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 generate 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).

Another case where we can unambiguously identify stem cells and the niche is the Drosophila male gonad, where the niche is specified during embryogenesis (Aboim, 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 (Aboim, 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 Chinno 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 (Broshier et al., 1998). Similarly, we
found that *neutralized* is important for GSC viability, and that the Notch pathway is also essential for hub cell specification (Okage 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**1 (FBal0011651), **lines**2 (FBal0117449), **lines**18 (FBal0155036), **exg66** (**lines**2 (FBal0039323), **drm** (FBal0121796), **bowl** (FBal005173)), **bowl** (UAS-Lines-Myc #8 III; UAS-Drm/MKRS; hh50 (FBal0034776); and Nos-GAL4VP16/TM3; EyaA3-GAL4; Upd-GAL4 UAS-GFP). All remaining lines were obtained from DSHB.

**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 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 mutant clone); see below). The aggregate was often located next to the hub (Fig. 1C, 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. 1C’).
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 cysts. 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 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. 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.

**Fig. 2. lines mutant aggregates express markers of hub fate.** (A, A′) lines138 mutant cells (arrows) do not accumulate Eya (A′, a clone at day 9). 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.
STAT accumulation in testes bearing activation is unclear, but it precludes us from interpreting the haze of right-hand side). Whether this is cross-reaction or uncharacterized STAT within differentiating germ cells (A; note spermatocyte nucleoli, along protein in somatic cells away from the hub (data not shown) as well as 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.

**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=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.
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 Eya′ 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 tests, 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 al., 2008).

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

<table>
<thead>
<tr>
<th></th>
<th>Endogenous* CySCs</th>
<th>Endogenous† CCs</th>
<th>Aggregate-associated‡ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes with control clones</td>
<td>0.18±0.023 (141)</td>
<td>0.01±0.01 (183)</td>
<td>nd</td>
</tr>
<tr>
<td>Testes with lines clones</td>
<td>0.13±0.04 (75)</td>
<td></td>
<td>0.18±0.03 (115)</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>Testes type</th>
<th>Endogenous EdU+ CySCs*</th>
<th>Aggregate-associated EdU+ cells*</th>
<th>Eya+ EdU+ cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>With control clones (pulse)</td>
<td>2.0 (11)</td>
<td>n/a</td>
<td>0 (98)</td>
</tr>
<tr>
<td>With control clones (2-day chase)</td>
<td>0 (10)</td>
<td>n/a</td>
<td>2.9 (122)</td>
</tr>
<tr>
<td>With lines/+ clones (pulse)</td>
<td>1.5 (11)</td>
<td>2.4 (11)</td>
<td>0 (132)</td>
</tr>
<tr>
<td>With lines/+ clones (2-day chase)</td>
<td>0.3 (10)</td>
<td>0.2 (10)</td>
<td>4.2 (178)</td>
</tr>
</tbody>
</table>

*Pulse-labeled cells per tests (average) with the total number of testes scored in parentheses.  
†Pulse-labeled cells per tests (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 lines⁵clones 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 Eya-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 Eya-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.

Table 3. Insignificant conversion of CySCs into Hub cells

<table>
<thead>
<tr>
<th>Experiment 1*</th>
<th>Days post heat-shock</th>
<th>Lineage-marked cell types (lacZ⁺)</th>
<th>CySC₁</th>
<th>CC₁</th>
<th>HC₁</th>
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<td></td>
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<tr>
<td>2</td>
<td>34</td>
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<tr>
<td>5</td>
<td>20</td>
<td>15 (0.8)</td>
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<tr>
<td>11</td>
<td>22</td>
<td>27 (1.2)</td>
<td>311 (14.1)</td>
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<th>Experiment 2†</th>
<th>Days post heat-shock</th>
<th>Lineage-marked cell types (lacZ⁺)</th>
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<td>6</td>
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<td>272 (13.8)</td>
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<td>10</td>
<td>20</td>
<td>21 (1.1)</td>
<td>246 (12.3)</td>
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<th>lines²/+++</th>
<th>Days post heat-shock</th>
<th>Lineage-marked cell types (lacZ⁺)</th>
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<th>CC</th>
<th>HC</th>
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<td>84 (7.6)</td>
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<th>Fng-Flog††</th>
<th>Age (days)</th>
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<td>6</td>
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<td>159 (8.0)</td>
<td>438 (21.9)</td>
<td>1 (0.05)</td>
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</table>

*Two-day-old flies, heat-shocked for 1 hour.
†Total CySCs marked; the average per testis scored is in parentheses.
‡Total Cyst cells marked; the average per testis scored is in parentheses.
§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 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’). 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; Aboim, 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-
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.

**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<2×10−4; 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<10−3; 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
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 drmA/bowl acts during hub cell specification, a simple model would be that Notch activation induces an antagonist of lines, for example, drmA. 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 drmA mutants had reduced hub cell number, we have not identified drmA-expressing cells within the forming gonad.

Thus, the relationship between Notch and the drmA/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 drmA (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,
tests, 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 (Vogel 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 to 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.

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Competing interests statement
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