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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Yorkie and Hedgehog independently restrict BMP production in escort cells to permit germline differentiation in the *Drosophila* ovary

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ABSTRACT

Multiple signaling pathways guide the behavior and differentiation of both germline stem cells (GSCs) and somatic follicle stem cells (FSCs) in the *Drosophila* germarium, necessitating careful control of signal generation, range and responses. Signal integration involves escort cells (ECs), which promote differentiation of the GSC derivatives they envelop, provide niche signals for FSCs and derive directly from FSCs in adults. Hedgehog (Hh) signaling induces the Hippo pathway effector Yorkie (Yki) to promote proliferation and maintenance of FSCs, but Hh also signals to ECs, which are quiescent. Here, we show that in ECs both Hh and Yki limit production of BMP ligands to allow germline differentiation. Loss of Yki produced a more severe germarial phenotype than loss of Hh signaling and principally induced a different BMP ligand. Moreover, Yki activity reporters and epistasis tests showed that Yki does not mediate the key actions of Hh signaling in ECs. Thus, both the coupling and output of the Hh and Yki signaling pathways differ between FSCs and ECs despite their proximity and the fact that FSCs give rise directly to ECs.

KEY WORDS: Yorkie, Hedgehog signaling, Stem cell niche, *Drosophila* ovary, Escort cell

INTRODUCTION

The physiological function of stem cells is to support tissue regeneration throughout adult life. This requires the maintenance of a stem cell pool, the production of non-stem cell derivatives at a suitable rate and the appropriate differentiation of those products. Each of these behaviors is substantially guided by exposing stem cells and their derivatives to position-specific combinations of external signals (Losick et al., 2011). Cells that provide these signals are sometimes described as constituting a stem cell niche or a differentiation niche (Xie, 2013). These support cells may be fabricated prior to adulthood to produce a stable niche architecture that is long-lived and exhibits invariant properties. Alternatively, some or all of the cells contributing to stem cell and differentiation niches may be renewed during adult life, sometimes from the very stem cells that they support (Hsu and Fuchs, 2012). We aim to understand the network of cell lineages and signaling communications that coordinate the activities of stem cells, their differentiating products and niche cells.

The germarium at the anterior end of *Drosophila* ovarioles provides an especially intriguing and accessible example of interactions among stem cells and niche cells (Nystul and Spradling, 2006; Losick et al., 2011; Xie, 2013). The germarium (Fig. 1A) maintains germline and somatic stem cells, supports the early differentiation of both types of stem cell derivative and ensures coordinated output. The net result is a new egg chamber, consisting of a 16-cell germline cyst surrounded by a follicle cell epithelium, emerging from the posterior of the germarium every ∼12 h in well-fed flies.

Two or three germline stem cells (GSCs) adhere to stable cap cells at the anterior end of the germarium (Fig. 1A) and generally divide along the anterior-posterior axis of the germarium with asymmetric outcomes to produce cystoblasts (CBs). GSC maintenance depends on two BMP signaling molecules, Decapentaplegic (Dpp) and Glass-bottom boat (Gbb), which are produced principally by cap cells in response to JAK-STAT pathway activation (Song et al., 2004; Lopez-Onieva et al., 2008; Xie, 2013). Several mechanisms ensure that BMP signaling is largely restricted to GSCs (Harris and Ashe, 2011; Xia et al., 2012; Xie, 2013). Germline cells posterior to the GSCs exhibit very little evidence of BMP signal transduction (measured by phospho-Mad antibody staining) or BMP target gene expression (conveniently monitored by *Dad-lacZ*). Crucially, this allows CBs and their descendants to initiate expression of an essential differentiation factor, Bag of marbles (Bam), that is directly transcriptionally repressed by BMP signaling in GSCs.

In contrast to GSCs, the stem cells that support follicle cell (FC) production, known as follicle stem cells (FSCs), are maintained by population asymmetry rather than by invariant divisions with asymmetric outcomes, they are not firmly anchored like GSCs but circulate within a larger territory of two or three rings lining the germarial wall, roughly midway along the germarium (Fig. 1A) (Reilein et al., 2017), and their maintenance depends on several signals produced by non-adjacent niche cells (Vied et al., 2012). Two key niche signals, Hedgehog (Hh) and Wnts, derive from cap cells and quiescent escort cells (ECs) anterior to the FSCs, whereas a third key signal, the JAK-STAT pathway ligand Unpaired, derives principally from specialized FCs, known as polar cells, posterior to FSCs (Vied et al., 2012; Sahai-Hernandez and Nystul, 2013; Reilein et al., 2017).

ECs surround GSCs and their derivatives as they mature into 16-cell cysts prior to displacement by FCs (Fig. 1A) (Decotto and Spradling, 2005; Morris and Spradling, 2011). ECs are required for normal germ cell differentiation and, reciprocally, EC morphology and survival depend on contact with differentiating germline cells (Kirilly et al., 2011). ECs are renewed throughout adult life and,

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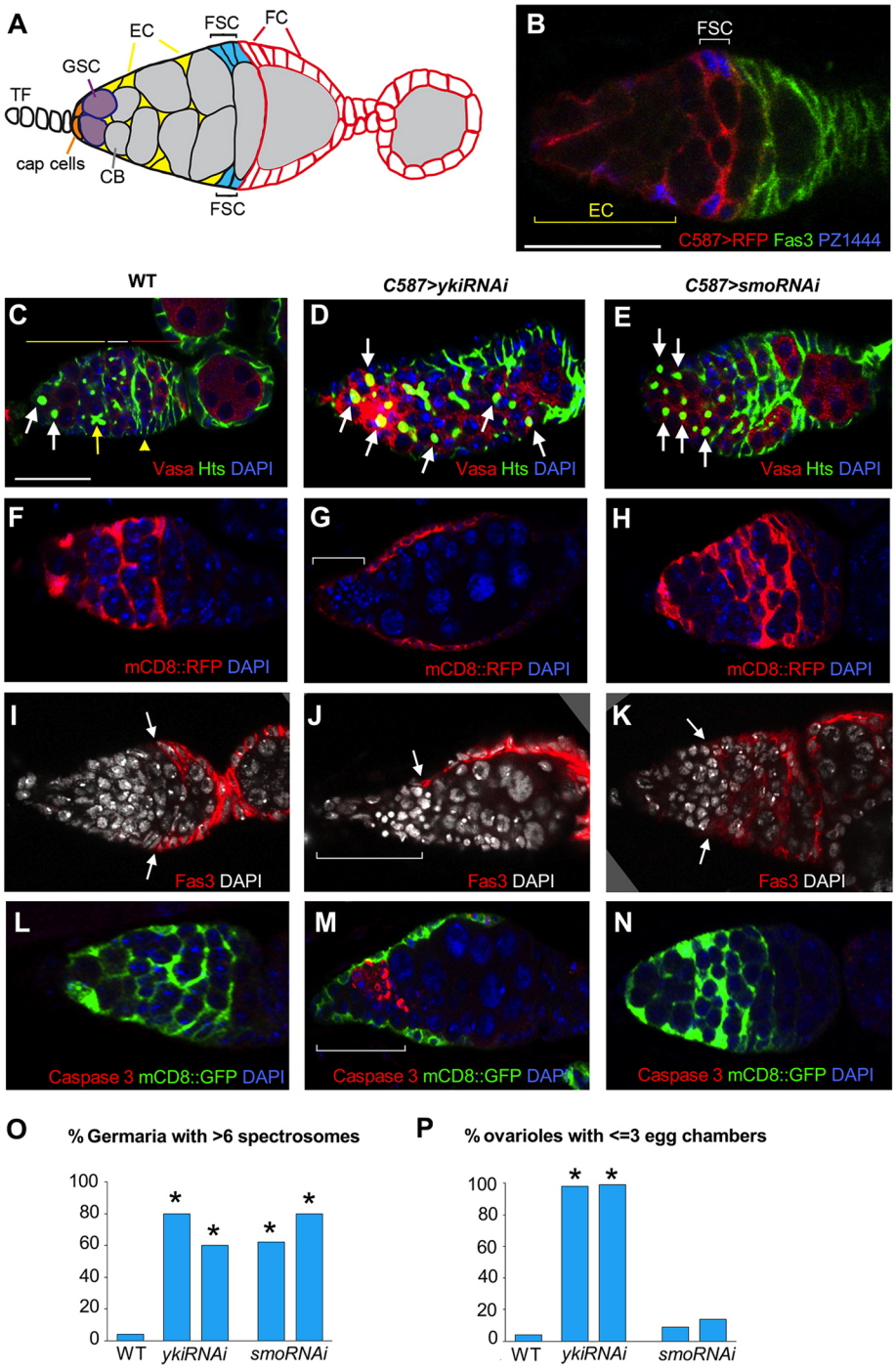

Fig. 1. Germline differentiation defects in germaria with reduced Yki or Smo activity. (A) Diagram of a germarium and (B) antibody staining for markers. Germline stem cells (GSCs) and cystoblast (CB) daughters (gray) have a spectrosome, whereas developing germline cysts (gray) have a fusome instead. Terminal filament (TF) and cap cells express *bab-GAL4*. Escort cells (ECs) and some follicle stem cells (FSCs) express *C587-GAL4* [driving *UAS-CD8-RFP* (red) in B] and *PZ1444-lacZ* (blue in B). Follicle cells (FCs) express Fas3 (red in A, green in B). (C-N) Germaria with *C587-GAL4* alone (C,F,I,L) or together with *UAS-yki RNA* (D,G,J,M) or *UAS-smo RNAi* (E,H,K,N) transgenes. (C-E) Vasa (red) antibody labels germline cells and Hts (green) antibody labels round spectrosomes (white arrows) and fusomes (yellow arrow), as well as the FC cortical cytoskeleton (yellow arrowhead). Horizontal lines indicate the EC (yellow), FSC (white) and FC (red) territory along the anterior-posterior axis (left to right). Reduction of (D) *yki* or (E) *smo* resulted in more spectrosome-containing cells [white arrows (not all marked)], abnormally far from the anterior (left), indicating impaired maturation of GSCs or CBs. (F-H) *UAS-CD8-RFP* driven by *C587-GAL4* (red) labels EC membranes. EC processes separate developing germline cysts (nuclei labeled in blue by DAPI) and collectively span the germarium (F). EC processes did not penetrate the interior in *yki* (G) but were mostly normal in *smo* (H) mutant germaria. (I-K) Fas3 (red) antibody marks early FCs and DAPI (gray) stains all nuclei. Arrows indicate the anterior limit of Fas3. The germarial region anterior to FCs was very small (J) only when *yki* was reduced. (L-N) Germaria labeled by *UAS-CD8-GFP* expression (green) using *C587-GAL4* were frequently stained with antibody to activated Caspase 3 (red) only when *yki* activity was reduced (M). Brackets (G,J,M) indicate a reduced germarium, fused to a compound unbudded egg chamber. Scale bars: 20 µm. (O,P) Percentage of germaria with more than six spectrosomes (O) and percentage of ovarioles containing three or more budded egg chambers (P) for wild-type (WT) and animals expressing two different RNAi transgenes directed to *yki* or *smo* using *C587-GAL4*. Left to right: (O) *n*=202, 120, 135, 196, 165 and (P) *n*=198, 99, 88, 120, 135 biologically independent ovarioles. **P*<0.0001, versus WT (Fisher's exact two-tailed test).

intriguingly, it was recently shown that they derive from FSCs (Reilein et al., 2017). Thus, ECs are produced by FSCs, respond to germline cells and provide niche functions for both FSCs and germline differentiation.

All of these EC behaviors are likely to be regulated by specific signals, some of which may have different roles in regulating nearby FSCs, GSCs and germline derivatives. How are these signals and their effectors organized to serve different purposes in different cells within the close-knit germarial community? Here, we examine the roles of Hh and Hippo/Yki pathway signaling in ECs and provide evidence for independent functions in limiting BMP ligand production in order to allow germline cell differentiation.

RESULTS

Yorkie and smoothened act in ECs to promote germ cell differentiation

FSC maintenance depends cell-autonomously on both Hh signaling and the activity of Yorkie (Yki), the regulated co-activator of the Hippo/Yki pathway (Huang and Kalderon, 2014). Moreover, excessive Hh pathway activity or excessive Yki activity renders FSCs hypercompetitive. Surprisingly, we found that Hh signaling acts principally by regulating Yki activity through transcriptional induction of *yki*, and that Yki acts by stimulating the proliferation rate of FSCs (Huang and Kalderon, 2014). Reporters of both Hh and Yki activity are also expressed strongly in ECs, suggesting that Hh

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and Yki might have different roles in ECs, which rarely, if ever, divide.

We used the *C587-GAL4* enhancer trap, which is mainly expressed in ECs of adult ovaries (Fig. 1B) (Song et al., 2004), to increase or decrease Yki activity. Yki activity is normally limited by the actions of two protein kinases, Hippo (Hpo) and Warts (Wts), while Scalloped (Sd) is a frequent DNA-binding partner, activating target genes when bound to Yki but repressing the same target genes in the absence of Yki (Staley and Irvine, 2012). Activation of Yki by expressing GAL4-responsive *yki* (*UAS-yki*) or RNAi against *hpo* or *wts* (*UAS-hpo RNAi*, *UAS-wts RNAi*) produced no discernibly abnormal phenotype (Fig. S1), using two independent transgenes for each. Similarly, reduction of *sd* in ECs produced no clear phenotype (Fig. S1).

By contrast, reducing Yki activity with *UAS-yki RNAi* produced a dramatic phenotype comprising several features. First, these germaria included many cells with round spectrosomes and few multicellular germline cysts with branched fusomes (Fig. 1D,O). In normal germaria, only GSCs and CBs at the anterior of the germarium have a round spectrosome, which is stained by Hts antibody and matures into the fusomes of differentiating germline cysts (Fig. 1C,O). Because GSCs are normally anchored to cap cells, we refer to these ectopic, more posterior spectrosome-containing cells as CB-like. Second, EC processes, which normally wrap around germline cells to form a web across the germarium were completely absent from the interior of germaria when Yki was downregulated in ECs; ECs still had processes, but they were aligned with the germarial wall (Fig. 1F,G). Third, the anterior portions of *yki*-deficient germaria were much smaller than normal, with a significantly reduced complement of ECs (Fig. 1I,J). The posterior germarial regions included large germline nuclei (Fig. 1G, J,M), indicating that these are advanced egg chambers that never separated from the germarium. Fourth, activated Caspase 3 staining revealed a high frequency of apoptotic cell death in both ECs and germline cells (Fig. 1L,M). Consistent with the virtual absence of mature germline cysts in *yki RNAi* germaria and an apparent failure of egg chamber budding (Fig. 1D,G,J,M), ovaries contained very few egg chambers (Fig. 1P) and were tiny.

Since alterations to Yki and Hh pathway activities have almost identical phenotypes in FSCs we then used *C587-GAL4* to express RNAi in order to deplete Smoothened (Smo), the essential transducer of Hh signaling, in ECs. We observed a large increase in the number of CB-like cells with round spectrosomes (Fig. 1E,O). However, unlike Yki-deficient germaria, these *UAS-smo RNAi* germaria included maturing germline cysts and the presence of at least three egg chambers per ovariole indicated continued budding (Fig. 1P). There was no evidence of increased apoptosis and these germaria were of roughly normal size (in fact, slightly fatter than normal; Fig. 1E,H,K,N). Furthermore, EC processes, revealed by CD8-RFP expression, contacted germline cell surfaces across the entire width of the germarium (Fig. 1H,N).

Both Yki and Smo function in ECs during adulthood

The *C587-GAL4* driver used to knock down Smo and Yki activities is expressed in a wider set of somatic cells, including developing terminal filament (TF) and cap cells, during ovary formation in larvae and pupae before becoming restricted to ECs and, more weakly, FSCs in adults (Zhu and Xie, 2003) (Fig. 1B). Driving the expression of *yki RNAi*, *smo RNAi* or *hpo RNAi* or the excess expression of the Hh pathway effector Ci with *bab-GAL4*, which is expressed in developing and adult TF and cap cells, or with *109-30-GAL4*, which is expressed in FSCs and early FCs (Fig. 1A), produced no abnormalities (Fig. S2), suggesting that loss of Yki and Hh signaling in ECs is crucial for the phenotypes observed with *C587-GAL4*.

To determine whether these phenotypes were due to Smo and Yki functions in adults or during ovary development we utilized the well-established increase of GAL4-specific activity at higher temperatures. The phenotypes described above for 6-day-old flies raised at 25°C were absent from flies raised at 18°C up to 12 days of age. We therefore raised flies at 18°C and moved half of the adults to 29°C. After 6 days at 29°C, at least 25% of germaria from both *yki RNAi* and *smo RNAi* samples included extra CB-like cells, and this proportion increased to over 60% by day 12 (Fig. 2A). In all cases, extra CB-like cells were slightly more frequent for *yki RNAi* than for *smo RNAi*, whereas controls maintained at 18°C throughout showed no such phenotypes. Thus, reduction of Hh pathway activity or Yki activity only in adults sufficed to produce a strong defect in germline cell differentiation.

We also tested the converse temperature shift, raising flies at 29°C from third instar onward and then moving half to 18°C after eclosion. Flies raised and maintained at 29°C throughout exhibited a high frequency of excess CB-like cells (although no more severe than for flies kept at 25°C throughout) but this phenotype was

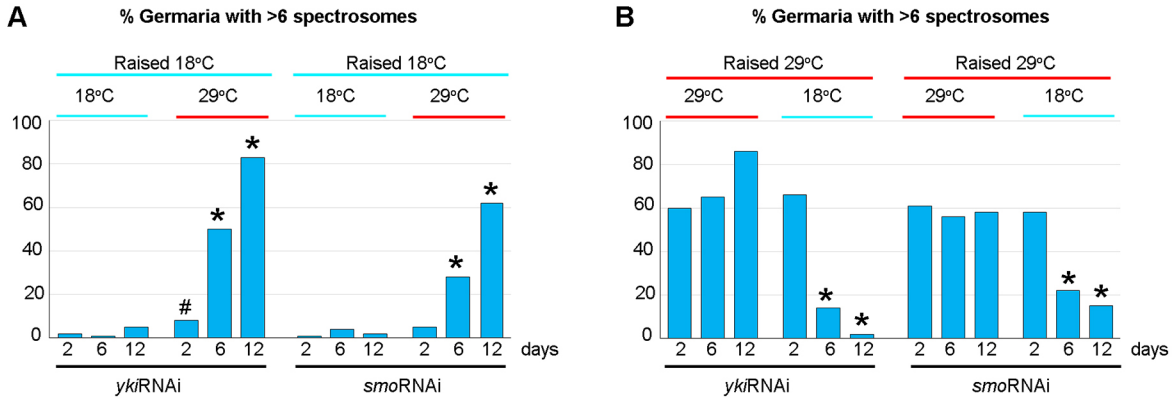

Fig. 2. Germline differentiation defects due to Yki or Smo inhibition can arise developmentally or in adults. (A) Animals carrying *C587-GAL4* and *UAS-yki RNAi* or *UAS-smo RNAi* were raised at 18°C up to eclosion and then maintained at 18°C or 29°C for the number of days indicated before ovary dissection. The percentage of germaria with six or more spectrosomes is shown. (B) An analogous experiment in which animals were maintained at 29°C from third larval instar until eclosion before moving half to 18°C. Left to right: (A) *n*=115, 98, 135, 122, 90, 88, 129, 115, 108, 99, 93, 76 and (B) *n*=83, 77, 107, 105, 123, 122, 85, 72, 92, 81, 120, 112 biologically independent germaria. **P*<0.0001, #*P*<0.05, for 18°C versus 29°C for the same time period (Fisher's exact two-tailed test).

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reduced to a penetrance of less than 25% by 6 days at 18°C and of 15% or less by day 12 for both *yki RNAi* and *smo RNAi*, showing that restoration of Yki and Smo function in ECs could restore normal germline differentiation (Fig. 2B). In animals transferred to 18°C, *yki RNAi* germaria and ovarioles also increased in size and multiple egg chambers accumulated.

Yki and Hh pathway activities in ECs restrict BMP signaling to GSCs

Normally, BMP signaling is restricted largely to GSCs within the germline. Consequently, high levels of the BMP target gene reporter *Dad-lacZ* are limited to two or three GSCs, with occasional staining of CBs (Fig. 3A) (Harris and Ashe, 2011; Xie, 2013). However, in germaria with reduced Yki activity in ECs, many more cells expressed high levels of *Dad-lacZ*. Ectopic staining was seen in up to ten cells (five to six on average), including some cells in the posterior half of the germarium, with aberrant *Dad-lacZ* staining seen in 30 of 96 germaria examined (31%) (Fig. 3B). A similar phenotype was seen in Smo-deficient germaria (21 of 83 germaria, 25%), with a similar number of ectopically staining cells and a similar intensity of ectopic *Dad-lacZ* staining (Fig. 3C).

Antibodies specific to phosphorylated Mad (pMad) reveal active BMP signal transduction and normally show a similar distribution of staining to *Dad-lacZ*, with clear signals only in GSCs and occasional CBs (Fig. 3D). Sporadic ectopic pMad staining was observed in additional CB-like cells throughout the germaria when either Yki (22 examples in 68 germarium, 32%) or Smo (20 examples in 100 germarium, 20%) activity was reduced in ECs (Fig. 3E,F).

A key consequence of BMP signaling is the repression of *bam* expression in GSCs, normally permitting expression and accumulation of Bam-GFP only in differentiating germ cells beyond the CB stage (Fig. 3G) (Kirilly et al., 2011). However, in germaria with reduced *yki* function many (38 out of 70, 54%) expressed very little or no Bam-GFP, consistent with aberrantly persistent repression by BMP signaling (Fig. 3H). Similarly, little or no Bam-GFP expression was observed in many (25 out of 60, 42%) germaria expressing *smo RNAi* in ECs (Fig. 3I).

Thus, both reduction in Hh signaling and in Yki activity show clear evidence of ectopic BMP signaling, which could plausibly account for the observed failure to derepress Bam expression and allow germline cell differentiation. The penetrance of ectopic BMP signaling and Bam-GFP silencing was between 20% and 54%, whereas the extra CB phenotype was closer to 70-80% penetrance. It is nevertheless possible that ectopic BMP signaling is entirely responsible for the extra CB phenotype, with different sensitivities of phenotype detection responsible for the apparent differences in penetrance.

Loss of Dpp and Gbb can partially rescue germline differentiation defects due to reduced Yki and Hh signaling

In *Drosophila* there are three BMP ligands: Dpp, Gbb and Screw (Scw) (Matsuda et al., 2016). Dpp and, to a lesser degree, Gbb have been shown to be important mediators of a niche signal, emanating principally from cap cells, that acts to maintain GSCs (Xie and Spradling, 1998; Song et al., 2004). Earlier studies showed that reduced activity of the small GTPase Rho or of Lysine-specific demethylase 1 [Lsd1; Su(var)3-3 – FlyBase] in ECs induces ectopic BMP pathway activity in germline cells, at least in part through ectopic expression of Dpp in ECs (Eliazer et al., 2011; Kirilly et al., 2011). By contrast, defects in EGF receptor signaling in ECs induce ectopic BMP signaling in germline cells through derepression of Dally, a glypican that can promote the spread of Dpp signaling (Liu

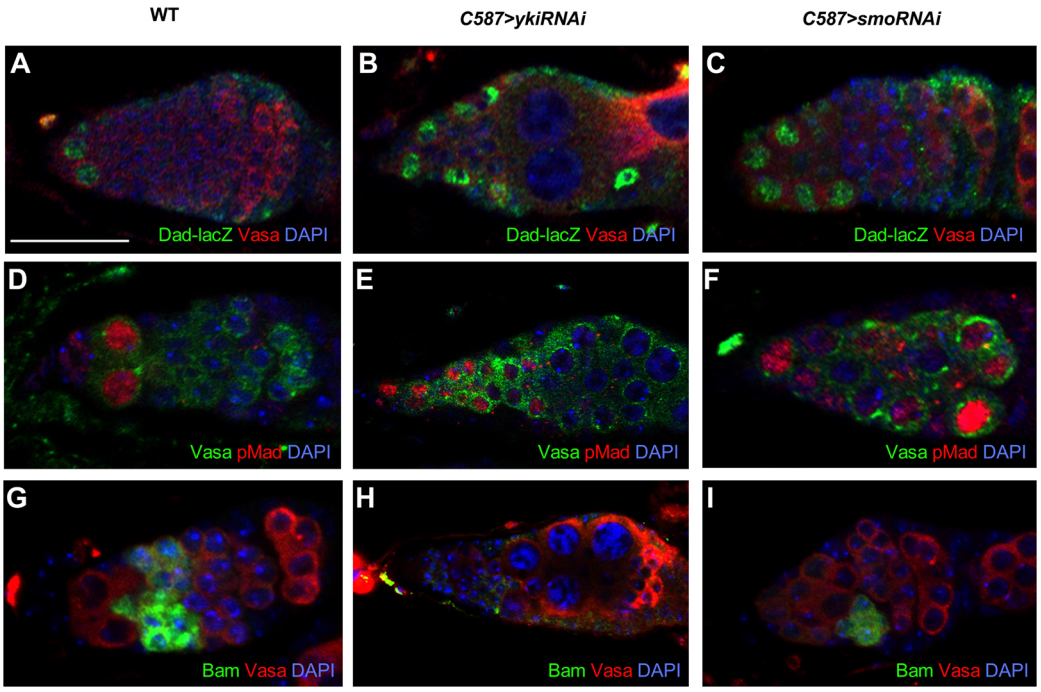

Fig. 3. Loss of Yki or Smo activity in ECs causes ectopic BMP pathway activity in germline cells. Germaria from wild-type females (A,D,G) and those expressing *UAS-yki RNAi* (B,E,H) or *UAS-smo RNAi* (C,F,I) using *C587-GAL4* were stained for markers of BMP pathway activity. (A-C) *Dad-lacZ* (green) in germline cells (marked by Vasa antibody, red) is (A) normally present only in GSCs but was present in additional cells when (B) *yki* or (C) *smo* activity was reduced in ECs. (D-F) pMad (red) immunostaining was similarly often observed in several cells beyond GSCs when (E) *yki* or (F) *smo* was inhibited. (G-I) Bam-GFP (green) is normally expressed in early differentiating cysts (Vasa, red) but was weak or absent in many germaria with reduced (H) *yki* or (I) *smo* activity in ECs. Scale bar: 20 µm.

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et al., 2010). We therefore tested whether the *yki RNAi* or *smo RNAi* phenotypes might result from ectopic expression of Dally or BMP ligands.

We found that co-expression of *dpp RNAi* using *C587-GAL4* strongly, but incompletely, suppressed the accumulation of excess CB-like cells normally observed for *yki RNAi* (Fig. 4A,B,G). This was accompanied by restoration of EC processes in a typical lattice pattern across some parts of the germarium with a similar penetrance of ~50% (Fig. 4I-L). Suppression of *yki RNAi* differentiation phenotypes by limiting *dpp* expression in ECs, which normally express little or no *dpp*, strongly suggests that a major contributor to the defect in germline differentiation is ectopic expression of *dpp* in ECs that lack Yki activity. Despite the clear amelioration of *yki* phenotypes evident from staining spectrosomes and EC processes, almost all germaria were small and of abnormal morphology, with very few showing any evidence of egg chamber budding (Fig. 4B).

Surprisingly, co-expression of *dpp RNAi* did not suppress the germline differentiation defect due to Smo reduction (Fig. 4D,G). The same results were observed for two different *UAS-dpp RNAi* lines, both of which substantially suppressed the *yki* differentiation defect. We therefore asked whether Gbb might be playing a role. Co-expression of *gbb RNAi* significantly suppressed the extra CB phenotype due to *smo RNAi* (reducing the penetrance from 74% to 34%; Fig. 4E,G). *gbb RNAi* also partially suppressed the *yki RNAi* CB differentiation phenotype but less effectively than observed for *dpp RNAi* (from 70% to 52% for *gbb RNAi* and 34% for *dpp RNAi*) and with no restoration of EC processes penetrating into the germarium or egg chamber budding (Fig. 4C,G,K,L). Co-expression of *dally RNAi* had no effect on the phenotypes induced by either *smo RNAi* or *yki RNAi* (Fig. 4F,H).

We also tested *lsd1 RNAi* expression. In our hands, downregulation of Lsd1 alone produced a phenotype intermediate between those of *yki RNAi* and *smo RNAi*. Most germaria (66%) had extra CB-like cells and appeared to include an unbudded egg chamber, as for *yki RNAi* (Fig. S3A). In contrast to the *yki* mutant phenotype, EC processes generally penetrated into limited regions of the germarium and most germaria were associated with one or more egg chambers that contained larger, more mature germline nuclei than typical, indicating occasional, much delayed budding (Fig. S3B). We found that these phenotypes, including excess CB-like cells (Fig. 4G), were suppressed significantly by *gbb RNAi* but barely at all by *dpp RNAi* (Fig. S3A-G).

We also tested whether *dpp* or *gbb* inhibition could reduce ectopic pMad staining. *dpp RNAi* reduced the frequency of ectopic pMad for Yki-deficient germaria, whereas *gbb RNAi* was effective for germaria with Smo or Lsd1 inhibition (Fig. 4M).

Finally, the induction of BMP ligands was tested directly by qRT-PCR of RNA extracted from germaria and early egg chambers of different genotypes. Yki inhibition significantly increased *dpp* and *gbb* RNA levels, whereas Smo and Lsd1 inhibition only significantly increased *gbb* RNA levels (Table 1).

Thus, inhibition of Hh signaling in ECs induced ectopic Gbb with little or no Dpp, whereas loss of Yki activity induced both Gbb and Dpp, with the latter contributing more to the consequent ectopic BMP signaling. Reduced Lsd1 activity inhibited germline differentiation by inducing Gbb. Limiting ectopic BMP signaling improved germarial morphology and egg chamber budding for Lsd1 but not for Yki inhibition.

Interactions among Lsd1, Hh and Yki

Lsd1 removes methyl groups from lysine 4 of histone H3 (H3K4) and is targeted to specific chromosomal locations as part of a Trithorax-related (Trr) complex. Conversely, Yki can associate with Trr complexes that include Nuclear receptor coactivator 6 (Ncoa6) to increase H3K4 methylation (Qing et al., 2014). Loss of Lsd1 in ECs was reported to induce ectopic Hh expression (Eliazer et al., 2014), while Hh signaling regulates Yki activity in FSCs (Huang and Kalderon, 2014). We therefore investigated whether the related phenotypes induced by inhibition of Lsd1, Smo and Yki in ECs reflect direct causal connections.

The *lsd1* mutant phenotype was reported to include ectopic induction of two characteristic markers of cap cells, Engrailed (En) and Lamin C (LamC), in ECs, suggesting a transformation of EC identity towards that of cap cells, which normally produce Dpp and Gbb ligands (Eliazer et al., 2014). We observed no ectopic expression of En in ECs expressing *yki RNAi* or *smo RNAi* (Fig. 5A-C). There was also clearly no ectopic LamC expression for *smo RNAi* (Fig. 5E,G). Germaria with reduced Yki function did include some ectopically positioned LamC-positive cells (Fig. 5F). However, LamC is normally expressed by some FCs as well as cap cells (Fig. 5E), and the extensive loss of ECs in *yki RNAi* germaria allows FCs to encroach further anterior than normal. Ectopic LamC expression was never seen immediately adjacent to cap cells, suggesting that the LamC-positive cells in Yki-deficient germaria were most likely invading FCs rather than ECs (Fig. 5F). These LamC-positive cells frequently expressed *UAS-CD8-RFP* driven by *C587-GAL4*, which is not normally expressed in FCs, but the normal pattern of *C587-GAL4* expression is also probably disturbed by the unusually anterior location of FCs. Thus, neither Smo nor Yki inhibition showed clear evidence of cap cell marker expression in ECs.

In our hands, *C587-GAL4*-driven expression of *lsd1 RNAi* also did not induce ectopic En or LamC expression in ECs (Fig. 5D,H) despite producing a very strong germline differentiation defect. It is possible that our *lsd1 RNAi* experiment, which allowed some egg chamber budding, produced lower Lsd1 inhibition than in previous analyses using the same RNAi approach and *lsd1* null mutations (Eliazer et al., 2014). The set of phenotypes that we observed allow us to deduce that reduction of Lsd1 can produce germline differentiation defects in the absence of an apparent transformation of ECs towards cap cell identity, as observed for loss of Smo and Yki function.

We then examined a reporter of Hh pathway activity. Hh signaling induces *patched* (*ptc*) expression in all tissues and *ptc-lacZ* reporters have consequently been used as universal and highly specific reporters of Hh pathway activity. *ptc-lacZ* is normally absent from TF and cap cells but it is highly expressed in ECs and FSCs, with declining levels in FCs towards the posterior of the germarium (Vied and Kalderon, 2009). In germaria with *smo RNAi* expressed using *C587-GAL4* at 25°C, *ptc-lacZ* expression remained in FCs but was essentially undetectable in ECs (Fig. 6C,F,Q). We conclude that Hh signaling is drastically reduced by *smo RNAi* in all germaria. It is therefore very unlikely that the retention of normal EC process distribution (which was also seen at 29°C), or the absence of ectopic En or LamC, could be due to residual Hh signaling. We saw no significant change in *ptc-lacZ* expression in germaria from flies expressing *lsd1 RNAi* in ECs (Fig. 6L,O), suggesting that Lsd1-deficient phenotypes are unlikely to be due to loss of Hh signaling in ECs.

Is Yki a key mediator of the Hh response in ECs?

Diap1-lacZ and *ex-lacZ* reporters are responsive to Yki in many tissues (Staley and Irvine, 2012; Huang and Kalderon, 2014). Both are expressed in cap cells, ECs, FSCs and FCs in the germarium

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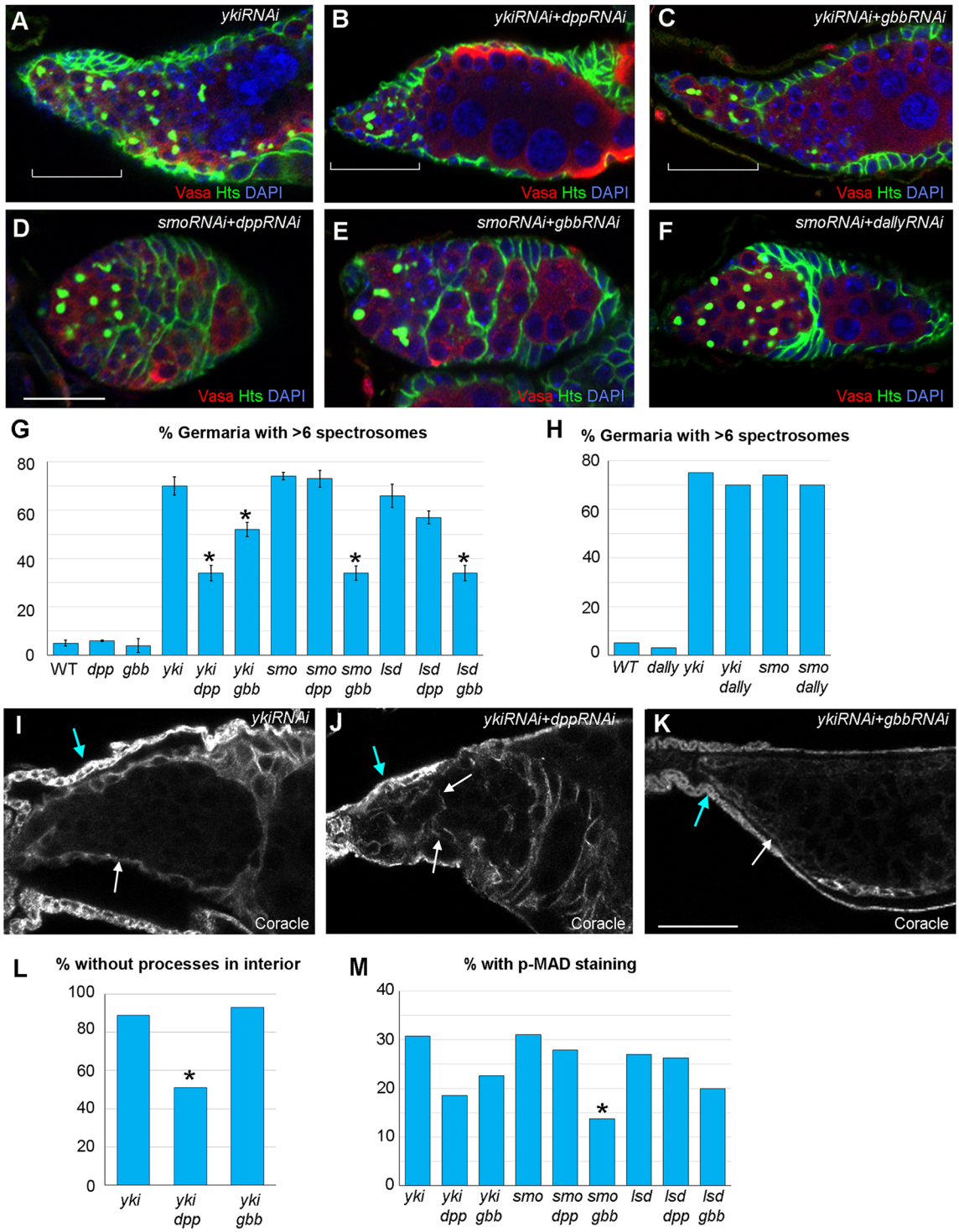

Fig. 4. Ectopic Dpp or Gbb production mediates germline differentiation defects for Yki and Smo dysfunction. (A-M) *C587-GAL4* was used to express *UAS-dpp RNAi*, *UAS-gbb RNAi* or *UAS-dally RNAi* alone or together with *yki RNAi* or *smo RNAi*. (A-F) Germaria stained for Hts (green) to visualize spectrosomes and fusomes, Vasa (red) to mark germline cells and DAPI (blue) to stain all nuclei. (A-C) Reduction of (B) *dpp* or (C) *gbb* reduced the number of spectrosomes in germaria expressing *yki RNAi* without restoring normal germarial size or egg chamber budding. (D-F) Ectopic spectrosomes were reduced in *smo*-deficient germaria by loss of (E) *gbb* but not by loss of (D) *dpp* or (F) *dally*. (G) Percentage of germaria with more than six spectrosomes for the indicated RNAi transgenes expressed with *C587-GAL4*. Mean and s.e.m. for three trials are shown using a total of (left to right) *n*=496, 467, 435, 338, 270, 340, 431, 332, 304, 238, 191, 206 biologically independent germaria. (H) Percentage of germaria with more than six spectrosomes for the indicated RNAi transgenes expressed with *C587-GAL4*. Left to right: *n*=106, 248, 72, 77, 113, 130 biologically independent germaria. (I-K) EC processes (white arrows) visualized by Coracle antibody staining (gray); blue arrows indicate muscle sheath staining. (L) Interior EC processes were absent for *yki RNAi* alone (I) or together with *gbb RNAi* (K) but were partially restored in germaria expressing *dpp RNAi* (J). *n*=36 (*yki*), 51 (*yki+dpp*) and 28 (*yki+gbb*) biologically independent germaria. (G,H,L) Significant differences imposed on the *yki RNAi* phenotype by *dpp RNAi* or *gbb RNAi* were calculated using Fisher's exact two-tailed test (*P<0.0001; all others, P>0.05). (M) Percentage of germaria with ectopic pMad staining upon expression of indicated the RNAi with *C587-GAL4*. Left to right: *n*=65, 54, 53, 58, 43, 58, 63, 57, 50 germaria. Significant differences due to *dpp RNAi* or *gbb RNAi* were calculated using Fisher's exact two-tailed test (*P<0.05). Scale bars: 20 µm.

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Table 1. *dpp* and *gbb* RNA levels measured by qRT-PCR in response to Yki, Smo and Lsd1 inhibition

		dpp RNA			*gbb* RNA		
	n	Mean/control	s.e.m.	*P*	Mean/control	s.e.m.	*P*
Control	3	1	0.06		1*	0.06	
yki RNAi	3	1.78	0.22	0.001	13.18	2.15	<0.001
smo RNAi	3	1.22	0.16	0.337	9.52	1.63	<0.001
lsd1 RNAi	3	0.71	0.13	0.173	193.21	69.32	<0.001

*Ratio of *dpp*/*gbb* RNA was 1458, 1584, 1067 in three trials (mean 1370, s.e.m. 270).

(Fig. 6A,B). *C587-GAL4* expression of *yki RNAi* eliminated detectable expression of these reporters in ECs while sparing expression in cap cells and FCs (where *C587-GAL4* is not expressed) (Fig. 6M-P). We conclude that both reporters provide very good measures of Yki activity in ECs and that *yki RNAi* is extremely effective at eliminating Yki activity in ECs.

We then tested whether *Diap1-lacZ* or *ex-lacZ* activity was affected by *smo RNAi*. There was no dramatic change evident by visual inspection (Fig. 6D,E) and quantitation confirmed that Smo inhibition had no substantial effect on these reporters of Yki activity in ECs (Fig. 6O,P). Both *Diap1-lacZ* and *ex-lacZ* activities were modestly increased by reducing Hpo activity (Fig. 6G,H,O,P), whereas *ptc-lacZ* activity was unchanged (Fig. 6I,Q). Lsd1 inhibition increased *Diap1-lacZ* and *ex-lacZ* expression (Fig. 6J, K,O,P).

In a complementary test, we found that Yki antibody staining of ECs showed no obvious difference in *smo RNAi* and *lsd1 RNAi* germaria, whereas it was much reduced in *yki RNAi* germaria (Fig. S4A-D). Thus, there is no indication that normal Yki protein levels or activity in ECs are promoted by Hh pathway activity.

Finally, we tested possible causal connections among Lsd1, Hh signaling and Yki genetically. Previously, we found that phenotypes due to loss of *smo* activity in FSCs were substantially rescued by expression of either a *tub-yki* transgene or a *UAS-yki* transgene (Huang and Kalderon, 2014). The penetrance of failed germline differentiation for *smo RNAi* was slightly reduced by *tub-yki* (from 79% to 69%) and not significantly altered by *UAS-yki*, using *C587-GAL4* to drive expression (Fig. 6R). The *lsd1 RNAi* differentiation defect was unaffected by *UAS-yki* or *tub-yki* (Fig. 6R). The *tub-yki* transgene fully rescued the *yki RNAi* phenotype, whereas *UAS-yki* provided very little rescue (Fig. S4E). Both transgenes contain sequences targeted by the *UAS-yki RNAi* transgene, so rescue of *yki RNAi* phenotypes is likely to drastically underestimate the increase in Yki protein provided in tests of suppression of *smo RNAi* and *lsd1 RNAi* phenotypes.

We also tested whether downregulation of Wts or Hpo could suppress germline differentiation defects. We saw no significant suppression of the *smo RNAi* or *lsd1 RNAi* phenotypes in either case (Fig. S4F). Thus, none of the four approaches for increasing Yki activity produced robust rescue of the *smo RNAi* differentiation defect, suggesting that the *smo* phenotype does not result from a reduction in Yki activity. This conclusion is consistent with the observed retention of substantial Yki activity in ECs with drastically reduced Smo function (Fig. 6O,P). We also found no evidence consistent with either Yki activity or Hh signaling serving as a downstream mediator of Lsd1 activity in ECs.

DISCUSSION

The intimate association of ECs with germline cells in the anterior half of the germarium immediately suggested functional interactions between the two cell types (Decotto and Spradling, 2005). Most contributions of ECs to the germline reported to date center on BMP signaling. The most anterior ECs may normally serve as a secondary source of BMP signals for GSCs (Rojas-Rios

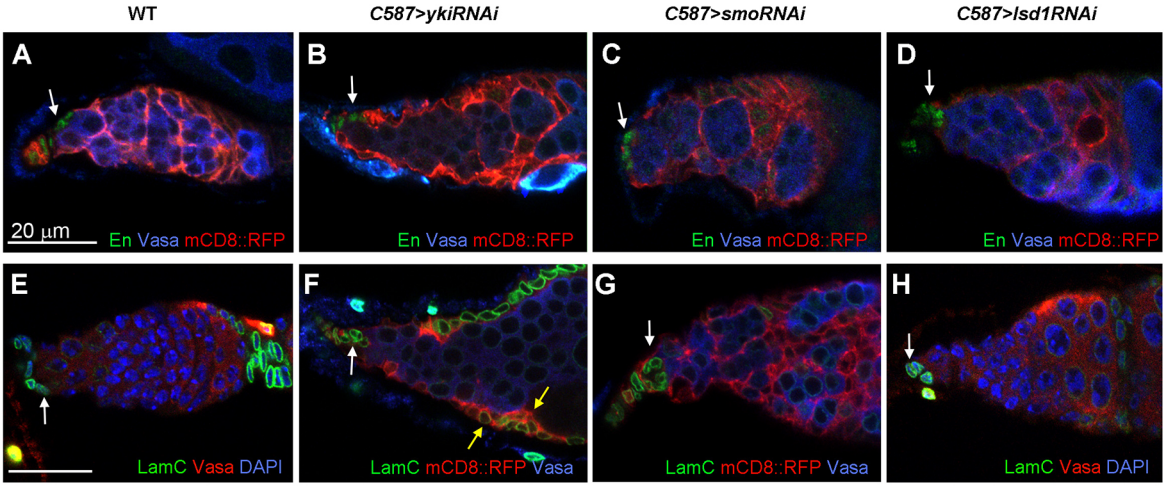

Fig. 5. Reduction of Yki or Smo activity in ECs does not show evidence of transformation to cap cell behavior. (A-D) Germaria from (A) controls or from females expressing (B) *yki RNAi*, (C) *smo RNAi* or (D) *lsd1 RNAi* together with CD8-RFP (red) in ECs using *C587-GAL4* all showed normal En antibody staining (green) in cap cells (arrows) without ectopic expression in ECs. Vasa antibody (blue) marks germline cells. Cap cells were recognized as being adjacent to TF cells, which have a characteristic morphology. (E-H) Ovarioles of the same genotypes were stained with LamC antibody (green). Vasa antibody (red in E,H; blue in F,G) marks germline cells and DAPI (blue in E,H) marks all nuclei. LamC is (E) normally expressed in cap cells (arrows) and some FCs with no changes when (G) *smo* or (H) *lsd1* activities were reduced in ECs. (F) Reduction of *yki* activity in ECs caused ectopic LamC staining in germarial regions, sometimes in cells that also expressed *UAS-CD8-RFP* (yellow arrows). Those cells were never adjacent to cap cells (white arrow) and are likely to represent FCs that encroach on territory normally occupied by ECs. Scale bars: 20 µm.

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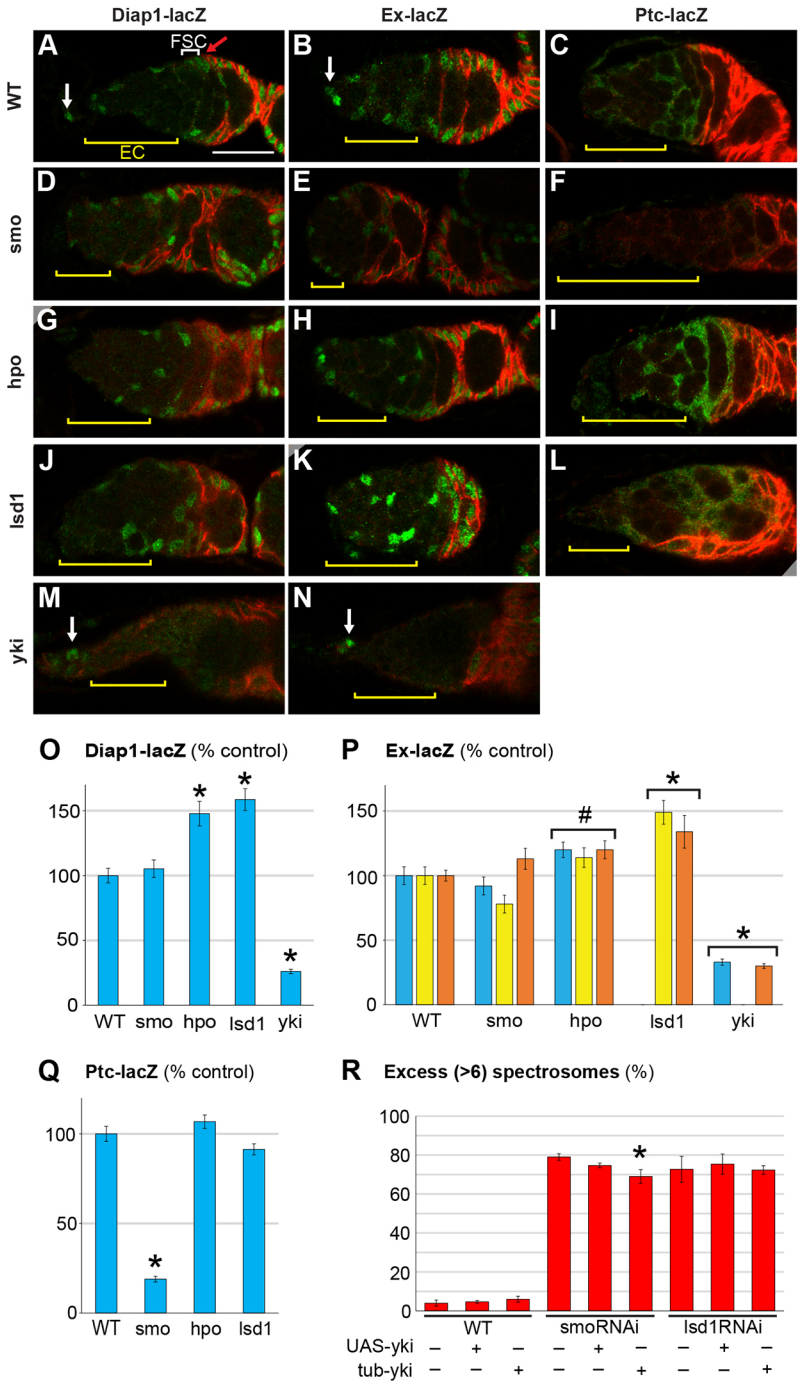

Fig. 6. Germline differentiation defect due to loss of Hh signaling in ECs is not mediated by changes in Yki activity. (A-Q) Yki activity was monitored by (A,D,G,J,M,O) *Diap1-lacZ* and (B,E,H,K,N,P) *ex-lacZ* reporters, while Hh pathway activity was monitored by (C,F,I,L,Q) *ptc-lacZ* in (A-C) controls and (D-N) females expressing the indicated *UAS-RNAi* transgenes using *C587-GAL4*. (A-N) Germaria stained with antibody to β-galactosidase (green) and Fas3 (red) to mark the border between FSCs and FCs. EC territory is indicated by a yellow bracket and cap cells are indicated by arrows. (O-Q) Mean staining intensity (with s.e.m.) of reporter gene products quantified in multiple ECs. Left to right: (O) *n*=49, 58, 60, 19, 30, (P) *n*=17, 42, 30, 30, 27, 27, 40, 24, 24, 20, 24, 18, 24 and (Q) *n*=32, 25, 44, 13 ECs. **P*<0.0001, #*P*<0.01 (for all others *P*>0.05) versus control values (Student's unpaired *t*-test), combining all measurements of *ex-lacZ* for a given genotype. (R) Percentage of germaria with more than six spectrosomes for animals containing the indicated transgenes in addition to *C587-GAL4*. Mean and s.e.m. for three trials are shown using a total of (left to right) *n*=476, 373, 425, 463, 291, 376, 429, 240, 331 biologically independent germaria. Significant differences imposed on the *smo RNAi* or *lsd1 RNAi* phenotype by *yki* transgenes were calculated using Fisher's exact two-tailed test (**P*<0.05). Scale bar: 20 µm.

et al., 2012; Liu et al., 2015) but the majority of ECs explicitly limit expression of BMP ligands and facilitators of BMP signaling to ensure that Bam expression and differentiation are not repressed in GSC derivatives. Several constituents of ECs are known to be involved in silencing BMP ligands and facilitators. These include molecules necessary for regulating the cellular processes that contact germline cells (as exemplified by Rho; Kirilly et al., 2011), EGFR (Liu et al., 2010), Wnt pathway components (Hamada-Kawaguchi et al., 2014; Luo et al., 2015; Wang et al., 2015; Mottier-Pavie et al., 2016), Piwi (Ma et al., 2014) and multiple regulators of chromatin modifications (Eliazer et al., 2011; Wang et al., 2011; Xuan et al., 2013; Maimon et al., 2014). Here, we report that the Hh and Hpo/Yki pathways also serve to suppress BMP ligand production in ECs.

What motivates this arrangement to prevent unwanted BMP signaling? One possibility is that ECs must retain some ability to express BMP ligands, either so that the most anterior ECs can constitutively supplement cap cell signaling to GSCs or so that all ECs can support the dedifferentiation of GSC derivatives to replenish GSCs in emergency situations (Liu et al., 2015). At the same time, significant constitutive BMP production from any but the most anterior ECs has severely disruptive consequences for germline differentiation. Hence, one suitable arrangement is for BMP expression to be poised in an inducible state in ECs but to be firmly repressed through the action of more than one pathway. Hh and Wnt signaling pathways are among those that repress BMP expression.

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Hh and Wnt ligands emanate from cap cells and ECs, and can therefore elicit strong signals in ECs. However, both signals also reach FSCs, where they have different roles in regulating proliferation and differentiation, respectively (Huang and Kalderon, 2014; Reilein et al., 2017). Likewise, Yki is active in both ECs and FSCs but it inhibits BMP ligand expression in ECs and promotes proliferation in FSCs; Yki activity is also significantly regulated by Hh signaling only in FSCs (Huang and Kalderon, 2014). Thus, it appears that both Hh and Wnt signals are exploited to regulate very different responses in ECs and FSCs. It remains to be discovered how Hh, Wnt and Yki pathway activities are directed to different targets and outcomes in these closely related and adjacent cell types.

Our study of the roles of Yki and Hh signaling in ECs shares common ground and exhibits significant differences compared with recently published findings. The only prior study of the effects of manipulating Yki activity in ECs (Li et al., 2015) described the principal phenotype of failed germline differentiation, but the altered disposition of EC processes, time of action, apoptosis and dramatic morphological changes in germaria are novel to our study. Most important, it was claimed that the germline differentiation defect did not involve ectopic BMP signaling (Li et al., 2015). Our evidence of ectopic BMP signaling, as manifested by *Dad-lacZ*, pMad antibody staining and Bam-GFP expression is overwhelming, as is our evidence of partial phenotypic suppression by inhibition of *dpp* and *gbb* expression in ECs. We are therefore confident in concluding that Yki activity in ECs promotes germline differentiation substantially by repressing Dpp and Gbb expression. Yki evidently has additional functions because Yki inhibition in ECs also led to EC apoptosis and a failure of egg chamber budding even when ectopic Dpp or Gbb production was suppressed.

Two recent papers showed, as we have found, that Hh signaling in ECs is required to limit BMP ligand production (Liu et al., 2015; Lu et al., 2015). All three studies directly contradict a prior conclusion that Hh signaling promotes BMP ligand production in ECs (Rojas-Rios et al., 2012). There are differences in the finer details of the findings. We found that inhibition of *gbb* expression in ECs partially suppresses the *smo RNAi* phenotype, whereas inhibition of *dpp* does not. Two of the other studies did not test the role of Gbb but did find significant suppression of germline differentiation phenotypes by inhibiting *dpp* expression in ECs (Liu et al., 2015; Lu et al., 2015). The differences in these results cannot easily be attributed to partial knockdown of Smo in our studies, since Hh pathway activity was shown to be drastically reduced using the *ptc-lacZ* reporter. Also, both *UAS-dpp RNAi* lines that we used were able to suppress the germline differentiation phenotype due to *yki RNAi*.

A second apparent difference in *smo RNAi* phenotypes is the reported disruption of EC process morphology (Lu et al., 2015), which we did not observe at any location other than the extreme anterior of the germarium. It is likely that defective germline differentiation is the primary deficit that prevents EC processes from penetrating into the germarium in several genotypes because this phenotype can be induced by loss of *bam* alone (Kirilly et al., 2011). Our studies of Smo, Yki and Lsd1 phenotypes support this hypothesis. EC processes were observed for all EC genotypes but penetration between germline cells ranged from zero (*yki RNAi*), intermediate (*lsd1 RNAi*) to almost normal (*smo RNAi*), which correlates with the extent of germline differentiation as measured by egg chamber budding. Thus, differences between our results and some previous descriptions of EC process phenotypes resulting from Lsd1 and Smo inhibition might be explained by the different severities of the germline differentiation defects observed (Eliazer et al., 2011; Lu et al., 2015).

How are the actions of Lsd1, Hh signaling and Hpo/Yki pathway activity in ECs connected? The Lsd1 phenotype was previously shown to be partially suppressed by reducing the activity of En or Hh, which is normally induced by En in cap cells, and was partially phenocopied by ectopic En expression but not by ectopic Hh (Eliazer et al., 2014). That study suggested that En induction might be one of several crucial effectors in ECs with reduced Lsd1. We observed highly penetrant germline differentiation defects by inhibiting Lsd1 in ECs without inducing any ectopic En expression, showing that there must be significant mediators other than En. We also observed no reduction of *ptc-lacZ* expression in these germaria, suggesting that a primary requirement for Lsd1 cannot be to modulate Hh signaling. Similarly, we observed no suppression of *lsd1 RNAi* phenotypes by provision of additional Yki, suggesting that Yki is also not a key mediator for Lsd1.

It was previously suggested that loss of Hh signaling in ECs disrupts germline differentiation because of a failure to maintain Yki activity in ECs (Li et al., 2015). That conclusion was based on the limited suppression of phenotypes that were due to deficient Hh signaling by manipulations expected to increase Yki activity, and on the basis of some protein interaction studies in heterologous cells. We found that Smo inhibition did not significantly alter Yki activity in ECs and that downregulation of Hpo pathway repressors of Yki activity (Hpo and Wts) did not suppress *smo RNAi* phenotypes at all. We observed only weak suppression of the *smo RNAi* phenotype by providing excess Yki. Our data therefore support the conclusion that Hh signaling and Yki act largely independently in ECs.

MATERIALS AND METHODS

Drosophila stocks

Drosophila stocks were maintained on standard cornmeal/molasses/agar medium at room temperature. The following strains were used: *C587-GAL4*, *C587-GAL4; UAS-mCD8-RFP*, *C587-GAL4; UAS-mCD8-GFP*, *C587-GAL4; ts-G80 FRT42D tub-lacZ/CyO*, *bab-GAL4*, and *UAS-ci*^{WT} (Vied et al., 2012; Zadorozny et al., 2015). *UAS-yki RNAi* (BL 31965 and 34067), *UAS-smo RNAi* (BL 27037 and 43134), *UAS-dpp RNAi* (BL 31530 and 31531), *UAS-gbb RNAi* (BL 34898), *UAS-dally RNAi* (BL 33952), *UAS-hpo RNAi* (BL 27661 and 33614), *UAS-wts RNAi* (BL 27662 and 34064), *UAS-sd RNAi* (BL 29352) and *UAS-wts* (BL 44250) lines were obtained from the Bloomington Drosophila Stock Center (BL). *UAS-yki* and *tub-yki* lines were provided by L. Johnston (Columbia University Medical School, New York, NY, USA). *UAS-hpo* was provided by N. Tapon (The Francis Crick Institute, London, UK). *Dad-lacZ* and *bam-GFP* lines were provided by T. Xie (Stowers Institute for Medical Research, Kansas City, MO, USA); *UAS-lsd1 RNAi* was provided by M. Buszczak (University of Texas Southwestern Medical Center, Dallas, TX, USA).

Immunohistochemistry

Ovaries were dissected in PBS and fixed in 4% paraformaldehyde in PBS for 20 min, rinsed with PBST (PBS containing 0.1% Triton X-100 and 0.05% Tween 20), blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories) in PBST, and stained overnight at 4°C with primary antibodies. The following antibodies were obtained from the Developmental Studies Hybridoma Bank: anti-Hts 1B1 (1:20) deposited by H. D. Lipshitz, anti-Vasa (1:10) deposited by A. C. Spradling and D. Williams, anti-Fasciclin 3 7G10 (1:250) deposited by C. Goodman, anti-Coracle C615.16 (1:100) deposited by R. Fehon, anti-Engrailed/Invected 4D9 (1:2) deposited by C. Goodman, and anti-Lamin C LC28.26 (1:50) deposited by P. Fisher. Additional primary antibodies were rabbit anti-Caspase 3 (1:100; D175, Cell Signaling), rabbit anti-β-galactosidase (1:500; NB600-305, Promega), rabbit anti-pS423/425 Smad3 (1:100; 1880-1, Epitomics), rabbit anti-Yki (1:500; kindly provided by D. Pan, UT,

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Southwestern Medical Center, TX, USA) and rabbit anti-GFP (1:1000; A6455, Thermo Fisher Scientific). Ovaries were then washed three times in PBST and incubated with Alexa Fluor 488 or Alexa Fluor 594 secondary antibodies (1:1000; Molecular Probes) for 2 h at room temperature. Ovaries were washed twice in PBST and once in PBS and mounted in DAPI Fluoromount-G (Southern Biotech, 0100-20). Fluorescence images were captured using 40×/1.3 NA or 63×/1.4 NA oil-immersion lenses on a Zeiss LSM 700 confocal microscope. Images were processed using ImageJ (National Institutes of Health) and Photoshop (Adobe).

Measurement of pathway reporter gene activities

Ovaries from flies of the various genotypes including the *Diap1-lacZ*, *ptc-lacZ* or *ex-lacZ* transgenes were stained with DAPI and antibodies to Vasa, β-galactosidase and Fasciclin 3 (Fas3) in order to identify different cell types. β-galactosidase was detected with the Alexa Fluor 488 secondary antibody. Images of germaria were taken at 63× using the same laser intensity and gain settings for each reporter gene. Using DAPI, Fas3 and Vasa staining as landmarks, individual ECs were circled with the Draw Spline Contour tool in Zen software (Carl Zeiss) and the intensity mean value in the 488 channel was recorded. Each cell was circled twice and the values averaged.

Statistical methods

Images shown are representative of at least ten examples. In most cases, the number is much higher and is given explicitly where relevant for statistical analysis of outcomes. No statistical method was used to predetermine sample size but we used prior experience to establish minimal sample sizes. No samples were excluded from analysis, provided staining was of high quality. The experiments were not randomized; all samples presented as groups in the results were part of the same experiment and treated in exactly analogous ways without regard to the identity of the sample. Investigators were not blinded during outcome assessment, but had no preconception of what the outcomes might be. Standard statistical tests are described in each figure legend, and sample sizes were appropriately large with appropriate distributions; relevant means, s.e.m. and *P*-values are provided together with explicit sample sizes, which always represent biological replicates.

Quantitative RT-PCR (qRT-PCR)

The germarium and early egg chambers (up to five) were dissected and RNA was isolated using the RNeasy Mini Kit (Qiagen, 74104). qRT-PCR was performed in the AriaMx real-time PCR system (Agilent Technologies) with the HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme, Q221-01). RT-PCR reactions were carried out for 30 min at 50°C, followed by 10 min at 95°C, then followed by 40 cycles of two-step PCR for 15 s at 95°C, 1 min at 60°C. Each reaction was performed in triplicate. *dpp* (primers 5′-TACCACGCCATCCACTCAAC-3′ and 5′-GCTCGTTACTCGATACGG CT-3′) and *gbb* (5′-CTGGATCATCGCACCAGAGG-3′ and 5′-GTCTGG ACGATCGCATGGTT-3′) RNA levels were normalized to *rp49* (*RpL32*) RNA (5′-CACCGGATTCAAGAAGTTCC-3′ and 5′-GACAATCTCCTTG CGCTTCT-3′) and relative concentration was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Significant differences were analyzed by one-way analysis of variance (ANOVA) using SPSS 17.0 software (IBM).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.H., D.K.; Methodology: J.H., D.K.; Formal analysis: J.H., A.R., D.K.; Investigation: J.H., A.R., D.K.; Writing - original draft: J.H., D.K.; Writing - review & editing: J.H., A.R., D.K.; Visualization: J.H., A.R.; Funding acquisition: D.K.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.147702.supplemental

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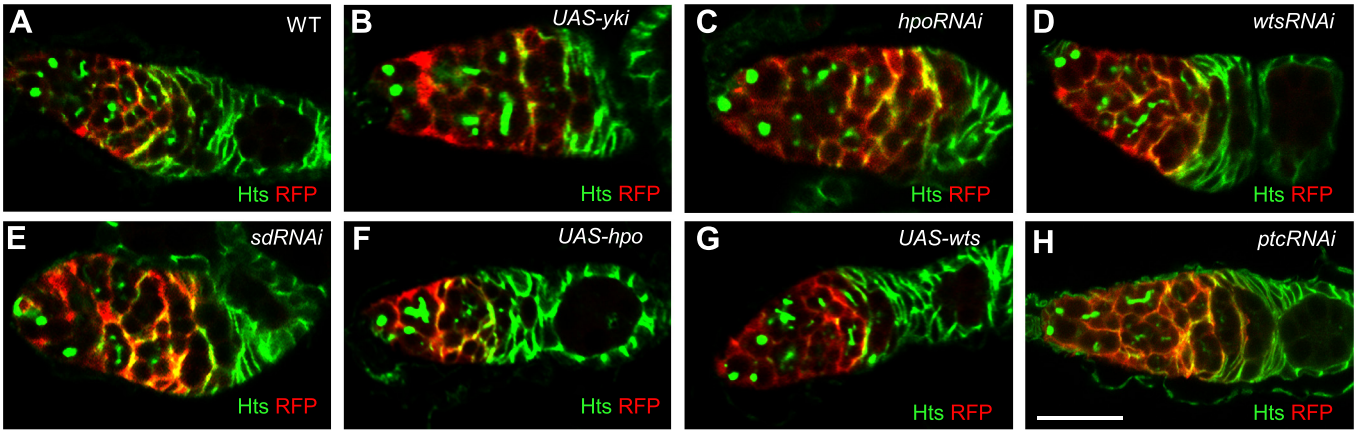

Fig. S1. Activation of Yki in ECs produces no significant defects.
(A-G) Germaria from adult females with UAS-CD8-RFP and C587-GAL4 (A) only or together with (B) UAS-yki, (C-E,H) the indicated UAS-RNAi transgenes, or (F) UAS-hpo or (G) UAS-wts. CD8-RFP (red) principally labels the surface of ECs and Hts (green) antibody labels round spectrosomes (arrowheads) and fusomes (arrows), as well as the cortical cytoskeleton of Follicle cells. No major differences were observed in germarial morphology or germline cyst maturation for any of these genotypes. Scale bar (same for all) is 20 μm.

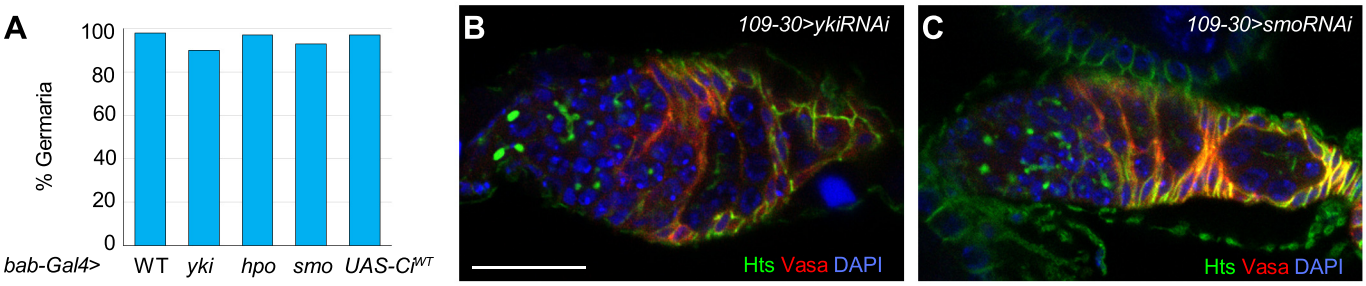

Fig. S2. Germline differentiation defects are not due to Yki and Smo actions in terminal filament, cap cells, or FSCs.

(A) Percentage of germaria with a normal complement of spectrosomes (less than or equal to six) when the indicated UAS transgenes were expressed in terminal filament and cap cells using bab-GAL4. n= 168, 88, 127, 133 and 129 biologically independent germaria, in the order shown.

(B,C) Germaria stained with antibody to Hts (green), Vasa (red) as well as DAPI (blue nuclei) and expressing (B) UAS-yki RNAi or (C) UAS-smo RNAi using 109-30-GAL4. No changes in germline differentiation are evident.

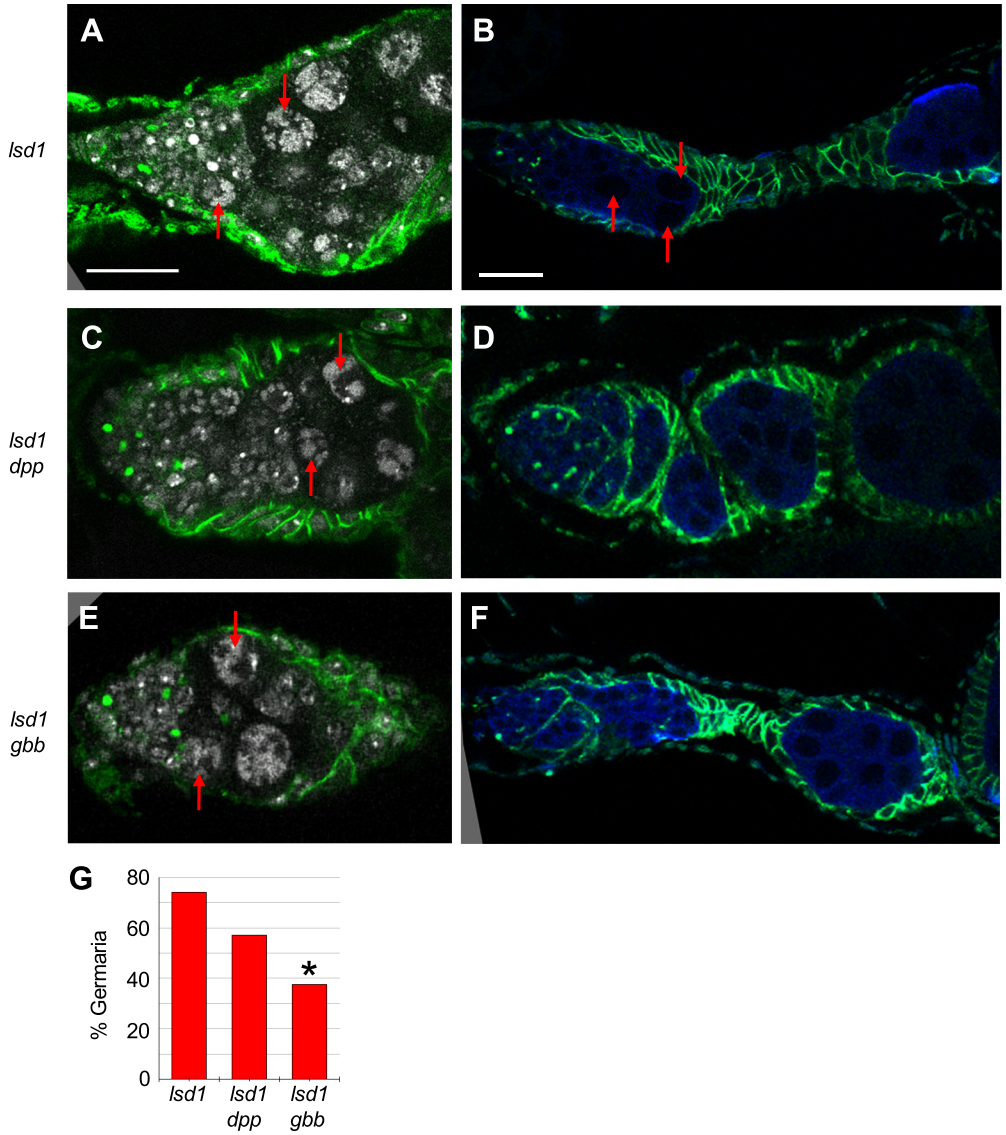

Fig. S3. Suppression of Lsd1-deficient phenotypes by Dpp or Gbb reduction.
(A-G) C587-GAL4 was used to express UAS-lsd1 RNAi (A, B) alone or together with (C, D) UAS-dpp RNAi or (E, F) UAS-gbb RNAi. (A,C,E) Germaria labeled with antibody to Hts (green) to visualize spectrosomes (anterior (left) dots) and DAPI (gray) to stain all nuclei exhibited severe morphological defects, including failure of cyst maturation and abnormally large germline nuclei (red arrows). (B,D,F) Germaria stained for Hts (green) and Vasa (blue). (B) Most lsd1 RNAi germaria could bud off egg chambers despite morphological defects. Red arrows indicated abnormally large germline nuclei. (D,F) Germaria were scored as morphologically normal if they did not contain abnormally large germline cells, even if (D) they did contain an abnormal number of spectrosomes. Both the proportion of ovarioles classified as morphologically abnormal due to the presence of large germline nuclei in the germarium (red arrows in A-C,E) and the severity of these defects were reduced by dpp RNAi and by gbb RNAi. Same magnification for (A, C, E) and (B, D, G); scale bars 20 μm. (G) The frequency of morphological defects was slightly reduced by dpp RNAi and more substantially by gbb RNAi. n= 58 (lsd1), 42 (lsd1+dpp) and 40 (lsd1+gbb) biologically independent germaria. Significant differences imposed on lsd1 RNAi phenotypes by dpp RNAi or gbb RNAi were calculated using Fisher's exact two-tailed test (* $p<0.01$).

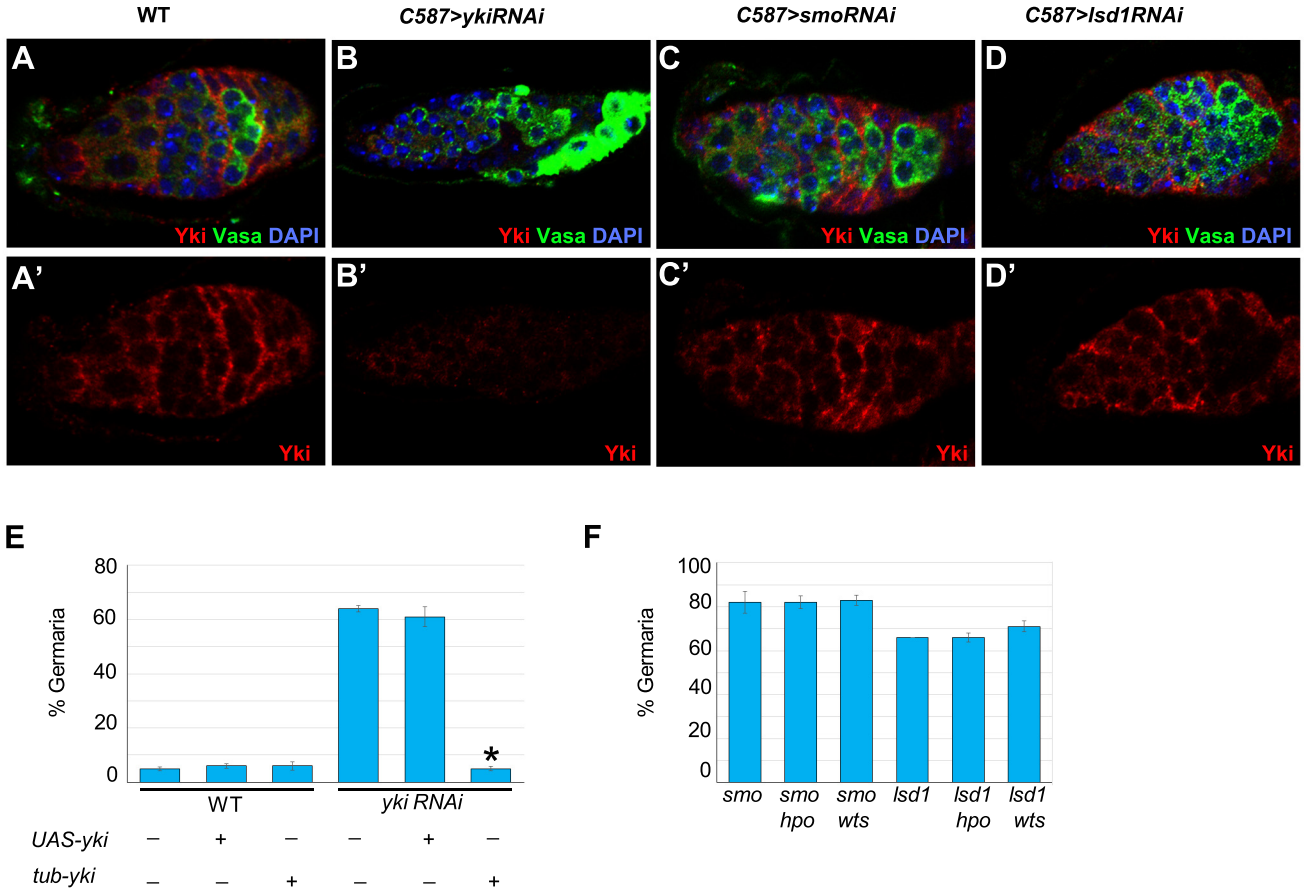

Fig. S4. Yki activity does not mediate Hh pathway actions in ECs that affect germline differentiation.

(A-D) Yki antibody staining (red) together with Vasa antibody staining of germline cells (green) or (A'-D') alone in (A, A') wild-type germaria was (B, B') greatly reduced in ECs by UAS-yki RNAi but unaltered by (C, C') UAS-smo RNAi or (D, D') UAS-lsd1 RNAi expression with C587-GAL4. Scale bar for all is 20 μm. (E) Percentage of germaria with 6 or more spectrosomes for females containing the indicated transgenes in addition to C587-GAL4. The yki RNAi transgene targets regions of yki that are present in both UAS-yki and tub-yki transgenes. Consequently, the rescue activity of those transgenes in the presence of UAS-yki RNAi is expected to be much lower than for genotypes where UAS-yki RNAi is absent (as in Fig. 6). n= 310, 282, 265, 333, 248 and 310 biologically independent germaria, in the order shown. Significant differences imposed on the yki RNAi phenotype by UAS-yki or tub-yki were calculated using Fisher's exact two-tailed test (* $p<0.0001$; for all others $p>0.05$). (F) Percentage of germaria with more than 6 spectrosomes for animals containing the indicated transgenes in addition to C587-GAL4. Mean and SEM for three (smo) or two (lsd1) trials are shown using a total of n= 216, 196, 316, 247, 166 and 172 biologically independent germaria, in the order shown. No significant differences ($p<0.05$) were seen for the effect of hpo RNAi or wts RNAi on smo RNAi or lsd1 RNAi phenotypes by Fisher's exact two-tailed test.