Supporting Information

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SI Materials and Methods

Cell Lines and Culture Conditions. J1 WT (129S4/SvJae), *Dnmt1*$^{c/c}$ (*Dnmt1*$^{-/-}$) (1), *Dnmt3a* and *Dnmt3b* double-negative (*Dnmt3a/b*$^{-/-}$), *Dnmt3a*$^{-/-}$ (clone 6aa), *Dnmt3b*$^{-/-}$ (clone 8bb) (2), TT2 WT (c57BL/6 × CBA), *G9a*$^{-/-}$ (clone 2-3), *G9a*$^{-/-}$*Tg* (clone 15-3) (3), *G9a*$^{-/-}$*Tg* C1168A (clone G4) (4), R1 WT (129 × 1/SvJ × 129S1), and *Suv39h1* and *Suv39h2* double-negative (*Suv39h1/2*$^{-/-}$) (5) mESCs were passaged every 48–72 h in DMEM supplemented with 15% FBS (HyClone), 20 mM Hepes, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 100 U/mL penicillin, 0.05 mM streptomycin, leukemia inhibitory factor, and 2 mM glutamine on gelatinized plates.

Viral Infections and Analysis of Proviral Load. For retroviral infections, ~1.5 × 10^6 Phoenix A cells were transfected with plasmid DNA, as described previously (6). At 48 h post-transfection, 500–1100 μL of viral supernatant was added to mESCs supplemented with 4 μg/mL of polybrene. Infected mESCs were centrifuged for 45 min at 1,000 × *g* in a Heraeus Labofuge 400 and cultured at 37 °C. Relative proviral copy number was determined by quantitative real-time PCR using genomic DNA isolated from infected pools and primers specific for the GFP gene, normalized to the endogenous *β-major* gene.

Flow Cytometry. Trypsinized cells were resuspended in 500 μL of phosphate-buffered saline supplemented with 2% bovine calf serum and 1 μg/mL of propidium iodide and analyzed by flow cytometry using a BD LSRII flow cytometer. Data on at least 10,000 viable cells (as determined by electronic gating in the forward and side scatter channels) were collected for each sample and analyzed using FlowJo software (Treestar). To generate a population of cells harboring silent provirus, mESCs were infected with MSCV-GFP and cultured for 14 d to allow for proviral silencing. GFP-negative cells were subsequently isolated by FACS.

Establishment of G9a CKO MEFs and mESCs. Primary MEF cultures were established from embryos carrying two conditional alleles of *G9a*, which contain *loxP* sites flanking exons 4–20 that encode the ankyrin repeat region (7). When deleted, the resulting frameshift mutation prevents transcription of the SET domain. A Cre-ERT2 expression vector was stably introduced into these cells, along with SV40T antigen, and immortalized clones were isolated. Conditional inactivation of G9a was achieved by treating these clones with 4-OHT, which promotes translocation of the Cre–ER fusion into the nucleus. Secondary *G9a*$^{-/-}$ subclones were subsequently isolated. Derivation of *G9a* CKO mESCs was as done described previously (8).

DNA Methylation Analyses. To analyze the methylation status of the introduced provirus, 0.2 μg of genomic DNA was subjected to sodium bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research) as described previously (9). Primers specific for the 5′ LTR region of the MSCV or MFG vectors or the GFP gene (listed in Table S1) were used in nested or seminested PCR reactions. PCR products were cloned via T/A cloning using the pGEM-T Easy Kit (Promega), and individual inserts were sequenced using BigDye v3.1 (Applied Biosystems). Sequencing data were analyzed using Sequencher software (Gene Codes). The mean number of methylated CpGs/molecule sequenced was calculated for each set of samples.

Western Blot Analysis. Nuclear and whole cell extracts were isolated as described previously (4, 10) and evaluated by Western blot analysis using the Odyssey Infrared Imaging System (LI-COR Biosciences), according to the manufacturer's protocol. Antibodies used included G9a (1:2,000; PPMX, A8620A), TFII-I (1:1,000; a kind gift from Ivan Sadowski, University of British Columbia), Dnmt1 (1:500; Imgenex, IMG-261A), and β-actin (1:2,000; MP Biomedical). To confirm the specificity of the antibodies raised against H3K9me2 (1:200; Upstate Biotechnology, 07-441) and H3K9me3 (1:200; Active Motif, 39161), Western blot analyses were conducted using whole cell extracts (generated by lysis directly in 2× Lamelli buffer) isolated from 10^4 *G9a*$^{-/-}$ and *Suv39h1/h2*$^{-/-}$ mESCs, which were found to have reduced levels of H3K9me2 and H3K9me3, respectively (3, 11, 12). An antibody specific for unmodified H3 (Abcam ab1791; 1:200) was used as a control.

RNA Extraction and RT-PCR. RNA was isolated using TRI Reagent (Sigma-Aldrich) according to the manufacturer's protocol. DnaseI-treated RNA was subjected to first-strand cDNA synthesis using the RevertAid H Minus Kit (Fermentas) in the presence or absence of reverse transcriptase. qRT-PCR using *GFP-, MLV-, G9a-,* and *Eset*-specific primers or *β-actin*–specific primers as an internal control (primer sequences listed in Table S1) was conducted with EvaGreen dye (Biotium) on an Opticon 2 thermal cycler (Bio-Rad). Relative expression levels were determined by normalizing to the endogenous β-actin gene.

Native ChIP. To generate chromatin for native ChIP, 1 × 10^{7}cells were harvested and washed in phosphate-buffered saline. Cells were resuspended in 250 μL of douncing buffer [10 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM $CaCl_2$, 1× protease inhibitory mixture (PIC)] and homogenized through a 25-gauge needle syringe for 25 repetitions. Subsequently, 1.25 μL of 50 U/mL of MNase was added to the nuclei and incubated at 37 °C for 7 min. The reaction was quenched by 0.5 M EDTA and incubated on ice for 5 min. Hypotonic lysis buffer (1 mL) [0.2 mM EDTA (pH 8.0), 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1.5 mM DTT, 1× PIC) was added, and the mixture was incubated for 1 h on ice. Cellular debris was pelleted, and the supernatant was recovered.

To generate preblocked beads for purification of immunoprecipitated material, 300 μL of protein A and protein G Sepharose beads were mixed and washed twice with 1 mL of IP buffer [10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 90 mM NaCl, 2 mM EDTA, 1× PIC]. Beads were blocked with 300 μg of sonicated salmon sperm DNA and 750 μg of BSA and rotated at 4 °C for 3 h. The beads were then washed once more with IP buffer and finally resuspended in 1× volume of IP buffer. To preclear chromatin, 100 μL of the blocked protein A/G beads were added to the digested chromatin fractions and rotated at 4 °C for 2 h. Then 100 μL of the precleared chromatin was purified by phenol-chloroform extraction, and DNA fragment sizes were analyzed on a 1.5% agarose gel.

Digested chromatin was divided into 1 × 10^6 cell equivalents per IP, and the volume was adjusted to 325 μL with IP buffer. Antibodies specific for H3K4me2 (3 μg; Abcam, ab7766), H3K9ac (5 μg; Upstate Biotechnology, 06-599), H3K9me2 (5 μg; Abcam, ab1220), and H3K9me3 (5 μg; Active Motif, 39161) were added to each tube and rotated at 4 °C for 1 h. The antibody–protein–DNA complex was precipitated by adding 20 μL of the blocked protein A/G beads and rotated at 4 °C overnight. The immunoprecipitated complex was

washed twice with 400 μL of ChIP Wash Buffer [20 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 1× PIC], followed by a single wash with ChIP Final Wash Buffer [20 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 1× PIC]. The protein–DNA complex was eluted by incubating the beads in 200 μL of elution buffer (100 mM $NaHCO_3$, 1% SDS) at 68 °C for 2 h. Immunoprecipitated material was purified using the QIAquick PCR Purification Kit (Qiagen) in 50 μL of elution buffer, according to the manufacturer's protocol. Purified DNA was diluted 1:4 and analyzed by qPCR (in triplicate) with EvaGreen dye and Hot-Start Taq polymerase (Fermentas) using 2 μL of template. Each experiment was performed at least twice with independent chromatin samples and yielded similar results.

1. Lei H, et al. (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development* 122:3195–3205.
2. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99: 247–257.
3. Tachibana M, et al. (2002) G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev* 16:1779–1791.
4. Tachibana M, Matsumura Y, Fukuda M, Kimura H, Shinkai Y (2008) G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. *EMBO J* 27:2681–2690.
5. Peters AH, et al. (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* 107:323–337.
6. Matsui T, et al. (2010) Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* 464:927–931.
7. Lehnertz B, et al. (2010) Activating and inhibitory functions for the histone lysine methyltransferase G9a in T helper cell differentiation and function. *J Exp Med* 207: 915–922.
8. Tachibana M, Nozaki M, Takeda N, Shinkai Y (2007) Functional dynamics of H3K9 methylation during meiotic prophase progression. *EMBO J* 26:3346–3359.
9. Appanah R, Dickerson DR, Goyal P, Groudine M, Lorincz MC (2007) An unmethylated 3′ promoter-proximal region is required for efficient transcription initiation. *PLoS Genet* 3:e27.
10. Nickerson JA, Krockmalnic G, He DC, Penman S (1990) Immunolocalization in three dimensions: Immunogold staining of cytoskeletal and nuclear matrix proteins in resinless electron microscopy sections. *Proc Natl Acad Sci USA* 87:2259–2263.
11. Peters AH, et al. (2003) Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol Cell* 12:1577–1589.
12. Rice JC, et al. (2003) Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* 12:1591–1598.
13. Dong KB, et al. (2008) DNA methylation in ES cells requires the lysine methyltransferase G9a but not its catalytic activity. *EMBO J* 27:2691–2701.

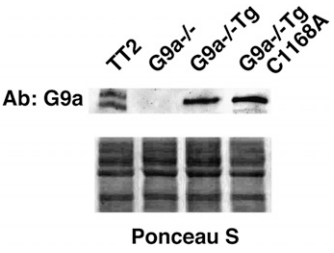

Fig. S1. Western blot analysis of G9a protein levels. Western blot analysis of *G9a* expression was conducted with whole cell extracts from WT (TT2), $G9a^{-/-}$, and $G9a^{-/-}$ mESCs rescued with a WT ($G9a^{-/-}$Tg) or a catalytically inactive ($G9a^{-/-}$Tg C1168A) *G9a* transgene. Ponceau S staining was used as a loading control.

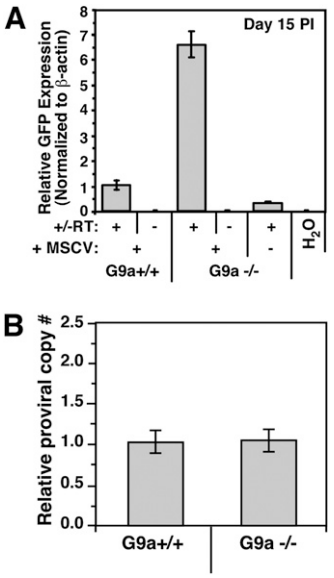

Fig. S2. qPCR analysis confirms that *GFP* expression is increased in $G9a^{-/-}$ mESCs and is not the result of a higher proviral load in the $G9a^{-/-}$ line compared with the WT line. (*A*) Proviral expression was determined independently at day 15 PI by qRT-PCR in the presence or absence of reverse transcriptase (± RT), using the *β-actin* gene as an internal control. (*B*) Proviral copy number was determined by qPCR using primers specific for the *GFP* gene, normalized to the endogenous *β-major* gene. The mean (± SD) proviral DNA content of the infected $G9a^{-/-}$ pool is presented relative to the corresponding infected TT2 WT pool.

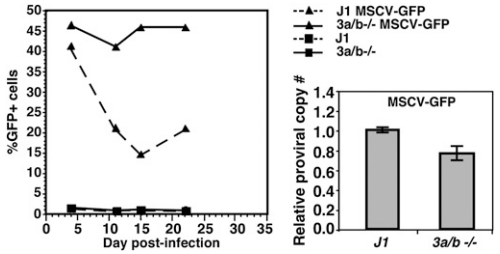

Fig. S3. *Dnmt3a/b*$^{-/-}$ mESCs show a defect in proviral silencing similar to that observed in *G9a*$^{-/-}$ cells. J1 (WT) and *Dnmt3a/b*$^{-/-}$ mESCs were infected in parallel with MSCV-GFP and analyzed by flow cytometry for GFP expression at successive time points, PI. The mean (± SD) proviral DNA content of the infected *Dnmt3a/b*$^{-/-}$ pool is presented relative to the corresponding infected J1 WT pool.

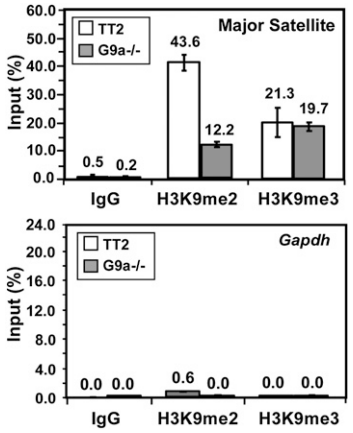

Fig. S4. H3K9me2, but not H3K9me3, is reduced in pericentromeric heterochromatin in *G9a*$^{-/-}$ mESCs. Chromatin isolated from MSCV-GFP–infected TT2 and *G9a*$^{-/-}$ mESCs was subject to ChIP using antibodies specific for H3K9me2 and H3K9me3. Nonspecific IgG was used as a control. A representative experiment is presented, showing the mean level of enrichment of technical replicates (conducted in triplicate), expressed as a percentage of input (± SD) material, as measured by qPCR. Primers specific for major satellite repeats or the *Gapdh* promoter region were used. Each experiment was performed at least twice with independent chromatin samples and yielded similar results. As expected, enrichment of H3K9me2, but not H3K9me3, was reduced in major satellite repeats, and no enrichment was observed in *Gapdh*.

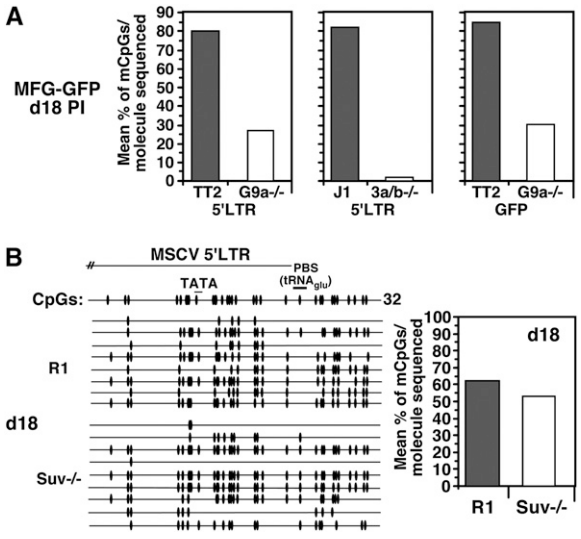

Fig. S5. DNA methylation analysis of MFG and MSCV proviral LTRs. (*A*) The mean percentage of methylated CpGs/molecule sequenced (a minimum of six molecules per cell line) is shown for an amplicon specific for the MFG 5′ LTR (described in ref. 13) and the *GFP* region at day 18 (d18) PI. (*B*) Bisulfite analysis was conducted in parallel on genomic DNA from MSCV-GFP infected *R1 and Suv39h1/h2*$^{-/-}$ mESCs isolated at d18 PI, using the same MSCV 5′ LTR-specific primers used in Fig. 5. Each row represents an individual sequenced molecule and the presence of a methylated CpG is indicated with a black oval. The mean percentage of methylated CpGs/molecule sequenced is shown.

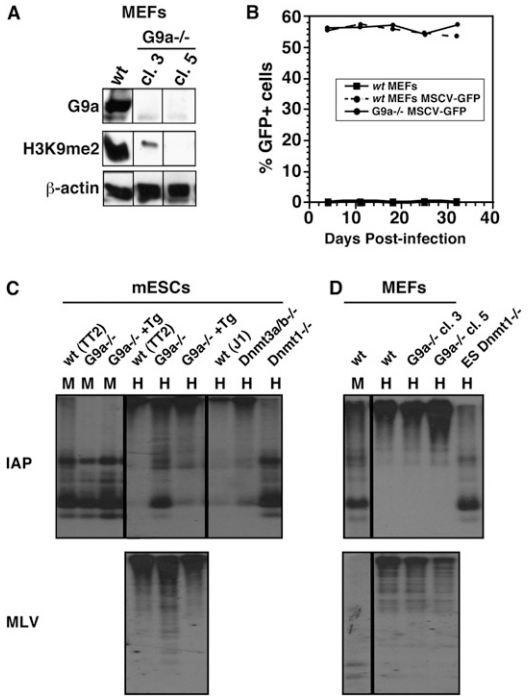

Fig. S6. G9a is not required for the maintenance of proviral DNA methylation in MEFs. MEFs harboring conditional *G9a* alleles were derived as described in *Materials and Methods*. (*A*) Western blot analysis of G9a expression and H3K9me2 in two clones after 4-OHT treatment reveals efficient depletion of G9a and concomitant loss of H3K9me2 in both. (*B*) Clone 3 was infected with MSCV-GFP virus and analyzed by flow cytometry as described for mESCs. (*C*) The methylation state of IAP and MLV ERVs in TT2 (WT), *G9a*$^{-/-}$, J1 (WT), *Dnmt1*$^{-/-}$, and *Dnmt3a/b*$^{-/-}$ mESCs was determined by Southern blot analysis using the methylation-sensitive restriction enzyme *HpaII* (H) and the methylation-insensitive isoschizomer *MspI* (M) as a control. Primers used to generate IAP- and MLV-specific probes are listed in Table S1. (*D*) The DNA methylation status of IAP and MLV ERVs in WT and *G9a*$^{-/-}$ MEFs (clone 3) was determined as described for *C*.

Table S1. Primer list

Primer name	Application	Sequence (5′ to 3′)
Gapdh F	ChIP	ATCCTGTAGGCCAGGTGATG
Gapdh R	ChIP	AGGCTCAAGGGCTTTTAAGG
Mage-a2 F	ChIP	TTGGTGGACAGGGAAGCTAGGGGA
Mage-a2 R	ChIP	CGCTCCAGAACAAAATGGCGCAGA
Major Sat F	ChIP	GACGACTTGAAAAATGACGAAATC
Major Sat R	ChIP	CATATTCCAGGTCCTTCAGTGTGC
MSCV LTR F	ChIP	TTCGGATGCAAACAGCAAGAGGC
MSCV LTR R	ChIP	AACCATCAGATGTTTCCAGGGTG
GFP F	ChIP/copy number	GGCGGATCTTGAAGTTCACC
GFP R	ChIP/copy number	ACTACAACAGCCACAACGTCTATATCA
β-Major F	Copy number	CTGCTCACACAGGATAGAGAGGG
β-Major R	Copy number	GCAAATGTGAGGAGCAACTGATC
MFG LTR F round 1	Bisulfite	TAGGTTTGGTAAGTTAGTTTAAGTAAYGTT
MFG LTR R round 1	Bisulfite	TAAAAAAATAATAACAATCTAACCCRAAC
MFG LTR F round 2	Bisulfite	GATTTATTATTGGGAGGTAAGTTGGTTAGT
MFG LTR R round 2	Bisulfite	TAAAAAAATAATAACAATCTAACCCRAAC
Gfp F round 1	Bisulfite	ATTATTTTTTAGATTGTTATGGTGAGTAAGGG
Gfp R round 1	Bisulfite	TAACTATTATAATTATACTCCAACTTATACC
Gfp F round 2	Bisulfite	GAGGAGTTGTTTATYGGGGTGGTGTTT
Gfp R round 2	Bisulfite	TAACTATTATAATTATACTCCAACTTATACC
MSCV LTR F round 1	Bisulfite	TAGGTTTGGTAAGTTAGTTTAAGTAAYGTT
MSCV LTR R round 1	Bisulfite	AATACAAAACTAATTAACTAACTAATACAA
MSCV LTR F round 2	Bisulfite	TTGTAAGGTATGGAAAATATATAATTG
MSCV LTR R round 2	Bisulfite	AATACAAAACTAATTAACTAACTAATACAA
Gfp F	RT-PCR	GGCGGATCTTGAAGTTCACC
Gfp R	RT-PCR	ACTACAACAGCCACAACGTCTATATCA
Mage-a2 F	RT-PCR	AAGGGAGGTCTCCATGCTGT
Mage-a2 R	RT-PCR	TCTCCCATCTCAGGCTTCTC
G9a CKO F	RT-PCR	ACCCCAACATCATCCCTGTCCGGG
G9a CKO R	RT-PCR	GTCCCAGAATCGGTCACCGTAGTC
Eset CKO F	RT-PCR	GCTGCTTGGATGATATTGCC
Eset CKO R	RT-PCR	CAGGCCTTCTTTGTCTGCAA
β-actin F	RT-PCR	TCATGAAGTGTGACGTTGACATCCGT
β-actin R	RT-PCR	CCTAGAAGCACTTGCGGTGCACGATGGAG
MLV LTR F	Southern Probes	CATGTGAAAGACCCCACCTGTAG
MLV LTR R	Southern Probes	AGTCGGATGCAACTGCAAGAGGG
IAP LTR F	Southern Probes	CAGAAGATTCTGGTCTGTGGTGTT
IAP LTR R	Southern Probes	GAATTCATACAGTTGAATCCTTCT