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# Hh signalling is essential for somatic stem cell maintenance in the *Drosophila* testis niche

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## SUMMARY

In the *Drosophila* testis, germline stem cells (GSCs) and somatic cyst stem cells (CySCs) are arranged around a group of postmitotic somatic cells, termed the hub, which produce a variety of growth factors contributing to the niche microenvironment that regulates both stem cell pools. Here we show that CySC but not GSC maintenance requires Hedgehog (Hh) signalling in addition to Jak/Stat pathway activation. CySC clones unable to transduce the Hh signal are lost by differentiation, whereas pathway overactivation leads to an increase in proliferation. However, unlike cells ectopically overexpressing Jak/Stat targets, the additional cells generated by excessive Hh signalling remain confined to the testis tip and retain the ability to differentiate. Interestingly, Hh signalling also controls somatic cell populations in the fly ovary and the mammalian testis. Our observations might therefore point towards a higher degree of organisational homology between the somatic components of gonads across the sexes and phyla than previously appreciated.

**KEY WORDS:** *Drosophila*, Hh, Cell competition, Somatic stem cell niche, Testis

## INTRODUCTION

Stem cells are specified and maintained by specific microenvironments called niches. The niches in the fly testis and ovary harbour germline stem cell (GSC) and somatic stem cell populations, the sizes and proliferation rates of which need to be coordinated. In the testis, the somatic cyst stem cells (CySCs) give rise to cyst cells (CyCs) that ensheath the differentiating germ cells. Both stem cell pools are arranged around a group of somatic cells termed the hub (Fig. 1A), which is the main source of niche signals for both lineages (Losick et al., 2011).

Germline and somatic stem cells divide asymmetrically. Daughter cells retaining contact with the hub keep their stem cell fate, whereas sibling cells that lose contact commit to differentiation. The differentiating germline daughter cells continue to divide, while the newly born CyCs stop dividing and exit the niche (Cheng et al., 2011; Hardy et al., 1979).

GSC stemness is determined via BMP pathway activation in both ovary and testis (Losick et al., 2011; Michel et al., 2011). Hub cells also secrete the cytokine-like ligand Upd, which mainly acts on the somatic cells (Flaherty et al., 2010; Issigonis et al., 2009; Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010; Singh et al., 2010). Despite differences in detail, the overall organisation of the testis GSC niche resembles that in the ovary (Decotto and Spradling, 2005; Lopez-Onieva et al., 2008).

In the ovary, Hedgehog (Hh) produced by the cap cells controls maintenance of the somatic follicle stem cells (FSCs) (Forbes et al., 1996; Margolis and Spradling, 1995; Zhang and Kalderon, 2001). Here we show that Hh is also an essential niche signal for somatic stem cells in the testis, implying a higher degree of organisational homology than previously appreciated.

## MATERIALS AND METHODS

### Fly strains

*smo*<sup>3</sup> (Chen and Struhl, 1998), *ptc*<sup>flw</sup> (Strutt et al., 2001), *hh*<sup>ts2</sup> (Ma et al., 1993), *hh::Gal4* (Tanimoto et al., 2000), *c587::Gal4* (Zhu and Xie, 2003), *nos::Gal4VP16* (Van Doren et al., 1998), *hh::lacZ=hh<sup>P30</sup>* (Lee et al., 1992), *ptc::lacZ=ptc<sup>10.8L</sup>* (Chen and Struhl, 1996), *UAS::Hh* (Burke et al., 1999), *UAS::RedStinger* (Bloomington 8545), *ci::GFP* (Quinones-Coello et al., 2007), and the MARCM strains (Lee and Luo, 1999) have been described previously. Clones were induced by three 1-hour heat shocks at 37°C at 1-hour intervals.

### Immunostaining and microscopy

Testes were imaged as described (Michel et al., 2011). Additionally, the following antibodies were used: anti-Hh (Panakova et al., 2005) 1:100, anti-Tj (Li et al., 2003) 1:1000, anti-β-Gal (Promega) 1:1000, anti-Zfh1 (Gilboa and Lehmann, 2006) 1:5000, anti-phospho-histone H3 (Ser10) (Upstate) 1:2000, and anti-BrdU488 (BD) 1:200. Anti-Ptc (1:100), anti-Smo (1:10) and anti-Eya (1:25) were obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

### Quantification and data analysis

Data were analysed using R (www.R-project.org). Significance was calculated using Fisher's exact test or ANOVA followed by Tukey's HSD or Kruskal-Wallis tests.

## RESULTS AND DISCUSSION

### Expression of Hh signalling components in the testis

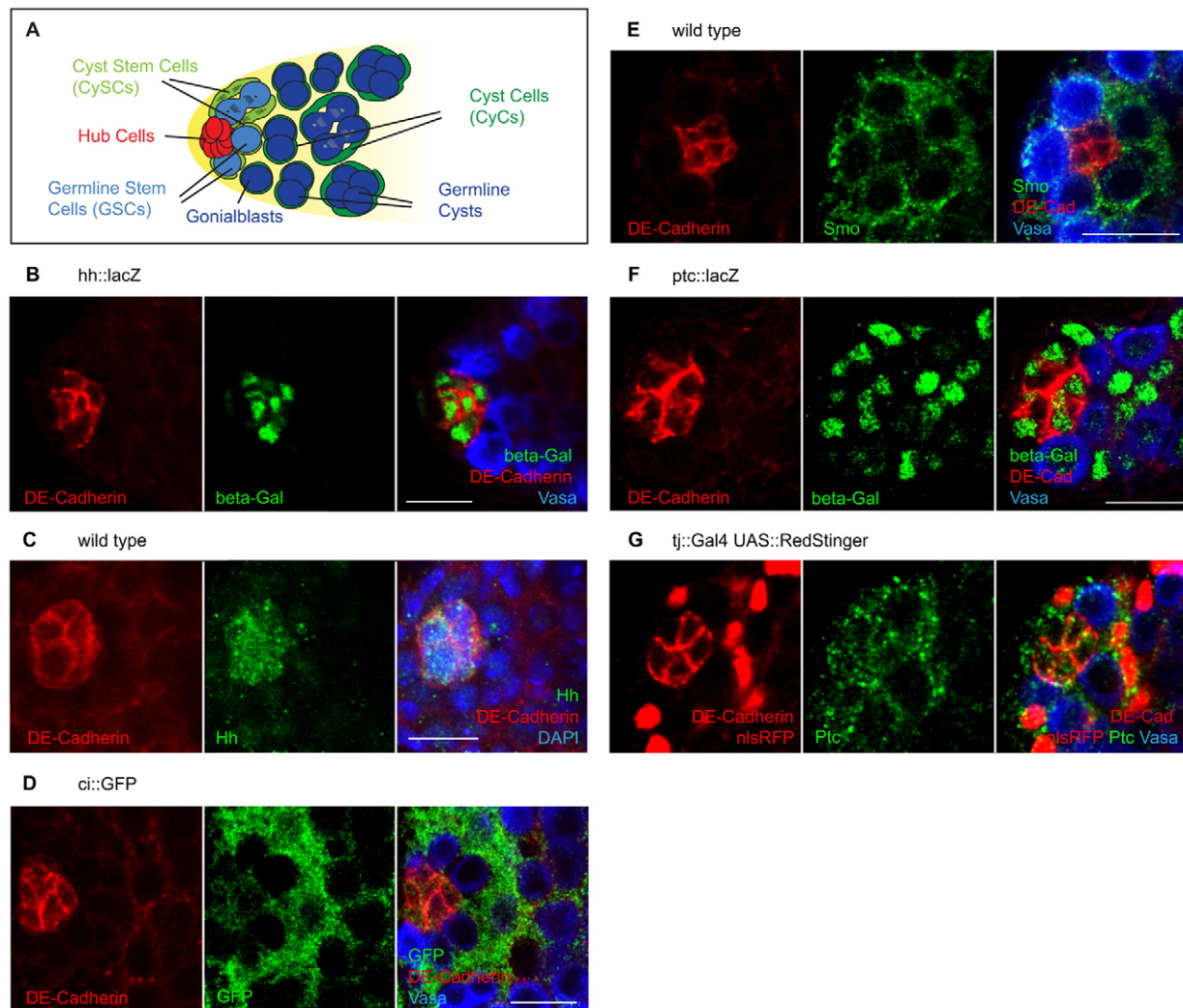
Hh signals via its receptor Patched (Ptc) and the GPCR-like signal transducer Smoothed (Smo) to activate the transcription factor Cubitus interruptus (Ci) (Ayers and Therond, 2010; Ingham et al., 2011). An *hh::lacZ* enhancer trap that reflects Hh expression in the larval testis and adult ovary (Forbes et al., 1996) and an *hh::Gal4* construct are both active in the adult hub (Fig. 1B; supplementary material Fig. S1A). However, the range of endogenous Hh protein appears to be limited (Fig. 1C).

Hh sensitivity is often controlled via Ci expression (Eaton and Kornberg, 1990; Sun and Deng, 2007). In the testis *Ci::GFP* (supplementary material Fig. S1B) is active in the CySCs (Dinardo et al., 2011) (Fig. 1D). CySCs also express Smo (Fig. 1E). However, we cannot exclude the possibility that these components are also present in the germline.

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**Fig. 1. The Hh pathway is present and active in the *Drosophila* somatic testis.** (A) Schematic representation of the testis niche. (B) An *hh::lacZ* transgene is active in the hub. (C) Endogenous Hh is produced in the hub. (D) Somatic cells express a *ci::GFP* reporter that is inactive in the germline. (E) Smo immunostaining labels somatic cells of the gonad surrounding the germline cells. (F) A *ptc::lacZ* transgene is expressed in the hub, somatic cyst stem cells (CySCs) and cyst cells (CyCs). (G) The punctate distribution of endogenous Ptc in somatic cells indicates ongoing Hh signal transduction. *tj::Gal4* marks somatic cells. (B-G) Hub is marked by DE-Cadherin and germline cells by Vasa. Scale bars: 10  $\mu$ m.

Since *ptc* is itself a transcriptional Hh target, Ptc expression (Fig. 1F) as well as its vesicular distribution in CySCs (Fig. 1G) suggest that Hh signalling is active in these cells (Chen and Struhl, 1996), consistent with the previously reported *ptc::Gal4* pattern (Schulz et al., 2002; Tazuke et al., 2002).

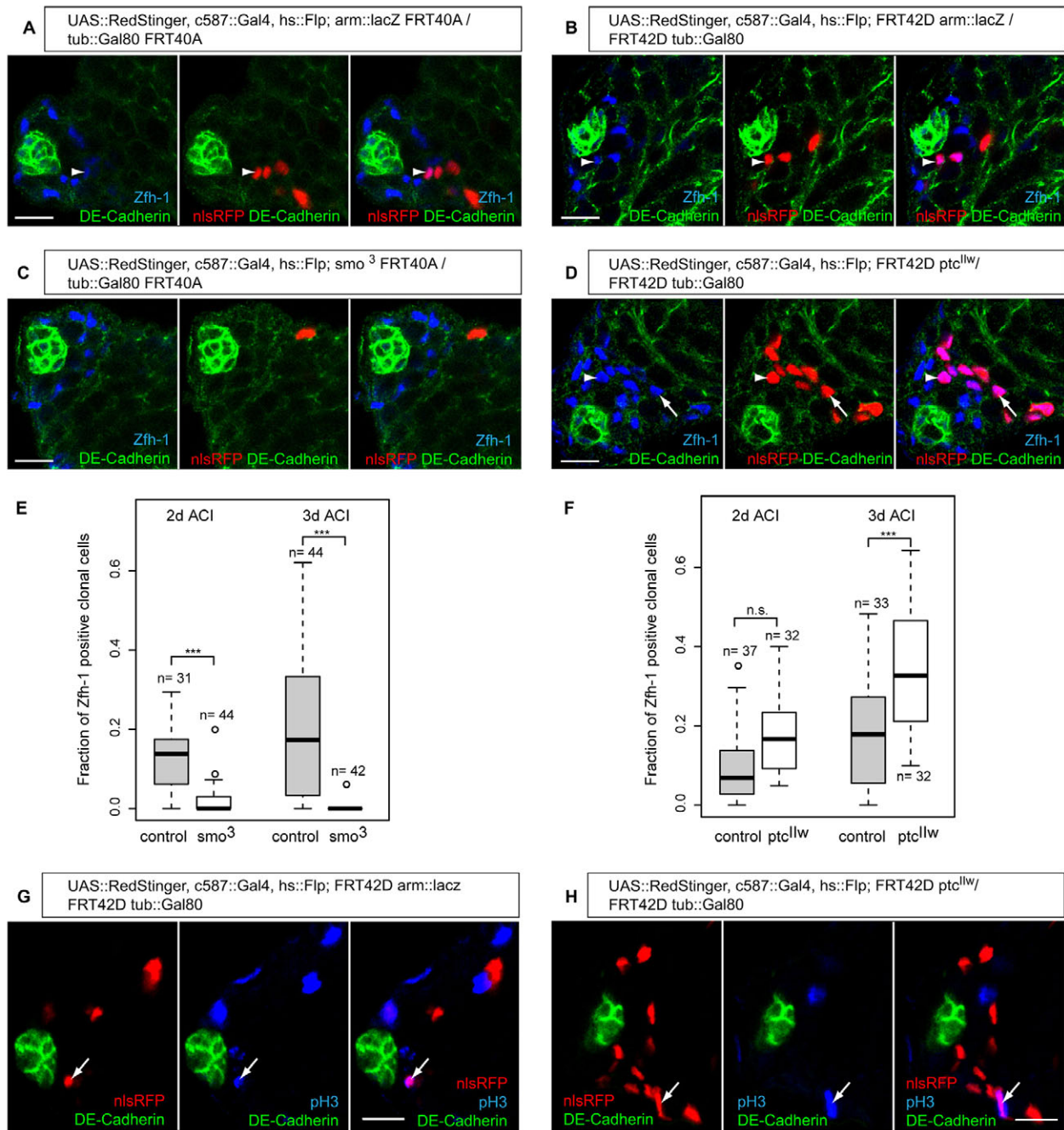
Thus, in the adult testis, Hh appears to signal from the hub to the adjacent somatic cells.

### ***smo* mutant CySCs are lost, whereas *ptc* clones overproliferate**

We generated cells homozygous for the amorphic alleles *smo*<sup>3</sup> or *ptc*<sup>low</sup> (Chen and Struhl, 1998; Strutt et al., 2001) using the MARCM system (Lee and Luo, 1999). Loss of Smo blocks Hh signalling, whereas *ptc* loss-of-function mutations activate the pathway (Ingham et al., 2011). Since, under normal conditions, CySCs are the only dividing somatic cells (Cheng et al., 2011; Hardy et al., 1979), all marked cells must be the product of a stem cell mitosis. CySCs were marked by the Jak/Stat target Zfh1, which is, however, also present in the second tier of somatic nuclei (Leatherman and Dinardo, 2008).

We generated control as well as *smo* and *ptc* mutant clones (Fig. 2A-D) with comparable efficiency (supplementary material Fig. S2). The fraction of testes with detectable clones increased between 1 and 2 days after clone induction (ACI). Since CySC cell-cycle length must be coupled to that of the GSCs, CySCs divide roughly once per day (Sheng and Matunis, 2011; Wallenfang et al., 2006). The lag between induction and detectability is presumably caused by the variable time before individual CySCs exit G2 phase and the time required for the Gal80 protein to decay after mitosis (Lee and Luo, 1999).

Compared with controls, the fraction of Zfh1-positive *smo*<sup>3</sup> cells was already reduced at 2 days ACI (1.8 $\pm$ 3.6% versus 12.8 $\pm$ 8.1% in controls,  $P < 0.001$ , ANOVA; Fig. 2E). By 3 days ACI, these cells had largely disappeared, although Zfh1-negative *smo*<sup>3</sup> cells were still present further out (Fig. 2C). Assuming equal induction rates for *smo*<sup>3</sup> and control CySCs, we fitted the ratios of Zfh1-positive *smo*<sup>3</sup> cells to control cells versus time with an exponential decay with a half-life of  $\sim$ 9 hours (supplementary material Fig. S3).

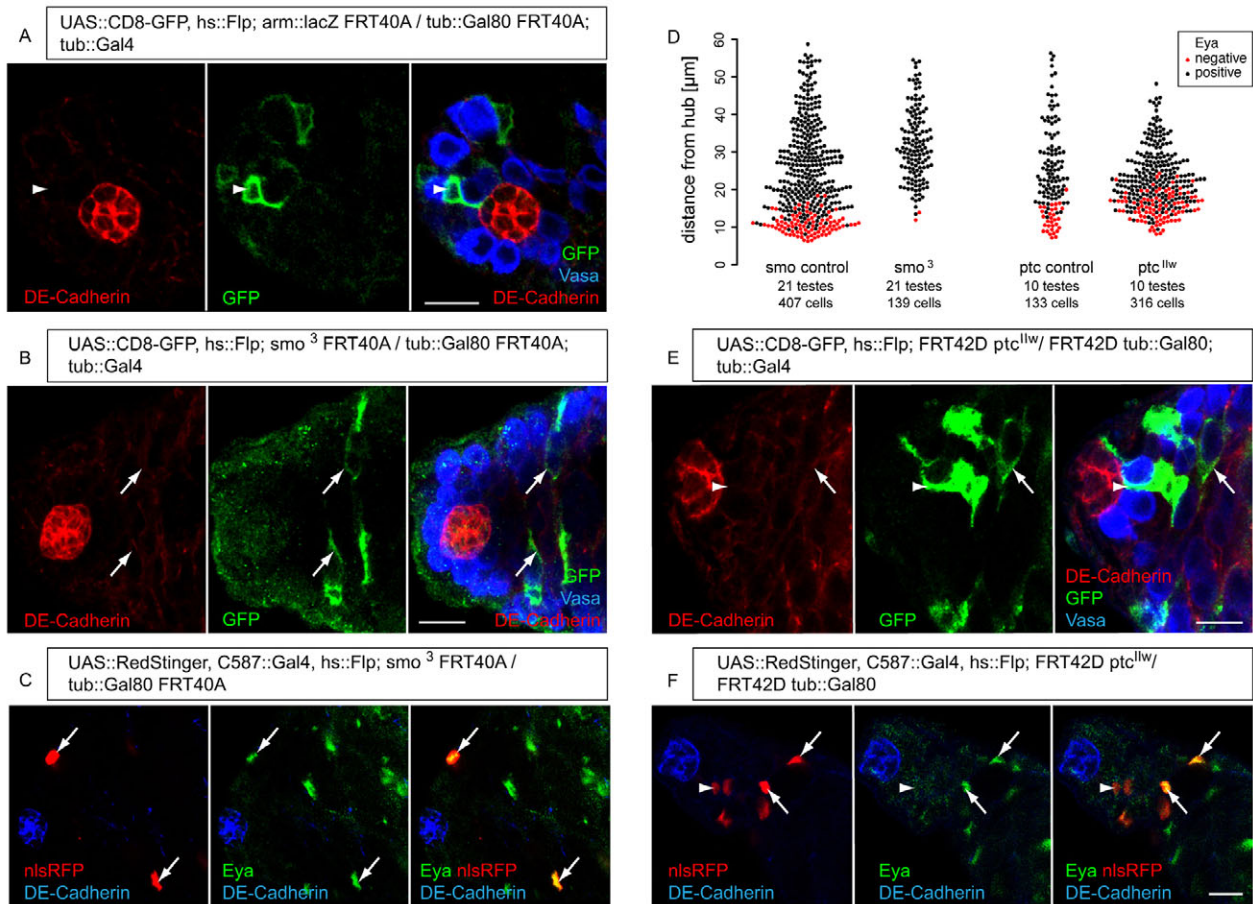


**Fig. 2. *smo*<sup>3</sup> CySCs are lost by differentiation, whereas *ptc*<sup>IIw</sup> clones overproliferate.** (A,B) Neutral control clones can retain CySC fate (arrowheads). (C) *smo*<sup>3</sup> homozygous cells are absent from the Zfh1-positive pool. (D) Homozygous *ptc*<sup>IIw</sup> cells retain Zfh1 staining distant from the hub (arrows) and cause an expansion of the Zfh1-positive pool. (E,F) Quantification of the fraction of homozygous mutant cells among the total Zfh1-positive population. Compared with controls, *smo*<sup>3</sup> clones (E) are diminished, whereas *ptc*<sup>IIw</sup> clones (F) expand over the same time window. Box-and-whisker plots indicate first and third quartile (box), median (horizontal line) and 1.5× interquartile range (whiskers). Circles indicate outliers. \*\*\**P*<0.001; n.s., not significant. *n*, number of testes. (G,H) Dividing homozygous control (G) and *ptc*<sup>IIw</sup> mutant (H) cells. Division of somatic cells is restricted to the CySCs adjacent to the hub (G, arrow), whereas pH3-positive *ptc*<sup>IIw</sup> mutant cells (H, arrow) are also found distant from the hub. MARCM clones are marked by nlsRFP (A-D,G,H), CySCs and their immediate progeny by Zfh1 (A-D). Scale bars: 10 μm.

By contrast, the fraction of *ptc*<sup>IIw</sup> cells among the Zfh1-positive pool increased relative to controls between 2 and 3 days ACI (2 days ACI, 17.5±9.7% versus 9.4±9.2% in controls, not significant; 3 days ACI, 34.1±15.4% versus 18.1±13.4%, *P*<0.001, ANOVA; Fig. 2F). Thus, by 3 days ACI, 67.6±18.6% of *ptc*<sup>IIw</sup> cells were Zfh1 positive in comparison with

35.1±20.7% of control clones (*P*<0.001, *t*-test; supplementary material Fig. S4A). This was not caused exclusively by the accumulation of additional *ptc* CySCs next to the hub. In controls, the average distance between the centre of Zfh1-positive cells and the hub did not change between 2 and 3 days ACI (12.3±3.1 μm versus 11.1±2.8 μm, *P*>0.05, ANOVA;





**Fig. 3. Both *smo* and *ptc* mutant cells can differentiate into CyCs.** (A) CD8-GFP-marked CySCs can be identified in control testes by cytoplasmic extensions contacting the hub (arrowhead). (B,C) *smo*<sup>3</sup> mutant somatic cells marked by CD8-GFP (B) or nlsRFP (C) differentiate into mature CyCs that ensheath germline cysts (B, arrows) and express Eya (C, arrows). (D) Quantification of number, position and Eya status of homozygous clonal cells. One point represents one cell. (E,F) Homozygous *ptc* mutant cells marked by CD8-GFP (E) or nlsRFP (F) can either retain CySC fate (E,F, arrowhead, which marks an Eya-negative *ptc* cell) or differentiate, ensheathing germline cysts (E, arrow) and expressing Eya (F, arrows). MARCM clones are marked by CD8-GFP (A,B,E) or nlsRFP (C,F), differentiating cells by Eya (C,F). Scale bars: 10  $\mu$ m.

supplementary material Fig. S4B). These distances are characteristic for CySCs, which are expected to lie between the first and second tier of germline cells found at  $8.3 \pm 1.0 \mu$ m and  $13.1 \pm 3.0 \mu$ m, respectively. By contrast, the average distance from the hub of Zfh1-positive *ptc*<sup>Iw</sup> mutant cells at 3 days ACI ( $20.6 \pm 6.3 \mu$ m) was increased relative to both mutant cells at 2 days ACI ( $16.5 \pm 5.8 \mu$ m,  $P < 0.001$ ) and controls at 3 days ACI ( $P < 0.001$ ).

We therefore examined proliferation of the *ptc* cells by phospho-histone H3 (pH3) staining. In neutral control clones,  $0.5 \pm 0.9\%$  (3 days ACI) to  $1.1 \pm 1.1\%$  (2 days ACI) of all homozygous cells were pH3 positive (Fig. 2G; supplementary material Fig. S5). As expected (Cheng et al., 2011; Hardy et al., 1979), all somatic mitoses occurred adjacent to the hub. By contrast, at both time points,  $\sim 2.5\%$  of all *ptc* mutant cells (2 days ACI,  $2.5 \pm 1.2\%$ ; 3 days ACI,  $2.5 \pm 0.5\%$ ) were pH3 positive (*ptc* versus control at 3 days ACI,  $P < 0.05$ , *t*-test; supplementary material Fig. S5). About one-third of these excess mitoses (27/71) occurred away from the hub (Fig. 2H). These results implicate Hh in CySC proliferation, resembling the situation in the ovary, where Hh signalling drives FSC proliferation (Forbes et al., 1996; Hartman et al., 2010; Zhang and Kalderon, 2001).

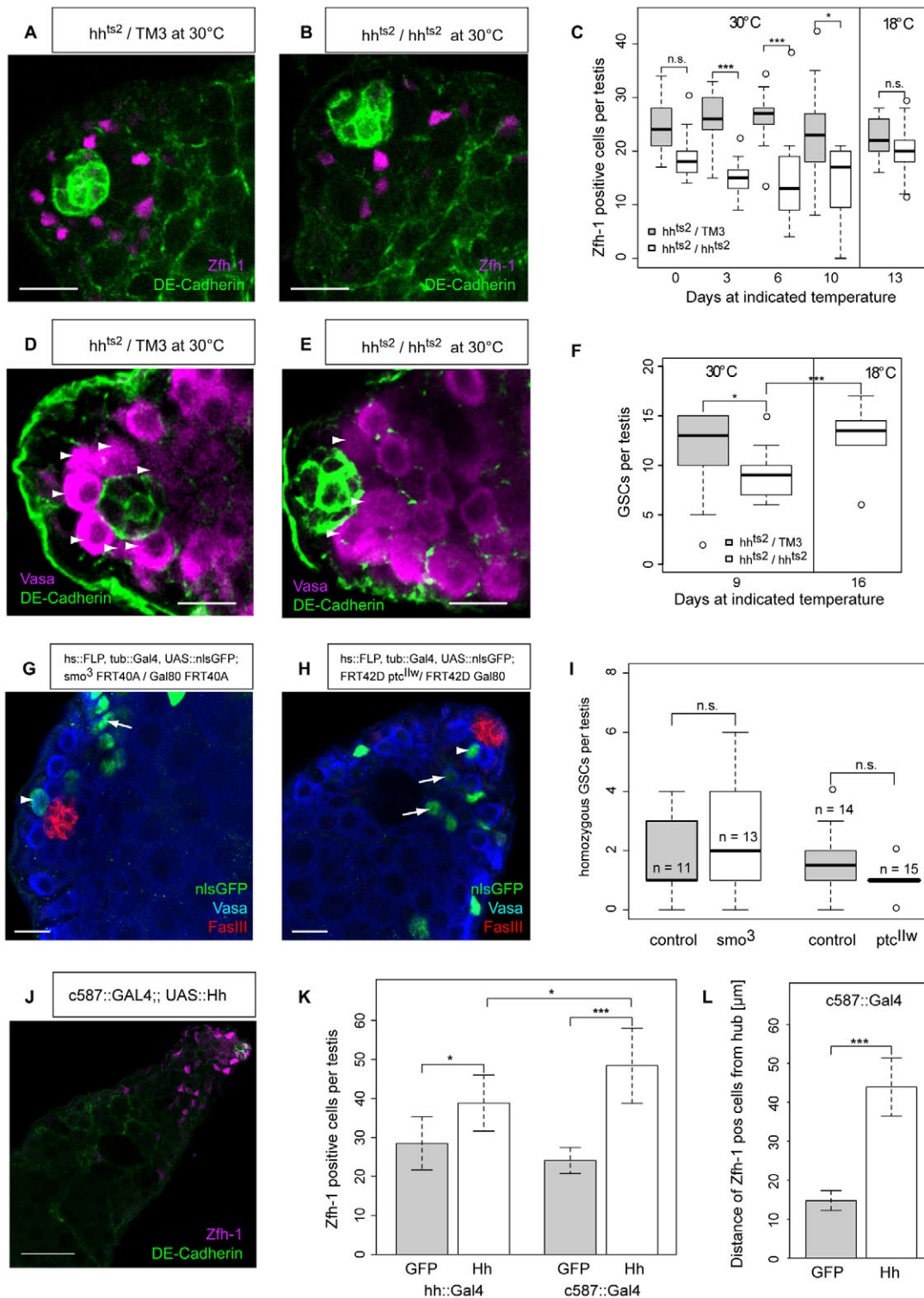
### *smo* mutant CySCs are lost by premature differentiation

Control CySCs marked by CD8-GFP could be identified by their processes extending towards the hub (Fig. 3A). *smo*<sup>3</sup> mutant cells were missing from the hub at 3 days ACI (Fig. 3B), but could be seen ensheathing germline cysts (Fig. 3B), suggesting that they had differentiated into functional CyCs.

Consistently, by 3 days ACI, almost all *smo*<sup>3</sup> cells expressed the CySC differentiation marker Eyes absent (Eya) (Fabrizio et al., 2003) (Fig. 3C), whereas control clones retained a sizeable Eya-negative population (*smo* clones, 1.4% Eya negative,  $n = 139$  cells; controls, 22.1% Eya negative,  $n = 417$  cells;  $P = 5.5 \times 10^{-11}$ , Fisher's exact test; Fig. 3D). The average number of homozygous cells per testis was about threefold higher in controls than in *smo*<sup>3</sup> clones ( $19.4 \pm 8.7$  versus  $6.6 \pm 3.9$ ,  $P < 0.001$ , *t*-test).

### *ptc* mutant CySCs overproliferate but can still differentiate

Homozygous *ptc*<sup>Iw</sup> CySCs were able to retain contact with the hub. However, other mutant cells ensheathed germline cysts (Fig. 3E) or expressed Eya (Fig. 3F). Both the number of homozygous cells per testis ( $31.6 \pm 13.2$  versus  $13.3 \pm 9.7$ ,  $P < 0.01$ ) and the size of the



**Fig. 4. Global loss of Hh activity affects both somatic and germline stem cells in the testis.** (A-C) Compared with controls (A), the number of Zfh1-positive cells is reduced in homozygous *hh<sup>ts2</sup>* testes (B) following 3 days at 30°C. (C) Quantification of the number of Zfh1-positive cells for control and homozygous *hh<sup>ts2</sup>* testes at the restrictive (30°C) and permissive (18°C) temperatures. Note the significant reduction from 3 days onwards at 30°C. (D-F) Relative to heterozygous controls (D), the number of germline stem cells (GSCs; arrowheads) around the hub is reduced in homozygous *hh<sup>ts2</sup>* testes (E) following 3 days at 30°C. (F) Quantification of GSC number in relation to Hh inactivation. (G-I) Homozygous *smo* (G) and *ptc* (H) germline cells (marked by nlsGFP, green) can both retain GSC fate (arrowheads) or differentiate into germline cysts (arrows). (I) Quantification of *smo* and *ptc* mutant versus control GSCs shows no significant differences. Hub is marked by FasIII. (J-L) Hh overexpression increases the Zfh1-positive pool. (K,L) Quantification of the number (K) and distance to the hub (L) of Zfh1-positive cells in testes overexpressing GFP or Hh, either at the normal source (*hh::Gal4*) or in all somatic cells (*c587::Gal4*). Box-and-whisker plots indicate first and third quartile (box), median (horizontal line) and 1.5× interquartile range (whiskers). Circles indicate outliers. \**P*<0.05; \*\*\**P*<0.001; n.s., not significant. *n*, number of testes. Scale bars: 10 μm in A,B,D,E,G,H; 25 μm in J.



Eya-negative fraction ( $28.1 \pm 12.5\%$  versus  $13.8 \pm 16.8\%$ ,  $P < 0.05$ ) were increased for *ptc* mutant cells relative to controls (Fig. 3D). Eya-negative *ptc* mutant cells were also found further away from the hub than corresponding controls (Fig. 3D), mirroring the expansion of the corresponding Zfh1-positive population (Fig. 2D,F). Thus, activation of the Hh pathway promotes CySC fate but does not prevent differentiation.

### Global loss of Hh activity affects both somatic and germline stem cells

To address whether systemic loss of Hh affected niche function, we turned to the temperature-sensitive allele *hh<sup>ts2</sup>* (Fig. 4A,B), which behaves similar to the wild-type allele at 18°C, but as an amorphous *hh* mutant at 30°C (Ma et al., 1993). The average number of Zfh1-positive cells did not differ between testes from homozygous *hh<sup>ts2</sup>* males raised at 18°C relative to their heterozygous siblings ( $P > 0.05$ , ANOVA) or males held at permissive conditions for 13 days (Fig. 4C). By contrast, CySC number was reduced in *hh<sup>ts2</sup>* males relative to heterozygous controls after 3 (Fig. 4A,B), 6 or 10 days at 30°C ( $P < 0.001$  for 3 and 6 days,  $P < 0.05$  at 10 days, ANOVA; Fig. 4C). However, CySCs did not completely disappear from the hub when Hh signalling was equally affected in all cells, and there was no significant difference in BrdU uptake between Zfh1-positive cells from *hh<sup>ts2</sup>* homozygous versus heterozygous testes (*hh<sup>ts2</sup>*,  $45 \pm 22\%$ ,  $n = 7$ ; control,  $49 \pm 14\%$ ,  $n = 9$ ;  $P > 0.05$ , *t*-test; supplementary material Fig. S6A,B).

We therefore propose that competition between the somatic stem cells (Rhiner et al., 2009) is largely, although not exclusively, responsible for the loss of homozygous mutant *smo* CySCs and the proliferative advantage of individual *ptc* mutant CySCs in an otherwise heterozygous testis.

### Hh signalling is dispensable for GSC maintenance and germline differentiation

GSC number was reduced in *hh<sup>ts2</sup>* testes following incubation at 30°C relative to heterozygous controls kept at the same restrictive conditions and to homozygous *hh<sup>ts2</sup>* males left at the permissive temperature of 18°C (Fig. 4D-F). Hub maintenance, however, was not affected in *hh<sup>ts2</sup>* males (supplementary material Fig. S7).

We induced *smo* and *ptc* germline clones marked by nlsGFP (Fig. 4G,H). Importantly, *smo<sup>3</sup>* and *ptc<sup>flw</sup>* mutant GSCs were maintained at the same level as control clones (Kruskal-Wallis test,  $P > 0.05$  for all comparisons; Fig. 4I). Thus, Hh signalling is not required in the germline, and the observed reduction in GSC number caused by systemic inactivation of Hh is presumably mediated via the somatic cells.

### Hh is a CySC niche signal

In summary, Hh provides a niche signal for the maintenance and proliferation of the somatic stem cells of the testis. CySCs that are unable to transduce the Hh signal are lost through differentiation, whereas pathway overactivation causes overproliferation. Hh signalling thereby resembles Jak/Stat signalling via Upd. Partial redundancy between these pathways might explain why neither depletion of Stat activity nor loss of Hh signalling causes complete CySC loss (Leatherman and Dinardo, 2010).

We have shown here that loss of Hh signalling in *smo* mutant cells blocks expression of the Jak/Stat target Zfh1 (Leatherman and Dinardo, 2008), whereas mutation of *ptc* expands the Zfh1-positive pool. Overexpression of Zfh1 or another Jak/Stat target, Chinmo, is sufficient to induce CySC-like behaviour in somatic cells irrespective of their distance from the hub (Flaherty et al., 2010);

Leatherman and Dinardo, 2010). By contrast, Hh overexpression in the hub using the *hh::Gal4* driver only caused a moderate increase in the number of Zfh1-positive cells relative to a GFP control. Ectopic Hh overexpression in somatic cells under *c587::Gal4* control increased this number further (Fig. 4J,K). However, unlike in somatic cells with constitutively active Jak/Stat signalling (Leatherman and Dinardo, 2008), the additional Zfh1-positive cells remained largely confined to the testis tip, although their average range was increased threefold (Fig. 4J,L). Thus, Hh appears to promote stem cell proliferation, in part, also independently of competition.

It is tempting to speculate that further stem cell expansion is limited by Upd range. Consistently, cells with an ectopically activated Jak/Stat pathway remain undifferentiated (Leatherman and Dinardo, 2008), whereas *ptc* cells can still differentiate. Future experiments will need to formally address the epistasis between these pathways. However, our observations already show that Hh signalling influences expression of the bona fide Upd target gene *zfh1*, and therefore presumably acts upstream, or in parallel to, Upd in maintaining CySC fate.

In addition, the reduction in GSC number following somatic stem cell loss implies cross-regulation between the different stem cell populations that presumably involves additional signalling cascades, such as the EGF pathway (Gilboa and Lehmann, 2006; Sarkar et al., 2007).

### How conserved is somatic niche organisation?

In recent years, research has focused on the differences between the male and female gonadal niches (Fuller and Spradling, 2007). We would instead emphasize the similarities: in both cases, Jak/Stat signalling is responsible for the maintenance and activity of cells that contribute to the GSC niche, and Hh signalling promotes the proliferation of stem cells that provide somatic cells ensheathing germline cysts. In the testis, both functions are fulfilled by the CySCs (Leatherman and Dinardo, 2010), whereas in the ovary the former task is fulfilled by the postmitotic escort stem cells/escort cells (Lopez-Onieva et al., 2008; Morris and Spradling, 2011) and the latter by the FSCs (Margolis and Spradling, 1995). Finally, male desert hedgehog (*Dhh*) knockout mice are sterile (Bitgood et al., 1996; Clark et al., 2000). *Dhh* is expressed in the Sertoli cells and is thought to primarily act on the somatic Leydig cells (Yao et al., 2002). However, the signalling microenvironment of the vertebrate spermatogonial niche is, as yet, not fully defined. Future experiments will need to clarify whether these similarities reflect convergence or an ancestral Hh function in the metazoan gonad.

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### 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.075242/-DC1>

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