signaling was inhibited for 11 days. Panel E is representative of about 3% of the tubule cross sections of each testis of Ret (V805A) mice that were examined 35 days after the last injection of NA-PP1. The black on white arrowhead points to a spermatocyte and the white arrow points to a spermatogonium. The rest of the tubules in this treatment group contained either no spermatogenic cells or only elongate spermatids (See Supplemental Fig. S9). Panel F shows one of the dense patches of Gfrα1^{+} A_{al} spermatogonia, which were dispersed along the tubules of Ret (V805A) mice that were examined 35 days after treatment. In this patch, the arrows point to 10 cells in a chain. Bar = 20 microns.

Figure 6. Some stem spermatogonia are lost when GDNF signaling is inhibited for only 2 days, resulting in a diversity of seminiferous tubule morphologies 44 days later. Panel A shows a normal stage VII seminiferous tubule containing all spermatogenic cell types; B shows a stage VII tubule lacking pachytene spermatocytes; C shows a stage VII tubule lacking round spermatids; D shows a stage I-VII tubule lacking elongate spermatids E shows a seminiferous tubule that contains only Sertoli cells and elongate spermatids; F shows a stage VII seminiferous tubule lacking both round spermatids and pachytene spermatocytes. White arrows point to spermatogonia or preleptotene spermatocytes. Black-on-white arrowheads point to pachytene spermatocytes. Black-on-white arrows point to round spermatids. White-on-black arrowheads point to nuclei of elongate spermatids. G. The mean percentage + SEM of stage I-VII seminiferous tubules exhibiting each of these morphologies. Testes from 4 separate animals were analyzed. Images in panels A-F are all at the same magnification and the bar in panel D equals 20 microns.

Savitt et al. Figure legends for supplemental figures.

Figure S1. Diagram of the targeting construct used to introduce the Ret (V805A) mutation into ES cells. The frt sites were removed by crossing mice carrying this construct with B6; SJL-Tg(ACTFLPe) 9205 DG M/J mice.

Figure S2. A diagram that shows the arrangement of mouse spermatogenic cells within the twelve stages of the cycle of the seminiferous epithelium. The cell types in each stage are depicted in columns. Each of the stages is identified by a roman numeral and the duration of each stage in hours is shown below the Roman numeral. Once cycle is completed in 8.6 days.

Un A = undifferentiated spermatogonia (i.e. A_s, A_{pr} and A_{al} spermatogonia).It is generally accepted that spermatogonial stem cells are a subset of A_s spermatogonia. If A_s spermatogonia initiate differentiation during their replication, they form A_{pr} cells, which are jointed to one another by a cytoplasmic bridge. Replication of A_{pr} cells forms a chain of A_{al} spermatogonia. These spermatogonia considered completely differentiated when they form Type A1 spermatogonia. Subsequent replications of differentiated spermatogonia leads to the formation of A2, A3, A4, Intermediate (In) and then B. Spermatocytes are identified as preleptotene (Pl), leptotene (L), zygotene (Z), pachytene (P), diplotene (D) and secondary (II). Spermatids progress through the sixteen steps of spermiogenesis, which are identified by Arabic numbers. A cell whose name is followed by a lower case ‘”m” undergoes a mitotic division or the meiotic divisions at that stage. There is a marked increase in the replication of undifferentiated spermatogonia at stage X and this replication continues through stage II (see arrows).

The linear process of spermatogenesis within any cross section of a seminiferous tubule can be appreciated by reading the chart from the left to right, bottom to top. In this chart, we define spermatogenesis in the mature testis as beginning with the mitosis of A_s spermatogonia at stages X through II (see arrows).

This figure is modified from Russell et al [1] according to Clermont [2], Oakberg [3] and de Rooij [4].

Figure S3. Sequence alignments showing conservation of the bulky residue at positions corresponding to v-Src residue 338 (shaded) in the ATP binding pocket of protein kinase subdomain V.

Figure S4. Kinase activity of Ret (V805A) but not wild-type Ret is inhibited by three different ATP competitive inhibitors. To assay Ret kinase activity, HeK293 cells were transfected with pRK5 plasmids encoding wild-type RET or RET V805A and phosphorylation of RET examined by use of immunoblots .
A: Immunoblot of phospho-RET in cells transfected with expression constructs encoding wild-type Ret and incubated with our without NA-PP1 or 1NM-PP1. Note these ATP competitive inhibitors have no effect on kinase activity of wild-type Ret.
B: Immunoblots of phospho-Ret and of total Ret in cells transfected with constructs encoding wild-type Ret or Ret (V805A). Cells expressing Ret (V805A) were incubated with or without 100 nM of four different ATP competitive inhibitors. Note that at 100 nM concentration, NA-PP1 substantially inhibits Ret kinase activity without significantly affecting the amount of total Ret in the cells.
C: Dose-dependent effect of NA-PP1 on kinase activity of Ret (V805A).
C. Structures of the ATP competitive inhibitors tested in these experiments.

Figure S5. Body and testis weights of wild type mice (A) and Ret (V805A) mice (B) treated for 30 days (A) or 20 Days (B) with vehicle or with NA-PP1 and sacrificed 24 hours after the last treatment. Data (n=4) are presented as means + SEM.

Figure S6. Treatment of of Ret (V805A) mice for 30 days with NA-PP1 has no effect on kidney or liver histology. Control mice were treated with vehicle for 30 days. Each arrow points to a glomerulus in the kidney section. Tissues were stained with hematoxylin and eosin. The bar in the lower left hand corner of each micrograph is equal to 20 microns (kidney) or 80 microns (liver). These data are representative of what was observed in the 5 treated and control animals.

Figure S7. Zbtb16^{+} spermatogonia on segments from a single seminiferous tubule that spanned one entire cycle of the seminiferous epithelium. Segments of living seminiferous tubules in PBS were transilluminated and examined by use of a dissecting microscope. The stages of the cycle of each

segment were identified by the pattern of light absorbance by the tubules, which is caused by the placement and the compaction of spermatid nuclei [5]. Note that stages X through II contain both strongly stained and faintly stained ZBTB16^{+} cells.

Figure S8. Morphologies of two stage VII seminiferous tubules in the same testis microenvironment from a Ret (V805A) mouse that was treated for 30 days with NaPP1-HCl and the testes analyzed 24 hours after the last injection. Arrowheads point to pachytene spermatocytes in one of the two tubules. Two representative pachytene spermatocytes also are shown at higher magnification in the insert. Bar = 20 microns

Figure S9. Inhibition of GDNF signaling for 11 days results in the loss of almost all spermatogonial stem cells. The micrographs show the morphologies of seminiferous tubules of a R(V805A) mouse treated for 11 days with NA-PP1 and testes examined 35 days thereafter. A. Low power image of a testis cross section. B and C. Two tubules, which are representative of approximately 97% of all of the tubules in each of 4 animals. In B, only Sertoli cells are within the tubule (Arrow head points to a Sertoli cell nucleus). In C, Sertoli cells and elongate spermatids are present but no other spermatogenic cell-type (Arrow head points to an elongate spermatid). Bar = 20 microns. The typical morphology of the remaining 3% of the tubules is shown in Figure 5E.

Figure S10. Inhibition of GDNF signaling for two days and its consequences 44 days later to: (A) the densities of GFRα1^{+} A_s, A_{pr} and A_{al} spermatogonia, (B) tubule cross sectional area and (C) the densities of GFRα1^{+} spermatogonia **X** tubule cross sectional area. Mice were injected daily for 2 days with NA-PP1 or with vehicle, and testes collected 44 days thereafter. Seminiferous tubules were isolated from one testis, fixed and immunostained for GFRα1 and the numbers of cells per mm^2 of tubule surface were determined. The other testis was fixed in glutaraldehyde, embedded in Epon and 1-micron

sections cut and stained and used to determine tubule cross-sectional areas. Data = means + SEM (n=4/group).

References for supplemental figure legends.

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