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A transit-amplifying population underpins the efficient regenerative capacity of the testis

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The murine spermatogonial stem cell (SSC) resides within the undifferentiated A-type spermatogonia that are defined by Asingle (A, isolated cells), Apaired (Ap, chain of two connected cells), and Aaligned (Aa, chains of 4, 8, or 16 cells) cells. These chains arise because, upon division, spermatogonia often fail to complete cytokinesis and remain connected by intercellular bridges. The A stem cell model posits that A represented the SSC, given that they reside at the apex of type A undifferentiated spermatogonia (Huckins, 1971; Oakberg, 1971). However, recent data have led to an expanded A model wherein the cell surface receptor GFRα1 identifies a subpopulation of Aa, Ap, and Aal (chain of four cells) cells that support long-term spermatogenic homeostasis in mice (Nakagawa et al., 2010; Hara et al., 2014). The GFRα1–positive cells are not an homogeneous population, as Id4 and Pax7 marks subsets of A, and these populations are endowed with SSC activity (Aloisio et al., 2014; Chan et al., 2014; Sun et al., 2015; Helsel et al., 2017). The relationship between the respective populations remains to be determined, but GFRα1–expression encompasses murine SSCs. Thereafter, SSCs down-regulate GFRα1 and express the transcription factor Ngn3 (Neurog3), which signifies the entry into an intermediate population of primitive spermatogonia that are competent to respond to retinoic acid and differentiate (Nakagawa et al., 2010; Ikami et al., 2015). Interestingly, this Ngn3–positive population acts as a transit-amplifying population under homeostatic conditions (Nakagawa et al., 2007). These Ngn3 potential SSCs can contribute to the pool of GFRα1–positive cells during regeneration (Nakagawa et al., 2010); however, the importance of this phenomenon to the regenerative capacity of the testis remains unknown. After the Aal8-16 stage, cells up-regulate the surface receptor c-kit to become differentiating spermatogonia that will undergo several further rounds of cell division and are committed to terminal differentiation (Yoshinaga et al., 1991). Here, we sought to identify novel spermatogonial populations and reveal their contribution to testicular physiology.

RESULTS AND DISCUSSION

Miwi2 expression defines a population of adult spermatogonia

Among the loci required for the maintenance of spermatogenesis, the gene encoding the Piwi protein Miwi2 caught our attention due to the slow progressive loss of germ cell phenotype observed in Miwi2−/− mice (Carmell et al., 2007; De Fazio et al., 2011). In addition, Miwi2's reported expression domain is restricted to fetal gonocytes rather than a population of adult spermatogonia (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). We therefore reasoned that Miwi2 could also be expressed in a tiny population of adult spermatogonia with SSC activity that has been overlooked by virtue of its rarity. To test this hypothesis, we generated a transcriptional reporter (Miwi2Tom) allele whereby the gene encoding the fluorescent tdTomato protein was knocked into the first coding exon of Miwi2. tdTomato faithfully recapitulated the spermatogonial stem cell (SSC) phenotype observed in Miwi2−/− mice (Carmell et al., 2000). After six months it is available under a Creative Commons License Attribution-Noncommercial-Share Alike 4.0 International license, as described at https://creativecommons.org/licenses/by-nc-sa/4.0/.

Abbreviations used: Aal, A aligned; As, A single; Apr, A paired; DTR, diphtheria toxin receptor; DTR, diphtheria toxin; SSC, spermatogonial stem cell.

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lates the expression of Miwi2 in Miwi2^{Tom} reprogramming gonocytes (Fig. 1 A and S1 C). Next, we examined by flow cytometry Miwi2-tdTomato (Miwi2-Tom) expression in the testsis gating out somatic populations with CD45 and CD51, we observed a tiny tdTomato-positive c-kit–negative (Miwi2-TomPos c-kitNeg) population (Fig. 1 B) and a larger c-kit–positive (Miwi2-TomPos c-kitPos) population that constitute proliferating EpCAM-positive differentiating spermatogonia (Fig. S1, E and F). Sorting of these respective populations revealed Miwi2 transcript in the Miwi2-TomPos c-kitNeg, but not in the Miwi2-TomPos c-kitPos populations (Fig. 1 C). We therefore concluded that the tdTomato expression in Miwi2-TomPos c-kitPos population reflects the extended life of the tdTomato protein rather than the active expression of the Miwi2 gene itself. c-kit negativity is a hallmark of SSC populations, we therefore focused our attention on the Miwi2-TomPos c-kitNeg population that represents ∼70,000 mostly quiescent or very slowly cycling cells per testis (Fig. 1, D and E). We next sought to define the surface phenotype of Miwi2-TomPos c-kitNeg cells, this population uniformly expresses all surface markers (CD9, CD49f, Thy-1, CD29, CD24, and SSClo) that enrich SSC activity in transplantation assays (Shinohara et al., 1999, 2000; Kubota et al., 2003; Kanatsu-Shinohara et al., 2004; Reding et al., 2010), whereas it is also negative for Sca1 (Fig. 1 F), whose expression has been shown to deplete for SSC potential (Kubota et al., 2003).
Tomato expression in A, Aa, and Aa revealed three distinct populations of Miwi2-Tomato− and GFRα1-expressing cells, the first group were positive for Miwi2-Tomato only (Miwi2-TomatoHi GFRα1Neg), the second class was solely GFRα1+ (Miwi2-TomatoNeg GFRα1Pos), and the third subset expressed low amounts of Miwi2-tomato, but were GFRα1− (Miwi2-TomatoLo GFRα1Pos; Fig. 1 G). The majority of Aa were Miwi2-TomatoNeg GFRα1Pos, with the other two populations present but contributing minimally (Fig. 1 H). An increased frequency of the Miwi2-TomatoHi GFRα1Neg and Miwi2-TomatoLo GFRα1Pos populations was observed with roughly all three groups contributing equally to Aa, spermatogonia (Fig. 1 H). At the Aa stage, the majority of cells had lost GFRα1 expression, and the Miwi2-TomatoHi GFRα1Neg dominated this population (Fig. 1 H). These data suggest that high levels of Miwi2-Tomato expression could be used to define a population of GFRα1− spermatogonia. Indeed, FACS analysis of Miwi2-TomatoHi c-kitNeg cells revealed their GFRα1 negativity, with increasing GFRα1 expression in Miwi2-Tomato low to dull cells (Fig. S1 G). In summary, Miwi2-TomatoHi c-kitNeg defines a population of GFRα1− undifferentiated spermatogonia that constitute ∼35,000 cells per testis (Fig. S1 H).

**Miwi2-expressing cells represent a novel subpopulation of Ngn3+ undifferentiated spermatogonia**

To further explore the molecular identity of Miwi2-TomatoHi c-kitNeg spermatogonia, we performed gene expression analyses. To this end, we also used the surface expression of CD9 as an additional sorting parameter, as it aids in eliminating contaminating autofluorescent cells from the tiny Miwi2-TomatoHi c-kitNeg population. Comparative gene expression analysis between Miwi2-TomatoHi c-kitNeg population and their Miwi2-TomatoHi c-kitPos descendants revealed enrichment for many genes that have been associated with SSC/SPC phenotype or function (e.g., Bcl6b, Lin28a, Plzf, Ret, Etv5, and Nanos2) in the Miwi2-TomatoHi c-kitNeg population (Buaas et al., 2004; Costoya et al., 2004; Chen et al., 2005; Naughton et al., 2006; Oatley et al., 2006; Sada et al., 2009; Zheng et al., 2009; Fig. 2, A and B). Interestingly, the Miwi2-TomatoHi c-kitNeg population also expressed Ngn3 that is associated with transit amplification and acquisition of differentiation competence under homeostatic conditions (Nakagawa et al., 2010; Ikami et al., 2015). To further define the Miwi2-TomatoHi c-kitNeg spermatogonial population, we performed single-cell qRT-PCR analysis using a panel of SSC, SPC, and differentiating spermatogonia markers (Fig. 2 C). Analysis of Miwi2-TomatoHi c-kitPos differentiating spermatogonia validated this approach with cells being negative for GFRα1, Miwi2, Nanos2, and Nanos3 while expressing c-kit mRNA (Fig. 2 C). Miwi2-TomatoHi c-kitNeg cells showed a distinct heterogeneity, with a small population registering positive for c-kit mRNA (Fig. 2 C), we posit that this cellular population is in the process of committing to differentiation and start to express mRNA but is not yet positive for the encoded protein. All Miwi2-TomatoHi c-kitNeg cells express the undifferentiated spermatogonia markers, Plzf, Lin28 and Oct4. Within the Miwi2-TomatoHi c-kitNeg population, a few cells express the GFRα1 mRNA although these cells are negative for expression of the protein. Most importantly all Miwi2-TomatoHi c-kitNeg cells expressed Ngn3 at the single cell level. To understand the overlap between Miwi2-TomatoHi c-kitNeg and Ngn3Pos populations, we generated Miwi2+TOM; Ngn3GFP− mice. Miwi2-TomatoHi c-kitNeg cells constituted a sub-population of Ngn3Pos spermatogonia (Fig. 2, D and E). The fraction of Ngn3Pos (GFRα1Pos) cells that express Miwi2-Tomato increases with chain length and by Aa stage all chains are positive for both Miwi2 and Ngn3 (Fig. 2, F and G). In summary, Miwi2 expression identifies a novel subpopulation of Ngn3+ undifferentiated spermatogonia.

**Miwi2-expressing spermatogonia are transit–amplifying cells under homeostatic conditions**

To determine the contribution of this novel Miwi2-TomatoHi c-kitNeg population to steady state or the long-term maintenance of spermatogenesis we opted for a lineage ablation strategy (Saito et al., 2001). To this end, the gene encoding the diphtheria toxin receptor (DTR) was placed into the Miwi2 locus (Miwi2DTR allele; Fig. 3 A and S2 A), thus enabling diphtheria toxin (DTx)-mediated lineage ablation experiments. Unfortunately, the DTR, antibody did not work for whole mount immunostaining, such that we could not resolve the DTR-expressing cells at the cellular Aa–Aa resolution. However, DTR expression was determined by FACS and was restricted to a tiny population of CD45Neg CD51Neg c-kitNeg (Miwi2-DTRPos c-kitNeg) cells, but not in any c-kitPos population within Miwi2+TOM tests (Fig. 3 B, top). Importantly, the overall percentages and numbers of Miwi2-DTRPos c-kitNeg and Miwi2-TomatoHi c-kitNeg were roughly equivalent (Fig. S1 H and S2 C). We administered three DTx doses spaced 2 d apart and analyzed 1 d after the last injection, here the Miwi2-DTRPos c-kitNeg population was reduced close to background levels (Fig. 3 B, bottom). This acute loss of Miwi2-DTRPos c-kitNeg population had no impact on GFRα1-expressing SSC populations (Fig. 3 C); however, strikingly, most differentiating spermatogonia (CD45Neg CD51Neg c-kitPos) were absent (Fig. 3 B, bottom). We interpreted this loss of c-kitPos cells as the failure to replenish the differentiating spermatogonia population upon their differentiation into meiocytes, and histological analysis supported this (Fig. 3 D). Indeed, a single administration of DTx and analysis 1 d after the injection revealed that the Miwi2-DTRPos c-kitNeg population was reduced, but without affecting other testicular populations inclusive of the c-kitPos populations (Fig. S2 D). We next followed the consequence of Miwi2-DTRPos c-kitNeg depletion on spermatogenesis over time. A single wave of spermatogenesis was lost in the Miwi2DTR+/ DTx-treated mice, with testicular weight being reduced by ∼30% 6 wk after injection, but recovering fully by 16 wk (Fig. 3, E and F). Concomitantly, fertility was lost at 6 wk and regained at 12 wk after DTx injection in the Miwi2DTR/+ mice (Fig. 3 G). Additionally, the reappearance of
Miwi2-DTR<sup>Pos</sup> c-kit<sup>Neg</sup> was observed in Miwi2<sup>−/−</sup>/DTR testis 8 wk after DTx treatment (Fig. S2 E). Thus, the Miwi2-DTR<sup>Pos</sup> c-kit<sup>Neg</sup> population behaves as transit amplifying population in this assay and the cells within this population are not broadly required for the long-term homeostasis of the testes.

**Miwi2-expressing spermatogonia are essential for the efficient regenerative capacity of the testes**

Miwi2 is member of the Piwi subclade of the Argonaute family, in planarians the Piwi proteins Smedwi2 and Smedwi3 are expressed in neoblasts and essential for the remarkable regenerative capacity of this organism (Reddien et al., 2005; Palakodeti et al., 2008). Additionally, whereas Ngn3<sup>+</sup> cells represent transit-amplifying cells under homeostasis, lineage-tracing studies have shown some ability of this population to contribute to the regeneration upon injury (Nakagawa et al., 2007). We therefore sought to understand if the Miwi2-DTR<sup>Pos</sup> c-kit<sup>Neg</sup> Ngn3<sup>Pos</sup> population not only contributes to regeneration but could also be critical for the regenerative capacity of the testis upon injury. Busulfan is a DNA-alkylating agent.
that is particularly toxic to spermatogonia, and at intermediate doses this chemical can be used to damage testis and induce regeneration (van Keulen and de Rooij, 1973). We subjected a cohort of Miwi2+/+ and Miwi2+/DTR adult mice to either vehicle control or 3× DTx injections, immediately followed by treatment of the mice with an intermediate dose of busulfan (Fig. 4A). The regeneration of vehicle control–injected mice was identical between the wild-type and Miwi2+/DTR genotypes (Fig. 4B). However, within the cohort where DTx was administered, the Miwi2+/DTR mice failed to recover with the same kinetics as control mice. At 12 wk after busulfan injection, the wild-type mice had reached maximal recovery, whereas the Miwi2+/DTR mice showed little or no recovery at this time point (Fig. 4, B–D). Only at 24 wk after injury did the DTx–injected Miwi2+/DTR mice mount a significant, but far from complete, recovery (Fig. 4, B–D).

A theoretical caveat of the aforementioned experiment is that the acute elimination of the Miwi2-DTR Pos c-kitNeg cells may provoke cell cycle entry in the GFRα1Pos population, rendering these cells more sensitive to busulfan treatment. We therefore did the reciprocal experiment; first inducing damage with busulfan, and then treating with DTx. This strategy resulted in a similar outcome with the Miwi2+/DTR mice, but not control, mice showing defective regeneration (Fig. S3). These data suggest that whereas the Miwi2Pos c-kitNeg population is transit amplifying under homeostatic conditions, they could harbor facultative stem cell ability under stress conditions. To directly test this, we used the transplantation assay,
where constitutively YFP-labeled Miwi2-Tom\textsuperscript{H} c-kit\textsuperscript{Neg} cells are FACS sorted and transplanted into germ cell–depleted testis. Indeed, the Miwi2-Tom\textsuperscript{H} c-kit\textsuperscript{Neg} population contained robust reconstitution activity (Fig. 4, E and F). Collectively, these data demonstrate that the Miwi2-expressing spermatogonial population that normally behaves as transit

Figure 4. **Miwi2-expressing cells are crucial for the regenerative capacity of the testis after injury.** (A) Experimental overview of DTx and busulfan injections, as well as time points analyzed. (B) Testicular weight of the indicated cohorts overtime is shown. n, number of mice analyzed per time point for the DTx-injected mice. P-values are shown; n.s., no statistical significance. (C) Histology of testis section stained with H&E for the same time points as indicated in A. Bar, 100 µm. (D) Enumeration of fully spermatogenic tubules in DTx-injected wild-type and Miwi\textsuperscript{DTx+} mice for each stage during the time course (n = 3 for each genotype and time point). P-values are shown; n.s., no statistical significance. (E) Representative image of reconstitution from CD45\textsuperscript{neg} CD61\textsuperscript{neg} Miwi2-Tom\textsuperscript{pos} c-kit\textsuperscript{neg} transplanted cell isolated from Miwi2Tom\textsuperscript{pos}; Rosa26\textsuperscript{YFP} mice. Colonies derived from the YFP-positive donor cells are shown. Bar, 1 mm. (F) Quantitation of colony formation for the indicated populations analyzed 3 mo after transplantation. Fold enrichment are calculated as colony forming units normalized per 10\textsuperscript{5} cells for the 10\textsuperscript{5} sorted CD45\textsuperscript{neg} Miwi2-Tom\textsuperscript{pos} c-kit\textsuperscript{neg} (n = 12) cells and 10\textsuperscript{7} testicular control cells transplanted (n = 4). Error bars represent SEM. (G) Immunofluorescence analysis of a transplanted YFP\textsuperscript{+} colony, spermatogonia that express GFR\textalpha\textsubscript{1} indicated (white boxes) after reconstitution. Bar, 50 µm (H) Total number of A\textalpha and A\textnu spermatogonia per 1,000 of sertoli cells before (CTR) and at day 5, 8, and 15 after busulfan treatment (left). Enumeration of Miwi2-Tom\textsuperscript{H} GFR\textalpha\textsuperscript{1neg}, Miwi2-Tom\textsuperscript{H} GFR\textalpha\textsuperscript{1pos}, and Miwi2-Tom\textsuperscript{H} GFR\textalpha\textsuperscript{1pos} populations is shown for the same time points (right). Stacked columns represent normalized percentages of the indicated populations per time point. Error bars represent SEM of three (CTR and +15D) and 4 (+5D and +8D) mice analyzed. Significance between CTR and the indicated populations is shown; *, P < 0.05; n.s., P > 0.05. (I) Normalized percentages of A\textalpha\textnu at indicated time points after busulfan treatment (left) as presented in H. Significance between CTR and the indicated populations is shown; ***, P < 0.001. Representative images of Miwi2-Tom\textsuperscript{H} GFR\textalpha\textsuperscript{1pos} and Miwi2-Tom\textsuperscript{H} c-kit\textsuperscript{neg} A\textalpha\textnu are shown for +8D (top) and +15D (bottom), respectively. Bar, 25 µm.
amplifying cells has stem cell activity in reconstitution assays and is essential for the efficient regenerative capacity of the testis after injury.

Given that all Miwi2\textsuperscript{pos} c-kit\textsuperscript{neg} cells express Ngn3, and Ngn3\textsuperscript{+} spermatogonia have a proven ability to revert to GFR\textalpha;expressing cells (Nakagawa et al., 2010), reversion may represent the mechanism by which Miwi2-expressing spermatogonia contribute to regenerative spermatogenesis. Although we cannot provide the formal proof of lineage tracing to observe the reversion of Miwi2\textsuperscript{pos} c-kit\textsuperscript{neg} cells to GFR\textalpha;expressing SSCs, we decided to look for evidence that would support this hypothesis. First, Miwi2\textsuperscript{pos} c-kit\textsuperscript{neg} cells can give rise to GFR\textalpha;expressing spermatogonia upon transplantation (Fig. 4 G). Next, we sought to understand whether the behavior of the respective Miwi2 and GFR\textalpha;expressing populations in the early stages of regeneration would be consistent with a reversal mechanism. We examined the acute impact of busulfan on A\textalpha; and A\textbeta; primitive spermatogonia. Damage to these populations reached a maximum at day 8, with the initiation of recovery observed as early as day 15 after busulfan administration (Fig. 4 H). We then analyzed the fractions of Miwi2-Tom\textsuperscript{Lo} GFR\textalpha;pos, Miwi2-Tom\textsuperscript{Lo} GFR\textalpha;pos, and Miwi2-Tom\textsuperscript{Lo} GFR\textalpha;pos in A\textalpha; and A\textbeta; populations during this time frame. Strikingly, we saw an increase in the Miwi2-Tom\textsuperscript{Lo} GFR\textalpha;pos A\textalpha; population at day 8 that preceded the qualitative recovery in the Miwi2-Tom\textsuperscript{Lo} GFR\textalpha;pos cells at day 15 after damage induction (Fig. 4 H). We also saw the emergence of Miwi2-Tom\textsuperscript{Lo} GFR\textalpha;pos A\textbeta; chains at day 5, with the presence of Miwi2-Tom\textsuperscript{Lo} GFR\textalpha;pos observed at day 15 after damage induction; importantly, neither of these A\textbeta; populations is observed in homeostatic testis (Fig. 4 I). GFR\textalpha;pos A\textbeta; have been observed in regenerating testis (Nakagawa et al., 2010) and could, in theory, also contribute to the quantitative recovery of the A, GFR\textalpha;population through fragmentation of the spermatogonial chains. Given that Miwi2\textsuperscript{pos} c-kit\textsuperscript{neg} cells express Ngn3 and can give rise to GFR\textalpha;pos cells in transplantation assays, and the observed expansion of Miwi2-Tom\textsuperscript{Lo} GFR\textalpha;pos cells before the onset of GFR\textalpha;cell recovery, it is reasonable to speculate that reversion could be the mechanism by which Miwi2-expressing spermatogonia undergo regenerative spermatogenesis. Interestingly, in the Drosophila germline, transit amplifying spermatogonial cells can revert to stem cell identity upon the ablation of germline stem cells (Brawley and Matunis, 2004).

Here, we show that Miwi2-expressing undifferentiated spermatogonia that normally behave as transit-amplifying cells constitute a cellular population that is essential for the efficient regenerative capacity of the testis. These Miwi2-expressing cells constitute a novel subpopulation of Ngn3\textsuperscript{+} spermatogonia. Although Ngn3\textsuperscript{+} spermatogonia are transit amplifying during homeostasis, they have been shown to retain stem cell potential and can contribute to regeneration by transplantation and lineage tracing, respectively (Nakagawa et al., 2007, 2010); however, their relative importance to the regenerative process is not defined. Here, we show by cell ablation that the Miwi2-expressing subpopulation of Ngn3\textsuperscript{+} spermatogonia is crucial for efficient regenerative spermatogenesis. Our data demonstrate that mouse spermatogenesis has adopted a strategy to expand the pool of spermatogonial cells with stem cell activity under regenerative conditions. The intestinal and airway epithelia upon injury adopt a similar strategy, where progenitor cells can dedifferentiate in vivo into stem cells and contribute to repair (van Es et al., 2012; Tata et al., 2013; Metcalfe et al., 2014; Zheng et al., 2014). This regenerative strategy is distinct from hematopoiesis, where repair is mediated by hematopoietic stem cells (Wilson et al., 2008; Buza-Vidas et al., 2009; Busch et al., 2015). The expansion of the effective SSC compartment to incorporate an undifferentiated transit amplifying spermatogonial population affords a protective mechanism ensuring efficient repair. It likely limits the amount of potentially damaging replication cycles that the homeostatic stem cells would be required to undergo to mediate repair, thus also protecting the genomic integrity of the germ line. The sensitivity of SSCs to the chemotherapeutic agents renders male cancer patients sterile; this is particularly acute for prepubescent patients where sperm cannot be frozen for future parenthood needs. Thus, our findings not only identify SSCs but also potentially spermatogonial transit amplifying cells as key target populations for cryopreservation and the basis of an autologous transplantation strategy for the restoration of fertility in cancer survivors.

**MATERIALS AND METHODS**

**Mouse strains**

For the Miwi2\textsuperscript{Tom} allele, a targeting construct was generated by recombineering, containing two homology arms to the Miwi2 locus. Within this vector, a synthetic intron and the coding sequence of tdTomato fluorescent protein, followed by bovine growth hormone polyA signal, as well as a ﬂr-ﬂanked neomycin (neo) cassette for ES-cell screening, was inserted into the starting codon of Miwi2. Southern blotting of the individual ES-cell-derived clones with a Miwi2 exon 3 external probe was used to identify homologous recombinants. Digestion of wild-type genomic DNA from tail biopsies with AseI generates a 9.7-kb DNA fragment; integration of the neo’-flank fragment introduces an additional AseI site, thus decreasing the size of the AseI DNA fragment recognized to 4.4-kb in the targeted allele. Flp-mediated recombination and excision of the neo’-flank cassette results in a 11.7-kb AseI DNA fragment recognized by the external exon 3 probe, which is diagnostic of the Miwi2\textsuperscript{Tom} allele.

For the Miwi2\textsuperscript{DTR} allele, within recombineered Miwi2 vector that contained the homology arms, a synthetic intron and the membrane-anchored form of human heparin-binding epidermal growth factor-like growth factor (HB-EGF), otherwise termed diphtheria toxin receptor (DTR), followed by BGH polyA signal, a ﬂr flanked neomycin (neo) cassette was inserted into the starting codon of Miwi2. Southern blotting of the wild-type Miwi2 locus digested with AseI gives
a 9.7-Kb band; Flp-mediated recombination and excision of the neo'-frt flanked cassette results in an 11-kb AseI DNA fragment recognized by the external exon 3' probe, which is diagnostic of the Miwi2<sup>ΔTTR</sup> allele. The Miwi2-targeting constructs were electroporated into A9 ES cell (De Fazio et al., 2011). Southern blotting as described for the individual ES-cell–derived clones was used to identify homologous recombinants. A9-targeted ES cells were injected into C57BL/6 8-cell-stage embryos for the generation of fully ES-cell–derived mice (De Fazio et al., 2011). The Miwi2<sup>ΔTomato-Neo/+</sup> and Miwi2<sup>ΔTTR-Neo/+</sup> targeted mice were then crossed to the FLPe transgenic mice (FLPeR; Farley et al., 2000) to remove the frt flanked neor cassette, resulting in the generation of Miwi2<sup>ΔTomato/+</sup> and Miwi2<sup>ΔTTR/+</sup> alleles, respectively. The mice analyzed in this study were on a mixed C57BL/6 and 129 genetic background. Primers used for the amplification of the exon 3 probe were Miwi2_Ex3_prb_F 5'-AAGGAGGATGTCGGTGT-3' and Miwi2_Ex3_prb_R 5'-ACACCCAACTTCTGGAAGTC-3'. Common primers used for screening Miwi2<sup>+</sup>, Miwi2<sup>Tom</sup>, and Miwi2<sup>ΔTTR</sup> alleles were Miwi2-Tom_GenoF1 5'-TACTCCCAAATCCGAGTC-3', Miwi2-Tom_GenoR1 5'-GTCGCTATTGCAGAAAGCCAAG-3', and Miwi2-Tom_GenoR2 5'-CTCCTAGCCAAGTGCCTT-3'. All mice were bred and maintained in EMBL Mouse Biology Unit, Monterotondo in accordance with current Italian legislation (Art. 9, 27 January 1992, number 116) under license from the Italian health ministry.

**Cell preparation and FACS staining**

After the removal of the tunica albuginea, the isolated testes were rinsed twice in DMEM enriched with 1 mM of sodium pyruvate and 1.5 mM of sodium lactate before enzymatic digestion. To achieve the complete single–cell suspension, the tubules were digested first with 0.5 mg/ml of type XI collagenase (e7657; Sigma-Aldrich) at 32°C for 7 min at 700 rpm shaking, followed by 0.05% Trypsin digestion (Gibco) in the presence of 0.05 mg/ml of DNase I (DN-25; Sigma-Aldrich). After digestion, cells are filtered through a 70-µm cell strainer and 1 antibody (Goat-IgG; Neuronics) diluted 1/12/tube) at 4°C. Tissues used in this study were as follows: CD45 (eBioscience; clone 103D11); CD34 (clone RAM34); CD117 (eBioscience; clone 2B8); Sca-1 (BioLegend; D7); CD49f (eBioscience; clone GoH3); and EpCAM (eBioscience; G8.8), plus isotype controls rat IgG2a, k PE-Cy7 (eBioscience 25–4321-81), rat IgG2a, k, FITC (eBioscience), Rat IgG2a, k, biotinylated (eBioscience), rat IgG2b, k, APC (BD), rat IgG2b, k, and Pacific Blue (BioLegend).

**Reverse transcription and quantitative PCR**

50 ng of total RNA from FACS-sorted cell populations were reverse transcribed with random hexamer primers using SuperScript II Reverse transcription (Invitrogen,18064-022) following manufacturer instructions. A quantitative real-time reaction was performed on a LightCycler 480 PCR instrument (Roche) using 2X SYBR green I master (Roche). All the expression data were normalized to the expression levels of β actin reference gene using the 2-ΔΔCt method (Livak and Schmittgen, 2001). The primers used for qRT PCRn were as follows: Miwi2_FW 5'-CGACCCGACGATGTCATGTT-3', Miwi2_RV 5'-ATCAAATCCGACCACTCA-3', β-actin F 5'-CACACCCGGCGACCGTTTC-3', and β-actin R 5'-CCATTCCACCACCATCACACC-3'.

**Whole mount immunofluorescence**

Tubules were dealbugined with 0.5 mg/ml collagenase in PBS as for FACS preparation and subsequently fixed in 4% paraformaldehyde for 8–12 h at 4°C. After fixation, tubules were quenched in 1 M Glycine for 30 min at room temperature, and then blocked/permeabilized in PBS/0.5% Triton X-100/5% donkey serum/0.5% BSA. Primary antibodies are incubated in PBS/0.5% BSA overnight at room temperature. After washes in PBS, the tubules were incubated with Alexa Fluor 488–, 647–, or 546–conjugated secondary antibodies in PBS/0.5% BSA, 0.1% Triton X-100. The tubules were then counterstained with DAPI (5 µg/ml) and mounted in prolong gold on glass slides. Antibodies used were α-RFP (Rockland), α-GFP (Aves), α-GFRα1 (Neuromics Goat-IgG), α-Plzf (EMD Millipore; mouse monoclonal), and c-kit (R&D Systems; goat polyclonal).

**Immunofluorescence staining on tissue sections**

Immunofluorescence double staining was performed on fetal testicular tissue samples. Fetal testes were dissected from E12.5 Embryos, fixed in 4% paraformaldehyde for 2 h, and then quickly PBS washed and immersed in cryoprotecting solution PBS/10% sucrose solution for 2 h at 4°C and in following PBS/20% sucrose from 2 h to overnight (until the tissue sinks at the bottom of the −12°tube) at 4°C. Tissues were OCT–embedded and cryosectioned in 7-µm-thick slides. Slides were washed in PBS, permeabized in PBS/0.1% Triton X-100 for 30 min, blocked with PBS/0.1% Triton X-100/5% donkey serum/0.5% BSA, and incubated o.n. at room temperature with primary antibody in blocking solution without Triton X-100. After washes, Alexa Fluor 488–,
647–, or 546–conjugated secondary antibodies were applied. Nuclei were finally counterstained with DAPI (5 µg/ml) and slides mounted in ProLong Gold antifade reagent (Thermo Fisher Scientific). Antibodies used were as follows: α-RFP (Rockland) and α-GFRα1 (R&D Systems).

**Lineage ablation**

DTx (Sigma-Aldrich) was dissolved in water to make high-concentration aliquots (1 mg/ml) that were kept as stocks at −80°C. For injections, the concentrated DTx stock was diluted in PBS for the working concentration (5 µg/ml). Miwi2DTR/+ mice were injected intraperitoneally three times, every 2 d (unless specified otherwise), with DTx at a concentration of 25 ng/gram of mouse body weight.

**Testicular damage and regeneration experiments**

Busulfan powder was dissolved in dimethyl sulfoxide (DMSO) prewarmed to 41–43°C. After the busulfan was dissolved, an equal volume of distilled water (41–43°C) was added to make the working solution. Miwi2+/+ or Miwi2DTR/+ mice were injected once with busulfan at a concentration of 20 mg/kg of mouse body weight as specified in the text. Analysis of regeneration was performed at the time points described in the text and figure legends.

**Histology**

Testes dissected from Miwi2DTR/+ mice were fixed in Bouin’s solution (Sigma-Aldrich), embedded in paraffin, and sectioned as follows: approximately six consecutive 7-µm-thick slices were cut and deposited on a glass slide, and then the specimen was trimmed for a total 500 length, this process was repeated twice to get three slides per animal at roughly the beginning, at 1/4, and at 1/2 of the whole testis longitudinal length. Slides were H&E stained and the best section was chosen for 2D image reconstruction using the mosaic function of the Microdissector 7000 microscope (Leica). Tubules from the sections of the testis were classified as normal or aspermatogetic.

**Gene expression profiling**

Gene expression analysis was performed using the Affymetrix Mouse Gene 2.0 ST Array from total RNA of FACS-sorted CD45Neg CD51Neg Miwi2-TomPos c-KiNeg and CD45Neg CD51Neg Miwi2-TomPos c-KitPos populations. Biological quadruplicates were processed for each population. Gene expression profiles have undergone the same treatment as the TomExp, c-KitNeg cells. The injection glass pipet was previously prepared by pulling 3-inch long borosilicate glass with a 1-mm outer diameter (World Precision Instruments # TW100-3) in a P-1000 pipette puller (Sutter Instruments). The tip of each pipette was ground to a sharp 40 degree beveled point on an EG-45 Microgrinder (Narishige Group). Before the injection, the pipette was loaded with cells and secured in a micropipette-holder (WPI Instruments) and used for delivery of the various cell populations. Surgery was performed under isoflurane–oxygen vapor anesthesia. The capillary needle was gently inserted into the rete-testis through the efferent duct of the recipient animal, and ∼10 µl of the cell solution was transplanted. The injection filled 75–85% of the tubules in each recipient testis. 50 µg anti-CD4 antibody (GK1.5) was gently inserted into the rete-testis through the efferent duct of the recipient animal, and ∼10 µl of the cell solution was transplanted. The injection filled 75–85% of the tubules in each recipient testis. 50 µg anti-CD4 antibody (GK1.5) was gently inserted into the rete-testis through the efferent duct of the recipient animal, and ∼10 µl of the cell solution was transplanted. The injection filled 75–85% of the tubules in each recipient testis.

**Preparation of germ cell–depleted mice as recipients for transplantation**

For the elimination of spermatogenesis to generate recipient mice for the transplantation assays, a single 40 mg/kg injection of busulfan was given to 4–5-wk-old C57BL/6n male mice. Busulfan powder was prepared as described in Testicular damage and damage experiments.

**Germ cell transplantation and reconstitution**

Donor cells were isolated from Miwi2Tom+/+; Rosa26YFP+ testis by FACS and sorted in PBS/10% FCS as described above without the addition of sodium azide. 10⁴ Miwi2-Tom+, c-KitNeg FAC-sorted cells were resuspended in a volume of 10 µl in fresh DMEM/0.05% Trypan blue for injection. The unsorted control cells (10⁴) were isolated from testicular cell suspensions passed through the FACS, but not sorted using any parameters except for viability such that the cells have undergone the same treatment as the TomExp, c-KitNeg cells. The injection glass pipet was previously prepared by pulling 3-inch long borosilicate glass with a 1-mm outer diameter (World Precision Instruments # TW100-3) in a P-1000 pipette puller (Sutter Instruments). The tip of each pipette was ground to a sharp 40 degree beveled point on an EG-45 Microgrinder (Narishige Group). Before the injection, the pipette was loaded with cells and secured in a micropipette-holder (WPI Instruments) and used for delivery of the various cell populations. Surgery was performed under isoflurane–oxygen vapor anesthesia. The capillary needle was gently inserted into the rete-testis through the efferent duct of the recipient animal, and ∼10 µl of the cell solution was transplanted. The injection filled 75–85% of the tubules in each recipient testis. 50 µg anti-CD4 antibody (GK1.5) was gently inserted into the rete-testis through the efferent duct of the recipient animal, and ∼10 µl of the cell solution was transplanted. The injection filled 75–85% of the tubules in each recipient testis. 50 µg anti-CD4 antibody (GK1.5) was gently inserted into the rete-testis through the efferent duct of the recipient animal, and ∼10 µl of the cell solution was transplanted. The injection filled 75–85% of the tubules in each recipient testis.
was intraperitoneally injected to recipient mice on days 0, 2, and 4 after transplantation to prevent potential immune rejection. To assay transplantation, recipient mouse testes were recovered ~11 wk after surgery and analyzed by observing the number of YFP fluorescent colonies formed. A cluster of germ cells was defined as a colony when it occupied the entire circumference of the tubule and was at least 0.1 mm long. Testes were then OCT embedded and frozen immediately. They were then cut in to 7-μm-thick slices and stained with α-GFP (Invitrogen) and α-GFRA1 (R&D Systems) antibody as described above.

Statistical analysis
An unpaired two-tailed Student’s t test was used for all statistical analysis.

Online supplemental material
Fig. S1 shows the validation of the Miwi2tdTomato/+ allele, as well as FACS analysis of the adult Miwi2-TomPoc-KitPos cells. Fig. S2 shows the Miwi2DTR/+ allele and the FACS after one Dtx injection and 8 wk after three Dtx injections. Fig. S3 shows the regeneration experiment with Busulfan administered before the three Dtx injections.

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