

Histone tails regulate DNA methylation by allosterically activating *de novo* methyltransferase

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Cytosine methylation of genomic DNA controls gene expression and maintains genome stability. How a specific DNA sequence is targeted for methylation by a methyltransferase is largely unknown. Here, we show that histone H3 tails lacking lysine 4 (K4) methylation function as an allosteric activator for methyltransferase Dnmt3a by binding to its plant homeodomain (PHD). *In vitro*, histone H3 peptides stimulated the methylation activity of Dnmt3a up to 8-fold, in a manner reversely correlated with the level of K4 methylation. The biological significance of allosteric regulation was manifested by molecular modeling and identification of key residues in both the PHD and the catalytic domain of Dnmt3a whose mutations impaired the stimulation of methylation activity by H3 peptides but not the binding of H3 peptides. Significantly, these mutant Dnmt3a proteins were almost inactive in DNA methylation when expressed in mouse embryonic stem cells while their recruitment to genomic targets was unaltered. We therefore propose a two-step mechanism for *de novo* DNA methylation – first recruitment of the methyltransferase probably assisted by a chromatin- or DNA-binding factor, and then allosteric activation depending on the interaction between Dnmt3a and the histone tails – the latter might serve as a checkpoint for the methylation activity.

Keywords: *de novo* DNA methylation; Dnmt3a; Histone H3 tail; PHD domain; allosteric stimulation

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Introduction

In plants and vertebrate animals, regulation of gene expression is mainly accomplished through the modification of DNA and its associated histones. Cytosine methylation is the best-known type of modification found in the genomes of these organisms. DNA cytosine methylation together with histone modifications present two profound epigenetic regulatory platforms essential for various developmental and disease processes [1].

In mammals, DNA methylation patterns are estab-

lished by the action of two *de novo* methyltransferases Dnmt3a and Dnmt3b during embryonic development and gametogenesis and then stably propagated by a maintenance methyltransferase Dnmt1 [2]. Missense mutations in DNMT3A are associated with acute myeloid leukemia [3]. A fundamental question concerns how a specific sequence is selectively methylated by the *de novo* enzymes in cells. Evidence for cross-talk between DNA methylation and histone modification has been found in various contexts of transcriptional regulation and chromatin functions [4–8]. In the filamentous fungus *Neurospora crassa*, either the replacement of histone H3 lysine 9 (H3K9) with other amino acids or deletion of the sole H3K9 methyltransferase DIM5 results in the loss of DNA methylation [9]. In mouse embryonic stem (ES) cells, deficiency in the histone H3K9 methyltransferases Suv39h1–Suv39h2 is associated with hypomethylation at a subset of repeat elements [10]. In the epigenome

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landscape, cytosine methylation is under-represented in regions containing histone H3K4 trimethylation [11, 12]. These observations, mostly correlative, suggest that chromatin cues might be a determinant of DNA methylation in mammals. Interestingly, all Dnmt3 members possess a plant homeodomain (PHD) domain that can specifically bind to the unmethylated H3 tail terminus [13-15]. This biochemical property has led to an idea that histone tails in chromatin might provide a docking site for region-specific methylation by the *de novo* methyltransferases [14, 15]. We recently found that the histone H3 tail is indeed essential for DNA methylation by providing a direct contact with Dnmt3a-Dnmt3L [16]. However, the exact mechanism for the transformation of a chromatin modification status into DNA methylation is left unexplored.

The current model of DNA methylation [7, 17] underscores the recruitment of Dnmt3 enzymes to genomic targets via direct or indirect binding to histone tails [13-15, 18]. While this model assumes that the histone tail with a specific modification state specifies a docking site for Dnmts as for many other chromatin binding/modifying proteins, it does not consider the possibility that the enzymatic activity of Dnmts might be regulated by histone tails and their modification. In this study, we explore the role of the histone tail-Dnmt3a interaction and provide evidence for allosteric regulation of Dnmt3a by histone tails in DNA methylation in mammalian cells.

Results

The enzymatic activity of Dnmt3a is stimulated in vitro by histone H3 peptides lacking lysine methylation

The PHD domain of Dnmt3a is able to bind histone tails *in vitro* [14, 15], but is dispensable for the enzyme association with chromatin *in vivo* [19, 20]. These observations suggest that recruitment of Dnmt3a to chromatin could be independent of the PHD domain-mediated binding to the H3 *N*-terminus. On the other hand, the PHD domain of Dnmt3a interacts with the catalytic domain as shown in both GST pull-down and yeast two-hybrid assays (Supplementary information, Figure S1), suggesting that binding of histone tails might play a regulatory role for the catalytic activity of Dnmt3a. To examine this possibility, recombinant Dnmt3a purified from transiently transfected 293T cells was used for *in vitro* methylation assay in the presence of histone peptides. Strikingly, the activity of Dnmt3a was stimulated by H3 peptides (amino acid 1-21) depending on the methylation status of lysine 4 (K4) (Figure 1A). While trimethylated peptides had little effect, di-, mono- and unmethylated peptides increased the Dnmt3a methylation activity by 2-, 4-, to 8-fold respectively. To explore the mechanism by which

H3 peptides stimulate Dnmt3a activity, we measured DNA methylation rates at different concentrations of S-

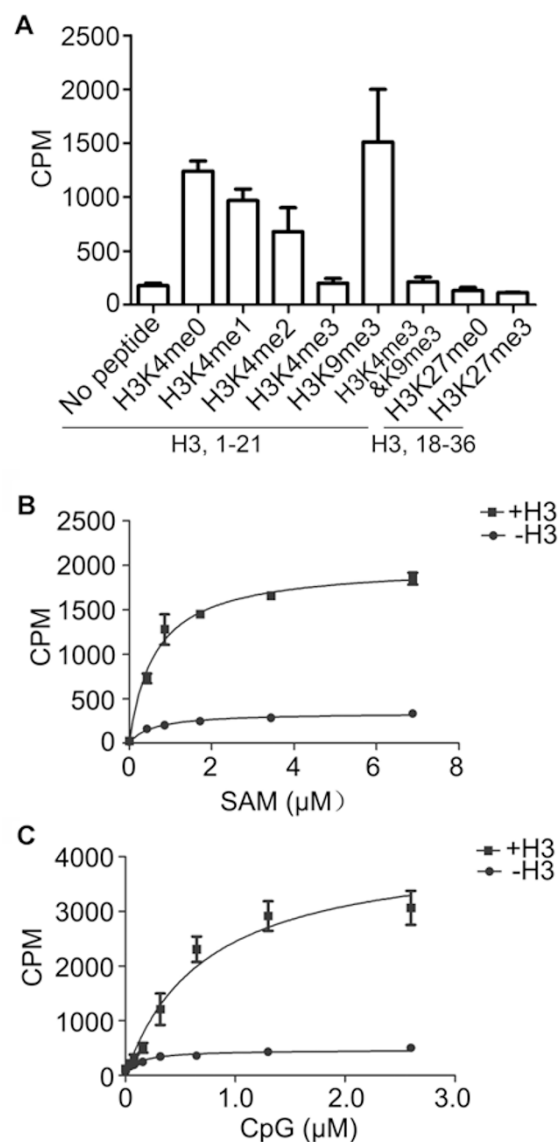


Figure 1 Histone H3 peptides stimulate methylation activity of Dnmt3a *in vitro*. **(A)** Methylation activity of Dnmt3a toward naked DNA in the presence of various peptides (amino acids 1-21 and 18-36 of H3) as indicated. Flag-tagged Dnmt3a was purified from transfected HEK293T cells (Supplementary information, Figure S3). CPM, counts per minute. Error bars indicate the deviation in three independent experiments. **(B)** Titration of methyl donor S-adenosyl-methionine (SAM) in the presence or absence of H3 peptides (amino acids 1-21). The curves represent global fits of the data to the Michaelis-Menten model. The SAM concentration is given below the X-axis. **(C)** Titration of DNA substrate in the presence or absence of H3 peptides (aa 1-21). The molar concentration of CpG dinucleotides in a 2.1-kb PCR fragment is given below the X-axis.

adenosyl-methionine (SAM) and DNA substrates. In the presence of H3, the maximal observed rate increased by 10.1-fold during titration of SAM (Figure 1B). A similar result was observed with titration of the DNA substrate (Figure 1C). Notably, the SAM concentration required to reach the half-maximal reaction rate was almost unchanged while this value increased by 7.2-fold for the DNA substrate. We also measured the dissociation constant (K_d) of DNA binding of Dnmt3a by SPR assay. The presence of H3 peptides did not lead to any change in the binding curve and thus binding affinity was unchanged (Supplementary information, Figure S2). These results suggest that H3 peptides stimulate the methylation reac-

tion by Dnmt3a mainly through increasing the turnover rate.

The binding ability to histone tails is essential for Dnmt3a to be stimulated in vitro and to methylate DNA in vivo

We next examined whether binding of histone H3 peptides to the PHD domain is involved in stimulation of Dnmt3a enzymatic activity by free H3 tail. Two residues at the H3 recognition interface in the PHD structure [14] were therefore chosen for mutational analysis. Asp308, which forms part of the H3 K4-binding pocket via its carboxylate, was substituted by alanine (A) to disrupt the

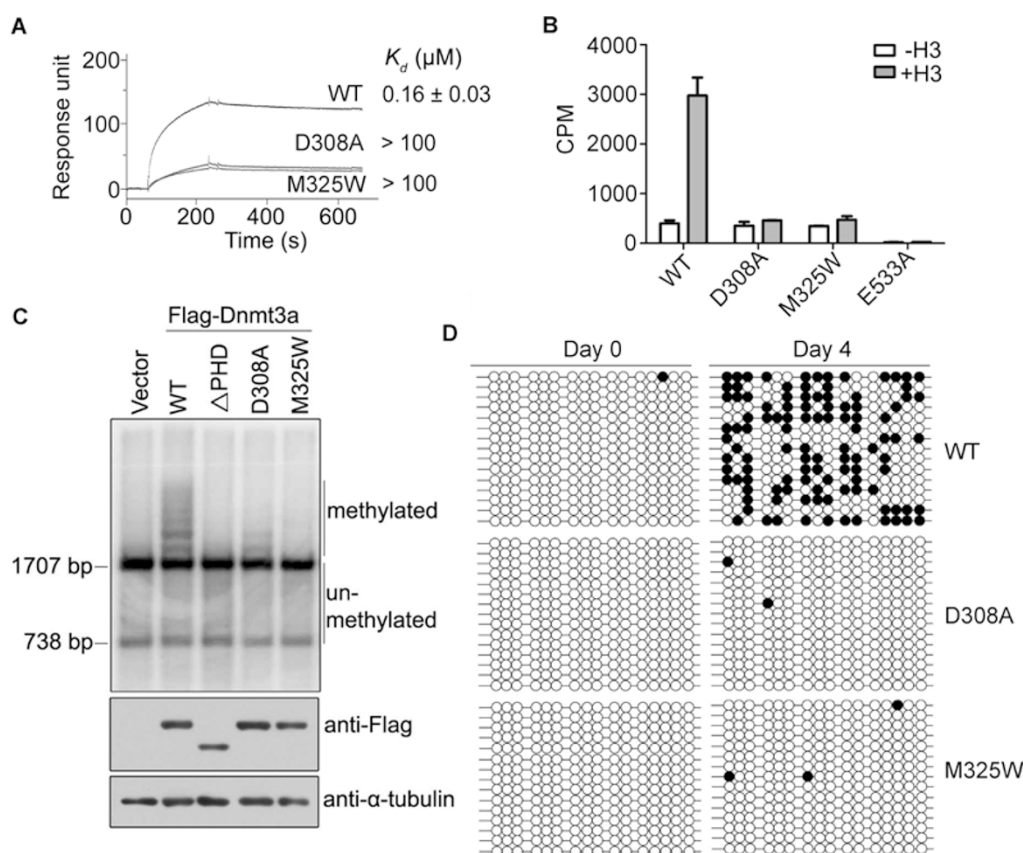


Figure 2 Dnmt3a mutants deficient in histone H3 binding fail to be stimulated by H3 peptides *in vitro* and are unable to methylate DNA *in vivo*. **(A)** Dissociation constants of GST-fusions with the wild-type (WT) PHD and the two mutants D308A and M325W in binding with H3 peptides (aa 1-21) measured by SPR assay. GST-PHD fusion proteins were purified from *E. coli* expressing strains (Supplementary information, Figure S3). **(B)** Lack of enzymatic stimulation in mutant Dnmt3a by histone peptides (aa 1-21). Dnmt3a proteins used were purified from transfected HEK293T cells (Supplementary information, Figure S3). Their basal methylation activity was determined in the absence of histone peptides (-H3). **(C)** Southern hybridization analysis of methylation activity of Dnmt3a. Flag-tagged Dnmt3a or its mutants as indicated was co-transfected with an assay plasmid p220.2 into HEK293 c18 cells. The recovered assay plasmid was digested with *Hha*I. Western blot analysis (bottom panels) shows the expression of Dnmt3a and α -tubulin in each transfection. Δ PHD is a deletion mutant lacking amino acids 252-391. **(D)** Bisulfite sequencing analysis of the *Oct4* promoter upon ES cell differentiation induced with retinoid acid (RA). PCR products amplified from bisulfite-treated genomic DNA were cloned and sequenced to determine the methylation status of individual CpG sites.

specific interaction. Met325, important for the recognition of H3 N-terminal residues, was replaced by bulky tryptophan (W) to create steric hindrance. As expected, the PHD domains bearing these mutations lost binding affinity for histone peptides (Figure 2A). The full-length Dnmt3a proteins bearing these mutations (Supplementary information, Figure S3) were also found to lack H3 binding ability in peptide pull-down assay (Supplementary information, Figure S4A). Unlike the catalytically inactive mutant E533A that lacked any enzymatic activity, the basal methylation activity of these two PHD mutants was similar to that of the wild-type Dnmt3a. However, these two mutants were no longer able to respond to the H3 peptide stimulation as the wild type (Figure 2B). These results suggest that the allosteric activation of Dnmt3a by H3 peptides is mediated by their binding to the PHD domain.

To evaluate the role of H3 binding by Dnmt3a for DNA methylation *in vivo*, we performed an episomal plasmid methylation assay in transfected human cells

[21]. Southern hybridization analysis showed that a significant portion of the episomal DNA became methylated when co-transfected with the wild-type Dnmt3a (Figure 2C). However, the three mutants affecting the PHD domain showed no or little methylation activity. A more biologically relevant functional assay was also conducted by introducing mutant proteins into mouse ES cells deficient in Dnmt3a and Dnmt3b [22]. Stable cell lines ectopically expressing wild-type and mutant Dnmt3a were established (Supplementary information, Figure S4B). Chromatin IP (ChIP) analysis demonstrated binding of the two D308A and M325W mutant proteins to the pluripotency gene *Oct4* promoter in ES cells (Supplementary information, Figure S4C). However, both mutants failed to methylate the promoter region of *Oct4* during its silencing (Figure 2D and Supplementary information, Figure S4D). These data suggest that allosteric activation by H3 binding might be required for the *in vivo* function of Dnmt3a.

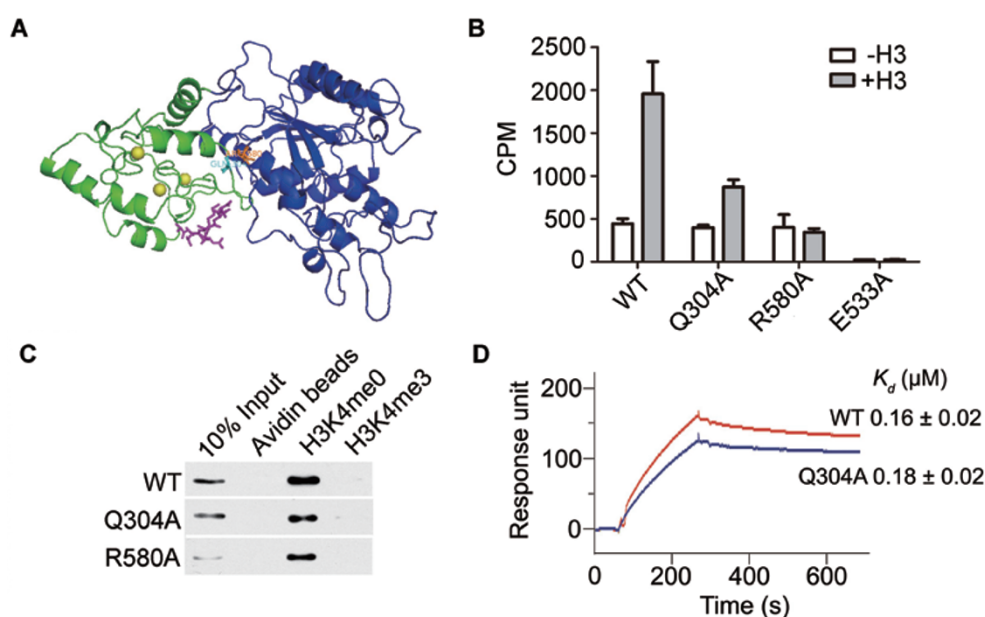


Figure 3 Design and characterization of mutations in Dnmt3a that specifically affect its response to histone peptide stimulation but not the peptide binding and basal catalytic activity. **(A)** Dnmt3a PHD-3aC structure model showing the interaction interfaces. Q304 in the PHD (green) and R580 in the catalytic domain (dark blue) are highlighted in light blue and orange, respectively. The bound H3 peptide (aa 1-6) is in purple. Zinc atoms bound in the PHD are in yellow. The PHD-3aC interface does not overlap with the interfaces [23] for Dnmt3a dimerization and its interaction with Dnmt3L. **(B)** Reduced response of the interface mutants to histone peptide stimulation. The batch of wild-type (WT), Q304A and R580A Flag-Dnmt3a proteins used here were purified from transiently transfected 293T cells in parallel (Supplementary information, Figure S3). **(C)** Unaltered binding of the interface mutants to H3 peptides. Peptide pull-down assay was performed for the Dnmt3a proteins using biotinylated H3 peptides (aa 1-21) either unmodified (H3K4me0) or trimethylated (H3K4me3) at K4. The bound proteins were detected by western blot analysis using anti-Flag antibodies. **(D)** Unaltered dissociation constant (K_d) of the GST fusion of PHD Q304A in binding with unmodified H3 peptides (aa 1-21) measured by SPR assay.

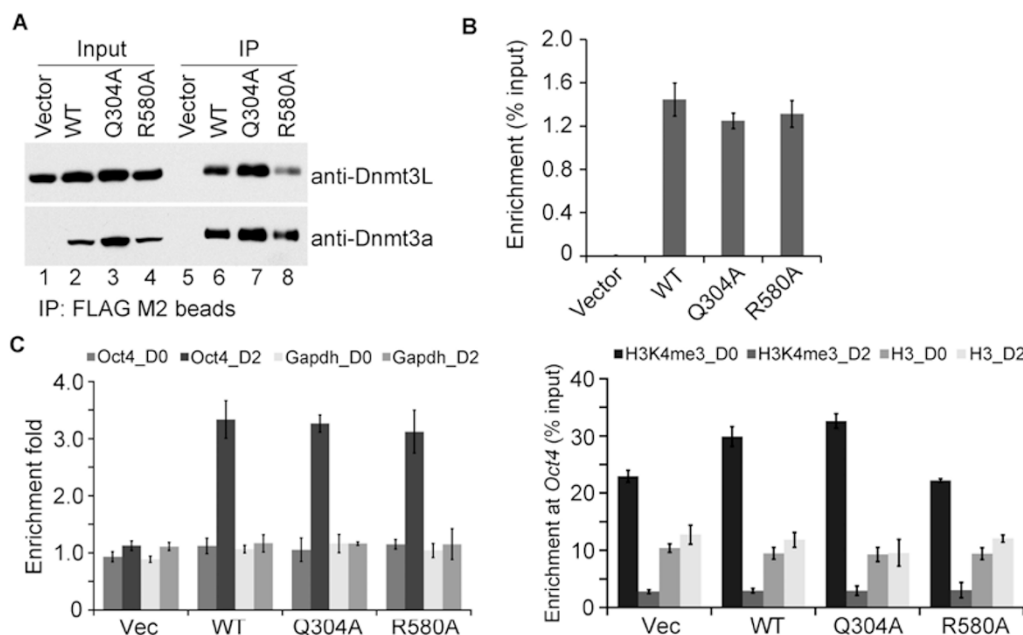


Figure 4 The interface mutants of Dnmt3a are unaltered in association with Dnmt3L and with genomic target sequences in ES cells. **(A)** Association of Flag-Dnmt3a Q304A and R580A mutants with the endogenous Dnmt3L in stably transfected TKO ES cell lines. Chromatin extract was prepared from the established ES cell lines and pulled down by FLAG M2 beads prior to competitive elution by FLAG peptides. The co-elution of Dnmt3L with Flag-HA-Dnmt3a or its mutants was revealed by western blot analysis (lanes 6–8). **(B)** Binding of wild-type Dnmt3a and its mutants at the major satellite DNA. The enrichment of major satellite DNA from stably transfected ES cells expressing wild-type and mutant Flag-HA-Dnmt3a was determined by real-time PCR. **(C)** Recruitment of wild-type Dnmt3a and its mutants to the *Oct4* promoter in RA-induced ES cells. The enrichment fold reflects the amount of the *Oct4* sequence immunoprecipitated with anti-Dnmt3a antibody, relative to the amount obtained with IgG. The *Gapdh* promoter, which is not bound with Dnmt3a served as a negative control. The right bar graph shows H3K4 demethylation at *Oct4* in the four compared ES cell lines during RA-induced differentiation. ChIP with anti-H3 served as a control. D0 and D2 denote undifferentiated ES cells and 2 days after RA-induction.

Characterization of mutations in Dnmt3a that abrogate its catalytic activation by histone peptides but not by the H3 binding and the basal methylation activity

As previous studies indicate that the Dnmt3a PHD domain is not required for its chromatin association [19, 20], we reasoned that the binding of H3 peptide via the PHD domain might regulate the Dnmt3a function through allosteric enzymatic activation rather than chromatin recruitment. As the allosteric effect of H3 binding might be mediated by interaction between the PHD and catalytic domains, we sought to characterize crucial residues at the interaction interfaces, whose substitutions would block allosteric activation of enzymatic activity but have no effect on the H3 binding and the basal catalytic activities. Based on the structures of PHD [14] and C-terminal catalytic domain [23] of Dnmt3a, and the structure of the full-length Dnmt3L [13], which covers both the PHD and C-terminal part, we modeled the potential interfaces by homology docking (Figure 3A). Residues on both interfaces were chosen for mutational

analysis. Among 13 residues we analyzed (Supplementary information, Table S1), alanine substitution of glutamine 304 (Q304) in the PHD domain severely reduced the degree of activation of Dnmt3a by histone peptides (Figure 3B), yet the histone peptide-binding property of the mutant protein remained unchanged (Figure 3C and 3D). Similarly, alanine substitution of arginine 580 (R580) in the catalytic domain rendered the enzyme insensitive to the stimulation by H3 peptides, while the basal methylation activity in the absence of H3 peptides (Figure 3B) and histone binding ability (Figure 3C) did not change. These data demonstrate the presence of interfaces involved in allosteric regulation that is independent of the histone tail binding and basal catalytic activities.

Mutations affecting allosteric activation by histone H3 do not affect genomic binding of Dnmt3a but diminish its methylation function in ES cells

Similar to the PHD mutations, the two interface mutations Q304A and R580A diminished the methylation

activity of Dnmt3a when expressed in cultured human cells (Supplementary information, Figure S5). We next determined the physiological importance of allosteric regulation of Dnmt3a by examining the impact of interface mutations on DNA methylation in ES cells. Stable cell lines expressing exogenous Dnmt3a at similar levels (Supplementary information, Figure S6A) were established from triple knockout (TKO) ES cells deficient in Dnmt1, Dnmt3a and Dnmt3b [24]. Cellular fractionation assay (Supplementary information, Data S1) confirmed that the mutant and wild-type proteins were similarly detected in the S1 and S2 fractions rich in euchromatin and heterochromatin, respectively (Supplementary infor-

mation, Figure S6B). Immunoprecipitation assay showed that the Dnmt3a proteins were associated with Dnmt3L (Figure 4A), consistent with the previous finding [13]. To examine whether the point mutation would change genomic localization of the Dnmt3a protein, we performed ChIP for two types of the known target sequences. Quantification by real-time PCR of co-immunoprecipitated DNA indicated that mutant proteins were enriched on the major satellite repeats (Figure 4B). Similarly, ChIP analysis revealed binding of the two mutant proteins to the *Oct4* promoter upon induction of cell differentiation, concomitant with H3K4 demethylation accompanying gene silencing (Figure 4C and 4D). These observations

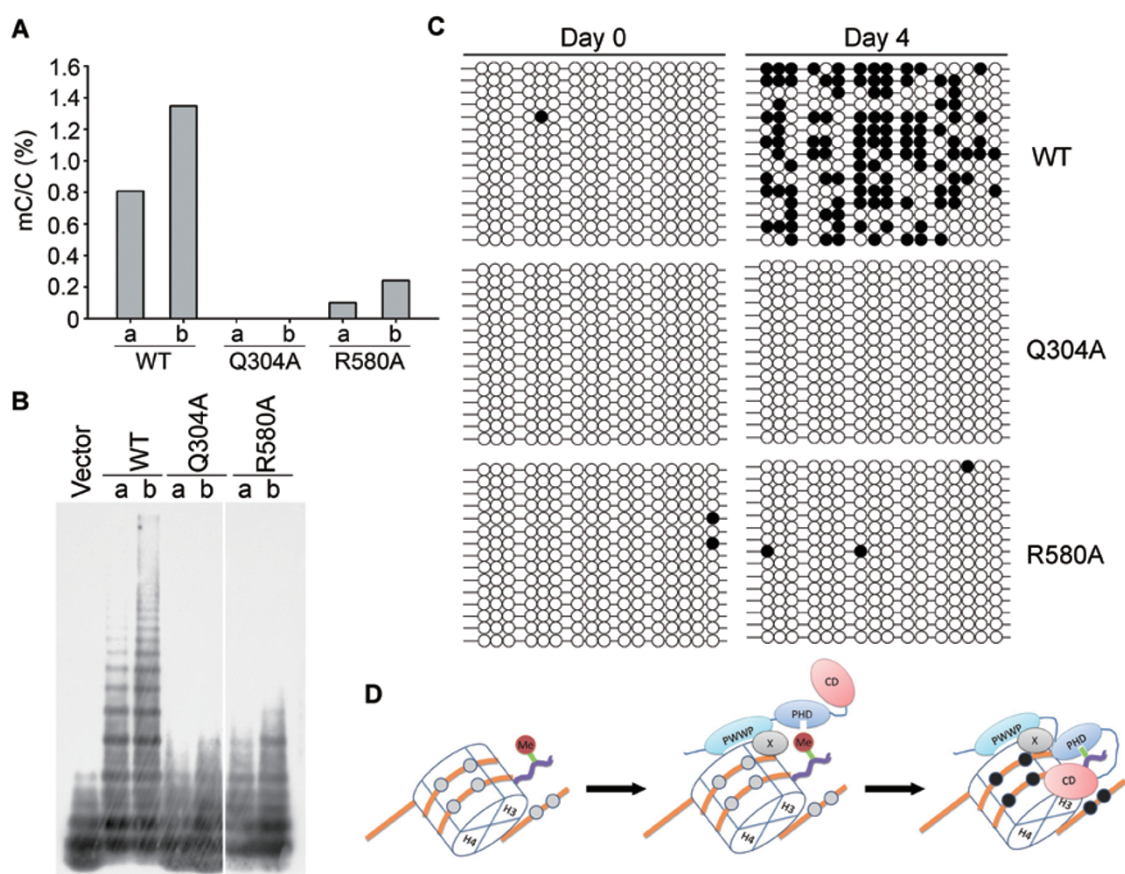


Figure 5 Function of Dnmt3a in ES cells depends on its capacity of allosteric regulation by histone H3. **(A)** Analysis of the overall DNA methylation level in TKO ES cell lines expressing ectopic wild-type (WT) and mutant Dnmt3a (Q304A and R580A). The mC/total C ratio in genomic DNA was determined by HPLC. For each sample, two independent cell lines were used. **(B)** Analysis of methylation at the major satellite repeats. Genomic DNA isolated from the TKO cell lines expressing wild-type and mutant Dnmt3a was digested with *HpyCH4IV* prior to Southern hybridization analysis. **(C)** Analysis of methylation at the *Oct4* promoter. Bisulfite sequencing was performed for DNA samples extracted before (Day 0) and after RA-induced differentiation (Day 4). **(D)** Model for DNA methylation. The methylation process takes two steps: enzyme recruitment and activation. The multi-domain methyltransferase is recruited to the specific genomic site through its PWWP domain by interacting with nucleosomal histones and/or DNA, or alternatively through other adaptor proteins such as Dnmt3L. The localized Dnmt3 is then activated by unmodified H3K4 histone tails that interacts with the PHD domain, to methylate the nearby cytosines.

suggest that the two interface mutants have unaltered interaction with other proteins and chromatin *in vivo*.

We next examined the function of the ectopic Dnmt3a in genome-wide methylation. Quantification of the methylcytosine content by HPLC indicated that expression of wild-type Dnmt3a in two independent cell lines could restore the methylation level to 0.8% and 1.4% while expression of the two mutant proteins resulted in no or much less methylation (Figure 5A). Analysis of specific target sequences extended this observation. As shown by Southern blotting analysis, major satellite repeats became substantially methylated upon ectopic expression of the wild-type Dnmt3a, while they were nearly unmethylated in cells expressing the Dnmt3a mutants, much like in the original TKO cells (Figure 5B). Similarly, both mutants were unable to methylate the promoter regions of the single-copy genes *Oct4* and *Sox30* during ES cell differentiation (Figure 5C and Supplementary information, Figure S7), while the wild-type Dnmt3a showed robust methylation. Therefore, point mutations that affect interfaces of both the PHD and catalytic domains and thus abrogate the allosteric effect of histones led to loss of the methylation function *in vivo*. As association of both mutant proteins with chromatin and their genomic targets did not appear to be affected, the failure in DNA methylation can be attributed to defective allosteric activation of Dnmt3a by histone tails *in vivo* in the chromatin context. Interestingly, one of the missense mutations, R803S, recently identified in human DNMT3A of acute myeloid leukemia patients [3], corresponds to the interface mutation R580A in sequence alignment. Thus, defective allosteric activation by histones can be implicated in the malfunction of mutant DNMT3A in human leukemogenesis.

Discussion

The data described in this and other previous work lead us to propose that occurrence of DNA methylation might take two steps – enzyme recruitment and activation (Figure 5D). In this model, the methyltransferase is recruited to a specific genomic site by directly recognizing nucleosomal DNA or histones [13–15, 18, 25], or alternatively through other adaptor proteins such as Dnmt3L [13, 23], G9a [26–28], Np95 [29] and PML-RAR [30]. The localized Dnmt3 is then allosterically activated to methylate nearby cytosines upon the binding of an unmodified H3K4 *N*-terminus to the PHD domain. Other sequences are excluded from methylation either because they are not loaded with Dnmts or their associated chromatin is not permissive due to K4 methylation. In agreement with our working model, epigenomic studies reveal that DNA cytosine methylation has no simple lin-

ear relationship with histone modification, except for the anti-correlation with H3K4 trimethylation [11–12, 31]. Requirement of allosteric activation by histone tails for Dnmt3a function might also account for the enrichment of DNA methylation in nucleosome-associated exons reported most recently [32].

The dual requirement for recruitment and activation provides an opportunity at the chromatin level to converge multiple cellular signals into long-term silencing of target genes. Presumably, there might be an advantage for patterns of histone modification that are not propagated efficiently during mitosis to be converted into patterns of DNA methylation that can be stably maintained. This is particularly relevant in case of pluripotency genes such as *Oct4* when this vitally important regulatory gene undergoes multi-step down-regulation during cell lineage commitment in response to intrinsic and extrinsic signals in early embryos [33, 34]. The repression process involves binding of transcription factor GCNF [34] and H3K9 methyltransferase G9a [28] to the *Oct4* promoter, both of which are necessary events for DNA methylation to ensue. G9a mediates the recruitment of Dnmt3 enzymes independent of its catalytic activity [28], arguing against the proposed role of H3K9 methylation as a signal for DNA methylation [33]. In agreement with this, histone tails required to activate the Dnmt3a only include a few *N*-terminal amino acids and the portion including K9 can even be deleted without reducing cytosine methylation by ectopic expressed Dnmt3a-Dnmt3L in yeast [16]. Moreover, inclusion of K9 trimethylation in the free H3 peptides (this work) or in the H3 tail of reconstituted chromatin [15] has little effect on the *in vitro* methylation activity. It is therefore unlikely for the DNA methyltransferase to respond directly to the change in H3K9 methylation. Given the implication of allosteric activation by H3 tails with lower methylation levels at K4, H3K4 demethylation instead appears to be a major checkpoint for DNA methylation. Consistent with this idea, mutations blocking the allosteric activation at the intra-molecule interfaces in Dnmt3a diminished its methylation activity in ES cells, but they did not change its complex formation with Dnmt3L, overall association with chromatin and the recruitment to major satellite repeats and the *Oct4* promoter. Thus, mere physical recruitment of the DNA methyltransferase to targets, while required, would not necessarily trigger DNA methylation. Histone modifications, especially H3K4 methylation, regulate the allosteric activation of recruited DNA methyltransferase. Further investigation is needed to reveal the mechanism underlying the coordinate actions of the factors that target Dnmts to chromatin, modify chromatin and trigger DNA methylation.

Materials and Methods

Protein purification

For purification of Flag-tagged full-length Dnmt3a, HEK293T cells were transiently transfected with expression constructs encoding Flag-tagged Dnmt3a (the ES form, amino acid 1-689) or its mutants by calcium phosphate transfection method. Cells were harvested 48 h post transfection and lysed in high salt lysis buffer (50 mM Tris-Cl, pH 7.5, 500 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease inhibitor cocktail (Roche)). After centrifugation at 100 000× g for 60 min at 4 °C, the clarified cell lysate was collected and incubated with FLAG-M2 agarose (Sigma) by gently rotating at 4 °C for 2 h. Following washing with lysis buffer three times, bound proteins were eluted by competition with FLAG peptides and dialyzed into storage buffer (10 mM HEPES-KOH, pH 7.5, 50 mM KCl, 1 mM EDTA and 50% glycerol).

For purification of GST fusion proteins, GST-tagged Dnmt3aPHD was overexpressed in *E. coli* strain BL21 (DE3) Codon-Plus-RIL (Stratagene). Cells were grown at 37 °C in LB medium complemented with 100 µM zinc sulfate to an optical density of 0.6-0.8 at 600 nm, and then induced with 0.2 mM IPTG at 20 °C overnight. Purification was performed using Glutathione Sepharose 4B (GE healthcare) according to the manufacturer's instructions.

In vitro methylation assay

In vitro methylation activity of Dnmt3a was determined as described [35] with slight modifications. In brief, purified Flag-Dnmt3a protein (200 ng, 0.13 µM) was pre-incubated with or without H3 peptides (100 ng, 2.3 µM) in 20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM EDTA, 50 µg/ml BSA and 1.25 mM ³H-SAM (80 Ci/mmol, Perkin Elmer) in a total volume of 19 µl at room temperature for 5 min. Biotinylated 2.2-kb DNA fragment (100 ng) (0.34 µM CpGs) amplified from the *EBNA1* region of p220.2 was added to the reaction and incubated for 20 min at 37 °C. Reactions were quenched by addition of excess of cold SAM. Incorporation of methyl-³H into the naked substrate DNA was determined as described [36].

In vivo methylation assay

In vivo activity of Dnmt3a was mainly analyzed in stably transfected ES cell lines. Methylcytosine content in genomic DNA was determined by HPLC as described [37]. Methylation level in major satellite repeats was assessed by Southern hybridization using non-radioactive method with biotin-labeled oligonucleotide (5'-biotin-TATGGCGAGGAACTGAAAAAGGTGGAAATTTA-GAAATGTCCACTGTAGGACGTGGAATATGGCAAG-3'). Methylation status at gene promoters was determined by bisulfite sequencing analysis as described [38]. The *in vivo* activity of Dnmt3a toward episomal plasmid in HEK293 c18 cells was analyzed essentially as described [21].

Establishment of stable ES cell lines and in vitro differentiation

DKO (*Dnmt3a* and *Dnmt3b* double knockout [39]) stable ES cells ectopically expressing Dnmt3a were established essentially as described [20]. TKO (*Dnmt31*, *Dnmt3a*, *Dnmt3b* TKO [24]) stable ES cell lines ectopically expressing Dnmt3a were established by infection with lentivirus carrying a Dnmt3a expression cassette

linked to the *EGFP* gene. ES clones expressing EGFP were picked and expanded. Positive clones expressing FLAG-HA-Dnmt3a were verified by western blotting. ES cell *in vitro* differentiation was performed as described [34].

Histone peptide binding assay

Histone peptide pull-down assays were performed as described [40]. Biotinylated histone peptides of high purity were synthesized by Scilight-Peptide Inc. Briefly, 1 µg of peptides were incubated with 1 µg of purified protein in binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40 and 1 mM PMSF) overnight at 4 °C. A volume of 20 µl of streptavidin beads (GE healthcare) were added into the mixture and incubated for another 2 h. After washing three times with washing buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% NP-40 and 1 mM PMSF), the bound proteins were eluted in 2× SDS loading buffer by heating at 100 °C for 5 min and subjected to western analysis.

Surface plasmon resonance measurements

Surface plasmon resonance binding experiments were performed on BIAcore 3000 biosensor at 25 °C as described previously [41]. Equivalent amount of H3 (1-21) peptides biotin-labeled at the C-terminus were immobilized in four different cells of one streptavidin-coated biosensor chip. The immobilization response unit of peptides containing unmethylated, mono-, di- and tri-methylated K4 was about 150. The wild-type and mutant GST-Dnmt3a PHD proteins were dialyzed in running buffer containing 10 mM HEPES, pH 7.5, 150 mM NaCl and 0.005% NP-20 at the concentration of 50 µM. A 3-min sample injection was performed under the flow rate of 30 µl/min and the disassociation time was 15 min. Chip was regenerated by washing with 10 mM NaOH. All measurements were repeated at least three times under the same condition.

Chromatin immunoprecipitation

The procedures are carried out mainly as previously described [42]. Briefly, the crosslinked chromatin of ES cells or embryonic body was extracted and sheared to average DNA size of 500 bp with Diagenode BioRuptorTM sonicator. The sheared extracts were diluted 10-fold by ChIP dilution buffer and precleared with 30 µl of protein A/G agarose beads. Following incubation with antibodies (anti-H3K4me3, ab8480; anti-H3, ab1791; anti-Dnmt3a, self-prepared) at 4 °C overnight, 30 µl of protein A/G agarose beads blocked with BSA and salmon sperm DNA were added and rotated for 2 h to capture the protein-DNA complex. After washing with a series of wash buffer, the bound protein-DNA complex was eluted with 100 mM NaHCO₃ and 1% SDS. After crosslink reversal and proteinase K treatment, the purified DNA was subjected to real-time PCR to determine the relative enrichment fold compared with IgG. Primers for *Oct4* promoter are as follows, Oct4_ChIP_f: 5'-TGGGGCATCCGAGCAACTGGT-3' and Oct4_ChIP_r: 5'-AGAGCTGTGGGGGTGGAGAACTGA-3'.

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