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The Wnt pathway limits BMP signaling outside of the germline stem cell niche in *Drosophila* ovaries

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Abstract

The mechanisms that modulate and limit the signaling output of adult stem cell niches remain poorly understood. To gain further insights into how these microenvironments are regulated *in vivo*, we performed a candidate gene screen designed to identify factors that restrict BMP signal production to the cap cells that comprise the germline stem cell (GSC) niche of *Drosophila* ovaries. Through these efforts, we found that disruption of *Wnt4* and components of the canonical Wnt pathway results in a complex germ cell phenotype marked by an expansion of GSC-like cells, pre-cystoblasts and cystoblasts in young females. This phenotype correlates with an increase of *decapentaplegic* (*dpp*) mRNA levels within escort cells and varying levels of BMP responsiveness in the germline. Further genetic experiments show that *Wnt4*, which exhibits graded expression in somatic cells of germaria, activates the Wnt pathway in posteriorly positioned escort cells. The activation of the Wnt pathway appears to be limited by the BMP pathway itself, as loss of *Mad* in escort cells results in the expansion of Wnt pathway activation. Wnt pathway activity changes within germaria during the course of aging, coincident with changes in *dpp* production. These data suggest that mutual antagonism between the BMP and Wnt pathways in somatic cells helps to regulate germ cell differentiation.

Keywords

Niche; Stem Cells; Wnt; BMP; Germline

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V. M-P. and V.P. designed and conducted experiments, interpreted the data and helped to write the paper. V. M-P. and V.P. carried out experiments and helped to edit the manuscript. S.E. conducted several of the initial genetic experiments. S.S. developed various transgenes and other reagents used throughout the study and M.B. designed the experiments, interpreted the data and helped to write the manuscript.

Introduction

Stem cells support tissue homeostasis by providing a continuous source of new cells that replace cells lost to damage, disease or normal turnover. Specialized microenvironments called niches govern the self-renewing activity of stem cells by producing a variety of factors that keep stem cells in an undifferentiated state (Losick et al., 2011). Stem cell daughters that remain within the niche remain stem cells, whereas daughters displaced away from the niche typically undergo differentiation. The size and signaling output of niches has a direct effect on stem cell number and activity. Therefore, changes in niche activity must be tightly regulated since inappropriate increases or decreases in niche function may contribute to tumor formation and age-related declines in tissue homeostasis, respectively.

The *Drosophila* ovary has provided a valuable platform for studying the mechanisms that influence niche formation and function *in vivo*. The germline stem cell (GSC) niche, comprised of terminal filament (TF) cells and cap cells, supports two to three GSCs. Anterior escort cells (ECs) that lie immediately adjacent to the cap cells may also assist with GSC maintenance (Rojas-Rios et al., 2012). Additional escort cells help support the early differentiation of germline cysts (Kirilly et al., 2011; Morris and Spradling, 2011).

The niche produces two bone morphogenetic protein (BMP) family members: Decapentaplegic (Dpp) and Glass Bottom Boat (Gbb) (Song et al., 2004; Xie and Spradling, 1998). These ligands initiate a canonical BMP signal transduction cascade by binding to a heterodimeric receptor on the surface of GSCs, resulting in the phosphorylation of Mothers against Dpp (Mad). pMad then binds to its partner Medea and translocates into the GSC nucleus. pMad and Medea directly repress the transcription of differentiation genes, including *bag of marbles* (*bam*) and induce the expression of canonical BMP targets such as *Daughters against dpp* (*Dad*) (Chen and McKearin, 2003a; Chen and McKearin, 2003b; Song et al., 2004).

In wild-type germaria, BMP signaling rapidly decreases in GSC daughters, known as cystoblasts (CBs), as they exit the cap cell niche. Recent efforts have sought to define and characterize mechanisms that establish this sharp gradient of BMP responsiveness between GSCs and their differentiating daughters. Intrinsic and extrinsic mechanisms, involving down-regulation of BMP pathway components, ensure that cystoblasts displaced away from the niche experience less BMP signaling (Harris et al., 2011; Liu et al., 2010; Schulz et al., 2002; Xia et al., 2010). However, mis-expression of *dpp*, but not *gbb*, results in the formation of GSC-like tumors (Song et al., 2004; Xie and Spradling, 1998), suggesting that despite the down-regulation of pathway components (Harris et al., 2011; Xia et al., 2010), germ cells remain competent to respond to BMP ligands on some level, even as they move posteriorly through the germarium.

Besides BMP ligands, the cap cells and escort cells of the *Drosophila* germarium express a variety of other signaling molecules including Unpaired (Upd), Hedgehog (Hh) and Wingless (Wg) (Decotto and Spradling, 2005; Forbes et al., 1996a; Forbes et al., 1996b; Lopez-Onieva et al., 2008; Sahai-Hernandez and Nystul, 2013; Wang et al., 2008). Recent results show that the expression of Wg, a member of the Wnt family of signaling molecules,

in escort cells regulates the activity of follicle stem cells (Sahai-Hernandez and Nystul, 2013). In addition to Wg, the *Drosophila* genome contains a number of other genes encoding Wnt ligand family members including Wnt2, Wnt4, Wnt6 and Wnt10, which act either through a canonical pathway, involving β -catenin dependent transcriptional regulation, or a non-canonical pathway (Coudreuse and Korswagen, 2007; Logan and Nusse, 2004). Besides Wg, disruption of *Wnt4* also results in a number of morphological defects in the ovary (Cohen et al., 2002). These phenotypes are likely caused by defects in the apical movement of somatic cells in the developing gonad, marked by the disruption of the normal expression and distribution of FAK (Cohen et al., 2002). More recently, Wnt4 has also been suggested to play a role in the regulation of the germline stem cell niche (Hamada-Kawaguchi et al., 2014; Wang et al., 2015).

Here we provide evidence that disruption of *Wnt4* and downstream components of the canonical Wnt signaling pathway in escort cells results in an expansion of BMP responsiveness in the germline and a subsequent increase in the number of GSCs, precystoblasts and cystoblasts. In addition, we find loss of Wnt pathway components is accompanied by an increase of *dpp* mRNA levels specifically within escort cells. Further genetic experiments show that Wnt4 tends to induce activation of the Wnt pathway in escort cells and early follicle cells of the germaria. Signaling within somatic cells of germaria appears to change during the course of aging. In older flies, *dpp* expression within the cap cell niche decreases. This coincides with a switch in Wnt pathway activation from the posterior escort cells to the terminal filament and cap cells. These results provide new insights into how cell-cell communication between specific somatic cell populations helps to modulate niche signaling within the *Drosophila* germarium.

Results

The canonical Wnt pathway non-autonomously promotes stem cell differentiation

In order to identify factors that act in escort cells to limit niche signaling and promote the differentiation of germ cells, we carried out a candidate gene RNAi screen. Targeted genes included various chromatin factors and signaling molecules. We conducted the screen by crossing available UAS-*gene specific* RNAi lines with the *c587-gal4* driver, which, in adult germaria, drives expression in the escort cells and early follicle cells (Song et al., 2007). Ovaries from the resulting females were stained for Vasa, to visualize the germline, and Hts, an adducin-like protein that localizes to an endoplasmic-like organelle called the fusome. In GSCs and cystoblasts, the fusome (also referred to as the spectrosome in single cells) usually appears round (de Cuevas and Spradling, 1998). This structure subsequently becomes branched within germline cysts progressing through their incomplete mitotic divisions. A substantial increase in the number of single cells with round fusomes indicates defects in germline differentiation. Through this initial small-scale screen, we found that knockdown of *Wnt4* using the *c587-gal4* driver resulted in an increased number of GSC-like cells with round fusomes, albeit with a low penetrance (15%, n=120 germaria) (Fig. 1B). To confirm the RNAi phenotype, we next stained ovaries carrying two loss-of-function *Wnt4* alleles: *Wnt4^{C1}*, which contains a 3 bp deletion that removes a highly conserved glutamate residue at position 299 and *Wnt4^{EMS23}*, which exhibits a premature stop codon at position

343 mutants (Cohen et al., 2002). Both of these alleles cause partial lethality and female sterility and are believed to be amorphic or strongly hypomorphic (Cohen et al., 2002). The initial characterization of these alleles showed that *Wnt4* mutant ovarioles were disorganized (Cohen et al., 2002). Delays in the migration of cells that form the muscle sheet surrounding the ovarioles during development likely contribute this phenotype (Cohen et al., 2002). In addition to these phenotypes, we noted that germaria from *Wnt4^{C1}/Df(2L)BSC291*, *Wnt4^{EMS23}/Df(2L)BSC291* and from transheterozygous *Wnt4^{C1}/Wnt4^{EMS23}* females also displayed an increased number of single cells with round fusomes (Fig. 1C,J). *Wnt4^{C1}/Df(2L)BSC291* and *Wnt4^{C1}/Wnt4^{EMS23}* germaria contained large germ cell tumors (containing more than 15 single germ cells with round fusomes) at 82% (n=103) and 85% (n=47) penetrance compared to *Wnt4^{EMS23}/Df(2L)BSC291*, which displayed large GSC/cystoblast-like tumors in 25% (n=77) of the germaria examined.

The *Wnt4* mutants display a reduction of the number of egg chambers compared to wild type (Cohen et al., 2002). The GSC/cystoblast-like tumor phenotype, the reduction of the number of egg chambers and female sterility of these alleles were rescued by a genomic HA-*Wnt4* tagged construct (Fig. 1J, S1). In addition, expression of a *Wnt4* cDNA driven by *c587-gal4* also partially rescued these phenotypes, supporting the model that *Wnt4* is required in the ECs and follicle cells to control the number of GSCs (Fig. 1J).

Previous results showed that *wingless (wg)* regulates the activity and differentiation of follicle cells within the germarium, but does not appear to greatly influence the earliest steps of germline development (Forbes et al., 1996b; Sahai-Hernandez and Nystul, 2013; Song and Xie, 2003). We also found that knocking down *wg*, using UAS-RNAi lines in combination with the *c587-gal4* driver does not result in an appreciable germ cell phenotype, consistent with the aforementioned studies. Furthermore, RNAi knock-down of the other *Drosophila* Wnt ligands using the same driver did not result in germ cell phenotypes. However, we cannot exclude the possibility that other Wnt ligands help to influence GSC differentiation, as the loss-of-function achieved through RNAi knock-down may be incomplete in our hands.

Given the phenotypes of *Wnt4* mutants, we next sought to determine whether loss-of-function of other canonical Wnt pathway components in escort cells and follicle cells results in an expansion of the number of single cells with round fusomes within germaria. RNAi knockdown of either *frizzled (fz)* and *frizzled 2 (fz2)* alone did not result in any obvious defects in the germarium, but knock-down of both (Fig. 1D) resulted in an increased number of GSC-like cells in 65% of the germaria examined (n=103). These results suggest that these receptors have redundant functions within somatic cells of the germarium, consistent with previous results that showed Fz and Fz2 are fully redundant for Wg-dependent signaling (Bhanot et al., 1999; Chen and Struhl, 1999). Knock-down of *arrow* (also named LRP5/6 co-receptor) and *dishevelled (dsh)* also resulted in the formation of ectopic GSC-like cells in 45% and 100% (n=100) of germaria respectively (Fig. 1E,F). The knockdown of the *Drosophila* β -catenin homolog *armadillo (arm)* led to a similar phenotype (100%; n=200) (Fig 1G). Moreover, overexpression of the kinase Shaggy (Sgg), which targets β -catenin for degradation, also resulted in a tumor phenotype in 94% of the germaria examined (Fig. 1H). In addition, we observed cyst formation defects when we knockdown *dsh* or overexpress *sgg*

using the *c587-gal4* driver, consistent with the role of the Wnt pathway in controlling follicle cell activity (Sahai-Hernandez and Nystul, 2013).

To further test whether the canonical Wnt pathway regulates germ cell differentiation, we overexpressed a constitutively active form of *arm* (*UAS-arm^{S10}*), using the *c587-gal4* driver in the *Wnt4* mutant background. This mutant form of Arm escapes from negative regulation by Sgg, and thus accumulates even in the absence of Wnt signal (Pai et al., 1997). When driven in the escort cells and early follicle cells, *arm^{S10}* partially rescued the *Wnt4^{C1}/Wnt4^{EMS23}* tumor phenotype (Fig. 1I,J), but did not fully rescue the sterility of these transheterozygotes. In addition, expressing *arm^{S10}* rescued the tumor phenotype observed in the *c587-gal4>UAS-dsh^{RNAi}* flies (Fig. 1J). These results indicate that a canonical β -catenin dependent pathway functions in escort cells to promote germ cell differentiation downstream of the GSC.

Next, we induced homozygous mutant clones to further corroborate that disruption of the Wnt pathway in somatic cells of the germarium results in expansion of undifferentiated germline cells. This analysis also allowed us to further map whether the Wnt pathway acted in the escort cells or follicle cells to promote cyst development. Because of its availability, we first generated positively marked mosaic analysis with a repressible cell marker (MARCM) clones using the *wg^{RF}* deficiency, which deletes a segment of genomic DNA that harbors many genes including *Wnt4*, *wg*, *Wnt10* and *Wnt6*. Escort cell clones of this deficiency resulted in the formation of germ cell tumors, marked by single cells with round fusomes (Fig. 2A). Next, we generated double homozygous clones of the *fz^{p21}* and *fz^{2C2}* mutations. Consistent with the phenotypes seen using RNAi knock-down, loss of *fz* and *fz2* in the majority of escort cells resulted in the expansion of GSC-like cells, marked by the presence of round fusomes (Fig. 2A). Of note, *wg^{RF}* and *fz^{p21}* and *fz^{2C2}* double mutant negatively marked follicle cell clones did not result in an expanded number of undifferentiated germ cells (Fig. 2B). These results were consistent with our RNAi knock-down data and suggest that loss of Wnt signaling in escort cells delays normal germ cell development.

To characterize the developmental state of the GSC-like cells in Wnt pathway mutants, we examined the expression of three molecular markers which are used to distinguish GSCs from their daughter cells: Nanos (Nos), Sxl (Sex-Lethal) and Bag-of-Marbles (Bam). GSCs and 16-cell cysts express Nos, a RNA binding protein that represses translation and helps to maintain GSCs (Forbes and Lehmann, 1998; Kobayashi et al., 1996; Wang and Lin, 2004). All the GSC-like cells in *Wnt4^{C1}/Wnt4^{EMS23}* and *c587-gal4>UAS-sgg* germline tumors express Nos at a level comparable to wild-type GSCs (n=80, Fig. 3A,D,G). We next analyzed the expression of Sxl and Bam, two differentiation-promoting factors. Wild-type GSCs, cystoblasts (CBs) and two cell cysts express Sxl, while cystoblasts and early differentiating cysts express Bam. In *Wnt4^{C1}/Wnt4^{EMS23}* and *c587-gal4>UAS-sgg* ovaries, the expression of Sxl expanded to all the single cells with round fusomes (Fig. 3E,H). By contrast, Bam remained largely undetected in *Wnt4^{C1}/Wnt4^{EMS23}* and *c587-gal4>UAS-sgg* germaria (only 35% and 12% of the tumorous germaria displayed Bam expression in subsets of germ cells) (Fig. 3F,I). These observations suggest that the majority of single cells that

arise upon Wnt pathway disruption remain in an undifferentiated GSC, pre-cystoblast-like or cystoblast-like state.

Wnt pathway functions in adulthood to promote germ cell differentiation

To assess the extent to which the Wnt pathway functions during development or in adulthood, we performed a series of temporally controlled knock-down experiments. We previously noted that the *c587-gal4* driver was less efficient at lower temperatures (Eliazer et al., 2011). We crossed the *c587-gal4* driver to a line carrying *UAS-arm^{RNAi}*. The majority of progeny from this cross did not exhibit a discernable phenotype when maintained at 18°C during development and in adulthood (Fig. 4A,E). In contrast, when female progeny resulting from this cross were kept at 29°C both during development and in adulthood, over 80% of their germaria displayed a striking expansion of single undifferentiated germ cells with round fusomes and a substantial decrease in the number of developing egg chambers (Figure 4B,E). Next, we set *c587-gal4>UAS-arm^{RNAi}* crosses either at 18°C, shifting the resulting progeny up to 29°C upon eclosion, or at 29°C, shifting the females down to 18°C when they emerged as adults. These temperature shift experiments showed that knocking down *arm* expression specifically in escort cells during adulthood resulted in the formation of germ cell tumors, accompanied by a reduction in the number of developing egg chambers (Fig. 4C,E,F). Similarly, restoring *arm* expression after developmental knock-down resulted in more phenotypically normal germaria, marked by a reduction in the size of the germline tumors and an increase in the number of developing egg chambers (Fig. 4D,E,F). These results indicate that the Wnt pathway functions in adult escort cells to promote the differentiation of germ cells. These findings also suggest that reducing Wnt pathway signal transduction in *c587-gal4* positive cells during larval and pupal development also results in an expansion of undifferentiated germ cells. However, loss of *Wnt4*, *dsh* or *arm* does not cause escort cells to adopt a cap cell-like fate (Fig. S2).

The canonical Wnt pathway limits Dpp signaling within the germarium

Previous studies have shown that expansion of *dpp* expression, changes in escort cell morphology and mutations in differentiation factors can all block normal germ cell development (Kirilly et al., 2011; McKearin and Spradling, 1990; Xie and Spradling, 1998). Given the loss of Bam expression and the cell autonomous function of the Wnt pathway in escort cells, we tested whether disruption of *Wnt4* and downstream pathway components resulted in escort cell morphological defects, ectopic expression of *dpp* or both. In germaria from young females, loss of *dsh* and *arm* or over-expression of *sgg* in escort cells resulted in an expansion of single germ cells with round fusomes despite the clear presence of some escort cell extensions as revealed by the mCD8::GFP reporter (Fig. S3). However, we do observe a loss of escort cell extensions over time upon loss of Wnt pathway activity. These observations suggest that the disruption of escort cell extensions by itself does not initiate the observed Wnt pathway mutant phenotypes. However changes in escort cell morphology likely do contribute to the complexity and severity of the terminal phenotype within the germaria of older females.

Next we tested whether disruption of the Wnt pathway in escort cells resulted in BMP pathway activation in germ cells outside of the cap cell niche. The Dad-LacZ enhancer trap

has been extensively used as a reporter of BMP signaling in the germline (Song et al., 2004). This reporter is normally limited to the GSCs and newly formed cystoblasts (Fig. 5A). In *Wnt4* mutants and *c587-gal4>UAS-dsh^{RNAi}* and *UAS-sgg* ovaries, the Dad-LacZ expression domain expands throughout the germarium (Fig. 5B-D).

Because the expression of Dad-LacZ may persist beyond the time when a cell directly experiences BMP signaling, we also examined the expression of phosphorylated Mad (pMAD), a second readout for BMP signal transduction. Like Dad-LacZ, pMAD is normally restricted to GSCs immediately adjacent to the cap cells of wild-type germaria (Figure S4). By contrast, we observed that within 67% of germaria (n=30) from *c587-gal4>UAS-dsh^{RNAi}* females a small subset of single germ cells with round fusomes outside of the normal niche expressed low levels of pMAD upon escort cell knock-down of *dsh* (Fig. S4). Other single cells with round fusomes within the same tumors did not express detectable levels of pMAD. While these results indicate that some germ cells experience ectopic BMP signaling transduction outside the normal niche upon disruption of Wnt signaling, and are thus similar to GSCs, others may be slightly more differentiated and exist within a pre-cystoblast or cystoblast-like state. To test this possibility, we examined the expression of a highly sensitive *bam* transcriptional reporter (*bamP-GFP*) (Chen and McKearin, 2003b) in both a wild-type and Wnt pathway loss-of-function background. In control germaria, we first detect the GFP expression of the reporter in cystoblasts. This expression continued to increase as cells entered into cyst differentiation. By contrast, ectopic single germ cells present upon escort cell knock-down of Wnt pathway components expressed either no or low levels of the *bam* transcriptional reporter (Fig. S4). Together, these results are largely consistent with recently published findings (Wang et al., 2015) and suggest that loss of Wnt signaling in escort cells results in a varying degree of ectopic BMP signal transduction in germ cells positioned away from the cap cells. This, in turn, results in an expansion of undifferentiated cells that exist within a GSC-like, pre-cystoblast and cystoblast-like state.

To determine whether the expanded domain of Dad-LacZ and pMAD expression reflected ectopic *dpp* expression, we employed a new, highly sensitive, *in situ* hybridization protocol that utilizes a series of end-labeled oligo probes. This method revealed an enrichment of *dpp* mRNA in the cap cells of wild-type ovaries (Fig. 5E). *Wnt4* mutants exhibited modest levels of ectopic *dpp* mRNA expression in escort cells (Fig. 5F). Likewise, RNAi knock-down of *dsh* and over-expression of *sgg* also resulted in an expansion of *dpp* mRNA within escort cells (Fig. 5G,H). To test the functional significance of the observed ectopic *dpp* expression, we tested whether loss of *dpp* in the escort cells modified the GSC expansion phenotype of the Wnt pathway mutants. Expressing *UAS-dpp^{RNAi}* in escort cells suppressed the *Wnt4* mutant and *c587-gal4>UAS-dsh^{RNAi}* mutant phenotypes, so fewer germaria contained greater than 5 single cells with round fusomes (Fig. 5I-K). These results together suggest that the Wnt pathway promotes germ cell differentiation, at least in part, by repressing *dpp* expression within the escort cells of adult ovaries.

Wnt signaling along the anterior-posterior axis of the germarium

To gain a better understanding of which cells produce *Wnt4* and which cells respond to this ligand, we examined the expression of the HA-tagged *Wnt4* in our rescuing genomic

construct (Fig. S1). Staining the resulting lines for the HA epitope and Traffic Jam (Tj), a marker for somatic cells in the germarium, revealed that cap cells and anterior escort cells expressed high levels of the Wnt4 tagged genomic transgene (Fig. 6A). Expression of Wnt4 was lower in more posteriorly positioned escort cells. The expression pattern of this tagged transgene was largely consistent with the expression of *Wnt4* mRNA, as visualized by in situ hybridization (Fig. 6B).

Next we examined the expression pattern of a previously described Wnt pathway reporter (6TH) within wild-type germaria (Chang et al., 2008). This reporter places the β -galactosidase gene under control of six TCF binding sites flanked by helper sites (Chang et al., 2008). We observed expression of this reporter in a small number of cells within each germarium. These Wnt reporter expressing cells tended to be positioned along the escort cell and follicle stem cell boundary (Fig. 6C), but occasionally more anteriorly positioned Wnt reporter positive escort cells were also noted (Fig. S5A). Loss of *Wnt4* or *dsh* extinguished expression of the reporter in all cells while over-expression of *arm^{S10}* increased the number of 6TH expressing cells (Fig. S5B-D). Again these positive cells tended to lie along the escort cells and follicle stem cell border, despite the expression of the *arm^{S10}* transgene throughout the escort cell population. These results suggest that unidentified mechanisms restrict the number of escort cells that respond to Wnt4, or that 6TH does not fully report all Wnt signaling within the germarium. Double labeling experiments using the HA-tagged Wnt4 genomic transgene and the Wnt reporter tended to show tight juxtaposition between cells expressing moderate levels of Wnt4 and those cells that expressed the reporter (Fig. 6C). Occasionally we observed individual cells that expressed Wnt4 and relatively high levels of the reporter. These data suggest that Wnt4 may tend to act in a paracrine manner to induce a response in more posteriorly positioned somatic cells. However, given that Wnt4 and the reporter are expressed together in individual escort cells, we cannot rule out the possibility that Wnt4 also acts in an autocrine fashion in certain circumstances.

The BMP pathway antagonizes the Wnt pathway within germaria

Previous studies have shown that the BMP and Wnt signaling pathways antagonize each other in specific contexts, resulting in mutually exclusive domains of pathway activation (Jiang and Struhl, 1996). Given our observations that *arm^{S10}* over-expression resulted in greater expression of the 6TH Wnt pathway reporter in posterior escort cells but not in anterior escort cells (Fig. S5), we considered the possibility that Dpp expressed by the cap cells activates the BMP signaling pathway in neighboring anterior escort cells of the germarium, which in turn represses Wnt signaling within these cells. To test this possibility, we disrupted BMP signaling within escort cells by knocking down the expression of *Mothers against dpp* (*Mad*) using the *c587-gal4* driver in combination with a UAS-*Mad^{RNAi}* transgene. Reduced levels of *Mad* resulted in an expansion of 6TH Wnt reporter expression, suggesting that loss of BMP signaling results in ectopic Wnt pathway activation within anterior somatic cells of the germarium (Fig. 7).

Decreased BMP signal production in aged flies contributes to a decline in GSC niche function (Pan et al., 2007). We speculated that changes in Wnt signaling response might contribute to age-related changes in niche function to some degree. To test this idea, we first

compared *dpp* expression in young and aged germaria using in situ hybridization (Fig. 8). Germaria from young females clearly exhibited high levels of *dpp* expression in cap cells and far lower and sporadic expression in the escort cells. In contrast, cap cells from aged females display reduced levels of *dpp* expression while escort cells exhibit increased expression (Fig. 8). These results are consistent with previous results (Rojas-Rios et al., 2012; Song et al., 2004). The changes in *dpp* mRNA expression within specific somatic cells within the germarium correlate with changes in the expression of the Wnt reporter. As noted above, in young flies expression of the 6TH Wnt reporter is observed in posterior escort cells and occasionally in anterior escort cells, but very rarely in cap cells or terminal filament cells (Fig. 6 and 7). Aged germaria exhibited a dramatic change in reporter expression so that fewer escort cells expressed the reporter while an increased number of cap cells and terminal filament cells exhibited very high levels of expression (Fig. 8). These results suggest that changes in both BMP and Wnt signaling may contribute to a decline in niche activity during the course of aging.

Discussion

The dynamic regulation of adult stem cell numbers and activity often depends on the size and signaling output of the specialized niches within which they reside. A growing body of evidence suggests that stem cell niches may exhibit a certain degree of plasticity, expanding and contracting in response to local homeostatic needs and systemic cues (Rojas-Rios and Gonzalez-Reyes, 2013). Characterizing the diverse mechanisms that affect niche size is critical for understanding the control of stem cell self-renewal, proliferation and differentiation *in vivo*. Here we provide evidence that the Wnt signaling pathway, driven by the Wnt4 ligand, and perhaps other Wnts as well (Wang et al., 2015), represses the normally niche cell specific expression of *dpp* in the escort cells of the *Drosophila* ovary. Interestingly, wild-type cap cells express high levels of both *dpp* mRNA and Wnt4, yet Wnt pathway activation is most readily seen in posterior escort cells and early follicle cells. These results indicate that the Wnt pathway plays an unexpected role in regulating BMP signal production in somatic cells of germaria and that cells neighboring the stem cell microenvironment influence whether one another can produce niche specific factors. Lastly, we show that the cells responding to Wnt signaling change with age, strongly correlated with a decrease in niche function in older females.

Wnt4 represses *dpp* expression in escort cells

Drosophila Wnt4 is most closely related to vertebrate Wnts 9, 14 and 15 (Prud'homme et al., 2002) and has been shown to be involved in the development of dorsal epidermis, retina, heart and ovaries as well as in the left-right asymmetric morphogenesis of the midgut (Cohen et al., 2002; Hamada-Kawaguchi et al., 2014; Inaki et al., 2007; Kuroda et al., 2012; Wu et al., 2013). Wnt4 was shown to bind to both the Fz1 and Fz2 receptors (Wu and Nusse, 2002) and to signal through both the canonical and non-canonical Wnt pathways, depending on cellular contexts (Cohen et al., 2002; Hamada-Kawaguchi et al., 2014; Wu et al., 2013). Importantly, Wnt4 has been previously shown to regulate morphological events during *Drosophila* gonad development (Cohen et al., 2002). Consistent with a recent report (Hamada-Kawaguchi et al., 2014), our data support a model in which canonical Wnt

signaling functions in the escort cells. However, unlike the aforementioned study, our findings suggest that Wnt signaling serves to limit *dpp* expression within somatic cells. We observe that loss of several different components of the Wnt pathway result in an expansion of single cells with round fusomes, more in line with a second recently published study (Wang et al., 2015). These undifferentiated cells express Dad-LacZ and do not express Bam protein, although variable expression of pMAD and a *bam* transcriptional reporter suggests that the germ cell tumors that arise upon loss of escort cell Wnt signaling contain a mixture of GSCs, pre-cystoblasts and cystoblast-like cells. We provide evidence that *dpp* expression expands in escort cells in the absence of Wnt signaling and that loss of *dpp* suppresses Wnt related phenotypes in the ovary. However, the complexity of the observed phenotypes may indicate that loss of Wnt signaling disrupts multiple Dpp-dependent and potentially Dpp-independent processes. For example, we observe an expansion of undifferentiated germ cells in germaria from young *c587-gal4>UAS-dsh^{RNAi}* females that still have escort cell extensions, suggesting that bulk loss of escort cell protrusions does not initiate the described phenotypes. However, over time the phenotype worsens, as escort cell extensions are lost, indicating that the ability of escort cells to maintain close contact with multicellular cysts plays an important role in the continued development of germ cells (Kirilly et al., 2011). Ectopic expression of *dpp* may disrupt the normal morphology of escort cells, giving rise to a complex phenotype involving both low levels of aberrant BMP signal transduction in germ cells and disruption of other processes that depend on contact between escort cells and germ cells (Kirilly et al., 2011; Wang et al., 2015). Future work will be needed to further clarify how germ cells and escort cells communicate and influence the activity and morphology of one another.

Somatic Cells Communicate with Each Other to Limit Niche-Specific Signaling

We observe that somatic cells of the germarium exhibit graded expression of Wnt4, from high levels in the terminal filament and cap cells to lower levels in the posterior escort cells. Despite high levels of Wnt4 expression in the anterior of the germarium, the 6TH Wnt reporter is most readily detected in posterior escort cells, follicle stem cells and early follicle stem cell daughters. Signal transduction reporters cannot always be expected to reflect the entirety of signaling in any given tissue because of the molecular and context dependent complexities that exist *in vivo* (Barolo, 2006). However, despite this caveat, the expression of the 6TH reporter in germaria appears at least responsive to levels of Wnt signal transduction at some level since it goes away in Wnt pathway mutants and increases in posterior escort cells upon *arm^{S10}* over-expression. Interestingly, the reporter did not come on in young anterior escort cells upon *arm^{S10}* over-expression or upon *arm^{S10}* over-expression coupled with TCF overexpression (data not shown), despite the expression of the *c587-gal4* driver throughout the escort cell and early follicle cell population. These observations hint at mechanisms whereby Wnt pathway activation is restricted in some way. The finding that loss of *Mad* results in expanded 6TH expression suggests that BMP signals emanating from the niche attenuate Wnt pathway activation in surrounding escort cells, and thereby also help to regulate escort cell function.

Wnt Signaling changes with Age

Previous results indicate that niche function within the *Drosophila* ovary declines with age (Pan et al., 2007). The influence of non-cell-autonomous factors on stem cell aging is not limited to invertebrates. For example, parabiotic studies show that extrinsic factors from young mice can enhance stem cell activity in older mice (Conboy et al., 2005; Conboy and Rando, 2005). Moreover, the ability of mice to regenerate muscle, which depends on the activity of satellite cells, decreases with age. This decrease in the capacity of satellite cells to respond to injury appears to be partially caused by a reduction in Notch gene expression and an increase in TGF-beta, which in the muscle, represses cell proliferation (Conboy et al., 2003). All of these data highlight the important influence that extrinsic factors have over stem cell maintenance and activity during the course of aging across species.

Results presented here extend our understanding of how *Drosophila* ovary niche function changes with age. High resolution in situ hybridization reveals that cap cells in young germaria specifically express high levels of *dpp*. As females age, *dpp* expression declines in cap cells, accompanied by a concomitant increase in Wnt signaling. Interestingly, we observe low levels of *dpp* expression in aged escort cells, suggesting that the balanced mutual antagonism between the BMP and Wnt pathways that exists in young escort cells becomes perturbed as these cells age. In regards aged cap cells, we speculate that increased Wnt signaling reinforces the observed loss of *dpp* expression. These findings suggest that the age-dependent decline in ovarian niche function is not merely a passive process, marked by a general reduction in basic cellular activity, but rather results from changes in signaling dynamics within niche cells and their close neighbors.

Materials and Methods

Fly stocks

Fly stocks were maintained at room temperature (RT) on standard cornmeal-agar medium unless otherwise indicated by experimental design. For RNAi, larvae were allowed to develop at RT for 5 days before being moved to a higher temperature (29°C) in order to increase the efficiency of knock-down. Adult flies were then kept at 29°C for 5 to 7 days before dissection of the ovaries. As the expression of *UAS-sgg* driven by *c587-gal4* during development is lethal, flies were raised at RT until eclosion, adults were then shifted to 29°C for a week before dissection of the ovaries.

The following fly strains were used in this study: *w¹¹¹⁸* was used as a control; the Wnt Reporter 6TH-LacZ (MK B-31) was provided by K. Cadigan (University of Michigan); the *y w hs-FLP¹²²*; *Sp/Cyo*; *fzp²¹Dfz2^{C2} ri FRT2A/TM2* and *y w hs-FLP¹²²*; *wg^{RF}*, *FRT40A/Cyo*; *TM2/TM6B* were a gift from G. Struhl (Columbia University); *c587-gal4* and *Dad-LacZ* lines were provided by A. Spradling (Carnegie Institute for Science, Baltimore, MD); *hs-FLP*, *Ubi-GFP*, *FRT19A* was a gift from Hugo Bellen (Baylor College of Medicine, Houston); *Wnt4^{CI}* (#6651), *Wnt4^{EMS23}* (#6650) alleles, *UAS-Wnt4^{RNAi}* (#29442), *UAS-fz^{RNAi}* (#31311), *UAS-fz2^{RNAi}* (#31390 and #27562), *UAS-Arrow/LRP5/6^{RNAi}* (#31473), *UAS-dsh^{RNAi}* (#31306 and #31307), *UAS-arm^{RNAi}* (#31304 and #31305), *UAS-Pan/dTCF^{RNAi}* (#26743), *UAS-sgg* (#5361), *UAS-Arm^{S10}* (#4782), *Df(2R)BSC291/Cyo*

(#23676), *y w dsh³ neoFRT19A/FM7* (#6331), *UAS-Mad^{RNAi}* (#31316) were obtained from the Bloomington Stock Center (stock # given in parentheses). The *Wnt4::HisHA* genomic construct was made according to Carreira-Rosario A. *et al*, 2013. Briefly, an *in vitro* recombineering step was performed between the P[acman] Bac CH321-78J12 containing the *Wnt4* locus and a cassette containing the His-HA tag followed by a RFP+ marker and flanked by *Wnt4* homology arms. Transgenic flies were then generated using the phiC31 integrase transgenesis system with 96E as the landing site. The RFP+ cassette was then excised using the Cre enzyme using the *w* ;; *MKRS, hs-FLP86E/TM6B, Cre* stock (Bloomington stock #1501).

Plasmids and Rescue

To construct the *pUAS-Wnt4-Flag* plasmid, full-length *Wnt4*-coding sequence was amplified by PCR from the RE26454 clone (Berkeley *Drosophila* Genome Project), cloned into pENTR/DTOPO (Invitrogen, Carlsbad, CA), and then recombined into pTWF (Terence Murphy, The *Drosophila* Gateway Vector Collection, Carnegie Institution of Washington, Baltimore, MD). The plasmid was injected into *w¹¹¹⁸* embryos by Rainbow Transgenics (Camarillo, CA) and flies were selected for the presence of the *w+* transgene. Rescue of *Wnt4* mutants was performed by driving *Wnt4-Flag* in ovaries with *c587-gal4*.

Generation of escort cell clones using MARCM

Escort cell mutant clones for *fz^{p21} fz2^{C2}* and *wg^{RF}* (a small deletion that removes *Wg, Wnt4, Wnt6* and *Wnt10*) were generated by FLP/FRT-mediated mitotic recombination using the MARCM system to positively mark the clones. For the double *fz^{p21} fz2^{C2}* mutant clones, 6 days after setting up the crosses larvae of the genotype *hs-FLP/tubgal4:UAS-GFP;;tubgal80, FRT2A/ fz^{p21} fz2^{C2}, FRT2A* were heat-shocked at 37°C for 1 hr twice a day for 3 consecutive days. For the *wg^{RF}* mutant clones, larvae of the genotype *hs-FLP/ tubgal4:UAS-GFP; tubgal80, FRT40A/ wg^{RF}, FRT40A* were heat-shocked as mentioned above. *hs-FLP/tubgal4:UAS-GFP;;tubgal80, FRT2A/FRT2A* and *hs-FLP/tubgal4:UAS-GFP; tubgal80, FRT40A/ FRT40A* were used as controls. The ovaries of the adults were dissected 7 days after eclosion.

Generation of follicle clones

Escort and follicle mutant clones for *dsh³* were generated by FLP/FRT-mediated mitotic recombination. 6 days after setting up the crosses larvae of the genotype *hs-FLP, UbiGFP, FRT19A/ dsh³, FRT19A* were heat-shocked at 37°C for 1 hr twice a day for 3 consecutive days, and adults were dissected 7 days after eclosion. *hs-FLP, UbiGFP, FRT19A/FRT19A* were used as controls. To generate follicle mutant clones for *dsh³* adult females of 1 to 5 days old of the genotype *hs-FLP, UbiGFP, FRT19A/ dsh³, FRT19A* were heat-shocked at 37°C for 1 hr twice a day for 3 consecutive days, and adults were dissected 14 days after the last day of heat-shock. Adults of the genotype *hs-FLP, UbiGFP, FRT19A/FRT19A* were used as controls. To generate follicle mutant clones for *wg^{RF}* adult females of the genotype *hs-FLP; UbiGFP, FRT40A/ wg^{RF}, FRT40A* were heat-shocked as mention above for the *dsh³* clones and the adults *hs-FLP; UbiGFP, FRT40A/FRT40A* were used as controls.

Immunostaining

Adult ovaries were dissected, fixed, and stained according to Eliazer *et al.* (2011), except that ovaries stained for pMAD were fixed for 30 minutes. The images were taken with Zeiss LSM 510 confocal microscope. The following primary antibodies were used: mouse anti-Hts (1B1 from Developmental Studies Hybridoma Bank, dilution at 1:20), rat anti-VASA (Developmental Studies Hybridoma Bank, DSHB, 1:20), mouse anti-Lamin C (LC28.26, DSHB, 1:20), anti-Engrailed (4D9, DSHB, 1:2), mouse anti-Sxl (M114 DSHB, 1:10), rabbit anti-pMAD (Abcam, 1:200) mouse anti-BamC A7 (gift from D. McKearin, 1:20), rabbit anti-Nanos (gift from A. Nakamura, RIKEN, Kobe, Japan), rabbit anti-Spectrin (gift from R. Dubreuil, University of Illinois at Chicago, 1:2,500), mouse anti- β -galactosidase (Promega, 1:1,000), rabbit anti-GFP (Invitrogen, 1:1,000), guinea pig anti-Traffic Jam (gift from D. Godt, University of Toronto, Toronto, ON, Canada, 1:5,000), rat anti-HA (Roche, 3F10). Cy3, Cy5, FITC (Jackson Laboratories) or Alexa 488 (Molecular Probes) fluorescence-conjugated secondary antibodies were used at a 1:200 dilution.

In situ hybridization—*In situ* hybridization on adult ovaries was performed mostly as described in Raj A. and Tyagi S., *Methods in Enzymology*, 2010 for *Drosophila* imaginal discs. This method involves probing target mRNAs using a larger number (>30) of shorter oligonucleotides (20 bases), each of which hybridize to a different portion of the target mRNA. Each of these oligonucleotides are labeled with a single fluorophore at its 3' end. For designing the oligonucleotides, we used the following web-based program (<http://www.singlemoleculefish.com/>, (Raj and Tyagi, 2010)). Briefly, ovaries were dissected in RNase free 1XPBS and then fixed in 3.7% formaldehyde for 45 min. After being washed 2 times for 5 min with 1XPBS, they were left in 70% EtOH overnight at 4°C. The next day, ovaries were washed briefly in the Wash Buffer containing 2XSSC RNase free and 10% of deionized formamide and then incubated overnight at 37°C in the dark with the *dpp* probe at 50nM diluted in the Hybridization Buffer containing 2XSSC, 10% Dextran sulfate (Sigma D8906), 1 μ g/ μ l of yeast tRNA (Sigma R8759), 2mM Vanadyl ribonucleoside complex (NEB, S142), 0.02% RNase free BSA (Ambion, AM2618) and 10% of deionized formamide. The last day, ovaries were washed 2 times 30 min each 37°C in the dark in the Wash Buffer and then resuspended in a drop of Vectashield containing DAPI (Vector Lab, H-1200).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Wnt pathway activation within escort cells promotes germ cell differentiation.
- Disruption of the Wnt pathway results in ectopic Dpp signaling within the germarium.
- Loss of BMP signal transduction in anterior escort cells results in enhanced Wnt pathway activity.
- In aged females, the Wnt pathway becomes active in niche cells, coincident with a decline in niche BMP production.

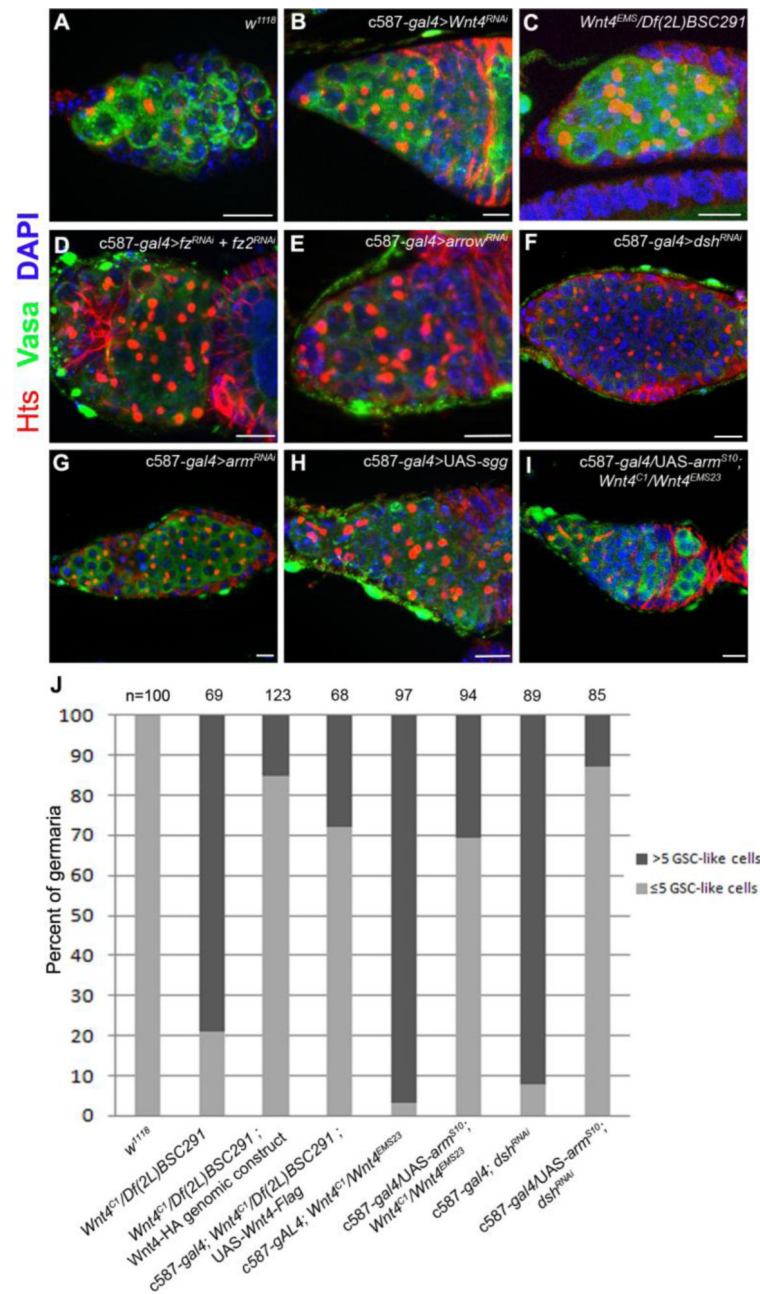


Fig. 1. Disruption of Wnt4 and canonical Wnt pathway components in escort cells results in the formation of GSC-like tumors

(A-I) Germaria stained for Vasa (green), Hts (red) and DNA (blue). (A) Control germaria contain two to three GSCs. (B) Germaria from *c587-gal4/+; UAS-Wnt4^{RNAi}/+* and from *Wnt4^{EMS}/Df(2L)BSC291* displayed an increase of GSC-like cells with round fusomes. RNAi knockdown of (D) the *fz* and *fz2* receptors, (E) the co-receptor *arrow*, (F) *dsh* or (G) *arm*, using the escort cell/early follicle cell *c587-gal4* driver, results in the accumulation of GSC-like cells. (H) Over-expression of *sgg* in escort cells and early follicle cells also results in an expansion of undifferentiated germ cells. (I) Overexpression of a constitutively activated form of *arm* (*arm^{S10}*) suppresses the *Wnt4^{C1}/Wnt4^{EMS23}* phenotype. (J) A graph

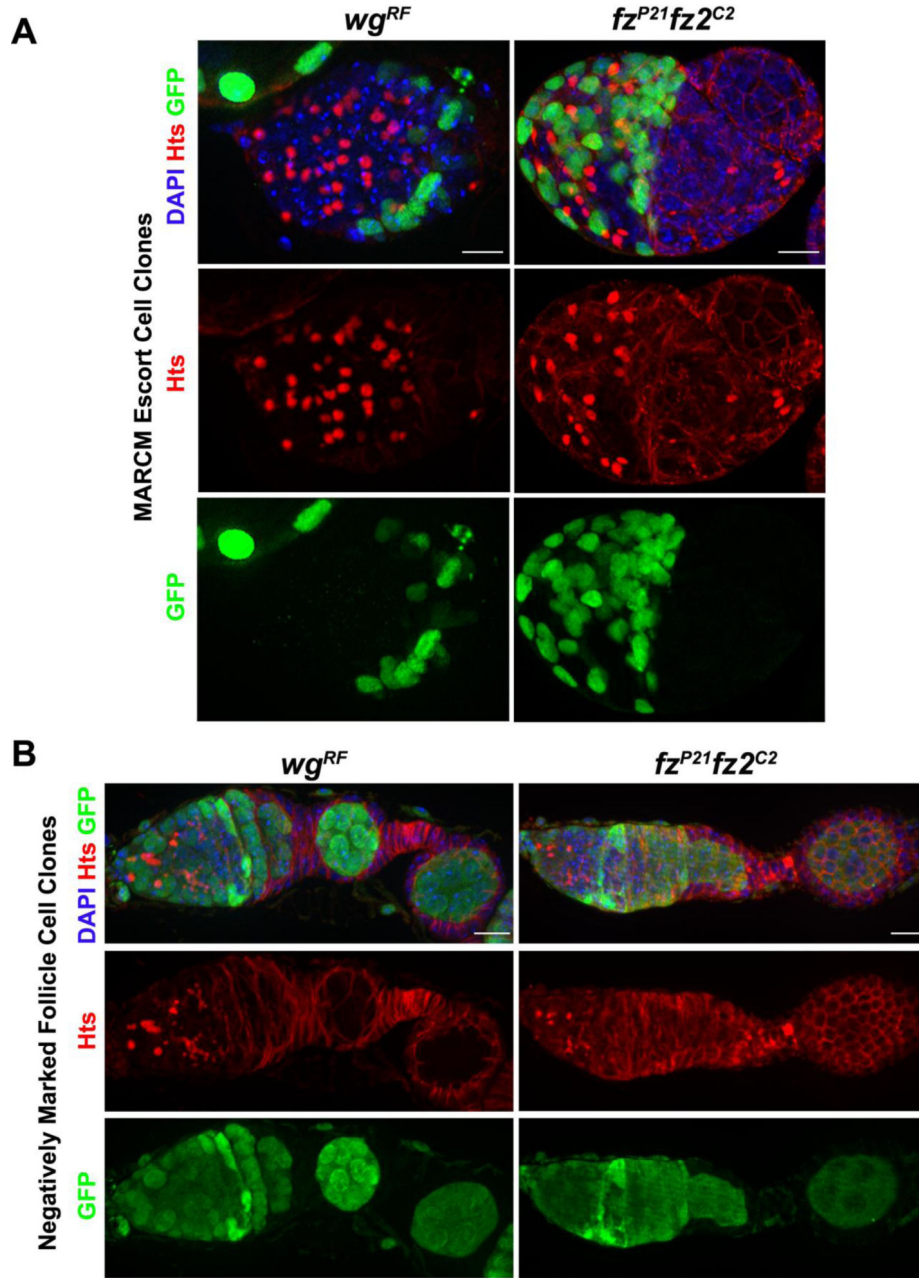
illustrating the percentage of germaria (Y-axis) that contained more (black bars) or less (light gray) than 5 round fusomes per germarium in the indicated genetic backgrounds (X axis). Note that the GSC-like tumor phenotypes observed after disruption of the canonical pathway are rescued by a genomic Wnt4 HA-tagged construct, as well as by expression of a *Wnt4* cDNA or by expression of *arm^{S10}* driven by *c587-gal4*. (Scale bars, 10 μ m)

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Fig. 2. Wnt pathway mutant escort cell clones are associated with undifferentiated germ cells (A) The MARCM system was used to generate *wg^{RF}* or double *fz^{p21} fz2^{C2}* mutant clones marked by GFP. Both samples were stained for GFP (green), Hts (red) and DNA (blue). Loss of Wnt pathway components in escort cells resulted in the appearance of many single cells with round fusomes (B) Negatively marked *wg^{RF}* or double *fz^{p21} fz2^{C2}* follicle cell clones stained for GFP (green), Hts (red) and DNA (blue). Loss of Wnt pathway components within follicle cells did not appear to perturb early germ cell differentiation. (Scale bars, 10 μ m).

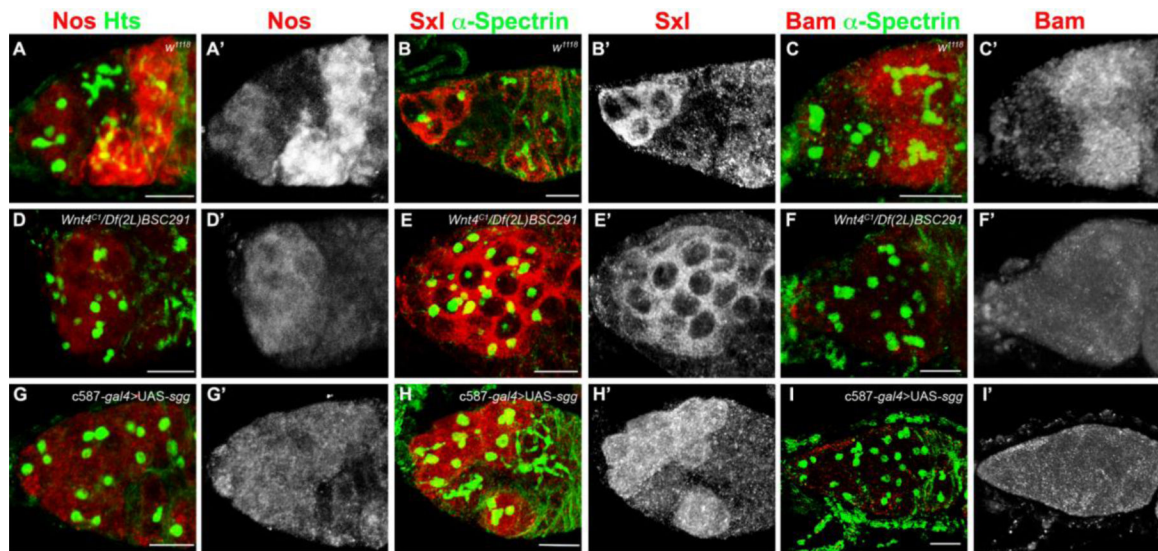


Fig. 3. Changes in germline markers upon disruption of the Wnt pathway in escort cells
 Germaria of (A) control, (D) *Wnt4^{C1}/Df(2L)BSC291* and (G) *c587-gal4/+; UAS-sgg/+* stained for Hts (green) and Nos (red). (B) Control, (E) *Wnt4^{C1}/Df(2L)BSC291* and (H) *c587-gal4/+; UAS-sgg/+* germaria stained for α -Spectrin (green) and Sxl (red). (C) Control, (F) *Wnt4^{C1}/Df(2L)BSC291* and (I) *c587-gal4/+; UAS-sgg/+* stained for α -Spectrin (green) and Bam (red). The GSC-like cells that accumulate upon Wnt pathway disruption express Nos and Sxl, but not the differentiation factor Bam. (Scale bars, 10 μ m).

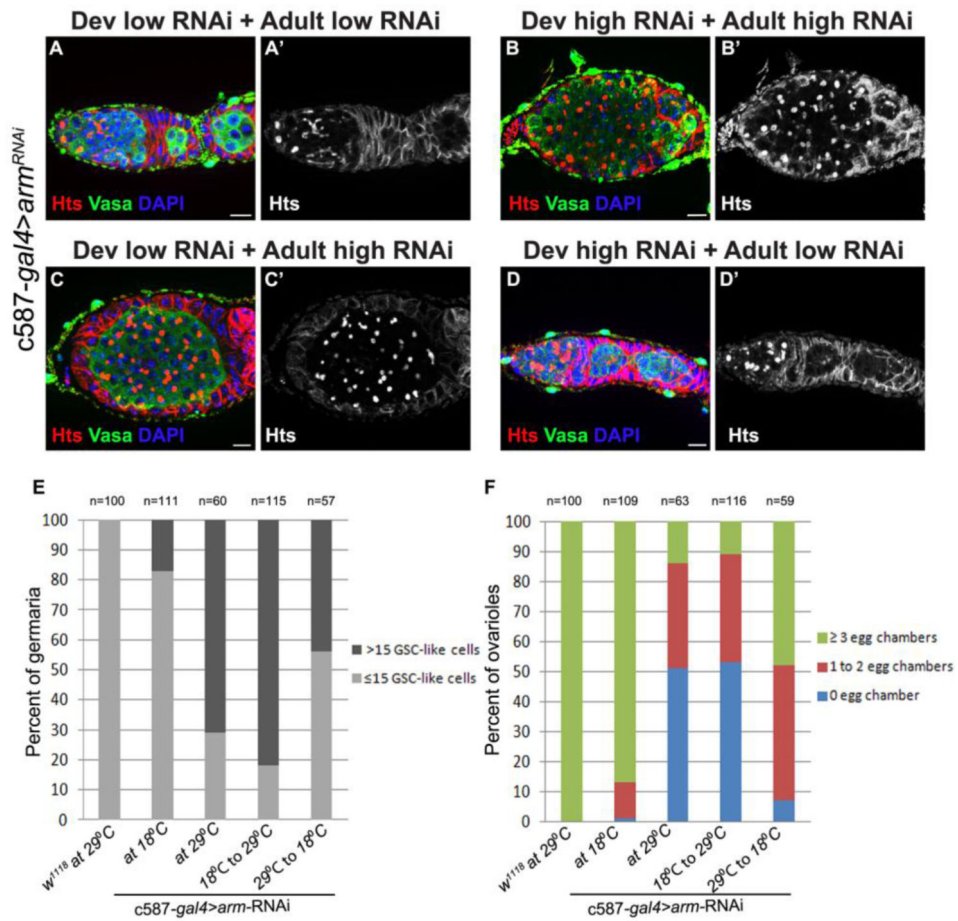


Fig. 4. The canonical Wnt pathway functions during adulthood to promote germ cell differentiation

(A–D) Germaria from *c587-Gal4/+; UAS-arm-RNAi/+* females raised at (A) 18°C, (B) 29°C, (C) shifted from 18°C to 29°C upon eclosion or (D) shifted from 29°C to 18°C upon eclosion stained for Vasa (green), Hts (red) and DNA (blue). Knockdown of *arm* in adulthood led to the formation of tumors containing more than 15 round fusomes (B and C) while knockdown of *arm* only during development led to the formation of smaller tumors (D). (E) A graph showing the percent of germaria (Y axis) that contain more (black bars) or less (light gray) than 15 round fusomes per germarium in *c587-gal4/+; UAS-arm^{RNAi}/+* females. (F) Graph showing the percentage of ovarioles that contain the indicated numbers of maturing egg chambers under each of the conditions. (Scale bars, 10 μm).

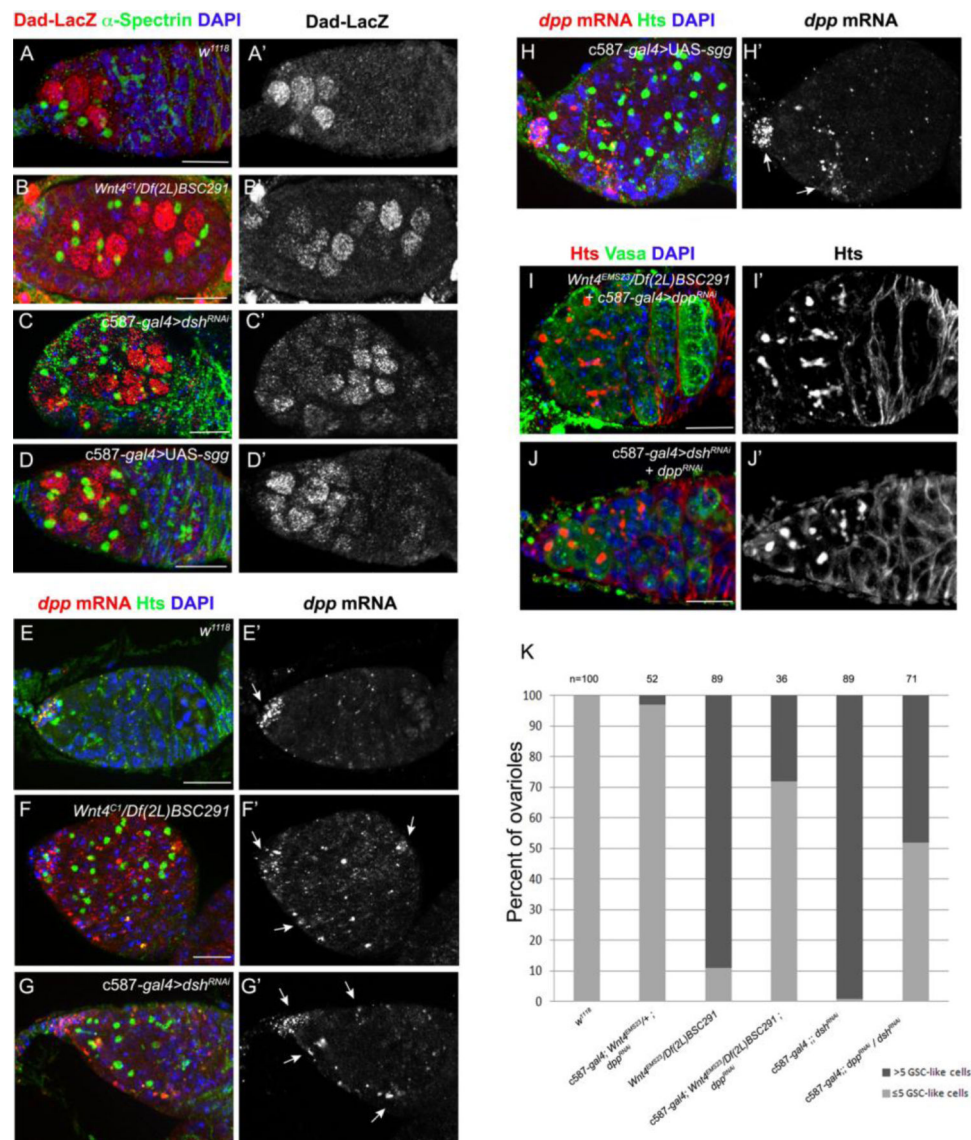


Fig. 5. Canonical Wnt pathway limits Dpp signaling within the gerarium
 (A) Control, (B) *Wnt4^{C1}/Df(2L)BSC291*, (C) *c587-gal4/+; UAS-dsh^{RNAi}/+* and (D) *c587-gal4/+; UAS-sgg/+* ovaries carrying a Dad-LacZ enhancer trap stained for Hts (green), LacZ (red) and DNA (blue). RNA in situ hybridization of *dpp* and Hts immunostaining were performed on (E) control, (F) *Wnt4^{C1}/Df(2L)BSC291*, (G) *c587-gal4/+; UAS-dsh^{RNAi}/+* and (H) *c587-gal4/+; UAS-sgg/+* ovaries. Arrows point to cells expressing *dpp* mRNA. (E) In control germaria, *dpp* transcripts were mostly detected in the cap cells. (F-H) Disruption of the canonical Wnt pathway led to an upregulation of *dpp* transcripts in the escort cells. RNAi knockdown of *dpp* partially rescued the tumorous phenotype of (I) *Wnt4^{C1}/Df(2L)BSC291* and (J) *c587-gal4/+; UAS-dsh^{RNAi}/+* germaria. (K) Quantification of the suppression of Wnt pathway mutants phenotypes by RNAi knockdown of *dpp*. (Scale bars, 10 μ m).

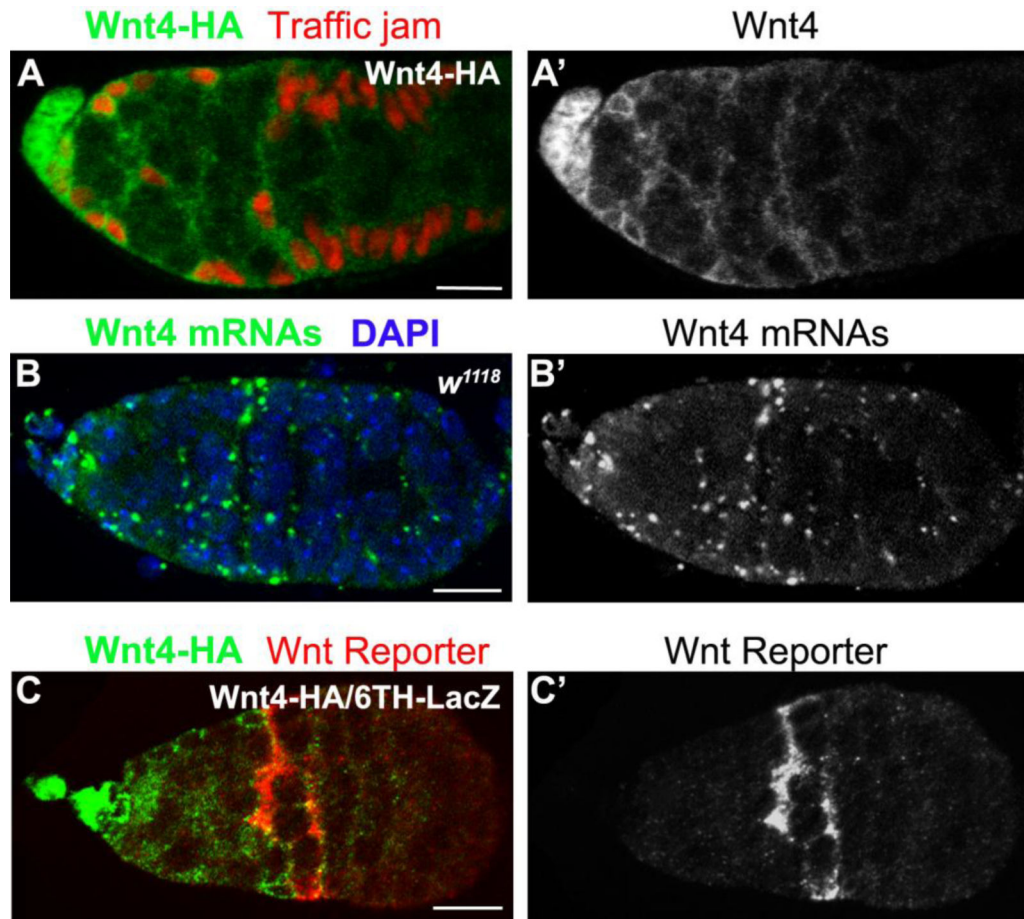


Fig. 6. Expression of Wnt4 and a Wnt pathway reporter in the germarium

(A) Germaria of transgenic flies harboring a genomic Wnt4 HA-tagged construct were co-stained for HA (green) and Traffic jam (red). Wnt4-HA was detected at high levels in the terminal filament, the cap cells as well as in the anterior escort cells and at lower levels in the more posterior escort cells. (B) The localization of Wnt4 mRNAs (green) correlated with the Wnt4-HA staining. (C) Germaria expressing the HA-tagged Wnt4 genomic transgene and a Wnt reporter 6TH-LacZ were co-stained for HA (green) and β -galactosidase (red). The 6TH-LacZ Wnt reporter labeled mostly escort cells and follicle stem cells located posterior to the cells expressing the Wnt4-HA transgene. However, we did observe cases where cells displayed high levels of both Wnt4-HA and of the Wnt reporter. (Scale bars, 10 μ m).

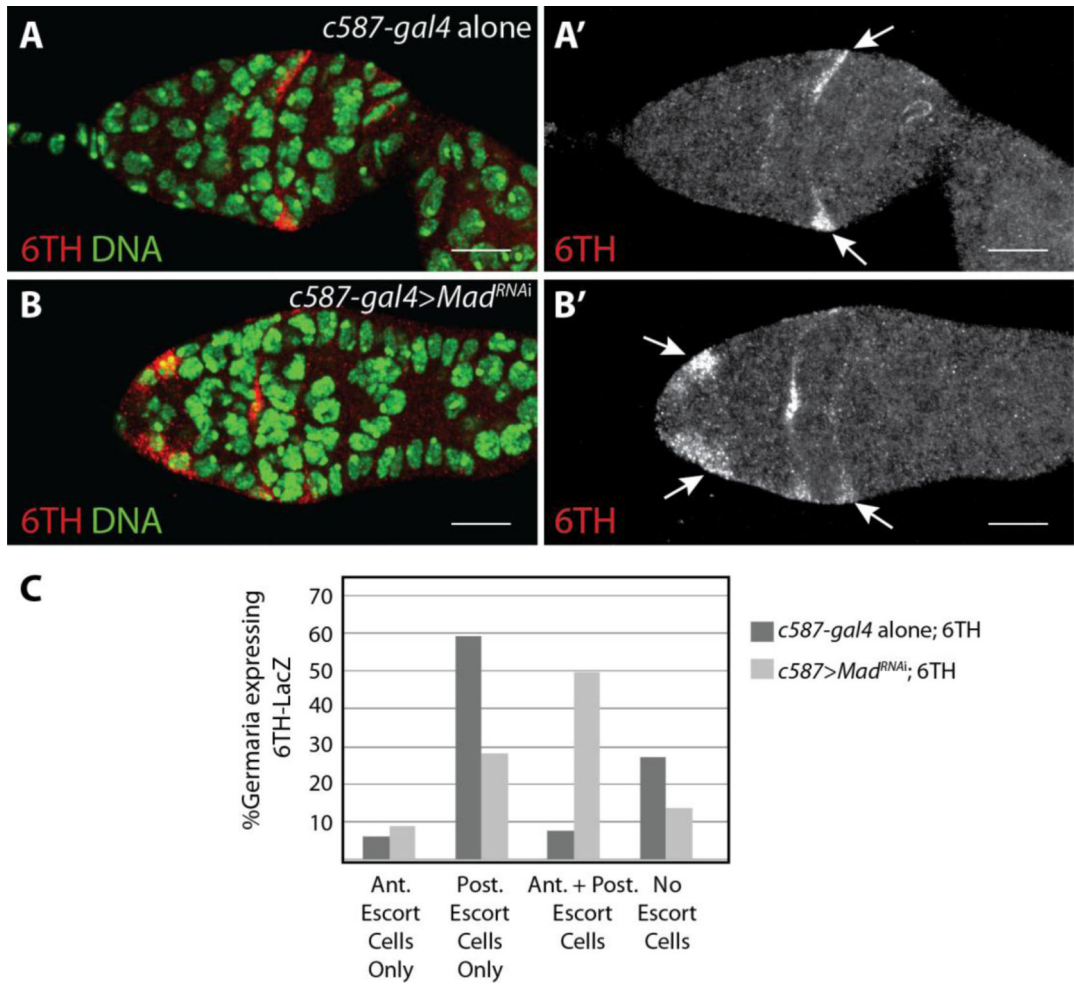


Fig. 7. Loss of Mad results in expanded Wnt signaling in the germarium

(A) *c587-gal4* alone control and (B) *c587-gal4>UAS-Mad^{RNAi}* knockdown germaria carrying the 6TH-LacZ Wnt reporter were stained for β -galactosidase (red). Knockdown of *Mad* led to an increase of the number of anterior escort cells expressing the Wnt reporter. Arrows point to cells expressing the 6TH reporter. (C) Graph showing quantification of the position of cells that express the 6TH Wnt reporter (*c587-gal4* control n=148; *c587-gal4>Mad^{RNAi}* n=135). (Scale bars, 10 μ m).

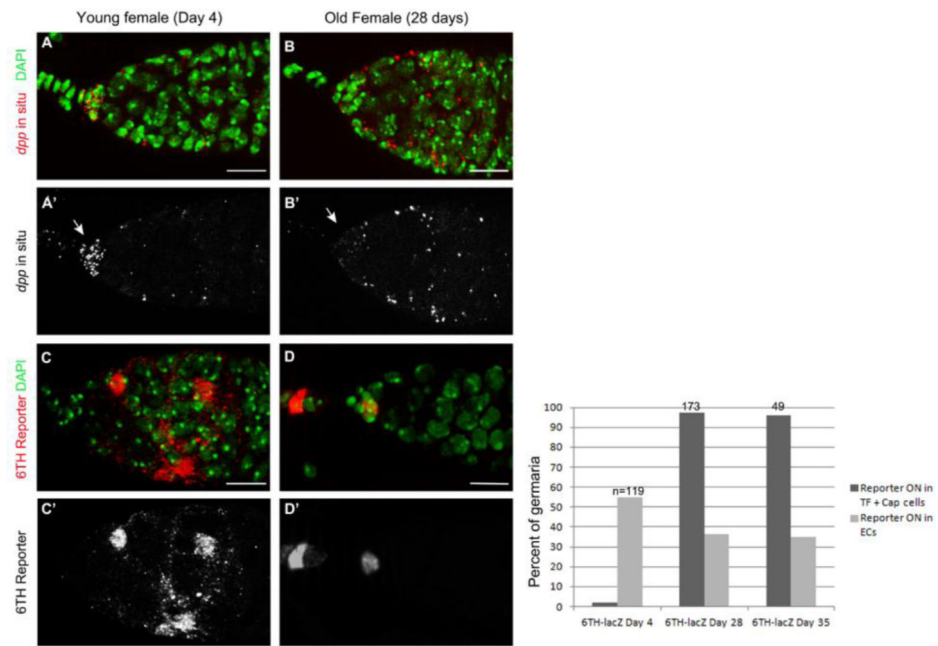


Fig. 8. Expression of *dpp* and a Wnt reporter change during aging

RNA *in situ* hybridization of *dpp* was performed on germlaria from females (A) aged for 4 days or (B) for 28 days. The level of *dpp* transcripts decreased in the cap cells and increased in the escort cells upon aging. Wnt reporter expression exhibits the opposite pattern with an increase of Wnt activity in the cap cells and a reduction in the escort cells in the germlaria of old females (D) compared to young females (C). The number of germlaria exhibiting Wnt activity either in the terminal filament (TF) and the cap cells or in the escort cells was scored in 4 day, 28 day and 35 day-old females (E). (Scale bars, 10 μ m).