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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 hetero-allelic *Stat92E* temperature-sensitive mutant (*Stat92E^{ts}*) 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 *Stat92E^{ts}* flies raised at the restrictive temperature rescues both germ line and somatic lineages (Leatherman and DiNardo, 2010). In the soma-rescued *Stat92E^{ts}* 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.,

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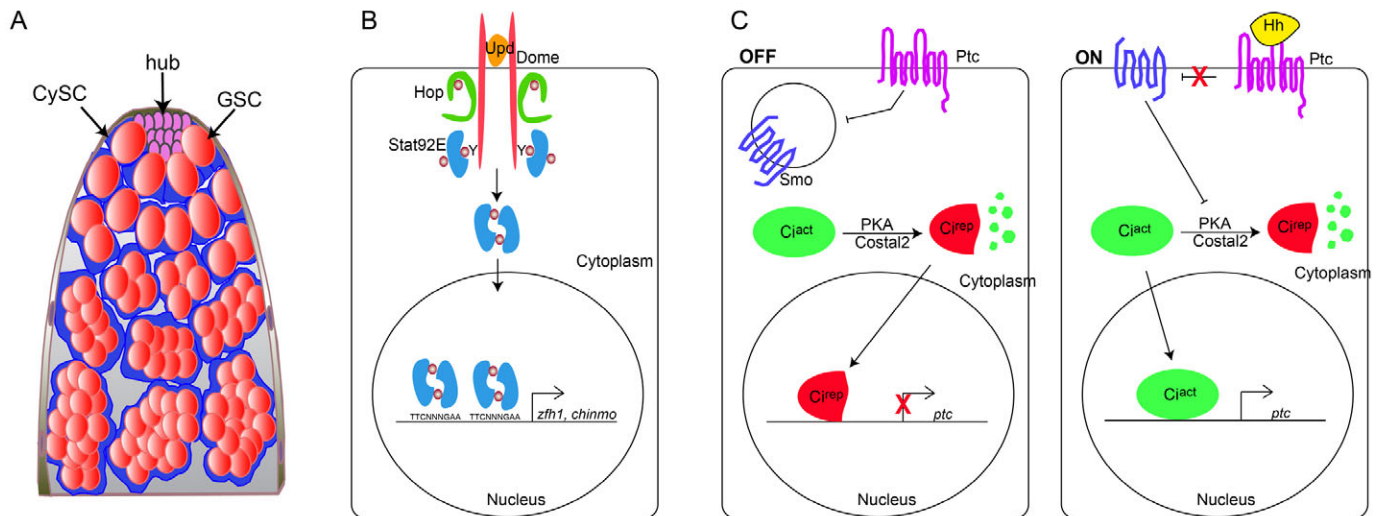


Fig. 1. The JAK/STAT and Hh pathways. (A) The *Drosophila* testis. See text for details. (B) JAK/STAT signaling in *Drosophila*. Binding of the secreted ligand Unpaired (Upd) to its receptor Domeless (Dome) causes phosphorylation and activation of the Janus kinase (JAK) Hopscotch (Hop). Hop in turn phosphorylates the transcription factor Stat92E, which dimerizes and enters the nucleus to promote the transcription of target genes, including *zfh1* and *chinmo*. (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^{act}) to a repressor (Ci^{rep}) 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^{act} enters the nucleus to activate transcription of target genes, such as *ptc*. GSC, germ line stem cell; CySC, cyst stem cell; Y, tyrosine residues.

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 complex that includes Protein kinase A (PKA) and the scaffolding protein Costal2 (Costa – FlyBase) (Kalderon, 2005). This results in a shortened form of Ci (called Ci^{rep}) 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 Ci (Fig. 1C, 'ON'). This results in a full-length Ci protein called Ci^{act} that induces transcription of target genes (Dominguez et al., 1996; Aza-Blanc et al., 1997). One such gene is *ptc*, which, in addition to encoding the Hh receptor, is also a well-established Hh pathway target (Hidalgo and Ingham, 1990; Tabata and Kornberg, 1994; Chen and Struhl, 1996; Goodrich et al., 1996).

Here, we dissect the role of Hh signaling in the *Drosophila* testis and describe a requirement for Hh signal reception in CySC maintenance, similar to the role that Hh signaling fulfills in somatic stem cells in the ovary. We find that Hh and JAK/STAT signaling act independently and cannot substitute for one another in CySC maintenance. We also demonstrate that, in contrast to JAK/STAT signaling, Hh pathway activity does not impart 'extended niche' function to CySCs and that Hh only regulates CySC self-renewal.

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. *eyaA3-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; FRT^{40A} ubi-GFP;
yw,hsflp¹²²; FRT^{40A};
yw,hsflp¹²²; UAS-nls-GFP, tub-gal4; tub-gal80, FRT^{40A};
yw,hsflp¹²²; UAS-CD8-GFP;; tub-gal4, FRT^{82B} tub-gal80.

Fly stocks

The following stocks were used and are described in FlyBase: Oregon-R, *FRT^{40A} smo²*, *FRT^{40A} smo^{119B6}*, *FRT^{40A} smo^{D16}*, *FRT^{40A} smo^{D16} Pka-CI^{H2}*, *FRT^{40A} chinmo¹*, *ptc-lacZ*, *hh^{P30} (hh-lacZ)*, *hh^{ts2}* (Ma et al., 1993); *Stat92E^F*, *FRT^{82B} Stat92E^{85c9}* (*Stat92E^F/Stat92E^{85c9}* corresponds to the *Stat92E^{ts}* mutation), *UAS-Ci^{act}* (*UAS-Ci5Ncm5*, *UAS-Ci5m30*; from D. Kalderon, Columbia University, NY, USA), *UAS-ptc RNAi* (Vienna *Drosophila* RNAi Center), *UAS-Ci^{rep}* (*UAS-CiN-ZN*; from R. Holmgren, Northwestern University, IL, USA), *UAS-3HA-Stat92E*, *UAS-mys*, *UAS-DE-Cadherin*, *UAS-Hop^{tim-1}*, *eyaA3-gal4* and *UAS-Hh* [from J. Treisman (Azpiroz 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 (1:50; 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,

Spain) and rabbit anti-Stat92E (1:500; Flaherty et al., 2010). Testes were dissected and fixed in 4% formaldehyde and processed as described (Flaherty et al., 2010).

RESULTS

Hh pathway components are expressed in the testis niche

It has been reported that an enhancer trap in the *hh* locus called *hh^{P30}* 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''). Ci, the transcription factor that mediates Hh signaling, is expressed in an overlapping, but larger, subset of somatic cells including the CySCs and their daughters (Fig. 2D-D''). The Ci antibody we used recognizes only full-length Ci (Motzny and Holmgren, 1995; Aza-Blanc et al., 1997), i.e. the Ci^{act} form that has not yet been cleaved into Ci^{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^{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^{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'', arrows) or (2) downregulated Zfh1 and began to express Eya (Fig. 3C-C'', arrow). By 7 dpci, we were unable to recover *smo*-deficient CySCs (Fig. 3E,E'; Table 1). By contrast, we were able to identify *smo* mutant GSCs at 7 dpci at rates similar to control MARCM clones (Fig. 3D-E', 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

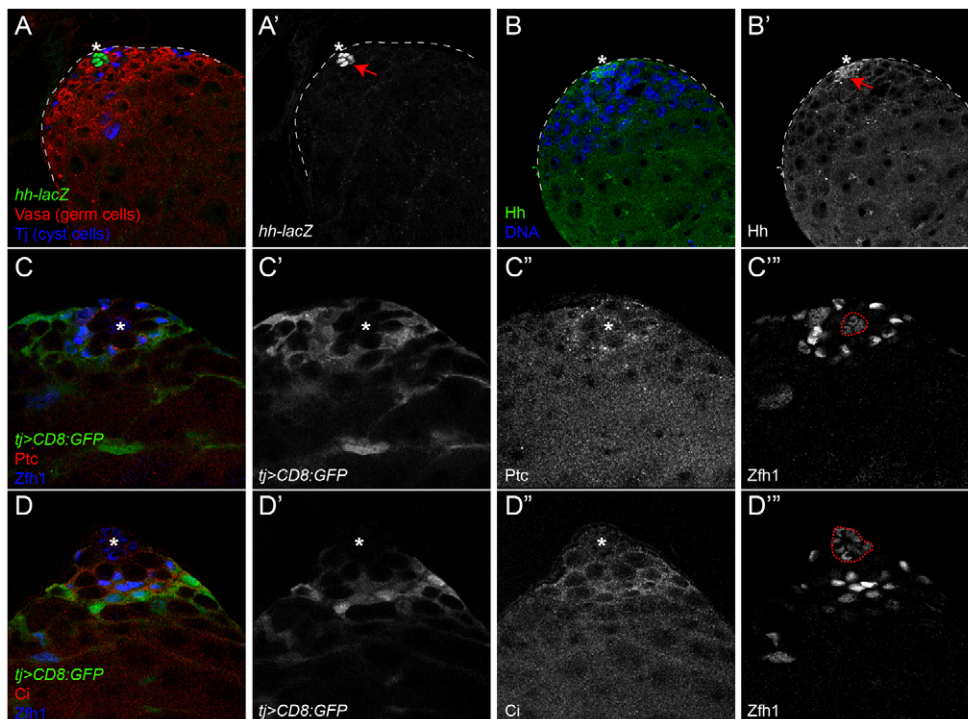
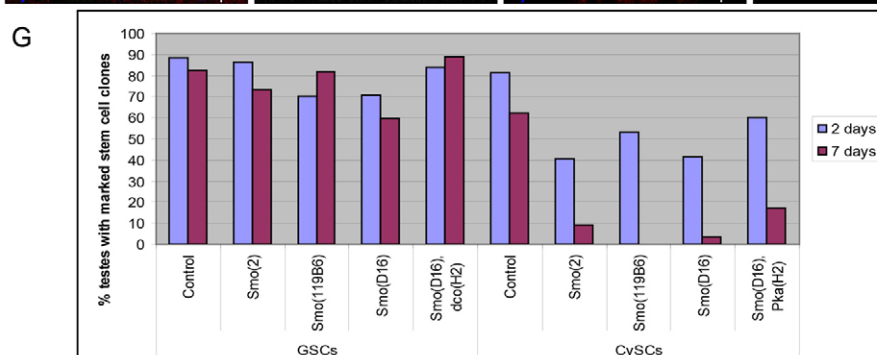
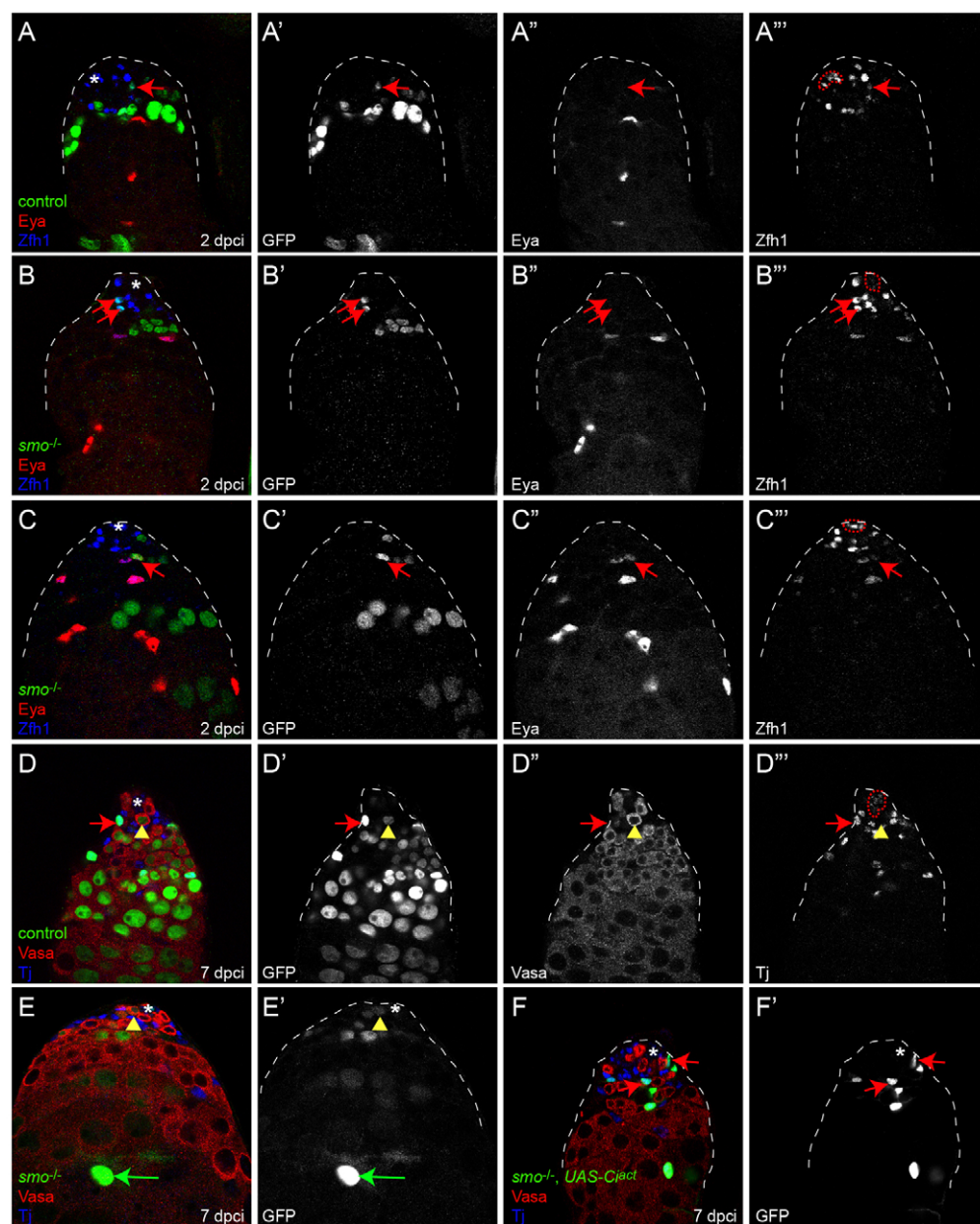


Fig. 2. Expression of Hh pathway components at the testis niche.

(A,A') Expression of *hh-lacZ* is seen in the hub (arrow in A'). (B,B') Hh protein is also found at high levels in and around the hub (arrow in B'). (C-D'') *tj-gal4>UAS-CD8::GFP* was used to label somatic cell membranes (single channel in C',D'). CySCs co-express Ptc (red; single channel in C'') and Zfh1 (blue; single channel in C'') adjacent to the hub. Ci (red; single channel in D'') is expressed in the cyst lineage, including Zfh1-positive CySCs. Asterisks indicate the hub, which is outlined (red dotted lines) in C''', D''', and D''''.



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 Ci^{act} , which behaves as a dominant-

Table 1. Percentage of testes containing one or more MARCM clones

Genotype	Dpci	CySC clones (%)	Differentiated cyst cells (%)	GSC clones (%)	Testes scored
Control (<i>FRT^{40A}</i>)	2	58.3	ND	50.0	24
	7	56.8	73.7	83.8	37
<i>smo^{119B6}</i>	2	36.4	ND	45.5	33
	7	2.2	8.3	77.8	45
<i>smo^{119B6}, UAS-Ci^{act}</i>	2	64.7	ND	58.8	17
	7	51.3	ND	28.2	39
<i>smo^{119B6}, UAS-Hop</i>	2	42.9	ND	61.9	21
	7	0.0	3.6	75.0	28
<i>smo^{119B6}, UAS-Mys</i>	2	ND	ND	ND	–
	7	0.0	6.7	43.3	30
<i>smo^{119B6}, UAS-DE-Cad</i>	2	ND	ND	ND	–
	7	0.0	11.1	71.1	45
<i>chinmo¹</i>	2	45.5	ND	81.8	22
	7	0.0	11.4	74.3	35
<i>chinmo¹, UAS-Ci^{act}</i>	2	75.0	ND	25.0	8
	7	0.0	56.0	80.0	25
Control (<i>FRT^{82B}</i>)	2	78.6	ND	0.0	28
	7	62.7	74.5	3.9	51
<i>Stat92E^{85c9}</i>	2	34.9	ND	0.0	43
	7	0.0	15.8	0.0	38
<i>Stat92E^{85c9}, UAS-Ci^{act}</i>	2	0.0	ND	36.4	22
	7	3.6	44.6	0.0	56

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 dpci (see text for details). We note that the MARCM stock used for *FRT^{82B}* clones works very poorly in GSCs, presumably due to very low expression of *UAS-GFP* in these cells after recombination.
ND, not determined.

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^{rep}*) 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 testes from *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*<9.7×10⁻⁸) (Fig. 4D,F). We observed a similar expansion of Zfh1-positive cells when we overexpressed *Ci^{act}* 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*<1×10⁻³ compared with control; Fig. 4D,E).

In order to address further the *in vivo* role of Hh in the adult testis, a temperature-sensitive mutant allele of *hh* (*hh^{ts2}*) 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^{ts2}* mutant animals (*n*=15, *P*<1.3×10⁻¹⁵; Fig. 4G–H,M). The autonomy and the specificity of this phenotype were tested by restoring Hh signaling downstream of the ligand using a somatic cell driver to express *Ci^{act}*. In this situation, CySC numbers were nearly restored to control levels (28±2.5; *n*=14, *P*<4.6×10⁻⁷ compared with *hh^{ts2}*; Fig. 4I,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^{ts2}* 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^{ts2}/+* heterozygous siblings with *hh^{ts2}* mutants raised under the same conditions. *hh^{ts2}/+* heterozygous siblings had 8.6±0.3 GSCs (*n*=18; Fig. 4K). By contrast, *hh^{ts2}* mutants had 6.3±0.4 GSCs (*n*=16), a statistically significant decrease (*P*<6.2×10⁻⁵; 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^{ts2}/+* (*n*=14) and 11.2±0.4 in *hh^{ts2}* (*n*=5); differences not significant by Student's *t*-test]. The reduction in GSCs in *hh^{ts2}* 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^{ts2}/+* animals and 11.1±0.5 hub cells in *hh^{ts2}* (*n*=15); differences not significant by Student's *t*-test]. We noted that, unlike *Stat92E* temperature-sensitive mutants (see Fig. 6B,B') (Leatherman and Dinardo, 2010), GSCs were still present in *hh^{ts2}* mutants. This is despite the reduction in CySCs,

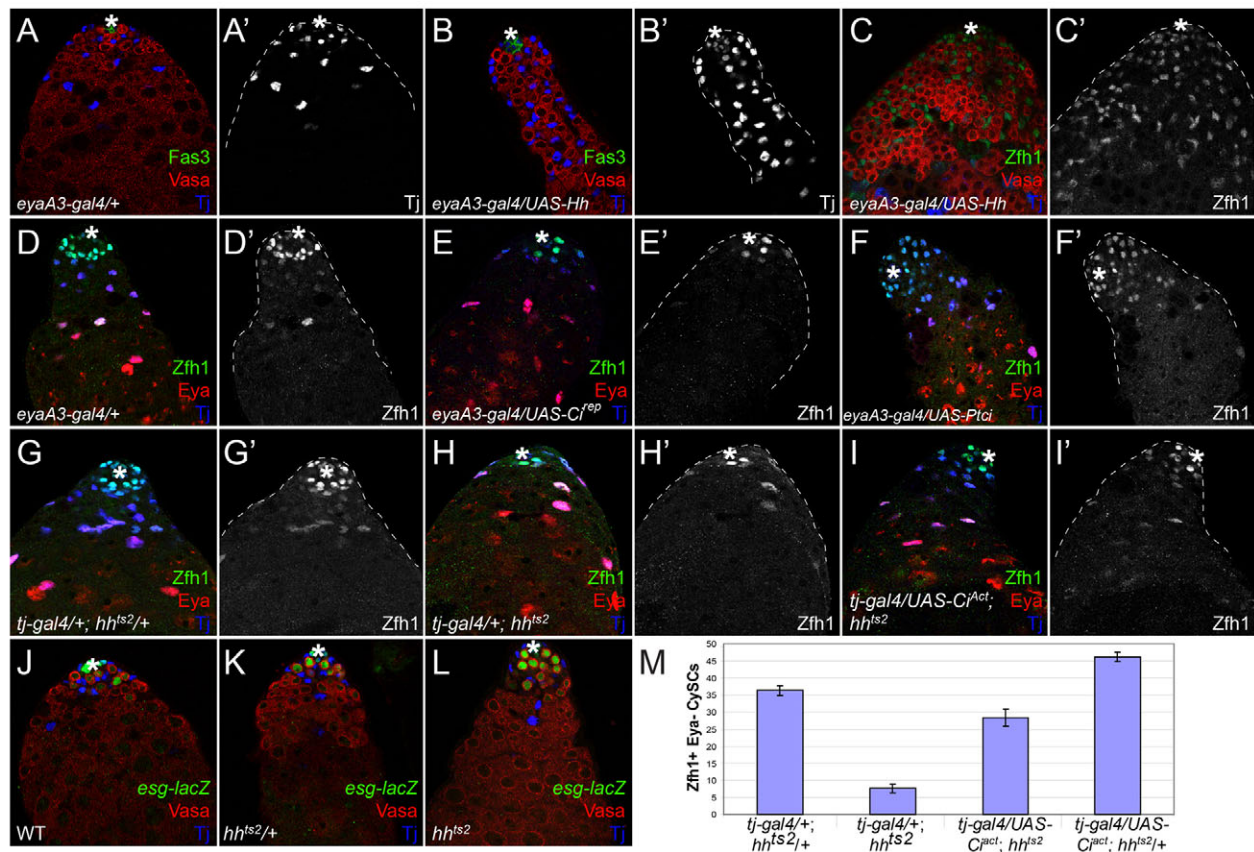


Fig. 4. Hh levels modulate CySC number. (A-B') Control *eyaA3-gal4*-expressing testis tip showing the hub (Fas3, green), germ cells (Vasa, red) and somatic cells (Tj, blue single channel in A', B'). Expression of Hh (*eyaA3>Hh*) leads to an increase in Tj-positive somatic cell number (B). (C, C') The additional somatic cells in *eyaA3>Hh* testes are stem cells as evidenced by expression of Zfh1 (green; single channel in C'); Vasa is red and Tj is blue in C. (D-F') Cell-autonomous modulation of Hh levels within somatic cells is sufficient to alter CySC number. Compared with *eyaA3-gal4* controls (D, D'), expression of *Ci^{rep}* (E, E') or knockdown of *Ptc* by RNAi (F, F') leads to loss or gain, respectively, of CySCs, assessed as Zfh1-positive, Eya-negative cells. (G, G') An *hh^{ts2/+}* sibling exhibits a normal complement of CySCs. (H, H') By contrast, an *hh^{ts2}* mutant displays a loss of CySCs after 10 days at the non-permissive temperature. (I, I') However, CySCs in the *hh^{ts2}* mutant can be rescued by expression of *Ci^{act}* in the somatic lineage. In D-I, Zfh1 is green (single channel in D'-I'), Eya is red and Tj is blue. (J-L) GSCs [Vasa-positive (red), *esg-lacZ*-positive (green)] are maintained in *hh^{ts2}* mutants (L) as compared with wild-type (WT) (J) and *hh^{ts2/+}* sibling controls (K). Tj is blue. The hub is indicated by an asterisk in all images. (M) Quantification of CySC numbers for the specified genotypes. CySCs were defined as Zfh1-positive, Eya-negative cells. Error bars indicate s.e.m.

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'', red and green arrows, respectively). This, combined with the fact that *Ci^{act}* 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* β PS-integrin homolog) in *smo* mutant MARCM 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 dpcl (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.

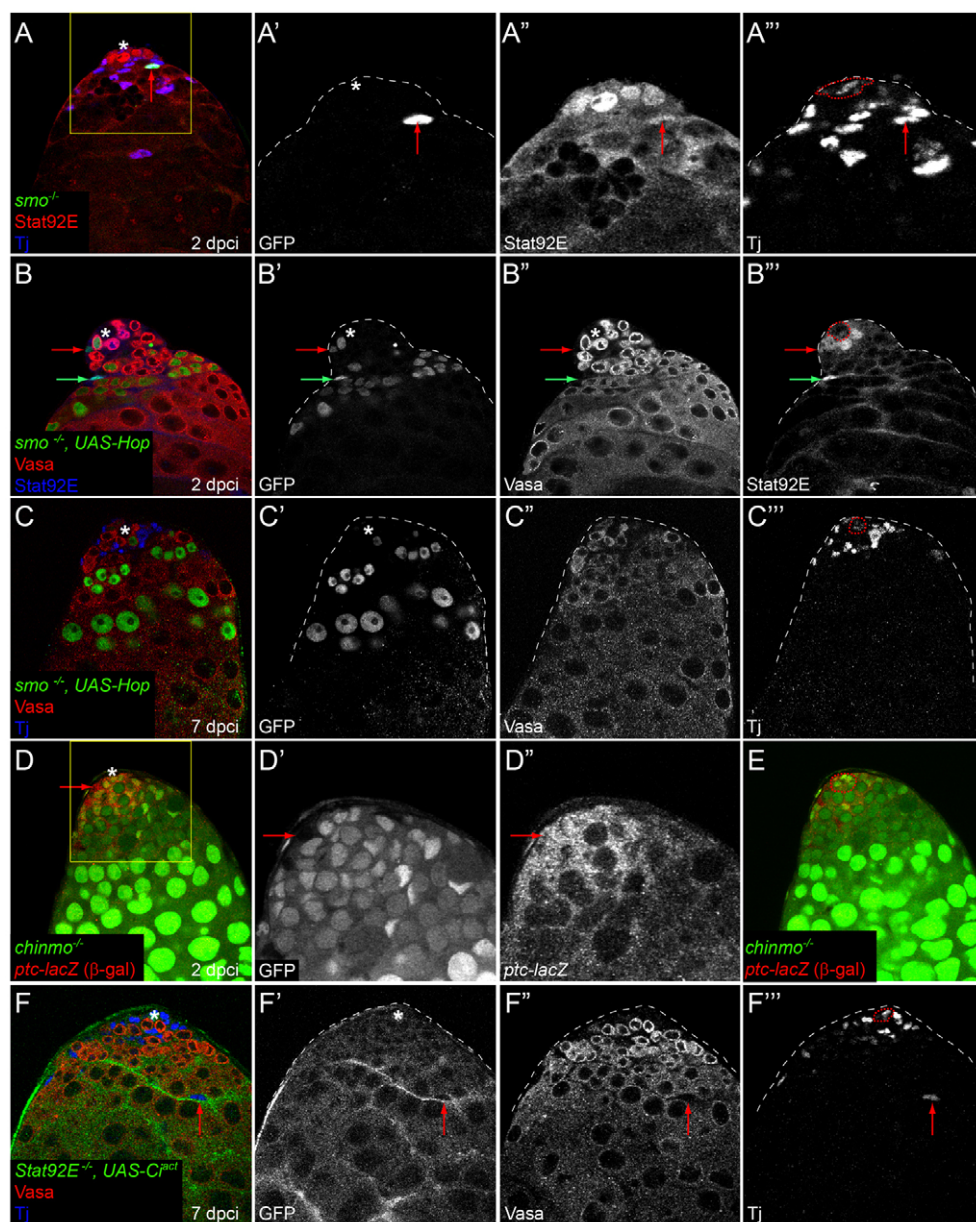


Fig. 5. Relationship between Hh and JAK/STAT signaling in CySC self-renewal. (A-A''') No change in Stat92E expression is detected in positively labeled *smd* mutant cells (A,A'''; arrow indicates *smd* mutant clone) at 2 dpci. Boxed area in A is enlarged in A'-A'''. GFP is green (single channel in A'), Stat92E is red (single channel in A'') and Tj is blue (single channel in A'''). (B-B''') Stat92E is stabilized in *smd* MARCM clones that express Hop. Red arrow indicates a *smd* mutant CySC and the green arrow indicates a differentiating cyst cell. GFP is green (single channel in B'), Vasa is red (single channel in B'') and Stat92E is blue (single channel in B'''). (C-C''') Activation of Stat92E by overexpression of Hop cannot rescue loss of *smd* MARCM mutants from the stem cell niche at 7 dpci, despite the stabilization of Stat92E protein in these clones (see B,B'''). GFP is green (single channel in C'), Vasa is red (single channel in C'') and Tj is blue (single channel in C'''). (D-D''') *ptc-lacZ* (red; single channel in D'') expression is unaltered in negatively marked *chinmo* mutants (arrow points to a *ptc-lacZ*-expressing mutant CySC in D-D'''). Boxed area in D is enlarged in single channels in D',D''. *chinmo* clones lack GFP (single channel in D'). (E) Adjacent optical z-section to D, showing where the hub (red dotted outline) is located. (F-F''') *Ci^{act}* expression cannot rescue *Stat92E* MARCM mutant CySCs at 7 dpci, but differentiated cyst cells can be recovered (red arrow indicates marked cyst cell; clones were labeled with membrane-targeted GFP). See also Table 1. The position of the hub is indicated with an asterisk or outlined in the Tj single channel.

We next tested whether readouts of Hh signaling were affected when the JAK/STAT pathway was disrupted. Since *ptc-lacZ* – the best-established readout of Hh signaling – and *Stat92E* reside on the same chromosome, we were unable to assess *ptc* levels in *Stat92E* clones. As a proxy, we examined *ptc-lacZ* in *chinmo* clones, as we previously showed that *chinmo* is key a functional effector of *Stat92E* in numerous cell types including CySCs (Flaherty et al., 2010). We found that *ptc-lacZ* was unchanged in *chinmo* mutant CySCs at 2 dpci, suggesting that Hh signaling is unaffected in CySCs lacking a key *Stat92E* functional effector (Fig. 5D-D'''). Consistent with this observation, we were unable to obtain significant rescue of either *Stat92E* or *chinmo* mutant CySCs by ectopic activation of Hh signaling through misexpression of *Ci^{act}* (Fig. 5F-F'''; Table 1). It should be noted that *Stat92E* and *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 *Stat92E* and *chinmo* mutant

cyst cells overexpressing *Ci^{act}* are readily recovered (Fig. 5F-F''', arrow; Table 1). This suggests that Hh activation can delay the loss of *Stat92E*- or *chinmo*-deficient CySCs, even though this is ultimately insufficient for their maintenance.

Furthermore, we were unable to detect a genetic interaction between *hh* and *Stat92E*. Animals that were transheterozygous for both mutations (*hh^{ts2}/Stat92E^{85C9}*) appeared to have a normal complement of stem cells at the testis niche (data not shown). In addition, CySC numbers in controls (*hh^{ts2}/+*) and transheterozygous animals were not statistically different (means of 36 and 34, respectively; $P < 0.6$). 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

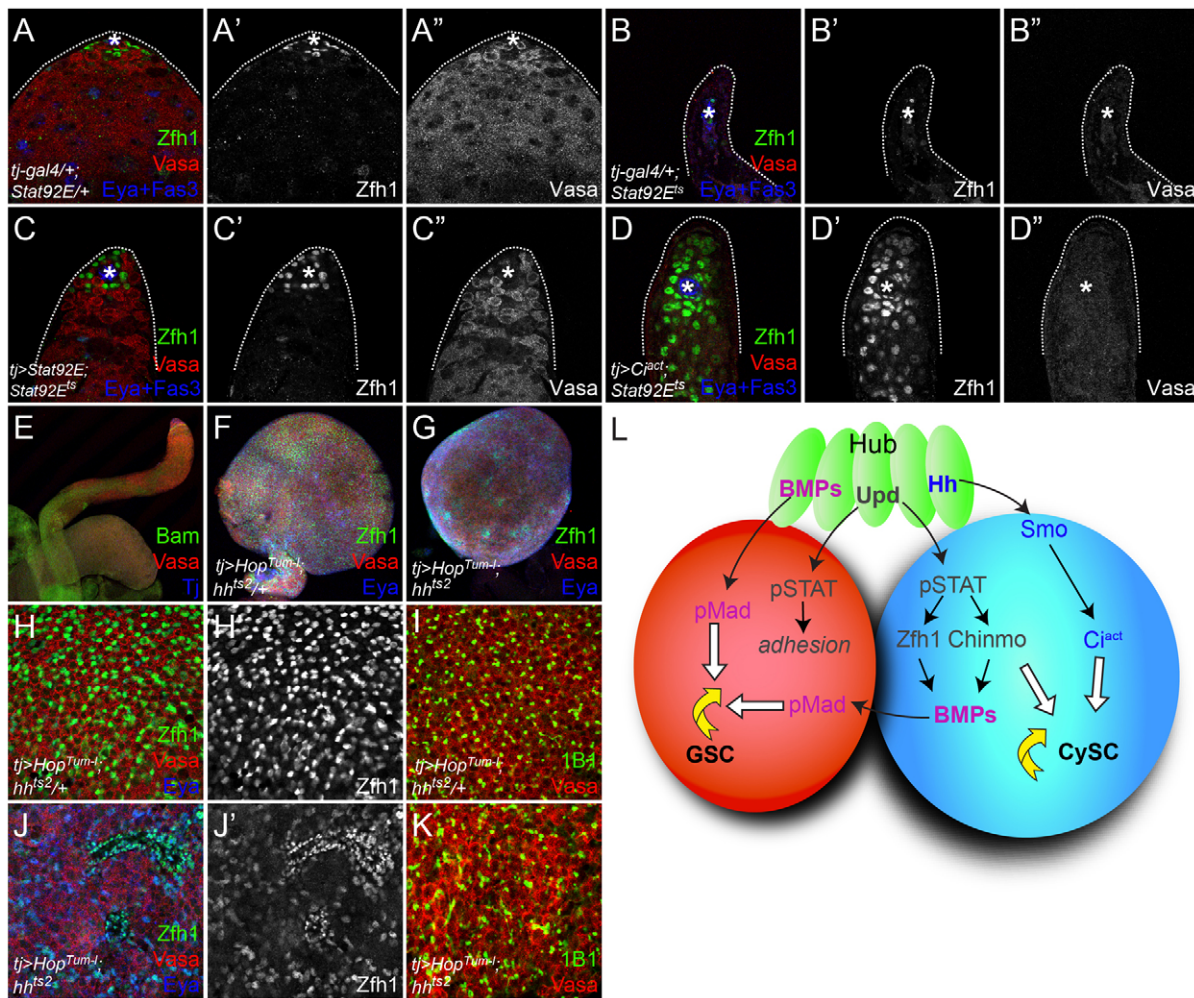


Fig. 6. Hh does not contribute to CySC niche function. (A-D'') *tj-gal4/+; Stat92E/+* control testes contain a normal complement of CySCs (determined by Zfh1 staining) and germ cells (as assessed by Vasa staining). CySCs and GSCs are lost in a *Stat92E* temperature-sensitive mutant (*Stat92E^{ts}*) (B). 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 *Ci^{act}* leads to rescue of Zfh1-expressing CySCs (D'), but not of GSCs (D''). 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 10× magnification. *Hop^{ts2}* overexpression in the somatic lineage causes tumors in both *hh* heterozygous (*tj>Hop^{ts2}; hh^{ts2}/+*; F) and *hh* mutant (*tj>Hop^{ts2}; hh^{ts2}/+*; G) 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 F,G. (H,H',J,J') The stem cell marker Zfh1 (green, single channel in H',J') is expressed at lower levels in *hh^{ts2}* (J,J') than in *hh^{ts2}/+* siblings (H,H'). Eya (blue) expression is largely absent in *hh^{ts2}/+* siblings (H) but is readily detectable in *hh^{ts2}* 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 *hh^{ts2}* mutant testes (K) are larger than in control siblings (I) and display large, branched fusomes (1B1, green) compared with the small, round fusomes in *hh^{ts2}/+* 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.

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, *Stat92E^{ts}* animals lose both CySCs and GSCs (Fig. 6A-B''). Both stem cell populations can be restored by resupplying wild-type *Stat92E* to the somatic lineage only (Fig. 6C-C''). We used *tj-gal4* to express *Ci^{act}* and activate Hh signaling in the somatic lineage of *Stat92E^{ts}* mutant testes (Fig. 6D-D''). Strikingly, we observed that CySCs are rescued in large numbers and fill up the testis tip, as assessed by Zfh1 expression (and the lack of Eya). These data suggest, in accordance with our

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 *Stat92E^{ts}* 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,

2008). Expressing Hop^{Tum-1}, a dominant-active form of Hop (Hanratty and Dearolf, 1993), in the somatic lineage is sufficient to cause overproliferation of stem-like cells, both cell-autonomously in CySCs and non-cell-autonomously in GSCs (Leatherman and Dinardo, 2008) (compare Fig. 6E with 6F). Markers of stemness of both populations are visible, including the presence of Zfh1 and absence of Eya for CySCs, and small, rounded germ cells with dot- or dumbbell-shaped fusomes for GSCs (Fig. 6H-I). Expressing Hop^{Tum-1} in the somatic lineage in an *hh^{ts2}* mutant animal also gave rise to expanded testes (compare Fig. 6G with 6E). However, the expanded population of somatic cells in these testes expressed lower levels of Zfh1 and higher levels of Eya than controls, suggesting that they have initiated differentiation (Fig. 6J). Similarly, germ cells in *hh^{ts2}* tumors are larger than in control tumors as they appear to be part of transit-amplifying cysts, and their fusomes display a branched morphology indicative of a more differentiated germ cell type (compare Fig. 6K with 6I). Thus, Hh 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, despite the drastic reduction in CySCs in *hh^{ts2}* 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, despite 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 conceivable 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 fulfil 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.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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