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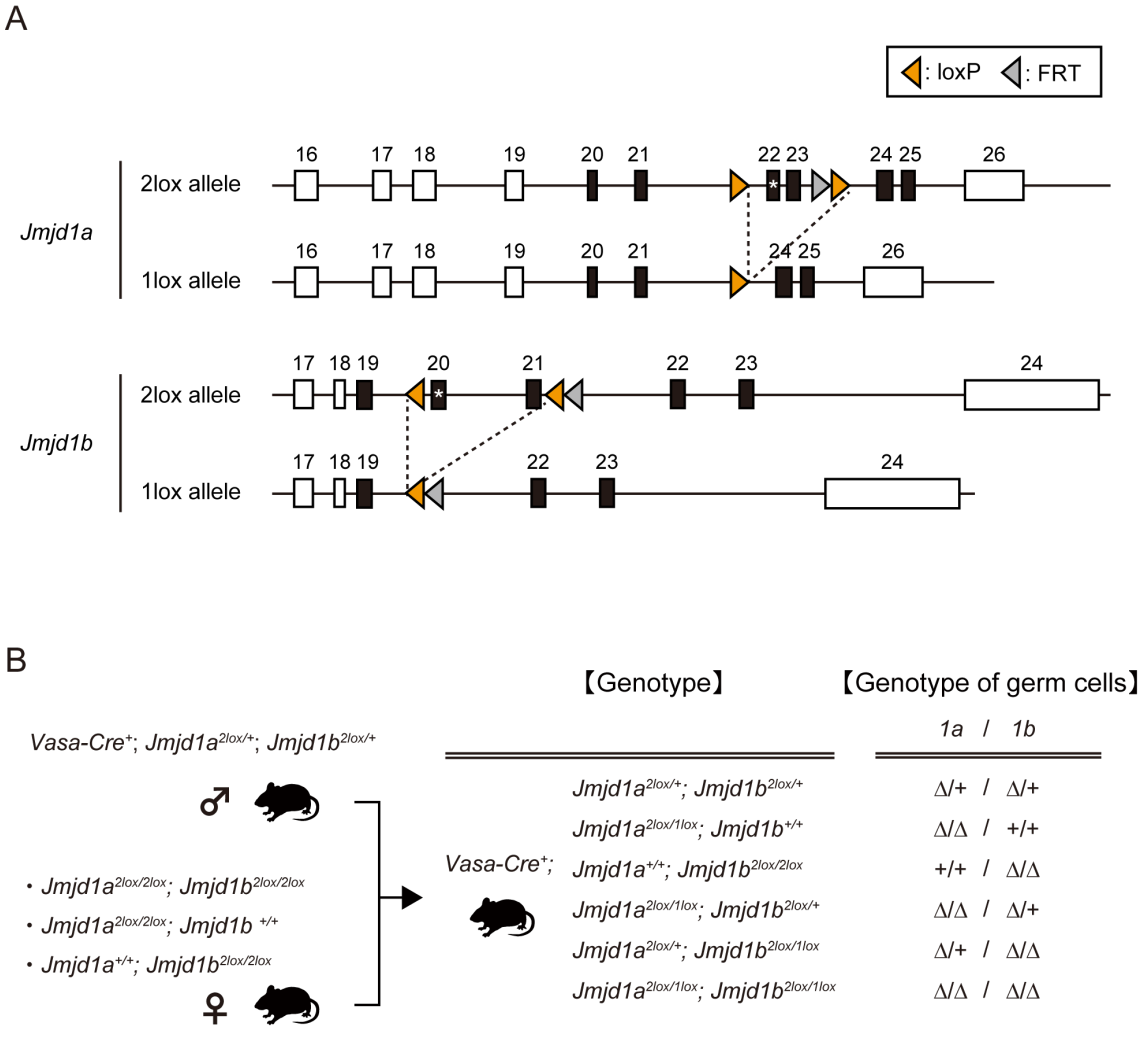
Supplemental Information

H3K9 Demethylases JMJD1A and JMJD1B Control Prospermatogonia to Spermatogonia Transition in Mouse Germline

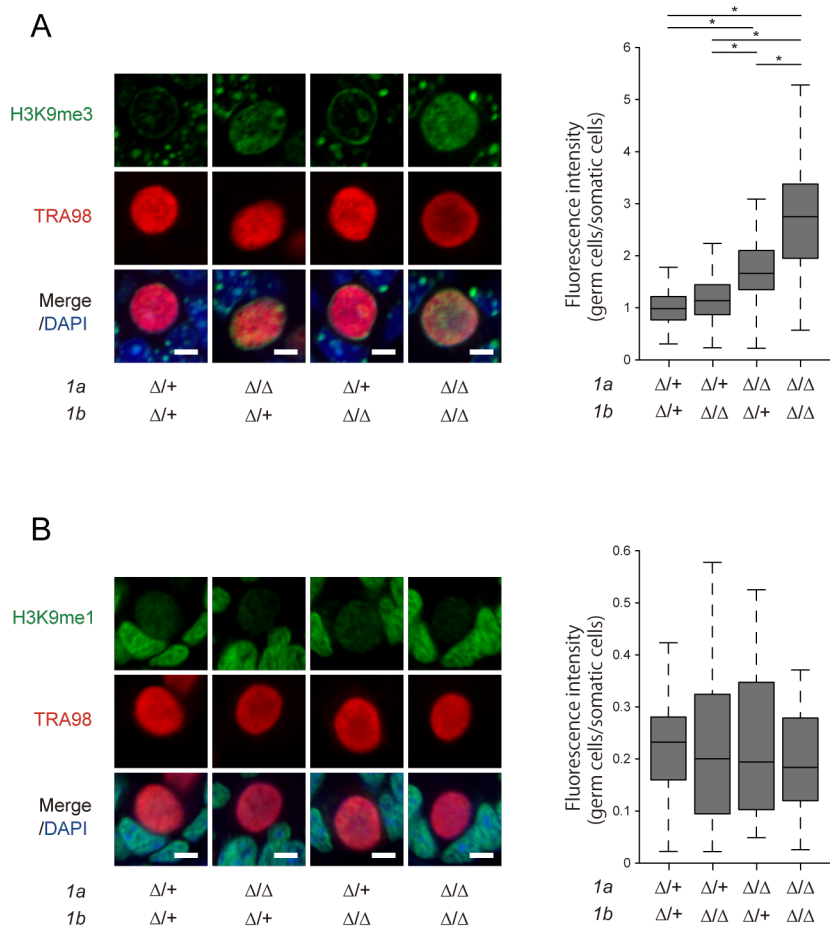
Shunsuke Kuroki, Ryo Maeda, Masashi Yano, Satsuki Kitano, Hitoshi Miyachi, Mikiko Fukuda, Yoichi Shinkai, and Makoto Tachibana



Supplementary Figure S1 (Related to Figure 2)

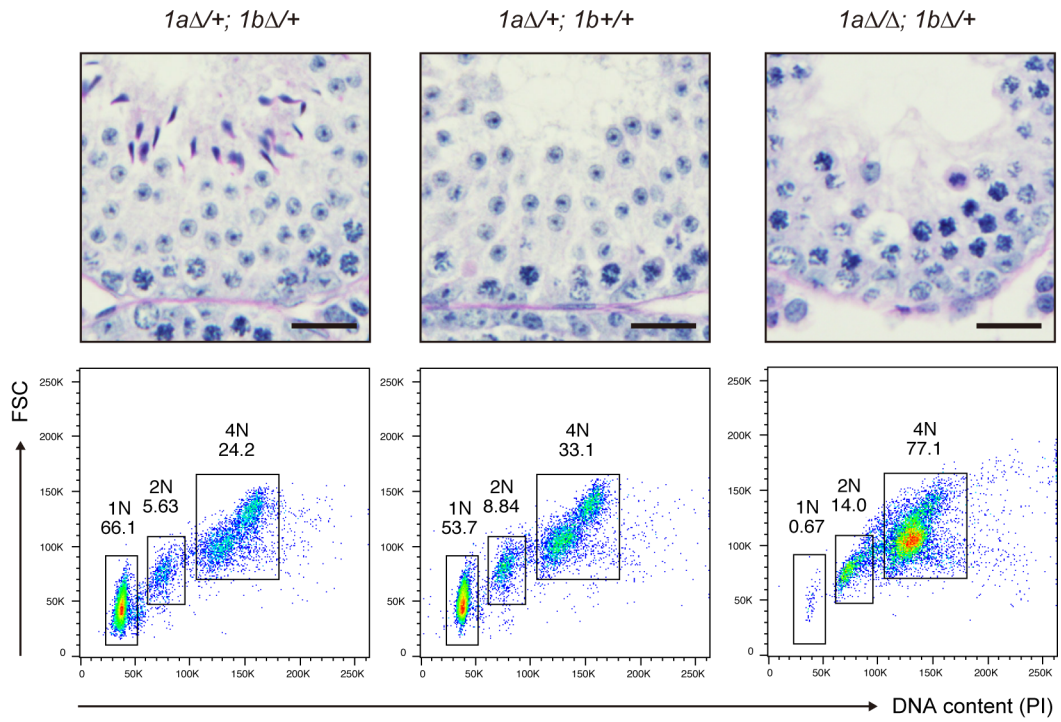
Generation of germline-specific knockout mice for *Jmjd1a* and *Jmjd1b*.

(A) 2lox conditional alleles and 1lox knockout alleles for *Jmjd1a* (top) and *Jmjd1b* (bottom). Black boxes represent exons corresponding to the JmjC domain. Asterisks represent exons corresponding to amino acid residues that are essential for Fe(ii) binding. (B) Scheme to generate a series of germline-specific *Jmjd1a* and/or *Jmjd1b* knockout mice.



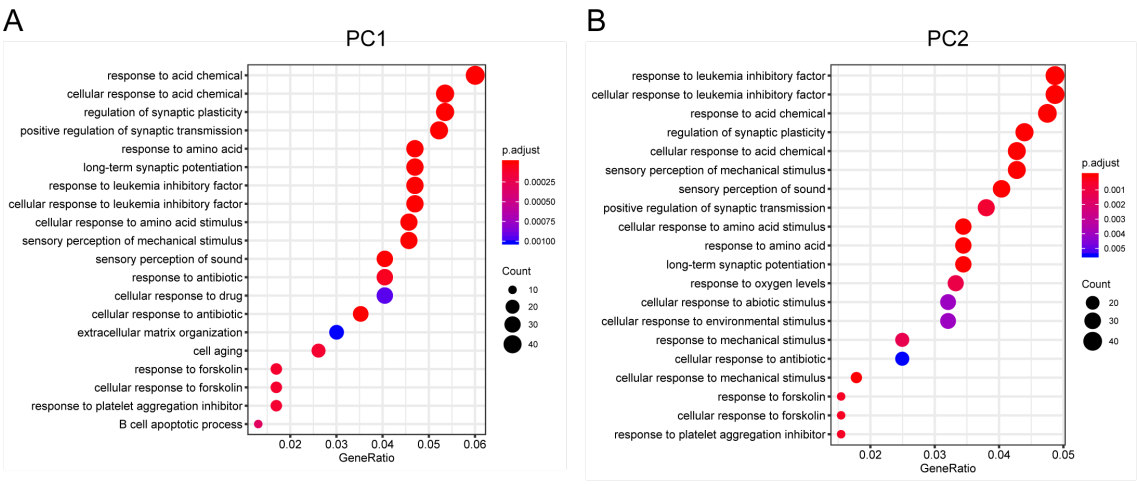
Supplementary Figure S2 (Related to Figure 2)
JMJD1A and JMJD1B Catalyze H3K9me3 Demethylation in Prospermatogonia

(A, B) Immunofluorescence analysis for H3K9me3 (A) and H3K9me1 (B) in P3 testes sections of the indicated genotypes. A TRA98 antibody was used to detect germ cells. Scale bars, 5 μm. Relative fluorescence intensity values were calculated by dividing the fluorescence intensities of germ cells by those of neighboring Sertoli cells (right panels). We examined >50 germ cells per genotype. *P < 0.05 (one-way ANOVA with Tukey's test).



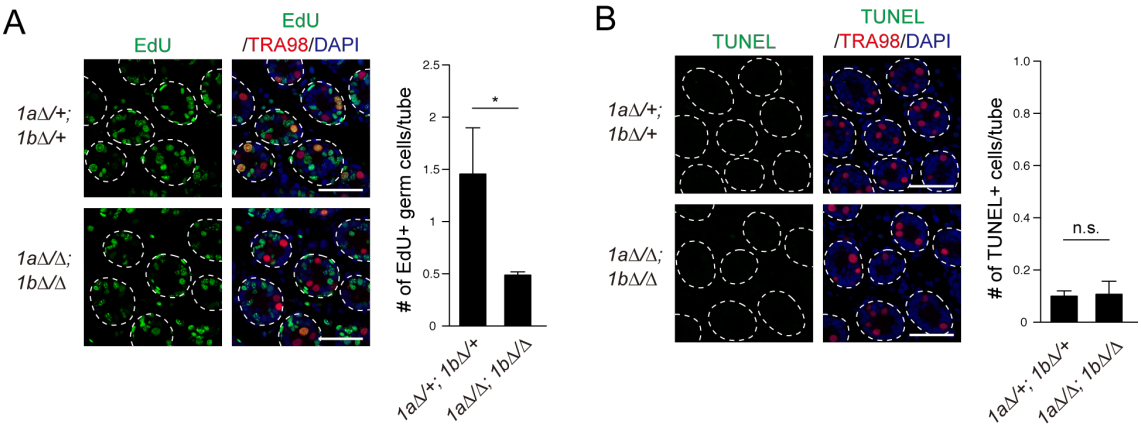
Supplementary Figure S3 (Related to Figure 3)

Role of JMJD1A and JMJD1B in meiotic progression. P28 testes sections of the indicated genotypes were stained with PAS-hematoxylin (top). Germ cells were dissociated from P28 testes of the indicated genotypes and then analyzed for DNA content (bottom). Briefly, testicular cells were fixed, stained with a TRA98 antibody and propidium iodide (PI), and then FACS sorted. Data show the DNA content of TRA98-sorted germ cells. Scale bars, 20 μm.



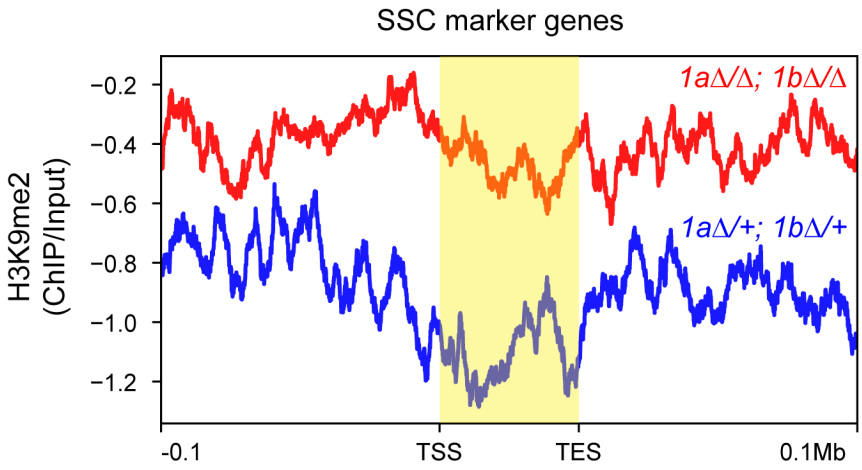
Supplementary Figure S4 (Related to Figure 4)

Gene Ontology analysis of genes contributing to PC1 and PC2 shown Fig. 4M. We calculated the loadings of PC1 and PC2 and used the top 5% of genes for Gene Ontology analysis.



Supplementary Figure S5 (Related to Figure 5)

(A) Proliferation analysis of JMJD1A/JMJD1B-depleted germ cells at P3. Cells incorporating Edu were counterstained with a TRA98 antibody (left). Scale bars, 50 μm. Average numbers of EdU+ cells per cross-tubular section are summarized (right). Error bars represent ± SEM (n=3 mice each) *P < 0.05 (t-test). (B) Detection of apoptotic cells in JMJD1A/JMJD1B-depleted germ cells at P3. TUNEL-stained section of P7 testis of the indicated genotypes were counterstained with a TRA98 antibody (left). Scale bars, 50 μm. Average numbers of TUNEL+ cells per cross-tubular section are summarized (right). Error bars represent ± SEM (n=3 mice each); n.s., not significant (P>0.05, t-test).



Supplementary Figure S6 (Related to Figure 6)

Distribution profile of H3K9me2 on SSC marker genes shown in Fig. 5F. Averaged H3K9me2 levels of the gene bodies and adjacent 100 kb regions for *Id4*, *Epcam*, *Cd9*, *Etv5*, *Plzf*, *Sohlh2*, *Pou3f1*, *Kit*, *Ret*, *Lhx1*, *Cdh1*, *Lin28a*, *Gfra1*, *Bcl6b*, *Pou5f1*, *Nanos3*, and *Stra8* are presented. Log2-transformed ratio of ChIP/Input is shown.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Genotyping

Genotyping of the Vasa-Cre Tg, *Jmjd1a*-deficient, and *Jmjd1b*-deficient mice was performed by standard PCR using Quick Taq HS Dyemix (Toyobo). The primers sets used were: 5′-ATTTGGGCCAGCTAAACATGCTTCATCG-3′ and 5′-CCTTCCAGGGCGCGAGTTGATAGC-3′ for *Vasa-Cre*, 5′-TCCTACCCAGTACAAGAGGGAGAGTGC-3′ and 5′-GGGAATTCCCACATAAACCATGACATTGGC-3′ for *Jmjd1a*^{2lox}, 5′-TCCTACCCAGTACAAGAGGGAGAGTGC-3′ and 5′-AGAACATGAGACCTTGGGGCCAAGG-3′ for *Jmjd1a*^{1lox}, 5′-GCACCAAGCACTGCCACGGAGCTGA-3′ and 5′-CATACACCATCACATTAACAGCGTCTGAC-3′ for *Jmjd1b*^{2lox}, 5′-GCACCAAGCACTGCCACGGAGCTGA-3′ and 5′-GCAGCCCAGGTTGGCCTCTATGATC-3′ for *Jmjd1b*^{1lox}, and 5′-GAAGAACATCATATACCACGCAGTG-3′ and 5′-GGAATGTGGCACCCTCGGCTTGAGT-3′ for *Jmjd1b*$^{Flag\text{-}KI}$.

Preparation of germ cells

Germ cells were isolated from testes of *Vasa-Cre; Jmj1a; Jmj1b* conditional knockout mice or *Oct4*-EGFP Tg mice by fluorescence-activated cell sorting. Testes of indicated genotypes were digested in Accutase (Nacalai) supplemented with 0.5 mg/ml DNaseI (Sigma) to obtain a single cell suspension. For the conditional knockout mice, suspended cells were incubated with an anti-EPCAM antibody followed by incubation with Alexa488-conjugated anti-rat IgG antibody. Just before analysis, 1 μg/ml propidium iodide was added and EPCAM + cells were sorted from the FSC^{high} population using a FACS Aria II flow cytometer (BD Biosciences). For *Oct4*-EGFP Tg mice, EGFP^{+} cells and EGFP^{-} cells were sorted as germ cells and somatic cells, respectively. The purity of the germ cells was checked by immunostaining with the anti-VASA antibody.

Histological analysis

Testes were fixed in Bouin’s solution, embedded in paraffin and sectioned at 4 μm. Sections were deparaffinized, rehydrated and then

stained with either hematoxylin-eosin or hematoxylin-periodic acid-Schiff (PAS).

Immunofluorescence analysis

Testes were fixed in 4% paraformaldehyde (PFA) for 12 h at 4°C. For immunohistological analysis, testes were embedded in OCT compound (Sakura Finetek) and cut into 8-μm-thick sections using a standard protocol. The sections were washed with PBS, and antigen-retrieved at 105°C for 5 min in 10 mM citric acid buffer (pH 6.0). For whole-mount immunostaining, segments of seminiferous tubules were dissected from testes, fixed in 4% PFA for 2 h and then permeabilized with PBS containing 0.5% Triton-X100 and 1% BSA for 20 min at RT. All sections were blocked with TBS containing 2% skimmed milk and 0.1% Triton X-100 for 1 h, and were then incubated with the primary antibodies overnight at 4°C, followed by incubation with Alexa Fluor dye-conjugated secondary antibodies for 1 h and counterstaining with DAPI (1 μg/ml). The sections were mounted in Vectashield (Vector) and analyzed by confocal scanning microscopy (LSM700, Carl Zeiss) using Zen 2011 imaging software (Carl Zeiss). Fluorescence intensity was measured using NIH ImageJ software.

Quantitative RT-PCR Analysis

Total RNA was purified using an AllPrep DNA/RNA Micro Kit (Qiagen). cDNA was synthesized with ReverTra Ace qPCR RT Master mix (Toyobo) according to manufacture's instructions. SYBR premix Ex Taq II (Takara) was used for quantitative PCR. The following primer sets were used. 5′-ACTCCAGAGGATCGGAAATATGGGACC-3′ and 5′-GGGAATTCCCACATAAACCATGACATTGGC-3′ for *Jmjd1a*, 5′-GGAGATGCTGATGAGGTGACCAAGC-3′ and 5′-GGATCTTCTCTGCATCCTTCGCTGC-3′ for *Jmjd1b*, and 5′-ATGAATACGGCTACAGCAACAGG-3′ and 5′-CTCTTGCTCAGTGTCCTTGCTG-3′ for *Gapdh*.

TUNEL assay

TdT-mediated UTP nick end labeling (TUNEL) was performed with immunostained sections using the In Situ Cell Death Detection Kit (Roche) according to manufacturer's instructions. Images were collected with an

LSM700 microscope (Zeiss). The number of TUNEL-positive cells in each seminiferous tubule was counted using Image J software. More than 50 tubular sections were analyzed per sample.

Cell proliferation assay

P3 or P7 pups of indicated genotypes were subcutaneously injected with 0.25 mg EdU (Sigma). Twelve hours after injection, testes were collected from the pups and immunofluorescence staining for TRA98 was performed as described above. After immunostaining, Edu was stained using a Click-it Plus Edu Imaging Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The percentage of Edu-positive cells among $TRA98^+$ cells was calculated using Image J software. More than 300 $TRA98^+$ cells were counted per sample.

FACS analysis

Testes were digested with Accutase (Nacalai) to obtain a single cell suspension. Suspended cells were fixed with 2% PFA in PBS for 10 min, permeabilized with ice-cold ethanol for 20 min, and blocked with 0.5% skimmed milk in PBS for 1 h. Cells were then stained with primary antibodies overnight at 4°C and subsequently incubated with Alexa Fluor Dye-conjugated secondary antibodies (Life Technologies) for 1 h at room temperature. For DNA content analysis, testicular cells were stained with TRA98 antibody using the above protocol and were then incubated with RNase and PI just before analysis. Data were collected using a FACS Aria II (BD Bioscience) and analyzed with FlowJo software (TreeStar).

Immunoblot analysis

Whole lysates of germ cells and somatic cells were fractionated by SDS electrophoresis and transferred to nitrocellulose membranes. Proteins on the membranes were visualized using an enhanced chemiluminescence (ECL) kit (Perkin Elmer).

RNA sequence analysis

Total RNA was extracted using an All Prep DNA/RNA Micro Kit (Qiagen). cDNA libraries were prepared from total RNA using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina). Quality control was performed using a

Bioanalyzer (Agilent). The libraries were sequenced on an Illumina Hiseq 1500 or 2000. Sequence reads were mapped to the mouse mm10 genome using TopHat2 (v2.1.1) with default parameters. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) were estimated using Cufflinks (v2.1.1). Differentially expressed genes between P0 and P3 or P7 germ cells were calculated using Cuffdiff. PCA analysis and Gene Ontology analysis were performed using R statistical software (v3.5.1). PC loadings were calculated using the prcomp() function. Gene Ontology analyses were performed with the enrichGO() function in the clusterProfiler package. Heatmaps were visualized using the Heatmap() function in the ComplexHeatmap package.

ChIP-seq analysis

ChIP of H3K9me2 was performed following a previously described protocol (Tachibana et al., 2008) with slight modification. Briefly, 0.7 x 10^5 cells were suspended in 25 µl 0.3 M sucrose-containing buffer 1 (60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 15 mM Tris-HCl pH 7.5, and protease inhibitor cocktail). The cells were then lysed on ice for 10 min by the addition of 25 µl 0.8% NP40. Four hundred microliters of 1.2 M sucrose-containing buffer 1 were then added and the chromatin was pelleted by centrifugation. The pellets were digested with micrococcal nuclease (0.05 U, Takara) in 10 µl digestion buffer (0.32 M sucrose, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5) by vortexing at 37°C for 15 min. Digestion was then stopped with EDTA. After centrifugation the supernatant was incubated with anti-H3K9me2-conjugated magnetic beads (Dynabeads Protein G, Invitrogen) in 50 µl incubation buffer (50 mM NaCl, 5 mM EDTA, 0.1% NP40, 20 mM Tris-HCl pH 7.5) at 4°C for 6 h. Then, DNA was extracted from the immune complex according to the standard protocol. DNA from input and ChIP fractions was processed using a SMARTer ThruPLEX DNA-seq Kit (TaKaRa) and sequenced using the Illumina HiSeq 1500 system according to the manufacturer's instructions. In brief, the DNA was end-repaired, ligated to sequencing adapters, amplified, and sequenced to generate single-end reads. Sequence reads were mapped to the mouse mm10 genome using Bowtie2 (v 2.1.0) with default parameters. Only uniquely mapped and non-redundant reads were used for further analysis. DROMPA software (v 3.2.6) (Nakato et al., 2013) was used for

processing the mapped reads and calculating gene density in the mouse mm10 genome. For visualizing H3K9me2 profiles across chromosome 5, the reads were processed using the PC_ENRICH command (with the -binsize 500000 -outputwig 1 -owtype 2 option, to output the normalized read density of ChIP and the corresponding input samples). The processed H3K9me2 profiles and the gene density were visualized using the plot() function in R or Integrated Genome Viewer (IGV). Plotting of averaged reads around gene bodies was performed using the bamCompare, computeMatrix, and plotProfile commands in DeepTools (v.3.2.1). The parameters for Fig. 6H and S6 are as follows: Fig.6H, bamCompare (-bs 100 --smoothLength 200 --operation ratio --ignoreDuplicates), computeMatrix (--beforeRegionStartLength 3000 --regionBodyLength 5000 --afterRegionStartLength 3000 --skipZeros), plotProfile (--perGroup); FigS6, bamCompare (-bs 1000 --smoothLength 5000 --operation log2 --ignoreDuplicates), computeMatrix (--beforeRegionStartLength 100000 --regionBodyLength 50000 --afterRegionStartLength 100000 --skipZeros), plotProfile (--perGroup).

SUPPLEMENTAL REFERENCES

Nakato, R., Itoh, T., and Shirahige, K. (2013). DROMPA: easy-to-handle peak calling and visualization software for the computational analysis and validation of ChIP-seq data. Genes Cells *18*, 589-601.

Tachibana, M., Matsumura, Y., Fukuda, M., Kimura, H., and Shinkai, Y. (2008). G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. The EMBO journal *27*, 2681-2690.