Hedgehog is required for CySC self-renewal but does not contribute to the GSC niche in the *Drosophila* testis

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**SUMMARY**

The *Drosophila* testis harbors two types of stem cells: germ line stem cells (GSCs) and cyst stem cells (CySCs). Both stem cell types share a physical niche called the hub, located at the apical tip of the testis. The niche produces the JAK/STAT ligand Unpaired (Upd) and BMPs to maintain CySCs and GSCs, respectively. However, GSCs also require BMPs produced by CySCs, and as such CySCs are part of the niche for GSCs. Here we describe a role for another secreted ligand, Hedgehog (Hh), produced by niche cells, in the self-renewal of CySCs. Hh signaling cell-autonomously regulates CySC number and maintenance. The Hh and JAK/STAT pathways act independently and non-redundantly in CySC self-renewal. Finally, Hh signaling does not contribute to the niche function of CySCs, as Hh-sustained CySCs are unable to maintain GSCs in the absence of Stat92E. Therefore, the extended niche function of CySCs is solely attributable to JAK/STAT pathway function.

**KEY WORDS:** Hh, Smo, Upd, JAK/STAT, Cyst stem cell (CySC), Germline stem cell (GSC), Hub, Niche, Testis, Self-renewal

**INTRODUCTION**

*Drosophila* has proven a useful model system in the study of stem cell biology, particularly in identifying the importance of the physical niche in maintaining stem cells and some of the signaling pathways involved. The germ line niche is well studied and has helped inform our understanding of niche-stem cell interactions (Losick et al., 2011; Spradling et al., 2011). The ovarian niche harbors two or three germ line stem cells (GSCs) and produces bone morphogenetic proteins (BMPs), which are essential for their maintenance (Xie and Spradling, 1998; Chen and McKearin, 2003). Proper development of oocytes requires two additional populations of somatic cells: the escort cells, which are dependent on JAK/STAT signaling for normal function (Decotto and Spradling, 2005; Morris and Spradling, 2011); and the follicle cells, which envelop the developing cysts. Follicle cells are produced from stem cells that require Hedgehog (Hh) signaling for self-renewal and proliferation (Zhang and Kalderon, 2000; Zhang and Kalderon, 2001).

The male germ line niche shares some features with the ovary, but it possesses a different architecture and different signals have been implicated in the self-renewal of resident stem cells (Losick et al., 2011). The niche, called the hub, supports two stem cell populations that are easily identifiable by their position relative to the hub and by molecular markers. GSCs give rise to sperm, whereas the somatic cyst stem cells (CySCs) divide to give rise to postmitotic cyst cells that envelop and support the development of germ cell cysts (Fig. 1A) (de Cuevas and Matunis, 2011).

The hub secretes the cytokine Unpaired (Upd; Upd1 or Os – FlyBase) to activate the JAK/STAT signaling pathway in both stem cell populations (Kiger et al., 2001; Tulina and Matunis, 2001). Upd activates Stat92E, which is the only *Drosophila* STAT transcription factor (Arbouzova and Zeidler, 2006), in both CySCs and GSCs (Fig. 1B). Clonal analyses revealed that GSCs and CySCs lacking Stat92E are not maintained in the niche and subsequently differentiate (Kiger et al., 2001; Tulina and Matunis, 2001; Leatherman and Dinardo, 2008). Furthermore, a heteroallelic Stat92E temperature-sensitive mutant (Stat92Ets) loses both stem cell populations – GSCs because they lose adhesion to the niche within 16 hours of Stat92E inactivation, and CySCs because they fail to self-renew (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010). Remarkably, restoration of wild-type Stat92E function only in the soma of Stat92Ets flies raised at the restrictive temperature rescues both germ line and somatic lineages (Leatherman and DiNardo, 2010). In the soma-rescued Stat92Ets flies, CySCs are found at locations next to the hub normally occupied by GSCs, presumably owing to increased Stat92E activation in the rescued CySCs. Regardless, these displaced GSCs are supported by CySCs, most likely through BMP ligands secreted by Stat92E-rescued CySCs, and give rise to mature spermatocytes.

In the CySCs, zfh1 and chinmo are targets of the JAK/STAT pathway that have important roles in self-renewal (Leatherman and Dinardo, 2008; Flaherty et al., 2010). In fact, Zfh1 is currently the best marker for the CySC state (Leatherman and Dinardo, 2008; Issigonis et al., 2009; Flaherty et al., 2010; Leatherman and Dinardo, 2010; Inaba et al., 2011). Hyperactivation of Stat92E or misexpression of either Chinmo or Zfh1 in the somatic lineage gives rise to stem cell tumors consisting of GSC-like and CySC-like cells (Leatherman and Dinardo, 2008; Flaherty et al., 2010; Leatherman and Dinardo, 2010), similar in constitution to the tumors observed when the ligand Upd is overexpressed (Kiger et al., 2001; Tulina and Matunis, 2001). The ability of CySCs to support and expand GSCs is most likely due to Stat92E-dependent production of BMPs, which act locally on GSCs to promote their self-renewal. Thus, a new model has emerged in which CySCs form part of the niche for GSCs together with the hub, and JAK/STAT signaling in CySCs is responsible for this support role (see Fig. 6L). Stat92E activation in GSCs is dispensable for self-renewal, and instead BMPs produced by the hub and by CySCs downstream of Stat92E and its targets Chinmo and Zfh1 regulate GSC self-renewal (Shivdasani and Ingham, 2003; Kawase et al.,...
2004; Flaherty et al., 2010; Leatherman and Dinardo, 2010). This is reminiscent of the female germ line niche, where BMP production also appears to be downstream of JAK/STAT signaling, albeit in different somatic cells (López-Onieva et al., 2008; Wang et al., 2008).

However, it is apparent that the genetic network is more complex in the testis niche. hh expression by hub cells has been known for some time (Forbes et al., 1996). In the absence of Hh, its receptor Patched (Ptc), a 12-pass transmembrane protein, inhibits the activation of Smoothened (Smo), a seven-pass transmembrane protein required for Hh signal transduction (Fig. 1C, ‘OFF’). (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996). With Smo inhibited, the full-length form of the transcription factor Cubitus interruptus (Ci) is cleaved by a protein Costal2 (Costa – FlyBase) (Kalderon, 2005). This results in a shortened form of Ci (called Cirep) that can translocate to the nucleus and bind regulatory elements of Hh target genes but represses their expression. When Hh binds to Ptc, Smo is no longer inhibited and it in turn can prevent the cleavage of Cirep. Hh ligand (‘ON’) prevents Ptc activity, allowing Smo to inhibit Ci cleavage, meaning that Cirep enters the nucleus to activate transcription of target genes, such as ptc, GSC, germ line stem cell, CySC, cyst stem cell; Y, tyrosine residues.

**Fig. 1. The JAK/STAT and Hh pathways.** (A) The Drosophila testis. See text for details. (B) JAK/STAT signaling in Drosophila. Binding of the secreted ligand Unpaired (Upd) to its receptor Domeless (Dome) causes phosphorylation and activation of the Janus kinase (JAK) Hopscotch (Hop). Hop in turn phosphorylates the transcription factor Stat92E, which dimerizes and enters the nucleus to promote the transcription of target genes, including zfh1 and chimaera. (C) The Hh pathway in the absence (‘OFF’) or presence (‘ON’) of ligand. When Hh is not present, Patched (Ptc) inhibits Smoothened (Smo), rendering it inactive. In the absence of Smo activity, the transcription factor Cubitus interruptus (Ci) is cleaved from a full-length transcriptional activator form (Ci^{att}) to a repressor (Ci^{lep}) by a complex that includes the Protein kinase A (PKA) enzyme and scaffolding proteins such as Costal2. Hh ligand (‘ON’) prevents Ptc activity, allowing Smo to inhibit Ci cleavage, meaning that Ci^{lep} enters the nucleus to activate transcription of target genes, such as ptc, GSC, germ line stem cell, CySC, cyst stem cell; Y, tyrosine residues.

**MATERIALS AND METHODS**

**Clonal analysis and fly husbandry**

Temperature-sensitive mutants were raised at 16°C. Adult males were collected every 3 days and shifted to 29°C for 10 days. eya3-gal4 crosses were kept at room temperature and adult males were shifted to 29°C for 10 days for maximum Gal4 activity. For clonal analysis, flies were raised at 25°C, collected for 2 days and heat shocked at 37°C for 1 hour and returned to 25°C until dissection, 2 or 7 days post-clone induction. The following stocks were used for clonal analyses: yw;hsflp122; FRT40A ubi-GFP; yw;hsflp122; FRT40A, yw;hsflp122; UAS-nls-GFP, tub-gal4; tub-gal80, FRT40A; yw;hsflp122; UAS-CD8-GFP, tub-gal4, FRT40A; tub-gal80.

**Fly stocks**

The following stocks were used and are described in FlyBase: Oregon-R, FRT40A smo^{D16}, FRT40A smo^{D16}; FRT40A smo^{D16}, FRT40A smo^{D16}; FRT40A Pka-C^{D12}, FRT40A chimino, ptc-lacZ, hh^{h290} (hh-lacZ), hh^{h425} (Ma et al., 1993); Stat92E^{F}, FRT40A Stat92E^{D16.9} (Stat92E^{D16.9} corresponds to the Stat92E^{F} mutation), UAS-Ci^{lep} (UAS-Ci5Ncm5, UAS-Ci5Sm30; from D. Kalderon, Columbia University, NY, USA), UAS-ptc RNAi (Vienna Drosophila RNAi Center), UAS-Ci^{lep} (UAS-Ci5Ncm5; from R. Holmgren, Northwestern University, IL, USA), UAS-Ha-Stat92E, UAS-mys, UAS-De-Cadherin, UAS-Hop^{lep}, eya3-gal4 and UAS-Hh [from J. Treisman (Azpiazu et al., 1996)].

**Immunohistochemistry**

The following antibodies were used: chicken anti-β-galactosidase (β-gal) (1:250; Immunology Consultants Lab), guinea-pig anti-Traffic jam (Tj) (1:3000; gift of D. Godt, University of Toronto, ON, Canada), goat anti-Vasa (1:400; Santa Cruz), guinea-pig anti-Zfh1 (1:1000; gift of J. Skeath, Washington University, MO, USA), rabbit anti-Zfh1 (1:5000; gift of R. Lehmann, New York University School of Medicine, NY, USA), rabbit anti-GFP (1:500; Invitrogen), mouse anti-GFP (1:500; Invitrogen), mouse anti-Eyes absent (Eya) [1:20; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Fas3 (1:500; DSHB), mouse anti-Adducin (Hts – FlyBase) (1B1, 1:20; DSHB), mouse anti-Ptc (1:200; DSHB), rat anti-Ci (1:20; DSHB), rabbit anti-Hh (1:50; gift of I. Guerrero, CBMSO, Madrid,
RESULTS
Hh pathway components are expressed in the testis niche
It has been reported that an enhancer trap in the hh locus called hhP30 is expressed in hub cells in the testis (Forbes et al., 1996; Dinardo et al., 2011). We confirm that hh is transcribed in hub cells (Fig. 2A,A′) and furthermore show that Hh protein is concentrated on and around the hub (Fig. 2B,B′). We also analyzed the expression of components required cell-autonomously for response to the Hh signal (see Fig. 1C). Colabeling somatic membranes with GFP reveals that Ptc, which is the Hh receptor and an Hh pathway target, is present in the somatic lineage, one cell diameter away from the hub, in presumptive CySCs (Fig. 2C-C′). The Ci antibody we used recognizes only full-length Ci (Motzny and Holmgren, 1995; Aza-Blanc et al., 1997), i.e. the Ci\textsc{rep} form that has not yet been cleaved into Ci\textsc{rep} and is competent for activation. Taken together, the patterns of Ptc and Ci show that Hh is secreted from the hub and that CySCs are competent to respond to it.

Hh signaling is required for CySC self-renewal
In order to test whether Hh signaling plays a role in stem cell self-renewal at the testis niche, we made clones of cells mutant for smo, which is essential for transduction of the Hh signal. Mutant negatively marked clones were generated by heat shock-induced recombination in adults and were scored at 2 or 7 days post-clone induction (dpci) to assess whether clones could be generated and maintained, respectively. We compared smo clones to neutral negatively marked clones made with the same FRT site but on a wild-type chromosome (i.e. FRT\textsuperscript{40A}). We used three different loss-of-function alleles of smo and obtained similar results. We scored GSCs as Vasa-positive cells that contacted the hub and scored CySCs as Zfh1-positive or Tj-positive cells with their nuclei one cell diameter away from the hub (i.e. on the other side of the GSCs). The hub was identified as a cluster of cells expressing low levels of Zfh1 or Tj surrounded by a rosette of Vasa-positive GSCs. In all cases, we were able to induce and recover smo mutant GSCs at comparable rates to control clones, indicating that Hh signaling is dispensable for GSC self-renewal (Fig. 3G; supplementary material Table S1). However, marked CySCs were recovered at low frequency at 2 dpci and very rarely at 7 dpci (Fig. 3G; supplementary material Table S1).

We also performed similar experiments using positively marked clones generated by the MARCM technique (Lee and Luo, 1999). Control FRT\textsuperscript{40A} MARCM clones in CySCs, which were identified by high levels of Zfh1, could be recovered at 2 dpci (Fig. 3A-A′). These control clones were maintained over time and could be readily identified at 7 dpci (Fig. 3D-D′; Table 1). smo MARCM clones in CySCs were recovered less frequently than controls (Fig. 3B; Table 1), but when they were recovered they either (1) expressed Zfh1 and lacked the differentiation marker Eya (suggesting that they are still CySCs) (Fig. 3B-B′, arrowheads; Table 1). Therefore, both negatively and positively marked clonal analyses confirm that GSCs are largely, if not entirely, unaffected by loss of Hh signaling, whereas CySCs require Hh for self-renewal.

We carried out several experiments to confirm that the lack of CySC recovery in smo mutant clones was due to specific loss of the Hh signal. Hh signaling activates the transcription factor Ci, which is cleaved in the absence of Hh to form a repressor. This cleavage requires the enzyme PKA. We reasoned that smo clones, which are unable to transduce Hh signaling, might be at least
partially rescued if the full-length Ci in these clones was unable to be cleaved to the repressor form. To test this hypothesis, we made clones that were doubly mutant for smo and Pka-C1 (the Drosophila gene that encodes PKA) and assessed CySC recovery rates in these clones at 2 and 7 dpci. Indeed, smo Pka-C1 double-mutant CySCs were present more often than single smo mutant CySCs at both 2 and 7 dpci (P<0.04; supplementary material Table S1). However, this is not a full rescue as PKA loss alone is insufficient to fully activate Ci (Price and Kalderon, 1999) and double-mutant clones are still lost with time. Finally, we used the MARCM technique to make smo mutant clones expressing an uncleavable form of Ci, termed Ciact, which behaves as a dominant-
active protein. In this case, CySCs could be recovered at 90% of control rates, indicating robust rescue of smo mutants (Fig. 3F,F'; Table 1).

Together, these data indicate that transcription downstream of Hh reception and signal transduction is required in CySCs, but not GSCs, for self-renewal.

**Hh levels control CySC number**

Next, we assayed the effect of Hh gain-of-function in the testis. Expressing Hh under the control of a somatic cell driver (eyaA3>gal4) causes an increase in the number of Traffic jam-positive somatic cells (Fig. 4B,B'; compare with 4A,A'). Further analysis showed that these excess somatic cells express Zfh1, which is most highly expressed in CySCs. These data suggest that the excess somatic cells in eyaA3>Hh are stem cells (Fig. 4C,C'). As Hh is secreted, this result could be due to cell-autonomous effects on cyst cells or to non-cell-autonomous signaling to the hub or germ cells. These possibilities were distinguished by expressing either the Ci repressor (Ci<sup>rep</sup>) or an RNAi against ptc (to block or enhance Hh signaling, respectively, within the somatic lineage. Loss of ptc results in ligand-independent Hh signaling (Ingham et al., 1991; Chen and Struhl, 1996). We counted 44.6±1.2 Zfh1-positive, Eya-negative cells in eyaA3-gal4/+ control males (n=20), consistent with the previously reported 36-50 Zfh1-positive cells per testis (Leatherman and Dinardo, 2010; Inaba et al., 2011). Ptc knockdown causes a statistically significant increase in the number of Zfh1-positive, Eya-negative CySCs to 73.2±3.1 (n=15, P<9.7×10<sup>-8</sup>) (Fig. 4D,F). We observed a similar expansion of Zfh1-positive cells when we overexpressed Ci<sup>rep</sup> using eyaA3-gal4 (not shown). Conversely, the number of CySCs was reduced again in a statistically significant manner when Hh signaling was impaired cell-autonomously (29.8±3.2 CySCs; n=10, P<1×10<sup>-3</sup> compared with control; Fig. 4D,F).

In order to address further the in vivo role of Hh in the adult testis, a temperature-sensitive mutant allele of hh (hh<sup>ts2</sup>) was analyzed. Adult males were allowed to develop normally by raising the embryos, larvae and pupae at the permissive temperature of 16°C and were then shifted after eclosion to the restrictive temperature of 29°C. CySC numbers were strongly reduced from 36±1.5 in heterozygous controls (n=19) to 7.6±1.3 in hh<sup>ts2</sup> mutant animals (n=15, P<1.3×10<sup>-15</sup>; Fig. 4G-H,M). The autonomy and the specificity of this phenomenon were tested by restoring Hh signaling downstream of the ligand using a somatic cell driver to express Ci<sup>rep</sup>. In this situation, CySC numbers were nearly restored to control levels (28±2.5; n=14, P<4.6×10<sup>-7</sup> compared with hh<sup>ts2</sup>; Fig. 4L,M). These data demonstrate that Hh signaling through Ci-activated transcription is necessary to specify the correct number of somatic stem cells at the testis niche.

We also investigated the presence of GSCs in hh<sup>ts2</sup> mutants at the restrictive temperature. We used the M5-4 enhancer trap line, which reports expression at the escargot (esg) locus and is expressed in GSCs, gonialblasts and hub cells in wild-type testes (Fig. 4J) (Gönczy and DiNardo, 1996). We compared GSCs (Vasa-positive, esg-lacZ-positive cells in contact with the hub) in hh<sup>ts2</sup>/+ heterozygous siblings with hh<sup>ts2</sup>/+ mutants raised under the same conditions. hh<sup>ts2</sup>/+ heterozygous siblings had 8.6±0.3 GSCs (n=18; Fig. 4K). By contrast, hh<sup>ts2</sup> mutants had 6.3±0.4 GSCs (n=16), a statistically significant decrease (P<6.2×10<sup>-5</sup>; Fig. 4L). This change is due to the requirement for Hh, as the same animals raised at the permissive temperature did not display significantly different numbers of GSCs [10.4±0.5 in hh<sup>ts2</sup>/+ (n=14) and 11.2±0.4 in hh<sup>ts2</sup> (n=5); differences not significant by Student’s t-test]. The reduction in GSCs in hh<sup>ts2</sup> mutants could be due to a reduction in hub size. We ruled out this possibility because the number of hub cells in mutant animals did not differ from that of controls [11.5±0.5 (n=17) for hh<sup>ts2</sup>/+ animals and 11.1±0.5 hub cells in hh<sup>ts2</sup> (n=15); differences not significant by Student’s t-test]. We noted that, unlike Star92E temperature-sensitive mutants (see Fig. 6B,B') (Leatherman and Dinardo, 2010), GSCs were still present in hh<sup>ts2</sup> mutants. This is despite the reduction in CySCs, Table 1. Percentage of testes containing one or more MARCM clones

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dpci</th>
<th>CySC clones (%)</th>
<th>Differentiated cyst cells (%)</th>
<th>GSC clones (%)</th>
<th>Testes scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (FRT&lt;sup&gt;90A&lt;/sup&gt;)</td>
<td>2</td>
<td>58.3</td>
<td>ND</td>
<td>50.0</td>
<td>24</td>
</tr>
<tr>
<td>smo&lt;sup&gt;1&lt;/sup&gt;1986</td>
<td>7</td>
<td>56.8</td>
<td>73.7</td>
<td>83.8</td>
<td>37</td>
</tr>
<tr>
<td>smo&lt;sup&gt;1&lt;/sup&gt;1986, UAS-Ci&lt;sup&gt;rep&lt;/sup&gt;</td>
<td>2</td>
<td>64.7</td>
<td>ND</td>
<td>58.8</td>
<td>17</td>
</tr>
<tr>
<td>smo&lt;sup&gt;1&lt;/sup&gt;1986, UAS-Hop</td>
<td>2</td>
<td>51.3</td>
<td>ND</td>
<td>28.2</td>
<td>39</td>
</tr>
<tr>
<td>smo&lt;sup&gt;1&lt;/sup&gt;1986, UAS-Mys</td>
<td>2</td>
<td>0.0</td>
<td>3.6</td>
<td>75.0</td>
<td>28</td>
</tr>
<tr>
<td>smo&lt;sup&gt;1&lt;/sup&gt;1986, UAS-DE-Cad</td>
<td>2</td>
<td>0.0</td>
<td>6.7</td>
<td>43.3</td>
<td>30</td>
</tr>
<tr>
<td>chinmo&lt;sup&gt;1&lt;/sup&gt;, UAS-Ci&lt;sup&gt;rep&lt;/sup&gt;</td>
<td>2</td>
<td>45.5</td>
<td>ND</td>
<td>81.8</td>
<td>22</td>
</tr>
<tr>
<td>Control (FRT&lt;sup&gt;92B&lt;/sup&gt;)</td>
<td>2</td>
<td>78.6</td>
<td>ND</td>
<td>0.0</td>
<td>28</td>
</tr>
<tr>
<td>Stat92E&lt;sup&gt;Scs&lt;/sup&gt;c</td>
<td>2</td>
<td>34.9</td>
<td>ND</td>
<td>0.0</td>
<td>43</td>
</tr>
<tr>
<td>Stat92E&lt;sup&gt;Scs&lt;/sup&gt;c, UAS-Ci&lt;sup&gt;rep&lt;/sup&gt;</td>
<td>2</td>
<td>0.0</td>
<td>3.6</td>
<td>75.0</td>
<td>28</td>
</tr>
</tbody>
</table>

Control or smo, chinmo or Stat92E mutant clones were generated by the MARCM technique in adult testes and scored for the presence of CySCs and GSCs at 2 or 7 dpc (see text for details). We note that the MARCM stock used for FRT<sup>90A</sup> clones works very poorly in GSCs, presumably due to very low expression of UAS-GFP in these cells after recombination. ND, not determined.
which constitute part of the niche for GSCs and are necessary to support their self-renewal (Leatherman and Dinardo, 2010).

**STAT and Hh act independently on self-renewal**

As JAK/STAT signaling is essential for CySC self-renewal (Leatherman and Dinardo, 2008), we examined whether Hh could be acting on stem cell maintenance by affecting the JAK/STAT pathway, or, conversely, whether JAK/STAT affects Hh signaling. Stabilization of the Stat92E protein is often used as a readout for JAK/STAT pathway activity (Chen et al., 2002; Flaherty et al., 2010). Mutant clones for smo accumulated Stat92E protein normally when found close to the hub, suggesting that JAK/STAT signaling is unaffected in these cells (Fig. 5A-A’). We tried to rescue smo-deficient CySCs by expressing Hop in them, which is sufficient to activate Stat92E when overexpressed (Ekas et al., 2006). We confirmed that Stat92E is indeed activated in smo MARCM clones expressing Hop both in CySCs and in differentiating cyst cells (Fig. 5B-B’). We confirmed that Stat92E is indeed activated in smo MARCM clones expressing Hop both in CySCs and in differentiating cyst cells (Fig. 5B-B’). This, combined with the fact that Ciact overexpression can rescue loss of CySCs in smo mutant clones (Fig. 3F,F’; Table 1), suggests that the MARCM technique is a valid tool for attempting to rescue CySC maintenance. We cannot rule out the possibility that perdurance of the Gal80 protein might prevent full CySC rescue. However, ectopic activation of the JAK/STAT pathway cannot rescue CySCs lacking smo (Fig. 5C-C’; Table 1).

We also addressed the possibility that smo-deficient CySCs are being outcompeted from the niche by wild-type CySCs. It has been reported that niche competition is due to differential Integrin-based adhesion downstream of Stat92E (Issigonis et al., 2009). We tested this hypothesis by overexpressing Myospheroid (Mys; the Drosophila βPS-integrin homolog) in smo mutant clones. CySC recovery was not observed in this experiment (Table 1). Additionally, we asked whether DE-Cadherin-based adhesion was responsible for the loss of smo mutant CySCs. We expressed DE-Cadherin (called Shotgun in Drosophila) in smo mutant clones by the MARCM technique, but CySCs were not recovered at 7 dpci (Table 1). These two experiments strongly suggest that smo-deficient CySCs are lost as a direct consequence of a failure to self-renew and not as a result of niche competition.
We next tested whether readouts of Hh signaling were affected when the JAK/STAT pathway was disrupted. Since \textit{ptc-lacZ} – the best-established readout of Hh signaling – and \textit{Stat92E} reside on the same chromosome, we were unable to assess \textit{ptc} levels in \textit{Stat92E} clones. As a proxy, we examined \textit{ptc-lacZ} in \textit{chinmo} clones, as we previously showed that \textit{chinmo} is a key functional effector of \textit{Stat92E} in numerous cell types including CySCs (Flaherty et al., 2010). We found that \textit{ptc-lacZ} was unchanged in \textit{chinmo} mutant CySCs at 2 dpci, suggesting that Hh signaling is unaffected in CySCs lacking a key \textit{Stat92E} functional effector (Fig. 5D–D'). Consistent with this observation, we were unable to obtain significant rescue of either \textit{Stat92E} or \textit{chinmo} mutant CySCs by ectopic activation of Hh signaling through misexpression of \textit{Ciact} (Fig. 5F–F'; Table 1). It should be noted that \textit{Stat92E} and \textit{chinmo} mutant CySCs differentiate very quickly (Leatherman and Dinardo, 2008; Issigonis et al., 2009; Flaherty et al., 2010), meaning that few differentiated cyst cells can be seen at 7 dpci (Table 1). By contrast, differentiated \textit{Stat92E} and \textit{chinmo} mutant cyst cells overexpressing \textit{Ciact} are readily recovered (Fig. 5F–F'; arrow; Table 1). This suggests that Hh activation can delay the loss of \textit{Stat92E}- or \textit{chinmo}-deficient CySCs, even though this is ultimately insufficient for their maintenance.

Furthermore, we were unable to detect a genetic interaction between \textit{hh} and \textit{Stat92E}. Animals that were transheterozygous for both mutations (\textit{hh}^{ts2}/\textit{Stat92E}^{85C9}) appeared to have a normal complement of stem cells at the testis niche (data not shown). In addition, CySC numbers in controls (\textit{hh}^{ts2}/+) and transheterozygous animals were not statistically different (means of 36 and 34, respectively; \textit{P} < 0.6). Thus, we conclude that the Hh and JAK/STAT pathways provide non-redundant signals for the self-renewal of CySCs.

**Hh signaling does not contribute to CySC niche function**

In a series of elegant experiments, Leatherman and DiNardo established that, in addition to being the source of cyst cells, CySCs...
have a second function, providing part of the ‘extended niche’ for GSCs (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010). We made use of their model to examine whether Hh signaling contributes to this extended niche function of CySCs. As shown by Leatherman and Dinardo, Stat92Ets animals lose both CySCs and GSCs (Fig. 6A-B). Expression of Stat92E in the somatic lineage using tj-gal4 can rescue both stem cell types (C). Activation of the Hh pathway in these cells by expressing Cact leads to rescue of Zfh1-expressing CySCs (D1), but not of GSCs (D2). Zfh1 is green (single channel in A-D), Vasa is red (single channel in A-D) and Eya and Fas3 are blue. (E-G) A wild-type testes taken at 10X magnification. HopK6M18 overexpression in the somatic lineage causes tumors in both hh heterozygous (tj>HopK6M18, hhts2/+) and hh mutant (tj>HopK6M18, hhts2) testes. Bam is green in E, Zfh1 is green in F,G; Vasa is red in E-G, Tj is blue in E and Eya is blue in E.G. (H, H1, J, J1) The stem cell marker Zfh1 (green, single channel in H, J1) is expressed at lower levels in hhts2 (J, J1) than in hhts2/+ siblings (H, H1). Eya (blue) expression is largely absent in hhts2/+ siblings (H) but is readily detectable in hhts2 mutants (J), where it is co-expressed with Zfh1. Vasa is red in H, J (I, K) The germ cells present in tumors induced in hhts2 mutant testes (K) are larger than in control siblings (I) and display large, branched fusomes (1B1, green) compared with the small, round fusomes in hhts2/+ siblings, which are indicative of a stem-like state in GSCs. (L) Model of genetic interactions occurring at the testis stem cell niche. The hub (green) produces Upd and Hh, which are required in the CySC (blue) for self-renewal. The CySC requires the Stat92E targets Zfh1 and Chinmo. Stat92E and its targets also regulate BMP production in the CySC, and these BMPs signal to the GSC (red) together with hub-derived BMPs for self-renewal, but Hh signaling does not contribute to this function.

Fig. 6. Hh does not contribute to CySC niche function. (A-D) tj-gal4/+; Stat92E/+ control testes contain a normal complement of CySCs (determined by Zfh1 staining) and germ cells (as assessed by Vasa staining). CySCs and GSCs are lost in a Stat92E temperature-sensitive mutant (Stat92Ets) (B). Expression of Stat92E in the somatic lineage using tj-gal4 can rescue both stem cell types (C). Activation of the Hh pathway in these cells by expressing Cact leads to rescue of Zfh1-expressing CySCs (D1), but not of GSCs (D2). Zfh1 is green (single channel in A-D), Vasa is red (single channel in A-D) and Eya and Fas3 are blue. (E-G) A wild-type testes taken at 10X magnification. HopK6M18 overexpression in the somatic lineage causes tumors in both hh heterozygous (tj>HopK6M18, hhts2/+) and hh mutant (tj>HopK6M18, hhts2) testes. Bam is green in E, Zfh1 is green in F,G; Vasa is red in E-G, Tj is blue in E and Eya is blue in E.G. (H, H1, J, J1) The stem cell marker Zfh1 (green, single channel in H, J1) is expressed at lower levels in hhts2 (J, J1) than in hhts2/+ siblings (H, H1). Eya (blue) expression is largely absent in hhts2/+ siblings (H) but is readily detectable in hhts2 mutants (J), where it is co-expressed with Zfh1. Vasa is red in H, J (I, K) The germ cells present in tumors induced in hhts2 mutant testes (K) are larger than in control siblings (I) and display large, branched fusomes (1B1, green) compared with the small, round fusomes in hhts2/+ siblings, which are indicative of a stem-like state in GSCs. (L) Model of genetic interactions occurring at the testis stem cell niche. The hub (green) produces Upd and Hh, which are required in the CySC (blue) for self-renewal. The CySC requires the Stat92E targets Zfh1 and Chinmo. Stat92E and its targets also regulate BMP production in the CySC, and these BMPs signal to the GSC (red) together with hub-derived BMPs for self-renewal, but Hh signaling does not contribute to this function.

previous results, that the Hh and JAK/STAT pathways independently regulate CySC fate. Surprisingly, no GSCs or early germ cell cysts were present in these Stat92Ets testes somatically supplied with ectopic Hh signaling, indicating that CySCs with activated Hh signaling alone are not able to support GSC maintenance.

Stem cell markers are lost in Hop-induced tumors in the absence of Hh

Next, we asked whether Hh signaling is required for the stem cell tumors observed when Upd or Stat92E is hyperactivated (Kiger et al., 2001; Tulina and Matunis, 2001; Leatherman and Dinardo,
Reduced numbers. The reduction in GSCs in
despite the presence of ~36 Zfh1-positive CySCs, elevated Stat92E
production of cyst progeny. This is consistent with the fact that,
activated Hh signaling that participate only in self-renewal and the
produce BMPs and act as a niche for GSCs, and others with
are distinct populations of CySCs, some with activated Stat92E that
This raises the question as to whether, in a wild-type animal, there
is not required for tumor formation but is required for those tumors
to be composed of stem cells.

DISCUSSION
We have shown that Hh from the Drosophila testis niche is a self-
renewal factor for CySCs and that Hh signaling does not contribute
to the role of CySCs as a niche for GSCs. This supports the model
that the Hh and JAK/STAT pathways act independently within
CySCs (Fig. 6L). Our results therefore confirm those recently
reported by another group (Michel et al., 2012), who showed that
Hh regulates CySC self-renewal, and extend their results by
demonstrating the genetic independence of Hh and the other
pathway (i.e. JAK/STAT) that is crucial in CySC function.

Separating niche and stem cell roles
It is notable that two signals regulate CySC self-renewal but only
JAK/STAT signaling contributes to the GSC niche. Moreover,
deprive the drastic reduction in CySCs in hh
2
 testes (from ~36 in controls to ~8), GSCs do remain in hh mutant animals albeit at
reduced numbers. The reduction in GSCs in hh mutants is not due to
changes in the size of the hub. These data suggest that most
CySCs are dispensable for their niche function and that only a few
BMP-producing CySCs are needed to maintain GSC self-renewal.
This raises the question as to whether, in a wild-type animal, there
are distinct populations of CySCs, some with activated Stat92E that
produce BMPs and act as a niche for GSCs, and others with
activated Hh signaling that participate only in self-renewal and the
production of cyst progeny. This is consistent with the fact that,
besides the presence of ~36 Zfh1-positive CySCs, elevated Stat92E
is only seen in a few CySCs (Fig. 5A-B”) (Leatherman and Dinardo, 2008; Flaherty et al., 2010). However, it is also
celvable that all Zfh1-positive CySCs are equivalent and that
high Stat92E correlates, for instance, with a specific phase of the
cell cycle, such as the repositioning of the spindle during anaphase
that brings the nucleus of the CySC closer to the hub interface
(Cheng et al., 2011) and might expose that CySC to more Upd
ligand. This possibility implies a much more dynamic stem cell
niche for the GSCs than has been previously appreciated.

Multiple signals for homeostasis
Our results indicate that the Hh and JAK/STAT pathways act
mostly in parallel, although activating Hh may delay the
differentiation of CySCs that are deficient for JAK/STAT pathway
components. It is unclear why the CySC would require both
signaling inputs to be maintained. However, it should be noted that
these inputs contribute different information, as JAK/STAT
signaling imparts niche potential (Leatherman and Dinardo, 2008;
Flaherty et al., 2010; Leatherman and Dinardo, 2010), and Hh
signaling additionally ensures that the right number of CySCs are present and provide cyst cells for normal spermatogonial
development (this study) (Michel et al., 2012). Future work will
establish whether self-renewal in CySCs depends on two sets of
genes controlled separately by the Hh and JAK/STAT pathways or
whether they converge on the same targets. The first possibility is
supported by the fact that Hh does not contribute to the niche
function of STAT in CySCs, indicating that different targets
(presumably BMPs) are regulated differently.

Conservation of the role of Hh
One consequence of this work is to lead us to re-evaluate the
differences between male and female gonad development in
Drosophila (Losick et al., 2011). Indeed, Hh signaling is an
essential regulator of the self-renewal and the number of follicle
stem cells, the offspring of which carry out a comparable function
to cyst cells by ensheathing germ line cysts (Forbes et al., 1996;
Zhang and Kalderon, 2000; Zhang and Kalderon, 2001). In the
ovary, as in the testis, JAK/STAT signaling in somatic cells is
required for the maintenance of GSCs via BMP production (López-
Onieva et al., 2008; Leatherman and Dinardo, 2010). However, in
the ovary, the escort cells and cap cells are the JAK/STAT-
responsive niche cells (López-Onieva et al., 2008; Wang et al.,
2008), implying that CySCs in the male gonad fulfill the function
of two cell types in the female gonad and require both the signals
used in the female to do so. Finally, our data evoke the interesting
possibility that Hh has a conserved ancestral role in male gonads.
Mutation in one of the three mammalian hh homologs, desert
hedgehog (Dhh), causes male sterility (Bitgood et al., 1996) and
a loss of somatic support cells called Leydig cells (Clark et al., 2000;
Yao et al., 2002). However, the cellular niche for spermatogenesis in
mammals is less well understood than in Drosophila and it
remains to be established whether the Hh pathway orchestrates
similar cellular functions.

Acknowledgements
We thank G. Struhl, D. Godt, R. Lehmann, I. Guerrero, J. Treisman, D.
Kalderon, R. Holmgren, F. Schöck, T. Harris, J. Skeath, the DSHB and the
Bloomington Stock Center for flies and antibodies; Y. Mavromatakis for helpful
comments on the manuscript; the NYU fly community for discussion; and we are
very grateful to Christian Bökel for communicating results.

Funding
This work was supported by grants from the National Institutes of Health
[R01-GM085075] and New York State Department of Health (NYSYSTEM) [C024284]
to E.A.B. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.086413/-/DC1

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Hh regulates CySC self-renewal


