Methylation of histone H4 by arginine methyltransferase PRMT1 is essential in vivo for many subsequent histone modifications

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PRMT1 is a histone methyltransferase that methylates Arg3 on histone H4. When we used siRNA to knock down PRMT1 in an erythroid cell line, it resulted in nearly complete loss of H4 Arg3 methylation across the chicken β -globin domain, which we use as a model system for studying the relationship of gene activity to histone modification. We observed furthermore a domain-wide loss of histone acetylation on both histones H3 and H4, as well as an increase in H3 Lys9 and Lys27 methylation, both marks associated with inactive chromatin. To determine whether the effect on acetylation was directly related to the loss of H4 Arg3 methylation, we performed an in vitro acetylation reaction on chromatin isolated from PRMT1-depleted cells. We found that nucleosomes purified from these cells, and depleted in methylation at Arg3, are readily acetylated by nuclear extracts from the same cells, if and only if the nucleosomes are incubated with PRMT1 beforehand. Thus, methylation of histones by PRMT1 was sufficient to permit subsequent acetylation. Consistent with earlier reports of experiments in vitro, H4 Arg3 methylation by PRMT1 appears to be essential in vivo for the establishment or maintenance of a wide range of "active" chromatin modifications.

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Within the nucleus, DNA is packed as chromatin that actively mediates dynamic changes in gene function and expression (Felsenfeld and Groudine 2003). Associated with these regulatory functions are a variety of histone modifications, largely at the N- and C-terminal tails, that can alter chromatin structure and chemical reactivity (Cheung et al. 2000a; Strahl and Allis 2000; Margueron et al. 2005). These include histone acetylation of lysine residues, methylation of lysine and arginine residues, phosphorylation of serine and threonine, ubiquitylation and sumoylation of lysine, and histone ADP ribosylation. Each of these kinds of modification has been implicated in regulation of gene expression through some chromatin-coupled set of mechanisms. For example, methylation of Lys9 of histone H3 (H3 Lys9), which creates a binding site for HP1, is involved in formation of repressive heterochromatin (Bannister et al. 2001; Lachner et al. 2001; Nakayama et al. 2001; Grewal and

Moazed 2003), whereas methylation of Lys4 histone H3 (H3 Lys4) correlates with transcriptionally active euchromatin (Litt et al. 2001a; Santos-Rosa et al. 2002; Schneider et al. 2004).

It has become increasingly clear that the various histone modifications can have distinct effects at different loci, perhaps because of interactions between them. Furthermore, there appears to be cross-talk between the modifications, so that, for example, acetylation of H3 Lys14 in yeast can be stimulated by H3 Ser10 phosphorylation (Cheung et al. 2000b; Lo et al. 2001; Agalioti et al. 2002; Clements et al. 2003). Similarly, ubiquitylation of histone H2B Lys123 is required for methylation of H3 Lys4 and Lys79 in *Saccharomyces cerevisiae* (Dover et al. 2002; Ng et al. 2002; Sun and Allis 2002; Ezhkova and Tansey 2004). Other results have shown that acetylation of H3 Lys18 appears before H3 Arg17 methylation during estrogen-regulated transcription, and that acetylation at Lys18 facilitates CARM1-mediated methylation at Arg17 (Bauer et al. 2002; Daujat et al. 2002). CARM1 also methylates arginine residues 2 and 26 on histone H3. A second enzyme, PRMT1, has been shown to act as a histone H4 Arg3-specific methyltransferase and is involved in nuclear-receptor-mediated transcriptional activation

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(Strahl et al. 2001; Wang et al. 2001). Studies in vitro have shown that methylation of Arg3 on H4 tail peptides facilitates p300-mediated histone H4 acetylation in vitro (Wang et al. 2001). Recently, an extensive examination of mechanisms of transcriptional activation by p53 has demonstrated the importance of recruitment by p53 of p300, PRMT1, and CARM1. These enzymes act synergistically, and the results are consistent with a mechanism in which the modifications occur in a defined sequence, with PRMT1 action as the first step (An et al. 2004).

In evaluating the role of histone modifications, we and others have made extensive use of the chicken β -globin locus to study the relationship between patterns of chromatin structure and developmental activation of transcription (Litt et al. 2001a,b; Schneider et al. 2004). This locus (Fig. 1) presents the opportunity to examine a variety of chromatin structures associated with various stages of gene activation. Upstream of the *globin* genes resides a gene for a developmentally regulated, erythroid-specific *folate receptor* (*FR*); it is controlled by its own upstream element, *HSA*, and separated from the *globin* genes by a 16-kb region of condensed chromatin (Prioleau et al. 1999). Between this 16-kb region and the β -globin genes lies an insulator element, 5'HS4, that recruits a variety of histone modifiers that we have suggested act as chain terminators to prevent the advance of repressive chromatin into the open *globin* locus (West et al. 2004).

In earlier studies we examined the changes in acetylation and methylation of histone lysine residues associated with gene activation or inactivation in the neighborhood of the *globin* locus (Litt et al. 2001a,b). Little is known, however, about the role of arginine methylation in these processes, and about its relationship to the other histone modifications. Given the recent interest in the relationship between histone H4 Arg3 methylation and other modifications, we undertook an investigation of the role of PRMT1 in regulation of *globin* gene chromatin structure and expression. Our results show that loss of PRMT1 through RNAi-mediated knock-down has major effects throughout the locus not only on H4 Arg3 methylation, but also, surprisingly, on the other modifications associated with active chromatin structures, as well as on gene expression. Such changes are not limited to the *globin* locus, and they involve histone H3 as well as H4. We further asked whether the effects of PRMT1 deficiency on histone acetylation arise directly from loss of H4 Arg3 methylation, or indirectly through down-regulation of other proteins, such as histone acetylases, in the PRMT1-deficient cells. We find that nucleosomes purified from these cells, and depleted in methylation at Arg3, are readily acetylated by nuclear extracts from the same cells, if and only if the nucleosomes are incubated with PRMT1 beforehand. Furthermore, inhibition of PRMT1 or depletion of the cofactor S-adenosyl methionine in the first step suppresses chromatin acetylation after addition of these nuclear extracts. However, if PRMT1 is allowed to act on nucleosomes before inhibitor is added, subsequent addition of the nuclear extract leads to histone acetylation. This is consistent with earlier findings in

Figure 1. Pattern of histone H4 methylation at Arg3 across the β -globin locus at different erythroid development stages. (A) Map of the chicken β -globin locus. The *folate receptor* gene is located at the 5'-end of the locus. *HSA* is a hypersensitive site associated with *FR* expression. A second hypersensitive site, *HS4*, located 5' upstream of the open *globin* domain, marks the insulator element of the β -globin. A 16-kb condensed chromatin segment is located between hypersensitive sites *HSA* and *HS4*. Downstream of *HS4*, there are three hypersensitive sites, *HS3* to *HS1*, comprising part of the *globin* locus control region. $\beta^{A/e}$ is a strong enhancer lying between the β^A - and β^E -globin genes. 3'HS marks the 3'-end of the β -globin locus and possesses enhancer-blocking properties only. The name and location of primer pairs and Taqman probes used for the ChIP analysis are shown below the map. Above the map are data for four chicken cell types representing different development stages: 6C2 is arrested in the CFU-E (colony-forming unit-erythroid) stage, and 10-d erythrocytes are taken from the embryonic circulation. Expression of the individual genes is indicated by + and -. Y indicates the presence of the hypersensitive sites. (B,C) Pattern of H4 Arg3 methylation across the chicken β -globin neighborhood. Results of ChIP with antibody specific to dimethyl Arg3 on histone H4; data from 6C2 and 10-d chicken embryonic erythrocytes. Chromatin preparation and IP procedures are described in Materials and Methods and in Litt et al. (2001b).

vitro that methylation of H4 N-terminal tails at Arg3 facilitated subsequent acetylation at other sites, and shows that the same requirement prevails in vivo.

Results

Distinct patterns of H4 Arg3 methylation over the β -globin locus during erythroid differentiation

In earlier studies we characterized patterns of histone acetylation and lysine methylation over the chicken β -globin domain as a function of development (Litt et al. 2001a,b). To obtain corresponding information about the distribution of H4 Arg3 methylation, we used chromatin immunoprecipitation (ChIP) to map this modification over the 53-kb β -globin domain at different erythroid developmental stages. The analysis was carried out by real-time PCR on mono- and dinucleosome fractions (Fig. 1A) obtained from 6C2 cells and 10-d embryonic erythrocytes. 6C2 cells are an erythroleukemia line arrested at the CFU-E stage of erythroid development, in which the *FR* gene is expressed but *globin* genes are quiescent (Prioleau et al. 1999); 10-d erythroid cells represent a later developmental stage (Felsenfeld 1993) in which the *globin* gene is activated but *FR* expression is shut off.

The pattern of Arg3 methylation in both cell types across the entire locus is shown in Figure 1B and C. There are marked enrichments of Arg3 methylation in 6C2 cells at HSA, the regulatory element close to the *FR* promoter, and at the 5'HS4 insulator. In 6C2 cells there is a large peak of H4 Arg3 methylation over the HS2 *globin* locus control region (LCR) (Fig. 1B). This may be related to the LCR's role in activation of the *globin* genes at a slightly later developmental stage. In contrast, in 10-d embryonic erythrocytes in which *globin* genes are active, the peak of Arg3 methylation is switched to a region near the β^H promoter. We note early reports that Arg3 methylation mediated by PRMT1 plays an important role in transcriptional activation (Wang et al. 2001; An et al. 2004). The pattern of histone H4 Arg3 methylation during erythroid differentiation thus may have implications for regulation of *globin*-locus chromatin structure by PRMT1. Some methylation is also evident within the 16-kb condensed chromatin region that lies between the *FR* and *globin* genes. By all other measures (such as DNA methylation, absence of histone acetylation and H3 Lys4 methylation) this region appears to behave as heterochromatin.

Histone H4 Arg3 methylation is required for histone acetylation and maintenance of an active chromatin domain in vivo

PRMT1 is a major H4 Arg3 methyltransferase (Strahl et al. 2001; Wang et al. 2001). We tested directly whether methylation of histone H4 at Arg3 is essential for transcriptional regulation by knocking down PRMT1 expression using stably expressed hairpin short interfering RNAs (siRNAs). We developed several stable lines; two of these displayed an ~80%–90% reduction in PRMT1 protein levels compared with parental 6C2 cells (Fig. 2A). We noticed that both PRMT1 knock-down cell lines grew extremely slowly during selection in comparison to the wild-type parental 6C2 cells, although after estab-

Figure 2. Both histone H3 and H4 acetylation are dependent on PRMT1 activity. (A) siRNA-mediated knock-down of PRMT1 expression in 6C2 cells. Western blot analysis of parental (WT) and two PRMT1 knock-down lines, 2G5 and 2H2. The expression of PRMT1 is reduced 80%–90% in both knock-down lines. (B) Results of ChIP with antibody specific to H4 dimethyl Arg3 on mono- and dinucleosomes purified from wild-type (green) or PRMT1 knock-down (2G5) (orange) 6C2 cell lines. (Blue) No antibody control. (C) H3 diacetylation at Lys9 and Lys14 across the β -globin locus. (D) H4 acetylation.

lishment the lines grew more normally. These cells differed morphologically from wild-type 6C2 cells (data not shown). If PRMT1 is the primary enzyme responsible for H4 Arg3 methylation, a marked reduction of this modification should be expected in these cell lines. Figure 2B shows the ChIP data for H4 Arg3 methylation across the β -globin locus for the knock-down cell lines as compared with wild type. Methylation at H4 Arg3 is almost abolished within the locus in both of the PRMT1 knock-down lines (Fig. 2B; Supplementary Fig. S1); the level of methylation is comparable to that of the no-antibody control. The effect of siRNA knock-down experiments appears to be specific, because the RNAi vector targeting the green fluorescent protein (GFP) gene doesn't affect chicken β -globin locus chromatin modifications (Supplementary Fig. S1), and USF1 siRNA experiments show only a very localized effect on chromatin modification in

this region (West et al. 2004). The results confirm that the histone H4 Arg3 residue is an *in vivo* substrate of the methyltransferase PRMT1 (Wang et al. 2001), and that this is the principal or only enzyme that carries out this reaction inside the cells.

We next asked whether other histone modifications were affected by Arg3 methylation in these PRMT1 knock-down cells. Compared with the wild-type parental 6C2 cells, histone H3 diacetylation at Lys9 and Lys14 is also dramatically decreased over the entire locus: Diacetylation at Lys9 and Lys14 is reduced by 50% and 75% in the *folate receptor* HSA element and 5'HS4 insulator, respectively (Fig. 2C; Supplementary Fig. S2A). The acetylation peak at the locus control region HS2 is almost entirely lost (Fig. 2C). Similarly to histone H3, we observed an almost threefold decrease of H4 acetylation at the *folate receptor* HSA element and a fivefold drop at the 5'HS4 insulator (Fig. 2D). The acetylation peak over the HS2 locus control element disappears almost entirely (Fig. 2D).

The data from the siRNA knock-down experiments are likely to reflect changes in PRMT1 specifically, because there were no effects on the expression of histone-modifying enzymes SET7/9 and PCAF (Supplementary Fig. S3B), or their abilities to acetylate oligonucleosomes (see Fig. 5 cf. lanes 5 and 7,8 [below]). There was also no effect on the expression of other proteins, including the transcription factor USF1 (which plays an important role at the insulator element HS4). In addition, the second PRMT1 knock-down clone (2H2) exhibits the same reduction as the first (2G5) in histone methylation and acetylation across the entire β -globin locus (Supplementary Figs. S1, S2).

Inhibiting PRMT1 expression has widespread genomic effects on these modifications. Although the same amounts of genomic mono- and dinucleosomes were used as input for ChIP with antibodies specific to dimethyl-Arg3, acetyl-histone H3 at Lys9 and Lys14, or acetyl H4, 32%–75% less antibody-bound total chromatin was recovered in PRMT1 knock-down cells compared with wild-type parental 6C2 cells. Furthermore, Western blot analysis of histones isolated from knock-down and parental 6C2 indicated that total acetylated histone H3 is decreased significantly in PRMT1 knock-down cells compared with wild-type parental 6C2 cells (Supplementary Fig. S3C), suggesting that PRMT1 affects global histone acetylation.

Loss of H4 Arg3 methylation leads to encroachment of repressive heterochromatin

We asked whether the recruitment of active histone modifications, such as H4 Arg3 methylation, played a role in the chromatin barrier activity. The 5'HS4 insulator is located immediately downstream of the 16-kb condensed chromatin region, which is enriched in di- and trimethylated Lys9 and Lys27 on histone H3 tails. These marks are characteristic of heterochromatin (Litt et al. 2001a; Cao et al. 2002; Peters et al. 2003; Plath et al. 2003). We have shown that the recruitment of histone

modification is essential for the endogenous 5'HS4 barrier activity (West et al. 2004). As noted above, depletion of PRMT1 results in significant decreases in H4 Arg3 methylation (Fig. 2B; Supplementary Fig. S1) and loss of acetylation of histone H3 and H4 (Fig. 2C,D) at the endogenous 5'HS4 element (Fig. 3C). We next measured the distribution of H3 dimethyl Lys9 and trimethyl Lys27 in wild-type parental 6C2 and PRMT1 knock-down cells (Fig. 3A,B). The H3 Lys9 methylation pattern in the PRMT1 knock-down lines was similar to that of wild-type 6C2 cells except for the 5'HS4 region, where there was significant accumulation of H3 Lys9 methylation immediately downstream of the insulator element (site 22.189) (Fig. 3A). The pattern resembles that seen in USF1 knock-down cells, which show a sixfold increase of H3 Lys9 methylation at the insulator site (West et al. 2004).

In contrast to the localized changes in Lys9 methylation, down-regulation of PRMT1 results in elevation of H3 Lys27 methylation over most of the 53-kb locus (Fig. 3B; Supplementary Fig. S3A), including the 16-kb con-

Figure 3. Loss of H4 Arg3 methylation leads to formation of repressive heterochromatin. Results of ChIP with antibodies specific to dimethyl Lys9 (A) and trimethyl Lys27 (B) with mono- and dinucleosomes purified from wild-type (Green) or PRMT1 knock-down (orange) 6C2 cell lines across the β -globin locus. (C) A summary of the ChIP data across the chicken β -globin locus showing results of PRMT1 knock-down. The arrows pointing up and down indicate increase or decrease of modifications, respectively. The minus signs indicate that there are no changes in histone modifications.

densed chromatin, 5'HS4 insulator, and β -globin domains.

H4 Arg3 methylation is important for transcriptional activation in vivo

Having demonstrated that H4 methylation at Arg3 is important for H4 acetylation, we further asked which residue(s) were affected. Antibodies specifically directed against acetyl Lys5, Lys8, and Lys12 were used in ChIP experiments across the entire *FR*- β -globin region to compare wild-type parental 6C2 with PRMT1 knock-down 6C2 cells (Fig. 4A–C). The cells with reduced PRMT1 abundance showed a significant decrease of H4 acetylation at Lys5 and Lys12 (Fig. 4A,C). We observed a 51% decrease of Lys5 acetylation at the *folate receptor* HSA element (peak value), an ~70% reduction at the 5'HS4 insulator element, and essentially complete loss of acetylation over the *globin* gene domain (Fig. 4A; Supplementary Fig. S2B). The decreases in Lys12 acetylation were somewhat less dramatic (32.5% over HSA, 40% over 5'HS4) (Fig. 4C). In contrast, Lys8 acetylation at the HSA and 5'HS4 elements is completely unaffected by the PRMT1 knock-down although acetylation over the *globin* genes and their upstream regulatory elements is abolished (Fig. 4B; Supplementary Fig. S2C), just as for Lys5 and Lys12.

As depleting PRMT1 resulted in decreases of both histone H3 acetylation and H4 acetylation on Lys5 and Lys12, especially over the *folate receptor* HSA element (Fig. 3C), we asked whether there was a reduction in *FR* expression. Quantitative RT-PCR results confirm that in the PRMT1 knock-down clones 2G5 and 2H2, *FR* mRNA expression is reduced by 80% and 87%, respectively (Fig. 4D). However, there is no reduction of *FR* expression in a USF1 knock-down cell line (Fig. 4D) that specifically affects the chromatin structure of the 5'HS4 insulator element located between the upstream 16-kb condensed chromatin and downstream open β -globin domain, but leaves the HSA element unaltered (West et al. 2004). The results for the *FR* gene are consistent with the notion that PRMT1-mediated H4 methylation at Arg3 can play an important role in transcriptional regulation, perhaps through acetylation of H3 and H4 tails.

Restoration of H4 Arg3 methylation in vitro potentiates histone acetylation

The effects of decreased PRMT1 expression on histone acetylation in vivo are consistent with previously published results of experiments in vitro, which showed that H4 Arg3 methylation facilitates acetylation. However, our in vivo data do not distinguish between direct effects of H4 Arg3 methylation on the ability of histones H3 and H4 to be acetylated, and indirect effects of loss of methylation, for example, involving down-regulation of expression of histone acetyltransferases or other enzymes in the gene activation pathway. To distinguish these possibilities, we purified oligonucleosomes from the wild-

Figure 4. Histone H4 Arg3 methylation is required for histone H4 acetylation at Lys5 and Lys12. (A–C) Results of ChIP with antibodies specific to H4 acetylation at Lys5, Lys8, and Lys12 on mono- and dinucleosomes purified from wild-type (green) or PRMT1 knock-down (orange) 6C2 cell lines across the β -globin locus. (D) Quantitative RT-PCR of *FR* mRNA expression in wild-type (WT), USF1 knock-down, and PRMT1 knock-down (2G5 and 2H2) cell lines.

type and a PRMT1 knock-down cell line and measured their ability to undergo histone acetylation under a variety of conditions (Fig. 5A). As shown in Figure 5B (cf. lanes 1,2 and 3,4), incubation of PRMT1 knock-down nucleosomes with nuclear extracts either from the same cells or wild-type extracts depleted in PRMT1 resulted in negligible acetylation of histones (Fig. 5B, lanes 1,2). When nuclear extracts from wild-type cells were used, a small amount of acetylation was detected both with PRMT1kd nucleosomes (Fig. 5B, lane 3) and with nucleosomes from wild-type 6C2 cells (Fig. 5B, lane 4). In contrast, pretreatment of PRMT1 knock-down nucleosomes with PRMT1 and S-adenosyl methionine before addition

Figure 5. Restoration of histone H4 Arg3 methylation potentiates histone acetylation in vitro. (A) Outline of experimental procedure. (B) The purified oligonucleosomes from wild-type parental 6C2 and siRNA knock-down 6C2 cells were incubated without (*left*) or with (*right*) purified PRMT1 in the presence of S-adenosyl methionine (SAM) for 1 h at 30°C. After methylation, the nuclear extracts purified from the same cells and [¹⁴C]acetyl-CoA were added and incubated for another 1 h at 30°C as shown. The reaction mixtures were then resolved on SDS-PAGE and visualized by autoradiography. (C) The purified oligonucleosomes from siRNA knock-down 6C2 cells were incubated with purified PRMT1 in the presence or absence of S-adenosyl methionine (SAM) for 1 h at 30°C. After methylation, the nuclear extracts purified from the same cells and [¹⁴C]acetyl-CoA were added and incubated for another 1 h at 30°C. The PRMT1 inhibitor AMI-1 (200 μM) was added either before methylation or after methylation.

of nuclear extracts from either PRMT1kd cells (Fig. 5B, lane 5) or wild-type 6C2 cells (Fig. 5B, lane 7) resulted in a marked increase in acetylation. Nucleosomes from wild-type 6C2 cells responded similarly to treatment with PRMT1 (Fig. 5B, lane 8). Addition of the arginine methyltransferase inhibitor AMI-1 (Cheng et al. 2004) before addition of PRMT1 abolished acetylation completely (Fig. 5B, lane 6). In addition, histone acetylation was significantly reduced in the absence of added S-adenosyl methionine (Fig. 5C, lane 13). The result strongly suggests that PRMT1-mediated Arg3 methylation potentiates histone H3 and H4 acetylation. This still left the possibility that, after the addition of nuclear extract,

PRMT1 was methylating some other protein that, in turn, controlled histone acetylation. To address this question, PRMT1 was incubated with PRMT1kd nucleosomes and then inactivated with AMI-1 before the addition of nuclear extract (Fig. 5C, lane 12). As shown in Figure 5C, histone acetylation was observed even though the PRMT1 could only have modified histones.

These results show that the effect of PRMT1 depletion on histone acetylation is a direct response to the loss of H4 Arg3 methylation. Taken together with the ChIP data obtained in vivo (Figs. 2, 4), they provide strong evidence for interdependent signals between H4 Arg3 methylation and acetylation of both histones H3 and H4. The methylation of Arg3 is thus not only required in *cis* for H4 acetylation, but also acts in *trans* to raise levels of H3 acetylation. These results also support the hypothesis that there are synergistic interactions between modifications on histone N-terminal tails (Jenuwein and Allis 2001).

Discussion

Our results demonstrate an important role in vivo for PRMT1, and the H4 Arg3 methylation that it catalyzes, in broad regulation of the patterns of histone acetylation over transcriptionally active loci. Earlier reports have shown that PRMT1 methylates Arg3 on histone H4, and that methylation of H4 tails in vitro facilitates subsequent acetylation of these tails by p300 (Wang et al. 2001; An et al. 2004). There have also been reports of synergy between histone acetyltransferases and protein arginine methyltransferases (Koh et al. 2001; Daujat et al. 2002). The interdependence of these modifications has been explored further in a cell-free system with reconstituted chromatin templates carrying a p53-dependent reporter gene (An et al. 2004); it was shown that PRMT1, the methyltransferase CARM1, and p300 act cooperatively to stimulate p53-dependent transcription. Furthermore, greater stimulation was observed when PRMT1 was added before p300 rather than simultaneously with it, and H4 Arg3 methylation facilitated histone acetylation.

Here we use the chicken β -globin locus and surrounding chromatin to study in vivo the distribution of H4 Arg3 methylation and its interplay with other histone modifications in the presence and absence of PRMT1. We show that depletion of PRMT1 results (as expected) in disruption of methylation at H4 Arg3, but also causes major decreases in histone acetylation in vivo at many of the normally enriched sites across the locus (Fig. 3C). Acetylation levels decrease not only at residues on H4, as might perhaps be expected from earlier studies (Fig. 2D; Wang et al. 2001), but on H3 Lys9 and Lys14 as well (Fig. 2C).

PRMT1 can be targeted to chromatin by a variety of transcription factors. PRMT1 interacts with nuclear hormone receptors (Koh et al. 2001), transcription factor YY1 (Rezai-Zadeh et al. 2003), and p53 (An et al. 2004). We have found (S. Huang, unpubl.) that PRMT1 also interacts with the regulatory factor USF1 at the 5'HS4

β -globin insulator. Thus, it seems reasonable to suggest that PRMT1 depletion might affect those genes normally activated by recruitment of this enzyme to the promoter, and that the absence of PRMT1 might work indirectly through a failure subsequently to recruit histone acetyltransferases because of the absence of methylation on H4 Arg3 (see below). A closer examination of the acetylation patterns over HSA and 5'HS4 reveals that PRMT1 depletion reduces the abundance of H4 Lys5Ac and H4 Lys12Ac but has no effect whatever on H4 Lys8 acetylation at these sites (Fig. 3C). The fact that H4 deacetylation over HSA and 5'HS4 is limited to Lys5 and Lys12 can be viewed in the light of the earlier observation that diacetylated H4 Lys5 and Lys12 are appropriately spaced for preferential binding by the two tandem bromodomains of TAF_{II}250, a major component of the TFIID complex (Jacobson et al. 2000). In this way, TFIID may be targeted to specific chromatin structures of the promoter to play its role in transcriptional regulation. TAF_{II}250, which itself harbors intrinsic histone acetyltransferase activity, may read these acetyl modifications and contribute to the establishment of an open chromatin domain. It was reported that PRMT1 and Arg3 methylation are linked to nuclear-receptor-mediated transcriptional regulation (Wang et al. 2001, 2004). Our finding that Arg3 methylation affects H4 Lys5/Lys12 acetylation, together with the potential for TAF_{II}250 recruitment, suggests one possible detailed molecular mechanism of PRMT1-mediated transcriptional activation.

We note that the specific H4 Lys5/Lys12 acetylation pattern over HSA and 5'HS4 does not extend into the *globin* domain (~23–30 kb), where Lys5, Lys8, and Lys12 are all acetylated in a PRMT1-dependent manner (Figs. 3C, 4A–C). This difference may result from recruitment by PRMT1 of different factors for 5'HS4/HSA and for the *globin* genes. Consistent with this distinction, the chromatin of the 5'HS4 insulator element is constitutively acetylated in all cell lines, whereas acetylation is not found over the *globin* domain in nonerythroid cells such as the DT40 lymphocyte line or those from brain (Litt et al. 2001a,b). In addition, despite the observed hyperacetylation of H3 and H4 throughout the majority of the 30-kb *globin* domain in 10-d erythrocytes (Litt et al. 2001b), p300/CBP and PCAF were recruited only to the 5'HS4 insulator site (West et al. 2004). We also note that the enzymes that acetylate H4 Lys5/Lys12 are different from that responsible for acetylating H4 Lys8/Lys16 (Makowski et al. 2001). This suggests that a different or additional mechanism may control acetylation in this region.

The effect of PRMT1 depletion on H4 Arg3 methylation confirms that in these cells PRMT1 is the principal or sole enzyme responsible for this modification. The additional effects on histone H3 and H4 acetylation, taken together with results of earlier studies in vitro (Wang et al. 2001; An et al. 2004), strongly suggest also that H4 Arg3 methylation is necessary for lysine acetylation of both H3 and H4. However the data in Figures 2 and 4 do not in themselves exclude other possibilities. For example, PRMT1 might regulate expression of his-

tone acetylases, either directly through modification of histones in the neighborhood of the corresponding genes, or of other upstream genes. (We show in Supplementary Fig. S3B that at least this cannot be a direct effect on the acetylase PCAF and methyltransferase SET7/9, since their levels are unaffected by depletion of PRMT1.) Alternatively, PRMT1 could methylate sites on important regulatory proteins other than histones (Kwak et al. 2003; Boisvert et al. 2005), with downstream effects on histone acetylase activity.

In order to distinguish among these possibilities, we carried out experiments under conditions such that methylation by PRMT1 had no other targets available but the nucleosomes isolated from PRMT1-depleted cells. Purified nucleosomes were treated with PRMT1 in vitro before the addition of nuclear extract from the same cells. As shown in Figure 5, the nucleosomal histones are acetylated in the extract, but only if PRMT1 is present in the preincubation step. Furthermore, acetylation does not occur even in the presence of PRMT1 if the inhibitor AMI-1 is added before the enzyme in the preincubation step. Therefore, the mere presence of PRMT1 is ineffective in the absence of arginine methylation. Finally, we showed that if PRMT1 is allowed to methylate nucleosomes and the inhibitor AMI-1 is added afterward, but before addition of the nuclear extract, histone acetylation occurs when the extract is added. Under these conditions there could be no effects, direct or indirect, on the level of expression of any genes or on the activity of the corresponding proteins, thus eliminating mechanisms of this kind for the role of PRMT1 in histone acetylation and supporting a model in which local H4 Arg3 methylation is a critical requirement for subsequent acetylation of histones H3 and H4. The data in Figure 5B (lane 4 vs. lane 8) also indicate that addition of PRMT1 even to wild-type 6C2 nucleosomes can stimulate histone acetylation, suggesting that many H4 Arg3 sites in these cells are normally unmethylated.

The data we have presented reveal the important role in vivo of PRMT1 and H4 Arg3 methylation in the establishment of an open chromatin domain, marked by histone acetylation and by the absence of histone methylation at residues associated with inactive chromatin, and suggest a role for H4 Arg3 methylation “upstream” of lysine acetylation. These results shed light on the intimate connection between Arg3 methylation and other histone modifications in the regulation of chromatin states. This methylation event appears to affect multiple modifications on both histones H3 and H4, either directly or through a cascade that it initiates. The separate question of how the H4 Arg3 methylation mark is recognized by proteins that affect the acetylation reactions remains open, and should be a major subject of future studies.

Materials and methods

Cell lines and siRNA knock-down

6C2 Cells were maintained as described (Prioleau et al. 1999). Ten-day chicken embryonic erythrocytes were harvested from

10-d embryos from fertilized White Leghorn chicken eggs (Truslow Farms). The oligonucleotide duplexes that specifically target different portions of the chicken PRMT1 were cloned into pSilencer3.1-H1 hygro vectors following the manufacturer's instructions (Ambion). The vectors containing one, two, and five oligonucleotide duplexes were transfected into 6C2 cells by electroporation. Stable transfectants were selected in the presence of 1 mg/mL of Hygromycin B. The stable hairpin-expressing cell lines were analyzed for PRMT1 expression by Western blotting.

Purification of nuclear extract and oligonucleosomes

The nuclear extracts were prepared as previously described (Bell et al. 1999). The oligonucleosomes from wild-type or PRMT1 knock-down 6C2 cells were purified as described by Litt et al. (2001b). For purification of PRMT1-depleted nuclear extract, the wild-type 6C2 nuclear extract was immunoprecipitated with 200 µg of PRMT1 antibody (Abcam) at 4°C overnight. The immunocomplexes were complexed with protein G agarose beads and were precipitated by centrifugation. The supernatant was then collected for HAT assays.

HMT and HAT assays

The oligonucleosomes were incubated with 0.25 µg of purified PRMT1 (Upstate Biotech.) in a total volume of 30 µL containing 20 mM Tris-HCl (pH 8.0), 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 1 µL of SAM (32 mM, NEB) at 30°C for 1 h. The methylation reaction mixtures were incubated with 24 µg of nuclear proteins from wild-type or PRMT1 knock-down 6C2 nuclear extracts for 1 h at 30°C in a 40-µL volume containing 50 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, and 1 µL [¹⁴C]acetyl-CoA (55 mCi/mmol; Amersham Pharmacia Biotech) as described (Huang et al. 2000). AMI-1, a kind gift of Dr. Mark Bedford (M.D. Anderson Cancer Center, Smithville, TX), was prepared in DMSO as described (Cheng et al. 2004).

Western blot analysis

Anti-PRMT1 antibody was purchased from Abcam. Anti-PCAF antibodies were purchased from Santa Cruz Biotechnology. Anti-SET7/9 antibody is a kind gift from Dr. Yi Zhang (University of North Carolina-Chapel Hill). The nuclear extract samples were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), and electrotransferred to polyvinylidene difluoride (PVDF) membranes. Blots were incubated with the indicated primary antibodies, and then with HRP-conjugated secondary antibodies. Proteins were visualized by enhanced chemiluminescence (Pierce).

ChIP analysis

Native ChIP assays for the modifications of core histones were performed in the absence of formaldehyde cross-linking as described previously (Litt et al. 2001b), using antibodies specific for various post-translational modifications of histone tails (anti-histone H4 dimethyl R3, #07-213; anti-histone H3 acetyl K9/acetyl K14, #06-599; anti-histone H4 tetra-acetyl K5/K8/K12/K16, #06-598; anti-histone H3 dimethyl K4, #07-030; anti-histone H4 acetyl K5, #06-759; anti-histone H4 acetyl K8, #06-760; anti-histone H4 acetyl K12, #06-761; anti-histone H3 dimethyl K9, #07-212 from Upstate Biotechnology) (anti-histone H3 trimethyl K27 is a kind gift from Dr. Thomas Jenuwein,

Vienna Biocenter, Vienna, Austria). The immunoprecipitated nucleosomes recovered from wild-type or PRMT1 knock-down 6C2 cells were purified and quantitated using the PicoGreen dsDNA Quantitation kit (Molecular Probes). DNA samples (0.5 ng each) were used to determine the relative concentration of DNA fragment in the DNA pool by real-time quantitative PCR on an ABI Prism 7700 Sequence Detector. Enrichment values were calculated as described previously (Litt et al. 2001b). Briefly, the enrichment of a given target sequence precipitated by a specific antibody was determined by dividing the concentration of target sequence in the IP fraction by the concentration of that sequence in input DNA.

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