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

Silvina Epsztejn-Litman[#1,2], **Nirit Feldman**[#1,2], **Monther Abu-Remaileh**[1], **Yoel Shufaro**[3], **Ariela Gerson**[1], **Jun Ueda**[4], **Rachel Deplus**[5], **François Fuks**[5], **Yoichi Shinkai**[4], **Howard Cedar**[1], and **Yehudit Bergman**[1]

[1]Departments of Experimental Medicine and Cellular Biochemistry Hebrew University Medical School Ein Kerem Jerusalem, 91120 ISRAEL

[3]IVF Unit, The Department of OB/GYN The Hadassah Human Embryonic Stem Cell Research Center Hadassah Hebrew University Medical Center Jerusalem, 91120 ISRAEL

[4]Experimental Research Center for Infectious Disease Institute for Virus Research Kyoto University Sakyo-Ku, Kyoto 606-8507 JAPAN

[5]Laboratory of Cancer Epigenetics Faculty of Medicine Free University of Brussels Brussels 1070 BELGIUM

[#] These authors contributed equally to this work.

Abstract

The pluripotency determining gene, *Oct-3/4* (also called *Pou5f1*) undergoes post implantation silencing in a process mediated by the histone methyltransferase (HMT) G9a. Microarray analysis now shows that this enzyme may operate as a master regulator that inactivates multiple early embryonic genes by bringing about methylated-histone H3K9 heterochromatinization and *de novo* DNA methylation. Genetic studies in differentiating ES cells demonstrate that a point mutation in the G9a SET domain prevents heterochromatinization, but still allows *de novo* methylation, while biochemical and functional studies indicate that G9a itself is capable of bringing about *de novo* methylation through its ankyrin (ANK) domain, by recruiting Dnmt3a/3b independently of its HMT activity. These modifications appear to be programmed for carrying out two separate biological functions, with histone methylation blocking target-gene reactivation in the absence of transcriptional repressors, while DNA methylation prevents reprogramming to the undifferentiated state.

Correspondence to: Howard Cedar.

Correspondence should be addressed to H.C. email: cedar@cc.huji.ac.il.

[2]These authors contributed equally to this work

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 IP experiments and Western blots and reversal experiments. Y.S. performed the nuclear transfer experiments. Y.U. and Y.S. generated the knockout and stable cell lines, R.D. and F.F. carried out some of the IP 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.

INTRODUCTION

Normal development appears to take place through a unidirectional process characterized by a step-wise 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. While 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 irreversibility2.

Oct-3/4 and *Nanog* are two genes that encode pluripotency-sustaining transcription factors that are expressed during early embryogenesis and in ES cells3. Oct-3/4 is regarded as pivotal because either its loss of function or its over expression completely abolishes self-renewal and induces differentiation4. Nanog is also regarded as a component of the core transcription factor network (along with Oct-3/4 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 LIF5,6. Expression of these genes is silenced both by histone modification and DNA methylation upon ES cell differentiation and subsequently in somatic cells7-10.

Recent studies on the murine *Oct-3/4* gene promoter have demonstrated that this post-implantation inactivation process is carried out in a multilayer manner that involves direct inhibition of transcription, heterochromatinization through the trimethylation of H3K9 and subsequent DNA methylation7. Interestingly, both of these latter epigenetic structures are actually put in place by the SET domain-protein, G9a, and it appears to be this event that causes its irreversible silencing in subsequent somatic cell lineages7.

This linkage between histone and DNA methylation appears to be a general phenomenon. Suv39h, for example, is singularly required for the inactivation of pericentric satellite DNA, while Ezh2 can bring about both H3K27 methylation as well as DNA methylation at selected genes in cancer cells11,12. In addition, heterochromatinization and DNA methylation have been shown to be genetically linked in plants13. In all of the cases, the mechanism for this linkage has not yet been fully elucidated.

Although *Oct-3/4* appears to be hermetically closed in somatic cells, this endogenous locus, as well as 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 iPS, and this is accompanied by chromatin opening and demethylation of promoter DNA14-17. These experiments suggest that embryonic cells may carry the molecular machinery needed to reverse the repressed state initially laid down when the embryo first undergoes differentiation, but very little is known about the components that take part in this process.

Since Oct-3/4 expression is known to be necessary but not sufficient for pluripotency5, there must be additional genes that undergo inactivation during embryonic cell differentiation, and it is likely that G9a plays 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

In order 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 *de novo* methylated during ES cell differentiation. To this end, we performed methylated DNA immunoprecipitation (mDIP) array on DNA from various ES cell samples and, in this way, identified genes that are unmethylated in undifferentiated cells, but appear methylated in cells exposed to 8 days of RA induction. As a further control, we selected only those genes that were also found to remain unmodified in differentiated mouse G9a$^{-/-}$ (also called *Ehmt2*) ES cells. By these criteria, we detected a set of 126 individual genes (Supplementary Table 1), and bisulfite analysis of a representative sample indicated that 75% (6/8) do in fact undergo G9a-dependent *de novo* methylation during ES cell differentiation (Fig. 1a and Supplementary Fig. 1). Much like *Oct-3/4*, all 6 of these new gene promoters were found to become heterochromatinized in differentiated ES cells as determined by ChIP analysis of H3K9me3 and HP1 (Fig. 1b). It is very likely that these genes also undergo structural inactivation *in vivo* as well, since 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 pre-implantation embryos or in germ line cells (Supplementary Table 1). It thus appears that G9a may actually direct the epigenetic silencing of a relatively large embryonic gene network.

G9a-dependent *de novo* 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 histone H3 lysine 9 may play a role in directing targeted DNA methylation, but the mechanism for this process is not known18. In order 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 tri-methylation of histone H3K9 (Ref. 19). Indeed, ChIP analysis clearly shows that, unlike the wild type gene, this G9a mutant does not bring about tri-methylation of lysine 9 nor the subsequent binding of HP1β at the *Oct-3/4* promoter, even in ES cells that have undergone RA-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 tri-methylation cannot be

due to blockage of H3K9 residues by acetyl groups (Fig. 2a). This experiment thus proves that H3K9 tri-methylation of *Oct-3/4* is indeed directly catalyzed by G9a.

Using bisulfite analysis, we then examined the methylation state of the *Oct-3/4* 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. Strikingly, however, we observed normal *de novo* methylation in the mutant (G9a$^{-/-}$/Tg*) (Fig. 2b), and similar results were obtained for additional G9a target genes. These findings clearly show that neither H3K9me3 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 *Oct-3/4* promoter in differentiated ES cells (Fig. 2c). Taken together, these results suggest that G9a may mediate *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 the pull-down material subjected to Western analysis using anti-Dnmt antibodies. These experiments clearly demonstrate that G9a is capable of specifically recruiting Dnmt3a and 3b, and this was confirmed by carrying out the reciprocal experiments, as well (Fig. 3a). Moreover, *in vitro* synthesized proteins were used 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 different gene sequences has been shown to be dependent on G9a20,21. It has also been demonstrated that G9a may interact with Dnmt1 in the replication complex22.

The G9a protein is made up of several different functional regions, including the enzymatic SET domain, several cysteine-rich regions and ANK repeats23 that were recently shown to interact with H3 mono- or di-methyl K9 (Ref. 24). In order 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 EGFP (Fig. 4a). Western analysis clearly revealed that the recruitment of Dnmt3a or 3b specifically requires the presence of an ANK repeat region, but is completely unaffected by removal even of the entire SET domain (Fig. 4a). It should be noted that since this SET domain also plays a role in generating heterodimers with the partner protein, GLP25, this later result suggests that, in this case, G9a itself is actually sufficient for recruiting *de novo* methylases. Using a reciprocal approach, we have also mapped the G9a-interacting domain to the catalytic regions of Dnmt3a and 3b. Interestingly, the non-catalytic Dnmt3L is unable to interact with G9a (Fig. 4b).

In light of these findings, we next employed a genetic approach to test whether the ANK domain is indeed responsible for *de novo* methylation. To this end, we transfected the

ΔANK construct into G9a$^{-/-}$ cells and then measured the initial rate of *Oct-3/4 de novo* methylation by bisulfite analysis on DNA from RA-induced ES cells, including time points at which methylation is still increasing monotonically. As can be seen in Fig. 5a and 5b, this construct carries out *de novo* methylation very slowly as compared to both wt and F1205Y G9a, even though there appears to be a relatively large excess of the ΔANK protein in the transfected cells (Fig. 5c). Despite this low level of DNA methylation, the *Oct-3/4* promoter was still found to be marked by H3K9me3 and HP1 (Fig. 5d). Since HP1 itself is able to recruit Dnmts26-28, this heterochromatinization may help explain the residual *de novo* methylation activity observed in this experiment. Similar results for methylation and heterochromatinization were obtained for the *Stk10* gene promoter, as well (Supplementary Fig. 2). From these biochemical and genetic studies, it thus appears that G9a can mediate the methylation of DNA by a mechanism that is independent of H3K9me/HP1β.

Separate functions of histone methylation and DNA methylation

Kinetic studies on Oct-3/4 repression and heterochromatinization as a function of differentiation in ES cells indicated that this process occurs in three distinct steps7. In the first stage Oct-3/4 expression is dramatically down-regulated by RA-induced transcriptional repressors29-31, and this is then followed, in a stepwise manner, by G9a-mediated heterochromatinization and *de novo* methylation at the promoter. These latter epigenetic changes appear to play a role in locking *Oct-3/4* into its repressed state, and once these structures are formed, transcription cannot be restored either by removing the original inducer (RA), or by recloning differentiated cells in LIF medium. In contrast, reprogramming to pluripotency occurs with high frequency in G9a$^{-/-}$ cells, and Oct-3/4, as well as other G9a target genes can be readily re-expressed upon removal of RA once the differentiation process is completed (Fig. 6a).

In order 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 Oct-3/4 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 days and RA was then removed. In contrast to the G9a$^{-/-}$ parent cells, Oct-3/4 remained completely repressed even after several days in culture. Since 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 Oct-3/4 under these conditions. Similar results were obtained for differentiated Dnmt3a/3b$^{-/-}$ or G9a$^{-/-}$/ΔANK ES cells in which the Oct-3/4 promoter undergoes normal heterochromatinization, but does not become *de novo* methylated. It thus appears that while the removal of RA allows the re-establishment of protein factors that work in *trans* to activate silenced genes in this non-dividing cell population, individual epigenetic marks such as heterochromatinization or DNA methylation that are 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 *Oct-3/4* to undo

their repressive epigenetic structure32,33. Evidence that this is indeed the case has come from experiments showing that, unlike the wild type, differentiated G9a$^{-/-}$ ES cells are able to revert to a proliferating undifferentiated phenotype when recloned in LIF medium7, and these cells appear 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). This reprogramming is undoubtedly made possible because these Oct-3/4 like genes lack both heterochromatin structure as well as DNA methylation in these cells. Since Dnmt3a/3b$^{-/-}$ (Ref. 7) and G9a$^{-/-}$/ΔANK (Fig. 6c) ES cells are also capable of generating undifferentiated clones in this system, it appears that heterochromatinization alone is not 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 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.

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, wt ES cell nuclei were unable to direct early development (0 blastocysts/28 cleavage stage embryos/48 reconstituted oocytes). In contrast, G9a$^{-/-}$ nuclei yielded 6 normal blastocysts from 32 cleavage stage embryos (48 reconstituted oocytes), and this is highly significant ($P<0.05$). These studies clearly indicate that G9a plays 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 reprogramming34,35.

DISCUSSION

G9a appears to carry out two distinct and different epigenetic activities involved in gene repression. Through the SET domain, it 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 that 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 HMT, Suv39h, is specifically required for the targeted *de novo* methylation of repeat DNAs associated with centromeres or telomeres36, and it was tacitly assumed that this reaction is mediated exclusively through histone H3K9 methylation in combination with HP1 heterochromatinization12. Since our findings on G9a suggest that H3K9me3 itself is not sufficient for bringing about full local *de novo* methylation, it is quite possible that in this case as well, *de novo* methylation is mediated by the HMT protein itself, and this is indeed supported by biochemical studies12. The same may be true for the enzyme KRYPTONITE which causes both histone and DNA methylation in Arabidopsis13. It was recently shown that Ezh2, a histone H3 lysine 27 specific methylase that brings about heterochromatinization through the binding of a chromodomain protein, is also capable of causing local *de novo* DNA methylation by directly recruiting Dnmts11, suggesting that this may represent a general mechanism. It should be noted that several studies have

demonstrated that HP1 itself can recruit Dnmts26-28, 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 has it been shown that an HP1-like molecule is actually required for local *de novo* methylation27.

Our studies show that G9a is a master structural regulator that plays an important role in early development by targeting a wide network of embryonic genes for post-implantation repression. This silencing process includes key genes, such as Oct-3/4, Nanog and Dnmt3L that are intimately involved in maintaining the embryonic stem cell phenotype3 and for establishing maternal imprints in mammalian germ cells37. G9a operates in a double manner, by employing its SET domain to generate heterochromatin, and utilizing its ANK domain to carry out *de novo* methylation. Each of these epigenetic marks appears 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 only be attained 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 appears to be faithfully maintained, thereby preventing reactivation. It is very likely that this silencing program actually represents the major barrier to embryonic reprogramming38,39.

METHODS

Cells, DNA and RNA analyses

Murine wild-type (wt), Dnmt3a/3b$^{-/-}$ (Ref. 40), G9a$^{-/-}$, G9a$^{-/-}$/Tg and G9a$^{-/-}$/Tg* (Ref. 23) ES cells were maintained as described previously40,41. The Tg* transgene (F1205Y) has a single nucleotide mutation in the SET domain that makes it inherently defective in di- and tri-methylation of H3K9, but may still be capable of mono-methylation at this site23. Cells were treated with 1 μM RA for the indicated times as previously described30,31. DNA methylation was analyzed by bisulfite treatment followed by PCR amplification using specific nested primers, cloning (Promega) and sequence analysis42. 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 RA 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 RA-differentiated ES cells was isolated using a kit (EZRNA, Biological Industries, Beit Haemek, Israel) and treated with DNaseI (Roche). Random-primed reverse transcription was performed at 37^{0}C using M-MLV reverse transcriptase (RT) (Promega). Control reactions lacking the RT enzyme were systematically verified for the absence of products. For each primer pair designed for quantitative real time PCR (Q-PCR), three different primer concentrations (300, 500 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 dimers products from specific products. Reactions that contained non-specific products of similar melting profile to the no-template controls were excluded from the subsequent analysis. Q-

PCR was carried out in 20 μl reaction using a kit (Finnzymes). Measurements were performed with SYBR Green Universal Mix 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 as well as Dnmt3a/3b$^{-/-}$ (Refs. 23, 25) were induced to differentiate with 1 μM RA (Sigma, Rehovot, Israel) for 8 days. Differentiated cells were washed and re-suspended 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.

ChIP analysis

Cells were cross-linked and chromatin extracted and immunoprecipitated using the ChIP assay kit as recommended by the manufacturer (Upstate Biotechnology, Lake Placid, NY). Antibodies were directed against acetylated histone H3K9,K14 (5 μg per 30 μg DNA), H3K9me3 (15 μl per 10 μg DNA) (Abcam, Cambridge, U.K.), rat anti-mouse HPIβ (50 μl per 10 μg DNA) (Serotec, Cambridge, U.K.) and anti-Dnmt3a and 3b (5 μg per 10 μg DNA) (Abcam, Cambridge, U.K.). For each immunoprecipitation there was always a difference between known active and inactive genes. Incubations with the various antibodies were followed by Salmon sperm DNA/protein A agarose −50% slurry (60 μl per 60 μg DNA) (Upstate Biotechnology, Lake Placid, NY). The bound and input fractions were quantified with SYBR Green Q-PCR (primer sequence available in Supplementary Table 2). Ratios of bound/input were calculated and were normalized to those of β-*actin* and β-*globin*.

Immuno-precipitation (IP) and Western blot analysis

293 cells were transiently transfected using the calcium phosphate procedure. The following expression vectors were used: pCAGGS-Flag-mG9as23, pcDNA3-Dnmt3a and pcDNA3-Dnmt3b1 (Ref. 43), pcDNA-HAG9a44 and pGal4-Dnmt3a45. cDNA of G9a full-length 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#1 and mG9a-S ΔSET were described previously25,46. Cells were harvested 48 hours after transfection and extracts were analyzed by Western blot analysis and processed for IP experiments. Agarose-conjugated anti-Flag (Sigma-Aldrich), anti-EGFP (Medical and Biological Laboratories, Japan) or anti-Gal4 (Santa Cruz Biotechnology) were used for the IP experiments. Anti-Dnmt3a (Santa Cruz Biotechnology), anti-Dnmt3b (Santa Cruz Biotechnology), anti-EGFP (Clontech), anti-Flag (Sigma-Aldrich), anti-HA (Abcam) or anti-G9a23 were used for Western blot analysis. ES cells were harvested and processed for Western blot analysis using antibodies specific for β-*actin* (Sigma-Aldrich) and G9a23. The following expression vectors for recombinant proteins were used; GST-Dnmt3a fragments, GST-Dnmt3b fragments expressed from pGEX4T1 (Pharmacia)47, GST-Dnmt3L48, His-Dnmt3a45, His-Dnmt3b (Dnmt3b was cloned by PCR using appropriate sets of primers in the pET30a vector (Novagen) and verified by DNA sequencing), and GST-G9a22. Recombinant proteins were expressed in and purified from E. coli B121. GST pull-down

experiments and *in vitro* translation, using the TNT system (Pharmacia), were performed as described47. Direct interaction assays were performed45, 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 the mG9a-S ΔANK 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 (mDIP) microarray

Sonicated chromatin (200-500 bp average size) was immunoprecipitated with anti-5-methylcytidine monoclonal antibody (EMD Biosciences (U.S.A), Serotec (U.K) and Eurogentech (Belgium)) and the bound fraction isolated by protein A column chromatography. Bound or input DNA fractions were labeled with cy-3(green) or cy-5(red) nucleotides using the random-primed Klenow Polymerase reaction in an overnight incubation at 37°C49. 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, Philadelphia) 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 prior to 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 pre-validated 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 RA-induced ES cells 3 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 normalized by Lowess50. A whole-ChIP error model49,51 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 was 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 described52 with some modifications. Oocytes are 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 kD) (Calbiochem, USA), 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, USA) with the assistance of a piezomicromanipulator (Primetech, Japan). After their activation, the reconstructed oocytes were cultured up to the 8 cell stage in Quinn's advantage cleavage medium (Sage, USA), and to blastocysts in Quinn's advantage blastocyst medium (Sage, USA). Using donor nuclei from cumulus cells, this procedure yielded blastocysts from 12% of transplanted oocytes.

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Supplementary Material

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a.

	ES(wt) 0 d RA	ES(wt) 8 d RA	G9a$^{-/-}$ 8 d RA	Somatic tissues
	Methylation (%)			
Oct-3/4	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

b.

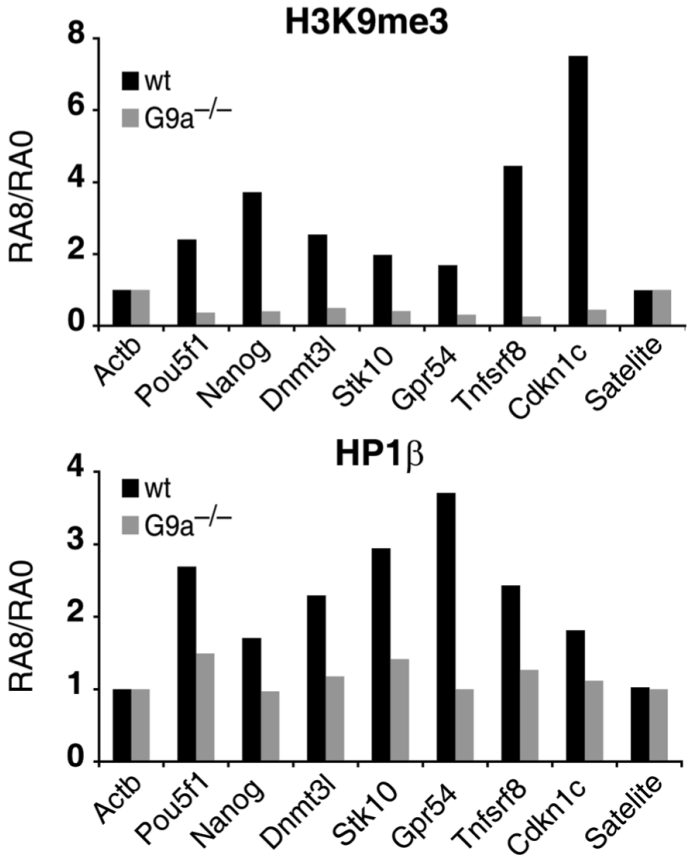


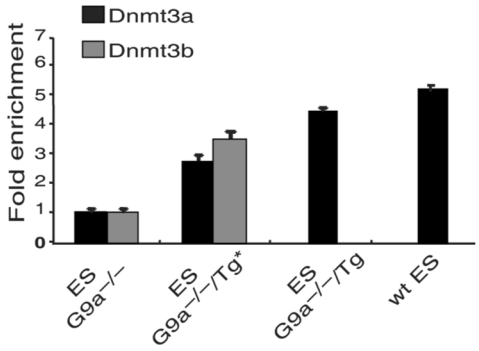
Figure 1. Genome-wide G9a-dependent *de novo* methylation

(a) DNA from 8 days RA-treated wt and G9a$^{-/-}$ ES cells and somatic tissues (spleen and kidney) was treated with Na bisulfite (Qiagen bisulfite kit, Cat. No. 59104), 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 different9 from that measured in previous studies7. **(b)** ChIP analysis of the genes promoters using antibodies specific for H3K9me3 and HP1β on 0 and 8 day RA-treated wt and G9a$^{-/-}$ ES cells.

a.

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

c.



b.

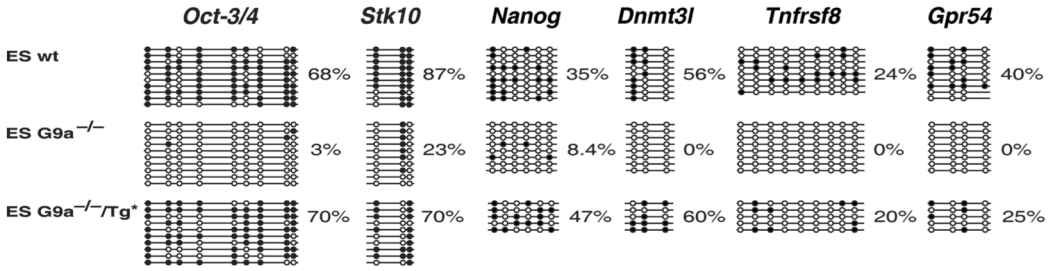


Figure 2. Heterochromatinization is prevented by point mutation in the G9a SET domain

(a) Chromatin immunoprecipitation (ChIP) analysis of the *Oct-3/4* promoter in wild type (wt), G9a$^{-/-}$ and G9a$^{-/-}$/Tg* (mutated in the SET domain, F1205Y) embryonic stem (ES) cells (RA-differentiated, 8 day) using antibodies specific for H3K9me3, HP1γ and H3K9,14ac. Quantitative real time PCR (Q-PCR) was used. The degree of enrichment was calculated as Bound (B)/Input (I) and normalized to β-actin or β-globin. **(b)** DNA from 8 day RA-treated ES cells was treated with Na bisulfite, amplified using specific promoter primers (Supplementary Table 2), cloned and subjected to sequence analysis. Nine and three CpG sites, respectively, were examined. **(c)** ChIP analysis of the *Oct-3/4* promoter using antibodies specific for Dnmt3a and Dnmt3b on 6 day differentiated ES cells. Enrichment values were normalized to pericentric satellite DNA in each sample (±SD).

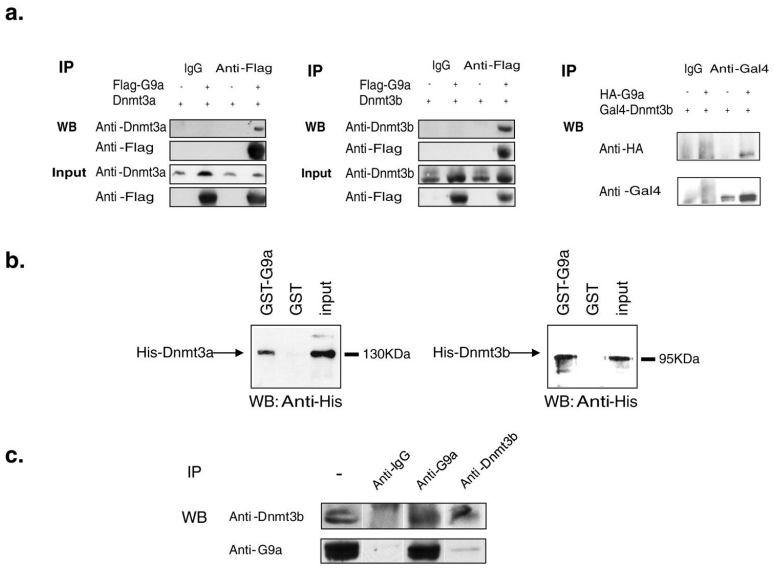


Figure 3. G9a recruits Dnmt3a and Dnmt3b

(a) 293 cells transiently transfected with Dnmt3a or Dnmt3b and G9a-Flag tagged, or with HA-G9a and Gal4-Dnmt3a (as indicated). Cell extracts were immuno-precipitated (IP) with IgG, specific antibodies against Flag or Gal4, and Western blot (WB) analysis performed with anti-Flag, anti-Dnmt3a, anti-Dnmt3b, anti-HA or anti-G9a, as indicated. **(b)** Direct interactions between G9a and Dnmts. A GST-fused G9a protein was incubated either with His-Dnmt3a or with His-Dnmt3b proteins. WB analysis 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. WB analysis was performed with anti-Dnmt3b or anti-G9a.

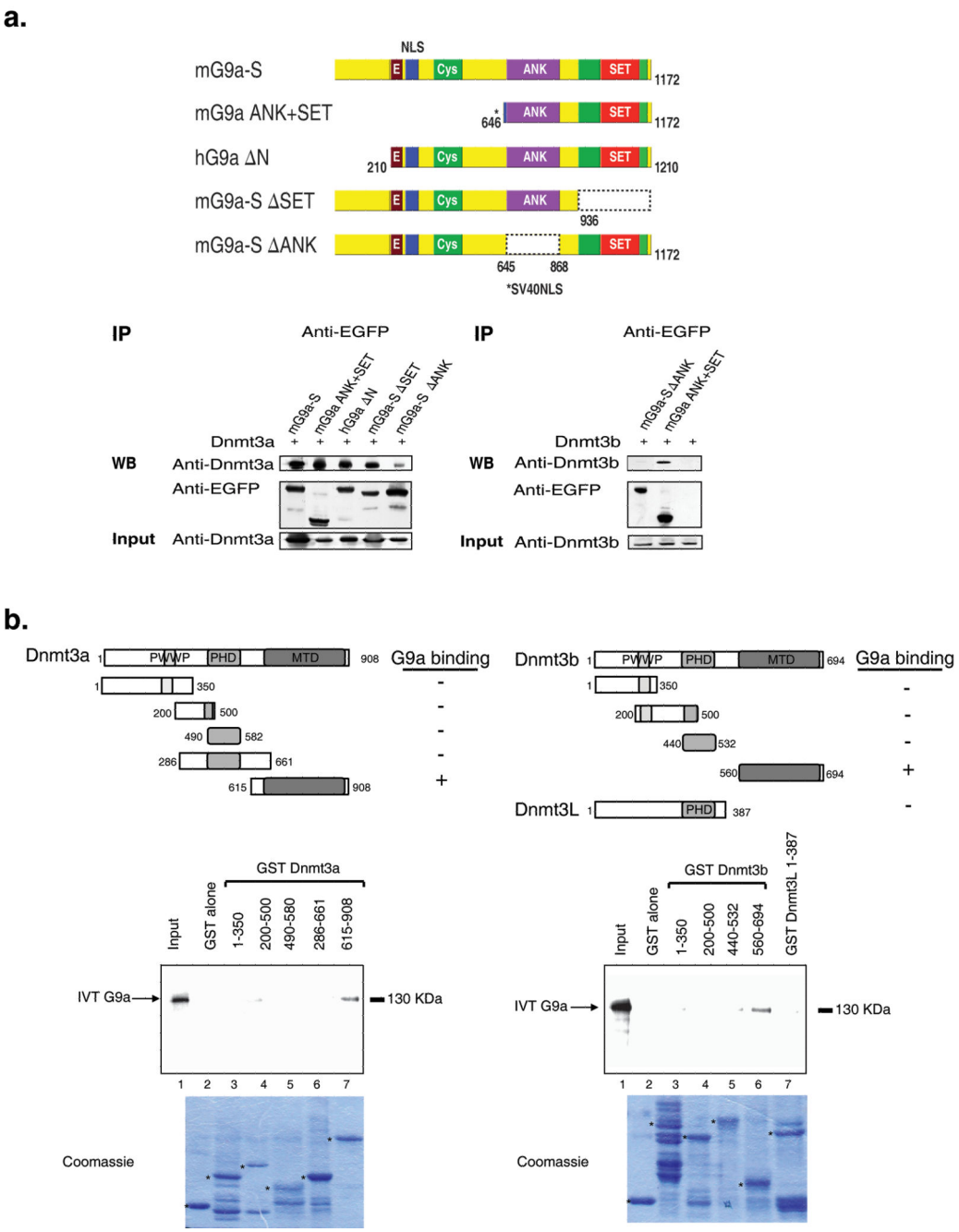


Figure 4. Mapping the G9a- and Dnmts-interacting domains

(a) Schematic representation of the plasmids used for the IP assay. 293 cells transiently transfected with various mutants of G9a EGFP-tagged plasmids. Extracts were immuno-precipitated with anti-EGFP and WB with antibodies specific for EGFP, Dnmt3a and Dnmt3b. **(b)** Schematic representation of GST-fusion constructs harboring full length Dnmt3L, different regions of Dnmt3a and 3b. The methyltransferase domain is labeled as MTD. *In vitro* translated (IVT) G9a was incubated with the indicated GST fusions of

Dnmt3a, 3b and 3L. The presence of G9a was visualized by WB analysis using anti-G9a. Expression of GST-Dnmts was assayed by SDS-PAGE gels stained with Coomasie-blue.

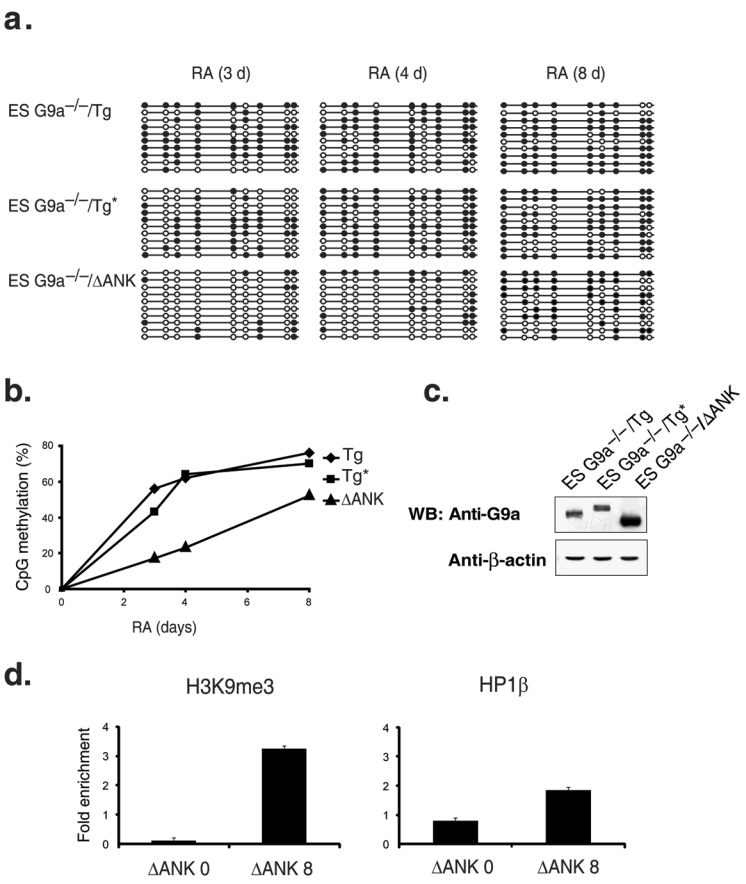
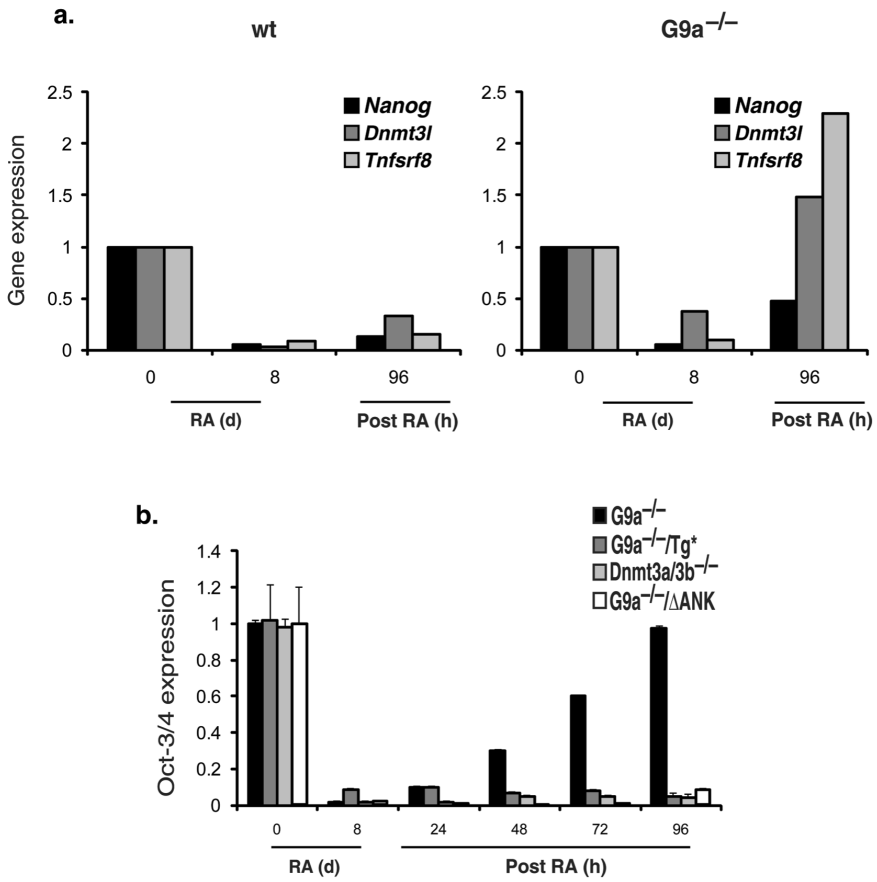


Figure 5. ANK-dependent *Oct-3/4* methylation

(a) Na bisulfite analysis of DNA from G9a$^{-/-}$ ES cells harboring the indicated G9a transgene constructs. **(b)** The data in (a) is redrawn 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 *Oct-3/4* promoter using anti-H3K9me3 or anti-HP1β on chromatin from G9a$^{-/-}$/ΔANK ES cells at 0 time and 8 days of RA-induced differentiation.



c.

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 *Oct-3/4, Nanog, Dnmt3L* and *Tnfsrf8* genes are affected by their epigenetic state

(a) wt and G9a$^{-/-}$ ES cells were treated with RA during 8 days, after which RA was removed and cells were isolated after 96 hours. Graph shows the level of expression for the indicated genes using Q-PCR analysis with *Ubiquitin C (UBC)* as the normalization control. **(b)** ES cells were treated with RA for up to 8 days, after which RA was removed and cells were isolated at various time points. Graph shows the level of Oct-3/4 expression (± SD) using Q-PCR analysis with *UBC* as the normalization control. **(c)** Number and percentage of reverted

single-cell clones established from wild type (wt) and mutant ES cells that were initially differentiated with RA for 8 days and re-cultured in 96-well plates for 2 weeks in the presence of LIF under conditions that were calibrated for each line to yield approximately one colony per well of 1 day RA-treated cells (10-20 cells per well). Each result represents the sum of 2-3 independent experiments. Normalization takes into consideration the clonability of each cell line, as determined by the number of colonies found after plating 1 day RA-treated cells.