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De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes

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The pluripotency-determining gene *Oct3/4* (also called *Pou5f1*) undergoes postimplantation silencing in a process mediated by the histone methyltransferase G9a. Microarray analysis now shows that this enzyme may operate as a master regulator that inactivates numerous early-embryonic genes by bringing about heterochromatinization of methylated histone H3K9 and *de novo* DNA methylation. Genetic studies in differentiating embryonic stem cells demonstrate that a point mutation in the G9a SET domain prevents heterochromatinization but still allows *de novo* methylation, whereas biochemical and functional studies indicate that G9a itself is capable of bringing about *de novo* methylation through its ankyrin domain, by recruiting Dnmt3a and Dnmt3b independently of its histone methyltransferase activity. These modifications seem to be programmed for carrying out two separate biological functions: histone methylation blocks target-gene reactivation in the absence of transcriptional repressors, whereas DNA methylation prevents reprogramming to the undifferentiated state.

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Normal development seems to take place through a unidirectional process characterized by a stepwise decrease in cell potency, and it is presumably this phenomenon that is mainly responsible for the difficulty in reprogramming differentiated somatic cells *in vivo*[1]. Changes in gene expression during development are accompanied or caused by epigenetic modifications such as methylation of DNA at CpG sequences, modification of histone tails and the presence of non-nucleosomal chromatin-associated proteins. As development proceeds, differentiated cells accumulate epigenetic marks that differ from those of the pluripotent cell type. Whereas some of these may have short-term flexibility that allows them to be easily removed, other marks are stably heritable even through multiple cell divisions and thus acquire a degree of irreversibility[2].

Oct3/4 and *Nanog* encode pluripotency-sustaining transcription factors that are expressed during early embryogenesis and in embryonic stem (ES) cells[3]. Oct3/4 is regarded as pivotal, because either its loss of function or its overexpression completely abolishes self-renewal and induces differentiation[4]. Nanog is also regarded as a component of the core transcription factor network (along with Oct3/4 and Sox2) that is required for the maintenance of pluripotency and it has a dominant function in supporting self-renewal of mouse ES cells in the absence of leukemia-inhibitory factor (LIF)[5,6]. Expression of these genes is silenced both by histone modification and DNA methylation upon ES cell differentiation and subsequently in somatic cells[7–10].

Recent studies on the mouse *Oct3/4* gene promoter have demonstrated that this postimplantation inactivation process is carried out in a multilayer manner that involves direct inhibition of transcription, heterochromatinization through the trimethylation of H3K9 and subsequent DNA methylation[7]. Notably, both of these latter epigenetic structures are actually put in place by the SET domain protein G9a (also known as Ehmt2), and it seems to be this event that causes *Oct3/4* irreversible silencing in subsequent somatic cell lineages[7].

This linkage between histone and DNA methylation seems to be a general phenomenon. Suv39h, for example, is singularly required for the inactivation of pericentric satellite DNA, whereas Ezh2 can bring about both H3K27 methylation and DNA methylation at selected genes in cancer cells[11,12]. In addition, heterochromatinization and DNA methylation have been shown to be genetically linked in plants[13]. In all cases, the mechanism for this linkage has not been fully elucidated.

Although *Oct3/4* seems to be hermetically closed in somatic cells, this endogenous locus and other pluripotency genes can actually be reactivated at low frequency when somatic cell nuclei are subject to reprogramming either by nuclear transplantation, fusion with ES cells or induction of pluripotency by defined transcription factors (iPS), and this is accompanied by chromatin opening and demethylation of promoter DNA[14–17]. These experiments suggest that embryonic cells may carry the molecular machinery needed to reverse the

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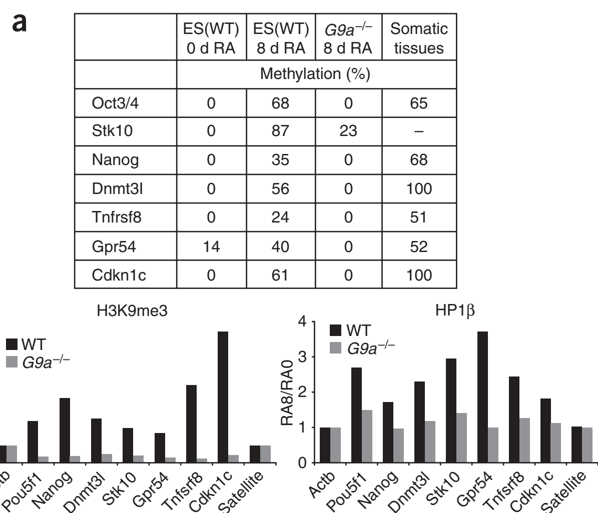


	ES(WT) 0 d RA	ES(WT) 8 d RA	*G9a*−/− 8 d RA	Somatic tissues
	Methylation (%)			
Oct3/4	0	68	0	65
Stk10	0	87	23	–
Nanog	0	35	0	68
Dnmt3l	0	56	0	100
Tnfrsf8	0	24	0	51
Gpr54	14	40	0	52
Cdkn1c	0	61	0	100

Figure 1 Genome-wide G9a-dependent *de novo* methylation. (**a**) DNA from wild-type (WT) and *G9a*$^{-/-}$ ES cells treated with retinoic acid (RA) for 8 d and from somatic tissues (spleen and kidney) was treated with sodium bisulfite (Qiagen), amplified using specific gene promoter primers, cloned and subjected to sequence analysis. It should be noted that *Nanog de novo* methylation was detected at a site different[9] from that measured in previous studies[7]. (**b**) ChIP analysis of the genes' promoters using antibodies specific for H3K9me3 and HP1β on 0-d and 8-d RA-treated wild-type and *G9a*$^{-/-}$ ES cells.

repressed state initially laid down when the embryo first undergoes differentiation, but little is known about the components that take part in this process.

As Oct3/4 expression is known to be necessary but not sufficient for pluripotency[5], there must be additional genes that undergo inactivation during embryonic cell differentiation, and it is likely that G9a has a role in this process. We set out to investigate the set of murine early-embryonic genes that G9a silences and to understand how G9a brings about heterochromatinization and *de novo* methylation. By understanding this inactivation process, it should be possible to gain further insights into the molecular logic of reprogramming as well.

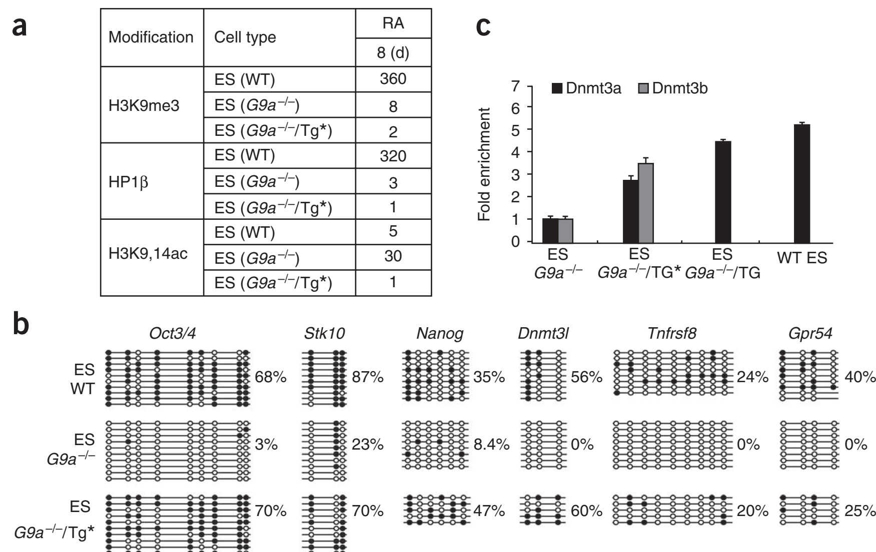
RESULTS

G9a-dependent methylation of a set of embryonic genes

To identify the set of genes that undergo G9a-mediated *de novo* methylation, we carried out differential microarray analysis aimed at identifying specific promoters that become methylated *de novo* during ES cell differentiation. To this end, we used methylated DNA immunoprecipitation (mDIP) array analysis on DNA from various ES cell samples to identify genes that are unmethylated in undifferentiated cells, but seem to be methylated in cells exposed to 8 d of retinoic acid induction. As a further control, we selected only those genes that were also found to remain unmodified in differentiated mouse *G9a*$^{-/-}$ ES cells. By these criteria, we detected a set of 126 individual genes (**Supplementary Table 1** online), and bisulfite analysis of a representative sample indicated that 75% (six of eight) do in fact undergo G9a-dependent *de novo* methylation during ES cell differentiation (**Fig. 1a** and **Supplementary Fig. 1** online). Much like *Oct3/4*, all six of these new gene promoters were found to become heterochromatinized in differentiated ES cells as determined by chromatin immunoprecipitation (ChIP) analysis of trimethylated (me3) H3K9 and heterochromatin protein 1 (HP1) (**Fig. 1b**). It is likely that these genes also undergo structural inactivation *in vivo*, as almost all of them were found to be methylated in somatic tissues (**Fig. 1a**). Although the actual functions of these genes during development are not known, transcription-pattern analysis of the 126 gene sequences identified in the original screen indicate that a high percentage are expressed preferentially either in preimplantation embryos or in germline cells (**Supplementary Table 1**). Thus, G9a may actually direct the epigenetic silencing of a relatively large embryonic gene network.

Methylation in the absence of a proper SET domain

Previous studies in a number of different organisms have demonstrated that enzymes involved in the methylation of H3K9 may have a role in directing targeted DNA methylation, but the mechanism for this process is not known[18]. To better understand how G9a brings about these events, we used *G9a*$^{-/-}$ ES cells that carry a G9a expression vector with a single-nucleotide mutation (F1205Y, designated *G9a*$^{-/-}$/Tg*) in its SET domain that makes it inherently defective in the di- and trimethylation of histone H3K9 (ref. 19). Indeed, ChIP analysis clearly shows that, unlike the wild-type protein, this G9a



Modification	Cell type	RA 8 (d)
H3K9me3	ES (WT)	360
	ES (*G9a*−/−)	8
	ES (*G9a*−/−/Tg*)	2
HP1β	ES (WT)	320
	ES (*G9a*−/−)	3
	ES (*G9a*−/−/Tg*)	1
H3K9,14ac	ES (WT)	5
	ES (*G9a*−/−)	30
	ES (*G9a*−/−/Tg*)	1

Figure 2 Heterochromatinization is prevented by point mutation in the G9a SET domain. (**a**) ChIP analysis of the *Oct3/4* promoter in wild-type (WT), *G9a*$^{-/-}$ and *G9a*$^{-/-}$/Tg* (mutated in the SET domain, F1205Y) ES cells (retinoic acid (RA)-differentiated, 8 d) using antibodies specific for H3K9me3, HP1γ, and H3K9 and acetylated H3K14 (14ac). qPCR was used. The degree of enrichment was calculated as Bound (B)/Input (I) and normalized to β-actin or β-globin. (**b**) DNA from ES cells treated with RA for 8 d was treated with sodium bisulfite, amplified using specific promoter primers (**Supplementary Table 2**), cloned and subjected to sequence analysis. Three to nine CpG sites were examined. (**c**) ChIP analysis of the *Oct3/4* promoter using antibodies specific for Dnmt3a and Dnmt3b on ES cells that were allowed to differentiate for 6 d. Enrichment values were normalized to pericentric satellite DNA in each sample (± s.d.).

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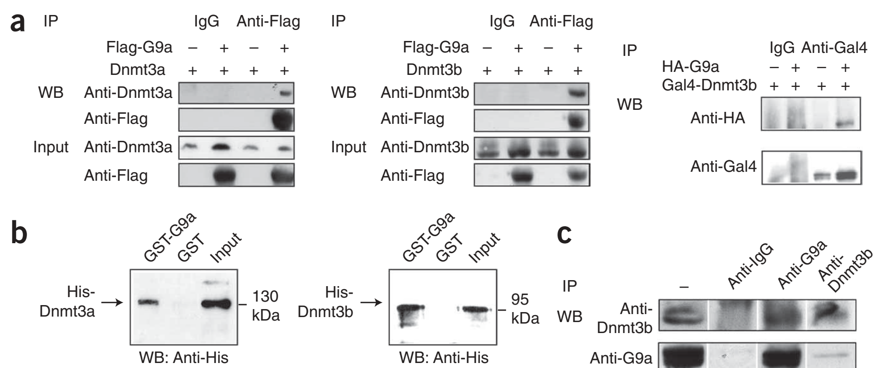

Figure 3 G9a recruits Dnmt3a and Dnmt3b. (**a**) 293 cells transiently transfected with Dnmt3a or Dnmt3b and G9a-Flag, or with hemagglutinin (HA)-G9a and Gal4-Dnmt3a (as indicated). Cell extracts were immunoprecipitated (IP) with IgG and specific antibodies against Flag or Gal4, and western blotting (WB) was performed with indicated antibodies. (**b**) Direct interactions between G9a and Dnmts. A GST-fused G9a protein was incubated either with His-Dnmt3a or with His-Dnmt3b proteins. Western botting was performed with anti-His. (**c**) Endogenous co-immunoprecipitations of G9a and Dnmts. Nuclear extracts prepared from differentiated ES cells were immunoprecipitated with anti-IgG, anti-G9a or anti-Dnmt3b. Western blotting was performed with anti-Dnmt3b or anti-G9a. All lanes are from the same gel.

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mutant does not bring about trimethylation of H3K9 nor the subsequent binding of HP1β at the *Oct3/4* promoter, even in ES cells that have undergone retinoic acid–induced differentiation (**Fig. 2a**). It should be noted that this point mutant is still capable of directing histone deacetylation, indicating that, in this case, the lack of H3 trimethylation cannot be due to blockage of H3K9 residues by acetyl groups (**Fig. 2a**). This experiment thus proves that H3K9 trimethylation of *Oct3/4* is indeed directly catalyzed by G9a.

Using bisulfite analysis, we then examined the methylation state of the *Oct3/4* promoter. As expected, this sequence was found to be highly methylated in wild-type cells and in *G9a*$^{-/-}$ cells carrying a wild-type transgene (data not shown), but unmethylated in the *G9a* knockout. Notably, however, we observed normal *de novo* methylation in the *G9a*$^{-/-}$/Tg* mutant (**Fig. 2b**), and similar results were obtained for additional G9a target genes. These findings clearly show that neither H3K9 trimethylation nor the presence of HP1β are required for targeted DNA methylation. In keeping with this, ChIP analysis demonstrated that, unlike the G9a knockout, the mutant is still capable of recruiting *de novo* methyltransferases (Dnmts) to the *Oct3/4* promoter in differentiated ES cells (**Fig. 2c**). Taken together, these results suggest that G9 mediates *de novo* methylation even in the absence of a proper SET domain, by directly recruiting DNA methyltransferases to the promoter.

G9a recruits Dnmt3a and Dnmt3b through its ANK domain

We next asked whether G9a is indeed capable of binding Dnmts at the biochemical level. To this end, we transfected plasmid vectors that express G9a-Flag and Dnmt3a or 3b into human 293 cells in culture. Extracts from these cells were then immunoprecipitated with anti-Flag, and we subjected the pull-down material to western analysis using anti-Dnmt antibodies. These experiments clearly showed that G9a is capable of specifically recruiting Dnmt3a and 3b, and this was confirmed by carrying out the reciprocal experiments (**Fig. 3a**). Moreover, we used proteins synthesized *in vitro* to show that there is direct interaction between G9a and Dnmt3 (**Fig. 3b**). Finally, using direct antibodies to G9a and Dnmt3b, we were able to demonstrate that the endogenous proteins themselves also form specific complexes *in vivo* in differentiated ES cells (**Fig. 3c**). This association could explain why DNA methylation at a number of gene sequences has been shown to be dependent on G9a[20,21]. It has also been demonstrated that G9a may interact with Dnmt1 in the replication complex[22].

The G9a protein is made up of several different functional regions, including the enzymatic SET domain, several cysteine-rich regions and ankyrin (ANK) repeats[23], which were recently shown to interact with mono- or dimethylated H3K9 (ref. 24). To determine which of these protein modules may be involved in the binding of Dnmt, we carried out a new set of pull-down experiments using transfected vectors carrying region-specific deletions of G9a linked to enhanced green fluorescent protein (EGFP) (**Fig. 4a**). Western analysis showed clearly that the recruitment of Dnmt3a or 3b specifically requires the presence of an ANK repeat region but is completely unaffected by removal of even the entire SET domain (**Fig. 4a**). It should be noted that, because this SET domain also has a role in generating heterodimers with the partner protein, Glp (also known as Ehmt1)[25], this last result suggests that in this case G9a itself is actually sufficient for recruiting *de novo* methylases. Using a reciprocal approach, we also mapped the G9a-interacting domain to the catalytic regions of Dnmt3a and 3b. Notably, the noncatalytic Dnmt3L was unable to interact with G9a (**Fig. 4b**).

In light of these findings, we next used a genetic approach to test whether the ANK domain is indeed responsible for *de novo* methylation. To this end, we transfected the ΔANK construct (in which the ankyrin repeat region is deleted) into *G9a*$^{-/-}$ cells and then measured the initial rate of *Oct3/4 de novo* methylation by bisulfite analysis on DNA from retinoic acid–induced ES cells, including time points at which methylation should still be increasing monotonically. This construct carries out *de novo* methylation slowly as compared to both wild-type and F1205Y G9a (**Fig 5a,b**), even though there seems to be a relatively large excess of the ΔANK protein in the transfected cells (**Fig. 5c**). Despite this low level of DNA methylation, the *Oct3/4* promoter was still found to be marked by H3K9me3 and HP1 (**Fig. 5d**). As HP1 itself is able to recruit Dnmts[26–28], this heterochromatinization may help explain the residual *de novo* methylation activity observed in this experiment. Similar results for methylation and heterochromatinization were also obtained for the *Stk10* gene promoter (**Supplementary Fig. 2** online). From these biochemical and genetic studies, it thus seems that G9a can mediate the methylation of DNA by a mechanism that is independent of H3K9me and HP1β.

Separate functions of histone methylation and DNA methylation

Kinetic studies on Oct3/4 repression and heterochromatinization as a function of differentiation in ES cells indicated that this process occurs in three distinct steps[7]. In the first stage, Oct3/4 expression is dramatically downregulated by retinoic acid–induced transcriptional repressors[29–31], and this is then followed, in a stepwise manner, by G9a-mediated heterochromatinization and *de novo* methylation at the promoter. These latter epigenetic changes seem to have a role in locking *Oct3/4* into its repressed state, and, once these structures are formed, transcription cannot be restored either by removing the

original inducer (retinoic acid) or by recloning differentiated cells in LIF-containing medium. In contrast, reprogramming to pluripotency occurs with high frequency in *G9a*$^{-/-}$ cells, and Oct3/4, as well as other G9a target genes, can be readily re-expressed upon removal of retinoic acid once the differentiation process is completed (**Fig. 6a,b**).

To decipher the individual roles of histone H3K9 methylation as opposed to DNA methylation in locking repression, we used a genetic approach. We first examined the ability of Oct3/4 to revert to its active state after ES cells have undergone differentiation (**Fig. 6b**). To this end, *G9a*$^{-/-}$ ES cells carrying the mutant *G9a* transgene (F1205Y) were differentiated *in vitro* for 8 d, and the retinoic acid was then removed. In contrast to what was observed with the *G9a*$^{-/-}$ parent cells, Oct3/4 remained completely repressed even after several days in culture. As the mutant brings about *de novo* methylation in the absence of H3K9 heterochromatinization it seems that DNA modification alone is sufficient to prevent the re-expression of Oct3/4 under these conditions. Similar results were obtained for differentiated *Dnmt3a/3b*$^{-/-}$ or *G9a*$^{-/-}$/ΔANK ES cells in which the Oct3/4 promoter undergoes normal heterochromatinization but does not become methylated *de novo* (**Fig. 6b**). It thus seems that, although the removal of retinoic acid allows the re-establishment of protein factors that work *in trans* to activate silenced genes in this nondividing cell population, individual epigenetic marks such as heterochromatinization or DNA methylation set up during the differentiation process prevent this from happening.

Many studies have demonstrated that differentiated cell nuclei do not easily undergo reprogramming to a totipotent phenotype even when placed in an early-embryonic environment, and this may largely be due to the inability of genes such as *Oct3/4* to undo their repressive epigenetic structure[32,33]. Evidence that this is indeed the case has come from experiments showing that, unlike wild-type cells, differentiated *G9a*$^{-/-}$ ES cells are able to revert to a proliferating undifferentiated phenotype when recloned in LIF-containing medium[7], and these cells seem to have recovered some degree of pluripotency, as judged by their ability to undergo normal differentiation when re-induced by retinoic acid (**Supplementary Fig. 3** online). This reprogramming is undoubtedly made possible because, in these cells, these *Oct3/4*-like genes lack both heterochromatin structure and DNA methylation. As *Dnmt3a/3b*$^{-/-}$ (ref. 7) and *G9a*$^{-/-}$/ΔANK (**Fig. 6c**) ES cells are also capable of generating undifferentiated clones in this system, heterochromatinization alone does not seem to be sufficient to prevent reprogramming. In contrast, differentiated *G9a*$^{-/-}$ ES cells carrying the mutant *G9a* transgene (F1205Y) could not produce any clones when replated in LIF-containing medium (**Fig. 6c**). When taken together, these results suggest that DNA methylation is not only necessary, but may also be sufficient to prevent reprogramming to a more pluripotent state.

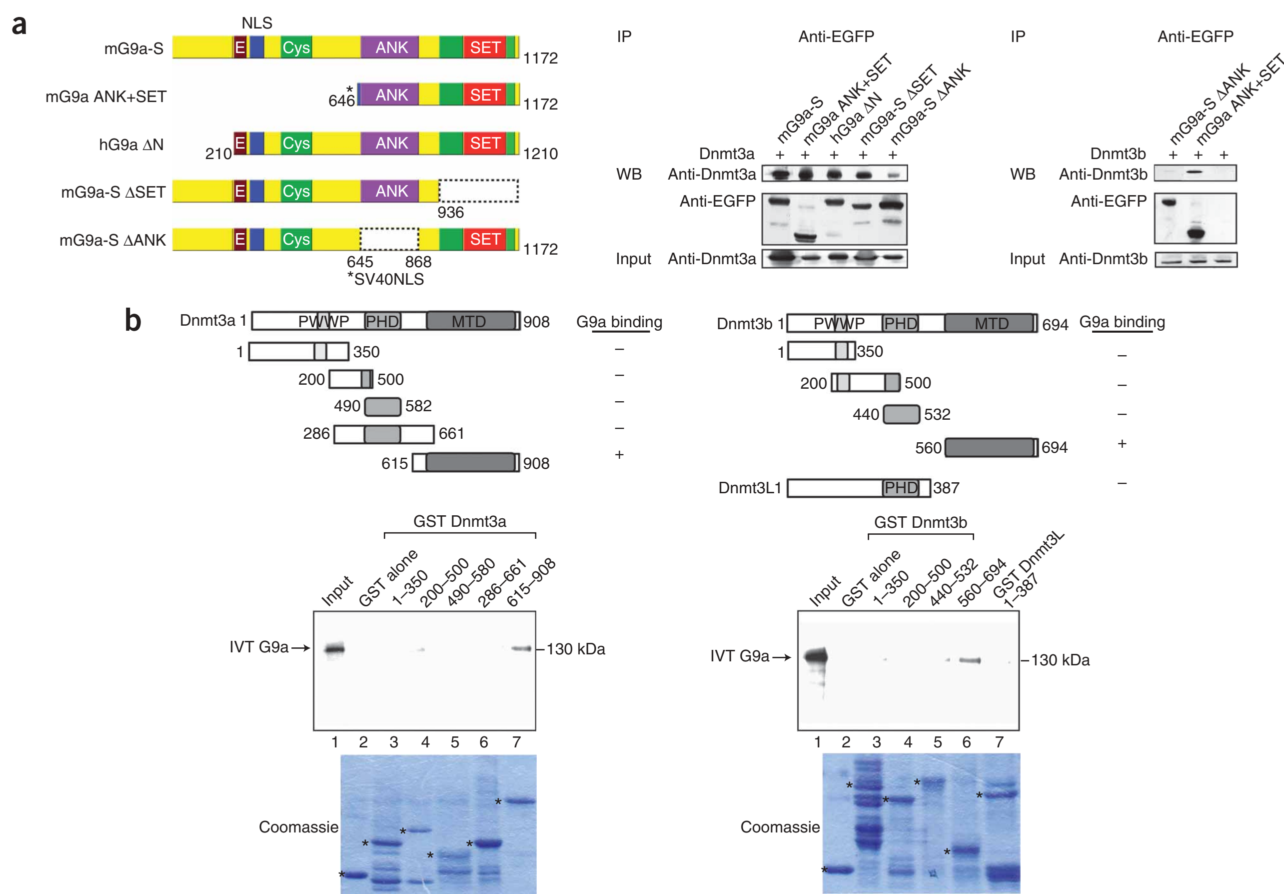

Figure 4 Mapping the G9a- and Dnmt-interacting domains. (**a**) Schematic representation of the plasmids used for the immunoprecipitation assay. 293 cells transiently transfected with plasmids encoding various EGFP-tagged mutants of G9a. Extracts were immunoprecipitated with anti-EGFP, and western blotting (WB) was performed with antibodies specific for EGFP, Dnmt3a and Dnmt3b. (**b**) Schematic representation of GST-fusion constructs harboring full-length Dnmt3L, or different regions of Dnmt3a and Dnmt3b. The methyltransferase domain is labeled as MTD. *In vitro*–translated (IVT) G9a was incubated with the indicated GST fusions of Dnmt3a, Dnmt3b and Dnmt3L. The presence of G9a was visualized by western blotting using anti-G9a. Expression of GST-Dnmts was assayed by SDS-PAGE gels stained with Coomasie blue.

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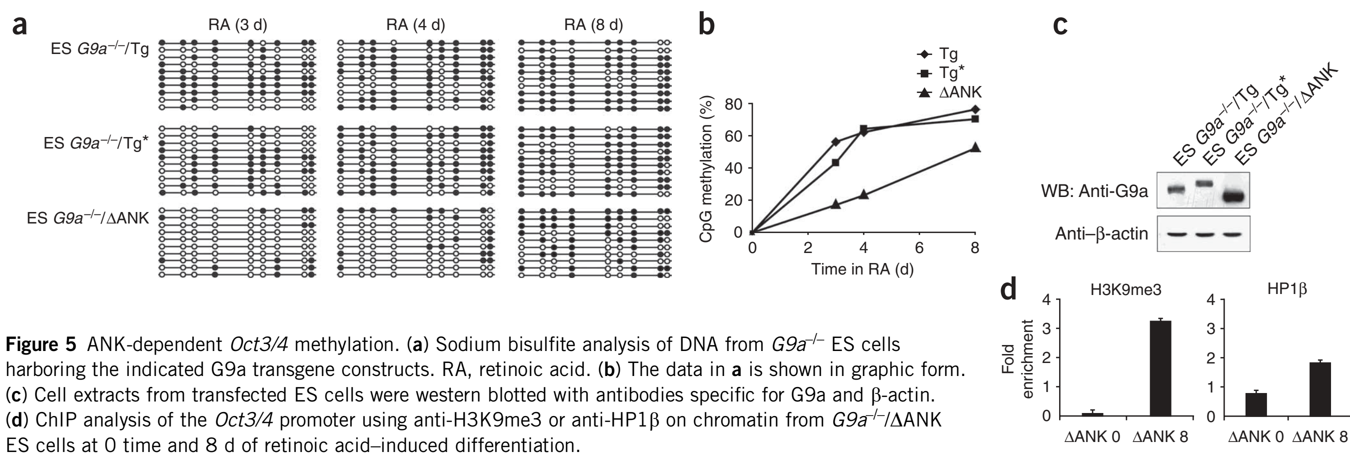


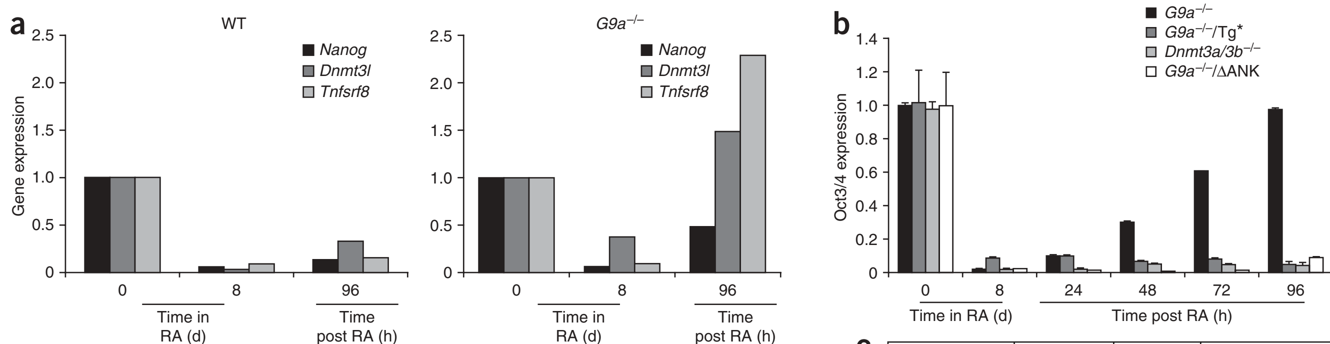
Figure 5 ANK-dependent *Oct3/4* methylation. (**a**) Sodium bisulfite analysis of DNA from *G9a*$^{-/-}$ ES cells harboring the indicated G9a transgene constructs. RA, retinoic acid. (**b**) The data in **a** is shown in graphic form. (**c**) Cell extracts from transfected ES cells were western blotted with antibodies specific for G9a and β-actin. (**d**) ChIP analysis of the *Oct3/4* promoter using anti-H3K9me3 or anti-HP1β on chromatin from *G9a*$^{-/-}$/ΔANK ES cells at 0 time and 8 d of retinoic acid–induced differentiation.

G9a-dependent reprogramming *in vivo*

In light of these results, we next asked whether differentiated *G9a*$^{-/-}$ ES cells are also good donors for reprogramming *in vivo*. To this end, we carried out nuclear transfer into enucleated oocytes and allowed the embryos to develop in culture. Under the conditions used for this experiment, wild-type ES cell nuclei were unable to direct early development (0 blastocysts from 28 cleavage-stage embryos from 48 reconstituted oocytes). In contrast, *G9a*$^{-/-}$ nuclei yielded 6 normal blastocysts from 32 cleavage-stage embryos (48 reconstituted oocytes), and this was highly significant ($P < 0.05$). These studies clearly indicate that G9a has an important biological role in the stable inactivation of pluripotency genes and could be a major influence in controlling the ability of somatic cells to undergo reprogramming[34,35].

DISCUSSION

G9a seems to carry out two distinct and different epigenetic activities involved in gene repression. Through the SET domain, G9a brings about local methylation of histone H3K9me3, which subsequently binds HP1, thus generating a local heterochromatin structure. In parallel, this same protein can recruit Dnmt3a and 3b, which then cause *de novo* methylation at the promoter. Using a mutant *G9a* gene we have shown that these two activities work largely independently of each other. Previous genetic studies have suggested that another histone methyltransferase, Suv39h, is specifically required for the targeted *de novo* methylation of repeat DNAs associated with centromeres or telomeres[36], and it was tacitly assumed that this reaction is mediated exclusively through histone H3K9 methylation in combination with HP1 heterochromatinization[12]. As our findings on G9a suggest that H3K9me3 itself is not sufficient to bring about full local *de novo* methylation, it is possible that, in this case as well, *de novo* methylation is mediated by the histone methyltransferase protein itself, and this is indeed supported by biochemical studies[12]. The same may be true for the enzyme KRYPTONITE which causes both histone and DNA methylation in *Arabidopsis thaliana*[13]. It was recently shown that Ezh2, an H3K27-specific methylase that brings



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Cell type	Reverted clones	(%)	Normalized (%)
WT	0/288	–	–
G9a$^{-/-}$ (10–20)	48/192	25	13.8
G9a$^{-/-}$ (2–3)	89/288	31	17.2
G9a$^{-/-}$/Tg*	3/288	1	0.8
G9a$^{-/-}$/ΔANK	29/192	15	8.5

Figure 6 Reactivation and reprogramming of *Oct3/4*, *Nanog*, *Dnmt3L* and *Tnfsrf8* genes are affected by their epigenetic state. (**a**) Wild-type (WT) and *G9a*$^{-/-}$ ES cells were treated with retinoic acid (RA) for 8 d, after which RA was removed and cells were isolated after 96 h. Graph shows the level of expression for the indicated genes using qPCR analysis with *Ubiquitin C* (*UBC*) as the normalization control. (**b**) ES cells were treated with RA for up to 8 d, after which RA was removed and cells were isolated at various time points. Graph shows the level of Oct3/4 expression (± s.d.) using qPCR analysis with *UBC* as the normalization control. (**c**) Number and percentage of reverted single-cell clones established from wild-type and mutant ES cells that were initially differentiated with RA for 8 d and recultured in 96-well plates for 2 weeks in the presence of LIF under conditions that were calibrated for each line to yield approximately 1 colony per well of cells treated with RA for 1 d (10–20 cells per well). Each result represents the sum of two or three independent experiments. Normalization takes into consideration the clonability of each cell line, as determined by the number of colonies found after plating cells treated with RA for 1 d.

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about heterochromatinization through the binding of a chromodomain protein, is also capable of causing local *de novo* DNA methylation by directly recruiting Dnmts[11], suggesting that this may represent a general mechanism. It should be noted that several studies have demonstrated that HP1 itself can recruit Dnmts[26–28], and this may provide an auxiliary mechanism for targeting and maintaining DNA methylation at heterochromatin regions in animal cells. Only in the case of *Neurospora crassa* has it been shown that an HP1-like molecule is actually required for local *de novo* methylation[27].

Our studies show that G9a is a master structural regulator with an important role in early development by targeting a wide network of embryonic genes for postimplantation repression. This silencing process includes key genes, such as Oct3/4, Nanog and Dnmt3L, that are intimately involved in maintaining the ES cell phenotype[3] and for establishing maternal imprints in mammalian germ cells[37]. G9a operates in a double manner, by using its SET domain to generate heterochromatin and its ANK domain to carry out *de novo* methylation. Each of these epigenetic marks seems to have a separate function *in vivo*. Following factor-mediated repression, heterochromatin is formed on the promoter, and this serves to protect against the possibility that changes in factor availability could reactivate the gene. Permanent silencing, however, can be attained only through *de novo* methylation of the promoter. Thus, when differentiated cells are placed anew into an embryonic environment, heterochromatin structure can probably be removed during the process of cell division, but DNA methylation seems to be faithfully maintained, thereby preventing reactivation. It is likely that this silencing program actually represents the main barrier to embryonic reprogramming[38,39].

METHODS

Cells, DNA and RNA analyses. Murine wild-type, *Dnmt3a/3b*$^{-/-}$ (ref. 40), *G9a*$^{-/-}$, *G9a*$^{-/-}$/Tg and *G9a*$^{-/-}$/Tg* (ref. 23) ES cells were maintained as described previously[40,41]. The Tg* transgene (F1205Y) has a single nucleotide mutation in the SET domain, which makes it inherently defective in di- and trimethylation of H3K9, but it may still be capable of monomethylation at this site[23]. Cells were treated with 1 μM retinoic acid for the indicated times as previously described[30,31]. DNA methylation was analyzed by bisulfite treatment followed by PCR amplification using specific nested primers, cloning (Promega) and sequence analysis[42]. In some cases, we studied the kinetics of *de novo* methylation during differentiation of ES cells by carrying out bisulfite analysis on DNA from cells treated with retinoic acid for several days. This rate analysis is more indicative of what happens *in vivo* where *de novo* methylation may take place within a short window of time.

Total RNA from undifferentiated or retinoic acid–differentiated ES cells was isolated using a kit (EZRNA, Biological Industries) and treated with DNaseI (Roche). Randomly primed reverse transcription was performed at 37 °C using M-MLV reverse transcriptase (Promega). Control reactions lacking the reverse transcriptase were systematically verified for the absence of products. For each primer pair designed for quantitative real time PCR (qPCR), three different primer concentrations (300 nM, 500 nM and 700 nM) were tested against a dilution series of template cDNA from each cell type and a no-template control. After 50 cycles of PCR, dissociation analysis was performed to distinguish primer-dimer products from specific products. Reactions that contained nonspecific products of similar melting profile to the no-template controls were excluded from the subsequent analysis. qPCR was carried out in 20 μl reactions and measurements performed with SYBR Green (Finnzymes) in triplicate and UBC transcript levels were used to normalize between samples. For each cDNA sample cycle, thresholds for the target gene were calculated relative to that of UBC.

For reversal experiments, wild-type, *G9a*$^{-/-}$ (clones 10-20, 2-3), *G9a*$^{-/-}$/ΔANK and *G9a*$^{-/-}$/Tg* ES cells and Dnmt3a/3b$^{-/-}$ (refs. 23, 25) cells were induced to differentiate with 1 μM retinoic acid (Sigma) for 8 d. Differentiated cells were washed and resuspended in LIF-supplemented medium. Limiting numbers of cells were plated in 96-well plates under conditions where the growth of 1 colony per well is achieved. The number of reverted clones was scored after 2 weeks in culture.

Chromatin immunoprecipitation analysis. Cells were cross-linked and chromatin extracted and immunoprecipitated using the ChIP assay kit as recommended by the manufacturer (Upstate Biotechnology). Antibodies were directed against acetylated H3K9, H3K14 (5 μg per 30 μg DNA), H3K9me3 (15 μl per 10 μg DNA) (Abcam), rat anti-mouse HPIβ (50 μl per 10 μg DNA) (Serotec) and anti-Dnmt3a and anti-Dnmt3b (5 μg per 10 μg DNA) (Abcam). For each immunoprecipitation there was always a difference between known active and inactive genes. Incubations with the various antibodies were followed by incubation with salmon sperm DNA and protein A agarose (Upstate Biotechnology) in a 50% slurry (60 μl per 60 μg DNA) (Upstate Biotechnology). The bound and input fractions were quantified by SYBR Green qPCR (primer sequences available in **Supplementary Table 2** online). Ratios of bound/input were calculated and were normalized to those of *β-actin* and *β-globin*.

Immunoprecipitation and western blot analysis. We transiently transfected 293 cells using the calcium phosphate procedure. The following expression vectors were used: pCAGGS-Flag-mG9as[23], pcDNA3-Dnmt3a and pcDNA3-Dnmt3b1 (ref. 43), pcDNA-HAG9a[44] and pGal4-Dnmt3a[45]. cDNA of full-length G9a and the indicated deletion mutants were subcloned into the EcoRI site of the expression vector pEGFPc3 (Clontech). mG9a-S ΔANK lacks amino acids 646 to 867, mG9a ANK+SET corresponds to amino acids 646 to 1,172 of mG9a-S. The expression vectors hG9a ΔN and mG9a-S ΔSET were described previously[25,46]. Cells were harvested 48 h after transfection and extracts were analyzed by western blotting and processed for immunoprecipitation experiments. Agarose-conjugated anti-Flag (Sigma-Aldrich), anti-EGFP (Medical and Biological Laboratories) or anti-Gal4 (Santa Cruz Biotechnology) were used for immunoprecipitation. Anti-Dnmt3a (Santa Cruz Biotechnology), anti-Dnmt3b (Santa Cruz Biotechnology), anti-EGFP (Clontech), anti-Flag (Sigma-Aldrich), anti-hemagglutinin (Abcam) or anti-G9a[23] were used for western blotting. ES cells were harvested and processed for western blot analysis using antibodies specific for *β-actin* (Sigma-Aldrich) and G9a[23]. The following expression vectors for recombinant proteins were used: GST-Dnmt3a fragments, GST-Dnmt3b fragments expressed from pGEX4T1 (Pharmacia)[47], GST-Dnmt3L[48], His-Dnmt3a[45], His-Dnmt3b (Dnmt3b was cloned by PCR using appropriate sets of primers in the pET30a vector (Novagen) and verified by DNA sequencing) and GST-G9a[22]. Recombinant proteins were expressed in and purified from *Escherichia coli* B121. GST pull-down experiments and *in vitro* translation, using the TNT system (Pharmacia), were performed as described[47]. Direct interaction assays were performed[45] using anti-His antibodies (Abcam, ab 15149).

Generation of stable cell lines. *G9a*$^{-/-}$ ES cells (clone 22-10) were co-transfected with the pCAGGS expression vector containing an mG9a-S ΔANK insert and a plasmid conferring Hygromycin B resistance (pGK-hygroB), using Lipofectamine 2000 reagent (Invitrogen). Resistant cells were selected in ES cell medium containing 150 μg ml^{-1} Hygromycin B and designated *G9a*$^{-/-}$/ΔANK.

Methylated DNA immunoprecipitation microarray. Sonicated chromatin (average size 200–500 bp) was immunoprecipitated with anti-5-methylcytidine monoclonal antibody (EMD Biosciences, Serotec and Eurogentech) and the bound fraction isolated by protein A column chromatography. Bound or input DNA fractions were labeled with cy3 (green) or cy5 (red) nucleotides using the randomly primed Klenow Polymerase reaction in an overnight incubation at 37 °C[49]. This resulted in a 10–20-fold amplification. Labeled samples were then hybridized to a microarray (Mouse Promoter Chip BCBC-5A, Department of Genomics, University of Pennsylvania) containing a nominal library of about 13,000 approximately 1-kb, PCR-generated promoter sequences. DNA methylation was determined directly by mixing bound and input DNA labeled with different fluorescent probes in equal quantities before hybridization. The ratio of the fluorescent probes gives an indication of the degree of methylation when displayed as a scatter plot. The mDIP assay was prevalidated by examining 20 presumed methylated or unmethylated promoters by bisulfite analysis. This yielded a false positive and false negative rate of 10%. mDIP was carried out on uninduced ES and retinoic acid–induced ES cells three independent times,

including one in which the dyes were swapped. We also carried out this same analysis on uninduced as well as induced *G9a*$^{-/-}$ ES cells. Scanned images were analyzed using GenePix (v4.1), to obtain background subtracted intensity values, and the data were normalized by Lowess[50]. A whole-ChIP error model[49,51] was then used to calculate confidence values for all spots on each microarray and to combine data for the replicates of each experiment to obtain a final average ratio and confidence for each promoter region. Genes were included in the set of 'methylated genes' if the *P*-value in the error model was <0.001 for induced ES cells and >0.1 in both uninduced ES cells and induced *G9a*$^{-/-}$ ES cells. Expression data were extracted from http://symatlas.gnf.org/SymAtlas/, a gene atlas project initiated by the Genomic Institute of the Novartis Research Foundation. Active genes are those with above-minimal expression levels.

Nuclear transfer. Somatic cell nuclear transfer was performed as previously described[52] with some modifications. Oocytes were picked up from superovulated $B_6C_3F_1$ females 13 h after hCG injection. Enucleation was performed in HEPES-buffered CZB medium, 0.1% (w/v) PVP (40 kDa) (Calbiochem), supplemented with 2 μg ml^{-1} Cytochalasin B (Sigma). The injection of the nuclei was performed in hypertonic (110% w/v) HEPES-buffered CZB, 1% (w/v) PVP. Enucleation and injection were carried out with Piezodrill micropipettes (Humagen) with the assistance of a piezomicromanipulator (Primetech). After their activation, the reconstructed oocytes were cultured up to the 8-cell stage in Quinn's advantage cleavage medium (Sage), and to blastocysts in Quinn's advantage blastocyst medium (Sage). Using donor nuclei from cumulus cells, this procedure yielded blastocysts from 12% of transplanted oocytes.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS
S.E.-L., N.F., M.A.-R. and A.G. performed the ChIP, mDIP microarray, bisulfite sequencing, RNA analyses, some of the immunoprecipitation experiments and western blots, and reversal experiments; Y. Shufaro performed the nuclear transfer experiments; J.U. and Y. Shinkai generated the knockout and stable cell lines; R.D. and F.F. carried out some of the immunoprecipitation and western blot analyses. H.C. and Y.B. planned and supervised the research and wrote the manuscript. All authors contributed to the preparation of the manuscript.

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