Development 138, 1687-1696 (2011) doi:10.1242/dev.057364


# *lines* and *bowl* affect the specification of cyst stem cells and niche cells in the *Drosophila* testis

**Stephen DiNardo*, Tishina Okegbe, Lindsey Wingert, Sarah Freilich and Natalie Terry**

## SUMMARY

To function properly, tissue-specific stem cells must reside in a niche. The *Drosophila* testis niche is one of few niches studied in vivo. Here, a single niche, comprising ten hub cells, maintains both germline stem cells (GSC) and somatic stem cells (CySC). Here, we show that *lines* is an essential CySC factor. Surprisingly, *lines*-depleted CySCs adopted several characteristics of hub cells, including the recruitment of new CySCs. This led us to examine the developmental relationship between CySCs and hub cells. In contrast to a previous report, we did not observe significant conversion of steady-state CySC progeny to hub fate. However, we found that these two cell types derive from a common precursor pool during gonadogenesis. Furthermore, *lines* mutant embryos exhibited gonads containing excess hub cells, indicating that *lines* represses hub cell fate during gonadogenesis. In many tissues, *lines* acts antagonistically to *bowl*, and we found that this is true for hub specification, establishing *bowl* as a positively acting factor in the development of the testis niche.

**KEY WORDS: Dedifferentiation, Gonadogenesis, Stem cell niche, *Drosophila***

## INTRODUCTION

Adult stem cells are necessary for tissue homeostasis, and in most cases are localized to specialized niches that are crucial for many aspects of stem cell function, such as protection from environmental insult and maintaining the proper balance between self-renewal and differentiation (Spradling et al., 2001). Additionally, the number of niche cells governs the number of stem cells available for tissue homeostasis, and vacated niches can recruit new stem cells (Barroca et al., 2009; Brawley and Matunis, 2004; Calvi et al., 2003; Kai and Spradling, 2004; Nakagawa et al., 2007; Song et al., 2007; Ward et al., 2006; Zhang et al., 2003). Finally, in vitro and in some instances in vivo, human ES cells, neural stem cells and some *Drosophila* stem cells are thought to generate new niche cells from other somatic cell populations (Bendall et al., 2007; Kuo et al., 2006; Voog et al., 2008; Wurmser et al., 2004). Given their significant effect on stem cells, understanding how niche cells are specified is important.

There are only a few cases where we can unambiguously identify stem cells and their niches (Morrison and Spradling, 2008). One case is the distal tip cell of the *C. elegans* gonad, which serves to maintain undifferentiated germline cells (Kimble and Ward, 1988). Identifying that cell as the niche has facilitated an understanding of its lineage and specification (Lam et al., 2006). Another particularly well-understood niche is in the fruitfly ovary (Koch and King, 1966; Song et al., 2002; Xie and Spradling, 2000). However, in contrast to the *C. elegans* gonad, the specification of the *Drosophila* ovarian niche cells is less well understood. Some signaling interactions have been implicated in niche specification, and these have so far involved feedback among somatic and germline cells (Gilboa and Lehmann, 2006; Song et al., 2007; Ward et al., 2006).

Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine and the Penn Institute for Regenerative Medicine, Philadelphia, PA 19104-6048, USA.

*Author for correspondence (sdinardo@zimbra.upenn.edu)

**Accepted 11 February 2011**

Another case where we can unambiguously identify stem cells and the niche is the *Drosophila* male gonad, where the niche is specified during embryogenesis (Aboïm, 1945; Gönczy et al., 1992; Le Bras and Van Doren, 2006; Sheng et al., 2009b; Tanentzapf et al., 2007). It supports two stem cell lineages in the steady-state testis: germline stem cells (GSCs) and somatic stem cells (called cyst stem cells, CySCs). Both stem cell types are radially arranged around somatic hub cells, which are a source of key self-renewal signals, such as the STAT-activating ligand, Upd/Os and several BMP ligands (Aboïm, 1945; Hardy et al., 1979; Kiger et al., 2001; Tulina and Matunis, 2001; Kawase et al., 2004; Shivdasani and Ingham, 2003). Additionally, these cells serve an architectural role by governing adhesion of the GSCs and CySCs to the source of these self-renewal signals (Yamashita et al., 2003; Yamashita et al., 2007; Issigonis et al., 2009; Leatherman and DiNardo, 2010; Wang et al., 2006). The CySCs are particularly intriguing as they serve as both as somatic stem cells and function as part of the niche for GSCs (Kawase et al., 2004; Leatherman and DiNardo, 2008; Leatherman and DiNardo, 2010). Additionally, the CySCs or their daughters can adopt hub cell fate in the adult steady-state testis (Voog et al., 2008), further suggesting that these two somatic populations are closely related.

Because the germline maintains spermatogenesis, much work has naturally focused on the renewal and adhesion of the GSCs to the hub. However, recent work has turned towards the CySCs. Modulation of STAT activation in CySCs has been shown to affect their competition with germline cells for niche occupancy, and Zfh-1 and Chinmo have been identified as factors that affect CySC renewal (Leatherman and DiNardo, 2008; Issigonis et al., 2009; Flaherty et al., 2010). In particular, our work on Zfh1 derived from microarray data in which we identified transcripts enriched in adult testes that contained excess stem cells (Terry et al., 2006). In mining that list, it has become clear that there are several genes that are required both during adult steady-state operation of the testis and early during gonadogenesis. For example, Zfh1 is also required in early gonadal mesoderm (Broihier et al., 1998). Similarly, we

found that *neuralized* is important for GSC viability, and that the Notch pathway is also essential for hub cell specification (Okegbe and DiNardo, 2011; Kitadate and Kobayashi, 2010).

The gene *bowl*, which encodes a transcription factor, appeared on our microarray list. It participates in a cassette of protein-protein interactions operative in many epithelia (Bras-Pereira et al., 2006; de Celis Ibeas and Bray, 2003; Green et al., 2002; Hao et al., 2003; Hatini et al., 2005; Iwaki et al., 2001; Johansen et al., 2003). In particular, the *lines* gene encodes an obligate antagonist of *bowl* function (Hatini et al., 2005). As *bowl* activity can be redundant with other members of the *odd-skipped* gene complex, but no redundancy has been observed for the *lines* gene (Bokor and DiNardo, 1996; Bras-Pereira et al., 2006; Green et al., 2002; Hao et al., 2003; Hart et al., 1996; Hatini et al., 2000; Iwaki et al., 2001), we focused on *lines* to explore the role of this cassette in the testis stem cell niche.

We found that *lines* played essential roles in the adult steady-state testis as well as during gonadogenesis. At steady-state, *lines* was a crucial CySC factor, and, in its absence, CySCs took on several characteristics of hub cells. This observation led us to explore the developmental relationship between hub cells and CySCs. We found that these cell types were generated from common precursors during gonadogenesis and that depletion of *lines* during gonadogenesis leads to excess hub cells. Consistent with the relief-of-repression model for this cassette, we found that *bowl* function was required for hub cell specification.

## MATERIALS AND METHODS

### Fly strains

Fly lines used were *lines*$^{2}$ (FBal0011651), *lines*$^{G2}$ (FBal0117449), *lines*$^{13B}$ (FBal0155036), *esgG66 lines*$^{2}$ (FBal0039323), *drm*$^{3}$ (FBal0121796), *bowl*$^{1}$ (FBal0051737), *bowl*$^{1}$ *esgG66*; UAS-Lines-Myc #8 III; UAS-Drm/MKRS; *hh*$^{P30}$ (FBal0034776); and Nos-GAL4VP16/TM3; EyaA3-GAL4; Upd-GAL4 UAS-GFP (Erika Bach, New York University, USA). For embryo and larval work, stocks were balanced over CyO Kr-GAL4 UAS-GFP, CyO Act-GFP or TM6 Ubi-GFP. We used three different methods for lineage tracing. To indelibly mark PS11 SGPs, we constructed Act5c<CD2stop<GAL4; UAS-GFP; UAS-FLP/Prd-GAL4 (FBst0001947) adult flies, using stocks obtained from E. Rulifson (UCSF, San Francisco, USA) (Zhang et al., 2007). Prd-Gal4 drives FLP and thereby recombines out the CD2stop cassette. Consequently, that generates heritable expression of GAL4, and thus GFP, by the Actin promoter. To indelibly mark all CySCs in adult testes, we substituted Fng-GAL4 (FBal0193084) for Prd-GAL4. Finally, we used Flp-mediated recombination to reconstitute a Tubulin promoter driving *lacZ* (Harrison and Perrimon, 1993). Using that setup, we found that re-introducing the source of FLP (Hs-FLP) at the start of our experiment increased the recombination frequency.

In mosaic experiments, mutant clones were positively marked using a UAS-GFP Tub-GAL4 HS-FLP; FRT42D Tub-GAL80 stock (Gary Struhl, Columbia University, New York, USA). We confirmed that clones in the somatic lineage are heat-shock dependent, but found that there is leaky clonal induction in germline lineages in these stocks. In all cases where we are using GAL4 to drive GFP, incubating embryos or flies at 29°C is essential to increase GAL4 function and more completely mark cells. All our experiments analyzing GFP use anti-GFP to help reveal the signal, as fixation reduces intrinsic fluorescence.

### Gonad and testis analysis

Embryos were collected for 3 hours and aged to the time of hatching. Larvae were dissected with tungsten needles in Ringers solution on a sylgard plate and the internal organs gently massaged out. Unhatched larvae were dechorionated, hand-devitellinized and dissected as above. After fixation for 15 minutes, primary antibodies were used overnight at 4°C; secondary antibodies were used at 1:400 (Alexa488, Cy3 or Cy5; Molecular Probes; Jackson ImmunoResearch) for 1 hour (room temperature). Male embryos and larvae were identified due to larger number of germ cells or (lack of) Sxl stain. To count hub cells, *z*-stacks were obtained through the depth of the larval gonad using an Axioplan equipped with an ApoTome.

For mosaic analysis, young adults were heat-shocked for 1 hour at 38°C to induce clones, and then were aged for 2, 5-6, 11-12 and 19-22 days; they were shifted to 29°C for 1 day prior to analysis to increase GFP levels. S-phase indices were obtained from an in vitro BrdU pulse as described previously (Wallenfang et al., 2006). For the in vivo pulse and chase, clones were first induced in young adults as above, and at 2 days after induction, we injected 5 mM EdU (Salic and Mitchison, 2008) mixed with green food coloring into the abdomen. Either two hours (pulse) or 2 days (chase) after injection, flies were processed for immunofluorescence and to reveal incorporated EdU using Alexa647 azide (Invitrogen, A10277).

We estimated the number of CySC divisions as follows: each cyst cell is generated by division of a CySC, and cyst cells no longer divide. It takes 2 days for a new cyst cell, associated with a gonial cell, to mature into a cyst cell associated with spermatocytes, during which time it has migrated away from the testis tip. Thus, we counted all marked cyst cells associated with gonial cells at each time point. As our time points were 4-5 days apart, which is significantly longer than the transit time into the spermatocyte region, we could be confidant that each time point sampled newly produced cyst cells.

We used rabbit antibodies against Vasa at 1:5000, Zfh1 at 1:500 (R. Lehmann, Skirball Institute, New York University, USA), Bowl at 1:1000 (de Celis Ibeas and Bray, 2003), STAT at 1:1000 (Flaherty et al., 2010) and GFP at 1:2000 (Molecular Probes); mouse antibodies against βgal at 1:10,000 (Promega), Wg (1:500), 1B1 (1:20), Cactus (1:20) and Sxl (1:25), all from DSHB; goat anti-Vasa at 1:400 (Santa Cruz); chick anti-Vasa at 1:5000 (K. Howard, University College London, UK); guinea pig anti-Traffic Jam at 1:5000 (Li et al., 2003); and rat anti-Filamin at 1:1000 (Sokol and Cooley, 2003).

## RESULTS

We used two approaches to deplete *lines* function from testis cells. First, we expressed a selective inhibitor of *lines* function, encoded by *drumstick*, in either the germline or the somatic lineage. Second, we induced mutant clones of *lines* using two different alleles. When *lines* was depleted from germline cells, we observed no phenotypes using either method, suggesting that *lines* was dispensable to the maintenance and behavior of GSCs (see Fig. S1 in the supplementary material and data not shown). By contrast, inhibiting *lines* function in the somatic lineage led to a striking accumulation of somatic cells (see Fig. S1D in the supplementary material). Mosaic analysis confirmed this, and allowed us to analyze this novel phenotype in some depth.

Control clones among CySCs were identified as GFP-positive, Traffic Jam-positive (Tj+) cells among the first somatic cells surrounding the hub (Fig. 1A, arrow), and were observed at about one CySC clone per testis. At the earliest time that we could identify clones (2 days post-induction), CySCs had already produced a variable number of progeny, and these were observed further along the testis, associated with later-stage germ cells as expected for cyst cells (Fig. 1A′, small arrowhead). When *lines* mutant CySCs were produced (Fig. 1B, arrow), their progeny tended to be grouped nearby (Fig. 1B′, bracket) rather than distributed throughout. Many of these *lines* mutant cells appeared to express Tj at relatively lower levels than nearby wild-type cyst cells (Fig. 1B″). By day six after clone induction, mutant clone size had increased (a median value of 90 cells per aggregate; Fig. 1C′, bracket), loss of Tj was more complete, and the mutant cells had withdrawn from the cell cycle (data not shown; see below). The aggregate was often located next to the endogenous hub, with one edge of the aggregate usually

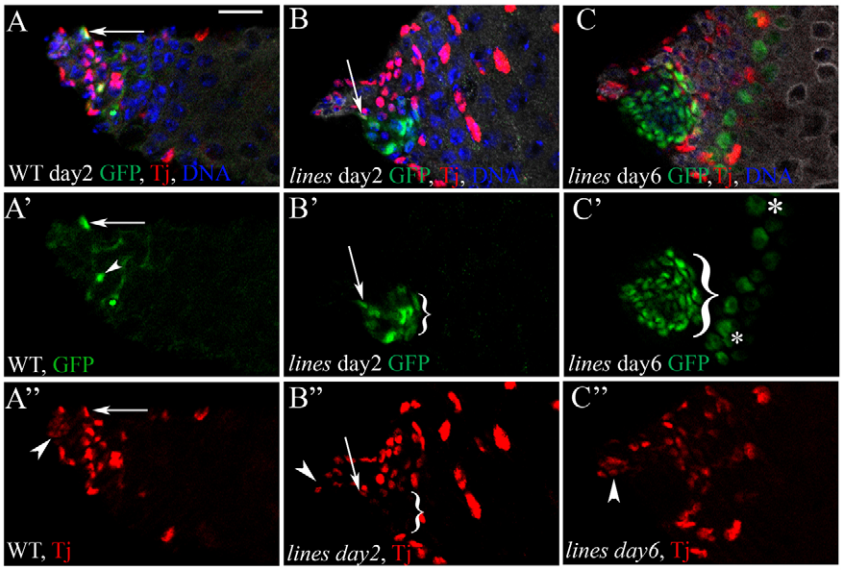


**Fig. 1. *lines* mutant CySCs initially proliferate and form aggregates.** (**A-A″**) A control CySC clone, 2 days post-induction. The clone is marked by GFP expression (A′, arrow) among the first tier of somatic cells (Tj; A″) adjacent to the hub (arrowhead, A″). One of several GFP+ progeny of this CySC is indicated (arrowhead, A′). (**B-B″**) A *lines* $^{g2}$ mutant clone, 2 days post-induction. A GFP$^{+}$ cell (arrow, B′) is adjacent to the hub (arrowhead, B″). This mutant CySC has generated about eight progeny since inception (GFP$^{+}$ cells, bracket, B′); mutant cells remain associated, and they tend to express lowered levels of Tj (B″, bracket). (**C-C″**) A *lines* $^{g2}$ mutant clone, 6 days post-induction. The aggregate is larger (bracket, C′), and the repression of Tj more complete (C″). The arrowhead in C″ marks the hub. This testis also contained a phenotypically normal germline clone, a portion of which is indicated (asterisks, C′). Scale bar: 25 μm.

positioned adjacent to the first tier of cells surrounding the endogenous hub. These aggregates accumulated Bowl protein (see Fig. S2A in the supplementary material), suggesting that *lines* normally targets Bowl protein for degradation in CySCs, just as it does in other tissues. Consistent with the idea that *bowl* should not be present nor required in CySCs, *bowl* mutant clones were recovered at the same frequency as control clones in these cells (data not shown). These data definitively establish that *lines* function is required in the CySCs.

### *lines* mutant cells express markers of hub cell fate

To determine whether *lines* mutant cells were differentiating normally, we examined expression of Eyes Absent (Eya). In wild-type somatic cells, Eya is undetectable in CySCs and early cyst cells, but first accumulates in cyst cells accompanying amplifying four-cell gonia, and to higher levels thereafter (Fabrizio et al., 2003; Leatherman and DiNardo, 2008). In testes with *lines* mutant clones, we did not detect Eya in GFP-positive cells comprising the aggregate (Fig. 2A′, arrow), suggesting that the *lines* mutant cells were not differentiating into cyst cells. Surprisingly, several markers of hub cell fate were expressed among the mutant cells. For example, the *lines* mutant aggregates expressed Hh-*lacZ* (Fig. 2B, arrows) (Forbes et al., 1996) and accumulated Cactus (Fig. 2C, arrows; two aggregates appear in these testes) (Leatherman and DiNardo, 2008). Additionally, the Hh-expressing aggregates led to activation of the Hh signaling pathway in and around the *lines* mutant cells, as judged by the accumulation of Patched and Ci proteins (see Fig. S2B-D in the supplementary material).

Although these markers are consistent with the *lines* mutant cells adopting hub cell fate, true hub cells should express the Os/Upd ligand, thereby causing accumulation of STAT in adjacent cells (Kiger et al., 2001; Tulina and Matunis, 2001; Chen et al., 2002). In wild-type testes, STAT accumulated among first tier germline cells, the GSCs, which also contained a dot fusome (Fig. 3A″, arrow). STAT also accumulated among first tier somatic cells, the CySCs (Fig. 3A′, arrowhead). In testes bearing *lines* mutant clones, STAT accumulation was always observed around the endogenous hub (Fig. 3B′, arrow). However, we never convincingly observed STAT accumulation in individual germline cells adjacent to the *lines* mutant aggregate (Fig. 3B′). In addition, we never observed germ cells containing dot fusomes adjacent to *lines* mutant aggregates (Fig. 3B″), although we did find differentiating germ cells with branched fusomes (data not shown). These data suggested that no new GSCs were recruited by the aggregates. Thus, although *lines* mutant cells expressed Hh and Cactus, they did not recruit GSCs and thus did not appear to be fully transformed into hub cells. However, we obtained strong evidence for the ectopic recruitment of new CySCs by *lines* mutant cells.

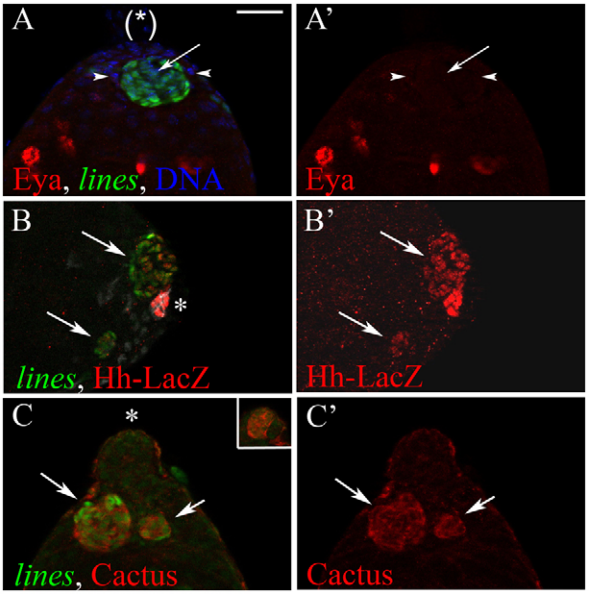


**Fig. 2. *lines* mutant aggregates express markers of hub fate.** (**A**,**A′**) *lines*$^{13B}$ mutant cells (arrows) do not accumulate Eya (A′; a clone at day 5). Wild-type somatic cells directly adjacent to the aggregates also do not accumulate Eya (arrowheads; see Fig. 6 and text). (**B-C′**) *lines*$^{g2}$ mutant aggregates (arrows) express Hh-*lacZ* (B′) and Cactus (C,C′; clones at days 4-6). (B) An example where both the endogenous hub (asterisk) and the aggregates were in the same focal plane (shown in inset). Each of these examples contains two separate aggregates; we do not know whether that reflects the initial induction of two *lines* mutant CySCs. Scale bar: 25 μm.

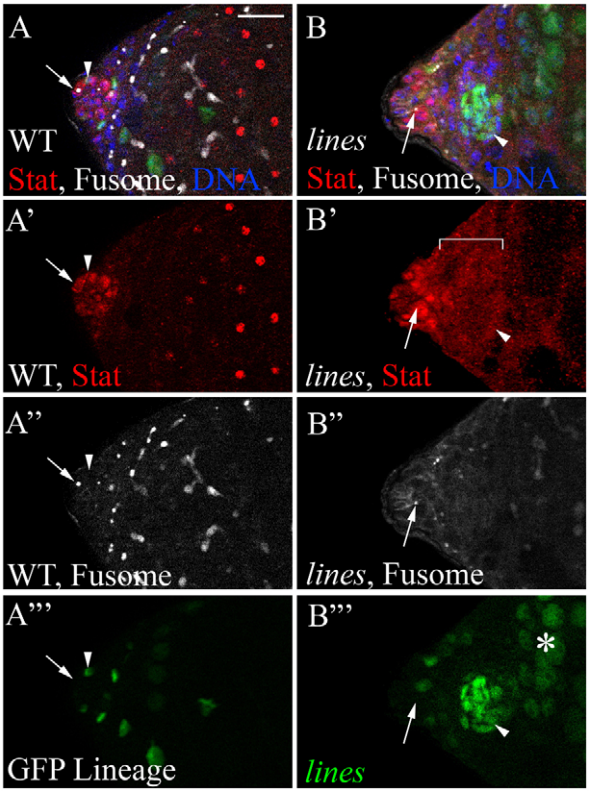


**Fig. 3. *lines* mutant aggregates do not recruit new GSCs.** Control (A-A‴) and *lines* $^{g2}$ (B-B‴) clones analyzed 5-7 days after induction. (**A**,**A′**) STAT accumulates among first tier germline (arrow, GSC) and somatic (arrowhead, CySCs) cells. (**A″**) GSCs contain dot fusomes (arrow). Arrowhead indicates a CySC. (**A‴**) There are several marked CySCs (e.g. arrowhead) and their descendants. (**B**,**B′**) Testes with *lines* $^{13B}$ or *lines* $^{g2}$ (shown) clones exhibit STAT enrichment (arrow) around the endogenous hub. Arrow marks the position of a GSC in B-B″. We also observe a haze of accumulation (B′, bracket) across regions of the testis containing *lines* mutant aggregates (arrowheads in B,B′,B‴). However, in wild type there is variable accumulation of STAT protein in somatic cells away from the hub (data not shown) as well as within differentiating germ cells (A; note spermatocyte nucleoli, along right-hand side). Whether this is cross-reaction or uncharacterized STAT activation is unclear, but it precludes us from interpreting the haze of STAT accumulation in testes bearing *lines*$^{-}$ clones. (**B″**) Although normal GSCs exhibited a dot fusome (arrow), we have never observed dot fusomes in cells around the distal arc of *lines*- aggregates (**B‴**). This testis also contains a phenotypically normal germline clone (asterisk). Scale bar: 25 μm.

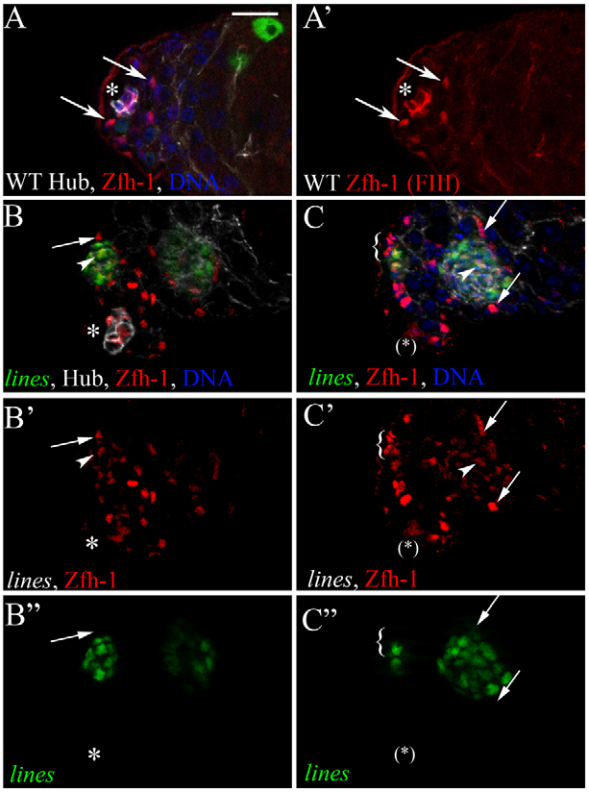


**Fig. 4. *lines* mutant aggregates are associated with Zfh1-expressing cells.** (**A**,**A′**) Control clone analyzed 5-7 days after induction. Zfh1 accumulates among CySCs (two are indicated by arrows) flanking the hub (asterisk; DE-Cadherin, white) and decays thereafter. This testis also contained a marked GSC clone (not in focus; two spermatocyte descendants are visible, upper right). (**B**,**C**) Testis bearing two *lines* $^{g2}$ aggregates, analyzed 5-7 days after induction. B was taken at a hub focal plane (asterisk; DE-Cadherin, white), while C was taken 15 μm deeper, with hub now out of focus (marked by parentheses). (**B′**,**B″**,**C′**,**C″**) Examples of *lines*$^{-}$ cells expressing Zfh1 and marked by GFP (arrowheads), confirming the somatic derivation of the mutant cells. However, far from endogenous hub there are also Zfh1-expressing cells flanking the aggregates (arrows in B-C″; bracket in C-C″). Scale bar: 25 μm.

## *lines* mutant cells recruit ectopic CySCs

In wild type, Zfh1 is highly expressed in CySCs, as well as at variable levels in hub cells (Fig. 4A, arrows), and it quickly decays in the progeny of CySCs (Leatherman and DiNardo, 2008). We found that the *lines* mutant cells themselves expressed Zfh-1, consistent with the fact that Zfh1 is expressed in endogenous hub cells (Fig. 4B,C, arrowheads). Invariably, we also observed Zfh1 expression among wild-type (GFP-negative) somatic cells adjacent to the aggregate. For example, Fig. 4B,C show two focal planes of the same testis, containing two *lines* mutant aggregates. The arrow indicates a Zfh-1-expressing cell on the distal side of one *lines* mutant aggregate (Fig. 4B, arrow), far from the hub (Fig. 4B, asterisk). Fig. 4C focused further away from the hub (*), where several Zfh-1-expressing cells now appear near the same aggregate (bracket), and other Zfh-1-expressing cells were observed distal to the second aggregate (Fig. 4C, upper arrow). We have never observed Zfh1-expressing cells at such distances from the endogenous hub (Fig. 4A) (Leatherman and DiNardo, 2008).

The ectopic Zfh-1-expressing cells also accumulated Wingless protein (Wg) (Leatherman and DiNardo, 2008), further suggesting they were potential CySCs. Arrows indicate several punctae of Wg among Tj-positive GFP-negative cells (Fig. 5A). We also tested whether these cells were cycling because CySCs are the only somatic cells that continually divide in the testis (Gönczy and DiNardo, 1996). After a pulse labeling, we observed BrdU$^{+}$ *lines*$^{+}$ cells adjacent to the *lines* mutant cells (Fig. 5B, arrows). The S-phase index of the somatic cells surrounding the *lines*-mutant aggregate was virtually identical to that of the endogenous CySCs located around the hub (Table 1; 0.18; $P \approx 0.46$, Student's *t*-test). These data suggest strongly that *lines* mutant aggregates recruited nearby wild-type somatic cells into an undifferentiated cycling state.

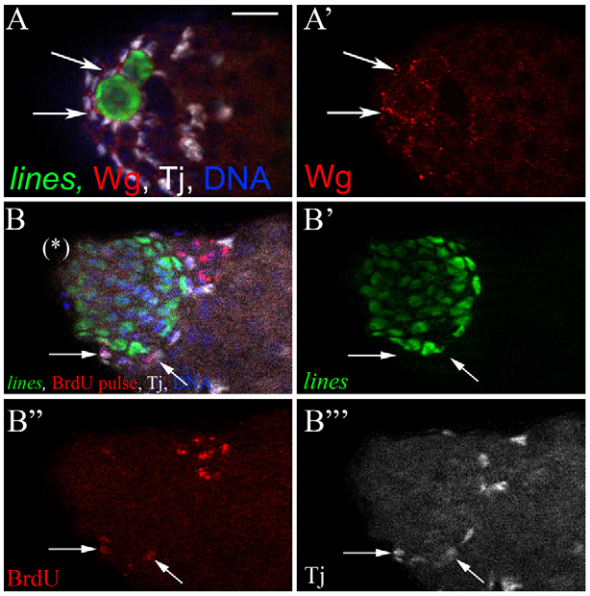


**Fig. 5. Aggregates recruit wild-type cells that express Wg and are cycling.** (**A**,**A'**) Arrows indicate Wg puncta (A') found in GFP-negative (A), and therefore heterozygous, wild-type cells associated with *lines* mutant aggregates. The endogenous hub is not in this focal plane. Clones were analyzed 4-6 days after induction. (**B-B‴**) A testis bearing a *lines* $^{g2}$ clone was pulse-labeled in vitro for 30 minutes 7 days post-induction. GFP-negative (B'), BrdU-positive (B″), Tj-positive (B‴) somatic cells are adjacent to the aggregate (arrows; for quantitation, see Table 1). The hub is in a different focal plane (B, asterisk). This testis also contains gonial cells in S-phase (upper right, B″). The *lines* mutant cells have withdrawn from the cell cycle, as we consistently observed no BrdU-positive GFP-positive cells. Why they arrest proliferation is unclear. Scale bar: 25 μm.

If these recruited cells were in fact CySCs, they should be able to generate differentiating progeny. We therefore performed an in vivo pulse-chase to determine whether the recruited, cycling somatic cells might generate differentiating progeny. We used the accumulation of Eya protein, as a marker for cyst cell differentiation. After inducing either control or *lines* mutant clones, we injected flies with EdU to label cycling cells. Testes were dissected from one half of the cohort after a 2-hour labeling period (the pulse), while the other half was aged for a further 2 days before being sacrificed (the chase). After the pulse-label, testes bearing control clones only exhibited EdU+ somatic cells adjacent to the hub. These are the endogenous CySCs, and were Eya negative, as expected (Table 2). Pulse-labeled testes bearing *lines* mutant clones had EdU-positive GFP-negative cells adjacent to the *lines*$^{-}$ aggregate (Fig. 6A, arrow). These cycling cells were invariably negative for Eya accumulation, confirming their undifferentiated state (Fig. 6A″, arrow; Table 2). However, after a chase, at positions distal to *lines*$^{-}$ aggregates we observed Edu$^{+}$ cells that now accumulated Eya (Fig. 6B″, arrow). The increased number of such cells in testes-bearing *lines*$^{-}$ clones (Table 2) suggested that these differentiating daughter cells were produced by the recruited CySCs.

In summary, *lines* mutant somatic cells recruited adjacent, wild-type somatic cells to become Zfh-1-expressing, to activate the Hh pathway, to accumulate Wg protein, to continue to cycle and to appear to produce differentiating progeny. These are all hallmarks of bona fide CySCs. Thus, *lines*-deficient cells are behaving as a niche and recruiting new somatic stem cells.

## Does *lines* influence the generation of hub cells from CySCs?

The fact that *lines* mutant CySCs shifted partially to hub fate led us to examine the steady-state and developmental relationship between CySCs and hub cells. For the steady-state testis, it was recently suggested by lineage tracing that daughters of CySCs can adopt hub fate as well as cyst cell fate (Voog et al., 2008). An attractive possibility is that *lines* is involved in such a circuit. However, we carried out lineage-tracing in flies heterozygous for *lines* and failed to detect any influence on the fate of CySC progeny (Table 3). It remains possible that *lines* function influences the potential of CySCs to produce niche cells under non-steady-state conditions, such as that encountered by stresses or by natural ageing.

In carrying out these lineage-tracing experiments in the wild-type background, we were surprised by the essentially negligible conversion to hub cells compared with what was reported (Voog et

**Table 1. S-phase index for genotypically wild-type (*lines*/+) cells associated with an aggregate**

| | Endogenous* CySCs | Endogenous† CCs | Aggregate-associated‡ cells |
|---|---|---|---|
| Testes with control clones | 0.18±0.023 (141) | 0.01±0.01 (183) | n/a |
| Testes with *lines* clones | 0.13±0.04 (75) | nd | 0.18±0.03 (115) |

*Wild-type cells positioned near the hub.
†Wild-type cells positioned greater than two tiers removed from hub.
‡Wild-type somatic cells within one tier of the *lines* mutant aggregate; we restricted counting to the distal two-thirds of the arc of the aggregate to avoid counting any cells that could have been under the influence of the endogenous hub.
Data are the average S-phase index in a 30-minute pulse±s.e.m. with the total number of cells scored in parentheses.
n/a, not applicable; nd, not determined.

**Table 2. Pulse-chase of CySC progeny into Eya$^{+}$ Cyst cells**

| Testis type | Endogenous EdU$^{+}$ CySCs* | Aggregate-associated EdU$^{+}$ cells* | Eya$^{+}$ EdU$^{+}$ cells† |
|---|---|---|---|
| With control clones (pulse) | 2.0 (11 ) | n/a | 0 (98) |
| With control clones (2-day chase) | 0 (10) | n/a | 2.9 (122) |
| With *lines*$^{g2}$ clones (pulse) | 1.5 (11) | 2.4 (11) | 0 (132) |
| With *lines*$^{g2}$ clones (2-day chase) | 0.3 (10) | 0.2 (10) | 4.2 (178) |

*Pulse-labeled cells per testis (average) with the total number of testes scored in parentheses.
†Pulse-labeled cells per testis (average) with total number of Eya$^{+}$ cells scored in parentheses.
The number of Edu$^{+}$ Eya$^{+}$ cells was higher in testes bearing *lines*$^{-}$ aggregates. Presumably, this was due to the increase in total CySC number, as testes with *lines* mutant clones contained endogenous plus ectopic CySCs.
n/a, not applicable.

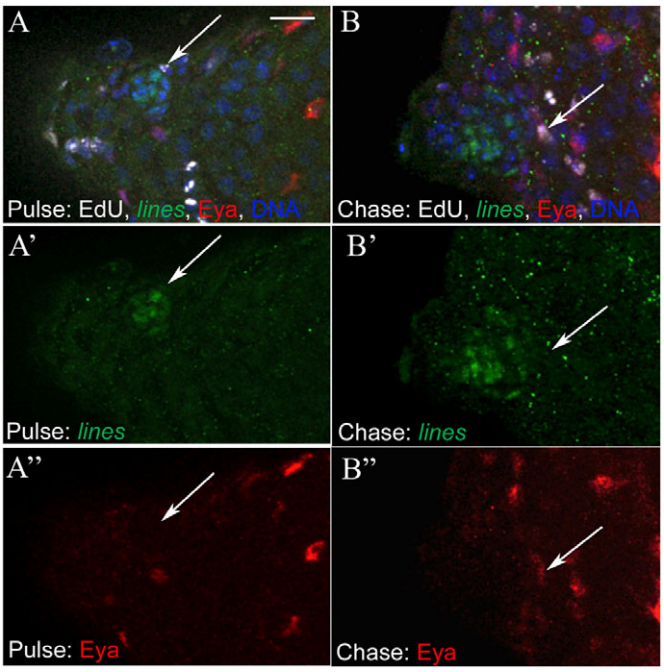


**Fig. 6. The recruited somatic cells chase into differentiating Eya$^+$ cyst cells.** (A-B″) An in vivo EdU pulse was analyzed 2 days after *lines*$^{g2}$ clones were induced (A-A″), and after a further 2-day chase (B-B″). (**A**,**A′**) An EdU-labeled cell (white, arrow) is adjacent to distal side of GFP$^+$ *lines*$^-$ aggregate. (**A″**) The EdU-positive cell is Eya negative. The endogenous hub is towards the left, out of focus. Other EdU+ cells in this image are germ cells. (**B**,**B′**) The EdU-labeled cell (white, arrows) is adjacent to distal side of GFP$^+$ *lines*$^-$ aggregate. (**B″**) The EdU-positive cell is Eya positive. The recruited CySC that must have generated this EdU$^+$ Eya$^+$ cyst cell is no longer expected to be visible, as it would have diluted its EdU label through division. The endogenous hub is towards the left, out of focus. The other EdU signal (B) is in a germ cell. Quantitation can be found in Table 2. Scale bar: 25 μm.

al., 2008). Our approach used the same FLP-FRT lineage-tracing method, and uses the FLP recombinase to reconstitute a general, tubulin promoter upstream of the *lacZ* gene (Harrison and Perrimon, 1993). The system is thought to work only in division-competent cells, and as hub cells are post-mitotic, none will express *lacZ* unless the cell was derived from the division or direct differentiation of a labeled CySC. As judged by assaying 2 days post-clone induction, we achieved a high efficiency of labeling CySCs, averaging 1.5 or 3.3 per testis (Fig. 7A, arrows). We analyzed cohorts at two, five to six, or ten to eleven days after clone induction, and as expected, the marked CySCs efficiently generated marked cyst cell daughters (Fig. 7B,B′, arrows). At each time point, we found an average of more than 12 marked cyst cells (Table 3), suggesting that we monitored more than 1600 CySC divisions (see Materials and methods). However, we observed only one marked hub cell. This differs significantly from that reported recently. For example, whereas 5-6 days after clone induction we observed no marked hub cells in 40 testes analyzed, while Voog et al. (Voog et al., 2008) reported that 46% of testes contained marked hub cells (*n*=74). We observed similarly negligible conversion using a distinct lineage-tracing method. In this case, we expressed FLP using Fringe-GAL4, which is expressed in all CySCs and their progeny (Fig. 7C, arrows and arrowhead, respectively). The cells also contained Actin5C>stop>GAL4 and UAS-GFP transgenes. Thus, the resulting

**Table 3. Insignificant conversion of CySCs into Hub cells**

| **Experiment 1*** | | **Lineage-marked cell types (*lacZ*$^+$)** | | |
|---|---|---|---|---|
| Days post heat-shock | *n* | CySC$^\dagger$ | CC$^\ddagger$ | HC$^\S$ |
| 2 | 34 | 50 (1.5) | 505 (14.8) | 0 |
| 5 | 20 | 15 (0.8) | 311 (15.5) | 0 |
| 11 | 22 | 27 (1.2) | 311 (14.1) | 0 |
| **Experiment 2¶** | | **Lineage-marked cell types (*lacZ*$^+$)** | | |
| Days post heat-shock | *n* | CySC | CC | HC |
| 2 | 23 | 75 (3.3) | 369 (16.0) | 0 |
| 6 | 20 | 40 (2.0) | 272 (13.8) | 0 |
| 10 | 20 | 21 (1.1) | 246 (12.3) | 1 (0.05) |
| ***lines*$^{2f}$/+**** | | **Lineage-marked cell types (*lacZ*$^+$)** | | |
| Days post heat-shock | *n* | CySC | CC | HC |
| 5 | 27 | 58 (2.1) | 181 (6.7) | 0 |
| 10 | 11 | 24 (2.2) | 84 (7.6) | 1 (0.1) |
| **Fng-Flog††** | | **Lineage-marked cell types (GFP$^+$)** | | |
| Age (days) | *n* | CySC | CC | HC |
| 6 | 17 | 170 (10) | 400 (23.5) | 0 |
| 15 | 20 | 159 (8.0) | 438 (21.9) | 1 (0.05) |

*Two-day-old flies, heat-shocked for 1 hour.
*n*, number of testes with at least one marked somatic cell in which marked cells were quantified.
$^\dagger$Total CySCs marked; the average per testis scored is in parentheses.
$^\ddagger$Total Cyst cells marked; the average per testis scored is in parentheses.
$^\S$Total Hub cells marked; the average per testis scored, if any, is in parentheses.
¶Two-day-old flies, heat-shocked for 2 hours on 2 consecutive days.
**Lineage-tracing in flies heterozygous for *lines*.
††Lineage-tracing using Fng-GAL UAS-FLP Act<stop<GAL4 UAS-GFP.

FLP-mediated recombination would remove the transcriptional stop signal and generate constitutive expression of GAL4 driven by the ubiquitously expressed Actin5c promoter. In turn, GFP would permanently mark all CySC descendants. The fact that marked cyst cells were now seen encasing transit amplifying gonia throughout the testis confirmed that the lineage tracing was working (Fig. 7D,D′). However, only one hub cell was marked with GFP, even though we sampled more than 830 CySC divisions (Table 3; see Materials and methods). These data indicate that in our hands CySCs rarely generated daughter cells that adopted hub fate.

## Hub and CySCs derive from a common precursor pool

Although *lines* dosage did not influence the production of new hub cells in the steady-state testis, our loss-of-function analysis strongly suggested that *lines* was essential to prevent CySCs from adopting partial hub cell character. This suggested that CySCs and hub cells might derive from a common lineage during embryogenesis. The gonad forms late in embryogenesis, when germ cells coalesce with somatic gonadal precursors (SGPs), which themselves arise from the mesoderm of parasegments 10, 11 and 12 (Gehring et al., 1976; Lawrence and Johnston, 1986; Szabad and Nöthiger, 1992; Aboïm, 1945; Boyle and DiNardo, 1995; Broihier et al., 1998; Brookman et al., 1992; Warrior, 1994). Sometime after coalescence, a subset of SGPs are specified as hub cells with the remaining SGPs presumably taking on CySC fate or directly differentiating as cyst cells (Boyle and DiNardo, 1995; Kitadate et al., 2007; Le Bras and Van Doren, 2006).

Previous lineage-tracing experiments suggest that some hub cells derive from parasegment 11 (PS11) SGPs (Le Bras and Van Doren, 2006; Sheng et al., 2009b). We wondered if CySCs were specified from among the same precursor pool. To test this, we lineage-

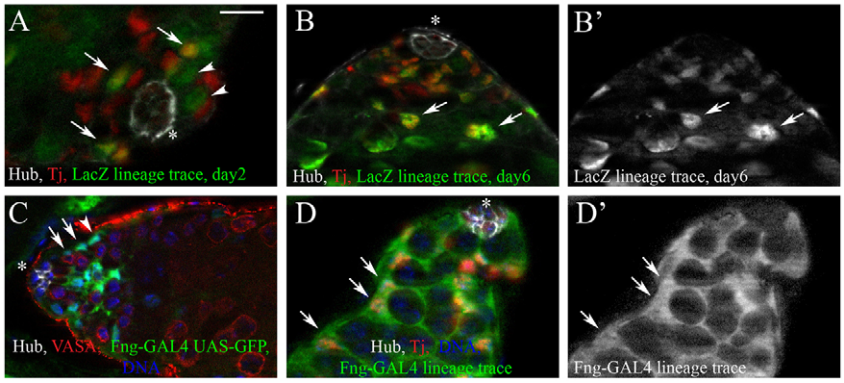


**Fig. 7. CySCs rarely generate hub cells.** (A,B) *lacZ* lineage tracing. (**A**) Two days after induction, several CySCs (arrows) and GSCs (arrowheads) are marked (hub, asterisk, DE-Cadherin). (**B**,**B′**) Six days after induction, differentiated cyst cells continue to express *lacZ* (arrows). No hub cells (asterisk, DE-Cadherin in B) are marked by *lacZ* (B′). (**C**) GFP driven by FngGAL4 accumulates in early tier somatic cells (arrows indicate some CySCs) and early cyst cells (one marked by an arrowhead). Asterisk indicates hub. (**D**,**D′**) Lineage tracing using Fng-GAL4 UAS-FLP Act5cFRTstopFRTGAL4. When Fng-expressing cells were indelibly marked, GFP accumulation is seen in the cytoplasm of cyst cells accompanying transit-amplifying spermatogonia and spermatocytes (arrows indicate a few of the labeled, late-stage cyst cells). No hub cells (D, asterisk, FasciII) are marked by GFP (D′). Scale bar: 25 μm.

traced the PS 11 SGPs by labeling them with GFP, and scoring the adult testis not only for marked hub cells but also for marked CySCs (Materials and methods). We found that 20% of the hub cells were GFP+ (25/122; $n$=12 testes; Fig. 8, yellow arrow), consistent with prior work. We also found that 65% of CySCs were GFP+ (86/133; $n$=12). For example, of two adjacent CySCs, one is GFP+ (Fig. 8, white arrow) and one is negative (Fig. 8, white arrowhead). Thus, some of the CySC lineage also derives from PS 11 SGPs. The lineage relationship between CySCs and Hub cells suggested a potential reason why *lines*$^{-}$ CySCs in the adult testis adopted partial hub character: perhaps *lines* was necessary during gonadogenesis for the proper number of hub cells to be specified, and the loss-of-function phenotype in adult testes was a hold-over from this embryonic role.

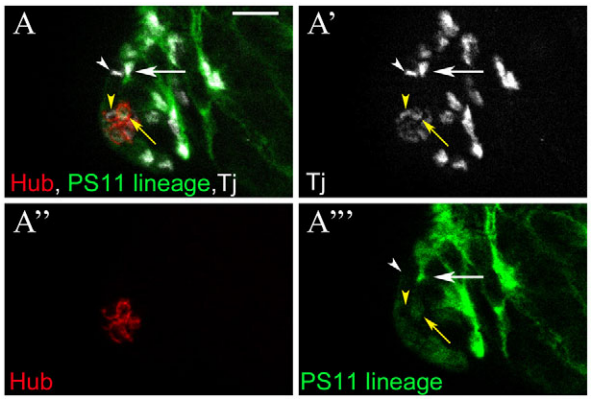


**Fig. 8. Hub and CySCs derive from a common precursor pool.** PS 11 SGPs were marked using Prd-GAL4, which is expressed in PS 11 mesodermal cells, to Flip out the transcriptional stop within Act5cFRTstopFRTGAL4; testes were analyzed from young adults. (**A-A‴**) Of two adjacent CySCs (white arrow and arrowhead, A,A′,A‴), one is marked by GFP while one is not (A‴; other CySCs in this focal plane are GFP$^{+}$). Of two adjacent hub cells (yellow arrow and arrowhead, A,A′,A‴), one is marked by GFP (yellow arrow, A‴), while the other is not (yellow arrowhead, A‴). Similar data were obtained using Zfh1 as a CySC marker (data not shown). Scale bar: 25 μm.

## Lines and Bowl influence hub cell specification

To address whether *lines* altered hub cell specification, we analyzed gonads at the end of embryogenesis. (Gönczy et al., 1992; Le Bras and Van Doren, 2006; Tanentzapf et al., 2007). As judged by a cytoskeletal marker, *lines* mutant gonads exhibited increased hub cell number (Fig. 9B,B′), averaging 14 hub cells (±0.8 s.e.m.) versus 10 (±0.3 s.e.m.) for sibling controls ($P<2\times10^{-5}$; see Fig. S3A in the supplementary material). This was confirmed using the gene expression hub markers *esg* and *upd* (see Figs S3B, S4 and S5 in the supplementary material). Thus, *lines* activity is necessary to restrict hub cell specification.

In all tissues where it has been examined, *lines* acts within a relief-of-repression hierarchy and normally antagonizes the activity of the *bowl* gene, unless *lines* itself is blocked from doing so by the action of *drumstick* (*drm*) (Green et al., 2002; Hatini et al., 2005). If this circuit acts during hub cell specification, mutations in either *drm* or *bowl* should have decreased hub cell number. Indeed, this was the case (see Fig. S3C,D in the supplementary material). Using Esg expression as a marker for hub fate confirmed this, as *bowl* mutant gonads contained only an average of five Esg$^{+}$ cells, compared with 12 in control siblings ($P<10^{-7}$; see Fig. S3 in the supplementary material). Finally, if this relief-of-repression hierarchy was operating to specify hub cell fate, then the *bowl lines* double mutant should also have fewer hub cells than wild type. We found this to be true, as average hub cell number was reduced from 12 (±0.7) to 7.3 (±0.8; $P<10^{-3}$). We next tested whether this decreased hub cell number affected the number of stem cells recruited. We cannot assay for CySCs in embryonic gonads, as the best CySC marker, Zfh1, was present in all SGPs, and has not become restricted to the CySCs at this stage (data not shown) (Broihier et al., 1998; Leatherman and DiNardo, 2008). However, we were able to test for effects on germline cells. Normally, the first tier of germline cells accumulates STAT protein as a consequence of Jak/STAT pathway activation, which is due to the association of germ cells with hub cells, and the recruitment of those germ cells as GSCs (Sheng et al., 2009b). First, we found a decrease in the number of first-tier germline cells, from an average of 10.1 (±0.6 s.e.m.) in sibling controls to 7.0 (±0.6 s.e.m.) in *bowl* mutants (Student's *t*-test: $P<10^{-4}$). Second, among the first-tier germline cells, STAT-positive germline cells were reduced from 8.9

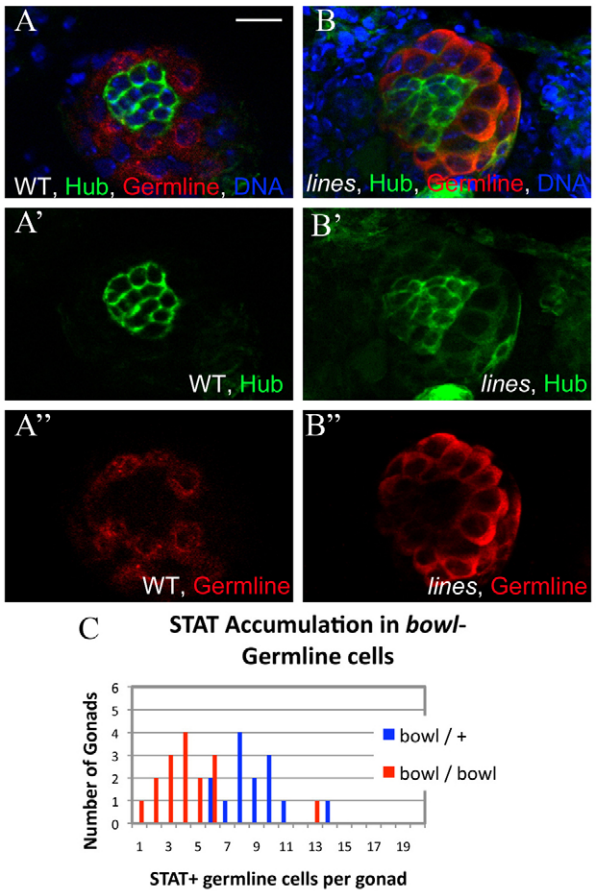


**Fig. 9. *lines* mutants contain excess hub cells, while *bowl* mutants contain fewer.** (**A**,**B**) Gonads from wild type (A; *lines*/CyO) and *lines*$^{2f}$ mutants (B). (**A′**,**A″**,**B′**,**B″**) Hub cells (Filamin, A′,B′), germ cells (Vasa, A″,B″), DNA (merge panels A, B). Hub cell number is increased in this focal plane (see Fig. S3 in the supplementary material for quantitation). Scale bar: 10 μm. (**C**) *bowl* mutants. Consistent with the reduction in hub cell number, there are significantly fewer STAT$^{+}$ germline cells in *bowl* mutants; four other *bowl*$^{-}$ gonads exhibited no detectable STAT. Data are absolute values.

(±0.6 s.e.m.) among control siblings to 3.9 (±0.7 s.e.m.) in *bowl* mutant gonads (Fig. 9C; Student's *t*-test: $P<5\times10^{-6}$). Thus, our data suggest a severe defect in hub cell specification and function in *bowl* mutants.

## DISCUSSION

We find that *lines* is an essential stem cell factor, and when depleted from CySCs these cells adopt niche qualities. This strongly suggested a close relationship between CySCs and their niche cells, and indeed we find that a subset of hub cells and CySCs derive from a common pool of gonadal precursors. These facts led us to establish that *lines* and *bowl* are genes necessary to specify proper hub cell number during gonadogenesis.

### Specification of hub cell fate

Our analysis together with previous lineage-tracing (Le Bras and Van Doren, 2006) shows that some hub cells and some CySCs are derived from the SGPs of PS11. The remaining CySCs could in principle derive from either PS10 or PS12. Currently, neither of those mesodermal parasegments can be uniquely lineage traced. However, the remaining hub cells probably derive from PS10 SGPs, as that would fit with the identification of receptor tyrosine kinase signaling as an antagonist of hub fate among posterior SGPs (Kitadate and Kobayashi, 2010; Kitadate et al., 2007).

Aside from pathways known to repress hub fate, work is also beginning to identify positive functions necessary to specify these cells. We find that *bowl* is one factor, as mutants had fewer hub cells, and those present appeared compromised for hub cell function. Still, the existence of residual hub cells suggests that Bowl is not the only factor required for hub cell specification, and, indeed, Notch signaling is a second positively acting component (Kitadate and Kobayashi, 2010; Okegbe and DiNardo, 2011).

It is of interest that both *Notch* and *bowl* are positively required for hub cell specification, as these two genes act together in several other tissues (de Celis Ibeas and Bray, 2003; Iwaki et al., 2001). However, the exact epistatic relationship between *bowl* and the *Notch* pathway can be complex. There is some evidence that Notch activation leads to Bowl accumulation (de Celis Ibeas and Bray, 2003; Greenberg and Hatini, 2009; Hao et al., 2003). As we found that Notch and also the relief-of-repression hierarchy consisting of *drm*/*lines*/*bowl* acts during hub cell specification, a simple model would be that Notch activation induces an antagonist of *lines*, for example, *drm*. This allows Bowl protein to accumulate in a subset of SGPs and to promote hub fate, while SGPs that retain functional Lines would adopt CySC fate. Attractive as this model is, we have had difficulty testing some of its predictions. We have been frustrated in attempts to visualize endogenous protein accumulation for Bowl and for Lines in the gonad. In addition, although *drm* mutants had reduced hub cell number, we have not identified *drm*-expressing cells within the forming gonad.

Thus, the relationship between Notch and the *drm*/*lines*/*bowl* cassette may be indirect, an outcome of the fact that both systems use the co-repressor Groucho. It has been suggested that conditions which alter the levels of available Bowl, such as in *drm* (down) or *lines* (up) mutants, could reciprocally affect the amount of Groucho available to Suppressor of Hairless, which requires this co-repressor to maintain repression of Notch target genes (Benitez et al., 2009). Whether or not the relationship between Notch and Bowl for hub cell specification is direct, loss of *Notch* has a stronger phenotype than loss of *bowl*. Thus, the *Notch* pathway must also engage a separate pathway that specifies some hub cells.

### Persistence of the developmental requirement into adulthood

During gonadogenesis, our model suggests that Lines represses hub fate and promotes CySC fate. It is intriguing that a requirement for *lines* persists in CySCs during the steady-state operation of the testis. Our analysis at this later stage suggests that *lines* plays a similar, though not identical, role. Although cells in gonads from *lines* mutant embryos fully adopt hub cell fate, in the testis the *lines*-depleted CySCs only partially adopt hub fate, as they do not recruit new GSCs. Thus, at steady-state, some additional regulation over the distinction between CySC and hub cell fate has been added on. Such a factor(s) remain to be identified.

Even the partial conversion of *lines* mutant CySCs into hub cells is an intriguing phenotype. Recently, a lineage relationship has been described for several stem cell-niche pairs, where stem cells can generate cells of their niche. These include production of Paneth cells in the mammalian intestine, the production of transient niche cells in the fruitfly intestine (Mathur et al., 2010; Sato et al., 2011), and the repair of ependymal cells by neural progenitors of the sub-ventricular zone (Kuo et al., 2006; Spassky et al., 2005). In the steady-state

testis, it was recently suggested that CySCs can efficiently generate new hub cells. Thus, we considered whether *lines* might be deployed at steady state to govern this transition, but we did not detect any increase in conversion in flies with decreased *lines* gene dose. In fact, in wild type we found that the conversion of cells into hub fate was insignificant compared with what has been reported (Voog et al., 2008). As one method used here was essentially identical to one used in the original report, we are unsure of the reason for the discrepancy. Our lineage-marking was very efficient. For example, two days after delivery of FLP by one heat-shock, 85% of testes possessed a labeled CySCs, with an average of 1.5 CySCs per testis. In the previous report, a similar regimen produced only 13% of testes with labeled CySCs. Still, it is not clear how an increase in marking efficiency could account for a decrease in apparent frequency of conversion of CySC progeny into hub cells.

Thus, as CySCs do not normally generate hub cells, why might *lines* function be maintained in CySCs so long after its embryonic requirement? Our favored model is that *lines* is deployed during steady-state for a distinct purpose. For example, recent work on the *lines*/*bowl* cassette suggests that it assists in signal integration (Benitez et al., 2009; Hatini et al., 2005). This idea is appealing as the niche cells and their local environment are subjected to the action of a number of signaling pathways, such as Hh, Wnt, BMP, Jak/STAT and EGFR. Currently, we do not fully understand how these pathways function in the steady-state operation of the niche, nor how signals from distinct pathways integrate to produce a single outcome. Even the dogma of the heavily studied Jak/STAT pathway continues be challenged and refined by recent data (Leatherman and DiNardo, 2008; Leatherman and DiNardo, 2010). Perhaps as newer data uncovers the nuanced roles of several of these pathways, the *lines*/*bowl* cassette will figure into the integration of those signals.

Finally, the fact that *lines*-depleted CySCs recruited neighboring wild-type somatic cells to adopt CySC fate is striking. Although we lack the imaging tools necessary to reveal which somatic cells are recruited to CySC fate, the fact of their recruitment suggests that under these mutant conditions cyst cells can de-differentiate into CySCs. It has been elegantly shown that maturing germ cells can de-differentiate, creating new GSCs (Brawley and Matunis, 2004; Cheng et al., 2008; Sheng et al., 2009a). As those maturing germ cells are encysted by the somatic cyst cells, during de-differentiation this grouping must break apart to release individual germline cells that repopulate the niche (Sheng et al., 2009a). Whether cyst cells de-differentiate to CySCs in these cases has not been directly assessed. If this happens under physiological conditions, it would be of great interest to study how cyst cells de-differentiation occurs, and testes harboring *lines*-deficient clones might aid in such studies.

**Acknowledgements**
We thank the fly community for their generosity, as well as the Bloomington Stock Center and the Developmental Studies Hybridoma Bank. We are also grateful to Victor Hatini, Stacie Dilks and members of the DiNardo and Ghabrial laboratories for helpful discussions and critical reading of the manuscript. This work was supported by the National Science Foundation pre-doctoral fellowship to T.C.O., by a NIH Training grant in Developmental Biology to N.T. and by NIH RO160804 to S.D. Deposited in PMC for release after 12 months.

**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.057364/-/DC1

**References**

**Aboïm, A. N.** (1945). Dévelopement embryonnaire et postembryonnaire des gonades normales et agamétiques de *Drosophila melanogaster*. *Rev. Suisse Zool.* **52**, 53-154.
**Barroca, V., Lassalle, B., Coureuil, M., Louis, J. P., Le Page, F., Testart, J., Allemand, I., Riou, L. and Fouchet, P.** (2009). Mouse differentiating spermatogonia can generate germinal stem cells in vivo. *Nat. Cell Biol.* **11**, 190-196.
**Bendall, S. C., Stewart, M. H., Menendez, P., George, D., Vijayaragavan, K., Werbowetski-Ogilvie, T., Ramos-Mejia, V., Rouleau, A., Yang, J., Bosse, M. et al.** (2007). IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature* **448**, 1015-1021.
**Benitez, E., Bray, S. J., Rodriguez, I. and Guerrero, I.** (2009). Lines is required for normal operation of Wingless, Hedgehog and Notch pathways during wing development. *Development* **136**, 1211-1221.
**Bokor, P. and DiNardo, S.** (1996). The roles of hedgehog, wingless and lines in patterning the dorsal epidermis in Drosophila. *Development* **122**, 1083-1092.
**Boyle, M. and DiNardo, S.** (1995). Specification, migration and assembly of the somatic cells of the Drosophila gonad. *Development* **121**, 1815-1825.
**Bras-Pereira, C., Bessa, J. and Casares, F.** (2006). Odd-skipped genes specify the signaling center that triggers retinogenesis in Drosophila. *Development* **133**, 4145-4149.
**Brawley, C. and Matunis, E.** (2004). Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. *Science* **304**, 1331-1334.
**Broihier, H. T., Moore, L. A., Van Doren, M., Newman, S. and Lehmann, R.** (1998). zfh-1 is required for germ cell migration and gonadal mesoderm development in Drosophila. *Development* **125**, 655-666.
**Brookman, J. J., Toosy, A. T., Shashidhara, L. S. and White, R. A. H.** (1992). The 412 retrotransposon and the development of gonadal mesoderm in *Drosophila*. *Development* **116**, 1185-1192.
**Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., Martin, R. P., Schipani, E., Divieti, P., Bringhurst, F. R. et al.** (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841-846.
**Chen, H. W., Chen, X., Oh, S. W., Marinissen, M. J., Gutkind, J. S. and Hou, S. X.** (2002). mom identifies a receptor for the Drosophila JAK/STAT signal transduction pathway and encodes a protein distantly related to the mammalian cytokine receptor family. *Genes Dev.* **16**, 388-398.
**Cheng, J., Turkel, N., Hemati, N., Fuller, M. T., Hunt, A. J. and Yamashita, Y. M.** (2008). Centrosome misorientation reduces stem cell division during ageing. *Nature* **456**, 599-604.
**de Celis Ibeas, J. M. and Bray, S. J.** (2003). Bowl is required downstream of Notch for elaboration of distal limb patterning. *Development* **130**, 5943-5952.
**Fabrizio, J. J., Boyle, M. and DiNardo, S.** (2003). A somatic role for eyes absent (eya) and sine oculis (so) in Drosophila spermatocyte development. *Dev. Biol.* **258**, 117-128.
**Flaherty, M. S., Salis, P., Evans, C. J., Ekas, L. A., Marouf, A., Zavadil, J., Banerjee, U. and Bach, E. A.** (2010). chinmo is a functional effector of the JAK/STAT pathway that regulates eye development, tumor formation, and stem cell self-renewal in Drosophila. *Dev. Cell* **18**, 556-568.
**Forbes, A. J., Lin, H., Ingham, P. W. and Spradling, A. C.** (1996). hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in Drosophila. *Development* **122**, 1125-1135.
**Gehring, W. J., Wieschaus, E. and Holliger, M.** (1976). The use of "normal" gynandomorphs in mapping the primordial germ cells and the gonadal mesoderm in *Drosophila*. *J. Embryol. Exp. Morphol.* **35**, 607-616.
**Gilboa, L. and Lehmann, R.** (2006). Soma-germline interactions coordinate homeostasis and growth in the Drosophila gonad. *Nature* **443**, 97-100.
**Gönczy, P. and DiNardo, S.** (1996). The germ line regulates somatic cyst cell proliferation and fate during Drosophila spermatogenesis. *Development* **122**, 2437-2447.
**Gönczy, P., Viswanathan, S. and DiNardo, S.** (1992). Probing spermatogenesis in *Drosophila* with P-element enhancer detectors. *Development* **114**, 89-98.
**Green, R. B., Hatini, V., Johansen, K. A., Liu, X. J. and Lengyel, J. A.** (2002). Drumstick is a zinc finger protein that antagonizes Lines to control patterning and morphogenesis of the Drosophila hindgut. *Development* **129**, 3645-3656.
**Greenberg, L. and Hatini, V.** (2009). Essential roles for lines in mediating leg and antennal proximodistal patterning and generating a stable Notch signaling interface at segment borders. *Dev. Biol.* **330**, 93-104.
**Hao, I., Green, R. B., Dunaevsky, O., Lengyel, J. A. and Rauskolb, C.** (2003). The odd-skipped family of zinc finger genes promotes Drosophila leg segmentation. *Dev. Biol.* **263**, 282-295.
**Hardy, R. W., Tokuyasu, K. T., Lindsley, D. L. and Garavito, M.** (1979). The germinal proliferation center in the testis of *Drosophila melanogaster*. *J. Ultrastruct. Res.* **69**, 180-190.
**Harrison, D. A. and Perrimon, N.** (1993). Simple and efficient generation of marked clones in *Drosophila*. *Curr. Biol.* **3**, 424-433.
**Hart, M. C., Wang, L. and Coulter, D. E.** (1996). Comparison of the structure and expression of odd-skipped and two related genes that encode a new family of zinc finger proteins in Drosophila. *Genetics* **144**, 171-182.

**Hatini, V., Bokor, P., Goto-Mandeville, R. and DiNardo, S.** (2000). Tissue- and stage-specific modulation of wingless signaling by the segment polarity gene lines. *Genes Dev.* **14**, 1364-1376.
**Hatini, V., Green, R. B., Lengyel, J. A., Bray, S. J. and Dinardo, S.** (2005). The Drumstick/Lines/Bowl regulatory pathway links antagonistic Hedgehog and Wingless signaling inputs to epidermal cell differentiation. *Genes Dev.* **19**, 709-718.
**Issigonis, M., Tulina, N., de Cuevas, M., Brawley, C., Sandler, L. and Matunis, E.** (2009). JAK-STAT signal inhibition regulates competition in the Drosophila testis stem cell niche. *Science* **326**, 153-156.
**Iwaki, D. D., Johansen, K. A., Singer, J. B. and Lengyel, J. A.** (2001). drumstick, bowl, and lines are required for patterning and cell rearrangement in the Drosophila embryonic hindgut. *Dev. Biol.* **240**, 611-626.
**Johansen, K. A., Green, R. B., Iwaki, D. D., Hernandez, J. B. and Lengyel, J. A.** (2003). The Drm-Bowl-Lin relief-of-repression hierarchy controls fore- and hindgut patterning and morphogenesis. *Mech. Dev.* **120**, 1139-1151.
**Kai, T. and Spradling, A.** (2004). Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. *Nature* **428**, 564-569.
**Kawase, E., Wong, M. D., Ding, B. C. and Xie, T.** (2004). Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing bam transcription in the Drosophila testis. *Development* **131**, 1365-1375.
**Kiger, A. A., Jones, D. L., Schulz, C., Rogers, M. B. and Fuller, M. T.** (2001). Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. *Science* **294**, 2542-2545.
**Kimble, J. and Ward, S.** (1988). Germ-line development and fertilization. In *The Nematode Caenorhabditis* (ed. W. B. Wood), pp. 191-213. Cold Spring Harbor, NY: Cold Spring Harbor Press.
**Kitadate, Y. and Kobayashi, S.** (2010). Notch and Egfr signaling act antagonistically to regulate germ-line stem cell niche formation in Drosophila male embryonic gonads. *Proc. Natl. Acad. Sci. USA* **107**, 14241-14246.
**Kitadate, Y., Shigenobu, S., Arita, K. and Kobayashi, S.** (2007). Boss/Sev signaling from germline to soma restricts germline-stem-cell-niche formation in the anterior region of Drosophila male gonads. *Dev. Cell* **13**, 151-159.
**Koch, E. A. and King, R. C.** (1966). The origin and early differentiation of the egg chamber of Drosophila melanogaster. *J. Morphol.* **119**, 283-303.
**Kuo, C. T., Mirzadeh, Z., Soriano-Navarro, M., Rasin, M., Wang, D., Shen, J., Sestan, N., Garcia-Verdugo, J., Alvarez-Buylla, A., Jan, L. Y. et al.** (2006). Postnatal deletion of Numb/Numblike reveals repair and remodeling capacity in the subventricular neurogenic niche. *Cell* **127**, 1253-1264.
**Lam, N., Chesney, M. A. and Kimble, J.** (2006). Wnt signaling and CEH-22/tinman/Nkx2.5 specify a stem cell niche in C. elegans. *Curr. Biol.* **16**, 287-295.
**Lawrence, P. A. and Johnston, P.** (1986). Observations on cell lineage of internal organs of *Drosophila*. *J. Embryol. Exp. Morphol.* **91**, 251-266.
**Le Bras, S. and Van Doren, M.** (2006). Development of the male germline stem cell niche in Drosophila. *Dev. Biol.* **294**, 92-103.
**Leatherman, J. L. and DiNardo, S.** (2008). Zfh-1 controls somatic stem cell self-renewal in the Drosophila testis and nonautonomously influences germline stem cell self-renewal. *Cell Stem Cell* **3**, 44-54.
**Leatherman, J. L. and DiNardo, S.** (2010). Germline self-renewal requires cyst stem cells and stat regulates niche adhesion in Drosophila testes. *Nat. Cell Biol.* **12**, 806-811.
**Li, M. A., Alls, J. D., Avancini, R. M., Koo, K. and Godt, D.** (2003). The large Maf factor Traffic Jam controls gonad morphogenesis in Drosophila. *Nat. Cell Biol.* **5**, 994-1000.
**Mathur, D., Bost, A., Driver, I. and Ohlstein, B.** (2010). A transient niche regulates the specification of Drosophila intestinal stem cells. *Science* **327**, 210-213.
**Morrison, S. J. and Spradling, A. C.** (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* **132**, 598-611.
**Nakagawa, T., Nabeshima, Y. and Yoshida, S.** (2007). Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. *Dev. Cell* **12**, 195-206.
**Okegbe, T. C. and DiNardo, S.** (2011). The endoderm specifies the mesodermal niche for the germline in Drosophila via Delta-Notch signaling. *Development* **138**, 1259-1267.
**Salic, A. and Mitchison, T. J.** (2008). A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci. USA* **105**, 2415-2420.
**Sato, T., van Es, J. H., Snippert, H. J., Stange, D. E., Vries, R. G., van den Born, M., Barker, N., Shroyer, N. F., van de Wetering, M. and Clevers, H.** (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* **469**, 415-418.
**Sheng, X. R., Brawley, C. M. and Matunis, E. L.** (2009a). Dedifferentiating spermatogonia outcompete somatic stem cells for niche occupancy in the Drosophila testis. *Cell Stem Cell* **5**, 191-203.
**Sheng, X. R., Posenau, T., Gumulak-Smith, J. J., Matunis, E., Van Doren, M. and Wawersik, M.** (2009b). Jak-STAT regulation of male germline stem cell establishment during Drosophila embryogenesis. *Dev. Biol.* **334**, 335-344.
**Shivdasani, A. A. and Ingham, P. W.** (2003). Regulation of stem cell maintenance and transit amplifying cell proliferation by tgf-beta signaling in Drosophila spermatogenesis. *Curr. Biol.* **13**, 2065-2072.
**Sokol, N. S. and Cooley, L.** (2003). Drosophila filamin is required for follicle cell motility during oogenesis. *Dev. Biol.* **260**, 260-272.
**Song, X., Zhu, C. H., Doan, C. and Xie, T.** (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. *Science* **296**, 1855-1857.
**Song, X., Call, G. B., Kirilly, D. and Xie, T.** (2007). Notch signaling controls germline stem cell niche formation in the Drosophila ovary. *Development* **134**, 1071-1080.
**Spassky, N., Merkle, F. T., Flames, N., Tramontin, A. D., Garcia-Verdugo, J. M. and Alvarez-Buylla, A.** (2005). Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. *J. Neurosci.* **25**, 10-18.
**Spradling, A., Drummond-Barbosa, D. and Kai, T.** (2001). Stem cells find their niche. *Nature* **414**, 98-104.
**Szabad, J. and Nöthiger, R.** (1992). Gyandomorphs of *Drosophila* suggest one common primarodium for the somatic cells of the female and male gonads in the region of abdominal segments 4 and 5. *Development* **5**, 527-533.
**Tanentzapf, G., Devenport, D., Godt, D. and Brown, N. H.** (2007). Integrin-dependent anchoring of a stem-cell niche. *Nat. Cell Biol.* **9**, 1413-1418.
**Terry, N. A., Tulina, N., Matunis, E. and DiNardo, S.** (2006). Novel regulators revealed by profiling Drosophila testis stem cells within their niche. *Dev. Biol.* **294**, 246-257.
**Tulina, N. and Matunis, E.** (2001). Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. *Science* **294**, 2546-2549.
**Voog, J., D'Alterio, C. and Jones, D. L.** (2008). Multipotent somatic stem cells contribute to the stem cell niche in the Drosophila testis. *Nature* **454**, 1132-1136.
**Wallenfang, M. R., Nayak, R. and DiNardo, S.** (2006). Dynamics of the male germline stem cell population during aging of Drosophila melanogaster. *Aging Cell* **5**, 297-304.
**Wang, H., Singh, S. R., Zheng, Z., Oh, S. W., Chen, X., Edwards, K. and Hou, S. X.** (2006). Rap-GEF signaling controls stem cell anchoring to their niche through regulating DE-cadherin-mediated cell adhesion in the Drosophila testis. *Dev. Cell* **10**, 117-126.
**Ward, E. J., Shcherbata, H. R., Reynolds, S. H., Fischer, K. A., Hatfield, S. D. and Ruohola-Baker, H.** (2006). Stem cells signal to the niche through the Notch pathway in the Drosophila ovary. *Curr. Biol.* **16**, 2352-2358.
**Warrior, R.** (1994). Primordial germ cell migration and the assembly of the Drosophila embryonic gonad. *Dev. Biol.* **166**, 180-194.
**Wurmser, A. E., Nakashima, K., Summers, R. G., Toni, N., D'Amour, K. A., Lie, D. C. and Gage, F. H.** (2004). Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. *Nature* **430**, 350-356.
**Xie, T. and Spradling, A. C.** (2000). A niche maintaining germ line stem cells in the Drosophila ovary. *Science* **290**, 328-330.
**Yamashita, Y. M., Jones, D. L. and Fuller, M. T.** (2003). Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. *Science* **301**, 1547-1550.
**Yamashita, Y. M., Mahowald, A. P., Perlin, J. R. and Fuller, M. T.** (2007). Asymmetric inheritance of mother versus daughter centrosome in stem cell division. *Science* **315**, 518-521.
**Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W. G., Ross, J., Haug, J., Johnson, T., Feng, J. Q. et al.** (2003). Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**, 836-841.
**Zhang, Y., Guo, H., Kwan, H., Wang, J. W., Kosek, J. and Lu, B.** (2007). PAR-1 kinase phosphorylates Dlg and regulates its postsynaptic targeting at the Drosophila neuromuscular junction. *Neuron* **53**, 201-215.