SUPPLEMENTAL INFORMATION FOR

PAX7 expression defines germline stem cells in the adult testis

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Supplemental Figures and Legends

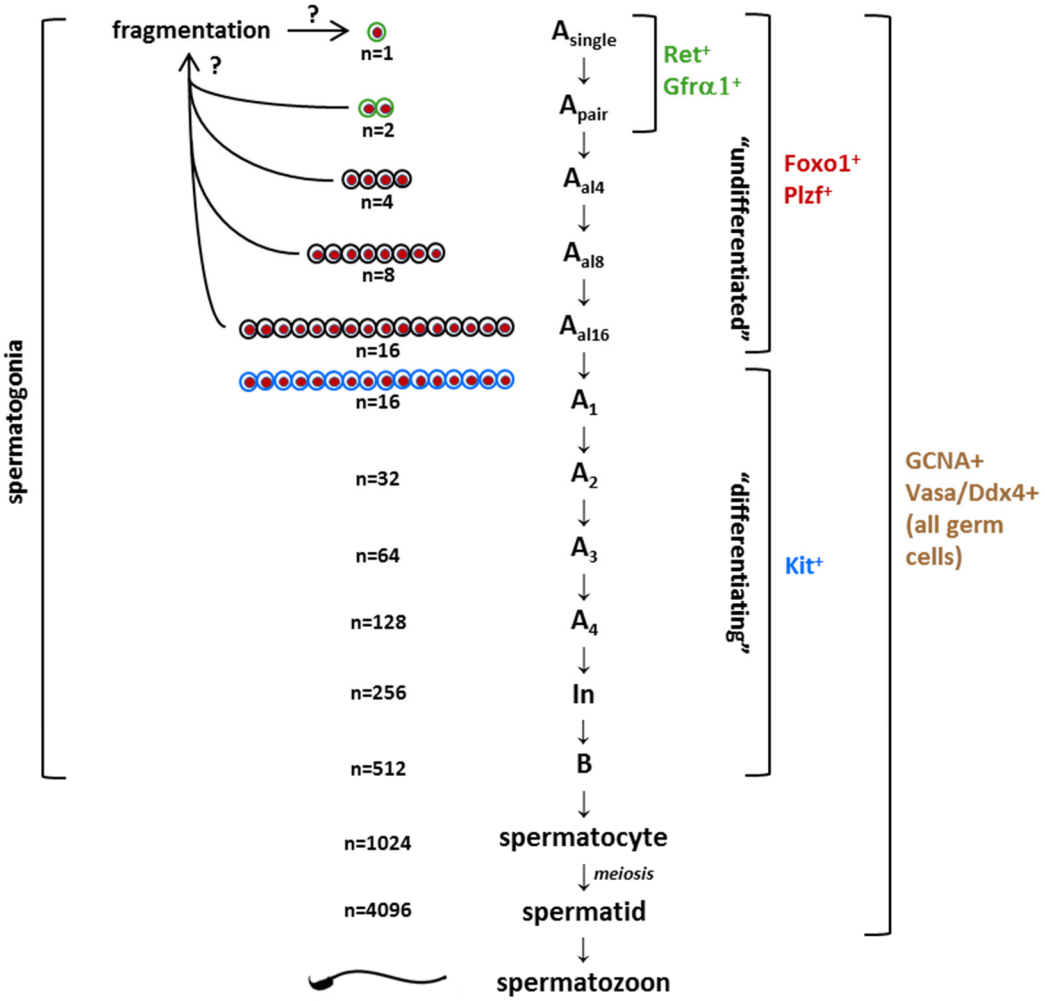


Fig. S1. Schematic of spermatogenesis in the mouse. A_{single} spermatogonia are the morphological precursors of spermatogenesis. Subsequent cell divisions occur with incomplete cytokinesis, producing intercellular bridges. The number of interconnected cells in a chain serves as a record of the number of previous mitotic divisions. Ret and Gfrα1 are coexpressed in most A_{single} and A_{pair} spermatogonia. Further rounds of mitotic divisions produce longer chains of 4, 8, and 16 spermatogonia, collectively termed $A_{aligned}$. These chains can undergo fragmentation, which may contribute to the pool of A_{single} spermatogonia, but this has not been formally established. A_{single} to $A_{aligned}$ cells are collectively called "undifferentiated" spermatogonia and express Foxo1 and Plzf. Kit^+ spermatogonia (A_{1-4} to B) are termed "differentiating"spermatogonia. B spermatogonia become spermatocytes that initiate meiosis to produce round spermatids, which elongate and eventually are released as spermatozoa.

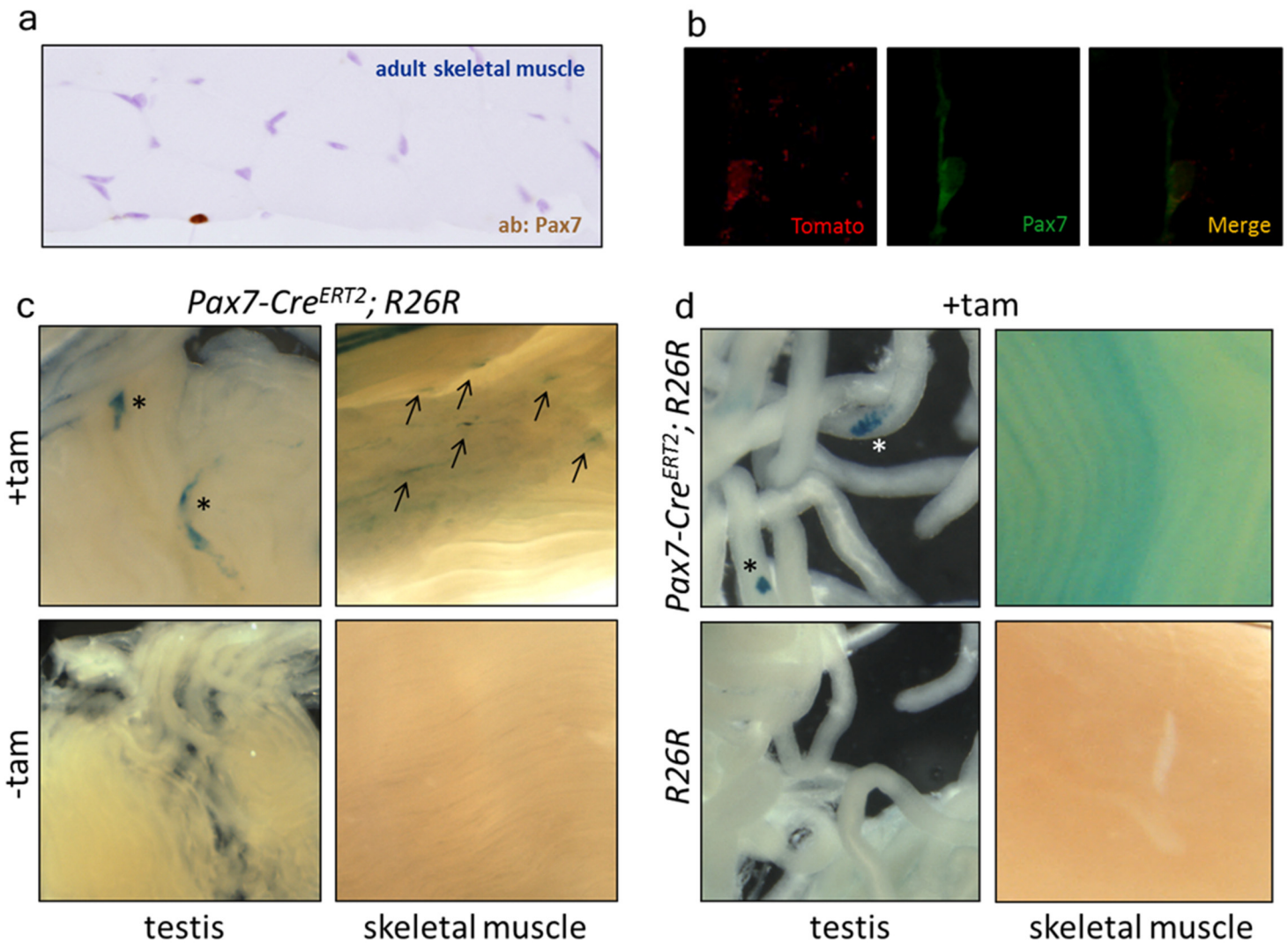


Fig. S2. Validation of reagents employed in this study. (**A**) α-Pax7 antibody shows expected pattern in adult skeletal muscle, with single labeled cell consistent with satellite cell in field shown (note nuclear localization). Immunohistochemistry was conducted by identical protocol used for testis. For B-C, seminiferous tubules were separated from the capsule and gently disaggregated with forceps. (**B**) Co-localization of endogenous Pax7 and reporter by immunofluorescence in mice harboring *Pax7-Cre*^{ERT2} and nuclear *tdTomato* reporter. Mice were treated with tamoxifen at PD14 and testes harvested at PD17. All (25/25) labeled clones analyzed by confocal microscopy showed Pax7 expression demonstrating faithful expression of *Pax7-Cre*^{ERT2} in Pax7^{+} spermatogonia. (**C**) Comparison of adult *Pax7-Cre*^{ERT2}; *R26R* mice (6 weeks of age) treated with tamoxifen versus untreated controls. Testes were harvested after 6 weeks. Note absence of labeled cells in untreated animals, confirming induction of Cre by tamoxifen. Asterisks denote labeled clones, while arrows point to muscle satellite cells. (**D**) Comparison of *Pax7-Cre*^{ERT2}; *R26R* mice vs. *R26R* controls treated with tamoxifen at PD2 and harvested at PD21. Asterisks denote labeled clones. Note absence of β-galactosidase activity in control samples. More diffuse pattern in skeletal muscle is consistent with early activation of the reporter.

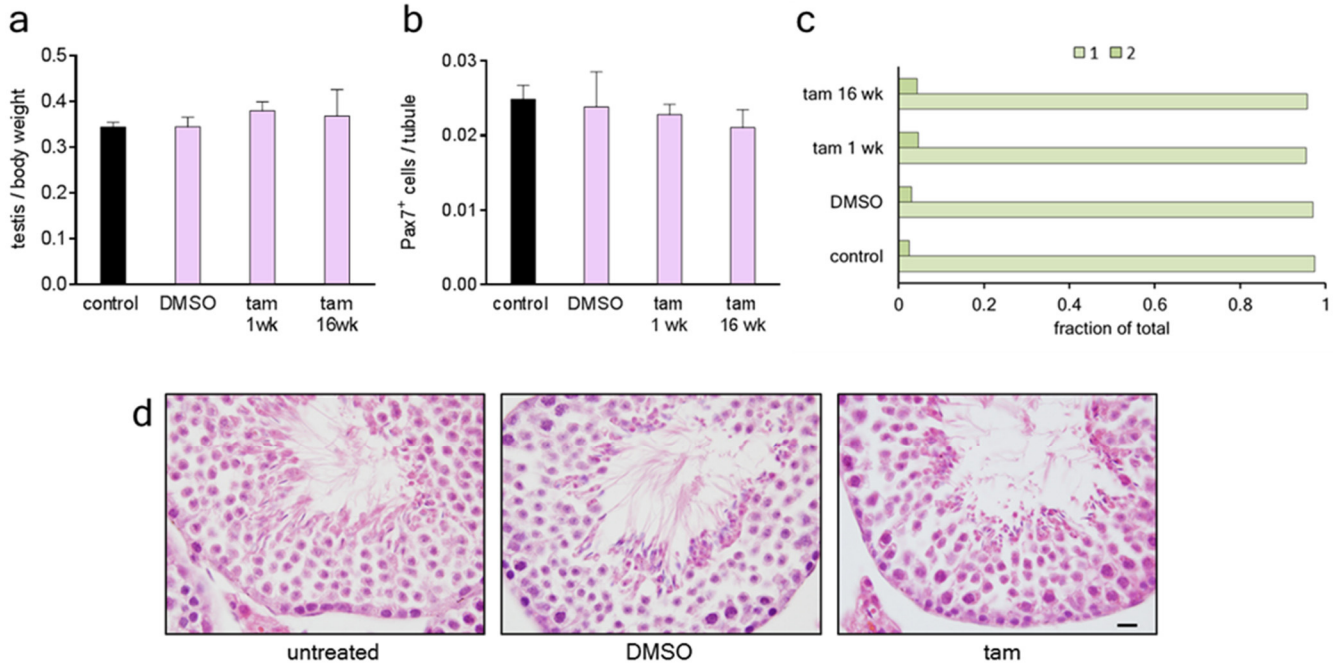


Fig. S3. Control experiments demonstrating that neither tamoxifen (1 or 16 wks) or DMSO (1 week) (carrier solvent for busulfan) have demonstrable impact on spermatogenesis or Pax7$^+$ spermatogonia. Animals were treated at 6 weeks of age and euthanized 1 or 16 weeks later (n=3 animals per observation; error bars=SEM). (**A**) Testis weight expressed as percent of total body weight. (**B**) Pax7$^+$ counts in testes from control and treated mice. (**C**) Pax7$^+$ cluster sizes in control and treated animals. (**D**) Testis morphology and spermatogenesis are unaltered by DMSO or tamoxifen. Bar=10 μ; all panels at same magnification.

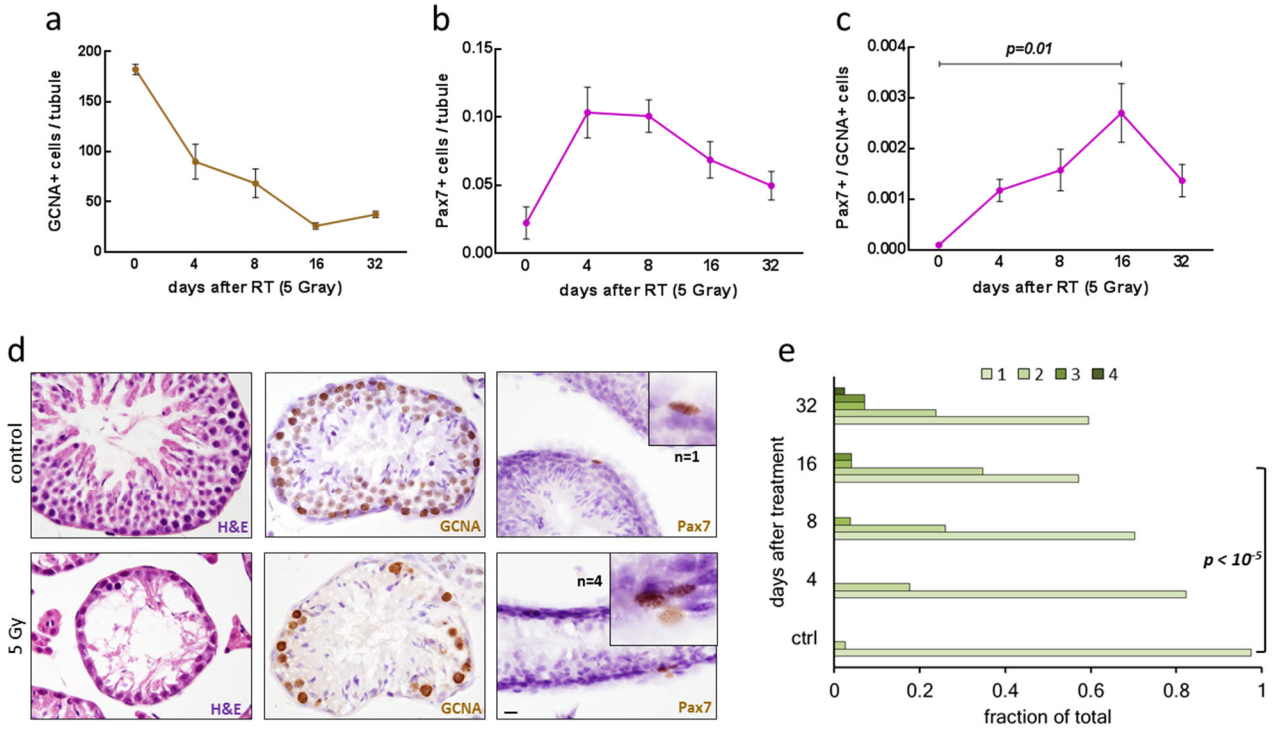


Fig. S4. Pax7$^+$ spermatogonia are selectively resistant to radiation treatment (RT). Mice were subjected to a nonfractionated dose (5 Gray) of X-rays at 6 weeks of age. Testes were harvested at timepoints shown. For A-C, error bars=SEM for n=3 animals per timepoint. (**A**) GCNA$^+$ cells per tubule. (**B**) Pax7$^+$ spermatogonia per tubule. (**C**) Pax7$^+$ spermatogonia normalized to GCNA counts; P value calculated by unpaired t-test. (**D**) H&E or immunostained slides 16 days after irradiation. Bar=10 μ; all panels at same magnification. (**E**) Fractions of Pax7$^+$ clusters of different sizes (0 to 4); $p<10^{-5}$ by Fisher Exact Test for clusters of 1 vs. 2 or more at 16 days.

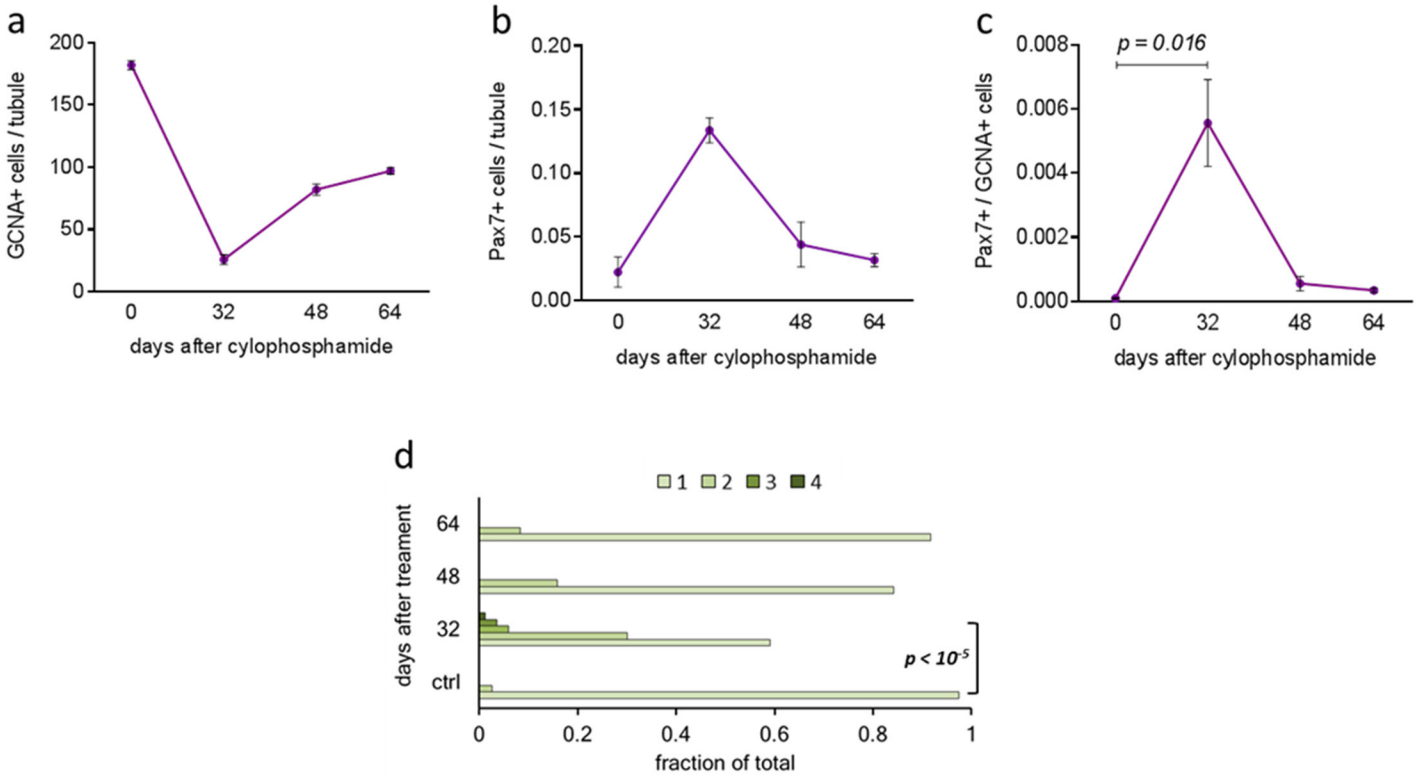


Fig. S5. Pax7$^+$ spermatogonia are selectively resistant to cyclophosphamide. Mice were treated with cyclophosphamide at 6 weeks of age. Cyclophosphamide is a relatively mild germ cell toxicant compared to busulfan, necessitating a multi-dose regimen (150 mg/kg every 5 days for 25 days). Animals were euthanized and testes harvested after the last dose. For A-C, error bars=SEM for n=3 animals per timepoint. (**A**) GCNA$^+$ cells per tubule. (**B**) Pax7$^+$ spermatogonia per tubule. (**C**) Pax7$^+$ spermatogonia normalized to GCNA counts. P value calculated by unpaired t-test. (**D**) Fractions of Pax7$^+$ clusters of different sizes (0 to 4); $p<10^{-5}$ by Fisher Exact Test for clusters of 1 vs. 2 or more at 32 days.

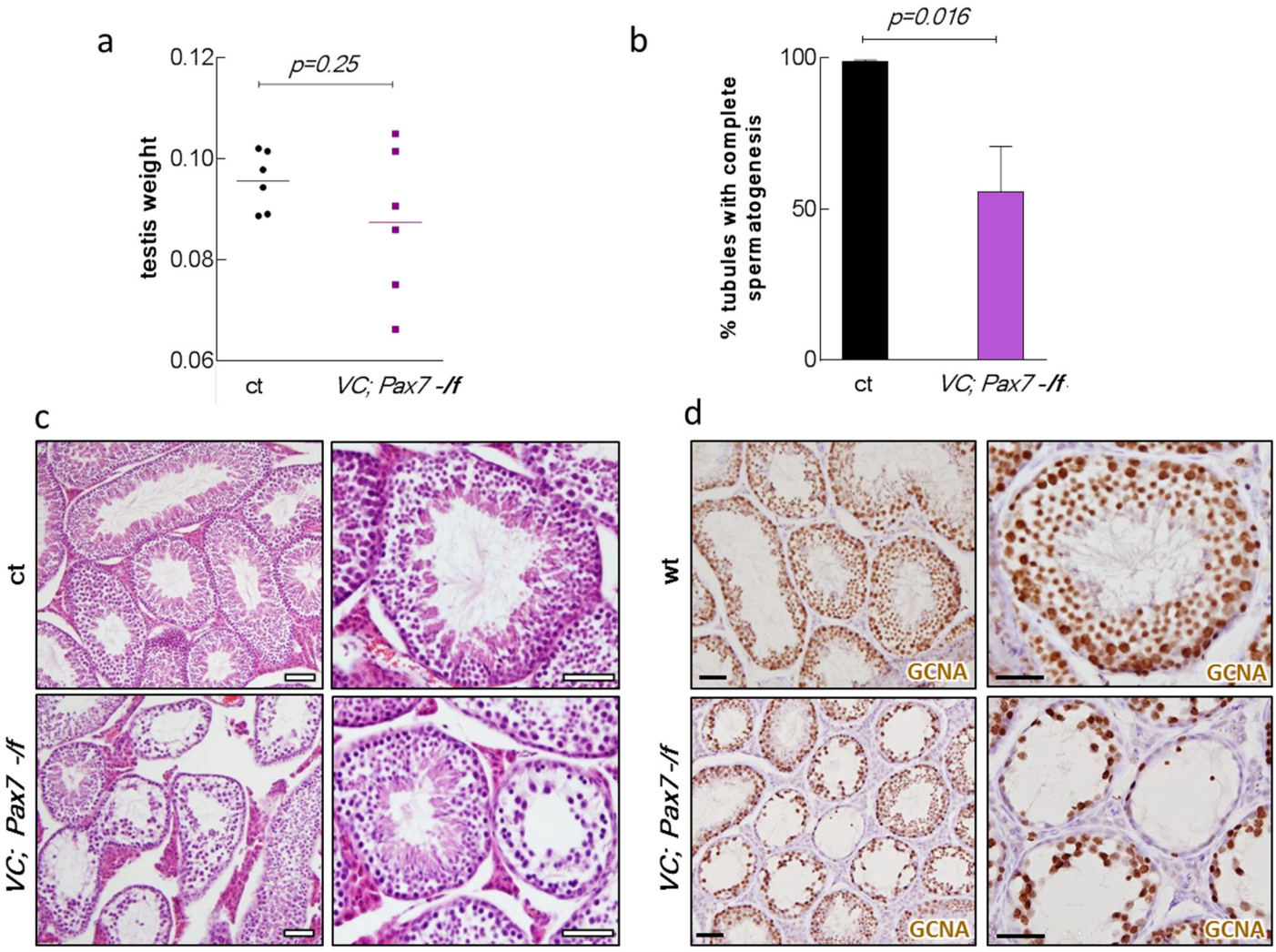


Fig. S6. Loss of Pax7 is associated with delayed spermatogenic recovery after busulfan. (**A**) Testes from adult (>3 months of age) controls and *VASA-Cre* (*VC*); *Pax7*$^{-/f}$ males were treated with a single dose of 20 mg/kg busulfan and allowed to recover for 8 weeks (testes from n=3 animals per genotype were analyzed). There was a trend towards lower testis weight in the conditional knockout animals and some of their testes were considerably smaller, but this did not achieve statistical significance. Bars=means. (**B**) Percent of tubules with complete spermatogenesis. *VC*; *Pax7*$^{-/f}$ males exhibited a significant lag in recovery of spermatogenesis, evidenced by higher numbers of tubules with decreased spermatogenesis, overall hypocellularity, and absence of full-lineage maturation (testes from n=3 animals per genotype). (**C**) Histological analyses 8 weeks after busulfan treatment. *VC*; *Pax7*$^{-/f}$ testes contained tubules with grossly abnormal spermatogenesis, although some tubules were more affected than others. Bars=50μ. (**D**) Immunohistochemical analyses 8 weeks after busulfan treatment. GCNA highlights complete or nearly complete recovery in control testes following busulfan. In contrast, in *VC*; *Pax7*$^{-/f}$ testes, germ cells were less abundant, with many tubules showing a much more limited recovery of spermatogenesis. Bars=50μ.

Supplemental Video Legends

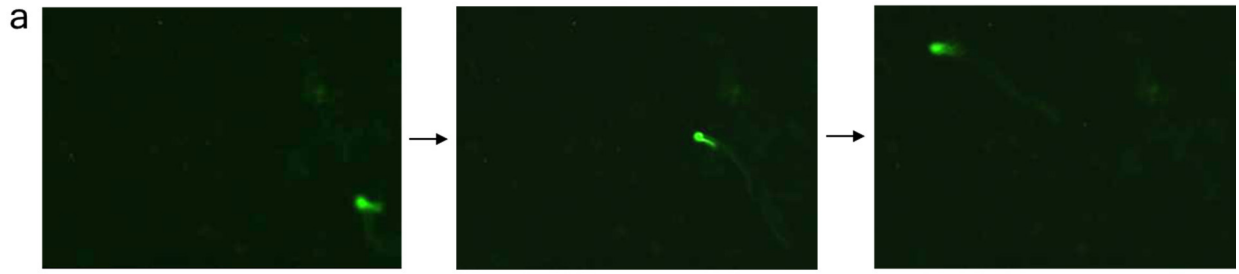
Video S1. Pax7$^+$ spermatogonia are a subset of A_{single} spermatogonia. Z-stack movie showing that Foxo1$^+$ Pax7$^+$ cell from Fig. 2C corresponds to an A_{single} spermatogonium; i.e. it is not part of a larger chain. A Foxo1$^+$ Pax7$^-$ cell is visible in the top right (as in Fig. 2C). Basement membrane staining is non-specific.

Video S2. Pax7$^+$ spermatogonia give rise to motile spermatozoa. Motile eGFP (mG)-expressing spermatozoon at 16 weeks post-tamoxifen. (**A**) Epididymides were dissected in PBS and mature sperm allowed to swim out onto the slide. Unlabeled motile spermatozoa (i.e. non-fluorescent in the green channel) were abundant in the field but cannot be seen. Comparably-fluorescent spermatozoa were not observed in control mice (i.e. observed fluorescence was not autofluorescence). See Supplemental Video 2. (**B**) Static photomicrograph by phase contrast microscopy (left) and epifluorescence (right) shows that the majority of sperm (white ellipses) were not labeled; only a single labeled sperm is present in this field (arrows).

Video S3. Pax7$^+$ spermatogonia are long-term progenitors for A_{single} spermatogonia. Z-stack movie showing portion of Pax7-descendant clone at 4 weeks post-tamoxifen with several isolated chains including (just to the left of center) an A_{single} spermatogonium. Z-stack is of the periphery of a much larger clone 4 weeks after treatment with tamoxifen.

Video S4. Pax7$^+$ spermatogonia are long-term progenitors for A_{single} spermatogonia. Three-dimensional reconstruction demonstrating that A_{single} spermatogonium in Movie S3 is physically separate from other descendants and thus indeed an A_{single} spermatogonium.

Video S2.



See Supplemental Video 2 in Supplemental Material