Title
The follicle stem cells require a dynamic population of stromal cells in the Drosophila ovary

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The follicle stem cells require a dynamic population of stromal cells in the Drosophila Ovary

by

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DISSERTATION

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Dedication:

I would like to dedicate my thesis to all my family, especially my grandparents, Lillian, Mariola, and Mrisa, for all the love, support, and encouragement they have given me.

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I would like to thank Dr. Todd Nystul for all the great mentorship and support he has given me while I have been in his lab.

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Abstract:

Epithelial stem cells are maintained within niches that promote self-renewal by providing signals that specify the stem cell fate. In the Drosophila ovary, epithelial follicle stem cells (FSCs) reside in niches at the anterior tip of the tissue and support continuous growth of the ovarian follicle epithelium. Here, we demonstrate that a neighboring dynamic population of stromal cells, called escort cells, are FSC niche cells. We show that escort cells produce both Wingless and Hedgehog ligands for the FSC lineage, and that Wingless signaling is specific for the FSC niche whereas Hedgehog signaling is active in both FSCs and daughter cells. In addition, we show that multiple escort cells simultaneously encapsulate germ cell cysts and contact FSCs. Thus, FSCs are maintained in a dynamic niche by a non-dedicated population of niche cells.

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I. Stem cells:

A multicellular organism is composed of diverse tissues that can be comprised of trillions of cells each with specialized functions. Throughout evolution, the advantage of multicellularity came from dividing work within a community of cells, which would make them more efficient at exploiting specific resources and adapting better to diverse environments. To achieve this feat, a single cell has within its genome the instructions to specify all the different cell types that make up the organism. Through diverse mechanisms, including a massive expansion from the initial single cell, to their subsequent differentiation into distinct cell types, an organism can achieve this remarkable task, generation after generation, in a precise and reproducible manner.

A *stem cell* can be defined as a progenitor cell that has the potential to self-renew and specify one or more different lineages. Stem cells are cells whose main function is to give rise to the variety of cell types that make up an organism. Stem cells can be divided into two broad categories: pluripotent stem cells and adult stem cells. Pluripotent stem cells are the primordial cells in a developing organism and have the ability to specify all cell types within the three main germ cell layer: ectoderm, mesoderm and endoderm. In turn, adult stem cells are mainly found in a mature organism and are much more specialized. Their function is primarily to replenish adult cell types that are lost through natural turn-over or from injury.

In *Drosophila*, there is no pluripotent stem cell population. However, due to their genetic tractability and simple tissue architecture, *Drosophila* has been used as a model for the study of adult stem cell biology. The most studied

stem cells in *Drosophila* have been: the germline stem cells, the intestinal stem cells, the follicle stem cells (in females) and the somatic cyst stem cells (in males).

II. Stem cell niche:

The stem cell niche hypothesis was initially proposed in the 1970s in the hematopoietic system. It started based on Dr. John Trentin's observation that the stromal cells have an inductive role in promoting the progeny of the hematopoietic stem cells (HSC) to differentiate into different blood cell lineages (Trentin J., 1971). He termed this phenomena, *hematopoietic inductive microenvironments*. In the late 70s, Ray Schofield expanded these initial observations and predicted that as there is a differentiation-inducing microenvironment, there should also be a fixed place where hematopoietic stem cells reside (Schofield R., 1978). This place, which he termed a niche, would in turn promote HSC self-renewal and prevent them from differentiating.

Experimental evidence for the niche at a single cell resolution first came in the female and male germline stem cells niches in *Drosophila* (Xie T., and Spradling A., 2000; Kiger et al., 2001). There the cap cells and hub cells, respectively, function to provide signaling ligands necessary for the germline stem cell self-renewal. Another useful model has been *C. elegans* germline stem cells and their distal tip niche-cells (Kimble and White, 1981). In mammals, stem niche characterization was initially slower due to their complex tissue architecture. However, recent work has characterized in detail many adult stem cell niches. In the HSCs, the osteoblasts were initially shown to be necessary for HSC self-renewal (Calvi et al., 2003). Since then, it has been shown that HSCs might self-renew through two main niche sites within the bone marrow: the endosteal- and the vascular- niche (Adams and

Scadden, 2006; Kiel et al., 2005). However, it is still not yet entirely clear which is the main site of hematopoiesis.

For other epithelial tissues, the stem cell niches have also recently started to become well characterized. In the intestine and colon, the Paneth and ckit^{+} cells have been shown to function as niche cells that support their respective lgr5^{+} stem cell population (Sato T. et al., 2011; Rothenberg et al., 2012). In addition, the epidermis, which is comprised of three major stem cell populations, has made strides in identifying their respective stem cell niches (reviewed in Wong et al., 2012). For the bulge stem cells, it has been shown that the niche signals necessary for hair follicle stem cell maintenance comes from multiple cell types including the dermal papilla, neurons, and adipocytes. In addition, the skin epidermal stem cells have been shown to secrete their own Wnt 4 ligands necessary for self-renewal (Lim X. et al., 2013). These findings paint a diverse picture for what are the components required for establishing a niche for a stem cell.

Interestingly, however, many common paradigms have been found between different stem cell-niche populations in these different organisms. Some of these include the local secretion of morphogen signals that promote the self-renewal of the stem cells; adhesion junctions between stem cell and their niches that are necessary for their retention; and the importance of the extracellular environment including the matrix and niche architecture, that provides necessary cues for their maintenance.

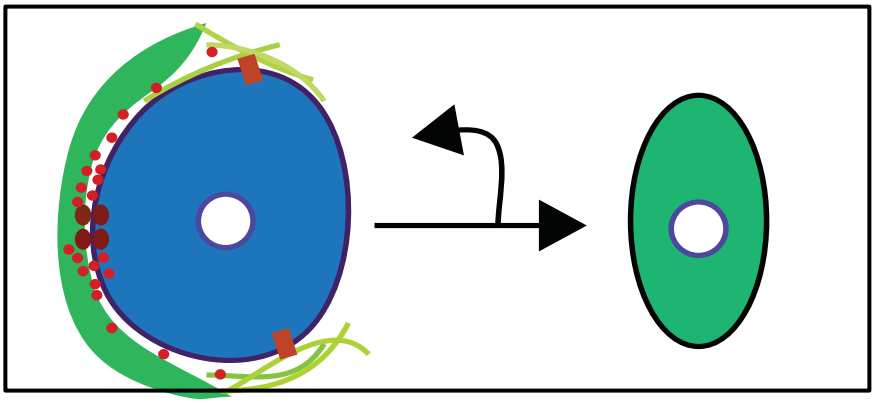


Figure 1. Emerging paradigms in stem cell-niche biology
Depicted here are several common features found in a 'canonical niche', in diverse tissues and organisms. A stem cell (shown in blue) is attached through adhesion junctions to its niche cell (shown next to it in green), necessary for its retention. Localized signaling occurs between the stem cell and its niche, allowing it to self renew. Extracellular membrane components, signal through integrin's, allowing stem cells to sense and respond to its extracellular environment. When the stem cell divides it produces two daughter cells, which recent evidence suggests that in many systems there are very small, if any, intrinsic differences between them. However, due to limited niche space, this usually results in one of the daughter cell to be retained in the niche while the other one initiates its differentiation program.

III. Significance of project:

The stem cell niche hypothesis was proposed by Ray Schofield in the 1970s. Experimental evidence for this first came in at the single cell resolution in the male and female germline stem cell-niches in *Drosophila* (Xie T., and Spradling A., 2000; Kiger et al., 2001). However, recent evidence suggests that various epithelial tissues lack a so-called 'canonical niche' (O'Brien L.E. and Bilder D., 2013) as the one depicted in Figure 1, suggesting that they lack a distinct, identifiable cell type that provides a unique microenvironment. We were consequently motivated to use the Drosophila ovary to better understand how an epithelial stem cell can be maintained in a distinct, dynamic environment and potentially serve as a model for epithelial stem cells found in other more complex systems. The female *Drosophila* contains two ovaries, each of which contains multiple structures of egg

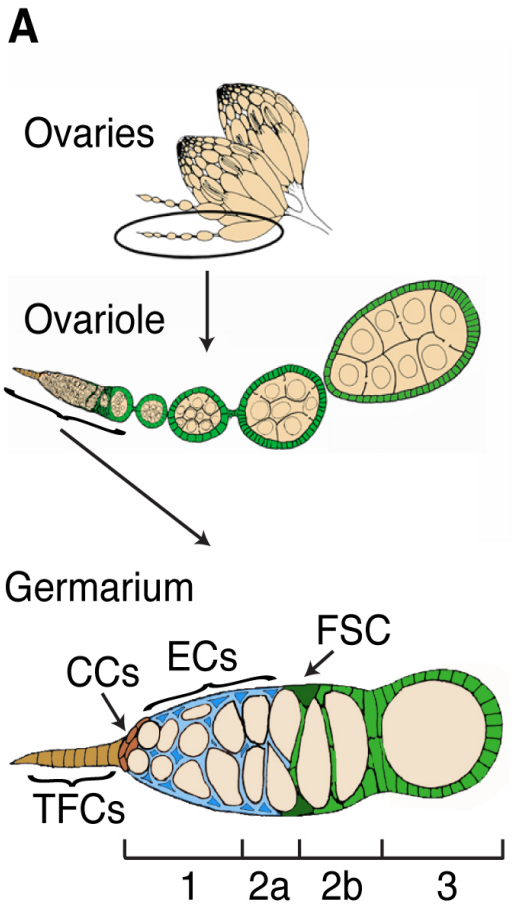


Figure 2. The Drosophila Ovary
A. A diagram of the Drosophila ovary. Each ovary is composed of multiple subunits, called ovarioles and each ovariole has a structure at the anterior tip, called the germarium. Each germarium has terminal filament cells (TFCs, orange) and cap cells (CCs, red) at the anterior tip, escort cells (ECs, blue) surrounding germ cell cysts in the anterior half, and two follicle stem cells (FSCs, light green) that produce follicle cells (dark green) that surround germ cell cysts in the posterior half. The germarium is divided into regions (1, 2a, 2b, and 3) that are defined by the stages of germ cell development.

producing chambers known as ovarioles (Fig. 2). At the anterior end of the ovariole there is a structure known as the germarium that houses the germline stem cells (GSCs) and the follicle stem cells (FSCs). The GSCs are attached to their niche cells, known as the cap cells, which provide adhesion junctions and BMP ligands necessary for their self-renewal and normal homeostasis. The GSCs divide perpendicular to their niche to give rise to daughter germ cell cysts, which move away from the niche and initiate their differentiation program. Germ cell cysts move through region 1 and 2a (Fig. 2) and get enveloped by a population of stromal cells known as the escort cells (ECs). Once the germ cells cyst reaches the 2a/2b border region, they are passed on to the follicle lineage. The follicle cells, which are a simple epithelium of cells that contain canonical epithelial features, wrap around the germ cell cysts and continue enveloping them until egg maturation. Previous work has shown that the follicle cells are maintained by two follicle stem cells, found at the 2a/2b border region (Nystul T. G. and Spradling A., 2007). Studying the

FSCs in the *Drosophila* ovary provide several advantages including its genetic tractability, a wide variety of tools available to manipulate gene expression in a spatial and temporal way, a short life span, and studying them at the single cell resolution. Together, with about 50% of their genome conserved with humans, we believe that this will prove a useful model in which to study epithelial stem cell-niche biology.

IV. Morphogens: Interpretation of gradients and theories of movement

As an organism grows from a single cell into a multicellular one, its cells need a way to determine their spatial distribution relative to others. This allows them to drive distinct gene programs that specify cell types with specific and unique functions. Over the last few decades, the term 'morphogen' has been coined for signals that give positional information to a cell (Wolpert, 1996). The current theory proposes that a signal produced in a localized location (known as an 'organizer cells'), forms a concentration gradient. The range at which this gradient acts depends on its production rate, spreading kinetics and half-life. A steady state gradient is reached when all these processes are balanced within a system, and the gradient is stable and unchanging. The graded signal can then specify unique gene programs in a concentration dependent manner. Wolpert proposed this idea in 1969 with the French flag model, which suggested that cells adopt different cell fates depending on the concentration information they obtain from a morphogen (Wolpert, 1969). This section will be subdivided into two segments: 1) the mechanisms by which cells interpret the morphogen gradients that drive specific transcriptional programs, and 2) the current theories by which these gradients are transmitted from a producing cell to a receiving cell in an organism.

Important note: The following section contains summarized excerpts from: The interpretation of morphogen gradient, Hilary L. Ashe and James Briscoe, Development, 2006:

In a strict sense, for a signal to be considered a morphogen it has to specify at least two distinct cell fates at different concentrations. Empirical evidence has shown that gradients typically can specify between three to seven different thresholds. In addition, small morphogen concentration changes can be easily sensed within an organism. For example: for sonic hedgehog and activin signaling, the full range of responses can be elicited by over a 50-fold concentration range, with a relatively small two-fold differences in concentration being enough to specify distinct cell fates (Green et al., 1992; Wilson et al., 1997; Ericson et al., 1997). Most morphogen are protein ligands that binds to an extracellular receptor and elicits a specific transduction pathway response, which results in the activation of a transcriptional effector and activates a specific gene program. Signaling pathways are considered linear, in the sense that the absolute number of activated receptor turns on a specific number of transcriptional effectors. Therefore, the linearity of signaling pathways assumes that a transduction pathway is able to transmit concentration-dependent information with enough fidelity to elicit a different response on cell types located in distinct locations.

Mechanisms on how morphogen gradients are interpreted:
Cells within a developing organism utilize different strategies throughout evolution that allows them to sense where they are in a tissue and determine the gene program that they need to turn on. This allows development of an organism to progress in a highly reproducible and predictable manner. Overall, strategies employed in the regulation of specific gene expression by cells sensing small differences to morphogen gradients, can be summarized by the following strategies: a) Binding–site affinity, b) combinatorial inputs,

c) feed-forward loops, d) positive feedback, e) cross repression and f) reciprocal repressor gradient. They are explained below and depicted in Figure 3.

a) Binding site affinity: An important mechanism that regulates distinct cell-type response to a graded morphogen, is by regulating the affinity by which a transcriptional effector of a pathway binds to different DNA-binding regions. By having both, low and high affinity binding regions, a transcriptional effector is able to activate distinct genes in different cell types (Stathopoulos and Levine, 2004). For example, low affinity binding sites can be activated only where morphogen concentration is highest. By contrast, high affinity binding sites can be activated at both low and high concentrations. Is important to note, however, that specific enhancer architecture ultimately determines the type of response (inhibitory or activating) (Stathopoulos and Levine, 2004). In addition, cooperative interactions between multiple effectors and cofactors can influence the robustness of the response.

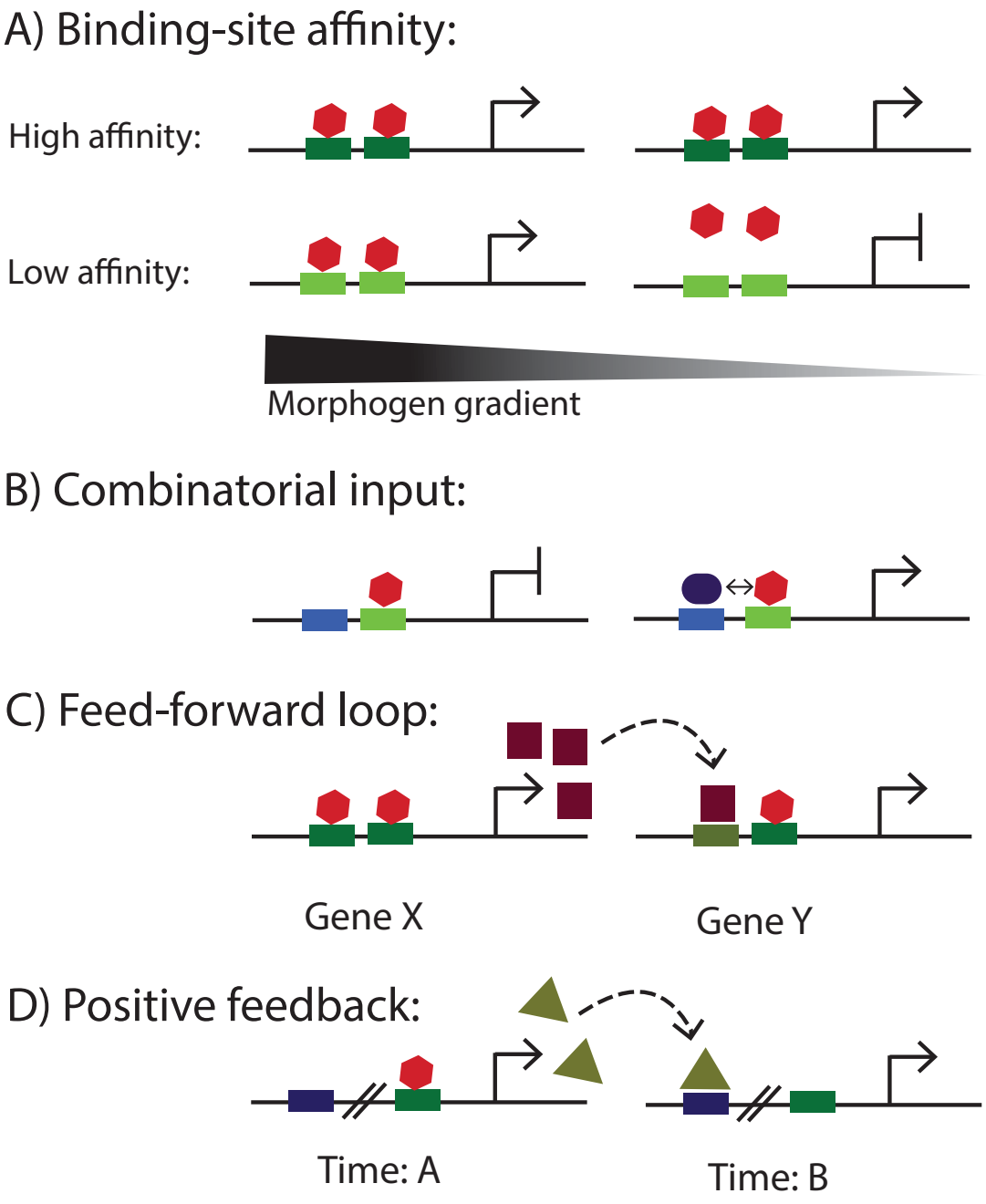
b) Combinatorial inputs: Binding affinity alone cannot explain the multiple gene response that is responsible for interpreting a concentration dependent gradient. For many genes it is not the absolute concentration of a transcriptional effector, but the integration of various positive and negative transcriptional inputs, that can determine the limits of an expression domain (Ochoa-Espinosa et al., 2005). Enhancer analysis has shown that high affinity enhancers usually have an affinity for another transcription factor, which is consistent with synergistic interactions (Papatsenko and Levin, 2005). In addition, studies show that multiple transcription factors acting together leads to sharper expression domains (Szymanski and Levin, 1995).

c) Feed-forward loops: Complex regulatory relationships can develop between genes responding to differential morphogen concentration. One such relation is the feed-forward loop, which is a regulatory network, by which transcription factor X activates a transcription factor Y and together, X and Y, activate gene Z. Furthermore, the coincidence requirement of a feed-forward loop can allow for a highly sensitive response to just small shifts in morphogen concentrations (Lee et al., 2002)

d) Positive feedback: Certain genes can have binding sites that can respond to transcription factors they encode, allowing for their auto-regulation. This usually works by a morphogen-induced transcriptional effector, X, turning on the transcription factor Y. In turn, the transcription factor Y has binding sites on its promoter for itself, so that a positive feedback circuit forms in gene Y. Subsequently, gene Y can be maintained in the absence of X. This can subsequently lead to an all or none response for gene Y, all the way up to the edge of a morphogen gradient.

e) Cross repression: This refers to the cross inhibitory response that different morphogen-activated-genes can have on each other, to create sharp distinct domains of expression. The different types of genes that can be induced by a gradient, say: A, B and C, would then inhibit one another to create sharp domains. If the inhibition is only one-way: A→B and B→C, it's known as asymmetric. If the inhibition is mutual: A→←B and B→←C, then is called symmetric. This mechanism creates a gradient of positional information into a discrete all or none changes in gene expression.

f) Reciprocal repressor gradient: Many morphogen gradients commonly form an inverse gradient of transcriptional repressor that is opposite to the transcriptional effector activated by the morphogen concentration. For example, the transcriptional effector for Wingless and H



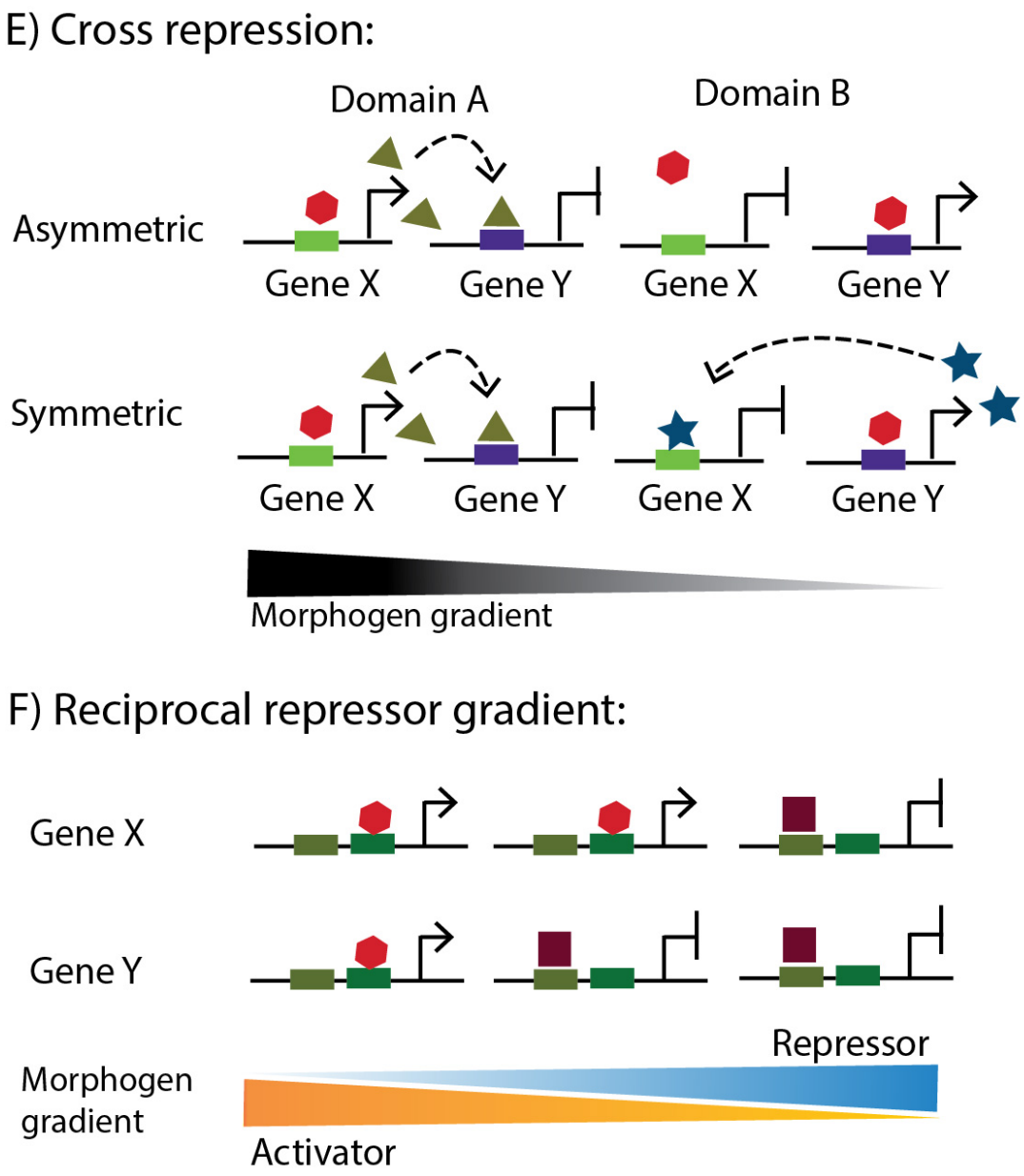


Figure 3: Mechanisms on how morphogen gradients are interepreted
Cells have generated throughout evolution different strategies that allows them to interpret morphopogens at distinct concentrations. This allows the cell to turn on the appropriate gene program that corresponds to it. The most common strategies are highlighted here (for detail see text).

Hedgehog transduced its signal via cubitus intereptus, and in the absence of Hh signal acts as a repressor (Giles et al., 2003; Jacob and Briscoe, 2003). This results in a transcriptional activator gradient with an opposing repressor gradient. This strategy can then increase the changes in the transcriptional activity mediated by the transcriptional effector of a morphogen. An alternative strategy to this, with an interesting mechanism, is the one used by the Decapentaplegic (Dpp) gradient in the Drosophila wing imaginal disc. A reciprocal gradient of Brinker is created to antagonize Dpp signaling. Mad and Madea directly repress Brinker, which in turn sets the limit of the Dpp threshold response. Interestingly, the *optomotor-blind* gene only requires the derepression of Brinker for its activation; no Mad input is necessary (Pyrowolakis et al., 2004; Muller et al., 2003).

g) Morphogen signaling can be noisy: A recent study (Xiong F et al., 2013) shows that in a developing tissue, which is actively proliferating and undergoing morphogenesis, can experience complex movements which affects the concentration by which cells respond to morphogens. Therefore, it is common for cells to be specified in domains outside of their own. Interestingly, cell sorting rearranges them into their correct locations (i.e. French flag model). It will be very interesting to determine how this cell rearrangement occurs, for example by the differential regulation of cadherin's. It also demonstrates that in theory many of these morphogen gradients are sharp, but in practice a graded signal may not be precise.

Mechanisms on how morphogens are dispersed:

Important note: The following section contains summarized excerpts from: Morphogen transport, Muller et al., Development, 2013:

Morphogens are produced at a localized location and is diffused within a target tissue. Morphogens can act up-to hundreds of micrometers from their initial site of production. In addition, they can act at different time periods and in diverse developmental contexts. There are certain canonical features that distinguish morphogen gradients, such as their graded distribution; different signaling ranges; and non-autonomous function. However, none of these features can directly implicate them to a specific transport mechanism. To explain these functions of a morphogen, certain models for their transport have been proposed over the last few decades. These transport models can be divided into two main groups: 1) intracellular-based mechanisms, where morphogens move throughout the cell into neighboring ones and 2) extracellular-based mechanisms, where morphogens move largely through diffusion. Below, these different models are further explained and depicted in Figure 4.

Extracellular diffusion:

a) Free diffusion: In the plainest case of morphogen dispersal, molecules simply move by free diffusion. However, loss of molecules needs to also to be considered, or otherwise all molecules will eventually be evenly distributed within a tissue. A molecule can be lost either in the form of degradation or being permanently trapped within a cell. The combination of morpohogen production, diffusion, and loss, results in a graded distribution over time. Notably, the amplitude of a gradient increases if the molecules have a long lifetime.

b) Hindered diffusion: This model is similar to free diffusion, but considers two other variable that influence molecule dispersion: 1) the obstacles of densely packed cells and 2) the transient binding to extracellular molecules such as receptors or basement membrane components. These two

factors affect the distance and the speed by which molecules travel. Interestingly, both free and hindered diffusion models can eventually result in the same steady-state morphogen gradient. However, the kinetics by which these gradient forms are different.

c) Facilitated diffusion and shuttling: This is an expansion of hindered diffusion model that considers positive and negative regulators of diffusion. Negative regulators can immobilize molecules until a positive regulator interferes with the interaction and allows the morphogen to move over long distances. In this model, proteoglycans can be considered both, positive and negative regulators of morphogens, depending in the context in which they act. Shuttling is a different mechanism in which a shuttle, which is able to bind and move morphogens, is generated in a localized source. This results in morphogens, binding to the shuttle, moving quickly through rapid diffusion, and finally being immobilized once the shuttle is destroyed. This eventually results in morphogens being concentrated far from the shuttle source, which generates a sharp gradient from the initial uniform morphogen distribution.

Intracellular diffusion:

d) Trancytosis: In this model, signaling molecules bind to the cell membrane, which results in their cellular uptake by endocytosis. The signaling molecules are subsequently released through exocytosis. By undergoing various rounds of uptake and release, the molecules eventually become dispersed within a tissue.

e) Cytonemes: The cytoneme model builds on the observation that cellular extensions serve to examine the environment to identify distant signals. The cytoneme model builds upon the knowledge that the long,

dynamic filopodia-like-structure that sometimes project from a cell, can contact and interact with morphogen producing ones. This then can induce a transduction signal through cytoneme-projecting cell. Cytonemes have been found to extend more than 50 um long (Kornberg, 2012).

The authors go on to hypothesize that *most* of the available data seems to support a diffusion-based model for morphogen dispersion.

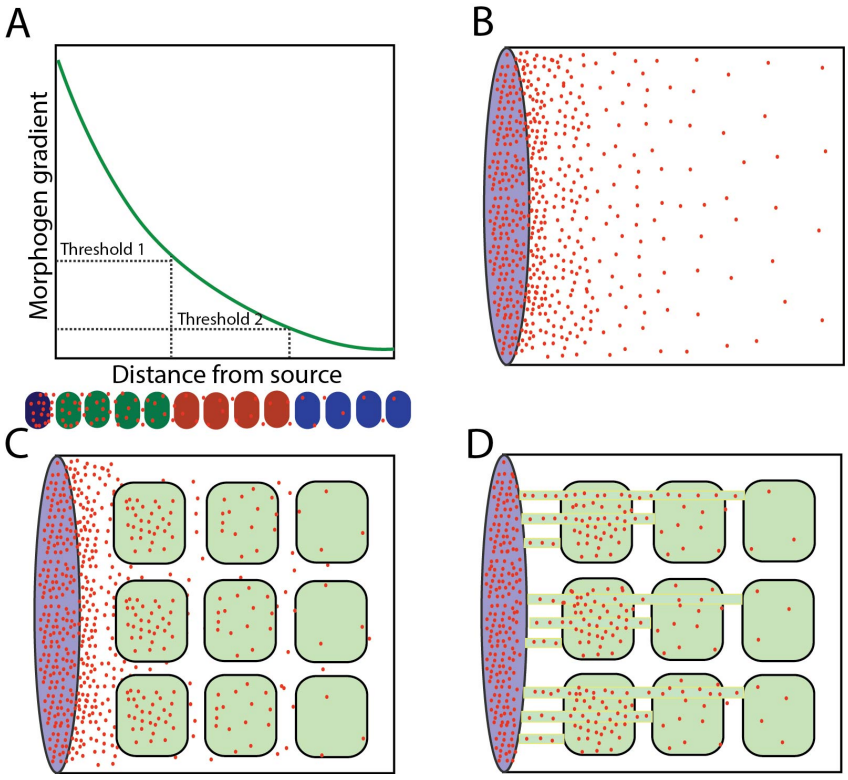


Figure 4. Theories on how morphogens are dispersed in a tissue. Morphogens originate from a specific cell type that is known as an *organizer cell*. After release, morphogen concentration decreases over distance. The morphogens rate of production, half-life, and spreading kinetics determine the range under which it acts. A. A depiction of a conical morphogen gradient. At different concentrations the gradient is capable of specifying different outcomes (i.e. cell fates). The gradients threshold generates these outcomes. B-D. The common theories by how gradients are dispersed in a tissue. B. The simplest of these is free diffusion. There are added complexities to this, as shown in the main text. C. Morphogens can also be dispersed by trancytosis, by subsequent cycles of uptake and release. D. Cytonemes have recently been shown to be a novel form of uptake by which distant cell types can release neuron-like protrusions that have been shown to reach more than 50 um long.

V. Wingless signaling in *Drosophila*.

Wnt gene families are a type of encoded glycoproteins that can act as a morphogen. Members of this family are defined by sequence homology to Wnt-1, rather than any functional homology. There are 19 homologs in vertebrates and 7 in *Drosophila*. Wingless (Wg), the fly homolog of Wnt-1, is necessary to pattern the adult wings and other adult structures (Graba Y., et al., 2000). The molecular mechanism by how the canonical Wg pathway, works has been elucidated mainly by using the *Drosophila* model. It's been found that in the absence of Wg signaling, the pathway effector, Armadillo (Arm; homolog to B-catenin), remains predominantly attached to its cytoplasmic binding partner DE-cadherin. The destruction complex, which is a negative regulator of the pathway, can easily degrade any unbound Arm. This results in pangolin (TCF homologue) remaining in its inhibitory form, and inhibiting Wg-activated genes. Binding of the Wg ligand results in Disheveled-mediated inactivation of the destruction complex. This results in the stabilization of Arm, which leads to its translocation to the nucleus, its association with TCF and the subsequent activation of Wg-target genes (Graba Y., et al., 2000).

Several handpicked topics relevant to my work that I'll write more about are:

1) Most Wnt proteins undergo posttranslational modification (Burrus and McMahon, 1995). They can undergo N-linked glycosylation as well as palmotylation, which are necessary for their secretion and function. Posttranslational lipidation of mammalian Wnts are also very important for their function. Mutants can be secreted but have little or no signaling activity. Consequently, unpalmitoylated Wnts cannot bind to Fz receptors (Komekado et al., 2007).

2) Most Wnts require Wntless for reaching the cell surface. Wntless is an integral membrane protein found in the Golgi, plasma membrane, and endosomes (Banziger et al., 2006). Current data suggests that Wls is a Wnt Chaperone, which guides WNTs from the Golgi to the cell surface (Belenkaya et al., 2008). Wls mutants result in the inhibition in the secretion of multiple Wnts from Wnt producing cells. Retromer is another protein that is necessary for Wnt recycling (Belenkaya et al., 2008). Retromer mutants result in the missorting of Wnt's into lysosomes, which results in their degradation.

3) Several factors can influence Wg movement: Glypicans are heparin sulfate proteoglycans anchored to cell membranes via a glycerol phosphatidylinositol (GPI) linkage (Blair 2005). Two glypicans, Dally and Dally-like, influence Wg signaling in the wing disc. Dally, promotes signaling and is suggested to facilitate Wg movement or act as a co-receptor (Franch-Marro et al. 2005; Han et al., 2005). Interestingly, Dally-like (Dly), have different functions than Dally. Loss of Dly, increases expression of short-range targets but decreases expression of long range Wg signaling. A recent study suggests that Dly mediates transcytosis of apically secreted Wg, transporting it then to the basolateral compartment, where it is then presumed to diffuse to activate long-range targets (Franch-Marro et al. 2005; Han et al., 2005).

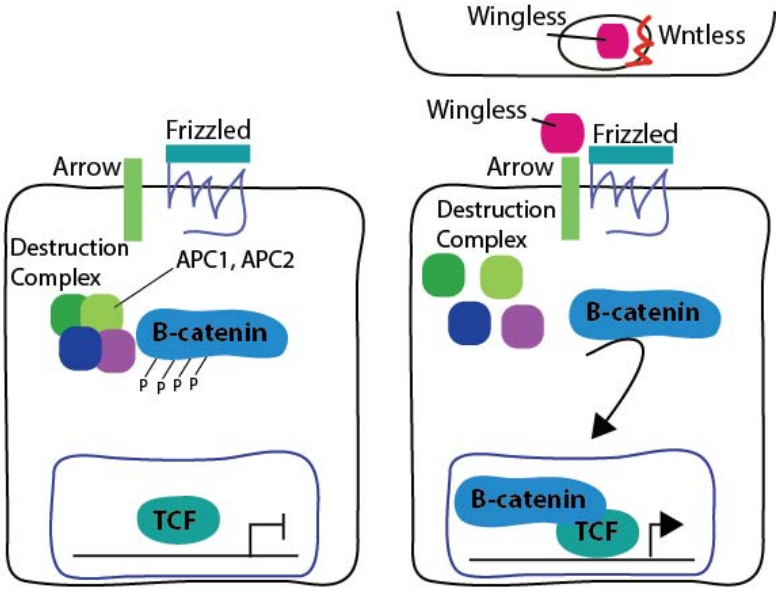


Figure 5. A simple diagram of how the Wingless Pathway functions in *Drosophila.* A. In the absence of Wingless (Wg), the destruction complex, composed of multiple proteins including APC-1 and -2, Shaggy, and Axin, phosphorylates cytoplasmic B-catenin and targets it for degradation. This results in pangolin (homologue of TCF), binding to Wg target genes and inhibiting their transcription. B. Wg is secreted in producing cells with the help of the chaperone protein Wntless (Wls). Wg subsequently binds to its receptors, Frizzled and Arrow, which leads to the inactivation of the destruction complex. Arm (homologue of B-catenin) is subsequently stabilized and makes it to the nucleus, binding to TCF, and activating Wg target genes.

VI. Hedgehog signaling in *Drosophila*.

Hedgehog (Hh) is another type of glycoprotein that acts as a morphogen. There is only one protein type in Drosophila but there are three homologs in mammals: sonic hedgehog, desert hedgehog and indian hedgehog. Drosophila has been a great model to elucidate the Hh signal transduction pathway. In the absence of Hh ligand, patched transmembrane receptor (ptc) represses the G-protein coupled receptor Smoothened (Smo) (Chen et al., 2000). Cubitus (Ci) is then retained in the cytoplasm through Costal-2 (Cos2) anchoring activity. It's subsequently phosphorylated by PKA, GSK3 and CK1, resulting in the suppressor form of Ci (CiRep). In addition, Suppressor of fused (Su(Fu)) also exerts its negative regulatory effects on Ci. The presence of CiRep results in the repression of target gene expression. Upon Hh signaling, ptc alleviates its repression of Smo, allowing it to accumulate at the cell membrane and signal downstream. Smo then physically interacts with its C-terminal tail with Cos2 and Fused (Fu). In addition, protein kinase A (PKA) phosphorylates Smo at several sites in its C-tail and enables additional phosphorylation by casein kinase 1 (CK1). These phosphorylations are important for Smo to accumulate at the cell surface. The degree of Smo phosphorylation correlates with the degree of accumulation and activity.

Then the interaction between Smo and Cos-2 alleviates Cos-2 interaction, which results in Fu activation. The formation of CiRep ceases and Su(Fu) can no longer anchor Ci to the cytoplasm, allowing full-length Ci to enter the nucleus and activate Ci transcriptional targets (Lum L. and Beachy P.A., 2004).

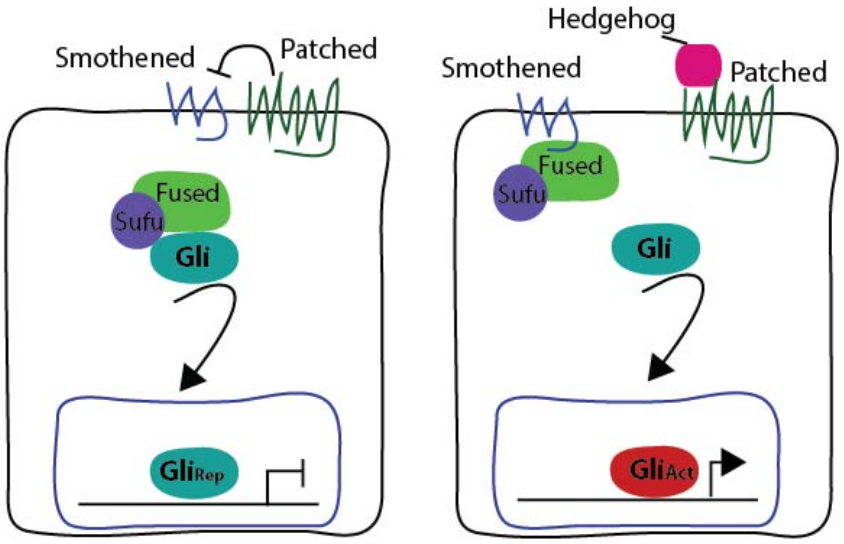


Figure 6. A diagram of how the Hedgehog Pathway functions in *Drosophila.* A. In the absence of Hedgehog (Hh), transmembrane receptor patched inhibits the G-protein coupled receptor, Smoothened. This inhibits the processing of full length cubitus (homologue of Gli) by suppressor of fused (Su(Fu)). The subsequent Gli that makes it to the nucleus, its in a repressive form, which results in the inhibition of Hh target genes. When Hh binds to its receptor Patched, it results in the disinhibition of smoothened. Suppressor of Fused is subsequently inhibited, which results in the full length Gli transiting into the nucleus and activating transcription of Hh-target genes.

Methodology

This section describes in further details the techniques that I used during my studies in better characterizing the follicle stem cell niche in *Drosophila* Ovary. Its divided into two main sections: 1) the first part describes in detail how the techniques work; 2) the second part explains the experimental procedures and how they were performed. With this section, I hope to provide a broader knowledge and a better understanding of the results section, and how the tools described below helped me to perform the studies to better understand the follicle stem cell niche.

I. Tools in Drosophila

A) P-elements and enhancer traps:

a) Identifying lines that drive expression in the germarium:

Transposable elements are DNA segments that have the ability to move to new sites within the genome of a host species. There is great variety of transposable elements and they have been put into groups according to their sequence similarities. They can be divided into two main types: During class I transposition, a new DNA copy is made from the original one by reverse transcription. In class II, elements can excise themselves from their original site and move into a new location. Transposable elements are found in all species and in some cases can comprise a large portion of their host's genome. The Drosophila genome has different families of transposable elements. The most intensive studied one's have been the P family, due to their ability to horizontally transfer across species, and also because of their technical applications, which has made them an invaluable tool for gene manipulation (Kaufman P.D. et al., 1989).

The P element structure is composed at the 3' and 5' end with a 31 base pair inverted repeat and another subterminal 11 base pair repeat. In between

these two repeats, there is a transposase-binding region. These repeats are necessary but still not sufficient for their transposition. Autonomous P elements encode within their structure a transposase gene; while nonautonomous one's do not. P elements are type II class transposable elements and do not require an RNA intermediate. Transposable element insertions are not totally random and carry certain insertion preferences such as: 1) within the euchromatin region, 2) in the non-coding upstream regions of genes, 3) target sites with the consensus octamer region, 4) near other P elements and 5) near the donor site. After transposition, a P element leaves behind a double strand break. Usually, homologous sequences flanking the DNA are used to repair the break, which can end up resulting in a net gain of one P element (Kaufman P.D. et al., 1989).

P elements have made a great tool in Drosophila, which has been used, in many different contexts, to better understand gene function. In general, some of the common uses for P elements have been a) mutagenesis, b) transgene expression and c) enhancer trapping.

i) Mutagenesis: The most efficient way of creating a P element mutagenesis is by crossing a parent fly containing the nonautonomous P element, to a different parent fly containing an immobile copy of the transposase gene. This can result in fly progeny that contains germ cell mutations. Afterwards, the transposase is crossed out in the next generation, to stabilize any mutants (Kaiser K and Goodwin S.F., 1990). Although, this has been the de facto way to generate mutations in flies, newer techniques such as TALENs and CRISPER, provides the advantage of generating mutants with just a single nucleotide change (Boch J., 2011; Cong L. et al., 2013).

ii) Transformation and transgene expression: In this procedure, a gene of interest, usually with a selectable marker such as mini white, is placed between P element ends and injected into an embryo in the presence of

transposase. The P element carrying the genes is then inserted into a random chromosomal site. Progeny are subsequently screened for the insertion using the selectable marker.

iii) Enhancer trapping: In this technique reporter and effector genes, such as lacZ and Gal4, are fused to a weak promoter and mobilized within a P element, to produce a collection of lines with particular expression patterns. These lines 'trap' enhancer elements of genes near where they land. Large collections of enhancer trap lines have been generated and have been used as tools to determine the expression pattern of specific genes, as well as to express transgenes in unique cell populations.

b) PhiC31 system: PhiC31 integrase is a site-specific recombinase naturally found in the genome of bacteriophages. It mediates the recombination between two 34-base-pair sequences known as attachment sites (att). This has been adapted to integrate attB-containing plasmids unidirectionally into the target genome of flies that has previously been inserted with an attP site. Because integration into the genome is site specific, the constructs are always inserted in a predetermined location. This limits genomic noise that can influence transgene expression. In addition, it is much more efficient than P element insertion, with frequencies reaching 70%, in contrast to 10-15% for P elements. For these reasons, PhiC31 is now commonly used and has been essential for the creation of various transgene libraries, including the enhancer trap collection known as the Janelia Farms Collection, and small interference RNA library known as the Transgenic RNAi Project (TRIP).

c) Janelia Farms Collection: The Janelia Farms enhancer trap collections were generated by the Rubin Lab at Janelia Farms. They were created differently from regular enhancer traps. They preselected a set of about 900 genes that are expressed in the brain. They subsequently cloned genomic fragments near these genes and sub-cloned them upstream of a Gal4 gene. Subsequently, they cloned the full construct into a phiC31 site-specific

integration system, and the transgenic lines were generated and screened for brain expression using a UAS::GFP. We obtained a subset of these lines and screened them for expression in the ovary that could help us manipulate gene expression in escort cells.

d) Transgenic RNAi Project: Most genes in the animal genome remains poorly characterized. *Drosophila*, one of the best-understood multicellular organisms, contains only about 25% of genes with known functions and with readily detectable phenotypes. This fact highlights that researchers have not been able to experimentally assay the role that the rest of the genes in the genome have. A technique that can help us better understand gene function is RNAi. This technique allows us to spatially and temporally knockdown gene expression, which is difficult to achieve using classical methods. Taking the advantage of small interference RNA library that targets thousands of genes in the genome can allow us to efficiently knockdown gene function and assess their role, in a relatively short period of time. The generation of siRNA lines in *Drosophila* has been relatively simple. The strategy consists of using computational program to generate small ~21bps sequences against the target gene. The construct is subsequently cloned into an entry vector and then into a destination vector that is used to integrate them into the genome. As much as RNAi has been an invaluable tool for Drosophila, there are two problems that should always be kept in mind: 1) knockdown efficiency and 2) target specificity. Sometime the knockdown efficiency can be poor (<50%), and therefore it is important to confirm knockdown by qRT-PCR. Secondly, it is important to determine if the phenotype observed is specific for the gene of interest Therefore, confirming the phenotype by using different siRNA lines that target different sites within the mRNA of interest, is recommended.

e) Flippase enhancer lines: In this approach, the Bing Zhang lab generated a library of about 1,000 enhancer trap lines. The P element construct was inserted with two Flippase genes. The resulting lines function

much like Gal4 enhancer traps, picking up the expression of genes near where they land. However, ET-FLP lines show expression pattern that are usually much more restrictive than regular Gal4 enhancer lines, due to the fact that FLP-FRT recombination are not 100% efficient.. The interest in screening these lines arose from the lack of Gal4 specific enhancer lines that are found in escort cells and follicle cells.

II) Detecting low abundance transcripts and protein:

a) Shibiri temperature mutants result in the paralysis at the non-permissive temperature that can be easily reversed. This results in endocytic vesicles being unable to separate from the parent membrane, resulting in a depletion of available vesicles. A different strategy that achieves similar results has been the use of Rab5 dominant-negative, which blocks endocytosis. The small GTPase Rab5 regulates the early pathway of endocytic uptake, including docking and fusion. These approaches have previously been used to detect greater protein abundance, in ligand producing cells. Specifically, they have been used in the wing disc to determine specifically the orientation of ligand secretion in polarized cells. However, our logic to use these mutants in the ovary, came from the idea that inhibiting the uptake of low-level-ligands in cells in the germarium, could help increase their availability in the extracellular space, and consequently make it possible to detect it through antibody.

b) Single-molecule fluorescence in situ hybridization (FISH): This method relies on generating a fluorescence tagged RNA analog that complements the target sequence. For RNA detection, previous techniques have focused on generating a long RNA. However, this method did not work very well in the germarium. By contrast, single molecule FISH relies in generating multiple small probes that are able to bind in tandem. Since only when most probes bind a target can one observe a signal at a significant level, this method dramatically reduces background. Furthermore,

transcripts could be detected at the single molecule level, which can be used as a quantitative approach to quantify mRNA levels.

III) Lineage tracing of stem cells: Lineage tracing has been used extensively during development to study lineage relationships between different cells. It relies upon genetically marking a progenitor cell with an inheritable marker, so that all daughters arising from it can be readily identifiable. Adult multicellular tissues can be quite complex, containing organs whose cells turn over at a fast rate. Previous work has shown that lineage analysis is the most powerful technique to identify a stem cell, and determine aspects of their behavior, such as total number of stem cells, their contribution to a tissue, and their rate of replacement.

For identifying a stem cell clone, a key step relies in actually labeling a stem cell. Most dividing cells that are marked in a tissue are not stem cells and therefore tend to be washed out over time. The size of these clones can vary, since they can be labeled at different stages during their expansion. However, a way to identify a stem cell clone is to analyze them at longer periods of time after they were labeled. Stem cells tend to be long-lived, and are able to label all cell types in their lineage.

There are multiple ways that can be used to label a stem cell in *Drosophila*. One of the most commonly used has been the site-specific recombination system, FLP-FRT, from yeast. This method makes possible to generate stem cell clones at a high frequency. And even though the cells in which the clones are generated can't be predetermined, analyzing a high percentage of clones circumvents this problem.

There are several factors to consider when performing lineage tracing of stem cells. There are various methods available and each carries advantages and

disadvantages. Two important factors to consider are: 1) background recombination and 2) G2/M requirement.

One of the earliest and most common used lineage labeling systems has been the FLP-FRT lacZ system, published by Harrison and Perrimon in 1993. It relies on FLP-induced FRT mitotic recombination, which leads to the fusion of a tubulin promoter and a lacZ gene. It was previously thought that it could only recombine under G2/M phase of the cell cycle; however, recent work has shown that stem cell division is not necessary (Kirilly et al., 2011). A different system that is also used is the 'flip-out' system, where two FRT sites flank a CD2-stop-codon, located next to a ubiquitous promoter. Once flippase is induced, it excises out the CD2 and allows the expression of any downstream reporter gene. In this system, cells do not require to be at the G2-M phase.

Other systems commonly used include the mosaic analysis with repressible cell marker method (MARCM), which relies on Gal4 expression together with a UAS controlled reporter gene. With this method, the Gal4 repressor, Gal80, is found on an FRT and is recombined to allow for Gal4 expression. Interestingly, this technique can also be combined with any transgene under the control of a UAS, which makes it a very useful to genetically manipulate a system.

Twin-Spot MARCM: This is a new system that allows for the differential labeling of daughter cells generated from a common progenitor. Most lineage labeling systems are only able to label reliably one of the daughter cells. However, this system starts out with no common labeling and upon FLP-FRT mediated recombination, one cell expresses a GFP label and the other one an RFP. This technique is very useful during development since it could be used to study the lineage relationship of cells.

IV) Signaling pathways reporter lines: The generation of different signaling pathway reporters that contain cis-responsive elements for a transduction pathway have been great tools to identify cells types that are responsive to them. Two reporter lines that I have used in my studies is the Hh-reporter line that was generated in the Kornberg lab, where cubitus (Gli homologue) binding sites were put upstream of a GFP-nls and was shown to be a sensitive reporter for cells that transduce the Hh transduction pathway. The other line I have used was generated in the Ken Cadigan Lab and contains promoter fragments of the Notum gene. This promoter fragment contains TCF binding together with TCF helper sites, which was shown in *Drosophila* to be required to active Wg-responsive genes.

II. Experimental Procedures

Fly stocks

Stocks were maintained on standard molasses food at 25C and adults were given fresh wet yeast daily. All progeny that contain tub-Gal80ts were kept at 18°C until eclosion and then shifted to 29°C for high RNAi expression. The following stocks were used:

1. yw, hsflp, UAS-CD8::GFP; FRT40a, tubGal80; tub-Gal4/TM6
2. y1w, hsflp, UAS-CD8::GFP, tubGal4; FRT82B,tub-Gal80/TM6
3. yw; Notum-LacZ (from Ken Cadigan)
4. yw; Hh-LacZ (from Allan Spradling)
5. yw; Traffic-Jam Gal4, (from Guy Tenentzapf)
6. hsFlp, FRT40A, UAS–Cd2::rfp, FRT40A, UAS–Cd8::gfp, UAS–Cd2-Mir/CyO,Y. (from Tzumin Lee).
7. yw*; Ptc-pelican (from Tom Kornberg).
8. yw;; UAS-Wg::GFP (from Vivian Budnik)
9. yw;; UAS-Hh::GFP (from Isabelle Guerrero)
10. yw*, hsBam (from Dennis McKearin)
11. yw;; TM1-GFP (from GFP Protein Trap Database)
12. Flp Enhancer trap lines (Bing Zhang)
13. yw, <CD2<Gal4; UAS-GFP (from Lawrence Zipursky)
14. Twin-spot MARCM (from Tzumin Lee)
15. Fucci-GFP (from Atsushi Miyawaki)

16. X-15-29 and X-15-33 (Harrison and Perrimon, 1993)
17. yw*; FRT40a/Cyo, y1, sc, v1; P{TRiP.HMS00844}attP2, y1 sc* v1; P{TRiP.HMS00492}attP2/TM3, Sb1, w[*];y1, sc, v1; P{TRiP.JF01261}attP2; P{w[+mW.hs]=GawB}bab1[Agal4-5]/TM3, Sb[1], y1 w*; P{GawB}109-30/CyO, w[*]; P{w[+mC]=tubP-GAL80[ts]}20; TM2/TM6B, Tb[1], w1118; P{GMR13C06-GAL4}attP2 (from the Bloomington Drosophila Stock Center)
18. yw; Wntless-RNAi (Vienna Drosophila RNAi Center, T-ID: 103812)

Immunostaining

Ovaries were dissected in 1x PBS, fixed in 1x PBS + 4% formaldehyde for 15 minutes, rinsed, and incubated with primary antibodies overnight at 4°C. Next, the tissues were washed 3 times over the course of an hour at room temperature, incubated with secondary antibodies for 2 hours at room temperature, and washed three times again over the course of an hour at RT. Finally, tissues were rinsed in 1x PBS, incubated in 1x PBS + 1 μg/ml DAPI for 5 minutes and mounted on glass slides in Vectashield (Vector Labs). 1x PBS with 0.3% Tween was used for all rinses and washes and to dilute antibodies. To detect Wg protein, the tissue was incubated with anti-Wg antibody for 30-60 minutes on ice prior to fixation (as described in Strigini and Cohen, 2000), and then fixed and processed as described above.

The following primary antibodies were used: mouse anti-Wg (1:4), mouse anti-Fas3 (1:100), and mouse anti-lamC (1:100) (from the Developmental Studies Hybridoma Bank); rabbit anti-Hh (1:500) (Taylor et al., 1993), rabbit anti-Wntless (1:1000) (from Konrad Basler), rabbit anti-GFP (1:2000) (Torrey Pines Biolabs), mouse anti-β-Galactosidase (1:1000) (Promega). The following secondary antibodies were used: anti-rabbit and anti-mouse conjugated to Alexafluor 488, 546 or 555 (1:1000) (Invitrogen).

Fluorescence In Situ Hybridization

Stellaris RNA FISH probes (Biosearch Technologies) were custom ordered for Drosophila Hh and Wg transcripts, and the manufacturer's protocol was modified for labeling of Drosophila ovaries. Briefly, ovaries were dissected in RNAse free 1x PBS (Invitrogen) and fixed in 1x PBS + 4% paraformaldehyde (Fixation Buffer) at room temperature. Samples were washed three times in 1x PBS, put in 70% ethanol for 2 hours at 4°C and rehydrated in 10% formamide in 2x SSC (Wash Buffer) at room temperature for 5 minutes. Next, the probe was diluted to 1 μM in 100 mg/ml dextran sulfate + 10% formamide in 2x SSC (Hybridization Buffer) and incubated with the tissue, first at 37°C for 15 minutes, and then at 30°C overnight. Next, the tissue was washed twice in Wash Buffer for 30 minutes per wash, with the last wash containing 1 μg/ml DAPI. Finally, the tissues were mounted on glass slides in Vectashield (Vector Labs) and imaged within 12 hours after

mounting. For RNase treatment, ovaries were incubated with 50 units of RNAse If (New England Biolabs) in NEB buffer 3 at 37°C for 1hr the fixation and wash steps. Then, the tissue was incubated at 70°C for 20 minutes to heat inactivate the RNase, and washed two times with 1x PBS,. The hybridization protocol was then continued as described above, starting with the ethanol dehydration step.

FISH Probe Sequences:

Wingless:

gaagatatagctgatatcca; acagggccatcaggcagatg; gatttctgtttgccctcgac; gtaatgttgttgggttcgcc; tgggtccatgtacatgatgg; ttctcaacgtagagtggatc; ctgaccaggcgtcgctgttt; ctcccagtacaccgggattg; aagttggcgcccttgaccag; gtgttggcactcgctaatgg; tccagcggcgatttctgaac; gagaagtttctcgtcgagca gccgaatagatttttgcccc; ggcagcctcgatcaacgatt; attgcgtaaatgaagctcgt; caggccctggcaatcgagtg aggactctatcgttccttca; gatctcgactggtggctgta; ccgcctggtggttcgcttgt; atgttgtcggagcagccgcc ggagaacttgaacccgaatc; tcgccggtatcgacgaattc; cttctcgcgcagattgcgac; cctcgttgttgtgcagattc catctccgcttggacgtgcg; catggcatttgcactcctgt; actgtacacgatccggacat; cagtcgcatccagcaggtct; cgccaatcacacggaagttg; atcgaagcgggccttcagat; gagactgttggtcacttgca; ctaactggggccagagcgtt aattcgagccggctgcattc; gaataatcaggccgttggag; ccgtagaccagaccagactg; gcatatggtcgttcagcata

ctgttctctagcaggatgtc; ggtgatggatcttgctgatc; cagcctggggcaaactgttg;

gacgacgtccatttcgtccg tatctattatgcttgcgtcc; ctccagatagacaaggtcct;

ggttcttctcgcagaagctc; catgggttcccaggatgccc agcgaggtctcattgcactg;

cagcaccagtggaaggtgca; ttttggtccgacacagcttg; tacagacacgtgtagatgac

Hedgehog:

gcgaatacgaatgcgagtat; ctcgcactgtgattgacaaa; tacggtcttaactgttctcc;

gaaatacttgagtcggcgaa; cttcacttttggcacacaga; ctgagctgtggttatccatg;

tttggagctggaactggaac; ctaaagaccatcggcaagac tgttcgtgtactcggataga;

gaaaaggatgtccctgttgt; cgtttagcttctccttgcag; gatggtagtcctcgtcccag

taggagacccaatcgaatcc; gggaactgatcgacgaatct; catgaagaggatcacttcgc;

ccaaacgctaaccaggtgag ttcttctcctcgatgcgatc; gttgatcaccgcatagcaac;

taccaatggatgccattctg; ggattccatctcaatcgtgg; tgctctttgctttatcgctt;

tcaaggacatttaacagtgt; ggatgatttaggatctgcgc; cagtaacagtcgtctgtgtt

EdU incorporation

For EdU incorporation experiments, ovaries were dissected and incubated in Schneider's Medium supplemented with 15% FBS and containing 20uM of 5-ethynyl-2´-deoxyuridine (EdU) (Click-it Cell Proliferation Assays, Invitrogen) at room temperature for 2 hours. Ovaries were then fixed in 4% paraformaldehyde for 15 minutes, washed two times in 1x PBS, permeabilized in 1x PBST for 30 minutes, washed two times in 1x PBS, and incubated in the reaction cocktail (Click-it Cell Proliferation Assays,

Invitrogen) for 30 minutes at room temperature. Finally, tissues were rinsed in 1x PBS, incubated in 1x PBS + 1 μg/ml DAPI for 5 minutes and mounted on glass slides in Vectashield (Vector Labs).

Clone induction

Clones were generated by culturing flies of the appropriate genotype with fresh wet yeast for at least two days, to ensure maturity to adulthood and then heat shocking in culture vials without food in a 37°C water bath either once for 15 minutes, to achieve labeling of single escort cells or once for 30 minutes to achieve labeling of multiple escort cells. To generate twin-spot MARCM escort cell clones, progeny were heat shocked once for 1 hour at the onset of pupation, 5-6 days after egg laying. For developmental-time point clone induction, flies were left laying eggs in vials for 12-hour period. Careful consideration was taken to prevent overcrowding of larvae. They were all heat shocked at once starting at 108 hrs. -196 hrs. after egg laying, and dissected as they eclosed at 2 days post eclosion. ET-FLP lines were crossed to a respective 'flp-out', kept at 25C and transferred to wet yeast immediately after eclosion. They were dissected at 2 dpe and 7 dpe.

Results:

Tools for expression of transgenes in subpopulations of somatic cells in the germarium

The germarium contains multiple somatic cell types, including terminal filament cells, cap cells, escort cells, and follicle cells. To begin our investigation into the source of FSC niche signals, we collected lines that express Gal4 in each of these different cell types. Bab1-Gal4-5 is commonly used to express transgenes specifically in terminal filament cells and cap cells (Cabrera et al., 2002), which can be identified by their position at the anterior tip of the germarium and bright lamin C staining on the nuclear membrane. As expected, when we combined this driver with UAS::CD8-GFP, we observed strong GFP expression specifically in terminal filament cells and cap cells (Fig 1B).

109-30-Gal4 has been reported to be expressed in the FSC lineage (Hartman et al., 2010) and indeed, we observed consistent, high levels of expression in the FSCs and all follicle cells in the germarium (Fig 1D), though we also occasionally observed GFP expression in 1-4 posterior escort cells adjacent to the FSCs. Likewise, we confirmed that TJ-Gal4 is expressed in escort cells, FSCs, and follicle cells (Hayashi et al., 2002; Morris and Spradling, 2011) (Fig. 1E).

Lastly, we identified one line from the Janelia Gal4 collection (Pfeiffer et al., 2008), 13C06-Gal4, that expresses Gal4 throughout the anterior half of the germarium. We found that 13C06-Gal4 drives high levels of expression in posterior escort cells, which surround cysts in Region 2a; FSCs; and prefollicle cells near the Region 2a/2b border. In addition, we observed low levels of expression in the escort cells in Region 1, and occasionally in 1-2 cap cells (Fig 1C).

Escort cells are the predominant source of wingless for the FSC lineage

Wg signaling is required for FSC self-renewal and proliferation (Song and Xie, 2003), and perturbations in Wg signaling lead to severe follicle formation defects (Li et al., 2010; Song and Xie, 2003). For example, when Wg^{ts} flies are shifted to the non-permissive temperature, follicle cell production is reduced, and germ cell cysts entering into the follicle epithelium fail to bud from the germarium as distinct follicles (Song and Xie, 2003). These phenotypes are likely due to a defect in somatic cells because essential genes in the Wg pathway are not required in the germline (Song et al., 2002). To determine which population of cells is the source of Wg for the FSC lineage, we used the Gal4 drivers described above in combination with a temperature sensitive Gal80 driven by a constitutive *tubulin* promoter (tub-$Gal80^{ts}$) to knockdown Wg expression by RNAi specifically during adulthood in distinct subsets of somatic cells in the germarium.

First, we examined Wg protein levels by immunofluorescence in each genotype. Consistent with previous studies (Forbes et al., 1996b; Song and Xie, 2003), we found that Wg protein was detectable in the terminal filament cells and cap cells in wild type germaria (Fig 2A). Likewise, Wg was detectable in the terminal filament cells and cap cells when Wg^{RNAi} was driven in escort cells or follicle cells (Fig 2C-D). In contrast, Wg was substantially reduced in the terminal filament cells and cap cells of most germaria when Wg^{RNAi} was driven in apical cells (Fig 2B). Next, we looked for follicle formation defects in each genotype. Surprisingly, we did not observe a significant number of follicle formation defects when Wg^{RNAi} was driven in apical cells (Fig. 3A, D). Indeed, we found that only 7.4% of ovarioles had follicle formation defects at 7 days after temperature shift (DATS) compared to 11.3% at 7 DATS in the control, and that this rose

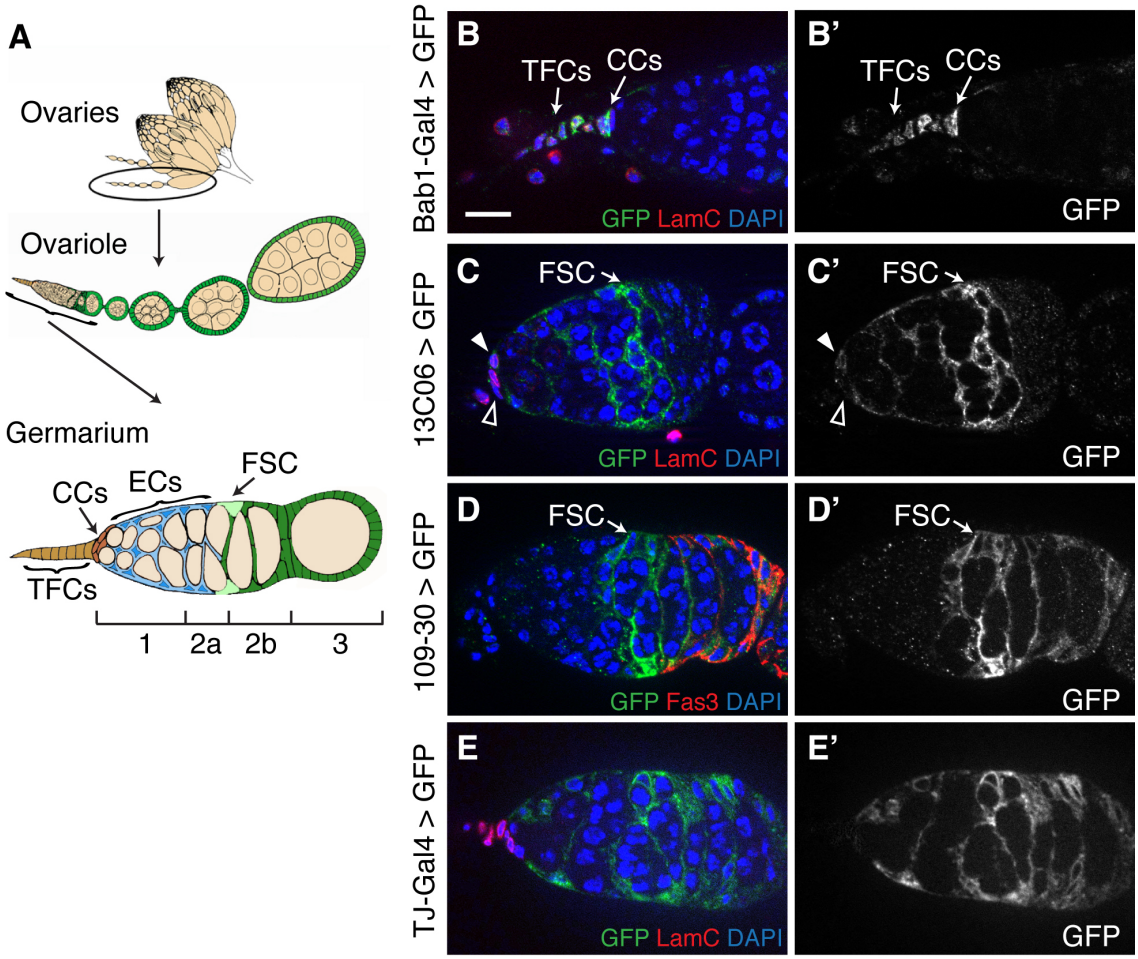


Figure 1. The germarium contains multiple types of somatic cells
A. A diagram of the Drosophila ovary. Each ovary is composed of multiple subunits, called ovarioles and each ovariole has a structure at the anterior tip, called the germarium. Each germarium has terminal filament cells (TFCs, orange) and cap cells (CCs, red) at the anterior tip, escort cells (ECs, blue) surrounding germ cell cysts in the anterior half, and two follicle stem cells (FSCs, light green) that produce follicle cells (dark green) that surround germ cell cysts in the posterior half. The germarium is divided into regions (1, 2a, 2b, and 3) that are defined by the stages of germ cell development. FSCs are always found at the Region 2a/2b border. B-E. UAS::CD8-GFP; Bab1-Gal4 (B), UAS::CD8-GFP, 13C06 (C), 109-30; UAS::CD8-GFP (D), or UAS::CD8-GFP; TJ-Gal4 (E), stained for GFP (green) to identify cells that express Gal4, LamC (B, C and E, red) to identify terminal filament cells and cap cells or Fas3 (D, red) to identify follicle cells, and DAPI (blue). B'-E' shows GFP channel only. Bab1-Gal4 is expressed strongly in terminal filament and cap cells. 13C06 is expressed strongly in posterior escort cells, FSCs, and some early follicle cells, weakly in anterior escort cells, and occasionally in 1-2 cap cells (solid triangle). Open triangle shows cap cells that do not express Gal4 at detectable levels. 109-30 is expressed strongly in FSCs and all follicle cells in the germarium. TJ-Gal4 is expressed in escort cells, FSCs, and follicle cells. Anterior is to the left. Scale bar represents 5 μm.

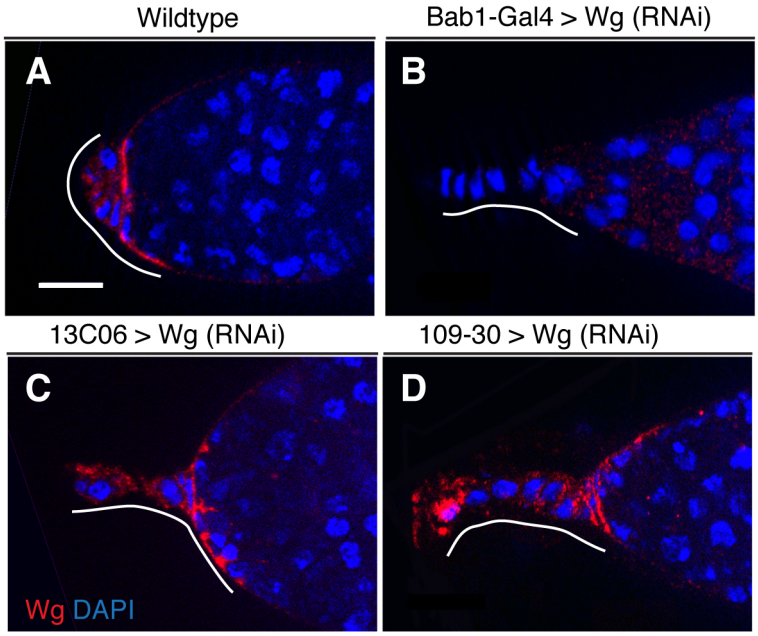


Figure 2. Wg^{RNAi} eliminates the Wg signal from terminal filament and cap cells
A-D. Germaria from wild type flies (A) or flies in which Wg^{RNAi} is driven by Bab1-Gal4 (B), 13C06-Gal4 (C), or 109-30-Gal4 (D), at 7 days after flies were shifted to 29°C to repress tub-$Gal80^{ts}$ and promote Gal4 activity. Tissue is stained for Wg (red) and DAPI (blue). White lines trace the position of the terminal filament cells and cap cells. Note that Wg protein is detectable in the cap and terminal filament cells when Wg^{RNAi} is driven by 13C06-Gal4 or 109-30-Gal4 but not when Wg^{RNAi} is driven by Bab1-Gal4. Anterior is to the left. Scale bar represents 5 um.

to only 15.6% by 21 DATS (vs. 9.3% in the control) (Fig. 3G). Likewise, we did not see a statistically significant phenotype when Wg^{RNAi} was driven by 109-30. Just 2.9% of the germaria had follicle formation defects at 7 DATS, and this increased to only 9.1% by 21 DATS (Fig. 3C, F, G). To determine whether oogenesis was proceeding in these ovarioles, we quantified the rate of follicle cell cycle progression using an EdU incorporation assay. We found that the frequency of EdU^{+} follicle cells in the germarium was not significantly different than the control when Wg^{RNAi} was driven in either apical cells or follicle cells (6.4% in the control versus 5.6% and 5.1% in flies with Bab1-Gal4 or 109-30 respectively, Fig. 3J). Collectively, these observations indicate that terminal filament cells, cap cells, and follicle cells are not significant sources of Wg for the FSC lineage. In contrast, we observed a dramatic phenotype when Wg^{RNAi} was driven in escort cells.

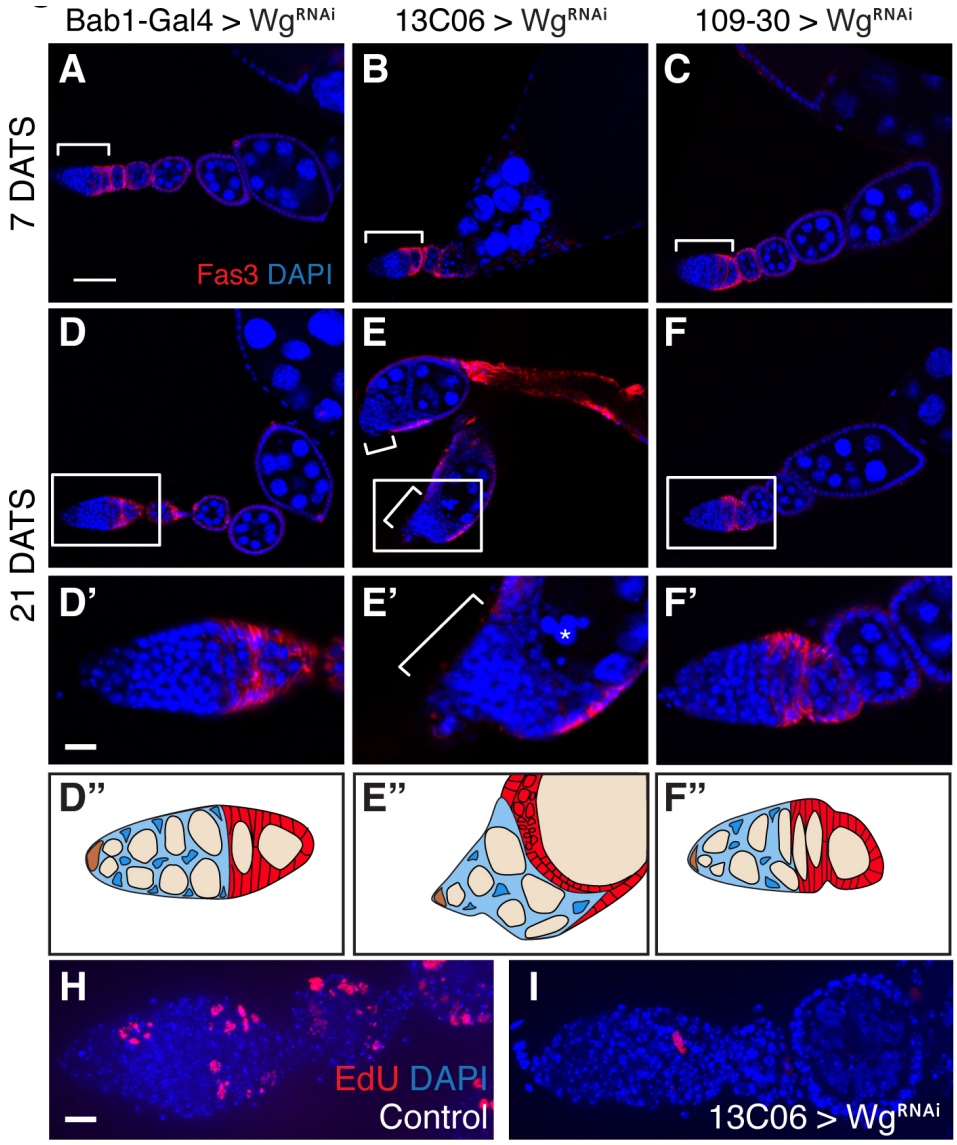


Figure 3. Wg produced by escort cells is required for follicle formation
A-F. Ovarioles in which Wg^{RNAi} is expressed in apical cells by Bab1-Gal4 (A, D), escort cells by 13C06 (B, E), or follicle cells by 109-30 (C, F), at 7 (A-C) or 21 (D-F) days after flies were shifted to 29°C to repress tub-$Gal80^{ts}$ and promote Gal4 activity. Tissue is stained for Fas3 (red) and DAPI (blue). White brackets indicate the extent of the germarium. Boxed regions in D-F are expanded in D'-F' and schematized in D"-F" (in diagrams, cap cells are orange, germ cells are tan, escort cells are blue, and the FSC lineage is red). Asterisk in E' indicates an apoptotic germ cell. H-I. Ovarioles from 13C06, tub-$Gal80^{ts}$; TM2/+ (control, H) or 13C06, tub-$Gal80^{ts}$; Wg^{RNAi}/+ (I) that were incubated with EdU for 2 hours, fixed, and stained for EdU (red), and DAPI (blue).

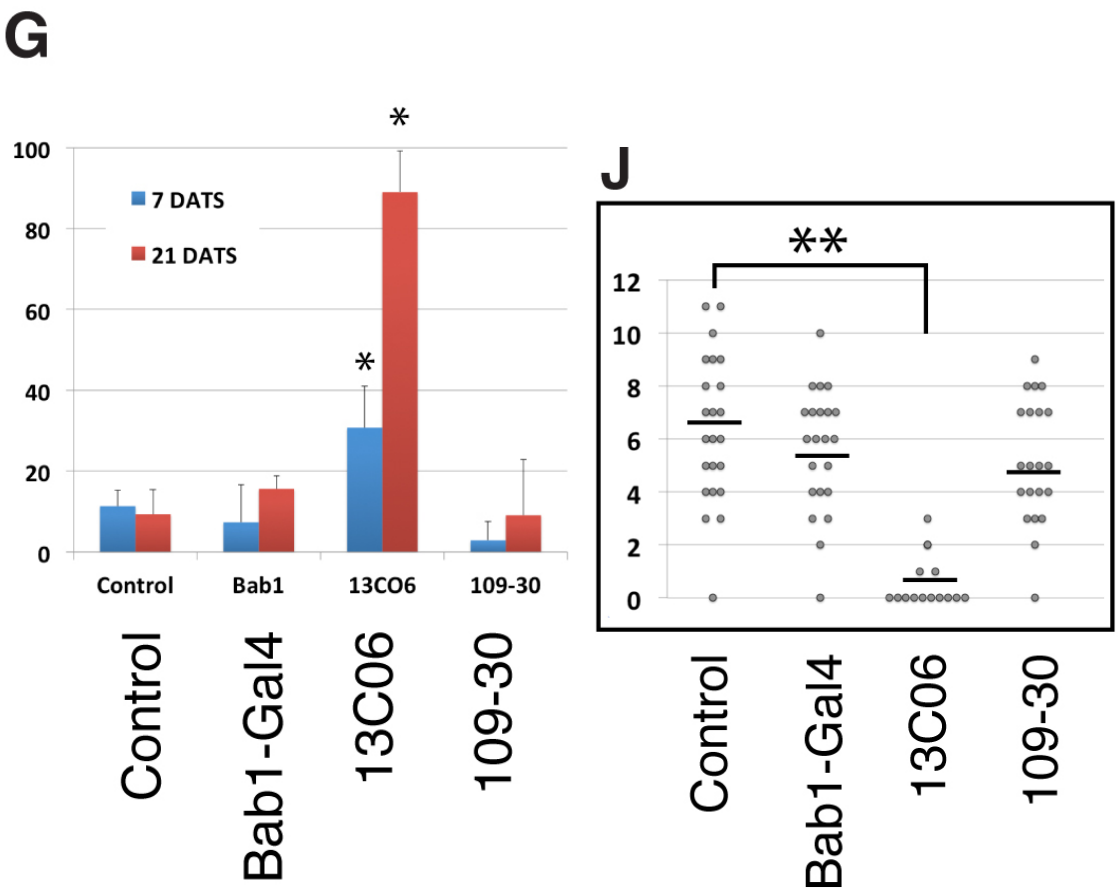


Figure 3. Wg produced by escort cells is required for follicle formation (continuation)
G. Quantification of follicle formation defects in ovarioles with each genotype. Ovarioles from 13C06, tub-Gal80^{ts}; TM2/+ flies were used as the control. Each data point is the mean of at least three replicates. Error bars represent the s.e.m.. Asterisk indicates a $p < 0.01$ compared to control. Total N values are greater than 200 ovarioles for each data point. J. Quantification of the number of EdU^{+} follicle cells in ovarioles with each genotype. Asterisks indicates a $p < 0.001$. Anterior is

By 7 DATS, 30.8% of the germaria had severe cyst formation defects (Fig. 3B, G). By 21 DATS, nearly all ovarioles (89.0%) had severe follicle formation defects (Fig. 3E, G). In these ovarioles, Regions 1 and 2a were still intact in most germaria, but Regions 2b and 3 were no longer identifiable, and these ovarioles lacked the typical chain of developing follicles downstream from the germarium. Instead, a few mid- and late-stage follicles were clustered

together and surrounded by a disorganized follicle epithelium (Fig. 3E). In addition, nurse cells frequently had fragmented nuclei (Fig. 3E', asterisk), suggesting that they were undergoing apoptosis. Moreover, we observed a significant reduction in the frequency of EdU^{+} follicle cells at 21 DATS (0.6% vs. 6.4% in the control, Fig. 3H-J).

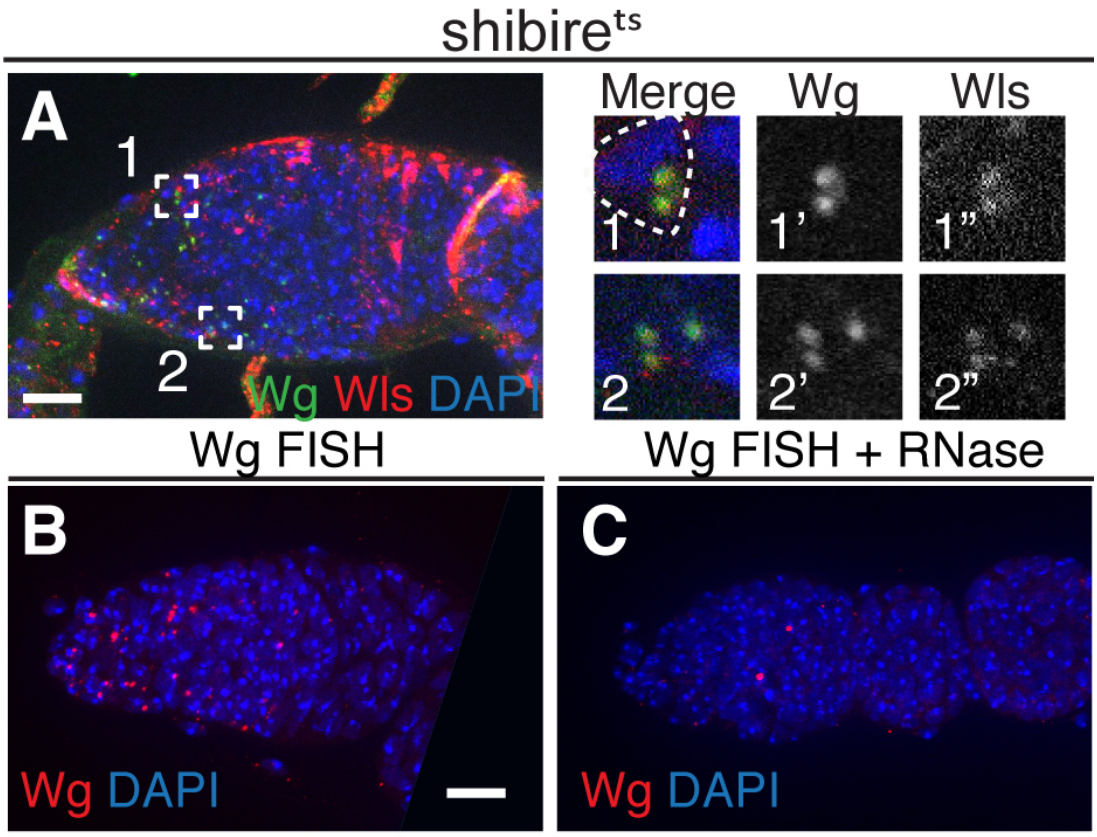


Figure 4. Wg is expressed in escort cells and Wg signaling is active specifically in FSCs.
A. A germarium from a *shibire^{ts}* fly that was shifted to the non-permissive temperature for 2 hrs. prior to dissection stained for Wg (green), Wntless (red) and DAPI (blue). Wg^{+} puncta are visible throughout the region containing escort cells and many co-localize with Wntless. Boxed regions 1 and 2 are expanded in panels to the right and shown as a merged image (1 and 2), Wg channel only (1' and 2') and Wntless channel only (1" and 2"). Dashed line in Box 1 shows a nucleus with a characteristic escort cell shape and position. B-C. Wild type germaria stained with a FISH probe for Wg transcript (red) and DAPI (blue). Pretreatment of tissue with RNase (C) eliminates the signal, demonstrating that the FISH probe is specific for an RNA target.

These phenotypes suggest that Wg is expressed in escort cells, but Wg expression has not been detected in escort cells by immunofluorescence. However, escort cells have a very large surface area, and Wg protein may not normally accumulate in sufficient quantity to be detectable. Therefore, we investigated Wg levels in germaria from *shibire^{ts}* flies, which are defective for Wg secretion at 29°C (Strigini and Cohen, 2000). We found that, by 2 hours after shifting to 29°C, Wg was visible as bright foci in both terminal filament cells and cap cells, and also throughout Regions 1 and 2a, often near nuclei with a characteristic escort cell shape and position (Fig. 4A). Many of these foci co-stained with an antibody against Wntless, which is known to be co-trafficked with Wg in vesicles (Fig. 4A) (Tang et al., 2012). This indicates that Wg protein is indeed present in escort cells.

As a complementary approach, we assayed for Wg transcript using a highly sensitive single-molecule fluorescence in situ hybridization (FISH) (Batish et al., 2011). We observed puncta throughout the anterior half of the germarium, and the signal was substantially diminished by pretreatment of the fixed tissue with RNAse (Fig. 4B, C). Collectively, these data indicate that escort cells are the relevant source of Wg for the FSC lineage.

Wingless is received specifically by FSCs and not follicle cells.

To determine which cells within the germarium have active Wg signaling, we assayed for LacZ expression in flies with a sensitive Wg activity reporter, Notum-LacZ (Liu et al., 2008). We noted consistent LacZ expression in terminal filament cells, cap cells, and outer muscle sheath cells, and sporadic low levels of LacZ expression in escort cells. Notably, germ cells were never LacZ^{+}, consistent with the observation that Wg signaling is not required in the germline. Lastly, we found that Notum-LacZ is highly expressed in 1-3 cells at the Region 2a/2b border. The shape and position of these cells

suggested that they were FSCs and recently produced prefollicle cells. Therefore, we used lineage analysis to investigate this possibility.

FSCs can be reliably identified as the anterior-most labeled cell on the side of the germarium in a mature FSC clone (Nystul and Spradling, 2007). Nearby escort cells sometimes resemble FSCs, so if a labeled escort cell is adjacent to a labeled FSC, it could appear to be part of the FSC clone. However, escort cells rarely divide in adult ovaries (Kirilly et al., 2011; Morris and Spradling, 2011) and are thus unlikely to be labeled by a lineage tracing system that requires mitosis to activate expression of the lineage marker. Thus, to determine whether Wg signaling is active specifically in FSCs, we first combined the Notum-LacZ reporter with a MARCM lineage tracing system (Lee and Luo, 2001), which labels the lineage of mitotically active cells with GFP. Then, we generated clones in adult flies, and assayed for LacZ and GFP expression. We found that in 65% (n = 87/134) of mature GFP^+ FSC clones, the FSC at the base of the clone was $LacZ^+$ (Fig. 4D, E) whereas in the remaining 35% of GFP^+ FSC clones, all GFP^+ cells, including the FSC, were $LacZ^-$. In addition, we found that, in most (91.7%) of the clones with a $LacZ^+$ FSC, the FSC was the only $LacZ^+$ cell in the clone.

The remaining (8.3%) clones contained just one additional $LacZ^+$ cell, which was always near the FSC (Fig. 5A). Given their proximity to the FSC niche, these cells are likely to be recently produced prefollicle cells in which the LacZ protein perdures. As expected, FSCs that were not part of a GFP^+ clone could also be $LacZ^+$. Specifically, we observed a GFP^-, $LacZ^+$ cell that was likely to be an FSC based on its shape and position in 42.0% of the germaria (Fig. 5B). Interestingly, we also noticed that a subset of $Delta^+$ intestinal stem cells (Ohlstein and Spradling, 2007), but not the surrounding enterocytes or enteroendocrine cells, were also $LacZ^+$ (Fig. 5C, D).

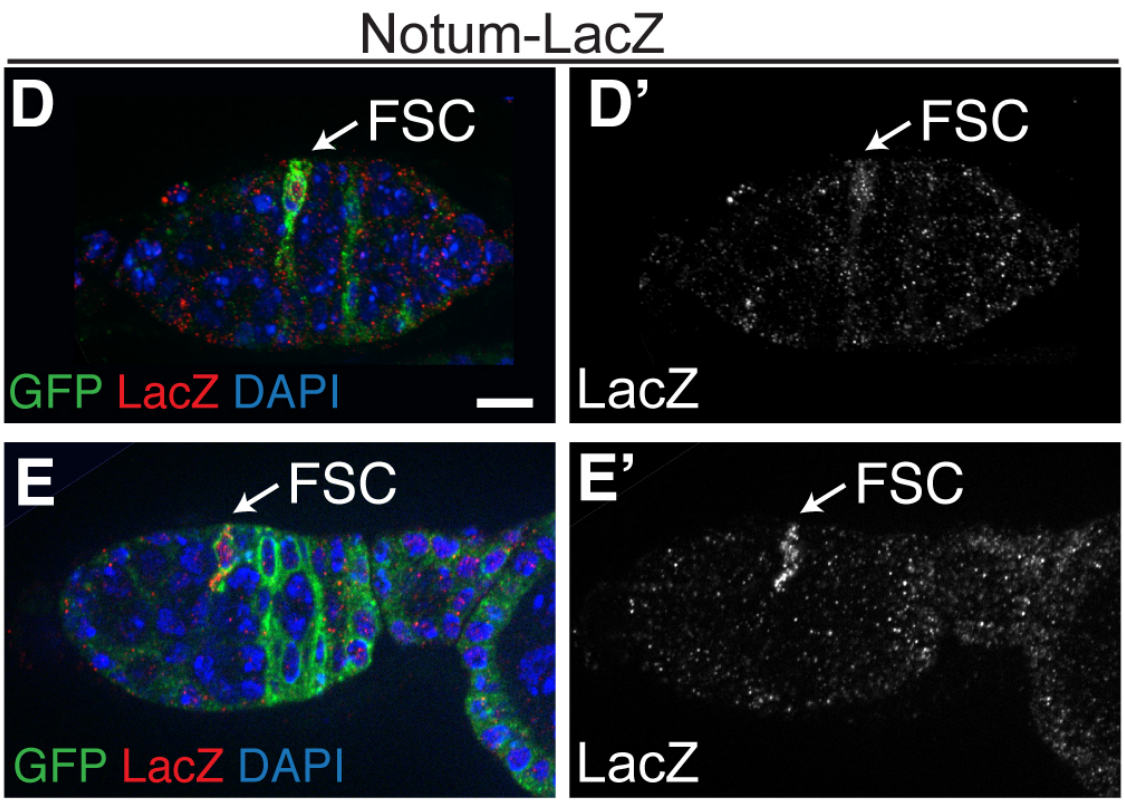
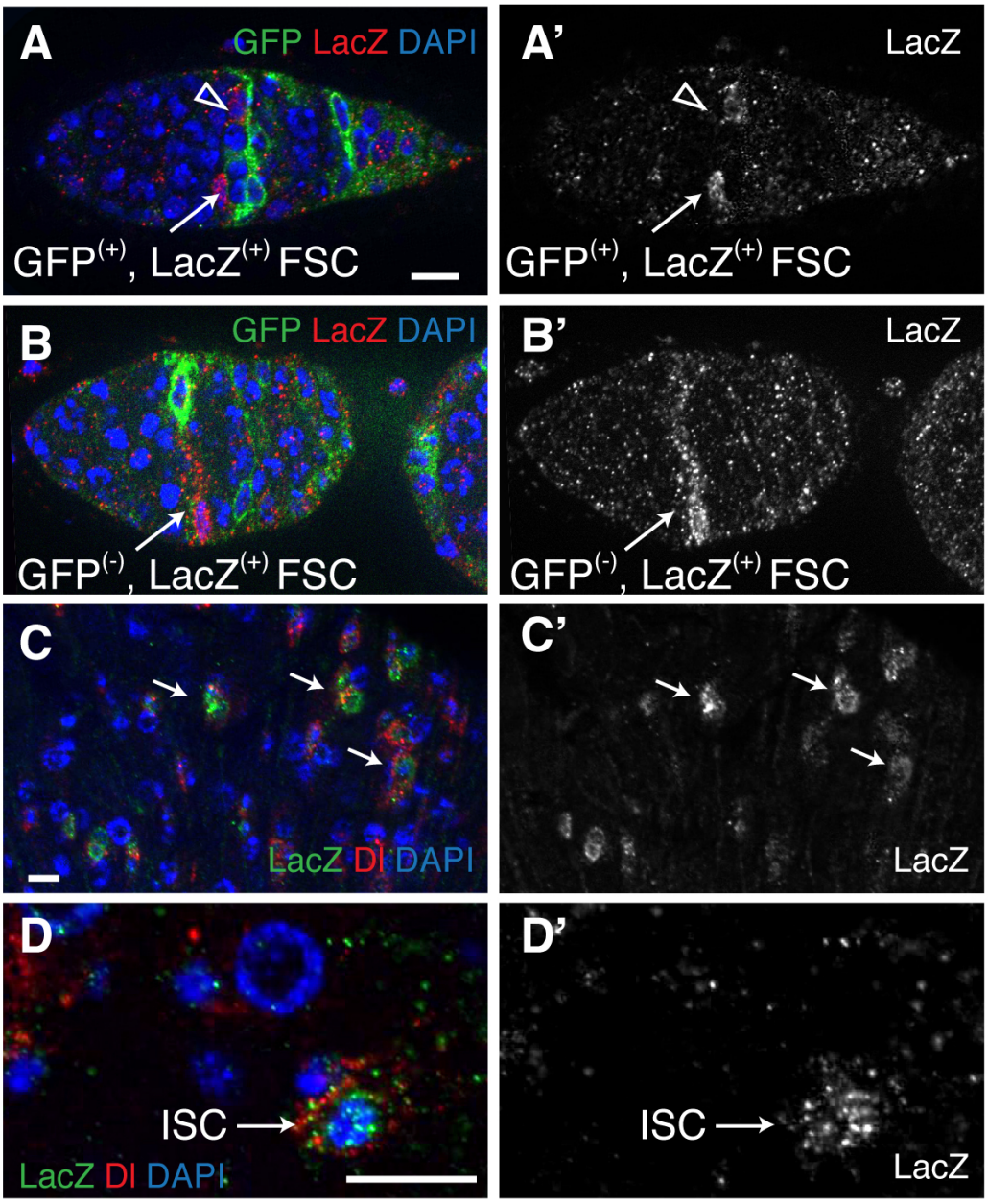


Figure 4 (continuation). Wg is expressed in escort cells and Wg signaling is active specifically in FSCs.
D-E. The Wg pathway activity reporter, Notum-LacZ, is expressed in the anterior-most labeled cell of a GFP+ FSC clone. Tissue is stained for GFP (green), LacZ (red), and DAPI (blue). D'-E' shows the LacZ channel only. Anterior is to the left. Scale bar represents 5 μm.

Figure 5. Notum-lacZ labels FSCs and ISCs
A-B. Germaria containing the Wg pathway activity reporter, Notum-lacZ, and a GFP+ FSC clone. In A, both the FSC (labeled) and an early FSC daughter (open triangle) are lacZ+. Because the FSC daughter is GFP+ and positioned near the FSC, we presume this is a recently produced daughter cell that has not yet fully downregulated or turned over the lacZ protein. In B, a lacZ+ FSC is GFP- because it is not part of a GFP+ clone. C-D. Sections of the posterior midgut containing the Notum-lacZ reporter stained with LacZ (green), Dl (red) to mark ISCs, and DAPI (blue). Dl+ cells are also lacZ+, indicating that Notum-lacZ is expressed in ISCs. The lacZ channels in A-D are shown in A'-D'. Anterior is to the left. Scale bar in panel A represents 5 μm and scale bars in B and C represent 10 μm.



Next, to determine whether Wg pathway activity is sufficient to activate Notum-LacZ expression in the FSC lineage, we assayed for Notum-LacZ expression in *Apc1*$^{-/-}$, *Apc2*$^{-/-}$ FSC clones. *Apc1* and *Apc2* are essential components of the β-catenin destruction complex, so the Wg pathway should be constitutively active in these cells. Indeed, we found that some FSC daughter cells within these clones ectopically expressed Notum-LacZ (Fig. 6D). This indicates that ectopic Wg pathway activation is sufficient to induce

activation of the Notum-LacZ reporter in at least a subset of FSC daughter cells within the germarium.

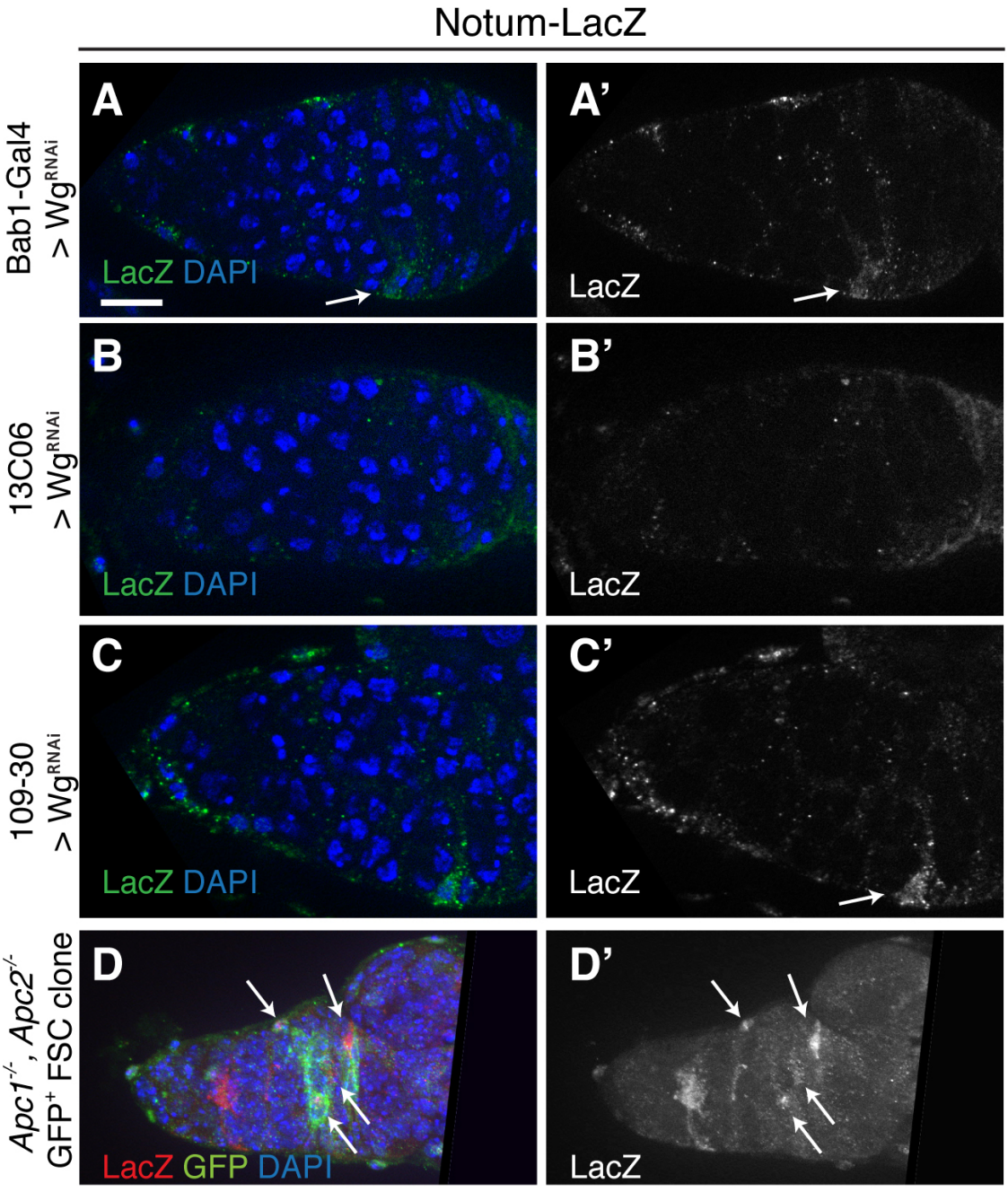


Figure 6. Wg produced by escort cells activates Notum-lacZ in FSCs
A-C. Ovarioles from flies 7 days after temperature shift that contain the Notum-lacZ reporter, UAS-WgRNAi, tub-Gal80ts, and either Bab1-Gal4 (A), 13C06-Gal4 (B), or 109-30-Gal4 (C). Tissue is stained for lacZ (green) and DAPI (blue). LacZ+ FSCs were present when WgRNAi is driven by Bab1-Gal4 or 109-30-Gal4 (white arrows), but not when WgRNAi is driven by 13C06-Gal4. Anterior is to the left. Scale bar represents 5 _m. D. A maximum image projection of a germarium with a mature GFP+ Apc1-/-, Apc2-/- FSC clone. Many mutant FSC daughter cells (white arrows) express Notum-lacZ. The tissue is stained for lacZ (red), GFP (green), and DAPI (blue).

Next, to determine which cells produce the Wg ligand that activates Notum-LacZ in FSCs, we combined the Notum-LacZ reporter with UAS-Wg^{RNAi} and either Bab1-Gal4, 109-30, or 13C06. We found that there was little or no effect on the frequency of $LacZ^+$ cells at the Region 2a/2b border when Wg^{RNAi} was driven in cap and terminal filament cells or in follicle cells (Table 1, Fig. 6A, C). In contrast, we observed a substantial reduction in the frequency of germaria with $LacZ^+$ cells at the Region 2a/2b border compared to the control population (35% vs. 75%, Table 1, Fig. 6B) when Wg^{RNAi} was driven in escort cells. Furthermore, we observed a significant correlation between the lack of Notum-$LacZ^+$ cells at the Region 2a/2b border and the presence of follicle formation defects when Wg^{RNAi} was driven in escort cells (Table 1). These observations indicate that, within the FSC lineage, Wg signal transduction is active specifically in FSCs, and therefore the defects we observe when Wg is removed from escort cells are due to a defect specifically in the FSCs.

Finally, to confirm the specificity of the phenotypes observed to the Wg pathway, we knockdown additional components of the pathway in the FSC-niche region. Wls is a conserved membrane protein that has been shown to be necessary for the secretion of Wg (Banziger et al., 2006). For example, Wg secretion is inhibited in Wls mutant cells, and replicates Wg mutant effects (Banziger et al., 2006). To determine if we see a similar phenotype to Wg^{RNAi}, we expressed Wls^{RNAi} in escort cells (Fig. 7A, A'). At 7 DATS, 63.7% of ovarioles had a follicle formation defect, compared to 30.8% for Wg^{RNAi} and

11.3% for controls (Fig. C). We next knockdown Arrow (Arr), another component of the Wg transduction pathway. Arr has been shown to be a necessary co-receptor, acting with Frizzled in Wg receiving cells (Wehrli et al., 2000). When we express Arr^{RNAi} in the FSCs using 13C06, at 21 DATS nearly all ovarioles (92.4%) had severe follicle formation defects (Fig. 7B, B'). These phenotypes replicated those of Wg^{RNAi} knockdown in escort cells (Fig. 3E, G). This data shows that other components of the Wg pathway are necessary for proper follicle formation. Wls seems to be important, most likely due to its effects on the secretion of Wg and other Wnt ligands that might also be expressed in escort cells. Very interestingly, we see a very similar effect when we knockdown Arrow in the FSCs, further supporting our work and previous work (Song and Xie, 2002), that has shown that Wg pathway is only required in the FSCs. Together, this data strongly argues that the phenotypes we see are due specifically to the inhibition of the Wg pathway

Table 1.

	1 or 2 $LacZ^+$ FSCs		0 $LacZ^+$ FSCs			
	Normal	Follicle Defect	Normal	Follicle Defect	N	Fisher Exact P-value
Wildtype	71.9%	3.1%	25.0%	0.0%	32	1
Bab1-Gal4 > Wg^{RNAi}	63.4%	7.3%	29.3%	0.0%	41	0.543
13C06 > Wg^{RNAi}	30.2%	4.7%	23.3%	41.9%	43	0.002
109-30 > Wg^{RNAi}	79.5%	0.0%	17.9%	2.6%	39	0.205

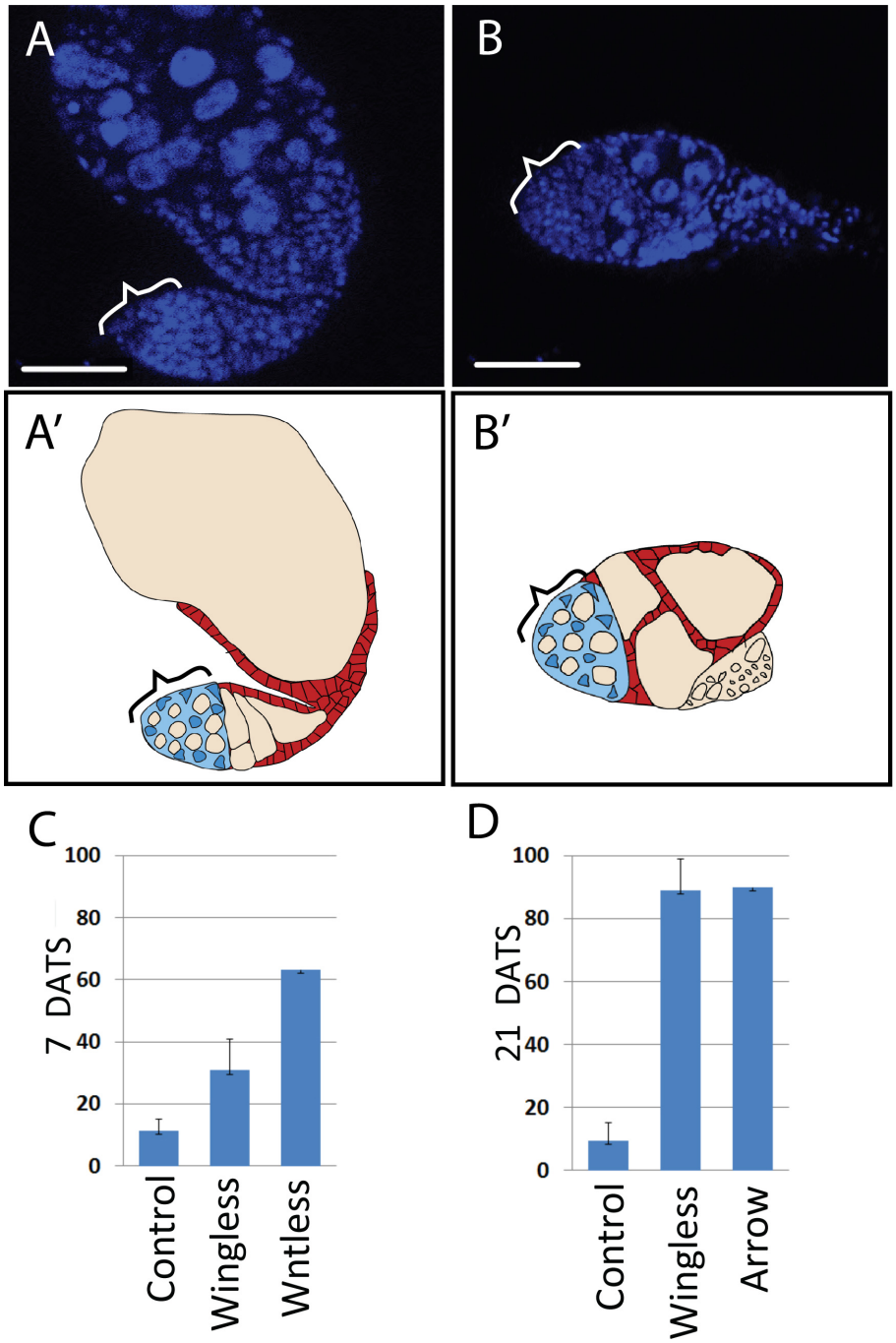


Figure 7. Wntless and Arrow produced by escort cells and prefollicle cells are required for follicle formation.

A-D. Ovarioles in which Wls^{RNAi} (A, C) or Arr^{RNAi} is expressed in the escort cell by 13C06 at 7 (A, C) or 21 (B, D) days after flies were shifted to 29°C to repress tub-$Gal80^{ts}$ and promote Gal4 activity. Tissue is stained for DAPI (blue). White brackets indicate the extent of the germarium. Images A and B are schematized in A' and D' (in diagrams, cap cells are orange, germ cells are tan, escort cells are blue, and the FSC lineage is red). C-D. Quantification of phenotype for Wntless (C) and Arrow (D), compared to Wingless. Wls^{RNAi} n > 30. Arr^{RNAi} n > 80.

Hedgehog is produced by multiple cell types

Hh signaling is required for both FSC self renewal (Vied and Kalderon, 2009; Zhang and Kalderon, 2001) as well as proliferation and differentiation of prefollicle cells. Consistent with previous studies (Forbes et al., 1996a; Zhao et al., 2008), we found that an enhancer trap located in the Hh locus (Hh-lacZ) is expressed at high levels in terminal filament cells, cap cells, and anterior escort cells, and at lower levels in posterior escort cells (Fig. 8A), but Hh protein is only clearly detectable in terminal filament cells and cap cells by immunofluorescence in wild type germaria (Fig. 8B) (Forbes et al., 1996a; Hartman et al., 2010). However, as with Wg, Hh protein may be sparse in escort cells and therefore difficult to detect. Therefore, we investigated Hh levels in germaria in which Hh protein trafficking in escort cells was blocked by the ectopic expression of $Rab5^{DN}$ (Callejo et al., 2011). We found that, by 2 days after the temperature shift to activate $Rab5^{DN}$ expression, Hh protein was clearly detectable in escort cells as well as cap cells and terminal filament cells (Fig. 8C). Importantly, this signal was substantially reduced by co-expression of Hh^{RNAi} with $Rab5^{DN}$ (Fig. 8D), which confirms that the staining was specific for Hh. Lastly, we assayed for the presence of Hh transcript in escort cells and prefollicle cells by FISH, as described above. Again, we observed bright puncta throughout the anterior half of the germarium (Fig. 8E) and found that this signal was eliminated by pretreatment of the tissue with RNase (Fig. 8F). Together, these data are consistent with the pattern of Hh-lacZ expression in the germarium and indicate that Hh is expressed in terminal filament cells, cap cells, and escort cells.

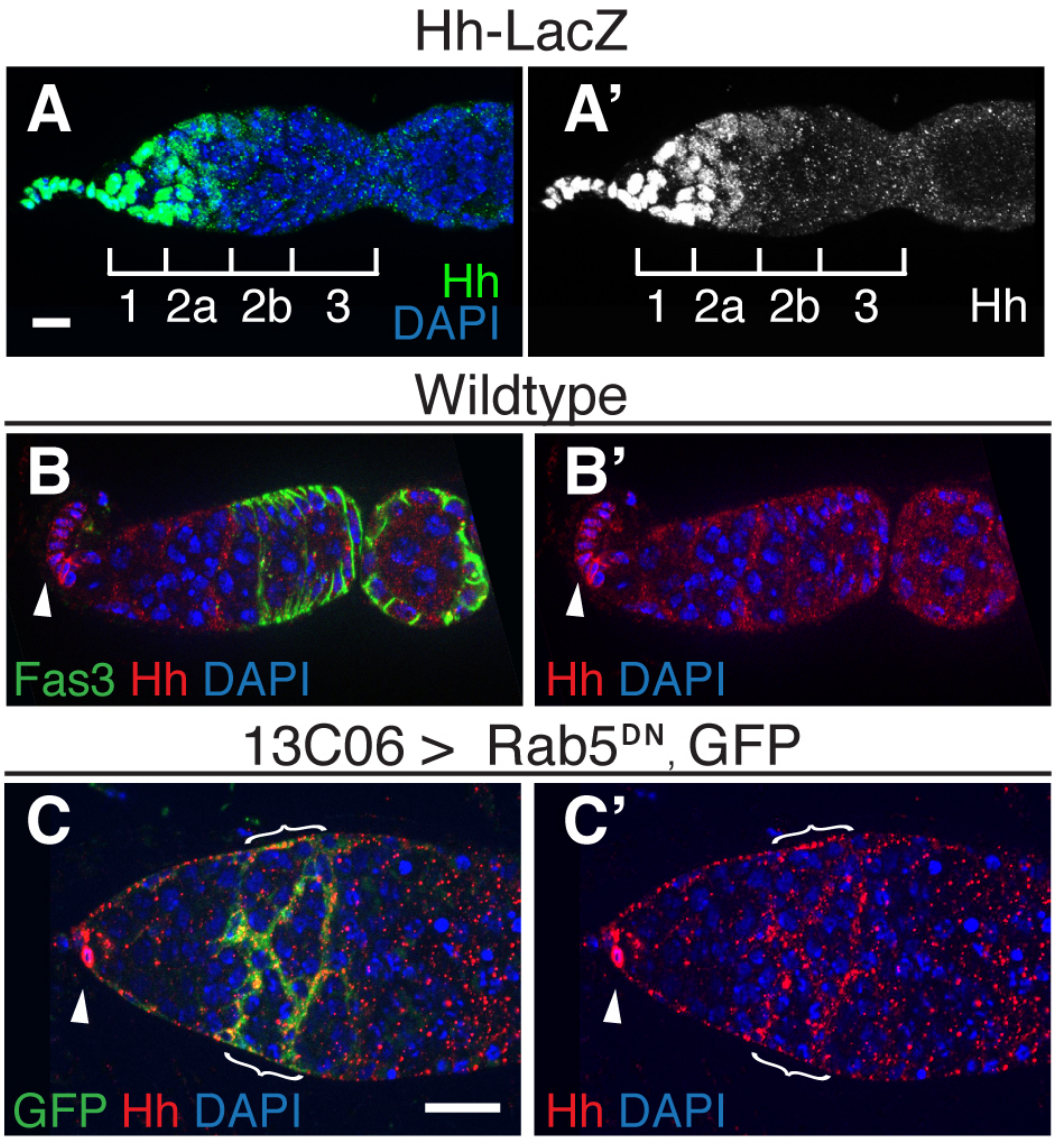


Figure 8. Hh is expressed in escort cells
A. Hh-LacZ expression in the germarium. Tissue is stained with LacZ (green) and DAPI (Blue). Regions 1, 2a, 2b and 3 are indicated. B. Wild type germarium stained for Fas3 (green) to highlight follicle cell membranes, Hh (red), and DAPI (blue). Hh signal Wild typed in cap cells (arrowheads) C, D. Germaria in which GFP and $Rab5^{DN}$ are driven in escort cells by 13C06 2 days after flies were shifted to 29°C to repress tub-$Gal80^{ts}$ and promote Gal4 activity. Tissue is stained for GFP (green) to visualize the extent of Gal4 expression, Hh (red) and DAPI (blue). (C) Hh puncta are abundant on escort cell membranes in region 2a, where Gal4 expression is high (white brackets), and sparse in region 1, where Gal4 is expression is lower. In addition, as in wild type germaria, Hh signal is bright in cap cells (arrowheads). Co-expression of Hh^{RNAi} with $Rab5^{DN}$

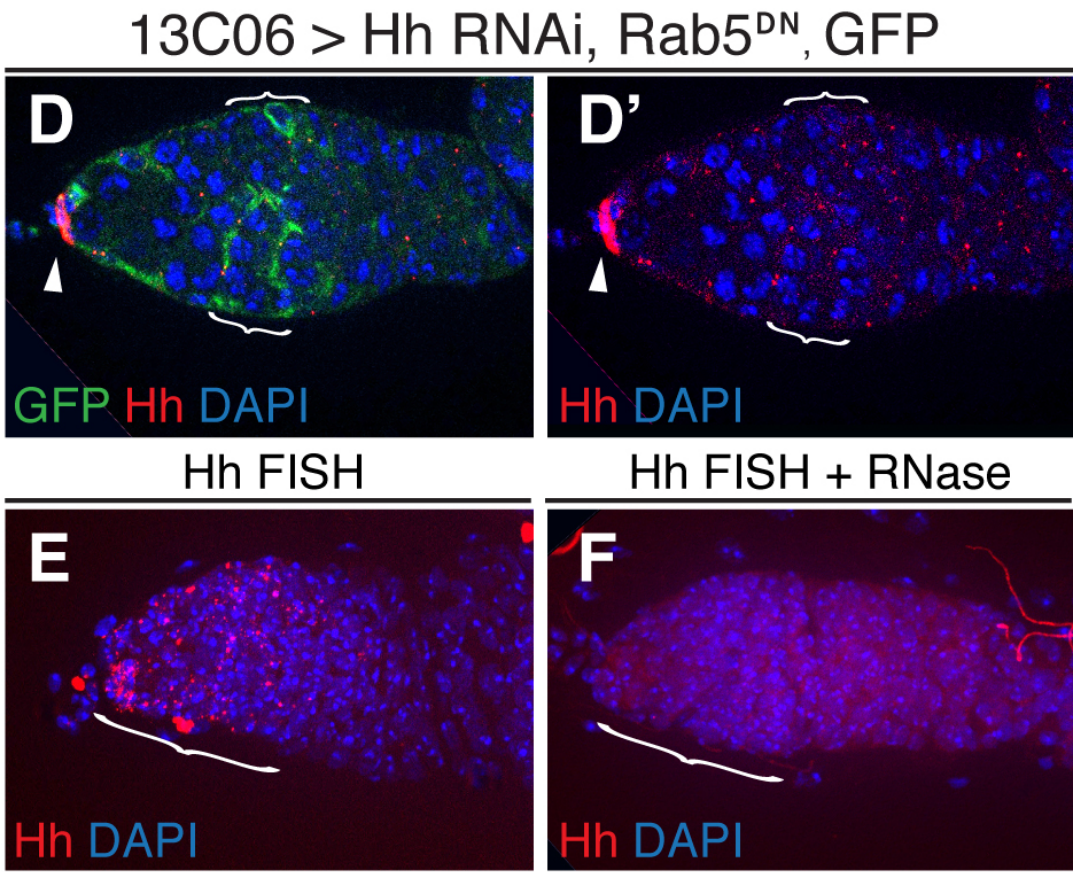


Figure 8. Hh is expressed in escort cells (continuation)
(D), significantly decreases Hh staining in escort cells (white brackets) but not cap cells (arrowhead). E, F. Wild type germaria stained with a FISH probe for Hh transcript (red) and DAPI (blue). Pretreatment of tissue with RNase (F) eliminates the signal, demonstrating that the FISH probe is specific for an RNA target. Anterior is to the left. Scale bar represents 5 μm.

Multiple cell types respond to Hedgehog signaling in the germarium

To determine which cells are the source of Hh ligand that acts on the early FSC lineage, we combined UAS-Hh^{RNAi} with tub-Gal80^{ts} and either Bab1-Gal4, 13C06, or 109-30 and assayed for follicle formation defects in adult ovaries at 7 and 21 DATS as described above. At 21 DATS, we found a significantly higher frequency of germaria with follicle formation defects compared to the control population when UAS-Hh^{RNAi} was expressed in apical cells or escort cells (32.1% and 53.3% for flies with Bab1-Gal4 or 13C06, respectively versus 12.4% for control) (Fig. 9A-F). Specifically, we observed

ovarioles with disorganized and discontinuous follicle epithelia (Fig. 9B, C), fused cysts (Fig. 9D, arrow), and defective follicle budding from the germarium (Fig. 9F, arrow). Next, we investigated the frequency of follicle formation defects when Hh^{RNAi} was driven more broadly throughout the germarium by either TJ-Gal4 alone or by TJ-Gal4 and Bab1-Gal4 in combination and found that this produced an even higher frequency of follicle formation defects (48.2% and 56.2%, respectively) (Fig. 9G-K).

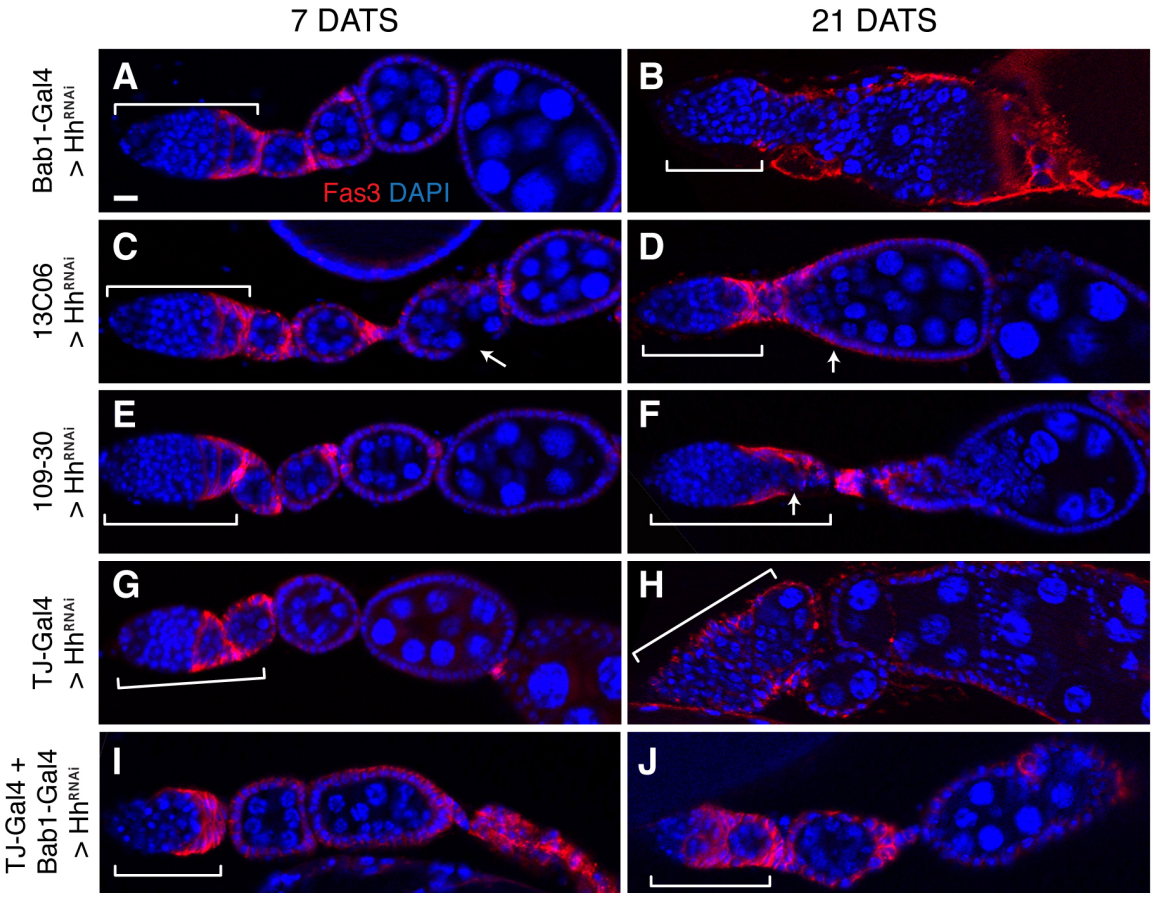
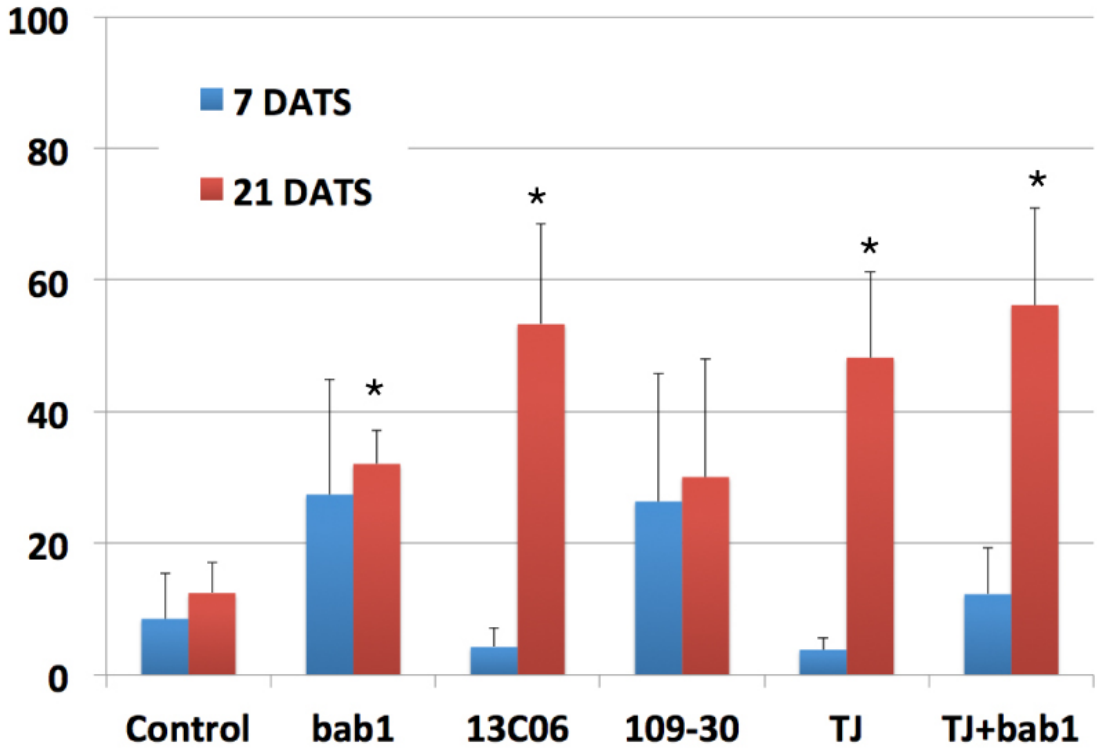


Figure 9. Hh produced by multiple sources is required for follicle formation
Ovarioles in which Hh^{RNAi} is driven by Bab1-Gal4 (A, B), 13C06 (C, D), 109-30 (E, F), TJ-Gal4 (G, H), or both Bab1-Gal4 and TJ-Gal4 (I, J) at 7 (A, C, E, G, I) or 21 (B, D, F, H, J) days after flies were shifted to 29°C to repress tub-$Gal80^{ts}$ and promote Gal4 activity. Tissue is stained for Fas3 (red) and DAPI (blue). White brackets indicate the extent of the germarium. K. Quantification of follicle formation defects in ovarioles with each genotype. Each data point is the mean of at least three replicates. Asterisks indicate $p < 0.05$ compared to the control and the error bars represent the s.e.m.. Total N values are greater than 200 ovarioles for each data point. Anterior is to the left. Scale bar represents 5 μm.



To determine which cells have active Hh signaling in the germarium, we used a sensitive reporter of Hh pathway activity, Ptc-Pelican-GFP(nls). This construct contains multiple *cubitus interruptus* binding sites upstream of GFP and activates expression of a nuclear-localized GFP specifically in cells with active Hh signaling (T. Kornberg, personal communication). To verify the fidelity of this reporter, we examined GFP expression in wing discs. As expected (Phillips et al., 1990), we observed bright stripe of GFP expression along the A/P boundary, with tapered expression toward the anterior (Fig. 9). In germaria, we found that Ptc-Pelican-GFP(nls) expression closely resembled Ptc-LacZ expression, but was brighter and more consistent. Specifically, we observed that escort cells, FSCs, and all follicle cells were GFP^{+}, and that the level of GFP expression tapered off in an anterior-to-posterior gradient (Fig. 10A).

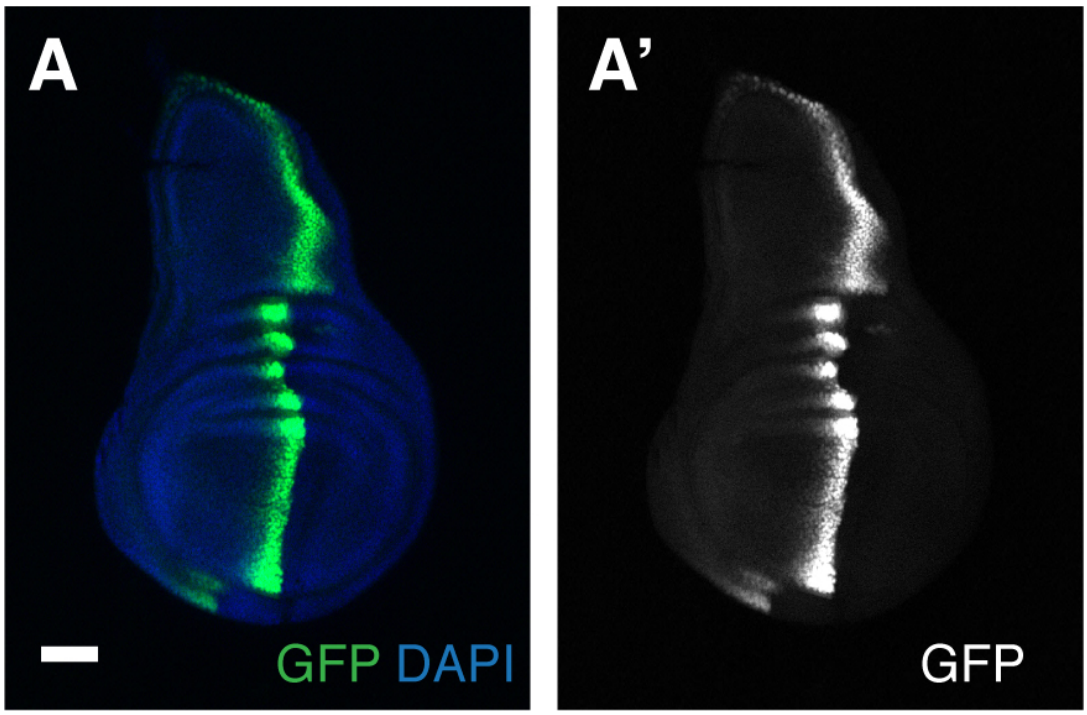


Figure 10. Ptc-pelican-GFP(nls) is a reporter for Hh activity in the wing disc
A. A wing disc that contains Ptc-pelican-GFP(nls). As expected for a reporter of Hh signaling activity, GFP expression is strong along the A/P boundary and tapers of toward the anterior. Anterior is to the left. Scale bar represents 50 μm.

Next, we examined Ptc-Pelican-GFP(nls) expression in germaria in which Hh^{RNAi} expression is controlled by tub-$Gal80^{ts}$ and each of the four Gal4 drivers alone as well as by TJ-Gal4 and Bab1-Gal4 combination at 7 DATS. We found that GFP expression was substantially decreased in all cases, particularly within the regions of the germarium in which the Hh^{RNAi} was expressed (Fig. 11B-E). Specifically, we found that GFP levels in escort cells were most affected when Hh^{RNAi} was expressed in apical cells or escort cells (Fig. 11B, C), whereas GFP levels in follicle cells were affected when Hh^{RNAi} was expressed in follicle cells (Fig. 11D). GFP expression was decreased throughout the germarium when Hh^{RNAi} was expressed broadly by TJ-Gal4 (Fig. 11E), or both TJ-Gal4 and Bab1-Gal4 together (Fig. 11F). Taken together, these results suggest that Hh is produced by multiple cell types in the germarium, and that the ligand secreted by these sources act in an additive manner on escort cells, FSCs, and prefollicle cells.

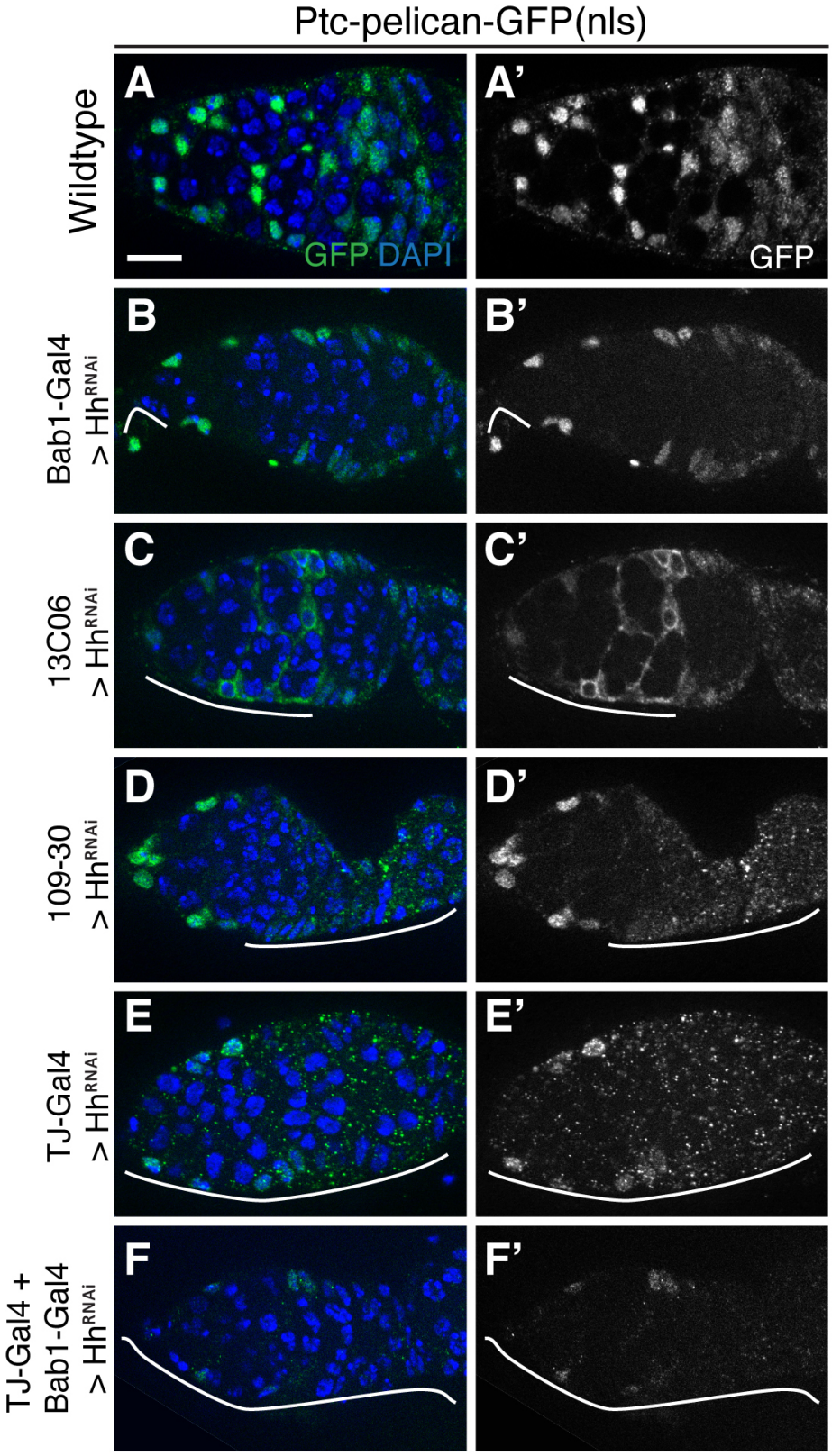

Ptc-pelican-GFP(nls)
Wildtype
A
GFP DAPI
A'
GFP
Bab1-Gal4 > Hh^RNAi
B
B'
13C06 > Hh^RNAi
C
C'
109-30 > Hh^RNAi
D
D'
TJ-Gal4 > Hh^RNAi
E
E'
TJ-Gal4 + Bab1-Gal4 > Hh^RNAi
F
F'

Figure 11. Multiple cell types have active Hh signaling
A-F. Ovarioles from flies 7 DATS that contain Ptc-pelican-GFP(nls), UAS-Hh^{RNAi}, tub-$Gal80^{ts}$, and either no Gal4 driver ("wild type," A), Bab1-Gal4 (B), 13C06 (C), 109-30 (D), TJ-Gal4 (E) or Bab1-Gal4 and TJ-Gal4 (F) stained with GFP (green) to visualize Ptc-pelican-GFP(nls) expression and DAPI (blue). In wild type, GFP expression is bright in escort cells and tapers off in toward the posterior in follicle cells. Germ cells are GFP^-. In panel C, the germarium also includes a UAS::CD8-GFP which labels the membranes of cells expressing Gal4. Note the absence of the nuclear Ptc-GFP signal in these cells. White lines in each panel indicate the approximate range of Gal4 expression. Anterior is to the left. Scale bar represents 5 μm.

FSCs contact multiple escort cells

The most well studied function of escort cells is to support germ cell development in Regions 1 and 2a. To better understand how escort cells interact with both germ cells and the FSC niche, we generated Twin-spot MARCM escort cell clones (Yu et al., 2009) by heat-shocking flies of the appropriate genotype during pupal development (Fig. 12). Consistent with previous studies (Kirilly et al., 2011), we found that posterior escort cells have long membrane extensions (Fig. 12A, B), and that multiple escort cells encapsulate each germ cell cyst in Regions 1 and 2a (Fig. 12D, E). In addition, we frequently observed germaria in which at least two escort cells produced membrane extensions that traversed the Region 2a/2b border, immediately adjacent to the follicle cell membranes (Fig. 12C). Lastly, we noticed that multiple escort cells frequently contacted a single FSC niche (Fig. 12F), and that the nuclei of these escort cells could be positioned anywhere throughout Region 2a, even as much as a full cyst diameter away (GFP^+ escort cell in Fig. 11A and E). Therefore, our data suggest escort cells throughout region 2a function in aggregate to support both germ cell development and FSC self-renewal.

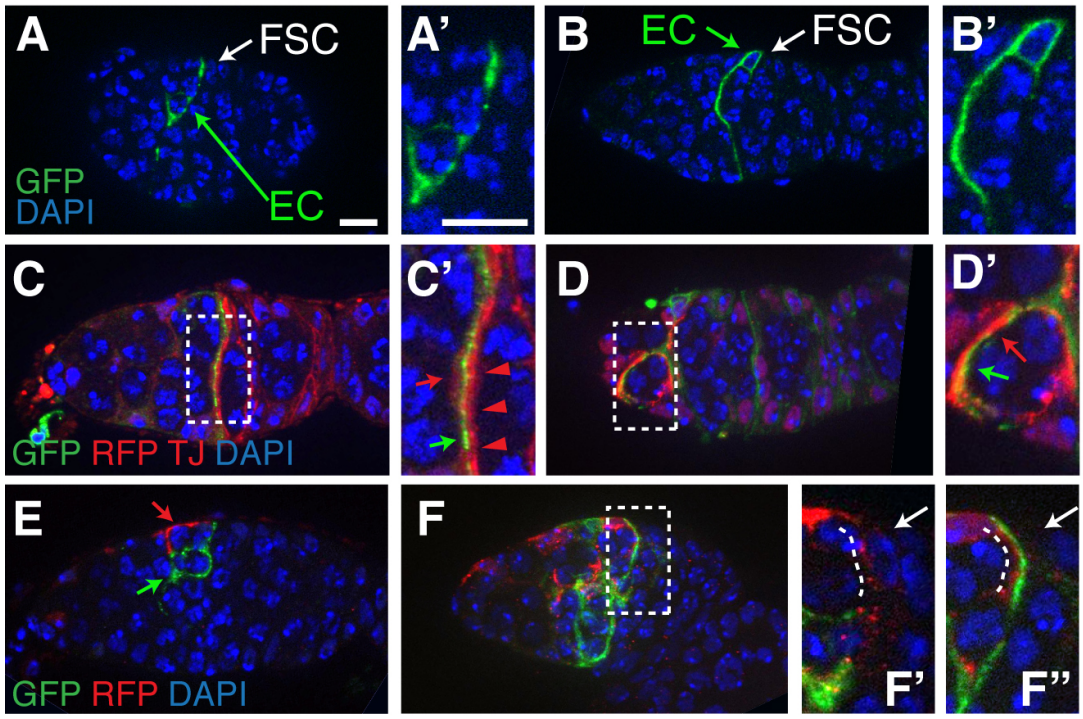


Figure 12. Multiple escort cells surround Region 2a cysts and contact the FSC niche

A-F. Germaria with twin-spot MARCM escort cell clones. A, B. Germaria with a single GFP$^+$ posterior escort cell. Although the escort cell nuclei can be in the center of the germarium (A) or on the edge (B), escort cells in this region always have extensive membranes that wrap around the region 2a cysts. The regions containing the single labeled escort cells are expanded in A' and B'. C-E. Germaria with both GFP$^+$ and RFP$^+$ escort cells reveal that multiple escort cell membranes (red and green arrows, C' and D') traverse the germarium at the Region 2a/2b border (C) and contact follicle cell membranes (red triangles, C'), and surround each cyst in Region 1 (D) and 2a (E). Boxed regions in C and D are expanded in C' and D'. F. Multiple escort cell membranes also contact the FSC niche. Boxed region in F is expanded in F' and F", which show single optical sections in which the membrane extension from the RFP$^+$ escort cell (F') or GFP$^+$ escort cell (F") is adjacent to the FSC niche. White dotted lines indicate the escort cell membranes and white arrows indicate the position of the FSC niche. Germaria are imaged for DAPI (blue) and GFP (green), RFP (red) and Traffic Jam (red, panels C and D only). All panels are single optical sections except panel D, which is a maximum projection of two optical sections. Anterior is to the left. Scale bar represents 5 μm.

N-cadherin and DE-cadherin are necessary in the FSC-niche region. DE-Cadherin is required for FSC maintenance and self-renewal and perturbations on it results in FSC loss (Song and Xie, 2002). In addition, previous work has shown that the requirement of FSC for cell adhesion is independent from its role in Wg signaling (Song and Xie, 2003). DE-cadherin is thought to work by retaining FSC to their niche. However, it is likely that they are playing additional roles in FSCs, such as maintaining spindle orientation and allowing for asymmetrical cell division (Inaba et al., 2010) or concentrating signaling ligands to the FSCs (Michel et al., 2011). Since generating mutant FSC clones for DE-Cadherin results in loss, it has not been possible to study the long-term effects it has on the follicle lineage. Therefore, to determine if adhesion junctions are necessary for FSC self-renewal when taken simultaneously away from both stem cells, I knocked down DE-cadherin by expressing DE-cad^{RNAi} using 13C06. We looked at the follicle formation defects at 10 DATS and 21 DATS (Fig.13). At 10 DATS, we observed that DE-Cadherin expression was significantly reduced in 13C06 regions, when assessed by antibody staining. However, all ovarioles looked normal at this time point (Fig. 13A, B). By contrast, at 21 DATS we see that most ovarioles (85.7%) had a downstream follicle defect, including germ cell cyst fusion (Fig. 13C, D), which is known to arise due to decreased follicle production.

Next, we wanted to determine if removing additional adhesion junction components, specifically N-cadherin (N-cad), also results in follicle formation defects. To knockdown N-cad, we expressed N-cad^{RNAi} using 13C06. At 14 DATS, we observe that 50% of the ovarioles analyzed had follicle formation defect (Fig. 14). We also saw a decrease in Fas III and 13C06 positive region (Fig. 14A), and mature germ cell cysts juxtaposed next to the germarium (Fig. 14C, D). Collectively, these results show that different types of adhesion

junction molecules in the FSC-niche region are required for proper follicle production.

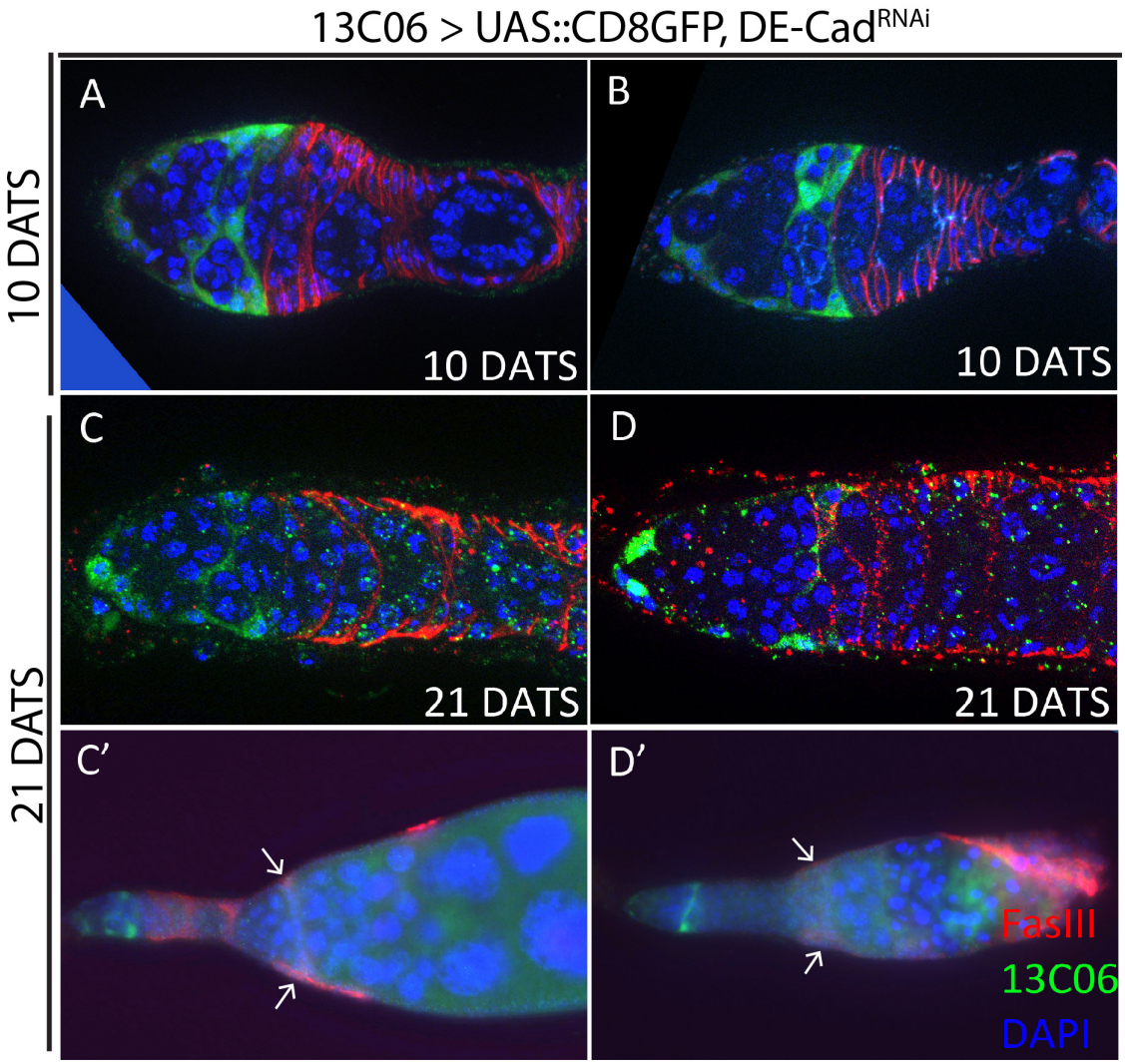


Figure 13. DE-Cad is required in FSC-niche region
Ovarioles in which DE-Cad^{RNAi} is driven by 13C06 at 10 (A, B) or 21 (C, D) days after flies were shifted to 29°C to repress tub-Gal80^{ts} and promote Gal4 activity. Tissue is stained for Fas3 (red), GFP (green) and DAPI (blue). White arrows indicate germ cell cyst fusion. N values equals to 20. Anterior is to the left.

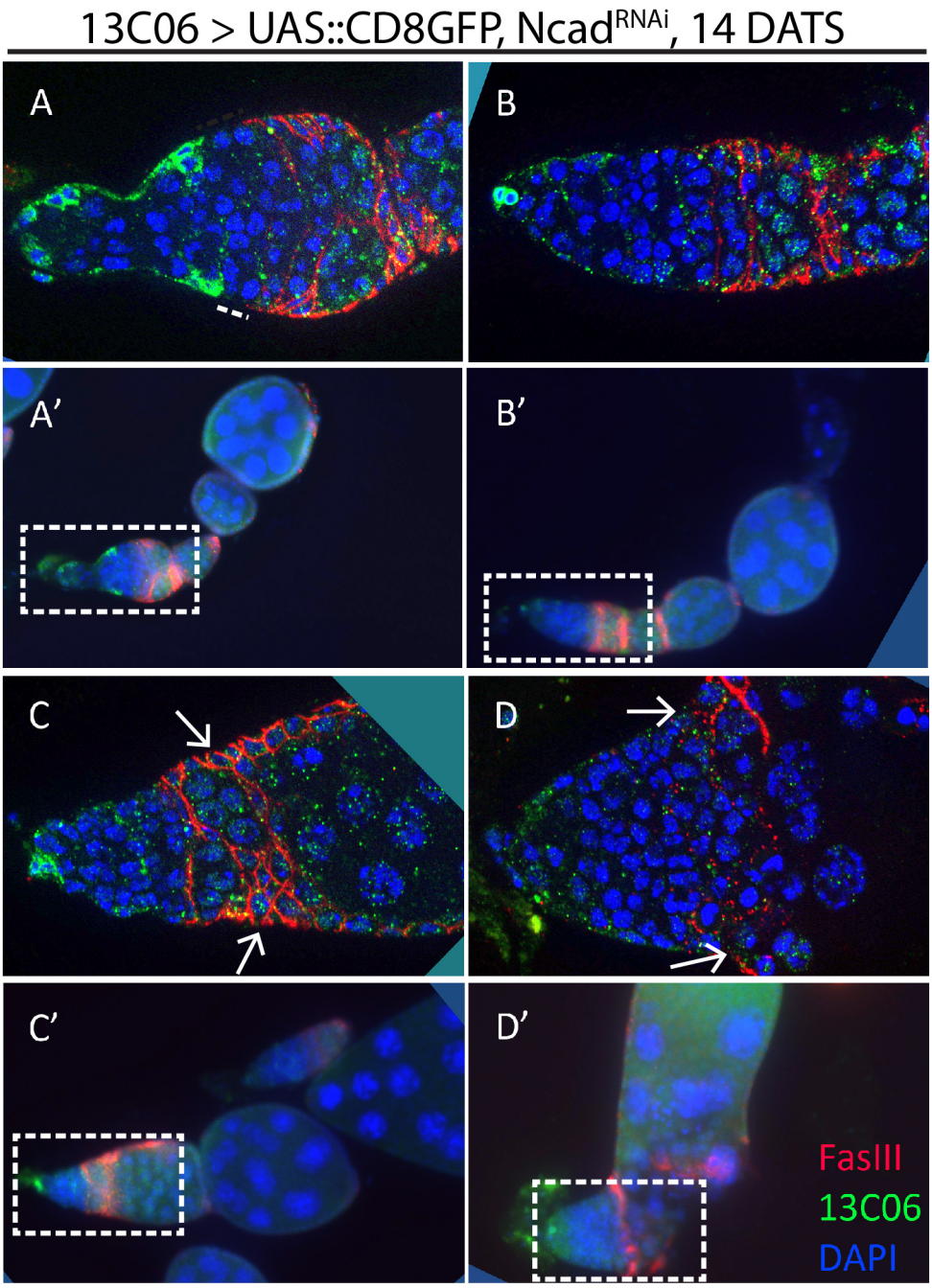


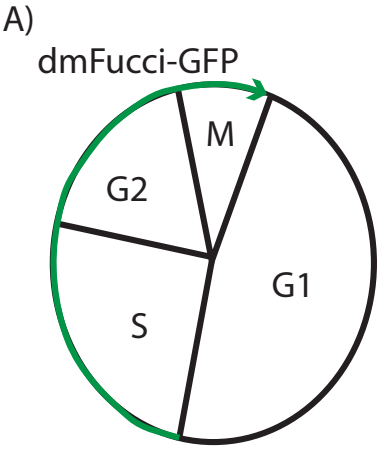
Figure 14. N-Cad is required in FSC-niche region
Ovarioles in which N-Cad^{RNAi} is driven by 13C06 at 14 days after flies were shifted to 29°C to repress tub-Gal80^{ts} and promote Gal4 activity. Tissue is stained for Fas3 (red), GFP (green) and DAPI (blue). A, B, C, D are zoomed in regions (white indented box) of A', B', C', D', respectively. Indented line in A, indicates a gap of 13C06 and Fas III expression. White arrows indicate lack of stalk cells that normally separates neighboring germ cell cysts. N values equals to 20. Anterior is to the left.

Follicle cells have a faster proliferation rate than escort cells.

Escort cells have previously been suggested to be maintained by an escort stem cell population and to have a high proliferation rate (Decotto and Spradling, 2005). However, a more recent study has shown that there are no escort stem cells and that the escort cells are a self-sustained population of cells with sparse division (Kirilly et al., 2012). To further look at this, we quantified the proliferation rate of escort cells and compared them to the follicle lineage in the germarium. We first quantified Fucci-GFP expression, which labels the S-G2-M phase of the cell cycle (Fig. 15A), and previously has been shown to be a proper reporter for this stage (Sakaue-Sawano et al., 2008; Makhijani et al., 2011). We find no expression in terminal filament and cap cells, as expected, since they are post-mitotic (data not shown). In addition, there is no expression past stage 6, the time when follicle cells starts endocycling (data not shown). In escort cells, we find that 17.5% of cells are in the S-G2-M phase of the cell cycle (n= 30) (Fig. 15B). Follicle cells, by contrast, had 54% of their cells at this stage. Next, we quantify the cells that are at the M phase of the cell cycle with phospho-histone-H3. We find that escort cell are labeled 0.30% of the time, while follicle cells are labeled 4.80% (n=30) (Fig. 15B). Collectively, these results support the recent finding that escort cells divide at a very slow rate and are unlikely supported by a stem cell population. In addition, it shows that follicles cells are highly proliferative in the germarium, and might be acting as a transit-amplifying population of cells that terminally differentiate at later stages in the ovariole.

Figure 15. Follicle cells have a faster cell cycle than escort cells.
A-B. Ovarioles were stained with dmFucci-GFP and phospho-H3 (PH3) to determine the proliferative rate of follicle and escort cells. A. A diagram of the stage in the cell cycle where dmFucci-GFP is expressed. B. Quantification of escort cells and follicle cells in the germarium that are dmFucci-GFP+ or PH3+.

A)



B)

	Escort cells	Follicle cells
Fucci-GFP	17.50%	54.00%
Phospho-H3	0.30%	4.80%

Escort cells and germ-cell cysts are required to maintain FSC-marker expression

Previous work has shown that in agametic germaria, follicle cells become localized next to cap cells and can proliferate for a very short time window at the time at which this occurs (Kai and Spradling, 2002). This was intriguing since it suggested that FSCs can respond to an ectopic niche. To further elucidate what are the effects in FSCs when the escort cells and GSCs are eliminated from the germarium, we looked at the expression of several follicle markers (Fig. 16, 17 and 18). We overexpressed heat shock-controlled bag of marbles (Bam), by heat shocking flies four times at 37°C, twice a day. Bam is a germline differentiation gene, and ectopic expression of it has previously been shown to induce GSC differentiation, followed by escort cell apoptosis (Kai and Spradling, 2002). We first look at the expression of 13C06, which is turned on in escort cells and FSCs (Fig. 1C), to determine how its expression changes in these conditions. We find that the number of 13C06 expressing cells is continuously reduced until its expression is gone by 18 days AHI (Fig. 16 A-D). In addition, the size of the germarium remains very small by 18 days AHI (Figure 16D), suggesting that not much proliferation occurs once escort cells *and* germ cell cysts leave the germarium. Next, we look at the expression of Fas III at 7 days AHI and observe that follicle cells have reached the cap cells (Fig. 17 A-D), as it has previously been reported (Kai and Spradling). Interestingly, germ cell cysts are still observed in the germarium and could be the ones responsible for promoting follicle cell proliferation at this stage (Fig. 17B). Lastly, we observe similar effects at 2, 7, and 10 days AHI, when we stain the germarium for traffic jam and TM1-GFP (Fig. 18A-D). Collectively, these results confirm the findings that when Bam is ectopically expressed, FSCs reach the cap cells. However, it also shows that FSCs need to be maintained in their normal environment to be able to retain FSC-marker expression.

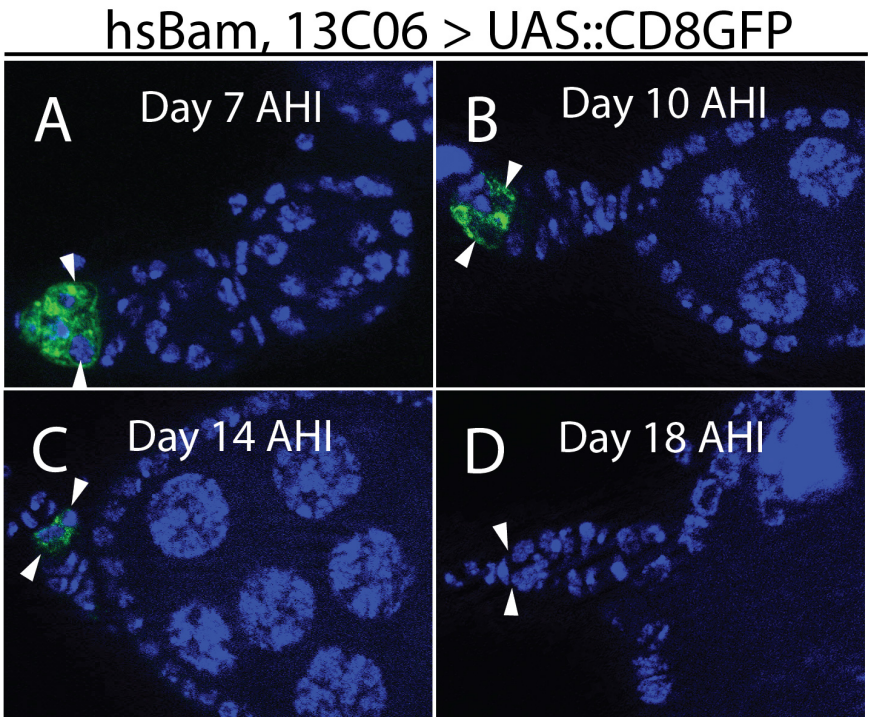


Figure 16. 13C06 expression ceases in agametic germarium.
Ovarioles that contain heat shock-induced bag of marbles (hsBam), where heat shocked twice at 37°C and dissected at 7, (A) 10, (B) 14 (C) and 18 (D) days after heat shock induction (AHI). Tissue is stained for GFP (green) and DAPI (blue). White arrowheads points to the FSCs.

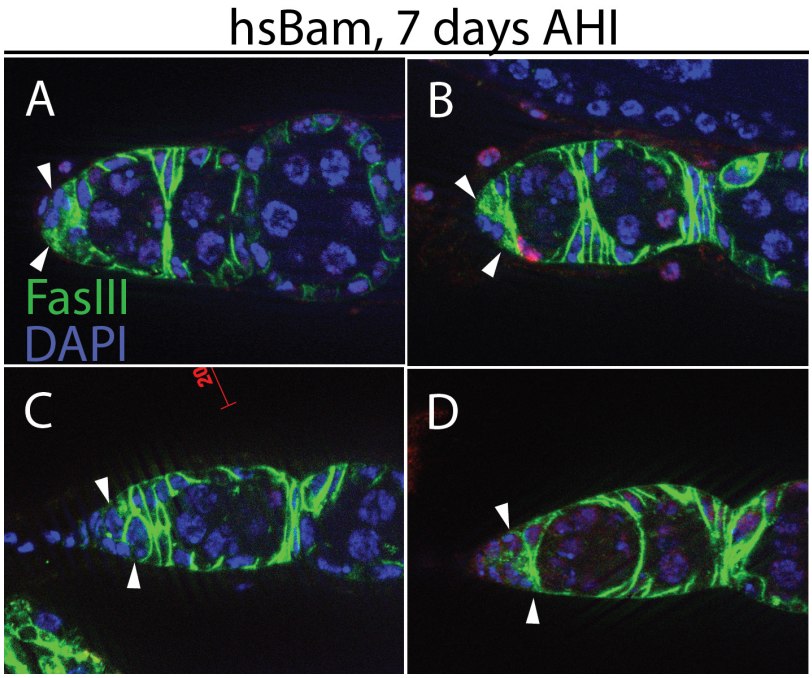


Figure 17. Follicle cells become localized next to the cap cells.
Ovarioles that contain heat shock-induced bag of marbles (hsBam), where heat shocked twice at 37°C and dissected at 7, days after heat shock induction. Tissue is stained for Fas III (green), PH3 (red) and DAPI (blue). White arrowheads points to the FSCs.

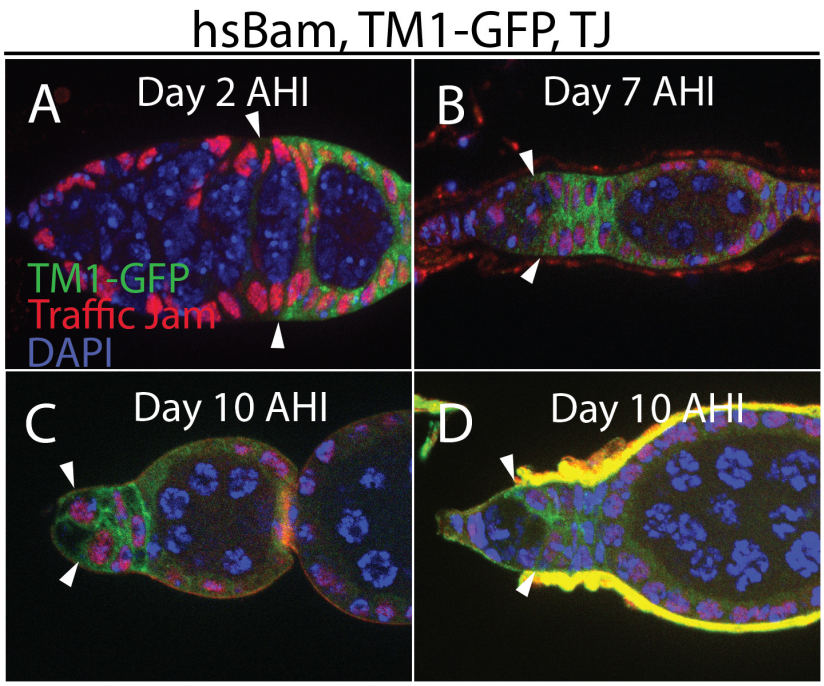


Figure 18. Follicle cells become localized next to the cap cells.
Ovarioles that contain heat shock-induced bag of marbles (hsBam), where heat shocked twice at 37°C and dissected at 2 (A) 7, (B) and 10 (C) days after heat shock induction. Tissue is stained for TM1-GFP (green), Traffic Jam (red) and DAPI (blue). White arrowheads points to the FSCs.

ET-FLP enhancer trap screen:

The use of Gal4 enhancer traps in Drosophila has been of great use to manipulate gene expression in a temporal and spatial manner. However, their expression is usually too broad and does not provide information of the developmental origins of cells. Recently, Bohm et al., has generated a collection of enhancer-trap recombinase flippase (ET-FLP) lines that provide tissue-specific expression and can be inherited from progenitor cells. When combined with a FRT-dependent Gal4 (flip-out) construct, which converts Gal4 into a tissue specific expression, one can express any transgene that contains the Gal4-upstream activating sequence (UAS), such as a UAS::GFP reporter. We decided then to screen through 202 lines from the collection as a way to learn more about the developmental relationship between somatic cells, and identify lines that would permits us to genetically manipulate the escort cell population to potentially learn more about the FSC-niche. We crossed the Flp-lines to two "flp-out" lines and looked at clones generated in the ovariole at 2 and 7 days post eclosion (dpe). Out of the 202 lines screened, we found that 168 of them produced clones in the ovary. This gave us a 69.8% rate of clone induction. Interestingly, the clones had a high correlation between 2 and 7 dpe, suggesting that most of them were induced during development and persisted as adults. Line 156 was an exception, since it produced clones at a 5.6% frequency at dpe, but increased to 44.4% by 7 dpe, suggesting that it was up regulated during adulthood. Interestingly, most of the lines produced clones in both escort cells and follicle cells, albeit only sporadically or in a low percentage of ovarioles. However, we found 6 lines that drive expression in a particularly interesting pattern in the ovary. These lines are highlighted below and representative images are shown in Fig. 19.

Line 168: Produced clones specifically in germ cells at a very high frequency. At 2 dpe, 60.6% (n = 72) contained GSC clones and at 7 dpe, 35.4% (n = 48) contained GSC clones. Since there is a decreased, it would be interesting to

know if this gene is important for GSC function during development. Somatic cells were rarely observed.

Line 688A: Produced clones in all cells in the ovary, but at a particularly high rate in the escort cells. At 2 dpe, 97.5% (n = 81) of ovarioles had 4 or more escort cells labeled and at 7 dpe (n = 56), all ovarioles had 4 or more escort cells labeled. Therefore line 688A labels escort cells at a high frequency.

Line 820A: Line 820A had a very interesting clone pattern which shows that it is particularly expressed in early FSC daughter cells. Cells that became the stalk, polar and anterior main body follicles were expressed at a very high level, 80.3% (n = 76) at 2 dpe and 70.1% (n=174) at 7 dpe. They were also labeled early on in the germarium, right before the FSCs, suggesting that they might label an early progenitor of these cells.

Line 165: Line 165 produced clones exclusively in the terminal filament cells. At 2 dpe, it had 25% (n = 56) of ovarioles labeled with one or more terminal filaments and at 7 dpe it increased to 63.3% (n = 49). This line mapped within the Ect4 gene.

Line 398A: This line produced escort cells, prefollicle cells and FSC clones at a 60% (n = 95) frequency at 2 dpe and at a 68.2% (n = 85) frequency at 7 dpe. We found that it localized within the chickadee gene. When we stained the ovarioles with chickadee antibody we found it to be highly expressed in posterior escort cells, FSCs and prefollicle cells.

Line 324A: This line showed a really high percentage of clones at the 2a/2b border region. Out of the ovarioles that had clones (n = 40), 40% were FSC specific clones, 32.5% had posterior escort cells and, 27.5% had clones that

labeled both cells. This suggests that this line might be expressed in a developmental precursor for both populations of cells.

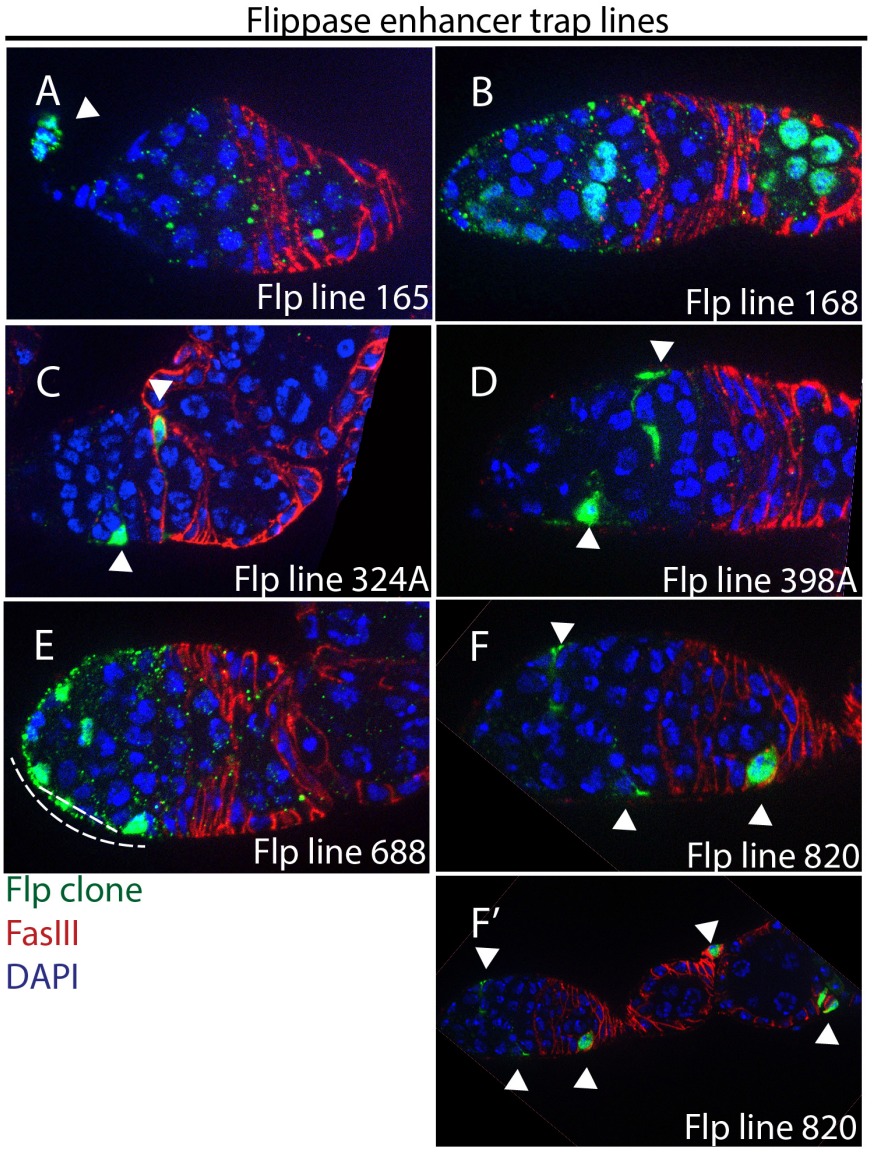


Figure 19. Flippase enhancer trap lines with interesting patterns in the germarium. A-F. Ovarioles which contains flippase enhancer trap lines, crossed to Actin>CD2>Gal4; UAS-GFP (C, D, F) or Actin>CD2>lacZ (A, B, E). They were kept at 25°C and dissected at 2 days or 7 days after eclosion. Tissue is stained for Fas3 (red), GFP or lacZ (green) and DAPI (blue). White arrowheads points to somatic cells that are labeled and presumably originate from the same clone. Dashed white line in D, outlines the escort cell population that are labeled from line 688.

Escort cells and follicle stem cells arise from a common progenitor

Results from the ET-FLP screen shows that escort cells and follicle cells are co-labeled at a very high frequency. In addition, line 324A specifically labels posterior escort cells and FSCs at a high rate. Since there is no increase in their labeling as adult flies, this strongly suggests that line 324A might be expressed in a common progenitor of both cells. To determine if this was in fact the case, we generated clones during development using two independent lineage marking system: Flp-FRT lacZ mitotic recombination system (Harrison and Perrimon, 1993) and twin-spot MARCM (Yu et al., 2009). Flies were put in vials in 12 hrs. intervals ranging from 108 hrs. to 196hrs. after egg laying (see methods). They were subsequently dissected progressively as they eclosed, at 2 days post eclosion (dpe). Relatively low clone induction frequency was generated, ranging between 2% - 20% for the $lacZ^+$ system and 4% - 38% for Dual-spot MARCM.

For the LacZ+ labeling system at 120hrs AEL, all clones that were generated contained escort cells and FSC clones (Fig. 20). Since the clone induction was low, this suggests that escort cells and FSCs arise from a common progenitor. Next, we looked at clones that were generated after the initiation of pupation, between 130 hrs. and 182 hrs. AEL. We see an increase in FSC specific clones between this period, culminating at 182 hrs., were most of the clones were FSC specific. This suggests that during pupation, the FSC-niche is established and the FSC is specified.

As a complementary approach, clones were generated using Twin-spot MARCM system. This system has the advantage of labeling both daughter cells of a progenitor, allowing one to follow their lineages. When flies were heat shocked before the onset of pupation, clones were observed to contain a high percentage of escort cells and follicle cells that were co-labeled. After the onset of pupation, escort cell marking became more restricted. Only a few

posterior escort cells, that also included FSCs, were labeled. At the middle of pupation, it was common to observe a most posterior EC, and a FSC, labeled with opposite colors. This is indicative of a progenitor cell getting labeled at the exact division where it generates an escort cell and a FSC. Lastly, at the latest time period looked at, 182hrs AEL, most clones that were generated were FSC specific (Fig. 20). These results confirm the findings that FSCs and escort cells arise from a common progenitor.

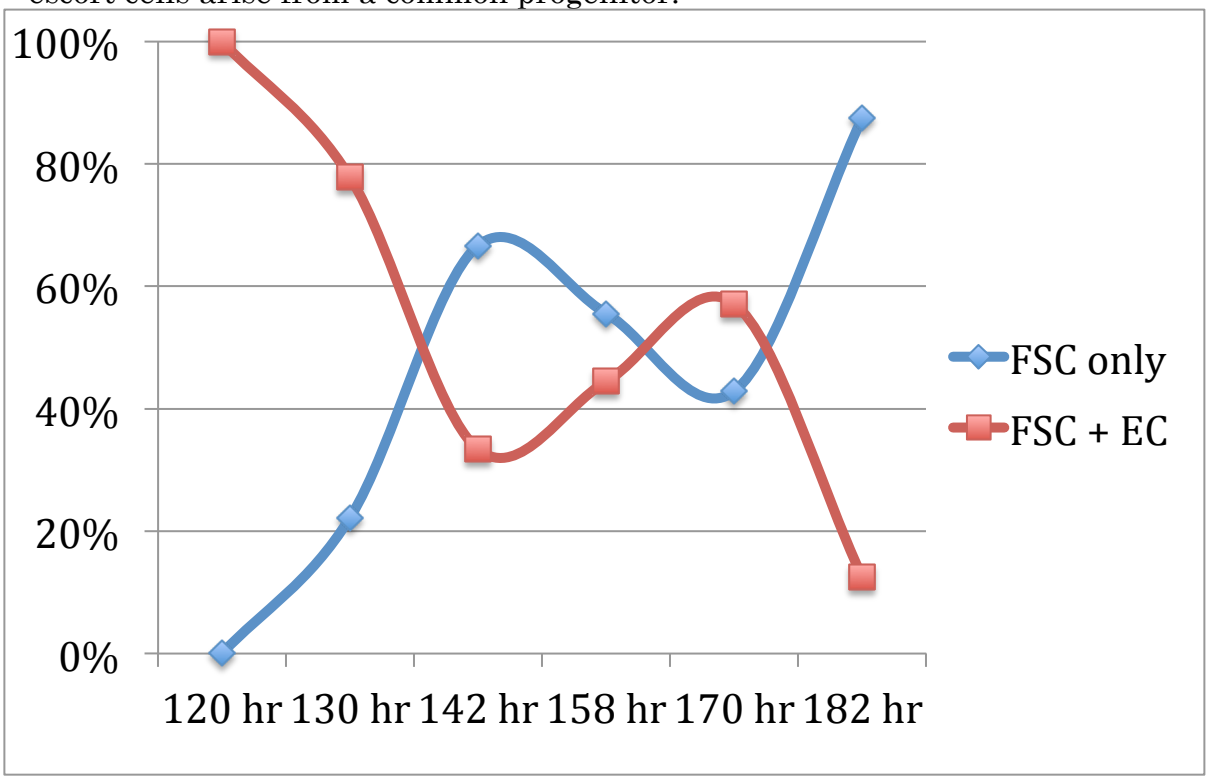


Figure 20. Escort cells and follicle cells share a common progenitor. LacZ+ clonal marking system was used to generate clones at different developmental stages during Drosophila larval and pupal development. They were scored at 2 days after eclosion for clones that only contain FSC (blue line), or clones that contain both FSC and escort cells (red line). Larval and pupae were kept at 25°C and heat shocked for 1 hour at 37°C, at the respective time points.

Dual Spot MARCM; 170-182 hrs AEL

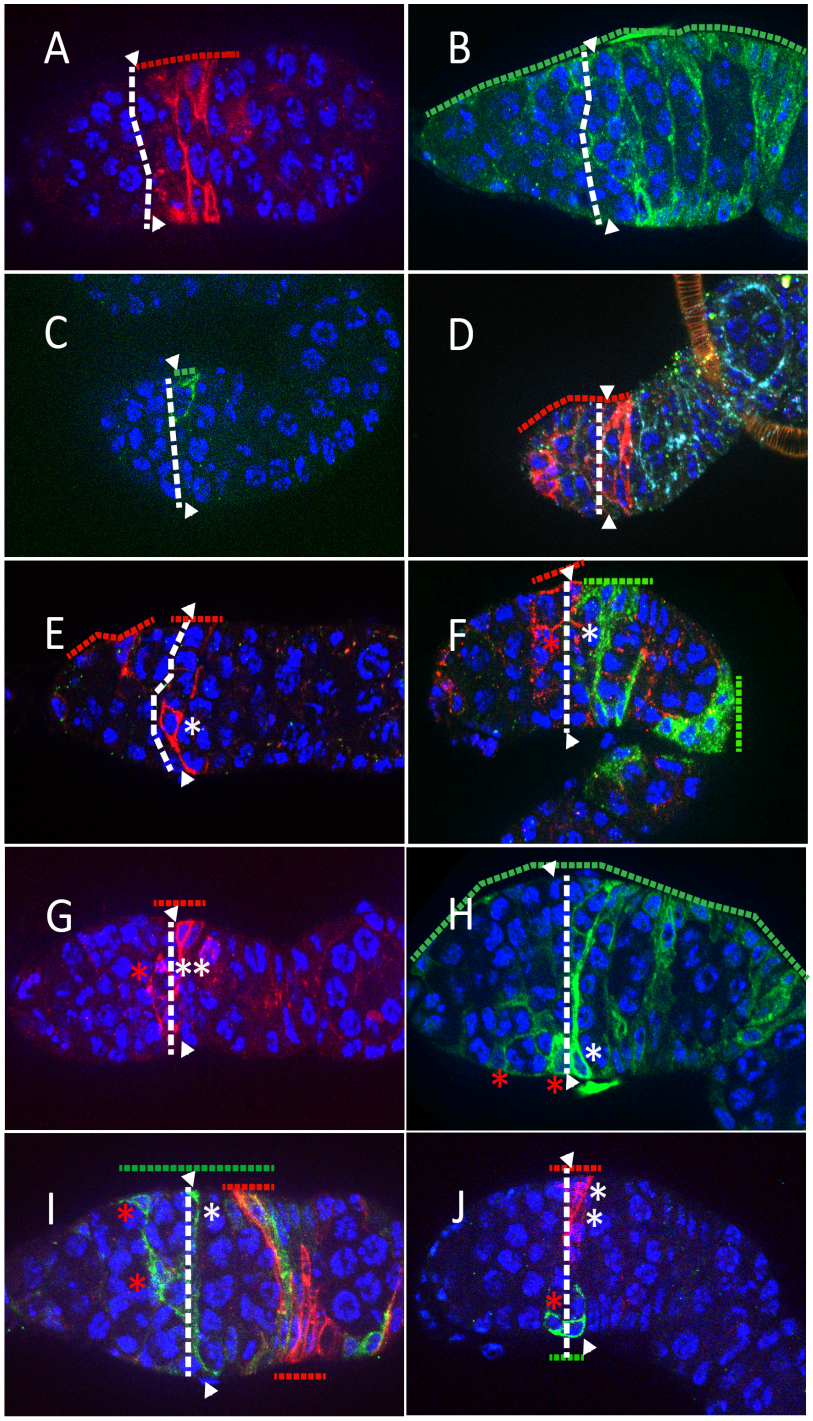


Figure 21. Escort cells and Follicle stem cells share a common progenitor. Larval and pupal ovaries from dual spot MARCM flies, were heat-shocked at for 37°C for one hour to generate clones. Both daughter cells from a common progenitor can become labeled, either red or green, after flippase-mediated recombination. Indented white line denotes the 2a/2b border region. Arrowheads represents the FSCs. White asterisk marks labeled FSCs and red asterisk marks labeled escort cells. Tissue is stained for GFP (green), RFP (red) and DAPI (blue).

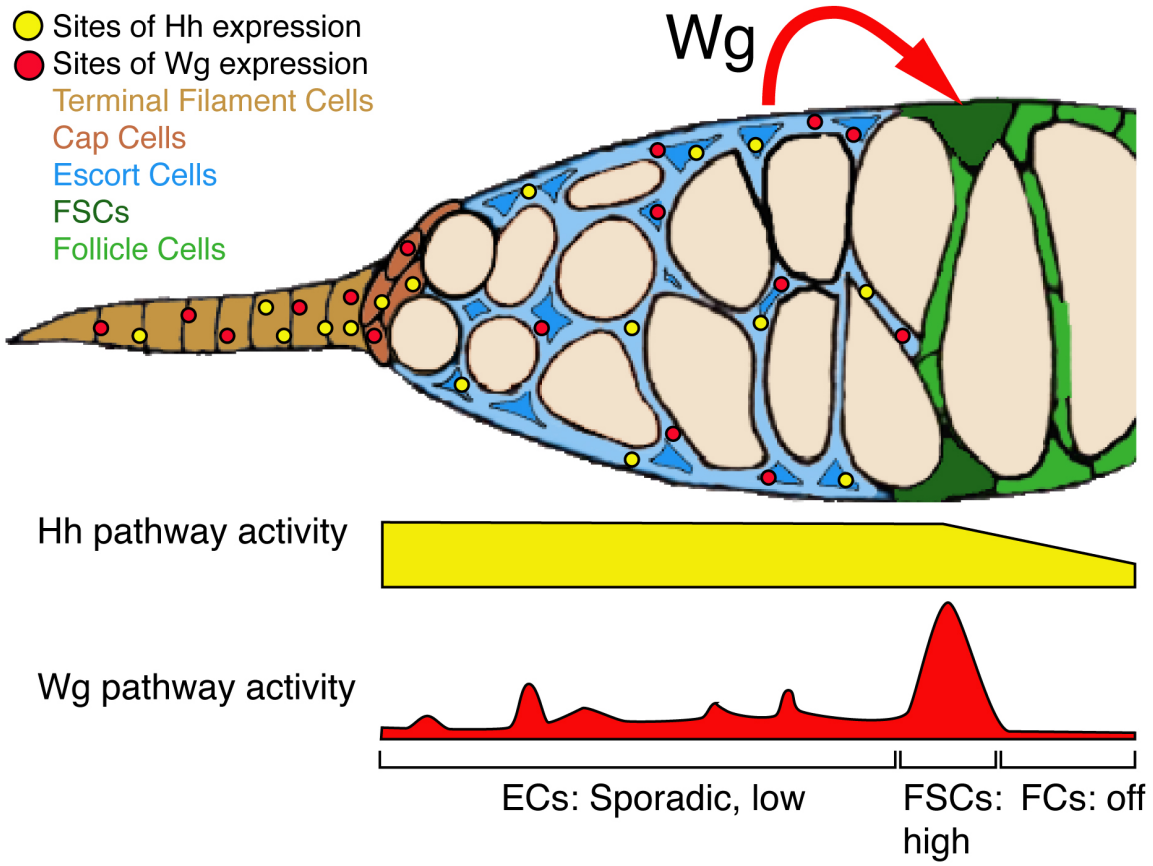


Fig. 22. Model on how the escort cells contribute to the follicle stem cell niche in *Drosophila* Ovary.
This work shows that escort cells produce both Wg and Hh ligands. Wg pathway acts as FSC-niche specific factor, while Hh acts more as a general factor, on follicle cells. Wg produced in escort cells is necessary for follicle production. By contrast, Hh produced by multiple cell types, including the escort cells, is necessary for follicle homeostasis. We don't find evidence for a escort cell specific niche cell, but rather our data supports a model that escort cells act in aggregate to provide a differentiation environment for germ cell cysts and microenvironment for the FSCs.

Discussion

Taken together, our results show that the escort cells are an integral and necessary component of the FSC-niche. Our data indicate that the FSC-niche has a canonical architecture in which at least some key niche signals are produced locally (Amoyel et al., 2013; Losick et al., 2011; Michel et al., 2012), although the FSC niche may also differ from other well-characterized niches in some ways, such as the extent to which it remodels during adulthood. Notably, our results do not contradict the observation that Hh protein relocalizes from apical cells to the FSC niche during changes from a poor to a rich diet (Hartman et al., 2013) since our flies were consistently maintained on nutrient-rich media. It will be interesting to investigate how such distantly-produced ligands interact with locally-produced niche signals to control FSC behavior during both normal homeostasis and in response to stresses.

In addition, our results confirm and extend the conclusion that Wg acts specifically on FSCs (Song and Xie, 2003) and ISCs (Lin et al., 2008), thus highlighting the role of Wg as a specific epithelial stem cell niche factor. As in other types of stem cell niches, this specificity could be achieved through multiple mechanisms, including local delivery of the Wg ligand to the niche and crosstalk with other pathways such as Notch and Hedgehog, which are known to interact with the Wg pathway (Dinardo et al., 1988; Muñoz-Descalzo et al., 2012; van den Brink et al., 2004). Although the precise function(s) of Wg signaling in FSCs is unclear, our observation that a reduction in Wg ligand results in a backup of cysts near the FSC niche at region 2a/2b border, and fused cysts downstream from the FSC niche suggests that one role is to promote FSC proliferation. In addition, the finding that FSC daughter cells with ectopic Wg signaling fail to form into a polarized follicle epithelium (Li et al., 2010; Song and Xie, 2003) suggests that Wg signaling may also promote self-renewal in FSCs by suppressing the

follicle cell differentiation program. Also, our result that Wls and Arr knockdown replicates Wg phenotype, further supports the findings that the Wg pathway is critical for FSC maintenance. In addition, since a stronger phenotype for Wls was seen at 7 DATS, it brings up the interesting possibility that other Wnts might also be playing a role in FSC maintenance. Future work should help determine if this is the case.

In contrast, our observations and published studies indicate that Hh signaling is not specific for the FSC niche, but instead, a more general signal that derives from multiple sources and regulates proliferation and differentiation in both FSCs and prefollicle cells. Consistent with this conclusion, Hh signaling is active throughout the germarium (Fig. 6A) (Forbes et al., 1996a), and is required both in FSCs to promote self-renewal (Vied and Kalderon, 2009; Zhang and Kalderon, 2001) and in prefollicle cells to promote development toward the stalk and polar lineages (Forbes et al., 1996a; Tworoger et al., 1999). Some of our results showed a variable phenotype when Hh was knockdown using different Gal4 lines. These results could be, at least in part, to external differences between different replicates, such as a variable incubator temperature (i.e. > 30°C). In addition, our results showed a different kinetics in phenotypes for bab1-Gal4 at 7 DATS. One possible explanation for this is that Hh is required for multiple cell types in the germarium, and could be having both direct and indirect effects, that sometimes manifest on the follicle lineage. One additional phenotype that we observed when Hh is knockdown in most or all cell types in the germarium, was the very small size of ovarioles that were generated. This is interesting since previous work has shown that Hh is a regulator of growth by directly binding to Cyclin E and Cyclin D promoter (Duman-Scheel et al., 2002). This supports a hypothesis that one of the main function of Hh in the germarium is to regulate proliferation and growth of the follicle cells. Therefore, when Hh is knockdown in different parts in the germarium, growth related

phenotypes are observed, such as cyst fusion. Future work should help address if the Hh pathway also directly regulates cyclin expression in the ovary.

Our DE-Cadherin and N-Cadherin knockdown supports previous work that has shown that FSCs require adherence junctions for their maintenance (Song and Xie, 2002). In addition, it provides greater insight into the role that adherence junctions have in FSCs. Our work suggests that the adherence junction might have additional roles in the niche. One possibility is the recent finding that they play an important role in concentrating signaling ligands to their stem cell (Michel et al., 2010). Therefore, when adherence junctions are knocked down in the niche, it might also inhibit the FSCs from obtaining the appropriate concentration of signaling ligands necessary for their self-renewal. Future work should help determine if adhesion junctions play a role in concentrating Wg and other ligands to the FSCs.

The ET-FLP enhancer screen provided greater insight into the developmental origin of FSCs and escort cells, as well as into the specification of the early progenitors of polar and stalks cells. Our data shows that most of the clones generated in the ovary come from development. In addition, the identification of line 324A, which showed a high frequency of FSCs and posterior ECs labeled, suggests that that this line might be expressed in a mutual progenitor of both cells. Line 820A provided greater insight into the lineage specification of polar and stalk cells. It will be interesting to determine what role PKA-R2 has on polar and stalk cell. In addition to providing greater insight into follicle biology, the screen identified novel tools that will help us to genetically manipulate selective population of cells in the ovary.

The developmental clonal analyses determinedly show that the escort and FSCs share a common developmental progenitor. It established that the FSC-

niche is formed, and the FSC is specific, during early to mid pupation. Interestingly, recent work has shown that during pupation, a basement membrane forms between the follicle cells, and the GSCs initiate their differentiation program (Vlachos et al., unpublished). This is very interesting since these two processes might be playing a role in niche formation. Future work can help determine the signals that promote the FSC-niche establishment, where they come from, and how the emergence of the basement membrane might contribute to asymmetrical divisions in the FSC.

Our agametic experiments show that in the absence of escort cells and germ cell cyst, FSCs become molecularly distinct and don't appear to proliferate at any significant level. This is not surprising since FSCs are found in an ectopic environment, which does not seem conduit for their maintenance and normal homeostasis. Future work can help establish what is necessary and sufficient for FSC maintenance. Being able to breakdown the components of escort, germline and cap cell and how they contribute to FSC maintenance, can provide insight into the requirements of epithelial stem cell-niches in other systems.

Our proliferation analyses of escort cells support recent work that escort cells are not maintained by a population of stem cells (Kirilly et al., 2012). It shows that compared to the follicle cells, escort cells proliferate at a much slower rate. Our multi-color labeling of somatic cells in the germarium indicated that multiple densely packed escort cell membranes surround Region 2a cysts and contact the FSC niche. Although we cannot rule out the possibility that one or more cells in this region are dedicated FSC niche cells, our observations strongly suggest that at least some escort cells contribute to both germ cell development and the FSC niche. Since these escort cells are dynamic (Morris and Spradling, 2011), constantly changing their shape and position to facilitate the passage of germ cell cysts, it is perhaps somewhat

surprising that the FSCs are so stable in the tissue. Indeed, the rate of FSC turn over is comparable to that of female GSCs, which are maintained by a dedicated and more static niche cell population (Margolis and Spradling, 1995). Thus, it will be interesting to investigate how this dynamic population of escort cells is able to maintain such a stable microenvironment for the FSCs. One possibility is that redundant sources of niche signals may allow niches of this type to partially break down and reform as needed to rapidly accommodate the changing demands of the tissue.

Our observations reinforce several themes that are emerging from recent studies of stem cell niches in different epithelial tissues. First, as in the FSC niche, the Wnt/wingless signaling pathway is a key stem cell niche signal in many Drosophila and mammalian epithelial tissues. Second, in several epithelial tissues, the stem cell self-renewal signals are also known to be produced by differentiated cells rather than a dedicated niche cell population. For example, Drosophila ISCs receive self-renewal signals from both nearby enterocytes (Jiang et al., 2009) and the surrounding visceral muscle (Lin et al., 2008; Lin et al., 2010). Likewise, mammalian ISCs at the base of the crypt receive self-renewal signals from Paneth cells (Sato et al., 2011), which are adjacent secretory cells with antimicrobial functions. Lastly, several epithelial niches have recently been shown to have a transitory capacity that may resemble the dynamic nature of the FSC niche. For example, stem cell niches can form de novo in the Drosophila intestine to accommodate increased food availability (O'Brien et al., 2011), and in the mammalian skin in response to hyperactive Wnt signaling (Celso et al., 2004). In addition, mammalian intestinal stem cells produce niche cells *in vivo* (Barker et al., 2007) and can spontaneously reform a niche in culture (Sato et al., 2009). In all of these examples, it seems likely that the relationship between the epithelial stem cell and its niche is not static, but instead flexible and dynamic. Further studies of the Drosophila FSC niche and these other

experimental models will continue to provide insights into the mechanism by which a dynamic epithelial stem cell niche functions.

Appendix:

I. Review of Relevant Follicle Stem Cell literature:

The seminal work by Margolis and Spradling in 1995 showed that a population of stem cells maintains the follicle cells in the *Drosophila* ovary. The follicle cells are an epithelial tissue that is necessary in the ovary to envelop and nurture the developing germ cell cyst starting from region 2b, and plays an integral role in their maturation. They are a multipotential population of stem cells that give rise to three main types of lineages: polar, stalk and main body cells, each of which have unique functions in the ovary.

As a way of introduction to my work, I have summarized and reviewed the body of literature that has been published and is relevant to my thesis. The work so far has shown that several major signaling pathways, including Hedgehog (Zhang and Kalderon, 2001), Wingless (Song and Xie, 2003), BMP (Kirilly et al., 2005), and Jak-Stat (Vied et al., 2012), are all necessary for FSC maintenance. These conclusions have stemmed from experiments where clones mutant for components of their respective signal transduction pathway (*i.e.* receptor, transcriptional effector) where generated in the FSC and where found to get lost at a faster rate than wild type controls. Together, these studies have demonstrated that the FSCs require a combination of signals to be able to be maintained in their niche. However, what are the cells that produce these ligands necessary for the FSC and its lineage, has not been known. Work so far has only shown that Hh (Forbes et al., 1996), Wg (Song and Xie, 2003) and Upd (Lopez-Onieva et al., 2008) are only expressed in the cap cells and terminal filaments. They have not genetically tested if ligands originating from them are required for the follicle cells.

Of these signaling pathways, Hh and Wg have been two of the most studied in the germarium. Work so far has only *suggested* that they might have

distinct roles in follicle cells. The Hh pathway has been shown to be active in all of the follicle cells in the germarium and to be necessary for follicle cell proliferation, as well as the differentiation of stalk cells (Zhang and Kalderon, 2000; Kirilly and Kalderon, 2009). By contrast, the Wg pathway has *only* been shown to be necessary for FSC maintenance and to have no effects in daughter cells (Song and Xie, 2003).

In addition, previous work has shown that adhesion junctions are necessary for FSC retention. This is in in accordance with work performed in other niche cells, which together demonstrate that stem cells require adhesion junction to be retained to their niche. Also, reviewed below is work performed in escort cells and what functions they have in the germarium. Several studies show that escort cells require germ cells for their maintenance. In addition germ cells secrete EGFR ligands, which act on escort cells and create their long membrane extension necessary for proper germ cell cyst differentiation. However, whether the escort cells have any additional functions in the FSC and its lineage is not known.

Margolis J. and Spradling A. *Identification and behavior of epithelial cells in the Drosophila ovary*. Development, 1995.

This is one of the seminal papers about the follicle stem cells (FSC) in the *Drosophila* ovary. Importantly, it's the first paper to show that persistent $lacZ^+$ clones, generated using the Flp/FRT recombination system, is a product of stem cell marking. $LacZ^+$ clones persist in the tissue by 26 days. They next show that the FSC clones do not extend into region 1 and 2a, suggesting then that the FSCs lie at the 2a/2b border position. Looking at proliferative cells using BrDU labeling, further confirmed this, since they rarely saw label incorporation on somatic cells in region 1 and 2a. Lastly, to examine how stem cell labeling varies with time after clones are generated, they ompared

ovarioles at 9-11 days and 20-22 days, and found that completely marked ovarioles rose from 0.5% to 5.8%; in turn, the numbers of mosaic ovarioles decreased from 28% to 11.2%. They misinterpret this data as stem cell loss due to aging (decreased overall number or activity of stem cells). Today, this data can be reinterpreted as showing the dynamics of stem cell replacement. Stem cells act as a population of cells and stem cell-daughters have the ability to replace neighboring stem cells. Therefore, when a labeled stem cell is lost in a tissue, is not necessarily because there are fewer stem cells, but rather because a daughter of a neighboring stem cell has replaced it.

Forbes et al., *Hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in Drosophila.* Development, 1996:

This paper is the first to show that hedgehog (Hh) signaling plays an essential role in regulating follicle cell proliferation. It starts out by showing that Hh is expressed only in terminal filaments and cap cells. The staining seen from the enhancer trap of Hh in the escort cells is attributed to an artifact of the line since antibody staining against Hh is not seen in these cells. Next, by using a transheterozygous allelic combination to generate a temperature sensitive Hh mutant (hh^{9k}/hh^{GSI}), they find that at 6 days at the restrictive temperature somatic cell invagination is reduced and germ line cysts fail to separate. They also observe that more than one cyst is often incorporated into an egg chamber. Next, they wanted to observe the effect that overexpressing Hh has in the ovary. By using a heat shock-Hh transgene, they find that stalk cell numbers are dramatically increased; follicle cell polarity is distinct from wild type; distal egg chambers degenerate; and germ-cell cyst budding is delayed. They next determined if the differentiation of polar and stalk cells were affected when Hh is overexpressed. They find that the stalk-cell specific marker, l(3)1344, fails to

turn on in ovaries suggesting that true-stalk specification does not occur. When using a polar-cell-specific enhancer traps, PZ80 and 8360, they find that it induces ectopic polar cell specification. Not surprisingly, this phenotype only occurs during early follicle differentiation. Follicles in egg chambers past stages 7 were not affected.

Zhang Y. and Kalderon D., *Regulation of cell proliferation and patterning in Drosophila oogenesis by Hedgehog signaling*. Development, 2000:

In this study they extend the observations of Hh made by Forbes et al., 1996. They first generate patched (ptc) mutant follicle clones and show through the expression of neuralized-lacZ expression, that polar cell differentiation is initially delayed from region 2 to 4, but subsequently supernumerary polar cells are formed. Next, by looking at a variety of markers (5A7 for border cells; BB127 for centripetal cells; and L53b for border cells and anterior stretched cells) in ptc mutant clones, they show that follicle cells fail to differentiate normally; these markers are both misexpressed and show decreased expression throughout the ovariole. Next, they observe a defect in oocyte positioning in stage 9 egg chambers and suggest that this is due to failure in follicle cell specification, presumably due to loss of germ line-soma communication. Next, they show that most of the ptc mutant defects in follicle cells are cell autonomous effects, except for the induction of border cell fate which seems to be induced on wild type follicle cells by ectopic polar cells found in the ptc mutant clone. They also find that most defects observed in ptc mutant cells need to be induced prior to follicle cells reaching region 3. They next induce various combinations of mutant clones, all in the Hh transduction pathway, that can be summarized in terms of severity as: ptc > PKA Su(fu) > cos2 >> PKA fu > PKA >> ptc fu. Next, they were interested in characterizing the phenotype that eliminating Hh signaling has in follicle cells. To this end, they generated Hh^{ts} animals and Smo mutant follicle cell

clones. They find that Hh signaling is not required for follicle cell specification but is required for their proliferation. In Hh^{ts} flies, budding of egg chambers seems to cease by day 7 after 29°C shift. In addition, Smo mutant follicle clones accumulated in region 3, indicative of arrested budding. In addition, no stalk cell between neighboring egg chambers were seen. Moreover, downstream germ cell cyst had encapsulation defects. This suggested that follicle cells mutant for Smo are unable to produce sufficient follicle cell, which results in their failure to envelop a germ cell cyst properly and separate them from neighboring cysts. Lastly, they show that follicle cell mutant for PKA is able to rescue Hh^{ts} phenotypes, and both polar cell fate and the positioning of oocyte at stage 9 egg chambers are normal.

Zhang Y. and Kalderon D., *Hedgehog acts as a somatic stem cell factor in the Drosophila ovary*, Nature 2001:

In this study, they perform different set of experiments to show that Hh is a specific stem cell factor and that increasing Hh levels by generating follicle stem cell (FSC) clones generates supernumerary stem cells. However, their experiments and subsequent work from the Kalderon lab, shows that this is not the case. Part of the problem from their experiments is that they identify a stem cell by the absence of Fas III expression on a follicle clone. Although FSCs do generally show lower levels of Fas III expression, their expression is variable and therefore this alone can't be used as a way to identify a stem cell. Next, they make an argument that the FSCs exclusively receive Hh expression in the follicle lineage. This is contradicted by subsequent work from this lab. In Vied and Kalderon, 2009, they show that in fact, all follicle cells in the germarium receive Hh signaling, since ptc-lacZ is expressed throughout the germarium. In addition, FSCs *do not* require higher levels of Hh expression than their daughter cells. The most likely phenomena that

they are observing is that FSCs over proliferate, which in turn makes them hypercompetitive, therefore replacing at a higher rate neighboring stem cells.

Besse F. et al.*, Fused-dependent Hedgehog signal transduction is required for somatic cell differentiation during Drosophila egg chamber formation*. Development, 2002*:*

In this paper they further build on previous Hh observations and find consistent phenotypes with previous work published on Hh. First, they look at ovaries in flies that are homozygous mutant for Fused (fu), and find that this leads to defective follicle cell encapsulation of germ cell cysts. Specifically, the long, thin prefollicular cell processes containing Fas III, are largely absent and stalk cells fail to form normally. However, polar and stalk cells specify normally. Next, they show that fu (transcript and protein) is expressed in both the germline and somatic cells in the ovary. They show that fused mosaic chambers contain mislocalized oocytes, similar to when Hh is taken away in follicle cells. They also find that there is no significant difference in the frequency or in the size of clones between fu mutants and control stem cell clones, suggesting that it does not affect proliferation. They find that fu functions as a Hh signal transducer in the ovary, being necessary for the downstream activation of the ovarian somatic ptc enhancer. In addition, they find that Su(fu) also functions to antagonize fu activity in the ovary. Next, they show that Ci overexpression is able to rescue Fu inactivation phenotype. All together, this shows that fu acts as a positive regulator of Hh signaling in the ovary. Finally, they show that by using different Hh trans-heterozygous allelic combinations (hh^{ts2}/ $hh^{ts2}$and hh^{AC}/ hh^{ts2}), which reduces Hh activity, they are able to mimic the fu ovarian phenotype.

Smith III J.E. et al. *daughterless coordinates somatic cell proliferation, differentiation and germline cyst survival during follicle formation in Drosophila*, Development 2002:

In this paper they show that daughterless (da), a bHLH type E protein, is necessary for follicle cell proliferation and differentiation. They start out by looking at recently eclosed da homozygous mutants ovaries (da^{lyh}/ da^{lyh}) and find that ovarioles were already defective, and normal follicle cells where generally not observed. Specifically, there is an increase in the number of germ line cysts in region 2a, which appear to be backing up. Also, in region 3 there is no constriction by follicle cells in the posterior region of the egg chamber, which is needed to complete follicle encapsulation. This could be a result of lack of follicle proliferation and failure to differentiate into the stalk cell lineage. Next, they find that two stalk cell markers, B1-93F and I(3)01344, are dramatically reduced in da mutants. When they look at polar cell differentiation, they find that they specify normally, but instead find that polar cell numbers increase by one or two cells. Next, they look at the consequence of overexpressing da by using a heat shock induced da transgene. They find that sustained da expression results in ectopic stalk cells that formed at the expense of the follicular epithelium, which results in the follicle cells 'squeezing' the underlying germ line cyst. Finally, they look at the genetic interactions between multiple signaling pathways. They find that females heterozygous for mutations in the da gene, and either hopscotch or STAT92E, showed da-like ovarian defects (when it is found in its homozygous form), which is consistent with the role that da might have in follicle differentiation and proliferation. Next, they look at genetic interactions of da and the Hh pathway. They find that da is *required* for Hh induced over proliferation. Conversely, elevated da *enhanced* Hh induced increase in interfollicular epithelium. It resulted in formation of branched stalks. It would have been interesting if the authors would have tested if

increasing da levels together with Hh resulted in true stalk cell formation since when Hh is overexpressed by itself, it results in extra stalk-like cells that do not turn on stalk cell markers.

Song X. and Xie T. DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the Drosophila Ovary, Proceedings of National Academy of Sciences, 2002.

This paper is one of the first to show that adherence junctions are important for stem cell maintenance. They start out by showing that armadillo and DE-cadherin (Shg) protein localizes at the interface between FSCs and a stromal population of cells known as escort cells. Next, when FSCs mutant clones for Shg are generated using a null and a hypomorph allele, Shg^{R69} and Shg^{10486}, they find that they are lost quickly from the tissue. At two weeks after clone induction (ACI) most FSC mutant clones are lost compared to controls (42.2% for WT vs. 0.3% for Shg^{R69}). This shows that Shg is necessary for FSC retention. Next, they are interested in determining if follicle cell proliferation is affected. They compare wild type and mutant Shg^{R69} follicle cell clone size and find no differences. Importantly, Shg^{R69} allele also affects wingless signaling. However, not surprisingly, Shg^{R69} did show a phenotype in their interactions with other cells. Lastly, they generate mutant pre-FSC clones, by heat shocking late-third instar larvae and looking at the presence of marked FSC clones in adults. They find that follicle clone patches were present in 34.3% of wild type germaria but only in 0.8% of Shg^{R69} mutants. All together, this shows that Shg is necessary for FSCs maintenance.

Song X. and Xie T., *Wingless Signaling regulates the maintenance of ovarian somatic stem cells in Drosophila*. Development, 2003:

In this paper they find that Wingless (Wg) signaling is necessary for maintaining FSCs in the *Drosophila* Ovary. First, they stain for Wg using an antibody and find only expression in the cap and terminal filament cells. Next, they look at Wg^{ts} flies and find that after 1 week at the restrictive temperature, mutant germarium carry much more germline cysts than in controls. Suggesting then that they are being backed up. Next, they increase the expression of the Wg pathway in the ovaries by overexpressing Fz2, dsh and an activated form of Arm, by using a heat shock Gal4. After four days of pulsed heat shock, they find that ovaries consistently produce more follicle cells that accumulated between egg chambers. These extra follicle cells formed long stalk with multiple rows of cells, suggesting that they are over proliferating and not differentiating normally. Next, they knockdown Wg signaling in the follicle cells by generating dsh, arm, sgg and axn mutant FSC clones, all positive regulators of the Wg transduction pathway. They find that all of these FSCs mutant clones were lost from the tissue, showing that Wg signaling is necessary for FSC maintenance. They next compare different alleles of arm mutants. They find that alleles that affected both Wg signaling and adhesion (arm^3 and arm^4) were lost more quickly than alleles that only affected Wg signaling (arm^2 and arm^8). Along with dsh, this data shows that Wg signaling is necessary for FSC maintenance. Interestingly, they also find that constitutive Wg signaling causes loss of FSCs (however, is important to note that follicle cell clones in where Wg is overexpressed causes tissue hyperplasia, eventually leading their disintegration. Therefore, the data in where they observed stem cell loss due to Wg overexpression can be based on the fact that mutant FSC clones were simply counted less frequently. Future work should help address this).

Next, they look at the size of twin-spot clones that generate homozygous mutants for the Wg signaling transduction pathway (twin-spot clones share a common progenitor, and after they are generated, one daughter is mutant

and the other is wild type). They find no significant differences between the sizes of the two, showing that Wg signaling is not essential for the proliferation of follicle cells in the egg chambers (consistent with Shg^{R69} data). Next, when they overexpress Wg pathway by generating axn and sgg mutant follicle cells clones, they find that they fail to integrate into the egg chambers and results in a disorganized cell mass. Also, mutant follicle cells had increased levels of Hts in their membranes. They find that in these mutants, there is a defect of over proliferation in follicle cells from the germarium and find that stalk cells, which are normally mitotically inactive, stained for PH3 in the mutant stalk cell patch, indicating that they are were dividing. Lastly, they find that sgg and axn mutant clones had differentiation defects. They specifically observe that mutants had a disorganized epithelium, with lower levels of Fas III in late egg chambers.

Kai T. and Spradling A. *An empty Drosophila niche reactivates the proliferation of follicle cells*. Proceedings of National Academy of Sciences, 2003:

In this paper they show that if escort cells are eliminated from the germarium, follicle cells are able to associate with cap cells, which supposedly makes them restart dividing, at least for a short period of time. They first start out by showing that Dad-lacZ, a reporter for Dpp signaling, is restricted to GSCs and to a lower level, their immediate daughters. They also find that this is recapitulated by antibody staining for pMad. Next, they observe that when they ectopically express bag of marbles (Bam), a gene that drives GSC differentiation, it leads all the GSCs to vacate their niche and differentiate. They find that when this happens, escort cells are also quickly lost from the germarium. Escort cell can no longer be found by 9 days after heat shock induction of Bam. This results in the follicle cells eventually reaching the vacated cap cells. Interestingly, they find that the cap and terminal filament

cells are able to remain stable after up to 18 days after GSCs are lost. But afterwards, cap cells lose expression of the enhancer lines PZ1444, PZ0078 and no longer stain for Hh.

Next, they find that escort cells are also able to turn on the expression of Dad-lacZ, after the GSCs are lost and the escort cells are found next to the cap cells. Afterwards, they show that the follicle cells also turn on the expression of Dad-lacZ, once it's their turn to reach the cap cells. But Dad-lacZ expression in the follicle cells ceases after cap cells are shut down. Surprisingly, they find that follicle cells are able to double in size once they reach the cap cells, since this supposedly makes them reenter the cell cycle (from 30 cells reaching more than >60 in about a week). Lastly, they find that follicle cells require, interestingly, Hh but not Dpp for this follicle cell expansion to occur, and this is because Hh is supposedly only produced in the cap and terminal filament cells.

Kirilly D. et al., *BMP signaling is required for controlling somatic stem cell self-renewal in the Drosophila ovary*. Developmental Cell, 2005:

In this paper the authors show that BMP signaling is necessary for FSC maintenance and self-renewal. They start out by showing that Dad-LacZ is expressed in about 5% of the FSCs and also in anterior escort cells close the cap. Next, they show that by generating follicle cell clones that overexpress an activated form of tkv, they are able to ectopically induce Dad-lacZ expression. Next, using a temperature sensitive allele for Gbb and Dpp in a agametic ovary that has been stripped of GSCs (by heat shock Bam), they find that Dad-lacZ expression is completely gone from follicle cells. Next, they ectopically express L3 larvae with hsBam and look at 10 days after the fly's eclosed. They find that in Gbb mutant ovaries, there are very few or no follicle cells. They could not test Dpp mutant alleles since they were too

strong and did not reach adulthood. They therefore conclude that Dpp/Gbb signaling is necessary to maintain them in their ectopic niche.

Next, they look at different components necessary for BMP signal transduction including punt, tkv, sax, mad and med. They found that FSC mutant for punt, tkv, and mad were lost faster than wild type clones. However, compared to other mutants such as Hh and Wnt signaling pathway components, they were not lost as fast and as severe. The strongest effect for the BMP receptors was seen with punt mutants. Mad mutants only had 12.6% vs. 38.4% in wild type at 21 days after clone induction (ACI) (a relative percentage change compared to clones from 1 week (RPC) of 73.4% for wild type vs. 23.8% for Mad mutant). The strongest effect overall was seen in Med mutants (1.9% for Med^{26} vs. 38.4% for wild type at 21 days ACI; a RPC of 3.9% for Med vs. 73.4% for wild-type). This suggests that Med might be involved in other signaling pathways in addition to BMP. Next, they show that they can partially rescue Med mutants, but not punt mutants, by overexpressing UAS-P35 (a RPC of 6.3% and 22.5% for Med^{26} and Med^{26};UAS-P35, respectively, compared to 73.8% for wild type controls). They show that overexpressing an activated form of tkv caused a very mild hypercompetitive phenotype, by clones persisting longer in the germaria than in controls (a RPC of 65.6% for tkv vs. 40.6% for wild type). Next, they wanted to determine if lack of BMP signaling affected follicle cell proliferation. They generated twin-spot clones that originate from the same follicle progenitor, one becoming mutant and the other remaining wild type. They found that clones mutant for tkv had the same clone size (a relative division rate (rdr) of 0.96%). By contrast, Med mutants had a rdr of 0.52%. This suggests that an unknown signaling pathway acting through Med, and BMP independent, controls follicle cell proliferation (or survival). Lastly, they show that overexpressing BMP signaling (UAS-tkvA*) can partially rescue

FSCs defective for Wg signaling (dsh^3) but not for Hh signaling (smo^3); (An RPC of 11.5% for dsh^3 vs. 40.3% for dsh^3; UAS-tkv*).

Overall, I thought that this paper was pretty good. Their life-span studies are impressive and it definitely shows that BMP signaling is necessary for FSC maintenance. However, I believe that their agametic experiments were distracting and do not contribute much to the understanding of how follicle cells are maintained in their normal environment.

Decotto, E and Spradling A, *The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals.* Developmental Cell, *2005:*

In this paper they make an argument that the population of escort cells is self-renewed by an escort stem cell population, located at the tip cells of the germarium. This has since then shown not to be true. Major flaws of the paper were assuming that the FRT based lacZ system is solely formed under mitotic recombination. In addition, looking at small numbers of germaria (small N's), and not at sufficient time points after clone induction (i.e. 1- and 2- weeks ACI), made them incorrectly conclude that there was an escort stem cell population.

However, some of the other data in the paper shows that escort cells increase in number when germ line Bam mutants accumulate in the germarium. This shows that even though escort cells have a slow division rate, they are still able to proliferate to adapt to the numbers of early germ line cysts. Next, they show that the STAT92E enhancer trap has expression in all of the somatic cells in the germarium, including escort and follicle cells. They find that in $STAT92E^{ts}$ mutants, GSCs are lost from their niche, where 48% ovarioles by 6 days after temperature shift, lacked GSCs entirely and escort cells showed abnormal morphology (this is consistent with work from Lopez-

Onieva et al., 2008, which showed that Jak-Stat is required in apical cells to maintain the GSCs). Interestingly, overexpressing UAS-Upd using c587 driver, caused the number of germ line cyst to significantly increase, and the germarium to become disorganized. Also, it appears that the enhancer trap for escort cells, Fax-GFP, also increased in number, suggesting that escort cells over proliferate when Upd is overexpressed. Lastly, they generate STAT92E mutant clones in both the germ line and soma. They show that when germ line mutant clones have no observable defect. In addition, they suggest that follicle cell clones mutant for STAT92E, don't have any defect in region 2, but have a defect in region 3 in stalk cell formation. However, they do not perform a life-span study. Overall, this paper suggests that Jak-Stat signaling might be important for escort cell maintenance and expansion, but further experiments need to be done to determine if this is the case.

Nystul T. and Spradling A. *An epithelial niche in the Drosophila ovary undergoes long-range stem cell replacement*, Cell Stem Cell, 2007*:*

In this paper, they show that there are only 2 FSCs per germarium. Using a clonal system with 3 possible genotypes, definitively proved this. With a moderate heat shock they generated clones that contained: 46% of the ovarioles with no FSC clones, 31.5% had a single recombinant FSC clone, 13% had two FSCs with two recombined genotype and 9.5% had all FSCs with the same recombined genotype. If there where 3 FSCs, 21% of the germaria would have been expected to have two FSCs recombined (and 1 remained unrecombined) and only 3.2% would have expected with all three recombined genotypes. However, 22.6% where seen of the former and none in the latter category, proving that there are only two FSCs. Next, the authors find that a pattern of follicle cell daughters alternate between posterior migration and cross-migration to the neighboring FSC side. This was shown since the pattern of migration of the follicle cells in region 2b, alternated each

time according to their genotype. Next, they show that the niche of the FSCs has to be different from that of the cap cells, since nearby escort cells are much more dynamic than that of the post-mitotic cap cells. Lastly, they show that the FSC's have the ability to replace each other. First, they noticed that FSCs turn over and have a half-life of about 12 days. But rather than the stem cell being lost, it resulted in loss of mosaicism, which strongly suggested that the daughters from each follicle stem cells are replacing each other.

Lopez-Onieva L. et al., *Jak/Stat signaling in niche support cells regulate Dpp transcription to control germline stem cell maintenance in the Drosophila ovary*. Development 2008:

In this paper the authors show the importance of Jak-Stat signaling in maintaining the GSCs, by directly regulating Dpp signaling in the cap cells and terminal filament cells (which is interesting, considering the role that Jak-Stat signaling plays in maintaining the GSCs in the testis). However, some of the findings are also relevant to the follicle cells, since they also require Jak-Stat signal transduction. The first experiment they do is to stain with anti-Upd. They find that it localizes in the cap cells and terminal filament cells. *However, interestingly, they also observe anti-Upd punctae throughout the escort cell region.* Next, they perform an RT-PCR in the ovary and find that all Upd ligands are expressed (upd-1,-2 and -3). They also find, using a reporter for JAK-STAT transduction pathway, that Jak-Stat is active in cap and terminal filament cells. Next, when they overexpress Upd using bab1-Gal4, they find that there is an increase in GSC numbers. Next, they look at strong hypomorphic conditions of the Jak/Stat pathway (hop^{25}, upd^{YM55}/hop^{27}) and find that GSCs numbers dropped to 0.9+-0.8 by 25 days and that 25% of germaria had no GSCs.

Next, they generated GSC clones mutant for hop, Stat92E, and dome, and found no effects in the germline, consistent with Decotto and Spradling, 2005. Next they overexpressed a dominant-negative form of the receptor dome by using bab1-Gal4; UAS-Flp, expressed during development (which generates mutant clones of cap cells). They found that mutant cap cells had no GSCs next to them. In addition they find that loss of Jak-Stat signaling during development does not affect specification of the cap cells, suggesting then that this is not due to a defect in cap cell development. Lastly, they show that when they overexpress an activated form of hop receptor using bab1-Gal4, it causes an increase in the expression of Dpp mRNA, (but not Gbb) in the ovary (shown by real-time PCR). They stained for pMad, the BMP pathway effector, and found that it was much more broadly expressed through early germ line cells, and not specific to the most posterior germ line cell, as seen in wild type. This then strongly suggests that Jak-Stat signaling controls Dpp expression in the cap cells, which is necessary for GSC maintenance.

Vied C. and Kalderon D., *Hedgehog-stimulated stem cells depend on non-canonical activity of the Notch co-activator Mastermind.* Development 2009:

In this paper the authors find that mastermind gene enhances Hh signaling in the follicle cells in a Notch-independent manner. They start out by performing a genetic screen for chromosome deficiencies to find a *dominant* suppressor that inhibited follicle proliferation when Hh is overexpressed in the ovary (through heat shock-Hh). They found only two deficiencies that had an suppressor effect and interesting both overlapped with the mastermind (Mam) gene, which had previously been studied mainly as an effector of the Notch signaling transduction pathway. To confirm this, they looked at heterozygous flies mutant for Mam and found that it also suppressed the hs-Hh phenotype. Next, they do a life span study, in where they perform clone induction in stem cells in a wild type and mutant background and look at

several time points to determine if stem cells are hyper-competitive or hypo-competitive for niche occupancy. They find that FSC mutant for Mam, are lost more quickly than in controls. However, their life span studies could have been done much better. First, they should have looked at many more germaria (more N's). In some time points they looked as little as 6, while overall they didn't have for any single time point more than 82. By comparison, Ting Xie's work looks on average at more than 300 germaria per time point, when they perform their life span studies. Second, looking at one more time point (3 instead of 2) would make their case much more solid and would had definitely supported better their conclusions. Lastly, it is incorrect to assume that the proportion of clones generated in wild type ovariole's and mutants are the same. Therefore, comparing relative clone frequency between wild type and mutant should not be done. A much better assay would have been to compare the percentage of change of the clone with the same genotype, to an earlier time point (i.e. 3 weeks vs. 1 week). This would give you a relative percentage of change, which could then be compared to wild type to determine if there is a hypo- or hyper- competitive phenotype.

With this in mind, their data suggests that Mam mutants might be getting loss at a higher frequency than in wild type. In addition, follicle cells within the mutant patch exhibited frequent egg chamber fusions, similar to phenotypes to when Hh signaling is decreased. The rest of their data just looks at only one time point with very few germaria and is hard to conclude much from them. However, they try to make the point that Mam fully suppresses ptc mutant follicle over proliferation. Next, they show that Suppressor of Hairless mutant FSCs are well maintained in the niche, suggesting that the Notch pathway doesn't play a role in FSC maintenance. In addition, loss of Nicastrin, which promotes cleavage of Notch into $Notch^{intra}$, does not impair FSC clone persistence. Lastly, they show that Mam function as an Hh effector is restricted to ovary and does not have the

same function in the wing disc. To conclude this, they look at the ptc-lacZ reporter in several mutant backgrounds including mam and ptc; mam double mutant. However, their data does not appear very solid. Overall, from their work I can conclude the Mam seems to be necessary for follicle cells but further work need to be done to determine if it in fact functions as an effector of the Hh signal transduction pathway in the ovary.

Li X. et al., *Polycomb group genes Psc and Su(z)2 restrict follicle stem cell self-renewal and extrusion by controlling canonical and noncanonical Wnt signaling*. Genes and Development, 2010

In this paper the authors show that the epigenetic silencers, the Polycomb group genes, allow for adult stem cell to differentiate by restricting follicle stem cell self-renewal in the germarium. The authors start out by showing that the Posterior sex combs, which encodes a core Polycomb-repressive complex 1 component (PRC1), functions redundantly with Suppressor of zeste two (Su(z)2), by using the $Su(z)2^{1.b8}$ allele, a chromosomal deficient line missing both genes. They generated FSC mutant clones mutant for $Su(z)2^{1.b8}$ and found that early follicle cells become basally extruded from the epithelium. Next they show convincing evidence that $Su(z)2^{1.b8}$ follicle cell clones form tumors once they are basally extruded. They become larger in size and continue to proliferate (shown by PH3 and CycA staining) past stage 6 (when wild type follicle cells stop proliferating). Next they make a set of experiments that suggests that $Su(z)2^{1.b8}$ follicle clones are unable to differentiate. They support this claim by showing evidence that $Su(z)2^{1.b8}$ mutant clones have no Fas III expression and have low levels of the transcription factor Cut. Most likely they are correct in their conclusions that follicle cells are unable to differentiate, but by using these markers alone, in my opinion, does not strongly support their conclusions. If, in addition, they

would have looked at the absence of stalk cell and polar cell markers, it would have made a more convincing argument overall.

Next they test the signaling pathways Hh, Wg and BMP, to determine if they are epistatic for the $Su(z)2^{1.b8}$ phenotype. They find that when clones mutant only for Wg pathway activity (by using a dominant negative form of TCF) is tumor growth of $Su(z)2^{1.b8}$ mutant clones significantly inhibited (BMP and Hh mutants had no effect on the $Su(z)2^{1.b8}$ phenotype). However, basal extrusion was not eliminated, suggesting that a separate mechanism promotes this. Next they expressed Frizzled (Fz) -RNAi in $Su(z)2^{1.b8}$ clones and find that more than half of the mutant FSC-derived clones do not become basally extruded, and instead develop into single-layered epithelial cells with normal morphology. Since Fz functions in both the canonical wingless pathway as well as the planar cell polarity pathway (PCP), they test the hypothesis that the PCP pathway might be responsible for the basal extrusion phenotype. They confirm these finding by generating clones mutant for $Su(z)2^{1.b8}$ together with disheveled (which is important for both pathways). Next, they test components of the PCP pathway (flamingo, four-jointed and dachsous), which function independent of the Wg canonical pathway, and find that the basal extrusion phenotype is inhibited in $Su(z)2^{1.b8}$ mutant clones. However, clones were not normal and developed small spherical tumor masses inside of the germarium. This experiments shows that both the canonical and the noncanonical Wg signaling pathway are necessary for the $Su(z)2^{1.b8}$ phenotype; each pathway controlling different features of the phenotype.

Next they show that follicle cells that become mutant for $Su(z)2^{1.b8}$ outside of the germarium, do not lead to tumorigenesis, suggesting then that for the phenotype to be expressed it has to occur in early follicle cells, presumably because differentiated cells have a more closed chromatin than the stem cells.

Lastly, they show that the tumor suppressive activity of PRC1 and Su(z)2 in FSCs is independent of the PRC1 complex function; and interesting they find that the Wg-lacZ enhancer trap is expressed within clones of $Su(z)2^{1.b8}$. Overall these studies were very well done, and support previous work (Song and Xie, 2003) that show that Wg signaling is required for FSCs maintenance and self-renewal.

Kirilly D. et al., *Self-maintained escort cells form a germ line stem cell differentiation niche*. Development, 2011:

In this paper, they definitively disprove the escort stem cell hypothesis and rather show that escort cells (ECs) are a self-sustained population that provides a differentiation environment for early germ line cysts. They start out by showing that ECs turnover at about 14.7% at the 2a/2b border and 4.3% in region 1 and 2a (of 8 day old germaria). Next, they perform a BrdU chase experiment. They first feed BrdU to their flies for 3 days, and look immediately afterward. They find that the germ line cells are labeled on average about 43% of the time, showing their fast proliferative nature. By contrast, ECs had a very small number of labeled cells localized mainly at the 2a/2b border region. After 15 days of chase (no feeding), no germ line cells where positive. By contrast 34.3% of germaria had 1 or more ECs positive at 2a/2b border and 13.8% in region 1 and 2a. These results show that ECs proliferate infrequently.

Next, they perform the same experiment from Decotto and Spradling, 2005. They use the Flp-FRT lacZ mitotic recombination system (Harrision and Perrimon) and perform a 30 min or 60 min heat shock regiment. After 1 week of clone induction (ACI), 34.5% and 56.2% carried at least one-$lacZ^{+}$ marked most anterior escort cells. However, these positively marked cells don't change within a 3-week span. In addition, 82.4% and 99.1% of germaria

carried an additional more posterior escort cells labeled, for the 30-min and 60-min heat shock regiment, respectively. These results might explain why it was previously thought that there was an EC stem cell population. However, the following reasons disprove the escort stem cell model. 1) The percentage of germaria that has escort cells labeled is the same after 1-, 2- or 3- weeks ACI. 2) Germaria with the most anterior escort cells labeled is found without any posterior escort cells labeled. 3) BrdU data don't support the high proliferation rates of the escort stem cell model. 4) Lastly, the tub-lacZ system can occur independent of division. Therefore they used 2 different lineage labeling system that do require cell division (PMML and MARCM). They found using the PMML system, that the most anterior escort cells was labeled only 0.9% or 2.5% for 30 min or 60 min heat shock, respectively; highly in agreement with their BrdU data. They saw similar results for MARCM. Lastly, they show that out of the 76 ovarioles with posterior escort cell labeled, 66 germaria had no other positive labeled ECs, further showing that there are no escort stem cells.

Lastly, they show that posterior ECs have long membrane extensions and that Rho is required for escort cells maintenance. Escort cells expressing Rho^{DN} show a decrease in EC number (on average, they decreased from 42 to 22 escort cells). In addition, they show that it was much more common to observe early germ line cysts containing spectrosomes, suggesting that they were not differentiating properly. To determine if in fact this was the case, they looked at Dad-lacZ and Bam-GFP expression. They found that Dad-lacZ was expressed more broadly while Bam-GFP was more restricted, suggesting that germ cell cyst were not differentiating properly.

Vied C. et al., 2012: *Regulation of stem cells by intersecting gradients of long-range signals.* Developmental Cell, 2012:

In this paper they argue that follicle stem cells (FSCs) require Jak-Stat signaling, and that multiple long-range signals coming from both cap cells and stalk cells, are required to maintain the FSCs at their location and at the right numbers. However, their data does not support this. In addition, my results contradict the idea that FSCs are exclusively maintained by long-range signals. First, they start out by showing that Jak-Stat is important for the follicle lineage by generating FSC mutant clones for STAT92E and hopscotch, and perform life span study. They conclude that Jak-Stat mutants are quickly lost from the tissue, suggesting that they are important for FSC maintenance. However, this has the typical shortcomings of a life span study from the Kalderon Lab: 1) Low N numbers, 2) look at only one developmental time point rather than at a minimum two, and 3) always assuming that wild type and mutant clones have the same clone induction rate.

Next, they look at a Jak-Stat signal transduction reporter (Jak-Stat reporter), and find that it is supposedly expressed highest in FSCs and prefollicle cells and that it decreases anteriorly, being absent in anterior escort cells (however, they don't mention that this reporter is on in cap cells and terminal filaments, as shown by Lopez-Onieva, 2008). Next, they knockdown Upd by using Upd-RNAi expressed with the Gal4 lines: bab1, c587 and 109-30. They find that the Jak-Stat reporter decreased 70% with only 109-30 Gal4 or neur-Gal4 (stalk cells). Interestingly, only 10% of the ovariole's showed a stalk cell defect with these Gal4's and they attribute this to incomplete knockdown of Upd in the germarium. This, however, is extremely worrying since they are making a very important conclusion of Upd movement, when in fact they're just observing a very low knockdown. Next, they generate FSC clones overexpressing UAS-hop and find that they are hypercompetitive. They conclude that since previous data shows that Hh is produced exclusively in cap cells, that is then the intersection of both these gradients that maintain the FSCs at the right numbers. However, they don't: 1) perform any further

experiments to show this and 2) don't mention or speculate why Upd produced in cap and terminal filament cells (Lopez-Onieva, 2008) do not play a role in this long range signal, while Hh and Wg do. The rest of the paper looks at life span studies using a combination different signaling pathways generated in both adults and larvae, which should be interpreted carefully. Perhaps the most interesting out of all of these is the results found in *cos2* and *smo; pka* double mutant clones in a *Su(fu)* mutant background. They find that FSCs are maintained well in the germarium and are not hyper or hypo-competitive. Therefore, this shows that FSCs *do not* need to have a higher Hh pathway activation than their follicle daughter cells.

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