ANNUAL REVIEWS

Annual Review of Biochemistry

Role of Mammalian DNA Methyltransferases in Development

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Annu. Rev. Biochem. 2020. 89:135–58

First published as a Review in Advance on December 9, 2019

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

https://doi.org/10.1146/annurev-biochem-103019-102815

Keywords

DNA methylation, DNMT, DNA methyltransferase, de novo methylation, DNA demethylation, mammalian development, 5mC, 5-methylcytosine

Abstract

DNA methylation at the 5-position of cytosine (5mC) plays vital roles in mammalian development. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), and the two DNMT families, DNMT3 and DNMT1, are responsible for methylation establishment and maintenance, respectively. Since their discovery, biochemical and structural studies have revealed the key mechanisms underlying how DNMTs catalyze de novo and maintenance DNA methylation. In particular, recent development of low-input genomic and epigenomic technologies has deepened our understanding of DNA methylation regulation in germ lines and early stage embryos. In this review, we first describe the methylation machinery including the DNMTs and their essential cofactors. We then discuss how DNMTs are recruited to or excluded from certain genomic elements. Lastly, we summarize recent understanding of the regulation of DNA methylation dynamics in mammalian germ lines and early embryos with a focus on both mice and humans.

Contents

INTRODUCTION

Development of a multicellular organism from a zygote relies on spatial and temporal regulation of tissue- and cell type-specific transcription programs. Although cell differentiation is largely directed by lineage-specific transcription factors, maintenance of the committed cell fates involves heritable epigenetic modifications. DNA methylation is one of the best characterized epigenetic modifications in which a methyl ($-CH_3$) group is covalently linked to the 5-carbon of the pyrimidine ring of cytosine. DNA methylation is highly conserved across different species and can be found in vertebrates, flowering plants, some fungi, and bacteria (1). As an epigenetic silencing mechanism of transposable elements (TEs), DNA methylation is vital for maintaining genome stability in eukaryotes. In mammals, DNA methylation has evolved additional functions beyond silencing TEs, including lineage specification, genomic imprinting, and X chromosome inactivation (2).

DNA methylation is catalyzed by a group of proteins known as DNA methyltransferases (DNMTs) (3). Addition of methyl groups to unmethylated cytosines is called de novo methylation and is catalyzed by DNMT3 family enzymes including DNMT3A, DNMT3B, DNMT3C, and DNMT3L (3–5). DNMT3A and DNMT3B are the major enzymes to establish DNA methylation in embryonic development (6). DNMT3L, although catalytically inactive, serves as an essential accessory protein for de novo methylation in germ lines (7–9). DNMT3C, a tandem duplicate of DNMT3B, is only found in rodents and is responsible for DNA methylation of a subset of repetitive elements in the male germ line (4, 5). After establishing DNA methylation,

the methylation pattern is maintained during DNA replication by the maintenance DNA methyltransferase DNMT1 (10). The faithful copy of DNA methylation pattern during cell divisions makes it an ideal mechanism to preserve epigenetic memory.

DNA methylation in mammals mostly takes places in palindromic CpG dinucleotides. Non-CpG methylation (e.g., CpA and CpT) is rare but can be found in cells with high de novo methylation activity such as oocytes, embryonic stem cells (ESCs), and brain tissues (2). De novo DNMTs are thought to be responsible for the small percentage of non-CpG methylation, which cannot be maintained by DNMT1 owing to the lack of sequence symmetry (2). In most human somatic cells, 60–80% of the ~28 million CpGs are methylated. The methylated CpGs are not randomly distributed but rather are enriched at certain genomic regions such as repetitive elements and gene bodies. In contrast, regions with high-density CpG dinucleotides, known as CpG islands (CGIs), are commonly located at promoters of developmental genes and are generally unmethylated (11). As none of the DNMTs has sequence specificity, how they are targeted to specific genomic regions to establish a developmental-specific DNA methylation pattern has been one of the central questions in the epigenetic field. Recent studies have unveiled some of the underlying mechanisms.

Once established in embryos, global DNA methylation is mostly static throughout life (12). However, two waves of genome-wide DNA demethylation and remethylation take place in germ lines and preimplantation embryos (13). In the germ line, DNA methylation is almost completely erased in early germ cells and then reestablished before mature gametes are formed. In early embryos, the genome also undergoes a global demethylation after fertilization, except for imprinted genes and certain repeats, and then gets remethylated after implantation (13). The development of low-input technologies charting histone modifications and base-resolution DNA methylation has unveiled the hidden rules of epigenetic reprogramming during these developmental windows.

DNMTs in nonmammalian species such as plants and fungi harbor extensive evolutionary differences from mammals and utilize distinct regulatory mechanisms for DNA methylation. For example, de novo methylation in plants is mediated by an RNA-directed DNA methylation pathway involving small interfering RNAs that is not conserved in mammals (14). Moreover, DNMT5 has been shown to be critical in mediating CpG methylation in some fungi for which no orthologous DNMT1 has been identified (15). As this review is mainly focused on mammalian DNMTs, the regulation of DNMTs in plants and fungi is not covered. The intrigued readers may refer to other recent excellent reviews on this topic (14, 16). In this review, we first describe the DNMTs and their cofactors essential for methylation reaction. The structures, protein domain organizations, and biochemical mechanisms of DNMTs are also discussed in this section. We then introduce a few established rules regarding how DNMTs are recruited to or excluded from certain genetic elements. Lastly, we explore the DNA methylation dynamics in germ lines and early embryos with a focus on the most recent advances in both mice and humans.

THE DE NOVO METHYLATION MACHINERY

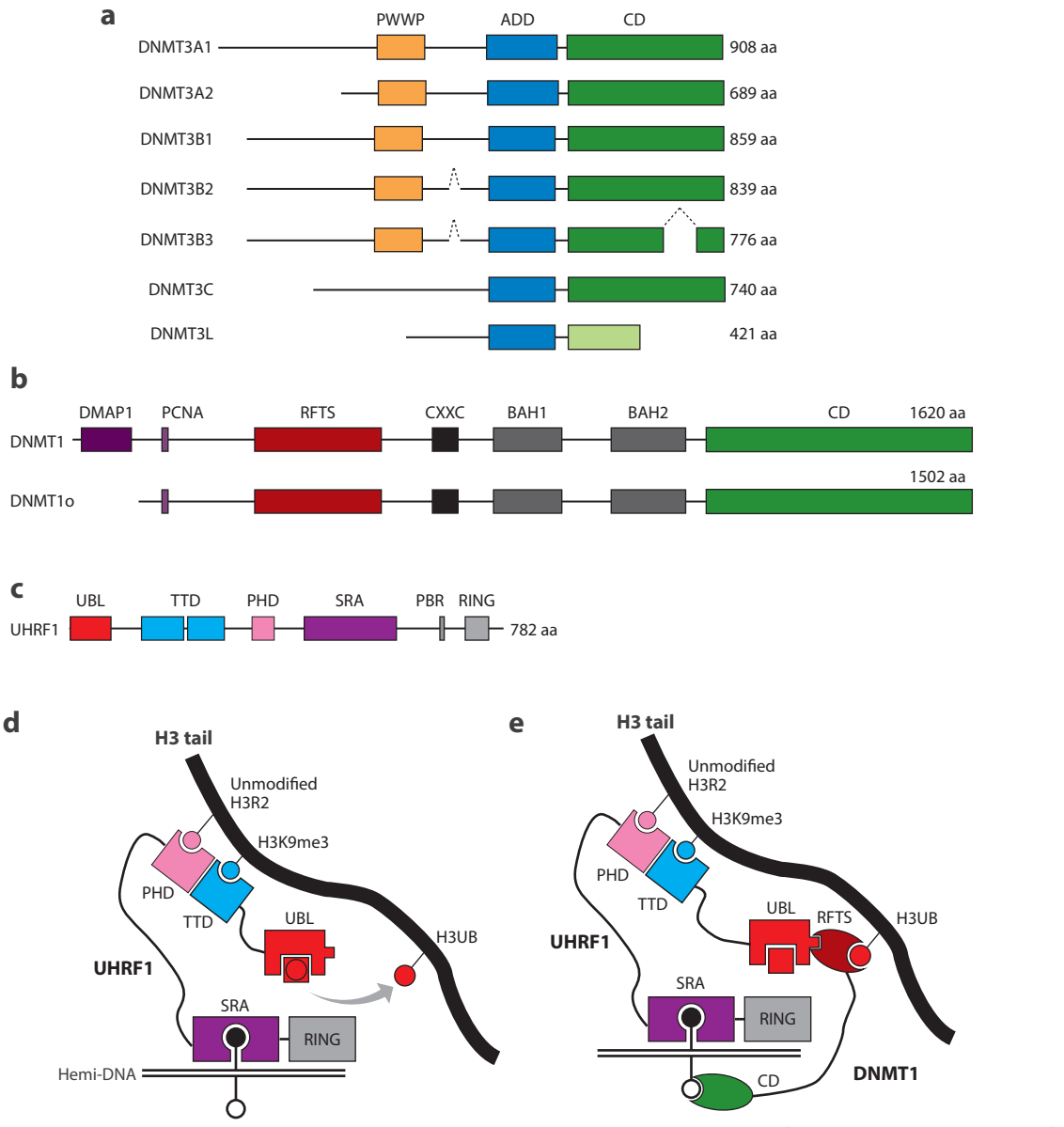
DNMT3 Domain Organizations and Their Functions

Of the four de novo DNMTs in mammals, DNMT3A and DNMT3B share similar domain structures, containing a Pro-Trp-Trp-Pro (PWWP) domain, an ATRX-DNMT3L-DNMT3A (ADD) domain, and a C-terminal catalytic domain (1) (**Figure 1*a***). Compared with DNMT3A/3B, DNMT3L does not have the PWWP domain and is catalytically inactive owing to a deletion from the conserved C-terminal methyltransferase motifs. The recently discovered DNMT3C shares the ADD and the catalytic domain but lacks the PWWP domain (4, 5).

The ADD domain has been reported to recognize unmethylated lysine 4 of histone 3 (H3K4) (17), which reveals a mechanism of histone-guided establishment of DNA methylation. The ADD

domain has also been shown to contribute to an autoinhibitory effect of DNMT3A (18). In the absence of an H3 tail, the ADD domain inhibits DNMT3A enzymatic activity by reducing DNA binding affinity of the catalytic domain. However, the H3 tail without methylation at lysine 4 can disrupt the interaction between the ADD and catalytic domain to release autoinhibition of DNMT3A (18). The structural evidence is consistent with the mutually exclusive distribution of H3K4 methylation and DNA methylation throughout the genomes (2).

The PWWP domain can recognize H3K36me3, a histone modification present in the gene body of actively transcribed genes (19, 20). Consistently, DNMT3B has been implicated in gene



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Domain organization of DNA methyltransferases (DNMTs) and the mechanisms of DNMT1/UHRF1–mediated methylation maintenance. (*a*) Domain structure of mouse de novo DNMTs. The catalytic domain (CD) confers the DNMT activity. Pro-Trp-Trp-Pro (PWWP) and ATRX-DNMT3L-DNMT3A (ADD) domains mediate histone-guided DNA methylation by recognizing H3K36me3 and unmethylated H3K4, respectively (17, 19, 20). For DNMT3A and DNMT3B, multiple isoforms including the catalytically inactive ones (e.g., DNMT3B3) are produced by alternative promoter usage and/or splicing (30). Note that not all DNMT3B isoforms are included here. (*b*) Domain structure of mouse DNMT1 and its oocyte-specific isoform DNMT1o. The DNMT1-associated protein (DMAP1) binding domain interacts with transcriptional repressor DMAP1 and histone deacetylase 2 (HDAC2) (32). The proliferating cell nuclear antigen (PCNA) binding domain is known to target DNMT1 to the replication foci (33). The replication foci–targeting sequence (RFTS) domain can occupy the catalytic pocket of DNMT1, acting as an inhibitor of the methyltransferase activity (37). After recruited to chromatin, RFTS binds to the ubiquitinated H3 tail (35, 36). The CXXC domain binds to unmethylated CpG to exclude it from the active site of the catalytic domain (38). Bromo-adjacent homology (BAH1/2) domains are essential for localization of DNMT1 at the replication foci (39). (*c–e*) Domain organization of mouse UHRF1 (also known as NP95) and the mechanisms of DNMT1/UHRF1–mediated methylation maintenance. The ubiquitin-like (UBL) domain recruits ubiquitin-conjugating enzymes (E2) to UHRF1 and targets ubiquitin to (*d*) the H3 tail (146, 147). UBL also directly interacts with the DNMT1 RFTS domain. (*d*,*e*) The tandem tudor domain (TTD) binds to H3K9me2/3 to facilitate UHRF1 chromatin localization (47). The TTD domain may also recognize the methylated DNA ligase 1 (LIG1) (148) (not shown in panels *d* and *e* for simplicity). Plant homeodomain (PHD) binds to unmodified H3R2 to facilitate chromatin localization of UHRF1 (46). The SET- and RING-associated (SRA) domain binds to hemi-methylated DNA (hemi-DNA) (43–45). The really interesting new gene (RING) domain deposits H3K14Ub, H3K18Ub, and H3K23Ub (denoted by H3UB), which can serve as docking sites for (*e*) the DNMT1 RFTS domain (34, 36). The poly-basic region (PBR) may associate with the linker between TTD domains in the absence of hemi-DNA, leading to a closed conformation of UHRF1 (not shown in panels *d* and *e* for simplicity) (149).

body DNA methylation, and depletion of the H3K36 methyltransferase SETD2 in mouse embryonic stem cells (mESCs) leads to reduced DNMT3B enrichment and DNA methylation levels at genic regions (21). Interestingly, recent genetics studies revealed that D329A or W326R point mutation of PWWP domain causes gain of function of DNMT3A and leads to hypermethylation of polycomb-associated DNA methylation valleys (22, 23). These results revealed a previously unrecognized role of the PWWP domain in restricting DNA methylation. Anchoring of DNMT3 to H3K36me3-enriched regions by the PWWP domain likely can avoid accidental methylation caused by extra freely available DNMT3 proteins.

Structural Basis of De Novo DNMT Complexes

Despite being catalytically inactive, DNMT3L serves as a cofactor of DNMT3A/3B to enhance their methyltransferase activities (24). The crystallographic structure revealed that DNMT3L and DNMT3A form a butterfly-shaped tetramer (DNMT3L-DNMT3A-DNMT3A-DNMT3L), which comprises two DNMT3L-DNMT3A interfaces and one DNMT3A-DNMT3A interface (25, 26). Although only the DNMT3A dimer directly binds to DNA, disruption of the interaction between DNMT3A and DNMT3L abolishes DNMT3A methyltransferase activity, which may be due to the loss of active DNMT3A conformation (25). DNMT3L has also been reported to facilitate DNA methylation by maintaining DNMT3A stability in mESCs (27).

As revealed by crystal structure, the DNMT3A homodimer in the tetramer complex targets two CpG sites on a DNA duplex in one binding event (25, 26). However, different base pair spacings (10 versus 14 bp) between the two CpG sites have been reported, and such discrepancy could be due to the intrinsic dynamic conformation of DNMT3A or different experimental conditions (25, 26). Of the DNMT3A residues that directly interact with DNA, Arg836 is of particular interest as it confers the preference of DNMT3A to methylate CpG versus CpA or CpT (26). Wild-type DNMT3A exhibits a 20-fold higher preference to methylate CpG-containing DNA than CpA- or CpT-containing DNA. In contrast, the DNMT3A R836A mutation can enhance the methylation of CpA and CpT by 4- to 5-fold while only slightly affecting CpG methylation (26).

Different Isoforms of De Novo DNMTs

Alternative promoter usage and splicing can give rise to different protein isoforms. In the case of DNMT3A, two isoforms are transcribed from two alternative promoters, resulting in a longer isoform, DNMT3A1, and a short isoform, DNMT3A2 (**Figure 1*a***). Analyses of the promoter usage indicate that DNMT3A2 is mainly expressed in fetal tissues, whereas DNMT3A1 can be detected at both fetal and adult stages (28). Compared with DNMT3A2, DNMT3A1 is more enriched at DNA methylation canyon edges and has been shown to compete with TET1 to regulate the expression of H3K27me3- and H3K4me3-containing bivalent genes in mESCs (28, 29). For DNMT3B, several isoforms that include the canonical full-length DNMT3B1, N-terminal truncated isoforms, and catalytically inactive isoforms have been reported (30) (**Figure 1*a***). The function of the catalytically inactive DNMT3B isoforms is largely unknown. Nevertheless, it has been reported that the inactive variant DNMT3B3 in humans is the major form of DNMT3B in differentiated ESCs (31) and may serve as an accessory protein like DNMT3L to stimulate gene body methylation in somatic cells (30).

THE MAINTENANCE METHYLATION MACHINERY

DNMT1 Domain Organizations and Their Functions

Although the C-terminal catalytic domain of DNMT1 is conserved with other DNMTs, its N-terminal regulatory region is unique and specifically required for methylation maintenance during DNA replication (3). The N-terminal regulatory region consists of a DNMT1-associated protein 1 (DMAP1) binding domain, a proliferating cell nuclear antigen (PCNA) binding domain, a nuclear localization signal (NLS), a replication foci–targeting sequence (RFTS) domain, a CXXC domain, and two bromo-adjacent homology (BAH) domains (3) (**Figure 1*b***). Of these domains, the DMAP1 binding domain is involved in interactions with the transcriptional repressor DMAP1 and histone deacetylase 2 (HDAC2) (32). The PCNA binding domain and RFTS are known to be involved in targeting DNMT1 to the replication foci during S phase (33–36). RFTS can also contribute to the autoinhibitory effect of DNMT1 by occupying the active site of the catalytic domain (37). Such autoinhibition mechanisms may help avoid aberrant de novo DNA methylation. The CXXC domain has been shown to bind and occlude unmethylated CpGs from the active site, limiting methylation only for hemi-methylated CpGs (38). Recently, BAH domains have also been implicated in localizing DNMT1 to the replication loci, but the underlying mechanism remains unclear (39).

UHRF1: A Cofactor Required for DNA Methylation Maintenance

DNMT1 cooperates with the E3 ubiquitin ligase UHRF1 (also known as NP95) to maintain DNA methylation in dividing cells (40, 41). UHRF1 is a modular multidomain protein containing a ubiquitin-like (UBL) domain, a tandem tudor domain (TTD), a plant homeodomain (PHD), a SET- and RING-associated (SRA) domain, a poly-basic region (PBR), and a really interesting new gene (RING) domain (42) (**Figure 1*c***). UHRF1 recognizes hemi-methylated DNA by the SRA domain and then recruits DNMT1 to the replication foci during S phase (43–45). UHRF1-deficient cells exhibit diffused nucleoplasmic localization of DNMT1 and undergo global loss of DNA methylation (40, 41). Similar to *Dnmt1* knockout (KO), *Uhrf1* null mouse embryos die shortly after gastrulation (40).

Structural Basis of UHRF1-Mediated DNMT1 Recruitment to Replication Foci

UHRF1-mediated recruitment of DNMT1 to the replication foci involves complex inter- and intramolecular interactions. In a simplified model, recruitment of UHRF1 to chromatin depends

on the binding of the SRA domain to hemi-methylated DNA as well as the cooperative binding of PHD/TTD domains to H3R2 and H3K9me2/3 (46, 47). Notably, binding of SRA domain to hemi-methylated DNA allosterically stimulates the E3 ligase activity of the RING domain to deposit mono-ubiquitin to K14, K18, and K23 on Histone 3 (34, 36) (**Figure 1*d***). The mono-ubiquitinated H3 tail then serves as a docking site for the RFTS domain of DNMT1, which consequently alleviates the autoinhibitory effect of DNMT1 by directing the RFTS domain away from the active site of the catalytic domain (35) (**Figure 1*e***). Moreover, it has been shown that UHRF1 can directly recruit DNMT1 through the UBL domain (48), adding another layer of complexity to how UHRF1 recruits DNMT1 to chromatin.

The role of USP7, a ubiquitin carboxyl-terminal hydrolase, in the methylation maintenance complex remains controversial. It has been reported that USP7 either helps stabilize DNMT1/UHRF1 or promotes UHRF1 into an open conformation (42). However, a recent study revealed that ablation of USP7 affects neither the steady-state DNMT1 level nor global DNA methylation, indicating that USP7 or its interactions with DNMT1 does not play a major role in methylation maintenance (49). The discrepancies might be due to the different cell lines or different USP7 ablation strategies used in these studies. Therefore, the role of USP7 in regulating DNMT1 and DNA methylation maintenance warrants further investigation.

Oocyte-Specific Isoform of DNMT1

DNMT1 has two isoforms derived from two alternative promoters (**Figure 1*b***). The somatic isoform is highly expressed in somatic cells but lowly expressed in oocytes and early embryos. In contrast, the oocyte-specific form (DNMT1o), which lacks 118 amino acids at the N terminus, is only present in oocytes and early embryos (50). DNMT1o is essential for maintaining parental-allele-specific DNA methylation at imprinted loci (50). Embryos derived from DNMT1o-deficient oocytes, in which the somatic DNMT1 is unaffected, exhibit partial loss of imprinting and mostly die before birth (50). Notably, embryos deficient of both isoforms of DNMT1 exhibit more complete loss-of-imprinting at the blastocyst stage (51). Therefore, the zygotically expressed somatic DNMT1 should also be involved in the maintenance of genomic imprints in preimplantation embryos despite its low abundance at the early developmental stages (51).

TARGETING OF DNMTs TO DISTINCT GENOMIC FEATURES

Exclusion of DNMTs from CpG Islands

Although most CpGs in the mammalian genome are methylated, CGIs at housekeeping and developmental gene promoters remain unmethylated regardless of the transcriptional status (11). There must be mechanisms to actively exclude DNMTs from the CGIs, as these regions maintain a hypomethylated state even when global de novo methylation takes place. In particular, some CGI promoters can extend beyond the proximal promoters to form a large hypomethylated domain known as the DNA methylation valley or canyon (52). Early studies suggested that sequence characteristics of the CGIs such as transcription factor binding motifs may explain their resistance to DNA methylation (53, 54). A survey of the DNA methylation valleys also revealed that they harbor binding motifs of key developmental transcriptional factors and these binding motifs are largely conserved across vertebrates (55). Recent genome-wide analyses revealed that the hypomethylated state is more likely a consequence of the interplay among CGI binding proteins, DNA methylation and demethylation machineries, and histone modifications (**Figure 2*a***).

CGI binding proteins harbor a CXXC domain that specifically recognizes and binds to unmethylated CpG dinucleotides. Of the CGI binding proteins characterized, FBXL10 (also known

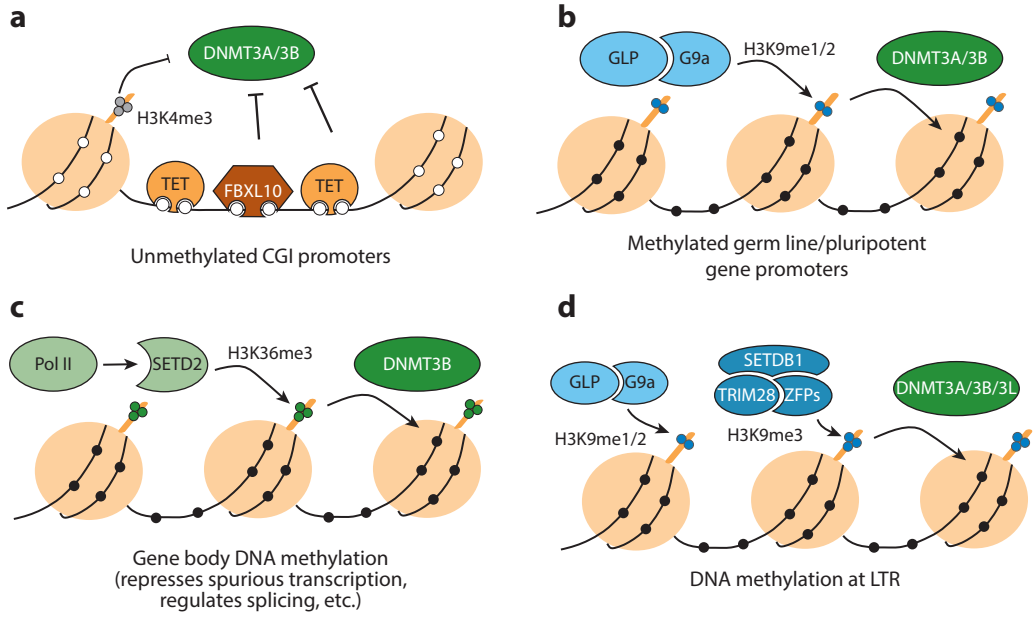


Figure 2

Targeting of DNA methyltransferases (DNMTs) to specific genomic features. (*a*) Exclusion of DNMTs from CpG island (CGI) promoters. Active chromatin mark H3K4me3 and CXXC protein FBXL10 are important to protect transcription start site (TSS)–associated CGIs from DNA methylation (17, 56). DNA demethylation mechanisms including TET binding to unmethylated CpGs also prevent de novo DNA methylation (29, 62–64). (*b*). Recruitment of DNMTs to germ line/pluripotent gene promoters. The G9a–GLP complex–mediated heterochromatin environment at gene promoters promotes de novo DNA methylation, which in turn reinforces transcriptional silencing (70–74). (*c*) Recruitment of DNMT3B to gene bodies. At transcribed gene bodies, RNA polymerase II (Pol II) associates with SETD2 to deposit H3K36me3 (19, 21). H3K36me3 is recognized by the Pro-Trp-Trp-Pro (PWWP) domain of DNMT3B to establish gene body DNA methylation (19, 21). Gene body DNA methylation may prevent spurious intragenic transcription and regulate alternative splicing (76, 77, 79, 80). (*d*) Recruitment of DNMTs to long terminal repeat (LTR) retrotransposons. Zinc finger protein ZFP809 recruits the TRIM28–SETDB1–H3K9me3 corepression complex and DNMTs to endogenous retroviruses (ERVs) for epigenetic silencing (83). In addition, the G9a–GLP complex contributes to de novo DNA methylation at ERVs, but the complex is not required to maintain repression of ERVs (84).

as KDM2B and CXXC2) binds to almost all CGI promoters and has been demonstrated to prevent a subset of CGI promoters from DNA methylation (56). In FBXL10-deficient mESCs, CGI promoters bound by both FBXL10 and polycomb repressive complexes (PRCs) undergo de novo methylation, whereas the FBXL10-bound CGI promoters without binding of PRCs still remain unmethylated (56). The likely explanation for why PRC-free CGI promoters are resistant to DNA methylation is that these regions are also enriched for RNA polymerase II (Pol II) and active chromatin modifications such as H3K4me3 that can antagonize DNMTs. In agreement with this notion, ectopic expression of DNMT3B in cell lines tends to methylate the regions that are lowly expressed and enriched for H3K27me3, whereas CGIs enriched for H3K4me3 are protected from aberrant DNA methylation (57). Moreover, polycomb targets are more likely to undergo de novo DNA methylation during mESC differentiation (58). It should be noted that not all CXXC domain–containing proteins have a role similar to that of FBXL10 in safeguarding CGIs from de novo methylation. Depletion of CFP1 (also known as CXXC1), another CXXC domain–containing protein that binds to CGIs, actually leads to loss of genomic DNA methylation (59).

Among the factors that bind to CGIs, TET proteins are known for their roles in DNA demethylation by oxidizing 5-methylcytosine (5mC) (60). TET1 contains a CXXC domain and is enriched at CpG-dense regions in mESCs (61). TET1 binding at the transcription start sites (TSS) is anticorrelated with DNMT3A enrichment, and depletion of TET1 leads to increased Dnmt3a occupancy and de novo DNA methylation at the TSS (29). However, depletion of all three TET proteins in mESCs only slightly affect the global 5mC level (62). Ectopic DNA methylation in the *Tet*1/2/3 triple knockout (TKO) mESCs largely occurs at enhancers and is able to modulate the transcription activity of the associated genes (62). In a *Tet*1/2/3 TKO mouse model, it was reported that TET-mediated demethylation counteracted de novo methylation and regulated gastrulation by modulating Nodal signaling (63).

In human *TET*1/2/3 TKO ESCs, regulatory regions such as promoters and enhancers gain DNA methylation without showing global 5mC differences (64). Interestingly, bivalent promoters exhibit the greatest magnitude of methylation increase in the human *TET*1/2/3 TKO ESCs (64). The silencing of a bivalent developmental gene *PAX6* by DNA hypermethylation may contribute to the impaired neuronal differentiation of the *TET* TKO ESCs, as locus-specific demethylation of *PAX6* can partially rescue the defects in differentiation to the neuroectoderm (64). Thus, despite the finding that TET proteins are not essential for self-renewal and pluripotent marker gene expression in ESCs, their competitive balance against de novo DNMTs is required to ensure the correct transcription program of ESCs during differentiation. In addition to the above-described epigenetic modifiers, formation of an R loop downstream of the CGI TSS has also been proposed to prevent de novo DNA methylation (65). Mechanistically, an R loop has been shown to trigger local DNA demethylation by recruiting the DNA demethylation machinery GADD45A and TET1 (66). Consistently, *Gadd45a/b/g* TKO mESCs exhibit locus-specific DNA hypermethylation (67).

Recruitment of DNMTs to Gene Promoters

Despite the intrinsic property of CGIs to repel DNA methylation, a small number of CGIs at the TSS still undergo de novo DNA methylation during postimplantation development, and most of these CGIs are located at germ-line gene promoters (68, 69). DNA methylation at germ-line gene promoters is repressive to transcription because reactivation of transcription in the germ line has been observed in embryos deficient for DNA methylation (69, 70).

How are germ-line gene CGI–containing promoters targeted by de novo DNMTs in differentiation or development? It has been suggested that G9a, a histone methyltransferase that deposits H3K9me1/2, may silence germ-line genes with DNA methylation in developing embryos (70) (**Figure 2*b***). In mouse *G9a* null embryos, de novo DNA methylation at germ-line gene promoters is impaired along with a decrease in H3K9me2 occupancy (70). However, H3K9me2 is unaffected at the gene promoters of *Dnmt3b* KO embryos, in which loss of DNA methylation correlates with gene reactivation (70). These data suggest that epigenetic silencing of these target genes is initiated by G9a/H3K9me2 and then taken over by DNA methylation. Moreover, targeting of G9a to these germ-line gene promoters can be linked to transcription factors such as E2F6, which is found to be in a complex containing G9a that represses germ-line gene expression by modulating DNA methylation (71).

Similar to postimplantation embryo development, mESC differentiation also couples with de novo DNA methylation at germ-line gene CGI promoters as well as pluripotent genes such as *Oct4* and *Dppa3* (68). Analogous to the findings in embryos, G9a-mediated H3K9 methylation and heterochromatin protein 1 (HP1) binding precedes DNA methylation to silence pluripotent genes during ESC differentiation (72). In addition, it has been shown that recruitment of DNMTs may

be independent of the enzymatic activity of G9a (73, 74). This might explain why some regions show loss of H3K9me2, but not DNA methylation, in G9a null embryos (70).

In addition to the epigenetic silencing of germ-line and pluripotent genes by DNA methylation in somatic tissues, extraembryonic lineage genes can be hypermethylated and transcriptionally repressed in the embryonic lineage. For example, visceral endoderm (VE) genes including apolipoprotein family *Apoa1/a4/b/c2* are hypermethylated in E6.5 epiblast but not in the VE (75). Moreover, a group of somatic tissue-specific genes related to signaling and extracellular matrix acquire DNA methylation when mESCs are differentiated into neurons (58). Therefore, promoter DNA methylation–mediated transcriptional silencing can orchestrate lineage specification during development.

Recruitment of DNMTs to Gene Bodies

In contrast to the CGIs at promoters, orphan CGIs located at intragenic regions are more frequently methylated (11). Methylation of intragenic CGIs is likely a consequence of DNMT3B-mediated genic DNA methylation. At an actively transcribed locus, elongating RNA Pol II can associate with the histone methyltransferase SETD2 to deposit H3K36me3, which in turn can be recognized by the PWWP domain of DNMT3B to establish gene body DNA methylation (19, 21) (**Figure 2*c***). Therefore, unlike DNA methylation at promoters, which is generally associated with transcriptional repression, gene body DNA methylation can be an indicator of active gene transcription.

What is the function of gene body DNA methylation? It was first proposed that intragenic CGIs could serve as alternative TSS. This notion is based on the evidence that these CGIs are enriched for H3K4me3, a marker of active gene promoters, and that tissue-specific intragenic DNA methylation is associated with alternative transcripts in a tissue-specific manner (76). A recent genome-wide TSS mapping study revealed a significant increase of TSS on the gene bodies in *Dnmt3b* KO mESCs, suggesting that intragenic DNA methylation may prevent spurious transcription initiation (77). Notably, although most spurious transcripts are degraded, at least some of them can be translated to generate aberrant proteins (77). The spurious transcription initiation might contribute to the etiology of human immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome caused by *DNMT3B* mutations (78). Indeed, study of cell lines from human ICF syndrome patients revealed that altered intragenic DNA methylation impairs the usage of alternative promoters in gene bodies (79). Collectively, these studies demonstrate an important role of gene body DNA methylation in suppressing spurious transcription initiation.

In addition to preventing spurious transcription, intragenic DNA methylation has also been linked to alternative splicing. For example, it has been shown that intragenic DNA methylation–mediated binding of the Brother of Regulator of Imprinted Sites (BORIS) leads to cancer-specific alternative splicing of the *pyruvate kinase* (*PKM*) gene (80). In cancer cells, BORIS preferentially binds to the methylated exon 10 and promotes local Pol II enrichment and spliceosome assembly to include exon 10 in the final *PKM* transcript (80). However, in normal cells, BORIS is absent from the unmethylated exon 10, leading to the exclusion of this exon in the *PKM* transcript. Importantly, BORIS-mediated alternative splicing has been identified at multiple loci, and such intragenic DNA methylation–dependent alternative pre-mRNA splicing might be a hallmark of breast cancer (80).

Recruitment of DNMTs to Repetitive Elements

The most conserved function of DNA methylation across different species is to safeguard genome integrity by suppressing TEs. Most TEs are truncated or mutated, thus losing their ability to

transpose or retrotranspose. However, some full-length TEs are capable of retrotransposition and can be harmful to genome integrity if insufficiently silenced. The role of DNA methylation in repressing TEs was first demonstrated in *Dnmt1* mutant embryos, in which intracisternal A particle retroviruses (IAPs) were elevated more than 50-fold (81). Subsequently, numerous studies have indicated that DNA methylation is essential for silencing different types of TEs including endogenous retroviruses (ERVs), non-long terminal repeats (non-LTR) (e.g., Line1), and pericentromeric repeats (82).

How is repetitive element silencing achieved? Typically, H3K9me3 is first recruited to target sequences to establish heterochromatin environment, which in turn brings in DNA methylation for long-term silencing. In the case of ERVs, a zinc finger protein ZFP809 recruits the TRIM28–SETDB1–H3K9me3 corepressor complex and DNMTs to achieve transcriptional silencing (83) (**Figure 2*d***). In addition, G9a, a major H3K9me1/2 methyltransferase, is required for de novo DNA methylation and the establishment, but not the maintenance, of ERV repression (84). For non-LTR such as LINE1, SUV39H1/2 are the major enzymes for depositing H3K9me3 to its 5′UTR in mESCs, and the repressive mark is then taken over by DNA methylation during ESC differentiation (85). This observation is in line with the notion that TE silencing in early embryos and ESCs is more tolerant to deficiency in DNA methylation (86, 87). Consistently, DNA methylation plays a more predominant role in silencing of repeats in somatic cells.

DNA METHYLATION DYNAMICS IN DEVELOPMENT

DNA Demethylation in Primordial Germ Cells

The progenitors of primordial germ cells (PGCs) are specified around embryonic day 6.25 (E6.25) in mice, and the global DNA methylation level at this stage is comparable with the adjacent epiblast lineage (68). After PGC proliferation and migration to the genital ridge by E13.5, the 5mC is almost completely erased with the exception of certain repeat elements such as IAPs (88–90). This DNA methylation reprogramming process comprises two sequential steps. Before E9.5, DNA demethylation is largely achieved through replication-dependent passive dilution as both TET proteins and 5mC oxidation products such as 5hmC are at a very low level during this period (91, 92). Notably, certain regions including imprinted loci and meiotic gene promoters maintain DNA methylation during this stage (88, 89, 92, 93). From E9.5 to E13.5, TET1 is responsible for the increase of the 5hmC level, and then the 5mC oxidation products are diluted through DNA replication or restored to unmodified cytosine by the base excision repair (BER) pathway (90, 92–94). Such two-stage DNA demethylation has been suggested to safeguard the germ line from precocious differentiation (95).

Despite the involvement of TET proteins in DNA demethylation in the second stage, the global methylation level is only slightly affected in PGCs deficient for TET enzymes (93). Therefore, along with the downregulation of DNMT1 cofactor UHRF1 and DNMT3A/3B, the DNA replication–mediated passive dilution is a main contributor for the DNA demethylation in PGCs (96, 97). However, certain loci such as meiotic genes in female germ cells and imprinted genes in male germ cells show aberrant hypermethylation in TET1 mutants, suggesting an essential role of TET1-mediated oxidation at specific genomic elements (92, 93) (**Figure 3*a*,*b***).

Recently, using an integrative approach, Hill et al. (98) identified a set of germ-line reprogramming-responsive (GRR) genes in PGCs that are critical for gametogenesis. The GRR genes are initially repressed by 5mC and PRC1 at E9.5/E10.5 and then reactivated upon the removal of the two repressive epigenetic modifications at E13.5/E14.5. Interestingly, although TET1 is recruited to the GRR gene promoters, it is not the major driver of bulk DNA demethylation. Instead, TET1 may play a critical role to prevent subsequent aberrant de novo

DNA methylation at these loci. Thus, protection from de novo methylation should be important to reinforce the newly acquired blank epigenetic state of GRR genes (98).

Similar to mouse cells, human fetal germ cells also exhibit a genome-wide DNA demethylation upon PGC specification (99–101). Given that the DNA methylation machinery is largely disabled and the *TET1* expression level is increased in the PGCs (101), a comparable DNA demethylation mechanism may be involved in the human fetal germ line.

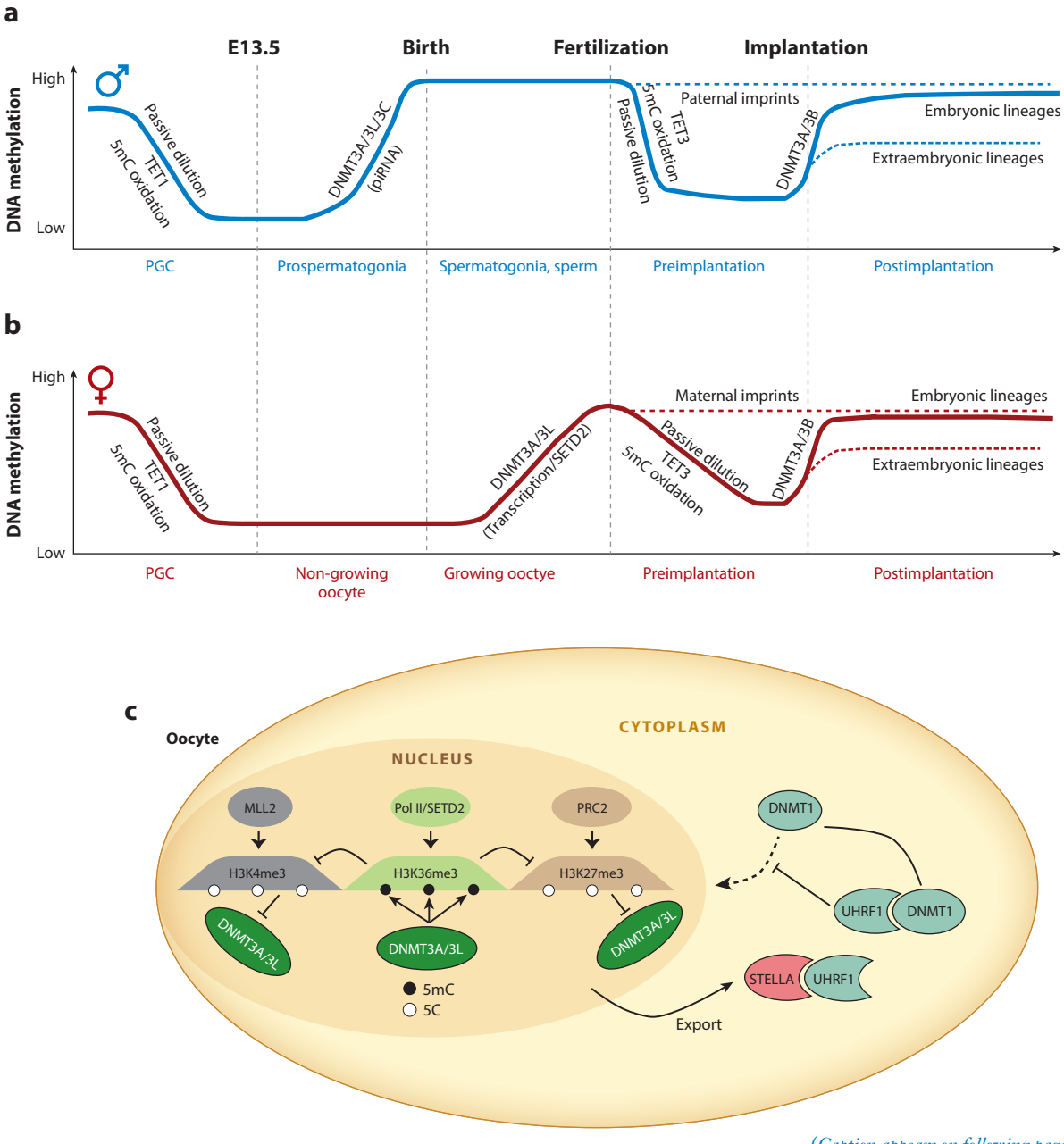


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Figure 3 (*Figure appears on preceding page*)

DNA methylation dynamics in mouse development and epigenetic modifiers that regulate the mouse oocyte epigenome. (*a*,*b*) DNA methylation dynamics in the germ line and embryos. Both male and female primordial germ cells (PGCs) undergo genome-wide DNA demethylation to reach a very low DNA methylation level at embryonic day 13.5 (E13.5) (88–90). The bulk demethylation is largely mediated by passive dilution with specific genomic regions depending on TET1-mediated oxidation (92–97). After E13.5, the male germ line is globally remethylated before birth, whereas the female germ line regains DNA methylation during oocyte growth after birth. DNMT3A and DNMT3L are essential for de novo DNA methylation in both germ lines (7–9, 102–104). In oogenesis, transcription-associated SETD2/H3K36me3 is responsible for the bulk oocyte DNA methylome (107, 111, 112). In spermatogenesis, the Piwi-interacting RNA (piRNA) pathway and DNMT3C contribute to DNA methylation at the young retrotransposons (4, 5, 119, 120). After fertilization, both genomes undergo passive dilution-mediated DNA demethylation, with the paternal genome undergoing more TET3-mediated 5mC oxidation (123–125). Parental-allele-specific DNA methylation at imprinted loci are preserved during this wave of global demethylation (104, 137). At implantation, remethylation of both parental alleles largely depends on DNMT3B, with less dependence on DNMT3A (6, 69). Notably, extraembryonic lineages are globally incompletely methylated and have a lower level of DNA methylation on average than the embryonic lineage (144). (*c*) Epigenetic modifiers involved in shaping the mouse oocyte epigenome. In full-grown oocytes, the genome is marked by consecutive broad domains of H3K4me3, H3K36me3, or H3K27me3 (109, 110, 112). Bulk H3K4me3 is deposited by MLL2 (150), whereas H3K27me3 establishment depends on PRC2. Both H3K4me3 and H3K27me3 are anticorrelated with DNA methylation in oogenesis (109, 110). In contrast, SETD2-deposited H3K36me3 is highly correlated with DNA methylation and contributes to the bulk oocyte DNA methylome (112). Moreover, H3K36me3 may be involved in H3K4me3 and H3K27me3 deposition in oogenesis (112). Loss of H3K36me3 in *Setd2* null oocytes leads to global redistribution of H3K4me3 and H3K27me3 including ectopic enrichment of H3K4me3/H3K27me3 at genomic regions that are normally enriched for H3K36me3 (112). Notably, DNMT1 is constantly exported to cytoplasm by STELLA and UHRF1 (129, 132). Aberrant nuclear localization of DNMT1 in STELLA-deficient oocytes leads to oocyte DNA hypermethylation (129, 130). Additional abbreviations: 5C, unmethylated cytosine; 5mC, 5-methylcytosine.

De Novo DNA Methylation in Oogenesis

Following the near complete erasure of genome-wide DNA methylation in PGCs, male and female germ cells undergo global remethylation in a sex-specific manner. Although de novo DNA methylation in male germ cells is fully established at birth, female germ cells acquire DNA methylation postnatally during oocyte growth. In addition to the different remethylation timing, mature sperm and oocytes also have very different methylomes. The sperm DNA are globally methylated with the exception of a few CGIs, whereas oocyte genomes consist of consecutive hyper- and hypomethylated domains (102).

DNMT3A and DNMT3L, but not DNMT3B, are responsible for de novo DNA methylation during oogenesis (102–104). DNMT3A or DNMT3L null oocytes fail to acquire methylation during oocyte growth and are not able to support embryonic development owing to defective maternal DNA methylation imprints (8, 9). The mechanism of how DNMT3A and DNMT3L target specific DNA sequences for methylation during oogenesis was first revealed by studying the establishment of maternal imprints. It was found that truncation of a transcript *Nesp* that is normally transcribed through the maternal-specific methylated region in oocytes disrupts de novo DNA methylation at this locus (105). Analysis of the oocyte transcriptome and DNA methylome further demonstrated that transcription correlates with DNA methylation and may account for the 85–90% of the oocyte methylome (106). Notably, it has been shown that species- and strain-specific LTR–initiated transcription in oocytes is associated with inter- and intraspecies divergent DNA methylation, respectively (107). These data support the notion that transcription-guided de novo DNA methylation occurs during oogenesis (**Figure 3*b***).

Then, how can transcription guide de novo DNA methylation? Transcription may create a chromatin environment that favors the recruitment of de novo DNA methylation machinery such as depositing H3K36me3 at transcribed regions (108). For example, transcription is responsible for the H3K36me3 enrichment at the *Zac1* imprinted locus (106). Ablation of transcription at this locus leads to reduced H3K36me3 and failure of de novo DNA methylation in oogenesis (106).

The recent technical advancement in low-input histone modification profiling (109–112) makes it possible to extend the study of imprinted loci throughout the genome and allows the study of the relationship between histone modifications and de novo DNA methylation during oogenesis. Consistent with the role of H3K4 methylation in repelling DNA methylation (17), noncanonical broad H3K4me3 domains in mouse oocytes are inversely correlated with DNA hypermethylation (109, 110). Furthermore, consistent with biochemical evidence that H3K36me3 promotes de novo DNA methylation, deposition of H3K36me3 in oocytes precedes DNA methylation and is highly correlated with DNA hypermethylation (107, 111, 112). Importantly, depletion of the primary H3K36me3 methyltransferase SETD2 in oocytes leads to loss of H3K36me3 and failure of global de novo DNA methylation (112) (**Figure 3*c***). Intriguingly, loss of H3K36me3 also causes ectopic DNA methylation at normally hypomethylated domains in oocytes (112). This observation is reminiscent of the aberrant DNA methylation of H3K27me3-enriched DNA methylation valleys by DNMT3A harboring mutations in the PWWP domain (22, 23). Together, these data suggest that gene body H3K36me3 can restrict DNMT3 enzymes from accidental methylation of nontranscribed regions.

Human oocytes exhibit a higher global DNA methylation level compared with that of mouse oocytes (~54% versus ~40%) (113). The distribution of 5mC in human oocytes follows a canonical bimodal pattern, and the methylated regions are also strongly enriched at the gene bodies (113). This observation suggests that transcription may also play a key role in recruiting de novo DNMTs in human oocytes. However, unlike in mice, DNMT3L may not be required for the human oocyte methylome because DNMT3L is not expressed in human oocytes (113, 114). DNMT3A alone might be sufficient to establish DNA methylation in the human female germ line.

De Novo DNA Methylation in Spermatogenesis

The de novo DNA methylation in the male germ line occurs by default at most genomic elements except the regions marked with H3K4me3 (115). Both DNMT3A and DNMT3L, but not DNMT3B, are required for de novo DNA methylation of the male germ line (7, 9). Male germ cells without DNMT3A or DNMT3L undergo meiotic catastrophe, which is associated with the derepression of retrotransposons including LINE-1 and IAP (7, 9). It has been suggested that activation of retrotransposons correlates with the recruitment of meiotic recombination machinery, which could cause impaired chromosome synapsis and finally lead to meiotic collapse (116).

In the male germ line, DNA methylation–mediated silencing of active retrotransposons also requires Piwi-interacting RNA (piRNA), a class of small (~26–30 bp) noncoding RNA (117). Depletion of the piRNA pathway mimics the meiotic catastrophe phenotype as observed in *Dnmt3l* KO male mice (116). piRNA can target retrotransposons cotranscriptionally by sequence complementarity to mediate de novo DNA methylation at the target regions (117). Although the underlying mechanism of how piRNA recruits DNMTs remains unclear, the piRNA pathway creates an H3K9me3-enriched heterochromatin environment at the target loci (118), which may facilitate establishment of DNA methylation. In addition, the recently discovered DNMT3C may be involved in piRNA-mediated de novo DNA methylation, as depletion of DNMT3C or the piRNA pathway leads to hypomethylation of the similar genomic elements including the young retrotransposons and the *Rasgrf1* imprinted locus (4, 5, 119, 120) (**Figure 3*a***).

Human sperm DNA is similarly methylated as that in mice (~75% versus 80%) (103, 114). However, the underlying de novo methylation mechanism may be different between these two species. Unlike in mice, sperm DNA methylome in humans may not require *DNMT3L*, as this gene is not expressed in the human male germ line (121). In addition, DNMT3C, the recently

discovered de novo DNMT essential for male mice fertility, is not present in human genome (5). Consistently, *RASGRF1* in humans does not contain a paternally methylated region like in mice (122), whereas DNMT3C is responsible for establishing the DNA methylation imprint at this locus during spermatogenesis (5).

Genome-Wide DNA Demethylation in Preimplantation Embryos

Following fertilization, genome-wide DNA demethylation takes place on both maternal and paternal genomes with different kinetics. Initially, immunostaining results suggested that the paternal genome is actively demethylated through TET3-mediated 5mC oxidation, whereas the maternal genome undergoes passive dilution by DNA replication (13). Subsequent bisulfite sequencing (BS-seq) analyses of one-cell embryos blocked for DNA replication indicated that DNA replication, but not TET3, is the major driving force for both maternal and paternal DNA demethylation (123, 124). Notably, TET3 also contributes to maternal demethylation, although the extent of TET3-mediated demethylation on the maternal genome is less pronounced compared with that of the paternal genome (123, 125) (**Figure 3*a*,*b***). Interestingly, evidence indicates that DNA demethylation can occur before DNA replication and TET3-mediated oxidation, suggesting that a TET3-independent demethylation mechanism may exist (126). Moreover, TET3 may also contribute to zygotic DNA demethylation by counteracting the de novo methylation activity of DNMT3A and DNMT1 (126).

Given that zygotic DNA demethylation is less robust in the maternal genome, how 5mC on the maternal genome is protected from TET3-mediated oxidation has attracted substantial attention. Earlier studies have suggested that maternally inherited STELLA (also known as PGC7 or DPPA3) may protect the maternal pronuclei (matPN) from 5mC oxidation in one-cell embryos (127, 128). In these studies, the 5mC signal detected by immunofluorescence is significantly reduced on the matPN of zygotes lacking STELLA. However, recent BS-seq and mass spectrometry–based assays revealed that matPN exhibit DNA hypermethylation in *Stella* maternal knockout (matKO) zygotes compared with the controls (129, 130). The abnormally high 5mC level in the matPN has been shown to be inherited from the *Stella* KO oocytes, which exhibit global DNA hypermethylation (~68% in *Stella* KO versus ~40% in wild type) as a result of de novo DNA methylation during oogenesis by ectopic nuclear Dnmt1 (129–131). Therefore, despite the finding that matPN undergo more dramatic demethylation in *Stella* matKO zygotes, global hypermethylation in the KO oocytes still causes higher levels of 5mC in matPN compared with that in wild-type embryos (129, 130).

The effect of STELLA on oocyte DNA methylation raises an intriguing question: How does STELLA control the cellular localization of DNMT1? It has been suggested that STELLA sequesters DNMT1 from the nucleus by nuclear export of its cofactor UHRF1 (129). With a mutated nuclear exporting domain, STELLA fails to restrict UHRF1 in the cytoplasm (**Figure 3*c***). Moreover, STELLA directly binds to the PHD domain of UHRF1, thus inhibiting the recruitment of UHRF1 to chromatin (132). In addition, although de novo methylation activity of DNMT1 was previously proposed (102, 131), strong in vivo evidence supporting this notion has been lacking. Thus, increased DNA methylation observed in *Stella* KO oocytes provides strong in vivo evidence of the ability of DNMT1 to target unmethylated DNA (129). Oocyte is a unique cell system, as it does not divide during oogenesis for weeks or months. The prolonged oocyte growth period may allow DNMT1-mediated gradual de novo methylation in *Stella* KO oocytes.

STELLA has also been reported to recognize the maternal-specific H3K9me2 to prevent 5mC oxidation (128). Ectopic removal of H3K9me2 in zygotes by overexpressing the H3K9me2 demethylase KDM3A resulted in loss of 5mC immunostaining signal on the matPN (128).

Consistently, embryos without maternal G9a, a H3K9me1/2 methyltransferase, exhibited significant loss of both H3K9me2 and 5mC on the matPN as revealed by immunofluorescence (133). However, whole-genome bisulfite sequencing (WGBS) revealed limited changes of global DNA methylation in *G9a* matKO embryos (134). Furthermore, low-input chromatin immunoprecipitation sequencing data indicated that H3K9me2 in oocytes is inversely correlated with DNA methylation and does not overlap with any imprinted loci for which the imprinting maintenance was previously reported to be dependent on maternal STELLA (127, 134). Thus, although G9a or H3K9me2 may be involved in preventing 5mC oxidation of the matPN, they are dispensable for protection against DNA demethylation in zygotes, as demethylation mainly occurs in a 5mC oxidation-independent manner.

In humans, global reprogramming of DNA methylation also takes place in the zygotic stage with similar active DNA demethylation taking place on the paternal allele (114, 135, 136). However, retention of the maternal methylation is much more pronounced in humans, which leads to higher average DNA methylation of the maternal genome throughout the preimplantation stages (136). Another unique feature of DNA methylation dynamics in human preimplantation development is the presence of two waves of de novo methylation at pronuclear and 4–8 cell stages, respectively (136). This feature is reminiscent of the reported de novo methylation activities of DNMT3A and DNMT1 at the one-cell stage in mice (126). However, the de novo methylated regions are generally demethylated again in the following stages (136), and whether this transient gain and loss of DNA methylation play any role in human development remains unclear.

Despite genome-wide DNA demethylation after fertilization, imprinted loci and some retrotransposons can escape such DNA methylation reprogramming (104, 137). For imprinted loci, the zinc finger proteins ZFP57 and ZFP445 act together to maintain parental allele–specific DNA methylation at imprinting control regions (ICR) (138, 139). Mechanistically, after binding to the ICRs, the zinc finger proteins further recruit DNMT1, KAP1 (also known as TRIM28), and H3K9me3 to protect the imprints from DNA demethylation (50, 51, 140). In humans, ZNF445 may play a more predominant role in imprint maintenance, as ZFP57 is not present in human oocytes and early embryos prior to zygotic genome activation (139).

In humans, a larger number of oocyte-derived differentially methylated regions (DMRs) are preserved at the blastocyst stage (113, 114, 136). Notably, some of these gametic DMRs can be specifically maintained in the placenta, with a subset correlating with allele-specific gene expression (141–143). Moreover, the imprinted methylation is variable across different individuals, supporting a polymorphic placenta-specific imprinting (141–143). Such placenta-specific DMRs are likely the consequence of incomplete erasure of maternally methylated DMRs. Whether polymorphic imprinting is associated with any phenotypic outcomes in placenta is currently unknown.

De Novo DNA Methylation at Implantation

After reaching a minimal methylation level at the blastocyst stage, the embryo proper then undergoes genome-wide remethylation to establish the canonical methylation landscape typically found in somatic cells (68, 104, 137). Because the de novo methylation at different genomic regions takes place with similar kinetics in a short time window (i.e., E4.5–E6.5 in mice), methylation likely is established by default along with the increased level of DNMT3 at implantation (75, 144) (**Figure 3*a*,*b***).

Although DNMT3A and DNMT3B cooperate for de novo DNA methylation at implantation, DNMT3B appears to play a dominant role at this stage, as *Dnmt3b* zygotic KO mice exhibit embryonic lethality, whereas *Dnmt3a* zygotic KO mice can survive to term and die at ~4 weeks of age (6). In addition, base-resolution DNA methylation profiling in *Dnmt3a* and *Dnmt3b* single E8.5

KO embryos revealed that depletion of DNMT3B has the more pronounced effect on methylation establishment (69). Unlike DNMT3A and DNMT3B, zygotic depletion of DNMT3L delays but does not abolish methylation establishment at a few genomic elements (145). Therefore, in contrast to its necessity in the germ line, DNMT3L is not required for de novo DNA methylation at implantation, and its deficiency at this stage may be compensated for by the increased level of DNMT3A or the ability of DNMT3A to methylate DNA without the help of DNMT3L (145).

After implantation, CpG methylation follows a canonical bimodal distribution that is common to multiple somatic tissues (137). Although most of the genomic features (e.g., gene bodies, TEs, and CpG poor promoters) become remethylated, CGIs at TSS generally remain unmethylated (2). Nonetheless, the promoters of germ-line genes and pluripotent genes acquire DNA methylation during implantation (68, 69, 75). Unlike the embryo proper, extraembryonic lineages such as extraembryonic ectoderm (ExE) and VE acquire a unique methylome, in which most CpGs are incompletely methylated and therefore do not follow the canonical bimodal 5mC distribution (75, 144). This large-scale partial DNA methylation is likely due to lower levels of DNMT3A/3B in extraembryonic lineages than the epiblast.

Recently, by analyzing genome-wide dynamics of DNA methylation in pre- and postimplantation embryos, Smith et al. (144) uncovered a number of CGI promoters that are generally protected from methylation in the embryo proper but are methylated in extraembryonic tissues. Interestingly, these CGIs are also pervasively methylated at orthologous regions in the human placenta and diverse human cancer types, suggesting that epigenetic regulation in the trophoblast lineage may resemble to some extent the somatic transition to cancer (144). However, it remains elusive why extraembryonic lineages acquire such a unique DNA methylome distinct from any other somatic lineages.

CONCLUSIONS AND FUTURE PERSPECTIVES

Over the past several years, considerable understanding has been achieved regarding the underlying mechanisms of de novo DNMT recruitment, DNA methylation maintenance, and DNA methylation dynamics in mammalian development. Despite these accomplishments, some questions still need to be addressed. First, it is striking that disruption of the interaction between the PWWP domain and H3K36me3 can cause DNMT3A gain of function to methylate regions normally unmethylated (22, 23). As the DNMT3A PWWP domain mutations have been linked to microcephalic dwarfism in humans (22), addressing this question is of great interest for both basic science and clinical medicine. Second, the function of numerous DNMT3B isoforms remains to be defined. Although it has been reported that some inactive DNMT3B isoforms can serve as accessory proteins for DNA methylation (30), some isoforms containing N-terminal truncations may be also subject to unique regulations under specific biological contexts. Third, the distinct expression patterns of DNMT3L in humans and mice are intriguing. In mice, DNMT3L is mainly involved in germ-line DNA methylation. However, *DNMT3L* is not expressed in the human germ lines, and whether it exerts function at distinct developmental stages remains to be investigated. Fourth, recent advancement of low-input profiling technologies has paved the way for studying the interplay between DNA methylation and histone modifications in early embryos with limited sample availability. These advances made it possible to study how the germ-line epigenome can mediate intergenerational or transgenerational inheritance in mammals. Lastly, the use of single-cell technologies has revealed cell-to-cell transcriptome heterogeneity in a variety of tissues. Simultaneous profiling of the transcriptome and DNA methylome at a single-cell level should deepen our understanding of how DNA methylation heterogeneity can contribute to the transcriptome and functional heterogeneity of cells.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work is supported by National Institutes of Health (R01HD092465). Y.Z. is an Investigator of the Howard Hughes Medical Institute. The authors sincerely apologize to colleagues whose work could not be cited owing to space limitations.

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