Supporting information

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SI Materials and Methods

DNA Methylation Analysis. Genomic DNA was isolated and treated with sodium bisulfite using the ZR Genomic DNA II and EZ DNA methylation kits (Zymo Research) according to the manufacturer's instructions and were analyzed on a Sequenom MassARRAY Epityper (Albert Einstein College of Medicine Core Facility). Detailed PCR amplification conditions are available on request.

Quantitative ChIP. Quantitative ChIP (qChIP) was carried out with the following specific modifications. Approximately 10^7 ES cells in 10-cm cell culture dishes were treated with 1% (vol/vol) formaldehyde for 10 min at 37 °C. Chromatin was prepared and fragmented by sonication on ice with four 30-s pulses with a Sonic Dismembrator (Fisher Scientific). A mixture of protein A- and protein G-agarose that had been pretreated with salmon sperm DNA was incubated with chromatin fractions. After removal of the agarose beads by centrifugation, the soluble fraction was incubated with specific antibodies or isotype control antibodies at 4 °C overnight and then incubated again with the pretreated protein A- and protein G-agarose for 1 h at 4 °C. After washing of the agarose beads and release of the bound fraction, DNA was isolated by phenol-chloroform extraction. The amounts of each specific DNA fragment were determined by quantitative PCR reactions.

Preparation and Purification of Chromatin Assembled in Vitro. A DNA fragment containing two copies of the nucleosome-positioning sequence from a sea urchin 5S RNA gene was amplified by PCR as described previously (1). The PCR product was purified using the QIAquick PCR Purification kit (Qiagen). For chromatin reconstitution, 25 pmol of DNA was mixed with 50 pmol of HeLa cell core histones (Millipore) in a buffer containing 2 M KCl, 10 mM Tris·HCl (pH 8.0), and 1 mM DTT. The mixture was subjected to salt dialysis as described previously (1). Dinucleosomes were purified by centrifugation in a 5–20% (wt/vol) sucrose gradient, concentrated using a Microcon-30 concentrator (Millipore), and dialyzed against a buffer containing 10 mM Tris·HCl (pH 8.0), 50 mM KCl, and 1 mM DTT. Then 10 pmol of purified recombinant H1d, prepared by thrombin cleavage of a GST-H1d fusion protein as described previously (2), was incubated with 5 pmol of purified dinucleosomes for 10 min on ice in a buffer containing 10 mM Tris·HCl (pH 8.0), 50 mM KCl, and 1 mM DTT. Reconstituted nucleosomes were analyzed by electrophoresis on nondenaturing 0.7% agarose gels in 0.5× Tris/borate/EDTA.

Histone Methyltransferase Assays and in Vitro qChIP with Reconstituted Nucleosomes. Histone methyltransferase assays were performed in 10 μL reactions containing 50 ng of purified SET7/9 (Abcam), 5 pmol of dinucleosomes reconstituted with or without H1, 50 mM Tris·HCl (pH 8.8), 5 mM $MgCl_2$, 4 mM DTT, and 160 μM S-adenosylmethionine (New England BioLabs). The reaction mixtures were incubated for 60 min at 30 °C. SET7/9 methyltransferase activity was measured by immunoblotting a portion of the reaction mixture with an antibody specific for methyl-histone H3 (Lys4). SET7/9 binding to dinucleosomes was analyzed by adding 1% (vol/vol) formaldehyde (final concentration) to the reaction mixtures, followed by the qChIP protocol described above.

DNA Methyltransferase Assays and in Vitro qChIP with Reconstituted Nucleosomes. DNA methyltransferase (DNMT) reactions contained 50 ng of purified DNMTs prepared from GST-DNMT fusion proteins as described previously (2), 5 pmol of dinucleosomes reconstituted with or without histone H1, 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5% (vol/vol) glycerol, 0.55 μCi of *S*-adenosyl-L-[methyl-^{3}H] methionine (Adomet) (PerkinElmer), and 100 μg/mL BSA in 20 μL. The reaction mixtures were incubated at 37 °C for 60 min. and treated with 10 μg of proteinase K (Invitrogen) for 60 min. at 45 °C. The DNA in the reaction mixtures was purified with the QIAquick system (Qiagen), and the amount of radioactivity incorporated into the DNA was measured by scintillation counting. DNMT binding to dinucleosomes was analyzed by adding 1% (vol/vol) formaldehyde (final concentration) to the reaction mixtures, followed by the qChIP protocol described above.

Plasmid Constructs. The H1d expression vector used to restore H1d expression in H1 triple-knockout (TKO) ES cells was constructed from the plasmid previously used for targeting the H1d locus by homologous recombination (3, 4). The 1.5-kb DNA fragment containing the *Puro* gene was excised by digestion with Asc I and replaced with a 1,503-bp Asc I fragment containing the *H1d* gene flanked by LoxP sites. The H1c expression vector used to restore H1c expression in H1 TKO ES cells was constructed by replacing the H1d coding sequence with the H1c coding region obtained by serial PCR reactions. The vector encoding the H1c.d chimeric fusion protein, consisting of residues 1–166 from H1c and residues 167–221 from H1d, and the vector encoding the truncated H1d protein (residues 1–159) were constructed by substituting their respective coding sequences for the H1d coding sequence in the H1d expression vector. The expression vectors encoding H1d with an HA tag and the truncated H1d proteins were constructed from a pEBB vector (5) by inserting corresponding coding sequences into XbaI and SpeI-ClaI enzyme sites, respectively. Plasmids used to express proteins in mammalian cells were pcDNA3-Myc-DNMT1[kindly provided by Kunio Shiota (University of Tokyo, Tokyo)]; pcDNA3-Myc-DNMT3A and pcDNA3-Myc-DNMT3B constructed by cloning full-length cDNAs encoding mouse DNMT3A or DNMT3B into the EcoRI and NotI sites of pcDNA3-Myc (Invitrogen), respectively; and p3×Flag-CMV-H1a, 1b, 1c, 1d, and 1e described previously (6). Plasmids used to express proteins in bacteria were pGEX-2T-DNMT1, pGEX-2T-DNMT3A, and pGEX-2T-DNMT3B constructed by subcloning PCR fragments of DNMT1, DNMT3A, and DNMT3B into the BamHI and EcoRI sites of pGEX-2T (Amersham Biosciences), respectively, and pGEX-2T-H1c, pGEX-2T-H1d, and the indicated H1d-deletion mutants constructed by cloning PCR fragments into the BamHI and EcoRI sites of pGEX-2T. The *pfu* DNA polymerase (Stratagene) was used for PCR reactions, and all constructs were confirmed by sequencing.

Antibodies. Anti-DNMT1 (ab13537), anti-DNMT3A (ab13888), anti-DNMT3B (ab13604), and anti–trimethyl-Histone H3 (Lys4) (ab8580) were purchased from Abcam. Anti-CTCF (07-729) and anti–trimethyl-Histone H3 (Lys9) (17-625) were purchased from Millipore. Anti-H3 (sc-8654), anti–c-Myc (sc-42), anti-HA (sc-805), and anti-Oct4 (sc-8628) were purchased from Santa Cruz. Anti-Flag (F1804) and anti-tubulin (T5168) were purchased from Sigma, and anti-SET7/SET9 and anti–dimethyl-Histone H3 (Lys4) were purchased from Cell Signaling. EZview red anti-HA (E6779) and anti-Flag (F2426) affinity gels for the immunoprecipitation assay were purchased from Sigma. Rabbit antisera specific for mouse H1c, H1d, and H1e were produced against the following specific peptides: H1c residues 178–191, H1d residues 25–36, and H1e residues 25–37.

Cell Culture and DNA Transfection. HEK 293T cells and H1 TKO ES cells (7) were transfected with plasmid DNA using Lipofectamine 2000 and were harvested after 48 h or 24 h, respectively. Stable transfectants of H1 TKO ES cells were generated by co-transfection with 10 μg of an exogenous H1 expression vector and 100 ng of pcDNA6/TR (Invitrogen), followed by selection in 10 μg/mL Blasticidin. H1 subtype stoichiometry was determined by HPLC analysis as described previously (7).

1. Martin C, Cao R, Zhang Y (2006) Substrate preferences of the EZH2 histone methyltransferase complex. *J Biol Chem* 281(13):8365–8370.
2. Rekhtman N, Radparvar F, Evans T, Skoultchi AI (1999) Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: Functional antagonism in erythroid cells. *Genes Dev* 13(11):1398–1411.
3. Fan YH, et al. (2003) H1 linker histones are essential for mouse development and affect nucleosome spacing in vivo. *Mol Cell Biol* 23(13):4559–4572.
4. Fan YH, Sirotkin A, Russell RG, Ayala J, Skoultchi AI (2001) Individual somatic H1 subtypes are dispensable for mouse development even in mice lacking the H1(0) replacement subtype. *Mol Cell Biol* 21(23):7933–7943.
5. Mizushima S, Nagata S (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* 18(17):5322.
6. Nishiyama M, et al. (2009) CHD8 suppresses p53-mediated apoptosis through histone H1 recruitment during early embryogenesis. *Nat Cell Biol* 11(2):172–182.
7. Fan Y, et al. (2005) Histone H1 depletion in mammals alters global chromatin structure but causes specific changes in gene regulation. *Cell* 123(7):1199–1212.

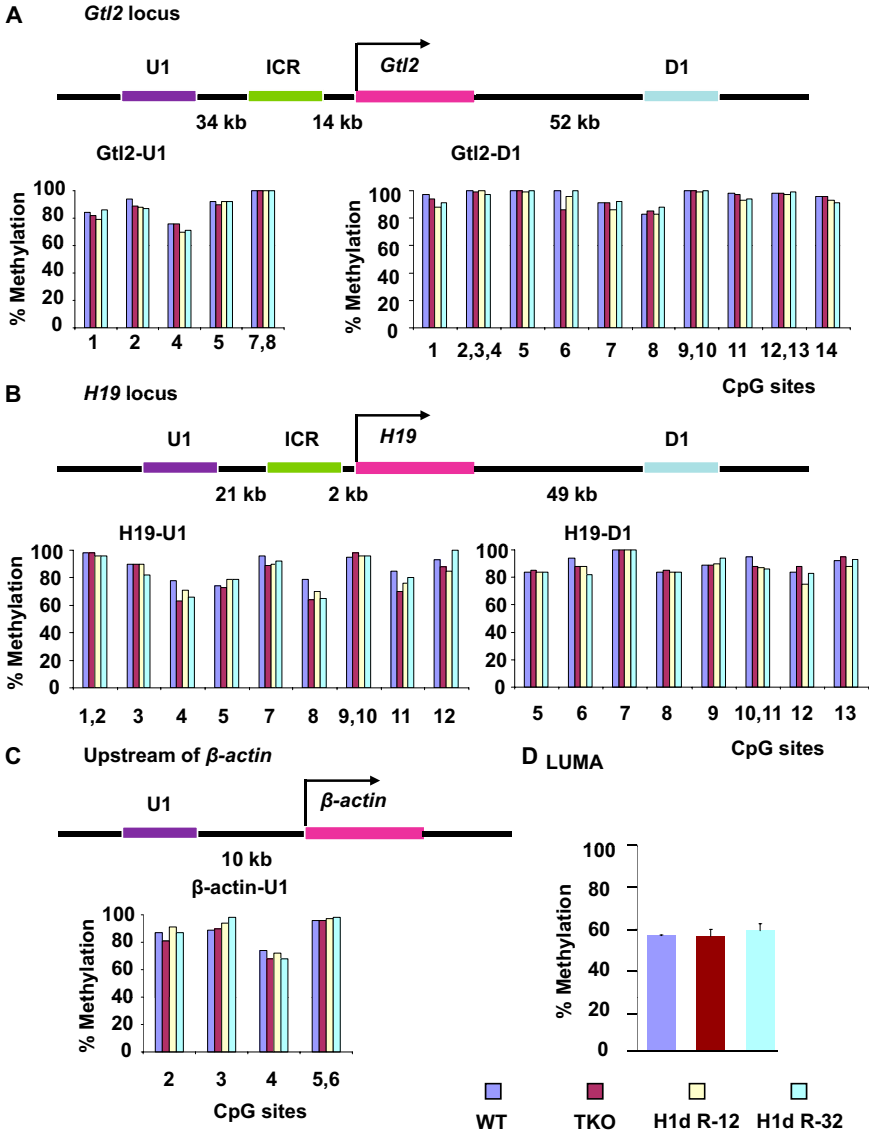
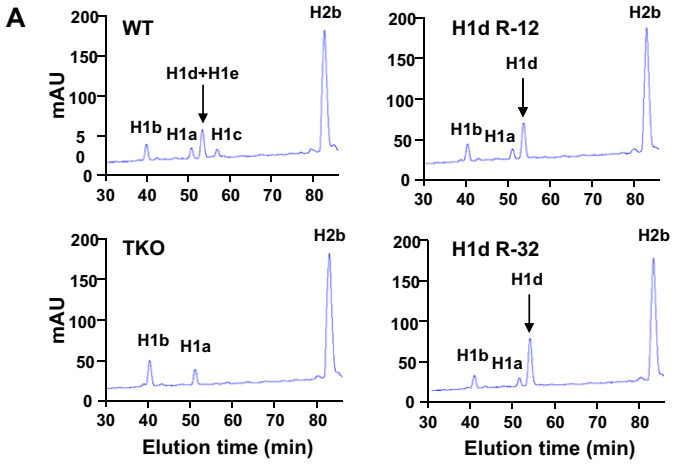


Fig. S1. DNA methylation analysis of regions upstream and downstream of the imprinting control regions (ICRs) and at the *β-actin* locus. The extent of methylation of individual CpGs within the indicated MassARRAY regions of the 5′-upstream (U1) and 3′-downstream (D1) of *Gtl2* (*A*), *H19* (*B*), and *β-actin* (*C*) in DNA from the cell lines described in Fig. 1 was determined by bisulfite treatment of genomic DNA and analysis of PCR products with a Sequenom MassARRAY Epityper. The results represent the average of analyses of at least two independent DNA preparations. The extent of methylation at all the indicated positions was not significantly different, as determined by two-tailed Mann–Whitney tests (all $P > 0.36$). The positions of the assayed regions and the ICRs are indicated with their approximate distances (in kilobases) from the transcription initiation sites of the corresponding genes shown below the line. The diagrams are not to scale. (*D*) Luminometric methylation assay (LUMA) was performed with the same cell lines described in Fig. 1.



B

Genotype	Molar Ratio of H1s per Nucleosome						Total H1 per Nucleosome
	H1°	H1a	H1b	H1c	H1d	H1e	
Wild-Type	0.010	0.062	0.097	0.075	0.150	0.057	0.45
Triple H1 knock-out	0.022	0.089	0.148	0	0	0	0.26
H1d Restored-Clone12	0.013	0.065	0.106	0	0.257	0	0.44
H1d Restored-Clone32	0.010	0.052	0.080	0	0.352	0	0.50

Fig. S2. Reverse-phase HPLC analysis of histone extracts from ES cell lines. (*A*) Approximately 100 μg of total histone extracts of chromatin from WT, H1 TKO homozygous *H1c*, *H1d*, and *H1e* null and two stably transfected TKO ES cell lines expressing exogenous H1d (H1d R-12 and H1d R-32) were analyzed as described previously (4). (*B*) H1 subtype stoichiometry in ES cell lines. Data were calculated from HPLC analyses like that shown in *A*, as described previously (4).

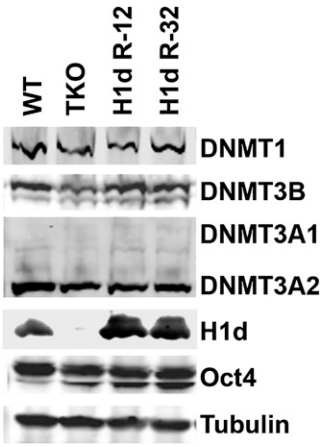


Fig. S3. Levels of DNMTs in ES cell lines. Immunoblots for the indicated proteins in WT, H1 TKO, and two stably transfected TKO ES cell lines expressing exogenous H1d (H1d R-12 and H1d R-32). Note that the levels of each specific DNMT are very similar in the four cell lines. Specificity of the H1d antiserum is shown in Fig. S5.

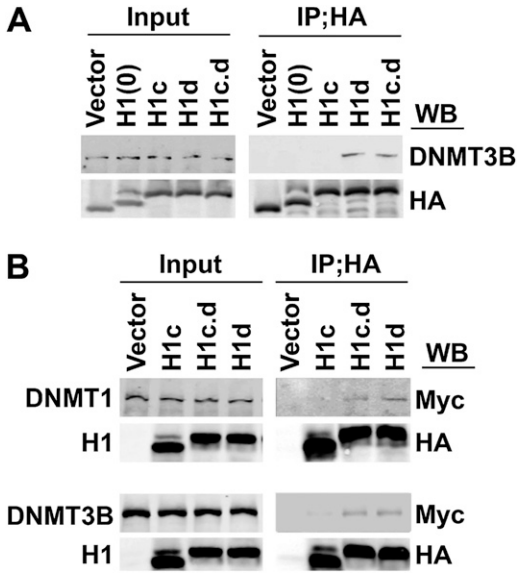


Fig. S4. The end of the H1d C-terminal domain confers binding of DNMT1 and DNMT3B to H1c. (*A*) Coimmunoprecipitation of endogenous DNMT3B with HA-tagged H1 proteins expressed in 293T cells. Immunoprecipitates with an anti-HA serum were analyzed by SDS/PAGE and immunoblotting for DNMT3B. Immunoblotting of immunoprecipitates with an anti-HA serum demonstrates approximately equal abundance of the four H1 proteins in the immunoprecipitates. Input lanes are 5% of extracts used for immunoprecipitation. The H1c.d chimeric protein consists of residues 1–166 of H1c and 167–221 of H1d. (*B*) Coimmunoprecipitation of HA-tagged H1 proteins with Myc-tagged DNMT1 and DNMT3B expressed in 293T cells. Lysates of transfected cells were immunoprecipitated with anti-HA serum and analyzed by SDS/PAGE and immunoblotting with an anti-Myc serum. Other details are as described in *A*. Input lanes are 5% of extracts used for immunoprecipitation.

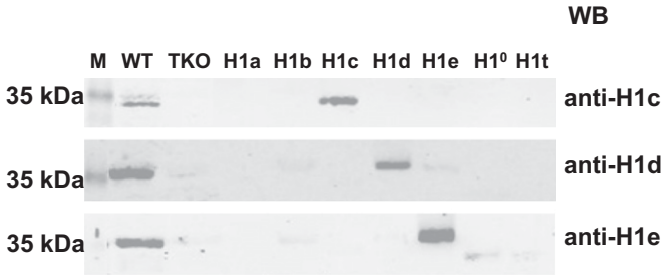


Fig. S5. Specificity of H1 subtype-specific antisera. Western blots using rabbit antisera raised against subtype-specific peptides from H1c (anti-H1c), H1d (anti-H1d), and H1e (anti-H1e) histones. Gel lanes were loaded with 0.2 μg of a total histone extract of chromatin from WT or H1 TKO (homozygous *H1c*, *H1d*, and *H1e* null) ES cells or ~0.2 μg of the indicated H1 subtype prepared by collecting individual peaks from fractions of reverse-phase HPLC chromatograms such as shown in Fig. S2. Because the H1d and H1e subtypes cannot be separated by this procedure, purified fractions of these subtypes were prepared from preparations of chromatin from H1e- and H1d-null mouse livers, respectively (1, 2).

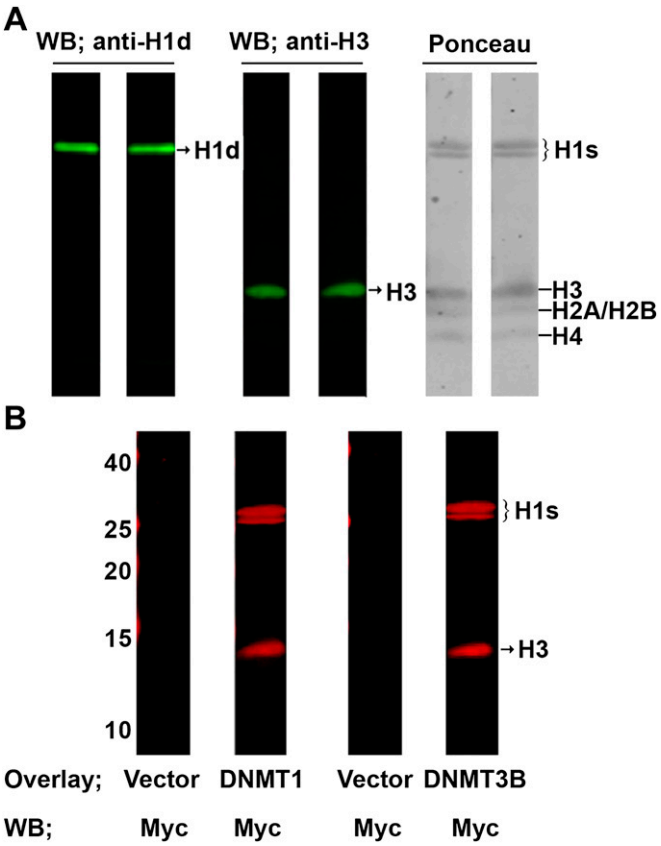


Fig. S6. Purified H1 and H3 histones interact with DNMT1 and DNMT3B by far-Western blotting analysis. (*A*) Total acid extracted histones from ES cell chromatin were separated by SDS/PAGE and transferred to membranes. The positions of histones H1 and H3 on the membranes were detected by immunoblotting (WB) with antisera to H1d and H3 antisera. Representative Ponceau S-stained membranes are shown at the right. (*B*) The membranes were treated as described in *SI Materials and Methods* and incubated with cell lysates (Overlay) from 293T cells expressing Myc-tagged DNMT1 or DNMT3B or from 293T cells transfected with an empty expression vector (Vector). Bound proteins were detected by immunoblotting (WB) with an anti-Myc serum.

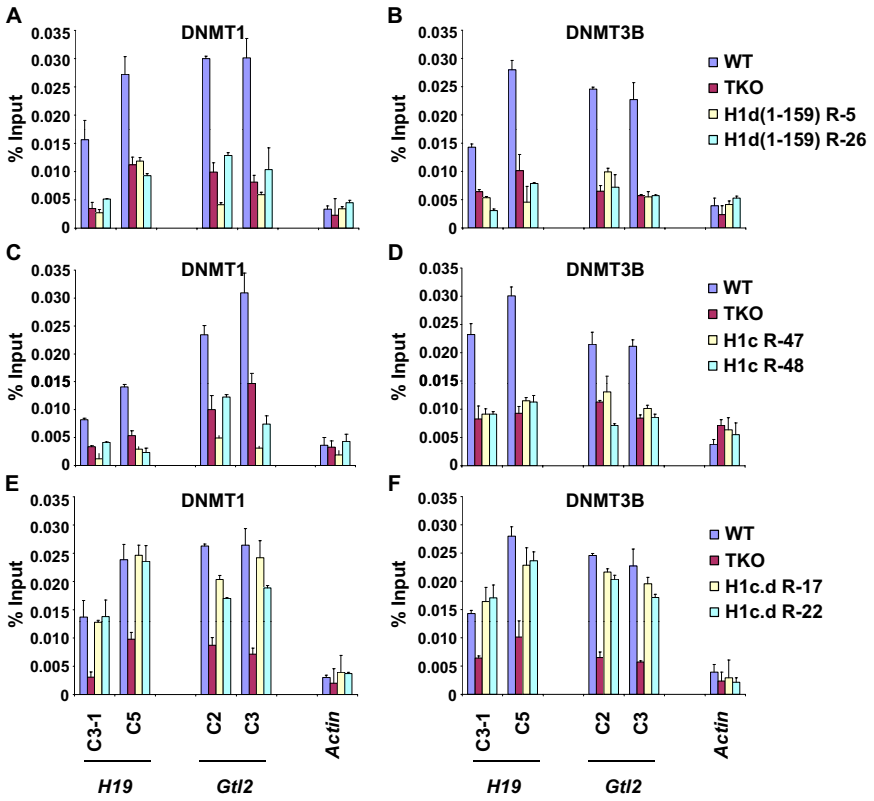
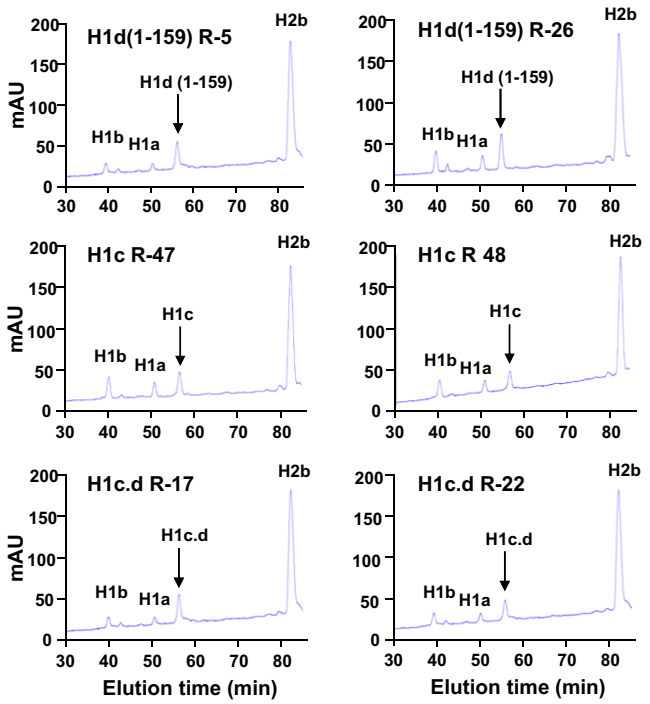


Fig. S7. The H1d C- terminal domain is required for recruitment of DNMT1 and DNMT3B to the *H19* and *Gtl2* ICRs in ES cells. qChIP was performed for DNMT1 and DNMT3B, as indicated, in WT, H1 TKO, and two stably transfected TKO ES cell lines expressing an exogenous H1d mutant lacking the C-terminal 62 residues [H1d(1–159) R-5 and H1d(1–159) R-26] (*A* and *B*) or two stably transfected TKO ES cell lines expressing exogenous H1c (H1c R-47 and H1c R-48) (*C* and *D*) or two stably transfected TKO ES cell lines expressing an exogenous chimeric H1 (H1c.d R-17 and H1c.d R-22) (*E* and *F*). The H1 histone subtype stoichiometries in these cell lines are shown in Fig. S8. Other details are as described in the legend of Fig. 2.

A



B

Genotype	Molar Ratio of H1s per Nucleosome					Total H1 per Nucleosome
	H1°	H1a	H1b	Exogenous H1	H1e	
H1d(1-159) R-5	0.012	0.067	0.105	0.194	0	0.38
H1d(1-159) R-26	0.010	0.074	0.107	0.270	0	0.46
H1c R-47	0.003	0.087	0.129	0.167	0	0.39
H1c R-48	0.006	0.087	0.132	0.146	0	0.37
H1c.d R-17	0.011	0.076	0.116	0.158	0	0.36
H1c.d R-22	0.012	0.084	0.120	0.131	0	0.35

Fig. S8. (*A*) Reverse-phase HPLC analysis of histone extracts from the ES cell lines used for the experiments shown in Fig. 5 and Fig. S7. Other details are as described in the legend of Fig. S2. (*B*) H1 subtype stoichiometry in ES cell lines.

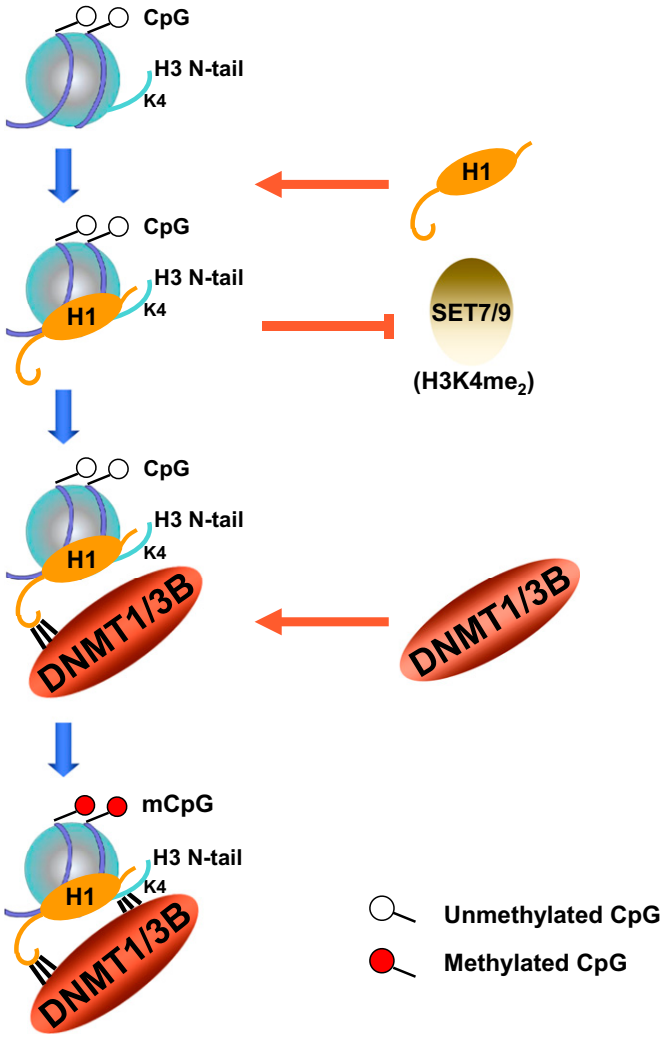


Fig. S9. H1 plays a dual role to promote gene silencing at the *H19* and *Gtl2* loci. The end of the C-terminal domain of H1 interacts directly with DNMT1 and DNMT3B, recruiting them to chromatin and promoting CpG methylation of the *H19* and *Gtl2* ICRs. H1 also interferes with binding of SET7/9 methyltransferase to chromatin, inhibiting methylation of H3 lysine 4, which may stabilize binding of DNMT1 and DNMT3B.

Table S1. RT-PCR primers

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
Gtl2	CATTTGCTGTTGTGCTCAGGTT	GTCCCACAGGAAATGTGCAA
Rian	CTGAGGGTCTGGTTGGTGAT	CACATCCAACCACTGGACTG
Mirg	GTGGATATGGGGATGGTCAG	GCTAGGTGCTGGTGTGACAA
H19	AGAGGACAGAAGGGCAGTCA	GTTCAAGGTAGGGGGAGGAG
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA

Table S2. ChIP-qPCR primers

Gene (region)	Forward (5′ to 3′)	Reverse (5′ to 3′)
H19		
C3–1	GACCATGCCCTATTCTTG	ACAGCATTGCCATTTGTG
C5	AGCTTTGAGTACCCCAGG	GCCTCTGCTTTTATGGCT
Gtl2		
C2	CCGCTACGGTTCATAGTG	GCGAAGCGGCATTAGTAC
C3	GATTCCTGACTCCCCTGA	TCTGAATTGTGCCTCAGT
Actin	CCTAAGGCCAACCGTGAA	GCTGAGAAGCTGGCCAAA

Table S3. MassARRAY PCR primers

Gene (region)	Forward (5′ to 3′)	Reverse (5′ to 3′)
H19		
M2	aggaagagagTGGAATTTATAAATGGTAATGTTGTG	cagtaatacgactcactatagggagaaggctACATAAACCCCTAACCTCATAAAAC
M3	aggaagagagTGATTTATAGGGTTTTGGGGTTAT	cagtaatacgactcactatagggagaaggctAAAATTCCTCAACTACCAAACTAACAA
M5	aggaagagagGGTTTGTTTATGATAATGTTTAAGGGT	cagtaatacgactcactatagggagaaggctTCCAAAAATACACACATCTTACCAC
U1	aggaagagagTGAGGAGTTTAGTTTTGATTTTGAG	cagtaatacgactcactatagggagaaggctACCTCAAACACCCTCACTTACTA
D1	aggaagagagAATATTTTTTGTTTGTTGGGTTTTG	cagtaatacgactcactatagggagaaggctCTCTTCTCAATCCCTTTCTAAAACA
Gtl2		
M4	aggaagagagTGTGTTGTGGATTTAGGTTGTAGTT	cagtaatacgactcactatagggagaaggctCATCCCCTATACTCAAAACATTCTC
M5	aggaagagagTTATGGTGTTGGGAGTTATGTTTTT	cagtaatacgactcactatagggagaaggctCCCCTATAACCAACAAACCTAAAATA
M2	aggaagagagTTGGGATTTAAAATTAAGGTTTTTTT	cagtaatacgactcactatagggagaaggctCCCCAACTAATAATTATTCTCAAAC
U1	aggaagagagTGGTTTTTTTAGAGAGGGTGATTTA	cagtaatacgactcactatagggagaaggcTTACACAAATCCACATCAAAACACT
D1	aggaagagagATTAATGTTTGGGTGGAATTTATTG	cagtaatacgactcactatagggagaaggctTCTCAACCTTCCTAAAAACCATATC
β-actin		
U1	aggaagagagAGTAGGGTTTAGTTTTGATTTTTTTG	cagtaatacgactcactatagggagaaggctCAACTAAATCTTTCTTAACACAAAACC