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DNA methylation in ES cells requires the lysine methyltransferase G9a but not its catalytic activity

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Histone H3K9 methylation is required for DNA methylation and silencing of repetitive elements in plants and filamentous fungi. In mammalian cells however, deletion of the H3K9 histone methyltransferases (HMTases) Suv39h1 and Suv39h2 does not affect DNA methylation of the endogenous retrovirus murine leukaemia virus, indicating that H3K9 methylation is dispensable for DNA methylation of retrotransposons, or that a different HMTase is involved. We demonstrate that embryonic stem (ES) cells lacking the H3K9 HMTase G9a show a significant reduction in DNA methylation of retrotransposons, major satellite repeats and densely methylated CpG-rich promoters. Surprisingly, demethylated retrotransposons remain transcriptionally silent in *G9a*$^{-/-}$ cells, and show only a modest decrease in H3K9me2 and no decrease in H3K9me3 or HP1α binding, indicating that H3K9 methylation *per se* is not the relevant trigger for DNA methylation. Indeed, introduction of catalytically inactive G9a transgenes partially 'rescues' the DNA methylation defect observed in *G9a*$^{-/-}$ cells. Taken together, these observations reveal that H3K9me3 and HP1α recruitment to retrotransposons occurs independent of DNA methylation in ES cells and that G9a promotes DNA methylation independent of its HMTase activity.

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Introduction

Retrotransposons, including long terminal repeat (LTR) and non-LTR elements, are widely dispersed in the euchromatic compartment in higher mammals (Kuff and Lueders, 1988; Medstrand *et al*, 2002), constituting ~37% of the mouse genome (Mouse Genome Sequencing Consortium, 2002). A subset of these elements are transcriptionally competent, placing a significant mutational load on their hosts (Maksakova *et al*, 2006). To minimize the likelihood of retrotransposition, a number of pathways that function at the transcriptional or post-transcriptional stages of the replicative cycle have evolved to inhibit the expression of these parasitic elements. DNA methylation for example, has an important function in transcriptional silencing of retrotransposons in mammalian cells (Li *et al*, 1992; Yoder *et al*, 1997; Walsh *et al*, 1998), as illustrated by the high level of expression of the intracisternal A particle (IAP) endogenous retrovirus (ERV) in mouse embryos deficient in the DNA methyltransferase (DNMT), Dnmt1 (Walsh *et al*, 1998). DNA methylation also has a critical function in transcriptional silencing of repetitive elements and their relics in filamentous fungi and plants (Goyon *et al*, 1996; Lindroth *et al*, 2001; Zhou *et al*, 2001), substantiating the importance of this epigenetic mark in suppressing transposable elements in distantly related eukaryotes.

Repetitive elements in eukaryotes are also marked by specific covalent histone modifications (Bernstein *et al*, 2007). Methylation of lysine 9 of the histone H3 tail (H3K9) in particular, has an important function in silencing of these elements in yeast (Nakayama *et al*, 2001), filamentous fungi (Tamaru and Selker, 2001), plants (Jackson *et al*, 2002) and animals (Martens *et al*, 2005). Recent genome-wide studies reveal that ERVs are marked by H3K9 dimethylation (H3K9me2) and/or H3K9 trimethylation (H3K9me3) in murine cells (Peters *et al*, 2003; Martens *et al*, 2005; Mikkelsen *et al*, 2007); however, the specific histone methyltransferases (HMTases) responsible have not been identified.

Intriguingly, the H3K9 HMTase *DIM-5* is required for CpG methylation in *Neurospora* (Tamaru and Selker, 2001) and the H3K9 HMTase *KRYPTONITE* is required for CpNpG methylation in *Arabidopsis* (Jackson *et al*, 2002), suggesting the existence of an evolutionarily conserved silencing pathway in which H3K9 methylation promotes *de novo* DNA methylation of repetitive elements (Freitag and Selker, 2005; Stancheva, 2005). However, the role, if any, that H3K9 methylation has in DNA methylation of retrotransposons in mammalian cells has not been systematically addressed.

Five HMTases in the 'Suv39' subfamily of SET (Suv39, Enhancer of Zeste, Trithorax) domain-containing proteins with H3K9 catalytic activity, including Suv39h1 and the closely related Suv39h2, G9a and the closely related GLP/EuHMTase1 and SETDB1/Eset, have been characterized in mammalian cells. On the basis of its sequence similarity to SETDB1, the sixth Suv39 family member, SETDB2/CLLD8, is

also likely to have specificity for H3K9 (Mabuchi *et al*, 2001; Kouzarides, 2007). Although Suv39h1 and Suv39h2 double-negative (*Suv39h1/2*$^{-/-}$) embryonic stem (ES) cells show a dramatic reduction in H3K9me3 and DNA methylation at major satellite repeats, IAP elements show no reduction in H3K9 methylation (Peters *et al*, 2003; Martens *et al*, 2005; Mikkelsen *et al*, 2007) and murine leukaemia virus (MLV) ERVs show no reduction in DNA methylation (Lehnertz *et al*, 2003) in these cells. Taken together, these results indicate that Suv39h1 and Suv39h2 do not have a major function in H3K9 methylation or DNA methylation of LTR retrotransposons in mammalian cells.

In contrast to the Suv39h HMTases, G9a and GLP/Eu-HMTase1, which form a heteromeric complex *in vivo*, are widely dispersed in the euchromatic compartment and deletion of either leads to a dramatic decrease in H3K9me1 and H3K9me2 in ES cells (Tachibana *et al*, 2002, 2005). A recent analysis revealed that ~300–400 genes show altered expression in *G9a*$^{-/-}$ cells (Sampath *et al*, 2007) and several studies have shown that G9a regulates the expression and/or DNA methylation status of specific genes (Feldman *et al*, 2006; Ikegami *et al*, 2007). However, experiments aimed at determining whether G9a influences the expression and/or DNA methylation states of interspersed repetitive elements have not been reported.

Here, we investigated the function that G9a and GLP have in DNA methylation and silencing of potentially active ERVs and non-LTR retrotransposons. We show that DNA methylation of these elements, and a subset of non-repetitive sequences including CpG-rich promoters, is reduced in *G9a*$^{-/-}$ cells and that Dnmt3a recruitment to retrotransposons is decreased in these cells. However, H3K9me3 enrichment and HP1α binding are unaltered, demonstrating that an alternative H3K9 HMTase marks ERVs and that H3K9 methylation *per se* is not sufficient to promote DNA methylation of these elements. In support of this model, we show that the introduction of two different G9a transgenes that lack catalytic activity into *G9a*$^{-/-}$ cells partially 'rescues' the observed DNA methylation defect, indicating that G9a promotes DNA methylation of retrotransposons independent of its catalytic activity.

Results

G9a is required for DNA methylation of retrotransposons

To establish whether G9a is required for DNA methylation of ERVs, genomic DNA isolated from TT2 wild-type (wt) and *G9a*$^{-/-}$ ES cells (Tachibana *et al*, 2002) (Supplementary Figure S1) was analysed by Southern blotting using the methylation-sensitive restriction enzyme *Hpa*II and probes specific for IAP and MLV ERVs, of which there are ~1200 and ~60 copies in the mouse genome, respectively (Figure 1A and B). Genomic DNA samples isolated from *Dnmt1*$^{-/-}$ (Lei *et al*, 1996), *Suv39h1/2*$^{-/-}$ (Peters *et al*, 2001) and the wt parent ES cell lines from which they were derived were analysed in parallel. A dramatic reduction in DNA methylation of both ERVs was detected in the *G9a*$^{-/-}$ line relative to the wt control. At the resolution of Southern blot analysis, this reduction in methylation is not distinguishable from that observed for the *Dnmt1*$^{-/-}$ ES line, or TT2 genomic DNA digested with the methylation-insensitive isoschizomer *Msp*I.

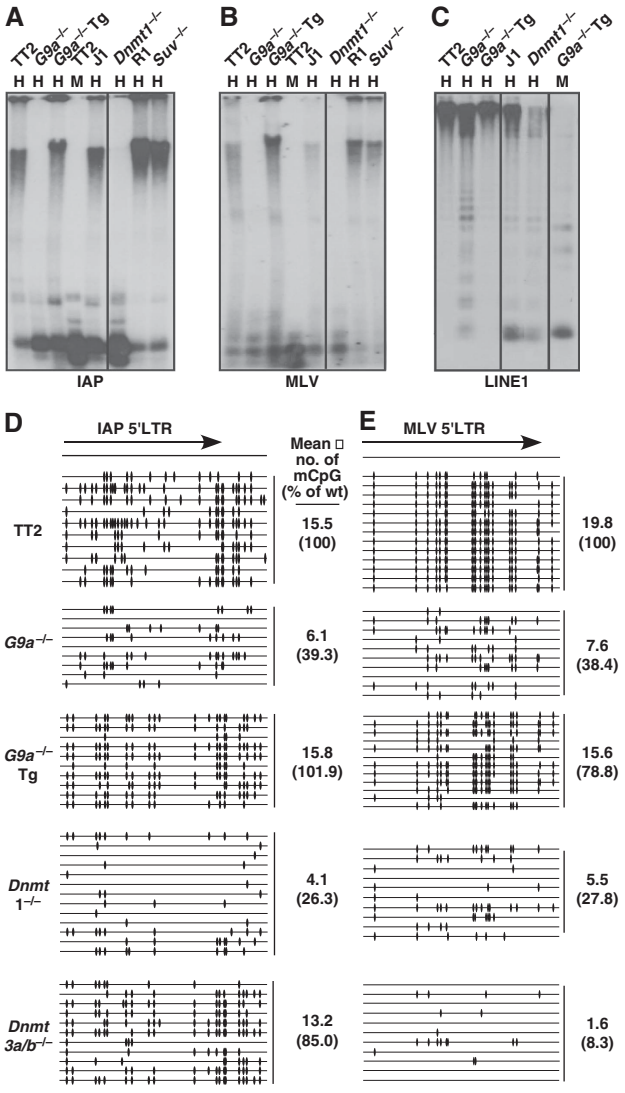

Figure 1 DNA methylation of MLV, IAP and LINE1 retrotransposons is reduced in *G9a*$^{-/-}$ cells. Genomic DNA isolated from *G9a*$^{-/-}$, *G9a*$^{-/-}$Tg, *Dnmt1*$^{-/-}$, *Suv39h1/2*$^{-/-}$ (*Suv*$^{-/-}$) and the wt parent lines TT2, J1 and R1, respectively, was digested with *Msp*I (M) or the methylation-sensitive restriction enzyme *Hpa*II (H) and subject to Southern blotting using probes specific for (**A**) IAP, (**B**) MLV or (**C**) LINE1 retrotransposons. The *G9a*$^{-/-}$ line shows a dramatic decrease in DNA methylation at each of these repetitive elements that is reversed in the *G9a*$^{-/-}$Tg line. In contrast, the *Suv39h1/2*$^{-/-}$ line shows no DNA methylation defect at IAP or MLV repeats. (**D**, **E**) Bisulphite analysis of the 5′LTR regions of IAP and MLV elements was conducted on TT2, *G9a*$^{-/-}$, *G9a*$^{-/-}$Tg (15-3), *Dnmt1*$^{-/-}$ and *Dnmt3a/b*$^{-/-}$ cells. For each molecule sequenced (horizontal bar), filled ovals represent the presence of an mCpG. The mean number of mCpGs per molecule sequenced is shown to the right of each set of sequenced samples. The mean % of mCpGs relative to the wild-type line is also shown (in parentheses).

To obtain a more accurate measure of the methylation status of these elements, high-resolution bisulphite sequencing analysis was conducted using primers specific for the CpG-rich 5′LTR regions of IAP and MLV elements. A >2.5-fold decrease in the mean number of mCpGs per molecule sequenced was detected in the *G9a*$^{-/-}$ line relative to the wt parent line (Figure 1D and E), with a subset of sequenced molecules in the *G9a*$^{-/-}$ line showing almost complete loss of methylation in these regions. A decrease in DNA methylation

across the LTR and downstream regions of the potentially active class II ERV MusD (Mager and Freeman, 2000), of which there are ~90 full-length copies in the mouse genome, was also detected in the *G9a*$^{-/-}$ line (Supplementary Figure S2). All three of these LTR retrotransposons show an even more severe DNA methylation defect in *Dnmt1*$^{-/-}$ cells (Figure 1D and E; Supplementary Figure S2). Consistent with the observations of Chen *et al* (2003), early passage *Dnmt3a/b*$^{-/-}$ cells (Okano *et al*, 1999) show a significantly more severe DNA methylation defect for MLV elements than IAP elements. Interestingly, whereas IAP elements show a less severe defect in *Dnmt3a/b*$^{-/-}$ cells than in *G9a*$^{-/-}$ cells, the reverse is true of MLV repeats. Thus, although it is clear that G9a is required for DNA methylation of distantly related ERVs in murine ES cells, the degree of demethylation is distinct from that observed for *Dnmt1*$^{-/-}$ or *Dnmt3a/b*$^{-/-}$ cells. In contrast, no reduction in DNA methylation of MLV or IAP elements was detected in *Suv39h1/2*$^{-/-}$ cells (Figure 1A and B), consistent with a previous report showing that Suv39h1 and Suv39h2 are not required for DNA methylation of MLV (Lehnertz *et al*, 2003).

DNA methylation of LINE1 (L1) elements, non-LTR retrotransposons that comprise ~20% of the mouse genome (Mouse Genome Sequencing Consortium, 2002), also depends on the presence of both Dnmt1 and Dnmt3a and/or Dnmt3b in ES cells (Liang *et al*, 2002). To determine whether G9a has a function in DNA methylation of this class of interspersed repeats, Southern blot analysis was conducted with a probe that spans the promoter region of the L1Md-A2 subfamily of L1 elements. A significant decrease in DNA methylation of L1 elements is also apparent in *G9a*$^{-/-}$ cells, although this defect is not as severe as that detected in the DNMT mutant lines (Figure 1C). Taken together, these observations indicate that G9a influences DNA methylation of both LTR and non-LTR retrotransposons in ES cells.

To determine whether G9a is also required for DNA methylation of tandem repeats in ES cells, Southern blot analysis using a probe specific for major satellite repeats (present at approximately 700 000 copies per cell) was conducted using the methylation-sensitive restriction enzyme HpyCH4IV (Supplementary Figure S3). Consistent with a previous report showing that Suv39h1 and Suv39h2 are required for methylation of major satellite repeats (Lehnertz *et al*, 2003), a dramatic reduction in DNA methylation of major satellite repeats was detected in *Suv39h1/2*$^{-/-}$ cells. Unexpectedly, a dramatic reduction in DNA methylation of this class of repeats was also detected in the *G9a*$^{-/-}$ line, revealing that G9a is required for DNA methylation of pericentromeric heterochromatin as well.

Introduction of a G9a transgene rescues the DNA methylation defect observed in G9a$^{-/-}$ *cells*

Reintroduction of Dnmt3a, Dnmt3a2 (the predominant isoform of Dnmt3a in ES cells; Chen *et al* (2002)) or Dnmt3b1 into *Dnmt3a/b*$^{-/-}$ ES cells restores DNA methylation of MLV and IAP elements (Chen *et al*, 2003), indicating that the *de novo* DNMTs are capable of reestablishing DNA methylation patterns in these cells. To determine whether reintroduction of G9a is capable of reversing the DNA methylation defect observed in *G9a*$^{-/-}$ cells, the methylation status of these elements was also analysed in a *G9a*$^{-/-}$ line stably expressing a wt G9a transgene (*G9a*$^{-/-}$*Tg*) (Tachibana *et al*, 2002) at a level similar to that of the endogenous protein (Supplementary Figure S1). Strikingly, the DNA methylation state of MLV, IAP, L1 (Figure 1) and MusD (Supplementary Figure S2) retrotransposons and major satellite repeats (Supplementary Figure S3) in the *G9a*$^{-/-}$*Tg* resembles that of the original wt parent line (TT2) rather than the *G9a*$^{-/-}$ line from which they were directly derived. These observations indicate that loss of DNA methylation in *G9a*$^{-/-}$ ES cells is not an irreversible process and that reintroduction of G9a is sufficient for the reestablishment of DNA methylation in G9a-deficient ES cells.

GLP is required for DNA methylation of retrotransposons

As G9a forms a complex with the closely related HMTase GLP, and both are required for the deposition of the H3K9me2 mark (Tachibana *et al*, 2005), we next determined whether GLP is also required for DNA methylation of retrotransposons. Genomic DNA isolated from wt TT2 and *GLP*$^{-/-}$ ES cells (Tachibana *et al*, 2005) was analysed by Southern blotting as above, using probes specific for IAP, MLV (Supplementary Figure S4) and L1 elements (data not shown). A significant DNA methylation defect is apparent for all three elements in the *GLP*$^{-/-}$ line as well, with IAP elements showing the most dramatic decrease. Furthermore, introduction of a wt GLP transgene into the *GLP*$^{-/-}$ line (generating the *GLP*$^{-/-}$*Tg* line (see Supplementary Figure S1; Tachibana *et al*, 2005) rescues the IAP DNA methylation defect and partially rescues the MLV methylation defect, revealing that DNA methylation can be reestablished on reintroduction of this HMTase as well. Consistent with these results, bisulphite sequencing analysis of polytrophic MLV elements reveals an ~40% reduction in DNA methylation across the 5′LTR in the *GLP*$^{-/-}$ line relative to the wt control, and a partial rescue of this methylation defect in the *GLP*$^{-/-}$*Tg* line (Supplementary Figure S4). Thus, both G9a and GLP have a function in DNA methylation of retrotransposons in ES cells.

DNA methylation at non-repetitive genomic regions is reduced in G9a$^{-/-}$ *cells*

To determine whether this DNA methylation defect extends to non-repetitive elements in the genome, we carried out MeDIP (Weber *et al*, 2005) on genomic DNA isolated from TT2, *G9a*$^{-/-}$ and *G9a*$^{-/-}$Tg ES cells and analysed the methylation status of 11 single-copy genomic regions, including 9 CpG-rich promoters shown previously to be methylated in ES cells (Mohn *et al*, 2008) (Figure 2A). Strikingly, all of the regions that are highly methylated in the TT2 line show a significant decrease in the *G9a*$^{-/-}$ line, including the germline-specific gene *Mage-a2*, shown previously to be aberrantly expressed in *G9a*$^{-/-}$ cells (Tachibana *et al*, 2002). As for the repetitive elements, DNA methylation is increased at most of these regions in the *G9a*$^{-/-}$Tg line. The DNA methylation defect was confirmed through bisulphite sequencing of the Dazl and Tuba3 promoter regions, both of which show an ~40% reduction in DNA methylation in the *G9a*$^{-/-}$ line (Figure 2B). The degree of demethylation across the Dazl promoter is similar to that observed in *Dnmt1*$^{-/-}$ and *Dnmt3a/b*$^{-/-}$ ES cells. In contrast, the degree of demethylation across the Tuba3 promoter in *G9a*$^{-/-}$ cells more closely resembles that observed in the *Dnmt3a/b*$^{-/-}$ line. Thus, although DNA

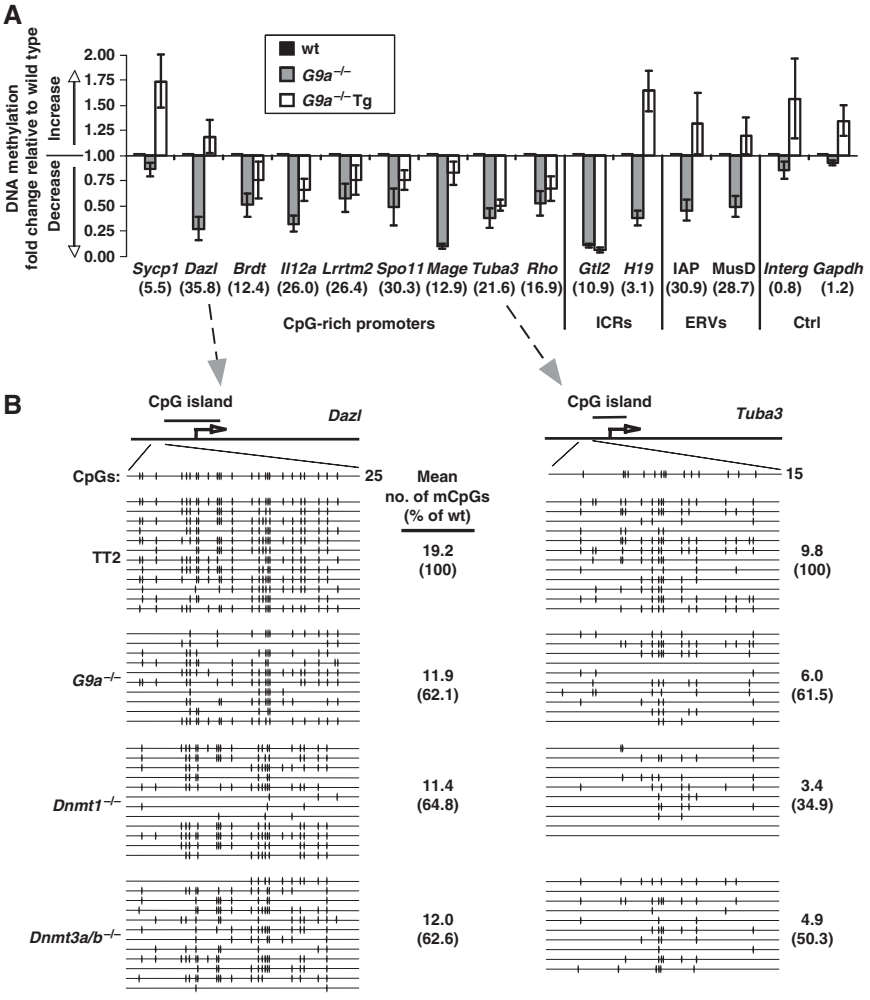

Figure 2 DNA methylation of promoter regions is reduced in *G9a*$^{-/-}$ cells. (**A**) MeDIP followed by quantitative PCR of nine CpG-rich promoter regions and two imprinting control loci (ICR) shown previously to be methylated in ES cells (Mohn *et al*, 2008) was conducted on wt, *G9a*$^{-/-}$ and *G9a*$^{-/-}$Tg lines. IAP and MusD amplicons were included as positive controls. An active housekeeping gene (Gapdh) and a CpG-poor intergenic region (Interg) were included as negative controls. A bar graph illustrating DNA methylation changes in *G9a*$^{-/-}$ and *G9a*$^{-/-}$Tg ES cells relative to wt ES cells (set to 1) is shown. The fold change is normalized to an unmethylated control gene (Hprt). Numbers in parentheses indicate the enrichment in MeDIP relative to Hprt. Error bars indicate the s.e.m. of at least three independent experiments. A lower level of methylation was detected in the *G9a*$^{-/-}$ line than the wt or rescued lines for all of the genes that show a high level of methylation in the TT2 parent line. (**B**) DNA methylation status of the germline-specific Dazl and Tuba3 genes in wt, *G9a*$^{-/-}$, *Dnmt1*$^{-/-}$ and *Dnmt3a/b*$^{-/-}$ cells was confirmed by bisulphite sequencing. The mean number of mCpGs per molecule sequenced is shown, along with the mean % of mCpGs relative to the wild-type line (in parentheses). Both promoters show an ~40% reduction in DNA methylation density in the *G9a*$^{-/-}$ line.

methylation of CpG-rich promoter regions is also dependent on G9a, the degree to which G9a influences DNA methylation state depends on the genomic context.

DNMT expression is not dramatically altered in G9a$^{-/-}$ *cells*

The observed DNA methylation defect prompted us to address whether Dnmt1, Dnmt3a, Dnmt3b or DNMT-like (Dnmt3L) are downregulated in *G9a*$^{-/-}$ ES cells. Quantitative RT–PCR analysis did not reveal a significant difference in mRNA levels of any of the DNMT family members in these lines (Figure 3A). Similarly, quantitative western blot analyses revealed a <2-fold difference in Dnmt1 or Dnmt3a2 expression levels and an ~2-fold higher level of Dnmt3b expression in the *G9a*$^{-/-}$ line than the wt parent line (Figure 3B). These data indicate that the DNA methylation defect observed in *G9a*$^{-/-}$ cells is unlikely to be a consequence of decreased DNMT expression.

Dnmt3a recruitment is reduced in G9a$^{-/-}$ *ES cells*

Introduction of Dnmt3a, Dnmt3a2 or Dnmt3b1 is sufficient to restore DNA methylation of retrotransposons in highly demethylated *Dnmt3a/b*$^{-/-}$ ES cells (Chen *et al*, 2003), indicating that *de novo* DNMT activity is required to maintain retrotransposons in a densely methylated state. To determine whether DNMT recruitment to such elements is perturbed in *G9a*$^{-/-}$ cells, chromatin immunoprecipitation (ChIP) was conducted using Dnmt1-, Dnmt3a/Dnmt3a2- or Dnmt3b-specific antibodies and unmodified histone H3 as an internal control. For Dnmt1 and Dnmt3b, ChIP experiments with two different antibodies specific for each did not yield conclusive results (data not shown). In contrast, a significant reduction in enrichment of Dnmt3a was detected in the LTR regions of MLV, IAP and MusD retrotransposons in the *G9a*$^{-/-}$ line (Figure 4A). Thus, the decrease in DNA methylation observed in *G9a*$^{-/-}$ cells can be attributed at

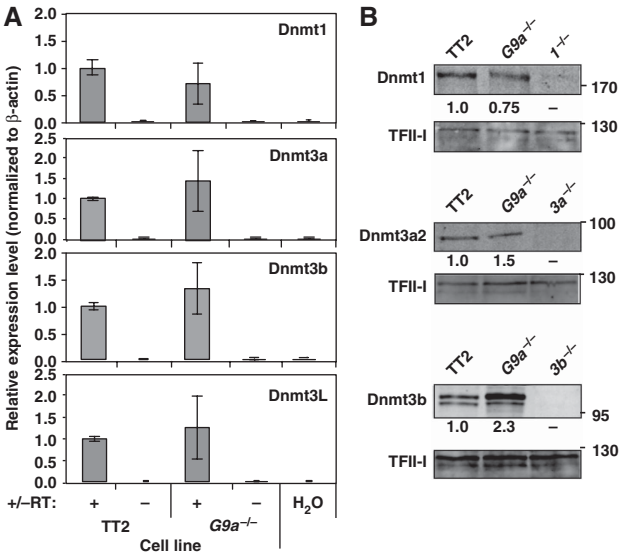

Figure 3 Expression of DNMTs in *G9a*$^{-/-}$ cells. (**A**) RNA was isolated from TT2 wt and *G9a*$^{-/-}$ cells and expression levels of Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L were determined by real-time quantitative RT–PCR, normalized to β-actin (RT–reverse transcriptase). Values represent the mean (±s.d.) expression level relative to the wild-type line, from three independent experiments. (**B**) Western blot analyses using quantitative two-colour fluorescence imaging was performed on nuclear extracts isolated from TT2 and *G9a*$^{-/-}$ cells, using antibodies specific for Dnmt1, Dnmt3a and DNMT3b and TFII-I as an internal control. Extract isolated from DNMT-deficient cells was used as a control for antibody specificity. Relative protein expression levels, normalized to TFII-I, are shown beneath each blot.

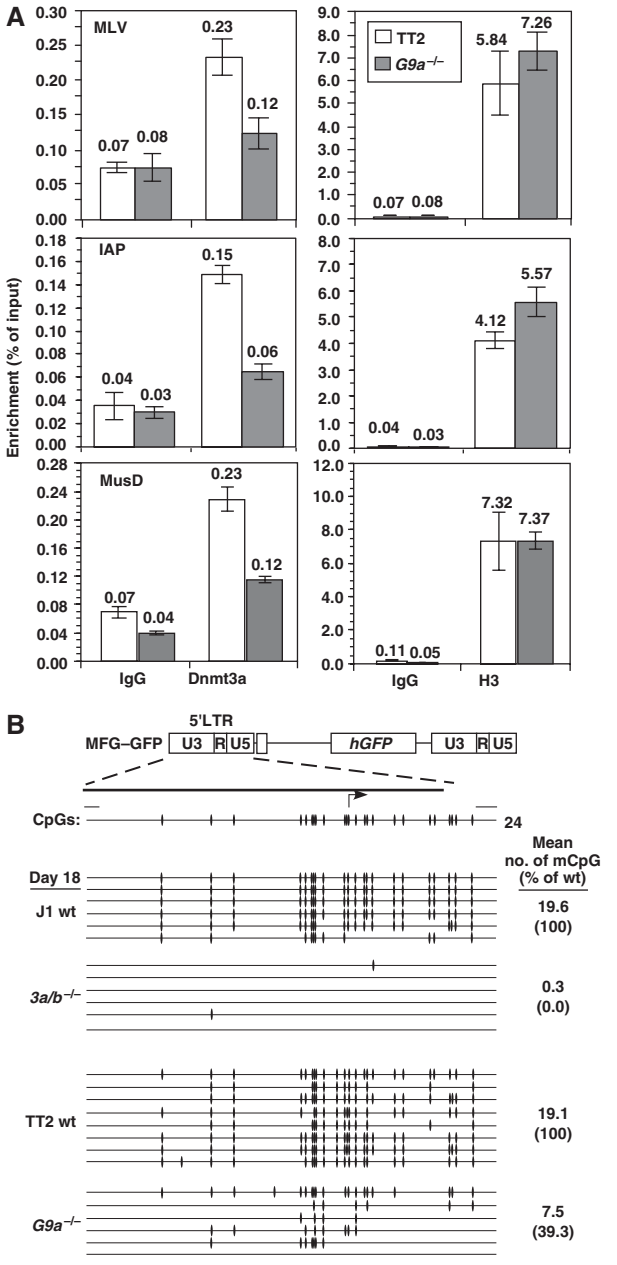

Figure 4 *G9a*$^{-/-}$ ES cells show defects in recruitment of Dnmt3a to ERVs and *de novo* methylation of introduced retroviruses. (**A**) Formaldehyde-fixed chromatin was isolated from TT2 and *G9a*$^{-/-}$ lines and ChIP was conducted using nonspecific IgG or antisera raised against Dnmt3a or unmodified histone H3. Real-time PCR using primers specific for the LTR regions of MLV, IAP and MusD ERVs was carried out and values are presented as percentage of input precipitated (±s.d.) relative to the input in the representative experiment shown. A significant reduction in Dnmt3a enrichment in the *G9a*$^{-/-}$ line relative to the wt control is clearly apparent. (**B**) TT2 wt, *G9a*$^{-/-}$, J1 wt and *Dnmt3a/b*$^{-/-}$ (*3a/b*$^{-/-}$) lines were infected with the retroviral vector MFG–GFP and passaged in the absence of selection. Genomic DNA was isolated on day 18 post-infection and analysed by bisulphite genomic sequencing.

least in part to a decrease in the efficiency of recruitment of *de novo* DNMT activity.

To independently assess whether deletion of G9a not only impairs maintenance of DNA methylation on already methylated loci but also influences the efficiency of *de novo* DNA methylation on previously unmethylated DNA, TT2 wt, *G9a*$^{-/-}$, J1 wt and *Dnmt3a/b*$^{-/-}$ ES cells were infected with the MLV-based retroviral vector MFG–GFP and the DNA methylation status of the proviral LTR was analysed at day 18 post-infection (Figure 4B). As expected, infected *Dnmt3a/b*$^{-/-}$ cells show virtually no DNA methylation at this time point. Strikingly, infected *G9a*$^{-/-}$ cells also show a significantly lower level of DNA methylation (>2.5-fold) than the parent line from which they were derived. Although not as dramatic as the methylation defect observed in the *Dnmt3a/b*$^{-/-}$-deficient cell line, this observation indicates that G9a is required for efficient *de novo* methylation in ES cells.

G9a is not required for transcriptional silencing of retrotransposons

As Dnmt1 was previously shown to be required for silencing of IAP elements in embryos (Walsh *et al*, 1998), we next determined whether the defect in DNA methylation of retrotransposons in *G9a*$^{-/-}$ cells is associated with aberrant expression of these potentially active endogenous elements. Expression of MLV and LINE1 elements was not detected above background levels in wt, *G9a*$^{-/-}$, *Dnmt1*$^{-/-}$ *or* *Dnmt3a/b*$^{-/-}$ lines by northern blotting (Figure 5A and data not shown). In contrast, IAP elements of each subtype (I, IΔ1 and II) (Kuff and Lueders, 1988) (Figure 5B) and MusD elements (Supplementary Figure S5) are expressed at a significantly higher level in the *Dnmt1*$^{-/-}$ line than the *G9a*$^{-/-}$ line, relative to the parent lines from which they were derived. Although not as dramatic as that observed in the *Dnmt1*$^{-/-}$ line, aberrant IAP expression was also observed in the *Dnmt3a/b*$^{-/-}$ line by RT–PCR (Figure 5C). As the G9a and

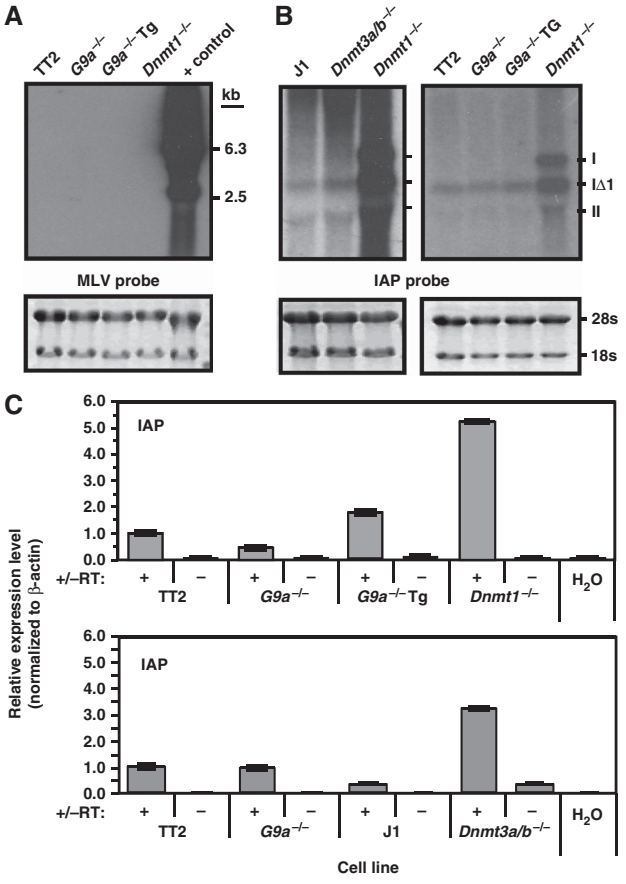

Figure 5 ERVs are not aberrantly expressed in $G9a^{-/-}$ cells. RNA was isolated from TT2 wt, $G9a^{-/-}$, $G9a^{-/-}$Tg, J1 wt, $Dnmt3a/b^{-/-}$ and $Dnmt1^{-/-}$ cells and analysed by northern blotting. RNA isolated from a cell line harbouring an active MLV-based retroviral vector was used as a positive control. 18 and 28s RNA loading controls are shown for each blot. (**A**) No expression of MLV was detected in any of the lines tested, using a probe specific for the MLV LTR region. (**B**) A high level of aberrant expression of the three subtypes (I, IΔ1 and II) of IAP elements was detected in the $Dnmt1^{-/-}$ line, but not the $G9a^{-/-}$ line, using a probe specific for the IAP LTR region. (**C**) Quantitative RT–PCR (+/−RT) using primers specific for the *Pol* region of full-length IAP elements revealed no increase in expression in the parent or $G9a^{-/-}$ lines, but a significant increase in expression in the $Dnmt1^{-/-}$ and $Dnmt3a/b^{-/-}$ lines.

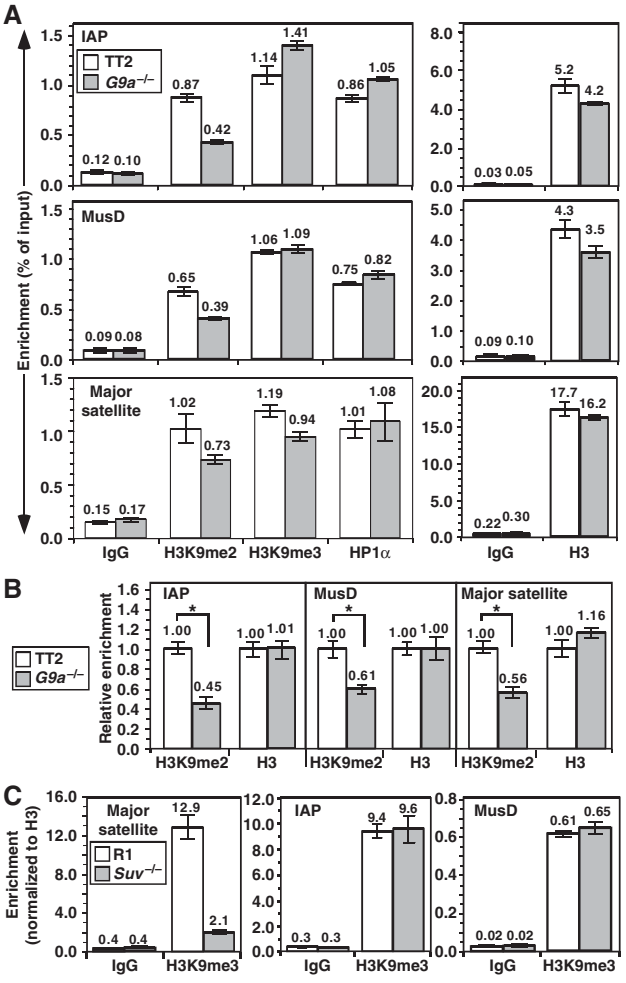

Figure 6 ERVs show a reduction in H3K9 dimethylation in $G9a^{-/-}$ cells, but no reduction in H3K9 trimethylation or HP1α binding. TT2 wt and $G9a^{-/-}$ ES cells were analysed using ChIP with specific for H3K9me2, H3K9me3, HP1α, unmodified H3 and nonspecific IgG (IgG) as a control. Quantitative real-time PCR was conducted using primers specific for IAP or MusD retrotransposons or major satellite repeats. (**A**) Mean enrichment values are presented as percentage of input precipitated (±s.d.), relative to the input in the representative experiment shown. (**B**) Plotting the mean relative enrichment (±s.d.) of H3K9me2 and H3 from three independent experiments reveals an ~2-fold decrease in H3K9me2 in the $G9a^{-/-}$ line relative to the parent line (*$P<0.05$, by Student's *t*-test) but no difference in H3 occupancy at these elements. (**C**) Relative to the R1 wt parent line, $Suv39h1/2^{-/-}$ ($Suv^{-/-}$) ES cells show a dramatic decrease in H3K9me3 only at major satellite repeats.

DNMT deletions were generated in ES cells of differing genetic backgrounds, it is not possible to attribute the differences in ERV expression exclusively to the genes deleted. Nevertheless, taken together with the DNA methylation data, these results indicate either that the level of residual DNA methylation is sufficient to maintain potentially active retroelements in a silent state in TT2 $G9a^{-/-}$ cells, or that an alternative repressive pathway exerts an effect on these elements independent of DNA methylation.

IAP and MusD ERVs show reduced H3K9 dimethylation in $G9a^{-/-}$ cells, whereas H3K9 trimethylation and HP1α binding are unaffected

Several groups have reported that ERVs and other repetitive sequences are marked by H3K9me2 and/or H3K9me3 in murine ES cells (Peters *et al*, 2003; Martens *et al*, 2005; Mikkelsen *et al*, 2007). As G9a is responsible for the majority of H3K9me2 in euchromatin (Tachibana *et al*, 2002), we next wished to determine whether ERVs show a decrease in either of these marks in $G9a^{-/-}$ cells. In addition, as methylation of H3K9 creates a binding site for the HP1 family of transcriptional repressor proteins (Lachner *et al*, 2001; Smallwood *et al*, 2007), we also wished to determine whether recruitment of HP1α is disrupted in the absence of G9a.

ChIP experiments using chromatin isolated from TT2 and $G9a^{-/-}$ cells and antisera specific for H3K9me2, H3K9me3a, HP1α and unmodified H3 revealed an ~2-fold reduction in H3K9me2 in the $G9a^{-/-}$ line in the LTR regions of IAP and MusD elements, in the absence of a significant change in H3 occupancy (Figure 6A and B). Consistent with a previous report indicating that H3K9me2 is decreased in the

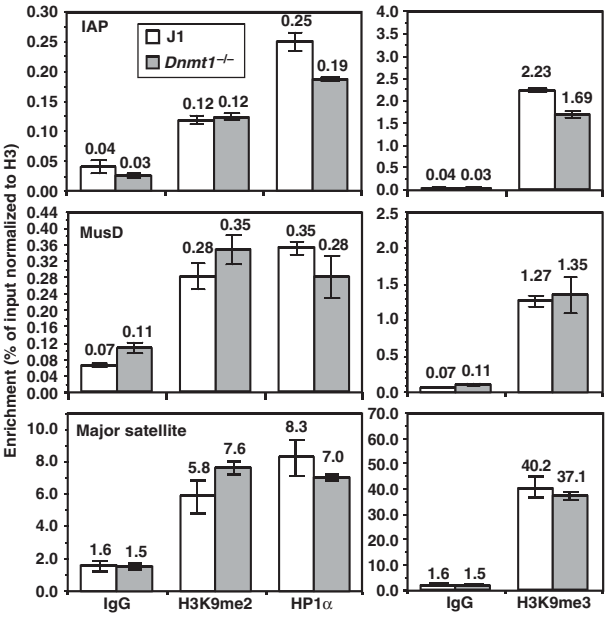

Figure 7 H3K9 methylation and HP1α binding at MusD, IAP and major satellite repeats in *Dnmt1*$^{-/-}$ cells. ChIP was conducted on J1 wt and *Dnmt1*$^{-/-}$ ES cells using antibodies specific for H3K9me2, H3K9me3, HP1α and unmodified H3. Nonspecific IgG was used as a control. Real-time PCR of reverse-crosslinked material using primers specific for IAP, MusD or major satellite repeats was conducted in triplicate and enrichment (±s.d.) is presented as the mean percentage of input material immunoprecipitated, normalized to unmodified H3. IAP elements show a modest reduction in H3K9me3 and HP1α binding in the *Dnmt1*$^{-/-}$ line, but no change in H3K9me2 enrichment. No significant difference in any of these features was detected at MusD or major satellite repeats.

pericentromeric compartment in *G9a*$^{-/-}$ ES cells (Rice *et al*, 2003), an ~2-fold decrease in H3K9me2 was also observed at major satellite repeats, indicating that G9a activity is not confined to the euchromatic compartment and perhaps explaining why major satellite repeats show a DNA methylation defect in *G9a*$^{-/-}$ cells. As we are simultaneously surveying the modification status of histones associated with multiple copies of each repeat, we cannot discriminate between complete loss of H3K9me2 at approximately half of the repeats, or a uniform ~2-fold decrease in this mark across all repeats. Nevertheless, these observations indicate that a significant number of IAP and MusD retrotransposons are direct targets of G9a.

Strikingly, no decrease in H3K9me3 or HP1α binding was detected at IAP or MusD retrotransposons in the *G9a*$^{-/-}$ line (Figure 6A), indicating that an alternative H3K9 HMTase is responsible for the deposition of the H3K9me3 mark at these elements. Although the HMTases Suv39h1 and Suv39h2 are required for H3K9me3 of major satellite repeats (Peters *et al*, 2003), they do not have a major function in the deposition of the H3K9me3 mark at IAP or MusD retrotransposons, as H3K9me3 enrichment is not reduced at these elements in *Suv39h1/2*$^{-/-}$ ES cells either (Figure 6C). In contrast, the promoter region of the G9a-regulated *Mage-a2* gene, which is significantly enriched for H3K9me2 in wt but not *G9a*$^{-/-}$ cells (Tachibana *et al*, 2002; Supplementary Figure S6), showed only very low levels of H3K9me3 enrichment in either line. Taken together, these results indicate that an HMTase with specificity for H3K9 other than G9a, Suv39h1 or Suv39h2 is responsible for H3K9 trimethylation of ERVs and that the deposition of this mark occurs independent of the DNA methylation state of these elements.

In contrast to *G9a*$^{-/-}$ cells, *Dnmt1*$^{-/-}$ cells show no decrease in H3K9me2 across the LTR regions of IAP and MusD elements when normalized to unmodified histone H3 (Figure 7), indicating that Dnmt1 is not required for the deposition of this mark by G9a. Surprisingly, HP1α binding and H3K9me3 across the LTR region of IAP and MusD elements also show only a modest or no reduction, respectively, despite the fact that these elements are hypomethylated and aberrantly expressed in *Dnmt1*$^{-/-}$ cells. The simplest explanation for this observation is that only a small number of proviruses of each class are actually transcribed in *Dnmt1*$^{-/-}$ cells and in turn depleted of H3K9me3 and HP1α. Alternatively, severely hypomethylated IAP and MusD elements may be transcriptionally active despite the presence of these repressive marks. In either case, these observations clearly demonstrate that H3K9me3 and HP1α binding at these elements are not dependent on the presence of dense DNA methylation.

Introduction of a catalytically inactive G9a transgene partially rescues the DNA methylation defect observed in G9a$^{-/-}$ *cells*

The observation of a decrease in DNA methylation of ERVs in *G9a*$^{-/-}$ cells, despite the presence of a high level of residual H3K9 methylation, is surprising, given the known function of K9 methylation in controlling DNA methylation in plants and filamentous fungi (Jackson *et al*, 2002; Freitag and Selker, 2005). To directly address whether DNA methylation of repetitive elements in mammalian cells is dependent on the catalytic activity of G9a, we took advantage of the previously described observation that the DNA methylation defect observed in *G9a*$^{-/-}$ cells is rescued by the introduction of a wt G9a transgene (see Figure 1).

Constructs encoding two G9a mutants (*G9a*$^{-/-}$Tg(C1168A) and *G9a*$^{-/-}$Tg(Y1120V;Y1207F)), each of which harbour amino-acid substitutions in the SET domain that reduce catalytic activity to <1% of that of wt G9a, but do not affect the ability of the encoded protein to form a complex with GLP (see Tachibana *et al*, this issue), were stably introduced into the *G9a*$^{-/-}$ line. Western blot analyses of cell lines stably expressing each of these transgenes revealed that the exogenous wt and mutant proteins are produced at the expected molecular weight (Figure 8A). Furthermore, quantitative western blot analysis reveals that GLP is expressed at similar levels in the wt, *G9a*$^{-/-}$ *and G9a*$^{-/-}$ Tg lines, confirming that the expression of G9a does not significantly influence the stability of its binding partner GLP (Tachibana *et al*, 2005) (Figure 8B). As expected, Southern blot analysis with an IAP-specific probe reveals that DNA methylation is reduced in the *G9a*$^{-/-}$ parent line and restored to wt levels in the *G9a*$^{-/-}$Tg(wt) line (Figure 8C). Strikingly, DNA methylation of IAP elements is also increased in cells expressing either of the catalytic mutants, albeit not to the same level as observed for the wt transgene (Figure 8C–E). DNA methylation levels at MusD (Supplementary Figure S7) and L1 repeats (data not shown) were also significantly increased in the *G9a*$^{-/-}$Tg(C1168A) line relative to the parent *G9a*$^{-/-}$ line.

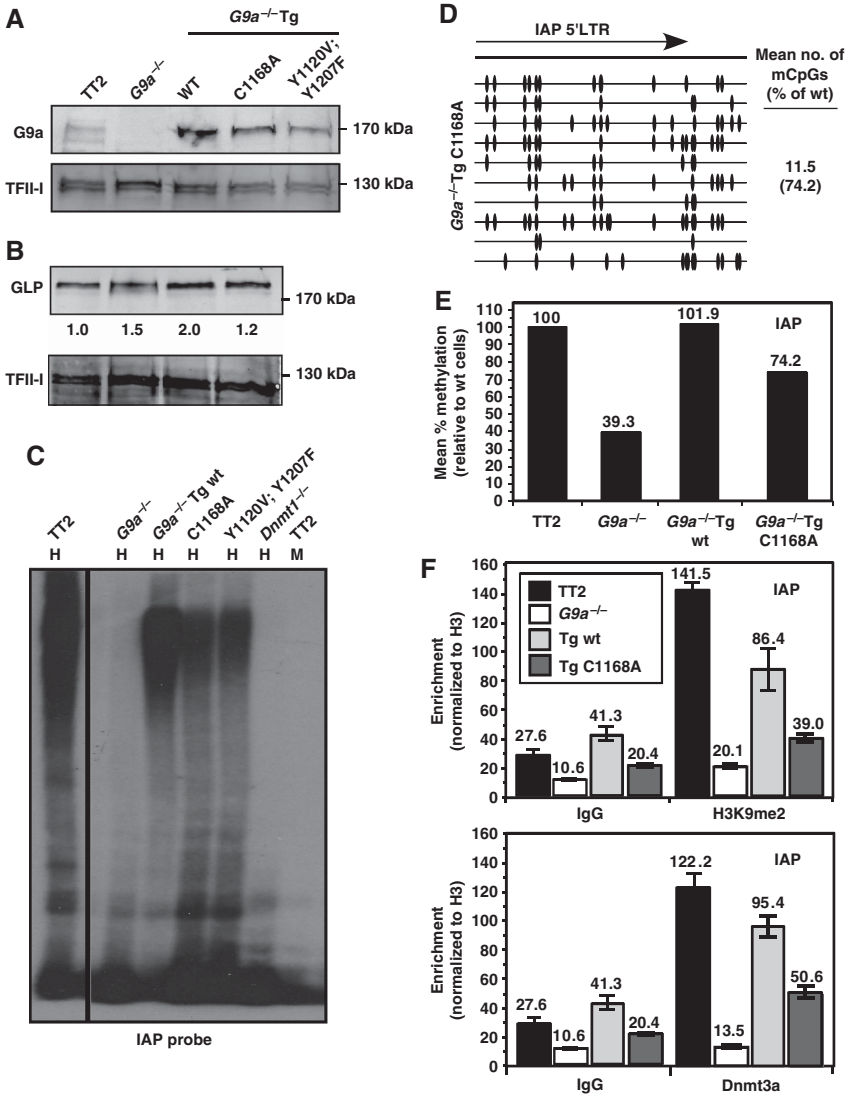

Figure 8 Stable expression of catalytically inactive G9a transgenes is sufficient to rescue the DNA methylation defect observed in $G9a^{-/-}$ ES cells. The $G9a^{-/-}$ line 2-3 was stably transfected with constructs encoding a wt G9a transgene $G9a^{-/-}$Tg(wt), or the mutant G9a transgenes $G9a^{-/-}$Tg(C1168A) and $G9a^{-/-}$Tg(Y1120V;Y1207F). Each of the latter transgenes harbour amino-acid substitutions in the SET domain that reduce the catalytic activity of the encoded protein to <1% of that of the wt protein. (**A**) Western blot analysis of cell lines expressing each of these transgenes reveals that the mutant proteins are expressed at the expected molecular weight. An antibody specific for TFII-I was used as a loading control. (**B**) Quantitative western blot analysis of GLP expression in these lines was determined by normalizing to the signal obtained for endogenous TFII-I on the same blot. (**C**) Genomic DNA isolated from wt (TT2), $G9a^{-/-}$ (2-3), $G9a^{-/-}$Tg(wt), $G9a^{-/-}$Tg(C1168A), $G9a^{-/-}$Tg(Y1120V;Y1207F) and $Dnmt1^{-/-}$ ES cells was digested with *Hpa*II (H) and subject to Southern blotting using an IAP-specific probe. *Msp*I (M) was used as a control. (**D**) Bisulphite analysis using primers specific for the IAP 5′LTR confirms that expression of catalytically inactive G9a partially rescues the DNA methylation defect. (**E**) A bar graph showing the mean no. of mCpGs per molecule sequenced is shown for the bisulphite data presented in (D) and Figure 1D. (**F**) ChIP was conducted on the wt (TT2), $G9a^{-/-}$ (2-3), $G9a^{-/-}$Tg(wt) and $G9a^{-/-}$Tg(C1168A) lines using antisera raised against H3K9me2, Dnmt3a or unmodified H3. Nonspecific IgG was included as a control. Real-time PCR of reverse-crosslinked material using IAP-specific primers was carried out in triplicate and enrichment (±s.d.) is presented as the mean percentage of input material immunoprecipitated, normalized to unmodified H3.

Importantly, the level of H3K9me2 enrichment in the $G9a^{-/-}$Tg(C1168A) line remains significantly below that observed for the TT2 and $G9a^{-/-}$Tg(wt) lines (Figure 8F). Furthermore, rescue of the DNA methylation defect in the $G9a^{-/-}$Tg(C1168A) line is accompanied by recruitment of Dnmt3a to the IAP LTR, although at a level lower than that observed in the wt parent line or the $G9a^{-/-}$ line rescued with the wt G9a transgene (Figure 8F). Taken together, these results reveal that independent of its catalytic activity, G9a promotes *de novo* DNA methylation through enhancing recruitment of Dnmt3a.

Discussion

We demonstrate that G9a is required for DNA methylation of representative LTR and non-LTR retrotransposons and a number of CpG-rich promoters in murine ES cells. However, unlike the H3K9 HMTases in plants and filamentous fungi (Jackson *et al*, 2002; Freitag and Selker, 2005), G9a promotes DNA methylation of repetitive elements independent of its catalytic activity. How might G9a influence DNA methylation, given that H3K9 methylation *per se* does not seem to be the predominant trigger? Although it is possible

that deletion of G9a influences DNA methylation through an indirect mechanism, given that H3K9me2 is decreased at each of the genomic regions analysed, we favour the possibility that G9a–GLP exert an effect *in cis* to promote DNA methylation. Indeed, two groups recently reported that in somatic cells, G9a interacts directly with Dnmt1 in a complex that includes PCNA (Esteve *et al*, 2006; Sharif *et al*, 2007), indicating that Dnmt1 and G9a coordinate H3K9 methylation and maintenance DNA methylation at the replication fork.

Our survey of the DNA methylation status of a number of repetitive and single-copy genomic sequences reveals that the extent of the defect in *G9a*$^{-/-}$ ES cells is generally not as severe as that in *Dnmt1*$^{-/-}$ ES cells. Although we did detect an interaction between G9a and GLP through co-immunoprecipitation, as described earlier (Tachibana *et al*, 2005), we were unable to detect an interaction between G9a and any of the DNMTs in ES cells (MCL and SL, unpublished data). On the other hand, we did find that Dnmt3a recruitment to the promoter regions of LTR retrotransposons was reduced in the *G9a*$^{-/-}$ line relative to the wt control.

Thus, although we cannot confirm whether G9a influences Dnmt1 activity in ES cells, we propose that G9a regulates DNA methylation in these cells at least in part by promoting *de novo* DNMT activity in *cis*. In support of this model, we find that introduced MLV-based retroviral vectors, which are unmethylated at the time of integration, are not efficiently *de novo* methylated in *G9a*$^{-/-}$ cells and show a defect in silencing similar to that observed in *Dnmt3a/b*$^{-/-}$ ES cells infected with the same virus (KBD and MCL, in preparation). As maintenance methylation by Dnmt1 is reported to be an inefficient process in ES cells (Liang *et al*, 2002), continual *de novo* methylation may be required to preserve DNA methylation homoeostasis. Alternatively, active demethylation by an as yet unidentified DNA demethylase may necessitate ongoing *de novo* methylation by Dnmt3a and/or Dnmt3b to maintain steady-state levels of this epigenetic mark.

Intriguingly, despite the fact that the potentially active IAP and MusD retrotransposons show a dramatic reduction in DNA methylation in *G9a*$^{-/-}$ cells, these interspersed repetitive elements remain transcriptionally inactive. As *G9a*$^{-/-}$ cells show a somewhat higher level of residual DNA methylation than *Dnmt1*$^{-/-}$ ES cells, in which these elements are aberrantly expressed, it is possible that potentially active ERVs are expressed only when DNA methylation density drops below a critical threshold. Consistent with this model, unlike Dnmt1-null mice, which show high levels of IAP expression (Walsh *et al*, 1998), compound heterozygous mice carrying a hypomorphic Dnmt1 allele over a null allele show genome-wide hypomethylation but no detectable IAP expression (Howard *et al*, 2008).

Alternatively, as H3K9me3 enrichment and HP1α binding at IAP and MusD elements are not dramatically reduced in *G9a*$^{-/-}$ or *Dnmt1*$^{-/-}$ cells, it is possible that an alternative repressive pathway maintains the vast majority of ERVs in a silent state independent of DNA methylation. Our observations clearly show that an HMTase with specificity for H3K9 other than G9a, GLP, Suv39h1 or Suv39h2 marks LTR retrotransposons in ES cells, leaving SETDB1, which shows di- and tri-methyl HMTase activity towards H3K9 *in vitro* and *in vivo* (Wang *et al*, 2003), or the closely related SETDB2, as the remaining candidates in the Suv39 family of HMTases for this activity.

Given the well-documented deleterious effects of retrotransposition on genomic integrity, the existence of a DNA methylation-independent silencing pathway may serve to minimize proviral expression during those stages in embryonic development when DNA methylation levels are relatively low, such as following fertilization or in the developing germ line. Intriguingly, deletion of the Piwi protein Mili leads to derepression of L1 and IAP ERVs and the loss of DNA methylation at L1 elements (Aravin *et al*, 2007), revealing that Piwi-interacting RNAs (piRNAs) generated by transposable elements in the germ line are required to maintain these elements in a silent state. Such piRNAs may have a function in the targeting of H3K9 HMTase activity to homologous repetitive elements prior to *de novo* DNA methylation of these elements.

We show that G9a is required for DNA methylation in ES cells not only of repetitive elements but also of the CpG-rich promoter regions of a number of genes that are normally densely methylated in ES cells. These results are consistent with those reported by Tachibana and colleagues (see accompanying paper by Tachibana *et al*), and indicate that in addition to the deposition of the H3K9me2 mark, the G9a–GLP complex may have a genome-wide influence on DNA methylation homoeostasis in ES cells. As GLP expression is downregulated in primordial germ cells, coincident with genome-wide DNA demethylation in these cells (Seki *et al*, 2005, 2007), it is possible that the G9a–GLP heteromeric complex has a function in the programmed changes in DNA methylation that occur not only following fertilization but also in the developing germ line.

Materials and methods

Cell lines

J1 wt (129S4/SvJae), *Dnmt1*$^{c/c}$ (Dnmt1$^{-/-}$) (Lei *et al*, 1996), Dnmt3a and Dnmt3b double-negative (*Dnmt3a/b*$^{-/-}$) (Okano *et al*, 1999), TT2 wt (c57BL/6xCBA), *G9a*$^{-/-}$ (clones 2-3 and 22-10), *G9a*$^{-/-}$*Tg* (clone 15-3) (Tachibana *et al*, 2002), *GLP*$^{-/-}$, *GLP*$^{-/-}$*Tg* (Tachibana *et al*, 2005), *G9a*$^{-/-}$Tg(wt), *G9a*$^{-/-}$Tg(C1168A) (clone G4), *G9a*$^{-/-}$Tg(Y1120V;Y1207F) (clone G7), R1 wt (129X1/SvJ × 129S1) and Suv39h1 and Suv39h2 double-negative (*Suv39h1/2*$^{-/-}$) (Peters *et al*, 2001) ES cells were passaged every 48–72 h in DMEM supplemented with 15% FBS (HyClone), 20 mM HEPES, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.05 mM streptomycin, leukaemia-inhibitory factor and 2 mM glutamine on gelatinized plates.

Bisulphite sequencing and MeDIP analyses

Genomic DNA was subject to sodium bisulphite conversion using the EZ DNA Methylation-Gold kit (Zymo Research) as described earlier (Appanah *et al*, 2007). MeDIP was conducted as described earlier (Weber *et al*, 2007). Detailed protocols are provided in the Supplementary data.

Northern and Southern blot analyses

Southern blot analyses, restriction digests, membrane transfers and preparation of the DNA probe were performed by standard methods. A detailed protocol is provided in the Supplementary data.

Quantification of proviral mRNA levels

RNA was isolated using Tri reagent (Sigma) according to the manufacturer's protocol. DnaseI-treated RNA was subject to first-strand cDNA synthesis using RevertAid H Minus kit (Fermentas) in the presence or absence of reverse transcriptase. Quantitative RT–PCR using MLV-, IAP- and MusD-specific primers, or β-actin-specific primers as an internal control (all primer sequences are listed in Supplementary Table 1), was conducted with EvaGreen dye (Biotium) on an Opticon 2 thermal cycler (Bio-Rad). Relative expression levels were determined by normalizing to the *β-actin* gene.

Antibodies and ChIP experiments

ChIP for histones (Appanah *et al*, 2007) and non-histone proteins (O'Geen *et al*, 2007) was conducted as described. Details are provided in the Supplementary data.

Western blot analysis

Nuclear extractions were conducted as described (Tachibana *et al*, 2002). Western blot analyses were conducted using the Odyssey Infrared Imaging System (LI-COR Biosciences), as described in the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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