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Supplemental Information

Spermatogonial Stem Cell Numbers Are Reduced by Transient Inhibition of GDNF Signaling but Restored by Self-Renewing Replication when Signaling Resumes

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Supplemental Figures

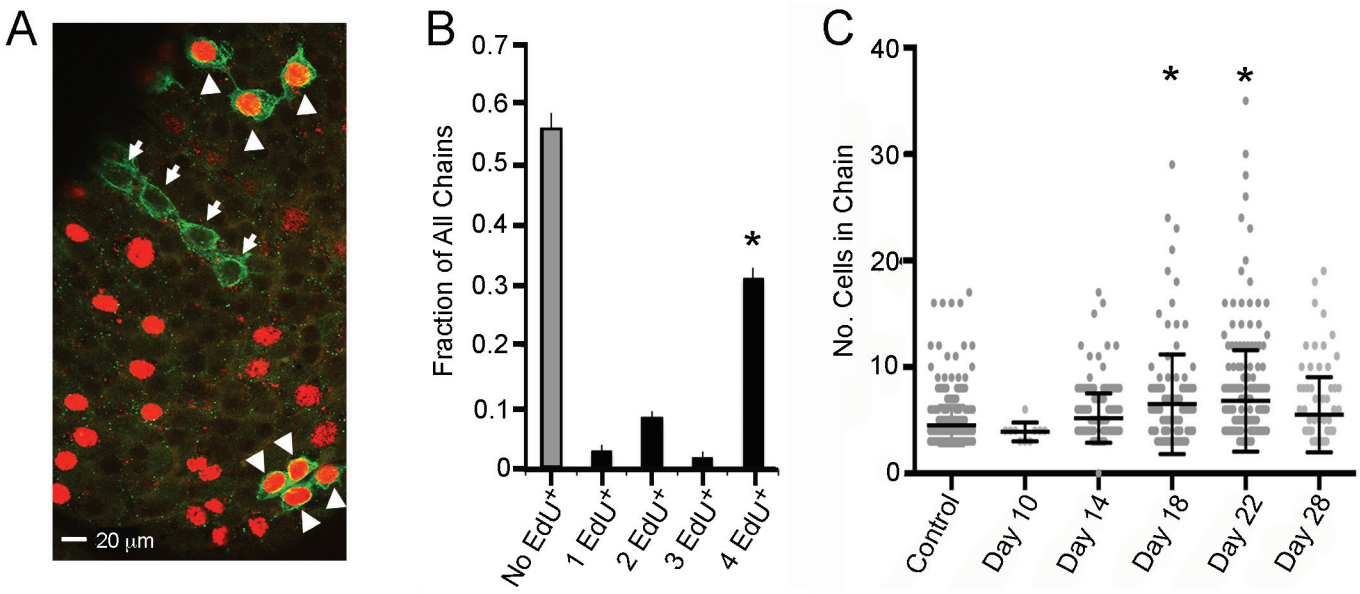

Figure S1. Characterization of chains of GFRα1$^+$ A_{al} spermatogonia. Related to Figure 2A. **A.** Confocal micrograph of a whole mount of a seminiferous tubule collected 24 hours after injecting a C57BL/6J mouse with EdU (red). A_{al} spermatogonia are identified by their expression of the cell surface stem cell marker, GFRα1 (green), and by the fact that their plasma membranes make direct physical contact. All cells in two of the chains incorporated EdU (arrow heads), none of the cells in the third chain incorporated EdU (arrows). **B.** Fraction of all chains of 4 A_{al} spermatogonia that contained no EdU$^+$ cells or 1 to 4 EdU$^+$ cells. Data (Mean+SEM) were obtained by analyzing tubules of 5 C57BL/6J mice. Asterisk indicates that there were significantly more chains with 4EdU$^+$ cells than with 1,2 or 3 EdU$^+$ cells. **C.** Numbers of GFRα1$^+$ A_{al} spermatogonia per chain in tubules of Ret(V805) mice that were injected with vehicle (control) or with 1NA-PP1 for 9 days. Tubules were collected on days 10, 14, 18, 22 and 28 of the experiment. Data are presented as numbers of cells in individual chains (gray dots) and as the mean + SEM chain length for the entire group. An asterisk over a specific experimental group identifies the mean number of cells per chain in that group as significantly greater than the mean number of cells per chain in control animals.

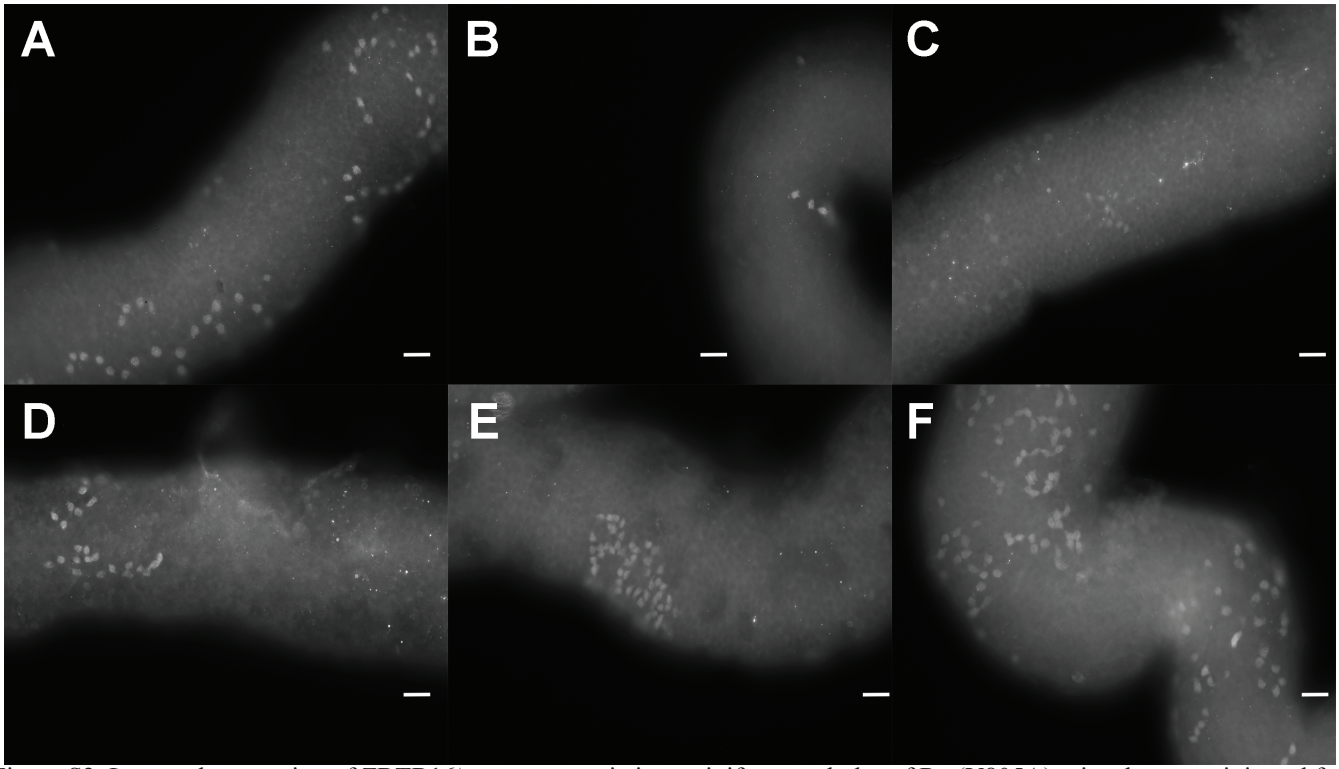

Figure S2. Loss and restoration of ZBTB16$^+$ spermatogonia in seminiferous tubules of Ret(V805A) mice that were injected for 9 days with vehicle (A) or with 1NA-PP1 and tubules collected on days 10 (B), 14 (C), 18 (D), 22 (E) and 28 (F) of the experiment. Images were captured by standard fluorescence microscopy. This figure is related to Figures 2A and 2B. Bars = 80μm

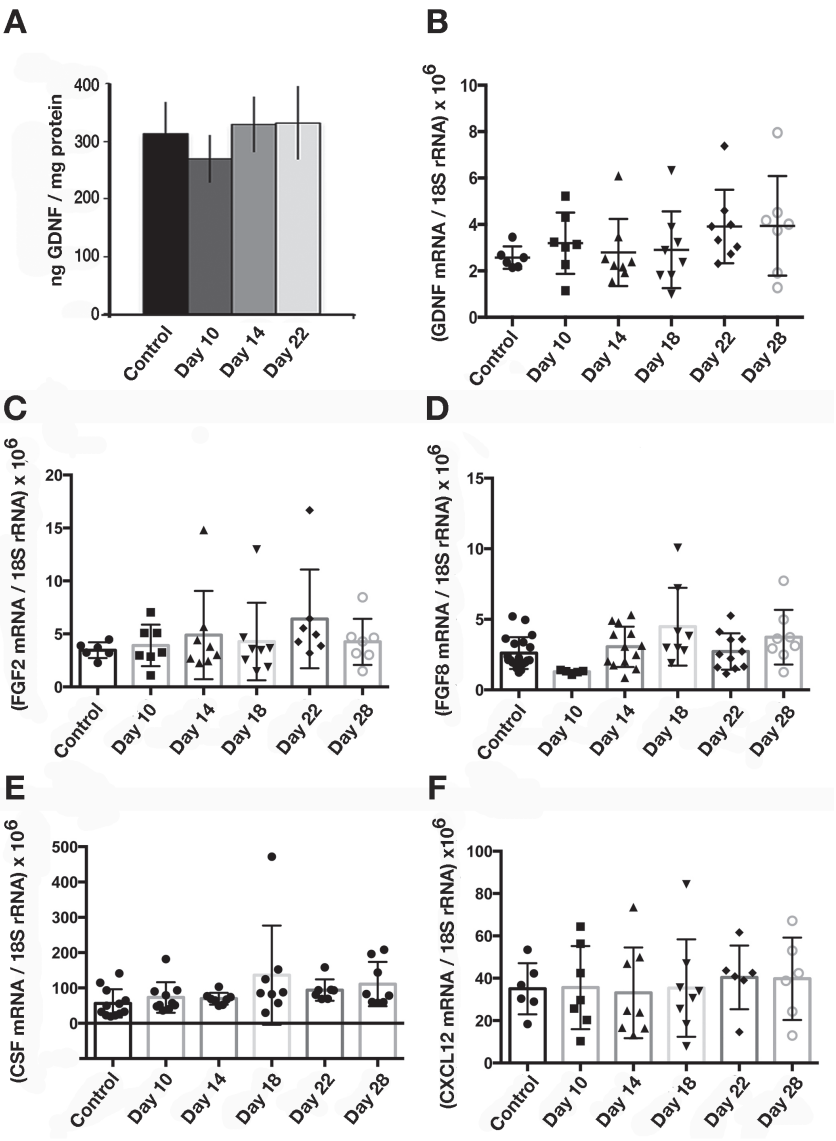

Figure S3. Testicular expression of GDNF protein (A) GDNF mRNA(B), FGF2 mRNA (C), FGF8 mRNA (D), CSF mRNA (E) and CXCL2 mRNA (F) in Ret(V805A) mice injected with vehicle or with 1NA-PP1 for 9 days. This figure is related to Figure 5. Animals were sacrificed on days 10, 14, 18, 22 and 28 of the experiment. GDNF protein levels were normalized to total cytosolic protein; transcripts levels were normalized to 18S rRNA. Data for transcript expression are presented both for individual testes and as mean ± SEM of the group.

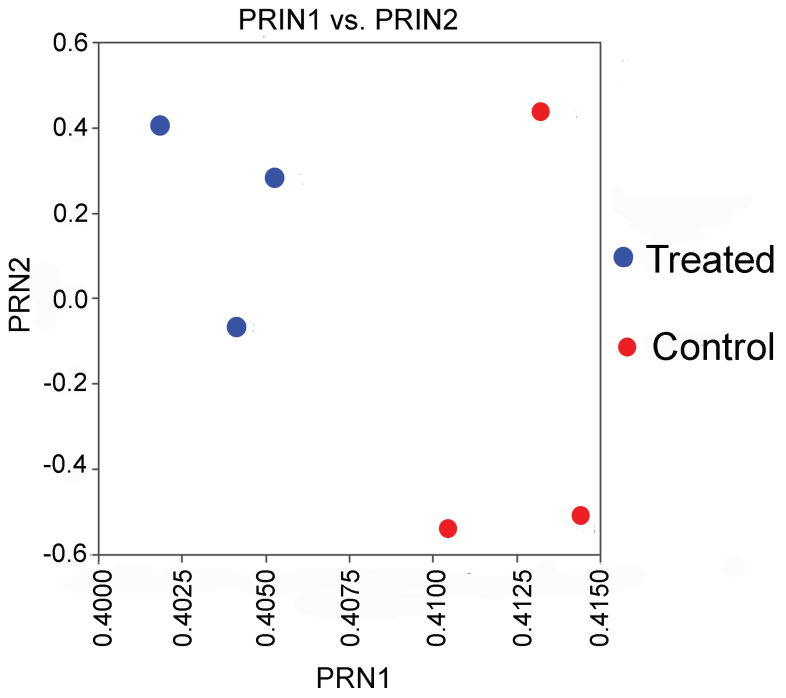

Figure S4. Principal component analysis of the testicular transcriptomes of Ret(V805A) mice that were injected for 9 days with vehicle (Control) or with 1NaPP1 (Treated). This figure is related to figure 5. Testes were collected and analyzed 5 days after the last injection.

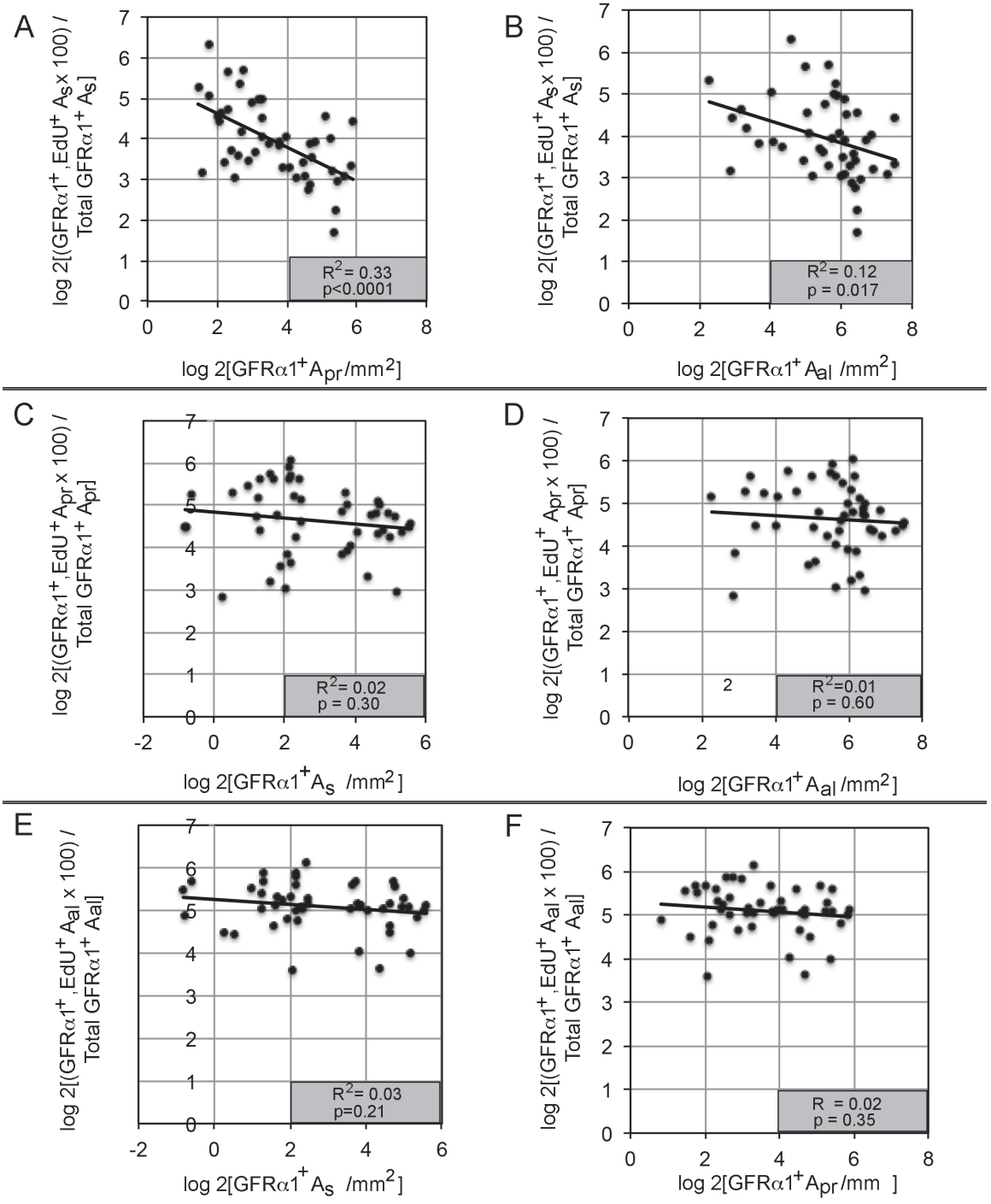

Figure S5. The replication of GFRα1$^+$ A_s spermatogonia decreases as densities of GFRα1$^+$ A_{pr} and A_{al} spermatogonia increase. This figure is related to Figure 5. For each control and treated animal (Fig. 2 B,C) densities of GFRα1+ A_s, A_{pr}, and A_{al} spermatogonia and the fractions of the other 2 cell types that were EdU$^+$ were log 2 transformed and replication of one cell type plotted against the densities of 1 of the other 2. Correlation coefficients and p values for each analysis are presented in each gray box.

Table S1: Production of New Cells from Replicating GFRa1+ As, Apr and Aal Spermatogonia During Each 3.2 Day Period Following 9 Days of Inhibited GDNF Signaling.

	Number of cells/mm^2** at start of each 3.2-day period			Fraction of cells replicating during the 24 hrs prior to collection***			Estimated number of new cells from replication of each cell type****			
Day*	As	Apr	Aal	As	Apr	Aal	As	Apr	Aal	Total
10	3.42	7.07	9.75	0.592	0.591	0.381	2.03	4.18	3.72	9.92
13.2	2.35	5.67	35.81	1.000	0.612	0.538	2.35	3.47	19.28	25.10
16.4	3.17	6.97	42.89	0.681	0.555	0.548	2.16	3.87	23.50	29.53
19.6	6.38	9.91	47.94	0.449	0.627	0.555	2.87	6.22	26.61	35.70
22.8	10.35	13.00	55.58	0.571	0.783	0.592	5.91	10.18	32.90	48.99
26	15.15	19.00	67.72	0.461	0.517	0.516	6.98	9.82	34.95	51.75
		Calculated total cell yield from replication:					**22.29**	**37.74**	**140.95**	**200.98**

* Time in days at the start of each 3.2 day period. Mice were treated with 1NaPP1-HCl from day 1 to day 9 of the experiment. The first samples were collected on day 10.

** Numbers of cells/mm^2 at the beginning of each 3.2 day period.

***Fraction of cells replicating = Fraction of cells that incorporated EdU / EdU-labeling efficiency. Numbers of cells and fraction of EdU positive cells calculated from Figure 2C.

****Yield of new cells from replication = (Number of Cells at start of cell cycle X percent of cells replicating)/100.

Table S1 is related to Table 1 in the text .

Table S2: Souces and Catologue Numbers of
Key Reagents and Kits and Working Concentrations of Antibodies

Primer	**Source**	**Catalogue No.**
Mouse GDNF	Applied Biosystems	Mm00599849_m1
Mouse GFRα1	Applied Biosystems	Mm00439086_m1
Mouse FGF2	Applied Biosystems	Mm01285715_m1
Mouse CXCL12	Applied Biosystems	Mm00445553_m1
Mouse Ret	Applied Biosystems	Mm00436304_m1
Mouse Kif26a	Applied Biosystems	Mm01339746_m1
Human 18S rRNA	Applied Biosystems	4319413E
Antibodies (Working Conc.)	**Source**	**Catalogue No.**
ZBTB16 [PLZF] (1:1000)	R&D Systems	AF2944
GFRa1 (1:200 and 1:100)	R&D Systems	AF560
c-Kit (1:50)	Novus Biologics USA	NB100-77477
BrdU(1:200)	Developmental Studies Hybridoma Bank	G3G4
Claudin 11(1:100)	Invitrogen	36-4500
Alexa Fluor 488-donkey anti-goat IgG (1:200)	Invitrogen	A11055
Alexa Fluor 546- donkey anti goat IgG (1:200)	Invitrogen	A11056
Alexa Fluor 488 donkey-anti rat IgG (1:200)	Invitrogen	A21208
Alexa Fluor 647 rabbit anti-mouse IgG (1:500)	Invitrogen	A21239
Alexa Fluor 555 donkey anti-rabbit IgG (1:500)	Invitrogen	A31572
Kits	**Source**	**Catalogue No.**
GDNF Emax ImmunoAssay System	Promega	G7621
Superscript III First Strand Synthesis	Invitrogen	18080-051
Rneasy Mini Kit	Qiagen	74104
Click-It EdU Alexa Fluor 555 Imaging Kit	Invitrogen	C10338

Table S2 is related to methods.

Supplemental Methods

Treatment of Mice. Mice were administered daily subscapular injections of 43.7 mg/kg of the ATP competitive inhibitor, 1NaPP1-HCl, which was synthesized and prepared for injection as previously described (Savitt et al., 2012). Controls were injected with vehicle (saline: cremophor EL (7:2)). Administration of drug occurred at the same time of day (± 1 hour). To estimate the relative numbers of cells that were replicating 24 hours prior to tissue collection, mice were injected intraperitoneally with 20 mg/kg of the thymidine analogue, 5'-ethynl-2'deoxyuridine (EdU, Invitrogen, Carlsbad, CA) (Parker et al., 2014).

To determine EdU-labeling efficiency mice were injected once or every hour for 24 hours with EdU. In this experiment, after inserting the needle into the abdomen but before injection of EdU, a slight negative pressure was applied to the syringe. If blood was observed in the syringe, the animal was immediately sacrificed. Labeling efficiency for each cell type was defined as: Fraction of GFRa1$^+$ A_s, A_{pr} and A_{al} spermatogonia that were EdU$^+$ after 1 injection / fraction of cells that were EdU$^+$ after 12 injections.

To determine the length of the cell cycle of GFRa1$^+$ A_s spermatogonia, mice were injected first with 140 mg/kg of the thymidine analogue, 5'-bromo-2'-deoxyuridine (BrdU) and then 60, 66, 72, 76 and 93 hours later, with 20 mg/kg of EdU. Mice were sacrificed 2 hours after the second injection and tubules processed for GFRa1and BrdU immunochtochemistry and for EdU-click chemistry.

Immunocytochemistry and Microscopy. Seminiferous tubules were isolated, fixed and immunostained for GFRa1, ZBTB16 and/or Kit as previously described (Parker et al., 2014; Savitt et al., 2012). In all experiments negative controls (nonimmune IgG) were run to document antibody specificity. We identified cells that incorporated the thymidine analogue EdU, as previously described (Parker et al., 2014). In comparing the numbers of EdU$^+$ cells in animals injected once or 12 times, we adjusted the laser power of the confocal microscope so that Alexafluor 555-fluorescence intensities were similar in both groups. To detect incorporation of the two-thymidine analogues, BrdU and EdU, by GFRa1$^+$ spermatogonia, we first used our standard GFRa1immunostaining protocol. Then tubules were washed twice for 10 minutes at room temperature (RT) with PBS and then incubated for 20 minutes at 37°C in 3N HCl. Tubules were immediately incubated at RT twice for 10 minutes in 0.1M Sodium borate, pH=8.0. Following three 10 minute RT washes in PBS, 1% BSA, 0.1% Triton X-100, tubules were incubated overnight at 4°C in 1:200 anti-BrdU. Tubules were then washed 6 times for 15 minutes in PBS, 1% BSA, 0.1% Triton X-100 at RT, incubated overnight at 4°C in Alexafluor 647-rabbit anti-mouse IgG and then washed 6 more times at RT. Incorporation of EdU was subsequently assayed by our standard protocol. During capture of images by confocal microscopy, Alexafluor 647 fluorescence was recorded as white light. Supplemental Table 2 lists the sources and working concentrations of all antibodies and kits.

To immunolocalize claudin 11, seminiferous tubules were fixed at RT for 4 hours in 4% formaldehyde and then washed exhaustively with PBS. Tubules were permiabilized at RT for 30 minutes by incubation in PBS, 1%BSA, 0.2% Tween 20, which was also the antibody diluent and wash buffer for the entire protocol. Tubules were incubated overnight at 4°C, in anti-claudin 11, washed 6 times at RT for 15 minutes, and incubated overnight at 4°C in 1:500 AlexaFluor 555 donkey anti-rabbit IgG. After 6 more washes, tubules were mounted in Vectashield with DAPI, in order to stain nuclei. Serial optical sections were captured for all tubules. Within a given plane of focus, the optical sections for claudin-11 and for DAP1 were 2.2 and 0.9 mm, respectively. Four micrometers separated

sequential optical sections.

For all but one experiment, whole mounts of seminiferous tubules were imaged using a Zeiss LSM 710 or 710 Confocal Microscope equipped with argon and helium-neon lasers. Depending on the experiment, optical sections of 1.8 or 2.2 microns were captured. We often captured serial, overlapping optical sections in order to clearly document direct physical contact of the plasma membranes of two or more GFR$\alpha 1^+$ spermatogonia. Laser strengths and the depth of optical sections were determined empirically at the beginning of the analysis of each experiment and then were used for analysis of all samples. Tubules processed for ZBTB16$^+$ cells were imaged by fluorescence microscopy as previously described (Savitt et al., 2012). We used previously described protocols to enumerate cells/mm^2 of tubule surface (Parker et al., 2014).

In one experiment, testes were emersion fixed at 4°C in 5% glutaraldehyde in cacodylate buffer, postfixed in osmium tetroxide, embedded in Epon 812, and 1-micron thick sections stained with Toluidine blue. Four to six different testis cross sections are evaluated and a minimum of 300 tubules per testis were classified as exhibiting normal spermatogenesis, incomplete spermatogenesis (missing 1-3 generations of spermatogenic cells) or Sertoli cell-only (absence of all spermatogenic cells).

Quantitative PCR. RNA was isolated with RNeasy columns (Qiagen). RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington DE). Libraries of cDNA were generated from 1 mg of RNA with random hexamers and the First Strand Synthesis Superscript III kit (Invitrogen). Transcript levels were quantified using Taqman gene expression assays and a StepOne Plus instrument (ThermoFisher Scientific). Standard curves of sequence-verified cDNAs were run in each assay. Data were normalized for the concentration of 18S rRNA in each sample. Supplemental table 4 provides catalogue number for all Taqman assays.

Quantification of GDNF protein from mouse whole testis lysates. GDNF protein was measured using the GDNF Emax Immunoassay System (Promega, Madison, WI). Preliminary control experiments showed that this assay could readily distinguish differences in the concentrations of GDNF in different fluids of mice and rats (serum, seminiferous tubule fluid, testis interstitial fluid) predicted to contain different levels of GDNF. Fresh testes (200 mg/ml) were immediately homogenized on ice in lysis buffer containing 20mM Tris, 137 mM NaCl, 10mg/ml aprotinin, 1mg/ml leupeptin, and 1mM PMSF. Homogenates were centrifuged for 30 minutes at 15,000 RPM at 4°C and supernatant stored at -80°C until use. A 5 ml aliquot was used to measure total protein (Bradford, 1976). Data were expressed as ng of GDNF/mg of total protein.

RNA Sequencing. Total testis RNA was isolated and quality assessed as previously described (Singh et al., 2017). Libraries were generated with the NEBNext [Directional] Ultra RNA Library Prep Kit (New England Biolabs) and sequenced using an Illumnia NextSeq500 instrument at 20 million single end, 90 nucleotide reads per library. The reference genome used for the cDNA libraries was the mouse (mm10) database on the UCSC genome browser (Zeisel et al., 2013). Database analyses were performed with cutadapt, bowtie2, tophat2, and cuffdiff2 (Chen et al., 2014; Langmead and Salzberg, 2012) (Trapnell et al., 2009; Trapnell et al., 2010). The level of detection in this analysis was defined as an FPKM $\geq$ 0.1. A significant difference between control and treated testes in transcript expression was defined as FDR$\leq$ 0.05. The RNAseq data were uploaded to GEO with accession #GSE111487.

Primers and PCR conditions for determining mouse genotypes.

Genomic DNA was isolated from tail biopsies, and mice homozygous for Ret V805A and heterozygous for the Rosa26 transgene were identified by polymerase chain reaction (PCR) using primers and assay conditions described below:

Ret (V805):

Ret F: 5'- GGA CGG AAC AGT GCT TCT TG - 3'

Ret R: 5'- CTC GGC GAC AGC CTA TCT TA – 3'

PCR conditions were 3 minutes at 95°C followed by 35 cycles of 1 minute at 95°C, 1 minute at 54.5°C, 2 minutes at 72°C, followed by a 2 minute incubation at 72°C.

LacZ:

LacZ -F: 5'-ATG GGT AAC AGT CTT GGC GG-3'

LacZ -R: 5'-GGC GTA TCG CCA AAA TCA CC-3'

PCR conditions were 3 minutes at 95°C followed by 35 cycles of 1 minute at 95°C, 1 minute at 55°C, 2 minutes at 72°C, followed by a 2 minute incubation at 72°C.

ID4-EGFP:

GFP F: 5'-AAG TTC ATC TGC ACC ACC G-3'

GFP R: 5'-TCC TTG AAG AAG ATG GTG CG-3' PCR conditions were 3 minutes at 94°C followed by 35 cycles of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 68°C, followed by a 2 minute incubation at 68°C.

Supplemental References

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