nature REVIEWS MOLECULAR CELL BIOLOGY

SUPPLEMENTARY INFORMATION

In format as provided by the authors

The diverse roles of DNA methylation in mammalian development and disease

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https://doi.org/10.1038/s41580-019-0159-6

Supplementary Box 1. The players of DNA methylation and de-methylation

Many studies, some of which are very recent, explain the mechanistic underpinnings of DNA methylation. Here we elaborate on our discussion of this topic in main text of the article.

De novo DNA methylation

The *de novo* DNMTs can methylate unmodified cytosines in all contexts, with the strongest preference for CpG dinucleotides, followed by CpA[1–3]. Such sequence specificity is achieved by the target recognition domain of DNMT3A, which contains an arginine residue that contacts CpG dinucleotides[4]. Given the conservation of the target recognition domain, a similar mechanism likely applies to DNMT3B. However, cell types with high DNMT3 expression exhibit substantial non-CpG methylation, such as mouse and human embryonic stem cells (ESCs)[1,5,6], neuronal cell types[7,8] and germline cells[9–11]. Therefore, the CpG preference does not absolutely preclude non-CpG methylation *in vivo*.

In addition to the active *de novo* DNA methyltransferases, there is also a catalytically inactive DNMT, DNMT3L. DNMT3L interacts with both DNMT3A and DNMT3B[12], and structural data indicate that DNMT3L exists in a heterotetrameric configuration with DNMT3A or DNMT3B[4,13,14]. DNMT3L stimulates the enzymatic activity of DNMT3A and DNMTB by inducing a conformational change that promotes binding to DNA and to the methyl-donor, S-adenosyl-L-methionine[15–17]. Intriguingly, DNMT3L is dispensable for *de novo* DNA methylation in the embryo, but is absolutely required for establishing DNA methylation in both the male and female germline[18,19]. As such, homozygous null *Dnmt3L* mice of both sexes are viable, but completely infertile. The explanation for the discrepancy in the requirement for DNMT3L between the embryonic and germline *de novo* DNA methylation programs is yet to be fully understood. However, it is noteworthy that embryonic *de novo* methylation takes place in dividing cells, whereas germline *de novo* DNMT3L-dependent methylation occurs in mitotically arrested cells in both sexes.

As discussed in the main text, the DNMT ADD domain binds to H3K4, but is sensitive to H3K4 methylation. Mutations generated in the ADD domain can bypass its repulsion by H3K4 methylation; in mouse ESCs, this leads to aberrant promoter DNA methylation of developmental genes (but not housekeeping genes) and failure to differentiate, underscoring the importance of ADD-based DNMT3 targeting[20]. Beyond its role in diverting DNMT3 from H3K4me3-marked promoters, the ADD is also sensitive to H3 threonine 3 phosphorylation (H3T3ph)[21], which is a modification found at mitotic centromeres[22–24]. DNMT3A with an engineered ADD domain that is insensitive to H3T3ph remains bound to centromeres during mitosis, leading to genome instability[20]. Thus, the ADD domain is a versatile module that prevents ectopic DNA methylation in different contexts.

The DNMT PWWP domain recognizes H3K36me3. Curiously, despite containing a PWWP domain, DNMT3A shows no preference for transcribed gene bodies (which are marked by H3K36me3) in mouse ESCs[25]. Yet in the female germline, there is strong evidence that it is DNMT3A that methylates gene bodies[26–31], although it is not known whether this depends on the PWWP domain. This may be another example wherein the *de novo* DNA methylation machinery has adopted different functions in the germline. In sum, the PWWP and ADD domains combine to direct and restrict the targeting of the DNMT3 enzymes targeting in mammalian genomes. In fact, simply expressing a murine DNMT3B in baker's yeast — which has a DNA methylation-free genome — largely recapitulates a mammalian DNA methylation landscape: enrichment at gene bodies and absence at gene promoters[32].

Considering the high degree of conservation between the PWWP, ADD and methyltransferase (MTase) domains of DNMT3A and DNMT3B, what could explain the different target specificities of the two proteins, especially in cell types in which both are highly expressed, such as ESCs? A likely possibility is that target specificity depends on interactions mediated by their less conserved amino-termini. Moreover, various isoforms of both DNMT3 proteins exist, potentially broadening their functional repertoires. DNMT3A has only two major isoforms, both of which are catalytically active. By contrast, DNMT3B has 30 reported isoforms, some catalytically active and some inactive[33–37], and the majority of them are conserved between mice and humans[38].

Recent work has begun to shed light on the functions of some specific DNMT3 isoforms. In a human colon cancer cell line, an MTase-less isoform of DNMT3B lacks catalytic activity but stimulates *de novo* DNA methylation as an accessory factor, akin to DNMT3L[37]. It remains to be determined in whether and in what healthy, physiological contexts this activity is relevant. The long isoform of DNMT3A (DNMT3A1) is the less expressed of the two DNMT3A isoforms in mouse ESCs. However, two studies indicate that DNMT3A1 has a specific role at the boundaries of bivalent promoters[39,40], which are promoters marked by both H3K4me3 and H3K27me3; the latter is deposited by Polycomb repressive complex 2. Bivalent promoters are developmentally important[41–43], and the studies point to an intriguing role of DNMT3A1 in their regulation. Although there seems to be some interplay between DNMT3A1and the Polycomb machinery, the precise function of DNMT3A1 at bivalent promoters has not been fully elucidated. DNMT3A2 appears to a have a specific neuronal function, and decreased expression of *Dnmt3A2* in aging mice is associated with reduced cognitive abilities[44]. Understanding the roles of the multitude of DNMT3 isoforms will be worthwhile moving forward and could potentially resolve the functional differences between *de novo* DNA methylation mechanisms in embryos, the germline and somatic tissue types. Additionally, expression of aberrant isoforms may help explain DNA methylation deregulation in cancer[37].

Maintenance DNA methylation

In the main text we discuss the interplay between DNMT1 and UHRF1 with histone post-translational modifications (FIG. 1c). Interestingly, DNA ligase 1 (LIG1), which is part of the DNA replication machinery, contains a sequence with high similarity to the histone H3 tail. This sequence also undergoes lysine methylation and as such, can recruit UHRF1[45]. UHRF1 can ubiquitylate non-histone proteins involved in replication, which potentially could diversify the modes of DNMT1 recruitment to the replication fork[46]. Non-histone proteins could assist in DNA methylation maintenance in histone poor regions and/or regions devoid of H3K9 methylation.

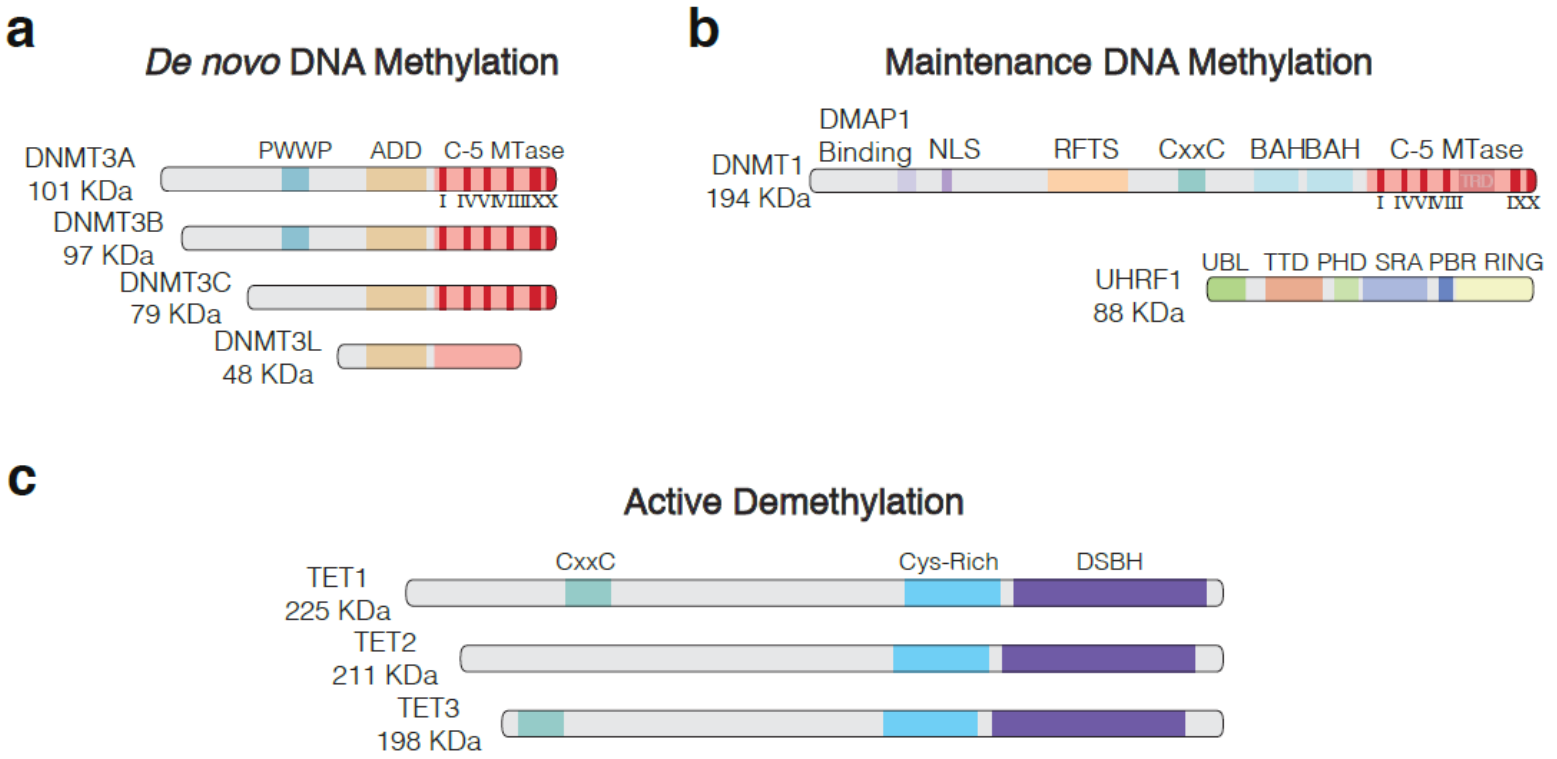
Finally, two recent studies used EdU and BrdU labeling followed by immunoprecipitation and bisulfite sequencing to determine the kinetics of maintenance of DNA methylation on newly synthesized DNA[47,48]. Even though both studies used the same human ESC line, their results were discordant: one study reported that maintenance methylation is rapid and takes 20 minutes to complete from the time of DNA synethesis, whereas the other indicated that the process takes several hours[48]. Further work is needed to resolve this discrepancy, as well assign possible functional relevance to these results. It should also be noted that studying DNA methylation maintenance in ESCs is complicated by the occurrence of *de novo* DNA methylation and demethylation in addition to the activity of the DNMT1 pathway[49].

DNA de-methylation

In mammals, there are three TET methylcytosine dioxygenases; they are all able to oxidize 5mC but are expressed in different stages of development. TET1 and TET3 contain a CxxC domain that binds to unmethylated CpG-rich regions[50] known as CpG islands (CGIs). Given that the majority of CGIs in mammals are unmethylated[51,52] and that TET1 and TET3 contain an intrinsic sequence-based recruitment module[53,54], it was suggested that TET proteins counteract DNA methylation at CGIs. TET-mediated regulation at CGI promoters may be important in some contexts[55]: human *TET1* mutant ESCs exhibit a moderate gain of DNA methylation at bivalent CGIs, and this leads to a defect in neural differentiation. However, in triple-*TET* knockout mouse ESCs, the biggest gain of 5mC is at enhancers, with only modest effect at CGI promoters[56,57]. The stemness properties of these cells are mostly unaffected, but differentiation is impaired[58], which likely reflects enhancer deregulation. These cellular phenotypes are borne out *in vivo*: triple- *TET* knockout mouse embryos exhibit pronounced defects at gastrulation, due to defective enhancer activation of *Lefty-Nodal* signalling[59]. In fact, TET-mediated regulation of enhancers during early embryogenesis appears to be a conserved feature across vertebrates: the phylotypic period (a highly conserved phase when the vertebrate body plan is shaped) is marked by wide-spread enhancer demethylation in mice, *Xenopus laevis* and zebrafish[60].

Finally, it should be noted that the TETs carry out a number of functions in addition to DNA demethylation (reviewed in [62]). Several readers specifically recognize 5hmC. Although some readers may be involved in the DNA demethylation process, there are indications that others may

affect gene regulation *per se*. For example, UHRF2 is a highly similar paralog to UHRF1, but preferentially binds 5hmC as opposed to 5mC[63,64]. *Uhrf2* knockout mice exhibit reduced 5hmC in the brain and behavioural abnormalities[65]. Importantly, TET1 seems to be an important regulator of embryonic development in mice, completely independently of its catalytic activity[61]. Incidentally, the same study noted that the developmental defects in *Tet* mutants are more pronounced in non-inbred mouse strains than in inbred strains[61]**.** Future work should distinguish the roles of TET enzymes in active DNA methylation from other means of transcriptional control.



Supplementary Figure 1. Major domains of DNA methylation and de-methylation proteins

a. Domain architecture of *de novo* DNA methyltransferases. DNMT3A and DNMT3B contain the H3K4-binding ADD domain and H3K36me3-binding PWWP domain, in addition to the MTase domain in the carboxy-terminus. Their amino-termini are more divergent. DNMT3C is a Muroidea-specific duplication of DNMT3B that has lost its PWWP domain. DNMT3L is a catalytically inactive co-factor that stimulates the catalytic activity of DNMT3A and DNMT3B.

b. Domain architecture of the key DNA methylation maintenance proteins. DNMT1 contains a number of conserved domains involved in DNA targeting, including the DMAP1-binding, RFTS and CxxC. The precise function of the bromo-adjacent homology (BAH) domains is unclear. UHRF1 is required for the recruitment of DNMT1 to DNA replication forks through an intricate mechanism.

c. Conserved domains of the three TET proteins. The carboxy-terminal Cysteine-Rich and double-stranded β-helix (DSBH) domains confer catalytic activity. TET1 and TET3 harbour a CxxC domain, which binds to unmethylated CpG-rich regions.

Supplementary Box 2: Low input and single-cell DNA methylation techniques

Whole genome bisulfite sequencing (WGBS) allows assessment of the DNA methylome in single nucleotide resolution. During chemical treatment with sodium bisulfite, unmethylated cytosines are deaminated, while methylated cytosines remain intact. Following a desulphonation treatment, deaminated cytosines are converted to uracils, which are read as thymines by the DNA polymerase during PCR amplification. Thus, upon sequencing, the remaining cytosines are considered as methylated (or hydroxymethylated). Although the procedure is straightforward and many commercial kits are available, a major drawback of WGBS is that during the process a large proportion of the DNA becomes degraded[66]. Thus, it is a challenge to generate WGBS libraries when the amount of available tissue is limited.

A number of techniques have been developed to circumvent this problem and enable the use of low amounts of cellular material in DNA methylome assays. Reduced representation bisulfite sequencing (RRBS) uses the restriction enzyme *MspI*, which recognizes the CCGG motif and is insensitive to CpG methylation to digest genomic DNA[67,68]. By excising small DNA fragments from gels, CpG-rich regions such as CpG islands (where *MspI* cuts more frequently) can be enriched for analysis. These fragments are subjected to adapter ligation, bisulfite conversion and sequencing. As extremely low input of DNA is required for RRBS, this technique is well-suited for working with low-cell numbers[69] and even with single cells[70]. Furthermore, it is more cost-effective than WGBS, because high genomic coverage can be achieved with less sequencing. However, although RRBS is superb for analysing CpG islands, a major drawback of the technique is that non-CpG-rich regions of the genome are depleted in the analysis. Thus, the resulting DNA methylome is incomplete.

In the original WBGS protocols, adapters are ligated prior to bisulfite conversion. Although this approach is convenient for amplifying properly bisulfite-converted DNA, a sizeable proportion of the adapters are degraded and many intact sequences are lost. In a technique named post-bisulfite adapter tagging (PBAT), bisulfite-treated DNA is amplified first and adapters are ligated afterwards[71]. Only very low input of DNA is therefore required for PBAT, making it compatible with single-cell analyses[72]. In a similar method, poly-C tails are added to post-bisulfite-treated DNA using terminal deoxynucleotidyl transferase[73]. The tails serve as primer binding sites, and adapters are ligated to double-stranded DNA. This technique, named small-scale TELP (tailing, elongation, ligation, PCR)-enabled methylome sequencing (STEM-seq), has also been applied with success on embryonic material[74].

Finally, a clever method has been developed to simultaneously assess the DNA methylome as well as chromatin accessibility and nucleosome phasing. Named nucleosome occupancy and methylation sequencing (NOMe-seq)[75], the technique uses the bacterial GpC methyltransferase *M.CviPI*, which preferentially methylates linker DNA between nucleosomes. Following bisulfite conversion, cytosines methylated in the GpC context designate inter-nucleosomal DNA; simultaneously, cytosines methylated in the CpG context reveal the endogenous DNA methylome. NOMe-seq has been paired with PBAT to reveal single-cell nucleosome occupancy and methylation patterns[76] (this is also called single-cell chromatin overall omic-scale landscape sequencing (scCOOL-seq))[77,78], and has even been combined with single cell RNA-seq in the same experiment[79]. Overall, the past several years have borne witness to increase in the amount of DNA methylation data that can be derived from using minimal amounts of cellular material.

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