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PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing

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Abstract

Mammalian gene silencing is established through methylation of histones and DNA, although the order in which these modifications occur remains contentious. Using the human β-globin locus as a model, we demonstrate that symmetric methylation of histone H4 arginine 3 (H4R3me2s) by the protein arginine methyltransferase PRMT5 is required for subsequent DNA methylation. H4R3me2s serves as a direct binding target for the DNA methyltransferase DNMT3A, which interacts through the ADD domain containing the PHD motif. Loss of the H4R3me2s mark through short hairpin RNA–mediated knockdown of PRMT5 leads to reduced DNMT3A binding, loss of DNA methylation and gene activation. In primary erythroid progenitors from adult bone

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AUTHOR CONTRIBUTIONS

Q.Z., G.R., L.C. and Y.T.T. performed the experiments; R.L.M. and R.J.S. performed and analyzed the mass spectrometry; Q.Z., G.R. and S.M.J. designed and analyzed the research; D.J.C., C.D.A., H.L., D.J.P. and J.M.C. provided ideas, reagents and discussion; Q.Z., G.R. and S.M.J. wrote the manuscript.

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marrow, H4R3me2s marks the inactive methylated globin genes coincident with localization of PRMT5. Our findings define DNMT3A as both a reader and a writer of repressive epigenetic marks, thereby directly linking histone and DNA methylation in gene silencing.

Covalent modification of DNA and histone molecules, the core components of chromatin, provides a heritable mechanism for regulating gene expression[1,2]. These histone marks function cooperatively to establish distinct repressive or active chromatin states that extend the information potential of the genetic code. Integral to this process are effector molecules, which interpret specific modifications to influence downstream events through recruitment or stabilization of chromatin-template machinery[3].

Although histone modifications and DNA methylation have also been shown to function cooperatively, in many settings the order in which these epigenetic marks are established remains unclear. In mammals, methylation of DNA is largely confined to position five of the cytosine ring in CpG dinucleotides and is most commonly associated with a repressed chromatin state and inhibition of gene expression[4,5]. Although some overlap exists[6], two general classes of cytosine DNA methyltransferases are known: the *de novo* methyltransferases, DNMT3A and DNMT3B, which are responsible for modifying unmethylated CpG sites, and the maintenance methyltransferase DNMT1, which copies pre-existing methylation patterns onto the new DNA strand during replication[7]. The precise sequence of events linking histone modifications and DNA methylation varies in different organisms, and at different gene loci, which suggests that it is context dependent. Evidence that DNA methylation can influence the histone modification pattern has been obtained in several model systems. Transgenes methylated *in vitro* before stable genomic integration associate with deacetylated histones, whereas an identical but unmethylated integrated transgene is enriched for acetylated his-tones[8,9]. Methyl-CpG–enriched regions may directly recruit histone methyltransferases[10], or are bound by methyl-CpG–binding proteins, which in turn recruit repressor complexes containing histone deacetylases[4] and histone methyltransferases[11,12]. Conversely, studies in fungi, plants and mammals have suggested that trimethylation of H3K9 (ref. 13), H3K27 (ref. 14) and H4K20 (ref. 15) is a prerequisite for subsequent DNA methylation. The functional link between these processes appears to be attributable to a physical association between the histone lysine methylation system and DNA methyltransferases, as has been established in the context of H3K9 and H3K27, where the relevant lysine histone methyltransferases, SUV39h1 and EZH2, have been shown to interact directly with DNMT1 and with DNMT3A and DNMT3B (refs. 16–18). Histone arginine methylation has also been implicated in gene silencing, but so far no links between arginine methyltransferases and DNA methyltransferases have been established.

Using the human β-globin locus as a model, we now report evidence of a link between arginine methylation of histones and DNA methylation and gene silencing. Central to this link is the protein arginine methyltransferase PRMT5, which symmetrically dimethylates H4R3. We show that this histone modification serves as a direct target for binding of DNMT3A, thus providing a new mechanism by which a repressive histone modification and DNA methylation are coordinated.

RESULTS

PRMT5 induces the H4R3me2s mark on the γ-globin gene

The β-globin locus has served as a paradigm for analyzing the role of epigenetic modifications in the regulation of tissue and developmentally specific gene expression. In both humans and primates, the genes encoding fetal (γ) globin are progressively silenced after birth, displaying methylation of a cluster of CpG dinucleotides in the proximal promoters and 5' untranslated regions in adult bone marrow[19]. To identify proteins involved in silencing of genes encoding γ-globin (γ-genes), we analyzed by mass spectrometry immunoprecipitates from an erythroid cell line (K562) expressing Flag-tagged NF-E4, a proximal γ-promoter–binding transcription factor implicated in both activation and repression of the γ-genes[20]. We identified a number of proteins specific to the Flag-NF-E4–expressing cells that were not detected in Flag immunoprecipitates from cells transfected with the control vector expressing the Flag epitope alone (**Fig. 1a**). The protein methyltransferase PRMT5 (ref. 21) was of particular interest, as the highly related protein PRMT1 has been shown to have a key role in activation of the adult β-globin gene[22]. PRMT5 has been implicated in gene silencing, which raises the possibility that the PRMT factors could have coordinate roles in regulating the fetal-to-adult switch in globin gene subtype. We confirmed the interaction between NF-E4 and PRMT5 by co-immunoprecipitation of endogenous proteins from untransfected cells (**Fig. 1b**). This interaction was direct, as demonstrated by glutathione S-transferase (GST) pulldown, and required the residues between 49 and 100 in the N-terminal half of NF-E4 (Supplementary Fig. 1a online). The two proteins were localized at the proximal γ-promoter (P-1, which contains the NF-E4 binding site) by chromatin immunoprecipitation (ChIP) using antibodies to the endogenous proteins (**Fig. 1c**). Binding of PRMT5 and NF-E4 to far upstream regions of the γ-promoter was not detected, but weak binding was detected to the promoter region adjacent to P-1 (P-2), and to regions downstream of the transcriptional start site (TSS) extending to exon 2 (P+1 to P+3) (Supplementary Fig. 1b). Analysis of the other proteins identified by mass spectrometry will be described elsewhere.

PRMT5 is a type II arginine methyltransferase that has been linked to gene silencing through establishment of repressive histone marks, including symmetrical dimethylation of H4R3, H2AR3 (refs. 21,23) and H3R8 (ref. 24). To identify the histone substrates of PRMT5 in K562 cells, we derived a cell line expressing Flag-tagged PRMT5 (PRMT5-f) to facilitate immunoprecipitation of the active enzyme. The Flag-immunoprecipitate from this line was subjected to a standard radioactive histone methyltransferase activity assay using free histones, which demonstrated radiolabeling of histone H4 (**Fig. 1d**, free histones). To ensure that methylation of this substrate was specific for PRMT5 methyltransferase activity, we also derived a line containing a Flag-tagged mutant form of PRMT5 in which five amino acids in the S-adenosyl-L-methionine binding motif had been deleted (PRMT5Δ-f)[25]. Methylation of histone H4 induced with the Flag-immunoprecipitate from this line was markedly reduced, and the weak signal we observed presumably reflects a small amount of endogenous PRMT5 dimerized to PRMT5Δ-f (ref. 25) (**Fig. 1d**, free histones). Comparable amounts of the tagged proteins were contained in the precipitates used in both assays **(Fig. 1d**, immunoblot: α-Flag). No methyltransferase activity was observed with the Flag-precipitate

from untransfected cells (data not shown). In this setting, methylation of the other known substrates of PRMT5 (histones H2A and H3) was not detected. This substrate specificity was identical when nucleosomes purified from K562 cells were used as the source of histones (**Fig. 1d**, K562 nucleosomes). To confirm that PRMT5 induced the H4R3me2s modification in this assay, we incubated the ^{3}H-methylated histones with an antibody against H4R3me2s or with normal rabbit IgG before immunoprecipitation. Substantial levels of radioactivity were detected in the precipitate generated with the H4R3me2s antibody from the PRMT5-f–labeled histones, but not from the PRMT5D-f reaction (**Fig. 1e**). Consistent with this finding, the repressive H4R3me2s mark was enriched at the γ-promoter (and binding extended downstream of the TSS) in cells expressing PRMT5-f but not PRMT5Δ-f, despite both proteins binding robustly to the proximal promoter site (**Fig. 1f** and Supplementary Fig. 1b).

PRMT5 mediates transcriptional silencing of the γ-gene

To ascertain whether perturbations to the levels or enzymatic activity of PRMT5 affect γ-gene expression, we performed quantitative real-time PCR (Q-RT-PCR) analyses on cells expressing PRMT5-f and cells expressing PRMT5Δ-f (**Fig. 2a**). Expression of the two tagged proteins was comparable (immunoblot: α-Flag) and equivalent to the level of endogenous PRMT5 in the vector control cells (immunoblot: α-PRMT5). Increased levels of PRMT5 resulted in a twofold to threefold downregulation of γ-gene expression, whereas enforced expression of PRMT5Δ-f induced a two-fold increase in γ-gene transcripts compared with the vector control (**Fig. 2a**, left). To examine the effect of reduced PRMT5 expression, we used stably expressed short hairpin RNAs (shRNAs) (PRMT5-kd). Cells transfected with an expression vector containing a scrambled sequence served as the control (Scr). Western blots confirmed that PRMT5 protein levels were reduced by more than 90% in the PRMT5-kd cells compared with the scrambled control, but no effect was observed on the control proteins tubulin, PRMT1 and GATA-1 (**Fig. 2b**, right). The knockdown of PRMT5 led to a sixfold induction of γ-gene expression compared with the scrambled vector (**Fig. 2b**, left). This effect was specific, as other markers of erythroid differentiation in K562 cells (GATA-1, glycophorin A and v-myb) remained unchanged in the PRMT5-kd cells compared with the Scr control (**Fig. 2b**, right; Supplementary Fig. 2 online). Consistent with the γ-gene expression data, the H4R3me2s repressive mark was markedly reduced at the γ-promoter in the PRMT5-kd cells, and RNA polymerase II localization was increased fivefold compared with the Scr control (**Fig. 2c**). We attempted to determine the role of NF-E4 in PRMT5 regulation of γ-gene expression by generating a knockdown of this factor. Despite the use of several different shRNA constructs, we were unable to modulate the levels of the endogenous protein.

PRMT5 and DNMT3A function cooperatively in gene silencing

Methylation of four CpG dinucleotides immediately flanking the transcriptional start site is essential for γ-gene silencing, and two of these CpGs reside within the PRMT5 binding region[19,26,27]. In view of this, we examined whether DNA methylation of the γ-gene was altered in PRMT5-f, PRMT5Δ-f, PRMT5-kd or scrambled control (Scr) cells using bisulfite DNA sequencing (**Fig. 3a**). Consistent with the observation that globin gene expression is not detectable in a large proportion of uninduced K562 cells, we observed methylation of the

four CpG dinucleotides immediately flanking the γ-globin transcriptional start site in 21% of clones derived from scrambled control cells. This frequency was increased in three of the four sites in the PRMT5-f cells, with 35% of the clones showing methylation. In contrast, methylation of all CpG dinucleotides was abolished in clones derived from the PRMT5-kd cells. It was also abolished in all clones derived from the PRMT5Δ-f cells, which suggests that the enzymatic activity of PRMT5, and not just its physical occupation of the γ-globin promoter, is essential for the epigenetic modification of DNA in this setting. No change in the high levels of DNA methylation (95%) at the six CpGs flanking the b-gene transcriptional start site (−415 to +110) was observed in either the PRMT5-kd, PRMT5Δ-f or scrambled control cells (Supplementary Fig. 3a online).

To determine the mechanism by which PRMT5 influences DNA methylation, we initially examined whether the protein methyltransferase is associated directly with a DNA methyltransferase. We analyzed Flag-antibody immunoprecipitates from PRMT5-f cells by immunoblot with antibodies to DNMT1, DNMT3A and DNMT3B, and demonstrated that DNMT3A was co-immunoprecipitated with PRMT5 (**Fig. 3b**). We confirmed this using antibodies to the endogenous proteins with extract from untransfected cells (**Fig. 3c**). The identification of DNMT3A as a PRMT5-interacting protein suggested that it might be responsible for DNA methylation in the context of PRMT5-induced fetal globin gene silencing. To address this, we examined the binding of DNMT3A to γ-promoter in PRMT5-f and PRMT5Δ-f cells by ChIP (**Fig. 3d**). Consistent with our methylation studies, binding of DNMT3A to the γ-gene was markedly reduced in the PRMT5Δ-f cells compared with cells expressing PRMT5-f. DNMT3A binding was also markedly reduced in the PRMT5-kd cells compared with the scrambled control cells (**Fig. 3e**). To assess the role of DNMT3A in γ-gene silencing, we used an shRNA approach to knock down the expression of the protein in K562 cells (DNMT3A-kd). A reduction in DNMT3A levels to 30% of the scrambled control (**Fig. 3f**, right) induced a fivefold increase in γ-gene expression levels (**Fig. 3f**, left). This was accompanied by a twofold reduction in CpG methylation at the γ-gene (**Fig. 3g**), but no change at the β-gene (Supplementary Fig. 3b).

DNMT3A binds specifically to histone H4R3me2s

The importance of PRMT5 enzymatic activity for the recruitment of DNMT3A to the γ-gene suggests that the methyltransferase domain of PRMT5 is involved in the interaction between the two proteins. To address this, we used a GST pulldown assay to analyze the binding of ^{35}S-radiolabeled *in vitro* transcribed and translated DNMT3A to GST-PRMT5 and GST-PRMT5Δ (**Fig. 4a**). Surprisingly, no difference was observed between the wild-type and mutant proteins, which implies that the enzymatic function of PRMT5 may lead to DNMT3A recruitment via an alternate mechanism. One possibility is that the PRMT5-induced H4R3me2s modification could directly recruit DNMT3A. To examine this, we performed a peptide pulldown assay using COOH-terminal biotin-tagged 20-mer N-terminal peptides of histone H4 with the Arg3 residue unmethylated, symmetrically methylated or asymmetrically methylated. We confirmed symmetric methylation by western blot with the H4R3me2s antibody (**Fig. 4b**, immunoblot: α-H4R3me2s). We incubated equivalent amounts of each peptide (**Fig. 4b**, Coomassie stain) coupled to streptavidin beads with nuclear extract from K562 cells, washed the beads and analyzed the eluate by immunoblot

with an antibody to DNMT3A (**Fig. 4b**, immunoblot: α-DNMT3A). Binding of DNMT3A was observed with the H4R3me2s peptide, but not with the unmethylated or asymmetrically methylated peptides. The DNMT3A protein contains a PWWP domain implicated in DNA and chromatin binding, an ATRXDNMT3-DNMT3L (ADD) domain that contains a plant homeo-domain (PHD) zinc finger motif that may mediate interactions to other proteins (including histones), and a C-terminal catalytic domain[3,28–30]. We demonstrated that the interaction between DNMT3A and H4R3me2s was direct and specific using pulldown assays with the three peptides and radiolabeled *in vitro* transcribed and translated DNMT3A (**Fig. 4c**). We then mapped the regions of DNMT3A required for binding to the H4R3me2s peptide. No binding was observed with the N-terminal third of the protein (amino acids 1–354). A larger N-terminal fragment (1–587) containing the GATA and PHD domains of ADD, but lacking an adjacent C-terminal helix, bound to levels comparable to those of the full-length protein (**Fig. 4c**). These findings suggested that binding of DNMT3A to the H4R3me2s peptide was mediated through a region between residues 354 and 587, which incorporates most of the ADD domain. We therefore repeated the peptide pulldown assays using the full-length ADD (479–610) and PWWP (281–424) domains generated as GST fusion proteins (**Fig. 4d**). Specific binding of the ADD domain was observed with the H4R3me2s peptide, but not with the unmodified or H4R3me2a peptides. No binding was observed with the PWWP domain. As an additional control, we included an unmodified N-terminal histone H3 peptide, as the ADD domain of DNMT3L has been shown to bind to histone H3 (ref. 31). Binding of the ADD domain of DNMT3A was also observed with this peptide. The significance of DNMT3A binding to histone H3 is unclear in this context, as loss of the H4R3me2s mark was sufficient to abolish DNMT3A binding to the γ-promoter in PRMT5-kd cells (**Fig. 3e**).

We compared these findings to studies examining the binding of DNMT3A truncation mutants to PRMT5 (Supplementary Fig. 4a online). In contrast to the H4R3me2s peptide, only very weak binding to PRMT5 was observed with a mutant containing the ADD domain (351–912). Binding to levels comparable to those of the full-length protein was seen with the N-terminal fragment (1–354). Analysis of the isolated PWWP and ADD domains confirmed these findings (Supplementary Fig. 4b).

Role of PRMT5 in developmental globin gene silencing

To determine whether the H4R3me2s mark was evident at the human γ-globin promoter in a developmentally specific pattern in primary human cells, we isolated erythroid progenitors from cord blood and adult bone marrow. As expected, the expression of the genes encoding γ-globin was higher in cord blood compared with bone marrow using Q-RT-PCR (Supplementary Fig. 5 online). ChIP analysis demonstrated a fourfold increase in the H4R3me2s mark at the γ-promoter in adult bone marrow erythroid progenitors compared with progenitors derived from cord blood (**Fig. 5a**). This was accompanied by a reduction in RNA polymerase II localized to the γ-promoter in the adult cells. We then examined the distribution of PRMT5 and NF-E4 binding, and the H4R3me2s mark across the β-globin locus in bone marrow erythroid progenitors using ChIP analyses with anti-PRMT5, anti-NF-E4 and anti-H4R3me2s antibodies (**Fig. 5b**). We used primer pairs spanning regions of the locus control region (LCR) hypersensitive sites (HS1–HS4), the e-globin, γ-globin and β-

globin promoters, and the intergenic region between the $^{G}\gamma$-genes and $^{A}\gamma$-genes. We detected high levels of PRMT5, NF-E4 and H4R3me2s at the transcriptionally inactive γ-promoters and e-promoters, but not at the b-promoter in these adult cells. We also observed increased levels at HS3 of the LCR, but not at the other hypersensitive sites or in the intergenic region.

To further examine the role of PRMT5 in developmental silencing of the γ-genes, we determined the cellular localization of the protein by immunofluorescence in cord blood and bone marrow erythroid progenitors (**Fig. 5c**). PRMT5 has previously been shown to translocate from the nucleus to the cytoplasm at the time of extensive epigenetic reprogramming of mouse germ cells[32]. We demonstrated that the protein was predominantly nuclear in the bone marrow progenitors, whereas it was primarily localized in the cytoplasm in the cord blood progenitors. These findings suggest an additional mechanism by which PRMT5 may play a developmentally specific role in regulating gene expression at the human β-globin locus.

DISCUSSION

In addition to their direct role in influencing chromatin compaction, an emerging paradigm suggests that post-translational modifications of histones also serve as direct targets of effector molecules. These effectors (or “readers”) induce downstream functional consequences, including (i) remodeling or stabilization of the chromatin structure, (ii) introduction of further post-translational modifications through interactions with “writers” and (iii) other gene regulatory effects[33]. Our studies provide an important addition to this paradigm by defining a direct link between the repressive histone modification H4R3me2s and DNA methylation. We show that DNMT3A functions as both a reader and a writer in this context, by binding directly to the H4R3me2s mark and inducing DNA methylation and gene silencing (**Fig. 6**). Central to this is PRMT5, which establishes the histone modification and also interacts directly with DNMT3A.

As with other effector molecules, DNMT3A targets the post-translationally modified histone through the ADD domain, which contains the PHD zinc finger region. PHD motifs have been shown in numerous factors to bind to methyllysine marks, including H3K4me2 and H3K4me3 (refs. 34–38), H3K9me3 (ref. 39), and H3K36me3 (ref. 40). Recently, the PHD domain of RAG2 was shown to bind coordinately to H3K4me3 and H3R2me2s[41]. Readers that recognize methylated arginine residues in histones have not been identified previously, and consequently no structure of a reader bound to this mark is available[3]. Tudor domain–containing proteins have been shown to recognize arginine-glycine–rich motifs in non-histone proteins in a methylarginine-dependent manner[42], and although DNMT3A contains a Tudor-like motif, this region of the protein appears not to have a role in the H4R3me2s interaction[29,43]. Notably, it is involved in the protein-protein interaction between DNMT3A and PRMT5.

Previous studies in the chicken β-globin locus have demonstrated that asymmetric methylation of H4R3 by the type I arginine methyltransferase PRMT1 is essential for the establishment and maintenance of a wide range of active chromatin modifications[22]. Taken

together with our findings, this suggests that arginine methyltransferases may play coordinated and contrasting roles in the developmental regulation of the β-globin locus. In addition to increased levels of the H4R3me2s mark at the γ-promoter, we also observed enrichment of this mark at HS3, the site proposed to preferentially activate the human embryonic and fetal globin genes[44]. It is conceivable that coordinated repressive marks established synchronously at the promoters and HS3 could interfere with the LCR–globin gene interaction[45,46], thereby inducing substantial changes in locus conformation in addition to the more localized modifications of chromatin structure. Alternatively, the complex could initiate at the LCR and spread across the locus leading to gene repression, much like the recent demonstration of epigenetic modifiers acting at a distance in the β-globin cluster in the setting of activation of β-gene expression[47]. The silencing of the genes encoding γ-globin after birth heralds the onset of b-thalassemia and sickle cell disease, which has prompted the search for therapies that will prevent or reverse this process. DNA methyltransferase inhibitors have been used for this purpose, with some effect[48]. An alternate approach that we are pursuing could involve the use of specific inhibitors of PRMT5 methyltransferase activity to block the H4R3me2s modification and subsequent recruitment of DNMT3A and DNA methylation.

Our results provide an important extension to the established links between repressive histone modifications and DNA methylation. Interactions between lysine methyltransferases and DNA methyltransferases[16–18,49], methyl-CpG–binding proteins[12] and methyl-CpG–enriched regions[10] have all been reported. Recently, the heterochromatin protein 1 (HP1) has been shown to translate methylation information from histone to DNA, to cement gene repression[50]. In this model, the G9a enzyme induces the repressive H3K9me2 mark at euchromatic sites, which is read by HP1. The bound HP1 functions as an adaptor by targeting DNMT1 enzymatic activity to these sites, thereby enhancing cytosine methylation. Further stabilization is achieved through a direct interaction between the G9a histone methyltransferase and DNMT1 (ref. 49). Our findings exhibit some parallels with this model —in particular, the dual role of PRMT5 in establishing the repressive mark and binding to DNMT3A, analogous to G9a. However, the recruitment of DNMT3A to H4R3me2s is direct, requiring no adaptor protein for the interaction. As such, it resembles the scenario in *Arabidopsis thaliana*, in which the DNA methyltransferase CMT3 is recruited directly to sites of repressive histone modifications[51]. Notably, other DNA methyltransferase subunits (for example, DNMT3L) fail to bind histone peptides that harbor activating histone marks such as H3K4me[31]. Thus, histone modifications, whether activating or repressing in nature, can influence DNA methylation signatures in positive or negative ways.

METHODS

Mass spectrometry

We resolved Flag immunoprecipitates from K562 cells expressing NF-E4-Flag on a 4–20% (w/v) gradient SDS-PAGE gel and stained with SimplyBlue Safestain (Invitrogen). We excised protein bands of interest from preparative one-dimensional gels and subjected them to electrospray–ion trap (ESI-IT) tandem mass spectrometry (MS/MS) (LCQ-Deca, Finnigan).

Cell culture and immunofluorescence

K562 cells were grown as described previously[20]. We isolated CD34+ cells from human cord blood using a MiniMACS magnetic cell sorting system (Miltenyi Biotec) and cultured them in Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% (v/v) fetal calf serum (FCS), SCF (100 ng ml^{-1}), erythropoietin (5 U ml^{-1}), insulin-like growth factor-1 (IGF-1, 40 ng ml^{-1}) and dexamethasone (1 μM) to induce erythroid differentiation. We cultured CD34+ cells isolated from fresh adult bone marrow (as above) in IMDM supplemented with 15% (v/v) FCS, SCF (100 ng ml^{-1}), IL-3 (10 ng ml^{-1}) and Flt-3 ligand (500 ng ml^{-1}) for 7 d, followed by erythropoietin (5 U ml^{-1}) alone for 5 d to induce erythroid differentiation. Cell surface marker analysis with CD71 and glycophorin A indicated that cultured cord blood and bone marrow cells were greater than 90% erythroid lineage. For immunofluorescence, cord blood and bone marrow cells were mounted on polylysine slides and permeabilized with 0.1% (v/v) Triton X-100. We incubated slides with mouse monoclonal anti-PRMT5 antibody overnight at 4 °C, washed and incubated with Texas Red–conjugated horse anti-mouse secondary antibody (Vector Laboratories) for 1 h at room temperature (25 °C). We then washed the slides and counterstained them with 4’,6-diamidino-2-phenylindole (DAPI) for 3 min before imaging with a Zeiss Axioplan microscope (Zeiss).

Protein interaction studies

Immunoprecipitation, immunoblotting and GST pulldown assays were performed as described previously[52]. We used the following antibodies in the immunoprecipitations: Flag (Sigma-Aldrich), PRMT5 (Abcam) and DNMT1, DNMT3A, DNMT3B, tubulin and GATA-1 (Santa Cruz Biotechnology). Peptide pulldown assays were performed as previously described[31]. Briefly, we coupled 2 μg of COOH-terminal biotin-tagged 20-mer N-terminal peptides of histone H4 with the Arg3 residue unmethylated, symmetrically methylated or asymmetrically methylated to streptavidin beads and incubated with K562 cellular extract prepared with high salt extraction (420 mM NaCl)[27]. We eluted specifically bound protein from stringently washed beads and visualized by western blot with anti-DNMT3A or anti-GST antibody after SDS-PAGE. For peptide pulldown assays with GST proteins, the molar ratio of peptide to GST protein was 10:1. The peptides were synthesized with a polyethylene glycol 4 (PEG4) linker between the histone sequence and the biotin moiety, and the N terminus was acetylated.

ChIP analysis

ChIP assays were performed as described previously[20]. We immunoprecipitated chromatin fractions from K562 cells with specific antibodies. No antibody and normal rabbit IgG served as the controls. Sequences of the primers for the globin locus are listed in Supplementary Table 1 online. We used the following antibodies: H4R3me2s and PRMT5 (Abcam), Flag (Sigma-Aldrich), and DNMT3A and RNA polymerase II (Santa Cruz Biotechnology). We calculated the relative enrichment using a method described previously[53]. We calculated the percentage of ChIP DNA relative to the input DNA. In all assays it ranged from 0.35% to 0.71%. The most substantial enrichment was assigned the value 1, and all other conditions were normalized to this value. For the NF-E4 and PRMT5

ChIP, 32 cycles were used, and for the H4R3me2s ChIP, 35 cycles were used. We performed each experiment at least twice independently.

In vitro methyltransferase assays

Beads from the immunoprecipitation assays from K562 cells transfected with PRMT5-f or PRMT5Δ-f were used as the enzyme source in *in vitro* methyltransferase assays as described previously[54], with slight modifications. Briefly, we incubated the beads with 10 μg of purified histone H2A, H2B, H3 and H4 (Roche), or purified nucleosomes[55], and 2 mCi of *S*-adenosyl-L-methyl-^{3}H-methionine (^{3}H-SAM, Amersham) as the methyl donor, in a mixture of 20 μl of HMTase buffer (25 mM NaCl, 25 mM Tris, pH 8.8) for 2 h at 30 °C. Proteins were resolved on a 14% (w/v) SDS-PAGE gel, stained with Coomassie blue and then dried and subjected to autoradiography.

Bisulfite sequence analysis

Bisulfite sequence analysis was performed as described previously[56]. Primers to amplify the bisulfite-treated γ-promoter are provided in Supplementary Table 2 online. We performed PCR with HiFi Taq polymerase (Roche) as follows: 30 cycles, 94 °C for 20 s, 55 °C for 20 s and 68 °C for 35 s. We cloned PCR products into pCRII (Invitrogen) and then sequenced nucleotides using the Big-Dye Termination method (Applied Biosystems). The significance of the differences between cell lines was calculated using the Fisher's exact test.

RNA interference and retroviral infection

The small interfering RNA target sequences for PRMT5 and DNMT3A were inserted into the pSUPER.retro.-neo+gfp retroviral vector according to the manufacturer's recommendations (OligoEngine). The oligo sequences are provided in Supplementary Table 3 online. Retrovirus production by 293T cells and infection of K562 cells were performed as described[20]. Transduced cells were selected for green fluorescent protein expression by fluorescence-activated cell sorting (FACS).

Q-RT-PCR

Total RNA was isolated from cells with Trizol reagent (Invitrogen). Complementary DNA was generated using the reverse transcription system (Promega). Q-RT-PCR primers are provided in Supplementary Table 4 online. The identities of the amplified bands were confirmed by sequencing. We performed Q-RT-PCR in a Rotorgene 2000 (Corbett Research) in a final volume of 20 μl. Reaction mixtures contained 1× reaction buffer, 2.5 mM $MgCl_2$, 0.05 mM deoxynucleotides (Roche), 0.1 μM gene-specific primers, 1 U Taq polymerase (Fisher Biotech), a 1:10,000 dilution of SYBR Green I (Molecular Probes) and 2 μl of sample or standard. Cycling conditions were 94 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s. Standard curves for hypoxanthine guanine phosphoribosyltransferase (HPRT), γ-globin and β-globin were generated from bone marrow and cord blood cells. The relative quantity of the transcripts was calculated for all individual cell lines. Each reaction was done in duplicate.

Supplementary Material

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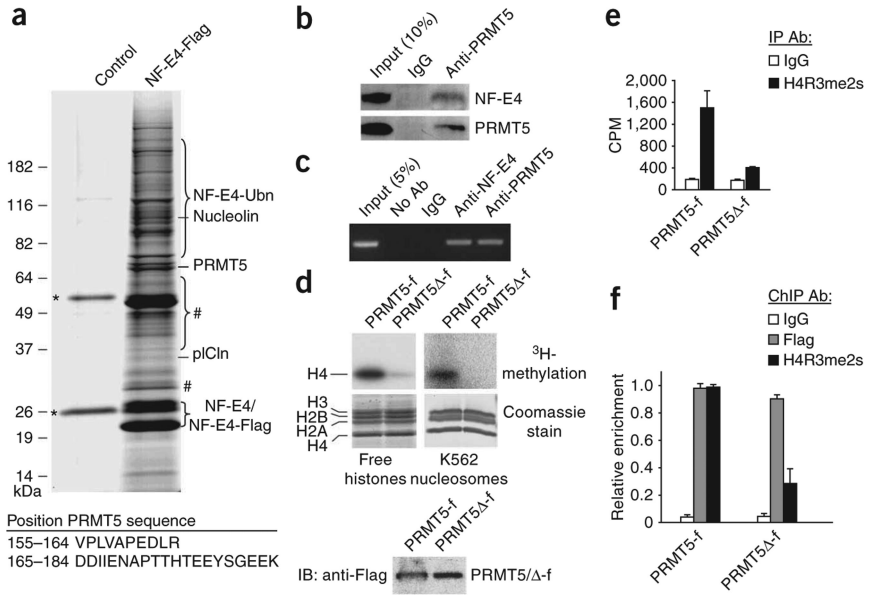

Figure 1.

PRMT5 symmetrically dimethylates histone H4R3 on the gene encoding γ-globin. (**a**) SimplyBlue Safestain of an SDS-PAGE gel of α-Flag antibody immunoprecipitates from K562 cells transfected with NF-E4-Flag, or vector alone (control) before analysis by mass spectrometry. The bands corresponding to PRMT5, NF-E4, polyubiquitinated NF-E4 (ref. 52) and the known PRMT5 partner proteins nucleolin and pICln are shown. Hash marks indicate bands that correspond to common background proteins including keratin, tubulins and ribosomal proteins. Asterisks indicate immunoglobulin chains. The PRMT5 peptide sequences identified are shown below. (**b**) Co-immunoprecipitation of endogenous PRMT5 and NF-E4 from K562 cells. (**c**) Interaction of the endogenous PRMT5 and NF-E4 with the γ-promoter by ChIP assays. (**d**) *In vitro* methyltransferase assays of Flag immunoprecipitates from K562 cells expressing PRMT5-f and K562 cells expressing PRMT5Δ-f. Autoradiographs (upper panels) and Coomassiestained gels (lower panels) are shown for each. (**e**) The HMTase assays with free histones detailed in **d** were immunoprecipitated with antibodies to histone H4R3me2s or normal rabbit IgG, and the radioactivity was quantitated in the respective precipitates. CPM, counts per minute. (**f**) H4R3me2s enrichment at the γ-promoter was measured by ChIP in K562 cells expressing PRMT5-f or PRMT5Δ-f. Error bars show s.d.

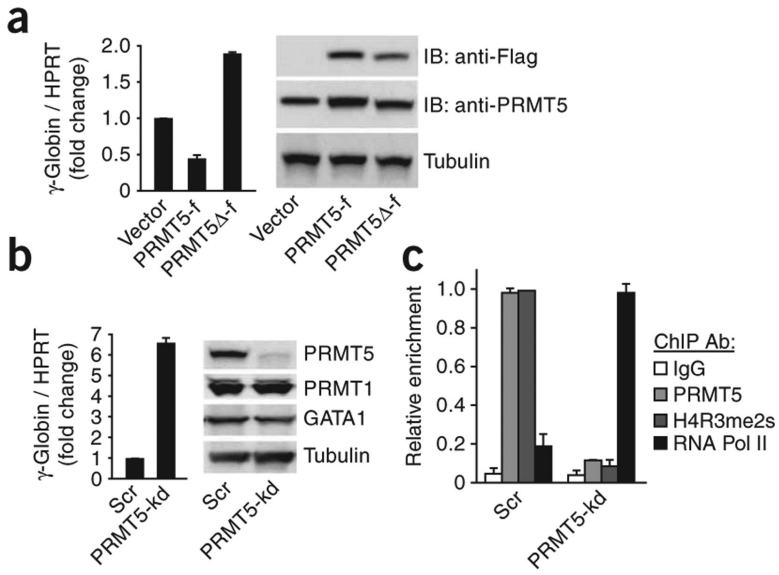

Figure 2.

PRMT5 mediates transcriptional silencing of the γ-gene. (**a**) Extracts from PRMT5-f, PRMT5Δ-f or vector control K562 cells were analyzed by western blot with anti-Flag or PRMT5 antibodies (right panel). RNA from these cells was analyzed by Q-RT-PCR with primers specific for the gene encoding γ-globin, and the signals were normalized against HPRT mRNA levels (left panel). (**b**) K562 cells expressing either an shRNA to PRMT5 (PRMT5-kd) or a scrambled sequence (Scr) were analyzed by western blot (with antibodies indicated, right panel) and Q-RT-PCR (left panel) as in **a.** Error bars show s.d. (**c**) H4R3me2s enrichment at the γ-promoter was measured by ChIP in PRMT5-kd and Scr cells. Error bars show s.d.

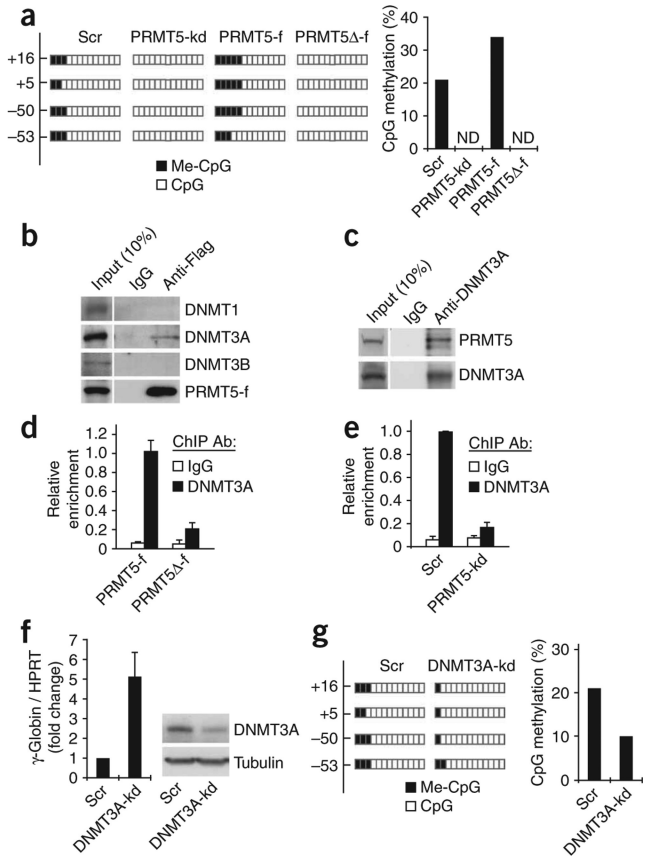

Figure 3.

PRMT5 and DNMT3A function cooperatively in gene silencing. (**a**) Effect of perturbed PRMT5 expression on DNA methylation at the human γ-gene. Each row shows the methylation status of individual CpG dinucleotides derived from sequence analysis of at least 40 individual cloned PCR products of the γ-genes following bisulfite modification from PRMT5-f, PRMT5Δ-f, PRMT-kd and the scrambled control (Scr) K562 cells. The differences between the three lines and the Scr were highly significant (P< o 0.01). The numbers on the left represent the positions of the CpG dinucleotides relative to the transcriptional start site of the γ-gene. The results are quantitated in the bar graph. ND, not detectable. (**b**) DNMT3A, but not DNMT1 or DNMT3B, co-immunoprecipitates with PRMT5-f from K562 cells. High salt extraction (420 mM NaCl) was used for the cellular extract preparation. (**c**) Co-immunoprecipitation of endogenous DNMT3A and PRMT5 from K562 cells. High salt extraction (420 mM NaCl) was used for the cellular extract preparation. (**d**) DNMT3A binding at the γ-promoter was measured by ChIP in PRMT5-f and PRMT5Δ-f cells. Error bars show s.d. (**e**) DNMT3A binding at the γ-promoter was measured by ChIP in PRMT-kd and Scr cells as above. (**f**) K562 cells expressing either an shRNA to DNMT3A (DNMT3A-kd) or a scrambled sequence (Scr) were analyzed by western blot, with indicated antibodies (right panel), and Q-RT-PCR with primers specific for the γ-globin genes, with the signal normalized against HPRT mRNA levels (left panel). (**g**) Effect of DNMT3A knockdown on DNA methylation at the human γ-genes as detailed in **a**. The difference between the two lines was significant (P < 0.02).

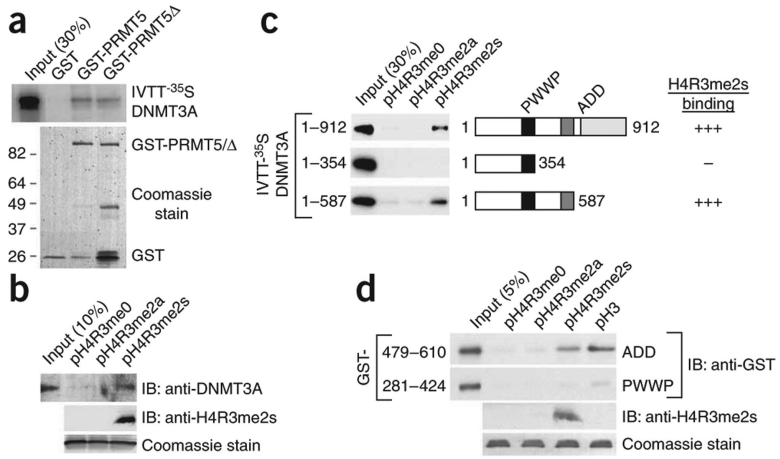

Figure 4.

DNMT3A binds specifically to histone H4 carrying the R3me2s modification. (**a**) Binding of ^{35}S-labeled *in vitro* transcribed and translated (IVTT) DNMT3A to purified GST, GST-PRMT5 and GSTPRMT5D. Top panel, autoradiograph; bottom panel, Coomassie. Input represents 30% of the *in vitro* translated DNMT3A used in the assay. (**b**) Binding of DNMT3A to N-terminal peptides of histone H4 with the Arg3 residue unmethylated, symmetrically methylated or asymmetrically methylated. Specifically bound protein was visualized by western blot with anti-DNMT3A antibody after SDS-PAGE. Input represents 10% of the cellular extract used in the assay. The H4R3me2s modification of the synthesized peptide was confirmed by immunoblot. Coomassie staining shows equivalent loading of the three peptides on a 20% (w/v) SDS-PAGE gel. (**c**) Peptide pulldown assays as described in **b**, with ^{35}S-labeled fragments of DNMT3A as shown in the schematic. Numbers refer to amino acids. The 1–354 construct contains the PWWP module but lacks its adjacent C-terminal helical motif. The 1–587 construct contains the GATA and PHD domains of ADD, but lacks an adjacent C-terminal helix. (**d**) Peptide pulldown assays as described in **b**, with purified GST, GST-DNMT3A (281–424, containing the PWWP domain) and GST-DNMT3A (479–610, containing the ADD domain). Specifically bound protein was visualized by western blot with anti-GST antibody after SDS-PAGE. Input represents 5% of the GST fusion proteins used in the assay. The H4R3me2s modification of the synthesized peptide was confirmed by immunoblot. Coomassie staining shows equivalent loading of the four peptides.

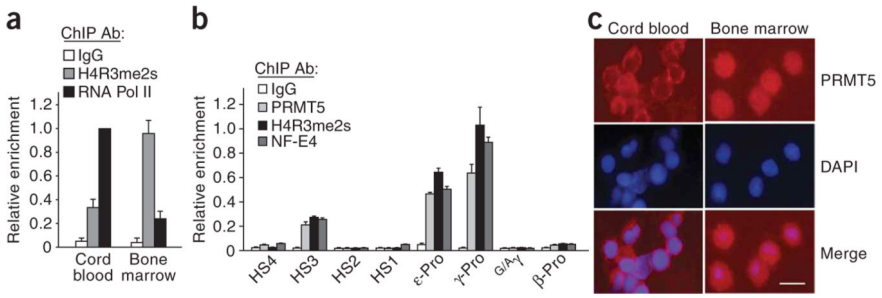

Figure 5.

Role of PRMT5 in developmental globin gene silencing. (**a**) H4R3me2s and RNA polymerase II enrichment at the γ-promoter was measured by ChIP in chromatin fractions from erythroid progenitors from cord blood and adult bone marrow. (**b**) Localization of PRMT5, NF-E4 and H4R3me2s across the β-globin locus measured by ChIP in chromatin fractions from erythroid progenitors from adult bone marrow. The precipitated DNA was amplified with primers specific for the indicated regions of the β-globin locus. HS, hypersensitive site; Pro, promoter; $^{G/A}$γ, intergenic region between ^{G}γ-globin and ^{A}γ-globin genes. Error bars show s.d. (**c**) Cellular localization of PRMT5 in erythroid progenitors from cord blood and adult bone marrow shown by immunofluorescence with anti-PRMT5 antibody and DAPI nuclear counterstaining. Scale bar, 10 μm.

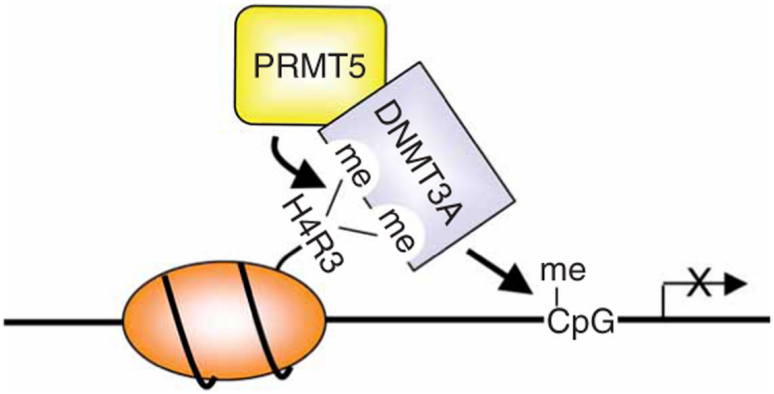

Figure 6.

Model of PRMT5-induced silencing of gene expression. Symmetric dimethylation of H4R3 by PRMT5 induces direct binding of DNMT3A, resulting in methylation of adjacent CpG dinucleotides and gene silencing.