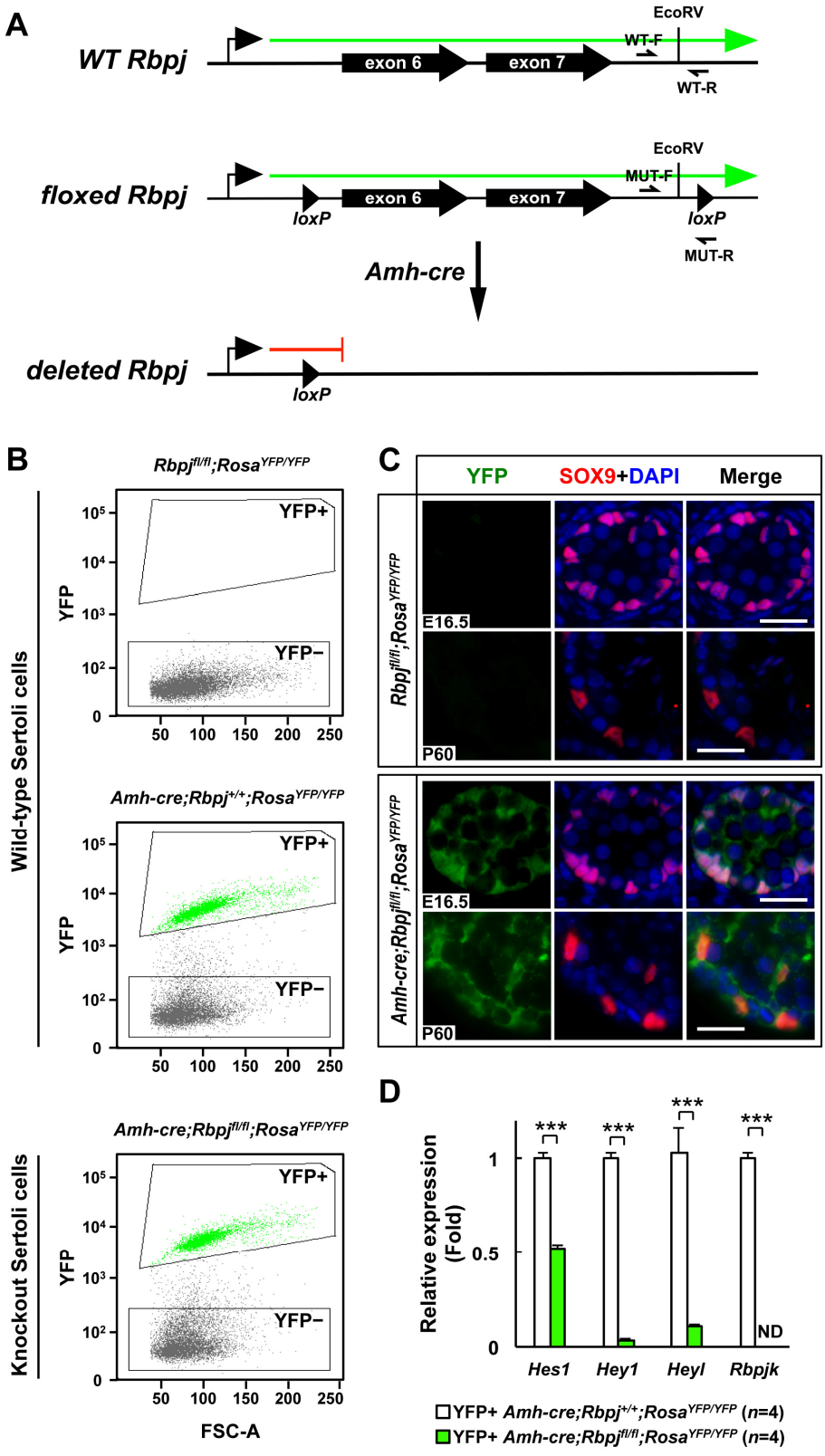
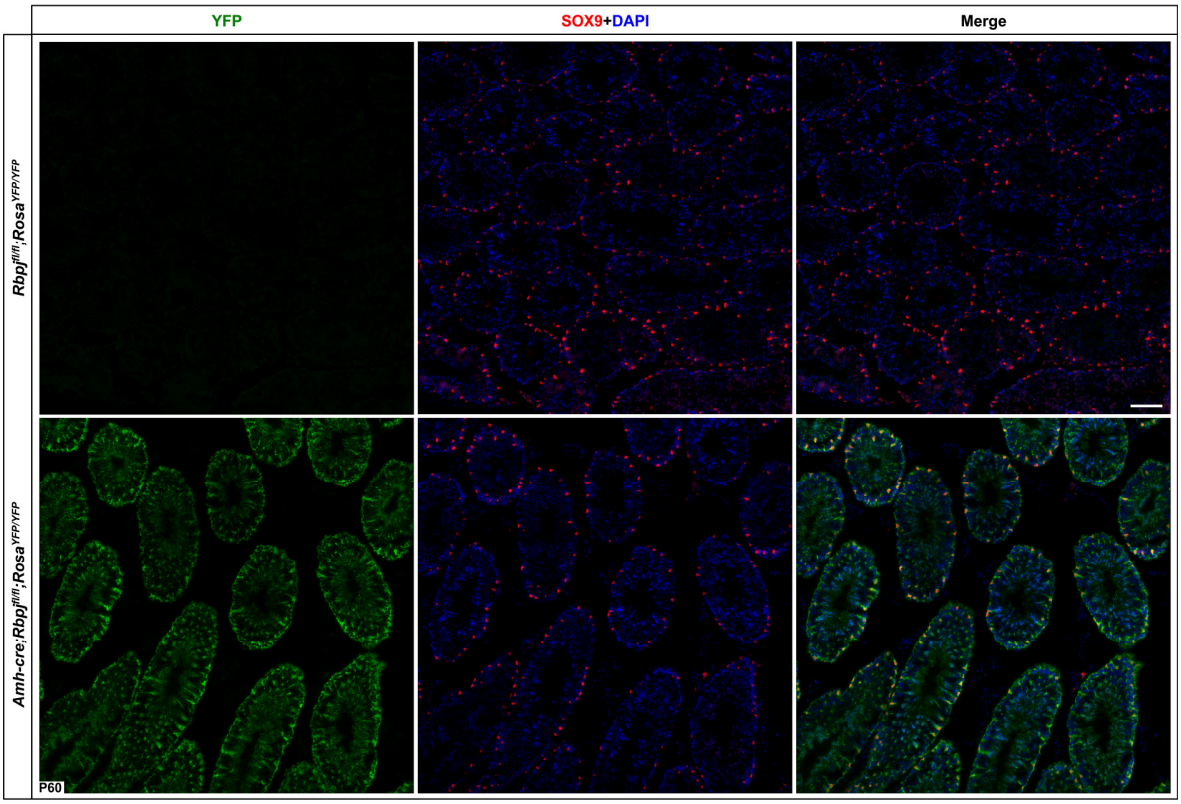
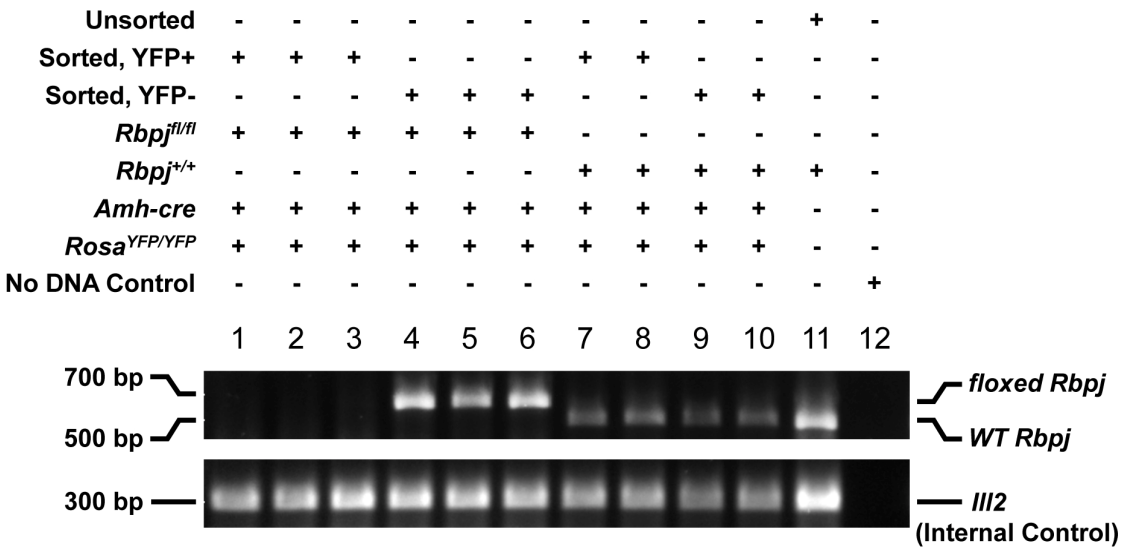

A
WT Rbpj
EcoRV
WT-F
exon 6
exon 7
WT-R
floxed Rbpj
EcoRV
MUT-F
loxP
exon 6
exon 7
loxP
MUT-R
Amh-cre
deleted Rbpj
loxP
B
Rbpjfl/fl;RosaYFP/YFP
Wild-type Sertoli cells
YFP+
YFP−
YFP
Amh-cre;Rbpj+/+;RosaYFP/YFP
Amh-cre;Rbpjfl/fl;RosaYFP/YFP
Knockout Sertoli cells
FSC-A
C
YFP
SOX9+DAPI
Merge
Rbpjfl/fl;RosaYFP/YFP
Amh-cre;Rbpjfl/fl;RosaYFP/YFP
E16.5
P60
D
Relative expression (Fold)
***
ND
Hes1
Hey1
Heyl
Rbpjk
YFP+ Amh-cre;Rbpj+/+;RosaYFP/YFP (n=4)
YFP+ Amh-cre;Rbpjfl/fl;RosaYFP/YFP (n=4)

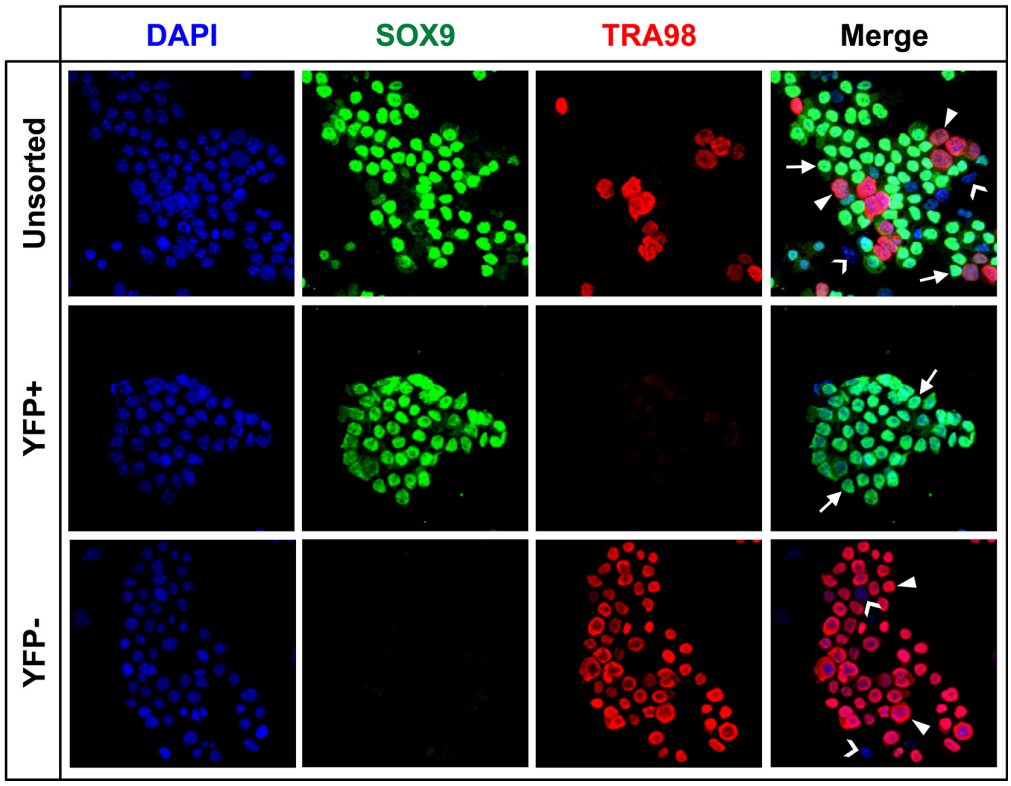
Supplementary Figure 1. Ablation of *Rbpj* gene function in Sertoli cells. (A) Schematic representation of exons 6 and 7 of the mouse *Rbpj* gene, highlighting the position of the loxP sites in the floxed animal, and the position of the wild-type and mutant primers used for genotyping ear/tail tissue as well as FACS-sorted cells. **(B)** Representative flow-cytometry profiles of wild-type and knockout Sertoli cells expressing yellow fluorescent protein (YFP) from introduction of the Cre-dependent $Rosa^{YFP}$ allele and used for fluorescence-activated cell sorting (FACS). **(C)** Representative tubule cross–sections from embryonic and postnatal control and knockout testes stained for YFP, SOX9 (Sertoli cell marker), and DAPI (DNA marker) showing Cre-mediated expression of YFP in Sertoli cells. **(D)** Real-time PCR analysis of YFP+ Sertoli cells isolated through FACS showing ablation of *Rbpj* transcripts and significant reductions in the expression of the canonical Notch target genes, *Hes1, Hey1,* and *Heyl* in knockout Sertoli cells relative to wild-type Sertoli cells. ***$P<0.005$. Scale bar = 100 µm.



Supplementary Figure 2. YFP staining of *Amh-cre;Rbpj$^{fl/fl}$;Rosa$^{YFP/YFP}$* sections show classic Sertoli cell cytoplasm staining pattern. Immunofluorescence images of *Rbpj$^{fl/fl}$;Rosa$^{YFP/YFP}$* and *Amh-cre;Rbpj$^{fl/fl}$;Rosa$^{YFP/YFP}$* testis sections at P60 as indicated. YFP (green), SOX9 (red), and DAPI (blue). Scale bars represent 100 µm.

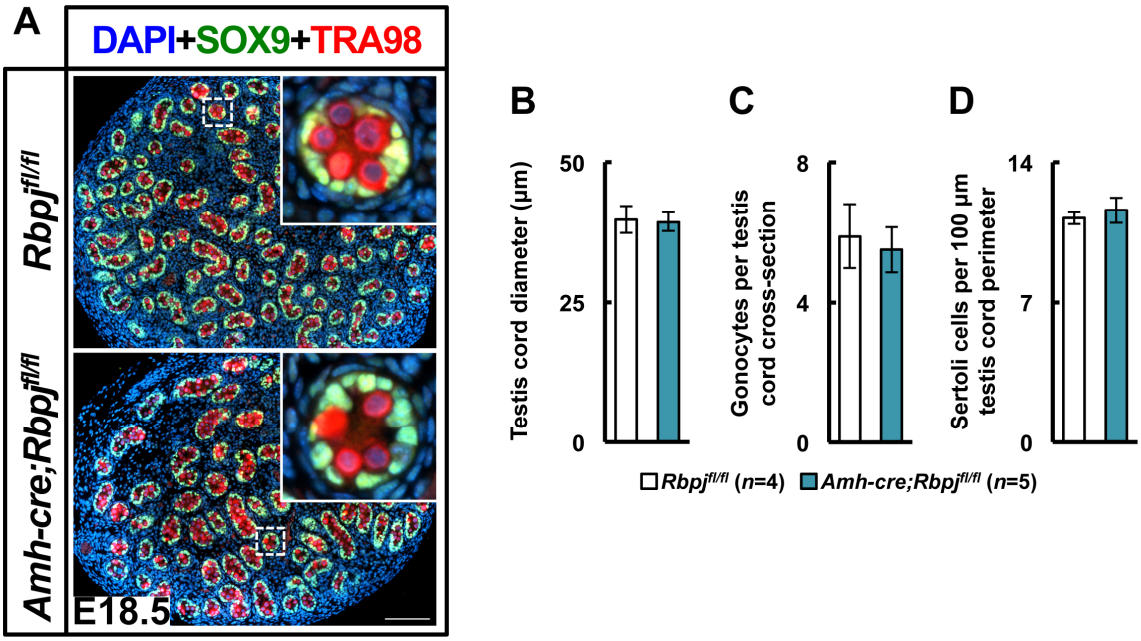


Supplementary Figure 3. Complete deletion of *Rbpj* floxed exons in mice containing the *Amh-cre* transgene. Genotyping as performed on ear and tail tissue for routine identification of mice was carried out on FACS sorted cells to determine efficiency of Cre-mediated recombination of floxed-*Rbpj*. Neither floxed nor WT alleles could be detected in YFP+ cells derived $Rbpj^{SCKO}$ mice demonstrating 100% recombination of the floxed allele. Results are given as means ± standard error of the mean.

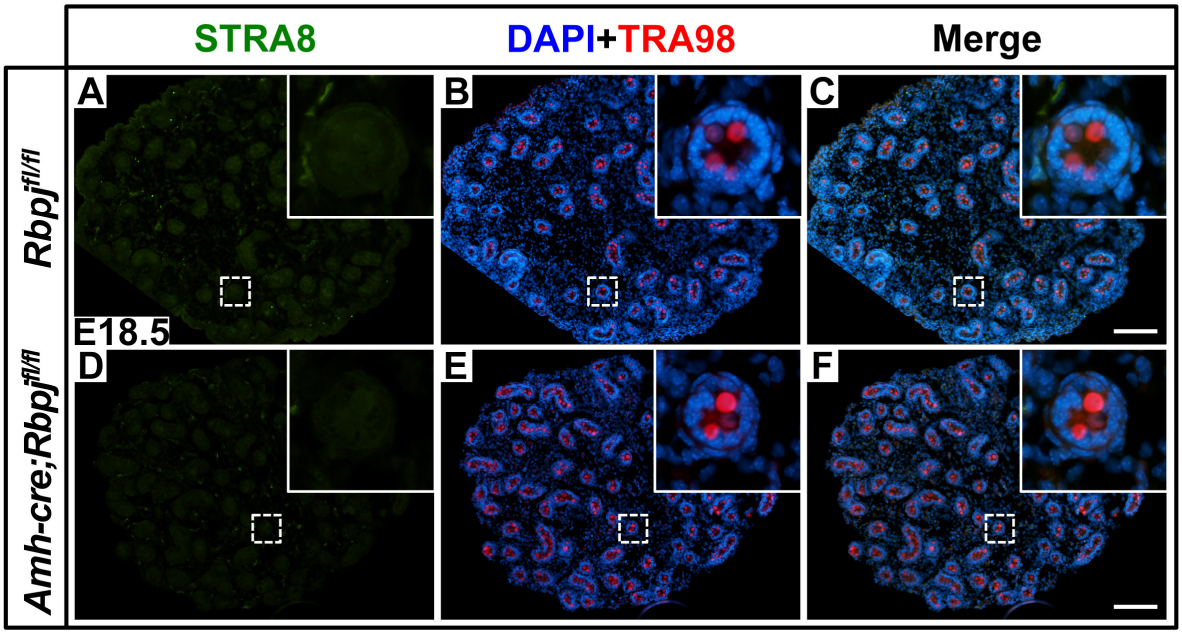


Supplementary Figure 4. Fluorescence-activated cell sorting of mice containing *Amh-cre* and *Rosa*^{YFP} transgenes yields pure populations of YFP+ Sertoli cells.

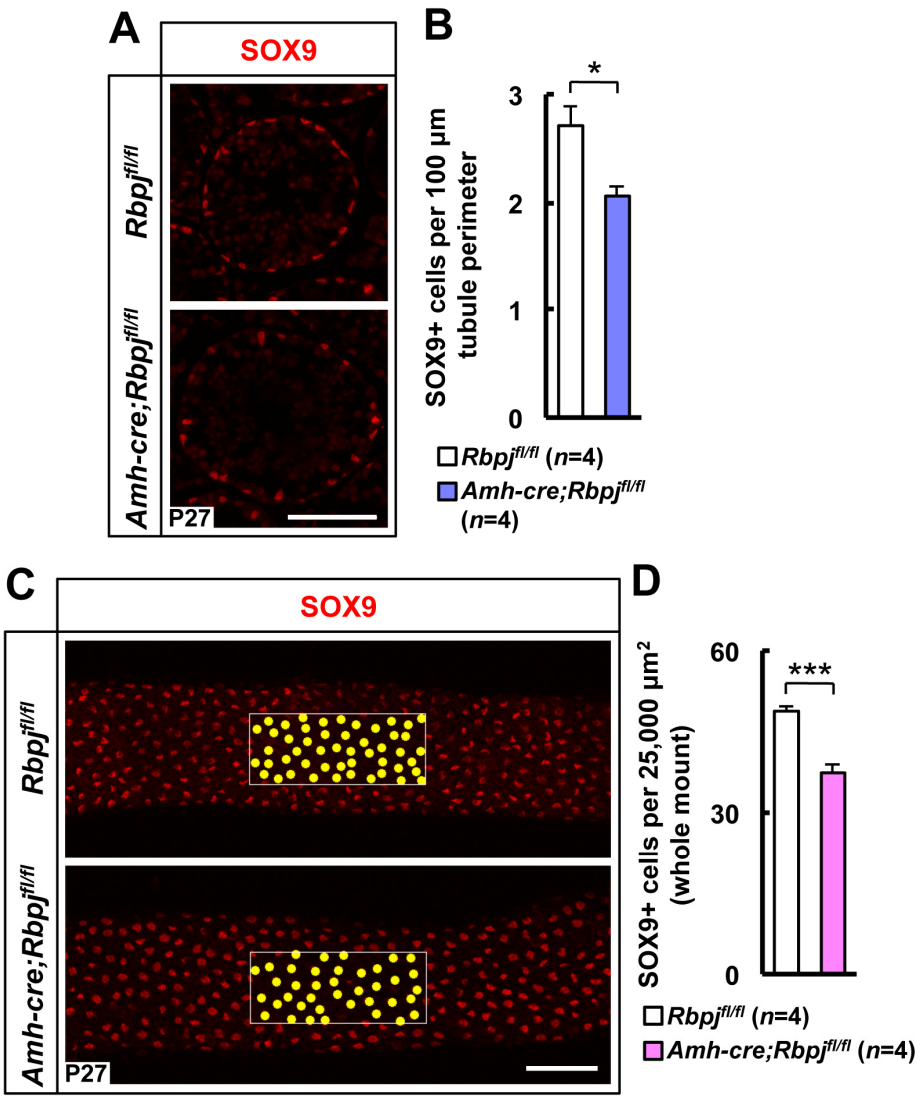
Immunocytochemistry was performed on unsorted (input) cells and YFP+ and YFP- (output) cells as isolated through FACS. After FACS, cells were adhered to microscope slides by cytocentrifugation, stained with the indicated primary antibodies, and then visualized through confocal microscopy with equivalent exposure levels set for like channels. Whereas unsorted cells contain a mix of DAPI+/SOX9+/TRA98- (Sertoli) cells, DAPI+/SOX9-/TRA98+ (germ) cells, and DAPI+/SOX9-/TRA98- (interstitial) cells, YFP+ cells contain DAPI+/SOX9+/TRA98- (Sertoli) cells only and YFP- cells contain a mix of DAPI+/SOX9-/TRA98+ (germ) cells and DAPI+/SOX9-/TRA98- (interstitial) cells. In the testis, SOX9 is widely accepted as a specific marker for Sertoli cells and TRA98 is widely accepted as a specific marker for germ cells. Therefore, YFP+ cells are Sertoli cells. Examples of Sertoli cells (arrows), germ cells (arrowheads), and interstitial cells (chevrons) as marked.



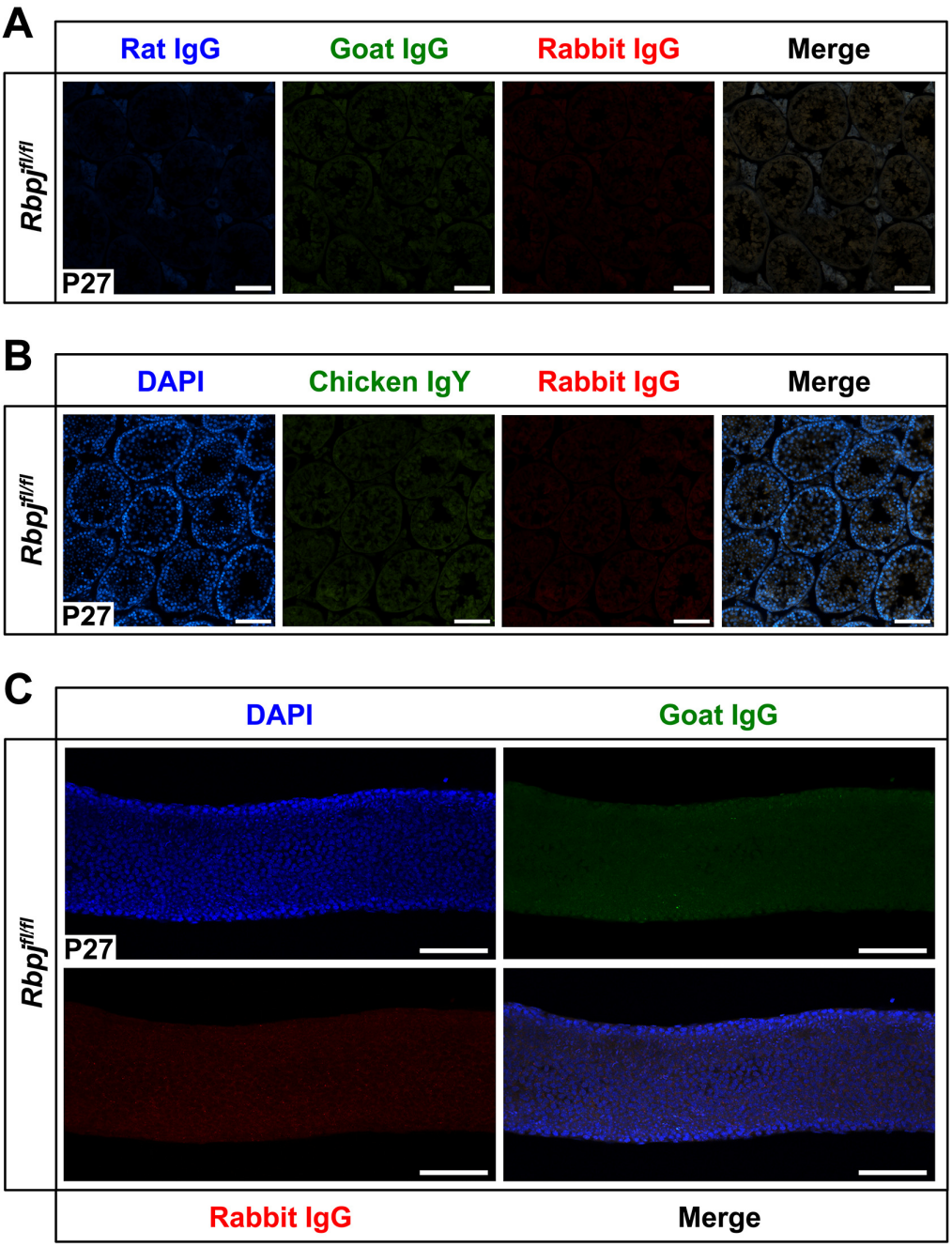
Supplementary Figure 5. Testis cord diameters and germ cell and Sertoli cell numbers are not altered in embryonic *Rbpj*^{SCKO} gonads. (A) Representative Immunofluorescence images of control (*Rbpj*$^{fl/fl}$) and *Rbpj*^{SCKO} (*Amh-cre;Rbpj*$^{fl/fl}$) gonad sections at E18.5. SOX9 (green), TRA98 (red), and DAPI (blue). Scale bars represent 100 µm. **(B-D)** In total, 252 circular cord cross-sections were evaluated, with an average of 14 circular cord cross-sections evaluated per gonad cross-section. Two gonad cross-sections were evaluated per fetus with one gonad cross-section coming from one gonad and the other gonad cross-section coming from the contralateral gonad. The average values (circular cord cross-section diameters, gonocytes per circular cord cross-section, and Sertoli cells per 100 µm cord perimeter) from one gonad cross-section were averaged with the average values of the contralateral gonad cross-section to obtain a mean value per fetus. This was done with 4 control and 5 *Rbpj*^{SCKO} fetuses to obtain the final means and standard error of the means depicted in the graphs **(B-D)**.



Supplementary Figure 6. *Rbpj*^{SCKO} gonocytes do not express the meiosis entry marker, STRA8, indicating appropriate maintenance in an undifferentiated state. (A-F) Immunofluorescence images of *Rbpj*$^{fl/fl}$ (A-C) and *Amh-cre;Rbpj*$^{fl/fl}$ (D-F) gonad sections at E18.5. STRA8 (green), TRA98 (red), and DAPI (blue). Scale bars represent 100 μm.

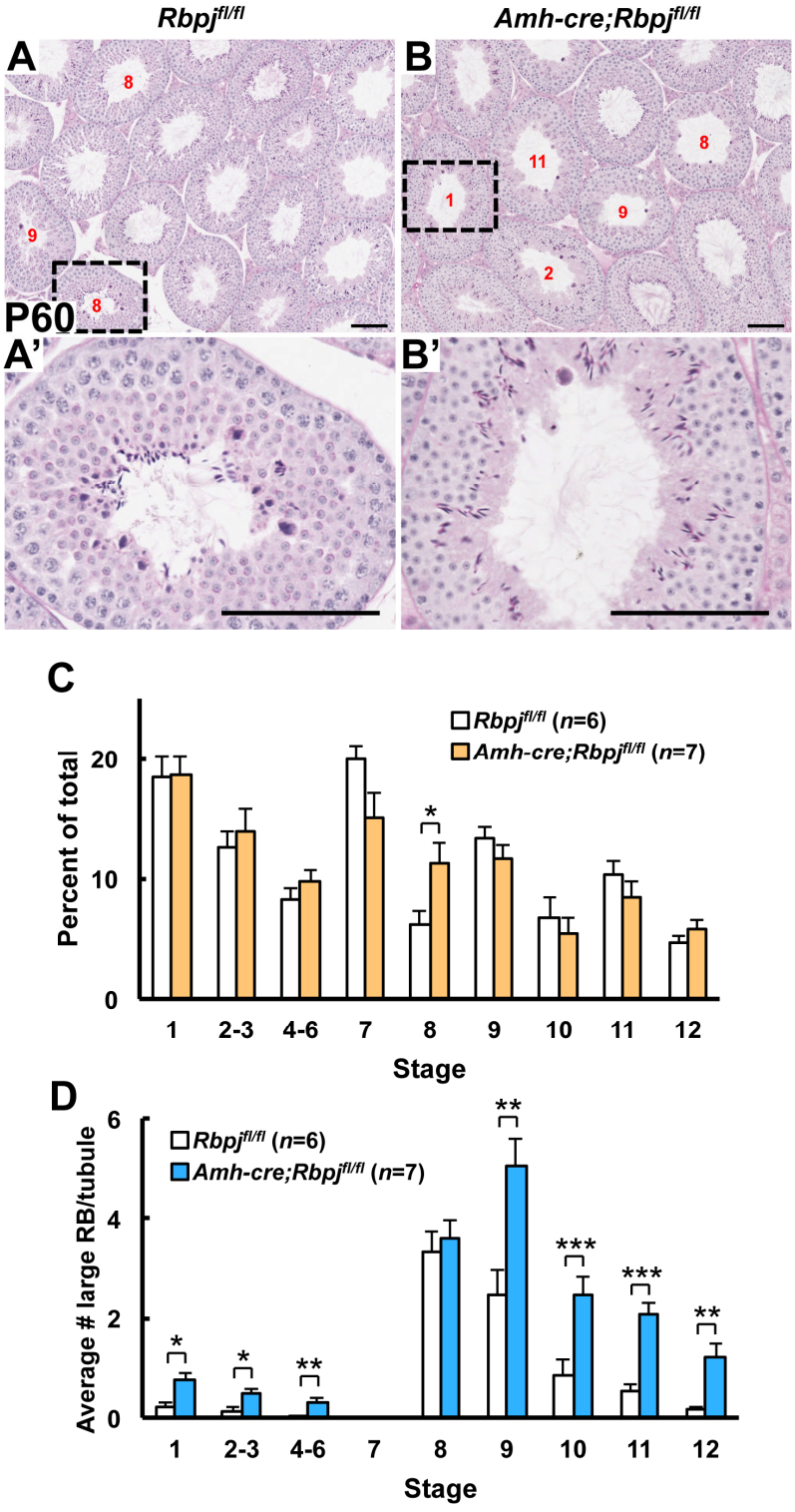


Supplementary Figure 7. Sertoli cells density is lower in *Rbpj*^{SCKO} mouse testes at postnatal day 27. (A) Representative tubule cross–sections from control and knockout testes stained for SOX9 (Sertoli cell marker). **(B)** Quantification of the number of SOX9+ Sertoli cells per 100 µm tubule perimeter. **(C)** In a representative example of anti–SOX9 antibody whole–mount staining of control and knockout tubules, yellow dots highlight individual Sertoli (SOX9+) cells within a 25,000–µm^2 (100 µm × 250 µm) tubule outer area. **(D)** Quantification of the number of SOX9+ Sertoli cells per 25,000 µm^2. Results are given as means ± standard error of the mean. *P<0.05 and ***P<0.005. Scale bar = 100 µm.

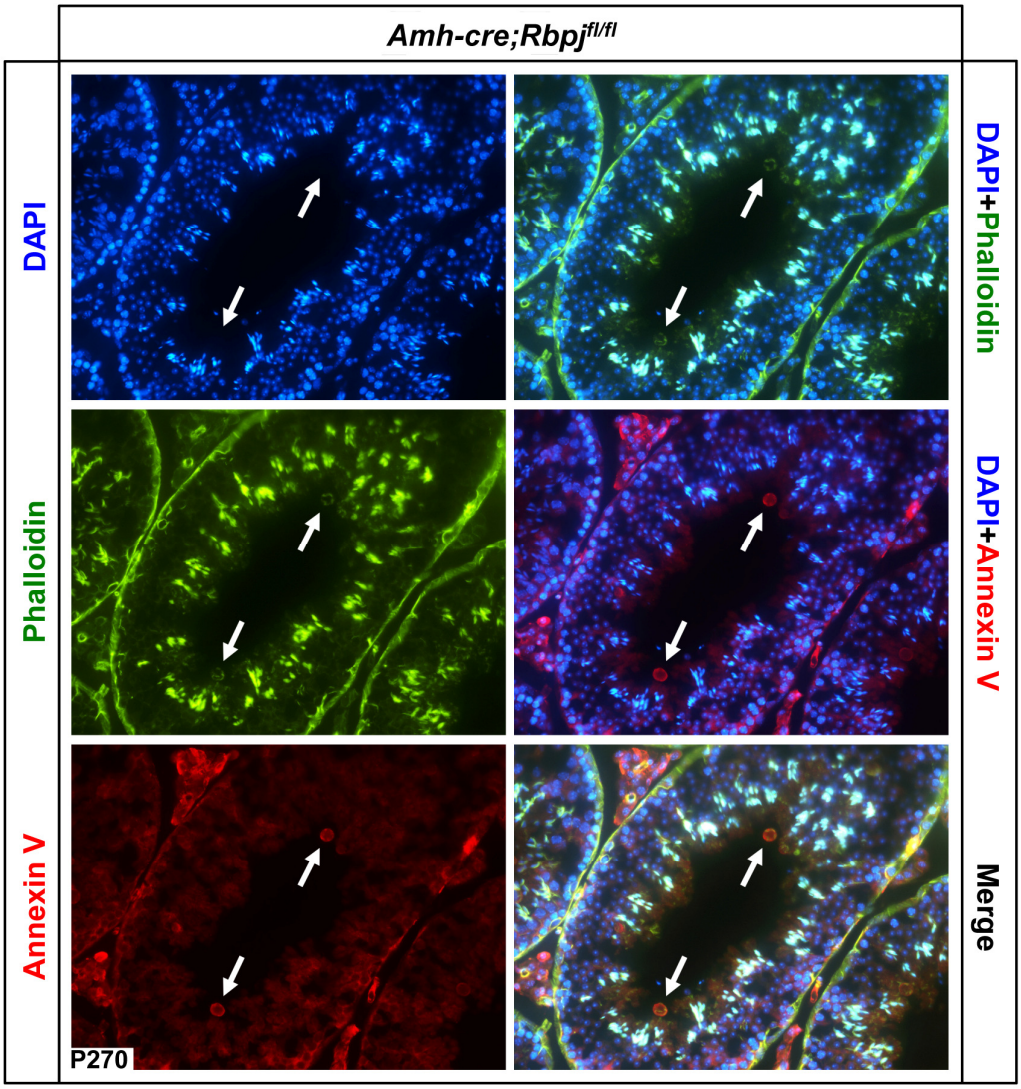


Supplementary Figure 8. Representative immunohistochemistry and whole mount staining with isotype control antibodies. (A-B) Postnatal day 27 testis sections from *Rbpj*$^{fl/fl}$;*Rosa*$^{YFP/YFP}$ control mice were incubated with the indicated isotype control IgGs, each at the highest concentration any target-specific primary antibody was used at during the study. For example, for immunohistochemistry, goat anti-GFRA1, goat anti-PLZF, and goat anti-JAG1 antibodies were used at 1:500, 1:500, and 1:75, respectively, which corresponded

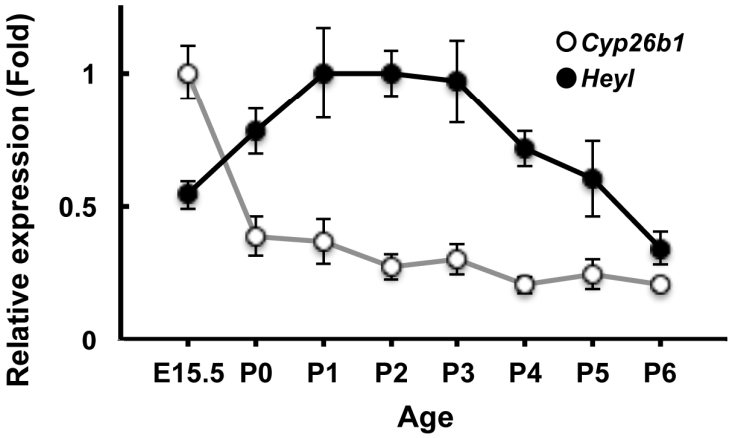
to concentrations of 2.0, 0.4, and 13.3 µg/ml, respectively. Therefore, as control, non-specific goat IgG antibodies were used at a concentration of 13.3 µg/ml. Similarly, chicken, rabbit, and rat isotype control IgG antibodies were used at 10.0, 2.0, and 4.0 µg/ml, respectively. **(C)** Isotype control whole mount staining of postnatal day 27 seminiferous tubules from *Rbpj*$^{fl/fl}$;*Rosa*$^{YFP/YFP}$ control mice. Since target-specific primary antibodies—rabbit anti-SOX9 and goat anti-GFRA1—were each used at 1:200 dilutions, corresponding to 5 µg/ml each, rabbit and goat isotype control antibodies were also used at 5 µg/ml. Scale bars = 100 µm.


Rbpj^fl/fl
Amh-cre;Rbpj^fl/fl
A
B
P60
A'
B'
C
Rbpj^fl/fl (n=6)
Amh-cre;Rbpj^fl/fl (n=7)
Percent of total
Stage
D
Average # large RB/tubule
Stage

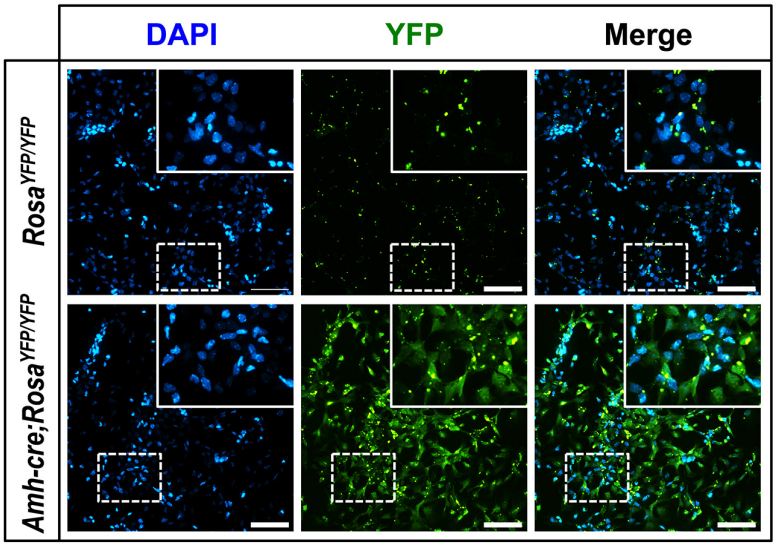
Supplementary Figure 9. The number of large, luminal residual bodies is increased at all stages of spermatogenesis in the non-atrophic knockout testes. Representative periodic acid–Schiff staining of control **(A)** and non–atrophic knockout **(B)** mouse testes. Residual bodies are normally present during stages 8 and 9 of spermatogenesis. **(A′–B′)** Higher magnification insets of A–B **(C)** Quantification of stages of spermatogenesis at P60 shows no gross alterations in the distribution of stages I through XII, except for a significant increase in the percentage of stage VIII tubules. **(D)** Stage-specific quantification of the mean number of large, luminal residual bodies (LLRBs) per tubule showing LLRBs are significantly increased at all stages of spermatogenesis.



Supplementary Figure 10. Excess intralumenal debris is likely excess residual bodies. **(A)** Staining with DAPI, anti-Annexin V, and Phalloidin confirming that spherical, intraluminal objects are devoid of nucleic acid, yet express the residual body markers consistent with their identity as residual bodies, or residual body-like extracytoplasmic debris. The arrows show representative residual bodies.



Supplementary Figure 11. *Heyl* and *Cyp26b1* transcript levels are inversely related in Sertoli cells in the perinatal testis. Perinatal gene expression analysis of pure GFP+ FACS–sorted Sertoli cells from *Amh–cre;Rosa*$^{mTmG/+}$ showing an inverse relationship between *Heyl* and *Cyp26b1* expression**.**



Supplementary Figure 12. Cytoplasmic expression of YFP in Sertoli cells cultured from mice containing *Amh-cre* and *Rosa*$^{YFP/YFP}$ transgenes. Primary isolated testicular cells from neonatal pups of the indicated genotypes were cultured for 3-5 days under growth conditions selective for Sertoli cells (as previously described), and then passaged into wells of Lab-Tek II Chamber Slides for further culture for 1-3 days. Prior to widefield fluorescence microscopy imaging, cells were washed with PBS, fixed with 4% paraformaldehyde, washed again, then coverslipped with an application of ProLong Gold with DAPI. *Amh-cre* transgene expression is specific to Sertoli cells, and YFP protein expression and fluorescence from *Rosa*^{YFP} occurs only in the presence of Cre-mediated recombination. Whereas cultured cells from *Rosa*$^{YFP/YFP}$ and *Amh-cre;Rosa*$^{YFP/YFP}$ mice both show small, punctate, non-specific fluorescence, only Sertoli cells from *Amh-cre;Rosa*$^{YFP/YFP}$ mice show cytoplasmic YFP fluorescence, verifying that greater than 95% of the cells in culture, under these culture conditions, are indeed Sertoli cells. Scale bar = 100 µm.

Table S1. Fertility of male mice over a 6 month period beginning at 2 months of age

	Number of males tested	Average no. of litters	Average no. of male pups per litter	Average no. of female pups per litter
Rbpj$^{fl/fl}$	9	5.56±0.53	5.47±0.50	4.36±0.40
Amh-cre;Rbpj$^{fl/fl}$	9	5.44±0.75	4.23±0.39	4.40±0.44
P-value		0.905	0.0678	0.948

One male was paired with one wild-type female for 6 months. *N* (male) = 9/group. If female failed to produce a litter after one full breeding cycle, she was replaced with a new virgin female. No statistically significant changes were observed between groups or over time throughout the duration of the study.

Table S2. Genotyping primers

Amh-cre	
GCG GTC TGG CAG TAA AAA CTA TC	Transgene
GTG AAA CAG CAT TGC TGT CAC TT	Transgene
CTA GGC CAC AGA ATT GAA AGA TCT	Internal Positive Control Forward
GTA GGT GGA AAT TCT AGC ATC ATC C	Internal Positive Control Reverse
Vasa-cre	
CAC GTG CAG CCG TTT AAG CCG CGT	Transgene Forward
TTC CCA TTC TAA ACA ACA CCC TGA A	Transgene Reverse
CTA GGC CAC AGA ATT GAA AGA TCT	Internal Positive Control Forward
GTA GGT GGA AAT TCT AGC ATC ATC C	Internal Positive Control Reverse
Rbpj^{fl}	
GAAGGTCGGTTGACACCAGATAGC	Mutant Forward
GCAATCCATCTTGTTCAATGGCC	Mutant Reverse
GTTCTTAACCTGTTGGTCGGAACC	Wild type Forward
GCTTGAGGCTTGATGTTCTGTATTGC	Wild type Reverse
Rosa^{YFP}	
AAG ACC GCG AAG AGT TTG TC	Mutant
AAA GTC GCT CTG AGT TGT TAT	Common
GGA GCG GGA GAA ATG GAT ATG	Wild type
Rosa^{NICD}	
AAA GTC GCT CTG AGT TGT TAT	Mutant Forward
GAA AGA CCG CGA AGA GTT TG	Mutant Reverse
CCA AAG TCG CTC TGA GTT GTT ATC	Wild type Forward
GAG CGG GAG AAA TGG ATA TG	Wild type Reverse
Rosa^{mTmG}	
CTC TGC TGC CTC CTG GCT TCT	Wild type Forward
CGA GGC GGA TCA CAA GCA ATA	Wild type Reverse
TCA ATG GGC GGG GGT CGT T	Mutant Reverse
TNR-GFP	
AAG TTC ATC TGC ACC ACC G	Transgene
TCC TTG AAG AAG ATG GTG CG	Transgene
CTA GGC CAC AGA ATT GAA AGA TCT	Internal Positive Control Forward
GTA GGT GGA AAT TCT AGC ATC ATC C	Internal Positive Control Reverse

Table S3. Primary antibodies used for immunohistochemistry

Primary Antibody	Host/isotype	Dilution / Concentration	Source	Catalog Number
anti-Annexin V	Rabbit Polyclonal IgG	1:250 / 2.0 µg/ml	Abcam	ab14196
anti-GFRA1	Goat Polyclonal IgG	1:500 / 2.0 µg/ml	Neuromics	GT15004
anti-JAG1	Goat Polyclonal IgG	1:75 / 13.3 µg/ml	Neuromics	GT15171
anti-PLZF	Goat Polyclonal IgG	1:500 / 0.4 µg/ml	R&D	AF2944
anti-SOX9	Rabbit Polyclonal IgG	1:1000 / 1.0 µg/ml	Millipore	ab5535
anti-STRA8	Rabbit Polyclonal IgG	1:1000 / 1.0 µg/ml	Dr. Michael Griswold	Zhou et al. 2008, PMID: 18032419
anti-TRA98	Rat Monoclonal IgG	1:250 / 4 µg/ml	Abcam	ab82527
anti-YFP	Chicken polyclonal IgY	1:1000 / 10.0 µg/ml	Abcam	ab13970
Isotype control	Chicken polyclonal IgY	10.0 µg/ml	Jackson ImmunoResearch	003-000-003
Isotype control	Goat IgG	13.3 µg/ml	Jackson ImmunoResearch	005-000-003
Isotype control	Rabbit IgG	2.0 µg/ml	Jackson ImmunoResearch	011-000-003
Isotype control	Rat IgG	4.0 µg/ml	Jackson ImmunoResearch	012-000-003

Table S4. TaqMan probes used for real-time PCR

Gene	Probe ID
4933402E13Rik	Mm01306153_m1
Amh	Mm03023963_m1
Cyp26b1	Mm00558507_m1
Ddx4	Mm00802445_m1
Dhh	Mm01310203_m1
Dll1	Mm01279269_m1
Dll3	Mm00432854_m1
Dll4	Mm00444619_m1
Edg7	Mm00469694_m1
Eif3l (housekeeping gene)	Mm00460859_m1
Gdnf	Mm00599849_m1
Hes1	Mm01342805_m1
Hey1	Mm00468865_m1
Heyl	Mm00516555_m1
Jag1	Mm00496902_m1
Jag2	Mm01325629_m1
Kitl	Mm00442972_m1
Plzf	Mm01176868_m1
Rbpj	Mm.PT.56a.13401125*
Rps3 (housekeeping gene)	Mm00656272_m1
Sox9	Mm00448840_m1
Vdr	Mm00437297_m1