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 Egless (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.
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. 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)
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 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 histidine (H) residue at position 135 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...
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 deoxyribonuclease-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; Lums 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...
and its mediated CSN knockout effect persists in adult ECs, as a result of its role in ECs. As expected, control (Fig. 3I,J). Consistently, the results in an accumulation of significantly more CBs than the overexpressing Ptc in ECs. Indeed, c587-driven ptc overexpression results in an accumulation of 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 knockouts 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 babi-gal4 to knock out hh in CPCs to determine if Hh is required in CPCs to promote germ cell differentiation. babi-gal4 is widely used to drive and knock out gene expression in CPCs (Bolivar 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 deenedylate 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.

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-CDS8FP* 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
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...
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 CSN knockout germaria carry significantly fewer CBs than the smo knockout germaria (Fig. 6O-Q). In summary, 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...
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 deneddylyase-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...
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 in ptc-lacZ expression is observed 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

**Production of plasmid constructs and transgenic flies**

The coding region for the CSN5 transcript was cloned and inserted into pValium20 vector by NheI and EcoRI restriction enzyme sites by standard protocol. To make the RNAi-resistant CSN5, which still encodes a wild-type CSN5 protein, the RNAi targeted sequence CGGAACCGATGTCATGAGAA was changed into AGGTACAGCTAAACGAAA 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-phosphorylated ERK1/2 (cat. #4370, 1:200, Cell Signalling 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. #A10129, A21424, A10304 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 knockdown 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 daily mRNA expression in CSN and smo knockout germaria, germaria were manually cut under a microscope and total RNA isolated 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 Quantitative PCR (qPCR) was performed with GoTaq qPCR Master Mix (Promega) on ABI SteponePlus Instruments. The ΔACt method was used to compare gene expression levels. The primers used were: dpp – TCCGAC- AACAAGATGGAAGGT and TTTACGTGGAAATGTCCCACTGAAA, daily – GAGCAGACTGATGAGATGCAAT and GTGCACTCAGA- GCCGGTTTACGCT; RNAi-resistant CSN5 and CSN5ED – AACGACT- AATCTACTGTGGAGGA and TTTCTATGATACCGTGTCG.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

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

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