Coordinated niche-associated signals promote germline homeostasis in the *Drosophila* ovary

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Stem cell niches provide localized signaling molecules to promote stem cell fate and to suppress differentiation. The *Drosophila melanogaster* ovarian niche is established by several types of stromal cells, including terminal filament cells, cap cells, and escort cells (ECs). Here, we show that, in addition to its well-known function as a niche factor expressed in cap cells, the *Drosophila* transforming growth factor β molecule Decapentaplegic (Dpp) is expressed at a low level in ECs to maintain a pool of partially differentiated germline cells that may dedifferentiate to replenish germline stem cells upon their depletion under normal and stress conditions. Our study further reveals that the Dpp level in ECs is modulated by Hedgehog (Hh) ligands, which originate from both cap cells and ECs. We also demonstrate that Hh signaling exerts its function by suppressing Janus kinase/signal transducer activity, which promotes Dpp expression in ECs. Collectively, our data suggest a complex interplay of niche-associated signals that controls the development of a stem cell lineage.

Introduction

Stem cells are characterized by their ability to self-renew and to give rise to differentiated progeny in line with the developmental programs of an organism. The proper development of a stem cell lineage is essential for maintaining tissue homeostasis, the disruption of which would lead to premature ageing or stem cell hyperplasia. The self-renewal of stem cells is sustained by specific factors produced from a local microenvironment in which stem cells reside, known as the stem cell niche (Lin, 2002; Fuchs et al., 2004; Li and Xie, 2005; Fuller and Spradling, 2007).

The *Drosophila melanogaster* ovary is an excellent model system for understanding the function of stem cell niches (Fig. 1 A). The germline stem cell (GSC) niche is located at the anterior tip of the germarium and formed by three different types of stromal cells, terminal filament (TF) cells, cap cells and escort cells (ECs, also known as inner germarium sheath cells or IGS; Xie and Spradling, 2000). In addition to providing positional information by anchoring GSCs via DE-Cadherin–mediated cell adhesion, cap cells are the major source of extrinsic factors/signals that promote GSC maintenance (Losick et al., 2011; Chen et al., 2013). ECs, which contact GSCs and their differentiating daughters, are proposed to support germ cell differentiation. However, the underlying mechanisms are yet to be fully understood. The primary niche-associated factor that maintains GSCs is Decapentaplegic (Dpp), a *Drosophila* homologue of vertebrate bone morphogenetic protein (BMP). Dpp is produced by cap cells and functions over a short (one cell diameter) distance to promote GSC self-renewal by suppressing the expression of the differentiation-promoting factor bags of marbles (Bam; Xie and Spradling, 1998, 2000; Kai and Spradling, 2003; Chen and McKearin, 2003a; Song et al., 2004). Although *dpp* transcripts are also detected in ECs, the functional relevance of Dpp in ECs is not known (Xie and Spradling, 2000; Song et al., 2004).

In *Drosophila*, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is initiated upon binding of ligands (Unpaired 1, 2, or 3) to its cognate receptor Domeless (Dome), which activates JAK (Hopscotch [Hop]) to phosphorylate the transcriptional effector Stat92e that subsequently translocates into the nucleus to regulate the transcription of its target genes. This pathway has been implicated in various stem cell systems, including hematopoietic stem cells and testicular GSCs (Gregory et al., 2008). In the germarium, JAK/STAT signaling is also essential for the maintenance of GSCs,

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Abbreviations used in this paper: AHS, heat-shock treatment; Bam, bag of marbles; CB, cystoblast; Ci, Cubitus interruptus; Dad, Daughters against dpp; Dad-lacZ, transcriptional reporter for Daughters against Dpp; Dome, domeless; Dpp, decapentaplegic; DSHB, Developmental Studies Hybridoma Band; EC, escort cell; FRT, Flippase recognition target; GSC, germline stem cell; Hh, Hedgehog; Hop, hopscotch; JAK/STAT, Janus kinase/signal transducer and activator of transcription; pMad, phosphorylated Mothers against Dpp; Ptc, Patched; Smo, Smoothened; Stat92e, signal-transducer and activator of transcription protein at 92E; TF, terminal filament; Upd, unpaired; WT, wild type.

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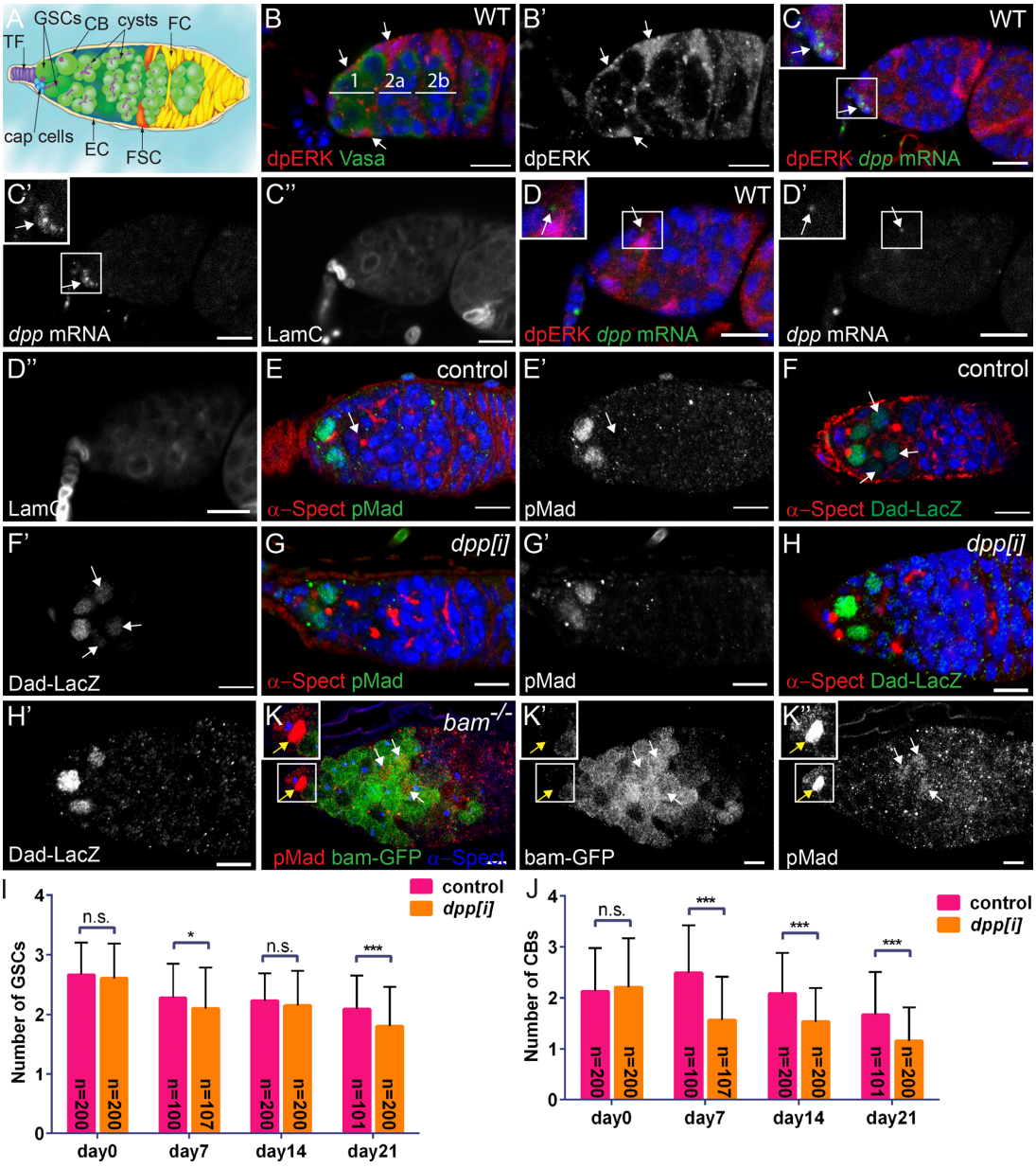

Figure 1. **Low levels of EC-expressed Dpp maintain germline homeostasis.** (A) Schematic of a *Drosophila* germarium. FSC, follicle stem cell. (B–H) TO-PRO-3 in blue. (B) A WT germarium showing diphosphorylated extracellular signal-regulated kinase in ECs (white arrows). Regions 1, 2a, and 2b are indicated by white lines. Vasa (green) is a germline cell marker. (C and D) Two different sections of a WT germarium showing strong *dpp* transcripts (green) in cap cells (C, arrow) and ECs (D, arrow). The inset shows magnified view of the boxed areas. (E) A *c587ts/+* germarium showing two GSCs expressing pMad; the indicated CB (marked by arrow) does not express pMad. (F) A *c587ts/+; dad-lacZ/+* germarium showing 5 Dad-lacZ–positive cells. Note that CBs (white arrows) express Dad-lacZ at a lower level. (G) A *c587ts/+; dpp*[BL25782]*/+* germarium showing pMad expression in GSCs. (H) A *c587ts/+; dpp*[BL25782]*/+* germarium showing 3 Dad-lacZ–positive cells (arrows). (I) Quantitation of GSC number in *c587ts/+* and *c587ts/+; dpp*[BL25782]*/+* germaria at different time points. (J) Quantitation of CB number in *c587ts/+* and *c587ts/+; dpp*[BL25782]*/+* germaria at different time points. (K) In a *bam* mutant germarium, some spectrosome-containing cells away from the niche (white arrows) expressing low levels of pMad (compared with strong pMad expression in GSCs, yellow arrow) and bam-GFP. The insert is the magnified view of the box area to show a GSC. *, $P < 0.05$; ***, $P < 0.001$. n.s., no significant difference. "n" indicates a single representative experiment out of three repeats. Error bars indicate SD. Bars, 10 µm.

highlighting the conserved role of this pathway in stem cell biology. However, unlike Dpp, which acts directly on GSCs, JAK/STAT signaling influences GSC maintenance indirectly via its function on cap cells and ECs. Whereas the removal of JAK/STAT activity from cap cells results in defects in Dpp production and consequent GSC loss, disruption of Stat92e activity in ECs leads to morphologic defects of the germarium and premature loss of GSCs (Decotto and Spradling, 2005; López-Onieva et al., 2008; Wang et al., 2008).

In addition to the JAK/STAT pathway, Hedgehog (Hh) signaling regulates the behavior of stem cells such as neuroblasts, testicular GSCs and ovarian follicle stem cells (Forbes et al., 1996; King et al., 2001; Zhang and Kalderon, 2001; Mandal et al., 2007; Chai et al., 2013; Sahai-Hernandez and Nystul, 2013). In the absence of Hh binding, Patched (Ptc, the Hh receptor) inhibits the activity of Smoothened (Smo, another transmembrane protein required for downstream signal activation), resulting in the proteolysis of Cubitus interruptus (Ci, the transcriptional

effector of Hh signaling) from the full-length activator form (Ci155) into a partially cleaved repressor form (Ci75). In contrast, the binding of Hh to Ptc leads to the activation of Smo, which stabilizes the full-length (active) form of Ci (Ci155); this form regulates downstream target gene expression.

In this study, we show that the expression level of Dpp in ECs is tightly regulated and that this optimum (low) level of Dpp plays an essential role in germline homeostasis by sustaining a pool of partially differentiated germline cells, which may dedifferentiate into GSCs. Specifically, the tight regulation of Dpp expression in ECs is controlled by two niche-associated signals. JAK/STAT signaling promotes Dpp expression, whereas Hh signaling suppresses Dpp expression by antagonizing JAK/STAT signaling.

Results

Dpp is expressed in ECs at a low level

Early studies reported that *dpp* is transcribed in both cap cells and ECs (Xie and Spradling, 2000; Song et al., 2004). Consistent with this finding, we observed the expression of *dpp* transcripts in some ECs, in addition to cap cells (Wang et al., 2008; Liu et al., 2010). To better understand the functional relevance of this EC-expressed Dpp, we examined the mRNA expression pattern of *dpp* in detail by fluorescence in situ hybridization. In the germarium, cap cells are located at the base of TFs and directly contact GSCs, whereas ECs, with triangular morphology and strong expression of di-phosphorylated extracellular signal-regulated kinase (Fig. 1 B; Decotto and Spradling, 2005), line the surface of region 1 and 2a. Consistent with previous reports, *dpp* transcripts were clearly detected in cap cells of wild-type (WT) germaria (Fig. 1 C and Fig. S1, A and B). However, 27% of those germaria ($n = 48$) exhibited weak yet unequivocal signals in some ECs (Fig. 1 D). Notably, these ECs with detectable *dpp* transcripts occupied varying positions in the examined ovaries (unpublished data), possibly reflecting the stochastic nature of *dpp* expression, both spatially and temporally.

EC-expressed Dpp maintains a population of partially differentiated germline cells

To further clarify this possibility, we investigated downstream signal activation in the germline cells. GSCs undergo self-renewal division to generate a GSC daughter and a cystoblast (CB) daughter, which is displaced outside the niche and undergoes differentiation. Both GSCs and CBs possess a spherical spectrosome that is enriched in cytoskeleton proteins, such as α–Spectrin. GSCs harbor a cap cell–contacting spectrosome, whereas CBs contain a non–cap cell–contacting spectrosome. In WT germarium, pMad (phosphorylated Mothers against Dpp, which is a signaling transducer and high-threshold signaling reporter) was expressed only in GSCs (Fig. 1 E), whereas Dad-lacZ (a transcriptional reporter for Daughters against Dpp [Dad], a target that responses to low levels of signal activation) was detected in both GSCs and CBs (Fig. 1 F).

To address whether the EC-expressed Dpp plays a role in germline development, we used dsRNA- or shRNA-mediated knockdown to compromise its activity in ECs. We first identified Gal4 drivers that are expressed in ECs but not in cap cells. As previously reported (Song et al., 2004; Eliazer et al., 2011; Jin et al., 2013), we found that *c587* drives reporter expression in ECs and early follicle cells, but not in cap cells of the adult ovary (see Materials and methods). Additionally, we identified *GMR25A11*, a driver that drives reporter expression specifically in ECs in regions 1 and 2a but not in other cell types in the germarium (unpublished data). Thus, we used these drivers in combination with a version of temperature-sensitive Gal80, which suppresses Gal4 activity under permissive temperatures but not under restrictive temperatures, to knock down Dpp expression in adult ECs and to examine the expression of downstream signaling reporters (Fig. S2, A–N). In WT, there were 2.28 ± 0.06 pMad-positive GSCs ($n = 100$ germaria) and 5.29 ± 0.13 Dad-lacZ–positive spectrosome-containing cells ($n = 142$ germaria). Although germaria with compromised Dpp activity in ECs (referred to as *dpp[i]* germarium; hereafter, the *[i]* suffix refers to as knocking down the gene of interest using *c587* or *GMR25A11* driver) contained a similar number of pMad-positive GSCs (2.1 ± 0.08; $n = 107$ for *dpp[i]* germaria; $P = 0.04$; Fig. 1 G), these germaria exhibited fewer Dad-lacZ–positive spectrosome-containing cells (4.02 ± 0.09; $n = 128$; $P < 0.001$; Fig. 1 H), suggesting that the EC-expressed Dpp has a positive influence on the expression of the low-threshold marker Dad-lacZ in the germline.

In line with the reduction of Dad-lacZ expression, we found that *dpp[i]* germaria contained fewer spectrosome-containing cells compared with control germaria (Fig. S1, C and D). Time-course analyses showed that GSCs in *dpp[i]* germaria could self-renew and were maintained in the first few weeks, similar to their WT counterparts; however, the germaria exhibited a weak GSC loss phenotype later (Fig. 1 I and Fig. S1, E and G), suggesting a role of EC-expressed Dpp in GSC maintenance. Interestingly, we found that *dpp[i]* germaria had fewer CBs from day 7 onward (Fig. 1 J and Fig. S1 F), preceding the GSC loss. Given that *dpp[i]* GSCs proliferated at a rate similar to that of control GSCs at this time point (Fig. S1, H and I; day 7) and given that CBs in *dpp[i]* germaria exhibited a higher propensity to undergo cell cycle progression (Fig. S1, J and K), these results suggest that these CBs differentiate faster. Collectively, our data suggest that low levels of EC-expressed Dpp function to stably maintain a population of partially differentiated germline cells, including CBs.

Dpp signaling activation in GSCs is known to suppress Bam expression, which is required for the differentiation of GSC daughters into CBs. In support of this, *bam*-deficient germline cells are arrested in differentiation and contain a spectrosome. P*bam-gfp* (a *bam* transcription reporter) is silenced in *bam* mutant GSCs but de-repressed in those arrested spectrosome-containing cells outside the niche (Chen and McKearin, 2003b). Examination of Dpp signaling revealed that Dad-lacZ was detected in some spectrosome-containing cells outside the niche in 90.2% of *bam* mutant germaria ($n = 102$; Fig. S1 L), indicating signal activation. This result is consistent with previous reports suggesting that Bam may play a role (redundant with dSmurf) in down-regulating Dpp signaling during germline cell differentiation (Casanueva and Ferguson, 2004; Lu et al., 2012). Because Dad-lacZ reporter is an enhancer trap line that disrupts the function of the Dad gene and its reporter (LacZ) is relatively stable and may perdure after signaling ceases, we next examined pMad expression in *bam* mutant germarium. Careful examination revealed that a small percentage (5%) of *bam* mutant germaria ($n > 100$) harbored some spectrosome-containing cells outside the niche that express a low level of pMad (compared with its expression level in GSCs; Fig. 1 K), indicating a low level of Dpp signal activation. Interestingly,

these pMad-positive cells also expressed bam-GFP (Fig. 1 K). Considering that these pMad-positive cells were located away from the niche, cap cell–expressed Dpp is unlikely to be responsible for signal activation. Instead, these results are in line with the hypothesis that a low level of Dpp is expressed in ECs. Supporting this, when Dpp was knocked down in the ECs of *bam* mutant germaria, only 5.8% of germaria (n = 103) exhibited Dad-lacZ–positive spectrosome-containing cells outside the niche (Fig. S1 M), suggesting that EC-expressed Dpp is responsible for its expression.

Dedifferentiation of partially differentiated germline cells into GSC-like cells

Dedifferentiation, or the reprogramming of differentiated cells into a pluripotent state, is widely observed in various systems. For instance, spermatogonia in the *Drosophila* testis were found to dedifferentiate into GSCs under physiologic conditions (Brawley and Matunis, 2004; Cheng et al., 2008). Additionally, differentiated four- to eight-cell cystocytes could revert into GSC-like cells in the *Drosophila* adult ovary upon ectopic Dpp expression in ECs (Kai and Spradling, 2004). Hence, we asked whether dedifferentiation also occurs in the adult ovary and whether these partially differentiated cells play a role in this process.

To answer these questions, we used an in vivo model to trace the dedifferentiated cells in the ovary based on an established strategy used in the *Drosophila* testis (Cheng et al., 2008). We combined the Gal4/UAS system with an *actin* flip-out cassette to label the differentiating germline cells using P*bam-gal4*, which initiates its expression only in CBs (Fig. 2 A). The expression of P*bam-gal4* in CBs drives Flippase expression, which removes the "STOP" element inserted between two Flippase recognition target (FRT) sites in the actin flip-out cassette, allowing the clonal expression of the *β-galactosidase* transgene throughout the Gal4/UAS system. Under the control of the *actin* promoter, β-galactosidase is constitutively expressed, thus permanently marking the lineage originating from the CBs.

In the ovaries of newly eclosed females, weak LacZ expression was detected in the CBs and two- to four-cell cysts; stronger LacZ expression was detected in four- to eight-cell cyst, whereas all GSCs were negative for LacZ expression (n > 2,000; Fig. 2 B). Interestingly, 0.20% of these germaria (n = 2,000) contained one LacZ-positive GSC within the niche at day 20 (Fig. 2 C), suggesting that a small number of GSCs could originate from CBs or cysts via dedifferentiation. The lower frequency of dedifferentiation observed in the ovary compared with the testis is likely due to the spatial constraint of the ovarian niche (which contains two to three GSCs in each ovarian niche compared with nine to 11 GSCs per testicular niche) and to normal GSC replacement by a clonal expansion mechanism (Xie and Spradling, 2000; Cheng et al., 2008). Nevertheless, our observations suggest that differentiating germline cells have the potential to revert into a GSC-like fate under physiologic condition in the *Drosophila* ovary.

To rule out the possibility that the low frequency of LacZ expression in the GSCs was caused by the spontaneous activation of the *bam* promoter in the GSCs instead of arising from the dedifferentiation of germline cells, we sought to increase the frequency of dedifferentiation by artificially inducing GSC loss. We stressed GSCs by feeding the flies with BrdU, a thymidine analogue that promotes chromosomal instability (Seecof and Dewhurst, 1976; Schneider and d'Adda di Fagagna, 2012; Sauer et al., 2013). Indeed, the frequency of LacZ-positive GSCs increased significantly to 1.71% (n = 644 germaria; Fig. 2 D) when flies were fed with BrdU-containing food for 3 d. These results suggest that these LacZ-positive GSCs were likely derived from the dedifferentiation of differentiated germline cells. In accordance, the frequency of LacZ-positive GSCs further increased to 4.64% (n = 560 germaria) when flies were fed with BrdU-containing food for a prolonged period of 6 d (Fig. 2 D). Collectively, these results reiterate that germline cells can dedifferentiate into GSC-like cells in the *Drosophila* ovaries.

EC-expressed Dpp promotes dedifferentiation of germline cysts

Next, we developed a *hs-bam*–mediated dedifferentiation assay to further investigate the role of EC-expressed Dpp. Dpp signal activation in GSCs represses the expression of *bam*, whose ectopic expression results in precocious GSC differentiation (Ohlstein and McKearin, 1997; Chen and McKearin, 2003a; Song et al., 2004). We induced differentiation in GSCs by expressing a moderate level of Bam using a *hs-bam* transgene and followed its effect on the GSCs. One day after heat-shock treatment (AHS), all control germaria contained two to three GSCs that were identifiable by their anteriorly positioned spectrosomes and by pMad expression (Fig. 2 E and Fig. S1 N). However, most germaria (97.6%) carrying one copy of the *hs-bam* transgene had only fusome-containing cysts within the niche and were devoid of pMad expression (Fig. 2 E and Fig. S1 O), consistent with the role of Bam in promoting GSC differentiation. Predictably, all control germaria contained GSCs at day 7 AHS. Whereas 53.7% of the germaria carrying the *hs-bam* transgene did not contain any GSC, as expected from irreversible GSC loss due to precocious differentiation, surprisingly, 46.3% of germaria regained their GSCs and managed to support germline development as the control germaria (Fig. 2 E). These results can be explained by the hypothesis that cystocytes of partially differentiated germline cysts can revert into functional GSCs under appropriate conditions (Kai and Spradling, 2004). In line with this hypothesis, our data show that *hs-bam*–induced differentiated cysts within the adult niche could dedifferentiate into GSCs under normal conditions.

We addressed whether the EC-expressed Dpp plays a role in this dedifferentiation process. To test this possibility, we knocked down Dpp activity in the adult ECs in a *hs-bam* background. Similar to the control germaria, 98.2% of the *dpp[i]* germaria carrying one copy of the *hs-bam* transgene examined at day 1 AHS did not contain GSCs but only harbored fusome-containing cysts within their niche. However, at day 7 AHS only 17% of those germaria contained GSCs, whereas 46.3% of control *hs-bam* germaria contained GSCs (Fig. 2 E), suggesting that a low level of EC-expressed Dpp is physiologic functional and promotes the dedifferentiation of cysts into GSCs in an *hs-bam* background.

We further investigated dedifferentiation in *bam* mutant germline cells, which exhibited low levels of Dpp signal activation outside the niche, by reintroducing Bam using *hs-bam* (see Materials and methods). As expected, most *bam* mutant germaria carrying one copy of *hs-bam* transgene harbored only fusome-containing germline cysts at 24 h after AHS, and only 1% of these germaria (n = 100) contained few spectrosome-containing cells (Fig. 2, F and J). Surprisingly, 71.8% of these germaria (n = 103) contained some single spectrosome-containing cells at 48 h after AHS (Fig. 2, G and J), indicating the dedifferentiation

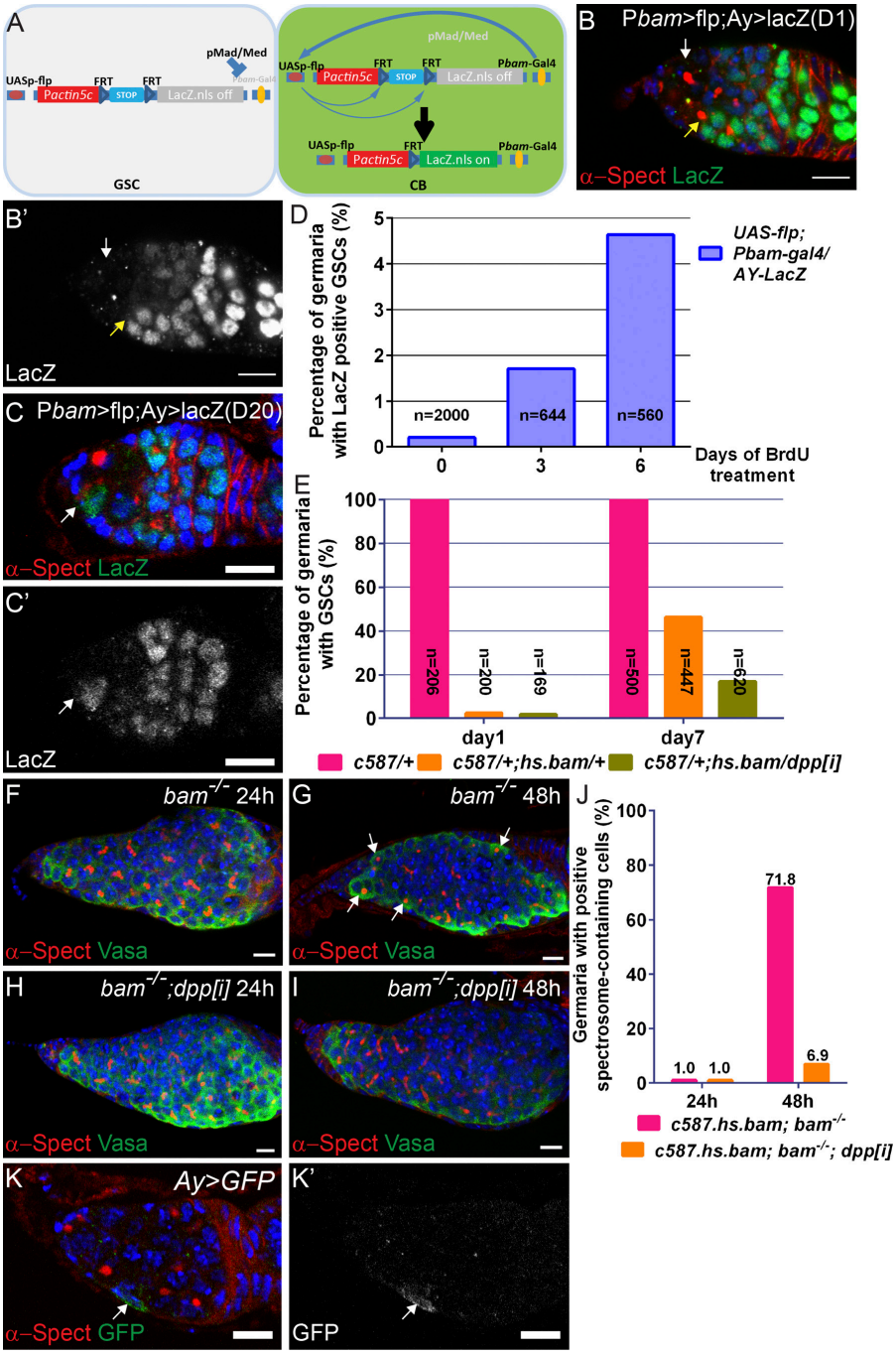

Figure 2. **Dedifferentiation of germline cells.** (A) Schematic showing the experimental design for tracking differentiated cells. In GSCs, Dpp signaling is required for the expression of pMad, which forms a complex with Medea (Med) to suppress the activation of the *bam* promoter. In CBs, Dpp signaling is down-regulated, and, consequently, the *bam* promoter becomes activated. (B, C, F–I, and K) TO-PRO-3 in blue. (B) A germarium (P*bam-gal4; Ay-lacZ*/UASp-*flp*) on day 1 (D1) showing that germline cysts (yellow arrow) but not GSCs (white arrow) expresses the LacZ reporter. (C) A germarium (P*bam-gal4; Ay-lacZ*/UASp-*flp*) at day 20 (D20) showing one GSC (arrow) expressing the LacZ reporter. (D) Percentage of germaria with LacZ-positive GSC(s) in (D20) flies fed with normal or BrdU-containing food. (E) Frequency of germaria containing GSC(s) in a *hs-bam* background at two different time points after AHS. (F–I) *c587.hs-bam/+; bam*$^{-/-}$ germarium harbors fusome-containing germline cysts at 24 h AHS (F) but contains some spectrosome-containing cells (G, arrows) at 48 h AHS. (H and I) *c587.hs-bam/+; bam*$^{-/-}$*.dpp[i]* germarium contains fusome-containing germline cysts at 24 h (H) and 48 h (I) AHS. (J) Frequency of germaria containing spectrosome-containing cells in F–I. (K) A germarium containing one Dpp-expressing EC (arrow) marked by GFP exhibits more spectrosome-containing cells. "n" indicates a single representative experiment of three replicates. Bars, 10 µm.

entiation of germline cysts into spectrosome-containing cells. However, when Dpp was further knocked down in the ECs in this background, only 6.9% of germaria (n = 101) harbored single spectrosome-containing cells at 48 h after AHS (Fig. 2, H and J), supporting a role of EC-expressed Dpp in promoting dedifferentiation in this genetic background.

These data show that Dpp, in addition to its expression in cap cells, is expressed at a low level in ECs and has a functional role. Notably, Dpp expression outside the niche has to be kept at a low level because a high level of ectopic Dpp expression even in a single EC with an *actin* driver was sufficient to produce ectopic spectrosome-containing cells, leading to a disruption of germline homeostasis (Fig. 2 K). Consistent with this finding, Song et al. (2007) previously reported that artificial induction of high levels of Dpp in multiple ECs using an *actin* driver results in the formation of many spectrosome-containing cells. Collectively, these findings suggest that the physiologic relevance of Dpp expression in ECs requires that its expression level remain low to maintain germline homeostasis.

Hh signaling suppresses Dpp expression in ECs

To elucidate the mechanism that controls *dpp* expression in ECs, we examined known niche-associated factors using Dad-lacZ reporter expression as readouts for the function of EC-expressed Dpp (Fig. 1, F and H) and identified several Hh signaling components that function in ECs to influence Dad-lacZ expression in germline cells.

First, we investigated the function of Hh signaling in the germarium. In addition to its strong expression in cap cells, low levels of *Hh* transcripts were detected in ECs (Fig. S3 A). Next, we examined the function of Hh in the ovary using the transheterozygote of two strong hypomorphic alleles (*hh[AC]* and *hh[TS2]*; Lee et al., 1992; Ma et al., 1993). Many of the mutant germaria exhibited an enlarged morphologic structure filled with fusome-containing cysts and were defective in proper segregation of individual germline cysts at region 2a/2b, consistent with the known role of Hh signaling in the regulation of follicular stem cells (Forbes et al., 1996; Zhang and Kalderon, 2001). Interestingly, a small proportion (<10%) of the germaria ($n > 100$) exhibited more spectrosome-containing cells (Fig. S3 B), suggesting that Hh may restrict the proliferation of early germline cells. We reasoned that the low penetrance of this phenotype could be due to the hypomorphic nature of the *hh* alleles used. To address this possibility and to avoid affecting early developmental stages that are Hh signaling dependent (Besse et al., 2005), we specifically knocked down Hh from its sources at the adult stage. Interestingly, we found that both cap cell– and EC-expressed Hh function redundantly to contribute to its signaling activities because only the removal of Hh ligands from both sources (but not from either source alone) resulted in the formation of ectopic spectrosome-containing cells (Fig. S3 C).

Consistent with the role of Hh signaling activation in ECs, *smo[i]* and *ci[i]* germaria contained ectopic spectrosome-containing cells (Figs. 3, A and C; and Fig. S3 D). In contrast, when Hh signaling was ectopically activated in the ECs by expressing Smo[Δ661-818] (a constitutively active form of Smo; Zhao et al., 2007) or Ci[Nc5m5m] (a noncleavable active form of Ci; Price and Kalderon, 2002) or by knocking down Ptc activity, those germaria harbored fewer spectrosome-containing cells compared with controls (Fig. 3, B and C). Thus, Hh signaling in ECs maintains germline homeostasis.

Next, we investigated whether Dpp signaling activity is affected by the Hh signaling pathway. Germaria with ectopic Hh signal activation in their ECs harbored fewer Dad-lacZ–positive spectrosome-containing cells compared with controls (3.84 ± 0.13, $n = 58$ germaria, $P < 0.001$ for Smo[Δ616-818] and 3.68 ± 0.15, $n = 47$ germaria, $P < 0.001$ for Ci[Nc5m5m] vs. 5.13 ± 0.13; $n = 142$ germaria for control; Fig. S3 E; unpublished data). In contrast, *smo[i]* and *ci[i]* germaria, as well as germaria with compromised Hh from both sources, contained more Dad-lacZ–positive spectrosome-containing cells (Fig. S3, F and G; 6.99 ± 0.16, $n = 87$ germaria, $P < 0.001$ for *ci[i]*; 6.8 ± 0.22, $n = 51$ germaria, $P < 0.001$ for *smo[i]* and 10.4 ± 3.0, $n = 120$ germaria, $P < 0.001$ for germaria lacking Hh activity). In addition, we identified one Dad-GFP protein trap line (a GFP fusion protein trap line for Dad generated by the Flytrap Project) and similarly found that *smo[i]* germaria contained more Dad-GFP–positive spectrosome-containing (unpublished data). Interestingly, many of these Dad-lacZ–positive spectrosome-containing cells also expressed a low level of bam-GFP, similar to CB in WT germaria (Fig. S3, H and I). Importantly, removing Hh from its sources or compromising its signaling activity in ECs resulted in more pMad-positive cells (3.9 ± 1.4, $n = 120$ germaria, $P < 0.001$ for *smo[i]* and 4.8 ± 1.25, $n = 120$, $P < 0.001$ for germaria lacking Hh from both sources; Fig. 3, D–F), with 58.3% of *smo[i]* germaria exhibited detectable pMad-positive spectrosome-containing cells outside the niche. In these germaria, only weak pMad expression was detected in the ectopic spectrosome-containing cells, indicating a lower level of Dpp signal activation compared with GSCs within the niche (Fig. 3, D and E). Furthermore, those pMad-positive cells were often located several cell diameters away from the niche (Fig. 3, D and E; arrows). These observations could be explained by the low level of Dpp expression in the ECs that we observed earlier. Interestingly, those ectopic pMad-expressing cells also expressed bam-GFP (Fig. 3, D and E). These data suggest that Hh signaling may influence the level and/or the activity of EC-expressed Dpp.

To assess whether Hh signaling can regulate Dpp expression in ECs, we performed fluorescence in situ hybridization to detect *dpp* transcripts in germaria. *dpp* transcripts were detected in some of the ECs in 27% of WT germaria (Fig. 1 D). However, when Hh signaling was compromised in the ECs, 86% of germaria ($n = 58$) exhibited detectable *dpp* transcripts in the ECs (Fig. 3, G and H). In contrast, when Ptc activity was compromised in the ECs, only 15% of the germaria ($n = 40$) had detectable *dpp* transcripts in the ECs (Fig. 3 G). To confirm this, we performed *dpp* transcriptional profiling in the ECs using a 4-thiouracil/phosphoribosyltransferase-based biosynthetic labeling (TU-tagging) method coupled with real-time quantitative PCR (qRT-PCR) in these backgrounds. *c587* was used to drive the expression of uracil phosphoribosyltransferase in the ECs. When coupled with 4-thiouracil (via feeding), these thio-substitute nucleotide analogues were incorporated into the newly synthesized RNA in the ECs, allowing them to be purified for subsequent analysis (Miller et al., 2009). Indeed, *dpp* transcripts (using two sets of primers detecting two different portions of the coding region) were increased in the EC samples with compromised Ci function, but slightly decreased in the EC samples with compromised Ptc activity (Fig. 3 I). Collectively, our findings suggest that Hh signaling activity functions to maintain Dpp expression at a low level in ECs.

To further demonstrate the link between Hh signaling and the elevated EC-expressed Dpp, we performed a genetic interaction analysis by knocking down Dpp in a *ci[i]* background. Our results showed that inhibiting Dpp activity in the ECs partially suppresses the formation of the ectopic spectrosome-containing cell phenotype in *ci[i]* germaria (Fig. 3 J). These results suggest that Hh signaling promotes the differentiation of germline cells by suppressing Dpp expression in ECs.

Hh signaling in ECs prevents the dedifferentiation of germline cyst

We next examined whether Hh signaling is also involved in the dedifferentiation of germline cells in the *hs-bam* germaria. When Ci function was compromised in the adult ECs in the *hs-bam* background, 53.5% of the examined germaria contained GSCs compared with 46.3% in controls at day 7 AHS (Fig. 4 A). In contrast, only 36% of the *hs-bam* germaria with compromised Ptc function in the ECs contained GSCs (Fig. 4 A). These data show that high Hh signaling activity in the ECs can suppress the dedifferentiation of germline cells in the *hs-bam* background, reminiscent of the depletion of EC-expressed Dpp.

In WT, GSCs undergo complete cytokinesis to produce a GSC daughter and a CB daughter. In contrast, CBs undergo incomplete cytokinesis to produce germline cysts interconnected by stable intercellular bridges called ring canals, whose stability is crucial for subsequent germline cyst development, concomitant with the down-regulation of Dpp signaling (Fig. 4, B and C). Consistent with this finding, ectopic Dpp expression leads to closure of the ring canals, followed by breakdown of the

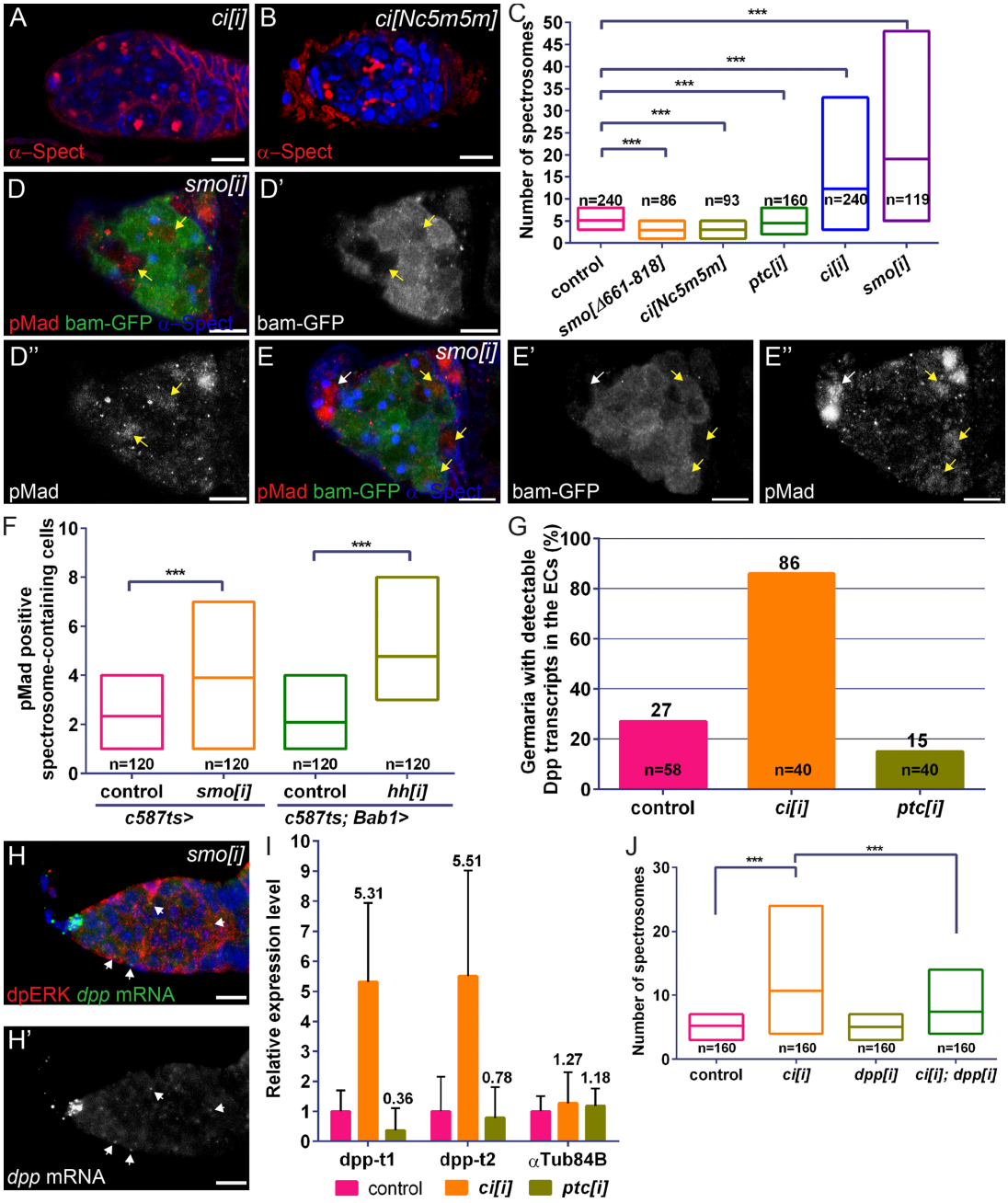

Figure 3. **Hh signaling suppresses Dpp expression in the ECs.** (A, B, and H) TO-PRO-3 in blue. (A) A *c587ts/+; ci*[v105620]*/+* germarium contains more spectrosomes. (B) A germarium expressing the active form of Ci (Ci[Nc5m5m]) exhibits less spectrosomes. (C) Quantitation of spectrosome-containing cells in germaria with aberrant Hh signaling. (D and E) Two different sections of a Z-stack image of a *c587ts/+; smo*[BL27037]*/+* germarium showing that some ectopic spectrosome-containing cells (yellow arrows) express low levels of pMad and bam-GFP. GSCs are marked by white arrows. (F) Quantitation of pMad-positive spectrosome-containing cells in control germaria and germaria with compromised Smo and Hh activity. Middle lines in column stand for the value of the mean. (G) Quantitation of germaria with *dpp* transcripts detected in ECs from various backgrounds. (H) A *c587ts/+; smo*[BL27037]*/+* germarium showing *dpp* transcripts detected in several ECs (arrows). (I) Relative expression levels of *dpp* transcripts in the EC samples derived from control (*c587ts/+*), *ci*[v51479] and *ptc*[BL28795] germaria. The relative expression levels in *ci*[v51479] and *ptc*[BL28795] are normalized by the expression level in *wt* control. Error bar represents standard deviation. (J) Quantitation of spectrosome-containing cells showing that *dpp* knock-down suppresses the formation of ectopic spectrosome-containing cells in *ci*[v51479] germaria. "n" indicates a single representative experiment of three replicates. ***, $P < 0.001$. Bars, 10 μm.

fusomes (signatures of the reversion of cysts into GSC-like cells; de Cuevas and Spradling, 1998; Kai and Spradling, 2004). In Hh signaling-defective germaria, CBs were found to undergo complete cytokinesis to produce two spectrosome-containing cells instead of a two-cell cyst (Fig. 4 D and Fig. S3 J). Furthermore, closure of the ring canals and breakdown of the fusomes were observed in differentiating cysts (Fig. 4 E). These data suggest that Hh signaling in ECs promotes the commitment of germline cells to differentiate by preventing the breakdown of germline cysts.

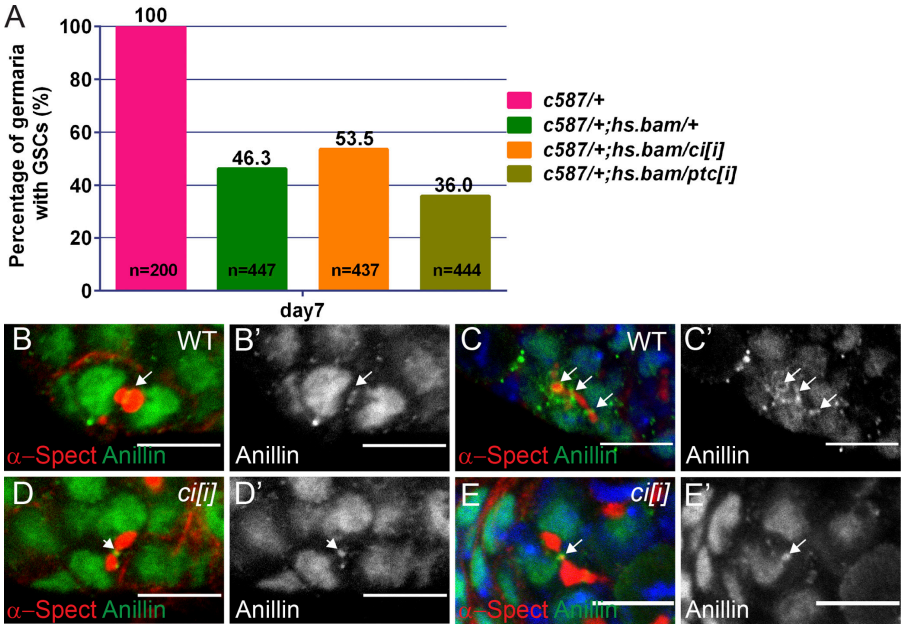

Figure 4. **Hh signaling inhibits the dedifferentiation of germline cells via EC-expressed Dpp.** (A) Frequencies of germaria containing GSCs in various backgrounds on day 7 AHS. (B) A WT CB undergoing incomplete cytokinesis; the ring canal is labeled by Anillin (arrow). (C) A WT cyst with a fusome interconnecting cystocytes with the ring canals labeled by Anillin and indicated by arrows. (D) A CB from a *c587ts/+; ci*[v105620]*/+* germarium undergoing complete cytokinesis as indicated by the closure of the ring canal (arrow). (E) A cyst from a *c587ts/+; ci*[v105620]*/+* germarium undergoing the breakdown process showing the closure of the ring canal (arrow). "n" indicates a single representative experiment of three replicates. Bars, 10 µm.

JAK/STAT signaling acts in ECs to promote germline Dpp signaling

Previously, JAK/STAT signaling has been shown to play a crucial role in the ovarian niche and to indirectly promote GSC maintenance (Decotto and Spradling, 2005; López-Onieva et al., 2008; Wang et al., 2008). In addition to Hh signaling, the JAK/STAT pathway was identified to act in ECs to modulate germline Dpp signaling in a non–cell-autonomous manner. *dome[i]* or *stat92e[i]* germaria contained fewer Dad-lacZ–positive spectrosome-containing cells, whereas germaria with ECs expressing Hop[Tum-1], a gain-of-function version of JAK, harbored more Dad-lacZ–positive and Dad-GFP–positive spectrosome-containing cells (Fig. S4, A–C; and not depicted). Further analyses showed that *dome[i]* or *stat92e[i]* germaria contained fewer pMad-positive GSCs (Fig. 5, A and C) and fewer spectrosome-containing cells (Fig. 5 D). Conversely, germarium-expressing Hop[Tum-1] in ECs harbored more spectrosome-containing cells and more pMad- and Dad-lacZ–positive cells (Figs. 5, B–D; and Fig. S4, C and D). To assess whether JAK/STAT signaling can also act in ECs to promote Dpp expression, we performed RNA in situ for *dpp*. Indeed, only 4.2% of *stat92e[i]* germaria (n = 24) exhibited detectable *dpp* signals outside cap cells, although *dpp* transcripts were consistently detected in cap cells (Fig. 5 E), whereas germaria expressing Hop[Tum-1] showed more detectable *dpp* transcripts in ECs (Fig. 5 F). These results, together with previously published data, indicate that the JAK/STAT pathway also acts in ECs to promote GSC maintenance by regulating Dpp expression, in addition its role in cap cells.

Hh signaling inhibits JAK/STAT signaling activity in ECs

Our data thus far show that the Hh pathway exerts its function in ECs to promote the differentiation of germline cells by dampening Dpp expression and that JAK/STAT signaling activates *dpp* transcription in ECs. Next, we asked whether these two signaling pathways cross-regulate each other to control Dpp expression.

We first investigated whether JAK/STAT signaling regulates Hh signaling activity, which can be monitored by its reporter *ptc-lacZ*, a transcription reporter of *ptc,* which is a target of Hh signaling. In WT, Ptc-LacZ was strongly expressed in ECs and, to a lesser extent, the prefollicular cells but not cap cells (Fig. S5 A). Notably, its expression was not altered in the ECs with ectopic Hop[Tum-1] expression or lacking Stat92e activity, indicating that Hh signaling is not controlled by the JAK/STAT signaling pathway (Fig. S5 B and not depicted).

We then addressed whether Hh signaling modulates JAK/STAT activity using the previously established S2-based luciferase assay (Baeg et al., 2005). As expected, expression of Unpaired (Upd, the ligand) strongly increased the *10XSTAT*-luciferase reporter activity in S2 cells (Fig. 6 A). Interestingly, whereas coexpression of Ci[5M], an active form of Ci variant that is resistant to PKA-mediated proteolysis (Price and Kalderon, 1999), reduced Upd-mediated reporter activity, coexpression of Ci[75], the repressive form, conversely enhanced its activity, suggesting that Hh signaling negatively regulates JAK/STAT signaling activity (Fig. 6 B).

To substantiate this, we further examined whether Hh signaling regulates JAK/STAT signaling in ECs. It has been reported that in mammalian culture system STAT shuttles constitutively between the cytoplasm and nucleus in resting cells, but activation of signaling leads to the phosphorylation and subsequent accumulation of STAT in the nucleus (Sekimoto et al., 1997). A similar translocation phenomenon was reported in *Drosophila* cell culture (Baeg et al., 2005). Anti-Stat92e antibody staining showed that Stat92e was expressed in the somatic cells, including the ECs (Fig. S5, C and D), consistent with previous observations using the reporter line *Stat92e*^{06936} (a *lacZ* enhancer trap line; Silver and Montell, 2001; Decotto and Spradling, 2005; López-Onieva et al., 2008). Additionally, this antibody also detected Stat92e in early germline cells, including GSCs (Fig. S5 C). In WT, colabeling with the nuclear membrane marker Lamin showed that Stat92e signal weakly accumulated in the nuclear region of the ECs (Fig. S5 E). However, Stat92e exhibited enhanced nuclear localization in the ECs expressing Hop[Tum-1] (Fig. S5 F). Interestingly, this nuclear enrichment of Stat92e was also observed in the ECs with compromised Hh signaling activity (Fig. S5 G). To

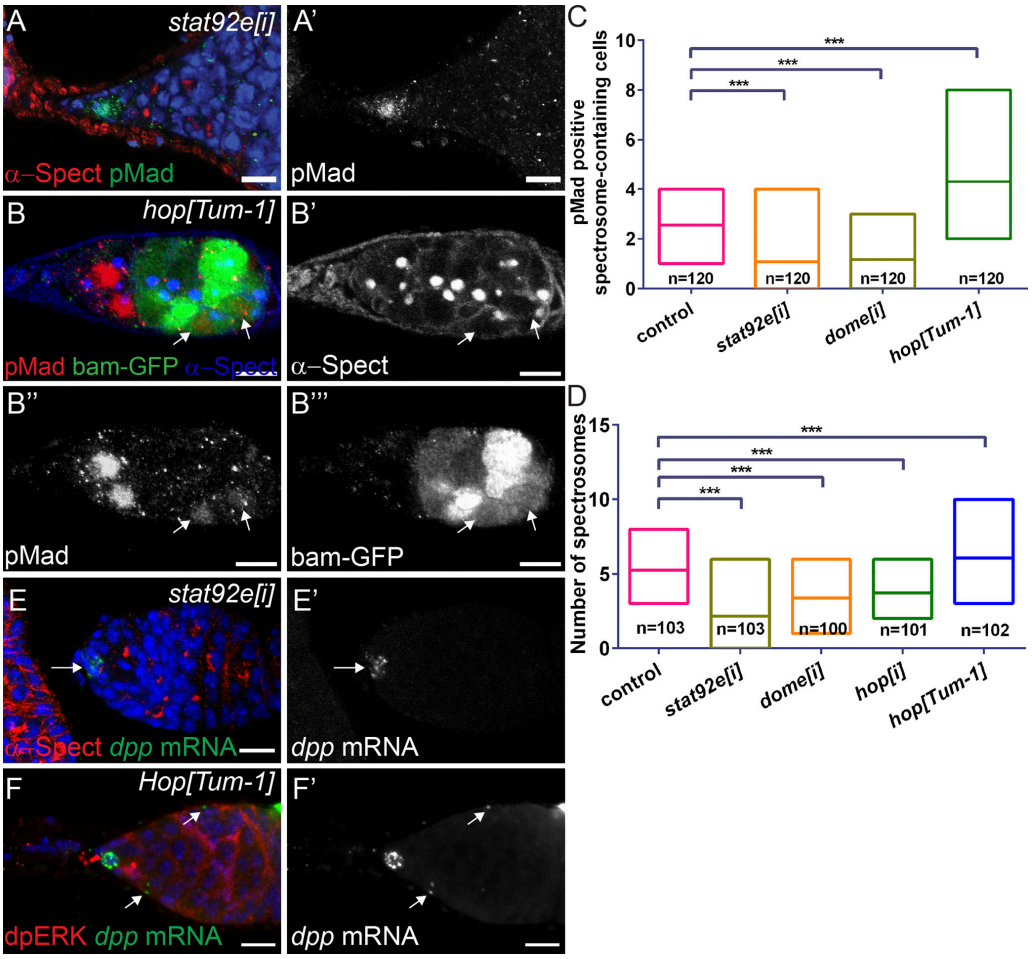

Figure 5. **The JAK/STAT pathway promotes Dpp signaling.** (A, E, and F) TO-PRO-3 in blue. (A) A *c587ts/+; stat92e*[BL33637]/+ germarium with one pMad-positive cell. (B) A *c587ts/+; UAS-hop[Tum-1]/+* germarium contains ectopic pMad-positive cells. Some pMad-positive cells (white arrows) express bam-GFP. (C) Quantitation of pMad-positive spectrosome–containing cells in *c587ts/+*, *c587ts/+; stat92e*[BL33637], *c587ts/+; dome*[BL53890]/+ and *c587ts/+; UAS-hop[Tum-1]/+*. Middle lines in the column stand for the value of the mean. (D) Quantitation of spectrosome-containing cells in germaria with compromised JAK/STAT signaling and germaria expressing Hop[Tum-1]. (E) A *c587ts/+; stat92e*[BL33637]/+ germarium showing *dpp* transcripts in the cap cells only (arrow). (F) A *c587ts/+; UAS-hop[Tum-1]/+* germarium showing *dpp* transcripts detected in ECs (white arrows). "n" indicates a single representative experiment of three replicates. ***, $P < 0.001$. Bars, 10 µm.

further validate this finding, we used the established *10XSTAT-GFP* reporter to monitor the JAK/STAT activity in the ECs (Bach et al., 2007). Whereas GFP was weakly detected in the ECs of the control germarium, the GFP signal was enhanced in the ECs in *smo[i]* and *ci[i]* germaria (Fig. 6, C and D; and Fig. S5 H). Collectively, these data suggest that Hh signaling suppresses JAK/STAT activity in ECs.

To further confirm this finding, we conducted genetic interactions between Hh signaling and the JAK/STAT pathway. Although compromising Hh signaling activity in the ECs resulted in the formation of ectopic spectrosome-containing cells, reducing Stat92e dosage by removing one copy of *stat92e* strongly suppressed this phenotype in either *ci[i]* or *smo[i]* germaria (Fig. 6 E). Supporting that the JAK/STAT pathway acts downstream of Hh signaling in ECs, knocking down JAK/STAT signaling components in ECs also strongly suppressed the formation of ectopic spectrosome-containing cells in *ci[i]* germaria (Fig. 6 F and Fig. S5, I–K).

Then, we examined whether components of these two pathways interact with each other using *Drosophila* Schneider cells (S2 cells). Immunoprecipitation assay showed that Stat92e formed a complex with both Ci[155] (the full-length form), and Ci[5M], but not with Ci[75] (Fig. 6 G and not depicted), suggesting a direct interaction between these signaling pathways in the regulation of Dpp expression. This finding is consistent with the notion that Hh signaling antagonizes JAK/STAT signaling by inhibiting the nuclear accumulation of Stat92e.

Collectively, these results strongly suggest that under normal conditions, JAK/STAT signaling promotes Dpp expression in ECs but that Hh signaling antagonizes JAK/STAT activity to maintain Dpp expression at an optimum range to ensure proper germline development.

Discussion

This study demonstrates that Dpp is also expressed at a low level in ECs to maintain a pool of partially differentiated germline cells that can dedifferentiate into GSCs under normal and stress conditions. Our study further reveals that Dpp expression in ECs is positively regulated by JAK/STAT signaling but negatively controlled by Hh signaling; both of these ligands

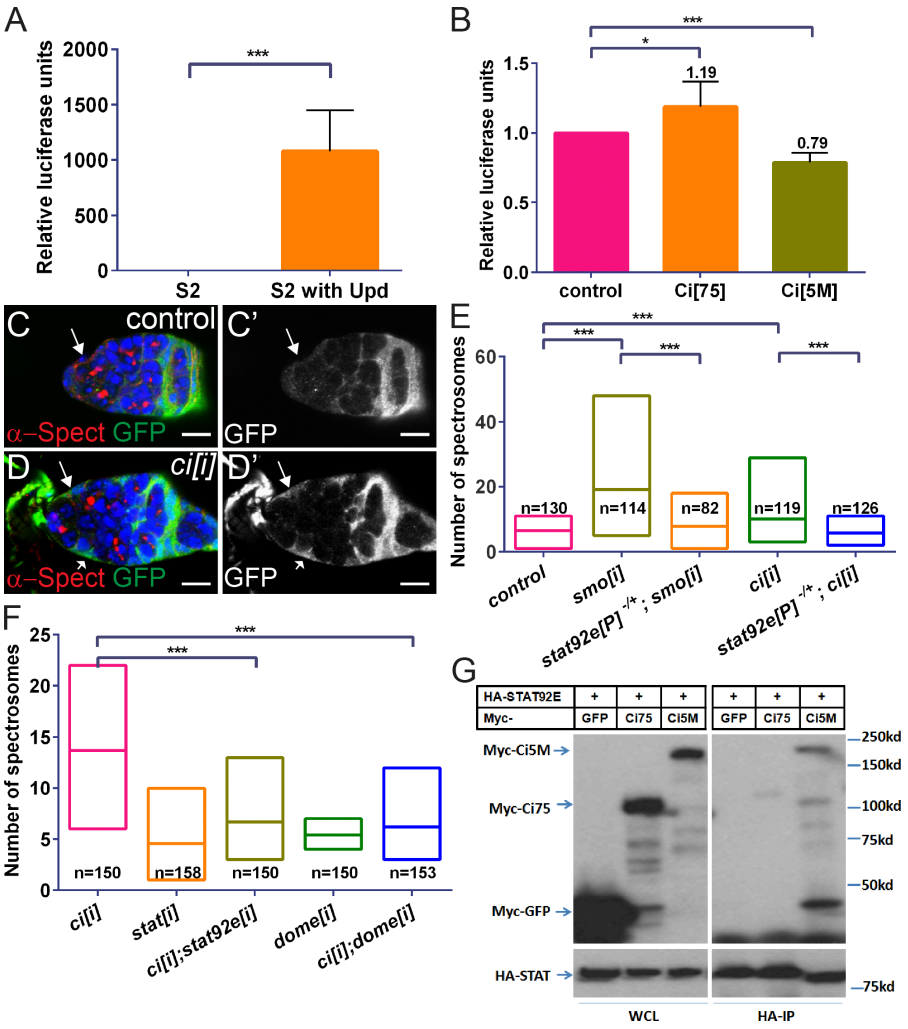

Figure 6. **Hh signaling negatively regulates JAK/STAT signaling in ECs.** (A) In S2 cells, coexpression of Upd dramatically increases 10XSTAT-luciferase activity. (B) Ci[75] expression enhances 10XSTAT-luciferase activity, whereas Ci[5M] reduces its activity. To measure the relative luciferase units, the ratio of firefly/*Renilla* is normalized by its value in S2 cells treated by Upd (control cells). Error bar represents the standard deviation. (C and D) TO-PRO-3 in blue. (C) 10XSTAT-GFP is weakly detected in the ECs (arrow) of a *c587ts/+;10XSTAT-GFP/+* germarium. (D) 10XSTAT-GFP is up-regulated in the ECs (arrows) of a *c587ts/+; 10XSTAT-GFP/+; ci*[v51479]*/+* germarium. (E) Quantitation of spectrosomes showing suppression of *smo[i]* (BL27037) and *ci[i]* (v51479) phenotypes by removing one copy of *stat92e*. (F) Quantitation of spectrosome-containing cells showing that knocking down *stat92e* (BL33618) and *dome* (BL34618) in ECs suppresses the *ci[i]* (v51479) phenotype. (G) Coimmunoprecipitation showing that Stat92e specifically binds Ci[5M] but not Ci[75]. WCL, whole cell lysate. "n" indicates a single representative experiment of three replicates. *, $P < 0.05$; ***, $P < 0.001$. Bars, 10 µm.

are expressed by the niche. Thus, our study suggests a complex yet coordinated action of niche-derived signals for maintaining *Drosophila* female germline homeostasis (Fig. 7).

Dedifferentiation of germline cells

In this study, we demonstrate that differentiated female germline cells can revert to functional GSCs to support normal germline development under *hs-bam* conditions and that the EC-expressed Dpp likely participates in this process by maintaining a population of partially differentiated germline cells. Hence, we propose that EC-expressed Dpp contributes to the maintenance of GSCs by allowing dedifferentiation to occur. In line with this hypothesis, we show that partially differentiated germline cells can dedifferentiate into GSC-like cells under physiological conditions. Dedifferentiation within the germarium is not unexpected, given that differentiated germline cysts in the *Drosophila* testis also undergo dedifferentiation to replenish testis GSCs under physiological conditions (Brawley and Matunis, 2004; Cheng et al., 2008) and that newly born neurons in the larval brain of *Drosophila* can dedifferentiate into neuroblast-like cells (Southall et al., 2014).

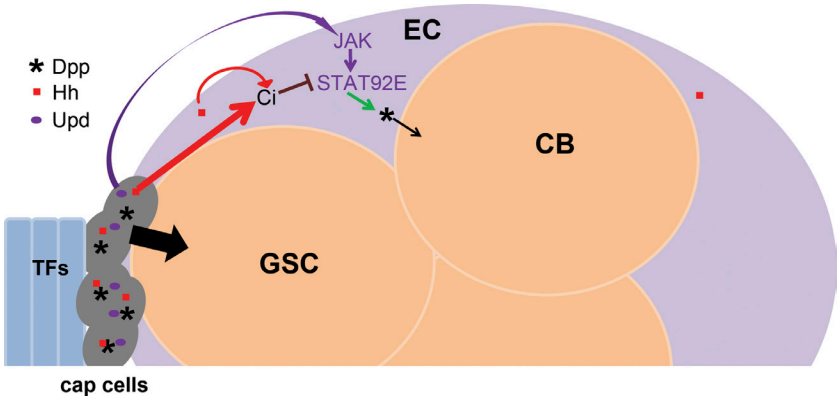

Figure 7. **The working model.** Several signaling molecules including Dpp, Hh, and Upd are expressed in the GSC niche. Whereas cap cell–expressed Dpp acts directly on GSCs to promote self-renewal (thick black arrow), cap cell–derived Upd activates the JAK/STAT pathway in ECs to promote low levels of Dpp expression in these cells (green arrow), and this EC-expressed Dpp functions to maintain a population of partially differentiated germline cells, including CBs (thin black arrow). In contrast, Hh signaling in the ECs dampens Dpp expression by antagonizing JAK/STAT signaling probably through the binding of Ci to Stat92e and influence its nuclear localization.

Regulation of Dpp expression in ECs

Although *dpp* transcripts were detected in both cap cells and ECs, the role of EC-expressed Dpp has been neglected because of the prominent role of the cap cell–expressed Dpp that directly promotes GSC self-renewal (Xie and Spradling, 2000; Song et al., 2004; Wang et al., 2008). Our data show that having an optimum level of Dpp expression in ECs is important for the development of the GSC lineage. Although excessive amounts of Dpp production in ECs arrest the differentiation of germline cells, decreased Dpp expression causes defects in long-term GSC maintenance. This tightly regulated Dpp expression is controlled by two signaling pathways, namely, the Hh and JAK/STAT pathways.

In addition to its role in positively regulating Dpp expression in cap cells, JAK/STAT signaling also functions in ECs to maintain the structure of germaria (Decotto and Spradling, 2005). We show here that this pathway is also required for maintaining a low level of Dpp in ECs. Whereas ectopic JAK/STAT signal activation in ECs results in ectopic Dpp signaling outside the niche, compromised JAK/STAT signaling leads to reduced Dpp signaling. However, its role in promoting Dpp expression is antagonized by Hh signaling. Our data suggest that Hh signaling likely exerts its inhibitory effect on the nuclear accumulation of Stat92e through the interaction between Ci and Stat92e. Loss of Hh activity in the ECs resulted in the elevated JAK/STAT signaling (Figs. 6, C and D; and Fig. S5 H) and ectopic (yet low levels of) Dpp signaling outside the niche (Fig. 3 F), which subsequently blocks germline differentiation. Consistently, reducing JAK/STAT activity (by removing one copy of *stat92e* or by knocking down Stat92e or Dome in ECs) strongly suppresses germline differentiation arrest in Hh signaling–deficient germanium (Fig. 6, E and F). In line with this, compromising Dpp in the ECs of *ci[i]* germaria partially suppressed the formation of ectopic spectrosome-containing cells (Fig. 3 J). This partial suppression also indicates that Hh signaling may have additional target(s) in the ECs.

A previous study (Rojas-Ríos et al., 2012) also showed that Dpp expression in the ECs plays a role in maintaining GSCs within the niche. Our study demonstrates that Hh signaling in ECs suppresses Dpp expression to promote germline differentiation, whereas Rojas-Ríos et al. (2012) suggested that Hh signaling in ECs maintains GSCs by promoting Dpp expression. Our conclusion is well supported by multiple lines of evidence, including the following: (a) Hh signaling–deficient germaria (including Hh mutants and RNAi knockdown using multiple constructs for Hh, Smo, and Ci; see Materials and methods) did not exhibit a GSC loss phenotype but contained more spectrosome-containing cells, and ectopic Dpp signal activation was detected outside the niche (Fig. 3, D–F). (b) *dpp* transcripts were up-regulated in EC samples derived from *ci*[*i*] germaria (Fig. 3 I). Although we used EC-derived samples to measure the expression levels of Dpp transcripts, Rojas-Ríos et al. used entire ovary samples to detect *dpp* transcripts. (c) Removing Dpp function from ECs of *ci*[*i*] germaria partially yet clearly suppressed the formation of ectopic spectrosome-containing cells (Fig. 3 J).

Dual role of ECs in GSC lineage development

ECs were initially regarded as a structural support for germline differentiation. However, a growing body of evidence indicates that these cells have an active role in controlling GSC lineage development. The ECs in direct contact with GSCs and cap cells form a niche, together with cap cells, to support GSC self-renewal (Chen et al., 2011). It has also been proposed that ECs can act as a differentiation niche to control GSC lineage development (Kirilly et al., 2011). Thus, ECs appear to have two opposing activities, with one promoting GSC self-renewal and the other initiating the differentiation of GSC daughters.

Several mechanisms have been identified to support the prodifferentiation role of ECs, many of which seem to prevent ectopic Dpp signaling outside the niche. First, Lsd1- and dBre1/dSet1-mediated suppression of *dpp* transcription in the ECs leads to the spatially restriction of Dpp expression to cap cells (Eliazer et al., 2011; Xuan et al., 2013). Second, whereas EGFR signal activation in ECs suppresses the expression of Dally to prevent the long-range function of cap cell-expressed Dpp, Wnt signaling in ECs promotes Tkv expression that constrains Dpp activity within the niche (Liu et al., 2010; Luo et al., 2015). Third, Eggless (a H3K9 methyltransferase) acts through a yet-to-be-identified mechanism to promote germline differentiation by repressing ectopic Dpp signal activation outside the niche (Wang et al., 2011). Fourth, ecdysone signaling acts in ECs to promote the differentiation of germline cells via unknown mechanism(s) (König et al., 2011). Last, EC-expressed Piwi promotes germline differentiation by repressing ectopic Dpp signal activation outside the niche, although the underlying mechanism is unclear (Rangan et al., 2011; Jin et al., 2013; Ma et al., 2014). In contrast, the pro-stemness activity of the ECs is less understood. JAK/STAT signaling is known to act in ECs to maintain GSCs (Decotto and Spradling, 2005); however, the underlying mechanism remains unknown. In this study, we show that JAK/STAT signaling functions to maintain a low level of Dpp expression in ECs, and that this source of Dpp is required for GSC maintenance. Our data further suggest that promoting the dedifferentiation of germline cysts into GSCs may be an alternative strategy to maintain the GSCs in a long term.

Interplay among niche-associated signals

The development of a stem cell lineage is a progressive process, and the differentiated daughters can dedifferentiate into functional stem cells, indicating the plasticity of the developmental process. Thus, both stem cells and their differentiated progeny are important for the maintenance of tissue homeostasis. How is this maintenance achieved? The *Drosophila* ovarian niche exemplifies one such solution. By producing Dpp (by the cap cells), the niche promotes GSC self-renewal that serves the purpose of long-term germline homeostasis (Xie and Spradling, 2000). In contrast, the niche also produces multiple Wnts that act on the ECs to regulate Tkv expression, which consequently restricts the activity of cap cell–produced Dpp (Luo et al., 2015). The niche-derived Wnt signaling, together with germline-derived EGFR signaling (by suppressing Dally expression in the ECs; Liu et al., 2010) effectively inhibits cap cell–derived Dpp activity outside the niche. Thus, the niche-associated pro-stemness activity is spatially restricted within the niche. Notably, the ovarian niche uses another set of signaling molecules to maintain a pool of partially differentiated daughters. This ovarian niche produces Upd (López-Onieva et al., 2008; Hayashi et al., 2012), a stress response cytokine and a ligand of the JAK/STAT signaling pathway. This study shows that JAK/STAT signaling in ECs promotes a low level of Dpp expression and that this source of Dpp is important for maintaining partially differentiated germline cells that can dedifferentiate under both normal

and stress conditions. Interestingly, our study shows that the niche produces another signaling molecule, Hh, which initiates downstream signaling in ECs. Ci, the downstream effector of Hh signaling, binds Stat92e and suppresses its activity by preventing its nuclear accumulation. These data show that Hh signaling in ECs acts to suppress JAK/STAT signaling to maintain Dpp expression at a low level that is essential for germline development. Thus, the *Drosophila* ovarian stem cell niche produces multiple signaling molecules that coordinate to achieve a single aim: germline homeostasis.

Although Dpp acts directly on GSCs to mediate self-renewal, Wnt, Upd, and Hh act through ECs to maintain germline homeostasis. Hence, the directional activation of niche-associated signals may be an important aspect of the development of stem cell lineage. Considering that the niche acts as an interaction hub that integrates a variety of signals to balance between self-renewal and differentiation, this model of directional signal activation is likely to be conserved in other stem cell systems.

Materials and methods

Fly stocks and culture

Drosophila genes, genetic symbols, and information about strains used in this study are described in the text or in FlyBase. All fly stocks were maintained at 25°C. The stocks used were *y[1]w[1118]*, FRT40A, FRT42D, P*actin*>Stop>*gal4*.UAS-*gfp* (J. Cheng and Y. Yamashita, University of Michigan, Ann Arbor, MI), *tub-gal80[ts]*, *ubi-gfp.nls*, $bam^{\Delta 86}$, *hh[TS2]*, *hh[AC]*, *ptc[S2]*, *ptc[16]*, *ptc[13]* (P. Ingham, Institute of Molecular and Cell Biology, Singapore), *smo[3]*, *smo[IA3]*, *ci[94]* (K. Basler, Institute of Molecular Life Sciences, University of Zurich, Zurich, Switzerland), *stat92e*[06346] (or *stat92e*[P]), UAS-*ci[Nc5m5m]* (D. Kalderon, Columbia University, New York, NY), UAS-*hh*, UAS-*smo[Δ616-818]* (J. Jiang, University of Texas Southwestern Medical Center, Dallas, TX), UAS-*hop*(*Tum-1*), *hs-bam*, P*bam-gfp* (D. Chen and D. McKearin, University of Texas Southwestern Medical Center, Dallas, TX), *hh-lacZ* (Bloomington stock no. 5530 or BL5530), *ptc-lacZ* (J. Hooper, University of Colorado, Aurora, CO), *c587-gal4* (or *c587* for short), P*bam-gal4* (J. Cheng and Y. Yamashita), *Bab1*-gal4 (*Bab1* for short, BL6802), *GMR25A11-gal4* (*GMR25A11* for short, BL49106), *dpp* RNAi stock (BL25782 and BL33618), *hh* RNAi stock (BL25794, BL31474, BL31042, BL32489, or v109454; v stands for Vienna Drosophila RNAi Center), *ptc* RNAi stock (BL28795), *ci* RNAi stock (v51479, v105602, BL28984, BL31320, or BL31321), *smo* RNAi stock (BL27037 or BL53348), *stat92e* RNAi stock (BL33637), *dome* RNAi stock (BL32860 or BL34618), *dad*-lacZ (*dad[1883]*; T. Tabata, University of Tokyo, Tokyo, Japan), and $bam^{\Delta 86}$.*dad-lacZ* stock (E. Ferguson, University of Chicago, Chicago, IL). The targeting sequencing for correspondent RNAi constructs can be found at TRiP at Harvard Medical School or Vienna Drosophila Resource Center.

dsRNA- or shRNA-mediated knock-down and misexpression

All crosses (unless otherwise stated) were set up and maintained at 16–18°C using a combination of tissue-specific driver and *tub-gal80[ts]*. Progeny with proper genotypes were collected within 24 h upon eclosion and fattened on freshly prepared yeast paste at 29–31°C for 5–10 d unless otherwise stated before dissection and immunostaining. Multiple RNAi stocks (listed in Fly stocks and culture) were tested and all showed consistent phenotypes.

To knock down a gene's function in ECs, *c587* or *GMR25A11* was used in combination with *tub-gal80[ts]* (referred to as *c587ts* or *GMR25A11ts, respectively*). To knock down a gene's function in TFs and cap cells, *Bab1* was used in combination with *tub-gal80[ts]* (referred to as *Bab1ts*). To knock down *hh* expression in TFs, cap cells, and ECs, a *c587* and *Bab1* (which drives reporter expression in TF and cap cells) double driver was used in combination with *tub-gal80[ts]* (referred to as *c587ts;Bab1*). For ectopic expression of *hh* or *smo[Δ616-818]*, *ci[Nc5m5m]* or *hop*[*Tum-1*] in adult ECs, similar strategies were performed.

To check for the *gal4* driver expression pattern, flies carrying the UAS-*gfp* construct were crossed with *c587*, *c587ts,* and *GMR25A11ts*, and the crosses were maintained at 16–18°C. Wandering larvae and white pupae were dissected to examine GFP expression at the larval and pupa stage, respectively. To check for GFP expression in newly eclosed adults at permissive temperatures, flies eclosed within 24 h were dissected and examined. To examine GFP expression at restrictive temperatures, flies were fattened at 29–31°C for desired before dissection and examination.

hs-bam–mediated dedifferentiation experiment

Crosses were maintained at 16–18°C and progeny with the desired genotypes (see below) were collected and kept in food vials for 4–5 d at 25°C (to inhibit a gene's function in ECs using an RNAi approach). The flies were heat-shock treated for 1 h 20 min at 37°C, followed by 2 h 20 min recovery at room temperature and one more AHS for 1 h 20 min at 37°C. After the AHS, flies were kept on food vials at 25°C before dissection and immunostaining. *c587*/+ was used as control and the genotypes of experimental groups were (1) *c587*/+;*hs-bam*/+, (2) *c587*/+;*hs-bam*/*dpp[i]*, (3) *c587*/+;*ci[i]*/+;*hs-bam*/+, and (4) *c587*/+;*hs-bam*/*ptc[i]*.

For dedifferentiation in the $bam^{\Delta 86}$ mutant background, progeny with the following genotypes were collected and aged on normal fly food for 3 d before being subjected to three HSs at 37°C with 3-h intervals at room temperature. After heat shock, the flies were fattened at room temperature before dissection and examination. Progeny with the following genotypes were collected and examined: *c587.hs-bam*/+;$bam^{\Delta 86}$ and *c587.hs-bam*/+; $bam^{\Delta 86}$.*dpp[i]*.

Dedifferentiation assay

To address dedifferentiation under normal conditions, crosses were maintained at 16–18°C and progeny with the desired genotypes (UASp*.flp*/+;;P*actin*>FRT-Stop-FRT>*lacZ-NLS*/P*bam.gal4*) were collected within 24 h upon eclosion and kept in food vials at 16–18°C before dissection and immunostaining. To address dedifferentiation under stress (BrdU feeding) conditions, progeny with the above genotypes were collected within 24 h upon eclosion and fed on BrdU-containing food modified from the recipe used by Yadlapalli and Yamashita (2013) (950 µl 100% apple Juice, 7 mg agar, and 50 µl of 100 mg/ml BrdU in a 1:1 mixture of acetone and DMSO) for a specific number of days at room temperature before shifting back to 16–18°C and maintained on normal food vials before dissection and immunostaining.

Generation of a single EC-expressing dpp

Crosses were set up and maintained at 16–18°C, and female progeny with genotype *hs-flp*/+;P*actin*>Stop>*gal4*.UAS-*gfp*/+;UAS-*dpp*/+ were collected and aged for 3 d before subjected to single heat shock (20 min at 37°C) and dissected in 5 d.

EdU incubation

Ovaries were dissected and ovarioles were separated in M3 insect medium (Sigma-Aldrich), then incubated for 1 h in M3 medium containing 0.25 µg/ml EdU (Invitrogen). After washing out the unincorporated EdU, ovaries were fixed for antibody staining according to the manufacturer's instruction.

Antibody generation

cDNA fragment corresponding to aa225-746 of the *stat92e*-RE isoform was amplified and cloned into pENTR-D/TOPO before being swapping into the destination vector pDEST-17 using Gateway Technology (Invitrogen) for His-tag fusion protein production. The fusion protein was purified using HisPur Ni-NTA resin and kits obtained from Thermo Fisher Scientific. Antibody was raised by injecting the fusion protein into guinea pigs, following a standard protocol.

Immunohistochemistry and imaging

For antibody staining, ovary samples were dissected in PBS, fixed in 4% PFA/PBS, and washed three times with PBST (0.1% Triton X-100 in PBS). The samples were then blocked in 5% NGS buffer (5% normal goat serum in PBST, Jackson ImmunoResearch Laboratories) for 30 min before incubation with primary antibody at 4°C overnight. The next day, the samples were washed three times with PBST and incubated with secondary antibody for 2 h before incubation with TOPRO-3/Hoechst.

For pMad staining, ovaries were fixed in 4% PFA, 0.1 M Hepes, pH 7.4, with 0.5% Triton X-100 for 50 min, followed by regular antibody staining.

For fluorescent RNA in situ, ovaries were dissected in PBS and immediately fixed in 4% PFA with protein phosphatase inhibitor (Sigma-Aldrich) at 4°C overnight. On the second day, ovary samples were washed three times with PBST (0.1% Tween 20 in PBS) before proteinase K (50 µg/ml in PBST; Sigma-Aldrich) treatment for 5 min. Samples were washed three times with PBST and refixed in 4% PFA, followed by prehybridization in hybridization solution (50% formamide, 5x SSC, 0.1% Tween-20, 50 µg/µl heparin, and 100 µg/ml salmon sperm DNA) for 1 h at 60°C. Samples were then hybridized with Dig-labeled probe overnight at 55°C–60°C. After the samples were rinsed with PBST, they were incubated with anti-Dig-POD (1:200; Roche), and the TSA Fluorescein system (Perkin Elmer) was used to develop in situ signals (Liu et al., 2010).

The following primary antibodies were used: mouse anti-α-Spectrin (3A9, 1:100; Developmental Studies Hybridoma Band [DSHB]), mouse anti-Lam C (LC28.26, 1:50; DSHB), Lam Dm0 (ADL67.10, 1:50; DSHB), rabbit anti-Anillin (1:500; a gift from C. Field and B. Alberts), rabbit anti-Hh (1:1,000; a gift from T. Kornberg [University of California, San Francisco, San Francisco, CA] and T. Tabata), rabbit anti-pMad (1:400; Cell Signaling), rabbit anti-GFP (1:5,000; Abcam), rabbit anti–di-phosphorylated ERK1/2 (1:200; Cell Signaling), rabbit anti–β-galactosidase (1:8,000; Cappel), and guinea pig anti-Vasa (1:3,000; a gift from T. Kai, Temasek Life Sciences Laboratory, Singapore). Alexa Fluor 488–, 555–, or 633–conjugated goat secondary antibodies against mouse, rabbit, and guinea pig (1:500; Molecular Probes) were used to detect the primary antibodies. TO-PRO-3 (Invitrogen) was used for DNA staining, and samples were mounted in Vectashield mounting medium (Vector Laboratories). Images were obtained using a Zeiss LSM 510 upright microscope with a NEOFLUAR 40×/1.3 NA oil objective lens or SPEII upright microscope (Leica) with an ACS APO 40×/1.15 oil objective lens at room temperature and were processed in Photoshop CS6 and Adobe Illustrator CS6 (Adobe). For the expression patterns of the *c587ts* (Fig. S2, G and H) and *GMR25A11ts* (Fig. S2, M and N) drivers, series of Z-stack images were obtained and 3D reconstruction images were processed with Imaris 7.0 (Bitplane) software. Acquisition software included LSM Image Browser (Cal Zeiss) and LAS X (Leica) The following primers were used to amplify the DNA templates: for *dpp* in situ, 5′-AGGACGATCTGGATCTAGATCGGT-3′ and 5′-ACTTTGGTCGTTGAGATAGAGCAT-3′; for *hh* in situ, primer set 1 (5′-GTGGATTTGGATCTGGCTATC-3′ and 5′-CAATTAGCCGCGATACAGCAC-3′) or primer set 2 (5′-ATTCGTCGATCAGTTCCCACGTGC-3′ and 5′-GATGGAATCCTGGAAGAGCGATCC-3′).

Constructs, S2 cell culture, coimmunoprecipitation, and Western blotting

Constructs for tissue culture cell transfection were generated using Gateway Technology (Invitrogen). The *ci*[75], *ci*[155], and *ci*[5M] plasmids were gifts from D. Kalderon. cDNAs of *ci*[75], *ci*[155], and *ci*[5M] were amplified and then inserted into pENTR-D/TOPO before being swapping into the destination vector pAMW.

All transfections were performed using Effectene Reagent (Qiagen) according to the manufacturer's instructions. In brief, 0.5 µg of each plasmid was transfected into S2 cells, and 2 d later, the cells were harvested and lysed. Each six-well plate was lysed in 250–300 µl of Nonidet P-40 lysis buffer containing 50 mM Tris, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, and 0.2 mM EDTA with protease inhibitor cocktail (complete, Roche) and phosphatase inhibitor added and incubated on ice for 30 min. The lysate was collected and cleared by centrifugation at 13,000 rpm for 5 min at 4°C. For coimmunoprecipitation, 200 µl of lysate was mixed with HA antibody–conjugated beads (Sigma-Aldrich) for overnight at 4°C. The beads were washed in lysis buffer three to five times and suspended in 25 µl of 2× SDS-PAGE sample buffer. The samples were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore). Rabbit anti-Myc antibody (1:2,000; Abcam) and Rat anti-HA antibody (1:2,000; Roche) were diluted in TBST with 5% nonfat dry milk.

Luciferase assay

S2 cells were cultured in M3 insect medium with 10% FBS at 25°C. For each transfection, 2×10^5 S2 cells were plated 24 h before plasmid transfection and then were transfected with 200 ng pAC-upd and 20 ng of pAC-Renilla, together with 200 ng 10XSTAT-luciferase reporter or luciferase reporter construct, using Effectene reagent (Qiagen) in 24-well plates. The luciferase activity was assayed 72 h after transfection using the duo-luciferase assay system (Promega) measured on a luminometer. Relative luciferase activity is the ratio between firefly and the constitutively expressed *Renilla* luciferase levels. Each experiment was performed in triplicate.

TU tagging and qRT-PCR analysis

TU tagging was performed as described in Miller et al. (2009) and Gay et al. (2014) with modifications. In brief, adult flies of several genotypes were induced with *c587-GAL4* to express uracil phosphoribosyltransferase in the escort cells of the germaria. These flies were collected over a period of 12 h after eclosion and fed with yeast paste infused with 1.0 mM 4-thiouracil for 24 h at 25°C. The germaria were then dissected and the total RNA was extracted from Trizol. As much as 75 µg total RNA from each sample was purified with Dynabeads (Invitrogen) to isolate the mRNA and to eliminate DNA contamination before being used for subsequent biotinylation (EZ-Link HPDP-Biotin; Thermo Fisher Scientific). Purification of biotinylated TU-tagged mRNA was performed with µMacs streptavidin kits (Miltenyi Biotec). Purified TU-tagged mRNA and 500 pg unbiotinylated mRNA from each sample were reversed transcribed and amplified using MessageBOOSTER cDNA Synthesis kit for qPCR (Epicentre).

The cDNA yielded from the preceding procedures were quantitated on Applied Biosystems 7900HT according to the manufacturer's instructions. Primer sets for the target and control genes were designed within 500 bp from the 3′ end of the mRNA transcript to prevent technical bias arising from oligo(dT) primed cDNA synthesis. For quantitative analysis, mRNA levels were first normalized to the geometric mean of five internal control genes (*tub*, *CG1115*, *gapdh-1*, *lt,* and *vps20*)

that are stably expressed in the ovary according to Genevestigator Expression Database (Vandesompele et al., 2002). The up- and down-regulations of TU-tagged mRNA were calculated as fold amplification relative to total germaria mRNA in the relevant fly lines. The purity of TU-tagged mRNA was evaluated with *nos* transcript level, which is highly expressed in the germline cells but not in the escort cells.

To examine the transcription level of *dpp*, primers 5′-ATC GATTCGTGCCTGATGTT-3′ and 5′-TTTCATCCTTGCTCCTGC AT-3′ were used to detect *dpp*-t1, and primers 5′-ACCCCCAT TAGCAAACACAC-3′ and 5′-TAGTTGAATGCGCAACGAAG-3′ were used to detect *dpp*-t2. Primers 5′-TCGATCTGATGTACGCCA AG-3′ and 5′-CTCGGAGAACTCTCCCTCCT-3′ were used to detect α-Tub84B for normalization.

Counting of spectrosome-containing cells

In all experiments, GSCs were defined by their direct contact with cap cells with an anteriorly positioned spectrosome, whereas the cells with a spectrosome that were not directly in contact with the cap cells were considered as CBs. In some experiments specified in the text (including *hs-bam*–mediated dedifferentiation), pMad expression was also used as a marker for GSC. Those germ cells contacting cap cells but without an anteriorly position spectrosome were not counted as GSCs. Because fusome morphology of GSC and CB changes periodically during cell cycle (de Cuevas and Spradling, 1998; Hsu et al., 2008), we found that using round spectrosome and the lack of cap cell contact as criteria for CB produced results with larger variation because these criteria did not apply to dividing (telophase) GSCs generating one GSC daughter and one CB daughter interconnected with elongated fusome. Thus, we counted the telophase GSC as one GSC and one CB. These criteria produced consistent results and thus were applied throughout this study. For counting of pMad-positive spectrosome-containing cells in various mutant backgrounds, ovary samples were stained with pMad and 3A9 and only spectrosome-containing cells expressing pMad were counted. Similarly, for Dad-lacZ–positive spectrosome-containing cells, only spectrosome-containing cells with LacZ expression were counted and those spectrosome-containing cells but negative for LacZ were not included.

Statistical analyses of spectrosome-containing cells

For statistical analyses of spectrosome-containing cells in this study, numbers of GSCs, CBs, pMad-positive spectrosome-containing cells and Dad-lacZ–positive spectrosome-containing cells were counted from randomly selected germaria under a fluorescence microscope. The spectrosome-containing cell number is counted according to α-Spectrin staining. Data processing was performed using Microsoft Office Excel 2007 (Microsoft Corp.) and Prism 6.0 (GraphPad Software). P values were determined by two-tailed Student's *t* tests. $P < 0.05$ represents a statistically significant difference. Error bars indicate the standard deviation. In all box plots, the upper and lower bounds represented the maximum and minimum values and the middle lines stand for the mean. To calculate the percentage of *dpp* transcripts in ECs, Z-stack images of germaria that underwent *dpp* in situ hybridization were acquired using a Zeiss LSM510 upright confocal. In situ signals in ECs were then examined in the Z-stack images.

Online Supplemental Material

Fig. S1 shows that the EC-expressed Dpp functions to maintain germline homeostasis. Fig. S2 shows the expression patterns of *c587ts* and *GMR25A11ts* during germline development. Fig. S3 shows Hh signaling acts in ECs to modulate Dpp expression. Fig. S4 shows that the JAK/STAT pathway functions in the ECs. Fig. S5 shows Hh signaling antagonizes JAK/STAT signaling to promote germline differentiation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201503033/DC1.

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References

Bach, E.A., L.A. Ekas, A. Ayala-Camargo, M.S. Flaherty, H. Lee, N. Perrimon, and G.H. Baeg. 2007. GFP reporters detect the activation of the *Drosophila* JAK/STAT pathway *in vivo*. *Gene Expr. Patterns.* 7:323–331. http://dx.doi.org/10.1016/j.modgep.2006.08.003

Baeg, G.H., R. Zhou, and N. Perrimon. 2005. Genome-wide RNAi analysis of JAK/STAT signaling components in *Drosophila*. *Genes Dev.* 19:1861–1870. http://dx.doi.org/10.1101/gad.1320705

Besse, F., D. Busson, and A.M. Pret. 2005. Hedgehog signaling controls Soma-Germen interactions during *Drosophila* ovarian morphogenesis. *Dev. Dyn.* 234:422–431. http://dx.doi.org/10.1002/dvdy.20537

Brawley, C., and E. Matunis. 2004. Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. *Science.* 304:1331–1334. http://dx.doi.org/10.1126/science.1097676

Casanueva, M.O., and E.L. Ferguson. 2004. Germline stem cell number in the *Drosophila* ovary is regulated by redundant mechanisms that control Dpp signaling. *Development.* 131:1881–1890. http://dx.doi.org/10.1242/dev.01076

Chai, P.C., Z. Liu, W. Chia, and Y. Cai. 2013. Hedgehog signaling acts with the temporal cascade to promote neuroblast cell cycle exit. *PLoS Biol.* 11:e1001494. http://dx.doi.org/10.1371/journal.pbio.1001494

Chen, D., and D. McKearin. 2003a. Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. *Curr. Biol.* 13:1786–1791. http://dx.doi.org/10.1016/j.cub.2003.09.033

Chen, D., and D.M. McKearin. 2003b. A discrete transcriptional silencer in the bam gene determines asymmetric division of the *Drosophila* germline stem cell. *Development.* 130:1159–1170. http://dx.doi.org/10.1242/dev.00325

Chen, S., S. Wang, and T. Xie. 2011. Restricting self-renewal signals within the stem cell niche: Multiple levels of control. *Curr. Opin. Genet. Dev.* 21:684–689. http://dx.doi.org/10.1016/j.gde.2011.07.008

Chen, S., M. Lewallen, and T. Xie. 2013. Adhesion in the stem cell niche: Biological roles and regulation. *Development.* 140:255–265. http://dx.doi.org/10.1242/dev.083139

Cheng, J., N. Türkel, N. Hemati, M.T. Fuller, A.J. Hunt, and Y.M. Yamashita. 2008. Centrosome misorientation reduces stem cell division during ageing. *Nature.* 456:599–604. http://dx.doi.org/10.1038/nature07386

Decotto, E., and A.C. Spradling. 2005. The *Drosophila* ovarian and testis stem cell niches: Similar somatic stem cells and signals. *Dev. Cell.* 9:501–510. http://dx.doi.org/10.1016/j.devcel.2005.08.012

de Cuevas, M., and A.C. Spradling. 1998. Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification. *Development.* 125:2781–2789.

Eliazer, S., N.A. Shalaby, and M. Buszczak. 2011. Loss of lysine-specific demethylase 1 nonautonomously causes stem cell tumors in the

Drosophila ovary. *Proc. Natl. Acad. Sci. USA.* 108:7064–7069. http://dx.doi.org/10.1073/pnas.1015874108

Forbes, A.J., H. Lin, P.W. Ingham, and A.C. Spradling. 1996. hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in *Drosophila*. *Development.* 122:1125–1135.

Fuchs, E., T. Tumbar, and G. Guasch. 2004. Socializing with the neighbors: Stem cells and their niche. *Cell.* 116:769–778. http://dx.doi.org/10.1016/S0092-8674(04)00255-7

Fuller, M.T., and A.C. Spradling. 2007. Male and female *Drosophila* germline stem cells: Two versions of immortality. *Science.* 316:402–404. http://dx.doi.org/10.1126/science.1140861

Gay, L., K.V. Karfilis, M.R. Miller, C.Q. Doe, and K. Stankunas. 2014. Applying thiouracil tagging to mouse transcriptome analysis. *Nat. Protoc.* 9:410–420. http://dx.doi.org/10.1038/nprot.2014.023

Gregory, L., P.J. Came, and S. Brown. 2008. Stem cell regulation by JAK/STAT signaling in *Drosophila*. *Semin. Cell Dev. Biol.* 19:407–413. http://dx.doi.org/10.1016/j.semcdb.2008.06.003

Hayashi, Y., T.R. Sexton, K. Dejima, D.W. Perry, M. Takemura, S. Kobayashi, H. Nakato, and D.A. Harrison. 2012. Glypicans regulate JAK/STAT signaling and distribution of the unpaired morphogen. *Development.* 139:4162–4171. http://dx.doi.org/10.1242/dev.078055

Hsu, H.J., L. LaFever, and D. Drummond-Barbosa. 2008. Diet controls normal and tumorous germline stem cells via insulin-dependent and -independent mechanisms in *Drosophila*. *Dev. Biol.* 313:700–712. http://dx.doi.org/10.1016/j.ydbio.2007.11.006

Jin, Z., A.S. Flynt, and E.C. Lai. 2013. *Drosophila* piwi mutants exhibit germline stem cell tumors that are sustained by elevated Dpp signaling. *Curr. Biol.* 23:1442–1448. http://dx.doi.org/10.1016/j.cub.2013.06.021

Kai, T., and A. Spradling. 2003. An empty *Drosophila* stem cell niche reactivates the proliferation of ectopic cells. *Proc. Natl. Acad. Sci. USA.* 100:4633–4638. http://dx.doi.org/10.1073/pnas.0830856100

Kai, T., and A. Spradling. 2004. Differentiating germ cells can revert into functional stem cells in *Drosophila melanogaster* ovaries. *Nature.* 428:564–569. http://dx.doi.org/10.1038/nature02436

King, F.J., A. Szakmary, D.N. Cox, and H. Lin. 2001. Yb modulates the divisions of both germline and somatic stem cells through piwi- and hh-mediated mechanisms in the *Drosophila* ovary. *Mol. Cell.* 7:497–508. http://dx.doi.org/10.1016/S1097-2765(01)00197-6

Kirilly, D., S. Wang, and T. Xie. 2011. Self-maintained escort cells form a germline stem cell differentiation niche. *Development.* 138:5087–5097. http://dx.doi.org/10.1242/dev.067850

König, A., A.S. Yatsenko, M. Weiss, and H.R. Shcherbata. 2011. Ecdysteroids affect *Drosophila* ovarian stem cell niche formation and early germline differentiation. *EMBO J.* 30:1549–1562. http://dx.doi.org/10.1038/emboj.2011.73

Lee, J.J., D.P. von Kessler, S. Parks, and P.A. Beachy. 1992. Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene hedgehog. *Cell.* 71:33–50. http://dx.doi.org/10.1016/0092-8674(92)90264-D

Li, L., and T. Xie. 2005. Stem cell niche: Structure and function. *Annu. Rev. Cell Dev. Biol.* 21:605–631. http://dx.doi.org/10.1146/annurev.cellbio.21.012704.131525

Lin, H. 2002. The stem-cell niche theory: Lessons from flies. *Nat. Rev. Genet.* 3:931–940. http://dx.doi.org/10.1038/nrg952

Liu, M., T.M. Lim, and Y. Cai. 2010. The *Drosophila* female germline stem cell lineage acts to spatially restrict DPP function within the niche. *Sci. Signal.* 3:ra57. http://dx.doi.org/10.1126/scisignal.2000740

López-Onieva, L., A. Fernández-Miñán, and A. González-Reyes. 2008. Jak/Stat signalling in niche support cells regulates dpp transcription to control germline stem cell maintenance in the *Drosophila* ovary. *Development.* 135:533–540. http://dx.doi.org/10.1242/dev.016121

Losick, V.P., L.X. Morris, D.T. Fox, and A. Spradling. 2011. *Drosophila* stem cell niches: A decade of discovery suggests a unified view of stem cell regulation. *Dev. Cell.* 21:159–171. http://dx.doi.org/10.1016/j.devcel.2011.06.018

Lu, W., M.O. Casanueva, A.P. Mahowald, M. Kato, D. Lauterbach, and E.L. Ferguson. 2012. Niche-associated activation of rac promotes the asymmetric division of *Drosophila* female germline stem cells. *PLoS Biol.* 10:e1001357. http://dx.doi.org/10.1371/journal.pbio.1001357

Luo, L., H. Wang, C. Fan, S. Liu, and Y. Cai. 2015. Wnt ligands regulate Tkv expression to constrain Dpp activity in the *Drosophila* ovarian stem cell niche. *J. Cell Biol.* 209:595–608. http://dx.doi.org/10.1083/jcb.201409142

Ma, C., Y. Zhou, P.A. Beachy, and K. Moses. 1993. The segment polarity gene hedgehog is required for progression of the morphogenetic furrow in the developing *Drosophila* eye. *Cell.* 75:927–938. http://dx.doi.org/10.1016/0092-8674(93)90536-Y

Ma, X., S. Wang, T. Do, X. Song, M. Inaba, Y. Nishimoto, L.P. Liu, Y. Gao, Y. Mao, H. Li, et al. 2014. Piwi is required in multiple cell types to control germline stem cell lineage development in the *Drosophila* ovary. *PLoS ONE.* 9:e90267. http://dx.doi.org/10.1371/journal.pone.0090267

Mandal, L., J.A. Martinez-Agosto, C.J. Evans, V. Hartenstein, and U. Banerjee. 2007. A Hedgehog- and Antennapedia-dependent niche maintains *Drosophila* haematopoietic precursors. *Nature.* 446:320–324. http://dx.doi.org/10.1038/nature05585

Miller, M.R., K.J. Robinson, M.D. Cleary, and C.Q. Doe. 2009. TU-tagging: Cell type-specific RNA isolation from intact complex tissues. *Nat. Methods.* 6:439–441. http://dx.doi.org/10.1038/nmeth.1329

Ohlstein, B., and D. McKearin. 1997. Ectopic expression of the *Drosophila* Bam protein eliminates oogenic germline stem cells. *Development.* 124:3651–3662.

Price, M.A., and D. Kalderon. 1999. Proteolysis of cubitus interruptus in *Drosophila* requires phosphorylation by protein kinase A. *Development.* 126:4331–4339.

Price, M.A., and D. Kalderon. 2002. Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by Glycogen Synthase Kinase 3 and Casein Kinase 1. *Cell.* 108:823–835. http://dx.doi.org/10.1016/S0092-8674(02)00664-5

Rangan, P., C.D. Malone, C. Navarro, S.P. Newbold, P.S. Hayes, R. Sachidanandam, G.J. Hannon, and R. Lehmann. 2011. piRNA production requires heterochromatin formation in *Drosophila*. *Curr. Biol.* 21:1373–1379. http://dx.doi.org/10.1016/j.cub.2011.06.057

Rojas-Ríos, P., I. Guerrero, and A. González-Reyes. 2012. Cytoneme-mediated delivery of hedgehog regulates the expression of bone morphogenetic proteins to maintain germline stem cells in *Drosophila*. *PLoS Biol.* 10:e1001298. http://dx.doi.org/10.1371/journal.pbio.1001298

Sahai-Hernandez, P., and T.G. Nystul. 2013. A dynamic population of stromal cells contributes to the follicle stem cell niche in the *Drosophila* ovary. *Development.* 140:4490–4498. http://dx.doi.org/10.1242/dev.098558

Sauer, S., S.S. Burkett, M. Lewandoski, and A.J. Klar. 2013. A CO-FISH assay to assess sister chromatid segregation patterns in mitosis of mouse embryonic stem cells. *Chromosome Res.* 21:311–328. http://dx.doi.org/10.1007/s10577-013-9358-8

Schneider, L., and F. d'Adda di Fagagna. 2012. Neural stem cells exposed to BrdU lose their global DNA methylation and undergo astrocytic differentiation. *Nucleic Acids Res.* 40:5332–5342. http://dx.doi.org/10.1093/nar/gks207

Seecof, R.L., and S.A. Dewhurst. 1976. A 5-bromodeoxyuridine-sensitive interval during drosophila myogenesis. *Differentiation.* 6:27–32. http://dx.doi.org/10.1111/j.1432-0436.1976.tb01464.x

Sekimoto, T., N. Imamoto, K. Nakajima, T. Hirano, and Y. Yoneda. 1997. Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. *EMBO J.* 16:7067–7077. http://dx.doi.org/10.1093/emboj/16.23.7067

Silver, D.L., and D.J. Montell. 2001. Paracrine signaling through the JAK/STAT pathway activates invasive behavior of ovarian epithelial cells in Drosophila. *Cell.* 107:831–841. http://dx.doi.org/10.1016/S0092-8674(01)00607-9

Song, X., M.D. Wong, E. Kawase, R. Xi, B.C. Ding, J.J. McCarthy, and T. Xie. 2004. Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the *Drosophila* ovary. *Development.* 131:1353–1364. http://dx.doi.org/10.1242/dev.01026

Song, X., G.B. Call, D. Kirilly, and T. Xie. 2007. Notch signaling controls germline stem cell niche formation in the *Drosophila* ovary. *Development.* 134:1071–1080. http://dx.doi.org/10.1242/dev.003392

Southall, T.D., C.M. Davidson, C. Miller, A. Carr, and A.H. Brand. 2014. Dedifferentiation of neurons precedes tumor formation in Lola mutants. *Dev. Cell.* 28:685–696. http://dx.doi.org/10.1016/j.devcel.2014.01.030

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:research0034-research0034.11. http://dx.doi.org/10.1186/gb-2002-3-7-research0034

Wang, L., Z. Li, and Y. Cai. 2008. The JAK/STAT pathway positively regulates DPP signaling in the *Drosophila* germline stem cell niche. *J. Cell Biol.* 180:721–728. http://dx.doi.org/10.1083/jcb.200711022

Wang, X., L. Pan, S. Wang, J. Zhou, W. McDowell, J. Park, J. Haug, K. Staehling, H. Tang, and T. Xie. 2011. Histone H3K9 trimethylase Eggless controls germline stem cell maintenance and differentiation. *PLoS Genet.* 7:e1002426. http://dx.doi.org/10.1371/journal.pgen.1002426

Xie, T., and A.C. Spradling. 1998. *decapentaplegic* is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. *Cell.* 94:251–260. http://dx.doi.org/10.1016/S0092-8674(00)81424-5

Xie, T., and A.C. Spradling. 2000. A niche maintaining germ line stem cells in the *Drosophila* ovary. *Science.* 290:328–330. http://dx.doi.org/10.1126/science.290.5490.328

Xuan, T., T. Xin, J. He, J. Tan, Y. Gao, S. Feng, L. He, G. Zhao, and M. Li. 2013. dBre1/dSet1-dependent pathway for histone H3K4 trimethylation has essential roles in controlling germline stem cell maintenance and germ cell differentiation in the *Drosophila* ovary. *Dev. Biol.* 379:167–181. http://dx.doi.org/10.1016/j.ydbio.2013.04.015

Yadlapalli, S., and Y.M. Yamashita. 2013. Chromosome-specific nonrandom sister chromatid segregation during stem-cell division. *Nature.* 498:251–254. http://dx.doi.org/10.1038/nature12106

Zhang, Y., and D. Kalderon. 2001. Hedgehog acts as a somatic stem cell factor in the *Drosophila* ovary. *Nature.* 410:599–604. http://dx.doi.org/10.1038/35069099

Zhao, Y., C. Tong, and J. Jiang. 2007. Hedgehog regulates smoothened activity by inducing a conformational switch. *Nature.* 450:252–258. http://dx.doi.org/10.1038/nature06225