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Review

Structural basis of DNMT1- and DNMT3A-mediated DNA methylation

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Abstract: DNA methylation, one of the major epigenetic mechanisms, plays critical roles in regulating gene expression, genomic stability and cell lineage commitment. Establishment and maintenance of DNA methylation in mammals is achieved by two groups of DNA methyltransferases: DNMT3A and DNMT3B, which are responsible for installing DNA methylation patterns during gametogenesis and early embryogenesis, and DNMT1, which is essential for propagating DNA methylation patterns during replication. Both groups of DNMTs are multi-modular proteins, containing a large N-terminal regulatory region in addition to the C-terminal methyltransferase domain. Recent structure-function investigations of the individual domains or large fragments of DNMT1 and DNMT3A have revealed the molecular basis for their substrate recognition and specificity, intramolecular domain-domain interactions, as well as their crosstalk with other epigenetic mechanisms. These studies highlight a multifaceted regulation for both DNMT1 and DNMT3A/3B, which is essential for the precise establishment and maintenance of lineage-specific DNA methylation patterns in cells. This review summarizes current understanding of the structure and mechanism of DNMT1- and DNMT3A-mediated DNA methylation, with emphasis on the functional cooperation between the methyltransferase and regulatory domains.

Keywords: DNMT1; DNMT3A; DNA methyltransferase; maintenance DNA methylation; de novo DNA methylation; allosteric regulation; autoinhibition

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1. Introduction

DNA methylation represents one of the major epigenetic mechanisms that critically influence gene expression and cell fate commitment [1,2,3,4,5,6]. In mammals, DNA methylation is essential for silencing of retrotransposons [7,8,9], genomic imprinting [10,11] and X-chromosome inactivation [12,13]. Mammalian DNA methylation predominantly occurs at the C-5 position of cytosine within the CpG dinucleotide context, accounting for ~70-80% CpG sites throughout the genome [14]. Establishment of DNA methylation is achieved by the closely related DNA methyltransferases 3A (DNMT3A) and 3B (DNMT3B), designated as de novo DNA methyltransferases, during germ cell development and early embryogenesis [15,16]. Subsequently, clonal transmission of specific DNA methylation patterns is mainly mediated by DNA methyltransferase 1 (DNMT1), designated as maintenance DNA methyltransferase, in a replication-dependent manner [17,18]. However, the classification of DNMT3A/3B as de novo methyltransferases and DNMT1 as maintenance DNA methyltransferase appears an oversimplification, as increasing evidence has revealed an important role of DNMT3A and DNMT3B in DNA methylation maintenance [19,20], while other studies have pointed to the de novo methylation activity of DNMT1 in specific loci [21,22]. A detailed understanding of the structure and regulation of DNMT1 and DNMT3A/3B is essential for elucidating their roles in DNA methylation establishment and maintenance in cells.

Both DNMT1 and DNMT3A/3B belong to the class I methyltransferase family [23], featured by a conserved catalytic core termed Rossmann fold, which consists of a mixed seven-stranded β-sheet flanked by three α-helices on either side [24]. These enzymes catalyze the methylation reaction in an S-adenosyl-L-methionine (AdoMet)-dependent manner, with the catalytic core harboring essential motifs for enzymatic catalysis and cofactor binding. In addition, a subdomain, termed target recognition domain (TRD), is inserted between the central β-sheet and the last α-helix of the catalytic core [24]. The TRD bears no sequence similarity between DNMT1 and DNMT3s; instead, it participates in DNA binding to ensure substrate specificity of each enzyme.

To ensure proper programming of DNA methylation patterns in cell linage commitment, the functions of DNMTs are subject to a stringent regulation during development [25,26]. Unlike their bacterial counterparts that contain only one methyltransferase (MTase) domain, both DNMT1 and

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DNMT3s are multi-modular proteins, containing a large regulatory region in addition to the C-terminal MTase domain (Fig. 1) [18,27]. Recent studies have generated a large body of structural and functional information on both groups of enzymes, including the molecular basis underlying their enzyme-substrate recognition, and the regulatory roles of their N-terminal segments in the substrate specificity, enzymatic activity as well as genomic targeting. This review provides an overview on the recent progress in structural and mechanistic understanding of DNMT1 and DNMT3A, with emphasis on how the regulatory and MTase domains of each enzyme cooperate in maintenance and de novo DNA methylation, respectively.

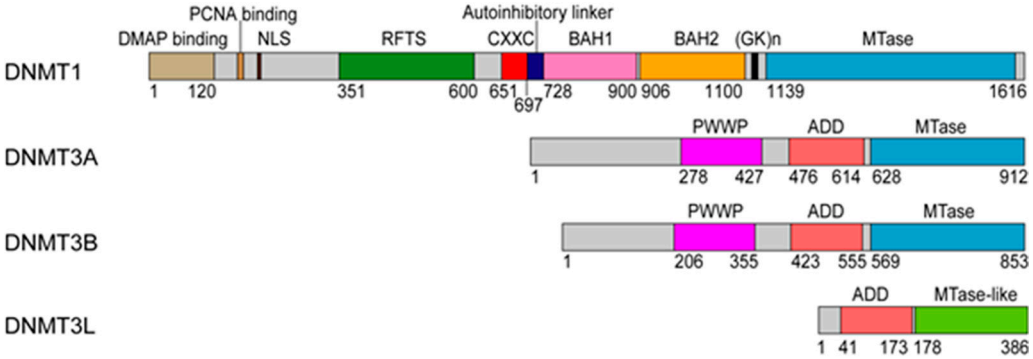

Figure 1. Domain architectures of human DNMT1, DNMT3A, DNMT3B and DNMT3L, with individual domains marked by residue numbers.

2. Structure and mechanism of DNMT1

DNMT1 is comprised of ~1600 amino acids, with an N-terminal regulatory region covering two thirds of the sequence, a highly conserved (GK)n repeat and a C-terminal MTase domain (Fig. 1). The regulatory region starts with a ~300 amino acid-long N-terminal domain (NTD) harboring a variety of protein and/or DNA interaction sites, followed by a replication foci-targeting sequence (RFTS) domain, a CXXC zinc finger domain and a pair of bromo-adjacent-homology (BAH) domains (Figure 1). The function of DNMT1 in replication-dependent DNA methylation maintenance is supported by its localization in replication foci during S phase, and in vitro a 3-40 fold enzymatic preference for hemimethylated CpG sites [18,28], an epigenetic mark enriched at replication foci [29]. How the regulatory domains of DNMT1 are coordinated in attaining its enzymatic and spatiotemporal regulations remains a long-lasting topic of interest. Nevertheless, recent structure-function study of various DNMT1 fragments under different DNA binding states

[30,31,32,33] have started to illuminate how different domains of this enzyme orchestrate its activity in maintenance DNA methylation.

Enzyme-substrate interaction of DNMT1. The crystal structure of a mouse DNMT1 fragment (mDNMT1, residues 731-1602) covalently bound to a 12-mer hemimethylated DNA duplex provides insight into the productive state of DNMT1 (Fig. 2A) [31]. The DNA molecule contains one central CpG site in which a 5-methylcytosine (5mC) and a 5-fluorocytosine (5fC) were installed on the template strand and target strand, respectively (Fig. 2B). The use of 5fC permits the formation of an irreversible, covalent complex between mDNMT1 and DNA [34]. The mDNMT1 fragment contains the pair of BAH domains (BAH1, BAH2) and the MTase domain.

The structure of mDNMT1 – DNA covalent complex reveals that the MTase domain, composed of a catalytic core and a large TRD (~200 amino acids), is organized into two-lobe architecture, creating a cleft to harbor the DNA duplex (Fig. 2A). The two BAH domains are separated by one α-helix, both with a tilted β-barrel fold that is reminiscent of other BAH domains (Fig. 2A) [35]. Both BAH domains are structurally associated with the MTase domain, forming an integrated structural unit. The BAH1 domain is attached to the MTase domain through antiparallel β-pairing as well as hydrophobic clustering, while the BAH2 domain interacts with the MTase domain mainly through hydrophobic contacts, with a long loop (BAH2-loop) protruding from one end of the β-barrel to join with the TRD at the tip (Fig. 2A). This mDNMT1 construct also contains two Cys3His-coordinated zinc finger clusters, one located in the TRD while the other associating BAH1 with the following α-helix (Fig. 2A). The mDNMT1 – DNA interaction spans eight base pairs, resulting in a buried surface area of ~2100 Å2. The target cytosine, 5fC, is flipped out of the DNA duplex and inserts into the active site of mDNMT1, whereby it forms a covalent linkage with the catalytic cysteine C1229, and hydrogen bonding interactions with a number of highly conserved residues, including R1313, R1315 and E1269 (Fig. 2C). The base flipping of 5fC creates a large cavity at the hemimethylated CpG site, which is in turn filled with bulky side chains of K1537 from the TRD and W1512 from the catalytic core (Fig. 2B). This protein – DNA intercalation further shifts the orphan guanine, which was otherwise paired with the flipped-out 5fC, one base down, resulting in the flipping out of a second nucleotide from the template strand (Fig. 2B). The interaction of mDNMT1 with the

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143 hemimethylated CpG site involves two loops from the TRD (TRD loop I: residues 1501-1516 and
144 TRD loop II: residues 1530-1537) and one loop from the catalytic site (catalytic loop: residues
145 1227-1243). Toward the DNA major groove, residues C1501, L1502, W1512, L1515 and M1535 from
146 TRD loop I form a concave hydrophobic surface, harboring the methyl group of 5mC (Fig. 2D). On
147 the other hand, residues M1535, K1537 and Q1538 from TRD loop II engage in base-specific
148 hydrogen bonding interactions with the CpG site (Fig. 2E). On the minor groove side, residues
149 G1234, N1236 and R1237 also form base-specific contacts with the CpG sites through hydrogen
150 bonding interactions (Fig. 2E). In addition, residues from both the TRD and catalytic core are
151 involved in salt bridge or hydrogen bonding interactions with the DNA backbone. The two BAH
152 domains are positioned distant to the DNA binding site. Nevertheless, residues from the tip of the
153 BAH2-loop contribute to the DNA binding through hydrogen bonding interactions with the DNA
154 backbone of the target strand (Fig. 2A).

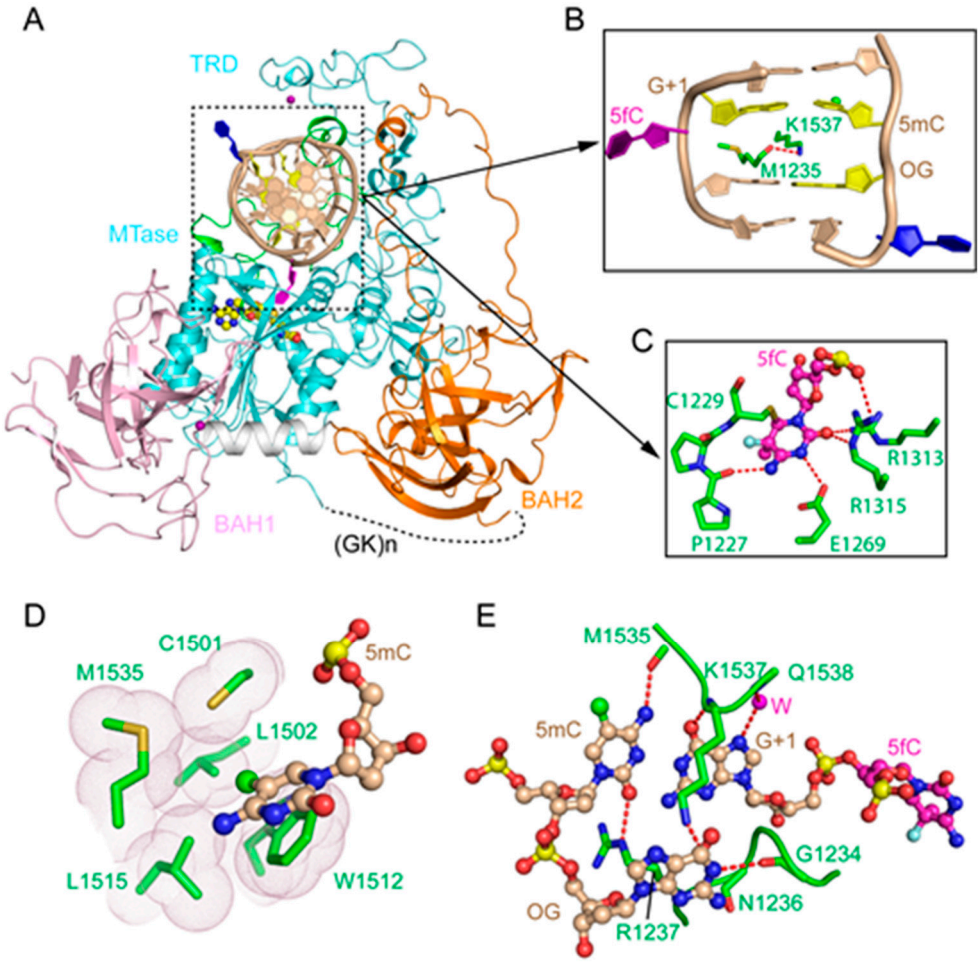

Figure 2. Structure of mDNMT1 – DNA productive complex. (A) Structural overview of mDNMT1 (731-1602) covalently bound to hemimethylated DNA. The zinc ions are shown in purple spheres. 5fC and another flipped-out cytosine from the template strand are colored in purple and blue, respectively. (B) The DNA cavity vacated by the base flipping is filled with mDNMT1 residues M1235 and K1537. (C) The flipped-out 5fC is surrounded by active site residues through covalent linkage or hydrogen bonding interactions. (D) Residues from the TRD loop II form a hydrophobic groove harboring the methyl group from 5mC. (E) CpG-specific interactions by the TRD loop I and the catalytic loop.

In summary, the structure of the productive mDNMT1 – DNA complex provides molecular basis for the substrate recognition of DNMT1. The extensive protein – DNA contacts underlie the processive methylation kinetics of this enzyme [36,37]. More importantly, it offers explanations on the strict substrate specificity of DNMT1 on the CpG sites, as well as on the marked substrate preference of DNMT1 toward hemimethylated CpG sites [18,28].

CXXC domain-mediated autoinhibition of DNMT1. The CXXC domain of DNMT1 belongs to one family of zinc finger domains that specifically bind to unmethylated CpG-containing DNA [30,38]. It manifests in a crescent-like fold, with two zinc finger clusters formed by conserved CXXCXXC motifs in cooperation with distal cysteines. The crystal structure of an mDNMT1 fragment (residues 650-1602), spanning from the CXXC domain to the MTase domain, in complex with a 19-mer DNA duplex containing unmethylated CpG sites provides insight into the functional role of this domain (Fig. 3A) [30]. In the structure, the CXXC domain is positioned on the opposite side of the MTase domain from the BAH domains, with a long CXXC – BAH1 domain linker (a.k.a autoinhibitory linker) running across the catalytic cleft (Fig. 3A). The mDNMT1 – unmethylated DNA complex contains two separate DNA-binding interfaces, one located in the CXXC domain and the other located in the MTase domain. At one end of the DNA, the CXXC domain interacts with the DNA molecule from both major groove and minor groove, with a loop segment (R684-S685-K686-Q687) penetrating into the CpG site for base-specific contacts (Fig. 3B,C). Notably, residues K686 and Q687 form side-chain hydrogen bonds with the two guanines on the CpG site, while the backbone carbonyls of Q687 and K688 are hydrogen bonded to the bases of two cytosine nucleotides, respectively (Fig. 3C). At the other end of DNA, the MTase domain interacts with the DNA backbone

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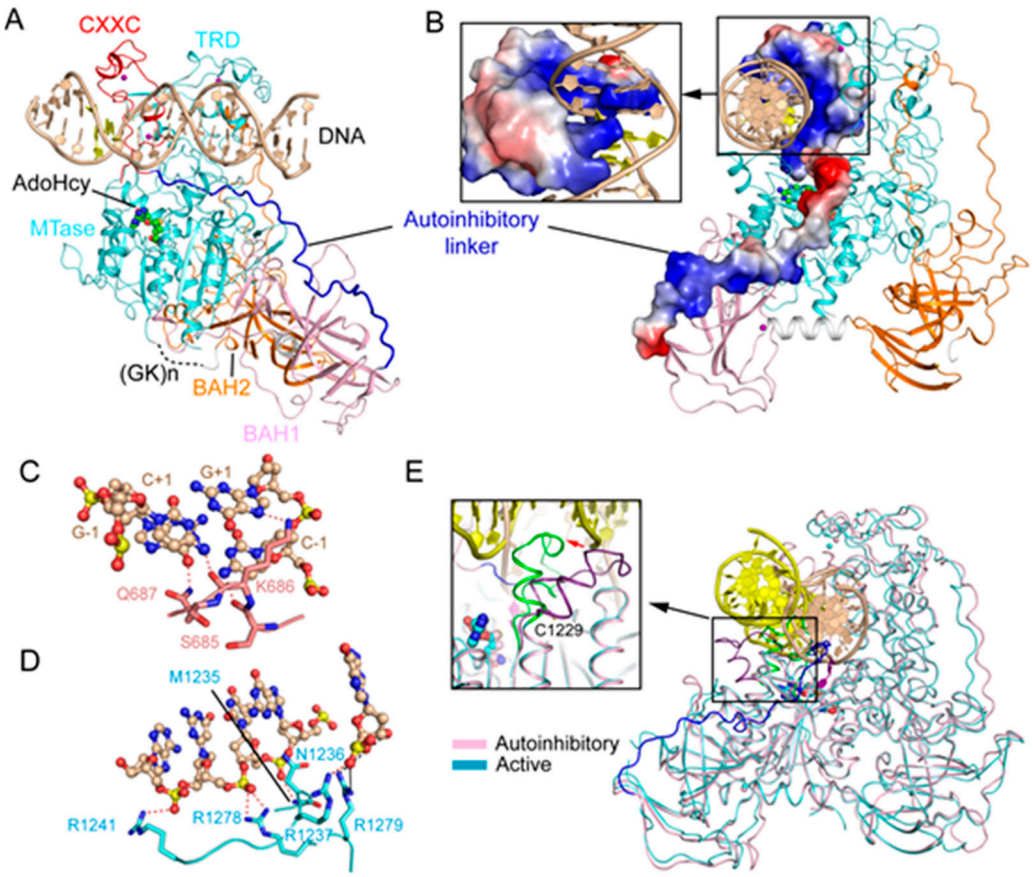

Figure 3. Structural analysis of the CXXC domain-mediated DNMT1 autoinhibition. (A) Structural overview of mDNMT1 (650-1602) bound to a 19-mer DNA duplex containing unmethylated CpG sites. (B) Surface views of the CXXC domain and the autoinhibitory linker in the complex of mDNMT1 with ummethylated CpG DNA. (C) base-specific interactions between the CXXC domain and the CpG site. The hydrogen bonding interactions are depicted as dashed lines. (D) The MTase – DNA interactions in the autoinhibitory complex. (E) Structural overlay between the active (light blue) and autoinhibitory (pink) complexes of mDNMT1, with the catalytic loops highlighted in the expanded view.

through the C-terminal portion of the catalytic loop (residues M1235, R1237 and R1241) and the adjacent α-helix (R1278 and R1279) (Fig. 3D). These protein – DNA interactions together localize the DNA molecule outside the catalytic cleft, resulting in an autoinhibitory conformation of DNMT1. Structural comparison of the autoinhibitory and active states of mDNMT1 reveals that the largest conformational change of mDNMT1 lies in the catalytic loop, which is poised in a retracted conformation in the autoinhibitory state but penetrates into the DNA minor groove in the active state (Fig. 3E). Furthermore, the α-helix following the catalytic loop undergoes a kinked-to-straight conformational transition, thereby regulating the contact between the catalytic loop and the DNA

minor groove (Fig. 3E). Indeed, a subsequent study indicates that disruption of this conformational transition leads to impaired enzymatic activity of DNMT1 [39], highlighting the importance of this conformational switch in DNMT1-mediated DNA methylation.

These structural observations therefore led to an autoinhibitory model of DNMT1: The CXXC domain specifically interacts with the unmethylated CpG site, which in turn stabilizes the positioning of the autoinhibitory linker over the catalytic cleft, leading to the extrusion of the unmethylated CpG DNA from the catalytic site. This model therefore assigns a regulatory role of the CXXC domain in inhibiting the de novo methylation activity of DNMT1, the major maintenance DNA methyltransferase. Indeed, enzymatic assays based on the mDNMT1(650-1602) constructs indicated that disruption of the CXXC – CpG interaction or deletion of the autoinhibitory linker both led to enhanced enzymatic activity of DNTM1 on unmethylated CpG DNA, but no significant change on hemimethylated substrates, lending support to the autoinhibitory mechanism. However, it is worth noting that a later study on full-length DNMT1 failed to identify any significant impact of the CXXC – DNA interaction on the substrate specificity of DNMT1 in vitro [40], suggesting that this autoinhibitory mechanism may need additional factors to ensure the substrate specificity of DNMT1.

RFTS domain-mediated autoinhibition of DNMT1. The crystal structures of DNA-free mouse and human DNMT1 fragments, spanning from the RFTS domain toward the MTase domain, reveal that the RFTS domain closely associates with the MTase domain, resulting in a compact fold (Fig. 4A) [32,33]. In both structures, the RFTS domain folds into two lobes, separated by a 24-amino acid long α-helix (Fig. 4A). The N-lobe is dominated by a zinc finger cluster, followed by a six-stranded β-barrel, while the C-lobe is assembled into a helical bundle (Fig. 4A). The N- and C-lobes form an acidic cleft, where the linker sequence downstream of the RFTS domain extends away from the RFTS domain (Fig. 4A). The intramolecular contact between the RFTS and MTase domains is underpinned by hydrogen bonding interactions between the residues from the C-lobe of RFTS (E531, D532, D548, D554, D583 and L593 in hDNMT1) and the residues from the TRD (N1493, T1503, Y1514, M1533, K1535 and R1574 in hDNMT1) (Fig. 4B), which partially overlap with the DNA binding surface of the TRD (Fig. 2A). The CXXC domain is positioned adjacent to the RFTS domain, adopting a

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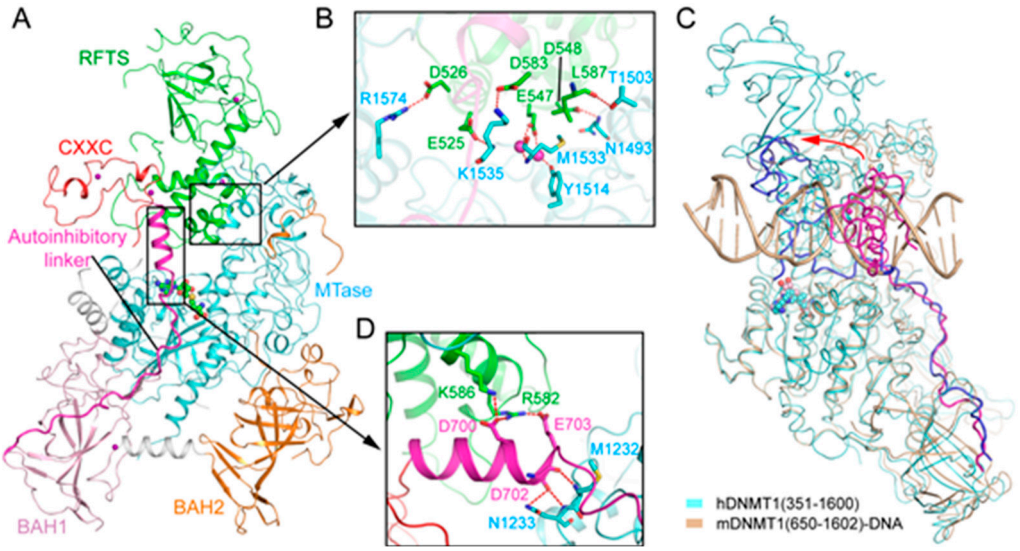

Figure 4. Structural analysis of the RFTS domain-mediated DNMT1 autoinhibition. (A) Structural overview of hDNMT1 (351-1602). (B) The intramolecular interactions between the RFTS (green) and MTase (aquamarine) domains. The hydrogen bonding interactions are depicted as dashed lines. The water molecules are shown as purple spheres. (C) Structural overlap between the CXXC- and RFTS-mediated autoinhibitory complexes, with the autoinhibitory linkers colored in blue and light magenta, respectively. The repositioning of the CXXC domain is indicated by a red arrow. (D) The interaction of the auto inhibitorylinker (magenta) with both the RFTS (green) and MTase domains.

213 conformation similar to its DNA-bound state (Fig. 4A). Structural comparison of free DNMT1 and its
214 unmethylated CpG DNA-bound state reveals a large conformational repositioning of the CXXC
215 domain: It sits on one side of the TRD in the structure of mDNMT1 – 19-mer unmethylated CpG
216 DNA, but moves to the front of the TRD in the structure of free DNMT1, resulting in a translocation
217 of ~30 Å (Fig. 4C). As a result, the autoinhibitory linker downstream of the CXXC domain undergoes
218 a large conformational change between the two complexes: It runs across the catalytic cleft in the
219 DNMT1 – unmethylated CpG DNA complex, but is released from the catalytic cleft in free DNMT1
220 (Fig. 4C). Intriguingly, this repositioning of the autoinhibitory linker is accompanied by a
221 loop-to-helix conformational transition: the N-terminal end of the linker assumes an extended
222 conformation in unmethylated CpG-bound DNMT1, but shows a helical structure in free DNMT1
223 (Fig. 4C). At the C-terminal end of this helix, residues D700 and E703 form salt bridges with residues
224 R582 and K586 from the RFTS domain, while residue D602 forms hydrogen bonds with residues
225 M1232 and N1233 from the catalytic core, which together help strengthen the interaction between
226 the RFTS and MTase domains (Fig. 4D). Consistently, deletion of residues 701-711 from the

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autoinhibitory linker led to significantly enhanced enzymatic activities of DNMT1 [33]. These data therefore suggest that the autoinhibitory linker not only plays a critical role in the CXXC domain-mediated DNMT1 autoinhibition, but also contributes to the RFTS domain-mediated DNMT1 autoinhibition.

Allosteric regulation of DNMT1. Crystal structures of DNMT1 in free state, in complex with unmethylated CpG DNA and in complex with hemimethylated CpG DNA together demonstrate that DNMT1 may adopt distinct conformational states under different DNA binding conditions, suggesting a multi-layered regulation of DNMT1 activity. It is conceivable that the interconversion between these states permits DNMT1 to discriminate the DNA substrates under different epigenetic environments, such as methylation-free CpG islands vs heavily methylated heterochromatic regions

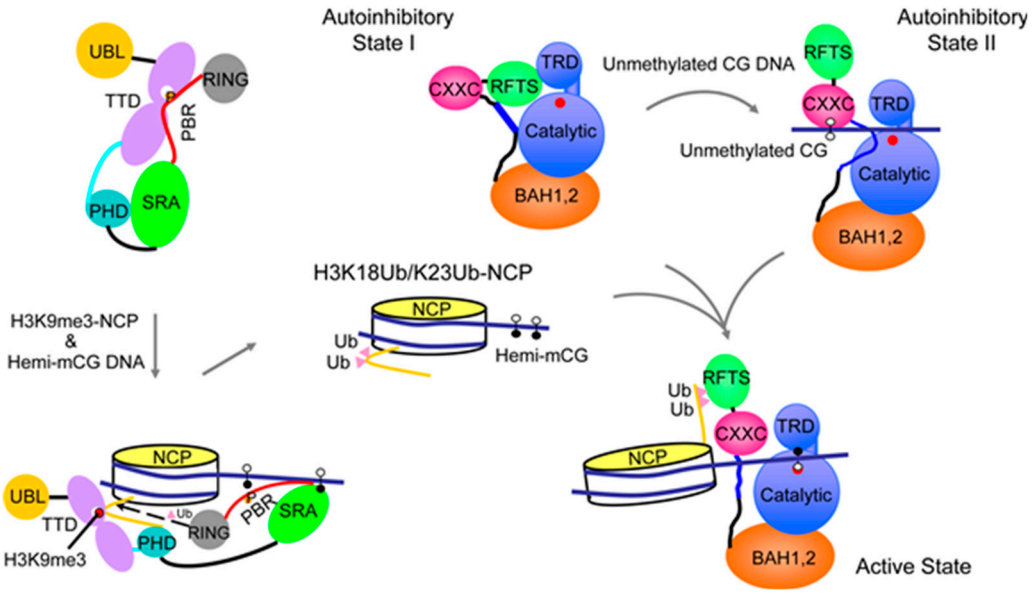

Figure 5. A model for the allosteric regulation of DNMT1-mediated maintenance DNA Methylation. Hemimethylated DNA and histone H3K9me3 serve as epigenetic signals to promote UHRF1-mediated ubiquitination of histone H3, which in turn shifts the conformation of DNMT1 from autoinhibitory states into an active state for maintenance DNA methylation. UHRF1 P656, which occupies the H3K9me3-binding cage of the TTD domain in the "closed" UHRF1 conformation, is indicated by a letter 'P'. The active site of DNMT1 is marked by a filled red circle.

(Fig. 5). Stabilization of each conformation is likely be achieved by distinct DNA or histone-binding mode of DNMT1 under different environments, ensuring DNMT1 to replicate DNA methylation pattern both faithfully and efficiently. Indeed, emerging studies have suggested a model in which DNMT1 mediates region-specific DNA methylation maintenance, rather than site-specific DNA

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methylation maintenance [41].

The RFTS domain mediates the localization of DNMT1 to replication foci and constitutive heterochromatin from late S throughout G2 and M phase [42,43]. Recent structural and functional characterizations of the interaction between the DNMT1 RFTS domain and histone modifications have further shed light onto the functional implication of the RFTS domain-mediated DNMT1 autoinhibition [44]. In particular, it has been shown that the DNMT1 RFTS domain binds to histone H3 ubiquitinated at lysine 14 (K14Ub), 18 (K18Ub) and/or 23 (K23Ub), with a preference for H3 with two mono-ubiquitination (H3Ub2) [44,45,46]. The crystal structure of the RFTS domain of hDNMT1 in complex with H3-K18Ub/K23Ub reveals that the two ubiquitin moieties engage hydrophobic interactions with two discrete surfaces of the N-lobe of RFTS, separated by a loop segment [44]. The N-terminal tail of H3 lies between the C-lobe and the ubiquitin molecule conjugated to H3K23, leading to the eviction of the linker sequence downstream of the RFTS domain out of the cleft between the N and C-lobes [44]. Consequent to these structural changes, the interaction of DNMT1 RFTS with H3Ub2 results in substantially elevated activity of DNMT1 [44], suggesting that H3Ub2 may serve as an epigenetic signal that relieves the RFTS-mediated autoinhibition of DNMT1. These studies have therefore established a link between the chromatin targeting and enzymatic activation of DNMT1, establishing the molecular mechanism for RFTS regulation (Fig. 5). It is worth noting that H3 K14Ub/K18Ub/K23Ub marks are the enzymatic products of UHRF1 (ubiquitin-like, containing PHD and RING finger domains, 1) [44,45,46], a key regulatory protein of DNMT1-mediated maintenance DNA methylation [47,48]. UHRF1 is also multimodular protein comprised of an N-terminal ubiquitin-like (UBL) domain, a tandem Tudor domain (TTD), a plant homeodomain (PHD), a SET- and RING-associated (SRA) domain, and a C-terminal RING-finger domain [49]. An intramolecular interaction between the TTD domain and the C-terminal polybasic region (PBR) of UHRF1 results in “a closed” conformation that occludes UHRF1 from chromatin association [50,51,52,53]. During the S phase of cell cycle, association of UHRF1 with histone H3 trimethylated at lysine 9 (H3K9me3) [54,55,56,57,58,59,60], a silencing histone mark [61], and hemimethylated CpG DNA [47,48,57,62,63,64,65] leads to the conformational “opening” [50,51,52], and enhanced E3 ubiquitin ligase activity of UHRF1 (Fig. 5) [66]. In this context, the DNMT1 RFTS

domain serves as an effector module that transmits the H3K9me3 signal into DNMT1-mediated DNA methylation (Fig. 5).

Regulatory role of DNMT1 NTD. The NTD appears not to affect the enzymatic activity of DNMT1. Instead, this region serves as a platform for the interaction between DNMT1 and proteins or DNA. Of particular note, the fragment equivalent to residues 159-171 of mouse DNMT1 (mDNMT1) is responsible for interacting with proliferating cell nuclear antigen (PCNA) [67], thereby contributing to the recruitment of DNMT1 to the replication foci during the S phase [67], or the DNA repair sites [68]. The NTD reportedly also interacts with other proteins, including DMAP1 [69], G9a [70], DNMT3A [71], DNMT3B [71], PKC [72] and CDKL [73] to regulate transcription repression, heterochromatin formation or the pathogenic processes of Rett syndrome. In addition, the DNA binding activity of the NTD has been reported [74,75,76]. However, due to lack of structural study, the functional implication of most of the NTD-associated interactions remains to be investigated.

Regulatory role of DNMT1 (GK)n repeats. The (GK)n repeat of DNMT1, highly conserved throughout evolution, links the regulatory domains to the MTase domain. Current structural studies indicate that this repeat is not involved in the DNA interaction. Rather, it constitutes a binding site for deubiquitinase USP7, an enzyme that plays a regulatory role in DNMT1-mediated maintenance DNA methylation [77,78,79,80]. The DNMT1 – USP7 interaction is subject to regulation by acetyltransferase Tip60 and deacetylase of HDAC1: Tip60-mediated acetylation of the (GK)n repeat leads to disruption of the DNMT1 – USP7 interaction, which can be restored by HDAC1-mediated deacetylation of the same site [78]. Due to lack of molecular details of DNMT1-mediated methylation in cells, the functional implication of the DNMT1 – USP7 interaction remains controversial [81].

3. Structural basis of DNMT3A-mediated DNA methylation

DNMT3A and DNMT3B mediate DNA methylation establishment during gametogenesis and embryogenesis [16,82], and subsequently participate in methylation maintenance [83,84,85]. The enzymatic activity of DNMT3A/3B in germ cells and embryonic stem cells is further regulation by DNMT3-like (DNMT3L) protein, which lacks DNA methylation activity but functions to stimulate the cofactor binding and enzymatic activity of DNMT3A/3B [7,86,87,88]. DNMT3A and DNMT3B

296 are highly related in sequence, both containing a largely disordered ND, followed by a
297 Pro-Trp-Trp-Pro (PWWP) domain, an Atrx-Dnmt3-Dnmt3l (ADD) domain, and a highly
298 homologous MTase domain (Fig. 1). DNMT3L contains an N-terminal ADD domain, followed by a
299 MTase-like domain, which is catalytic inactive due to lack of essential motifs for enzymatic activity
300 (Fig. 1) [89,90].

301 **Enzyme-substrate interaction of DNMT3A.** The crystal structure of the MTase domain of DNMT3A
302 in complex with the C-terminal domain of DNMT3L (DNMT3L-C) provides first atomic details of
303 the DNMT3A – DNMT3L complex [91]. The DNMT3A MTase domain forms a tetrameric fold with
304 DNMT3L-C, in the order of 3L-3A-3A-3L, resulting in two DNMT3A – DNMT3L heterodimeric
305 interfaces and one DNMT3A – DNMT3A homodimeric interface. Homodimerization of DNMT3A is
306 mediated by a network of salt bridge and hydrogen bonding interactions, while heterodimerization
307 of DNMT3A and DNMT3L is mainly driven by hydrophobic stacking interactions between two
308 pairs of phenylalanine residues [91]. Notably, the active sites between the two DNMT3A monomers
309 are separated by ~40 Å, a distance equivalent to one helical turn of DNA. This observation provided
310 the basis for the "CpG spacing" model, in which the DNMT3A dimer is capable of methylating two
311 CpG sites located across the opposite strands of one DNA duplex, separated by ~10 base-pair (bp)
312 DNA, in one binding event. This model predicts the prevalence of ~10 bp methylation periodicity in
313 cells, which has been supported by a number of biochemical and cellular studies [91,92]. However,
314 the observation that the 10 bp-methylation periodicity also occurs in plants later prompted
315 alternative explanations for the methylation periodicity [93].

316 Recently, the crystal structure of DNMT3A-DNMT3L in complex with a DNA duplex
317 containing two separate CpG sites, in which the target cytosine is replaced with Zebularine [94], a
318 cytosine analogue, has been determined [95]. The structures reveals a productive state of the
319 DNMT3A – DNA complex, with two CpG/ZpG sites separately targeted by the two DNMT3A
320 monomers of the DNMT3A-DNMT3L tetramer (Fig. 6A), therefore confirming the notion of
321 DNMT3A-mediated DNA co-methylation. The structure of DNA-bound DNMT3A – DNMT3L
322 tetramer resembles that of free DNMT3A – DNMT3L (Fig. 6B), with an RMSD of 1.1 Å over 826
323 aligned Cα atoms. The most notable structural difference arises from a loop from the TRD (TRD

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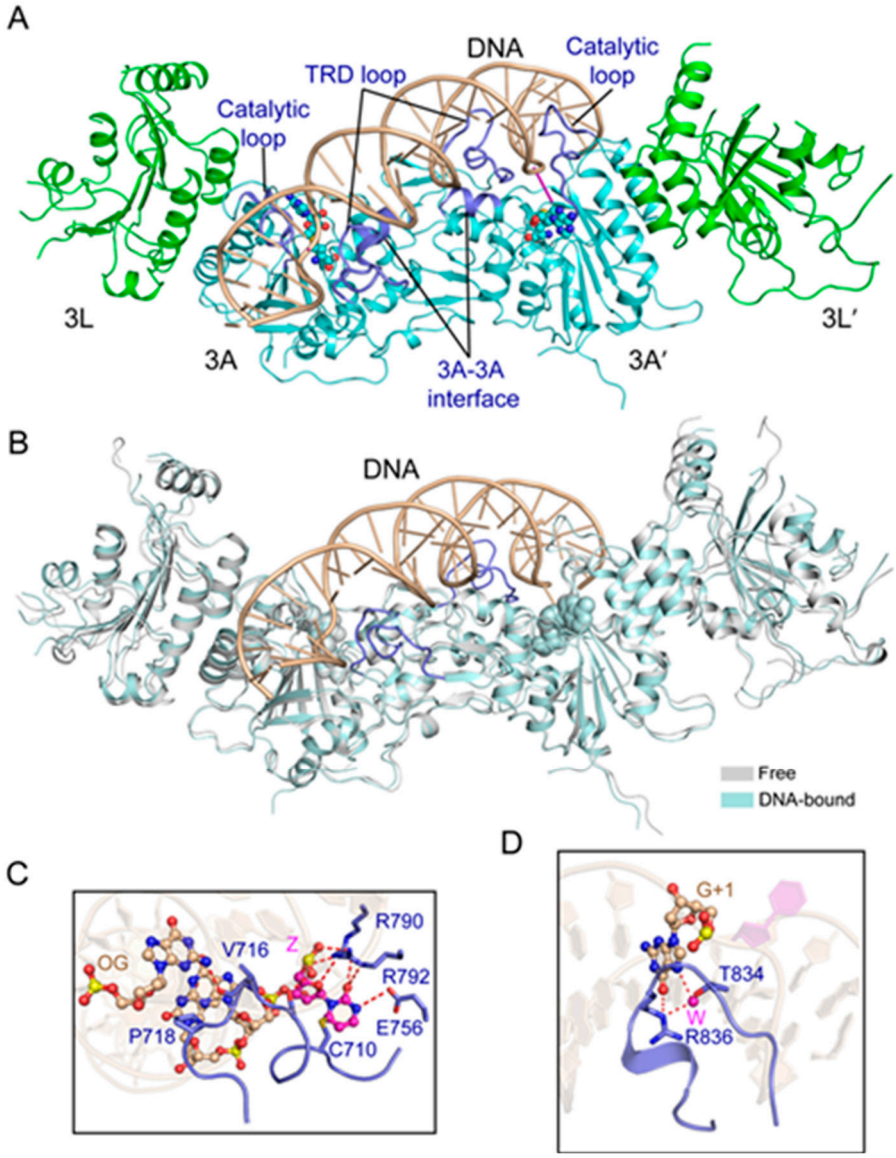

Figure 6. Structural analysis of the productive complex of the DNMT3A – DNMT3L tetramer with CpG DNA. (A) Structural overview of the DNMT3A – DNMT3L tetramer covalently bound to a 25-mer DNA duplex containing two CpG/ZpG sites (Z: Zebularine). The flipped-out Zebularines are colored in purple. (B) Structural overlap between DNA-bound and free DNMT3A – DNMT3L tetramer. The TRD loops, which undergo disorder-to-order transition upon DNA binding, are colored in blue. (C) The DNA interactions involving the catalytic loop and other catalytic residues. (OG: orphan guanine). The hydrogen bonding interactions are depicted as dashed lines. (D) Residues T834 and R836 from the TRD loop (blue) engage in base-specific recognition of the CpG site. The water molecules are shown as purple spheres.

loop), which undergoes a disorder-to-order transition upon DNA binding (Fig. 6B). The interaction between DNMT3A and DNA is mediated through the catalytic loop, the TRD loop and the DNMT3A – DNMT3A homodimeric interface (Fig. 6A), which together create a continuous DNA-binding surface. The Zebularines were flipped out of the DNA duplex and insert deep into the

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328 catalytic pocket of DNMT3A, where they are covalently anchored by the catalytic cysteine C710 and
329 recognized by E756, R790 and R792 through hydrogen bonding interactions (Fig. 6C) [95]. Similar to
330 that in the productive mDNMT1 – DNA complex, the catalytic loop and TRD loop of DNMT3A
331 approach the DNA molecule from minor groove and major groove, respectively, with residue V716
332 from the catalytic loop intercalating into the DNA cavity vacated by base flipping (Fig. 6C). At the
333 minor groove, the backbone carbonyl of V716 forms a hydrogen bond with the orphan guanine,
334 while side chains of residue P718 engages van der Waals contact with the guanine on the target
335 strand (Fig. 6C). At the major groove, residues R836 and T834 from the TRD loop also interact with
336 the guanine on the target strand through direct and water-mediated hydrogen bonding interactions
337 (Fig. 6D). Consistent with these structural observations, introduction of mutations onto these
338 CpG-interacting site leads to either dramatically decreased activity (for V716G) or altered
339 methylation specificity (for R836A) in vitro and in cells [95].

340 It is worth noting that the structure of DNMT3A – DNMT3L – DNA complex reveals that the
341 active sites between the two DNMT3A monomers are separated by 14 bp DNA, instead of the 10 bp
342 as previously proposed. Whether this observation arises from the inherent structural property of
343 DNMT3A or its conformational dynamics remains to be investigated.

344 **ADD domain-mediated autoinhibition of DNMT3A.** The ADD (Atrx-Dnmt3-Dnmt3l) domain of
345 DNMT3A is comprised of an N-terminal GATA-like zinc finger, a plant homeodomain (PHD) finger
346 and a C-terminal α-helix [96], together packing into a single globular fold. This domain has been
347 characterized as a reader module that specifically binds to histone H3 unmethylated at lysine 4
348 (H3K4me0) [96,97]. Association of the DNMT3A ADD domain with H3K4me0 is mediated by
349 antiparallel β-pairing between the two-stranded β-sheet of the ADD domain and residues A1-T6 of
350 H3, with the side chain of H3K4me0 engaging hydrogen-bonding interactions with D529, D531 and
351 Q534 from ADD [96]. In addition, a downstream loop of the ADD domain undergoes a
352 disorder-to-order transition to close up on the N-terminus of H3, supporting the specific ADD – H3
353 association [96].

354 Recent studies have further revealed that the ADD domain regulates the activity of DNMT3A
355 through an H3-dependent, autoinhibitory mechanism [98,99]. The structure of a DNMT3A fragment,

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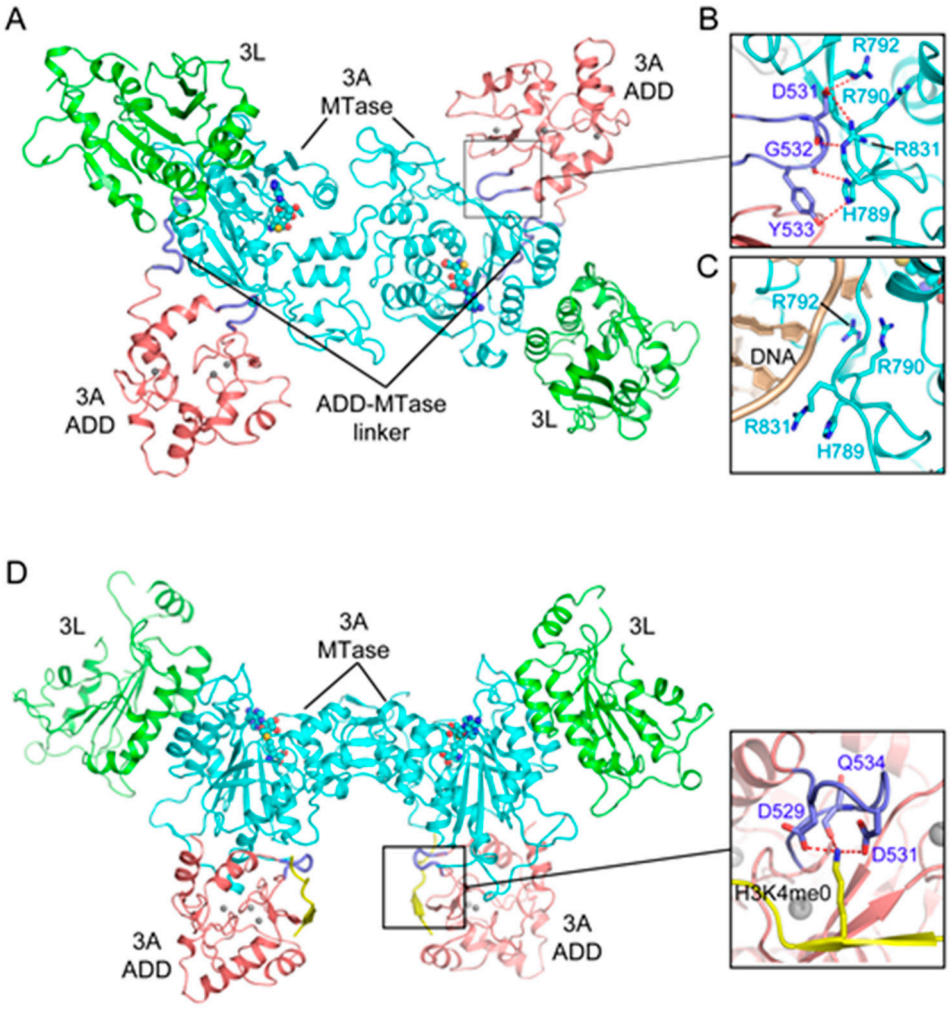

Figure 7. Structural analysis of the ADD domain-mediated DNMT3A autoinhibition. (A) Structural overview of the DNMT3A – DNMT3L tetramer, with the DNMT3A fragment comprised of both the ADD and MTase domains. (B) Intramolecular interactions between the ADD loop (blue) and the MTase domain (aquamarine) of DNMT3A. The hydrogen bonding interactions are depicted as dashed lines. (C) The ADD-binding site of the DNMT3A MTase overlaps with its DNA binding site. (D) Structure of the DNMT3A – DNMT3L tetramer bound to histone H3K4me0 peptide, with the interaction between H3K4me0 and the ADD domain shown in expanded view.

spanning the ADD and MTase domains, in complex with DNMT3L-C reveals an intramolecular interaction between the ADD and MTase domains of DNMT3A (Fig. 7A). In particular, the linker sequence following the ADD domain initiates a hydrophobic contact with the MTase domain, which then guides the insertion of a loop (residues 526-533) of the ADD domain into the catalytic cleft, whereby it engages salt bridge interactions with DNA binding sites (R790, R792, H789, and R831) (Fig. 7B), thereby inhibiting the substrate binding of DNMT3A (Fig. 7C) [98]. In contrast, the

structure of the DNMT3A – DNMT3L – H3 complex demonstrates that, upon binding to H3 (Fig. 7D, E), the DNMT3A ADD domain is repositioned from the catalytic cleft onto a difference surface of the MTase domain, engaging a distinct set of hydrogen bonds and hydrophobic interactions (Fig. 7D) [98]. Structural comparison of the H3-free and H3-bound DNMT3A –complexes therefore provide a dynamic view on how the H3 binding switches the conformation of DNMT3A from an autoinhibitory state to an active state. Note that the residues involved in the autoinhibitory regulation of DNMT3A are highly conserved in DNMT3B, suggesting a conserved allosteric regulation mode of DNMT3 methyltransferases.

The observation that the intramolecular ADD – MTase interaction interplays with the intermolecular ADD – H3 interaction establishes a direct coupling between the enzymatic activity and chromatin targeting of DNMT3A. Similar to the RFTS domain-mediated allosteric regulation of DNMT1, as described above, this regulatory mechanism of DNMT3A ADD domain ensures a precise spatial regulation of DNMT3A [97,98,99], which is essential for installing lineage-specific DNA methylation patterns across the genome.

Functional regulation of DNMT3A by the N-terminal tail (NTD) and PWWP domain. The NTD segment defines the most divergent region between DNMT3A and DNMT3B. This region has been shown to regulate the DNA binding and cellular localization of DNMT3A [100,101,102]. Unlike full-length DNMT3A that is predominantly localized to the heterochromatic region, DNMT3A2, an isoform of DNMT3A lacking residues 1-221 of the NTD, becomes enriched in the euchromatic region, with reduced DNA binding affinity [102]. The precise regulatory role of this domain remains to be investigated.

The PWWP domain, named after a characteristic proline-tryptophan-tryptophan-proline motif, belongs to the Royal super-family of domains that recognize histone tail with various modifications [103,104]. The PWWP domain of DNMT3A and DNMT3B mediates their chromatin association through specific recognition of histone H3 trimethylated at lysine 36 (H3K36me3) [105,106], which is essential for directing the de novo methylation activity of DNMT3A/3B at the pericentric heterochromatin [107]. Structural studies of the DNMT3A/3B PWWP domain reveals a β-barrel followed by a C-terminal helical bundle, similar to other PWWP domains (Fig. 8A, B) [108,109,110].

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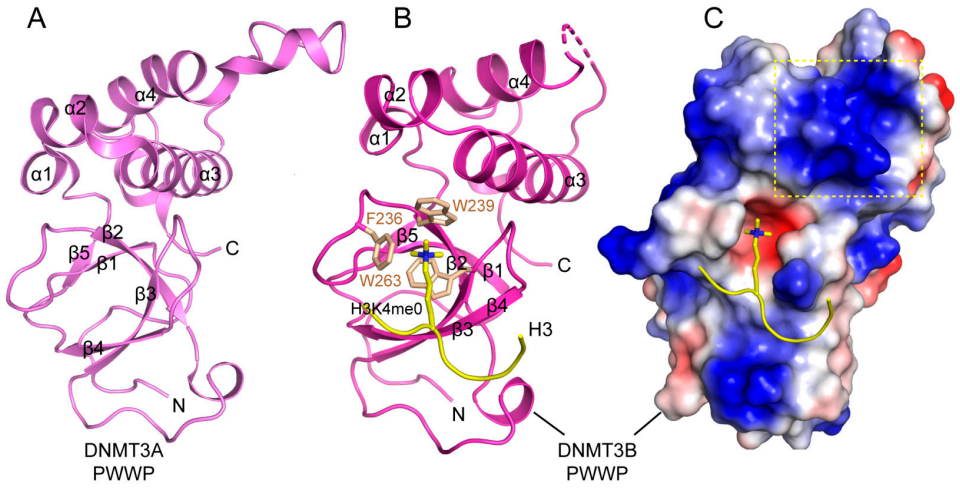

Figure 8. Structures of DNMT3A/3B PWWP domains. (A) Crystal structure of DNMT3A PWWP domain. (B) Crystal structure of DNMT3B PWWP bound to histone H3K36me3 peptide, with the side chains of H3K36me3 and the cage residues of the PWWP domain shown in stick representation. (C) Electrostatic surface view of DNMT3B PWWP domain bound to histone H3K36me3 peptide. The putative DNA binding surface is boxed with dotted lines.

The β-barrel is comprised of five β-strands, with the signature "PWWP" motif replaced by a "SWWP" motif at the beginning of the second β-strand. The structure of DNMT3B PWWP domain in complex with an H3K36me3 peptide reveals that the histone peptide occupies a surface groove formed by residues from the β1 strand, the β1-β2 loop, and the β4 strand, with the side chain of H3K36me3 inserting into the aromatic cage formed by F236, W239 and W263 through hydrophobic and cation-π interactions (Fig. 8B) [109]. The H3K36me3 binding also induces a conformational change of the β1-β2 loop, which moves to close up the aromatic cage, thereby enhancing the specific H3K36me3 recognition. In addition, both the DNMT3A and DNMT3B PWWP domains present a positively charged surface that confers their DNA binding activity (Fig. 8C) [108,109,111]. The cooperative engagement of both DNA and H3K36me3 by the DNMT3A/3B PWWP domains provides a mechanism for targeting these two enzymes to heterochromatic regions [106,107] or actively transcribed gene body in nucleus [112].

4. Structural comparison of the DNMT1 – DNA and DNMT3A – DNA complexes

Structural comparison of the DNMT3A – DNMT3L – DNA complex and mDNMT1 – hemimethylated DNA complex provides insights into the distinct molecular basis between DNMT3A-mediated de novo DNA methylation and DNMT1-mediated maintenance DNA

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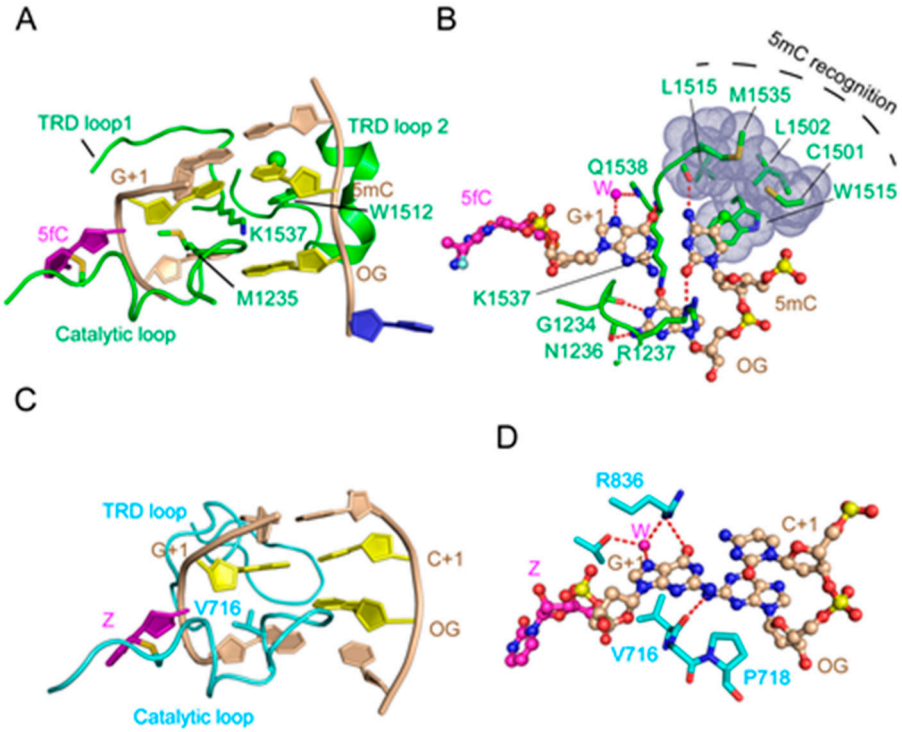

Figure 9. Structural comparison of mDNMT1-DNA and DNMT3A-DNA interactions. (A) Recognition of hemimethylated CpG DNA by mDNMT1 (green) (PDB 4DA4). The hemimethylated CpG site, containing a 5-methyl group (green sphere), is colored in yellow or purple (5-fluorocytosine, fC'). The flipped out cytosine on the template strand is colored in blue and the rest of the DNA in wheat. (B) Recognition of unmodified CpG DNA by DNMT3A (light blue). (C-D) Detailed interactions between mDNMT1 and hemimethylated CpG site (C), and between DNMT3A and unmodified CpG site (D). The hydrogen bonds are depicted as dashed lines.

406 methylation. Despite with conformational similarity in their catalytic loop for accessing the DNA
407 minor groove, mDNMT1 and DNMT3A enter the DNA major groove differently for CpG
408 recognition (Fig. 9A-D). First, mDNMT1 interacts with the DNA major groove through two of its
409 TRD loops, with one (TRD loop 1) engaging the CpG dinucleotide through hydrogen bonding
410 interactions and the other (TRD loop 2) forming a hydrophobic concave harboring the methyl group
411 of 5mC along the template strand (Fig. 9A,B). In contrast, while DNMT3A interacts with the DNA
412 major groove through a loop similar to the TRD loop 1 of DNMT1, it lacks the DNMT1 TRD loop
413 2-equivalent segment for 5mC recognition (Fig. C,D). These observations explain why DNMT1, but
414 not DNMT3A, shows enzymatic preference for hemimethylated over unmethylated substrates.
415 Second, the DNA molecules bound to mDNMT1 and DNMT3A also exhibit different conformational
416 adjustments. In mDNMT1-bound DNA, the base flipping leads to one-base translocation of the
417 orphan guanine and a large distortion of the CpG site, with the DNA cavity filled by two bulky

418 protein residues (M1235 and K1537) (Fig. 9A). In contrast, in DNMT3A-bound DNA, the orphan
419 guanine remains in space, resulting in a smaller DNA cavity occupied by one small residue of
420 DNMT3A (V716) (Fig. 9C). In addition, the large TRD of DNMT1 permits an extensive protein –
421 DNA interaction, resulting in buried surface area of ~2100 Å2, whereas the DNA binding of
422 DNMT3A, with a much smaller TRD, only leads to buried surface area of ~1300 Å2 for each
423 DNMT3A monomer. This limited DNA binding of each DNMT3A monomer is nevertheless
424 overcome by the presence of two DNMT3A monomers in the DNMT3A – DNMT3L tetramer, which
425 provides an enlarged protein – DNA contact surface to ensure the efficiency of DNA methylation.
426 Together, these observations highlight different molecular basis underlying DNMT3A-mediated de
427 novo methylation and DNMT1-mediated maintenance methylation.

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429 **Conflicts of Interest:** The authors declare no conflict of interest."

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