes place. TET1 expression is mainly confined to embryonic stem cells, whereas TET2 and TET3 expressions are more ubiquitous (123, 125). In mouse embryonic stem cells, TET1 and TET2 are necessary for cellular commitment and identity (29, 49, 59). In the zygote, TET3 is responsible for the conversion of 5mC into 5hmC of the paternal pronucleus (34, 48). TET1 binds preferentially near transcription start sites (TSSs) and at promoter regions with high CpG density (138, 141, 144). This could be explained by the presence of a CXXC zinc finger domain in TET1 that recognizes unmethylated CpG. Interestingly, 5hmC shows a broader

distribution across the genes than TET1 (29, 93, 138, 141, 144) and is found at the TSS and within genes. Accumulation at the TSS and promoter region is preferentially detected among genes with bivalent chromatin, containing activating and repressive marks, whereas in actively transcribed genes 5hmC is distributed throughout gene bodies, particularly at exons. A similar distribution has been reported in mouse cerebellum and human brain (54, 120).

As with methylation of cytosine, the formation of 5hmC from 5mC causes an alteration in molecular recognition. The MBD-containing MECP2 protein exhibits a 50-fold reduced recognition of 5hmC as compared with 5mC (134). This loss in recognition is also observed for DNMT1, which in turn promotes passive demethylation, as DNMT1 does not recognize hemimethylated CpGs (133), and thereby provides a bridge between passive and active DNA demethylation.

Recent studies reported that TET proteins could further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (43, 50), raising the possibility that TET proteins could carry out active DNA demethylation by successive oxidation of 5mC. The TET enzyme's efficiency decreases for successive oxidation (50), and it will be interesting to know whether this is an important enzymatic mechanism for controlling 5hmC or is due to the lack of yet-to-be-identified cofactors. This could be analogous to the sumoylation of TDG at the bound substrate, which allows for turnover kinetics to proceed (40).

To date, no enzyme has been identified that can promote loss of the formyl or carboxyl group on C5, which would lead to complete demethylation of 5mC to C. The enzymatic steps in the TET pathway leading to C formation are of course analogous to those described almost 20 years ago (129), with the work focusing on the oxidation and removal of the methyl group of thymine. Thymine hydroxylase from *Neurospora* and *Rhodotorula* oxidizes the methyl group of free thymine by three successive steps to form 5-hydroxymethyluracil, 5-formyluracil, and 5-carboxyuracil. The carbon-carbon bond of 5-carboxyuracil is then cleaved by a separate carboxylase.

As mentioned above, it may not be necessary to remove these C5 moieties to obtain biological function. Further oxidation may preclude binding of proteins that usually recognize 5hmC (yet to be identified) or prevent the reversion of the TET reaction (5hmC to 5mC), or 5hmC may be sufficient to hide 5mC. Interestingly, the presence of high concentrations of 5hmC in the absence of 5mC has been correlated with elevated transcription rates (29). However, complete oxidation of 5hmC to 5fC and 5caC may provide a substrate for TDG, as the level of 5caC is elevated in the genome of TDG deficient cells (43), and in at least one in vitro system, TDG can act on 5fC:G and 5caC:G pairs (73).

Activation-Induced Deaminase and Base Excision Repair

AID was first characterized for its function in the diversification of immunoglobulins (Ig) (98). AID targets cytosine in single-stranded DNA (ssDNA) at the Ig loci and induces deamination to create U:G mismatches (99), which, due to hijacked DNA repair (mis)function, lead to mutation and recombination. Although the process of diversification is highly mutagenic, AID's mutator activity is orders of magnitude lower outside the Ig locus than within it (70).

As transcription of the *AID* gene can be driven by estrogen (94), its expression is not limited to immune cells. Interestingly, in some nonimmune tissues, such as oocytes, embryonic germ cells, and stem cells (82), DNA demethylation is highly active and correlates with AID expression. To address the possibility that the AID-initiated deamination is a mechanistic pathway for DNA demethylation, AID was tested for the ability to use 5mC as a substrate (82). In vitro and in bacteria, AID was shown to deaminate 5mC to generate a T:G mismatch. Genetic evidence for AID involvement in DNA demethylation came from zebrafish, where overexpression of AID induces global DNA demethylation (102).

Furthermore, in nondividing heterokaryons the reprogramming of fibroblast nuclei was induced by AID expression, changing the methylation status of pluripotency genes *oct4* and *nanog* (12). Importantly, in PGCs of AID-deficient mice, the 5mCpG content is increased by 30%, suggesting that AID is involved in genome-wide demethylation of PGCs (101).

Mechanistically (**Figure 3**), AID may function in demethylation by combining its deamination activity with various DNA repair pathways. As already shown, during immune diversification AID-initiated damage elicits a response from BER proteins, mismatch repair (MMR), and translesion synthesis and nonhomologous end-joining pathways (98). Similar pathways may be utilized at sites of AID deamination leading to DNA demethylation. Deamination of 5mC generates T, and in the context of dsDNA the resulting T:G mismatch is a preferred substrate for TDG and MBD4 (82). As described above, TDG and MBD4 could repair the mismatch that leads to cytosine incorporation using factors from the BER pathway.

A number of models have been proposed in which AID and TDG/MBD4 form a complex to promote demethylation. Genetic association between MBD4 and AID has been suggested in zebrafish (102), yet biochemical analysis needs to confirm this as well as explain how APOBEC2 (like AID, a member of the APOBEC DNA deaminase family, but lacking catalytic activity) can induce demethylation in zebrafish. More recently, AID has also been proposed to form a complex with TDG or SMUG1 (22, 35) in the context of the TET-induced demethylation pathway. Unfortunately, proper biochemical evidence is missing, and it is unlikely that AID will function on 5hmC, 5fC, or 5caC. Recent work from our lab (103) and others (83) has indicated that the binding pocket of AID cannot accommodate C5 side chains that are larger than methyl groups. This suggests that DNA deaminases and TET proteins utilize different initiation pathways for DNA demethylation, whereas lesion resolution may proceed through common intermediates.

Activation-Induced Deaminase and Mismatch Repair

During immune diversification, cytosine deamination by AID leads to mutations at neighboring As and Ts. Known as phase 2 of somatic hypermutation, key enzymes of the MMR pathway are responsible for this effect, including MSH2, MSH6, and EXO1, as well as the error prone DNA pol η.

Linking MMR factors to DNA demethylation could provide a number of unique insights (**Figure 3**). Mechanistically, AID-induced mismatches would be recognized by MMR proteins (e.g., MSH2/6), which in classical MMR would lead to recruitment of EXO1 and resection of 200–2,000 bases of ssDNA (81). This gap would then be resynthesized by DNA pol δ/ε, incorporating cytosines into the newly formed strand. Therefore, a single AID-induced lesion would lead to the removal of 5mC over a stretch of 1–2 kB of DNA. This type of processive DNA polymerase–dependent DNA demethylation would be more efficient and less mutagenic than targeting a single 5mC by the BER pathway. Future work will need to determine the key factors that distinguish error-free MMR (classical) and mutagenic MMR (as observed in AID-induced Ig diversification) (96), and how these pathways are controlled during AID-induced demethylation. For AID, the possibility of extended demethylation from a single site is especially intriguing, as AID is known to travel with the stalled/restarted elongating RNA pol II (95, 122; K.L. Willmann, S. Milosevic, S. Pauklin, K.-M. Schmitz, G. Rangam, M.T. Simon, S. Maslen, M. Skehel, I. Robert, V. Heyer, E. Schiavo, B. Reina-San-Martin, S.K. Petersen-Mahrt, unpublished observation) and to deaminate C and 5mC in limited sequence contexts (8, 15). Hence, inducing local DNA demethylation cannot always be achieved by a static mechanism that targets AID to all individual CpGs.

Currently, it is not clear what determines DNA repair pathway choice upon induction of lesions by AID. Importantly, the relevant repair pathways are interlinked through substrates

(U:G for BER and MMR) and intermediates as well as specific proteins (e.g., PCNA). It is likely that the efficiency of AID-induced demethylation may depend on the number of T:G mismatches versus U:G mismatches, the activity of TDG or UNG, and the activation of MMR.

Non-5mCpG Targeting

One of the more important caveats of a model postulating a role for MMR in AID-induced DNA demethylation is that it is not essential to target 5mCpG to achieve DNA demethylation. Deamination of C to U also generates a mismatch, and activity of MMR at this mismatch could initiate DNA demethylation at adjacent positions. By extension, any DNA repair pathway that utilizes a processive DNA polymerase—not only MMR but also nucleotide excision repair (NER) or long-patch BER (see below)—could induce DNA demethylation without specifically targeting 5mCpG. Therefore, targeted DNA damage near CpGs induces DNA demethylation. How these targeted lesions arise, aside from AID and TET-coupled BER, is yet to be determined, but could include targeting mechanisms of transcription, DNA replication, chromatin remodeling, or transposon silencing (**Figure 1**; and see below).

DNA damage–induced DNA demethylation was proposed prior to the discoveries of AID or TET (139). The function and chromatin association of these 5mC modifying enzymes, rather than the random environmental damage of physical or chemical agents, make the understanding of the current pathways even more pertinent.

Other DNA Damage

Recently, it was proposed that DNMTs participate in active DNA demethylation. This was based on the in vitro observation that DNMT3a/b cannot perform C5 methylation when the concentration of the methyl donor (i.e., SAM) for 5mC formation is below a certain threshold but instead initiates deamination at an already formed 5mC (78). Those experiments showed a requirement for TDG function as well as a genetic requirement for DNMTs in the cyclic DNA demethylation of the *TFF1* promoter (56, 78). In vivo data seem to suggest that the necessary 30–600-fold drop in SAM concentration to induce deamination by methyltransferases is unlikely to be a global event (31). It is also not clear why the chosen conditions allow DNMT3 to act on their product (5mC) rather than just their substrate C. However, the possibility that a DNMT enzyme induces deamination on a cytosine leading to DNA demethylation is plausible if this activity is linked to DNA repair, and an interaction of DNMT3 and TDG has been demonstrated by various labs (13, 21, 67). Furthermore, DNMT-dependent demethylation could be quite efficient if a processive polymerase-dependent repair pathway is involved.

Aside from AID, most mammals contain at least two other active DNA deaminases, and humans and large primates contain eight additional DNA deaminases that are members of the APOBEC family (20). APOBEC1 evolved to deaminate C to U in RNA but also has the ability to target DNA (42, 100), whereas the APOBEC3 subfamily has antiviral activity, with some APOBEC3 members functioning by deaminating retroviruses during first strand cDNA synthesis (41). APOBEC1 is known to act on 5mC (82), but it is not known if the other APOBEC members can, although it is likely they do. Future work will determine if these mostly cytoplasmic proteins are involved in 5mC demethylation.

As the above pathway predicts that DNA demethylation can also be initiated by events not targeted to 5mCpG, other physiological DNA damaging proteins may also induce demethylation. To this end, two reports have shown that drug-induced inhibition of the DNA topoisomerases can lead to a change in DNA methylation (89, 111). Although the effects of these drugs are likely to be indirect, camptothecin (inhibitor of DNA topo I) induced methylation, whereas etoposide

(inhibitor of DNA topo II) did not, indicating that DNA structural changes do not always lead to global demethylation.

The dependence on DNA repair to achieve DNA demethylation also carries a price for mutation and random demethylation. If targeted DNA damage can induce demethylation, what precludes random DNA damage from also inducing changes in the epigenome? UV light, oxidative damage, or any number of environmental DNA damaging agents may induce local or, in the worst case, global DNA demethylation, possibly through a single lesion. Although these lesions will be repaired and will not leave a genetic mark (mutation), they may leave an epigenetic mark, which can also lead to pathologies, including cancer. To circumvent too much change due to the environment, backup systems in the form of other epigenetic marks (e.g., histone modification, polycomb complex binding) will coordinate with the methylome.

INDIRECT DNA DEMETHYLATION

AID, TET, TDG, MBD4, and DNMTs act at or near 5mC, yet a number of proteins with no apparent DNA modification activity have been identified that can, at least genetically, induce DNA demethylation (**Figure 2**).

Transcription

Although somewhat paradoxical, transcription may induce active DNA demethylation. Because of the high oxidative potential, the process of transcription induces DNA lesions (6). The damaged bases have to be repaired, and if this involves processive polymerases, 5mC can be replaced.

In the past, TDG has been thought of not only as a glycosylase but also as a transcription factor that is inducible by retinoic acid (130, 132). It can interact with the retinoic acid receptor as well as CBP/p300. In light of the involvement of TDG in DNA demethylation, it needs to be determined if TDG's function as a transcription factor is a means for targeting it to promoters, a means to recruit transcription factors to an inactive promoter, or a means to induce DNA demethylation during chromatin remodeling.

As mentioned above, AID can be part of the elongating RNA pol II complex, possibly inducing DNA demethylation during transcription. Although direct associations between AID and SPT5 (95), as well as AID and PAF1 (polymerase associated factor) (K.L. Willmann, S. Milosevic, S. Pauklin, K.-M. Schmitz, G. Rangam, M.T. Simon, S. Maslen, M. Skehel, I. Robert, V. Heyer, E. Schiavo, B. Reina-San-Martin, S.K. Petersen-Mahrt, unpublished observation), have been demonstrated during transcription, AID mutational activity is found in a limited region starting from 100 bp to a maximum of 2,000 bp downstream of the TSS (25). Analogously TET proteins are usually associated with promoters, but their products can also be detected within the coding region and upstream of the TSS. It still needs to be determined if this association occurs pre- or post-transcription. The possible difference between AID and TET in patterns of distribution along a gene, especially with respect to the TSS, could point toward a differential usage of the two DNA demethylation pathways. AID and TDG also exhibit a difference in temporal association with RNA pol II, in that TDG seems to be associated with transcription initiation factors (130, 132), whereas AID seems to be associated with elongation/restart factors.

The RNA pol II elongator complex was also shown to play a role in partial genome-wide demethylation of the paternal genome in zygotes (90). Knockdown of ELP3 (part of the holo-elongator complex), as well as ELP1 and ELP4, partially impaired paternal DNA demethylation. Although domain mapping of ELP3 indicated that the Fe-S-radical SAM domain was critical for demethylation [which would lead to a more direct attack on 5mC via DNMTs (142)], recruitment of other factors may also play a significant role in ELP3-dependent DNA demethylation.

After transcription has been initiated, DNA demethylation of a promoter can still be governed by the transcription machinery. In the

case of the 5mC to C cycling at the pS2/TFF1 promoter, it was shown that TDG coordinates with DNMT3A/B and BER pathway proteins to induce strand-specific demethylation during estrogen-induced transcriptional cycling (78).

A possible link between promoter activation, DNA demethylation, and NER was shown by knockdown of the constitutive NER factors XPC, XPA, and ERCC1, leading to promoter methylation (62, 85). Those experiments demonstrated a correlation between histone modification, gene activation, and NER proteins as well as loss of 5mC demethylation at a promoter.

DNA Damage Repair

That NER could play an important part in DNA demethylation was hypothesized (although no data were presented) a number of years ago (19). In mammals, NER can be divided into two subpathways: global genome repair (GGR) and transcription-coupled repair (TCR). During NER (**Figure 4**), the regions

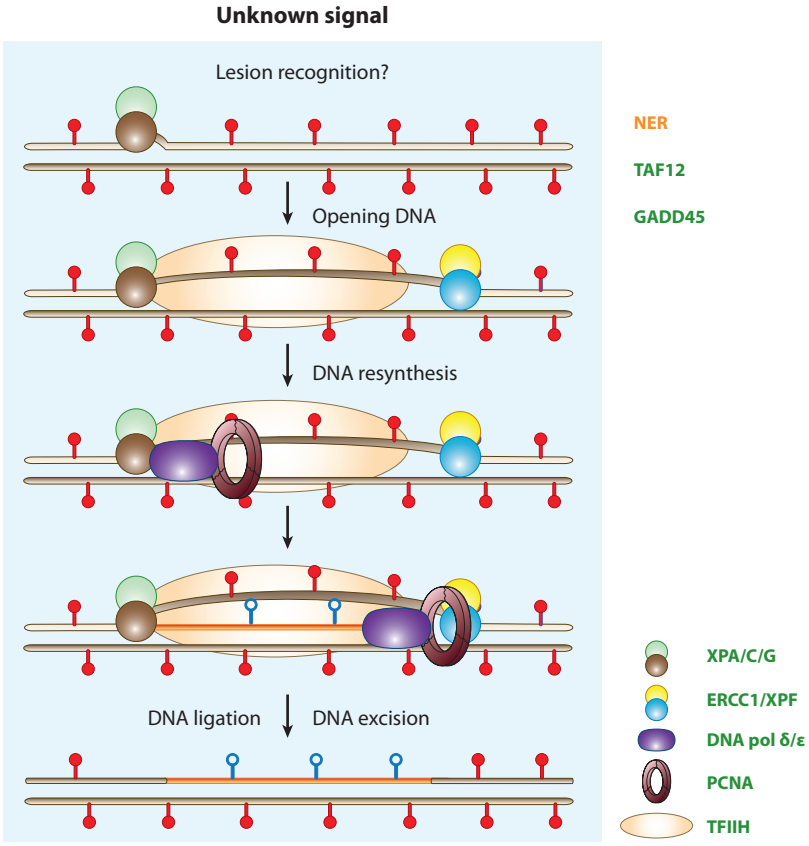

Figure 4

Schematic of nucleotide excision repair (NER) leading to DNA demethylation. Lesions are recognized by yet to be identified signals that initiate NER, possibly recruiting TAF12 (TBP-associated factor) and/or GADD45a (growth arrest and DNA damage-inducible 45 alpha), and in turn bringing XPC, XPA, and endonuclease XPG to the lesion. XPC binding serves as a landing platform for TFIIH and the endonuclease ERCC1/XPF and the bound complex defines the region (approximately 30 nt) to be subsequently unwound and excised. Endonucleolytic cleavage of the target strand provides an entry for the PCNA/DNA pol δ or ε complex. During DNA resynthesis and ligation the excised 5mC will be replaced by an unmodified cytosine, resulting in processive polymerase-dependent short stretch DNA demethylation.

flanking damaged DNA are incised and a 24–32 nucleotide oligomer is excised. Subsequently, DNA polymerases (similar to MMR) fill the gap and DNA ligase seals the ends. The three chromatin-associated steps of NER are (*a*) recognition, or initial damage detection (XPC-RAD23B), (*b*) damage verification (TFIIH, XPB, XPD), and (*c*) enzymatic action of the excision machinery (XPG, XPF-ERCC1) on the damaged DNA substrate and resynthesis (PCNA, DNA pol δ, ligase III-XRCC1) (85). As base recognition may be rate limiting during NER (71), lesion identification is paramount for DNA demethylation. That NER did induce DNA demethylation was shown after GADD45a (growth arrest and DNA damage-inducible 47 alpha) was isolated from a screen for potential DNA-demethylating proteins (7). Furthermore, GADD45a interacts with XPG, and involvement in DNA demethylation was demonstrated for both proteins (7).

The involvement of GADD45a in DNA demethylation was also demonstrated on the rDNA locus (112). TAF12 plays a key role in keeping rRNA genes free from methylation. This TBP-associated factor (part of the RNA pol I- and pol II-specific TBP-TAF complexes) binds to promoters and recruits GADD45a and XPG to induce NER-dependent demethylation, thereby providing the first molecular connection between TCR and DNA demethylation. Unlike GGR, classical TCR is independent of GADD45a; therefore, the boundaries between these NER pathways may be blurred. Although, GADD45a may participate in other DNA demethylation systems (102), a specific lesion that stimulates GADD45a for demethylation is yet to be identified, either in vivo or in vitro.

Recent work demonstrated the necessity of lesions (of unknown origin) to induce global DNA demethylation (140). By staining mouse zygotes for DNA breaks via γH2A.X phosphorylation, it was possible to monitor the dynamics of DNA strand breaks/DNA repair events and correlate them with active DNA demethylation during distinct developmental stages. Colocalization of PARP1 with sites of DNA damage indicated the utilization of BER and NER as well as other pathways that respond to DNA nicks. PARP1 is critical during BER, as it seems to play a differentiating role for short-patch BER (SP-BER) and long-patch BER (LP-BER) (2). To induce LP-BER, a stretch of DNA has to be removed after DNA glycosylase activity, which is facilitated by FEN1 (a flap endonuclease). The FEN1-induced nick is 2–13 nucleotides away from the initial lesion. DNA pol δ/ε, in conjunction with PCNA, performs DNA resynthesis and strand displacement. Unlike SP-BER, the gaps are religated by DNA lig I. As with MMR, LP-BER allows for DNA demethylation not targeted to 5mCpG.

PARP1 was first implicated in active DNA demethylation during studies on PGCs (38). Between embryonic stages E10.5 and E12.5, mRNA encoding factors associated with BER (and to a lesser extent NER) were detected, and PARP1 activity, based on its chemical inhibition, could be correlated with the appearance of DNA demethylation. The authors of that study suggested that AID was not involved. This was in contrast to others who identified a role for AID during PGC development (101), although technical differences may account for these different results. Interestingly, although the effect of PARP1 inhibition on DNA demethylation was observed globally as well as locally, APE (a key protein in BER) inhibition seemed to influence only global DNA methylation status, suggesting that different proteins from the same pathway share unequal responsibilities during DNA demethylation (38). Furthermore, the authors also saw a correlation of DNA demethylation with increased TET protein mRNA (38). More detailed analysis delineating which DNA demethylation pathway is active at what time, on which locus, and to what extent will provide us with the proper insight.

Chromatin and Demethylation

Discussion of chromatin and changes in the epigenetic states cannot proceed without including histone modifications. Rather than exploring all the possible histone marks that could lead to

repressive or active chromatin, we focus on those modifications that show a more direct link to DNA demethylation. Interplay between DNA methylation and histone modifications has been extensively demonstrated (17). As shown by the evolutionary loss of 5mC in certain species (44), the regulatory functions of DNA methylation and demethylation can be substituted by other, possibly non-DNA-dependent pathways. This observation may also be relevant to the understanding of DNA demethylation in mammals, as histone modifications may substitute for DNA methylation and not reveal the complete molecular mechanism of 5mC biogenesis.

Specific epigenetic environments might be required for DNA demethylation. During DNA reprogramming in fertilized zygotes, only the paternal genome is targeted for DNA demethylation, whereas the maternal genome seems to be protected. Although an active process by PGC7/Stella has been suggested for the protection of the maternal genome (86), asymmetric chromatin marks distinguishing the two genomes could also play a role. Absence of PGC7/Stella results in aberrantly targeted DNA demethylation in both the maternal and paternal pronuclei. In zygotes from PGC7/Stella null females, the activation of BER components is detectable in both parental pronuclei. After fertilization, the paternal genome undergoes histone variant H3.3 incorporation during protamine exchanges (131, 135). Moreover, H3K27 (mono-, di-, and trimethylation) and H3K9 (di- and trimethylation) are barely detected in the paternal genome, but are identified in the maternal pronucleus (5, 23, 26, 69, 110, 135). This indicates that even on the global scale, histone modification and DNA demethylation are interdependent.

Similarly, during migration to, and once in, the genital ridge, PGCs are associated with dynamic changes of histone modifications (80). Interestingly, at the time AID seems to be required for DNA demethylation, the chromatin undergoes changes similar to those observed in the male pronucleus, losing repressive marks H3K9me2, H3K9me3, H3K27me3, H3K64me3, and the linker histone H1, and gaining permissive modifications H3K9ac and H3K4me (24, 36, 113, 114). Whether these changes precede or occur concomitant with DNA demethylation, or whether they are a prerequisite, is not clear, but creating a more permissive chromatin may provide accessibility for the DNA demethylation pathway. Accordingly, TET1 occupancy positively correlates with H3K4me3, and depletion of TET1 results in increased CpG island methylation (138, 141, 144). Although TET1 occupancy presents an inverse correlation with the presence of H3K27me3 (138, 141, 144), TET1 also binds genes presenting a bivalent chromatin, (H3K4me3 and H3K27me3). In ES cells, bivalent genes represent 20% of the genes with high CpG content promoter and are usually developmentally regulated genes (10). Surprisingly, knockdown of TET1 did not only downregulate gene expression but also caused gene upregulation (138, 141, 144), suggesting a broader role for TET1 in gene transcription regulation. In another context, TET1 was found to colocalize with the polycomb repressor complex 2 (PRC2) (138, 141), and to associate with the corepressor complex Sin3A (138). Therefore, TET1 can both activate and repress transcription, and its activity is regulated via histone modification as well as presence of polycomb complexes.

Should DNMTs be directly involved in DNA demethylation, then histone H3K4 modification could also influence DNA demethylation (57, 65). The plant homeodomain-like domain in DNMT3A/B can recognize the histone tail, which is where modification occurs, and depending on its methylation status can induce DNMT3 activity. Furthermore, DNMT3L, the guiding partner of DNMT3A/B, can be excluded from chromatin by the presence of the specific histone mark H3K4me3 (92). Hence, DNA would become available for de novo methylation only when the full epigenetic landscape of the genome has changed.

Recently, AID has been shown to interact with members of the RNA pol II elongation

complex and chromatin remodeling factors (95, 122; K.L. Willmann, S. Milosevic, S. Pauklin, K.-M. Schmitz, G. Rangam, M.T. Simon, S. Maslen, M. Skehel, I. Robert, V. Heyer, E. Schiavo, B. Reina-San-Martin, S.K. Petersen-Mahrt, unpublished observation). Importantly, part of AID targeting was due to the presence of histone H3K4 trimethylation. It was shown that AID interacts with SPT5, which is needed to induce phosphorylation of ser5 on the CTD to restart RNA pol II after pausing. This serves as a platform for the AID-PAF complex (K.L. Willmann, S. Milosevic, S. Pauklin, K.-M. Schmitz, G. Rangam, M.T. Simon, S. Maslen, M. Skehel, I. Robert, V. Heyer, E. Schiavo, B. Reina-San-Martin, S.K. Petersen-Mahrt, unpublished observation), which in turn can promote histone H2B monoubiquitination. This ubiqutination allows for Set1/Set2-dependent trimethylation of H3K4 and H3K36. At the same time, the FACT (facilitates chromatin transcription) complex facilitates efficient RNA pol II elongation (9). FACT (SSRP1/SUPT16H) cooperates in chromatin remodeling as a histone chaperone—possibly via its ability to destabilize histone nucleosomes and to enhance cotranscriptional chromatin marks (115, 122). As the FACT complex also plays a key role in other chromatin modification pathways (9), AID may be recruited for DNA demethylation outside of transcription. Furthermore, recent data indicate that the evolutionarily conserved FACT complex plays a direct role in DEMETER-induced DNA demethylation (47), identifying an orthologous pathway between plants and mammals.

Just as AID has been linked with the chromatin modifying FACT complex, so has TDG been shown to interact with the histone acetyltransferase (HAT) CBP/p300 (130). Because of the possible duality in function, it was suggested that a TDG knockout would exhibit a phenotype combining altered DNA demethylation and histone modification, especially with regards to expression of bivalent modified genes (21).

Chromatin status is not only important for targeting the TMD or TLD factors to 5mCpG and initiating demethylation, but also for determining the efficiency and possibly choice of the downstream processing machinery. Recently, it has been shown that nucleosome modification and remodeling can alter the activity of BER (77, 87). DNA pol β and APE are inhibited by the presence of nucleosomes (77, 87), whereas UNG is unaffected (87). Importantly, the SWI/SNF remodelers could reactivate the BER pathway but were dispensable if an H2A.Bbd variant was used (77).

Clearly, even from these few examples, the inter- and intraplay among pathways leading to DNA methylation, nucleosome modification, and DNA demethylation provides a rich source of regulatory interactions as well as potential drug targets in diseases.

Demethylation and Mutation

The effect of loss of DNA methylation in cancer has been extensively documented and the reader is kindly asked to consult comprehensive reviews for more details (e.g., 124). We would like to highlight the following important aspects. Spontaneous DNA damage, including deamination of cytosine, is a major source of mutations in cancer (68). In dsDNA, 5mC has a 2.2-fold higher spontaneous deamination rate than cytosine (117), yet this small difference cannot explain the observed number of oncogenic mutations that occur at CpGs, which are estimated to be more than 30% of all point mutations. It is therefore likely that active direct or indirect DNA demethylation (physiological or pathological) contributes to the development of tumorgenic mutations.

Furthermore, pharmacological agents that induce DNA demethylation, leading to reactivation of tumor suppressor genes, have received a lot of attention, have moved through the clinical trial phases, and are becoming important cancer-therapy drugs. Currently, this approach has centered on inactivation of remethylation (e.g., 5-aza-cytidine), but our evolving understanding of how proteins and enzymes enhance DNA demethylation will provide more direct, proliferation-independent drug targets.

CONCLUSION

DNA demethylation has become an integral focus of epigenetic research. Long lagging behind methylation and the methyltransferases, demethylation and the various enzymes involved are now gaining attention and will provide new approaches for understanding genes, cells, health, and disease. It will be interesting to see the details of each DNA demethylation pathway and how those pathways interact. Future work will delineate how classical DNA repair pathways are used or hijacked by the DNA demethylation machinery. This would be analogous to how the AID-induced immune diversification machinery has altered DNA repair efficiency while relying on the function of DNA repair proteins.

We are just at the beginning of understanding the multitude of DNA demethylation pathways and their physiological consequences. As alluded to above, for each purpose a physiological balance is in the making. As shown in **Figure 5**, 5mCpG demethylation has to be wary of efficiency, efficacy, persistence, and mutability, as well as whether it is global or local and whether it is static or dynamic. At times, those aspects are mutually exclusive, although there may also be necessary evils when associating one with the other (i.e., increased mutability). Importantly, future work will provide insight into molecular details of each DNA demethylation pathway as well as provide us with the understanding of what the connecting factors are and of general principles that govern DNA methylation/demethylation dynamics. Understanding DNA demethylation pathways will serve not only the epigenetics field but will also provide new ideas to the DNA repair community.

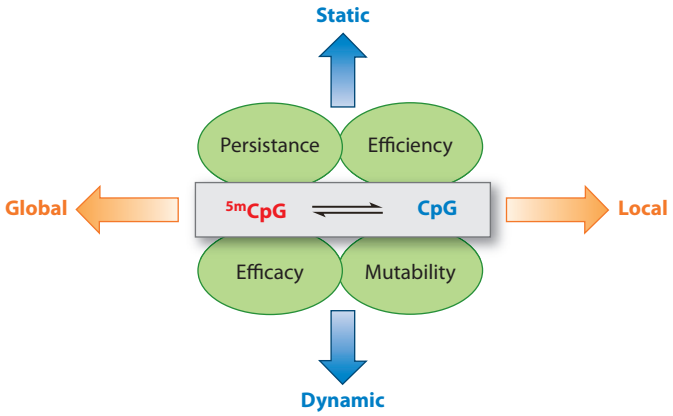

Figure 5

Dynamics of 5mC. Factors influencing the steady-state levels of DNA methylation and demethylation. Steady-state methylation patterns are a balance between methylation and targeted demethylation, and can range from almost static to highly dynamic. The interconversion of 5mC and cytosine can be restricted to a specific genetic locus or be genome wide. Achieving this balance of methylated and unmethylated marks will be defined by the persistence, efficiency, and efficacy of the various pathways, and how they avoid mutability.

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