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The interplay between DNA and histone methylation: molecular mechanisms and disease implications

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Abstract

Methylation of cytosine in CpG dinucleotides and histone lysine and arginine residues is a chromatin modification that critically contributes to the regulation of genome integrity, replication, and accessibility. A strong correlation exists between the genome-wide distribution of DNA and histone methylation, suggesting an intimate relationship between these epigenetic marks. Indeed, accumulating literature reveals complex mechanisms underlying the molecular crosstalk between DNA and histone methylation. These *in vitro* and *in vivo* discoveries are further supported by the finding that genes encoding DNA- and histone-modifying enzymes are often mutated in overlapping human diseases. Here, we summarize recent advances in understanding how DNA and histone methylation cooperate to maintain the cellular epigenomic landscape. We will also discuss the potential implication of these insights for understanding the etiology of, and developing biomarkers and therapies for, human congenital disorders and cancers that are driven by chromatin abnormalities.

Keywords cancer; chromatin; developmental disorder; DNA methylation; histone methylation

Subject Categories Cancer; Chromatin, Transcription & Genomics; Molecular Biology of Disease

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See the Glossary for abbreviations used in this article.

Introduction

Methylation of the 5-position of cytosine is a highly conserved chromatin modification among vertebrates. The majority of cytosine methylation occurs in the context of CpG dinucleotides. Nevertheless, in certain tissue and cell types such as brain and embryonic stem cells, non-CpG methylation is readily detectable (He & Ecker, 2015). In mammals, 60–80% of CpGs are methylated (Ehrlich *et al*, 1982; Lister *et al*, 2009), which are non-randomly distributed in repetitive sequences, gene bodies, and intergenic regions (Suzuki & Bird, 2008; Jones, 2012). At these regions, CpG methylation is believed to play a primary role in restricting chromatin accessibility, leading to silencing of retrotransposon elements, prevention of cryptic transcription (Neri *et al*, 2017), and regulation of transcription factor binding (Zhu *et al*, 2016). In contrast, genomic regions containing high density of CpGs, known as CpG islands, are normally free of methylation. CpG islands are often located within gene promoters and *cis*-regulatory elements, and their low methylation levels are thought to facilitate a transcriptionally permissive state for their target genes.

The landscape of DNA methylation is shaped by the collective action of DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B, and enzymes involved in DNA demethylation, including the TET family of methylcytosine dioxygenases. The two *de novo* DNA methyltransferases DNMT3A and DNMT3B catalyze CpG methylation at previously unmethylated CpGs. Once established, during replication DNMT1 serves to copy pre-existing CpG methylation to newly synthesized daughter strand with high fidelity, thus preserving the patterns of CpG methylation through cell division (Goll & Bestor, 2005). However, it should be noted that in certain contexts, DNMT3A/B could facilitate maintenance DNA methylation (Jones & Liang, 2009), while DNMT1 could mediate *de novo* DNA methylation (Yarychkivska *et al*, 2018; Li *et al*, 2018b); therefore, their functional distinctions are not univocal. On the other hand, TET1-3 enzymes catalyze stepwise oxidation of methylcytosine, generating 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine that contribute to either active or passive DNA demethylation (Wu & Zhang, 2014).

In the cycle of mouse development, there are two major waves of global genome demethylation and re-methylation: one occurring following germ cell specification and the other during early embryonic development after fertilization (Zeng & Chen, 2019). While the bulk levels of CpG methylation are otherwise relatively stable, it is evident that DNMTs and TETs continuously act to shape focal CpG methylation to yield tissue- and development-specific DNA methylomes that are strongly correlative with chromatin accessibility and enhancer activation (He *et al*, 2020). Accordingly, genetic knockout

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Glossary

ADD	ATRX-DNMT3-DNMT3L
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ChIP	chromatin immunoprecipitation
cryo-EM	cryogenic electron microscopy
DIPGs	diffuse intrinsic pontine gliomas
DLBCLs	diffuse large B-cell lymphomas
DNMTs	DNA methyltransferases
ERVs	endogenous retroviruses
H3Kx	histone H3 lysine number x
hm-DNA	hemi-methylated DNA
ICF	immunodeficiency, centromeric instability, facial anomalies syndrome
MDSs	myelodysplastic syndromes
MEFs	mouse embryonic fibroblasts
mESCs	mouse embryonic stem cells
MPNs	myeloproliferative neoplasms
MPNST	malignant peripheral nerve sheath tumor
OGID	overgrowth and intellectual disability
PMDs	partially methylated domains
PRC1	polycomb repressive complex 1
PRC2	polycomb repressive complex 2
RFTS	replication foci targeting sequence
SRA	SET- and RING-associated
TBRS	Tatton-Brown–Rahman syndrome
TCL	T-cell lymphoma
TET	ten-eleven translocation
TTD	tandem Tudor domain
UBL	ubiquitin-like
UHRF	ubiquitin-like, containing PHD and RING finger domains
UIM	ubiquitin-interacting motif
WGBS	whole-genome bisulfite sequencing

of DNMTs arrests embryonic or neonatal mouse development (Li *et al*, 1992; Okano *et al*, 1999), and tissue-specific ablation of DNMTs and TETs affects organ homeostasis and regeneration (Challen *et al*, 2011; Rinaldi *et al*, 2016; Bowman & Levine, 2017). Moreover, germline and somatic mutations in DNA methylation modifiers are associated with human developmental disorders (Hansen *et al*, 1999; Tatton-Brown *et al*, 2014; Heyn *et al*, 2019) and cancers (Abdel-Wahab *et al*, 2009; Ley *et al*, 2010). These findings suggest that precise regulation of the dynamics of CpG methylation is key to proper cell fate specification and represents a barrier to neoplastic transformation.

A central question in the field is how DNA methylation machineries interact with other chromatin components to ensure faithful establishment and maintenance of genome-wide CpG methylation. Whereas the roles of transcription factors and non-coding RNAs in shaping cellular methylome have been excellently reviewed elsewhere (Zhu *et al*, 2016; Zhao *et al*, 2016b), in this review we focus on the interplay between DNA and histone arginine and lysine methylation. Indeed, early genome-wide profiling of CpG methylation noted a strong correlation between DNA methylation and histone methylation, including a positive correlation with histone H3K9 methylation and a negative correlation with H3K4 methylation (Meissner *et al*, 2008). Furthermore, recent studies have uncovered that various regulatory domains within DNA methylation modifiers and their associated factors possess intrinsic affinity to histones in a modification-dependent manner (Table 1). We will summarize the progress made toward a better understanding of the molecular mechanisms underlying the crosstalk between DNA and histone methylation and discuss their implications for human diseases driven by dysregulation of these chromatin marks.

Table 1. Annotated regulatory domains of DNMTs and associated proteins.

Gene (-Domain)	Interaction	Location	Functional significance
DNMT1- RFTS	H3K18ub (+) H3K23ub (+)	Replication fork	Maintenance methylation
DNMT1- RFTS	H3K9me3 (+)	Heterochromatin	Maintenance methylation
UHRF1-TTD/ PHD	H3K9me3 (+)	Heterochromatin	Replication-uncoupled maintenance methylation
UHRF1-PHD	H3R2me2a (−)	Active genes	Prevent aberrant maintenance methylation
DNMT3L/A/B-ADD	H3K4me3 (−)	Active gene promoter	Prevent aberrant *de novo* methylation
DNMT3B-PWWP	H3K36me3 (+)	Active gene body	Genic *de novo* methylation
DNMT3A-PWWP	H3K36me2 (+)	Intergenic region	Intergenic *de novo* methylation

Interaction between DNMT1/UHRF1 and histone methylation facilitates maintenance DNA methylation

DNMT1 plays an indispensable function in propagating patterns of CpG methylation across cell cycles by reinstalling hemi-methylated CpG to full methylation state. UHRF1, a RING E3 ubiquitin ligase also known as NP95 in mouse or ICBP90 in human, is essential for DNMT1-mediated maintenance of DNA methylation. UHRF1 is required for DNMT1's loading to replicating heterochromatin and genetic knockout of *Uhrf1* phenocopied loss of *Dnmt1*, leading to DNA hypomethylation and early developmental arrest (Sharif *et al*, 2007). Furthermore, UHRF1 specifically recognizes hemi-methylated DNA through its SET- and RING-associated (SRA) domain, therefore bridging DNMT1 to its substrate to maintain DNA methylation (Bostick *et al*, 2007; Sharif *et al*, 2007). The recruitment can occur directly as UHRF1 physically interacts with DNMT1 during the S phase. Moreover, the E3 ubiquitin ligase activity of UHRF1 catalyzes the ubiquitination of histone H3K18 and H3K23, which can be recognized and bound by DNMT1 via its ubiquitin-interacting motif (UIM) within the replication foci targeting sequence (RFTS) domain (Nishiyama *et al*, 2013; Qin *et al*, 2015). Recent crystal structures revealed that the RFTS domain of DNMT1 binds simultaneously to

both H3K18 and H3K23 mono-ubiquitination, which not only facilitates DNMT1 chromatin targeting but also stimulates its methyltransferase activity (Ishiyama *et al*, 2017; Li *et al*, 2018a). Therefore, through direct and indirect mechanisms, UHRF1 can target DNMT1 onto newly synthesized DNA substrates during semi-conservative DNA replication.

In addition to SRA domain and RING domain, UHRF1 contains multiple functional domains including a tandem Tudor domain (TTD) and PHD finger connected by a linker region. When analyzed individually *in vitro*, the TTD of UHRF1 was reported to preferentially recognize histone H3K9 methylation (Rottach *et al*, 2010; Nady *et al*, 2011), while the PHD finger specifically recognizes the N-terminal region of histone H3 with unmodified arginine 2 (H3R2) (Hu *et al*, 2011; Rajakumara *et al*, 2011; Wang *et al*, 2011). Interestingly, the TTD recognition of H3K9 methylation is independent of H3S10 phosphorylation (H3S10P) (Rothbart *et al*, 2012). H3S10 phosphorylation (H3S10P) is a mitotic "phospho-methyl switch" and acts to antagonize H3K9 methylation readers such as HP1 during cell cycle (Fischle *et al*, 2003, 2005). Structural analysis suggested that compared to other H3K9me3-interacting domains, UHRF1 TTD, enabled by Asn147, readily accommodates H3S10P. Indeed, mutating Asn147 to negatively charged glutamate abolishes UHRF1's insensitivity toward H3K9me3-to-S10P switch, which is important for UHRF1's association with chromatin during mitosis and maintenance of DNA methylation especially at late-replicating genomic regions (Rothbart *et al*, 2012).

Tandem Tudor domain and PHD finger of UHRF1 are only separated by a 17-aa linker region, raising the possibility that the two domains may operate as a single functional unit to recognize combinatorial histone modifications. Structural and biochemical studies of the linked TTD-PHD finger module demonstrated that while the recognition of N-terminal histone H3 by PHD seems to be independent of TTD, the binding to H3K9 methylation by TTD is markedly enhanced by the presence of PHD finger (Arita *et al*, 2012; Xie *et al*, 2012; Cheng *et al*, 2013; Rothbart *et al*, 2013). The linker region also appears to play a role, as its mutation and phosphorylation disrupt the higher order structure and the binding to H3K9me3 (Arita *et al*, 2012). The coordinated recognition of histone tails by TTD-PHD finger module *in vitro* suggests that UHRF1's recruitment to chromatin is dependent on histone engagement through cooperation between multiple reader domains. Indeed, point mutations in either the TTD, PHD, or linker regions reduce UHRF1's association with chromatin in cells and compromise UHRF1's ability to maintain DNA methylation (Rothbart *et al*, 2013). This is further illustrated by a recent study that identified a splicing variant of mouse UHRF1, which has additional 9 amino acids inserted in the TTD-PHD finger linker region compared to canonical mouse and human UHRF1 (Tauber *et al*, 2020). As a result, the variant UHRF1 had distinct H3K9me3 binding profiles and displayed enhanced ubiquitination activity toward H3K9me3-modified nucleosomes.

Beyond TTD and PHD finger, recent reports also pointed to the importance of SRA domain, which recognizes hemi-methylated DNA (hm-DNA), in directing the histone engagement and substrate specificity of UHRF1 (Fang *et al*, 2016; Harrison *et al*, 2016). It was shown that prior to binding histones and DNA, UHRF1 adopts a "closed" conformation, in which the histone-binding (e.g., PHD finger) and DNA-binding (e.g., SRA) modules are physically associated. Upon chromatin recruitment, the inter-domain rearrangement of UHRF1 allows it to transit into "open" conformation in which the SRA binds to the hm-DNA and TTD-PHD finger to H3K9me2/3 via a positive feedback mechanism. Furthermore, the recognition of hm-DNA by SRA domain allosterically activates and directs the ubiquitin ligase activity of UHRF1 toward H3K18 and H3K23. Collectively, these biophysical and structural data suggest a highly sophisticated mechanism involving extensive inter-domain communication and cooperation in directing UHRF1's chromatin engagement and activating its enzymatic activity (Fig 1A). Indeed, yet another domain—ubiquitin-like domain (UBL)—was found to interact with other UHRF1 domains to facilitate histone/DNA binding and H3 ubiquitination (DaRosa *et al*, 2018; Foster *et al*, 2018).

In parallel to *in vitro* studies of the multidomain-mediated UHRF1 binding to histones, *in vivo* analyses have generated interesting insight into the functional importance of these reader domains and the interacting histone modifications (Fig 1B). Veland *et al* (2017) identified PRMT6 as the arginine methyltransferase for histone H3R2me2a. Overexpressing PRMT6 in mouse embryonic stem cells (mESCs) resulted in dissociation of UHRF1 from chromatin and global DNA hypomethylation, supporting the notion that unmodified H3R2 facilitates UHRF1 chromatin recruitment and maintenance of CpG methylation (Veland *et al*, 2017). In mouse embryonic fibroblasts (MEFs) deficient for *Suv39h* genes encoding H3K9 methyltransferases, the decrease in H3K9 methylation at pericentric heterochromatin is correlated with a reduced localization of UHRF1 (Karagianni *et al*, 2008), suggesting an important role of H3K9 methylation for UHRF1 chromatin recruitment. Using the same system, it was shown that the PHD finger and SRA domain are required for UHRF1 to localize to pericentric heterochromatin. These two domains are also necessary for UHRF1 to restore or maintain DNA methylation in various cell types (Qin *et al*, 2015; Harrison *et al*, 2016; Kong *et al*, 2019).

In contrast, the role of TTD seems to be more context-dependent. Whereas a point mutation (Y188A) that abolishes TTD's interaction with H3K9me2/3 is unable to complete CpG re-methylation following UHRF1 re-expression in UHRF1-depleted cells (Rothbart *et al*, 2012), the same mutation in TTD does not affect UHRF1's function in maintaining CpG methylation in colorectal cancer cells (Kong *et al*, 2019). Moreover, a knock-in TTD mutant mouse model has no overt phenotypes and shows modest ($\sim$10%) reduction in CpG methylation (Zhao *et al*, 2016a). Similarly, the RING domain appears to be essential for *de novo* or re-methylation, but dispensable for maintaining CpG methylation (Qin *et al*, 2015; Harrison *et al*, 2016; Li *et al*, 2018a; Kong *et al*, 2019).

A recent in-depth analysis of the kinetics and fidelity of DNMT1-mediated maintenance methylation offers critical insights into the function of UHRF1-TTD in the process (Ming *et al*, 2020) (Fig 1C). By developing a novel method—Hammer-seq—that combines EdU labeling, biotin-mediated enrichment, and hairpin bisulfite sequencing technologies, Ming *et al* (2020) uncovered two distinct maintenance kinetics: replication-coupled and replication-uncoupled maintenance phases. The TTD is required for both phases, yet through interactions with different ligands: The interaction of TTD with methylated replication fork protein LIG1 (Ferry *et al*, 2017) is required for replication-coupled maintenance phase, whereas TTD-H3K9me2/3 for replication-uncoupled maintenance phases. At steady state, the inactivation of TTD causes delays in replication-coupled CpG methylation maintenance that could be compensated

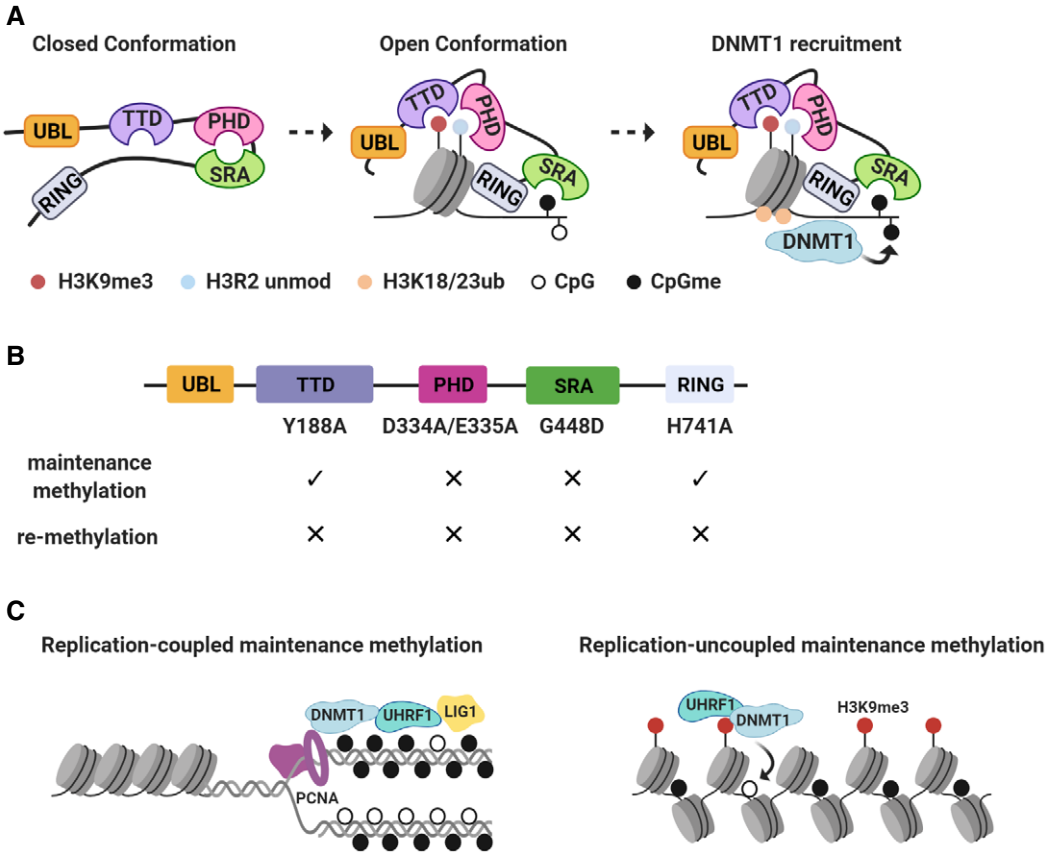

Figure 1. Interaction between DNMT1/UHRF1 and histone methylation during maintenance DNA methylation.

(A) In the absence of chromatin interaction, UHRF1 adopts a closed conformation, where the linker region between SRA and RING domains binds to the TTD domain and the SRA domain binds to PHD finger. Upon engagement with histone and hemi-methylated DNA, inter-domain conformational change will enable the TTD-PHD module to recognize H3K9me3 and H3R2, and SRA domain to bind to hemi-methylated DNA. This open conformation will facilitate the ubiquitination of histone H3K18 and H3K23 by UHRF1, which in turn recruits DNMT1 to catalyze maintenance DNA methylation. (B) Schematics summarizing the functional impact of various domain-inactivating UHRF1 missense mutations on maintenance DNA methylation or re-methylation following global demethylation. (C) Recent study suggests two distinct modes of maintenance methylation by DNMT1-UHRF1. At the replication fork, interactions between DNMT1 and PCNA, and UHRF1-TTD and methylated LIG1, facilitate replication-coupled maintenance methylation. The interaction between UHRF1-TTD and H3K9me3, on the other hand, facilitates replication-uncoupled maintenance methylation.

by other mechanisms, resulting in modest reduction in bulk CpG methylation. However, when replication-coupled phase is compromised, or when the genome is largely devoid of CpG methylation (e.g., UHRF1 depletion), TTD mutation will significantly impact the rate of replication-uncoupled re-methylation. In support of this notion, genomic regions of high H3K9me2 display greater loss of CpG methylation in UHRF1-TTD mutant cells (Ming *et al*, 2020). It is possible that the RING domain of UHRF1 plays a similar function in replication-uncoupled maintenance of CpG methylation.

Finally, in addition to recognizing H3K18 and H3K23 ubiquitination catalyzed by UHRF1, a recent study demonstrates that the RFTS domain of DNMT1 also specifically binds to H3K9me3 (Ren *et al*, 2020). It was proposed that the direct interaction between DNMT1-RFTS and H3K9me3 in conjunction with H3 mono-ubiquitination could compensate for the loss of UHRF1-TTD to maintain CpG methylation. While DNMT1 does not harbor any canonical histone-binding domains, it would be interesting to explore whether DNMT1 directly interacts with additional histone modifications via non-canonical mechanisms.

Role of histone methylation in targeting *de novo* DNMTs

In mammals, *de novo* CpG methylation is catalyzed by DNMT3A and DNMT3B. During embryogenesis, DNMT3L, which shares partial sequence homology with DNMT3A/B yet lacks the catalytic domain, acts as an essential accessary protein for establishing genomic methylation (Bourc'his, 2001). DNMT3L is absent or expressed at extremely low levels in adult tissues. Its function, however, can be partially compensated by catalytically inactive isoforms of DNMT3B such as DNMT3B3 (Weisenberger *et al*, 2004; Duymich *et al*, 2016; Zeng *et al*, 2020). DNMT3A and DNMT3B have distinct mechanisms of DNA substrate engagement and show differential preference for the flanking sequence of target CpG (Handa & Jeltsch, 2005; Dukatz *et al*, 2020; Gao *et al*, 2020; Mallona *et al*, 2021), and we point readers to the excellent reviews of the molecular and structural basis for the catalysis of *de novo* methylation by DNMT3 (Jurkowska & Jeltsch, 2016; Ren *et al*, 2018). Furthermore, DNMT3A/B as well as DNMT3L can interact with histone tails through shared regulatory domains. Thus, pre-existing histone

methylation plays a plausible role in shaping the non-random targeting of *de novo* DNMTs and distribution of CpG methylation throughout the genome. Indeed, recent meta-analysis of whole-genome bisulfite sequencing (WGBS) and ChIP-seq datasets from 35 human cell types revealed that CpG methylation is negatively correlated with H3K4 and H3K27 methylation and positively correlated with H3K9 and H3K36 methylation (Fu *et al*, 2020).

ADD domain-mediated interaction with H3K4 methylation

Genome-scale profiling study revealed a strong anti-correlation between CpG methylation and H3K4 methylation that is particularly pronounced at CpG islands (Meissner *et al*, 2008). Immunoprecipitation and *in vitro* biochemical assays found that DNMT3L directly binds to N-terminal tail of histone H3 and the binding is abolished with methylation of H3K4 (Ooi *et al*, 2007). Crystal structure revealed a direct interaction between the first seven amino acids of H3 and the cysteine-rich region within the ATRX-DNMT3-DNMT3L (ADD) domain of DNMT3L. As expected from the significant sequence homology, the ADD domains of DNMT3A/B interact with H3 tails in a similar manner (Otani *et al*, 2009; Zhang *et al*, 2010). In addition to H3K4 methylation, this interaction is sensitive to phosphorylation of H3T3, H3S10, or H3T11 (Zhang *et al*, 2010). Furthermore, unmodified but not H3K4-methylated histone H3 peptide can stimulate the activity of DNMT3A up to 8-fold (Li *et al*, 2011). Therefore, it appears that H3K4 methylation, a mark for active transcription, antagonizes the recruitment as well as the allosteric activation of DNMT3A/B-DNMT3L complex to prevent *de novo* CpG methylation and gene silencing.

The opposing action between H3K4 methylation and *de novo* DNMTs is also supported by *in vivo* evidence. By introducing DNMT3A and DNMT3L into yeast, Hu *et al* (2009) reported that the histone H3 N-terminal tail is required for ectopic genomic methylation (Hu *et al*, 2009). Notably, in yeast stains lacking H3K4 methyltransferases, the levels of *de novo* methylation are substantially increased in a manner dependent on the ADD domain of DNMT3L. The H3K4 demethylase KDM1B is highly expressed in growing oocytes, and its ablation results in increased H3K4 methylation and subsequent failure of *de novo* DNA methylation and establishment of genomic imprints (Ciccone *et al*, 2009). These findings were confirmed by Stewart *et al* (2015), which further nominated another H3K4 demethylase KDM1A/LSD1 in regulating DNA methylation during oogenesis (Stewart *et al*, 2015). Similarly, during the development of male germline, H3K4me2-enriched CpG islands are protected from *de novo* methylation (Singh *et al*, 2013). A point mutation in the ADD domain of DNMT3L that abolishes its histone-binding activity is sufficient to impair establishment of both CpG and non-CpG methylation, spermatogenesis and fertility (Vlachogiannis *et al*, 2015). In the context of embryogenesis, complete loss of maternal KDM1A/LSD1 arrests embryo at the maternal-to-zygotic transition stage, whereas partial loss of maternal KDM1A leads to developmental and behavioral abnormality that is associated with DNA hypomethylation and misexpression of imprinted genes (Wasson *et al*, 2016). It should be noted, however, that the maternal effects of KDM1A during embryogenesis may be independent of H3K4 methylation, since it has been demonstrated that KDM1A also directly demethylates DNMT1 which in turn affects maintenance CpG methylation (Wang *et al*, 2009). Finally, through structural modeling and protein engineering, Noh *et al* (2015) identified point mutations that render the ADD domain of DNMT3A insensitive to either H3K4 methylation or H3T3 phosphorylation (Noh *et al*, 2015). Re-expression of H3K4 methylation-insensitive mutant DNMT3A in DNMT triple knockout mESCs results in accumulation of DNA methylation at H3K4me3/2-enriched regions and a defect in mESC differentiation. Expression of H3T3 phosphorylation-insensitive mutant DNMT3A, on the other hand, leads to chromosomal instability. Together, these functional analyses corroborate with *in vitro* studies and strongly support a pivotal role of ADD-H3K4me3/2 antagonism for protecting transcriptionally active promoter CpG islands from *de novo* methylation (Fig 2A).

PWWP domain-mediated interaction with H3K36 methylation

In contrast to promoters, actively transcribed genes have high levels of CpG methylation at their gene bodies (Jones, 2012). These regions are also enriched for tri-methylation of histone H3K36 (H3K36me3), and as expected DNA methylation has been shown to be associated with the deposition of H3K36me3 (Fu *et al*, 2020). Interestingly, comparative studies of DNA methylomes found that H3K36me3-rich genes in *Drosophila* can predict CpG methylation and content of orthologous genes in other organisms, suggesting that the crosstalk between H3K36me3 and CpG methylation is highly evolutionarily conserved (Nanty *et al*, 2011). Both DNMT3A and DNMT3B harbor PWWP domain, which in other proteins such as BRPF1 has been shown to bind to H3K36me3 (Vezzoli *et al*, 2010). Indeed, the PWWP domains of DNMT3A and DNMT3B interact with H3K36me3 peptide *in vitro* (Dhayalan *et al*, 2010; Baubec *et al*, 2015). Furthermore, ChIP-seq revealed preferential localization of DNMT3B in the gene bodies of highly expressed, H3K36me3-enriched genes in mESCs, which is dependent on its PWWP domain and the H3K36me3 "writer" enzyme SETD2 (Baubec *et al*, 2015). Similarly, when DNMT3B is heterologously expressed in yeast, its binding and *de novo* methylation correlate with H3K36me3 and are abolished upon deletion of SET2—the yeast homologue of SETD2 (Morselli *et al*, 2015). More recently, it has been shown that maternal depletion of SETD2 causes loss of H3K36me3, followed by decreased genic CpG methylation yet aberrant gain of intergenic methylation, which contributes to the defects in oocyte maturation and embryonic development arrest at 1-cell stage (Xu *et al*, 2019). These findings agree with prior observations from growing murine oocytes and 195 human/mouse DNA methylomes, where a positive correlation between co-transcriptionally deposited H3K36me3 and DNA methylation across the gene body has been noted (Morselli *et al*, 2015; Stewart *et al*, 2015; Salhab *et al*, 2018). The function of H3K36me3-mediated gene body CpG methylation (Fig 2B) remains unclear, though one hypothesis is that it ensures the efficiency and fidelity of transcriptional elongation through preventing spurious intragenic transcription initiation (Neri *et al*, 2017). While further investigation is needed, this notion is consistent with the role of H3K36me3 in yeast where it prevents cryptic transcription through histone deacetylation (Carrozza *et al*, 2005).

Interestingly, the binding pattern of DNMT3A differs substantially from that of DNMT3B and is enriched in the intergenic regions of euchromatin (Wu *et al*, 2010; Baubec *et al*, 2015). Indeed, whereas DNMT3B-PWWP preferentially interacts with H3K36me3, DNMT3A-PWWP can recognize both H3K36me3 and di-methylated H3K36 (H3K36me2) with a slightly higher affinity for H3K36me2 (Sankaran *et al*, 2016; Dukatz *et al*, 2019; Weinberg *et al*, 2019; Xu

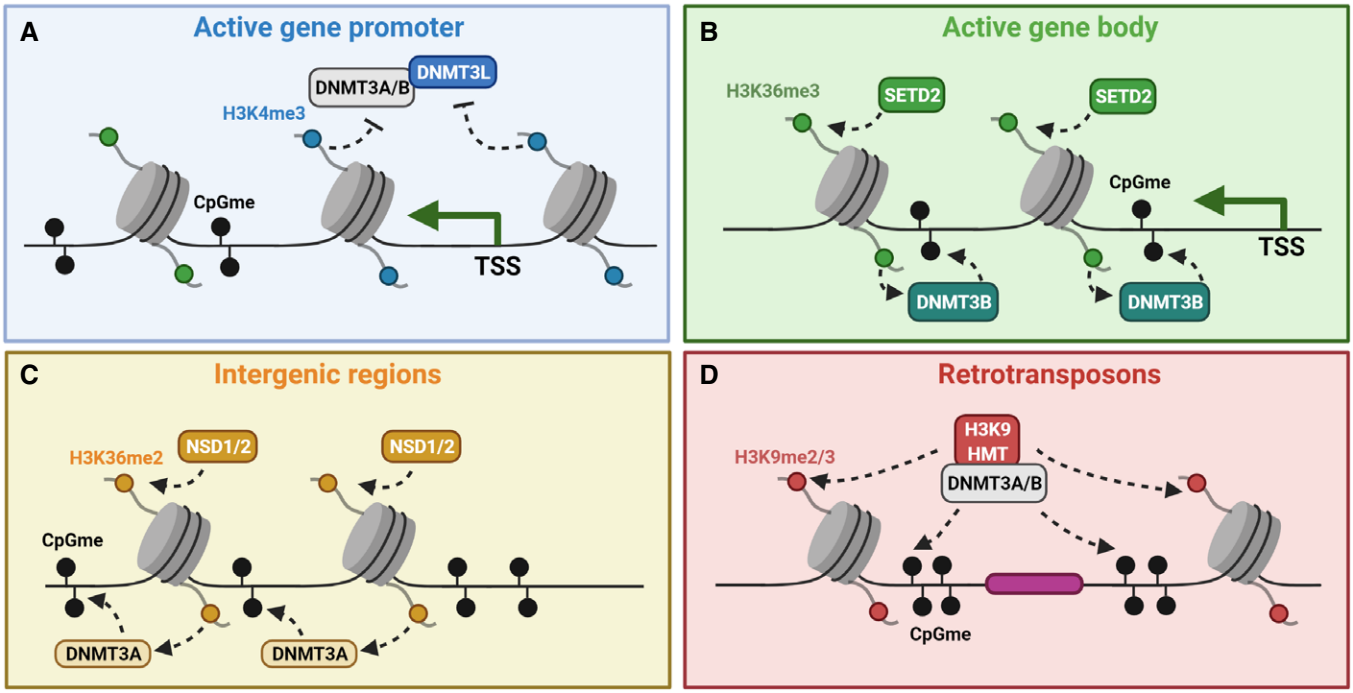

Figure 2. Various mechanisms underlying targeting of *de novo* DNMTs by histone methylation.

(A) At promoters of actively transcribing genes, high levels of H3K4me3 oppose ADD domain and the binding of DNMT3L-DNMT3A/B to prevent *de novo* CpG methylation. (B) At gene bodies of actively transcribing genes, high levels of H3K36me3 interact with PWWP domain of DNMT3B and facilitate its genic localization. (C) A parallel pathway operates at the intergenic region, where H3K36me2 interacts with PWWP domain of DNMT3A and facilitates its intergenic localization. (D) At repetitive elements and retrotransposons, interactions between H3K9 methyltransferases and DNMT3A/B enable co-localization of H3K9me3 and CpG methylation for transcriptional silencing.

et al, 2020a; Xu *et al*, 2020b). While closely related, H3K36me2 is a highly abundant chromatin modification which is catalyzed by a distinct set of methyltransferases (NSD1-3 and ASH1L) (Kuo *et al*, 2011). It is spatially separated from H3K36me3 and mainly enriched in the intergenic regions (Rao *et al*, 2005). Genome-wide DNMT3A binding and DNMT3A-mediated *de novo* CpG methylation show positive correlation with H3K36me2 (Weinberg *et al*, 2019). Genetic knockout of NSD1/2 depletes intergenic H3K36me2 and CpG methylation, and redistributes DNMT3A to H3K36me3-enriched genic regions (Weinberg *et al*, 2019). Conversely, overexpression of NSD2 in multiple myeloma cells is coupled with elevated intergenic CpG methylation (Xu *et al*, 2020a; Xu *et al*, 2020b). Taken together, these findings support a model in which H3K36me2-DNMT3A interaction complements and competes with H3K36me3-DNMT3B to establish intergenic and genic CpG methylation at euchromatin, respectively (Fig 2C). It remains unclear what is the structural basis for the valence-specific recognition of H3K36 methylation by DNMT3A-PWWP versus DNMT3B-PWWP. A crystal structure of DNMT3B-PWWP bound to H3K36me3 is available (Rondelet *et al*, 2016), and it would be interesting to compare and contrast that to DNMT3A-PWWP.

Consistently, the H3K36me2 methyltransferase NSD1 has been linked to DNA methylation in several developmental and disease contexts. NSD1-mediated H3K36me2 is critical for guiding *de novo* methylation and establishing paternal imprints in the male germline (Shirane *et al*, 2020). *Nsd1* knockout male mice exhibit defects in spermatogenesis and are infertile. This is in sharp contrast to the role of SETD2/H3K36me3 in oocyte development and *de novo* methylation (Xu *et al*, 2019), and suggests that the sex-specific landscape of CpG methylation in the germline could result from distinct state of H3K36 methylation. Germline NSD1 mutations define Sotos syndrome which is characterized by developmental overgrowth and intellectual disability (Douglas *et al*, 2003). Blood samples from Sotos patients display profound genome-wide CpG hypomethylation compared to the controls (Choufani *et al*, 2015). Interestingly, this pattern mimics aging-induced loss of DNA methylation, leading to the hypothesis that NSD1 depletion accelerates the "epigenetic clock" (Martin-Herranz *et al*, 2019). Similarly, somatic mutations and deletions of NSD1, which are common in squamous cell carcinomas of the head and neck and other body sites (Papillon-Cavanagh *et al*, 2017), are associated with CpG hypomethylation (Brennan *et al*, 2017; Bui *et al*, 2018) that are particularly pronounced at the intergenic regions (Lee & Wiemels, 2016; Weinberg *et al*, 2019). The mechanism by which loss of intergenic H3K36me2 and DNA methylation affects gene regulation remains elusive, although recent reports suggests that the dosage of NSD2 and H3K36me2 is linked to the binding of methylation-sensitive genome architecture protein CTCF, reprogramming of 3D chromatin organization and distal enhancer activation (Lhoumaud *et al*, 2019).

Mechanisms of DNMT3A/B localization to constitutive heterochromatin

Both DNA methylation and H3K9 methylation are involved in the formation and maintenance of constitutive heterochromatin and the silencing of retrotransposons. In some organisms including

Neurospora crassa and *Arabidopsis thaliana*, DNA methylation is strictly guided by methylation of H3K9 (Tamaru & Selker, 2001; Jackson *et al*, 2002). Although there is a genome-wide positive correlation between H3K9 and CpG methylation (Meissner *et al*, 2008; Fu *et al*, 2020), the interplay between these two repressive marks seems to be complex in mammalian cells. There are three groups of methyltransferases, Suv39h1/2, G9a/GLP, and Setdb1, which catalyze H3K9 methylation at various parts of the genome. In mESCs, double knockout of *Suv39h1/2* abolishes the localization of DNMT3B as well as DNA methylation at pericentric satellite repeats (Lehnertz *et al*, 2003). DNMT3B was also found to interact with H3K9 methylation "readers" HP1α and HP1β. Similarly, DNMT3A was co-purified with H3K9 methyltransferase activity in HeLa cells and a direct interaction between DNMT3A and Suv39h1 and HP1β was observed (Fuks, 2003). This interaction appears to be mediated through the ADD domain of DNMT3A, although it is noteworthy that the PWWP domains of DNMT3A/B are required to direct their localization to pericentric heterochromatin (Chen *et al*, 2004). Therefore, additional histone-binding domains could be involved. SETDB1, which is often complexed with KRAB-Zinc Finger proteins and KAP1 to silence endogenous retroviruses (ERVs), also facilitates *de novo* DNA methylation. Depletion of either SETDB1 or KAP1 abolishes *de novo* methylation of natural or ectopically introduced ERVs (Rowe *et al*, 2013). Consistently, Leung *et al* (2014) observed that deletion of *Setdb1* in mESCs induces hypomethylation and misexpression of LTR retrotransposons and class I and II ERVs, as well as several imprinted genes (Leung *et al*, 2014). This seems to be mediated through active rather than passive demethylation, as TET-mediated 5-hydroxymethylcytosine was transiently found prior to demethylation. Again, a direct interaction between DNMT3A (ADD domain) and SETDB1 (N-terminal region) could be the underlying recruitment mechanism (Li *et al*, 2006). Finally, G9a-dependent *de novo* CpG methylation has been shown in mESCs during silencing of imprinted genes (Xin *et al*, 2003), retrotransposons (Dong *et al*, 2008) and provirus (Leung *et al*, 2011). The proviral silencing defect in *G9a* knockout cells could be phenocopied by knockout of *Dnmt3a*, suggesting that the recruitment of *de novo* methylation activity is important for initiating transcriptional repression (Leung *et al*, 2011). In differentiating mESCs, methylation of several pluripotent genes is dependent on G9a (Epsztejn-Litman *et al*, 2008), and in $G9a^{-/-}$ embryos, promoter methylation of germline-specific genes is decreased which is coupled by increased gene expression (Auclair *et al*, 2016). This G9a-dependent *de novo* methylation seems to be independent of H3K9 methylation, since catalytically inactive G9a is sufficient to restore or maintain CpG methylation (Dong *et al*, 2008; Epsztejn-Litman *et al*, 2008; Tachibana *et al*, 2008). Instead, several models have been proposed: G9a's ankyrin repeat region can directly interact with DNMT3A/B's catalytic domain (Epsztejn-Litman *et al*, 2008); alternatively, Ga9 may indirectly recruit DNMT3A/B, which can be bridged by either the HUSH complex member MPP8 (Chang *et al*, 2011) or UHRF1 (Meilinger *et al*, 2009). Collectively, these studies suggest that while DNMT3A/B do not harbor functional reader domains that recognize H3K9 methylation, they can be recruited either directly or indirectly by H3K9 methyltransferases to promote methylation and reinforce silencing at heterochromatin regions (Fig 2D).

Complex interplay between de novo CpG methylation and H3K27 methylation

Histone H3 lysine 27 methylation, established by the polycomb repressive complex 2 (PRC2), is a hallmark of facultative heterochromatin and associated with silencing of genes involved in cell cycle and differentiation (Comet *et al*, 2016). The enzymatic component EZH1/2, along with three core components (SUZ12, EED, and Rbbp4) and various accessary units of PRC2, catalyze the mono-, di-, and tri-methylation of H3K27 (Yu *et al*, 2019). PRC2 closely interacts and cooperates with polycomb repressive complex 1 (PRC1) for gene silencing: Canonical PRC1 recognizes H3K27me3 to mediate chromatin compaction and transcriptional repression, while variant PRC1 complex could initiate polycomb-mediated gene silencing through catalyzing H2AK119 ubiquitination (Holoch & Margueron, 2017).

The relationship between DNA methylation and H3K27 methylation is complex and highly dynamic. Genome-wide studies in stem cells and cancer cells demonstrate that H3K27me3 and DNA methylation are anti-correlated and have no obvious co-localization (Kondo *et al*, 2008; Meissner *et al*, 2008; Lister *et al*, 2009; Hon *et al*, 2012; Fu *et al*, 2020). This is particularly evident at the so-called DNA methylation valleys or canyons—large conserved genomic regions with very low (< 10%) levels of CpG methylation (Xie *et al*, 2013; Jeong *et al*, 2014). These domains are highly enriched for H3K27me3 and binding of polycomb proteins and recently have been shown to form megabase-long chromatin contact loops (Zhang *et al*, 2020). The overall mutual exclusivity between DNA and H3K27 methylation could be attributed to a role of CpG methylation in antagonizing PRC2. In multiple cell types, depletion of CpG methylation via the deletion of various DNMTs leads to pervasive increases of H3K27me3 at previously methylated regions of the genome (Brinkman *et al*, 2012; Lynch *et al*, 2012; Reddington *et al*, 2013). Similarly, in the transitioning of mESCs from naïve to 2i-induced ground state (Ying *et al*, 2008), there is a marked decrease in DNA methylation that is accompanied by a gain of H3K27me3 (van Mierlo *et al*, 2019). Interestingly, these genome-wide increases and redistributions of H3K27me3 can result in a "titration" effect on the localization of PRC1 and therefore have a negative impact on polycomb-mediated gene silencing (Reddington *et al*, 2013) or 3D loop formation (McLaughlin *et al*, 2019). Conversely, re-expressing wild-type but not catalytically dead DNMTs in methylation-deficient mESCs restore patterns of H3K27me3 as well as activity of H3K27ac-associated enhancers (King *et al*, 2016). Importantly, by inserting artificial sequences into the genome, two studies identified the *cis*-elements that are sufficient to induce accumulation of H3K27me3 and found that CpG methylation directly counteracts H3K27me3 recruitment (Jermann *et al*, 2014; Wachter *et al*, 2014). These results are consistent with prior studies *in vitro* showing that CpG methylation of nucleosomal DNA inhibits PRC2 binding to H3K27me3-marked nucleosomes (Bartke *et al*, 2010; Wu *et al*, 2010). Indirect mechanisms are also likely to be at play. For example, MTF2, an accessary subunit of PRC2, binds to CpG islands in a methylation-dependent manner (Perino *et al*, 2018). Similarly, KDM2B, a component of the variant PRC1.1 that facilitates the recruitment of PRC2 (Holoch & Margueron, 2017), carries a CXXC domain that recognizes unmethylated CpGs (Blackledge *et al*, 2010). Finally, while less characterized, studies have suggested a reciprocal role of PRC2 in opposing CpG methylation. For example, deletion of

EED leads to aberrant accumulation of CpG methylation in DNA methylation valleys in mESCs (Li *et al*, 2018c). This effect is recapitulated by triple knockout of TET1-3 enzymes, consistent with the observation that PRC2 directly interacts with TET1 and regulates levels of 5-hydroxymethylcytosine (Neri *et al*, 2013a).

It should be noted, however, that despite the well-documented antagonism between H3K27 and DNA methylation, these two marks can co-occur in low CpG regions in more differentiated cells (Statham *et al*, 2012). Moreover, dynamic switch from H3K27me3-marked to DNA-methylated CpG islands has been observed at some imprinted genes during embryonic development (Chen *et al*, 2019) and at pluripotency and germline-specific genes during mESC-to-neuron differentiation (Mohn *et al*, 2008). The molecular mechanisms underlying these transitions remain poorly understood. PRC2 may directly recruit DNMT1 and DNMT3A/B (Viré *et al*, 2006). It was also suggested that DNMT3L competes with DNMT3A/B for the binding to PRC2 (Neri *et al*, 2013b). Hence, variations in DNMT3L expression could represent a mechanism for controlling *de novo* methylation of PRC2 target genes. Consistently, the long isoform of DNMT3A, DNMT3A1, preferentially localizes to H3K27me3/H3K4me3 bivalent CpG islands in mESCs (Manzo *et al*, 2017). Interestingly, aberrant CpG methylation of polycomb target genes has been observed when the PWWP domain of DNMT3A is inactivated (Heyn *et al*, 2019; Sendžikaitė *et al*, 2019) or when DNMT3B is overexpressed (Zhang *et al*, 2018a). Since the levels of H3K36 methylation are low at CpG islands (Blackledge *et al*, 2010), it appears that the interaction between H3K36me2/3-PWWP domain could normally serve to "trap" DNMT3A/B away from PRC2-regulated CpG islands.

Interplay of DNA and histone methylation in disease development and therapy

Developmental disorders

DNMT3B and ICF syndrome

Consistent with the importance of CpG methylation in development, germline mutations in all three DNMTs and TET3 have been associated with human congenital disorders (Hansen *et al*, 1999; Klein *et al*, 2011; Winkelmann *et al*, 2012; Tatton-Brown *et al*, 2014; Heyn *et al*, 2019; Beck *et al*, 2020). DNMT3B is the first DNA-modifying enzymes implicated in human diseases (Hansen *et al*, 1999). Genetic alterations in DNMT3B are found in patients with immunodeficiency, centromeric instability, facial anomalies syndrome (ICF; OMIM 602900). ICF syndrome is a rare, autosomal recessive disorder characterized by distinct facial features, absence of B and plasma cells and chromosome instability, often leading to death during early childhood. The majority of ICF-associated DNMT3B mutations are loss of function, including nonsense, splice-site, or missense mutations within the catalytic domain, consistent with hypomethylation of pericentromeric satellite 2 and 3 repeats in the genomes of affected patients (Jeanpierre *et al*, 1993). Intriguingly, homozygous missense mutations (S282P) in the PWWP domain of DNMT3B have been reported (Shirohzu *et al*, 2002), in agreement with a role of PWWP domain in mediating DNMT3B's localization to pericentromeric heterochromatin (Chen *et al*, 2004). Furthermore, the homologous (S277P) mutation in mESCs abolishes DNMT3B's interaction with H3K36me3 and intragenic localization (Baubec *et al*, 2015), and recent findings have shown that ICF-associated *DNMT3B* mutant cells exhibit defects in intragenic DNA methylation and mRNA splicing (Gatto *et al*, 2017). Therefore, DNA hypomethylation at both repeat regions and gene bodies could contribute to the etiology of ICF syndrome. In addition to DNMT3B, three genes have been linked to ICF syndrome: *ZBTB24*, *CDCA7*, and *HELLS* (Velasco *et al*, 2018). Patients carrying mutations in these genes have overlapping clinical features and share hypomethylated pericentromeric repeats with *DNMT3B*-mutant patients, suggesting their involvement in *de novo* CpG methylation. Indeed, in MEFs or mESCs deficient for HELLS (also known as LSH), maintenance of DNA methylation is not affected but establishing methylation at retroviral transgene is impaired (Zhu *et al*, 2006). HELLS directly interacts with DNMT3A/B in mESCs. In more differentiated cell types, it seems that HELLS recruits and cooperates with H3K9 methyltransferase G9a/GLP to promote methylation at select loci (Myant *et al*, 2011). A few patients with ICF syndrome-like feature do not have mutations in the known genes (Weemaes *et al*, 2013), and it would be interesting to examine whether any histone methylation modifiers are altered in these patients.

DNMT3A and Tatton-Brown–Rahman syndrome

Germline heterozygous mutations in DNMT3A, on the other hand, define Tatton-Brown–Rahman syndrome (TBRS, OMIM 615879) (Tatton-Brown *et al*, 2014). TBRS is characterized by tall stature, macrocephaly, intellectual disability, and distinctive craniofacial features. Intriguingly, TBRS belongs to a group of genetic disorders known as overgrowth and intellectual disability (OGID) syndromes, which share many overlapping clinical features (Tatton-Brown *et al*, 2017). Among them, germline deletions/mutations in NSD1 are associated with Sotos syndrome (OMIM 117550) (Kurotaki *et al*, 2002; Douglas *et al*, 2003). PRC2 complex members—EZH2, EED, and SUZ12—are associated with Weaver syndrome (OMIM 277590), Cohen–Gibson syndrome (OMIM 617561), and Weaver-like syndrome, respectively (Tatton-Brown *et al*, 2011; Gibson *et al*, 2012; Cohen & Gibson, 2016; Imagawa *et al*, 2017; Cyrus *et al*, 2019). Furthermore, germline duplications of 5q35 involving *NSD1* and missense mutations in the PWWP domain of *DNMT3A* correlate with opposite developmental characteristics, including dwarfism and microcephaly (Dikow *et al*, 2013; Rosenfeld *et al*, 2013; Heyn *et al*, 2019). These *DNMT3A* PWWP domain mutations (W330R and D333N) are putatively gain of function, as they impair DNMT3A-H3K36me2/H3K36me3 interaction yet cause DNA hypermethylation at key developmental genes enriched for H3K27me3 (Heyn *et al*, 2019; Sendžikaitė *et al*, 2019). The fact that alterations in NSD1, PRC2, and DNMT3A cause developmental disorders with considerable phenotypic overlap offers strong human genetic evidence in support of the molecular crosstalk between these chromatin enzymes (Deevy & Bracken, 2019). Indeed, consistent with the finding that H3K36me2 is required for DNMT3A targeting and intergenic DNA methylation (Weinberg *et al*, 2019; Xu *et al*, 2020a; Xu *et al*, 2020b), NSD1 mutations/deletions in Sotos patients are associated with a profound DNA hypomethylation phenotype in the blood (Choufani *et al*, 2015). Furthermore, TBRS-associated missense mutations in *DNMT3A* fail to interact with chromatin and cause protein instability (Heyn *et al*, 2019; Weinberg *et al*, 2019). The DNA methylomes from the blood of TBRS patients cluster closely with that of Sotos patients (Weinberg *et al*, 2019) and both display signatures of accelerated aging (Jeffries *et al*, 2019), suggesting

that impaired NSD1-H3K36me2-DNMT3A interplay represents a common mechanism underlying the pathogenesis of TBRS and Sotos syndrome. A similar mechanism could be at play for mutations in SETD2, which have been found in patients with "Sotos-like" syndromes (Luscan *et al*, 2014).

In contrast, OGID syndrome mutations in *EZH2*, *EED*, and *SUZ12* do not cause aberrant DNA hypomethylation found in TBRS and Sotos syndrome patients (Choufani *et al*, 2015; Martin-Herranz *et al*, 2019; Weinberg *et al*, 2019). Instead, as described above, patterns of DNA methylation could directly or indirectly affect PRC2 activity. Moreover, H3K36 methylation has a well-documented role in opposing H3K27me3. Nucleosomes decorated with H3K36me2/3 can directly inhibit PRC2 activity *in vitro* (Schmitges *et al*, 2011; Yuan *et al*, 2011). In multiple myeloma and acute lymphoblastic leukemia (ALL) with overexpression or gain-of-function mutations of NSD2, global increases in H3K36me2 are accompanied by a loss of H3K27me3 (Martinez-Garcia *et al*, 2011; Jaffe *et al*, 2013). Conversely, genetic ablation of *Nsd1* in mESCs or expression of an oncohistone H3.3K36M mutation leads to a reduction of H3K36me2 and a gain of H3K27me3 (Lu *et al*, 2016; Streubel *et al*, 2018). Similarly, in SETD2-deficient oocytes and NSD1-deficient sperms, in addition to abnormal CpG methylation there is also an "invasion" of H3K27me3 into former H3K36me2/3 territories (Xu *et al*, 2019; Shirane *et al*, 2020). Several accessary units of PRC2 contain "reader" domains for H3K36me3. PHF19 (PHD finger protein 19) and PHF1 are known to facilitate the recruitment of PRC2 through their Tudor domains (Hunkapiller *et al*, 2012). Both Tudor domains of PHF1 and PHF19 act as readers for H3K36me3, and it is believed that the recognition of H3K36me3 by PHF1/19 initiates PRC2 targeting, H3K27me3 deposition, and silencing of actively transcribed genes (Ballaré *et al*, 2012; Brien *et al*, 2012; Musselman *et al*, 2012; Cai *et al*, 2013). Moreover, a direct sensing mechanism of H3K36 methylation state by EZH2 has recently been reported (Jani *et al*, 2019). Biochemical and structural analysis shows that EZH2 contains a specific sensing pocket for H3K36 that allows the complex to distinguish between modified and unmodified H3K36 residues, altering enzymatic activity accordingly to preferentially methylate the unmodified nucleosome substrate. Interestingly, a Weaver syndrome-associated EZH2 mutation (K634E) renders the enzyme less sensitive to the inhibition by H3K36 methylation (Jani *et al*, 2019). Notably, the majority of mutations affecting *EZH2/EED/SUZ12* in OGID syndromes are missense. While studies have suggested that the mutations reduce the catalytic activity of PRC2 (Cohen *et al*, 2016; Imagawa *et al*, 2017), it remains to be determined if change-of-function mutations exist that alter the interaction between PRC2 and H3K36/DNA methylation.

Taken together, it is plausible that several OGID syndromes share a common etiology linked to the dysregulated crosstalk between NSD1, PRC2 and DNMT3A (Fig 3). Future efforts are required to validate this hypothesis and determine how the imbalance between these chromatin marks contributes to disease development. To this end, it is noteworthy that mice with heterozygous loss of *Ezh2*, *Eed*, *Suz12*, *Nsd1*, or *Dnmt3a* are overall developmentally normal, while homozygous knockouts lead to prenatal or neonatal lethality (Faust *et al*, 1995; Okano *et al*, 1999; O'Carroll *et al*, 2001; Rayasam *et al*, 2003; Pasini *et al*, 2004). Therefore, alternative models, such as tissue-specific knockout mice or patient-derived induced pluripotent stem cells, are needed to better model and study chromatin-related OGID syndromes.

Additional developmental disorders linked to abnormal DNA and histone methylation

Additional histone-modifying enzymes are linked to human congenital disorders which are associated with abnormal CpG methylation landscape. Loss-of-function mutations in *KMT2D* (also known as MLL2), an H3K4 methyltransferase, and *KDM6A* (also known as UTX), an H3K27 demethylase, are the primary cause of Kabuki syndrome (Ng *et al*, 2010; Lederer *et al*, 2012). Kabuki syndrome patients are characterized by developmental delays, congenital abnormalities, and globally altered CpG methylation (Sobreira *et al*, 2017). Mutations in H3K4 demethylase *KDM5C* (also known as JARID5C) are linked to X-linked intellectual disability (Iwase *et al*, 2007), and blood samples from patients carrying *KDM5C* mutations display CpG hypomethylation at several genomic loci (Grafodatskaya *et al*, 2013; Schenkel *et al*, 2018). Frameshift mutations affecting *HIST1H1E*, a gene encoding histone H1, are found in another OGID syndrome known as Rahman syndrome (Tatton-Brown *et al*, 2017; Takenouchi *et al*, 2018) which exhibits a specific DNA hypomethylation signature (Ciolfi *et al*, 2020). Future studies are required to determine whether the aberrant CpG methylation profiles associated with these disorders are directly caused by altered histone methylation or reflect patterns of gene expression. Nevertheless, the unique DNA methylation signatures can be reliable molecular biomarkers to classify genetic variants of uncertain significance (Aref-Eshghi *et al*, 2017, 2019; Choufani *et al*, 2020) and facilitate accurate diagnosis of clinically overlapping disorders caused by mutations in distinct chromatin enzymes (Aref-Eshghi *et al*, 2018).

Cancers

Dysregulation of DNA and histone methylation has been widely implicated in various types of human cancers. Whereas the mechanistic details are being actively investigated, several lines of evidence support an intimate interaction between DNA and histone methylation in driving cancer initiation and progression. First, like OGID syndromes, mutations in DNA- and histone-modifying enzymes are found in closely related cancer types, particularly within hematological malignancies. In myeloid neoplasms, *DNMT3A* and *TET2* are frequently mutated in acute myeloid leukemias (AML), myelodysplastic syndromes (MDSs), and myeloproliferative neoplasms (MPNs) (Delhommeau *et al*, 2009; Ley *et al*, 2010; Stegelmann *et al*, 2011; Haferlach *et al*, 2014). Inactivating mutations in *EZH2* are also frequently found in MDS and MPN (Ernst *et al*, 2010), whereas loss-of-function mutations in *SETD2* and chromosomal translocations of *NSD1* and *NSD3* were recurrently identified in acute leukemias (Jaju *et al*, 2001; Rosati *et al*, 2002; Zhu *et al*, 2014). In lymphoid malignancies, DNMT3A and TET2 mutations have been linked to adult acute lymphoblastic leukemias (ALL) (Grossmann *et al*, 2013) and T-cell lymphomas (TCL), in particular angioimmunoblastic T-cell lymphomas and peripheral T-cell lymphomas, not otherwise specified (PTCL, NOS) (Couronné *et al*, 2012; Lemonnier *et al*, 2012; Sakata-Yanagimoto *et al*, 2014). Gain-of-function mutations in NSD2 are found in 14% of *ETV6/RUNX1*-fusion pediatric ALL (Jaffe *et al*, 2013), and modifiers of histone methylation and acetylation (*KMT2D, SETD2*, *KMT2A*, *KDM6A*, *EP300*, *CREBBP*) are collectively

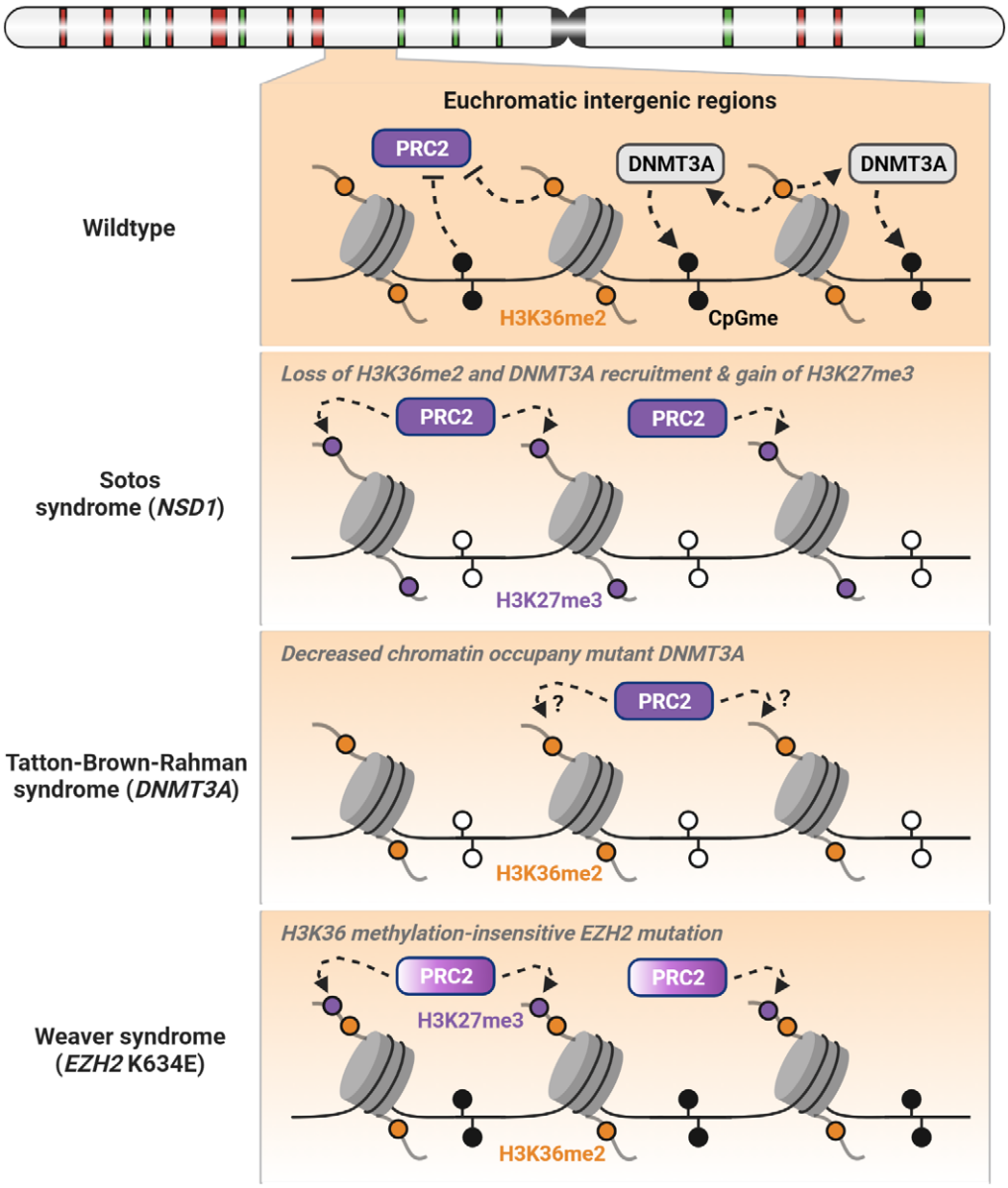

Figure 3. Dysregulated interplay between DNA and histone methylation in human OGID syndromes.

During normal development, H3K36me2 facilitates the deposition of CpG methylation by recruiting DNMT3A at euchromatic intergenic regions, and these two modifications act together to antagonize PRC2 and H3K27me3. In Soto syndrome, *NSD1* mutations and deletions lead to reduced H3K36me2 and CpG methylation, and a resulting gain of H3K27me3. In TBRS, loss-of-function mutations in *DNMT3A* reduce CpG methylation, although its impact on H3K36me2 and H3K27me3 is unclear. Some Weaver syndrome patients carry missense mutation of EZH2 (e.g., K634E) that renders the PRC2 insensitive to inhibition by H3K36 methylation, which could potentially leads to accumulation of H3K27me3 at intergenic regions despite the presence of H3K36me2. The significant overlap in clinical features of Soto, Weaver, and TRBS patients suggests that an imbalance of H3K36me2, H3K27me3, and CpG methylation could represent a common pathogenic mechanism.

mutated in up to 36% of patients with PTCL, NOS (Ji *et al*, 2018; Watatani *et al*, 2019). On the other hand, mutations in *KMT2C*, *KMT2D*, and *EZH2* are common events in diffuse large B-cell lymphomas (DLBCLs) (Morin *et al*, 2010, 2011), where *DNMT3A* and *TET2* mutations have also been reported albeit at lower frequencies (Asmar *et al*, 2013; Reddy *et al*, 2017). Furthermore, neomorphic mutations in the genes encoding metabolic enzymes IDH1 and IDH2, which produce an oncometabolite 2-hydroxyglutarate that competitive inhibits both histone and DNA demethylation (Figueroa *et al*, 2010; Lu *et al*, 2012), are frequently found in AML and TCL (Mardis *et al*, 2009; Cairns *et al*, 2012). The widespread mutations affecting DNA/histone methylation machinery genes are consistent with the findings that genetic perturbations of these enzymes result in abnormal hematopoietic self-renewal and differentiation (Challen *et al*, 2011; Mochizuki-Kashio *et al*, 2011; Zhang *et al*, 2018b; Leonards *et al*, 2020), and epigenetic drugs, such as inhibitors of DNA methyltransferase, histone deacetylases, and IDH1/2, are approved to treat leukemias and lymphomas (Bates, 2020). Future investigations are required to

address whether, and how, alterations in histone and DNA methylation pathways act redundantly or cooperatively to drive hematopoietic malignancies.

Second, although somatic mutations affecting DNMTs and TETs are rare in solid tumors, mutations in histone-modifying enzymes and histones themselves can have a direct effect on reprogramming DNA methylome. As described above, *NSD1* mutations and deletions in squamous cell carcinomas result in global DNA hypomethylation as a consequence of defective DNMT3A recruitment by H3K36me2 (Lee & Wiemels, 2016; Brennan *et al*, 2017; Papillon-Cavanagh *et al*, 2017; Bui *et al*, 2018; Weinberg *et al*, 2019; Farhangdoost *et al*, 2021). Histone H3 lysine 36 to methionine (H3K36M) mutations deplete H3K36 methylation by inhibiting the catalytic activities of H3K36 methyltransferases, and H3K36M mutations are mutually exclusive with *NSD1* loss-of-function mutations in head and neck squamous cell carcinomas (Lu *et al*, 2016; Papillon-Cavanagh *et al*, 2017). Consistently, H3K36M mutation also leads to global decreases in DNA methylation (Rajagopalan *et al*, 2021). In contrast, *SETD2* mutations in renal cell carcinoma and other cancer types are associated with global DNA hypermethylation (Tiedemann *et al*, 2016). This unexpected finding could be recapitulated in cellular models and is hypothesized to result from ectopic gain of H3K36me3 at large intergenic regions upon SETD2 inactivation. While counterintuitive, similar observation has been made in female germline following *Setd2* deletion (Xu *et al*, 2019). Genetic inactivation of PRC2 complex is associated with malignant peripheral nerve sheath tumors (MPNST), and Wojcik *et al* (2019) demonstrated that PRC2-null MPNST cells exhibit genome-wide hypermethylation (Wojcik *et al*, 2019). This is likely due to a decreased ratio of H3K27me3/H3K36me2, as knockdown of NSD2 could reverse the changes in gene expression by PRC2 inactivation. Interestingly, oncohistone H3 lysine 27 to methionine (H3K27M) mutation—found in diffuse intrinsic pontine gliomas (DIPGs)—dominantly inhibits PRC2 activity and reduces global H3K27me3 yet results in DNA hypomethylation (Bender *et al*, 2013; Lewis *et al*, 2013). Furthermore, *EZHIP/CXORF67*, a germline-specific gene, encodes a protein that binds to and blocks PRC2 spreading from H3K27me3-occupied CpG islands through an H3K27M-like mechanism (Jain *et al*, 2019; Ragazzini *et al*, 2019). *EZHIP* is silenced through promoter methylation in adult tissues, and its de-repression upon DNA hypomethylation results in PRC2 inhibition (Bayliss *et al*, 2016; Piunti *et al*, 2019). These findings again highlight the complex interplay between H3K27me3 and DNA methylation. Lastly, a recent study points to a role of H3K4me1 in predisposing CpG island methylation during cancer progression (Skvortsova *et al*, 2019). In breast and prostate cancers, the invasion of methylation at the edges of CpG island is linked to enrichment of H3K4me1. Inactivation of *KMT2D* leads to a concurrent loss of H3K4me1 and CpG methylation. It would be interesting to determine the impact of DNA methylation reprogramming on gene expression and tumor development driven by mutations in HMTs and, consequently, whether these tumors display altered sensitivity to DNA hypomethylating therapies.

Third, altered DNA methylation is a well-established pan-cancer molecular hallmark. DNA hypomethylation across intergenic regions and DNA hypermethylation at promoter CpG islands have been described in many cancer contexts, independently of specific genetic mutations and tissue types (Baylin & Jones, 2016). Interestingly, both features can be predicted from pre-existing histone methylation marks in corresponding normal cells (Fig 4). Promoter CpG islands that gain DNA methylation in cancers are marked by H3K27me3 in embryonic or tissue stem/progenitor cells (Ohm *et al*, 2007; Schlesinger *et al*, 2007; Widschwendter *et al*, 2007; McGarvey *et al*, 2008). More recently, it was shown that the ratio of H3K27me3 to H3K4me3 at bivalent promoters can predict the likelihood of cancer-associated DNA hypermethylation (Dunican *et al*, 2020). On the other hand, the loss of DNA methylation in cancers is found to mainly occur at partially methylated domains (PMDs) (Zhou *et al*, 2018). PMDs are associated with nuclear lamina and late-replicating regions (Berman *et al*, 2012). Recent evidence suggests that DNA hypomethylation at PMDs is coupled to mitotic cell division (possibly due to ineffective maintenance methylation) and gain of heterochromatic histone marks such as H3K9me3 (DEEP Consortium *et al*, 2018; Zhou *et al*, 2018). In contrast, regions of H3K36me3 where *de novo* DNMTs are targeted are protected from progressive hypomethylation (Zhou *et al*, 2018). Collectively, these correlative studies raise an intriguing possibility that by comparing DNA methylomes of cancer and histone methylomes of normal cells, one may be able to delineate the potential tissues and cells of origins for certain difficult-to-diagnose tumors.

Therapeutic implication

The close interplay between DNA and histone methylation enzymes in development and human diseases represents an opportunity for designing new therapeutic approaches. One strategy is to develop inhibitors of the "reader" domains that are key for enzyme interaction and activation. For example, two studies screened for inhibitors that prevent the binding between UHRF1's TTD-PHD finger and H3K9me3 (Houliston *et al*, 2017; Senisterra *et al*, 2018). These compounds can be used as powerful chemical probes and have the potential to become a new class of DNA hypomethylating agents with distinct mechanisms of action from that of catalytic inhibitors of DNMT1 for cancer therapy. Chemical probes for the NSD2-PWWP and NSD3-PWWP domains were recently reported (Böttcher *et al*, 2019), and similar approaches could be adopted to identify inhibitors of the DNMT3A/B PWWP domains to modulate *de novo* DNA methylation. Compared to inhibitors targeting the catalytic domains, reader domain inhibitors are expected to specifically correct disease-associated mislocalization of methyltransferases while leaving their normal function unaffected, therefore providing a higher therapeutic index.

As another therapeutic strategy, combined inhibition of histone and DNA methylation has demonstrated synergy in cancer therapy. In gastric cancer, inhibition of EZH2 following DNA demethylation fully activates the expression of tumor suppressor *RUNX3*, suggesting a redundancy between H3K27me3 and CpG methylation in silencing certain genetic loci (Kodach *et al*, 2010). Indeed, in mESCs exposed to DNA hypomethylating culture condition, the initial de-repression of retrotransposons is quickly silenced by H3K27me3 (Walter *et al*, 2016). Similarly, in breast cancer cells, global DNA hypomethylation seems to be compensated by gains in H3K9me3 and H3K27me3 with a remarkable allelic-specific mutual exclusivity between these epigenetic marks (Hon *et al*, 2012). Accordingly, in colorectal cancer cells or chemotherapy-resistant breast cancer lines, genetic or pharmacological inhibition of H3K27 or H3K9 methyltransferases significantly augments the effects of DNA hypomethylation on de-repressing retrotransposons (Ohtani

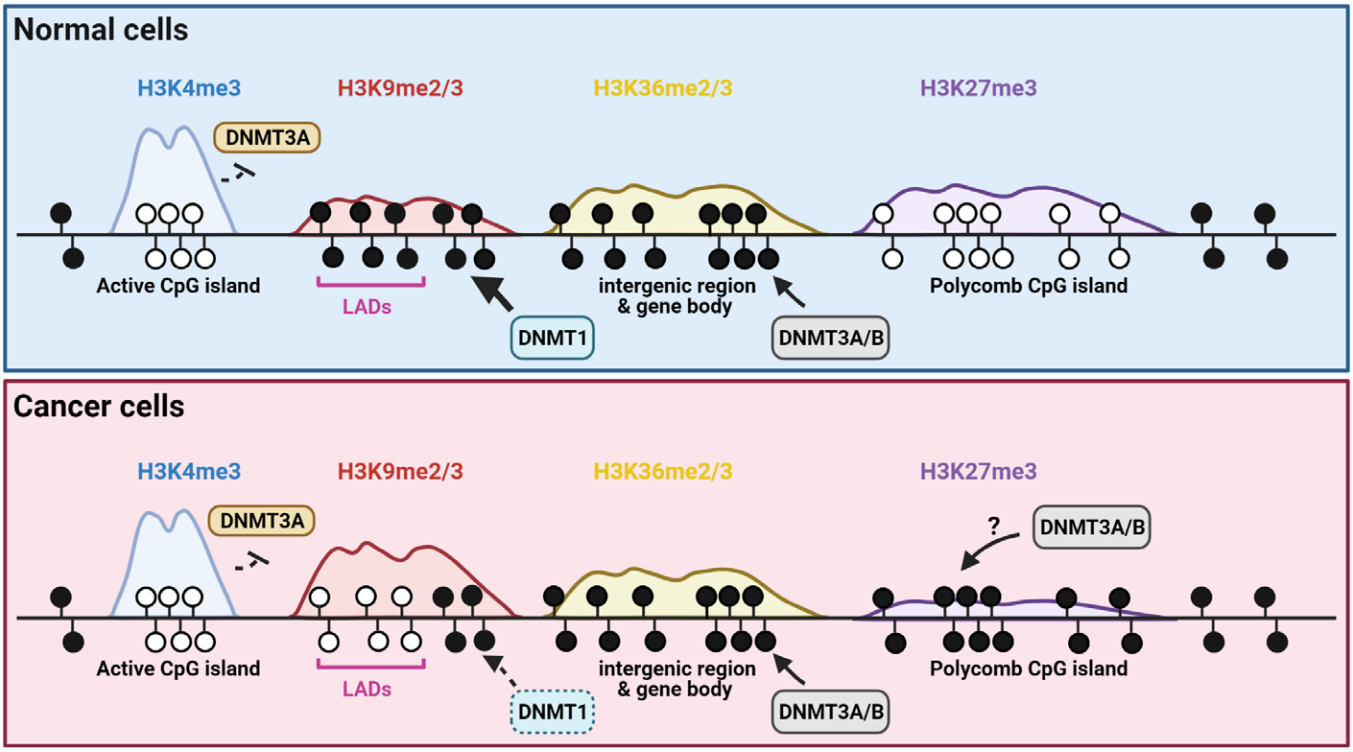

Figure 4. Reprogramming of DNA methylation during cancer progression.

The transition from normal to cancerous state is associated with changes in genome-wide patterns of DNA methylation. While promoter CpG islands of active genes marked by H3K4me3 remain free of DNA methylation, polycomb-regulated promoter CpG islands become hypermethylated, possibly due to aberrant targeting of DNMT3A/B through an unknown mechanism. The gene-poor, H3K9 methylation-rich, late-replicating lamina-associated domains undergo progressive loss of maintenance methylation during cancer cell replication. Gene body and intergenic regions marked by H3K36 methylation are protected from such mitotically linked DNA hypomethylation, presumably due to the preferential targeting and activity of *de novo* methyltransferases DNMT3A/B.

et al, 2018; Deblois *et al*, 2020). Since the expression of these transposable elements induces a "viral mimicry" that increases tumor infiltration (Chiappinelli *et al*, 2016), combined treatment of repressive HMTs and DNMTs may represent an effective strategy to sensitize poorly immune-infiltrated tumors to immune checkpoint inhibitors.

Concluding remarks

It is increasingly evident that DNA and histone methylation marks cooperate to maintain patterns of epigenome in a stable and sometimes mitotically heritable manner. The molecular mechanisms, in large part, seem to involve the recognition (or the lack thereof) of chromatin modifications by the "reader" or "writer" domains of DNA- and histone-modifying enzymes. As exciting advances in chromatin biochemistry and epigenomics continue to surface, researchers are equipped with powerful tools to gain a more comprehensive and deeper appreciation of how these epigenetic marks communicate (see also Box 1).

First, the majority of biochemical and structural analysis thus far involves isolated domains of chromatin enzymes and histone tail peptides. However, as illustrated by studies of UHRF1, extensive inter-domain remodeling occurs during enzyme–histone engagement. Furthermore, since nucleosomes are the physiological substrates for chromatin-modifying enzymes, the use of histone peptides may lead to incomplete or misguided mechanistic insights (Vaughan *et al*, 2018). Recent innovations in synthesizing chemically modified "designer" nucleosomes and nucleosome arrays

Box 1. In need of answers.

i Are there additional functional domains in DNMTs and TET family enzymes that interact with modified histone to guide the genomic targeting of these DNA modifiers? Conversely, which histone-modifying complexes are sensitive to CpG methylation and how?

ii How do multiple functional domains of DNMT cooperate to recognize histone modifications in the nucleosome context? What is the underlying biochemical and structural basis?

iii How does the imbalance between NSD1/H3K36me2, PRC2/H3K27me3, and DNMT3A/CpG methylation contribute to dysregulated gene expression during development and OGID syndromes?

iv Does aberrant histone–DNA methylation crosstalk contribute to cancer-associated chromatin abnormalities? If yes, how?

v What is the therapeutic potential for inhibitors of "reader" domains of DNA and histone methylation? How do we rationally combine DNA and histone methyltransferase inhibitors as more effective treatment strategy for human cancer and developmental diseases?

provide ways to overcome this limitation (Holt & Muir, 2015). In addition, the revolution of cryogenic electron microscopy (cryo-EM) has offered unprecedented opportunities to visualize the structure of fully assembled large chromatin complexes bound to nucleosomes (Jang & Song, 2019), such as the recently reported structure of DNMT3A2-DNMT3B3-nucleosome (Xu *et al*, 2020). We anticipate that these technologies will help reveal critical new insights into the molecular basis of DNA-histone methylation crosstalk and uncover new functional domains, such as the recently described nuclear localization signal within the N-terminal domain of DNMT3A1 (Zeng *et al*, 2020).

Second, our study of the histone and DNA methylation interplay is heavily based on bulk tissue cultures which offer limited physiological relevance. However, the recent explosion of new epigenome-profiling technologies, such as CUT&RUN and CUT&Tag, enables analysis of genome-wide histone modifications with extremely low cell numbers or even single cell (Ai *et al*, 2019; Carter *et al*, 2019; Hainer *et al*, 2019; Kaya-Okur *et al*, 2019). Single-cell CpG methylome analysis is also possible (Karemaker & Vermeulen, 2018). With these tools, we are now able to profile the epigenome in rare cell populations such as early embryos and adult tissue stem cells. Furthermore, we expect the rapid development of technology for simultaneous single-cell analysis of histone and CpG methylation, which will allow interrogation of their interplay at unprecedented scale and resolution.

Finally, we look forward to the discovery and optimization of highly specific inhibitors that block the binding of chromatin "reader" domains to DNA and histone methylation. These inhibitors will serve as powerful probes to study the kinetics of chromatin interplay at high temporal resolution. Furthermore, compared to inhibitors of catalytic domains, "reader" domain inhibitors may be more effective in correcting mis-targeting of chromatin enzyme activities, thus serving as potent and less toxic drug candidates for human developmental disorders and cancers driven by epigenetic abnormality.

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Conflict of interest

The authors declare that they have no conflict of interest.

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