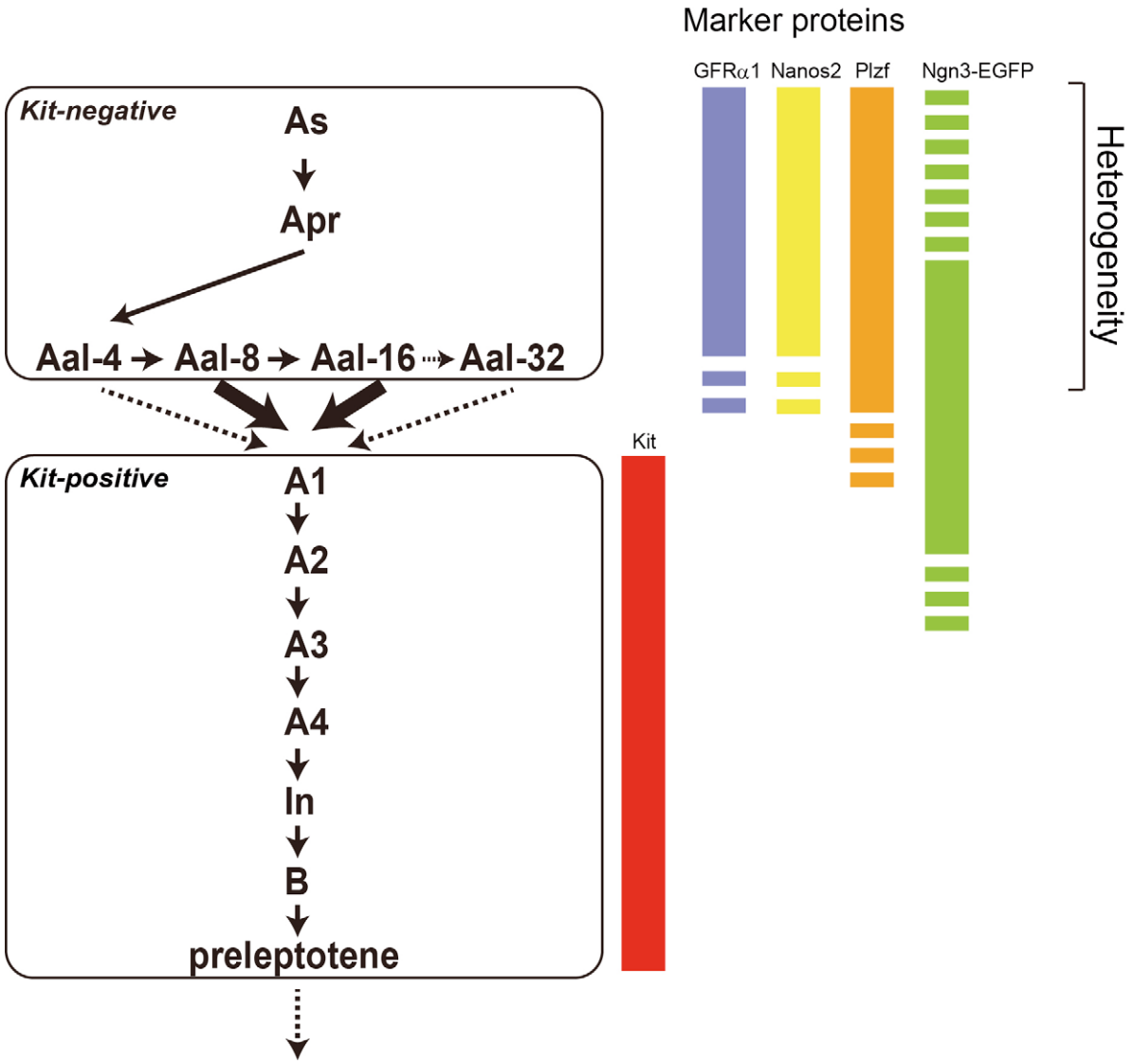


Fig. S1. Schematic representation of spermatogonial differentiation at the adult stage and markers used in this study. Spermatogonial differentiation from Kit^{neg} (A_s-A_{al}) to Kit^{pos} [A_1-A_4, intermediate (In), B] in the adult mouse. Kit expression commences in A_1 cells (Yoshinaga et al., 1991), and is an indicator of differentiated status and loss of stem cell activity (Ohbo et al., 2003; Shinohara et al., 2000). A_1 cells are thought to be the first differentiating spermatogonia, which go through six rounds of synchronous interconnected division (A_1, A_2, A_3, A_4, intermediate and B spermatogonia). GFRα1, Nanos2 and Plzf are expressed mainly in spermatogonia from A_s to A_{al}. In adults, chains of most Ngn3-$EGFP^{pos}$ spermatogonia are longer than those of $GFR\alpha1^{pos}$ and $Nanos2^{pos}$ spermatogonia. Spermatogonial heterogeneity in A_s to A_{al} was revealed by the expression patterns of marker proteins (Suzuki et al., 2007; Nakagawa et al., 2010).

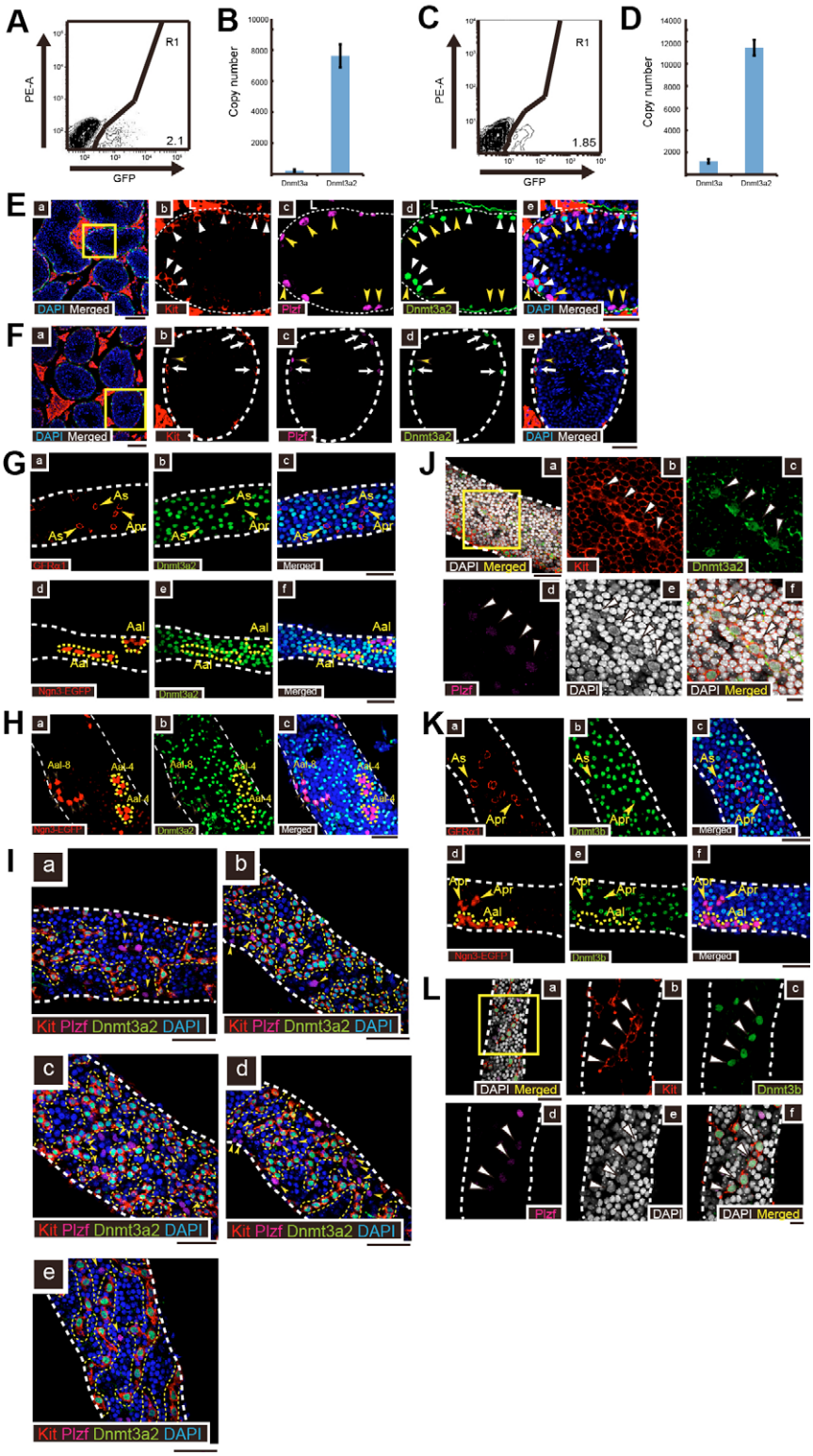


Fig. S2. Dnmt3a2 and Dnmt3b are expressed in Kit^{pos} spermatogonia during neonatal and adult stages. (**A**) Representative FACS plots of P7.5 Oct4-GFP^{pos} mouse testicular cells. Signals from the PE channel served as autofluorescence controls. Dead cells were identified by propidium iodide (PI) staining. GFP^{pos} cells (R1) were sorted and used for qPCR assays. The values presented indicate the percentage of the gated cell population. (**B**) Absolute qPCR using sorted Oct4-GFP^{pos} spermatogonia. Values shown are the mean±s.d. (**C**) Representative FACS plots of 5-week-old Ngn3-EGFP mouse testicular cells. Signals from the PE channel served as autofluorescence controls. Dead cells were eliminated by PI staining. GFP^{pos} cells (R1) were sorted and used for qPCR. The presented numbers denote the percentage of the gated cell population. (**D**) Absolute qPCR using sorted Ngn3-EGFP^{pos} spermatogonia. Values shown are the mean±s.d. (**E**) Immunohistochemical analysis of Dnmt3a2 using adult testis sections. (a) The boxed area is enlarged in b-e. Dnmt3a2 (green) is expressed in Plzf^{heg}/Kit^{pos} (white arrowheads) but not in Plzf^{bos}/Kit^{neg} (yellow arrowheads) spermatogonia. Kit, red. Plzf, magenta. DAPI, blue. L, Leydig cells (Kit^{pos}). Scale bars: 50 μm. (**F**) Immunohistochemical analysis of Dnmt3a2 using adult testis sections. (a) The boxed area is enlarged in b-e. Dnmt3a2 (green) is expressed in Plzf-weak positive/ Kit^{pos} (white arrows) but not in Plzf^{bos}/Kit^{neg} (yellow arrowheads) spermatogonia. Kit, red. Plzf, magenta. DAPI, blue. L, Leydig cells (Kit^{pos}).

Scale bars: 50 μm. (**G**) Whole-mount immunohistochemical analysis of Dnmt3a2 (green) and two stem cell markers (red): GFRα1 (a-c) and Ngn3-GFP (d-f). The white dashed lines outline seminiferous tubules. TOPRO3, blue. (a-c) Wild-type mice. (d-f) Ngn3-GFP mice. Scale bars: 50 μm. (**H**) A subset of $A_{al\text{-}8}$ Ngn3-EGFP^{pos} spermatogonia shows weak Dnmt3a2 expression. Whole-mount immunohistochemical analysis of Ngn3-EGFP mouse testis for Dnmt3a2 and EGFP revealed that Dnmt3a2 is weakly expressed in $A_{al\text{-}8}$ spermatogonia (yellow arrowheads), whereas $A_{al\text{-}4}$ spermatogonia (solid yellow dotted line) do not express Dnmt3a2. White dashed lines outline seminiferous tubules. EGFP, red; Dnmt3a2, green; TOPRO3, blue. Scale bar: 50μm. (**I**) Five representative images of Plzf^{heg}/Kit^{pos} spermatogonia longer than eight chains of cysts (a-e). Yellow dashed lines indicate Plzf^{heg}/Kit^{pos} spermatogonia longer than eight chains of cysts, which are Dnmt3a2^{pos}. Plzf^{pos}/Kit^{neg} spermatogonia lack Dnmt3a2 expression (yellow arrowheads). Dnmt3a2 (green), Plzf (magenta), Kit (red) and DAPI (blue). The white dashed lines outline seminiferous tubules. Scale bars: 50 μm. (**J**) A subpopulation of chains of four Plzf-weak positive/Kit^{pos} spermatogonia at stages VIII-IX expressed Dnmt3a2. The boxed area in a is enlarged in b-f. Kit, red; Dnmt3a2, green; Plzf, magenta; DAPI, gray. Scale bars: 50 μm in a; 10 μm in f. (**K**) Whole-mount immunohistochemical analysis of Dnmt3b (green) and two stem cell markers (red): GFRα1 (a-c) and Ngn3-GFP (d-f). The white dashed lines outline seminiferous tubules. TOPRO3, blue. (a-f) Wild-type mice. (g-i) Ngn3-GFP mice. Scale bars: 50 μm. (**L**) A subpopulation of chains of four Plzf-weak positive/Kit^{pos} spermatogonia at stages VIII-IX expressed Dnmt3b. The boxed area in a is enlarged in b-f. Kit, red; Dnmt3b, green; Plzf, magenta; DAPI, gray. Scale bars: 50 μm in a; 10 μm in f.

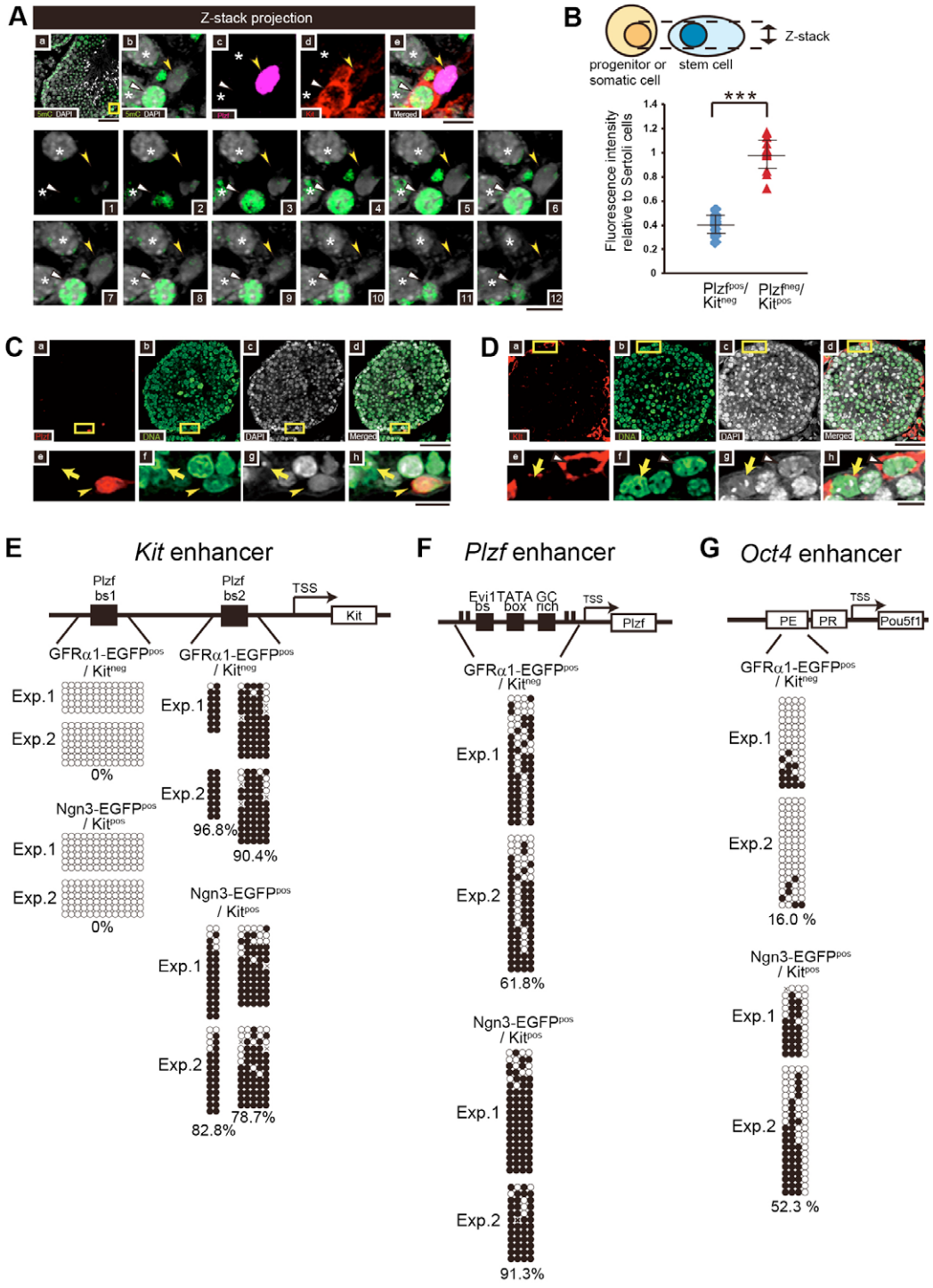


Fig. S3. Immunohistochemical analysis of global DNA methylation and bisulfite sequencing analysis of marker genes for spermatogonia. (**A**) *Z*-stack projection of confocal images (a-e) of Plzf^{pos}/Kit^{neg} and Plzf^{neg} /Kit^{pos} spermatogonia stained with the anti-5mC antibody. Boxed area (a) is enlarged in b-e. Plzf^{pos}/Kit^{neg} spermatogonia (yellow arrowheads) show lower global 5mC levels than Plzf^{neg}/Kit^{pos} spermatogonia (white arrowheads). A *z*-series of twelve confocal images is shown in 1-12. Plzf, magenta; Kit, red; 5mC, green; DAPI, gray. Asterisks indicate spermatocytes. Scale bars: 50 μm in a; 10 μm in e and 12. (**B**) Diagram of *z*-stack confocal analysis. Nuclei from the top to the bottom of spermatogonia and somatic cells were stacked and evaluated for signal strength using NIH Image. Quantification of 5mC signal strength shown as a ratio of 5mC signals from Plzf^{pos}/Kit^{neg} and Plzf^{neg} /Kit^{pos} spermatogonia relative to those of Sertoli cells. *P* values are derived from *t*-tests; ***$P<0.001$. Values shown are mean±s.d. (**C**,**D**) Accessibility of an antibody to genomic DNA under denatured conditions is similar between Plzf^{pos} and Kit^{pos} spermatogonia. Immunohisochemical analysis under denatured conditions using anti-DNA antibody (green) plus anti-Plzf (red in C, yellow arrowheads) or anti-Kit (red in D, white arrowheads) indicates similarly stained nuclei in both cell types. Yellow arrows indicate Sertoli cells. Boxed areas (a-d) are enlarged in e-h. Scale bars: 50 μm in d; 10 μm in h. (**E-G**) Methylation status of *kit* enhancer (E), *plzf* enhancer (F) and *oct4* enhancer (G) in GFRα1-EGFP^{pos}/Kit^{neg} and Ngn3-EGFP^{pos}/Kit^{pos} spermatogonia. Open and filled circles represent unmethylated and methylated cytosines, respectively. Each row of circles represents one bisulfite-sequenced clone. Results of two independent experiments for each region are shown.

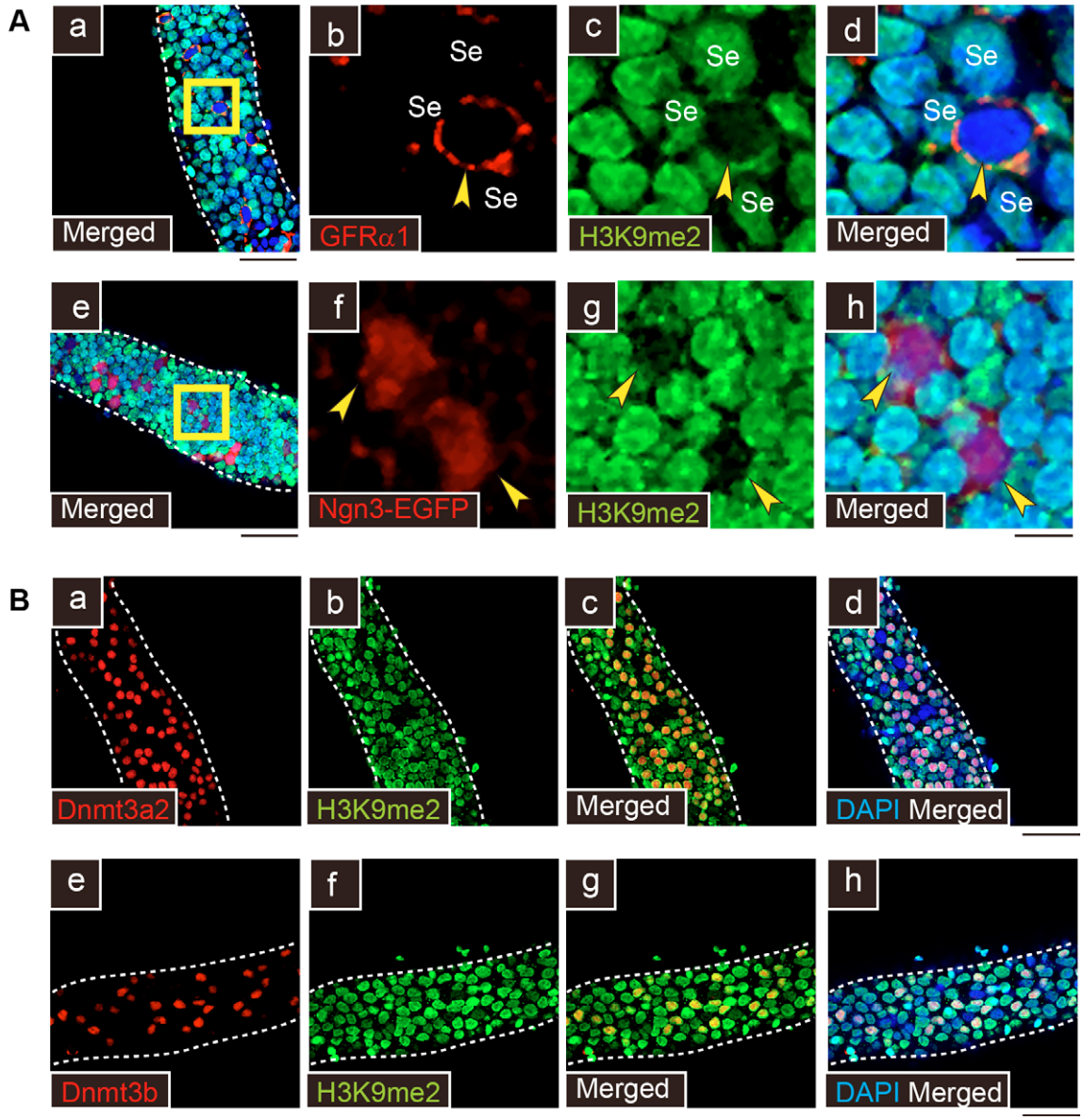


Fig. S4. Concomitant increase in H3K9me2 modification and expression of Dnmt3a2 and Dnmt3b in spermatogonia. (**A**) Spermatogonia expressing GFR 1 (yellow arrowheads) (a-d) and Ngn3-EGFP (yellow arrowheads) (e-h) lack H3K9me2 modification (yellow arrowheads). The boxed areas in a and e are enlarged in b-d and f-h, respectively. Dashed lines outline seminiferous tubules. Se, Sertoli cells. DAPI, blue. Sertoli cells were identified by the pattern of DAPI staining. Scale bars: 50 μm in a,e; 10 μm in d,h. (**B**) Whole-mount immunohistochemical analysis of H3K9me2 (green) co-stained with anti-Dnmt3a2 (red; a,c,d) and anti-Dnmt3b (red; e,g,h) antibodies in wild-type mouse seminiferous tubules. Dnmt3a2^{pos} and Dnmt3b^{pos} spermatogonia are also positive for H3K9me2. Dashed lines outline seminiferous tubules. DAPI, blue. Scale bars: 50 μm.

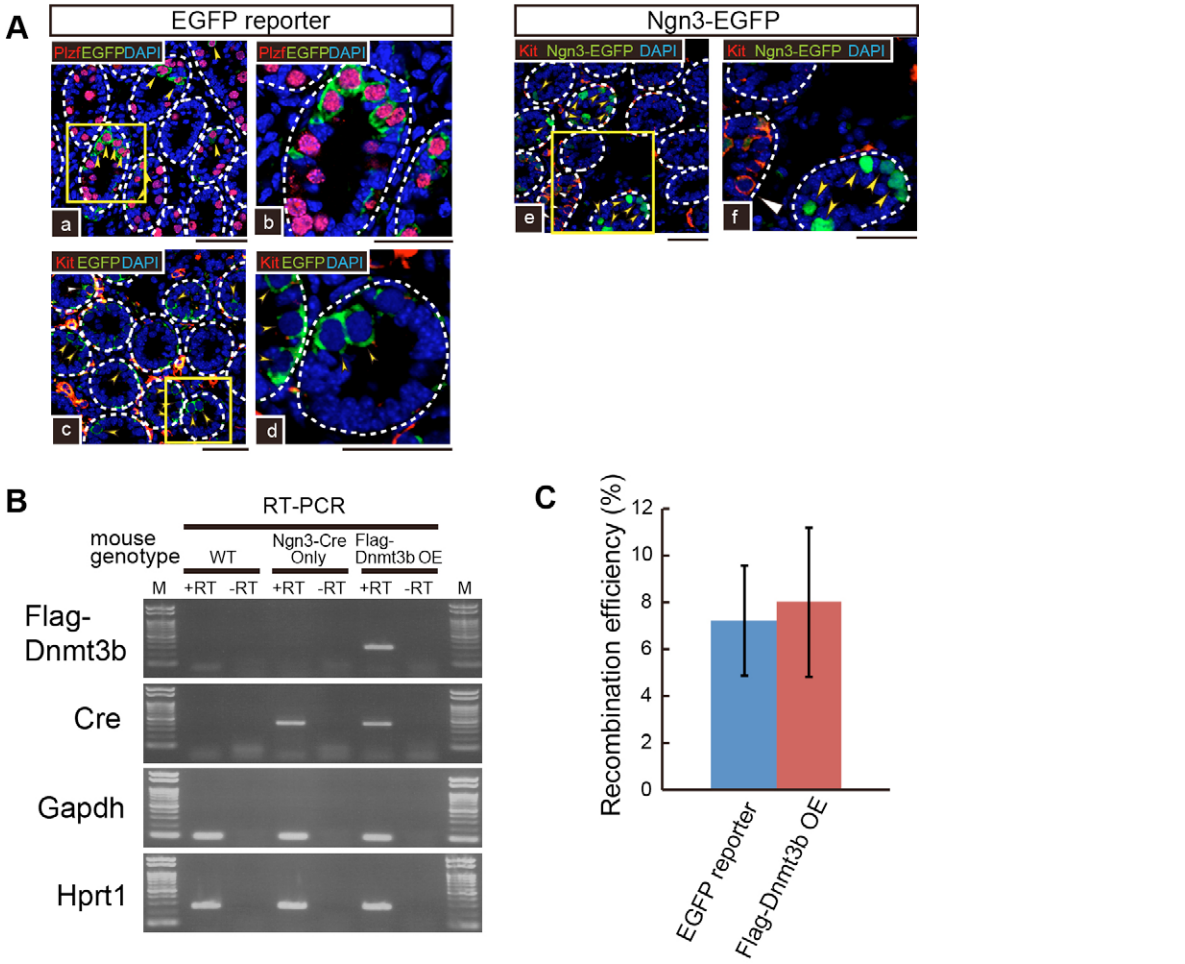


Fig. S5. Confirmation of Flag-Dnmt3b expression and Cre recombination frequency. (**A**) All EGFP-expressing spermatogonia (yellow arrowheads) are Plzf^{pos} at neonatal stages (a-d). Plzf, red; EGFP, green; TOPRO3, blue. Scale bars: 50 μm. White dashed lines outline seminiferous tubules. Most spermatogonia recombined by Ngn3-Cre at P6.5 are Kit negative (c,d). In addition, Ngn3-EGFP^{pos} spermatogonia at P6.5 are mostly Kit negative (e,f). Kit, red; EGFP, green; DAPI, blue. Scale bars: 50 μm. White dashed lines outline seminiferous tubules. (**B**) Efficient transgene excision by Ngn3-cre. RT-PCR analysis of Cre and Flag-Dnmt3b in P6.5 testes with or without reverse transcriptase. M, molecular weight. Only the mice carrying Ngn3-cre:CAG-DsRed-Flag-Dnmt3b transgenes (Flag-Dnmt3b OE mice) expressed Flag-Dnmt3b. (**C**) Comparison of recombination frequency in control EGFP reporter mice and Flag-Dnmt3b OE mice at P6.5. The proportion of EGFP^{pos} cells to TRA98^{pos} cells (blue column) in EGFP reporter mice, and Flag-Dnmt3b^{pos} cells to TRA98^{pos} cells (red column) in Flag-Dnmt3b OE mice was very similar (7±2% and 8±3%, respectively). TRA98 is a germ cell marker (Tanaka et al., 1997). Values are presented as mean±s.d.

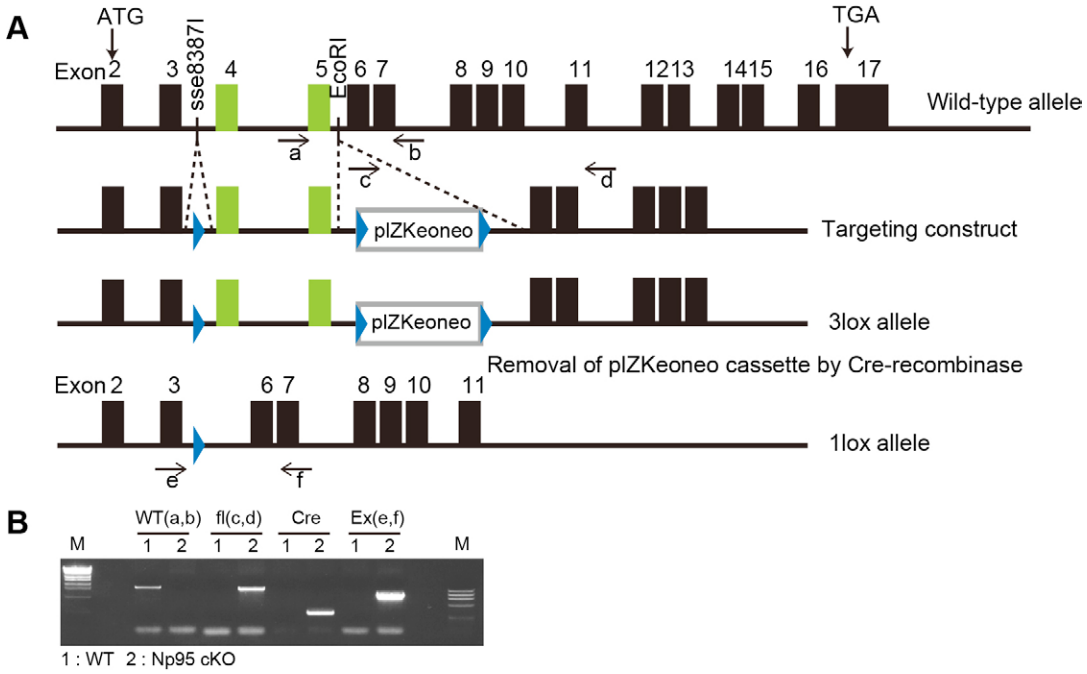


Fig. S6. Generation of Np95 cKO mice. (**A**) Strategy for conditional mutagenesis, and positions of primers for genotyping. (**B**) Confirmation of deletion of the Np95 allele by genomic PCR after tamoxifen treatment. Primers a and b amplify the wild-type allele (WT, 1569 bp), whereas primers c and d detect the floxed allele (fl, 1472 bp). After deletion, primers e and f were used to amplify the deleted allele (Ex, 1006 bp) but the floxed allele is not amplifiable with these primers owing to the extended length. Cre, $CreER^{T2}$ (762 bp). Lane 1, wild-type mice; lane 2, Np95 cKO mice. The positions of primers are shown in A.

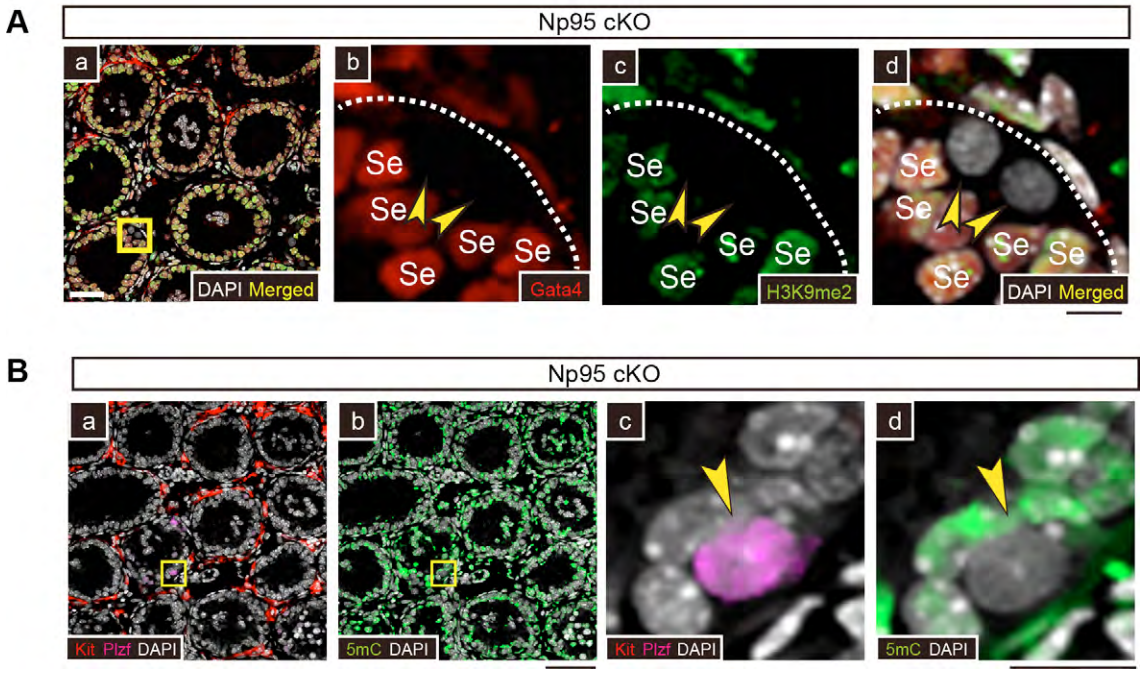
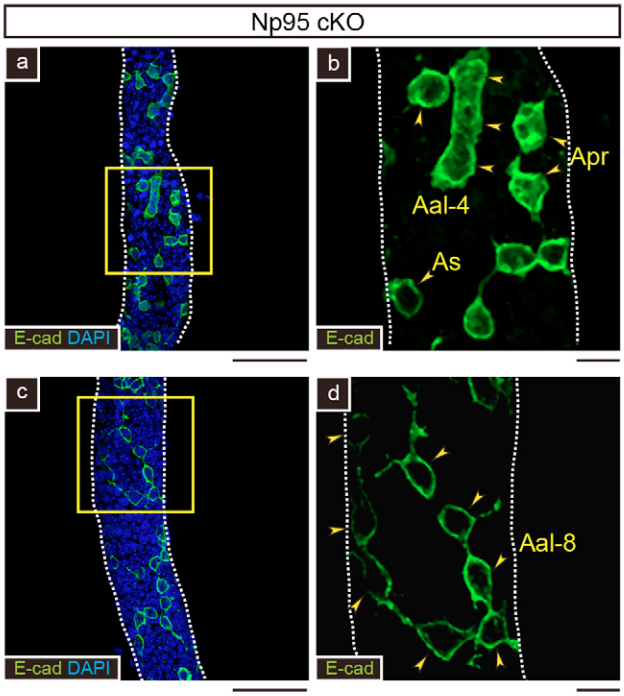


Fig. S7. Spermatogonia in Np95 cKO mouse testis lack H3K9me2 modification and show weak 5mC signals. (**A**) Spermatogonia (yellow arrow) do not exhibit H3K9me2 signals (green) in Np95 cKO mouse testis. $Gata4^{pos}$ Sertoli cells (red, Se) show signs of H3K9me2 modification. Broken white lines outline seminiferous tubules. DAPI, gray. Scale bars: 50 µm (a, white) or 10 µm (d, black). (**B**) Confocal images of Np95 cKO spermatogonia stained with the anti-5mC antibody. Boxed areas (a,b) are enlarged in c,d. Plzf, magenta; Kit, red; 5mC, green and DAPI, gray. Scale bars: 50 µm in b; 10 µm in d.

A



B

number of Ecad^{pos} chained cell	1	2	4	8	more	total
Control	83	79	71	60	102	495
Np95 cKO	72	75	69	15	1	232

Fig. S8. Residual spermatogonia in Np95 cKO mice were primarily made up of single cells, paired cells, and chains of four cells and eight cells. (**A**) Majority of the residual spermatogonia in Np95 cKO mice are A_s, A_{pr}, A_{al-4} or A_{al-8}. Chained spermatogonia were visualized by E-cadherin (green) staining. Boxed areas in a and c are enlarged in b and d, respectively. DAPI, blue. Scale bars: 50 μm in a, c; 10 μm in b,d. (**B**) The number of Ecad^{pos} chained cells in control and Np95 cKO mice was counted to compare the distribution of chain length.

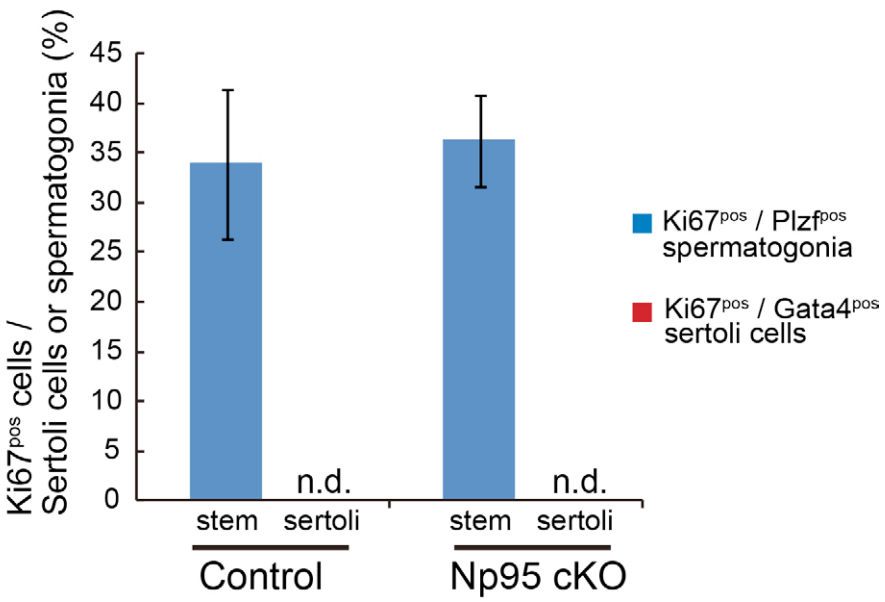


Fig. S9. Cell cycle analysis of Plzf^{pos} spermatogonia in Np95 cKO mice. Cell cycle analysis of Plzf^{pos} spermatogonia and Sertoli cells in Np95 cKO or control mouse testes. The frequency of Ki67^{pos} cells in Plzf^{pos} spermatogonia in 4-week-old Np95 cKO mice was 36%, similar to that seen in littermate control mice. No Ki67^{pos} cells were detected among Gata4^{pos} Sertoli cells in control or Np95 cKO mice. Values are presented as the mean±s.d.

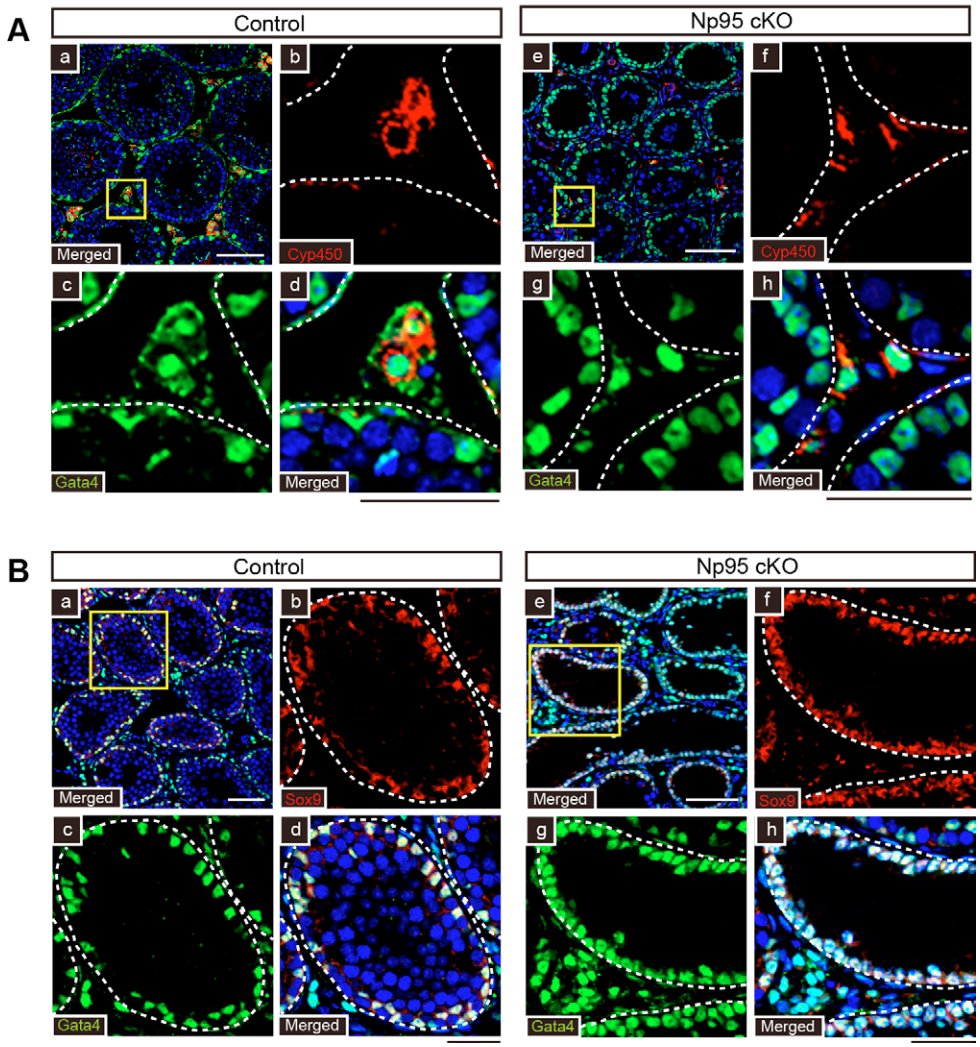


Fig. S10. Analysis of residual somatic cells in Np95 cKO mouse testis. (**A**) Leydig cells express Leydig-specific markers in Np95 cKO mouse testes. The expression level and pattern of Cyp450 (red) and Gata4 (green) were similar between littermate controls (left panels) and Np95 cKO (right panels). Gata4 is also expressed in Sertoli cells. The boxed areas in a and e are enlarged in b-d and f-h, respectively. DAPI, blue. Scale bars: 100 μm (white); 50 μm (black). (**B**) Sertoli cells express Sertoli-specific markers in Np95 cKO mice testes. The expression level and pattern of Sox9 (red) and Gata4 (green) were similar between littermate controls (left panel) and Np95 cKO (right panel). The boxed areas in a and e are enlarged in b-d and f-h, respectively. DAPI, blue. Scale bars: 100 μm (white); 50 μm (black).

Table S1. Primers used in this study

	Forward primer	Reverse primer
Absolute qPCR		
Dnmt3a1	CGACCCATGCCAAGACTCACCTTCCAG	AGACTCTCCAGAGGCCTGGT
Dnmt3a2	CCAGACGGGCAGCTATTTAC	AGACTCTCCAGAGGCCTGGT
RT-PCR		
Flag-*Dnmt3b*	CAAAGACGATGACGACAAGC	CTGTCATGTCCTGCGTGTAA
Cre	GACCATGTCCAATTTACTGACCGTACAC	TTTTGCAGGTTCACCGGCATCAACG
Gapdh	CAATGTGTCCGTCGTGGATCT	GCCTGCTTCACCACCTTCTT
Hprt1	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC
Genotyping		
Np95 cKO WT	GGCCCCTGCCCTTATGGCTTTGCTGGACTCT	GGGGCTGAGCCTGGGCTTCTTCACCCAGGTTGA
Np95 cKO Fl	GCCGGGGCAGGATCTCCTGTCATCTCAC	GGGGCTGCAACAGGCCACCATGCCACCTGGCATTG
Np95 cKO Ex	GCGCCAGAGTCTGGCACTGCCTCTCAGTAC	GCCCACCACACACATGGCAGGCACACT
Np95 cKO CreER^{T2}	GCCTGCATTACCGGTCGATGCAACGA	GTGGCAGATGGCGCGGCAACACCATT
Flag-*Dnmt3b* OE	ACCAGAGGCCGCAGGTCAAGC	CCACTGTACCCAGCGCATTCC
EGFP reporter	TACGGCAAGCTGACCCTGAA	TGTGATCGCGCTTCTCGTTG
Ngn3-Cre	GACCATGTCCAATTTACTGACCGTACAC	TTTTGCAGGTTCACCGGCATCAACG
Bisulfite sequencing		
Kit enhancer Plzf bs1	GTTTACGTGGTTTTTTTTTTTTATTGGGAG	TCCTAACGAACCTTTAATACTACC
Kit enhancer Plzf bs2-1	GTTTATATTTTTAAGTATTTTGTAGGGTTG	AAATTCTTTAAATCCCAAAATTATACAC
Kit enhancer Plzf bs2-2	GGGATTTAAAGAATTTTTAAAATAAA	CCCAAATACTAAAATTAAAATCATAC
Plzf enhancer	ATTGGTTTTTGTTTTTATAGTGTTGG	AAACTCCAAAACATTTCCACTTAAC
Oct4 enhancer	TAAGATGGAATATTGTGTTTTGAAAA	TCAACCATCCTCTAAAAAACCTAAA