© 2015. Published by The Company of Biologists Ltd | Development (2015) 142, 4242-4252 doi:10.1242/dev.124768

STEM CELLS AND REGENERATION

RESEARCH ARTICLE

COP9-Hedgehog axis regulates the function of the germline stem cell progeny differentiation niche in the *Drosophila* ovary

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ABSTRACT

Both stem cell self-renewal and lineage differentiation are controlled extrinsically as well as intrinsically. Germline stem cells (GSCs) in the *Drosophila* ovary provide an attractive model in which to study both stem cell self-renewal and lineage differentiation at the molecular and cellular level. Recently, we have proposed that escort cells (ECs) form a differentiation niche to control GSC lineage specification extrinsically. However, it remains poorly understood how the maintenance and function of the differentiation niche are regulated at the molecular level. Here, this study reveals a new role of COP9 in the differentiation niche to modulate autocrine Hedgehog (Hh) signaling, thereby promoting GSC lineage differentiation. COP9, which is a highly conserved protein complex composed of eight CSN subunits, catalyzes the removal of Nedd8 protein modification from target proteins. Our genetic results have demonstrated that all the COP9 components and the *hh* pathway components, including *hh* itself, are required in ECs to promote GSC progeny differentiation. Interestingly, COP9 is required in ECs to maintain Hh signaling activity, and activating Hh signaling in ECs can partially bypass the requirement for COP9 in GSC progeny differentiation. Finally, both COP9 and Hh signaling in ECs promote GSC progeny differentiation partly by preventing BMP signaling and maintaining cellular processes. Therefore, this study has demonstrated that the COP9-Hh signaling axis operates in the differentiation niche to promote GSC progeny differentiation partly by maintaining EC cellular processes and preventing BMP signaling. This provides new insight into how the function of the differentiation niche is regulated at the molecular level.

KEY WORDS: COP9, Hh, Differentiation, Niche, Stem cells

INTRODUCTION

Stem cells in adult tissues can continuously self-renew and produce differentiated cells for replenishing lost cells caused by natural turnover, injury or diseases (Li and Xie, 2005; Morrison and Spradling, 2008). Their self-renewal is regulated by combined actions of intrinsic factors and extrinsic signals from the niche. Stem cell differentiation is generally considered as a developmental 'default' state, the consequence of departure from the self-renewing niche. Germline stem cells (GSCs) in the *Drosophila* ovary have provided an attractive model for studying how the niche and intrinsic factors synergistically control GSC self-renewal (Xie, 2013). In contrast to the idea of the developmental default state, a differentiation niche has been proposed to control GSC progeny differentiation extrinsically in the *Drosophila* ovary (Kirilly et al., 2011). However, it remains largely unclear how the function and maintenance of the differentiation niche are regulated at the molecular level.

In the *Drosophila* ovary, two or three GSCs are located at the ovariole tip (known as the germarium), and directly contact with cap cells (CPCs) anteriorly and escort cells (ECs) laterally. CPCs and lateral ECs form the self-renewing niche for controlling GSC self-renewal by anchorage and signals (Xie, 2013). CPCs produce the short-range BMP signal, contributed by Dpp and Gbb, for directly activating BMP signaling only in GSCs to control self-renewal and proliferation (Song et al., 2004; Xie and Spradling, 1998). The major function of BMP signaling is to repress the transcription of *bam*, a key differentiation factor, in GSCs, thereby promoting self-renewal by preventing differentiation (Chen and McKearin, 2003a; Song et al., 2004). In order for GSCs to self-renew continuously, GSCs must remain in contact with CPCs. Indeed, E-cadherin-mediated adhesion between CPCs and GSCs is crucial for keeping GSCs in the niche for continuous self-renewal (Song et al., 2002). In addition, various intrinsic factors have been identified to work with BMP signaling and E-cadherin-mediated cell adhesion to control GSC self-renewal (Xie, 2013).

Following the GSC division, the immediate differentiating GSC daughter, also known as the cystoblast (CB) is positioned one cell away from CPCs, and is wrapped by cellular processes of adjacent ECs (Kirilly et al., 2011; Morris and Spradling, 2011). The CB continues to divide four more times synchronously with incomplete cytokinesis to produce mitotic cysts (2-cell, 4-cell, 8-cell cysts) and 16-cell cysts, which are also wrapped by long cellular processes of posterior ECs. Bam is a key factor expressed in CBs and mitotic cysts for controlling CB differentiation and cyst division (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). Disruption of cellular process-mediated interactions between ECs and differentiating CBs leads to an accumulation of mixed GSC-like and CB-like undifferentiated GSC progeny (Kirilly et al., 2011). One of the EC functions is to prevent BMP signaling in differentiating GSC progeny. For example, inactivation of Eggless (encoding a H3K9 methyltransferase), EGF signaling, Lsd-1, Piwi and Rho1 in ECs leads to elevated BMP signaling activity and thus a GSC differentiation defect (Eliazer et al., 2011; Jin et al., 2013; Kirilly et al., 2011; Liu et al., 2010; Ma et al., 2014; Wang et al., 2011). Interestingly, the Woc-Stat-Zfh1 axis (Stat is also known as Stat92E) is also required in ECs to maintain EC cellular processes and promote germ cell differentiation (Maimon et al., 2014). It still remains poorly understood how the function of the differentiation niche is regulated molecularly.

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Received 13 April 2015; Accepted 29 October 2015

Development (2015) 142, 4242-4252 doi:10.1242/dev.124768

The Skp, Cullin, F-box containing complex, often known as the SCF complex, is a multi-protein E3 ubiquitin ligase complex catalyzing the ubiquitination of proteins destined for proteasomal degradation (Vodermaier, 2004). Cullin family proteins are responsible for organizing E3 ubiquitin ligase complexes and are also modified and regulated by conjugation of the ubiquitin-like polypeptide Nedd8 to conserved lysines. Deneddylation, the removal of Nedd8, is catalyzed by COP9 (also known as the CSN complex) (Cope and Deshaies, 2003; Wei et al., 1998). COP9 is composed of eight CSN proteins, which are highly conserved from plants to animals (Wei et al., 1998). CSN proteins have been shown to be important for cyst division and oocyte axis determination during *Drosophila* oogenesis (Doronkin et al., 2003; Oron et al., 2002). Our recent study has shown that COP9 is required intrinsically for controlling GSC self-renewal and differentiation (Pan et al., 2014). This study shows that COP9 is required in ECs to promote GSC progeny differentiation, and also reveals a surprising role of autocrine Hh signaling in ECs to promote GSC progeny differentiation partly by preventing Dpp/BMP signaling. Interestingly, COP9 is required in ECs to maintain active Hh signaling, thereby promoting GSC progeny differentiation. Therefore, this study has revealed crucial roles of COP9 and Hh signaling in the differentiation niche to promote GSC progeny differentiation partly by preventing BMP signaling.

RESULTS

COP9 is required in ECs to promote GSC progeny differentiation

To gain a better understanding of how the function of the differentiation niche is regulated, we used *c587-gal4* and available *UAS-RNAi* transgenic lines from the Tsinghua Fly Stock Center to carry out systematic RNAi screens to look for important EC regulators. *c587-gal4* is expressed specifically in ECs and early follicle cell progenitors (Song et al., 2004). In the screens, multiple CSN genes were identified for their essential roles in ECs for promoting GSC progeny differentiation. In *Drosophila*, the COP9 complex is composed of proteins CSN1b (*CSN1a* is a pseudogene), CSN2 (also known as Alien) and CSN3-8. To verify EC-specific RNAi-mediated CSN knockout phenotypes, we designed at least two shRNA lines against different regions for each CSN gene according to the published procedures (Ni et al., 2008, 2011) (Table S1). The control and CSN knockout ovaries are labeled for Hu-li tai shao (Hts) protein to visualize germ cell-specific organelles, known as fusomes (Lin et al., 1994). GSCs and CBs contain a spherical fusome, known as the spectrosome, whereas mitotic cysts and 16-cell cysts carry a branched fusome. Thus, the fusome morphology can be reliably used to distinguish CBs from cysts. In the control germarium, zero to two CBs can be identified based on the existence of the spectrosome one cell away from GSCs (Fig. 1A). By contrast, CSN knockout germaria (*CSN1b-i* to *CSN8-i*)

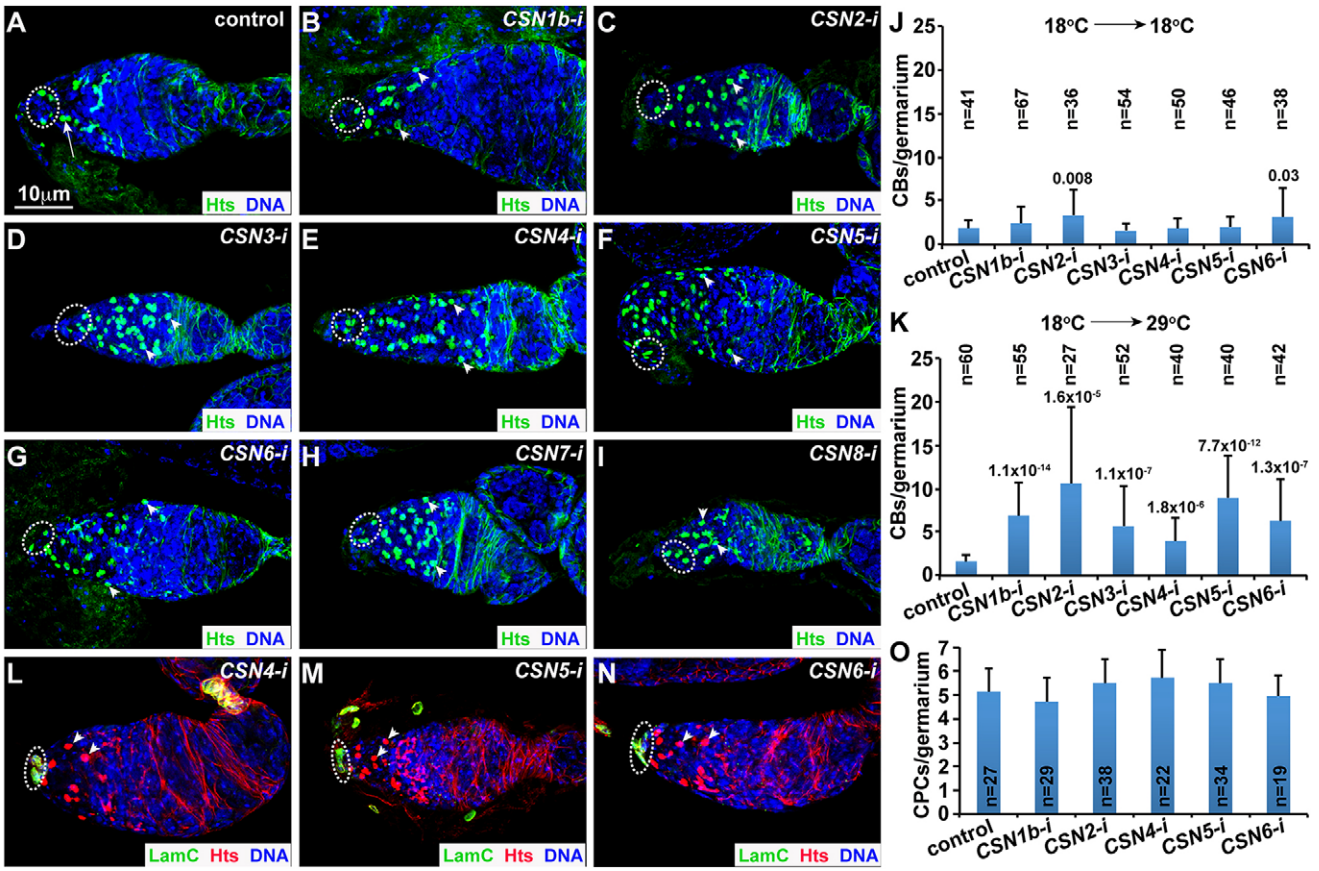

Fig. 1. COP9 components are required in ECs to control germ cell differentiation. (A) Control germarium containing a two-cell cyst (arrow) but no CBs. (B-I) EC-specific knockout of *CSN1b* (B), *CSN2* (C), *CSN3* (D), *CSN4* (E), *CSN5* (F), *CSN6* (G), *CSN7* (H) and *CSN8* (I) leads to excess CBs. (J,K) Quantitative results show that inactivation of CSN gene functions in adult ECs at the restrictive temperature (K) leads to excess CBs, but no or low CSN gene inactivation in adult ECs at the permissive temperature (J) does not. (L-O) EC-specific knockout of *CSN4* (L), *CSN5* (M) and *CSN6* (N) does not transform ECs into Lamin C-positive cap cells and does not change the number of endogenous cap cells (quantified in O). Broken ovals show cap cells/GSCs in A-I and cap cells in L-N; arrowheads indicate CBs. Error bars represent s.d., *P*-values are given above columns of statistically significant results.

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accumulate many single CB-like germ cells carrying a spectrosome a few cells away from GSCs, indicative of the germ cell differentiation defect (Fig. 1B-I). To simplify presentation, we refer to these CB-like cells collectively as CBs hereafter. These results indicate that COP9 is required in ECs to promote GSC progeny differentiation.

Because *c587-gal4* is also expressed in the somatic cells of the developing female gonad (Zhu and Xie, 2003), the GSC differentiation defect could be caused by abnormal EC differentiation or loss of adult EC function. To further determine the requirement of COP9 in adult ECs for promoting GSC progeny differentiation, we took advantage of the temperature-sensitive nature of the GAL4-UAS system to knock out the functions of CSN genes specifically in adult ECs. This system works effectively to knock out target genes at 29°C but poorly at 18°C. Indeed, at 18°C (permissive temperature), the germaria carrying *c587-gal4* and a *UAS-RNAi* against one of the CSN genes (*CSN1b-i* to *CSN6-i*) contain similar numbers of CBs to the control germaria (Fig. 1J). By contrast, at 29°C (restrictive temperature), the CSN knockout germaria (*CSN1b-i* to *CSN6-i*) contain significantly more CBs than the control germaria, indicating that they exhibit a germ cell differentiation defect (Fig. 1K). Therefore, these results demonstrate that the COP9 complex is required in adult ECs to promote GSC progeny differentiation.

To further determine if COP9 is indeed required in ECs to promote GSC progeny differentiation, we used a newly identified GAL4 driver, *13c06-gal4*, which was recently reported to be specific to ECs (Sahai-Hernandez and Nystul, 2013), to knock out *CSN4* and *CSN5*. Based on the expression of *UAS-RedStinger*, *13c06-gal4* is indeed expressed in ECs at high levels as well as in early follicle progenitors at much lower levels (Fig. S1A,B). *13c06*-driven *CSN4* and *CSN5* knockout yields a similar germ cell differentiation defect, but is weaker than *c587*-driven knockout (Fig. S1C-E,H). The weak phenotype could result from either GAL4 expression levels or contributions from early follicle cell progenitors. These results have further confirmed that COP9 is required at least in ECs to promote germ cell differentiation.

The germ cell differentiation defect caused by EC-specific CSN knockout could be caused by EC-to-CPC transformation. We tested this possibility by examining the expression of the well-established CPC marker Lamin C (LamC) in the CSN knockout germaria. As in the control germarium, LamC expression is restricted to endogenous CPCs, but is not present in ECs, in the *CSN4*, *CSN5* and *CSN6* knockout germaria (Fig. 1L-N). In addition, the numbers of endogenous CPCs in the CSN knockout germaria are comparable to those in the control germaria (Fig. 1O). These results rule out the possibility that the germ cell differentiation defect in CSN knockout germaria is caused by either EC-to-CPC transformation or the expansion of endogenous CPCs.

COP9 functions in ECs to regulate GSC lineage differentiation in both a deneddylation-dependent and -independent manner

The function of the COP9 complex is to remove Nedd8 modification from target proteins by the catalytic activity of CSN5 (Cope and Deshaies, 2003; Wei et al., 1998). To determine if neddylation is important for EC function, we knocked down *Nedd8*, which encodes the only Nedd8 protein in *Drosophila*, specifically in ECs by *c587*-driven expression of an RNAi line against *Nedd8*. Interestingly, the *Nedd8* knockout germaria also accumulate significantly more CBs than the controls (Fig. 2A,B). This result indicates that Nedd8 is required in ECs to promote germ cell differentiation, and further suggests that protein neddylation is important for EC function in promoting GSC lineage differentiation.

To further determine if the catalytic activity of CSN5 is required in ECs to promote germ cell differentiation, we generated transgenes carrying RNAi-insensitive wild-type and catalytically dead mutant *CSN5* under the control of a *UAS* promoter, *UAS-CSN5* and *UAS-CSN5ED* (enzyme dead) which are inserted on the same attp40 site on the second chromosome. To generate the RNAi-insensitive *CSN5*, the RNAi target sequences were mutated by changing the DNA sequence, but with the replacement codons encoding an unchanged amino acid sequence. For the *CSN5ED* mutation, the histidine (H) residue at position 135 was changed to alanine (A), which has been shown to completely inactivate the enzymatic activity of CSN5 in yeast (Cope et al., 2002). Indeed, *c587*-driven expression of RNAi-insensitive *CSN5* can almost completely rescue the germ cell differentiation defect caused by *CSN5* knockout in ECs, indicating that RNAi-mediated *CSN5* knockout is very specific (Fig. 2C,D,F). Intriguingly, *c587*-driven expression of

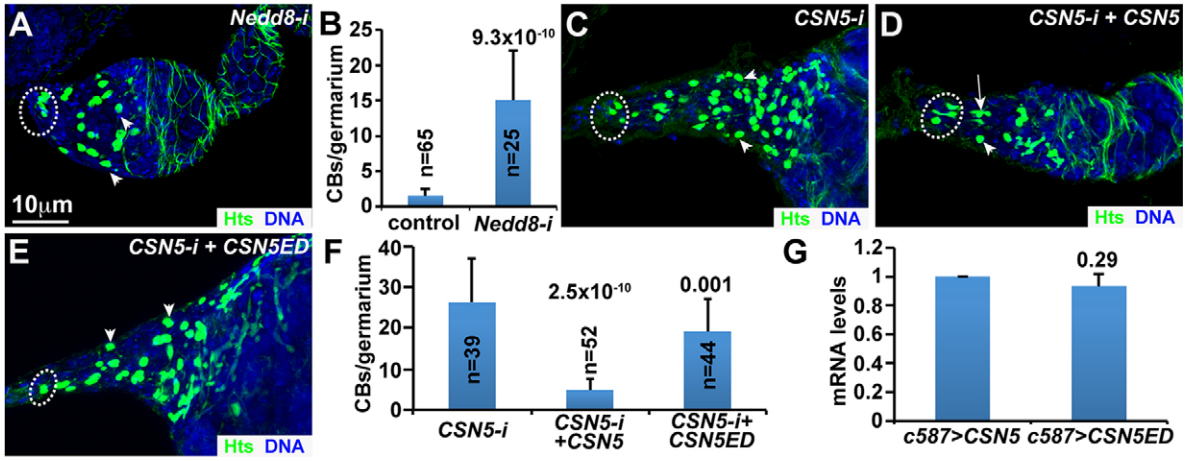

Fig. 2. The dedennylase activity of CSN5 is required in ECs to control germ cell differentiation. (A,B) Knockout of *Nedd8* in ECs leads to significantly more CBs than the control. (C-F) EC-specific expression of wild-type *CSN5* (D) and deneddylase-dead *CSN5* (E) can fully and partially rescue CBs to the normal level, respectively (quantified in F). The arrow in D indicates a differentiated cyst. (G) qRT-PCR results show that EC-specific expression of wild-type *CSN5* and *CSN5ED* transgenes inserted at the same chromosomal location have similar expression levels. Broken ovals show GSCs; arrowheads indicate CBs. Error bars represent s.d., *P*-values are given above columns of statistically significant results.

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CSN5ED can only partially rescue the differentiation defect caused by *CSN5* knockout in ECs (Fig. 2E,F). As these two transgenes are under the same regulatory sequence and inserted in the same genomic location, we would expect that their mRNA and protein expression levels should be similar. Using PCR primers specific to the RNAi-resistant *CSN5* and *CSN5ED*, we performed quantitative RT-PCR (qRT-PCR) to confirm that wild-type *CSN5* and *CSN5ED* have comparable mRNA expression levels (Fig. 2G). Our results indicate that CSN5 functions in ECs to promote germ cell differentiation in both deneddylase-dependent and -independent manners.

Hedgehog (Hh) signaling is required in ECs to control GSC progeny differentiation

Hh signaling has been proposed to directly signal GSCs to control their maintenance (King et al., 2001). It has also been proposed to control GSC maintenance indirectly by promoting BMP signaling in the niche (Rojas-Ríos et al., 2012). In the *Drosophila* Hh pathway, upon binding of the ligand Hh to its receptor Patched (Ptc) and the subsequent release of its inhibition, the co-receptor Smoothened (Smo) becomes active and thus promotes the formation of an active form of downstream transcriptional activator Cubitus interruptus (Ci), which then initiates target gene expression (Ingham et al., 2011; Kalderon, 2000; Lum and Beachy, 2004). Surprisingly, our RNAi screens identified *hh*, *smo* and *ci* as being required in ECs to promote GSC progeny differentiation because their knockout germaria (*hh-i1*, *smo-i1* and *ci-i1*) accumulate excess spectrosome-containing CBs (Fig. 3A-C). This result prompted us to carefully re-examine the functions of the Hh pathway in ECs. Our RNA sequencing results on the purified wild-type ECs show that *hh*, *smo*, *ptc* and *ci* are indeed expressed in ECs

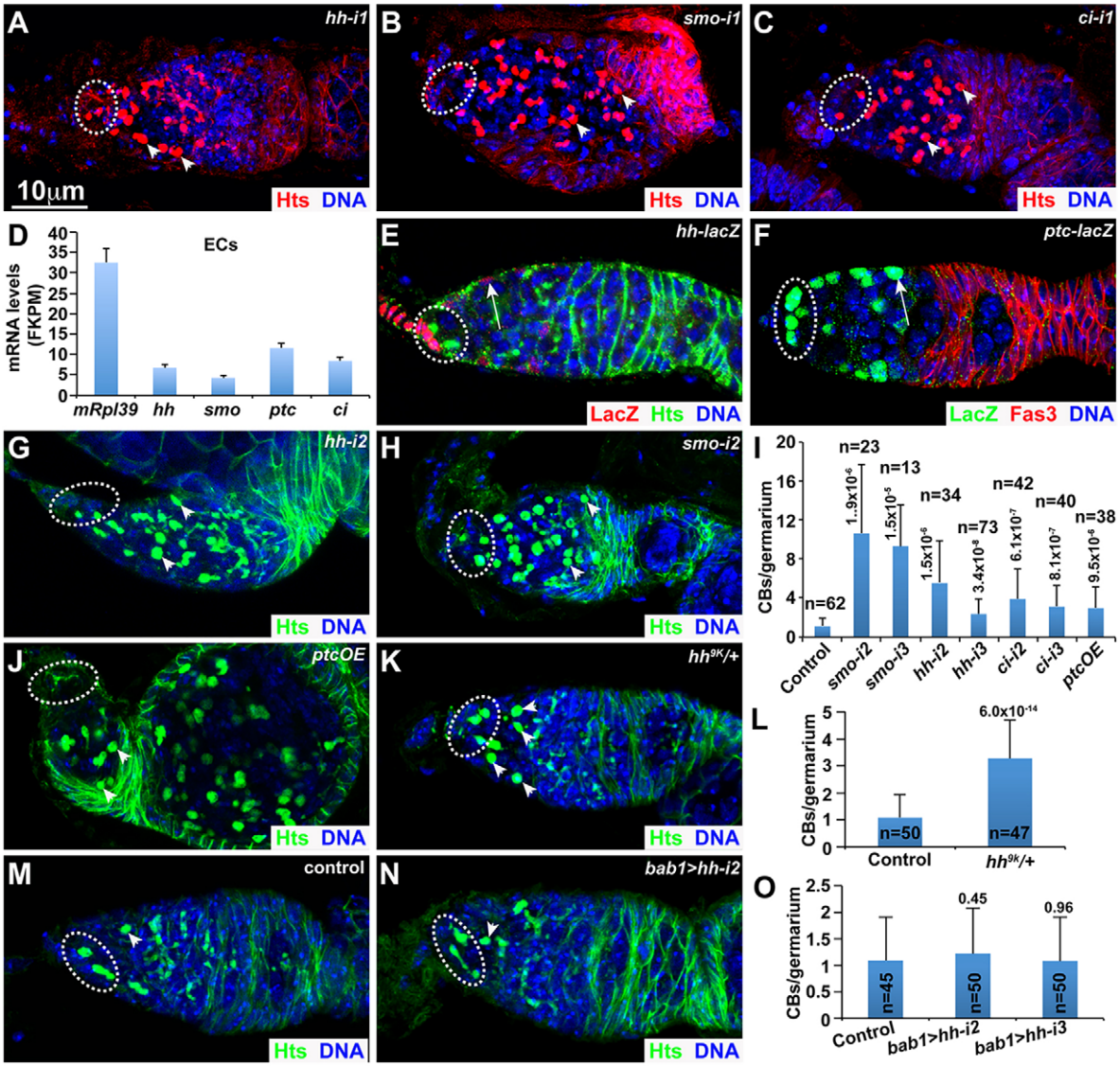

Fig. 3. Canonical Hh signaling is required in ECs to control germ cell differentiation. (A-C) EC-specific knockout of *hh* (A), *smo* (B) and *ci* (C) results in excess CBs. (D) RNA-sequencing results show that purified adult ECs express *hh*, *smo*, *ptc* and *ci*. (E) *hh-LacZ* is expressed in terminal filament and cap cells at high levels and in ECs (arrow) at low levels. (F) *ptc-LacZ* is expressed in cap cells and ECs (arrow) at high levels. (G-I) EC-specific knockout of *hh* (G), *smo* (H) and *ci* by additional RNAi lines causes similar germ cell differentiation defects (quantified in I). (J) EC-specific overexpression of *ptc* (*ptcOE*) leads to excess CBs. (K,L) hh^{9k}/+ germarium carries four CBs in addition to three GSCs. (M-O) Both control (M) and cap cell-specific *hh* knockout germarium (N) carry two GSCs and one CB (CBs quantified in O). Broken ovals show GSCs; arrowheads indicate CBs. Error bars represent s.d., *P*-values are given above columns of statistically significant results.

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(Fig. 3D). In addition, enhancer trap lines *hh-lacZ* and *ptc-lacZ* are also expressed in ECs in addition to CPCs and terminal filament cells, further confirming that Hh signaling components are present in ECs (Fig. 3E,F).

To verify the specificity of *hh*, *smo* and *ci* knockout, we generated two additional RNAi lines against different sequences for each gene. Their knockout germaria by *c587-gal4* accumulate significantly more CBs than the control germaria (Fig. 3G-I). It is worth noting that *smo* knockout consistently leads to a more severe germ cell differentiation defect than *hh* and *ci* knockout (Fig. 3I). This difference could result from different RNAi efficiencies or the existence of additional redundant factors. To further verify the requirement of Hh signaling in ECs, we inhibited Hh signaling by overexpressing Ptc in ECs. Indeed, *c587*-driven *ptc* overexpression results in an accumulation of significantly more CBs than the control (Fig. 3I,J). Consistently, the hh^{9K}/+ heterozygous germaria contain significantly more CBs than the control germaria (Fig. 3K,L). Thus, these results demonstrate that Hh signaling is active and required in ECs for promoting GSC progeny differentiation.

To further determine if *hh* is required in ECs to promote germ cell differentiation, we used *13c06-gal4* to knock out *hh* and *smo* in ECs. As expected, *13c06*-driven *hh* and *smo* knockout in ECs results in a similar germ cell differentiation defect, confirming the requirement of Hh in ECs for promoting germ cell differentiation (Fig. S1F-H). *hh* is highly expressed in CPCs, and has also been suggested to be important for GSC maintenance (King et al., 2001; Rojas-Ríos et al., 2012). Then we used *bab1-gal4* to knock out *hh* in CPCs to determine if Hh is required in CPCs to promote germ cell differentiation. *bab1-gal4* is widely used to drive and knock out gene expression in CPCs (Bolívar et al., 2006). Surprisingly, CPC-specific *hh* knockout does not affect the numbers of GSCs and CBs, indicating that CPC-derived Hh signaling is dispensable for GSC maintenance and germ cell differentiation (Fig. 3M-O). These results further demonstrate that EC-derived Hh, but not CPC-derived Hh, is responsible for germ cell differentiation.

COP9 promotes GSC progeny differentiation partly by maintaining active Hh signaling in ECs

COP9 activity has been shown to deneddylate cullin1 (Cul1) and cullin3 (Cul3) proteins, and promote the turnover of the substrate receptor F-box protein Slimb in the SCF complex involved in Ci proteolysis, promoting low to intermediate Hh signaling in *Drosophila* imaginal disc cells (Wu et al., 2005, 2011).

Our earlier results show that both COP9 and Hh signaling are required in ECs to promote GSC progeny differentiation (Figs 1, 3). *ptc* not only encodes an Hh receptor but also is an Hh target gene (Ingham et al., 2011; Kalderon, 2000; Lum and Beachy, 2004). Here, the enhancer trap line *ptc-lacZ* is used as a reporter to monitor Hh signaling activity in CSN knockout ECs (Forbes et al., 1996). In comparison with the control ECs, the CSN knockout ECs demonstrate decreased *ptc-lacZ* expression (Fig. 4A-C′). In addition, our β-galactosidase quantification results show that the CSN knockout ECs exhibit a significant decrease in *ptc-lacZ* expression compared with the control (Fig. 4D). It is worth noting that *ptc-lacZ* expression in CPCs is also drastically decreased in the CSN knockout germaria in comparison with controls, although *c587* is not expressed in adult CPCs. Our earlier studies have shown that *c587* is ubiquitously expressed in somatic gonadal precursors, and its *piwi*-mediated RNAi knockout effect can persist to adult ECs (Ma et al., 2014; Song et al., 2007). It is probable that the *c587*-mediated CSN knockout effect persists in adult ECs, as a result of its mitotically inactive nature. These results indicate that COP9 is required in ECs to maintain active Hh signaling.

ptc and *pka* are negative regulators of Hh signaling, and their inactivation leads to constitutively active Hh signaling (Ingham et al., 2011; Kalderon, 2000; Lum and Beachy, 2004). Thus, we tested if inactivating *ptc* or *pka* function in ECs could rescue the germ cell differentiation defect caused by CSN knockout. Interestingly, *c587*-driven *ptc* or *pka* knockout does not result in any obvious germ cell differentiation defect, but instead causes egg chamber budding defects (Fig. 4E,H). This is consistent with the known role of Hh signaling in regulating follicle cell differentiation (Forbes et al., 1996; Zhang and Kalderon, 2001). By contrast, *ptc CSN1b* or *pka CSN1b* double knockout germaria contain significantly fewer CBs than those CSN single knockout germaria after one week at restrictive temperature (29°C), indicating that decreased Hh signaling in the CSN knockout germaria indeed contributes to the germ cell differentiation defect (Fig. 4E-J). Interestingly, the follicle cell defect in the double knockout germaria still persists (Fig. 4F,I). These results show that COP9 regulates GSC progeny differentiation at least partly by modulating Hh signaling in ECs.

Both COP9 and Hh signaling are required for maintaining EC long cellular processes, but not ECs themselves

Our previous studies showed that a severe EC loss could also cause the germ cell differentiation defect (Ma et al., 2014; Wang et al., 2011). To determine if COP9 is required for EC maintenance, we used the enhancer trap line PZ1444, a commonly used EC marker, to quantify the EC number in control and CSN knockout germaria. PZ1444 is expressed in cap cells and ECs, which can be easily distinguished by their location and nuclear morphology (Margolis and Spradling, 1995). One-week-old CSN knockout germaria contain a similar number of ECs in comparison with the control germaria, indicating that COP9 is dispensable for EC maintenance (Fig. 5A-E). Similarly, the one-week-old *hh*, *smo* and *ci* knockout germaria also appear to have a normal EC number in comparison with the control (Fig. 5F-I). These results indicate that COP9 and Hh signaling are dispensable for EC maintenance.

As shown previously by us, cellular process-mediated interactions between ECs and germ cells are important for proper germ cell differentiation (Kirilly et al., 2011). We used *UAS-CD8GFP* in combination with *c587-gal4* to specifically label EC cellular processes with membrane GFP. In contrast to the control germarium, in which long EC cellular processes wrap the cysts underneath, the CSN knockout ECs lose their EC processes, leaving the germ cells beneath unwrapped (Fig. 5J-L,N). Similarly, the *smo* knockout ECs also lose their processes, failing to wrap differentiating germ cells beneath (Fig. 5M,N). These results indicate that COP9 and Hh signaling are required for maintaining long EC cellular processes and wrapping of germ cells.

Because properly differentiated GSC progeny are required for maintaining long EC cellular processes (Kirilly et al., 2011), we then investigated if COP9 is required intrinsically in ECs to control their cellular processes by using the AyGAL4 flip-out system (Ito et al., 1997). In this system, heat shock-induced flipase expression leads to the removal of the transcriptional stop between the cytoplasmic actin promoter and the yeast *gal4* gene, leading to the GAL4-driven expression of GFP and RNAi under the control of the UAS promoter. By controlling the heat shock duration, we could detect a number of GFP-labeled control ECs with cellular processes (Fig. 5O). Interestingly, GFP-labeled *CSN1b*, *CSN4* and *CSN6* knockout ECs mostly lacked cellular processes (Fig. 5P-S). These

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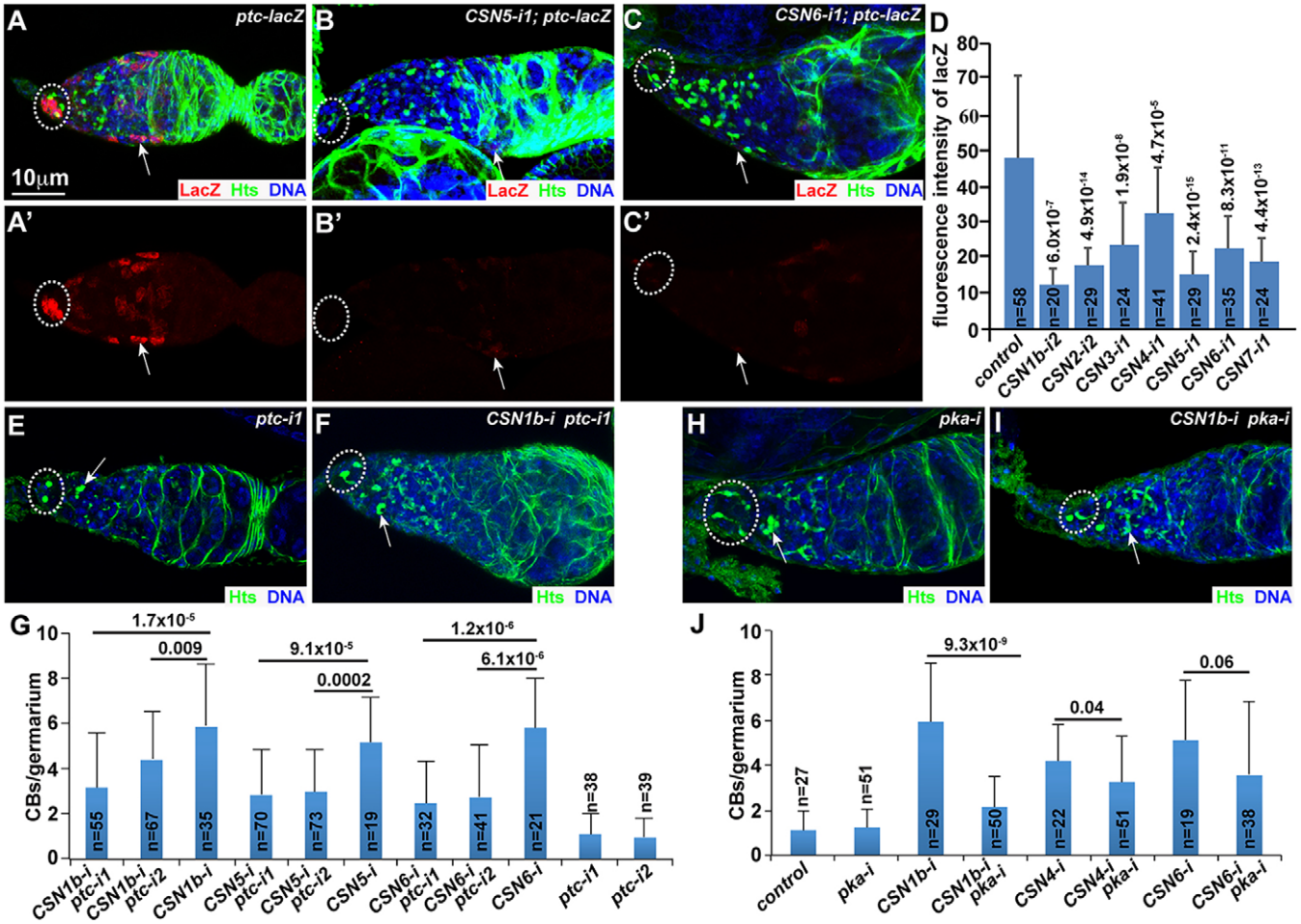

Fig. 4. COP9 is required in ECs to promote Hh signaling and thus germ cell differentiation. (A-D) *CSN5* (B) and *CSN6* (C) knockout ECs show a significant reduction (quantified in D) of *ptc-lacZ* expression in comparison with control ECs (A). (E-J) EC-specific knockout of *ptc* (F) or *pka* (I) can significantly rescue the germ cell differentiation defect caused by *CSN1b* knockout (quantified in G and J), whereas EC-specific *ptc* (E) or *pka* (H) knockout does not interfere with normal germ cell differentiation (quantified in G and J). Broken ovals show GSCs; arrows indicate ECs (A-C) or differentiated cysts (E-I). Error bars represent s.d., *P*-values are given above columns of statistically significant results.

results indicate that COP9 is required intrinsically to maintain EC cellular processes.

COP9 and Hh signaling are required in ECs to promote GSC differentiation partly by preventing BMP signaling

Previous studies suggested that one of the major functions of ECs is to prevent Dpp/BMP from spreading to the germ cell differentiation zone (Eliazer et al., 2011; Jin et al., 2013; Kirilly et al., 2011; Liu et al., 2010; Ma et al., 2014; Wang et al., 2011). To determine if BMP signaling is indeed active in the germ cells in the differentiation zone, we used two BMP activity reporters, *Dad-lacZ* and *bam-GFP*, to monitor BMP signaling in the COP9 and Hh signaling-defective germaria. In the control germaria, *Dad-lacZ* expression is activated in GSCs by BMP signaling, and turned off in CBs and mitotic cysts (Fig. 6A) (Casanueva and Ferguson, 2004; Kai and Spradling, 2003; Song et al., 2004; Xie and Spradling, 1998). By contrast, the *CSN1b*, *CSN6* or *smo* knockout germaria contain *Dad-lacZ*-positive CB-like cells a few cells away from GSCs in addition to *Dad-lacZ*-positive GSCs, indicating that COP9 and Hh signaling are required in ECs to prevent BMP signaling from spreading to the germ cell differentiation zone (Fig. 6B-E). Importantly, only a small fraction of the accumulated single germ cells express *Dad-lacZ*, and those expressing *Dad-lacZ* show much lower levels of expression than GSCs, suggesting that those single germ cells represent a mixture of CBs at different differentiation stages (Fig. 6E). In the control germarium, *bam-GFP* is repressed in GSCs by BMP signaling, and gains expression in CBs and mitotic cysts as a result of the loss of BMP signaling (Fig. 6F) (Chen and McKearin, 2003a,b; Song et al., 2004). Consistently, *bam-GFP* is weakened or repressed in some single germ cells a few cells away from GSCs in the *CSN1b*, *CSN6* or *smo* knockout germaria (Fig. 6G-J), further supporting the idea that COP9 and Hh signaling are required in ECs to prevent BMP signaling in the germ cell differentiation zone. It is important to note that the accumulated single germ cells represent a mixture of CBs at different developmental stages: those with no *bam-GFP* expression representing CBs freshly separated from GSCs (or pre-CBs), those with low *bam-GFP* expression representing early differentiating CBs and those with high *bam-GFP* expression representing late CBs. For this reason we have referred to accumulated single germ cells caused by defective COP9 and Hh signaling collectively as CBs. Thus, these results demonstrate that COP9 and Hh signaling are required in ECs to prevent BMP signaling.

Defective ECs can increase BMP signaling activity in the differentiating germ cells by upregulating *dpp* expression (Eliazer et al., 2011; Jin et al., 2013; Kirilly et al., 2011; Liu et al., 2010; Ma

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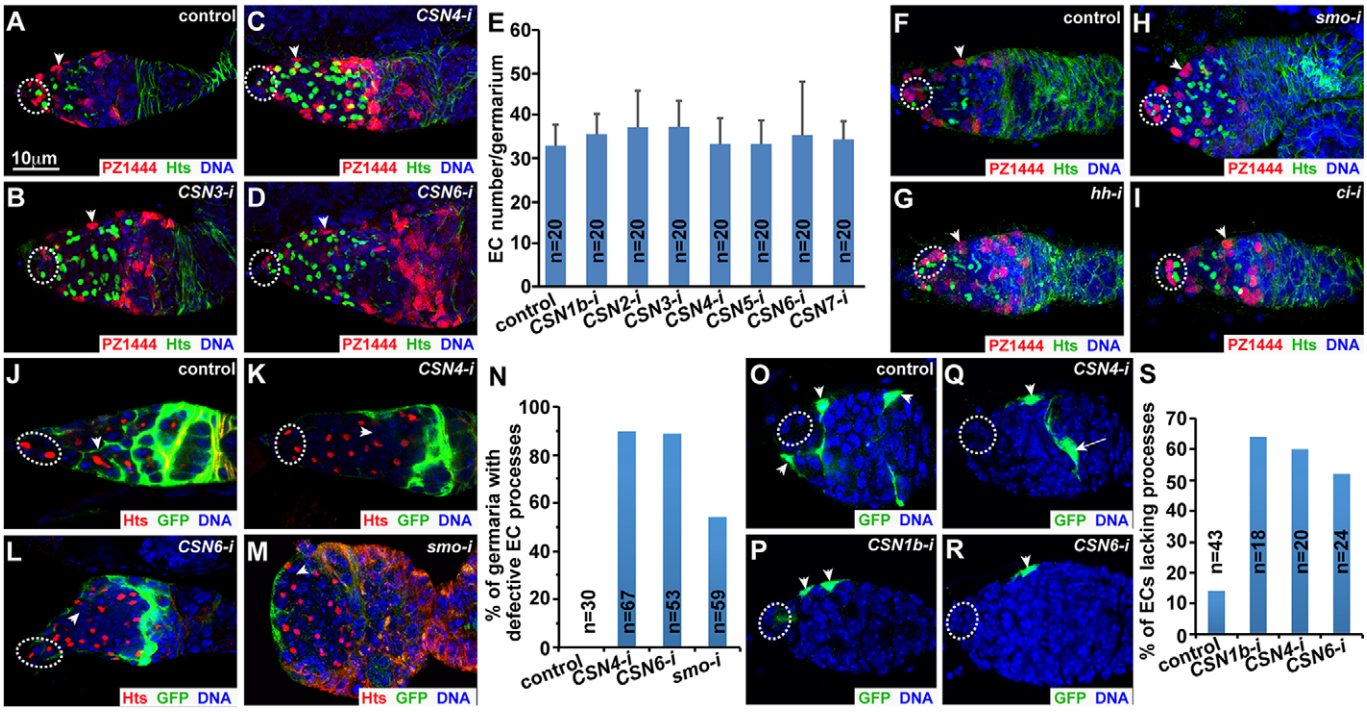

Fig. 5. COP9 and Hh signaling are required for maintaining long EC cellular processes, but not ECs themselves. (A-E) EC-specific knockout of CSN genes (B-D) does not affect the EC number (quantified in E) in comparison to the control (A). (F-I) EC-specific knockout of *hh* (G), *smo* (H) and *ci* (I) genes does not affect EC maintenance in comparison to the control (F). (J-N) EC-specific knockout of *CSN4* (K), *CSN6* (L) and *smo* (M) results in the loss of EC cellular processes wrapping differentiated germ cells (quantified in N) existing in the control ECs (J). (O-S) GFP-labeled individual *CSN1b* (P), *CSN4* (Q) and *CSN6* (R) knockout ECs (arrowheads) show the loss of cellular processes (quantified in S) in comparison with the marked control ECs (O). The arrow in Q indicates the GFP-labeled *CSN4* knockout EC with cellular processes. Broken ovals show GSCs; arrowheads indicate ECs in A-D and F-I, EC cellular processes in J, missing cellular processes in K-M, GFP-labeled control ECs in O, and GFP-labeled CSN knockout ECs in P-R. Error bars represent s.d., *P*-values are given above columns of statistically significant results.

et al., 2014; Wang et al., 2011). To determine if COP9 is required in ECs to repress *dpp* expression, we performed qRT-PCR to determine *dpp* mRNA expression levels in the purified GFP-labeled control, *CSN4* and *CSN5* knockout ECs. Indeed, *dpp* mRNA levels increase in the *CSN4* or *CSN5* knockout ECs compared with the control ECs (Fig. 6K). To further determine if *dpp* mRNA upregulation in *CSN4* or *CSN5* knockout ECs is responsible for the germ cell differentiation defect caused by *CSN4* or *CSN5* knockout, we performed *dpp CSN4/5* double knockout experiments in ECs. *dpp CSN4* or *dpp CSN5* double knockout germaria carry significantly fewer CBs than *CSN4* or *CSN5* knockout germaria, whereas *dpp* knockout germaria have the normal CB number, indicating that *dpp* upregulation in the CSN knockout ECs contributes to the germ cell differentiation defect (Fig. 6L-N). Consistent with the idea that COP9 modulates Hh signaling activity in ECs, the *dpp smo* double knockout germaria also carry significantly fewer CBs than the *smo* knockout germaria (Fig. 6O-Q). In summary, COP-regulated Hh signaling in ECs prevents BMP signaling from spreading to the differentiation zone, thereby providing a permissive environment for GSC progeny differentiation.

Both COP9 and Hh signaling are dispensable for MAPK signaling in ECs

MAPK signaling is active in ECs, and has been shown to be important for EC cellular process formation and germ cell differentiation (Liu et al., 2010; Schultz et al., 2002). In the control germarium, only ECs express nuclear pERK (also known as Rl or Mapk) at high levels (Fig. 7A). The CSN knockout ECs still exhibit comparable expression levels of nuclear pERK to those control ECs (Fig. 7A-D). Our quantification results have further confirmed that there are no significant differences in nuclear pERK expression levels between the control and CSN knockout ECs (Fig. 7E). Similarly, the *smo* knockout ECs also show comparable pERK expression to those control ECs (Fig. 7F,G). These results indicate that the COP9-Hh axis is dispensable in ECs to maintain pERK expression.

Defective EGFR-ERK signaling in ECs is known to cause an upregulation of *dally* expression (Liu et al., 2010). *dally* encodes a proteoglycan protein, which is important for Dpp/BMP diffusion in the GSC niche (Akiyama et al., 2008; Belenkaya et al., 2004; Guo and Wang, 2009; Jackson et al., 1997). Consistently, in CSN or *smo* knockout germaria, qRT-PCR revealed that *dally* expression is not upregulated in comparison with controls (Fig. 7H). These results further suggest that COP9 and Hh signaling control germ cell differentiation independently of MAPK signaling in ECs.

DISCUSSION

Although ECs have been proposed to function as a niche for promoting GSC progeny differentiation (Kirilly et al., 2011), it remains poorly understood how its function is regulated. In this study, we show that the COP9 complex is required in ECs to promote germ cell differentiation by positively regulating Hh signaling (Fig. 7I). RNAi-mediated knockout of COP9 and Hh pathway components in adult ECs causes similar germ cell differentiation defects, but does not affect EC numbers, indicating that both COP9 and Hh signaling regulate EC function in promoting GSC progeny differentiation. Interestingly, COP9 and Hh signaling are important for maintaining long EC cellular processes encasing

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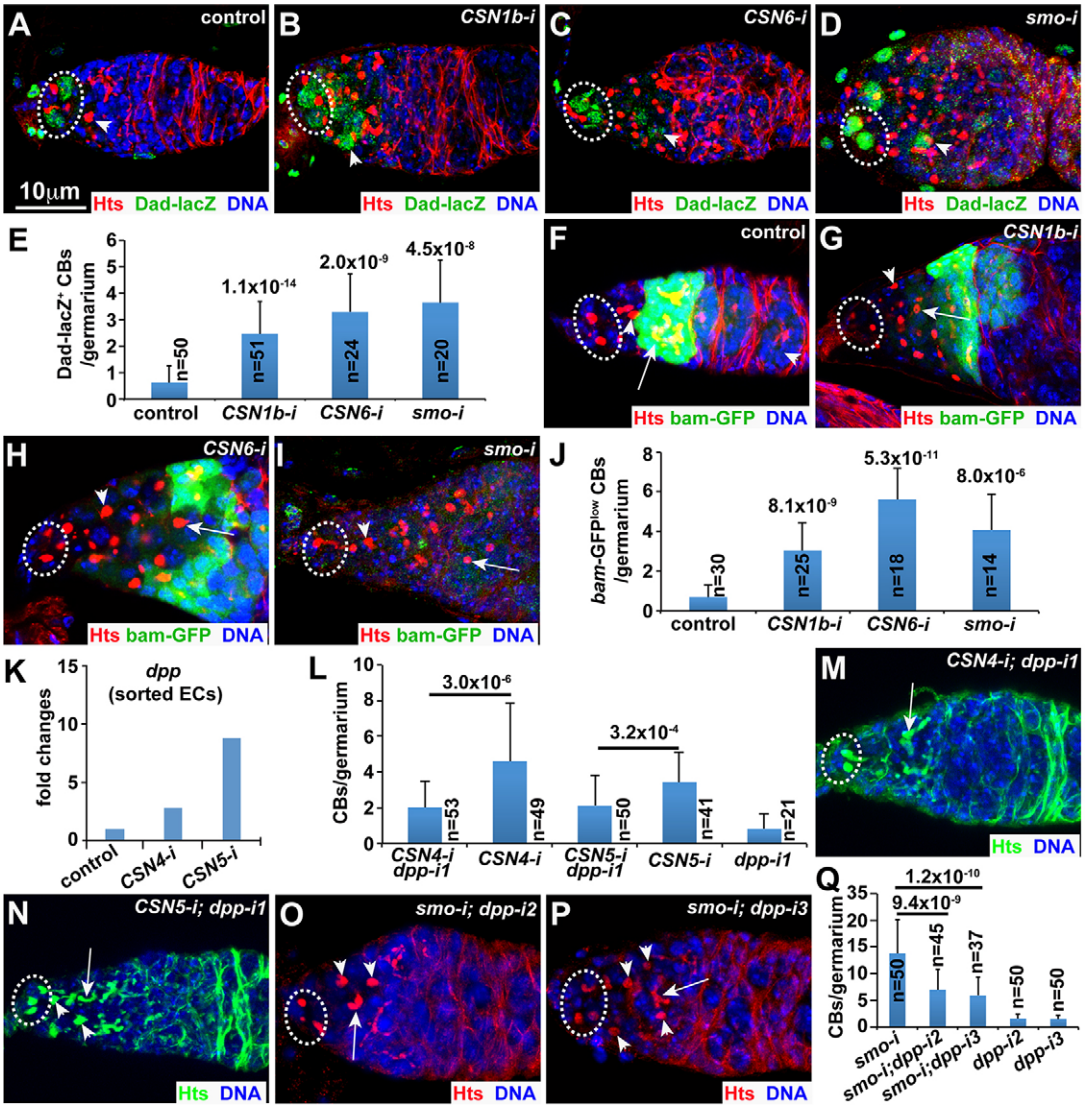

Fig. 6. COP9 and Hh signaling are required in ECs to prevent BMP signaling in differentiated germ cells by repressing *dpp* expression. (A-E) EC-specific knockout of *CSN1b* (B), *CSN6* (C) and *smo* (D) upregulates *Dad-lacZ* expression in CBs (arrowheads) (*Dad-lacZ*$^{+}$ CB-like cells quantified in E) compared with control CBs which lack *Dad-lacZ* expression (A). (F-J) *bam-GFP* expression remains repressed in some CBs a few cells away from GSCs in *CSN1b* (G), *CSN6* (H) and *smo* (I) knockout germaria, whereas germ cells (arrow, F) in similar positions in the control germarium express *bam-GFP* (bam-GFP^{low} CBs are quantified in J). (K) *dpp* mRNA levels significantly increase in the purified *CSN4* and *CSN5* knockout ECs in comparison to the control ECs. (L-Q) EC-specific knockout of *dpp* significantly decreases the excess CBs caused by *CSN4* and *CSN5* (M,N, quantified in L) and *smo* (O,P: two independent *dpp* RNAi lines, quantified in Q) knockout by promoting them to differentiate into cysts (arrows). Broken ovals show GSCs; arrowheads indicate CBs. Error bars represent s.d., *P*-values are given above columns of statistically significant results.

differentiated GSC progeny, but are dispensable for MAPK signaling activation. Finally, both COP9 and Hh signaling are required in ECs by repressing *dpp* expression, thereby preventing BMP signaling in differentiating GSC progeny. Consistent with the findings in this study, a new study shows that COP9 is also required in somatic cyst cells to regulate their cellular processes encasing differentiated GSC progeny and promote germ cell differentiation in the *Drosophila* testis (Qian et al., 2015). However, our findings are in contrast to a recent study reporting that Hh signaling is required in CPCs to maintain GSCs by promoting Dpp signaling (Rojas-Ríos et al., 2012). This present study has identified COP9 and Hh signaling as two important pathways in the regulation of EC function and GSC progeny differentiation, further supporting the concept that ECs function as the differentiation niche for GSC progeny in the *Drosophila* ovary (Fig. 7I).

Our recent study has demonstrated that COP9 is required in GSCs and their progeny to control self-renewal and differentiation (Pan et al., 2014). This study demonstrates that COP9 is required in ECs to promote germ cell differentiation partly by maintaining Hh signaling and preventing BMP signaling. Firstly, the whole COP9 complex is required in adult ECs to promote GSC progeny differentiation; RNAi-mediated inactivation of all eight COP9 components in adult ECs leads to an accumulation of excess undifferentiated single germ cells. In addition, we show that COP9 controls EC function in deneddylase-dependent and -independent manners to promote GSC progeny differentiation. Secondly, COP9 is dispensable for EC maintenance and MAPK signaling activation in ECs. CSN knockout ECs are well maintained, and still retain active MAPK signaling. Thirdly, COP9 is required in ECs to maintain encasing of differentiating germ cells by their long cellular

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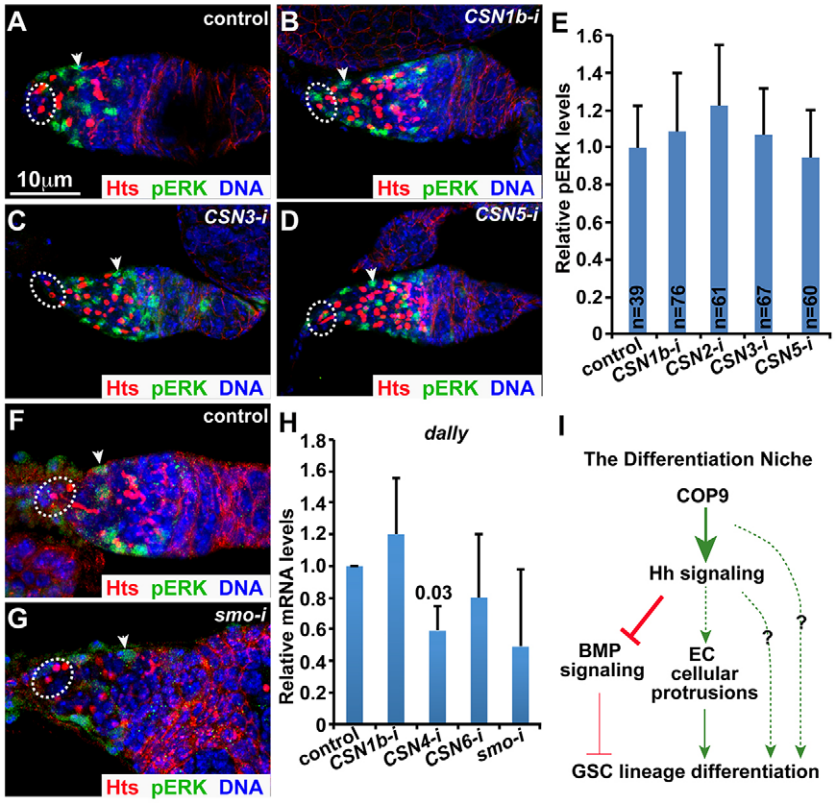

Fig. 7. Both COP9 and Hh signaling are dispensable for pERK and dally expression in ECs. (A-E) *CSN1b* (B), *CSN3* (C) and *CSN5* (D) knockout ECs express normal levels of pERK (quantified in E) in comparison to control ECs (A). (F,G) *smo* (G) knockout ECs express pERK at the levels comparable to control ECs (F). (H) The dissected CSN and *smo* knockout germaria express similar levels of *dally* mRNAs to the control germaria. Broken ovals show GSCs; arrowheads indicate ECs. Error bars represent s.d., *P*-values are given above columns of statistically significant results. (I) A working model showing that COP9 controls Hh signaling in ECs, which in turn regulates GSC lineage differentiation by repressing BMP signaling and also possibly by maintaining EC cellular processes. The dashed lines represent other possible pathways COP9 and Hh signaling could regulate.

processes, which are known to be important for GSC progeny differentiation (Kirilly et al., 2011). Active MAPK signaling is known to be important for maintaining EC cellular processes (Liu et al., 2010; Schultz et al., 2002). Interestingly, COP9 is required intrinsically to maintain EC cellular processes independently of MAPK signaling. Finally, COP9 is also required in ECs to prevent BMP signaling in differentiating germ cells. Taken together, our results demonstrate that COP9 is required in ECs to promote GSC progeny differentiation, possibly by maintaining EC cellular processes and preventing BMP signaling.

In contrast to a recent study proposing that Hh signaling is required in the GSC niche to maintain Dpp expression and thus promote GSC maintenance (Rojas-Ríos et al., 2012), in this study, we show that Hh signaling is required in ECs to promote GSC progeny differentiation by preventing BMP signaling. Firstly, all *hh* pathway components, *hh*, *ptc*, *smo* and *ci*, are expressed in ECs and also required in ECs to promote germ cell differentiation, with their knockout in ECs all leading to similar germ cell differentiation defects. Secondly, Hh signaling is dispensable for EC maintenance and MAPK signaling, which are both known to be important for germ cell differentiation (Liu et al., 2010; Ma et al., 2014; Schultz et al., 2002; Wang et al., 2011). Thirdly, Hh signaling is required in ECs to maintain the wrapping of differentiating germ cells by their long cellular processes, as *smo* knockout ECs lose their long cellular processes. Finally, Hh signaling is required in ECs to prevent BMP signaling in the differentiation zone, thereby promoting GSC progeny differentiation. EC-specific *dpp* knockout can significantly rescue the germ cell differentiation defect caused by *smo* knockout, indicating that elevated *dpp* expression in Hh signaling-defective ECs causes the germ cell differentiation defect. Our results have unequivocally demonstrated that autocrine Hh signaling is required in ECs to control the function of the differentiation niche, partly by preventing *dpp* expression. In addition, CPC-specific *hh* knockout does not result in GSC loss as reported by Rojas-Ríos et al. (2012). A possible explanation for the discrepancies is the utilization of problematic reagents in the published study.

In the *Drosophila* imaginal disc, COP9 is known to be required to maintain low-to-intermediate Hh signaling by regulating the stability of Ci (Wu et al., 2005). This study shows that Hh signaling functions downstream of COP9 to control GSC progeny differentiation. Firstly, using *ptc-lacZ* as an Hh signaling activity reporter, a significant and drastic reduction *ptc-lacZ* expression is in CSN knockout ECs showed that COP9 is required in ECs to maintain Hh signaling activity. Secondly, *ptc* and *pka* inactivation in ECs can partially and significantly rescue the germ cell differentiation defect caused by CSN knockout. Taken together, Hh signaling functions downstream of COP9 to control GSC progeny differentiation primarily by regulating the function, but not the maintenance, of the differentiation niche. Although COP9 is required intrinsically for both GSC maintenance and progeny differentiation, its targets in GSCs and their progeny have not yet been identified. As Hh signaling is dispensable intrinsically for GSC maintenance and germ cell differentiation, it is unlikely that COP9 regulates Hh signaling intrinsically to control GSC maintenance and differentiation. Therefore, in the future, it is important to further investigate how COP9 regulates Hh signaling mechanistically in ECs, and how COP9 controls GSC self-renewal and their progeny differentiation at the molecular level.

MATERIALS AND METHODS

Drosophila stocks and RNAi-mediated knockout

Flies were maintained at 25°C on standard cornmeal media supplied with live yeast unless otherwise specified. The information about the following strains are available from http://flybase.org/: *c587-gal4*, *bam-gfp*, *Dad-lacZ*, *UAS-CD8GFP*, *PZ1444*, *ptc-lacZ*. For gene-specific knockout in ECs, the following RNAi lines were used for this study (for more detailed

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information, see Table S1): *CSN1a* (TH00055.N), *CSN1b* (TH00057.N, TH00058.N), *CSN2* (TH00065.N, TH00066.N), *CSN3* (TH00052.N, THU0736), *CSN4* (TH00059.N, TH00060.N), *CSN5* (TH00061.N, TH00062.N), *CSN6* (TH00063.N, TH00064.N), *CSN7* (TH00053.N, THU0610), *CSN8* (TH00054.N), *hh* (TH00654.N, THU0935), *smo* (TH00655.N, TH00656.N), *ci* (TH00657.N, TH00658.N), *ptc* (TH00660.N), *dpp* (THU0083). The following RNAi lines were obtained from the Bloomington Stock Center: *ptc* (JF03223), *smo* (JF02363) and *dpp* (JF01090 and JF01091).

Production of plasmid constructs and transgenic flies

The coding region for the *CSN5* transcript was cloned and inserted into pValium20 vector by *Nhe*I and *Eco*RI restrictive enzyme sites by standard protocol. To make the RNAi-resistant *CSN5*, which still encodes a wild-type CSN5 protein, the RNAi targeted sequence CGGAACCGATGTCAATGAGAA was changed into AGGTACAGACGTAAACGAAAA to maximally disrupt the matched sequences. The *CSN5* mutant (*CSN5ED*), which encodes an enzyme-dead CSN5 protein, was made by mutating the 135th histidine into alanine (CAC to GCC) on the RNAi-resistant *CSN5* gene. Both constructs were made by standard site-directed mutagenesis. The plasmids were sequenced, extracted and injected into recipient fly embryos carrying the *attp40* attR site and a recombinase-expressing transgene to generate transgenic flies.

Immunohistochemistry and fluorescence microscopy

Ovaries were dissected, fixed and stained according to the method described previously (Xie and Spradling, 1998). The following primary antibodies were used: monoclonal anti-Hts (1B1, 1:200, DSHB), chicken anti-GFP antibody (cat. #A10262, 1:400, Life Technology), rabbit anti-β-galactosidase (cat. #559761, 1:10,000, MP Biomedicals), rabbit anti-phosphorylated ERK1/2 (cat. #4370, 1:200, Cell Signaling Technology). Secondary antibodies used were: Alexa Fluor 488 or Alexa Fluor 555 conjugated goat anti-mouse IgG antibody and Alexa Fluor 488 or Alexa Fluor 568 conjugated goat anti-rabbit IgG antibody (cat. #A11029, A21424, A11034 and A11011, respectively, 1:400, Life Technology), Alexa Fluor 488 conjugated goat anti-mouse IgG antibody and Alexa Fluor 488 conjugated donkey anti-chicken IgY antibody (cat. #115-545-062 and 103-545-155, respectively, 1:400, Jackson ImmunoResearch Laboratories). All pictures were taken with Leica SP5 confocal microscope and processed with Leica SP5 or Photoshop (Adobe) software.

Measurement of fluorescence intensity

To compare fluorescence intensity between control and gene knockout ECs, all images were measured under the same parameters at the same time using Leica SP5 quantification software. The selected regions within ECs were randomly picked, and the mean fluorescence intensity was normalized to the background, calculated and compared.

RNA extraction and qRT-PCR

To detect *dally* mRNA expression in CSN and *smo* knockout germaria, germaria were manually cut under a microscope and total RNA isolated using Trizol according to the manufacturer's manual. To detect *dpp* mRNA expression in ECs, GFP-labeled ECs were sorted by FACS, and total RNA was extracted using Trizol. RNA was amplified using CellAmp Whole Transcriptome Amplification Kit (TAKARA). Quantitative PCR (qPCR) was performed with GoTaq qPCR Master Mix (Promega) on ABI SteponePlus Instruments. The ΔΔCt method was used to compare gene expression levels. The primers used were: *dpp* – TCGGCCAACACAGTGCGAAGTTT and TTCACGTCGAAGTGCAGCCGAAA, *dally* – GAGCAACAGCAGATGCACACGAAT and GTGCACTTCAAGGGTTTCACGGTT; RNAi-resistant *CSN5* and *CSN5ED* – AAGCAGTACTATCCCTTGGAAA and TTCTCATTG ACATCGGTTCCG.

Acknowledgements

We would like to thank Developmental Studies Hybridoma Bank and Bloomington *Drosophila* Stock Center for reagents, and the Ni and Xie lab members for comments and discussions.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Manuscript preparation: T.L., S.W., J.N. and T.X.; experimental design: T.L., S.W., J.N. and T.X.; data acquisition and interpretation: T.L., S.W., Y.G., Y.M., Z.Y., L.L. and X.S.

Funding

This work was supported by Tsinghua-Peking Joint Center for Life Sciences (T.L.); the Ministry of Science and Technology of the People's Republic of China [2015BAI09B03 and 2013CB35102 to J. N.]; and the Stowers Institute for Medical Research (T.X.).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.124768/-/DC1

References

Akiyama, T., Kamimura, K., Firkus, C., Takeo, S., Shimmi, O. and Nakato, H. (2008). Dally regulates Dpp morphogen gradient formation by stabilizing Dpp on the cell surface. *Dev. Biol.* **313**, 408-419.

Belenkaya, T. Y., Han, C., Yan, D., Opoka, R. J., Khodoun, M., Liu, H. and Lin, X. (2004). *Drosophila* Dpp morphogen movement is independent of dynamin-mediated endocytosis but regulated by the glypican members of heparan sulfate proteoglycans. *Cell* **119**, 231-244.

Bolívar, J., Pearson, J., López-Onieva, L. and González-Reyes, A. (2006). Genetic dissection of a stem cell niche: the case of the *Drosophila* ovary. *Dev. Dyn.* **235**, 2969-2979.

Casanueva, M. O. and Ferguson, E. L. (2004). Germline stem cell number in the *Drosophila* ovary is regulated by redundant mechanisms that control Dpp signaling. *Development* **131**, 1881-1890.

Chen, D. and McKearin, D. (2003a). Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. *Curr. Biol.* **13**, 1786-1791.

Chen, D. and McKearin, D. M. (2003b). A discrete transcriptional silencer in the *bam* gene determines asymmetric division of the *Drosophila* germline stem cell. *Development* **130**, 1159-1170.

Cope, G. A. and Deshaies, R. J. (2003). COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. *Cell* **114**, 663-671.

Cope, G. A., Suh, G. S. B., Aravind, L., Schwarz, S. E., Zipursky, S. L., Koonin, E. V. and Deshaies, R. J. (2002). Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**, 608-611.

Doronkin, S., Djagaeva, I. and Beckendorf, S. K. (2003). The COP9 signalosome promotes degradation of Cyclin E during early *Drosophila* oogenesis. *Dev. Cell* **4**, 699-710.

Eliazer, S., Shalaby, N. A. and Buszczak, M. (2011). Loss of lysine-specific demethylase 1 nonautonomously causes stem cell tumors in the *Drosophila* ovary. *Proc. Natl. Acad. Sci. USA* **108**, 7064-7069.

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

Guo, Z. and Wang, Z. (2009). The glypican Dally is required in the niche for the maintenance of germline stem cells and short-range BMP signaling in the *Drosophila* ovary. *Development* **136**, 3627-3635.

Ingham, P. W., Nakano, Y. and Seger, C. (2011). Mechanisms and functions of Hedgehog signalling across the metazoa. *Nat. Rev. Genet.* **12**, 393-406.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. (1997). The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124**, 761-771.

Jackson, S. M., Nakato, H., Sugiura, M., Jannuzi, A., Oakes, R., Kaluza, V., Golden, C. and Selleck, S. B. (1997). Dally, a *Drosophila* glypican, controls cellular responses to the TGF-beta-related morphogen, Dpp. *Development* **124**, 4113-4120.

Jin, Z., Flynt, A. S. and Lai, E. C. (2013). *Drosophila piwi* mutants exhibit germline stem cell tumors that are sustained by elevated Dpp signaling. *Curr. Biol.* **23**, 1442-1448.

Kai, T. and Spradling, A. (2003). An empty *Drosophila* stem cell niche reactivates the proliferation of ectopic cells. *Proc. Natl. Acad. Sci. USA* **100**, 4633-4638.

Kalderon, D. (2000). Transducing the Hedgehog signal. *Cell* **103**, 371-374.

King, F. J., Szakmary, A., Cox, D. N. and Lin, H. (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.

Kirilly, D., Wang, S. and Xie, T. (2011). Self-maintained escort cells form a germline stem cell differentiation niche. *Development* **138**, 5087-5097.

Li, L. and Xie, T. (2005). Stem cell niche: structure and function. *Annu. Rev. Cell Dev. Biol.* **21**, 605-631.

Lin, H., Yue, L. and Spradling, A. C. (1994). The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* **120**, 947-956.
Liu, M., Lim, T. M. and Cai, Y. (2010). The *Drosophila* female germline stem cell lineage acts to spatially restrict DPP function within the niche. *Sci. Signal.* **3**, ra57.
Lum, L. and Beachy, P. A. (2004). The Hedgehog response network: sensors, switches, and routers. *Science* **304**, 1755-1759.
Ma, X., Wang, S., Do, T., Song, X., Inaba, M., Nishimoto, Y., Liu, L.-P., Gao, Y., Mao, Y., Li, H. 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.
Maimon, I., Popliker, M. and Gilboa, L. (2014). Without children is required for Stat-mediated *zfh1* transcription and for germline stem cell differentiation. *Development* **141**, 2602-2610.
Margolis, J. and Spradling, A. (1995). Identification and behavior of epithelial stem cells in the *Drosophila* ovary. *Development* **121**, 3797-3807.
McKearin, D. and Ohlstein, B. (1995). A role for the *Drosophila* bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development* **121**, 2937-2947.
McKearin, D. M. and Spradling, A. C. (1990). *bag-of-marbles*: a *Drosophila* gene required to initiate both male and female gametogenesis. *Genes Dev.* **4**, 2242-2251.
Morris, L. X. and Spradling, A. C. (2011). Long-term live imaging provides new insight into stem cell regulation and germline-soma coordination in the *Drosophila* ovary. *Development* **138**, 2207-2215.
Morrison, S. J. and Spradling, A. C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* **132**, 598-611.
Ni, J.-Q., Markstein, M., Binari, R., Pfeiffer, B., Liu, L.-P., Villalta, C., Booker, M., Perkins, L. and Perrimon, N. (2008). Vector and parameters for targeted transgenic RNA interference in *Drosophila* melanogaster. *Nat. Methods* **5**, 49-51.
Ni, J.-Q., Zhou, R., Czech, B., Liu, L.-P., Holderbaum, L., Yang-Zhou, D., Shim, H.-S., Tao, R., Handler, D., Karpowicz, P. et al. (2011). A genome-scale shRNA resource for transgenic RNAi in Drosophila. *Nat. Methods* **8**, 405-407.
Ohlstein, B. and McKearin, D. (1997). Ectopic expression of the *Drosophila* Bam protein eliminates oogenic germline stem cells. *Development* **124**, 3651-3662.
Oron, E., Mannervik, M., Rencus, S., Harari-Steinberg, O., Neuman-Silberberg, S., Segal, D. and Chamovitz, D. A. (2002). COP9 signalosome subunits 4 and 5 regulate multiple pleiotropic pathways in *Drosophila melanogaster*. *Development* **129**, 4399-4409.
Pan, L., Wang, S., Lu, T., Weng, C., Song, X., Park, J. K., Sun, J., Yang, Z.-H., Yu, J., Tang, H. et al. (2014). Protein competition switches the function of COP9 from self-renewal to differentiation. *Nature* **514**, 233-236.
Qian, Y., Ng, C. L. and Schulz, C. (2015). CSN maintains the germline cellular microenvironment and controls the level of stem cell genes via distinct CRLs in testes of *Drosophila melanogaster*. *Dev. Biol.* **398**, 68-79.
Rojas-Ríos, P., Guerrero, I. and González-Reyes, A. (2012). Cytoneme-mediated delivery of hedgehog regulates the expression of bone morphogenetic proteins to maintain germline stem cells in *Drosophila*. *PLoS Biol.* **10**, e1001298.
Sahai-Hernandez, P. and Nystul, T. G. (2013). A dynamic population of stromal cells contributes to the follicle stem cell niche in the *Drosophila* ovary. *Development* **140**, 4490-4498.
Schultz, C., Wood, C. G., Jones, D. L., Tazuke, S. I. and Fuller, M. T. (2002). Signaling from germ cells mediated by the rhomboid homolog stet organizes encapsulation by somatic support cells. *Development* **129**, 4523-4534.
Song, X., Zhu, C.-H., Doan, C. and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the *Drosophila* ovary niches. *Science* **296**, 1855-1857.
Song, X., Wong, M. D., Kawase, E., Xi, R., Ding, B. C., McCarthy, J. J. and Xie, T. (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.
Song, X., Call, G. B., Kirilly, D. and Xie, T. (2007). Notch signaling controls germline stem cell niche formation in the *Drosophila* ovary. *Development* **134**, 1071-1080.
Vodermaier, H. C. (2004). APC/C and SCF: controlling each other and the cell cycle. *Curr. Biol.* **14**, R787-R796.
Wang, X., Pan, L., Wang, S., Zhou, J., McDowell, W., Park, J., Haug, J., Zueckert-Gaudenz, K., Tang, H. and Xie, T. (2011). Histone H3K9 trimethylase eggless controls germline stem cell maintenance and differentiation. *PLoS Genet.* **7**, e1002426.
Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M. and Deng, X.-W. (1998). The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. *Curr. Biol.* **8**, 919-924.
Wu, J.-T., Lin, H.-C., Hu, Y.-C. and Chien, C.-T. (2005). Neddylation and deneddylation regulate Cul1 and Cul3 protein accumulation. *Nat. Cell Biol.* **7**, 1014-1020.
Wu, J.-T., Lin, W.-H., Chen, W.-Y., Huang, Y.-C., Tang, C.-Y., Ho, M. S., Pi, H. and Chien, C.-T. (2011). CSN-mediated deneddylation differentially modulates Ci (155) proteolysis to promote Hedgehog signalling responses. *Nat. Commun.* **2**, 182.
Xie, T. (2013). Control of germline stem cell self-renewal and differentiation in the *Drosophila* ovary: concerted actions of niche signals and intrinsic factors. *Wiley Interdiscip. Rev. Dev. Biol.* **2**, 261-273.
Xie, T. and Spradling, A. C. (1998). *decapentaplegic* is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. *Cell* **94**, 251-260.
Zhang, Y. and Kalderon, D. (2001). Hedgehog acts as a somatic stem cell factor in the *Drosophila* ovary. *Nature* **410**, 599-604.
Zhu, C.-H. and Xie, T. (2003). Clonal expansion of ovarian germline stem cells during niche formation in *Drosophila*. *Development* **130**, 2579-2588.

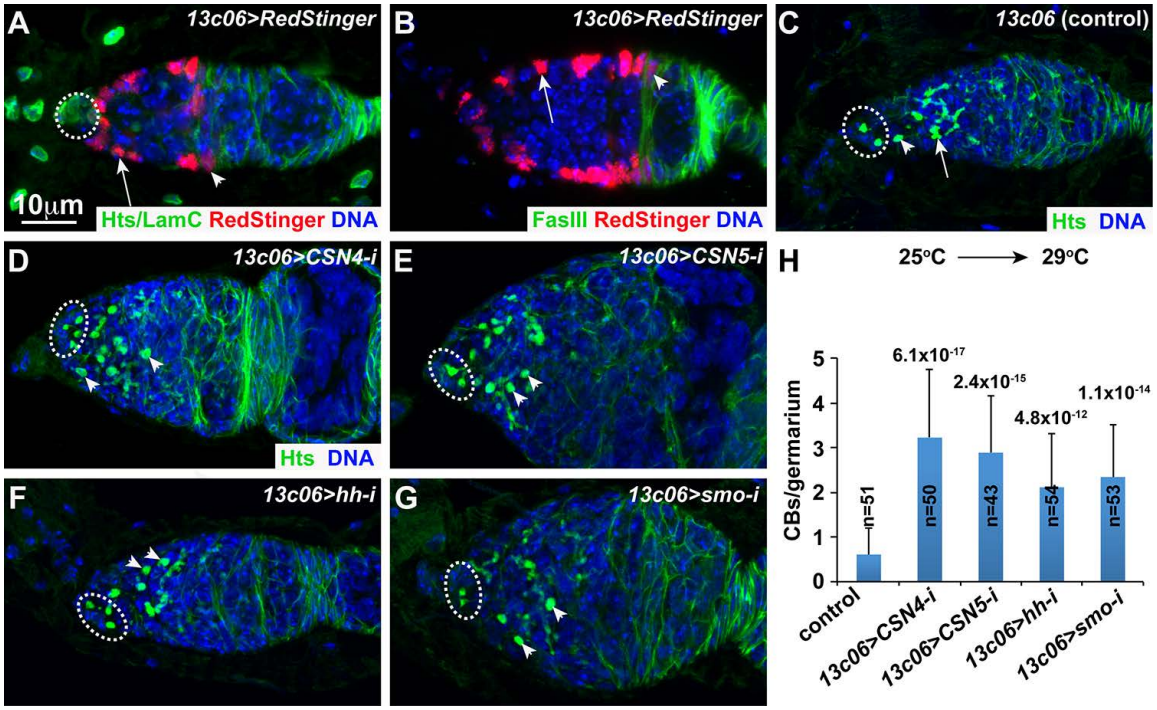

Fig. S1. EC-specific csn4, csn5, hh and smo knockdown leads to similar germ cell differentiation defect. (**A**, **B**) *13c06-gal4* drives the expression of *UAS-RedStinger* in ECs (arrows) at high levels but in early follicle progenitor cells (arrowheads) at much lower levels. Its expression is absent in cap cells (broken circle). Cap cells are labeled by LamC (**A**), whereas follicle cell progenitors are marked by Hts (**A**) or FasIII (**B**). (**C**-**H**) *13c06-gal4*-driven *csn4* (**D**), *csn5* (**E**), *hh* (**F**) and *smo* (**G**) knockdown in ECs leads to the accumulation of CB-like cells in the knockdown germaria in comparison with the control germarium (**C**) (**H**: CB-like cell quantification results).

Table S1. Information on RNAi lines against various components of COP9 and Hh pathway

Gene	Symbol	CG#	Target sequences	Vector	Location
csn1a	TH00055.N	CG4697	CACCTACAATGTCAAGTTGT	VALIUM20	attp2 III
csn1b	TH00057.N	CG3889	CAGCATCGATCTCGAAGTGT	VALIUM20	attp2 III
csn1b	TH00058.N	CG3889	TTCAAGTTCTACGAAAGTAA	VALIUM20	attp2 III
csn2	TH00065.N	CG9556	TCGGCTGTGGTTTAAGACCAA	VALIUM20	attp2 III
csn2	TH00066.N	CG9556	CTGCAGAAGATACTAAAGCAA	VALIUM20	attp2 III
csn3	THU0736	CG18332	CTCACCGGAGATGTTCCTCAA	VALIUM20	attp2 III
csn3	TH00052.N	CG18332	CGGTATGGTGTTGTTCAAAG	VALIUM20	attp2 III
csn4	TH00059.N	CG8725	TCGACTCTATCTGGAGGACA	VALIUM20	attp2 III
csn4	TH00060.N	CG8725	TCCGAGGAGTTGCAGGTTCT	VALIUM20	attp2 III
csn5	TH00061.N	CG14884	CGGAACCGATGTCAATGAG	VALIUM20	attp2 III
csn5	TH00062.N	CG14884	CAGCTCGGGTCTGCTCACC	VALIUM20	attp2 III
csn6	TH00063.N	CG6932	CCCCATCATGCTACAGCTG	VALIUM20	attp2 III
csn6	TH00064.N	CG6932	AACAGTGATCAACAAGGACT	VALIUM20	attp2 III
csn7	TH00053.N	CG2038	ACGCGAATGCCGAGAAATCT	VALIUM20	attp2 III
csn7	THU0610	CG2038	TTGCATCGAAATGCAGATTAA	VALIUM20	attp2 III
csn8	TH00054.N	CG42522	CAGCGCATATATGTCCATTT	VALIUM20	attp2 III
hh	TH00654.N	CG4637	AAGCAGACAATTCCCAATCTA	VALIUM20	attp2 III
hh	THU0935	CG4637	TCGATCGATACTATTTATTTA	VALIUM20	attp2 III
smo	TH00655.N	CG11561	TCCGGCAGTAATTGTGTTCTA	VALIUM20	attp2 III
smo	TH00656.N	CG11561	CAGCATATGTCTTCTGTCTAA	VALIUM20	attp2 III
ci	TH00657.N	CG2125	CCGCTTGGATGAGTATATTAA	VALIUM20	attp2 III
ci	TH00658.N	CG2125	TACGCAAGATGAACTTGTTAA	VALIUM20	attp2 III
ptc	TH00660.N	CG2411	ACCAAGCTGATTCTCAAGAAA	VALIUM20	attp2 III
dpp	THU0083	CG9885	CGTTCAGTGATAGTGATAAA	VALIUM22	attp2 III
nedd8	THU1211	CG10679	GAGGAGGTGATTCAATCCTAA	VALIUM20	attp2 III