Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9

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Histone H3 Lys 9 (H3-K9) methylation is a crucial epigenetic mark for transcriptional silencing. G9a is the major mammalian H3-K9 methyltransferase that targets euchromatic regions and is essential for murine embryogenesis. There is a single G9a-related methyltransferase in mammals, called GLP/Eu-HMTase1. Here we show that GLP is also important for H3-K9 methylation of mouse euchromatin. GLP-deficiency led to embryonic lethality, a severe reduction of H3-K9 mono- and dimethylation, the induction of Mage-a gene expression, and HP1 relocalization in embryonic stem cells, all of which were phenotypes of G9a-deficiency. Furthermore, we show that G9a and GLP formed a stoichiometric heteromeric complex in a wide variety of cell types. Biochemical analyses revealed that formation of the G9a/GLP complex was dependent on their enzymatic SET domains. Taken together, our new findings revealed that G9a and GLP cooperatively exert H3-K9 methyltransferase function in vivo, likely through the formation of higher-order heteromeric complexes.

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In all eukaryotes, genetic information is stored as chromatin, which consists of genomic DNA, histones, and a wide array of chromosomal proteins. The most fundamental unit of chromatin is the nucleosome, which is built from an octamer of core histones, H2A, H2B, H3, and H4, wrapped around 147 bp of DNA (Luger et al. 1997). The N-terminal tails of core histones are subject to a variety of covalent modifications such as phosphorylation, acetylation, methylation, ubiquitination, and ADP-ribosylation. These modifications play critical roles in the nuclear functions of chromatin. The “histone code” hypothesis (Strahl and Allis 2000; Turner 2000; Jenuwein and Allis 2001) predicts that different modifications at specific amino acid residues in histones or combinations of these modifications are translated into functionally distinct nuclear processes. For example, lysine methylation on histone H3 or H4 is involved in a variety of biological processes including transcriptional regulation, DNA elimination, DNA methylation, heterochromatin formation, and X-chromosome inactivation [Lachner and Jenuwein 2002; Lachner et al. 2003; Cao and Zhang 2004a]. Distinct effector molecules can read different methyl-lysine codes through specific interactions with the methylated histone. For example, heterochromatin protein 1 [HP1] and Polycomb [Pc] mediate transcriptional repression via their high-affinity interactions with histone H3 methylated at K9 and K27, respectively [Bannister et al. 2001; Lachner et al. 2001; Fischle et al. 2003].

SET domain-containing proteins (SET domain proteins) are potential histone methyltransferases (HMTases), and there are >50 putative SET domain protein genes in the mouse genome. Enzymatically characterized SET domain proteins are classified into subgroups by their putative substrate specificities. Among the histone H3 Lys 9 (H3-K9) methyltransferase group genes, at least Suv39h1 [Rea et al. 2000], Suv39h2 [O’Carroll et al. 2000], and Suv39h3 [Ueda et al. 2004] are known to be involved in the regulation of gene expression. These genes are expressed in a variety of tissues and are essential for development and cell proliferation.
Methylation of H3-K9 by G9a is an integral component of the transcriptional silencing program for many genes. We have reported that a null G9a-mutation in embryonic stem (ES) cells led to a loss of H3-K9 dimethylation at the Mage-a promoter and reversed transcriptional repression of Mage-a genes (Tachibana et al. 2002). Targeting of G9a and induction of H3-K9 methylation at antigen receptor gene segments suppressed their germline transcription and V(D)J recombination (Osipovich et al. 2004). Furthermore, G9a-mediated H3-K9 methylation has been implicated in the silencing of developmentally regulated genes via interaction with CDP/cut (Nishio and Walsh 2004), the plasma cell transcription factor Blimp-1 (Gyory et al. 2004), and the neuron-restrictive silencing factor NRSF/REST (Roopra et al. 2004).

Mammals possess only one closely related homolog of G9a, termed GLP/Eu-HMTase1. Recent biochemical studies have shown that several transcriptional silencing complexes, such as E2F6 (Ogawa et al. 2002), CtBP-1 (Shi et al. 2003), and CDP/cut (Nishio and Walsh 2004), contained not only G9a but also Eu-HMTase1. However, genetic studies have shown that mutation of G9a was sufficient to drastically reduce H3-K9 mono- and dimethylation at euchromatic regions. Thus, the in vivo contribution of GLP/Eu-HMTase1 as an H3-K9 HMTase and its functional interaction with G9a remained unknown.

To address these questions, we generated GLP-deficient mice and ES cells. Surprisingly, the phenotypes observed for germline mutations of the G9a and GLP genes were identical in many respects, including embryonic lethality, drastic reduction of H3-K9 mono- and dimethylation, induction of Mage-a genes, and HP1 relocalization in ES cells. These findings indicate that G9a and GLP function cooperatively rather than redundantly to mediate H3-K9 methylation at euchromatin. Moreover, we show that G9a and GLP form a stoichiometric heteromeric complex in vivo, which is likely important for the HMTase function of these proteins during gene repression.

Results

Molecular characteristics and expression profiles of GLP

Figure 1A compares the molecular features of GLP (1296 amino acids) and mouse G9a-S (G9a short form, 1172 amino acids). GLP and G9a exhibit a high overall sequence similarity and a conserved domain structure. The most conserved domain is the SET domain (75% identities, 87% positives). The human counterpart of GLP, Eu-HMTase1, has been shown to express H3-K9 HMTase activity in vitro (Ogawa et al. 2002). To further investigate the substrate specificity and the enzymatic activity of GLP versus G9a, we generated GST-fusion proteins carrying their respective SET domains (amino acids 878–1172 for mG9a-S and 1002–1296 for GLP). The GST-fusion proteins were incubated with S-adenosyl methionine and several recombinant GST-histone H3 tail substrates (amino acids 1–57) (Tachibana et al. 2001). As reported previously, the GST-G9a SET protein preferentially methylated K9 [Fig. 1B, left panel, H3N(K9)] and less efficiently K27 [H3N(K27)] in our in vitro assay system. The GST-GLP SET protein also methylated K9 preferentially and K27 weakly [Fig. 1B, right panel]. Thus, the enzymatic activities of GLP were quite similar to those of G9a.

To explore the biological function(s) of GLP, we examined the intracellular localization profile of GLP as compared with G9a. Newly generated monoclonal antibodies specific for G9a or GLP (epitopes shown in Fig. 1A) were used for detection of the corresponding endogenous proteins using immunohistochemistry. G9a and GLP displayed similar intracellular localization profiles in mouse ES cells and embryonic fibroblasts (Fig. 1C, top and bottom, respectively). Both enzymes were detected exclusively in nuclei, but did not accumulate at DAPI-dense heterochromatin, and were excluded from nucleoli.

We also examined RNA expression profiles of G9a and GLP by multi-tissue Northern blot hybridization in adult mice (Fig. 1D). G9a and GLP were ubiquitously expressed with some variations at steady-state mRNA levels when comparing different tissues. It was notable that the relative levels of GLP transcripts among the examined tissues were quite proportionate to those of G9a, except in testis, where G9a transcripts were elevated. Collectively, these results suggest that G9a and GLP may possess similar functions as H3-K9 HMTases in vivo.

Generation of GLP-deficient mice and ES cells

To generate GLP<sup>−/−</sup> mice and cells by genetic mutation, we designed a targeting construct for replacement of exon 25 and a portion of exon 26 with the neomycin gene cassette [Fig. 2A]. The replaced sequences encode the catalytic core region of the GLP SET domain [as shown in Fig. 1A, underlined]. The targeting construct was introduced into TT2 mouse ES cells (Yagi et al. 1993) and selected by G418/Gnc. From the three hundred clones
screened, we identified eight that carried a correctly targeted GLP allele and no extra copy of the construct. Three clones (#94, #138, and #233) were injected into the morula stage of mouse embryos to generate chimeric animals. Figure 2B shows the establishment scheme of GLP-deficient ES cells. At first, we introduced a Flag-tagged GLP cDNA flanked with two loxP sites into GLP+/− ES cells (#94) and then generated GLP−/− ES cells (118) expressing exogenous Flag-tagged GLP molecules. Finally, using Cre-recombinase treatment of 118 cells, we generated GLP-deficient ES cells (118+) by homologous recombination. The successful generation of GLP−/− ES cells was confirmed by both Southern blot (Fig. 2C) and immunoblot (Fig. 2D) analyses. Anti-GLP antibody could not detect any truncated polypeptides in 118+, indicating that our GLP targeting results in a null mutation. Interestingly, GLP-deficiency caused a reduction in the steady-state levels of G9a protein to approximately one-third of those observed in wild-type ES cell, TT2 (Fig. 2D, middle panel, TT2 vs. 118+). RNA hybridization analysis of GLP-deficient ES cells demonstrated no decrease in G9a-transcript levels, indicating that the reduction in G9a protein occurred post-transcriptionally (data not shown).

**Embryonic lethality in GLP-deficient mice**

To address the in vivo functions of GLP, we established three lines of GLP−/− mice from the #94, #138, and #233 chimeras. The GLP−/− offspring were intercrossed and the resultant GLP−/− mice (embryos) were subjected to phenotypic analyses. No significant differences were identified between GLP−/− and GLP+/− mice. However, as shown in Table 1, the heterozygous crosses produced no GLP−/− pups from three distinct mouse lines. Thus, we conclude that the GLP−/− genotype results in embryonic death. We further examined lethality in the GLP−/− embryos using dissection at different developmental stages. At E12.5, embryo proper genotyped as GLP−/− could not be detected, and putative GLP−/− embryos were already adsorbed completely. Live GLP−/− embryo proper could be detected at E9.5, but were reduced from the expected Mendelian ratio. Approximately one-half of the expected...
GLP−/− embryos were dead at E9.5. Some of these embryos were completely adsorbed and the remainder consisted only of a residual yolk sac, suggesting that GLP−/− embryos die around E9.5. Notably, viable GLP−/− embryos at E9.5 could be distinguished from GLP+/+ and GLP+/− littermates by their morphological properties, because they exhibited severe growth retardation. The gross morphology of typical E9.5 GLP−/− embryos is shown in Figure 3A (left). The cell mass of these GLP−/− embryos was estimated at approximately one-fifteenth that of wild type (data not shown). The E9.5 GLP−/− embryos developed only to the 4–6 somite stage (Fig. 3A, upper left) as opposed to the 21–25 somite stage in wild-type siblings. In addition, the neural groove of GLP−/− embryos was closed only at the tail region but not at the anterior region. These morphological characteristics of the E9.5 GLP−/− embryos resembled those of the E8.0–E8.5 wild-type embryos, and no organ-specific abnormalities were recognized. Most importantly, levels of H3-K9 dimethylation in GLP−/− embryos were drastically reduced when compared with those observed for wild-type embryos (Fig. 3B). The latter data demonstrate that GLP is also a critical H3-K9 HMTase in vivo. In conclusion, the phenotypes of GLP−/− embryos were mostly identical to those of G9a−/− embryos (Tachibana et al. 2002), suggesting that G9a and GLP coordinate common functions during embryonic development.

Drastic reduction of mono- and dimethylated H3-K9 and relocalization of HP1 proteins in GLP−/− ES cells

To clarify the impact of GLP deficiency on H3-K9 methylation, we compared the levels of this histone modification among wild-type (TT2), G9a−/− [2-3], and GLP−/− (CD10) ES cells (Fig. 4A,B). As reported previously [Peters et al. 2003, Rice et al. 2003], Western blot analysis clearly showed that H3-K9 mono- and dimethylation was severely diminished in the G9a−/− ES cells (Fig. 4A).
The GLP−/− ES cells also exhibited a drastic reduction in mono- and dimethylated H3-K9 but no alteration in H3-K9 trimethylation. Due to our previous data for H3-K9 dimethylation in G9a−/− ES cells (Tachibana et al. 2002), we estimated that the reduction of H3-K9 dimethylation in GLP−/− ES cells was also approximately one-eighth. Immunostaining analysis confirmed the specific disappearance of mono- and dimethylated H3-K9 signals in G9a−/− and GLP−/− ES cells, which were detected mostly at euchromatic regions in wild-type nuclei (Fig. 4B). These data further indicate that G9a and GLP are equally required for overall H3-K9 mono- and dimethylation in vivo.

The enrichment of HP1 proteins at pericentric heterochromatin is strictly dependent on H3-K9 methylation by Suv39h family HMTases (Lachner et al. 2001). However, the HP1 proteins also localize to euchromatic loci (Eisenberg and Elgin 2000). Thus, we examined whether deletion of GLP or G9a affects the nuclear localization of HP1 proteins (HP1α, HP1β, and HP1γ). In wild-type ES cells, dense signals for HP1α and HP1β were detected on pericentric heterochromatin, whereas HP1γ was widely dispersed on euchromatin (Fig. 4C, Eisenberg and Elgin 2000). Significant amounts of HP1α and HP1β signals were also detected in the euchromatic regions. In contrast, both G9a−/− and GLP−/− ES cells showed distinct HP1 nuclear localization profiles. All of the HP1 proteins accumulated into pericentric heterochromatin regions and were significantly reduced at euchromatic regions (Fig. 4C, 2-3 and CD10 panels). Total amounts of HP1 proteins were unaltered among these ES cells (data not shown). Thus, G9a- and GLP-mediated methylation of H3-K9 affects the subnuclear distribution of HP1 to euchromatin.

**Table 1.**Embryonic lethality of GLP-deficient mice

| Genotype | +/+ | +/- | -/- | Complete resorption |
|----------|-----|-----|-----|---------------------|
| Newborn  | #94 | 18  | 18  | 0                   |
|          | #138| 10  | 26  | 0                   |
|          | #233| 17  | 38  | 0                   |
| Embryo   | E12.5| 8   | 17  | 2a                  | 9 |
|          | E10.5| 5   | 9   | 31b+2a              | 3 |
|          | E9.5 | 12  | 23(1a) | 8(6b+2a) | 4 |

*aNo embryo proper and only yolk sack remained.

**H3-K9 hypomethylation induces Mage-a gene expression in GLP−/− ES cells**

Next, we examined the expression of Mage-a family genes in GLP−/− ES cells, since the expression of certain Mage-a genes was induced in G9a-deficient ES cells (Tachibana et al. 2002). As shown in Figure 4D (left panel), Mage-a gene(s) expression was also induced in all three GLP−/− ES cell lines examined (CD10–CD12) but was undetectable in wild-type TT2 cells and GLP cDNA positive S1M2 cells. We have also reported that G9a deficiency caused a significant decrease in H3-K9 dimethylation and an increase in H3-K4 dimethylation on the Mage-a2 promoter region in ES cells (Fig. 4D, right panel, Tachibana et al. 2002). Accordingly, we analyzed the methylation status of H3 on the Mage-a2 promoter region in GLP−/− ES cells. Chromatin immunoprecipitation analysis clearly demonstrated that H3-K9 dimethylation was reduced and H3-K4 dimethylation was increased on the Mage-a2 promoter region in the GLP-deficient cells (Fig. 4D, right panel). This result provides further support to our model that GLP functionally overlaps with G9a, not only on a chromosome-wide level but also for a specific genetic locus.

Finally, we complemented the GLP−/− ES cells with a cDNA encoding the wild-type protein. As shown in Figure 4E, all phenotypes observed for the GLP deficiency, including G9a protein instability (left middle panel), loss of H3-K9 dimethylation (center middle panel), and derepression of Mage-a transcripts (right upper panel), were rescued, indicating that the observed GLP−/− phenotypes were intrinsic.

**G9a and GLP exist as heteromeric complexes in vivo**

Our data not only demonstrate that G9a and GLP exhibit significant functional overlap, but also that G9a protein stability is at least partially dependent on GLP. One potential explanation for these results is that the two HMTases function as components of a higher-order complex. Indeed, a subset of transcriptional suppressive complexes in human cells contained both G9a and EuHMTase1 (Ogawa et al. 2002; Shi et al. 2003; Nishio and

**Figure 3.** GLP-deficiency results in embryonic lethality. (A) The gross morphology of a typical GLP-deficient embryo (left) and a wild-type sibling (right) at E9.5. GLP-deficient embryos were severely growth-retarded, containing only 4–6 somites (enlarged in upper left), while wild-type siblings had 21–25 somites. (B) Acid-extracted histones from E9.5 whole embryos were separated by SDS–PAGE and followed by immunoblotting with anti-dimethyl H3-K9 (top) or anti-H4 antibodies (bottom). The levels of H3-K9 dimethylation were drastically reduced in GLP-deficient whole embryos.
Walsh 2004). As such, we examined the association of endogenous G9a and GLP by coimmunoprecipitation assays (Fig. 5A). In wild-type TT2 cells, both G9a and GLP coprecipitated efficiently with antibodies specific for either G9a or GLP (Fig. 5A, lanes 4, 7). Control immunoprecipitations confirmed the specificity of this interaction. GLP failed to precipitate with anti-G9a antibodies from G9a−/− ES cell extracts. Likewise, G9a was not detected in anti-GLP precipitates from GLP−/− ES cells (Fig. 5A, lanes 5, 9). The G9a/GLP interaction was stable under highly stringent conditions using the detergent buffer RIPA (Fig. 5A), or even in high salt (0.5 M) conditions (data not shown).

To determine the stoichiometry of G9a/GLP complexes, we first purified each recombinant protein from insect cells infected with baculovirus vectors encoding mouse G9a-S or GLP (Fig. 5B, left panel). These recombinant proteins were used as standards to compare G9a and GLP concentrations in immunocomplexes. Immunoprecipitates with anti-G9a or anti-GLP Abs were prepared from wild-type ES cells and subjected to immunoblot analysis using the same antibodies (Fig. 5B, right panel). Western blot analysis with the recombinant standards showed that both anti-G9a and anti-GLP immunoprecipitates contained nearly equal amounts of each HMTase. Next, we carried out sequential immunodepletion analysis with anti-G9a and GLP antibodies using the wild-type ES cell extract (Fig. 5C). Two consecutive immunoprecipitations with anti-G9a resulted in an efficient depletion of both G9a and GLP from the cell extracts. The ratio of G9a/GLP signal intensities was main-

Figure 4. Phenotypes of GLP-deficient ES cells. [A,B] The H3-K9 methylation status of TT2 (wild type), G9a-deficient [2-3], and GLP-deficient cells (CD10) was analyzed by Western blot (A) and immunostaining analyses (B). [A, left] GLP protein was absent from in CD10 cells. The reduction in levels of H3-K9 mono- and dimethylation in GLP-deficient cells were indistinguishable from that observed in 2-3 cells. Overall H3-K9 trimethylation at pericentric heterochromatin was unaffected in the mutant cells. [C] The G9a or GLP mutations alter the nuclear distribution of HP1 proteins. Mutant cell nuclei were stained with specific antibodies against three HP1 isoforms (α, left; β, middle; γ, right panels). In G9a- and GLP-mutant cells, euchromatic staining profiles of HP1 had significantly disappeared, but were enriched at pericentric loci. [D] GLP suppressed Mage-a gene expression. [Left] Five micrograms of total RNA were separated and probed with radiolabeled Mage-a cDNA. Fixed and sonicated chromatin of TT2, 2-3, and CD10 ES cells were immunoprecipitated with the indicated Abs and applied to the semiquantitative assay. [Right] H3-K9 dimethylation was reduced and H3-K4 dimethylation was increased on the Mage-a2 promoter region in G9a- and GLP-deficient cells. [E] GLP-cDNA introduction can rescue GLP-deficient phenotypes. A GLP-cDNA driven by the chicken β actin promoter was introduced stably into GLP-deficient cells. [Left panels] In rescued clones L2 and L6, which expressed GLP at levels comparable to those observed in wild-type cells, the protein stability of G9a was recovered. Similarly, dimethyl H3-K9 levels and Mage-a gene suppression were restored (middle and right panels, respectively).
lysates from adult thymocytes, E13.5 primary fibroblasts, the NIH3T3 cell line, the C2C12 myocyte cell line, and the myeloma cell line P3U1 were subjected to coimmunoprecipitation studies. As shown in Figure 5D, the G9a/GLP heteromeric complex was clearly detected in all cell types examined. The signal ratios of G9a/GLP in most immunocomplexes were strikingly similar to those observed for wild-type ES cells. Moreover, G9a/GLP heteromers were readily detected in the human cell lines HeLa and HEK293T (Fig. 5D; data not shown). These data strongly suggest that the association of G9a and GLP to form heteromeric complexes is highly conserved in mammalian cells.

**Formation of G9a/GLP heteromeric complexes requires their SET domains**

Next, we analyzed the specificity of the interactions between G9a and GLP. For this purpose, we coexpressed EGFP-tagged H3-K9 HMTases (Suv39h1, ESET, or GLP) with Flag-tagged G9a-S in HEK 293T cells (Fig. 6A). Flag-G9a-S coprecipitated EGFP-GLP but not EGFP-Suv39h1 or EGFP-ESET (Fig. 6A, lane 3). An identical approach was applied to test interactions between EGFP-tagged Suv39h1, ESET, or G9a and Flag-tagged GLP (Fig. 6B). Again, Flag-GLP only coprecipitated EGFP-G9a but not other H3-K9 HMTases. However, interactions between Flag-G9a-S or Flag-GLP and their partner molecules lacking their C-terminal catalytic domains were drastically reduced (Fig. 6A,B; EGFP-GLPΔSET and EGFP-G9aΔSET lanes). These findings strongly suggest that the SET domains of G9a and GLP are essential not only for their enzymatic activities but also for heterodimerization. Finally, we examined whether G9a and GLP can form complexes directly. To test this possibility, Sf9 insect cells were coinfected with baculoviruses encoding G9a and GLP. After 2 d in culture, nuclear extracts from the coinfected Sf9 insect cells were prepared and subjected to immunoprecipitation analysis using anti-G9a or anti-GLP Abs. As shown in Figure 6C (lanes 3,4), recombinant G9a and GLP ubiquitously form a heteromeric complex. Anti-G9a and anti-GLP immunocomplexes were prepared from mouse adult thymocytes, E13.5 primary fibroblasts, the NIH3T3 cell line, the C2C12 myocyte cell line, the myeloma cell line P3U1, and the human cell line HeLa and were subjected to Western blot analysis. G9a and GLP formed a heteromeric complex in all murine and human cells tested and this heteromeric complexes were the predominant form.
tagged G9a or GLP and EGFP-tagged G9a, GLP, or their SET domain-deletion mutants in HEK 293T cells. As shown in Figure 6D, both G9a and GLP clearly formed homomeric complexes in a SET domain-dependent fashion. To further evaluate the dependence of complex formation on G9a- and GLP-SET domains, we constructed two different epitope-tagged versions of each SET domain and introduced these expression plasmids into HEK 293T cells (Fig. 6E). As shown in Figure 6F, all combinations of the SET domains interacted as complexes. Specifically, Myc-tagged SET domains were coimmunoprecipitated by anti-Flag antibody but not by control antibody [Fig. 6F, upper panel]. As a specificity control, we showed that Flag-tagged EGFP did not associate with Myc-tagged SET domains in this assay [Fig. 6F, lower panel; data not shown]. Together, these biochemical data strongly suggest that the SET domains of G9a and GLP are necessary and sufficient to mediate hetero- or homomeric interactions.

**Discussion**

**G9a and GLP form functional dimeric complexes in vivo**

Our biochemical studies clearly revealed that G9a and GLP form homo- and heteromorphic complexes dependent on their SET domains. However, G9a and GLP preferentially form heteromorphic complexes when cells express both of these HMTases [Fig. 5B,C]. In this regard, we found that steady-state levels of G9a decreased in cells lacking GLP [Figs. 2D, 4E]. These data suggest that G9a...
Regulation of global HP1 targeting by G9a/GLP-mediated H3-K9 methylation

Loss of H3-K9 mono- and dimethylation by mutation of G9a or GLP resulted in a gross relocalization of HP1 [Fig. 4C]. In null cells, all HP1 proteins accumulated at pericentromeric heterochromatin without a corresponding induction of H3-K9 trimethylation [Fig. 4B]. These data strongly suggest that G9a/GLP-mediated methylation of H3-K9 methylation also directs the global recruitment of HP1 proteins to euchromatin. HP1 binding to "heterochromatin domains" is not static but highly dynamic (Cheutin et al. 2003; Festenstein et al. 2003). HP1 isoforms interact with the H3 N terminus peptides containing di- and trimethylated H3-K9 with similar binding constants in vitro (Fischle et al. 2003). Therefore, the balance of Suv39h- and G9a/GLP-mediated H3-K9 methylation is one of the critical factors for HP1 dynamics and its binding to specific chromatin domains. However, in general, HP1α and HP1β seem to be more abundant at pericentromeric heterochromatin where the density of H3-K9 trimethylation is relatively high. HP1 also interacts with Suv39h and Suv4-20h HMTases, both of which localize to pericentromeric heterochromatin (Aagaard et al. 1999; Schotta et al. 2004). These factors in combination probably enhance the retention of HP1α and HP1β to pericentromeric heterochromatin.

G9a and GLP cooperatively exert H3-K9 methyltransferase function in vivo

The current studies show that G9a and GLP are equally critical for euchromatic H3-K9 mono- and dimethylation and do not function redundantly in this regard. In support of this conclusion, we have also established G9a/
a unique function in specific cell types such as murine testicular cells where the G9α gene is predominantly expressed. Finally, it will be of great interest to determine the physiological relevance of the G9α/GLP dual-enzyme regulation on euchromatic H3-K9 methylation.

Materials and methods

**Generation and genotyping of the GLP-mutant embryos and ES cells**

A mouse GLP cDNA fragment corresponding to amino acids 1002 to its termination was used as a probe to screen a TT2 genomic library. To make a GLP targeting construct, a 6.0-kb genomic fragment, spanning from the AatII site in exon 26 to the Xhol site downstream of exon 26, was inserted into the modified Sall site of twoloxP-containing plasmid, pLNTK, and then a 1.8-kb Xbal genomic fragment located in the intron between exons 24 and 25 was further subcloned into the Xhol site. The GLP targeting construct replaces exon 25 and a portion of exon 26 of mouse GLP gene with the pgk-neomycin gene.

TT2 ES cells (1 x 10⁷) were transfected with 20 µg of the Xhol-linearized GLP-targeting construct and screened as described previously [Tachibana et al. 2002]. Homologous recombinant clones (#94, #138, and #233) were identified by Southern blot analysis of BamHI/HindIII digested DNA probed with a PCR-amplified genomic fragment located between exons 24 and 25. Southern blot analysis with a fragment of neomycin gene confirmed that there were no extra copies of the targeting construct integrated randomly into the genome of the recombinant cells. The recombinant cells were injected into the morula stage of ICR mouse embryos. Established chimeric male mice derived from the three clones described above successfully generated F1 ICR mouse embryos. Established chimeric male mice derived from the targeting construct replaces exon 25 and a portion of genomic fragment, spanning from the AatII site in exon 26 to its termination was used as a probe to screen a TT2 ES cell genomic library. To make a GLP targeting construct, a 6.0-kb genomic fragment, spanning from the AatII site in exon 26 to the Xhol site downstream of exon 26, was inserted into the modified Sall site of twoloxP-containing plasmid, pLNTK, and then a 1.8-kb Xbal genomic fragment located in the intron between exons 24 and 25 was further subcloned into the Xhol site. The GLP targeting construct replaces exon 25 and a portion of exon 26 of mouse GLP gene with the pgk-neomycin gene.

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**Antibodies**

For detection of G9α, hamster monoclonal antibody #14-1 (MBL) or mouse monoclonal antibody #8620 was used. For detection of GLP, hamster monoclonal antibody C7-5 or mouse monoclonal antibody #422 was used. Anti-G9α #8620 was generated to recognize an epitope embedded within amino acid positions from 1 to 81 of the GLP polypeptide. These two monoclonal antibodies were raised by immunizing mice with recombinant baculovirus particles displaying amino acid sequences of G9α or GLP shown above as fusion proteins to the viral surface glycoprotein gp64 as previously described [Tanaka et al. 2002]. For detection of epitope tagged-protein, rabbit anti-GFP (MBL) and anti-Flag M5 (Sigma) were used. The methylation status of H3-K9 was analyzed with anti-monomethyl H3-K9 [Upstate, #07-395], anti-dimethyl H3-K9 (#07-212), and anti-trimethyl H3-K9 (#07-523) antibodies. To control for protein loading, anti-histone H4 (Upstate, #07-108), anti-tubulin (Oncogene, CP06) were used. For HP1 detection, mouse monoclonal antibodies against HP1α [Euromedix, 2HP-1H5 and 2HP-2G9], HP1β (Euromedix, 1MOD-1A9), and HP1γ (Euromedix, 2MOD-1G6) were used. We also used different antibodies against mono-, di- and trimethylated H3-K9 gifted by Thomas Jenuwein (IMP, Vienna, Austria), and confirmed the same results.

**Immunofluorescence analysis**

Cytospun cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and incubated with the primary antibodies described above at 37°C for 40 min. Anti-rabbit IgG or anti-mouse IgG conjugated with Zenon Alexa 568 Fluor [Molecular Probes] were used for detection. The nuclei were counterstained with DAPI, observed under fluorescence microscopy, and analyzed with AxioVision software (Zeiss).

**Immunoprecipitation**

For immunoprecipitation of endogenous G9α and GLP, ES cells were harvested with PBS containing trypsin (0.05%) and EDTA (0.2 mM). After removing the cytoplasmic fraction with buffer A (10 mM HEPES-KOH at pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 10 min incubation on ice), nuclear pellets were lysed with one ml of RIPA buffer [PBS containing 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor cocktail; Nakalai] for 30 min at 4°C with rotation. Nuclear extracts were then prepared by removing chromatin pellets after centrifugation. Typical conditions for immunoprecipitation were as follows. Nuclear extracts from 10⁷ ES cells nuclei were incubated with 2 µg of either anti-G9α (#8620) or anti-GLP (#422) overnight and immune complexes were collected with 20 µL of protein G slurry [1:1 ratio] for 1 h. The immune complexes were washed three times with 500 µL of PBS containing 0.1% Nonidet P-40. Several murine cells shown in Figure 5C were also treated as described above.

For transient G9α and GLP interaction analyses, HEK293T cells were transfected with combinations of Flag-tagged cDNAs inserted into the pcDNA3 vector [Invitrogen] or pEGFP-C vectors (Clontech) containing corresponding cDNAs. After 2 d in culture, whole cell extracts were prepared with RIPA buffer and used for immunoprecipitation as described above. Antibodies used were anti-Myc [9E10], anti-Flag M2 [Sigma], and anti-GFP (MBL). EGF-tagged SET domain-deletion mutants of G9α and GLP were made by removal of cDNA portions coding pre-SET, SET, and post-SET domains by digestion of restriction enzyme PmlI for G9α and Scal for GLP and Kpnl. The cDNAs for ΔSET versions of G9α and GLP encode 936 and 1058 amino acids, respectively.

**Immunoblot analyses**

Acid-extracted histones [Tachibana et al. 2002] corresponding to 5 x 10⁴ nuclei were separated by SDS-PAGE, transferred to
nitrocellulose membranes, blocked with 5% milk, and probed with the specific antibodies described above. Following washes, blots were incubated with an HRP-conjugated anti-rabbit antibody (Amersham) prior to addition of ECL (Amersham). For detection of the mouse monoclonal primary antibody, we used an HRP-conjugated anti-mouse IgG-Fc portion (Cappel).

In vitro HMTase activity assays
HMTase activity assays were performed as described (Tachibana et al. 2001).

Chromatin immunoprecipitation
Chromatin immunoprecipitation analysis was performed as described (Tachibana et al. 2002).

Baculovirus expression
Insect cells SF9 were infected with baculovirus encoding either Flag-His-tagged mouse G9a-S or His-tagged GLP cDNA and cell extracts were prepared 2 d post-infection. Purified protein amounts were measured by coomassie staining in comparison with BSA.

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