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G9a-mediated irreversible epigenetic inactivation of *Oct-3/4* during early embryogenesis

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Oct-3/4 **is a POU domain homeobox gene that is expressed during gametogenesis and in early embryonic cells[1,2], where it has been shown to be important for maintaining pluripotency[3]. Following implantation, this gene undergoes a novel multi-step programme of inactivation. Transcriptional repression is followed by a pronounced increase in histone H3 methylation on Lys 9 that is mediated by the SET-containing protein, G9a. This step sets the stage for local heterochromatinization via the binding of HP1 and is required for subsequent *de novo* methylation at the promoter by the enzymes Dnmt3a/3b. Genetic studies show that these epigenetic changes actually have an important role in the inhibition of *Oct-3/4* re-expression, thereby preventing reprogramming.**

The *Oct-3/4* gene has already been shown to be expressed at high levels in embryonic germ (EG), embryonic stem (ES) and embryonic carcinoma cells such as P19 (refs 2, 4), but undergoes rapid repression both at the level of mRNA and protein following the addition of retinoic acid (RA) to the growth medium (Fig. 1a)[4–6]. This inactivation is probably mediated directly by *trans*-acting repressors, such as ARP-1, COUP-TF1 and GCNF, that are induced transiently at the onset of differentiation[6,7]. We have used chromatin immunoprecipitation (ChIP) analysis to follow changes in the histone-modification pattern that accompany this repression (Fig. 1b). In undifferentiated cells, the *Oct-3/4* promoter is packaged with nucleosomes that contain the highly acetylated histone H3(K9, K14), but the gene promoter slowly becomes deacetylated as differentiation proceeds (Fig. 1a), which is similar to the pattern that is seen in somatic cells (data not shown). Similar results were obtained for me-H3(K4), another marker of active chromatin[8] (Fig. 1a, b). These marked changes occur after the initial repression has taken place and parallel a general reduction in chromatin accessibility, as detected by DNaseI sensitivity (see Supplementary Information, Fig. S1).

The process of targeted gene repression during development often involves changes in chromatin structure and histone modification that mimic heterochromatin. To examine whether *Oct-3/4* is subject to this type of regulation, we carried out ChIP analysis using an antibody that is specific for 2me- or 3me-H3(K9), the latter being a definitive marker of pericentric heterochromatin both in *Drosophila* and mammals (reviewed in ref. 9). To be able to measure the level of these modifications, we compared the degree of *Oct-3/4* precipitation to that of a constitutively active gene, *β-actin*. *Oct-3/4* was found to be relatively unenriched (30-fold) in undifferentiated cells (see also ref. 10), but following induction, *Oct-3/4* underwent a pronounced increase in both 2me- and 3me-H3(K9) (to 300-fold). This was accompanied by a similar increase in the binding of heterochromatin protein 1 (HP1) (Fig. 1c), which is a chromodomain protein that is thought to be involved in organizing higher-order chromatin structure (reviewed in ref. 9).

We next used Southern blotting to assay DNA methylation. Although initially unmethylated, all of the *Hpa*II and *Hha*I sites in the *Oct-3/4* promoter underwent *de novo* methylation during differentiation[6,11], ultimately reaching a level similar to that seen in somatic cells (Fig. 1d). This process took place with delayed kinetics and, therefore, lagged behind the changes in histone modification and chromatin structure (Fig. 1a). Unlike blastocysts, cultured embryonic cells (P19, F9 and ES) mimic the DNA-methylation potential of embryos at the implantation stage in that they have already acquired the ability to carry out genome-wide *de novo* methylation[12]. It has been previously demonstrated that the *Oct-3/4* promoter remains unmodified in this environment, probably by virtue of specific *cis*-acting sequences that protect it from global methylation in a manner that is similar to that seen in CpG islands[11]. Therefore, the *de novo* methylation of *Oct-3/4* that was observed following differentiation of P19 cells reflects a separate developmental event that specifically targets selected embryonically expressed genes.

To analyse the developmental dynamics of *Oct-3/4*, we carried out ChIP analysis on post-implantation embryos that were 8.5 days post-conception, as well as *ex vivo* tissue specimens (Fig. 2a). These results show that histone H3 Lys 9 is highly methylated and binds HP1 in nucleosomes that cover the *Oct-3/4* promoter in all of these cell types. These experiments also demonstrate conclusively that *Oct-3/4* heterochromatinization by means of histone H3 methylation does not only

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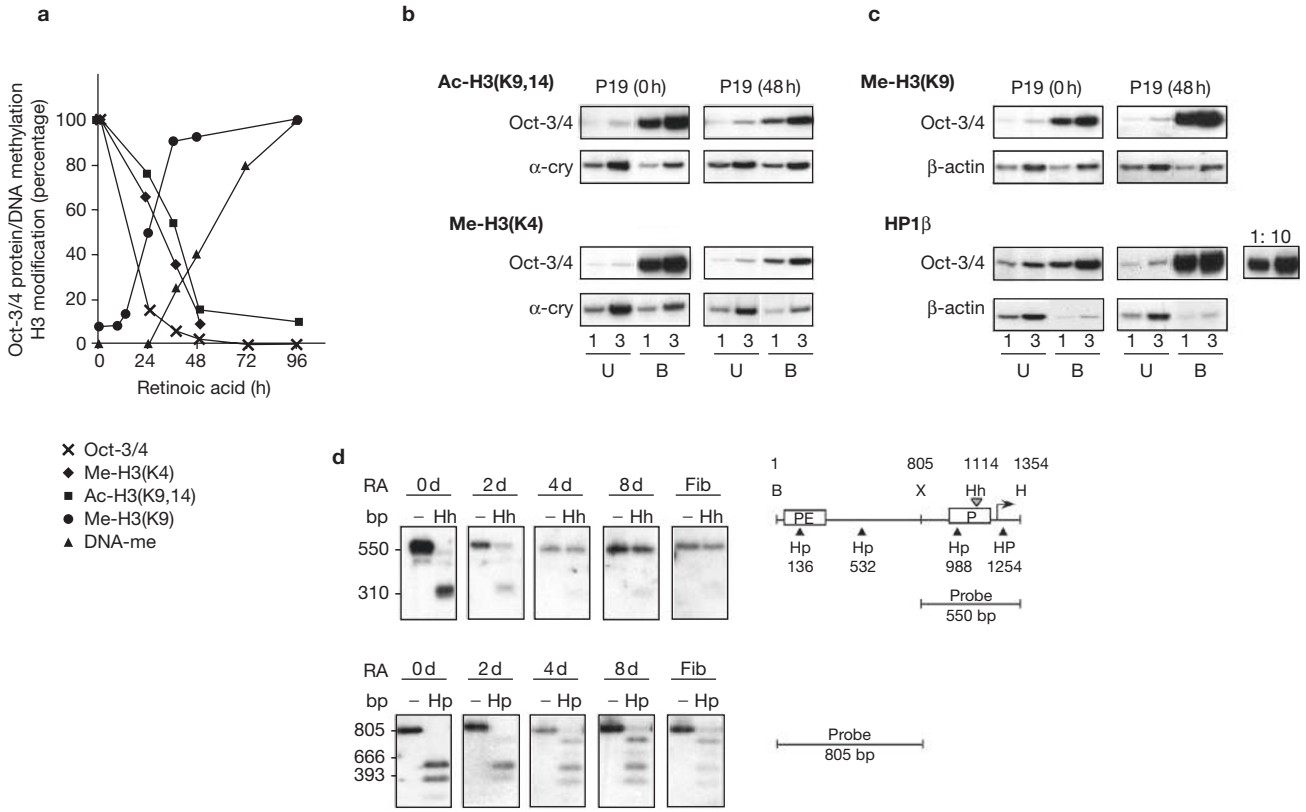


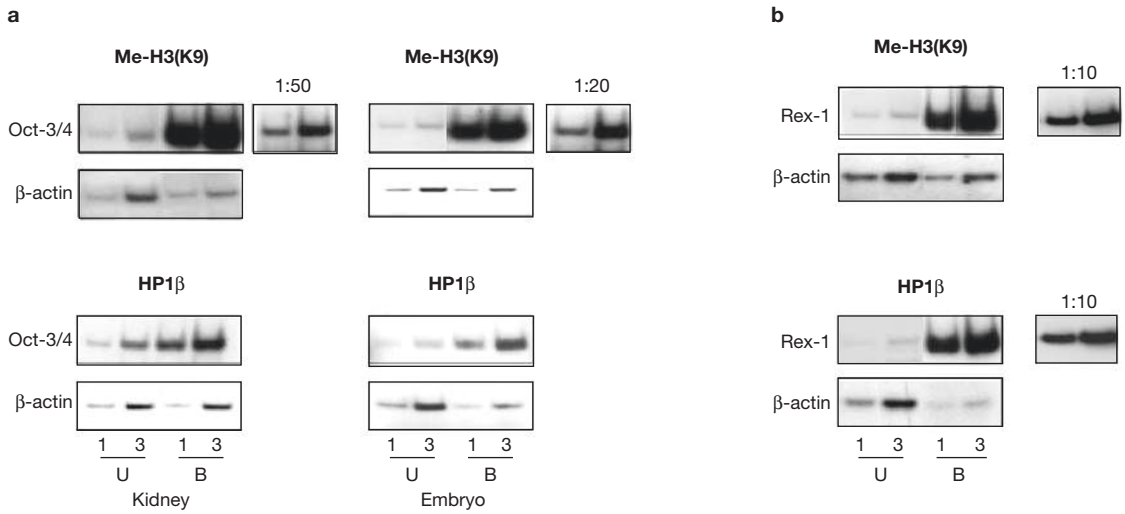
Figure 1 Programmed inactivation of *Oct-3/4* in P19 cells. P19 cells were treated with retinoic acid (RA) and isolated at various times. (**a**) Graph shows the level of Oct-3/4 protein as measured by electrophoretic mobility shift assays (percentage of initial level in non-induced cells normalized to Oct-1 levels; crosses), changes in the histone modification patterns by chromatin immunoprecipitation (ChIP) analysis (diamonds, squares and circles) and methylation at the gene promoter (percentage of level in somatic cells; triangles). ChIP data is presented as the percentage of the level in undifferentiated P19 cells. (**b**) ChIP analysis on mononucleosomes isolated from P19 cells using antibodies to Ac-H3(K9, K14) or Me-H3(K4) (shown in **a**). Semi-quantitative PCR using specific primers from the *Oct-3/4* promoter was carried out at three different concentrations (two are shown) of bound (B) or unbound (U) fractions. The degree of enrichment was calculated as B/U and normalized to α-crystallin (set at 1), which serves as a negative control and is presented (in **a**) as the percentage of the level at 0 h (Ac-H3(K9,K14) (40-fold) and Me-H3(K4) (50-fold). (**c**) ChIP analysis of the *Oct-3/4* gene using antibodies specific for 3me-H3(K4) and HP1β in P19 (wild-type undifferentiated and RA-differentiated). The *β-actin* gene is shown for comparison. Semi-quantitative PCR reactions were performed as described in **b**. For the HP1β sample, the bound fraction was also assayed after a 10-fold dilution to measure enrichment in the linear range. Fold enrichment: 3me-H3(K9) (wild type, 30; RA, 320; real time PCR yielded the same values), HP1β (wild type, 100; D, 300). Similar results were obtained using antibodies against HP1α or HP1γ (data not shown), indicating that all of these may work together as markers for gene silencing. (**d**) DNA from untreated, RA-treated P19 and fibroblast (Fib) cells was digested with *Xba*I(X)/*Hind*III(H) or *BamH*I(B)/*Xba*I with or without *Hha*I (Hh) or *Hpa*II (Hp), respectively, and subjected to Southern blotting using probes spanning the promoter or the proximal enhancer (PE) region (see map). Although unmethylated in undifferentiated cells, the promoter and the PE reach a level of methylation similar to that of somatic cells after 4–8 d of RA treatment. bp, base pairs.

take place in differentiating embryonic cells in culture, but also occurs *in vivo* during normal development, both at the promoter, as well as at the coding regions (data not shown).

We next asked whether other gene sequences are also marked for silencing through heterochromatinization that involved histone H3 methylation. *Rex-1*, for example, is a zinc-finger-containing transcription-factor gene that is a direct target of *Oct-3/4* and that also becomes downregulated following implantation[13]. Strikingly, *Rex-1* was also found to be packaged into nucleosomes containing me-H3(K9), as well as its chromodomain partner HP1, in differentiated P19 (Fig. 2b) and somatic cells (data not shown). Other embryonic or homeotic genes, such as *Dppa3/Stella/PGC7*, *Nanog*, *Sox-2*, *Hox-B5* and *Hox-D11* (which were also analysed in ES cells) were not found to undergo promoter inactivation by this mechanism (data not shown). These data therefore indicate that histone H3(K9) methylation and heterochromatinization represent a highly specific mechanism for epigenetic silencing of select embryonic genes.

To confirm that histone H3 methylation represents a specific marker for genes that undergo targeted inactivation during embryonic development, we compared the histone-modification pattern of these genes to that of several tissue-specific genes that are constitutively repressed in the same cells. Like *Oct-3/4*, these genes are packaged in a closed chromatin conformation that is characterized by deacetylated histones H3 and H4, as well as depletion of me-H3(K4) (data not shown). Strikingly, however, these repressed gene sequences are not marked by the tri-methylation of histone H3 Lys 9 to any appreciable extent and are not associated with the corresponding chromodomain-containing partner, HP1 (Fig. 2c). It therefore seems that histone H3 methylation is, indeed, a specific tag for the process of targeted inactivation in embryonic cells.

Although DNA methylation of the *Oct-3/4* promoter contributes to the maintenance of gene silencing in somatic cells, the kinetics of inactivation in EC cells indicate that the *de novo* methylation event is actually secondary to transcriptional and chromatin changes that take place early in this process (Fig. 1). To confirm this hypothesis, we examined the characteristics of



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Gene	Fold enrichment	
	Me-H3(K9)	HP1β
Oct-3/4	320	300
Rex-1	250	250
CD4	5	1
α-cry	10	2
Pepck	5	2
β-globin	12	2

Figure 2 Heterochromatinization of *Oct-3/4* and *Rex-1*. (**a**) Chromatin immunoprecipitation (ChIP) analysis of the *Oct-3/4* gene using antibodies specific for 3me-H3(K9) and HP1β in kidney or whole 8.5 days post-conception embryos. For the 3me-H3(K9) samples, the bound (B) fractions were also analysed after a 50-fold (kidney) and 20-fold (embryo) dilution to measure enrichment in the linear range. Fold enrichment: embryo (3me-H3(K9), 100; HP1β, 10); kidney (3me-H3(K9), 100; HP1β, 10). Similar results were obtained using chromatin from liver and spleen. U, unbound fraction. (**b**) ChIP analysis of *Rex-1* using antibodies to 3me-H3(K9) or HP1β were carried out on differentiated P19 cells (48 h). For all ChIPs in this experiment, the bound fraction was diluted 1:5 and then 1:10 to measure *Oct-3/4* by polymerase chain reaction in the linear range. *β-actin* is measured using undiluted bound DNA. Fold enrichment: 3me-H3(K9), 250; HP1β, 250. Similar results were obtained on chromatin from kidney. (**c**) Table showing the specificity of heterochromatinization by assaying histone H3(K9) methylation and HP1β for *Oct-3/4* and *Rex-1* compared with other inactive genes in differentiated (48 h) P19 cells.

Oct-3/4 silencing in ES cells that carry homozygous deletions of both *Dnmt3a* and *Dnmt3b*, the genes that are thought to be largely responsible for *de novo* methylation during early development[14]. In both mutant and wild-type ES cells, the *Oct-3/4* gene undergoes repression, with kinetics being slightly slower than those seen in P19 embryonal carcinoma cells (data not shown). Strikingly, this gene still undergoes histone H3 methylation at Lys 9, as well as heterochromatinization through the binding of HP1 in *Dnmt3a/3b*$^{-/-}$ ES cells (Fig. 3a), despite the absence of any *de novo* methylation at the promoter even after 8 d of RA treatment (Fig. 3b). These experiments clearly show, for the first time, that it is indeed *Dnmt3a/3b* that is responsible for modification of *Oct-3/4* and also prove conclusively that DNA methylation represents a second-line epigenetic change, the role of which may be restricted to the maintenance of repression in somatic cells.

Several different genes in the animal genome have the ability to methylate histone H3 at Lys 9. It seems, for example, that *Suv39h1/2* is responsible for methylation in pericentric heterochromatin, whereas *G9a* may be involved in the methylation of histone H3(K9) in the euchromatic fraction of the genome[15] (reviewed in ref. 9). To determine the cause of histone H3 methylation specifically at the *Oct-3/4* promoter, we followed the molecular events that take place in wild-type and *G9a*$^{-/-}$ ES cells during RA-induced differentiation. In both cell types, *Oct-3/4* expression, as measured by RNA (Fig. 4a) and protein (EMSA) (data not shown), underwent a rapid decrease within 1–2 d of treatment. In wild-type cultures, this was followed by the deacetylation of histone H3, a marked increase in 3me-H3(K9) and the binding of HP1β at the *Oct-3/4* promoter as expected, but none of these changes were observed

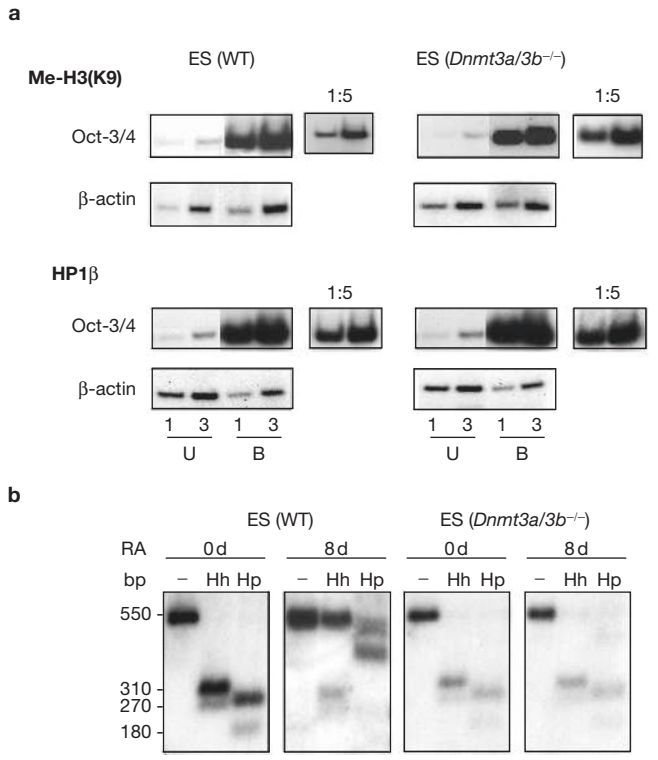

Figure 3 Role of Dnmt3a/3b in *Oct-3/4* repression. (**a**) Chromatin immunoprecipitation analysis of *Oct-3/4* using antibodies to 3me-H3(K9) and HP1β in retinoic-acid (RA)-differentiated wild-type (wt) and *Dnmt3a/3b*$^{-/-}$ embryonic stem (ES) cells. β-actin was used as a negative control. The bound (B) fractions were also assayed by PCR after a 5-fold dilution to measure enrichment in the linear range. Fold enrichment: 3me-H3(K9) (wild type, 30; *Dnmt3a/3b*$^{-/-}$, 40); HP1β (wild type, 150; *Dnmt3a/3b*$^{-/-}$, 150). Note that methylation of H3(K9) and HP1β are equally enriched in the wild-type and mutant cells. U, unbound fraction. (**b**) Methylation analysis of the *Oct-3/4* promoter in undifferentiated and differentiated (8 d) wild-type and *Dnmt3a/3b*$^{-/-}$ ES cells. DNA was digested with *Xba*I/*Hind*III with or without *Hha*I (Hh) or *Hpa*II (Hp), and subjected to Southern analysis using the radioactive *Oct-3/4* promoter region probe shown in Fig. 1d. Note that no *de novo* methylation is observed in the mutant. bp, base pairs.

in the mutant cells either for *Oct-3/4* (Fig. 4b; and see Supplementary Information, Fig. S2) or *Rex-1* (data not shown), indicating that *G9a* is necessary for the observed heterochromatinization.

Our data indicate that G9a may play multiple roles in this process. First, this protein seems to be involved in histone deacetylation at the *Oct-3/4* promoter region, as has been shown in other systems[15,16]. Once H3(K9) is deacetylated, G9a may then instigate di- and tri-methylation of Lys 9, either directly[17] or through the recruitment or activation of other histone methylases[18]. In this regard, it should be noted that G9a actually forms a stochiometric heterodimeric complex with another histone methylase, GLP1/Eμ-Hmtase1, and both probably function cooperatively to mediate K9 modification[19]. It is unlikely that Suv39h plays any role in this process, as *Oct-3/4* was found to undergo completely normal repression and heterochromatinization in *Suv39h1/h2*$^{-/-}$ ES cells following RA treatment (data not shown).

As genes involved in H3(K9) methylation have been shown to be required for *de novo* DNA methylation in several systems (reviewed in ref. 18), we next analysed the methylation state of the *Oct-3/4* promoter in *G9a*$^{-/-}$ ES cells following 8 d of differentiation. As expected, wild-type cells became fully methylated at this stage. In striking contrast, no DNA methylation was observed in the *G9a*$^{-/-}$ cells (Fig. 4c), indicating that the histone-modification machinery is required for targeted *de novo* methylation in this system as well. ChIP experiments, using an antibody against Dnmt3a, showed that G9a probably carries this out by attracting *de novo* DNA methylases to the *Oct-3/4* promoter, either directly or through downstream effectors such as HP1 (reviewed in ref. 18) and, in its absence, Dnmt3a is not recruited (Fig. 4d). Although this type of heterochromatin-associated DNA-methylation pathway has been described previously for repeated sequences in a number of different organisms (reviewed in ref. 18), this represents the first time that it has been shown for a specific developmentally controlled gene.

It should be noted that this process is different from the non-sequence-specific *de novo* global methylation that takes place at the time of implantation, and gene sequences that are subject to this global process (for example, β-globin, α-crystallin or Pepck) actually retain their fully methylated state in *G9a*$^{-/-}$ ES cells (see Supplementary Information, Fig. S3). Further evidence that this is the case comes from experiments in *Dnmt1*$^{-/-}$ proliferating fibroblasts. Despite the fact that almost all gene sequences in these cells are unmethylated, the *Oct-3/4* promoter remains highly modified (Fig. 4c), presumably because it is able to constitutively recruit *de novo* methylases, and is therefore not dependent on Dnmt1 to maintain its methylation pattern. When taken together, these results clearly indicate that *Oct-3/4* is specifically targeted for G9a-mediated *de novo* methylation by Dnmt3a/3b during embryonic cell differentiation.

Our studies demonstrate that the *Oct-3/4* gene undergoes rapid transcriptional inactivation at the beginning of the differentiation process, and only afterwards is subject to epigenetic changes that lead to heterochromatinization and DNA methylation at the promoter (see Fig. 2). Presumably, these latter events are necessary to ensure that silencing is maintained even after the initial repression factors are no longer available. To test this idea, we took cells that had undergone differentiation for 8 d in the presence of RA and re-plated them in medium lacking this inducer. Whereas Oct-3/4 remained silenced in wild-type cells, we observed a reappearance of *Oct-3/4* expression to almost normal levels in *G9a*$^{-/-}$ cells (Fig. 4a). This clearly indicates that the initial RA-induced repression is indeed a transient phenomenon and also shows that G9a plays a role in secondary silencing, probably by mediating heterochromatinization and DNA methylation.

With these results in mind, we then tested whether, in the absence of histone H3 and DNA methylation, long-term *Oct-3/4* transcriptional repression may allow differentiated *G9a*$^{-/-}$ cells to actually revert to a more pluripotent state. To test this hypothesis, we re-plated limited numbers of 8 d RA-differentiated ES cells into microtitre plates containing LIF medium, under conditions in which we expected the growth of no more than one colony per well. Whereas wild-type cells were almost completely unable to revert to the undifferentiated phenotype (1/624 wells), two independent *G9a*-knockout lines produced colonies in more than 20% of the wells (Fig. 5a). Reverse transcription polymerase chain reaction (RT-PCR) analysis of these clones revealed that this was accompanied by the re-expression of two pluripotential markers, *Oct-3/4* and *Nanog*, to normal levels (Fig. 5b). This effect is due to the absence of G9a, as the replacement of this gene in the mutant ES cells (*G9a*$^{-/-}$/Tg) brought about a marked reduction in the ability of these cells to revert to the undifferentiated phenotype (Fig. 5a).

To distinguish between the effects of chromatin as opposed to DNA methylation, we carried out a similar experiment with *Dnmt3a/3b*$^{-/-}$ ES

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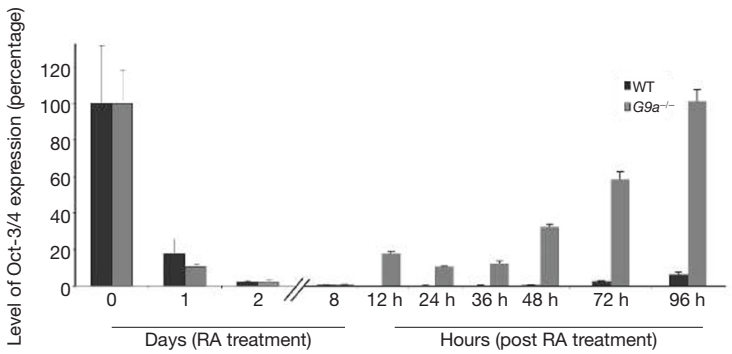

b

Modification	Cell type	RA treatment	
		0 d	8 d
Me-H3(K9)	ES (WT)	30	360
	ES (*G9a*$^{-/-}$)	10	8
Hp1β	ES (WT)	10	320
	ES (*G9a*$^{-/-}$)	1	3
Ac-H3(K9, 14)	ES (WT)	50	5
	ES (*G9a*$^{-/-}$)	30	30

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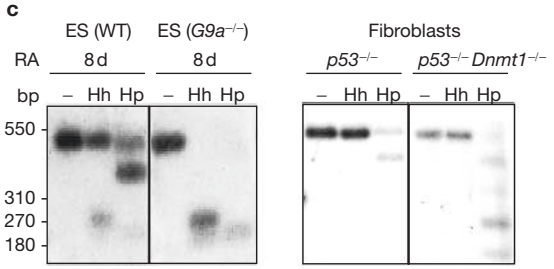

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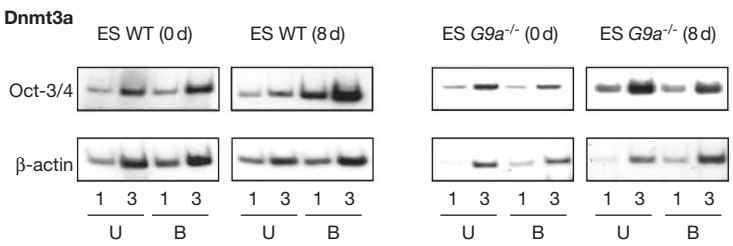


Figure 4 G9a-mediated *Oct-3/4* H3(K9) methylation. (**a**) *G9a*$^{-/-}$ and WT embryonic stem (ES) cells were treated with retinoic acid (RA) for up to 8 d, after which RA was removed and cells were isolated at various time points. Graph (± SD) shows the level of *Oct-3/4* expression using quantitative real-time RT–PCR analysis with β-actin as the normalization control. (**b**) Chromatin immunoprecipitation (ChIP) analysis of the *Oct-3/4* promoter in wild-type and *G9a*$^{-/-}$ ES cells (undifferentiated, 0 d; RA-differentiated, 8 d) using antibodies specific for me-H3(K9), HP1β or Ac-H3(K9). Fold enrichment was determined by semi-quantitative PCR as described in Fig. 1b. (**c**) DNA was digested with *Xba*I and *Hind*III with or without *Hha*I (Hh) or *Hpa*II (Hp), and subjected to Southern blotting using a probe spanning the promoter region (see map in Fig. 1d). bp, base pairs. (**d**) ChIP analysis of *Oct-3/4* in wild-type and *G9a*$^{-/-}$ (undifferentiated and RA-differentiated) ES cells using antibodies specific for Dnmt3a. Semi-quantitative PCR reactions were performed as described in Fig. 1b. Differentiated cells show a 30-fold enrichment, no enrichment is seen in the *G9a*$^{-/-}$ mutant. B, bound fraction; U, unbound fraction.

cells (designated 7aabb). Despite the fact that, in these cells, *Oct-3/4* becomes fully heterochromatinized following differentiation (see Fig. 3a), these *Dnmt3a/3b*$^{-/-}$ cells were still able to undergo reversion (Fig. 5a), albeit with somewhat slower kinetics. When taken together, these results strongly indicate that, whereas histone H3 methylation may slow down *Oct-3/4* reactivation, DNA methylation of the promoter seems to be a locking mechanism that serves to stably inhibit re-expression of *Oct-3/4* and to prevent proliferation under conditions that are conducive to the undifferentiated phenotype.

In this paper, we demonstrate that *Oct-3/4* is inactivated in a multi-step process (Fig. 5c) that involves factor-mediated transcriptional repression which, although transient in nature, triggers structural changes that generate a small island of self-maintaining constitutive heterochromatin within the euchromatin fraction of the nucleus[20,21]. This mode of heterochromatinization is different from that involved in X-chromosome inactivation, which does not use either tri-methylation of H3(K9) or the binding of HP1 (reviewed in ref. 18). It is also unlike the process observed in the immune system whereby selected genes can be transiently inactivated by being physically recruited to pericentric heterochromatic regions within the nucleus[22]. Therefore, although heterochromatinization may represent a common pathway for gene repression during development, there seems to be a variety of different strategies for carrying out this process.

It is well established that *Oct-3/4* is necessary for the totipotent phenotype in ES cells and, as such, seems to be absolutely required for the reprogramming of transplanted somatic nuclei in cloning experiments[23,24]. It is, perhaps, for this reason that the gene initially undergoes such a complex process of inactivation that involved multiple layers of repression (Fig. 5c). Our studies demonstrate, for the first time, that these epigenetic changes do indeed act to prevent reactivation of the gene even in newly differentiated cells. Furthermore, by genetic analysis we were able to decipher the individual components of this process, and have shown that it is mainly promoter methylation that serves as the major barrier to *Oct-3/4* reactivation-mediated dedifferentiation. Therefore, although germ cells and early embryos seem to have an innate mechanism for recognizing and demethylating *Oct-3/4* (refs 11, 25), it becomes irreversibly locked in a repressed state once it undergoes inactivation and *de novo* methylation post-implantation.

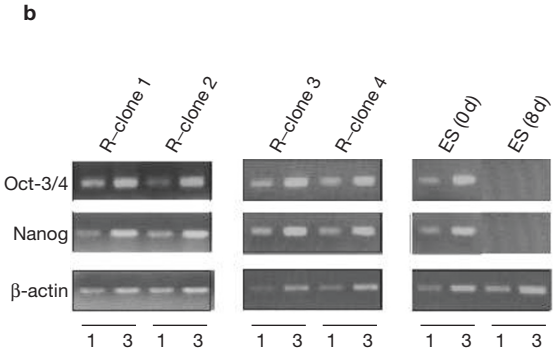
METHODS

Cells, DNA and RNA analyses. Murine P19[4], *p53*$^{-/-}$, *p53*$^{-/-}$*Dnmt1*$^{-/-}$ fibroblasts[26], wild-type (wt), *Dnmt3a/3b*$^{-/-}$[14], *G9a*$^{-/-}$ and *G9a*$^{-/-}$/Tg[15] ES cells were maintained as described previously[4,14]. Cells were treated with 1 μM RA for the indicated times, as previously described[6,7]. Suv39h1/2$^{-/-}$ embryonic stem cells were kindly provided by T. Jenuwein. Nuclear extracts were prepared and analysed by electrophoresis mobility shift assay, as described previously[4], using the OCTA double-stranded oligonucleotide 5′-CGTACTAATTTGCATTTCTA-3′. Genomic DNA was digested with restriction enzymes and analysed by Southern blotting using the radioactive *Oct-3/4* promoter (549 base pairs) and the proximal enhancer (805 base pairs) regions as probes. RNA was extracted using a kit (Biological Industries, Beit Haemek, Israel) and analysed by semi-quantitative and quantitative real-time PCR (further details are given in the Supplementary Information).

For reversal experiments, wild-type, *G9a*$^{-/-}$ (clones 10–20, 2–3) and *G9a*$^{-/-}$Tg ES cells, as well as *Dnmt3a/3b*$^{-/-}$[15,19], were induced to differentiate with 1 μM

a

Cell type	Reverted clones	(Percentage)	Normalized (percentage)
WT	1/624	0.2	0.3
G9a$^{-/-}$ (10–20)	133/624	21	11.7
G9a$^{-/-}$ (2–3)	89/288	31	17.2
G9a$^{-/-}$ /TG	1/288	0.3	0.2
Dnmt3a/3b$^{-/-}$	42/768	5.5	10.6



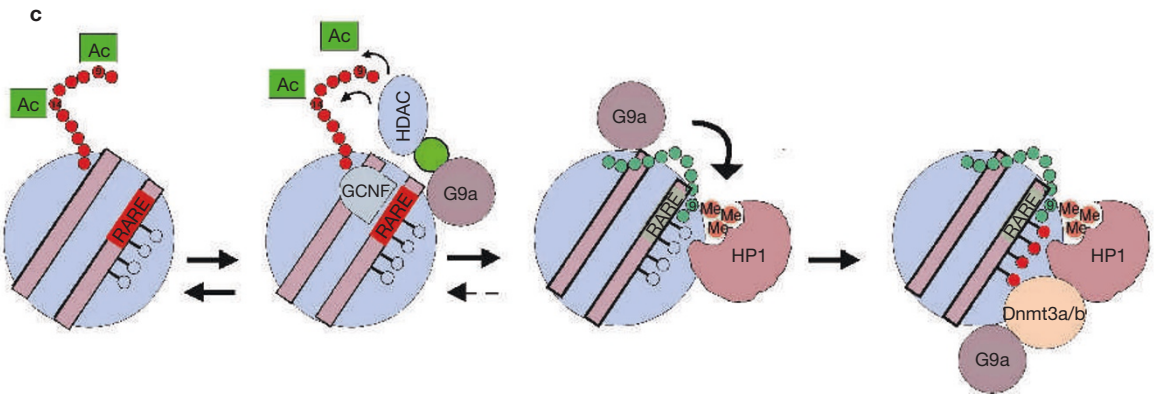


Figure 5 Chromatin structure affects reversal of ES cell differentiation. (**a**) Number and percentage of reverted single-cell clones established from wild-type (wt) and mutant embryonic stem (ES) cells that were initially differentiated with retinoic acid (RA) for 8 d and then re-cultured in 96-well plates for 2 weeks in the presence of LIF under conditions that were calibrated for each line to yield approximately one colony per plate of 1 d RA-treated cells (10–50 cells per well). Each result represents the sum of 2–3 independent experiments. Two *G9a*$^{-/-}$ and one *Dnmt3a/3b*$^{-/-}$ ES cell lines were tested, as well as *G9a*$^{-}$ ES cells containing a G9a expression transgene (*G9a*$^{-/-}$/Tg)[28]. *Dnmt3a/3b*$^{-/-}$ revertants grew at a slightly lower rate than *G9a*$^{-/-}$ cells. Normalization takes into consideration the clonability of each cell line, as determined by the number of colonies found after plating 1 d RA-treated cells. (**b**) RT–PCR analysis of *Oct-3/4* and *Nanog* is shown in undifferentiated and RA-differentiated cells, as well as in two representative *G9a*$^{-/-}$ reverted clones (clone 1 and 2, out of 133) and two *Dnmt3a/3b*$^{-/-}$ reverted clones (clone 3 and 4 out of 42) that were obtained by re-culturing in LIF-containing medium. β-actin was used as an internal control. (**c**) Model for *Oct-3/4* heterochromatinization. Nucleosomes over the active *Oct-3/4* promoter initially contain histone H3 that is acetylated at Lys 9 and Lys 14 (refs 10, 29). When differentiation is initiated, repressors (including GCNF, which binds the RA receptor element, RARE) associate with the *Oct-3/4* promoter, causing transient transcriptional repression (Fig. 4a). This presumably brings about the binding of G9a, which recruits histone deacetylase molecules (HDACs) by an, as yet, uncharacterized mechanism. Once deacetylated, H3(K9) becomes a substrate for methylation, either by G9a itself, or through the involvement of additional histone methylases. 3me-H3(K9) can then bind the chromodomain protein HP1. DNA methylation is catalysed by Dnmt3a, and perhaps Dnmt3b, which are recruited to the promoter through the involvement of G9a and other effectors, such as HP1 (ref. 30). Prior to *de novo* methylation, Oct-3/4 can be reactivated (double arrows) when cells are returned to early pre-differentiation conditions. Following this step, however, repression seems to be irreversible (Fig. 5a). Note that this model may not include all of the epigenetic factors that are involved in Oct-3/4 inactivation.

RA (Sigma, Rehovot, Israel) for 8 d. Differentiated cells were washed and re-suspended in LIF-supplemented medium. Limited numbers of cells were plated in 96-well plates under conditions in which growth of one colony per well was achieved. The number of reverted clones was scored after 2 weeks in culture, and *Oct-3/4, Nanog* and β-actin expression were assessed by RT-PCR (PCR details are listed in Supplementary Information).

ChIP analysis. We prepared monomeric nucleosome fractions from tissue culture cells or fresh tissues and carried out ChIP analysis as described previously[27]. Alternatively, cells were cross-linked and chromatin was extracted and immunoprecipitated using the ChIP assay kit as recommended by the manufacturer (Upstate Biotechnology, Lake Placid, NY). Antibodies were directed against acetylated histone H3(K9, K14) (5 μg per 30 μg DNA), Me-H3(K4) (5 μg per 30 μg DNA), (Upstate Biotechnology), 2,3me-H3(K9) (15 μl per 10 μg DNA) (Abcam, Cambridge, UK), rat anti-mouse HPIβ (50 μl per 10 μg DNA) (Serotec, Cambridge, UK) and anti-Dnmt3a (5 μg per 10 μg DNA) (Abcam). To certify the presence of 3me-H3(K9), we also carried out ChIP experiments using highly purified antibodies (10 μl of 0.1 mg ml^{-1} per 10 μg DNA) that were affinity selected to remove any cross-reactivity with non-specific methylated forms of H3. For each immunoprecipitation, there was always a difference between the known active and inactive genes.

Incubations with the various antibodies were followed by protein A-Sepharose (5 μg per 60 μg DNA) (Sigma). Because we usually precipitated <1% of nucleosomes, PCR analysis of the bound fraction was compared to a 1:100 dilution of the input DNA. To amplify DNA molecules from bound and unbound fractions, serial 3-fold dilutions of nucleosomal DNA (1-, 3- and 9-fold) were analysed using appropriate pairs of oligonucleotides (sequences available on request). PCR (1 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 60°C for *Oct-3/4* and *Rex-1* and 55°C for CD4 and Pepck, and 30 s at 72°C; and finally 1 min at 72°C) was carried out in 50 μl containing 0.1 μl of ^{32}P-dCTP (Amersham Pharmacia, Little Chalfont, UK). PCR conditions for *β-actin*, *α-cry* and *β-globin* have been described previously[27].

Almost all of the ChIP analyses were carried out by semi-quantitative PCR using three different concentrations of DNA (only two are shown in the figures).

In each case, we tried to use concentrations that were in the linear range. As the *Oct-3/4* gene promoter was hyper-enriched compared with the negative control for several of the markers, in some instances it was necessary to analyse additional dilutions of the bound fraction to get linear PCR reactions for these particular sequences, and some of these are shown in Figs 1 and 3. Although there are some variations between individual ChIP experiments, most of the results were reproducible to within about 20%. In numerous cases, we checked our results using real-time PCR (examples are described in figure legends).

It should be noted that, in ChIP experiments using antibodies against 3me-H3(K9), we observed enrichment of more than 300-fold for *Oct-3/4* compared with the negative control (*β-actin*). If this high level is indicative of nucleosomes that have been fully methylated on both H3 molecules, we must assume that gene regions that show lower levels of enrichment are only partially methylated. Previous studies[27] demonstrated that tissue-specific genes are only enriched by 2–10-fold in similar assays, indicating that although these nucleosomes may be deacetylated at the H3(K9) moiety, few are actually methylated.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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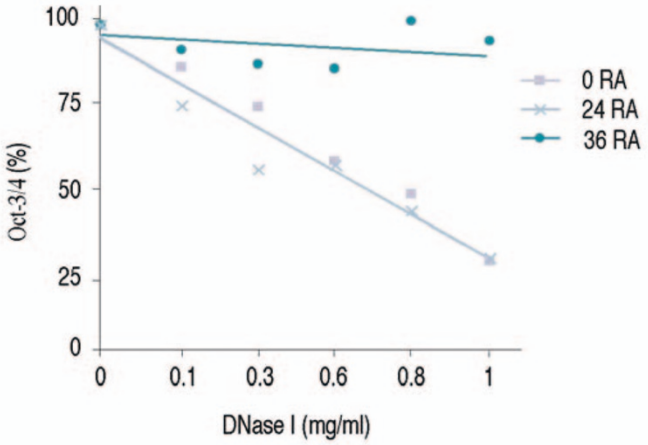
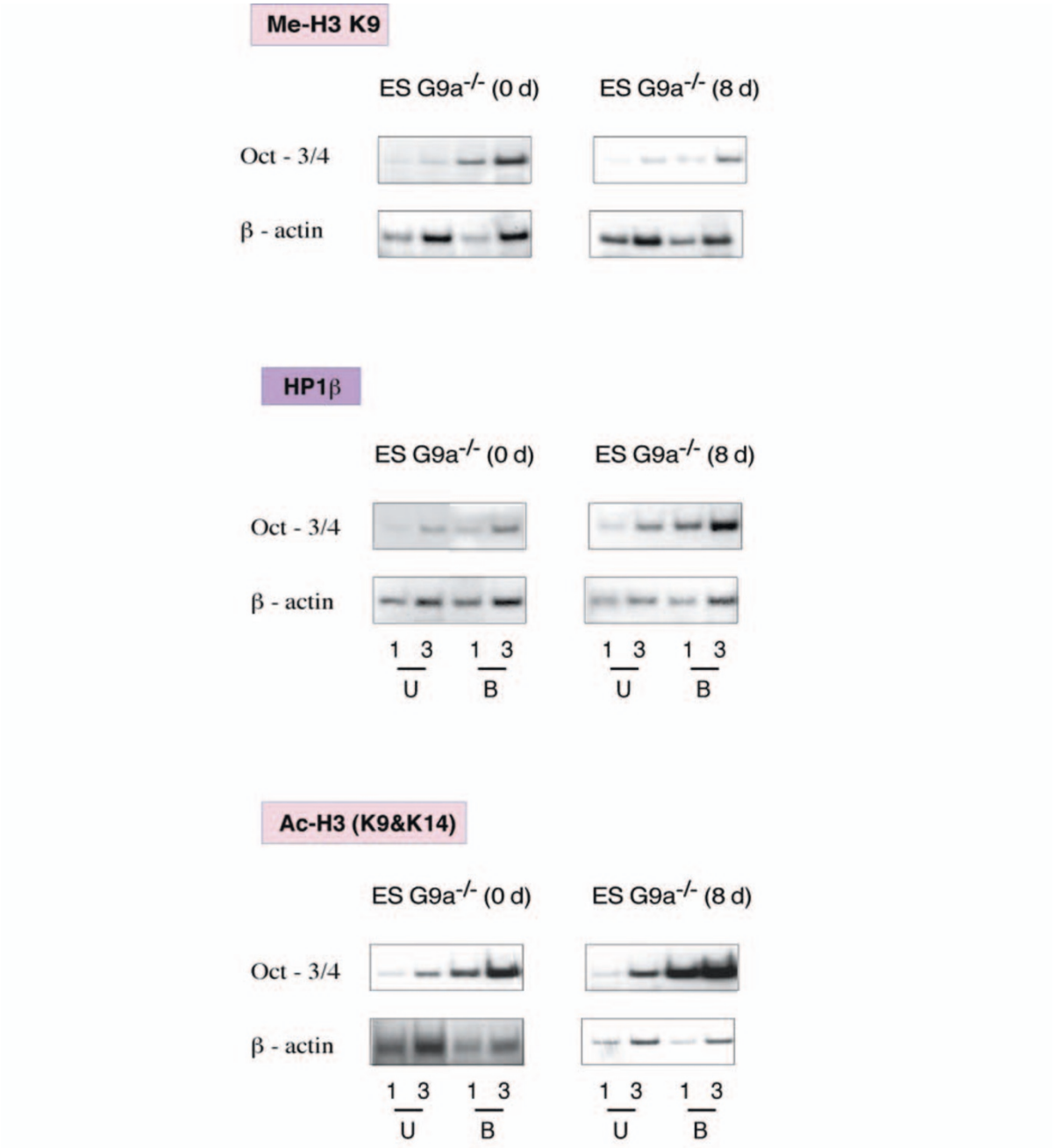


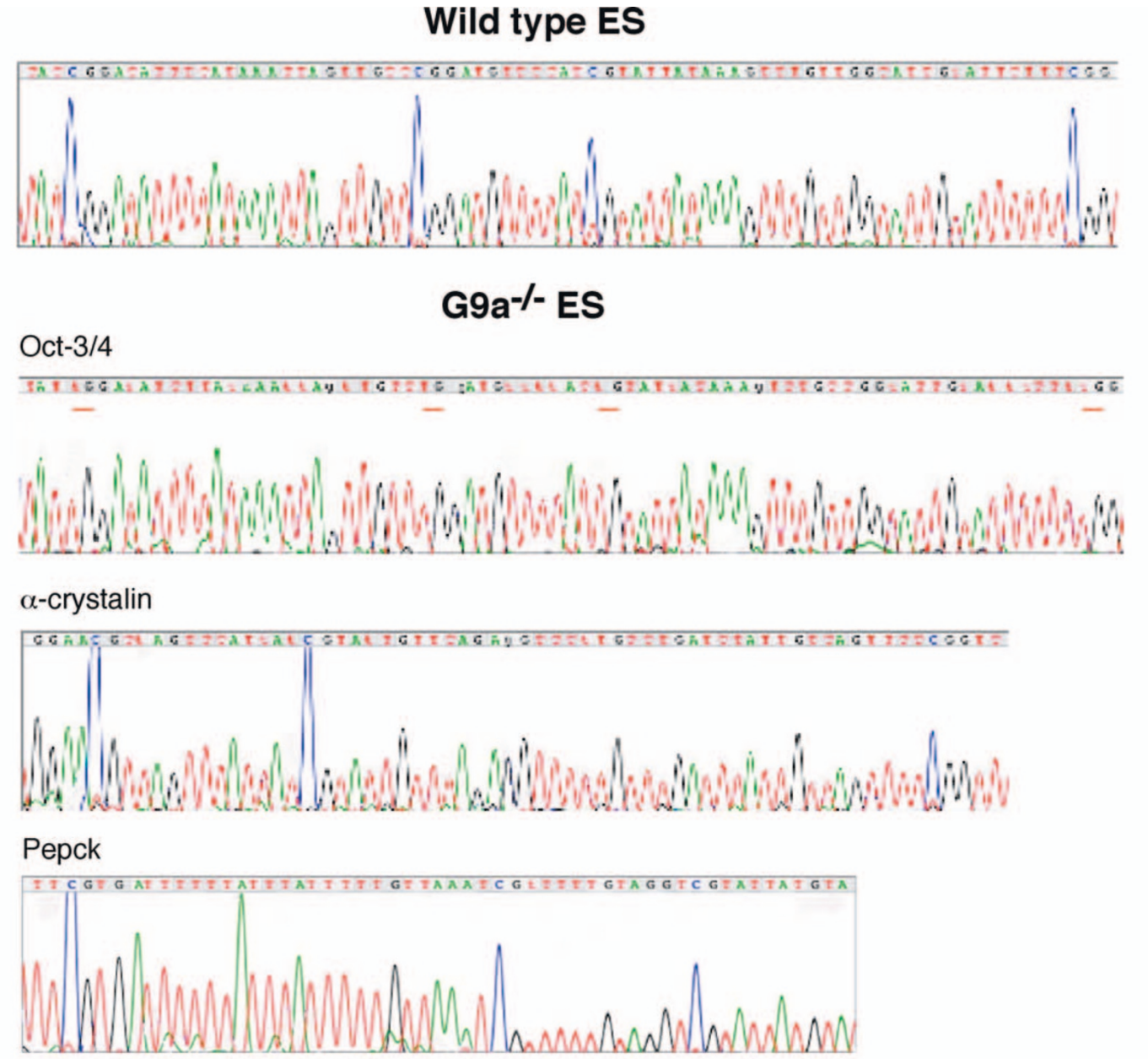
Supplementary Figure 1. DNaseI sensitivity of the Oct-3/4 promoter.

10^8 P19 cells before or after treatment with retinoic acid were prepared, washed in cold PBS, centrifuged at 1000 rpm for 5 min and lysed in NP-40–containing lysis buffer (20 mM Tris pH 7.0, 3 mM, $CaCl_2$, 2 mM $MgCl_2$, 0.3% NP-40). The mixture was kept on ice for 10 min and centrifuged at 1000 rpm for 5 min. The resulting nuclei were resuspended in a solution of 10 mM Tris pH 7.0, 3 mM $MgCl_2$, 10 mM NaCl to the final concentration of 10^8 nuclei per ml. Aliquots of 10-150 ml nuclei were then digested with increasing concentrations of DNaseI (0.1-3 mg/ml) for 15 min at 37°C and digestion was stopped by adding an equal amount of 2x lysis buffer containing 20 mM Tris pH 7, 200 mM NaCl, 2 mM EDTA, 2% SDS. The lysates were treated with Proteinase K (200 mg/ml) overnight at 37°C, DNA was isolated by phenol-chloroform extraction and Southern blotting then carried out using the probe depicted in Figure 1 (percent of starting material).



Supplementary Figure 2.

ChIP analysis of the Oct-3/4 promoter using antibodies specific for 3me-H3(K9), HP1β and Ac-H3(K19 & K14), in undifferentiated (0d) and RA-differentiated (8d) G9a$^{-/-}$ cells. β-actin was used as the normalization control. Semi-quantitative PCR reactions were performed as described in Figure 1 B and fold enrichment was determined and shown in Figure 4B.



Supplementary Figure 3. Bisulfite analysis of genes in ES cells.

DNA from 8d RA treated wild type or G9a$^{-/-}$ ES cells was treated with Na bisulfite, amplified using specific nested primers (sequences available upon request) and then subjected to whole population sequence analysis without cloning. Note that all CpGs in the Oct-3/4 promoter have been converted to TG in the differentiated G9a$^{-/-}$ cells, while the same sites are methylated in wild type cells. The original CpG sites are marked with a red line. All together we examined 9 CpG sites in the Oct-3/4 promoter region, but only a portion of this sequence is shown in the figure. These sites were >80% methylated in wild type cells and unmethylated in the mutant. In addition, we analyzed the promoters of α-crystallin (5 CpG sites), Pepck (3 CpG sites) and β-major globin (4 CpG sites). Almost all were found to be >80% methylated in wild type and G9a$^{-/-}$ cells. One site in the globin promoter was found to be <10% methylated in both cell types.