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A Notch Above Bowl: Specification of Niche Cells in the Drosophila Testis

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Abstract
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We also uncover a role for the *bowl* pathway in influencing niche cell specification, where *bowl* promotes niche cell fate, while its antagonist, *lines,* promotes cyst cell fate. Additionally, we present data suggesting that *bowl* functions as a transcriptional repressor to restrict cyst cell gene expression in precursor cells, thereby inducing niche cell specification. Ultimately since niche cells influence stem cell behavior, understanding how niche cells develop and dissecting the interactions between niches and their resident stem cells is paramount if we seek to use stem cells as tools in regenerative medicine.

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ABSTRACT

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Stephen DiNardo

Niche cells exercise elaborate control over the behavior of many tissue-specific stem cells. However, in no system do we fully understand how niche cells are specified, develop and then begin producing the signals necessary to properly regulate stem cells. Here, we take advantage of the paradigmatic stem cell-niche system of the *Drosophila* testis to address these fundamental questions. We first find that the Notch signaling pathway is necessary for niche cell specification and that its activity in precursor cells prevents those cells from adopting the alternative somatic cyst cell fate. We also discover that the Notch-activating ligand, Delta, is presented from the neighboring endoderm, rather than from within the gonad "proper." Moreover, we show that niche specification occurs very early during gonadogenesis, before the expression of extant niche cell markers.

We also uncover a role for the *bowl* pathway in influencing niche cell specification, where *bowl* promotes niche cell fate, while its antagonist, *lines,* promotes cyst cell fate. Additionally, we present data suggesting that *bowl* functions as a transcriptional repressor to restrict cyst cell gene expression in precursor cells, thereby inducing niche cell specification. Ultimately since niche cells influence stem cell behavior, understanding how niche cells develop and dissecting the interactions between

niches and their resident stem cells is paramount if we seek to use stem cells as tools in regenerative medicine.

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INTRODUCTION

Overview

At its core, the field of developmental biology seeks to understand how a cell, a tissue, and an organism come to be. Fascinatingly, the development of most multicellular organisms begins with a single cell zygote, which divides mitotically to give rise to all of the cells within the body. These initially equivalent cells must eventually differentiate to contribute to the distinct tissues and organs that make up the body. The question of how this is accomplished has intrigued developmental biologists for centuries and has led to a series of sub-questions including: how does morphogenesis (the creation of ordered form) proceed, how is cell growth and division regulated, how do the egg and sperm become specialized cell types, how do changes in development drive evolution and how do environmental cues influence development?

Historically, model organisms have been used to address some of these fundamental questions. Using simple model systems, great advances in our understanding of basic developmental principles have occurred. For example, early studies on the chick embryo in the 1800's revealed that vertebrate embryos contain three germ layers, the endoderm, the ectoderm and the mesoderm, which produce the distinct organ systems of all three-layer organisms [Reviewed in (148)]. Additional studies on organisms as diverse as the frog, worm, mouse and fly have provided insight on topics as distinct as patterning of a body plan to the development and regulation of various tissues and organs to understanding diseases caused by genetic mutations.

One aspect of development that is particularly interesting is that some tissues and organ systems never stop developing, even after an adult organism is fully formed. In humans, for example, skin cells are replenished daily. Similarly, a continuous source of

blood cells must be supplied from the bone marrow to sustain life. In addition, simpler organisms, such as planaria, or amphibians such as the salamander, can regenerate severed body parts [Reviewed in (86)]. It is now appreciated that these phenomena are due to a pool of stem cells that have the capacity to self-renew and produce differentiating daughter cells throughout the course of an organism's lifetime. These adult stem cells are distinct from embryonic stem cells, which give rise to all of the cells of the body during development (Figure 1.1; from nih.gov) [Reviewed in (192)]. In my discussion below, I will focus on the increasingly studied branch of stem cell biology concerning adult stem cells.

Stem cells

Stem cells have been heralded as a potential cure-all for numerous diseases and maladies. However, we are just truly beginning to uncover the mechanisms that govern stem cells. Adult stem cells have the long-term capacity to self-renew, and in doing so maintain the integrity of many tissues and organs by replenishing lost cells [Reviewed in (56)]. Stem cells that give rise to all the cell types of a particular tissue are known as multipotent, such as hematopoietic stem cells (HSC), which produce all blood cells (Figure 1.2) [Figure taken from (7); (74)]. Unipotent stem cells give rise to only one cell type. A prime example are male germline stem cells, which only produce sperm (see Figure 1.1, lavender box) [Reviewed in (114)]. Both stem cell types reside in specialized microenvironments known as niches and must delicately balance the process of self-renewal– to produce more stem cells, with differentiation– to produce lineage-committed daughter cells (Figure 1.3) [Reviewed in (156)].

Defining stem cells *in vivo*

Historically, in mammalian systems, stem cells were proposed based on how well the cells retained label after being pulsed with a marker of DNA replication and chased for a number of weeks (41). This technique relied on the presumption that true stem cells divide infrequently and therefore would retain label. These cells came to be classified as label-retaining cells (LRC). However, there were several caveats associated with this methodology. First, if presumptive stem cells were quiescent during the pulse, the dye would not be incorporated into the cell. Second, since cells had to be permeabilized to allow for quantitative measurements of dye retention, the cells could not be studied further [Reviewed in (56)]. Now, with advances in genetic lineage-tracing, this new method has become the gold standard in defining previously unidentified or ambiguous stem cell populations *in vivo* in *Drosophila* as well as mice. By genetically marking stem cells and their descendants, this methodology has led to the unearthing of mammalian spermatagonial stem cells, muscle satellite cells, epidermal stem cells and intestinal stem cells, among others (15, 40, 104, 127).

Since invertebrate systems are typically simpler, it has proven easier to identify stem cells within a single-cell resolution, compared to mammalian systems with more complicated tissue architecture. For some invertebrate models, including the *Drosophila* ovarian and testis germline system, the *Drosophila* intestine and the germline of *C.elegans,* we can now define stem cells based on gene expression markers coupled with knowledge of their anatomical location [Reviewed in (126); (12, 42, 88, 95, 134)]. Taking into account the anatomical location of stem cells is important since transit-

amplifying daughter cells may share a similar gene expression pattern for a time shortly after division [Reviewed in (126)].

Cell divisions in stem cell systems

During steady-state operation of a stem cell system, stem cells balance self-renewal with differentiation through asymmetric cell division to properly maintain tissue homeostasis [Reviewed in (189)]. Typically, a stem cell division produces a differentiating daughter cell, which is displaced from the self-renewing source, while the other cell remains close to the niche cells and thus maintains stemness (Figure 1.4 A) (104, 139, 190). Asymmetric division can also be achieved via asymmetric segregation of molecular determinants into daughter cells, whereby a set of differentially inherited molecular cues promotes stemness (129). This is thought to occur during neuroblast division in *Drosophila* where one daughter cell remains a self-renewing neuroblast while the other daughter cell becomes a terminally differentiated glial or neuronal cell [Reviewed in (198)].

Stem cells also have the potential to divide symmetrically, giving rise to two stem cells or alternatively to two daughter cells (Figure 1.4 B). An asymmetric division that produces two stem cells is thought to occur when stem cell numbers need to be increased, namely during embryonic development and tissue repair (125). However, this mechanism is also likely to be causative in inducing cancer, where stem cells divide unregulated, forming tumors. In fact, so called “cancer stem cells” share many similarities with normal somatic stem cells, such as maintaining an undifferentiated state and the ability to produce lineage-committed daughter cells (Figure 1.4 C) [Rev in (20); (39)].

Another interesting quality of some stem cell systems is the ability of transit-amplifying daughter cells to de-differentiate to return to a stem cell-like state (Figure 1.4 D). This has been found to occur under experimentally induced conditions in both the male and female *Drosophila* germline systems as well as in the murine germline (25, 87, 128, 152). This reveals the potential plasticity of a stem cell system, whereby daughter cells can repopulate an empty niche if stem cells are lost. This also has a strong implication about the differentiated state: that at least early on, daughter cells from the stem cell are not irreversibly committed to differentiate. This mechanism could contribute to the replenishment of stem cells damaged or destroyed by environmental toxins, harsh chemical treatments or during the aging process (87).

Intrinsic and extrinsic self-renewal requirements

The process of stem cell self-renewal requires both intrinsic and extrinsic inputs (Figure 1.5). As such, stem cell self-renewal is a result of an intrinsic gene expression program that is modulated by extrinsic cues from the local microenvironment. We are just beginning to uncover factors necessary for intrinsic self-renewal and it appears that these regulators may function in a cell-type specific manner. A classic example for the requirement of intrinsic inputs for self-renewal lies in the *Drosophila* central nervous system (CNS) [Reviewed in (193)]. In the developing CNS, a neuroblast asymmetrically divides to produce a neuroblast daughter cell as well as a differentiating daughter cell. These distinct cell fates are determined based on the asymmetric segregation of a number of cell fate determinants, such as Prospero and Numb (99, 144). Cells that receive Prospero and Numb differentiate into a ganglion mother cell, which ultimately give rise to neurons or glia. Cells that do not accumulate these proteins remain as neuroblasts and

continue to divide in a stem cell-like fashion. Though studies of *Drosophila* neuroblasts may offer some insight, for many stem cell systems, the molecular mechanisms required for intrinsic self-renewal still remain a mystery. Understanding the principles that guide intrinsic self-renewal is key if we seek to use stem cells as therapeutic tools.

Finally, extrinsic cues emanate from supportive niche cells, which typically reside adjacent to the stem cell populations they support. These extrinsic cues can take the form of soluble signaling factors, membrane-bound factors or even the extracellular matrix [Reviewed in (154)]. I will discuss stem cell niches and their regulation of stem cells in further detail below.

Stem cell niches

Stem cell niches have recently been uncovered for numerous stem cell systems. A niche consists of the surrounding microenvironment where stem cells reside and acts to direct stem cell behavior and maintain tissue homeostasis [Reviewed in (126, 178)]. A niche typically produces several signals that are necessary to promote stem cell maintenance and self-renewal. Due to this, niche cells are critically important in maintaining the integrity of a stem cell system.

To date, two types of stem cell niches are thought to exist based on the physical relationship with the resident stem cells: “stromal” niches and “epithelial” niches (Figure 1.6) [Reviewed in (126)]. Stromal niches tend to develop independently of stem cells and maintain their morphology even in the absence of stem cells. These niches develop in precise anatomical locations adjacent to stem cells and provide short-range signals important for self-renewal. An example of such a niche can be found in the germlines of

both the *Drosophila* female and male in the form of cap cells in the ovary and hub cells in the testis (93, 157, 172, 182). In contrast, an epithelial niche is typically devoid of distinct niche cells and instead the stem cells contact the basal lamina, and/or other mature cells of the lineage, which regulate stem cell self-renewal. Mammalian muscle satellite cells reside in epithelial niches, such that the cells directly contact the basal lamina and the muscle fiber [Reviewed in (103)]. The basal lamina– a major component of the extracellular matrix, consists of mainly collagen, laminin and proteoglycans– and provides largely undefined, but important regulatory cues for these stem cells.

In many cases, the niche is not simply static, but is dynamic. The niche must respond to changes in the stem cell environment, such as in the case of tissue damage and subsequent regeneration. For example, in mammals, most hematopoietic stem cells (HSCs) are normally localized to the bone marrow, which acts as its niche. However, it has been shown that HSC number and hematopoiesis can drastically increase in other organs, such as the spleen and liver, in response to stress or bone marrow malignancies to produce more circulating blood cells (92). Additionally, researchers have shown that hair follicles can form *de novo* after wounding by establishing a stem cell population and co-opting neighboring cells to function as the niche (80). These examples exemplify the idea of a facultative niche, whereby the surrounding microenvironment takes on niche fate to support a new stem cell population (126). More work is still needed to be done, however, to fully understand how cells are transformed to function as facultative niche cells.

Defining the niche *in vivo*

Although supporting niche cells were proposed to exist since the 1970's (149), only recently do we have the tools necessary to prove their existence in some stem cell

systems. Therefore, recent work has hinged upon identifying the precise locations of niche cells using genetic manipulations and laser ablation techniques. The germline of the nematode, *C.elegans*, has emerged as a useful model. The distal tip cell (DTC) functions as the niche in the germline and signals via the Notch pathway to maintain germline stem cells (GSCs) (10, 42). Laser ablation of this single niche cell results in the loss of adjacent GSCs, proving its important role in regulating stem cell behavior (95).

Recent work has shown just how important niche cells are to regulate stem cell behavior. Studies in the *Drosophila* testis that genetically manipulate the number of niche cells, modulate the amount of signal produced from the niche cells, or assay changes in the niche in aging flies have confirmed that these cells directly regulate the number of stem cells present (23, 93, 97, 98, 111, 112, 172, 180). Furthermore, similar types of manipulative experiments have been performed in the hematopoietic stem cell (HSC) system, where osteoblasts are thought to comprise a critical component of the support niche (31, 194). In instances where the number of osteoblasts is increased, a larger population of HSCs is maintained. Even with evidence that osteoblasts contributed to the HSC niche, the definitive identity of all the cells that make up the niche remained unclear in this system. Recently, however, another piece of the puzzle has been solved, in that mesenchymal stem cells also regulate HSCs and are thought to form an important component of the HSC niche (122). Identifying all the cells that make up this niche will be necessary to have a complete understanding of the signals that regulate HSCs.

<u>An aberrant niche: The cancer stem cell niche</u>

Since the niche functions as the master regulator of stem cells, its activity must be tightly regulated to maintain tissue homeostasis and to prevent aberrant stem cell

behavior. As mentioned above, the ideas of a "cancer stem cell" (CSC) as well as a "cancer stem cell niche" have recently been proposed whereby a stem cell transforms and divides unregulated and can thus populate an abnormal niche [Reviewed in (20)].

Interestingly, the CSC niche appears to play a dual role in relation to CSCs. Similar to a normal niche, the CSCs rely on its extrinsic cues to maintain stemness (20). In addition, however, the CSC niche can play a protective role by sheltering the CSCs from toxic insults, associated with extant cancer therapies. This protection likely contributes to the therapy resistance found in some patients (53, 77). Although in most cancers it is unclear which cells make up the CSC niche, it is hypothesized that this tumor microenvironment also promotes metastasis by inducing an epithelial-to-mesenchymal transition in CSCs, allowing for tumor cell invasion throughout the body [Reviewed in (20)]. Thus it will be interesting to determine the mechanism of how the CSC niche permits or directs tumorigenesis by regulating CSCs. Elucidating the cells which function as the niche as well as the self-renewal signals they provide to CSCs could aid in developing more effective and targeted cancer therapeutics.

Signaling pathways in stem cell-niche systems

Although stem-cell niche systems vary from the simple to the complex many systems share common signaling pathways. I will explore the role of the four most commonly employed pathways- Notch, Wnt, Hegdgehog (Hh), and bone morphogenetic protein (BMP) -below.

Signaling within stem cell-niche systems is important since signals that emanate from niche cells regulate stem cell behavior. In fact, integrin- and cadherin-mediated

adhesion of stem cells to the niche cells is critical to ensure that the stem cells receive the proper self-renewal signals both in fly stem cell systems (23, 79, 111) as well as in mammalian systems. One prime example of a mammalian stem cell system that requires integrin-mediate adhesion is in the niche of epidermal stem cells– cells that eventually give rise to the epidermis [Reviewed in (117); (137)]. In instances where adhesion is lost in these systems, signaling is interrupted, stem cells differentiate and the integrity of the stem cell system is compromised.

Some signaling pathways that are commonly employed in stem cell systems include the Notch, Wnt (known as Wingless in *Drosophila*), Hedgehog (Hh) and bone morphogenetic protein (BMP) pathways [Reviewed in (132)]. In addition to localized signals emanating from niche cells, signaling can occur across cell types in stem cell niches where multiple cell types coexist. Since there are numerous regulatory pathways employed in stem cell systems, even within a single niche, it is critical to understand the crosstalk that occurs *in vivo* to specify and maintain a properly functioning system. Although the field has uncovered some roles for these pathways, more work still needs to be done to fully understand how these pathways function in stem cell-niche systems. Below, I will briefly highlight what is known and try to point out gaps in our knowledge.

<u>The Notch pathway</u>

Notch signaling is a developmentally conserved pathway in metazoans that mediates cell-cell interactions via a transmembrane receptor and ligands, Delta and Serrate [Reviewed in (9)]. Upon ligand binding, the Notch receptor is cleaved allowing the intracellular domain (N_{icd}) to translocate into the nucleus. N_{icd} can then bind to the Notch responsive transcription factor, Su(H) and activate target gene expression

[Reviewed in (27)]. Notch signaling ultimately regulates binary cell fate choices and allows the induction of numerous gene expression programs (51).

This pathway is necessary for stem cell maintenance and differentiation in several mammalian systems, including the neural, hematopoietic, and hair follicle (bulge cells) stem cell systems [Reviewed in (37); (8, 30)]. For example, in the adult brain, Notch is required to regulate the cell cycle to balance neural stem cell maintenance with daughter cell production [Reviewed in (1)]. However, although the vasculature likely contributes, it still remains unclear which cells make up the definitive niche for these neural stem cells (59). In flies, Notch is required for the maintenance of intestinal and germline stem cells (134, 157). Even so, from these studies it still remains unclear how the Notch pathway initially becomes activated in these systems.

Furthermore, distinct from its role in maintaining stem cells cell-autonomously, Notch signaling is also necessary for niche cell formation in the *Drosophila* ovarian niche (157, 182). Although this is true, in this system, it still remains ambiguous which tissue provides the Notch-activating ligand, when the niche cells are specified and how they begin to regulate their resident stem cells.

The Wnt pathway

The Wnt family of secreted proteins consists of growth factors that bind to and activate cell surface receptors of the Frizzled family [Reviewed in (141)]. During canonical Wnt signaling, β-catenin accumulates in the nucleus, interacts with the TCF/LEF family of transcription factors and is then able to promote target gene expression. Recent studies have shown that canonical Wnt signaling can direct HSC self-renewal *in vivo* as well as *in vitro* (142, 187). Similarly, Wnts play an important role in

maintaining and regulating stem cells in the crypts of mammalian intestines [Reviewed in (191)]. Although the intestinal stem cell (ISC) niche had been studied for a number of years, due to complicated tissue architecture only recently do we have an idea of which cells are "true" stem cells and which cells make up the supportive niche (15, 146). Interestingly, recent work has suggested that mammalian ISCs give rise to their niche cells, in the form of differentiated daughter cells, known as Paneth cells (146). Even so, it remains to be determined how Paneth cell number and their slow turnover rate is regulated.

Moreover, the Wnt pathway has been found to be deregulated in many cancers, including colon cancer, indicating its profound affect on stem cell behavior [Reviewed in (143); (174)]. In fact, adenomas were found to develop in intestinal crypts when stem cells were hyperactivated for the Wnt pathway in a murine model (14). Additionally, although a role has yet to be uncovered for Wnts in the *Drosophila* testis stem cell niche, Wingless protein accumulates in somatic stem cells adjacent to the niche (48, 112).

Lastly, in addition to its role in stem cells, Wnt signaling is also required to specify the niche in the nematode, *C.elegans.* Wnt signaling and the coordinate expression of the transcription factor Nkx2.2 is essential for the specification of the distal tip cell (DTC), which functions as the niche. However, the source of the Wnt ligand remains unknown (109).

The Hedgehog pathway

The Hh pathway plays an essential patterning role during the development of many organisms, as diverse as *Drosophila* and humans [Reviewed in (57)]. When the pathway is active, the Hh ligand binds to the cell surface transmembrane protein, Patched

(Ptc). This binding inhibits Ptc activity, allowing the transmembrane receptor, Smoothened (Smo) to accumulate and thus preventing the degradation of the pathway responsive transcription factor, Cubitus interruptus (Ci). Ci is then able to accumulate in the nucleus and promote target gene expression. Work from the *Drosophila* ovary has shown that Hh produced from the niche cells is necessary for follicle stem cell proliferation (196). Additionally, Sonic Hedgehog (Shh), the mammalian homolog of Hh, regulates neural stem cells in the adult mouse brain (5). Hh activation in these systems is important for proper stem cell regulation, yet how the Hh pathway becomes activated is unknown.

We also find that Hh protein accumulates in niche cells of the *Drosophila* testis (48, 54). However, it still remains unclear what role Hh is playing in this system. Finally, Hh signaling has also recently been implicated in tumor progression in lung cancer as well as intestinal cancer (17, 183). Although the exact mechanism has not been elucidated, tumors likely result due to abnormal activation of the pathway, which allows normal stem cells to adopt cancer stem cell fate [Reviewed in (57)].

The BMP pathway

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily of ligands [Reviewed in (3)]. Signal transduction begins when BMPs bind to a type II receptor, which recruits and phosphorylates a type I receptor. The type I receptor then phosphorylates a SMAD, which forms a complex with a co-SMAD. This complex can then translocate into the nucleus and activate downstream target genes. The BMP pathway has been shown to promote the self-renewal of mouse embryonic stem cells, but repress the proliferation of intestinal stem cells (67, 75, 138).

Additionally, the BMP pathway plays a critical role in promoting the self-renewal of somatic stem cells (SSCs) and germline stem cells (GSCs) in the *Drosophila* ovary (34, 96, 158). In GSCs, BMP activity acts to repress the expression of differentiation genes, such as *bag of marbles* (34, 158). However, the BMP targets necessary for SSC self-renewal remain largely unknown. Similarly, BMP activity is necessary to maintain germline stem cells in the *Drosophila* testis hinting that conserved mechanisms may be at play in the two germline stem cell systems (90, 111, 155). Although this is true, BMP does not seem to regulate somatic stem cell maintenance in the testis, also illustrating differences (111). Furthermore, in the testis niche, a few issues remain unclear. It is unknown which *Drosophila* BMP ligand(s) are required for GSC maintenance, how the BMP ligands are regulated and which cells produce the necessary pathway activating ligands.

Though, it is clear that we have uncovered many principles guiding stem and niche cell biology, there are still very large gaps in our current knowledge of stem cell-niche systems. As stated previously, though niche and stem cell identification still remains a difficult task in most mammalian systems, we now have the capacity to identify niche and stem cells at a single cell resolution in invertebrate model organisms, such as the fly and worm. Given these tools, we can delve deeper into understanding the mechanisms within these systems and perform experiments on a finer scale. In our lab, we therefore take advantage of the well-characterized *Drosophila* male germline system to further our understanding of stem cell and niche cell biology.

The *Drosophila* male germline system: A model niche

The adult testis niche

The *Drosophila* male germline has emerged as an excellent model system to study *in vivo* adult stem cells. The testis is a stem cell-based tissue, operating at steady state to sustain spermatogenesis. The niche is localized to the apical tip of the testis and is comprised of two distinct populations of stem cells, germline stem cells (GSCs) and cyst stem cells (CySCs), which cluster around a population of terminally differentiated somatic cells, called the hub (Figure 1.7) (68). There are typically 10-15 GSCs, 20-30 CySCs and 10-12 hub cells. A GSC usually divides asymmetrically through an oriented division giving rise to a daughter cell that remains adjacent to the self-renewal source, and thus a stem cell, and a daughter that is displaced from the niche and differentiates as a gonialblast (GB) (190). GBs then undergo four rounds of transit amplification giving rise to spermatogonia that differentiate and enter meiosis to produce a mature population of sperm. The CySCs appear to also have an oriented division and produce both stem cells and somatic daughter cells (36). These daughter cells encyst the differentiating germline cells and provide key signals for progression through spermatogenesis (50, 68, 94, 120, 150, 171).

Several signals implicated in stem cell maintenance and self-renewal emanate from hub cells. The ligand Unpaired (Upd) activates the Jak-STAT pathway in the immediately adjacent tier of germline and cyst cells. STAT activation in a cell, which promotes adhesion to the hub, is essential for its maintenance as a GSC or CySC (79, 93, 112, 172). Not only is STAT required, but its activation is sufficient for the renewal of

CySCs (93, 112, 172). In contrast, STAT activation is not sufficient for GSC renewal, suggesting another signal at play.

In fact, it appears that both the hub cells and CySCs function as the niche for GSCs (111). The BMP ligands *decapentaplegic* (*dpp)* and *glass bottom boat (gbb)* might regulate GSC self-renewal since they are both produced by hub cells and CySCs. BMP pathway activation is essential in maintaining GSCs and acts to repress the expression of the gene *bag of marbles (bam)*, which promotes GSC differentiation (111). Loss of critical BMP pathway components in GSCs results in de-repression of *bam*, precocious differentiation and a loss of stem cells (90, 150, 155).

Though the adult testis has been studied for a number of years and is one of the most well understood stem cell-niche systems, we in no way have a complete grasp on all the signaling interactions or mechanisms necessary that allow this tissue to be maintained. Furthermore, we are just beginning to understand the development of this testis niche and uncover the signals required to establish each of the cell populations. In the next section I will describe what is known about gonad formation and initial niche specification, as well as highlight gaps in our current knowledge.

Gonad formation and niche specification

In order to truly understand how a stem cell-niche system is organized, one must study the development of the organ. This germline stem cell-niche system is established during embryogenesis in the male gonad, the developmental precursor to the adult testis. Although the adult testis stem cell niche has been studied in greater detail, there is a similar structural architecture found within the gonadal stem cell niche (Figure 1.8) (110). Bilaterally symmetric gonads are formed during mid-embryogenesis from two distinct

lineages: primordial germ cells (PGCs) and mesodermally-derived somatic gonadal precursor cells (SGPs) (2). Germ cells develop at the posterior pole of the embryo and are internalized in the posterior midgut (PMG) during gastrulation (32). They then migrate through the endoderm to reach the mesoderm. While germ cells are migrating, the SGPs are specified from the lateral mesoderm in parasegments 10-12, and begin associating with germ cells at stage 11 (Figure 1.9) (21, 22, 29, 159). The SGPs and the germ cells then migrate together anteriorly, arrest and finally coalesce at stage 14 within parasegment 10 (21, 22, 38). Although activity of the homeotic gene, *abdominal A*, is necessary to halt migration in PS10, it is not clear what guidance cues prompts these cells to migrate (22). SGPs then extend cellular processes to ensheath the germ cells, resulting in a spherical, compacted gonad (84).

Though hub cells were identified as the regulators of GSCs some years prior, only recently do we have an idea of where these cells originate. Lineage-tracing experiments have demonstrated that hub cells derive from the anterior two-thirds of SGPs, definitively from parasegment (PS) 11. The remaining hub cells likely derive from parasegment 10, given that the hub eventually compacts in PS10 (110). Since hub cells derive (at least) from PS11, these hub-specified cells must migrate anteriorly to properly coalesce and compact with other hub-specified cells in PS10. The mechanisms by which this guided migration and hub cell compaction occur still remain unclear.

Only some of the PS10 and PS11 SGPs become hub cells, while the remainder likely adopt cyst cell fate (48). This suggests that SGPs give rise to both hub and cyst cells, although, it is not known which signaling pathways are responsible for inducing these differential cell fates. It has been shown, however, that receptor tyrosine kinase

(RTK) signaling mediated by the Boss/Sevenless and Epidermal growth factor receptor (EGFR) pathways inhibit hub cell formation among posterior SGPs, while permitting formation in the anterior (97, 98). Even so, it is not clear how these pathways become activated among posterior cells and how they act to prevent hub cell specification.

As mentioned above, SGPs also give rise to cyst cells. Cyst cells initially specified during gonadogenesis can remain as cyst cells or adopt cyst stem cell (CySC) fate. Cyst cells that lie adjacent to the hub and therefore the self-renewing ligand, *upd*, likely adopt CySC fate; those further away from the source of *upd*, remain as cyst cells and begin to differentiate. Although no marker currently exists to unambiguously identify CySCs during gonadogenesis, we know that *upd* activates the Jak-STAT pathway in germline cells adjacent to the hub prompting them to adopt GSC fate (153). Since both stem cell types rely on Jak-STAT pathway activity for proper maintenance in the testis, it is likely that it is also necessary for initial stem cell specification (111, 112).

Hub cells have been thought to be specified late in embryogenesis, since they are not visible until near hatching of the first larval instar (110). Hub cells can then be visualized as a tight cluster of somatic cells at the anterior end of each gonad, by using either cell surface or gene expression markers, such as *Drosophila* E-cadherin or *upd* (46, 63, 110, 165, 184). Although we could visualize hub cells once the stem cell-niche system had been established, it was unclear how hub cells were specified. Until recently, no pathway necessary to promote hub cell fate had been identified (97). In that study, the authors showed that the Notch pathway was necessary for hub cell specification. However, it still remained unclear when hub cells were specified, how hub cell number was tightly regulated, how hub cells aggregated at the anterior, what genes are activated

downstream of Notch signaling, how hub cells begin to upregulate the expression of the extant hub cell markers and how they begin to express factors that ultimately regulate stem cell behavior.

Given the importance of hub cells to stem cell survival, it is important to know how they become specified during embryogenesis. Additionally, given the developmental relationship between hub cells, which function as the niche, and cyst cells, a subset of which can function as stem cells, how hub cells come to be specified is important to understand. Essentially, a single developmental pathway could control niche versus stem cell fate in the *Drosophila* testis and this balance must be maintained to ensure a properly functioning stem cell-niche system.

Project Summary

Through my thesis work, I have sought to understand what signaling pathways are important for specifying a critical component of the *Drosophila* testis niche, the hub cells. Previous microarray expression data from our lab allowed me to take a candidate approach to identify pathways involved in hub cell specification. In Chapter 2, I show that the Notch signaling pathway is necessary for this process. Surprisingly, I found that hub cells were specified much earlier than previously presumed and before the expression of extant markers. I further show that the Notch-activating ligand, Delta, is presented from a tissue source outside of the gonad: the neighboring endoderm. Chapter 3 reveals that the *bowl* pathway also influences hub cell specification, although its exact relationship to the Notch pathway still remains ambiguous. Finally, in Chapter 4 I provide an extensive summary and discussion of my work as a whole and attempt to place my data into the context of the stem and niche cell biology field.

Overall, our data provides key insight into the specification of an *in vivo* niche. Understanding which signaling pathways specify niche cells and by extension regulate stem cells in vivo is paramount if we seek to use stem cells as tools in regenerative medicine.

Figure 1.1:

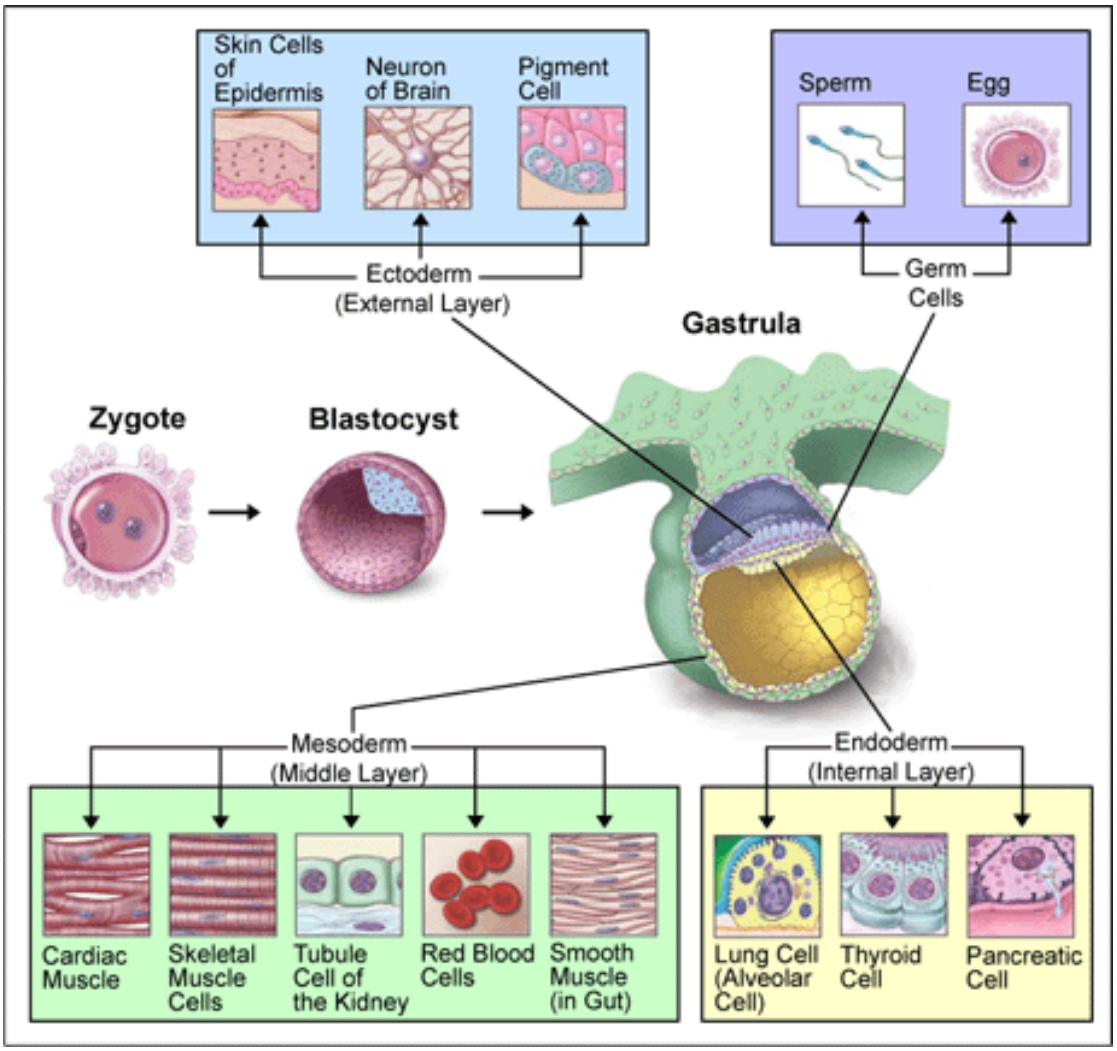


Figure 1.1: Differentiation of human tissues

In the first few hours after fertilization, the single cell zygote divides, giving rise to identical cells. These cells begin to specialize, forming the blastocyst. Inside the hollow sphere of the blastocyst lies a cluster of cells called the inner cell mass (light blue). The inner cell mass can give rise to germ cells (lavender box) as well as specialized cells derived from the three germ layers (endoderm, yellow box; mesoderm, green box; ectoderm, blue box). (Figure taken from NCBI/NIH)

Figure 1.2:

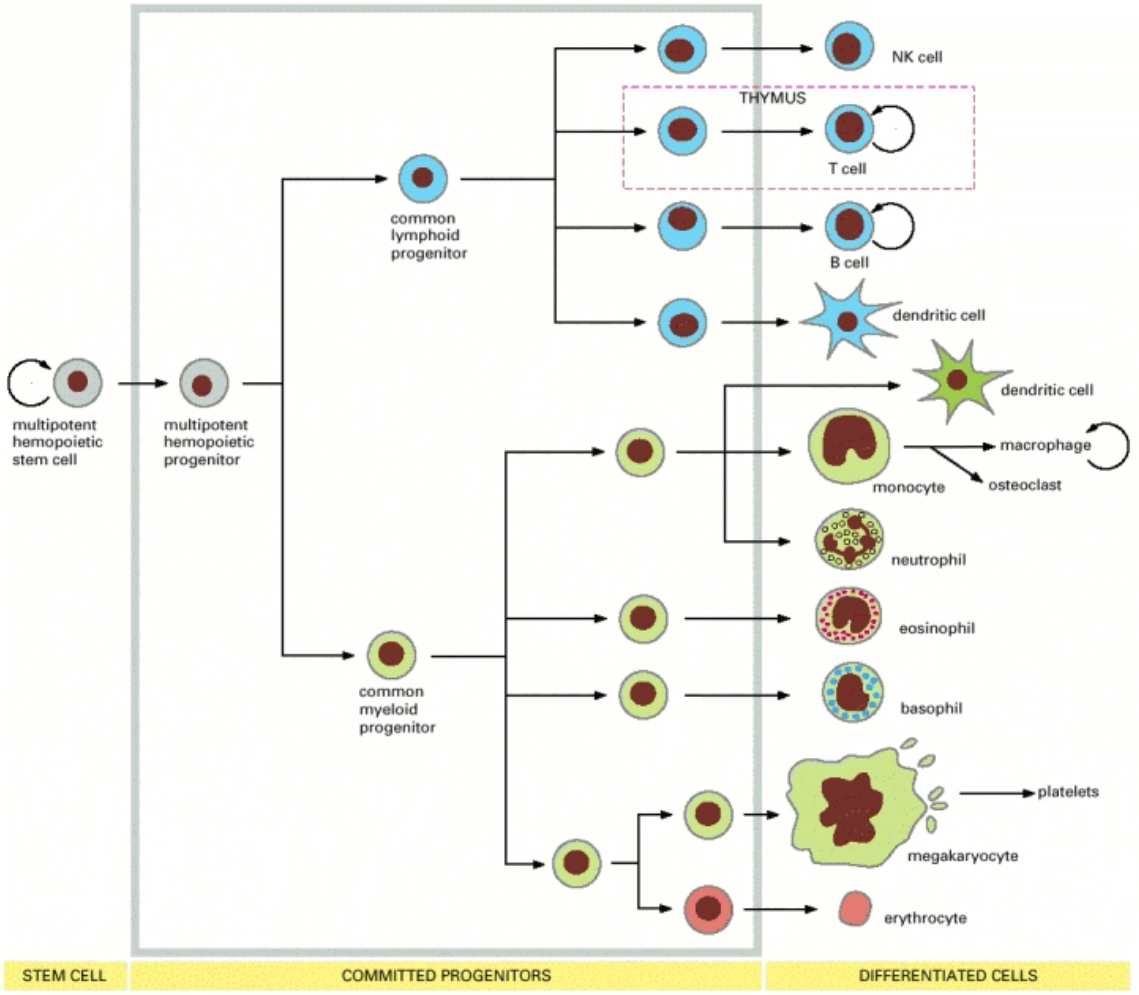


Figure 1.2: A scheme of hematopoiesis

The multipotent hematopoietic stem cell normally divides infrequently to generate more multipotent stem cells or to give rise to committed progenitors. These progenitors give rise to all the specialized cell types of the blood lineage. [Figure taken from (7)]

Figure 1.3:

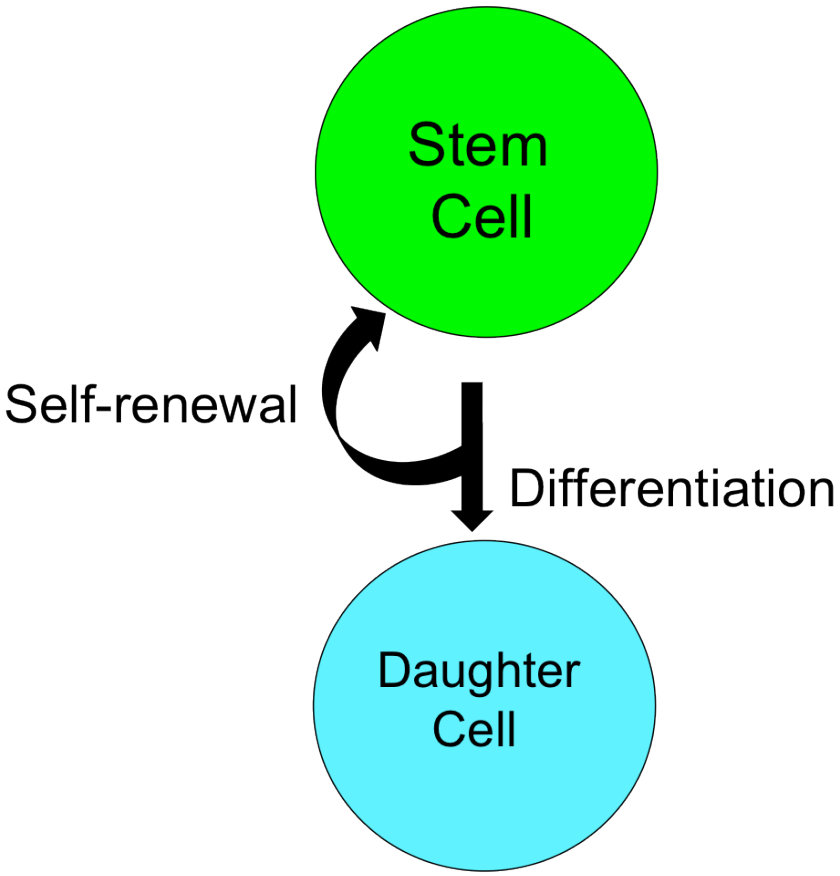


Figure 1.3: Stem cells balance self-renewal with differentiation

Stem cells must balance the process of self-renewal, to produce more stem cells, with differentiation, to produce lineage-committed daughter cells.

Figure 1.4:

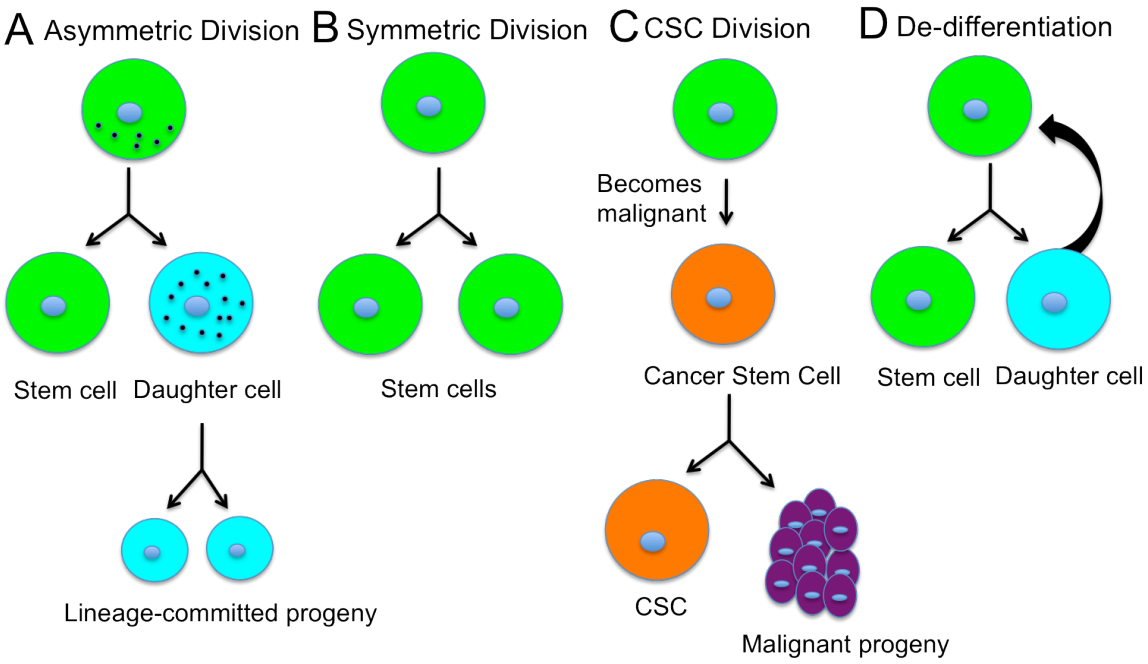


Figure 1.4: Modes of stem cell division

(A) A stem cell (green) can divide asymmetrically to give rise to a stem cell and a differentiating daughter cell (blue), which produces lineage-committed progeny. Often times, asymmetrical segregation of cell fate determinants (small dots) into one cell lead to this asymmetric division. (B) A stem cell undergoes a symmetric division, giving rise to two stem cell progeny. Note that a stem cell can also divide symmetrically to give rise to two differentiating daughter cells. (C) A normal stem cell (green) is transformed into a malignant cell and a cancer stem cell (CSC, orange) is born. Similar to normal stem cells, a CSC can divide to produce a self-renewing stem cell daughter or differentiating malignant progeny (purple). (D) Instead of producing lineage-committed progeny, if necessary, the daughter of a stem cell division can de-differentiate to adopt stem cell fate. This illustrates the plasticity of transit-amplifying daughter cells.

Figure 1.5:

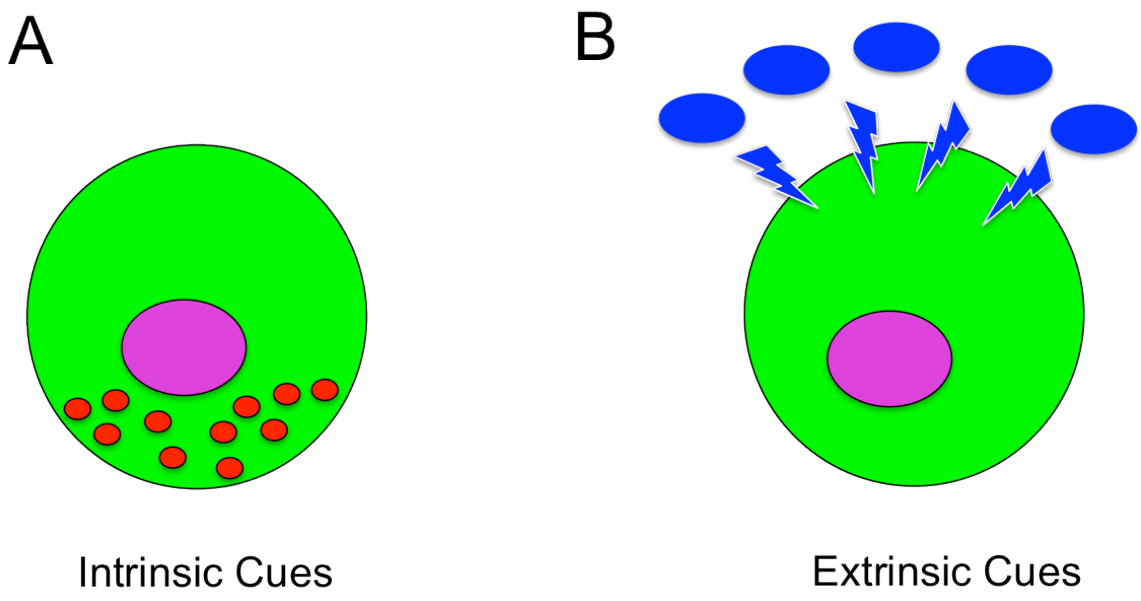


Figure 1.5: Intrinsic versus extrinsic cues for stem cell renewal

Stem cells are depicted in green. (A) Intrinsic cues, in the form of asymmetrically segregated proteins (red dots) are necessary for proper regulation of stem cell self-renewal. For example, *Drosophila* neuroblasts divide asymmetrically by segregating cell fate determinants, such as Numb and Prospero. These proteins determine which daughter cell self-renews and which daughter cell differentiates. (B) Extrinsic cues emanate from neighboring niche cells (blue) and are required to regulate stem cell behavior.

Figure 1.6:

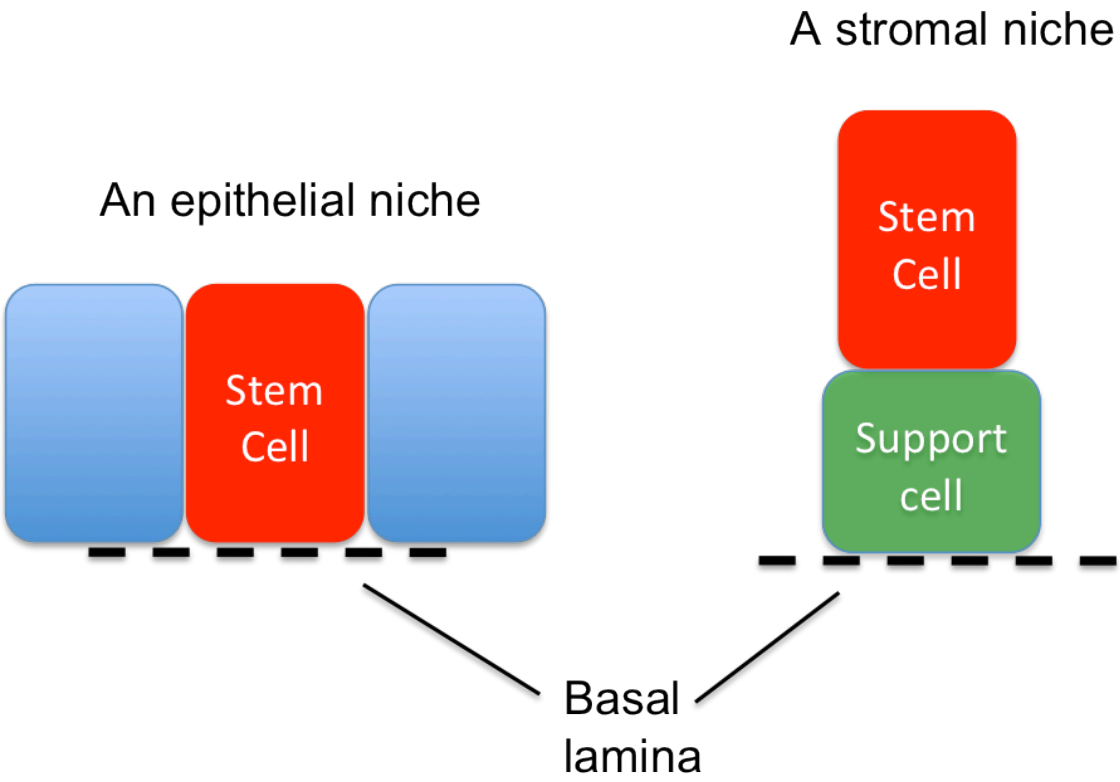


Figure 1.6: The two basic types of niches

In an epithelial niche (left), the stem cell is in direct contact with the underlying basal lamina and contacts neighboring cells (blue). In a stromal niche (right), the stem cell contacts a support cell that contacts the basal lamina. [Modified from (118)]

Figure 1.7:

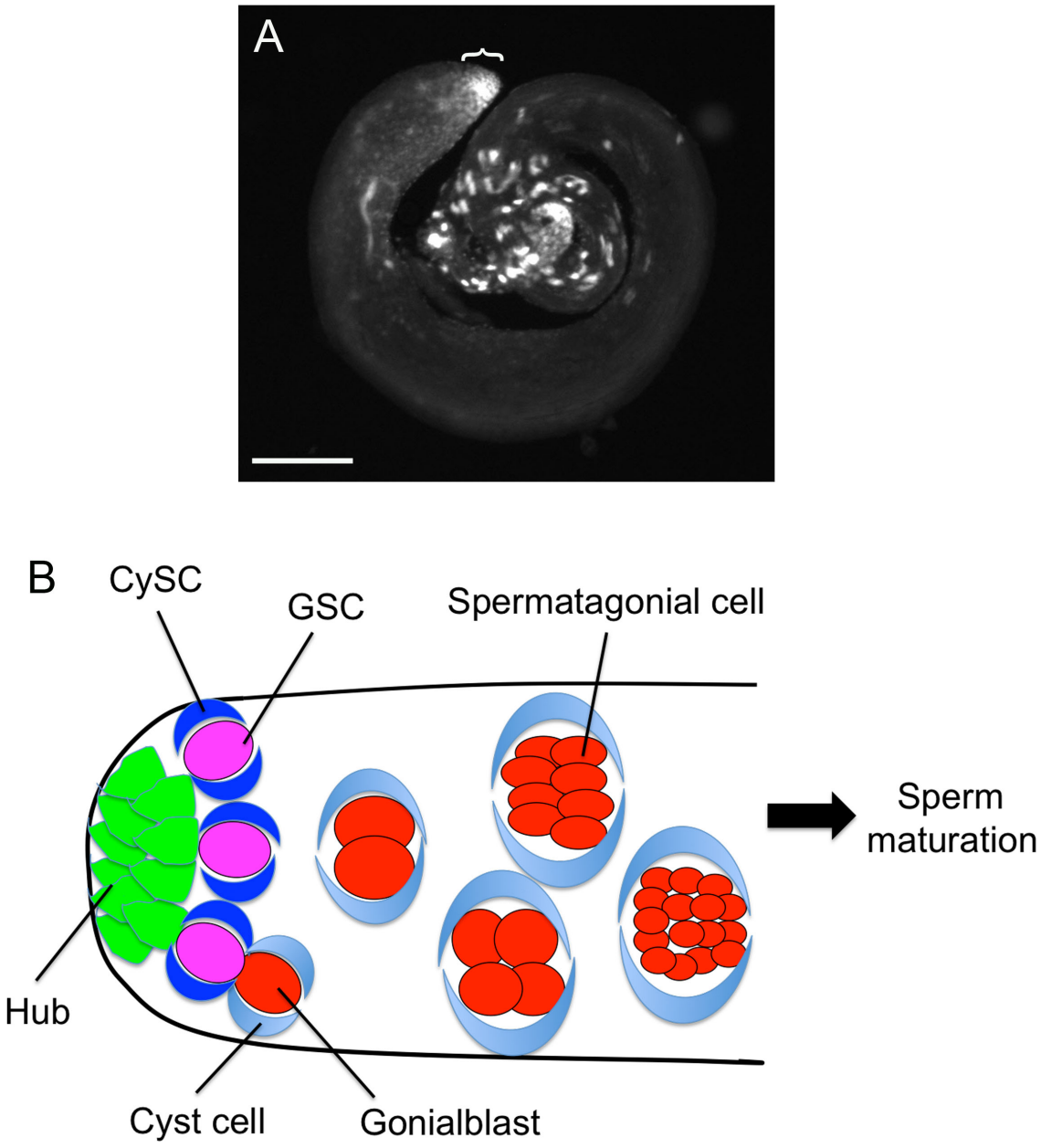

A
B
CySC
GSC
Spermatagonial cell
Sperm
maturation
Hub
Cyst cell
Gonialblast

Figure 1.7: The *Drosophila* testis stem cell niche

(A) In a wild type testis bright DNA-positive cells are found at the testis apex (bracket). Note that there are also DNA-bright cells at the other end of the testis (center of image), which are compacted haploid sperm nuclei. Scale bar is 150 μm. (B) Model of the testis stem cell niche. At the testis tip, germline stem cells (GSCs, magenta) and cyst stem cells (CySC, dark blue) circumscribe the hub (green). A GSC and its associated CySCs divide to produce a differentiating daughter cell, known as a gonialblast (GB, red), which is encysted by daughter cyst cells. The GB then mitotically divides four times producing spermatagonia, which eventually produce mature sperm, all the while encysted by supporting cyst cells.

Figure 1.8:

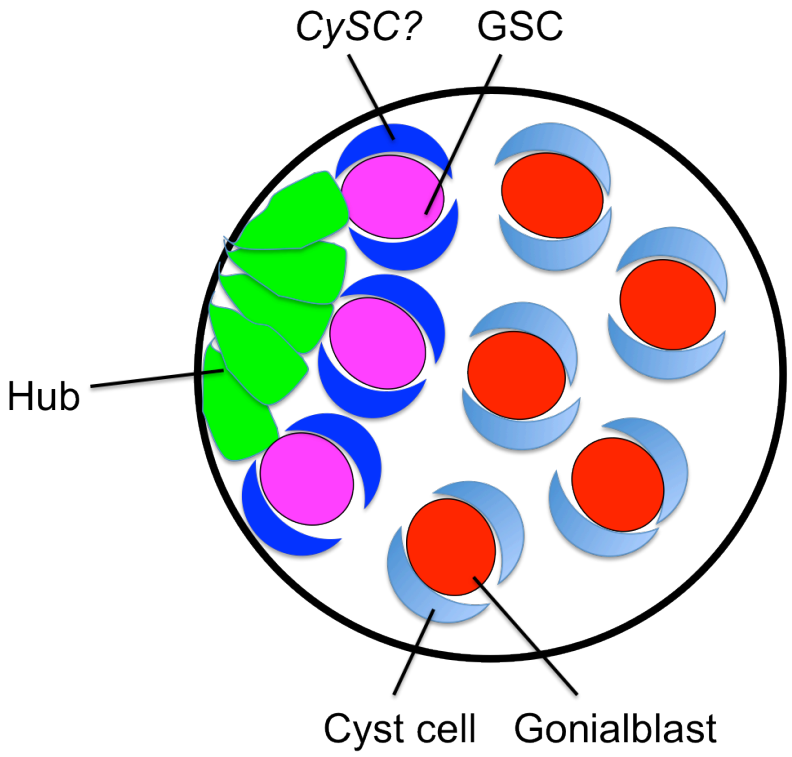


Figure 1.8: Model of the *Drosophila* gonadal stem cell niche

Note that similar cell types are found in the gonadal stem cell niche, the precursor of the adult testis niche. Stromal hub cells (green) are anchored at the anterior pole of the gonad and are surrounded by germline stem cells (GSCs, magenta) and likely cyst stem cells (CySCs, dark blue), although no markers exists to conclusively identify CySCs at this early stage. GSCs divide to produce gonialblast daughters and differentiating spermatagonia (red) encysted by support cyst cells (light blue), which eventually populate the adult testis niche. Only the 1st tier of germline cells receive the self-renewing signal, Unpaired, from the hub and are fated as GSCs.

Figure 1.9:

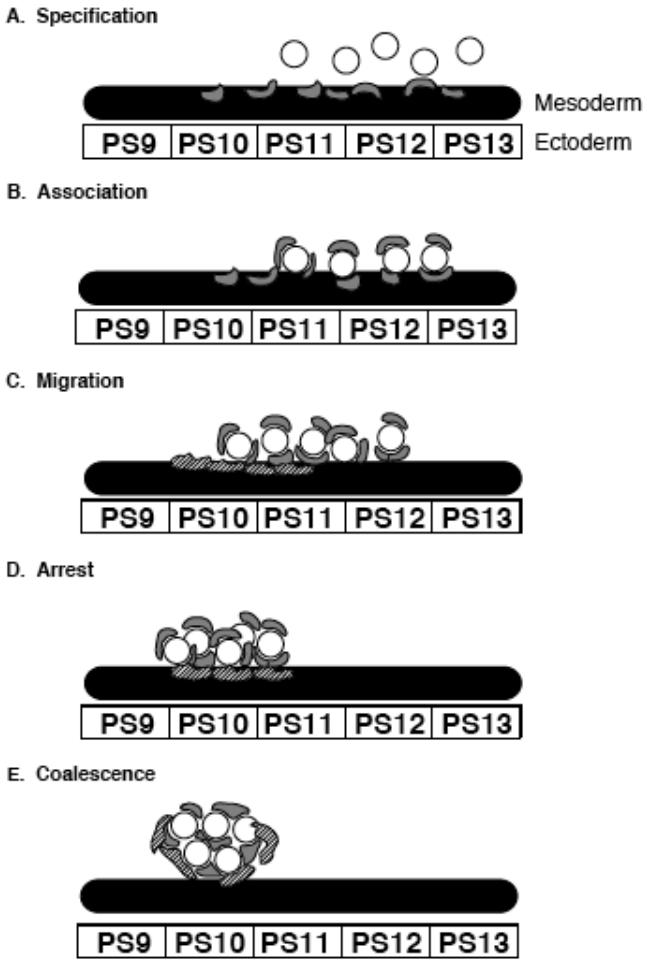


Figure 1.9: The stages in *Drosophila* gonad formation

In each panel, the lower boxes represent the ectoderm of parasegments (PS) 9-13, while the black solid area above represents the overlying mesoderm. (A) During stage 11, somatic gonadal precursors (SGPs, gray) are specified in PS10 through PS13. (B) After germ cells (white circles) exit the midgut, they associate with SGPs. (C) SGPs and germ cells migrate anteriorly. (D) SGPs and germ cells arrest migration in PS10. (E) SGPs and germ cells coalesce as a gonad at stage 14, within PS10. SGPs extend cellular processes to ensheath germ cells, resulting in a spherical, compacted gonad. [Figure taken from (22)]

Chapter Two:

THE ENDODERM SPECIFIES THE MESODERMAL NICHE FOR THE GERMLINE IN *DROSOPHILA* VIA DELTA-NOTCH SIGNALING*

*Portions of this chapter were published as: **Okegbe, T.C.** and DiNardo, S. (2011) The endoderm specifies the mesodermal niche for the germline in *Drosophila* via Delta-Notch signaling. *Development.* **138**, 1259-1267.

Summary

Interactions between niche cells and stem cells are vital for proper control over stem cell self-renewal and differentiation. However, there are few tissues where the initial establishment of a niche has been studied. The *Drosophila* testis houses two stem cell populations, which each lie adjacent to somatic niche cells. Although these niche cells sustain spermatogenesis throughout life, it is not understood how their fate is established.

Here we show that Notch signaling is necessary to specify niche cell fate in the developing gonad. Surprisingly, our results indicate that adjacent endoderm is the source of the Notch-activating ligand, Delta. We also find that niche cell specification occurs earlier than anticipated, well before the expression of extant markers for niche cell fate. This work further suggests that endoderm plays a dual role in germline development. The endoderm assists both in delivering germ cells to the somatic gonadal mesoderm, and in specifying the niche where these cells will subsequently develop as stem cells. Since in mammals primordial germ cells also track through endoderm on their way to the genital ridge, our work raises the possibility that conserved mechanisms are employed to regulate germline niche formation.

Introduction

Interactions of tissue-specific stem cells with their local microenvironment, or niche, are vital for proper stem cell self-renewal and differentiation [Reviewed in (126)]. Although rough locations for numerous stem cell niches have been elucidated in mammals and invertebrates, in many cases we do not understand how the niche is specified, nor can we molecularly identify niche cells in vivo (126). An understanding of the principles of niche cell development will be key in order to use stem cells effectively in therapeutics, as niche cells regulate important aspects of stem cell behavior. For example, in the absence of a self-renewal signal from niche cells, *Drosophila* germline cells differentiate, preventing stem cell maintenance and proper tissue homeostasis (93, 98, 158, 172, 182). Similarly, when ectopic or excess niche cells are induced, extra cells adopt stem cell characteristics, leading to the proliferation of stem-like cells, and potentially tumors (182). Therefore, it is important to fully understand which signaling pathways are necessary to establish a niche.

We have a partial understanding of niche cell development in two tissues maintained by germline stem cells, however unanswered questions remain. Studies from the *Drosophila* ovary have shown that Notch signaling is required during development to properly specify cap cells, which function as the niche (157, 182). However, it remains ambiguous how the cap cells become activated for Notch and which neighboring cells present the signaling ligand. In the development of the *C.elegans* germline, the distal tip cell (DTC) functions as the niche (18, 95). Although it appears that Wnt signaling and the coordinate expression of the transcription factor Nkx2.2 is essential for DTC specification, the source of the Wnt ligand remains unknown (109).

As the *Drosophila* testis stem cell-niche is amenable to the study of signaling pathways (93, 98, 172) we have chosen to investigate how the niche is specified in this model system. To understand what signaling pathways are at play *in vivo,* a microarray experiment was previously performed in our lab using testes genetically enriched for the niche and its stem cells (169). In examining the list for coordinate enrichment among components of a given signaling pathway, the Notch pathway emerged as one candidate to explore. This was promising because Notch has been implicated in various stem cell systems (134, 147, 157, 182).

Preliminary data from our lab suggested a role for Notch signaling in the somatic cell population of the *Drosophila* adult testis stem cell-niche. Importantly, Notch reporter expression is detected in somatic cell types and is lost within the hub cell population when Notch signaling is inactivated indicating that this pathway acts within the hub (Terry, unpublished result). Furthermore, overexpression of the Serrate ligand induces ectopic hub cell formation (Terry and Kelliher, unpublished results). However, our lab was unable to uncover a requirement for continual Notch signaling in the adult steady-state niche. Since it is possible that one pathway can play a distinct role in the development versus the maintenance of a system, we wondered whether Notch had an earlier role during the formation of the niche.

The Notch signaling pathway is an evolutionarily conserved developmental pathway that mediates cell-cell interactions [Reviewed in (9)]. Notch signaling is mediated through the Notch receptor, which is a single-pass membrane tethered receptor containing a large extracellular domain with 36 EGF-like repeats (186). In *Drosophila*, there is one Notch receptor and two ligands, Delta and Serrate, which are similarly single

pass transmembrane proteins that interact and activate the Notch receptor through their extracellular DSL (Delta/Serrate/Lag-2) domain (52, 166). Notch activation, which occurs when the receptor and ligands interact on adjacent cells, mediates three well-studied development processes: lateral inhibition, boundary formation and cell fate assignment [Reviewed in (26)]. Ligand activation of the receptor leads to Notch cleavage in the signal-sending cell, allowing its intracellular domain (N_{icd}) to translocate into the nucleus. N_{icd} then binds to Su(H), the Notch responsive transcription factor, permitting this complex to activate downstream target genes.

Here we show that Notch signaling is required for niche cell specification, exemplifying its role in the developmental process of cell fate assignment. We uncover a key role for Notch signaling in the initial allocation of SGPs to hub cell fate [see also (97)]. Additionally, our results suggest that the posterior midgut cells are the source of the ligand, Delta, which induces hub cell fate. Finally, we show that a subset of SGPs is activated to take on hub cell fate shortly after initial SGP specification and before gonad coalescence, much earlier than previously thought.

Results

Notch signaling specifies hub cell fate

To test whether the Notch pathway was necessary to specify hub cell fate, we examined Notch mutants. We scored hub cell number shortly after larvae hatch, in animals aged 22-25 hours after egg lay (AEL; see Materials and Methods) (110). Gonads were stained for germ cells (Vasa), for somatic cells (Traffic jam) and for hub cells, using either a cytoskeletal or gene expression marker. For instance, in wild type gonads, hub cells accumulate high levels of the f-actin-binding protein, Filamin (165), and are

circumscribed by a rosette of germ cells (Figure 2.1 B, green). We quantitated total hub cell number by stepping through z-slices in the image stack (see Materials and Methods). In controls, we observed an average of 11 hub cells per gonad (11 ± 0.3 (s.e.m.); n=12; Figure 2.1 B). However, Filamin-positive hub cells were not detected in gonads from $N^{264.39}$ mutant larvae (n=35; Figure 2.1 C). In addition, larvae carrying a hypomorphic mutation of Notch, N^{ts1}, exhibited reduced hub cell number when grown at non-permissive temperature compared with controls (Figure 2.1 H; 8 ± 0.6 versus 12 ± 0.4, respectively; $p<0.0001$; we consistently found slight differences in the average hub cell number among various control genotypes, and attribute this variation to differences in genetic background. Consequently, we always report the data compared to sibling controls). Importantly, in Notch mutants the proper number of somatic gonadal precursor cells (SGPs) were specified as stage-matched $N^{264.39}$ mutants and wild-type embryos had comparable numbers of Tj-positive cells (Figure 2.1 A; averaging 39 ± 2.3 versus 42 ± 0.9, respectively; $p=0.3$). This indicates that although the precursor population is properly specified, SGPs cannot adopt hub cell fate in the absence of Notch. Additionally, *Notch* mutations did not affect the specification of posterior male-specific SGPs (data not shown). This reveals that SGPs can properly differentiate into other specialized somatic cell types within the gonad. Thus, Notch signaling appears to be specifically required for hub cell specification.

As an additional test for a role of Notch in hub cell specification, we assayed larval gonads using an enhancer trap at *escargot* (*esg*), a gene expression marker of hub cell fate (110). In control gonads, all Filamin-enriched cells were *esg*-positive (Figure 2.1 D). In contrast, we observed a drastic reduction in the number of *esg*-expressing cells

specified in $N^{264.39}$ mutant gonads. Approximately 50% of gonads exhibited no *esg*-lacZ expression (8/17), while the remainder had two or fewer *esg*-lacZ-expressing cells (Figure 2.1 E). It is known that *esg* is detected in a number of anterior SGPs before its expression becomes restricted down to the hub during late embryogenesis (63, 110). Given this, it is possible that the absence of Notch activity results in the loss of some early expressing *esg*-positive cell types, but there exist no specific markers for such cells to definitively establish this.

Finally, hub cells express Unpaired (93, 172) which activates the Jak-STAT pathway in adjacent somatic and germline cells (152). One readout of pathway activation is the stabilization and accumulation of STAT protein (35). In controls, STAT protein accumulated at high levels in somatic and germline cells adjacent to the hub, as well as in hub cells themselves (Figure 2.1 F) (152). In contrast, in $N^{264.39}$ mutant gonads, STAT accumulation was undetectable (Figure 2.1 G). Taken together, we conclude that Notch signaling is necessary for proper hub cell specification.

Notch is activated within the SGP population

To determine if SGPs within the developing embryonic gonad were activated for the Notch pathway, and whether such cells eventually contribute to the hub, we employed a Notch reporter. We used a reporter construct encoding a chimeric Notch-GAL4-VP16 receptor (under control of a *hsp70* promoter) (162). Upon heat shock, the chimera will be expressed on all cells. Subsequently, in any cells activated for Notch, processing of its intracellular domain will also release Gal4-VP16, which can induce expression of a UAS-lacZ transgene. By the time of gonad formation during embryonic stage 13, we were able

to detect reporter activation in a subset of SGPs (Figure 2.2 A). Indeed, if such embryos were aged until the hub formed, and stained for LacZ protein, we found that Notch-activated SGPs could become hub cells (Figure 2.2 B, arrows; 50% of hub cells were lacZ-positive; n=16). These data showed that Notch is activated in a subset of SGPs, and that such cells can contribute to the hub. Interestingly, we also noted that Notch-activated cells were not restricted to the anterior of the developing gonad, but were also found in the middle and posterior (Figure 2.2 A). However, receptor tyrosine kinase (RTK) pathways active in the posterior of the gonad antagonize Notch, likely preventing these middle and posterior activated cells from adopting hub cell fate [see Discussion; (97)].

Hub cells are specified before gonad coalescence

We next wanted to identify the stage of gonadogenesis at which Notch is required to specify hub cell fate. It was previously thought that hub cell specification occurred after gonad coalescence, once germ cells and SGPs had formed a contiguous tissue (110). To perform our experiments, we again took advantage of the *hsp70*-Notch-GAL4-VP16 chimera, which functions as a wild-type receptor. In fact, delivering three heat shocks was sufficient to rescue formation of the ventral epidermis in *Notch* mutant embryos (162). We expressed the transgene in a Notch mutant background and assayed for the rescue of hub cell specification in larval gonads. To activate the receptor globally we delivered three 40 min heat shocks, each followed by a 45 min recovery period at 25°C. Embryos that received the first heat pulse at 8-9 hours AEL (mid-stage 12) appeared similar to non-heat shocked controls. In both cases, more than two-thirds of the gonads analyzed lacked any hub cells (Figure 2.3, compare yellow with blue bars). Note that a

few hub cells were observed among non-heat shocked *Notch* null embryos that carried the *hsp70*-Notch transgene (never more than 7 specified per gonad). As this is the same *Notch* null allele as in Figure 2.1, the occasional hub cell was likely due to leaky expression of the *hsp70*-Notch transgene. The slightly different distribution we observed comparing non-heat shocked and late heat shocked embryos (8-9 hrs AEL) is likely attributable to subtle variation in the leaky transgene expression. In contrast, we found that embryos that received the first heat pulse at 5-6 hours AEL (early-mid stage 11) exhibited significant rescue of hub cells (Figure 2.3, red). In fact, 65% of gonads had 5 or more hub cells specified (19/29 gonads), and almost half reached our observed wild type range of hub cells (9-14 hub cells, 13/29 gonads; Figure 2.3, red). The fact that significant rescue only occurred upon early expression of Notch, suggested that hub cell specification occurred much earlier than previously appreciated, likely late-stage 11 and 12.

Serrate and Delta both contribute to hub cell fate

In *Drosophila,* there are two ligands that can activate the Notch receptor, Delta and Serrate. To determine their respective contribution to hub specification, we assayed larval gonads singly mutant for either ligand. We could not confidently score hub cell number in doubly mutant embryos due to a severe germ cell migration defect. Germ cell migration was also severely disrupted in *Delta* null mutant embryos, preventing the confidant analysis of hub cell phenotypes (124, 175). We therefore assayed larval gonads that were homozygous for a hypomorphic mutation in *Delta*, Dl^{RF}. *Delta*-deficient larvae had a 70% reduction in hub cell number compared with control gonads (Figure 2.4 A-C;

averaging 5 ± 0.8 versus 14 ± 0.6, respectively; p<0.0001). The effects of *Serrate* mutations were more modest in our hands, exhibiting a 30% decrease in hub cell number (Figure 2.4 D-F; averaging 8 ± 0.4 for Ser^{RX106} versus 12 ± 0.3, respectively, p<0.0001; data not shown for Ser^{RX82}). This suggests that while both ligands contribute to hub cell specification, Delta has a more prominent role in this process. Furthermore, we find that reducing the protein levels of *neuralized*, an E3 ubiquitin ligase important for ligand endocytosis and productive Notch signaling (106-108), in heterozygous animals results in a decrease in hub cell number (Figure 2.5; averaging 11 ± 0.5 versus 13 ± 0.5 in controls, p= 0.01). This further confirms the role of Serrate and Delta in this process.

The posterior midgut activates Notch in developing SGPs

We next attempted to identify the source of the Notch ligand(s). We observed that forced expression of Delta using a mesodermal driver, Twist-Gal4, led to a 14% increase in hub cell number compared with controls (averaging 14 ± 2.2, n=30 versus 12 ± 2.5, n=18; p=0.027). Similarly, misexpressing Serrate from germ cells using the Nanos-Gal4 driver led to an increase in hub cell number compared with controls (averaging 14 ± 1.5 versus 11 ± 1.3, respectively; p=0.01). While these gain-of-function experiments supported the notion that activation of the Notch pathway among SGPs could direct them to select hub cell fate, they do not establish which cells normally express the ligand(s). In fact, in our hands, neither Serrate nor Delta expression was detectable within the gonad (Figure 2.6) [see, however (97)]. We thus turned our attention to adjacent tissues as potential sources.

Beginning at stage 13, the gonad coalesces in very close proximity to the developing trachea, which expresses a high level of both Delta and Serrate mRNA and protein (Figure 2.6). We found, however, that the loss of the trachea in *trachealess* or *breathless* mutants did not appear to affect hub cell number (data not shown). This suggests that signaling from the trachea is not necessary to specify hub cell fate.

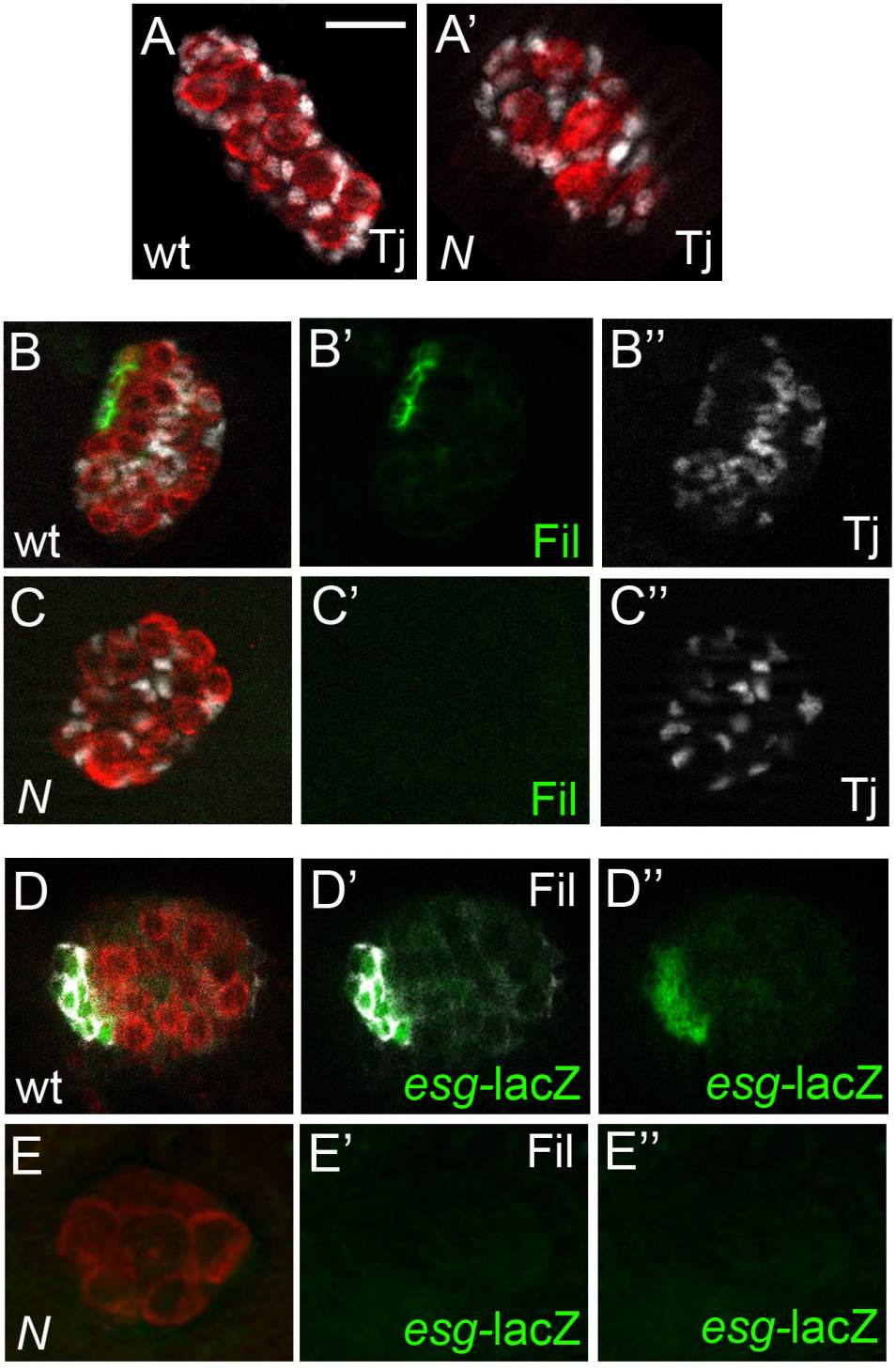
It is known that Delta is highly expressed in the posterior midgut (PMG; Figure 2.7, arrows) (168). SGPs, as identified by the nuclear protein *eyes absent* (*eya*) (21), are positioned very close to the PMG beginning at stage 11 when they are initially specified and through the end of germ band retraction at late stage 12 (Figure 2.7 D). During this period, the SGPs passively move past the gut, and PMG cells and SGPs are found in the same focal plane (Figure 2.7 D). The SGPs closest to the PMG are only three to six μm away, well within the range of distances reported for productive Delta-Notch signaling (up to 15μm) (45). This data suggests that the PMG cells are close enough to activate Notch in SGPs.

We first attempted an endoderm-specific knockdown of Delta. Driving Delta dsRNA using either a midgut (Figure 2.7 H) or an endoderm driver (data not shown) led to an approximately 20% reduction in hub cell number. This small decrease was perhaps due to the inefficiency of knockdown, as we observed residual Delta protein on gut cells (data not shown). For example, embryos expressing dsRNA to Delta driven by Drm-Gal4 averaged 13 ± 0.4 hub cells compared with 16 ± 0.7 for Uas-Dl-RNAi alone, and 15 ± 0.6 for Drm-Gal4 alone (Figure 2.7 H; $p<0.05$).

As an independent test whether Delta-expressing PMG cells contribute to hub cell specification, we assayed *folded gastrulation* (*fog*) mutants (168). In *fog* mutant embryos

the posterior midgut is not internalized and instead develops on the exterior of the embryo (Figure 2.7 B), although all other cell types develop normally. Such *fog* mutant gonads displayed a 70% decrease in hub cell number, scored using either Filamin or *esg-lacZ* (Figure 2.7 E-G; 12 ± 0.4 versus 3 ± 0.9, respectively; $p<0.0001$; data not shown). Importantly, the phenotype was selective for hub cells, as a distinct intragonadal cell type, msSGPs, were specified normally in *fog* mutants (Figure 2.7 C). In addition, normal numbers of SGPs were specified, as sibling controls and *fog* mutant embryos at stage 13 had a similar number of Traffic jam-positive SGPs (32 ± 1.5 versus 31 ± 0.9, respectively; $p = 0.71$). Thus, the absence of hub cells in *fog* mutants was consistent with the proposal that the proximity of endoderm to the SGPs was essential for hub specification. Furthermore, overexpressing Delta from the endoderm resulted in a 20% increase in hub cell number over controls (Figure 2.7 I; averaging 16 ± 1.8 versus 13 ± 1.6, respectively; $p<0.005$). This indicates that Delta specifically expressed from the PMG is not only necessary for hub cell specification, but its overexpression can cause an increase in hub cell number. Additionally, we attempted to rescue the hub cell defect in *Delta* mutant larvae by restoring *Delta* specifically to the endoderm. However, since the endoderm is not properly specified in *Delta* mutant embryos (135), no significant rescue in hub cell number was observed (data not shown). Taken together, our findings implicate the endoderm in delivery of Delta to activate Notch for hub cell specification among SGPs.

Figure 2.1: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Tj
A'
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B'
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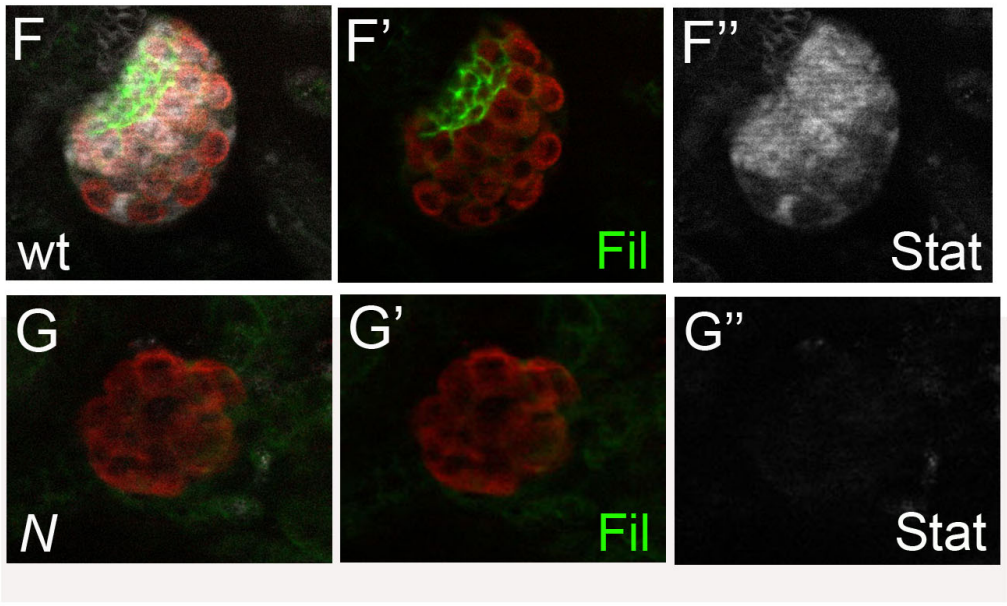
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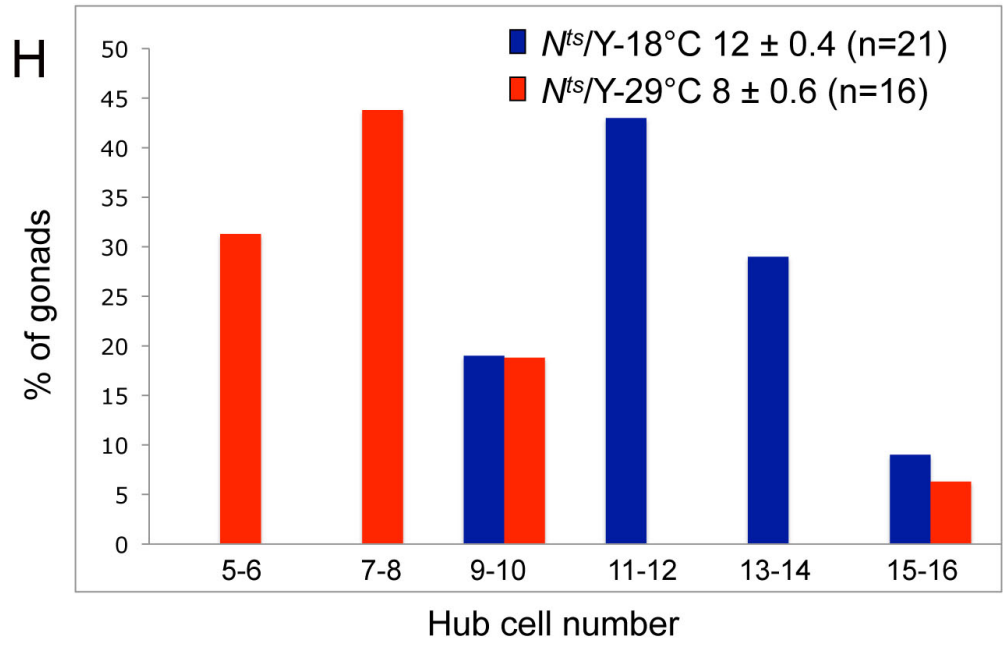

H
Nts/Y-18°C 12 ± 0.4 (n=21)
Nts/Y-29°C 8 ± 0.6 (n=16)
% of gonads
50
45
40
35
30
25
20
15
10
5
0
5-6
7-8
9-10
11-12
13-14
15-16
Hub cell number

Figure 2.1: Notch signaling specifies hub cell fate

Anterior is to the left in each panel. Gonads were stained with Vasa (red, germ cells). (A, A') Stage 13 male embryos showing Traffic jam (white, SGPs) at the onset of coalescence. (A) Controls, +/Y (n=18) and Notch mutants, $N^{264.39}$/Y, (n=14) have a similar number of SGPs specified (41.5 and 39.3, respectively; p = 0.30). (B-C'') 1st instar larval gonads showing Filamin (green, hub) and Traffic jam (white, somatic cells). In controls, +/Y (B-B''; D-D''; F-F'') the hub is outlined by Filamin. In $N^{264.39}$/Y gonads (C-C'') the hub is absent (C'); however somatic cells are still present (C, C''). (D'-E'') +/Y and $N^{264.39}$/Y larval gonads expressing an esg^{G66B} enhancer trap. Gonads were stained with Filamin (white) and anti-βgal (green). Note that in control gonads both Filamin and *esg* detect hub cells. However, in $N^{264.39}$/Y gonads (E-E''), most *esg*-positive cells are lost and Filamin staining is rarely observed. (F-G'') Gonads were stained with Filamin (green) and Stat (white). In +/Y gonads, (F-F'') Stat protein accumulates in neighboring somatic and germline cells and in the hub. In $N^{264.39}$/Y gonads (G,G'') Stat accumulation decreases drastically, indicating the lack of productive *upd* signaling. Scale bar is 10μm. (H) The distribution of the number of Filamin positive hub cells per gonad. Note the significant shift to lower hub cell numbers under non-permissive conditions for N^{ts} (red) compared to control gonads (blue) raised at the permissive temperature ($p<0.0001$). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 2.2:

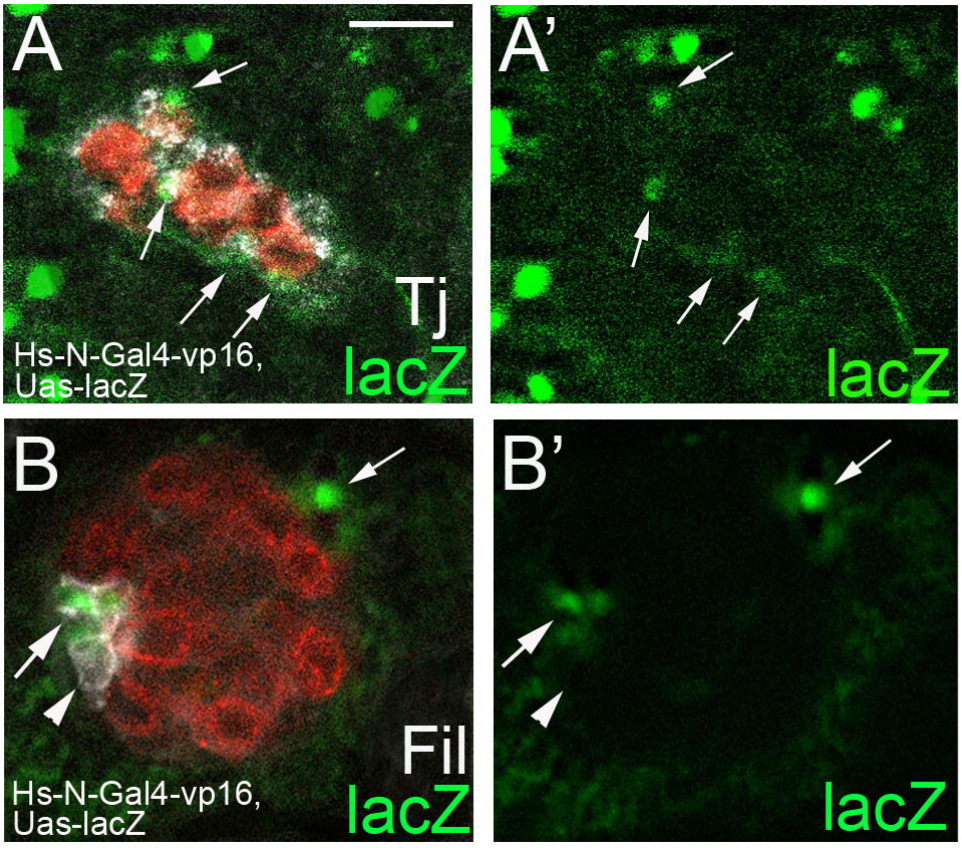


Figure 2.2: Notch-activated SGPs contribute to the hub

(A, A') Stage 13 male embryonic gonad. Notch reporter activation was assessed using the *hsp70*-Notch–Gal4-vp16; Uas-lacZ-nls reporter construct. Gonad showing Notch-activated lacZ positive cells (green) that co-stain with Traffic jam (white) (arrows, figure A, A') and Vasa (red, germ cells). Note that lacZ-positive cells are dispersed throughout the gonad. (B, B') Cells activated for Notch during embryogenesis (green) contribute to the hub (Filamin, white) in the 1st larval instar gonad. Arrows denote lacZ-positive cells. Arrowhead denotes lacZ-negative hub cells. Note that a lacZ-positive cell is also found at the posterior of the gonad. Thus, it is possible that Notch signaling also contributes to some gonadal sheath cells. Scale bar is 10μm.

Figure 2.3:

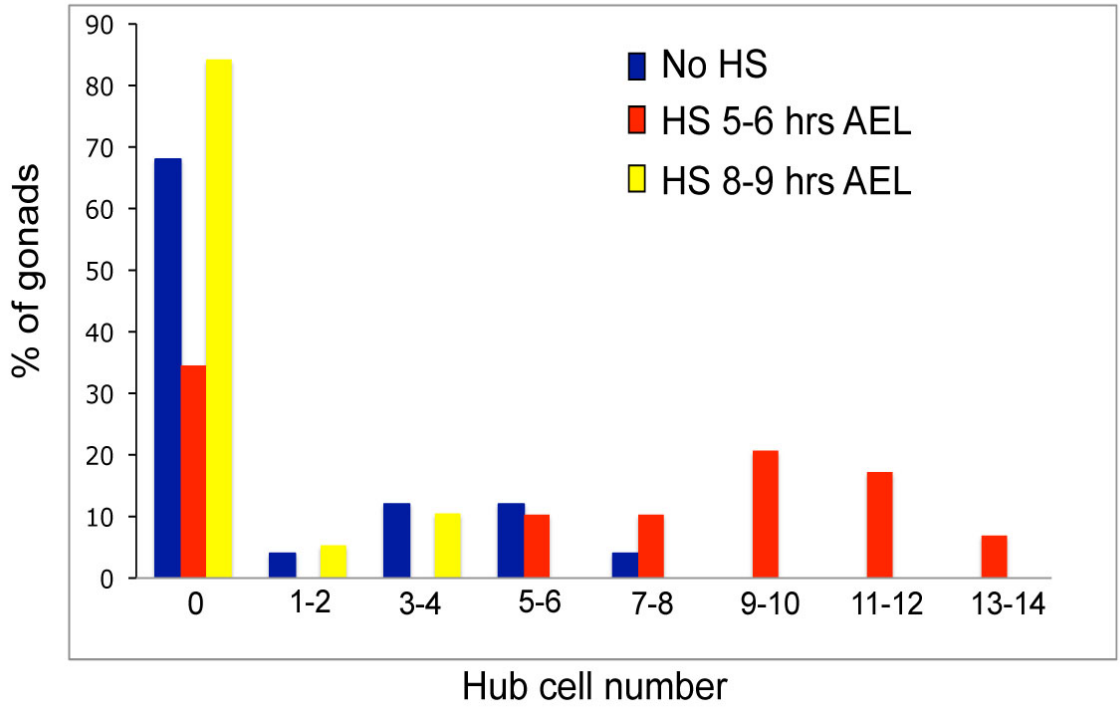


Figure 2.3: Notch activity is required before gonad coalescence to specify hub cell fate

A graph of the number of $N^{264.39}$/Y; *hsp70*-Notch-Gal4-VP16 gonads with Filamin-positive hub cells. In this background, control gonads receiving no heat shock (blue, n=25) still have a small number of hub cells specified, indicating leaky transgene expression. The rescuing heat shock began at 5-6 hours (red, n=29) or 8-9 hours (yellow, n=19) after egg lay (AEL). Note that there is a significant rescue of hub cells when the heat shock occurs at 5-6 hours AEL (red).

Figure 2.4:

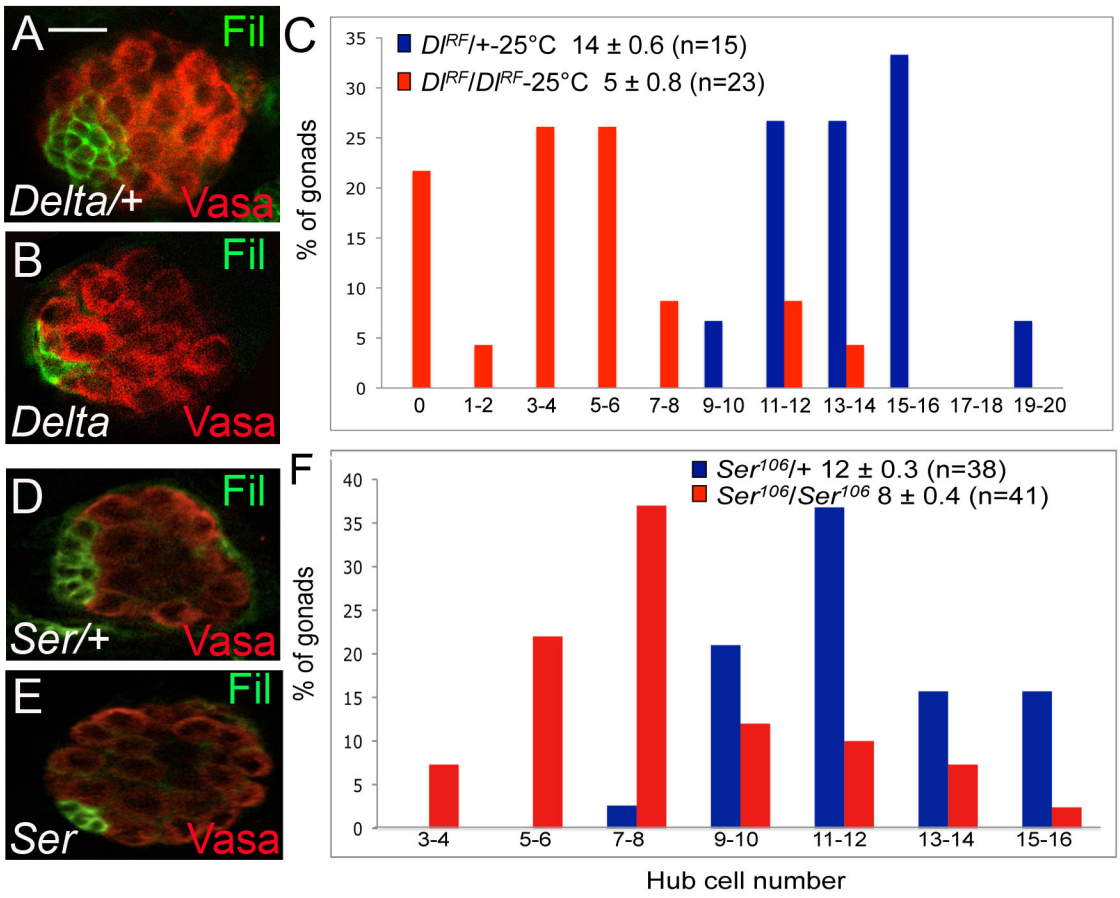


Figure 2.4: Serrate and Delta both contribute to hub cell fate

(A, B, D, E) 1st larval instar gonads from (A) Dl^{RF}/+, (B) Dl^{RF}/Dl^{RF}, (D) Ser^{106}/+ and (E) Ser^{106}/Ser^{106} raised at 25°C. Filamin (green, hub) and Vasa (red, germ cells). Scale bar is 10μm. (C, F) Distribution of the number of hub cells in Dl^{RF}/+ (blue) and Dl^{RF}/Dl^{RF} (red) gonads (C, $p<0.0001$ by Student's t-test) and Ser^{106}/+ (blue) and Ser^{106}/Ser^{106} (red) gonads (F, $p<0.0001$) is shown. The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 2.5:

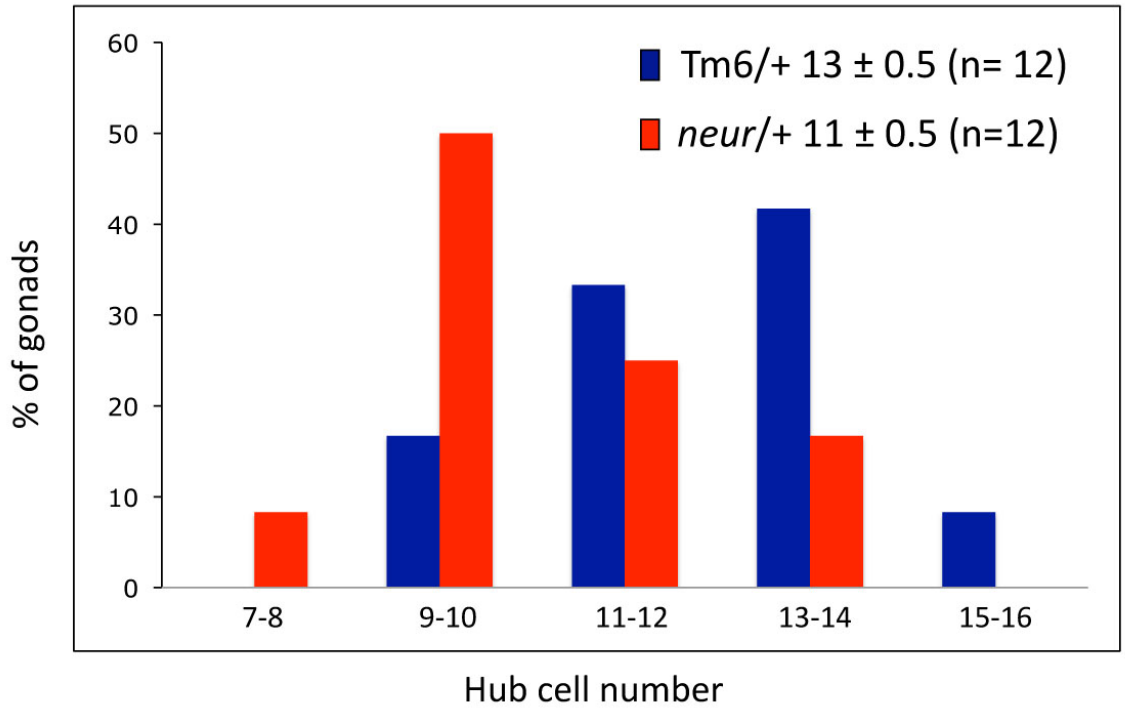


Figure 2.5: Ligand endocytosis is necessary for proper hub cell specification

The distribution of the number of Filamin positive hub cells per gonad. Note the shift to lower hub cell numbers in *neur*/+ heterozygotes (red) compared to Tm6/+ control gonads (blue) (p=0.01). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 2.6:

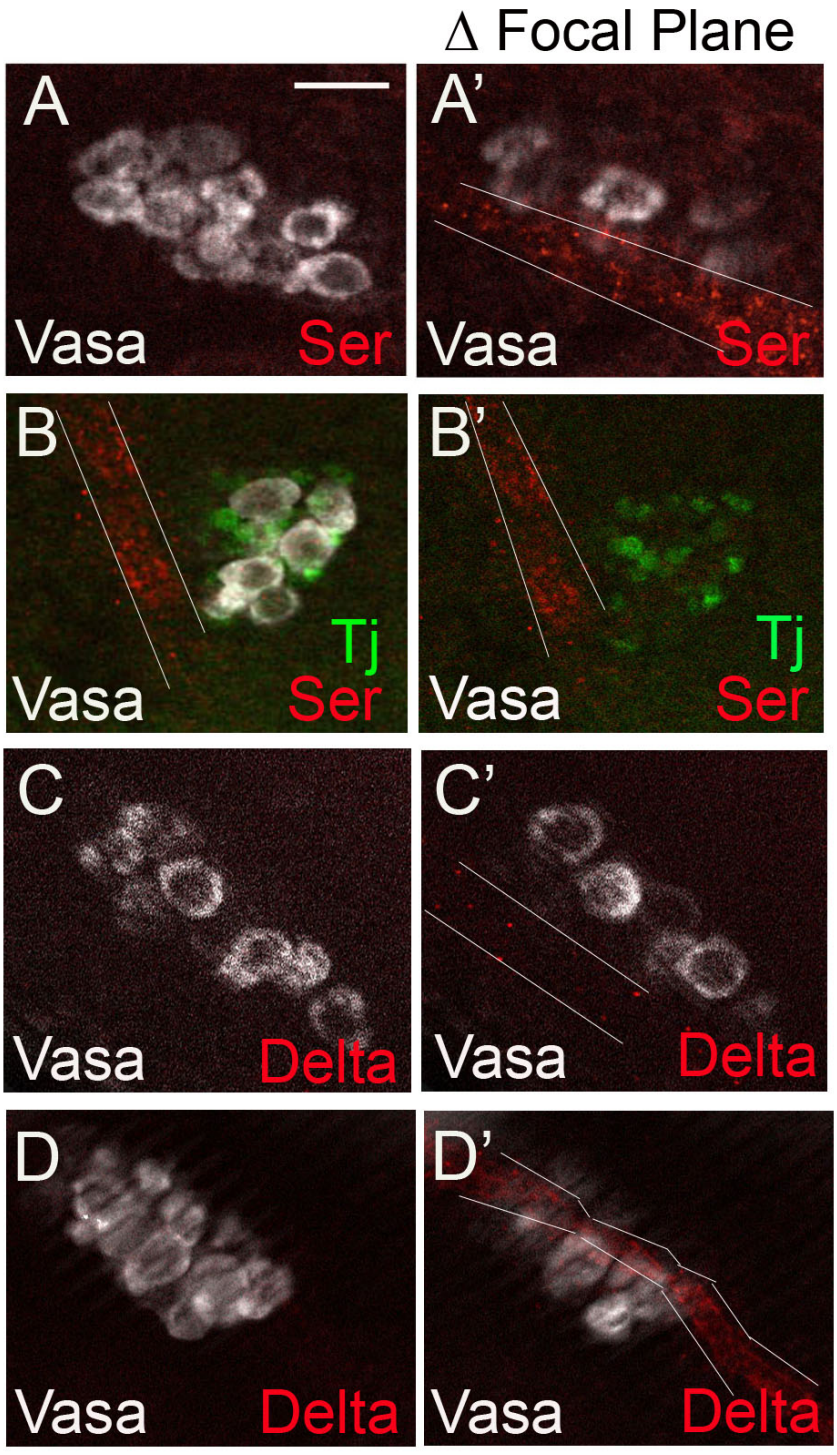
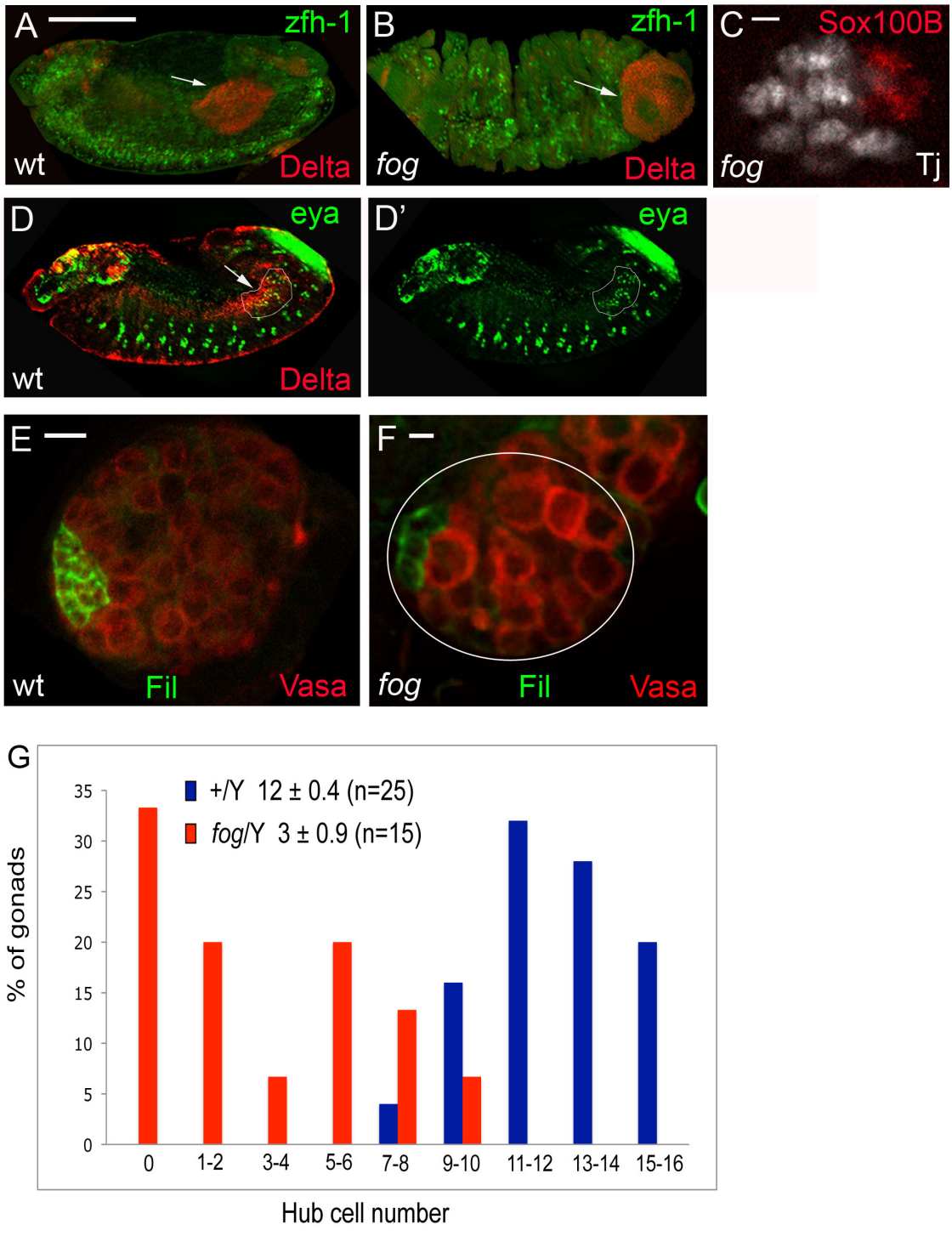

Δ Focal Plane
A
Vasa
Ser
A'
Vasa
Ser
B
Vasa
Tj
Ser
B'
Vasa
Tj
Ser
C
Vasa
Delta
C'
Vasa
Delta
D
Vasa
Delta
D'
Vasa
Delta

Figure 2.6: Notch ligands are expressed on neighboring tracheal cells

(A-D) Wild type male gonads showing Vasa (white) to reveal germ cells. Ligand-expressing tracheal cells are highlighted with white lines. (A, A') A stage 14 male gonad showing Vasa and fluorescent in situ hybridization to Serrate mRNA (red). (B, B') A stage 15 male gonad showing Vasa, Traffic Jam (green, somatic cells) and Serrate (red). (C, C') A stage 14 male gonad showing Vasa and fluorescent in situ hybridization to Delta mRNA (red). (D, D') A stage 14 male gonad showing Vasa and Delta (red). Note that Serrate mRNA (A), Serrate protein (B), Delta mRNA (C) and Delta protein (D) are not detected within the gonad proper, but are expressed from an adjacent stripe of tracheal cells in a different focal plane (A', B', C', D'). Scale bar is 10μm.

Figure 2.7:


A
zfh-1
wt
Delta
B
zfh-1
fog
Delta
C
Sox100B
fog
Tj
D
eya
wt
Delta
D'
eya
E
wt
Fil
Vasa
F
fog
Fil
Vasa
G
+/Y 12 ± 0.4 (n=25)
fog/Y 3 ± 0.9 (n=15)
% of gonads
35
30
25
20
15
10
5
0
0
1-2
3-4
5-6
7-8
9-10
11-12
13-14
15-16
Hub cell number

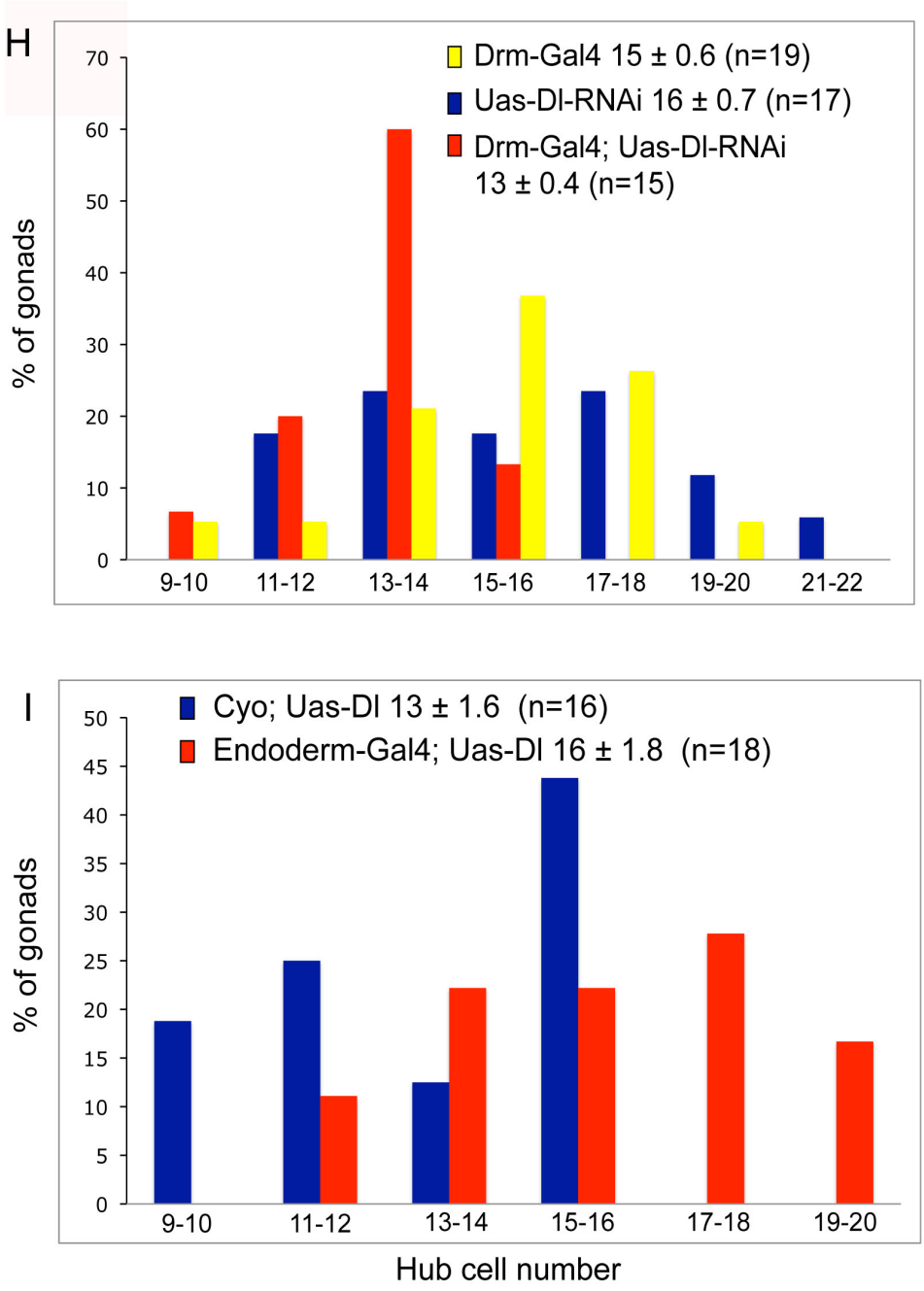

H
Drm-Gal4 15 ± 0.6 (n=19)
Uas-Dl-RNAi 16 ± 0.7 (n=17)
Drm-Gal4; Uas-Dl-RNAi
13 ± 0.4 (n=15)
% of gonads
0
10
20
30
40
50
60
70
9-10
11-12
13-14
15-16
17-18
19-20
21-22
I
Cyo; Uas-Dl 13 ± 1.6 (n=16)
Endoderm-Gal4; Uas-Dl 16 ± 1.8 (n=18)
% of gonads
0
5
10
15
20
25
30
35
40
45
50
9-10
11-12
13-14
15-16
17-18
19-20
Hub cell number

Figure 2.7: The posterior midgut (PMG) is necessary for proper hub cell specification

(A, B) Lateral view of a Z-slice through stage 12 male embryos from (A) wild type and (B) *fog* showing Delta (red, PMG; arrows) and zfh-1 (green, somatic cells). Note that in the *fog* mutant (B) the PMG develops on the exterior of the embryo. Scale bar is 100μm in A-B. (C) Stage 16 *fog* mutant male embryo showing Sox100B (red, msSGPs) and Traffic jam (white, SGPs). Scale bar is 10μm. (D, D') Lateral view of a Z-slice through a stage 12 wild type male embryo showing Delta (red, PMG; arrows) and eyes absent (green, SGPs; encircled in white). Z-slice= 0.7μm. Scale bar is 100μm. (E, F) 1st larval instar male gonads from +/Y (D) and *fog*/Y (E). Filamin (green, hub cells) and Vasa (red, germ cells). One gonad is outlined in E; a second lies just up and to the right. Note that fewer germ cells contribute to the *fog*/Y larval gonad. Scale bar is 10μm in D and 5μm in E. (G) Distribution of the number of hub cells in +/Y (blue) and *fog*/Y (red) is shown ($p<0.0001$). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown. (H) Distribution of the number of Filamin positive hub cells in Uas-Dl-RNAi (blue), Drm-Gal4 (yellow) and Drm-Gal4; Uas-Dl-RNAi (red) gonads is shown. Note the decreased hub cell number in Drm-Gal4; Uas-Dl-RNAi gonads ($p < 0.05$) compared to controls, Drm-Gal4 and Uas-Dl-RNAi gonads. The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown. (I) Distribution of the number of hub cells in cyo;Uas-Dl (blue) and Endoderm-Gal4;Uas-Dl (red) is shown ($p<0.005$) . The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 2.8:

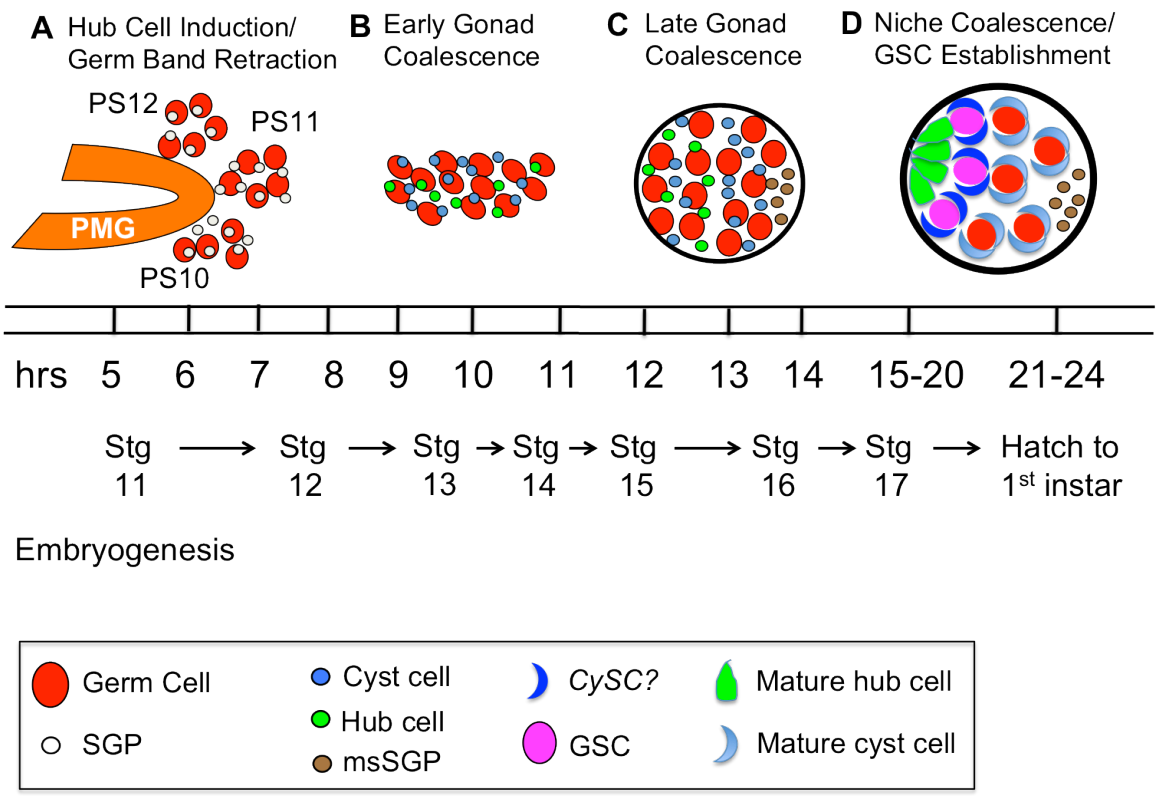


Figure 2.8: Model for hub cell specification in the male gonad

(A) SGPs (white) that originate from parasegments (PS) 10-12 become Notch-activated as they passively travel by Delta-expressing PMG cells (orange) during germ band retraction. (B) During early gonad coalescence as germ cells (red) and SGPs form a contiguous tissue, SGPs begin to differentiate into either hub (green) or cyst cells (light blue). (C) During late gonad coalescence, Notch-activated hub cells must migrate towards the anterior. Sox100B-positive male-specific SGPs (brown) join the gonad. (D) During the last stage of embryogenesis, stage 17, the hub cells execute a mesenchymal-to-epithelial transition, upregulate cell adhesion molecules and induce Unpaired expression, establishing germline stem cells (GSC, purple) and possibly cyst stem cells (CySC, dark blue).

Discussion

Stem cell niches are inferred to exist for many tissues. However, the difficulty in unambiguously identifying niche cells has left unanswered when and how these niches are specified. Here, we have identified the Notch pathway as key in the specification of a crucial component of the *Drosophila* male testis niche, the hub cells. We find that hub cells are specified before gonad coalescence, earlier in development than previously appreciated. Furthermore, our data suggest that Delta-expressing endoderm cells are critical for proper hub cell specification. This demonstrates tissue non-autonomous regulation of this niche.

The role of Notch signaling in hub cell specification

Our data reveal that Notch signaling is necessary to specify hub cell fate. A similar conclusion has recently been reached by Kitadate and Kobayashi (97). It is interesting to note that in three well-characterized stem cell-niche systems in *Drosophila*, including the transient niche for adult midgut progenitors, the female gonad and now the developing male gonad, Notch signaling is directly responsible for niche cell specification (97, 119, 157, 182). Moreover, Notch has been found to play a role in the maintenance of various mammalian stem cell populations, including neural stem cells, HSCs and hair follicle stem cells [Reviewed in (37); (30, 147, 176)]. However, due to difficulty in performing lineage-specific knockouts in these systems, it remains unclear which cells require Notch activity. Since the various cases in *Drosophila* all require direct Notch activation for niche cell specification, perhaps this reveals a conserved role for Notch signaling in other, more complex stem cell systems.

Notch signaling specifies niche cells in both the male and female *Drosophila* gonad, however, it is important to note that there are still some differences. For the ovary, only Delta is required to activate the Notch receptor for proper niche cell specification (157, 182). For the testis, we find that both ligands contribute to the process, although, here, too, it appears that Delta is the dominant ligand employed (Figure 2.4). Interestingly, depleting Delta or (genetically) separating the endoderm from SGPs both led to a 70% reduction in hub cell number, while depleting Serrate yielded a 30% reduction. Perhaps Delta-Notch signaling from the endoderm accounts for two-thirds of hub cell specification while Serrate-Notch signaling accounts for only one-third of this process. Although we were unable to identify the source of Serrate, Kitadate and Kobayashi (97) have shown that Serrate mRNA is expressed from SGPs after gonad coalescence. Perhaps, this late expression accounts for the modest role Serrate plays in hub specification. Those authors did not explore in detail a potential role for Delta in hub specification, and our data suggests that that role is carried out at earlier stages, and from outside the gonad proper.

Secondly, in the ovary, cells within the developing gonad appear to present the Notch-activating ligand, although it is unclear whether germ cells or somatic cells are the source of Delta (157, 182). Here, our data suggests that cells from a distinct germ layer, the endoderm, present Delta to SGPs in the male gonad. These differences may indicate distinct evolutionary control over gonadal niche development between the sexes.

Hub cell specification occurs early, before gonad coalescence

While the gonad first forms during mid-embryogenesis, hub cells only become identifiable just prior to hatching of the larvae, some 6 hours later (110). At that time, hub cells begin to tightly pack at the anterior of the gonad, upregulate several cell adhesion and cytoskeletal molecules (Fascilin 3, Filamin, DN-Cadherin, DE-Cadherin) as well as induce Upd expression and other markers of hub fate (110, 165). Surprisingly, our data reveal that most hub cells are specified well before these overt signs of hub cell differentiation, as judged by Notch reporter activation and Notch rescue (Figure 2.2 and 2.3). While it was previously thought that SGPs were equivalent at the time of gonad coalescence (110) it is now clear that due to Notch activity, the SGPs are parsed into a group of either hub cells or cyst cells before gonad coalescence occurs.

Thus, we believe that a series of steps must occur before the hub can function as a niche. First, the PMG presents Delta, leading to Notch activation in some SGPs as they are carried over these endodermal cells during germ band retraction (Figure 2.8). Activation might be dependent on, for instance, length of time in contact with passing PMG cells. At the present time, it is unclear if all SGPs are activated for Notch (97), or only some (this work). Second, after gonad coalescence, activated SGPs must then migrate anteriorly (this work) (97, 110). While it is known that integrin-mediated adhesion is required to maintain the hub at the anterior (165), no cues have been identified that could guide the migration of the Notch-activated SGPs. Third, as the cells reach the anterior of the gonad they must execute a mesenchymal-to-epithelial transition, as evidenced by the upregulation of cell adhesion molecules and preferential associations between hub cells (46, 110). This step occurs independently of the integrin-mediated

anchoring at the anterior. Finally the hub cells must induce Upd expression and recruit neighboring cells to adopt stem cell fate (152). The apparent delay between the activation of the Notch pathway and the initiation of the hub cell gene expression program might suggest that initiating that hub program first requires that the cells coalesce into an epithelium. Such a mechanism would prevent precocious or erroneous stem cell specification within the gonad.

Although our data reveal Notch-activated SGPs at all positions within the gonad and that some of these become hub cells, it is unclear how hub cell number is tightly regulated. Potentially, SGP migration over endodermal cells could induce Notch activation among SGPs throughout the forming gonad, potentiating these cells to become hub cells. However, solely relying on that mechanism could lead to the specification of too many hub cells. It appears, though, that specification is regulated by EGFR pathway activation (97). The authors have recently shown that EGFR protein is observed on most SGPs throughout the embryonic gonad beginning at gonad coalescence (stage 13). The EGFR ligand, *Spitz*, is expressed from all germ cells during gonad coalescence and activates EGFR among posterior SGPs. This activity antagonizes Notch and that appears to regulate final hub cell number. How EGFR activation is restricted or enhanced only among posterior SGPs is at present unclear [see Discussion in (97)].

Given that we find that hub cell specification occurs prior to gonad coalescence, it is also possible that Notch and EGFR act in a temporal sequence. In this case, early Notch-activated SGPs, perhaps even those in the posterior will adopt hub cell fate. But, as EGFR becomes activated, further induction of the Notch pathway in the posterior is antagonized, prohibiting the specification of too many hub cells. Such a temporal

inhibition might be important as Serrate is expressed on the SGPs (97) both Delta and Serrate are robustly expressed on tracheal cells (Figure 2.6), whose activity might otherwise lead to excess hub cell induction. Lastly, perhaps during later stages of gonadogenesis (stages 14-16) a small number of anterior SGPs become Notch-activated due to the activity of Serrate-Notch signaling from other SGPs, supplementing the hub cells previously specified by Delta-Notch signaling.

Endoderm induction of hub cells

Given that niche cells in the *Drosophila* ovary become activated via Delta-Notch signaling by neighboring somatic cells, we initially expected that Notch would be activated in a subset of SGPs by ligand presented from other SGPs (157). However, we could not detect Delta nor Serrate expression among SGPs. Furthermore, although nearby tracheal cells expressed both ligands robustly, that expression appears later than our Notch rescue suggests would be necessary, and genetic ablation of tracheal cells did not influence hub cell number.

Instead, we found that a critical signal for niche cell specification is presented from the endoderm, as Delta is expressed robustly on posterior midgut cells, at a time consistent with the requirement for Notch function. Furthermore, these endodermal cells are close enough to SGPs for productive Delta-Notch signaling to occur (Figure 2.7 D). While visceral mesodermal cells are also close to the PMG and the SGPs (11, 21, 28, 168), this tissue does not affect hub specification, as we found that *brachyenteron* mutants exhibited normal hub cell number (data not shown). In contrast, in mutants that

do not internalize the gut (*fog*), and thus would not present Delta to SGPs, we found a drastic reduction in hub cell number.

Additionally, we note that absolute hub cell number varies amongst animals, and by genetic background (this work) (98, 180). We attribute this to normal biological variation, just as germline stem cell number varies (180). Potentially, this variation could be caused by how robustly the Notch pathway is activated in SGPs as they are carried over the midgut cells. It will be interesting to test this hypothesis by genetically manipulating the number of midgut cells or the time of contact between endoderm and SGPs. Additionally, the antagonistic effects of EGFR signaling might account for some of the observed variation. In fact, gonads heterozygous for Star, a component of the EGFR pathway, exhibit increased hub cell number (97).

Finally, it is interesting to consider why the endoderm would be critical for the proper specification of the GSC niche. In *Drosophila,* as in many animals, there is a special relationship between the gut and the germ cells. Primordial germ cells in mammals and in *Drosophila* must migrate through the endoderm to reach the gonadal mesoderm [Reviewed in (145)]. In fact, in *Drosophila*, the gut exercises elaborate control over germ cell migration. As the germ cells begin their transepithelial migration and exit from the midgut pocket, tight connections between midgut cells are dissolved, allowing for easy germ cell passage (82, 83). Germ cells then migrate on the basal surface of endodermal cells and midgut expression of wunens (encoding lipid phosphate phosphatases) repels germ cells, driving them into the mesoderm (161, 195). Thus, the endoderm not only delivers germ cells to the somatic mesoderm, but our work reveals that the same endoderm specifies niche cells from among the somatic mesoderm wherein

germ cells can subsequently develop into stem cells. In mammals, while the exact make-up of the spermatogonial stem cell niche has not been determined, it must (in part) derive from cells of the genital ridge. It will be interesting to determine if proximity to the gut endoderm is important for the specification of this niche.

Material and Methods

Fly Stocks

Heterozygous siblings or w^{1118} were used as controls as appropriate. We analyzed gonads from the following mutants, or involving these transgenic lines: $N^{264.39}$ (FBal0029934), N^{ts1} (FBal0012887), *paired*-Gal4 (FBal0048793), Dl^{RF} (135), Ser^{RX82} (FBal0030223), Ser^{RX106} (FBal0030221), *nanos*-Gal4-vp16 (Erica Selva), $Dl^{Rev10}Ser^{RX82}$ (FBal0029366/FBal0030223), $neur^{11}$ (FBal0012950), $trachealess^{10512}$ (FBal0009624), $trachealess^{2}$ (FBal0017037), fog^{S4} (168), *hsp70*-Notch-Gal4-VP16 (146), *hsp70*-Dl (Gary Struhl), Uas-lacZ-nls (Bloomington Stock Center), *esg*-lacZ (63), Uas-Dl-dsRNA (FBgn0000463), *drm*-Gal4 (64), P[GawB]48Y-Gal4 for endoderm expression (FBti0004594), Twist-Gal4 (FBal0040491). Stocks were balanced over CyO P[w+ Ubi-GFP] or TM6 Hu P[w+ Ubi-GFP].

Immunostaining

Embryos were collected on apple agar plates and aged 22-24 hours in a humidified chamber to 1st instar larvae. Hatched larvae were dissected in half with tungsten needles in Ringers solution and the internal organs were gently massaged out. Unhatched larvae were dechorionated, hand-devitellinized and dissected as above. Tissue was fixed in 4% formaldehyde, Ringers and 0.1% Triton-X-100 for 15 minutes, washed

in PBTX and blocked one hour at room temperature in 2% normal donkey serum/normal goat serum. Primary antibodies were used overnight at 4°C. Secondary antibodies were used at 1:400 (Alexa488, Cy3 or Cy5; Molecular Probes; Jackson Immuno Research) or 1:1000 (biotinylated; Invitrogen) for 1 hour at room temperature. DNA was stained with Hoechst 33342 (Sigma) at 0.2 μg/ml for 2 minutes.

Immunostaining for testes was performed as previously described except 1X PBS was substituted for Buffer B (169). For embryo studies, embryos were collected, aged for the appropriate time in a humidified chamber, fixed in 4% paraformaldehyde and heptane for 15 minutes and devitellinized with methanol.

The following primary antibodies and concentrations were used: rabbit anti-Vasa 1:5000 (R. Lehmann), goat anti-Vasa 1:400 (Santa Cruz), chick anti-Vasa 1:5000- 10,000 (K. Howard), guinea pig anti-Traffic Jam 1:10,000 (Dorothea Godt), mouse anti-βgal 1:10000 (Promega), rabbit anti-STAT 1:1000 (Erica Bach), rat anti-Filamin-N terminal 1:1000 (Lynn Cooley; recognizes full length isoforms), rat anti-Filamin-C terminal 1:1000 (Lynn Cooley; recognizes C-terminal isoform), rat anti-Serrate 1:1000 (K. Irvine), mouse anti-Delta C594.9B (Developmental Studies Hybridoma Bank), , Streptavidin-HRP 1:400 (Chemicon), mouse-anti Biotin 1:1000, rabbit anti-Sox100B 1:1000 (S. Russell), mouse anti 1B1 1:20 (DSHB); mouse anti-Sxl 1:25 (DSHB).

Tyramide amplification was used to increase the anti-lacZ staining. Samples were incubated with a biotinylated secondary antibody for 1 hour, washed and followed by a 25-minute incubation in SA-HRP. After a final washing, a 15-minute incubation in tyramide-Fluorescein was employed (PerkinElmer).

Sex identification, genotyping and staging of embryos

Male embryos and larvae without a gonad coalescence defect were unambiguous due to larger size of the gonad. For other cases, embryo or larvae sex was determined by immunostaining with Sex lethal. Balancer chromosomes containing a GFP-transgene P[w+ TM6 Hu Ubi-GFP] or P[w+ Ubi-GFP] were used to distinguish between heterozygous and homozygous mutant larvae. Larvae and embryos mutant for *Notch* or *Delta* were identified by their obvious neurogenic phenotype. Embryos were staged according to Campos-Ortega and Hartenstein (32).

In situ hybridization

Biotin-labeled probes (not size-reduced) were synthesized from cDNA plasmids obtained from the BDGP collection or the DGRC. In situ hybridizations were performed as described in Terry et al. (169). Hybridization signal was revealed by immunofluorescent detection using anti-Biotin (1 hour), washed four times (20 minutes each) in PBS containing 0.1% Tween-20, and incubated in a Cy3 secondary antibody (1 hour). Embryos were then blocked for at least 30 minutes and then immunostained for various antigens.

Counting the number of hub cells and germline stem cells

To count hub cell number, larval gonads were stained as needed, and also with anti-Filamin and Hoechst, and z-stacks were obtained through the depth of the gonad using a Zeiss Axioplan with an ApoTome attachment. Nuclei that were surrounded by a Filamin signal were counted as hub cells.

To count germline stem cells, larval gonads were double stained with anti-Vasa and anti-STAT or anti-1B1 antibodies. Germ cells that were directly adjacent to the hub and that accumulated STAT protein or had a dot spectrosome were scored as stem cells.

Notch rescue

We noticed that in the absence of a heat shock, hub cells were specified at a low frequency, indicating that there is leaky expression of the *hsp70*-Notch-Gal4-VP16 transgene. We therefore delivered a set of three heat shocks to induce robust expression of the receptor. Embryos were collected for 1 hour and aged at 25°C until the heat shock. Heat shocks at 37°C were delivered to embryos beginning at either 5-6 hours after egg lay (AEL) or 8-9 hours AEL. A recovery period of 45 minutes followed each 40-minute heat shock. Embryos were processed after aging at 25°C until they reached hatching stage.

Measuring cell size and distance

SGP cell nuclei and cell distances between SGPs and PMG cells were measured by using the Length tool in AxioVision. During stages 11-12, the diameter of the SGP nucleus is approximately 5-6μm in size.

Acknowledgements

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Chapter Three:

THE *BOWL* PATHWAY INFLUENCES HUB CELL SPECIFICATION IN THE *DROSOPHILA* TESTIS

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Summary

In chapter two we show that Notch signaling is necessary to specify hub cell fate in the *Drosophila* testis. Given that Notch is a master regulator of gene expression, we were interested in identifying potential targets of the pathway that also function in this process. Here, we have identified another factor that promotes hub cell specification: *bowl.*

Somatic gonadal precursors (SGPs) are thought to give rise to both hub cells and cyst cells, however it is not understood how this binary cell fate decision is made. Here we show that the *bowl* signaling pathway influences hub cell specification during gonadogenesis. Our data reveal that a *bowl* antagonist, *lines,* acts to promote cyst cell fate. Conversely, we find that *bowl* acts to promote hub cell fate, while likely restricting cyst cell fate. Furthermore, our data suggests that *bowl* functions as a repressor to limit cyst cell gene expression by recruiting the general co-repressor, *groucho (gro)*. Since a subset of cyst cells can eventually take on CySC fate given their proximity to the hub, this pathway may function to ultimately distinguish niche versus stem cell fate.

Introduction

Our lab previously carried out a microarray experiment to identify genes that were enriched within the testis stem cell niche (169). *Brother of odd with entrails limited (bowl)* was one such gene we found to be upregulated in testes genetically enriched for stem cells, their amplifying daughters and niche cells compared to wild type testes. *bowl* encodes for a Zinc-finger protein and is a member of the *odd-skipped* family of transcription factors, which includes *odd-skipped (odd), sister of odd and bowl (sob),* and *drumstick (drm)* (24, 43, 73, 81, 85). The family members share significant homology within their zinc finger domains (64, 69). These genes function in a post-translational relief-of-repression hierarchy, along with an antagonist of *bowl*, *lines.* It has been shown that the *odd* family members *odd, sob* and *drm* share a similar expression pattern and can function redundantly in some tissues, while *bowl* appears to act uniquely as evidenced by its broader expression domain (43, 85). Similarly, no such redundancy has been observed for *lines* (69, 73).

The most common form this hierarchy takes is as the *drm/lines/bowl* regulatory cassette, whereby *drm* activity sequesters *lines* in the cytoplasm thereby relieving its repression of *bowl*, allowing nuclear *bowl* accumulation and the expression of target genes. For example, *bowl* is active when it is nuclear. However, when *drm* is absent, *lines* is able to shuttle from the cytoplasm (where it is non-functional) into the nucleus where it can repress *bowl* activity (73). Therefore, *lines* also accumulates in the nucleus when it is functional.

The *bowl* pathway is employed reiteratively throughout development in several *Drosophila* epithelia. During gut development, the *drm/lines/bowl* cassette regulates

morphogenesis by controlling the spatial expression of *unpaired*, the ligand for the Jak-Stat pathway (81, 85). In the eye disc, the pathway regulates proper retinogenesis by controlling the activation of hedgehog (Hh) protein (24). In the *Drosophila* embryo, it is required for proper epidermal cell differentiation (73). Finally, during imaginal disc development the pathway is required downstream of Notch activation in the leg disc (43, 66). Given the myriad roles of this pathway in regulating tissue morphogenesis, our lab previously tested the role of this regulatory cassette in the testis stem cell niche.

Mosaic analyses indicated that *lines* was required for CySC maintenance in the adult testes (48). In fact, CySCs depleted for *lines* activity began to aggregate, accumulate *bowl* protein and take on hub cell characteristics. For instance, *lines* mutant aggregates upregulated several markers of hub cell fate, including Hh and Cactus (112). Aside from the induction of markers of hub cell fate, these *lines*-depleted cells acted like functional niche cells as they recruited neighboring cyst cells to adopt CySC fate.

We next pondered why *lines*-depleted CySCs would adopt hub cell fate. We had shown that there was a lineage relationship between hub cells and CySCs: SGPs could generate both cell types. This, in turn suggested that *lines* was acting in the assembly of the niche during gonadogenesis. To investigate this further, we turned our attention to the *Drosophila* gonad. Here, we show that the *bowl* pathway influences hub cell specification. More specifically, we find that *bowl* promotes this process, while the *bowl* antagonist, *lines*, restricts hub cell fate. Finally, our data suggests that *bowl* functions as a repressor by recruiting the co-repressor, *groucho*, and restricts the cyst cell gene expression program in SGPs.

Results

The *bowl* pathway influences hub cell specification

To determine whether the *bowl* pathway contributes to hub cell specification, we examined mutants of each component of the circuit: *drumstick, lines* and *bowl*. We scored hub cell number in larval gonads shortly after hatching (see Materials and Methods) by staining with the cytoskeletal marker, Filamin, which accumulates in hub cells (165). Larval gonads were also stained with Vasa to recognize germ cells and Traffic jam to recognize somatic cells. We quantitated total hub cell number by stepping through z-slices in the image stack (see Materials and Methods) and in each instance compared the mutants to heterozygous sibling controls. As *drm* positively regulates the accumulation of *bowl* (81, 85), we expected to observe a decrease in hub cell number in *drm* mutants, as well as *bowl* mutants. Indeed, we found that *drm* mutant larval gonads exhibited a significant reduction in hub cell number compared with controls (Figure 3.1; averaging 7 ± 0.5 versus 10 ± 0.7, respectively, $p=0.005$). Furthermore, in *bowl* mutant larval gonads, we detected a 50% reduction in hub cell number in mutants compared with sibling controls (Figure 3.2; averaging 6 ± 0.5 versus 13 ± 0.2, respectively, $p<0.0001$). Finally, this reduction in *bowl* mutants was confirmed by assessing hub cell number using two gene expression markers of hub fate, *esg* and *upd* (Figure 3.2 C-D, F; data not shown).

Conversely, as *lines* normally antagonizes *bowl* function (16, 69, 73, 81, 130), we hypothesized that in the absence of *lines*, which would lead to excess bowl, hub cell number would be increased. Indeed, we observed a substantial increase in hub cell number in *lines* mutants compared with controls (Figure 3.3, from Sarah Freilich;

averaging 14 ± 0.8 versus 10 ± 0.3, respectively, $p<0.001$). Moreover, this increase in hub cell number was confirmed by assessing *upd*- and *esg*-positive hub cells in *lines* mutant gonads compared with controls (data not shown; Figure 3.3 C-D). Lastly, to confirm that the epistatic relationship previously observed in other tissues for *lines* and *bowl* holds true in the gonad (73, 81, 85, 130), we analyzed *linesbowl* double mutants. In these mutants, we would expect to observe a similar reduction in hub cell number as observed in *bowl* mutants. We found that compared to sibling controls, hub cell number in *linesbowl* mutants was decreased (Figure 3.4; averaging 12 ± 0.7 versus 7 ± 0.8, respectively, $p<0.001$), implying that *bowl* is epistatic to *lines* in the *Drosophila* gonad as well. Altogether, these data indicate that the *bowl* pathway contributes to the process of hub cell specification. More specifically, it suggests that *bowl* and *drm* function as positive regulators of this process, promoting hub cell fate, while *lines* functions to restrict hub specification and instead promotes cyst cell fate.

Modulation of *bowl* pathway activity affects GSC number

Given that the hub functions as a critical component of the germline stem cell (GSC) niche and fluctuations in niche cell number can disrupt normal tissue homeostasis (25, 93, 172) we wanted to assess the affect on GSC number when components of the *bowl* pathway were compromised. *Unpaired*-producing hub cells normally recruit adjacent first tier germline cells to become GSCs (93, 172). These hub cells activate the Jak-STAT pathway in neighboring germline cells (93, 153, 172) allowing them to adopt stem cell fate (153). Upon Jak-STAT pathway activation, STAT protein accumulates and is stabilized in the nucleus and this accumulation serves as a useful readout for pathway

activation and a marker for GSCs (35). However, although STAT also accumulates in neighboring cyst cells, we do not have a unique marker for cyst stem cells (CySCs) during gonadogenesis, so changes in CySC recruitment could not be analyzed.

Since the hub is smaller in *bowl* mutants, we expected to observe a correlative decrease in GSC number given that fewer stem cells could potentially be accommodated around a smaller hub. Overall, we found that there were fewer first-tier germline cells recruited in *bowl* mutants compared with controls (Figure 3.5 A-C; averaging 7 ± 0.6 versus 10 ± 0.6, respectively, $p<0.001$). Moreover, among the first-tier germline cells, the number of STAT-positive germline cells adjacent to the hub was also reduced in *bowl* mutants compared with sibling controls (Figure 3.5 D; averaging 4 ± 0.7 versus 9 ± 0.6, respectively, $p<0.001$). These data indicate that a significant reduction in GSC number results from compromised *bowl* pathway activity, such that when hub cell number decreases, GSC number follows. Furthermore, it suggests that the physical space around the hub may account for the number of GSCs recruited. If fewer hub cells are specified, fewer cells can receive an *upd* signal and thus be recruited as stem cells.

Determining the localization of *bowl* pathway components in the gonad

Since the *bowl* pathway is required for proper hub cell specification, we next sought to determine the protein and mRNA localization of pathway components to further understand their roles in this process. To elucidate the mRNA expression patterns of *drm, lines,* and *bowl* we performed RNA in situ hybridizations to each using mid-to-late stage wild type embryos. Unfortunately, however, we were unable to detect mRNA

expression of any of the pathway components within the embryonic gonad (data not shown).

We also attempted to assess the localization of *drm* using a reporter construct consisting of the endogenous promoter of *drm* fused to Gal4 (*drm*-Gal4) (64). We drove nuclear localized gfp (Uas-gfp-nls) and assessed embryonic and first instar larval gonads for gfp expression. Since *drm* positively regulates *bowl* accumulation, we expected to observe *drm* expression in a subset of SGPs: those that are presumptive hub cells. Unfortunately however, we did not detect any gonadal *drm* expression using this reporter assay (data not shown).

Given that *drm* and *bowl* belong to the *odd-skipped* gene family, whose family members *odd-skipped (odd)* and *sister of odd and bowl (sob)* can function redundantly with *drm* (43, 69), we wanted to similarly assess their mRNA localization pattern. Therefore, we performed in situ hybridization to *odd* and *sob*, again using mid-to-late stage wild-type embryos. Here, as well, we were unable to detect any expression within the embryonic gonad (data not shown).

We next turned our attention to understanding the protein localization of the pathway components. As these proteins are only active when localized in the nucleus, determining the sub-cellular localization of the pathway components could indicate the cells where these proteins are functionally required (73). We performed a series of antibody stains against *bowl* in wild-type embryonic and larval gonads, as well as in adult testes. In embryonic gonads prior to niche (hub) compaction, we expected to observe nuclear *bowl* accumulation in a subset of anteriorly-localized SGPs– those SGPs that would presumably take on hub cell fate. In larval gonads and the adult testis, we expected

to detect nuclear *bowl* accumulation in hub cells. Again however, we were unable to detect any protein expression in any of the tissues (data not shown). We took advantage of the fact that *lines* normally represses *bowl* and we examined *lines* mutants, which would presumably express ectopic *bowl*. However, we were still unable to detect endogenous *bowl* protein in *lines* mutant gonads, although we observed robust expression in other embryonic tissues where *bowl* is known to accumulate (data not shown). Although we were unsuccessful in our attempts to detect endogenous protein or mRNA in the *Drosophila* gonad and testes, we were comforted by the fact that historically it has been difficult to detect these components in various tissues (73). Presumably, this is because they are expressed at very low levels, undetectable even to our most sensitive assays.

Since it proved difficult to detect either endogenous protein or mRNA, we took advantage of a series of *bowl* pathway reporters. We began by examining the expression of *lines* in embryonic gonads using an epitope-tagged version of the protein, Uas-*lines*-myc (73) driven by the SGP driver, Traffic jam-Gal4 (Tj-Gal4). Although misexpressing this construct does not indicate the endogenous protein expression domain, the sub-cellular localization could suggest where this protein is normally required. For example, in cells where *lines* is active, the protein would accumulate in the nucleus. Conversely, in cells where *lines* is inhibited by *drm* activity, the protein would accumulate in the cytoplasm. In Tj-Gal4>Uas-*lines*-myc gonads, by stage 13, we were able to detect nuclearly localized *lines*-myc, suggesting that *lines* could be active in a subset of SGPs (Figure 3.6 A-A’’).

In a similar fashion, we probed the sub-cellular localization of *bowl* in embryonic gonads using an epitope-tagged version of the protein, Uas-*bowl*-flag (73). We found that a fraction of SGPs in Tj-Gal4>Uas-*bowl*-flag gonads also accumulated nuclear *bowl*-flag beginning at stage 13 (Figure 3.6 B-B''). This data suggests that *bowl* could function in some SGPs to specify hub cell fate. Although this is true, we were unsuccessful in our attempts to assess the localization of both epitope-tagged proteins within the same gonad, therefore it is difficult to conclude which SGPs normally show a requirement for either protein. Assessing the co-localization of the proteins would allow us to determine which subset of cells accumulate nuclear *bowl* and have cytoplasmic *lines*– these would be presumptive hub cells. Conversely, those cells that only accumulate nuclear *lines* would eventually adopt cyst cell fate. These results could predict which cells normally require the activity of either protein for eventual hub or cyst cell differentiation.

***bowl* is required within SGPs to specify hub cells**

Although endogenous *bowl* is undetectable in the gonad, it is robustly expressed in the *Drosophila* hindgut and its activity is necessary to properly pattern a hindgut derivative, the small intestine (81). Though the published literature mostly shows *bowl* gut expression restricted to the hindgut (81, 85), our antibody staining suggests that *bowl* could also be expressed in the endodermally-derived posterior midgut (PMG; data not shown). Additionally, the positive *bowl* regulator *drm* accumulates in PMG cells during embryonic stages 11-13 and its expression is subsequently lost in this tissue (64). Given the close proximity of the PMG to the developing gonad (see Figure 2.7 D) and the fact that the Notch-activating ligand Delta is employed from the PMG, we wanted to

definitively exclude the PMG as a candidate source of *bowl*. We therefore sought to knock down *bowl* expression from the endoderm. We misexpressed Uas-*lines,* using Endoderm-Gal4, in an attempt to inhibit *bowl* accumulation. We found that a comparable number of hub cells was specified in control gonads compared with Endoderm-Gal4>Uas-*lines* larval gonads, in which endodermal *bowl* was inhibited (Figure 3.7 A; averaging 14 ± 0.4, n=10 versus 12 ± 0.6, n=20, respectively, p=0.17). These data strongly suggest that *bowl* activity is not required within the neighboring endoderm for proper hub cell specification.

To address whether *bowl* was required within the gonad proper, and specifically within SGPs, we first inhibited *bowl* by overexpressing Uas-*lines* within SGPs, using *Twist24B*-Gal4 (110). Compared to control gonads, inhibiting bowl within the SGP population yields a small, but statistically significant decrease in hub cell number (Figure 3.7 B; averaging 15 ± 0.4 versus 13 ± 0.5, respectively, p=0.02). This small decrease could be attributed to the fact that endogenous *drm* may yet be present within cells. In this case, it is possible that overexpressing *lines* is not sufficient to override the inhibitory influence of *drm*.

To conclusively determine if *bowl* is required within SGPs, we attempted to rescue the hub cell defect in *bowl* mutant gonads by restoring *bowl* to a subset of SGPs. SGPs are derived from the lateral mesoderm within parasegments (PS) 10-12 (21, 22, 29, 159). We took advantage of the Prd-Gal4 driver, which drives selective expression within PS11 SGPs. Recent lineage-tracing experiments in the adult testis have shown that PS11 SGPs can give rise to either hub cells or CySCs (48, 110).

We first wanted to determine whether overexpressing Uas-*bowl* using Prd-Gal4 was sufficient to increase hub cell number. We found that Prd-Gal4>Uas-*bowl* larval gonads that had sustained *bowl* misexpression throughout embryogenesis did not show a statistically significant increase in hub cell number compared to controls (11 ± 0.4 versus 11 ± 0.5, respectively, p=0.3). Intriguingly however, we found that 85% of *bowl* mutant larval gonads expressing the Prd-Gal4>Uas-*bowl* rescue construct exhibited a wild-type spread of hub cell number, ranging from 9-14, with an average of 11 hub cells per gonad compared to *bowl* mutant controls (without *bowl* restoration) which averaged only 6 hub cells per gonad **(**Figure 3.8; averaging 11 ± 0.7 versus 6 ± 0.5, respectively, $p<0.001$). These results indicate that *bowl* is required within SGPs for proper hub cell specification. In addition, it appears that simply overexpressing *bowl* is not sufficient to increase hub cell number. Furthermore, the data suggests that *bowl* is responsible for specifying hub cell fate primarily within PS11 SGPs, since wild-type hub cell number is virtually restored in rescued gonads.

Towards understanding the interplay between the Notch and *bowl* pathways

Given that the Notch and *bowl* pathways both positively regulate hub cell specification, we sought to more fully understand the relationship between the two. In particular, we wanted to elucidate the epistatic relationship between Notch and *bowl* in the gonad. *bowl* is required downstream of Notch signaling to properly specify tarsal segments of the *Drosophila* leg during development (43, 65, 66). Even so, *bowl* has also been shown to regulate Notch pathway activity by regulating the expression of both pathway-activating ligands, Serrate (81) and Delta (65). The fact that *bowl* can sometimes

act upstream of Notch signaling in some tissues, but downstream in others made understanding this complex relationship even more intriguing. Unfortunately, due to complications associated with unhealthy fly stocks, we were unable to address whether *bowl* was required downstream of Notch signaling to properly specify hub cells.

We then set out to test whether Notch activity was required downstream of *bowl* by performing a rescue experiment. Similar to above, we first wanted to determine whether overacting the Notch pathway by expressing a constitutively active version of the Notch intracellular domain, $UasN^{ICD}$, during embryogenesis could lead to an increase in hub cell number. We found that Prd-Gal4>$UasN^{ICD}$ larval gonads did show a statistically significant increase in hub cell number compared to controls (16 ± 1.3 versus 13 ± 0.5, respectively, $p<0.001$). Furthermore, in preliminary data we found that hub cell number was virtually restored to the wild-type range in *bowl* mutant larval gonads expressing the Prd-Gal4>$UasN^{ICD}$ rescue construct, with an average of 10 hub cells per gonad compared to *bowl* mutant controls which averaged only 6 hub cells per gonad (Figure 3.9; averaging 10 ± 0.8 versus 6 ± 0.5, respectively, $p<0.001$).

Since *bowl* is presumably absent from these PS11 cells in this experiment, this data suggests that Notch is able to engage the unknown pathway, "pathway X," downstream to induce hub cell fate. However, given that pathway X has yet to be identified and because we cannot assess *bowl* pathway accumulation in SGPs, it still remains unclear where these proteins are normally required for proper hub cell specification. Finally, since Notch activation is presumably able to engage pathway X, given this data it is difficult to interpret the relationship of Notch relative to *bowl.*

bowl and Su(H) genetically interact

Our data indicate that there are at least two important transcriptional regulators functioning within the hub: *bowl* and *Su(H)*. *Su(H)* is an integral part of the Notch signaling pathway and it activates downstream target genes in response to Notch activation (13). Considering that both the Notch and *bowl* pathways positively influence hub specification, we wanted to confirm that the transcriptional regulators displayed a genetic interaction.

To assess the genetic interaction, we analyzed hub cell number in *bowl* and *Su(H)* heterozygous adults compared to *bowl/Su(H)* transheterozygotes. Fortuitously, hub cells are a post-mitotic population (68). Due to this, the number of hub cells specified during larval stages remains relatively static during adulthood. Either *bowl* or *Su(H)* heterozygous testes averaged 14 hub cells (Figure 3.10; 14 ± 0.6 and 14 ± 0.4, respectively, $p>0.05$). As predicted, we observed a synergistic decrease in hub cell number in *bowl;Su(H)* transheterozygotes, such that only 11 hub cells were specified on average (Figure 3.10; 11 ± 0.4, $p<0.001$ compared to either heterozygous condition). This data indicates that the transcriptional regulators, *bowl* and *Su(H)*, genetically interact and are both positive-acting factors promoting hub cell specification.

bowl and *groucho* interact to properly specify hub cells

Although Bowl is a known transcriptional regulator (69, 181), it remains unclear whether it functions as an activator or a repressor in the process of hub cell specification. Bowl contains three putative transcriptional activation domains (181). Additionally, it also contains an engrailed homology 1 (eh1) domain that recruits the general co-

repressor, Groucho (Gro), to mediate the repressive affect of Bowl (60). Given this, we wanted to understand whether Bowl was acting in SGPs as an activator– to promote hub cell gene expression, or as a repressor– to restrict the cyst cell gene expression program.

Groucho is a ubiquitously expressed co-repressor that silences gene expression when it is recruited to target promoters. It cannot bind DNA itself and thus must interact with a number of DNA-binding transcriptional regulators to induce its repressive affect. Gro functions in multiple signaling pathways, including Wnt (Wingless in the fly), Hh, EGFR, Dpp and Notch (72). When these pathways become activated via ligand induction, Gro and its associated co-repressors are replaced on target DNA by an activator complex. As mentioned above Gro also forms a repressor complex with Bowl and acts to repress Bowl targets. Since Gro interacts with diverse pathway regulators, mutations in this gene can lead to ectopic target gene expression and pleiotropic affects.

To determine if Bowl was functioning as an activator or a repressor, we tested whether the Bowl interaction with Gro was necessary for proper hub cell specification (60). We hypothesized that if Bowl interaction with Gro was necessary for proper hub number, Bowl would function as a repressor in this system. We took advantage of a Bowl protein that has a deleted eh1 domain, Uas-*bowl*$^{eh1-}$, and thus does not interact with Gro (16). Driving expression of the native Bowl protein as a control using the SGP driver, *Twist24B*-Gal4, led to a statistically significant increase in the number of hub cells specified compared to the Uas-only and the driver-only controls (Figure 3.12 A; 16 ± 0.7 versus 10 ± 0.5 and 11 ± 0.6, respectively, $p<0.0001$ for both). If the Bowl interaction with Gro was necessary for this increase in hub cell number, we would expect no change in hub number when misexpressing Uas-*bowl*$^{eh1-}$. Surprisingly however, we found that

there was a decrease in hub cell number in *Twist24B*-Gal4>Uas-*bowl*$^{eh1-}$ gonads compared to the Uas-only control (Figure 3.12 B; 12 ± 0.7 versus 15 ± 0.5, respectively, p=0.003). We interpret this to mean that this Uas-*bowl*$^{eh1-}$ protein is behaving as a dominant negative and as such, is interfering with the normal repressive function of wild-type Bowl in SGPs. *bowl*$^{eh1-}$ is able to bind to target DNA in place of wild-type Bowl, but without the eh1 domain, Gro is not recruited to Bowl repressor complexes. This suggests that the normal role of Bowl may be to restrict cyst cell fate in SGPs by repressing the cyst cell gene expression program. The repression of cyst cell fate in turn promotes hub cell specification.

lines and *groucho* genetically interact

Our data shows that Notch and *bowl* activity in a subset of SGPs ultimately leads to hub cell specification. Presumably, those SGPs that remain inactive for Notch also accumulate nuclear *lines*, which prevents hub specification, and instead promotes cyst cell fate. Given that the absence of *lines* results in increased hub number and that depletion of *gro* can lead to derepression of Notch target genes, we wanted to determine if *lines* and *gro* genetically interacted. We therefore compared *lines* and *gro* heterozygous adult testes to *lines/+;gro/+* transheterozygotes. If these two genes interact, we would expect to observe a synergistic increase in hub cell number because SGPs that would normally be inactive for Notch and *bowl* are now able to accumulate ectopic Notch target genes and ectopic *bowl*. Indeed, we found that there was a strong genetic interaction between *lines* and *gro* as hub cell number increased to an average of 16 per testes (Figure 3.11; 16 ± 0.5) compared to an average of 10 (10 ± 0.3, p<0.0001) for *lines* heterozygotes

and 11 for *gro* heterozygotes (11 ± 0.2, $p<0.0001$). This data indicates that *lines* and *gro* genetically interact and suggests that some SGPs that would normally be cyst cells, instead developed as hub cells.

Figure 3.1:

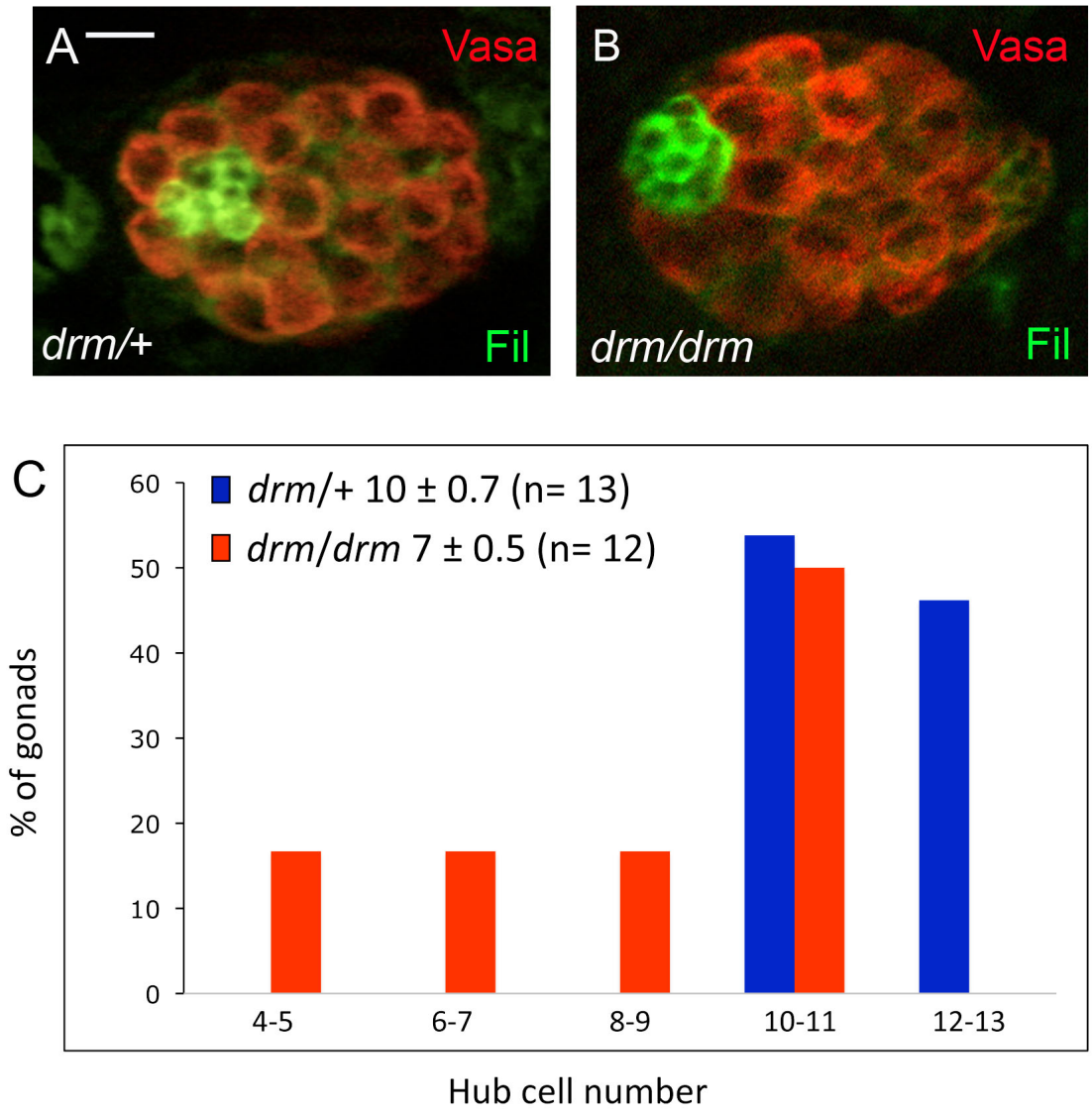
A
Vasa
drm/+
Fil
B
Vasa
drm/drm
Fil
C
drm/+ 10 ± 0.7 (n= 13)
drm/drm 7 ± 0.5 (n= 12)
% of gonads
60
50
40
30
20
10
0
4-5
6-7
8-9
10-11
12-13
Hub cell number

Figure 3.1: *drm* contributes to hub cell specification

Anterior is to the left in each panel. (A, B) 1st larval instar male gonads from control (A; *drm/+*) and *drm* mutants (B) were stained with Vasa (red, germ cells) and Filamin (green, hub cells). (B) Hub cell number is decreased in this focal plane compared to A. Scale bar is 10μm. (C) The distribution of the number of Filamin positive hub cells per gonad. Note the significant shift to lower hub cell numbers in *drm* mutants (red) compared to control gonads (blue; p=0.005). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 3.2:

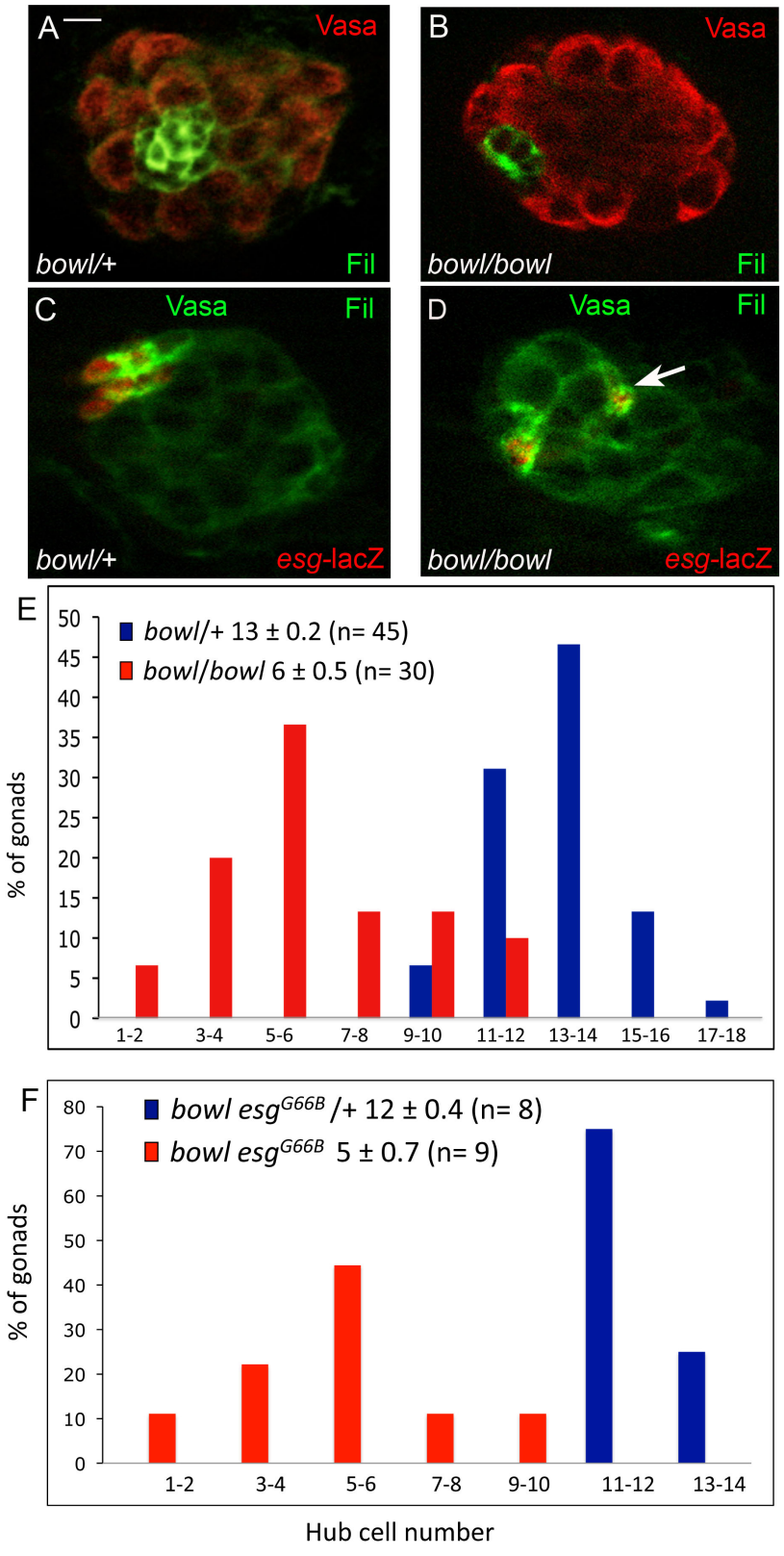
A
Vasa
bowl/+
Fil
B
Vasa
bowl/bowl
Fil
C
Vasa
Fil
bowl/+
esg-lacZ
D
Vasa
Fil
bowl/bowl
esg-lacZ
E
bowl/+ 13 ± 0.2 (n= 45)
bowl/bowl 6 ± 0.5 (n= 30)
% of gonads
50
45
40
35
30
25
20
15
10
5
0
1-2
3-4
5-6
7-8
9-10
11-12
13-14
15-16
17-18
F
bowl esg^G66B /+ 12 ± 0.4 (n= 8)
bowl esg^G66B 5 ± 0.7 (n= 9)
% of gonads
80
70
60
50
40
30
20
10
0
1-2
3-4
5-6
7-8
9-10
11-12
13-14
Hub cell number

Figure 3.2: *bowl* mutant gonads exhibit a reduced hub cell number

1st larval instar gonads were stained with Filamin (green, hub cells). (A, B) Control (*bowl*/+) and *bowl* mutant gonads were stained with Vasa (red) to detect germ cells. Note the decrease in hub cell number in a *bowl* mutant (B) compared to a control gonad (A, C). (D, E) Control (*bowl*/+) and *bowl* mutant gonads expressing an esg^{G66B} enhancer trap. Gonads were stained with anti-βgal (red) to detect hub cells and Vasa (green) to detect germ cells. The decrease in hub number in *bowl* mutants (E) compared to controls (D) was confirmed by this enhancer trap. An arrow denotes an *esg*-positive hub cell away from the main hub, which lies in another focal plane. Single hub cells are observed at a low frequency in *bowl* mutants. Scale bar is 10μm. (C, F) The distribution of the number of Filamin positive (C) and *esg*-positive (F) hub cells per gonad is shown. There is a significant shift to lower hub cell numbers in *bowl* mutants (red) compared to control gonads (blue) ($p<0.0001$) in both. The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 3.3:

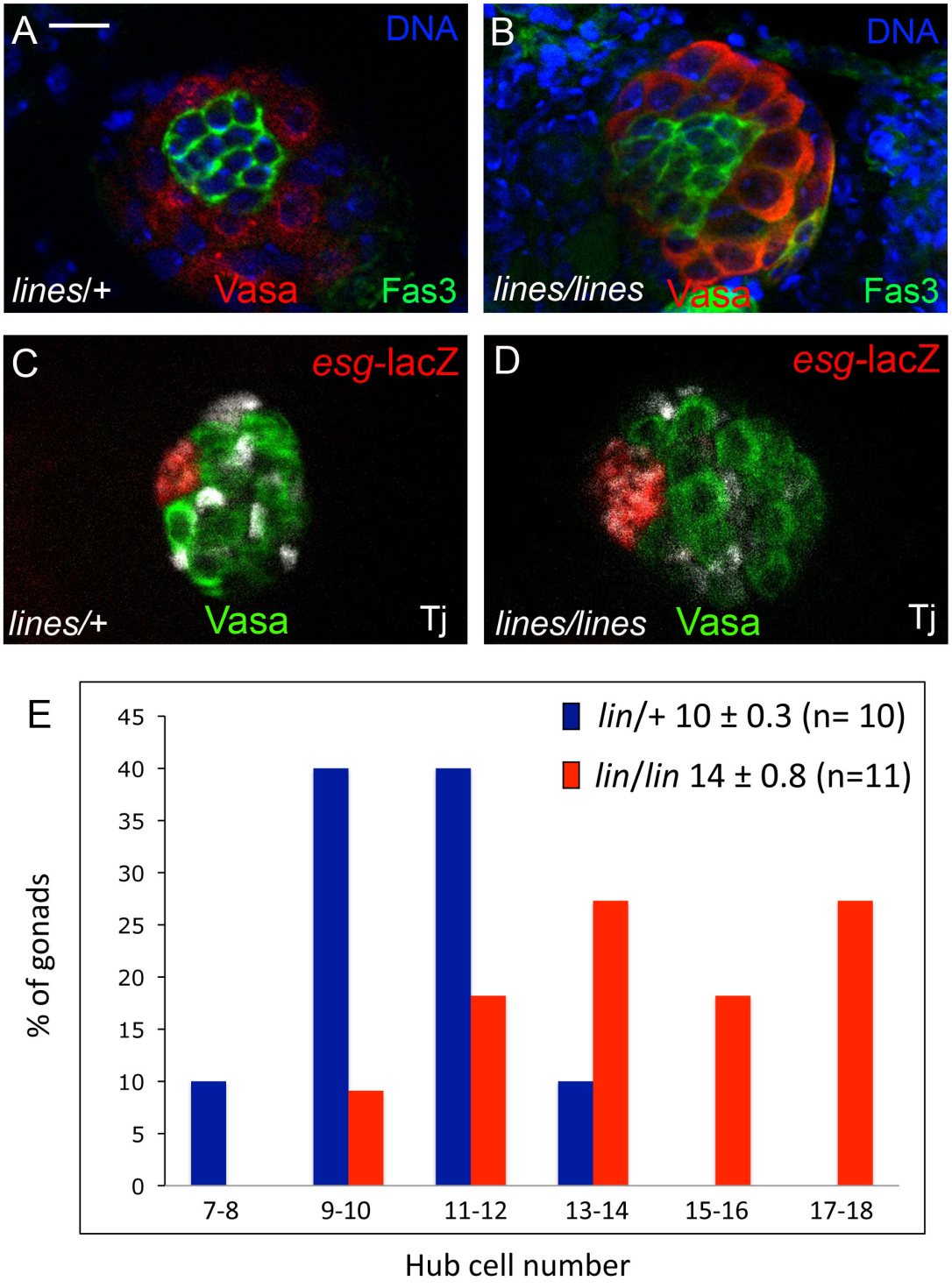
A
DNA
lines/+
Vasa
Fas3
B
DNA
lines/lines
Vasa
Fas3
C
esg-lacZ
lines/+
Vasa
Tj
D
esg-lacZ
lines/lines
Vasa
Tj
E
lin/+ 10 ± 0.3 (n= 10)
lin/lin 14 ± 0.8 (n=11)
% of gonads
45
40
35
30
25
20
15
10
5
0
7-8
9-10
11-12
13-14
15-16
17-18
Hub cell number

Figure 3.3: *lines* mutant gonads contain excess hub cells

(A, B) 1st larval instar gonads from control (*lines*/+) and *lines* mutant gonads were stained with Vasa (red, germ cells), Fascilin 3 (green) to detect hub cells and Hoechst (blue, DNA). Note the increase in hub cell number in this focal plane of a *lines* mutant (B) compared to a control gonad (A). (C, D) Control (*lines*/+) and *lines* mutant gonads expressing an esg^{G66B} enhancer trap. Gonads were stained with anti-βgal (green) to detect hub cells. The increase in hub number in *lines* mutants (D) compared to controls (C) was confirmed by this enhancer trap. Scale bar is 10μm. (E) The distribution of the number of Fascilin 3 positive (E) hub cells per gonad is shown. Note the significant shift to higher hub cell numbers in *lines* mutants (red) compared to control gonads (blue) ($p<0.001$). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown. [Sarah Freilich, (48)]

Figure 3.4:

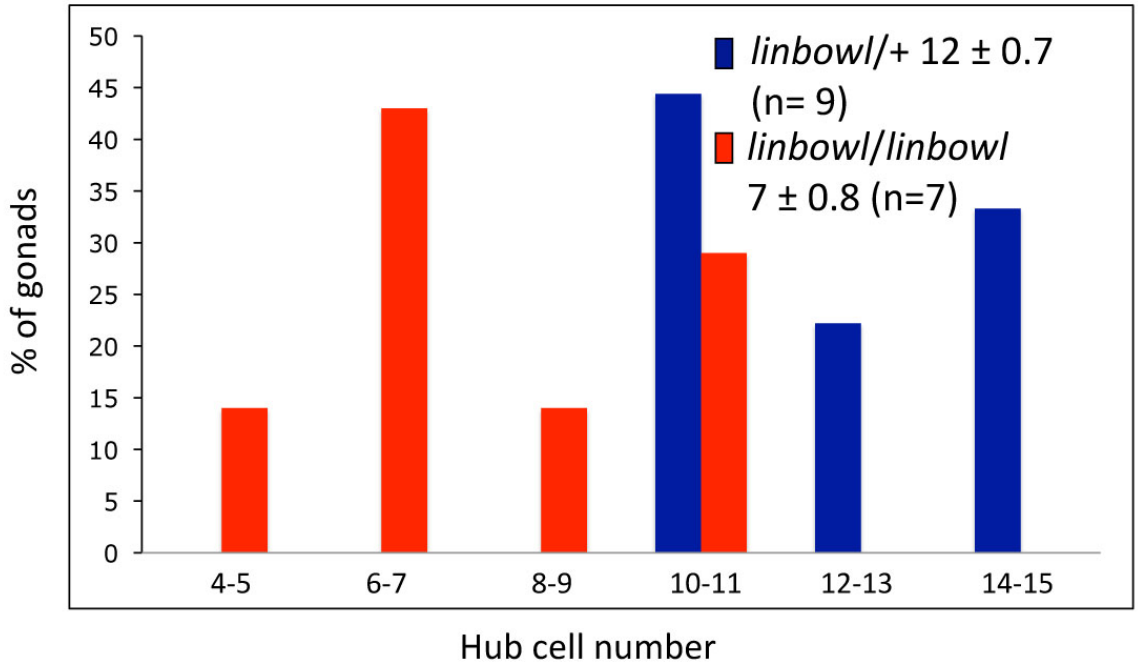

Figure 3.4: *bowl* is epistatic to *lines* in the gonad

The distribution of the number of Filamin positive hub cells per gonad in controls (*linesbowl*/+; blue) compared to *linesbowl* mutant (red) gonads is shown. Note that the reduction in hub cell number in *linesbowl* mutant gonads is similar to the reduction observed in *bowl* mutants ($p<0.001$). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 3.5:

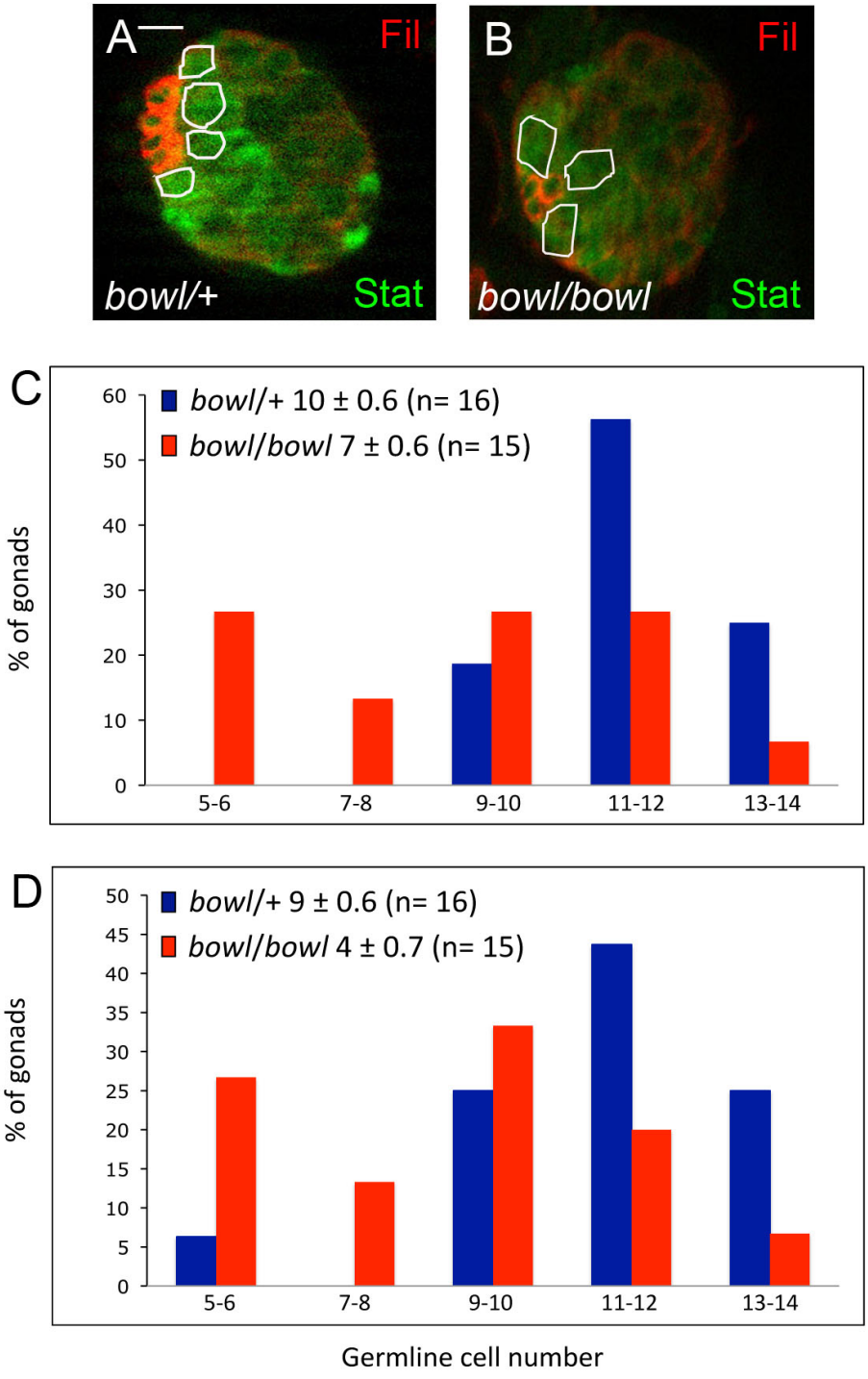
A
Fil
bowl/+
Stat
B
Fil
bowl/bowl
Stat
C
bowl/+ 10 ± 0.6 (n= 16)
bowl/bowl 7 ± 0.6 (n= 15)
% of gonads
60
50
40
30
20
10
0
5-6
7-8
9-10
11-12
13-14
D
bowl/+ 9 ± 0.6 (n= 16)
bowl/bowl 4 ± 0.7 (n= 15)
% of gonads
50
45
40
35
30
25
20
15
10
5
0
5-6
7-8
9-10
11-12
13-14
Germline cell number

Figure 3.5: GSC number is reduced in *bowl* mutant gonads

(A, B) 1st larval instar male gonads from control (A; *bowl*/+) and *bowl* mutants (B) were stained with Filamin (red, hub cells) and STAT (green). Note that there are fewer first tier germline cells (B). Circles highlight GSCs. Scale bar is 10μm. (C) The distribution of the number of first tier germline cells per gonad in controls (*bowl*/+; blue) compared to *bowl* mutant (red) gonads is shown ($p<0.001$). (D) The distribution of the number of STAT-positive GSCs per gonad in controls (*bowl*/+; blue) compared to *bowl* mutant (red) gonads is shown ($p<0.001$). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 3.6:

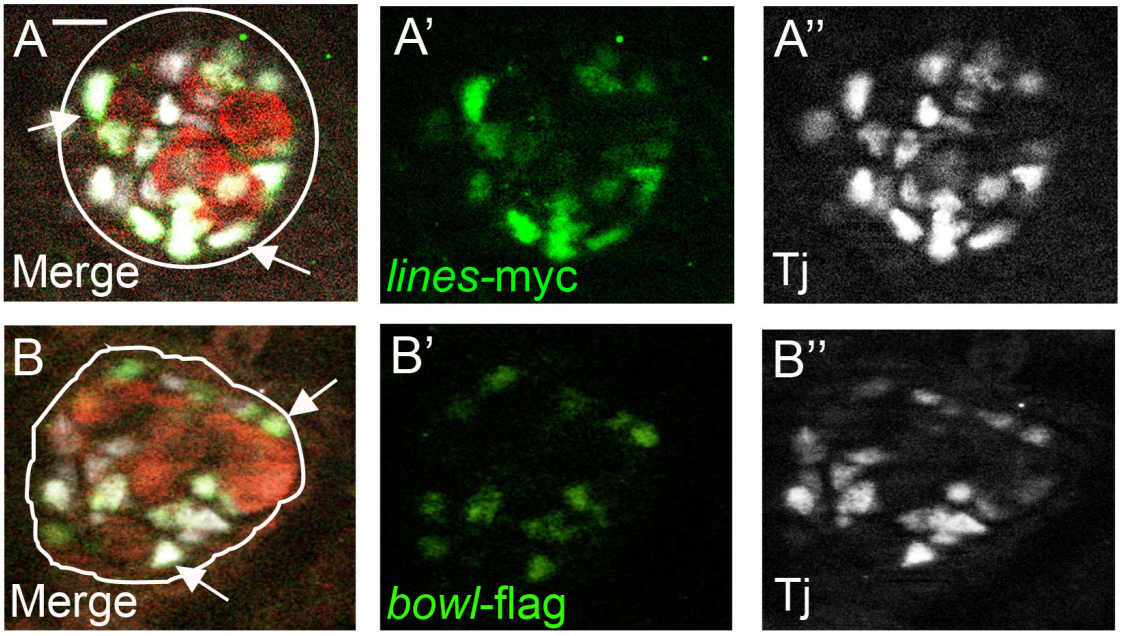

Figure 3.6: Epitope-tagged *lines* and *bowl* protein accumulate in the nucleus of SGPs

Stage 15 male embryonic gonads were stained for Vasa (red, germ cells) and a nuclear SGP marker, Traffic jam (Tj, white). (A-A'') A Tj-Gal4>Uas-*lines*-myc embryonic gonad accumulates nuclear *lines*-myc (A', green) and co-stains with Tj. (B-B'') A Tj-Gal4>Uas-*bowl*-flag embryonic gonad accumulates nuclear *bowl*-flag (B', green) and co-stains with Tj. Scale bar is 10μm. Arrows highlight a few cells that accumulate both nuclear Tj and the epitope-tagged proteins. A circle outlines the gonad.

Figure 3.7:

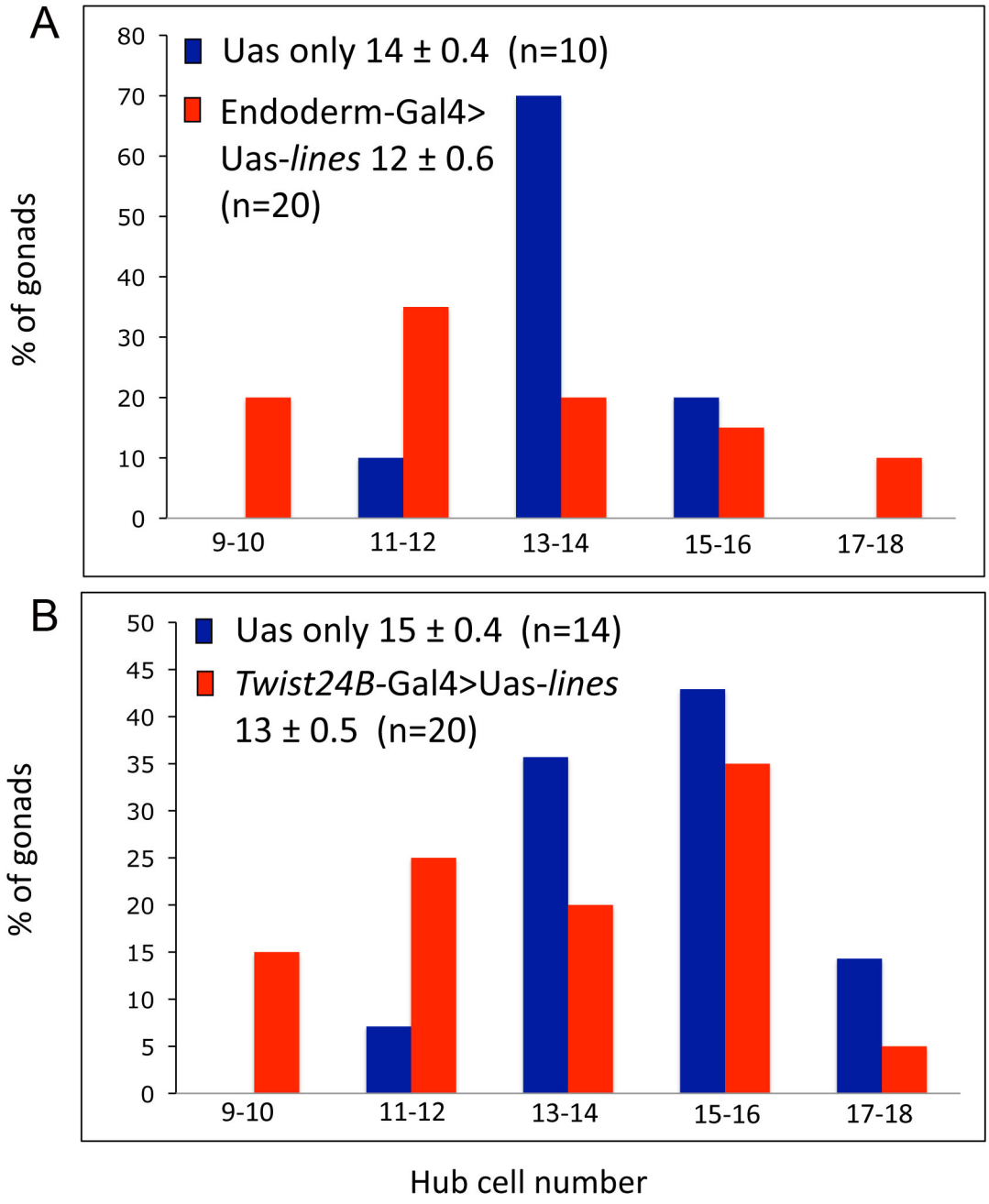
A
80
70
60
50
40
30
20
10
0
% of gonads
Uas only 14 ± 0.4 (n=10)
Endoderm-Gal4>
Uas-lines 12 ± 0.6
(n=20)
9-10
11-12
13-14
15-16
17-18
B
50
45
40
35
30
25
20
15
10
5
0
% of gonads
Uas only 15 ± 0.4 (n=14)
Twist24B-Gal4>Uas-lines
13 ± 0.5 (n=20)
9-10
11-12
13-14
15-16
17-18
Hub cell number

Figure 3.7: Inhibiting *bowl* in SGPs, but not in the endoderm reduces hub cell number

(A) The distribution of the number of Filamin positive hub cells per gonad in controls (Uas-*lines*/CyO; blue) compared to Endoderm-Gal4>Uas-*lines* (red) gonads is shown (red; p=0.17). (B) The distribution of the number of Filamin positive hub cells per gonad in controls (Uas-*lines*; blue) compared to *Twist24B*-Gal4>Uas-*lines* (red) gonads is shown. Note that there is a modest decrease in hub cell number when *bowl* is inhibited in SGPs (red; p=0.02). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 3.8:

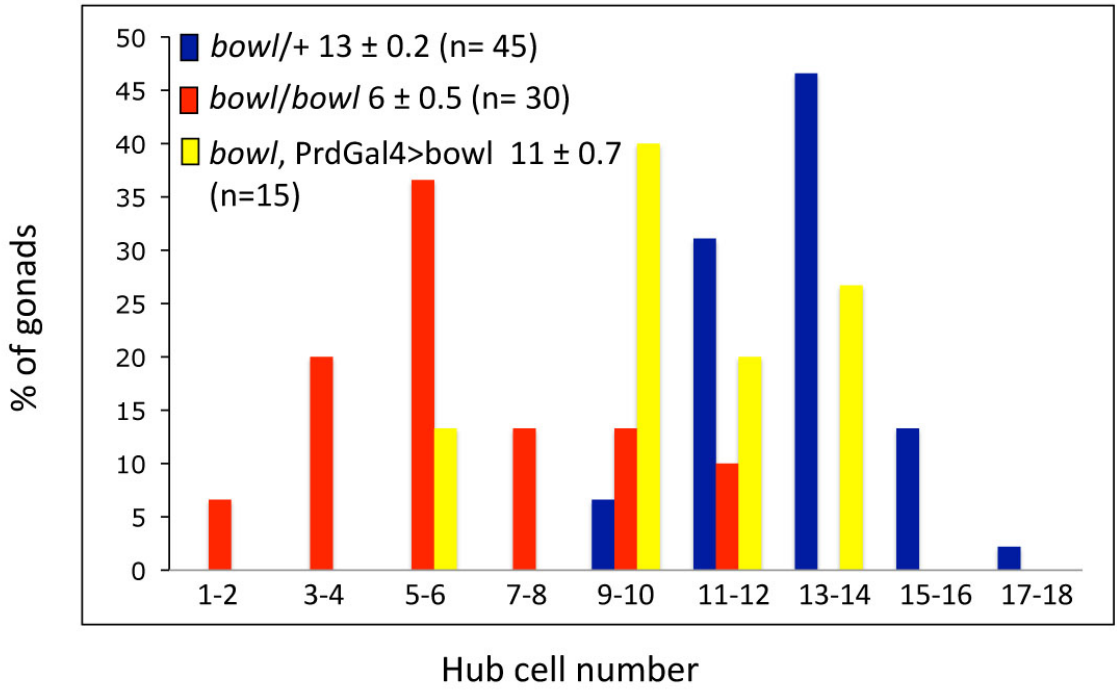

Figure 3.8: Restoring *bowl* in PS11 SGPs rescues the hub cell defect

The distribution of the number of Filamin positive hub cells per gonad in heterozygous siblings (*bowl*/+; blue), *bowl* mutant (red) and *bowl*, Prd-Gal4>Uas-*bowl* (yellow) gonads is shown. Note that there is a significant rescue of hub cell specification to almost wild type numbers (blue) in *bowl*, Prd-Gal4>Uas-*bowl* gonads (yellow) compared to *bowl* mutants (red; $p<0.001$). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 3.9:

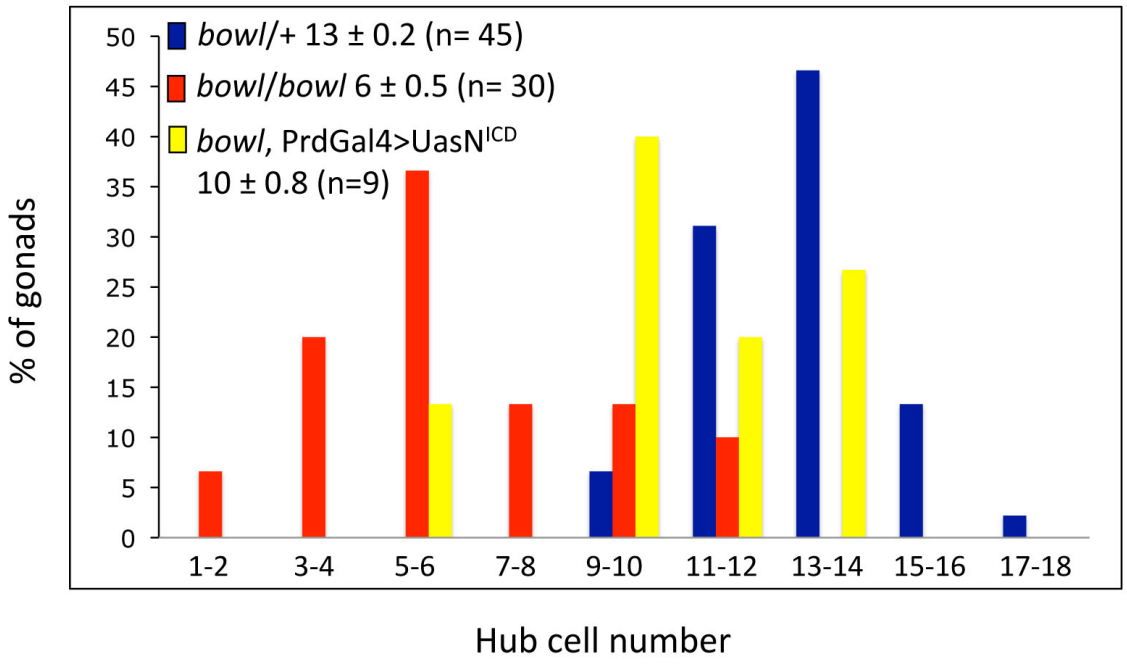

Figure 3.9: Constitutively activating Notch in PS11 SGPs rescues the hub cell defect

The distribution of the number of Filamin positive hub cells per gonad in heterozygous siblings (*bowl*/+; blue), *bowl* mutant (red) and *bowl*, Prd-Gal4>UasN^{ICD} (yellow) gonads is shown. Note that there is a significant rescue of hub cell specification to almost wild type numbers (blue) in *bowl*, Prd-Gal4>UasN^{ICD} gonads (yellow) compared to *bowl* mutants (red; $p<0.001$). The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 3.10:

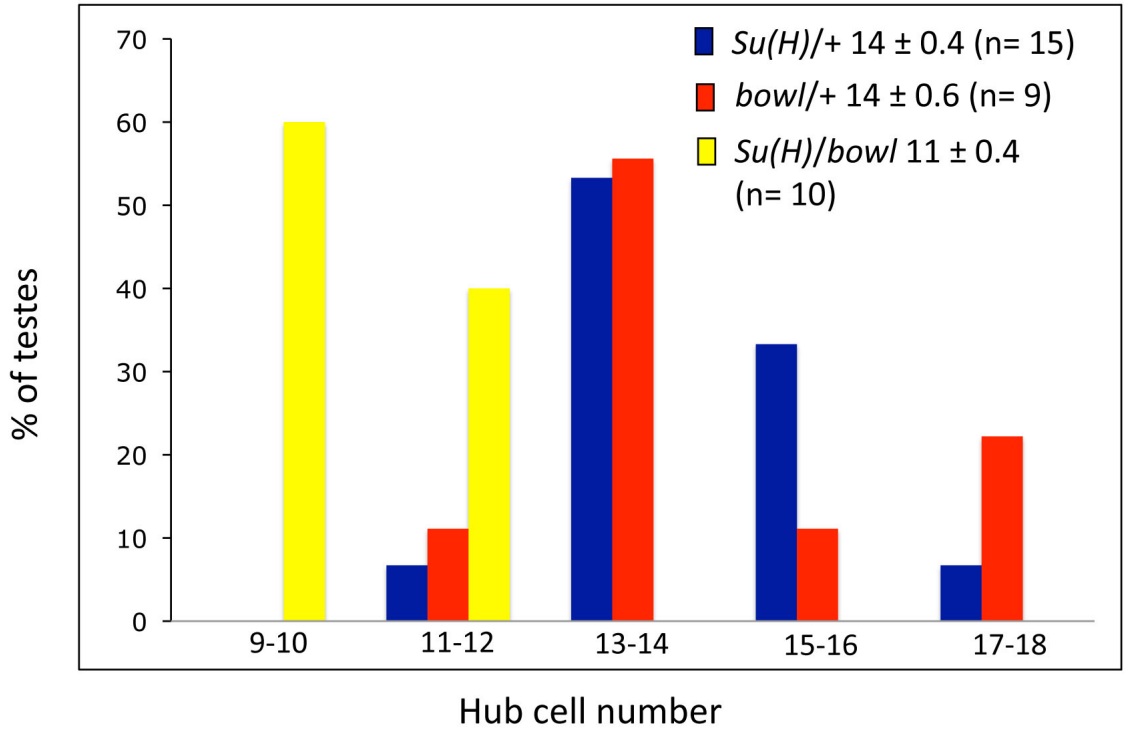

Figure 3.10: *bowl* and *Su(H)* genetically interact and promote hub cell specification

The distribution of the number of Filamin positive hub cells per testes. Note the shift to lower hub cell numbers in *bowl*/*Su(H)* transheterozygotes (yellow) compared to *Su(H)*/+ (blue) and *bowl*/+ (red) control testes ($p<0.001$ for both heterozygous conditions). The average number of hub cells per testis ± s.e.m. and the number of testes (n) observed is also shown.

Figure 3.11:

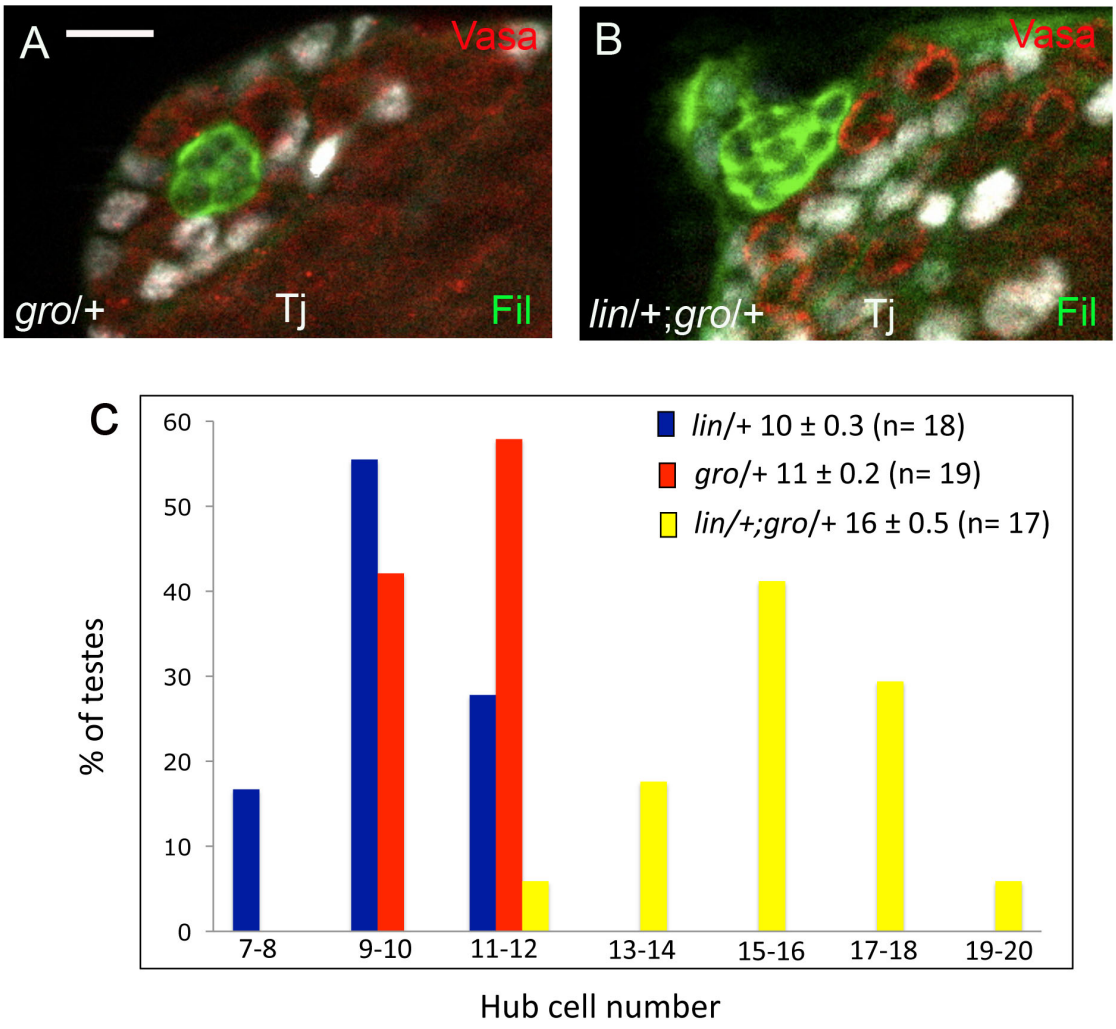
A
Vasa
gro/+
Tj
Fil
B
Vasa
lin/+;gro/+
Tj
Fil
C
lin/+ 10 ± 0.3 (n= 18)
gro/+ 11 ± 0.2 (n= 19)
lin/+;gro/+ 16 ± 0.5 (n= 17)
% of testes
60
50
40
30
20
10
0
7-8
9-10
11-12
13-14
15-16
17-18
19-20
Hub cell number

Figure 3.11: *lines* and *gro* show a genetic interaction

(A, B) Adult testes from control (A; *gro*/+) and *lin*/+;*gro*/+ transheterozygotes (B) were stained with Vasa (red, germ cells), Traffic jam (Tj, white, somatic cells) and Filamin (green, hub cells). Note the expanded hub size in *lin*/+;*gro*/+ transheterozygotes testes (B) compared to controls (A). Scale bar is 30μm. (C) The distribution of the number of Filamin positive hub cells per testes. Note the shift to higher hub cell numbers in *lin*/+;*gro*/+ transheterozygotes (yellow) compared to *lin*/+ (blue) and *gro*/+ (red) control testes ($p<0.0001$ for both heterozygous conditions). The average number of hub cells per testis ± s.e.m. and the number of testes (n) observed is also shown.

Figure 3.12:

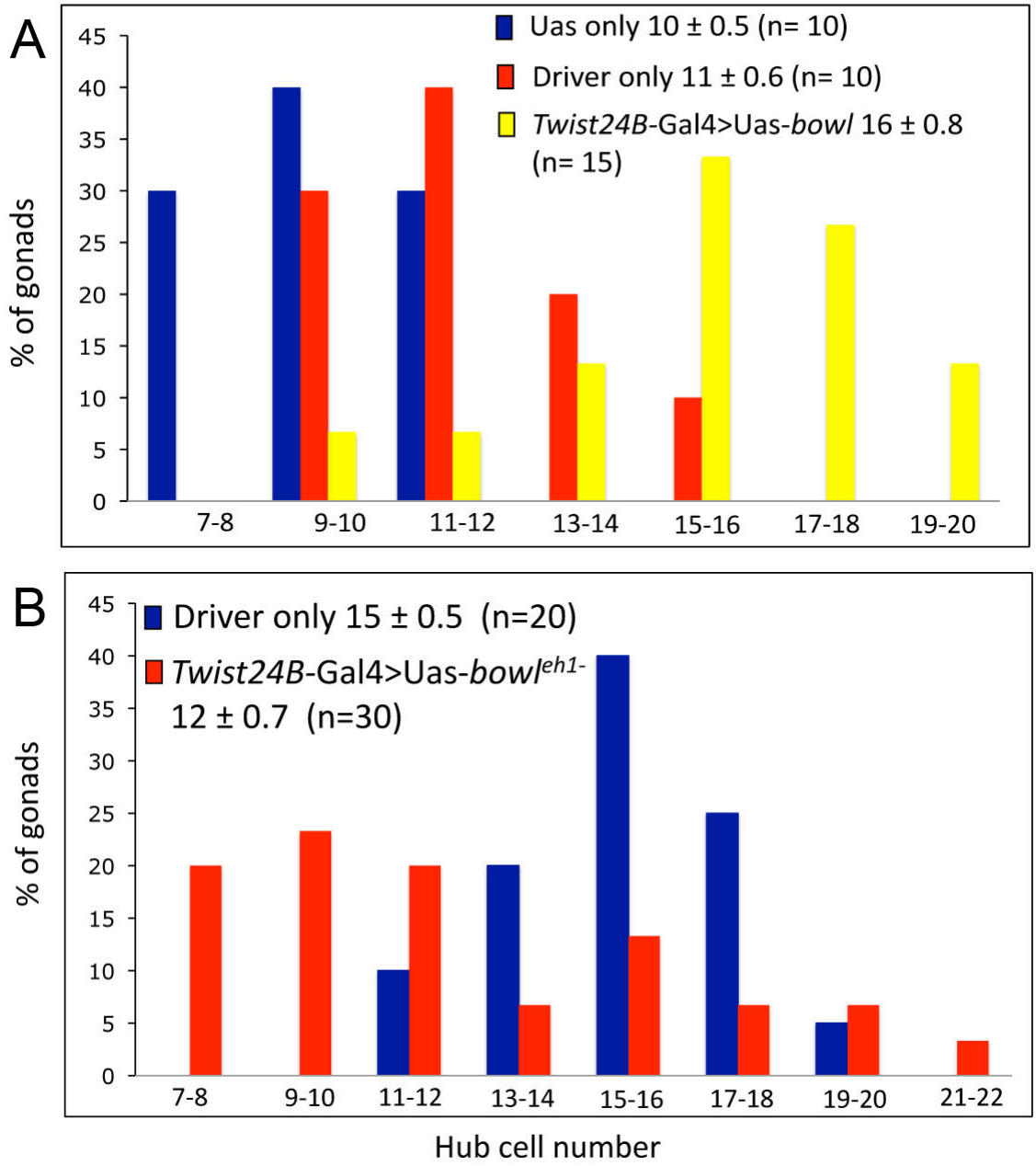
A
Uas only 10 ± 0.5 (n= 10)
Driver only 11 ± 0.6 (n= 10)
Twist24B-Gal4>Uas-bowl 16 ± 0.8 (n= 15)
% of gonads
45
40
35
30
25
20
15
10
5
0
7-8
9-10
11-12
13-14
15-16
17-18
19-20
B
Driver only 15 ± 0.5 (n=20)
Twist24B-Gal4>Uas-bowl^eh1- 12 ± 0.7 (n=30)
% of gonads
45
40
35
30
25
20
15
10
5
0
7-8
9-10
11-12
13-14
15-16
17-18
19-20
21-22
Hub cell number

Figure 3.12: *bowl* recruits *gro* to regulate hub cell specification

(A) The distribution of the number of Filamin positive hub cells per gonad in controls Uas-*bowl* (blue) and *Twist24B*-Gal4 (red) compared to *Twist24B*-Gal4>Uas-*bowl* (yellow) gonads is shown ($p<0.0001$). Note that there is a significant increase in hub cell number in *Twist24B*-Gal4>Uas-*bowl* gonads. (B) The distribution of the number of Filamin positive hub cells per gonad in controls (Uas-$bowl^{eh1-}$/CyO; blue) compared to *Twist24B*-Gal4>Uas-$bowl^{eh1-}$ (red) gonads is shown. Surprisingly, there is a considerable decrease in hub cell number when the eh1 domain is deleted (red; $p=0.003$) indicating that the *bowl*/*gro* interaction is necessary for proper hub specification. The average number of hub cells per gonad ± s.e.m. and the number of gonads (n) observed is also shown.

Figure 3.13:

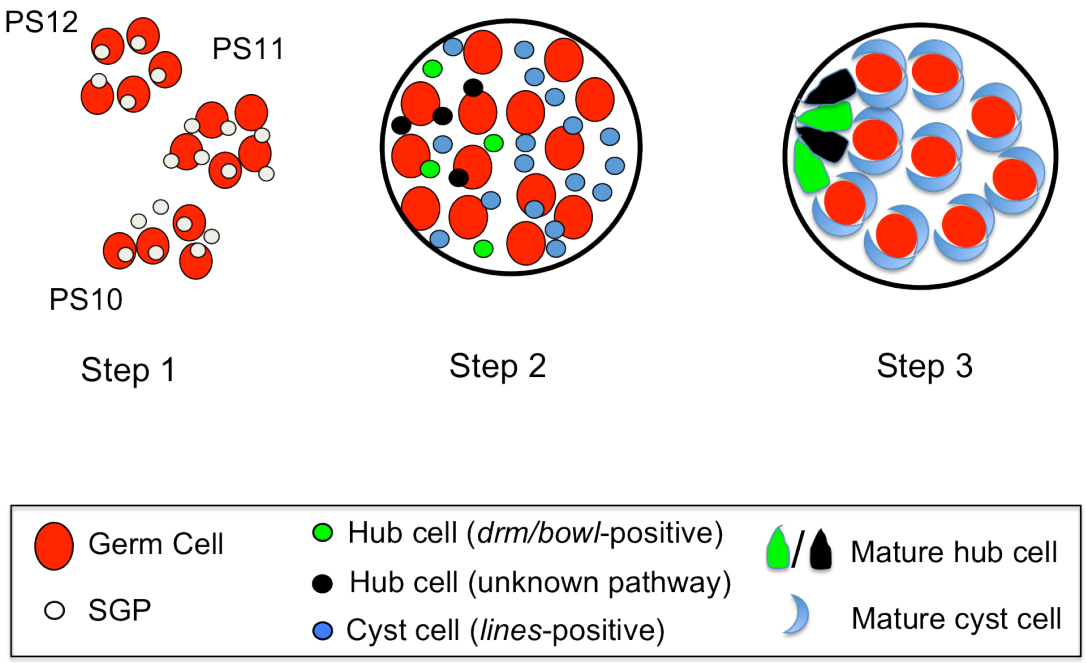

Figure 3.13: Model for *bowl* pathway activity in the gonad

In step 1, SGPs (white) that originate from PS10-12 are initially equivalent. In step 2, activation of *drumstick (drm)* positively regulates the nuclear accumulation of Bowl in a subset of SGPs by inhibiting Lines, fating them to become hub cells (green). Those cells that remain inactive for *drm*, accumulate nuclear Lines and adopt cyst cell fate (blue). In step 3, *drm/bowl*-positive SGPs differentiate as mature hub cells (green), while *lines*-positive SGPs differentiate as mature cyst cells (blue). Note that since *bowl* activity only accounts for ~50% of hub cells specified, hub cells specified by an unknown pathway, presumably downstream of Notch activation, are depicted in black. Also note that for the sake of simplicity, stem cells nor male-specific SGPs are highlighted in this diagram.

Discussion

Here we show that the *bowl* pathway influences hub cell specification in the *Drosophila* testis. We find that the genes *drm* and *bowl* function as positive acting factors promoting hub specification, while the gene *lines* represses hub cell fate. Furthermore, a genetic interaction is observed between *bowl* and the Notch responsive transcription factor *Su(H)*, confirming that these two pathways both promote hub fate. Lastly, our data suggests that *bowl* may function as a repressor, restricting cyst cell fate in SGPs, while allowing hub cell specification. This data furthers our understanding of how a crucial component of the niche, the hub, is initially specified in this classical model system.

The role of the *bowl* pathway in hub cell specification

The components of the *bowl* pathway influence hub cell specification: *drm* and *bowl* promote hub cells, while *lines* restricts hub cell fate, instead promoting alternative cyst cell fate. This leads to an interesting possibility, whereby these factors parse out SGPs to differentiate as either hub or cyst cells. Since both cell types, cyst and hub, are derived from SGPs (48, 110), it is not difficult to imagine that this circuit could direct eventual cell fate. In fact, the *bowl* pathway is commonly employed to regulate binary cell fate decisions (81, 85, 130). Further support for this idea comes from work recently published by our lab. We showed that CySCs mutant for *lines* in the adult testes accumulate *bowl* protein, begin to dedifferentiate and take on characteristics of hub cells (48). Taken together, we propose the following model. Initially, it is possible that all SGPs are equivalent (Step 1). However, activation of an antagonist of *lines, drm* for example, leads to nuclear accumulation of *bowl* in a subset of SGPs (Step 2). Those SGPs

active for *bowl* are fated to differentiate as hub cells, while cells that retain *lines* activity differentiate as cyst cells (Step 3; Figure 3.13).

Residual hub cells are present in *bowl* mutant gonads

Interestingly, we find that there is an ~50% reduction in hub cell number in *bowl* mutants and that those remaining cells appear compromised for normal hub function. This is intriguing for two reasons. First, there was only a 30% reduction in hub number in gonads mutant for the positive *bowl* regulator, *drm*. This may indicate that another member of the *odd-skipped* family, either *sob* or *odd*, functions redundantly with *drm* in this tissue to regulate *bowl* activity. Although there are no extant mutant alleles for *sob*, a deficiency line exists which uncovers *drm, sob* and *odd* (64). It would be of interest to determine if gonads mutant for all three genes exhibit a greater reduction in hub cell number, comparable to *bowl* mutant gonads.

Second, in chapter two we show that Notch signaling is necessary for this process and that in its absence, neither hub cells nor GSCs are specified. Considering this, it is interesting that there is only a 50% reduction of hub cell number in *bowl* mutants. This suggests that another signaling pathway, possibly functioning downstream of Notch activation, accounts for the remainder of hub cells specified. In fact, our preliminary data suggests just that (Figure 3.9). In an attempt to rescue hub cell number in *bowl* mutant gonads, we misexpressed a constitutively active version of the Notch receptor, $UasN^{ICD}$, solely in PS11 SGPs using Prd-Gal4. We found that hub cell number was virtually restored to wild-type numbers, implying that Notch was able to engage "pathway X" downstream to induce hub cell fate. However, this data does not define where pathway X

is normally required; it only demonstrates that it can be activated in PS11 cells when the Notch pathway is constitutively activated. At present, though it remains unclear what other pathway could be required for this process, elucidating it will be paramount to have a complete understanding of hub cell specification.

Furthermore, this data could also suggest that constitutive activation of the receptor in PS11 SGPs forces cells that would normally be unresponsive to Notch, to become Notch-activated. Kitadate and Kobayashi have shown that hub cell fate is inhibited by the activity of EGFR signaling (97), and it is known that Notch and EGFR commonly antagonize each other (163). Perhaps constitutive activation of the Notch receptor is able to override the inhibitory affect of EGFR activity. This Notch activity could force cells that would normally develop as cyst cells to instead develop as hub cells, by engaging pathway X.

Does *bowl* solely govern hub cell specification in PS11 SGPs?

From our work and others, we have uncovered several new principles guiding hub cell specification in the *Drosophila* testis. First, hub cells derive from both PS10 and PS11 (48, 110). Second, Notch activity is required for this process and pathway activation in a subset of SGPs potentiates them to differentiate as hub cells [this work; (97)]. Finally, our work suggests that *bowl* activity contributes to ~50% of hub cells specified.

In an attempt to rescue the hub cell defect in *bowl* mutants, we restored *bowl* expression uniquely to PS11 SGPs, using *Paired*-Gal4. We found that *bowl* supplied solely to PS11 SGPs is sufficient to rescue the hub cell defect in *bowl* mutants (Figure

3.8). Interestingly however, we found that overexpressing *bowl* from PS11 was not sufficient to increase hub cell number. This suggests that simply overexpressing *bowl* in SGPs is insufficient to alter cell fate, likely because the *bowl* antagonist, *lines,* is still present within a subset of cells. Those cells that express *lines* will still differentiate as cyst cells. The data also implies that *bowl* is normally required to specify hub cell fate specifically in PS11 SGPs. It appears that restoring *bowl* to those SGPs that would normally accumulate it allows the proper number of hub cells to be specified. This is an unexpected result because we hypothesized that, similar to Notch activation, *bowl* would stochastically accumulate among some PS10 and PS11 SGPs directing them toward hub fate, rather than its effect being confined to only one parasegment.

To clarify whether *bowl* is required solely in PS11 or in both PS10 and PS11, we could perform a lineage-tracing experiment in *bowl* mutant gonads where PS10 cells are GFP-labeled. If we find a similar number of GFP+ hub cells specified in *bowl* mutants compared to controls when PS10 SGPs are indelibly marked, this would suggest that *bowl* activity within PS10 is not responsible for hub cell fate, and *bowl* acts solely among PS11 cells. If however, there is a reduction in the number of GFP+ hub cells in *bowl* mutants compared to controls, this would suggest that *bowl* activity is also required within PS10 cells for proper hub specification. This would clarify whether *bowl* is required solely in PS11 or if its activity in both PS10 and PS11 SGPs contributes to proper hub cell specification.

Finally, if we find that Bowl is only required in PS11, it still remains unclear how a subset of these SGPs begin to accumulate Bowl. It is possible that regional parasegment identity set up during early embryogenesis is responsible for the differential accumulation

of Bowl. We could test a series of downstream targets of these early embryonic regulators to determine if they influence Bowl accumulation uniquely in PS10 versus PS11 SGPs. If however, we find that both PS10 and PS11 SGPs require *bowl* activity to become hub cells, it is possible that Notch activation triggers Bowl accumulation in those cells fated to become hub. However, given the difficulty in detecting *bowl* protein and mRNA expression in the gonad, these predictions are difficult to test with current tools.

bowl likely functions as a repressor

Bowl is a known transcriptional regulator and can potentially function as an activator or a repressor. Although its role as an activator has yet to be functionally proven, it has been shown to function as a repressor by recruiting the co-repressor, Groucho (60). We examined the relationship between *bowl* and *gro* in the process of hub cell specification by analyzing testes partially depleted for *lines* (therefore excess Bowl) and *gro.* We observed a significant increase in hub cell number in *lines*/+;*gro*/+ transheterozygotes and found that there was a genetic interaction between the two (Figure 3.11). Since more hub cells are specified in this partially depleted genetic background with excess Bowl, our data could suggest that Bowl normally functions as a repressor to restrict cyst cell fate.

To determine if *bowl* was functioning as a repressor we tested whether the interaction between Bowl and Gro was necessary for hub cell specification. Surprisingly, we found that overexpressing a Bowl missing its Gro interaction domain led to a statistically significant decrease in hub cell number (Figure 3.12 B). Upon closer examination, we believe that $bowl^{eh1-}$ may function as a dominant negative, interfering

with the normal activity of Bowl. It is thought that Bowl recruits Gro to silence gene expression (60). In the absence of this *gro*-interacting domain, *bowl*$^{eh1-}$ binds to DNA, potentially supplanting normal Bowl. As a consequence, such target genes are no longer repressed and therefore more cyst cells are specified instead of hub cells. It is interesting that both hub cells and cyst cyst cells derive from the same precursor pool, since hub cells eventually function as the niche inducing some cyst cells to adopt stem cell fate. Thus, our data suggests that we have identified a pathway that regulates the early niche versus stem cell decision.

Dissecting the interaction between the Notch and *bowl* pathways

Although we were unable to confirm that Notch signaling regulates *bowl* activity in the gonad, this is still a very attractive and simple model. Since both pathways positively regulate hub cell specification, it is easy to speculate that Notch activity leads to *bowl* accumulation and therefore repression of cyst cell gene expression. In fact, *bowl* is required downstream of Notch to properly pattern the *Drosophila* leg (43, 65, 66). Even so, recent work from the Guerrero lab has shown that the two pathways can intersect indirectly at the level of the general co-repressor, *gro* (16).

According to this alternative sequestration model, *bowl* binds *gro* via its eh1 domain and can titrate it away from co-repressor complexes of the Notch, Hh and Wg pathways, causing derepression of target genes (16). Therefore, *lines* activity is critical to modulate proper nuclear *bowl* accumulation. In *lines*/+;*gro*/+ transheterozygous testes, we find that there is a strong genetic interaction, yielding a 50-60% increase in hub cell number (Figure 3.11). A simple way to interpret this data under the sequestration model

is that reduced *gro* sensitizes Notch target genes towards derepression, and that excess *bowl* accumulation then titrates residual *gro* definitively shifting the balance to derepression. Moreover, since it is not known what other pathway contributes to the process in addition to *bowl* and Notch, it is possible that ectopic expression of Hh or Wg targets, due to depleted *gro*, also promotes hub specification. This is especially interesting to posit since both Hh and Wg accumulate in this system, yet functional roles for the pathways have yet to be uncovered. Ultimately, although *bowl* could function as a repressor, given our data, we cannot rule out this potential secondary role of *bowl* in regulating hub specification: by modulating the amount of *gro* bound to repressor complexes.

Finally, hub cells upregulate numerous genes, including *upd* and *hh* (48, 54, 93, 172). Intriguingly, *bowl* regulates the expression of *upd* during gut morphogenesis and *hh* during retinogenesis (24, 81, 85). Therefore, it will be of interest to determine which genes are regulated by *bowl* in this system and how this regulation leads to differential cell fate specification.

Materials and Methods

Fly stocks

Heterozygous siblings or w^{1118} were used as controls as appropriate. We analyzed gonads and testes from the following mutants, or involving these transgenic lines: gro^{1} (FBal0005217), $lines^{2}$ (FBal0011651), $lines^{G2}$ (FBal0117449), drm^{3} (Fbal0121796), $bowl^{1}$ (Fbal0051737), *esgG66* (63), $bowl^{1}$*esgG66*, $lines^{2}$*esgG66*, Uas-*lines*-myc #8 III, Uas-*bowl*-flag, Uas-*bowl*-flag #21 (Victor Hatini, Tufts University), *upd*-Gal4 Uas-GFP

(Erika Bach, NYU), *paired*-Gal4 (FBal0048793), UasN^{ICD} (58), *Twist24B*-Gal4 (A gift from Ruth Lehmann, Skirball Institute), Tj-Gal4 (DGRC, Kyoto Institute of Technology, Japan). Stocks were balanced over CyO P[w+ Ubi-GFP] or TM6 Hu P[w+ Ubi-GFP].

Immunostaining

Embryos were collected on apple agar plates and aged 22-24 hours in a humidified chamber to 1st instar larvae. Hatched larvae were dissected in half with tungsten needles in Ringers solution and the internal organs were gently massaged out. Unhatched larvae were dechorionated, hand-devitellinized and dissected as above. Tissue was fixed in 4% formaldehyde, Ringers and 0.1% Triton-X-100 for 15 minutes, washed in PBTX and blocked one hour at room temperature in 2% normal donkey serum/normal goat serum. Primary antibodies were used overnight at 4°C. Secondary antibodies were used at 1:400 (Alexa488, Cy3 or Cy5; Molecular Probes; Jackson Immuno Research) for 1 hour at room temperature. DNA was stained with Hoechst 33342 (Sigma) at 0.2 μg/ml for 2 minutes.

Immunostaining for testes was performed as previously described except 1X PBS was substituted for Buffer B (169). For embryo studies, embryos were collected, aged for the appropriate time in a humidified chamber, fixed in 4% paraformaldehyde and heptane for 15 minutes and devitellinized with methanol.

The following primary antibodies and concentrations were used: rabbit anti-Vasa 1:5000 (Ruth Lehmann, Skirball Institute), goat anti-Vasa 1:400 (Santa Cruz), chick anti-Vasa 1:5000- 10,000 (K. Howard, University College London), guinea pig anti-Traffic Jam 1:10,000 (Dorothea Godt, University of Toronto), mouse anti Fascilin III 1:25 (Developmental Studies Hybridoma Bank), mouse anti-flag 1:1000 (Sigma, pre-absorbed

on embryos for 1 hr at RT), mouse anti-βgal 1:10000 (Promega), chick anti-GFP 1:1000 (Aves Labs), rabbit anti-myc 1:1000 (Santa Cruz), rabbit anti-bowl 1:5000 (Victor Hatini, Tufts University, pre-absorbed on embryos for 1 hr at RT); rabbit anti-bowl 1:2000 (Sarah Bray, University of Cambridge), guinea pig anti-oddskipped 1:1000 (John Reintiz, SUNY); rabbit anti-STAT 1:1000 (Erica Bach, NYU), rat anti-Filamin-N terminal 1:1000 (Lynn Cooley, Yale University; recognizes full length isoforms), rat anti-Filamin-C terminal 1:1000 (Lynn Cooley; recognizes C-terminal isoform), mouse anti-1B1 1:20 (DSHB).

Sex identification, genotyping and staging of embryos

Male embryos were unambiguous due to larger size of the gonad. Balancer chromosomes containing a GFP-transgene P[w+ TM6 Hu Ubi-GFP] or P[w+ Ubi-GFP] were used to distinguish between heterozygous and homozygous mutant larvae. Embryos were staged according to Campos-Ortega and Hartenstein (32).

In situ hybridization

Digoxigenin-labeled probes (not size-reduced) were synthesized from cDNA plasmids obtained from the BDGP collection or the DGRC. In situ hybridizations were performed as described in Terry et al. (169). An anti-dig-AP antibody 1:1000 (Roche, pre-absorbed on embryos for 1 hr at RT) was used and the signal was developed with NBT/BCIP.

Counting the number of hub cells and germline stem cells

To count hub cell number, larval gonads were stained as needed, and also with anti-Filamin and Hoescht, and z-stacks were obtained through the depth of the gonad

using a Zeiss Axioplan with an ApoTome attachment. Nuclei that were surrounded by a Filamin signal were counted as hub cells.

To count germline stem cells, larval gonads were double stained with anti-Vasa and anti-STAT or anti-1B1 antibodies. Germ cells that were directly adjacent to the hub and that accumulated STAT protein or had a dot spectrosome were scored as stem cells.

Acknowledgements

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Chapter Four:

DISCUSSION:

SUMMARY AND SPECULATIONS

Summary

The studies presented here provide greater insight into the initial specification of niche cells in an *in vivo* stem cell system. The stem cell biology field has just begun to truly appreciate the importance of niche cells in regulating stem cell behavior. Although it is appealing to study niche cells in mammalian systems, complicated tissue architecture has proven difficult in the quest to unambiguously identify stem cells and their supportive niche cells. Therefore, seminal studies expanding our knowledge of stem cell-niche systems have been carried out in invertebrates, such as *C.elegans* and *Drosophila.* Here, we have taken advantage of the *Drosophila* male germline system to conclusively identify two pathways necessary to promote niche cell fate: the Notch and *bowl* pathways. Given the evolutionary conservation of the pathways employed in specifying hub cells in the *Drosophila* testis, basic principles learned may be applicable to the development of niche cells in higher organisms, once they have been conclusively identified. In the following discussion, I will attempt to place my work into the greater context of the field and provide a series of follow-up studies targeted to address a number of remaining questions in the future.

A model for niche cell specification in the *Drosophila* testis

From my work, we have established a working model of hub cell specification in the *Drosophila* testis (Figure 4.1). First, the endodermally-derived posterior midgut (PMG) presents Delta, leading to Notch activation in some SGPs as they are carried over these endodermal cells during germ band retraction. At present, the mechanism guiding Notch activation remains unclear as well as how many SGPs become Notch-activated during this activation process [this work; (97)]. Given the lack of conclusive localization data for *bowl*, it is difficult to determine when *bowl* is required in this system for hub cell specification and its relationship to the Notch pathway. However, for the sake of a simplified model, we propose that it is required in SGPs after Notch activation, in the second step of this process. Interestingly, however, *bowl* activity only appears to contribute to ~50% of the total number of hub cells specified. This may indicate that another pathway is required downstream of Notch activation.

Third, after gonad coalescence the Notch-activated cells must then migrate anteriorly (97, 110). No cues have yet been identified that guide this anterior migration. In the fourth step of this process, Notch-activated cells compact at the anterior of the gonad and are anchored at the anterior pole via integrin-mediated adhesion (165). These cells also undergo a mesenchymal-to-epithelial transition (MET), as evidenced by the upregulation of cell adhesion molecules and preferential associations between hub cells (46, 110). However, it remains unclear if this MET is required for hub cells to terminally differentiate and to express genes indicative of niche cell fate. Finally, as the stem cell-niche system is established, the hub cells induce Upd expression and recruit neighboring

cells to adopt stem cell fate (153). It remains unclear, however, how *upd* gene expression is initiated in hub cells.

Endoderm induction of mesodermal SGPs

Presentation of Delta from neighboring PMG cells activates Notch in a subset of SGPs inducing hub cell specification. Since the PMG is an endoderm derivative and because SGPs are mesodermally-derived, this indicates that a cross-germ layer signaling mechanism is at play. In vertebrates, such as the mouse, zebrafish and *Xenopus*, as well as in *Drosophila*, it is known that conserved signaling inputs from the mesoderm induce endoderm specification and differentiation [(33); Reviewed in (164)]. Now it also appears that the reverse is true: an inductive signal from the endoderm can cause mesodermal cells to differentiate into a specialized cell type: hub cells.

In fact, recent work in the chick shows that endoderm and mesoderm reciprocal signaling establishes pancreatic progenitor cells, those cells that will differentiate into mature cells of the pancreas (89). In the chick embryo, angioblasts (the cells from which blood vessels arise) reside in the mesoderm and adjacent to the gut endoderm from which pancreatic progenitors arise. Angioblasts, which are attracted to the endoderm via chemokine signaling, signal back to the gut endoderm inducing the expression of *Pdx1*, and establish the pancreatic cell fate. In the absence of proper signaling, neither the pancreas nor blood vessels develop normally. This work indicates that cross-germ layer signaling may be a more common phenomenon than previously appreciated.

Interestingly, our work may have parallels in regards to development of the mammalian spermatagonial stem cell niche. Similar to *Drosophila*, in mammals, the

primordial germ cells must migrate through the endoderm to reach the gonadal mesoderm (Figure 4.2) [Figure adapted from (105); Reviewed in (145)]. In *Drosophila*, this involves a series of steps that ultimately results in germ cells being repelled from the midgut and driven into the neighboring mesoderm (82, 83, 161, 195). Thus it appears that in fruitflies the endoderm plays two important roles in respect to germ cells. First, the endoderm delivers the germ cells to the somatic gonadal mesoderm. Second, this same endoderm specifies niche cells from among the somatic mesoderm wherein germ cells can subsequently develop into stem cells.

Although the exact makeup of the mammalian spermatagonial stem cell niche has yet to be uncovered, it must in part derive from cells of the genital ridge, the mesodermal precursor to somatic gonads. In fact, undifferentiated spermatagonia (among which are spermatagonial stem cells, SSCs) lie along the basement membrane of seminiferous tubules and in close contact with somatic Sertoli cells (167). This hints that important regulatory cues emanate from at least a subset of these mesodermally-derived Sertoli cells to maintain SSCs. Given that the endoderm also exercises elaborate control over germ cell migration in mammals, it will be interesting to determine if it plays an additional role in specifying the niche cells for this germline stem cell system.

Notch signaling regulates a binary cell fate decision to specify hub cells

Our data, as well as work from Kitadate and colleagues (97), show that Notch signaling is necessary to specify hub cell fate during gonadogenesis. In the absence of Notch activity we find that only cyst cells are specified, suggesting that these cells are the default cell fate. Since SGPs give rise to both hub cells and cyst cells, Notch activity acts

to regulate this binary cell fate decision. This is not a novel role for Notch, as it regulates cell fate choices in many developing systems. One of the most well studied examples is found in the *Drosophila* peripheral nervous system (PNS).

In the fruitfly PNS, a sensory organ precursor (SOP) cell ultimately gives rise to four daughter cells: shaft, socket, sheath and neuron (Figure 4.3) [Reviewed in (26); (71)]. Notch activity is required to regulate the two lineage decisions in this process. Its activity first distinguishes the "a" versus "b" cell fate in daughter cells arising from the SOP, and then it is necessary for one of the progeny resulting from a "b" cell division to develop as a glial cell. At each step, Notch is activated in only one daughter cell, due to the activity of the asymmetrically inherited protein, Numb (144, 173). Numb antagonizes the Notch receptor, so that in its presence, Notch activity is inhibited (55, 160). The absence of Notch activity at any point in this process results in the specification of the default cell fate.

Although the two systems are similar in that a lineage decision occurs, there are also some differences observed. First, SGPs do not undergo cell divisions to give rise to daughter cells that will adopt differential fate, as SOPs do. Instead, Notch is stochastically activated in a subset of cells among the pool of initially specified SGPs. Second, given that SGPs do not divide, asymmetric segregation of Numb may not occur. It is possible that Numb accumulates in some SGPs, however, to date, Numb has only been observed during asymmetric cell divisions [Reviewed in (62)]. Interestingly though, recent work in the murine neural stem cell niche (4) has shown that EGFR antagonizes Notch activity by upregulating Numb protein cell autonomously. Given that EGFR signaling acts to restrict hub cell specification (97), this may hint that it does so by

upregulating Numb protein in EGFR-activated SGPs. This will be an interesting idea to test in the future.

Developmental relationship between hub cells and cyst cells

The early developmental decision initiated by Notch and *bowl* signaling is critical because it ultimately determines whether SGPs will eventually adopt niche fate– as hub cells, or stem cell fate– as cyst stem cells. During niche establishment, hub cells coalesce as an epithelium at the anterior of the gonad and induce Upd expression and other markers of hub cell fate (110, 153, 165). Those cyst cells that lie adjacent to the hub, and thus closest to the *upd* signal, likely adopt cyst stem cell (CySC) fate. Although likely to be induced by Jak-STAT pathway activity, it remains an open question how and when CySCs are specified in this system. Even so, the need for tight regulation of hub cell number remains, so as not to induce the specification of too many stem cells.

The developmental relationship between a stem cell and its supportive niche cell is interesting. Recent work has shown that several stem cells can give rise to their niche cells. Examples include the production of transient niche cells in the *Drosophila* intestine and the production of Paneth cells in the mammalian intestine (119, 146). In our system, the developmental relationship between hub and CySC could hint at the plasticity of these cells in the adult testes, where they could replenish each other, if necessary. The hub cells and CySCs reside in close proximity to each other at the testis tip, therefore it is not hard to imagine that they could signal to each other, prompting a cell fate switch to properly maintain the tissue. In fact, a recent study from Voog et al., suggested that CySCs could generate new hub cells in adult testes under wild-type conditions (177).

However, follow-up studies from our lab show that this phenomenon is rare in wild type testes (48). Moreover, decreasing the gene dose of *lines* (which promotes cyst cell fate), also does not affect CySC conversion to hub cells. However, it is important to note that CySCs depleted for *lines* in the adult testis revert to partial hub character (48). Therefore, hub-to-cyst cell or cyst cell-to-hub conversion could still potentially occur in extreme cases of tissue damage. It will be interesting to test this hypothesis in the future.

Hub cell number regulation

How hub cell number is tightly regulated remains an open question. Interestingly, it appears that Notch-activated SGPs are found at all positions within the gonad and are not simply confined to PS10 or PS11. Our work suggests a "salt and pepper" speckling of Notch-activated SGPs, while Kitadate et al., report that all SGPs become activated for Notch (97). Whatever the case, this data still suggest that a regulatory mechanism is in place to limit the number of SGPs that take on hub cell fate.

In our hands, we find that only a subset of SGPs become Notch-activated and therefore adopt hub fate. Perhaps, hub cell number is initially limited by the number of SGPs that can be activated as they transiently pass the Delta-expressing posterior midgut (PMG) cells. In this regard, the surface area of the PMG or the amount of time SGPs are in contact with PMG cells might account for the normally observed fluctuations in hub cell number. We can test this hypothesis by analyzing mutants that affect the size of the PMG. One such candidate is a *caudal* mutant.

In *Drosophila,* the digestive system is divided into three parts: the foregut, the midgut and the hindgut (Figure 4.4) [Figure adapted from (70); Reviewed in (113)].

Caudal is initially expressed in the primordia that gives rise to both the midgut and hindgut but is only necessary for the internalization and maintenance of the hindgut primordium, which lies adjacent to the developing PMG (188). Perhaps, the absence of the hindgut in these mutants would lead to an increase in the size of the PMG. A larger PMG (and therefore more PMG cells) could lead to an increase in the number of SGPs that encounter Delta, and thus become Notch-activated. Decreasing the size of the PMG and therefore the number of PMG cells encountering SGPs could have the reciprocal affect, in reducing hub cell number. In fact, our data already show that preventing the internalization of the PMG and thus contact with SGPs, in *fog* mutants, leads to a decrease in hub cell number.

Although, it may prove difficult to modulate the contact time between PMG cells and SGPs and still maintain the integrity of the tissue, this remains a plausible hypothesis for regulating hub cell number. Increased contact time between cells would allow for more productive signaling and potentially an increase in Notch-activated SGPs, resulting in higher hub cell number.

However, even with a PMG-specific regulatory mechanism in place, too many hub cells could still be specified. As mentioned above, active EGFR signaling in posterior SGPs acts to restrict hub cell fate. Therefore, it is likely that the antagonistic affects of EGFR signaling accounts for some of this observed variation. Although it is not clear how EGFR becomes activated in posterior SGPs or how pathway signaling antagonizes Notch activity (97), it is clear that in the absence of EGFR pathway activity, a substantial increase in hub number is observed. It will be important to dissect the interactions between the pathways in the future.

Is *bowl* required specifically in PS11 SGPs for hub cell specification?

Our data reveal that there is an ~50% reduction in hub cell number in *bowl* mutants. Furthermore, preliminary data suggests that *bowl* activity is required specifically within PS11 SGPs for proper hub cell specification. Given that hub cells derive from both PS10 and PS11, this would also suggest that these parasegments contribute a relatively equal number of SGPs for eventual hub cell fate. If *bowl* is indeed only required in PS11, then it is interesting to ponder how *bowl* is uniquely regulated in this subset of SGPs.

One way to address this question is to assess the upstream regulators in each of the parasegments and determine if differential regulatory mechanisms result in distinct gene expression. The fly body is patterned as a series of segmental units (Figure 4.5) [Figure taken from (7)]. A host of genes control early embryonic development and are required to lay out a properly segmented body plan, which consists of fourteen parasegments [Reviewed in (136)]. One such class of genes is the pair-rule genes, which act to define alternating parasegments in the embryo (Figure 4.6) [Figure taken from (6); (131)].

The fact that hub cells derive from both an even (PS10) and an odd (PS11) parasegment may indicate that they initially have distinct cellular identities, controlled by the expression of the pair-rule genes. For example, the pair-rule gene *paired (prd)* is responsible for establishing odd-numbered parasegments, while *fushi tarazu (ftz)* is responsible for establishing even-numbered parasegments (78, 131). Perhaps, expression of distinct target genes downstream of these pair-rule genes in the parasegments accounts for *bowl* repression in PS10, but accumulation in PS11. If this is indeed the case, this could suggest that *bowl* accumulation in SGPs is independent of Notch activation. It

would also suggest that this separate and earlier input is ultimately required for hub cell specification. If, however, *bowl* does accumulate in response to Notch activation, it still remains possible that a uniquely expressed factor in PS10 SGPs represses *bowl* activity or that a uniquely expressed factor in PS11 SGPs allows *bowl* activity. In the future, it would be of interest to assay downstream targets of the pair-rule genes to determine if this early regional identity potentiates hub cell fate.

Niche cell migration

The hub is found at a stereotyped position at the anterior of the gonad and is anchored there via integrin-mediated adhesion (110, 165). Since Notch-activated SGPs –presumptive hub cells– are found in all positions throughout the gonad, these cells must migrate to the anterior and compact to form a functional niche. The cues that guide this anterior migration and compaction have yet to be identified. Recent live imaging data from our lab show that at least PS11 SGPs migrate anteriorly to join the hub, confirming that a directed migration indeed occurs (Wingert, unpublished results). It also appears that the presumptive hub cells migrate individually, rather than as a collective unit, suggesting that the cells are mesenchymal in nature. Perhaps, a combination of attractive cues from the anterior pole and repulsive cues from the posterior pole promote the directed migration of Notch-activated cells. I will discuss potential guidance cues in the next section.

Typically, in cell migration, remodeling of the actin cytoskeleton occurs. Actin-rich protrusions extend from the front of the cell in the direction of the gradient, driving migration, and actomyosin filaments generate contractile forces at the sides and rear of a

cell [Reviewed in (19)]. A recent model predicts that actin polymerization at the front of a cell is regulated by Rac, while actomyosin contractility at the rear is regulated by Rho, two members of the Rho family of GTPases (140). It would be interesting to assess the localization of Rac and Rho in migrating Notch-activated cells via live imaging of the fluorescently-labeled proteins. Stereotypical accumulation of the proteins in migrating cells should hint at the direction and/or location of the guidance cue. It would also be of interest to assess the migration of Notch-activated cells in these mutants via live imaging to determine if Rac and Rho GTPases are indeed necessary for this process.

Potential cues guiding hub cell migration

A recent mutagenesis screen performed by the Van Doren lab has uncovered several previously unknown genes required for proper gonad formation (185). One particularly interesting finding is that the Slit/Roundabout (Robo) pathway plays an essential role in this process, promoting the fusion of the three SGP clusters, from PS10-12, and gonad compaction. The Slit/Robo pathway is best known for its role in regulating axon guidance in *Drosophila*, but also regulates cell migration in the trachea, salivary gland and heart tube (49, 91, 101, 116). Slit, a secreted protein, can act as an attractive or repellant signal and functions as a ligand for the three Robo receptors in *Drosophila*, Robo, Robo2 and Robo3 (91).

In Weyers et al., the authors observed Robo and Robo2 accumulation on SGPs in the gonad beginning at stage 13, with the levels increasing as gonad coalescence proceeded (185). Intriguingly however, Slit does not accumulate in the gonad or in immediately surrounding tissues, so the source of Slit still remains unclear. Potential

sources of Slit, identified by immunostaining and enhancer trap analysis, include the CNS midline, the ectoderm at muscle attachment sites and the gut. This is interesting because the gut already plays a critical role in hub cell specification by presenting the Notch-activating ligand, Delta. Perhaps, the gut also secretes the ligand, Slit, and provides a directional cue to guide Robo-expressing, Notch-activated cells to the anterior of the gonad. Additionally, since the affect on hub cell specification was not assayed in these mutants, it will be interesting to determine if loss of Slit or the Robo family of receptors influence hub cell number.

A delay in niche cell gene expression

SGPs become Notch-activated during stages 11 and 12 of embryogenesis, yet the hub cell gene expression program is not initiated until several developmental stages (and hours) later. This data suggests that the identity of early Notch-activated cells is slightly different than the identity of terminally differentiated hub cells, which express markers of niche cells (such as *upd* and *escargot*) (110). However, it is not understood how early Notch-activated cells transition to fully functional niche cells and why there is a delay in the initiation of hub cell gene expression.

It is interesting to ponder what these cells are doing after they become Notch-activated, but before functioning as a niche. It is possible that Notch activation leads to a series of downstream events that prepares these cells for their eventual hub fate, which happens over a course of a few hours. Once it is time for the cells to function as a niche, an internal signal simply induces the hub cell gene expression program. Alternatively, maybe Notch-activation potentiates these cells to develop as hub, but only contact with

other Notch-activated cells allows them to differentiate as functional niche cells. This is an intriguing thought, because data show that hub cell gene expression only occurs after cells compact at the anterior of the gonad and upregulate several cell adhesion and cytoskeletal molecules, such as Fascilin 3, Filamin, DN-Cadherin and DE-Cadherin (110, 165). The upregulation of these molecules hints at a mesenchymal-to-epithelial transition (MET) occurring within hub cells.

It is possible that hub-hub association could initiate intercellular signaling and lead to a MET. If epithelialization is a prerequisite for hub cell gene expression, we could assay a series of mutants that disrupt MET, such as β-catenin –a known adherens junction protein– to determine if the transition is required for terminal hub differentiation. In mutants where MET is disrupted, we would assay *escargot* or *upd* expression as a readout of functional niche cells. A mechanism such as this, where Notch-activated cells first have to find each other, adhere, and coalesce at the anterior before the hub can function as a "true" niche, would prevent precocious or erroneous stem cell specification within the gonad.

Identifying Notch target genes required for hub cell specification

Now that we know that Notch activity specifies hub cells, we can begin to look for Notch targets, which function downstream of the pathway and which might also serve as useful markers for early hub cells. Our data show that Notch activity is absolutely necessary for hub cell specification, while *bowl* contributes to ~50% of this process. Although it remains unclear in our system if *bowl* accumulates downstream of Notch, it is a plausible hypothesis since it does so in other developmental contexts (43, 66). Even so,

this data suggests that there is another pathway at play, and that this pathway should act downstream of Notch for hub specification.

Given that *Suppressor of Hairless (Su(H)),* functions as the Notch responsive transcription factor, it would be fruitful to scan a list of *Su(H)* targets to determine if any function downstream of Notch in hub cell specification (151). The Enhancer of Split, E(spl), complex of genes are probably the best characterized Notch targets. The E(spl) locus includes seven genes that encode related basic helix loop helix proteins (47, 100). During neurogenesis, these genes function as repressors and primarily act by suppressing genes of the *achaete-scute* pro-neural complex (76, 133). Given the characteristic upregulation of E(spl) complex genes downstream of Notch, it would therefore be interesting to determine if these genes play a role in hub specification. A deficiency line exists which uncovers all seven genes of the cluster. Analyzing this line first would easily allow us to determine if the complex plays a role. If we find that hub cell number is affected, we can then scan individual E(spl) complex mutants to narrow down the responsible gene.

Additionally, recent chromatin immunoprecipitation (ChIP) data, published by the Bray lab, identifies a number of direct *Su(H)* target genes, some previously known and many unknown (102). The short-term transcriptional response to Notch activation was assessed in DmD8, a *Drosophila* adult muscle progenitor cell line. Over 200 genes were found to be upregulated in response to Notch activation in these cells. Scanning this list may allow us to identify and test promising candidates that regulate hub cell specification downstream of Notch signaling. This could also aid in the recognition of individual presumptive hub cells before they coalesce into an epithelium at the anterior.

Interestingly, both positive and negative regulators of the EGFR pathway were found to be upregulated. This may suggest that in our system, Notch activated cells directly inhibit EGFR pathway activity, allowing hub cell fate.

Does Bowl function as a repressor to regulate hub cell specification?

Our data suggest that Bowl functions as a repressor to regulate hub cell specification. We arrived at this preliminary conclusion given the result of overexpressing a *bowl* protein with a deleted eh[1], *groucho*-interacting domain. We found that overexpression of this protein within SGPs caused a decrease in hub cell number, while overexpressing a wild-type version of *bowl* increased hub cell number. This suggested that under normal circumstances, the interaction with the co-repressor *gro* was necessary for hub cell fate. It also suggested that the *bowl*-eh[1-] protein was potentially behaving as a dominant negative and interfering with normal Bowl activity. To confirm these results, it would be necessary to assess the behavior of *bowl*-eh[1-] in another tissue. A prime candidate tissue is the *Drosophila* dorsal epidermis. In this tissue, *bowl* regulates the proper specification of three dorsal cell fates (73). If overexpressing this protein leads to a dominant negative phenotype, we would observe a dorsal epidermal cell pattern that looks more similar to a *bowl* null mutant.

If Bowl does indeed function as a repressor –to restrict cyst cell gene expression and to promote hub cell fate– it will be clarifying to determine its downstream targets. In several tissues, *bowl* positively regulates the expression of genes such as *unpaired* and *hedgehog*, however, it remains unclear which genes *bowl* directly regulates (24, 81, 85). Given that Bowl likely functions as a repressor, it may be difficult to narrow down direct

targets that promote hub cell fate, since Bowl will probably repress the expression of a repressor of a hub gene. Still, it remains interesting that *upd* and *hh* are regulated by *bowl* in other contexts, since they are both expressed uniquely in hub cells. Moreover, it would be useful to identify the targets that Bowl represses to prevent cyst cell fate, since this regulation may be more direct. Potential candidate genes include *Traffic jam (Tj)* and *Zinc-finger homeodomain-1 (Zfh-1).* These proteins, which are initially expressed in all SGPs, are downregulated in the hub and become restricted to cyst cells in the mature adult niche (28, 112). Perhaps, Bowl represses the expression of Tj and Zfh-1, allowing hub cells to be specified.

Potential applications in regenerative medicine

Ultimately, researchers seek to apply the knowledge learned from stem cell model systems to develop therapeutic treatments for a number of diseases. Therefore, an understanding of the basic principles guiding stem cell biology is critical. Since many signaling pathways are conserved from flies to humans, the pathways that I have uncovered that regulate niche cell specification in the *Drosophila* testis may also act to specify niche cells in the mammalian testis. If so, these signaling pathways could be targeted by pharmacological agents to stimulate stem cell production or continued stem cell self-renewal in male patients suffering from infertility.

Concluding Remarks

In this work, I have taken advantage of the *Drosophila* germline stem cell niche to understand the development and specification of an *in vivo* stem cell-niche system.

Though this story is far from complete, our work provides an in-depth mechanistic insight into the specification of niche cells in a stem cell system. In the future, it will be of great interest to understand how these niche cells begin expressing factors critical to specify and maintain stem cells. Although, the exact make-up of stem cell-niche systems vary from tissue to tissue and among organisms, basic principles learned here may be applicable to the process of niche cell specification in higher organisms.

Figure 4.1:

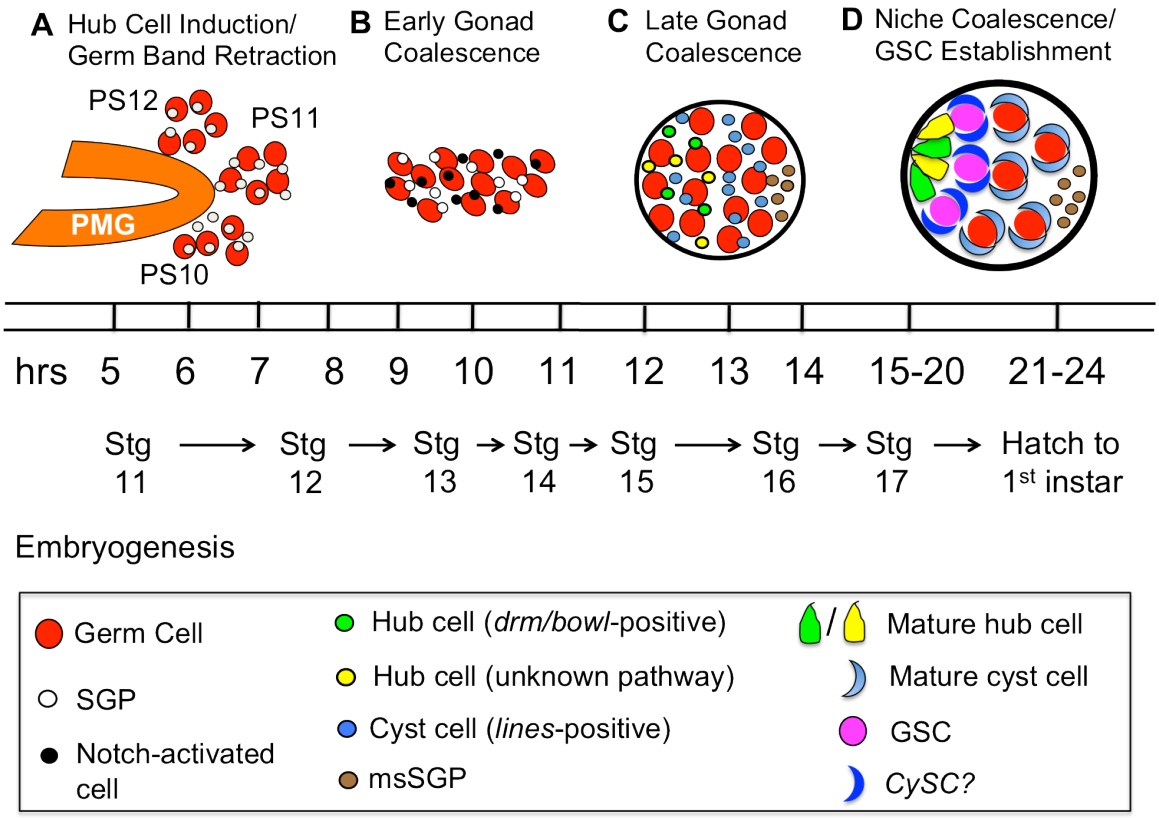

A Hub Cell Induction/ Germ Band Retraction
B Early Gonad Coalescence
C Late Gonad Coalescence
D Niche Coalescence/ GSC Establishment
PS12
PS11
PMG
PS10
hrs 5 6 7 8 9 10 11 12 13 14 15-20 21-24
Stg 11 → Stg 12 → Stg 13 → Stg 14 → Stg 15 → Stg 16 → Stg 17 → Hatch to 1st instar
Embryogenesis
Germ Cell
SGP
Notch-activated cell
Hub cell (drm/bowl-positive)
Hub cell (unknown pathway)
Cyst cell (lines-positive)
msSGP
Mature hub cell
Mature cyst cell
GSC
CySC?

Figure 4.1: A model for niche cell specification in the *Drosophila* testis

(A) SGPs (white) that originate from parasegments (PS) 10-12 become Notch-activated as they passively travel by Delta-expressing PMG cells (orange) during germ band retraction. (B) During early gonad coalescence, germ cells (red), Notch-activated SGPs (black) and non-Notch-activated SGPs (white) form a contiguous tissue. During the transition from early to late gonad coalescence, it is possible that components of the *bowl* pathway become active. *Drumstick* activity in a subset of Notch-activated cells allows nuclear *bowl* accumulation and these SGPs begin to differentiate into hub cells (green). The activation of a yet unidentified pathway, likely downstream of Notch, also allows SGPs to differentiate into hub cells (yellow). Those cells that accumulate the *bowl* antagonist, *lines*, differentiate as cyst cells (light blue). (C) During late gonad coalescence, hub cells (green and yellow) must migrate towards the anterior and are anchored at the anterior pole via integrin-mediated adhesion. Sox100B-positive male-specific SGPs (brown) also join the gonad. (D) During the last stage of embryogenesis, stage 17, the hub cells (green and yellow) execute a mesenchymal-to-epithelial transition, upregulate cell adhesion molecules and induce Unpaired expression, establishing germline stem cells (GSC, purple) and possibly cyst stem cells (CySC, dark blue).

Figure 4.2:

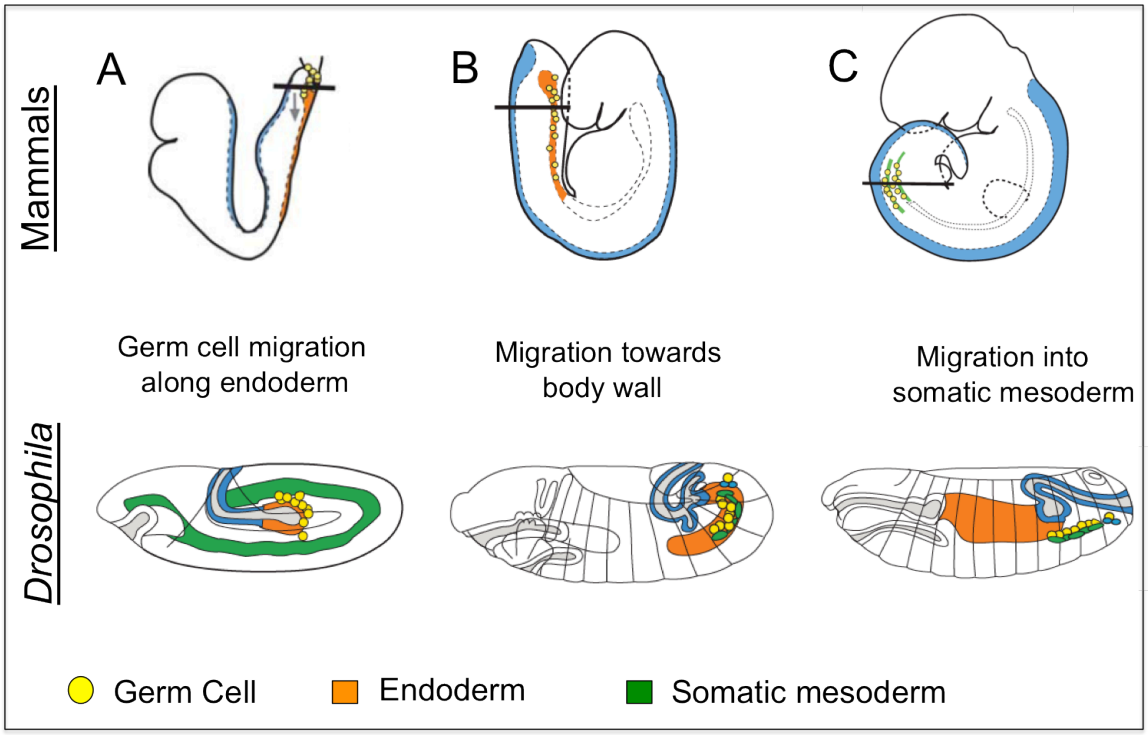


Figure 4.2: Germ cell migration in mammals and *Drosophila*

Mammals and flies share strikingly similar mechanism of germ cell migration and gonad formation. Germ cells are shown in yellow, the endoderm in orange and the somatic mesoderm in green. In mammals after germ cells are specified, they migrate from the primitive streak to the endoderm. They then migrate bilaterally towards the body wall and finally reach the genital ridge (somatic mesoderm) where they form a gonad. In *Drosophila* after specification, primordial germ cells are carried into the embryo by the midgut primordium. The germ cells then migrate through the endoderm (specifically the midgut) and reorient on the midgut towards the mesoderm. The germ cells then migrate bilaterally towards the somatic gonadal precursors (SGPs, mesoderm derivatives) and finally coalesce with SGPs to form the gonad. [Modified from a figure in (105)]

Figure 4.3:

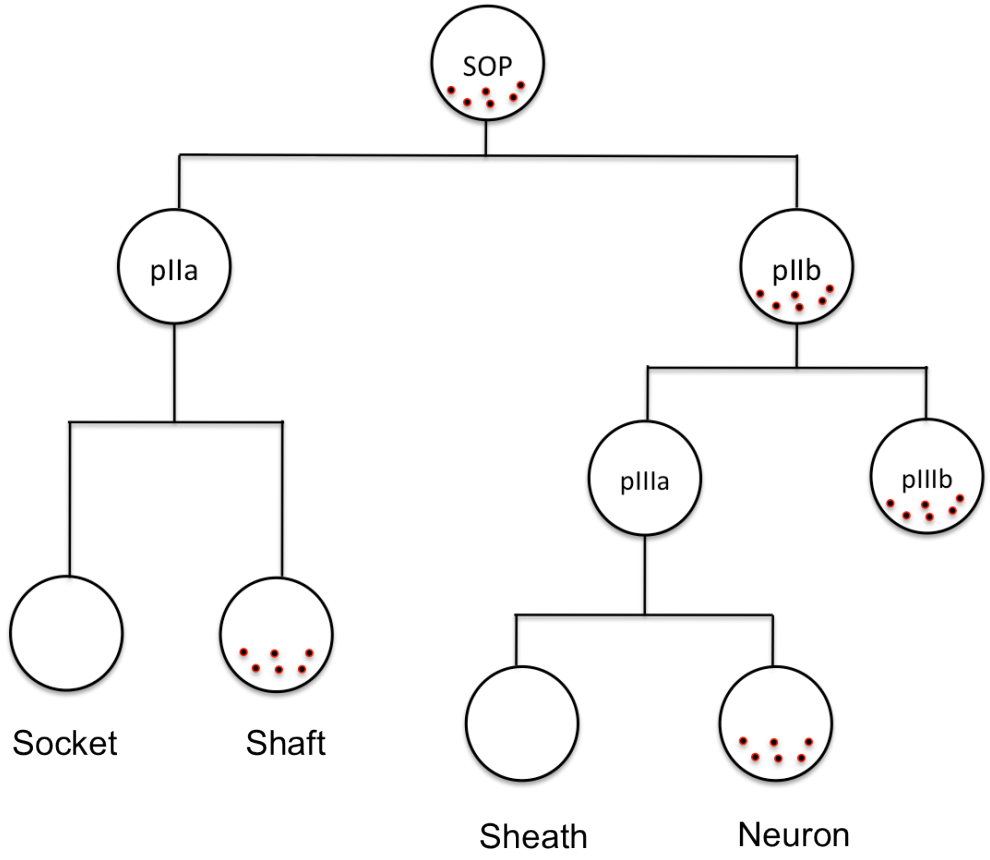


Figure 4.3: Asymmetric division in the SOP lineage

The adult mechanosensory lineage in *Drosophila*. A sensory organ precursor (SOP) undergoes a series of asymmetric divisions to give rise to four differentiated daughter cells: a socket, shaft, sheath and neuron. This asymmetric division is mediated by lateral inhibition of the Notch pathway, such that cells that receive Numb (red dots) remain unresponsive to Notch activation, while those that do not accumulate Numb become Notch-activated. At each stage, this lateral inhibition mediates a competitive interaction that forces adjacent cells to adopt different cell fates.

Figure 4.4:

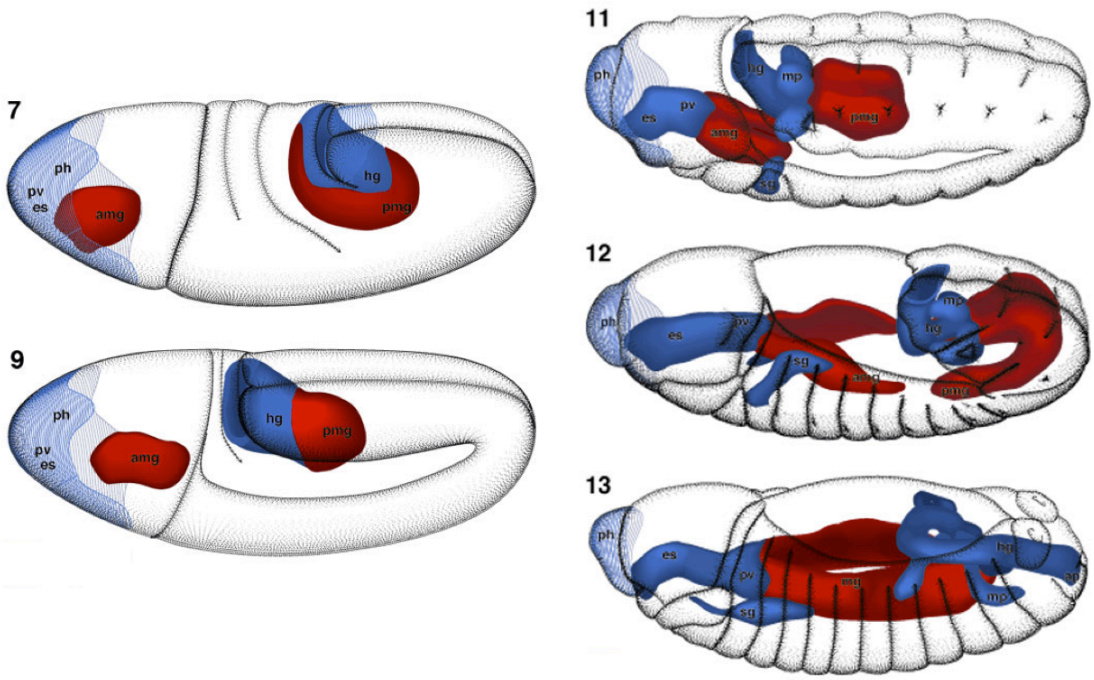


Figure 4.4: The *Drosophila* digestive system

The *Drosophila* digestive system through stages 7-13 of embryonic development. The digestive system is divided into three parts: the foregut (in stages 11-13, blue at the anterior), midgut (red) and hindgut (in stages 7 & 9, blue; in stages 11-13, blue at the posterior). The midgut and hindgut primoridia, which lie adjacent to each other, begin invaginating during stage 7 and complete this process by stage 9. The foregut primordial invaginates during stage 10 (not shown) and attaches to the midgut at the anterior. During stages 11-13, migration and reorganization of the gut structures occur. According to our model, somatic gonadal precursors migrate past midgut cells and are activated for Notch during germ band retraction (stages 11 and 12). [Figure modified from (70)]

Figure 4.5:

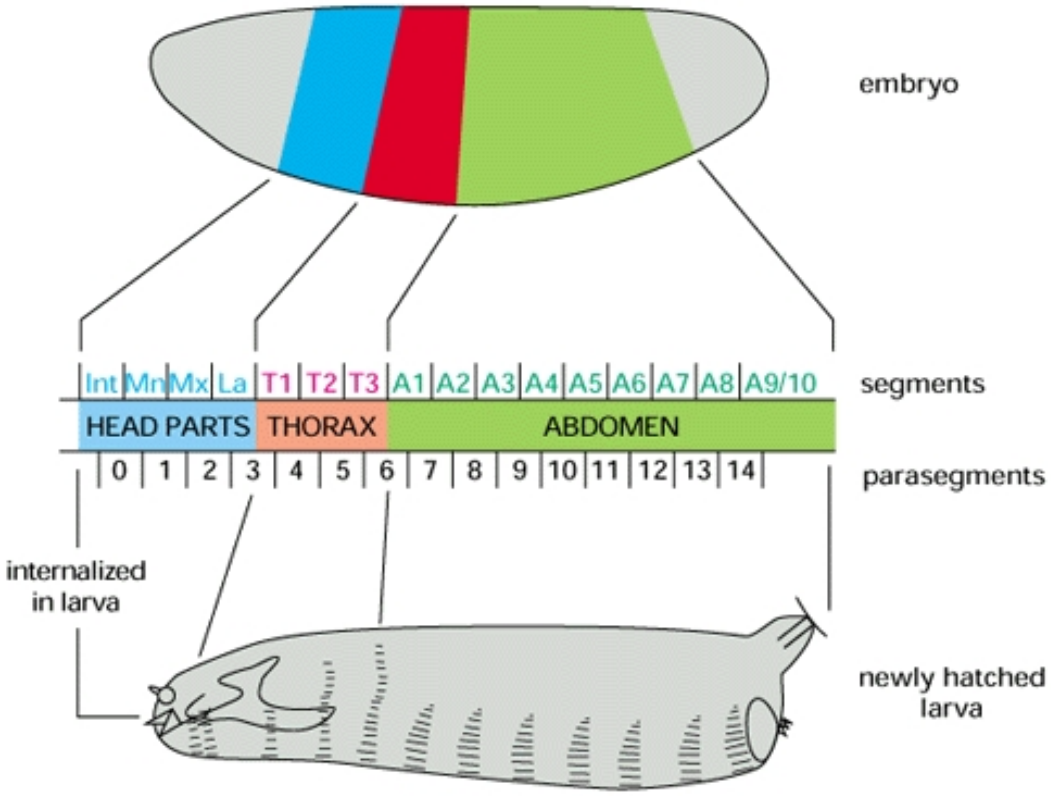


Figure 4.5: Segmentation of the *Drosophila* embryo and larva

The parts of the embryo that become organized into segments are shown in color and their corresponding segments are shown in the larva. The embryo can be divided into segments as well as parasegments, which often correspond to patterns of gene expression. The relationship between the two is shown in the middle of the diagram. [Figure taken from (7)]

Figure 4.6:

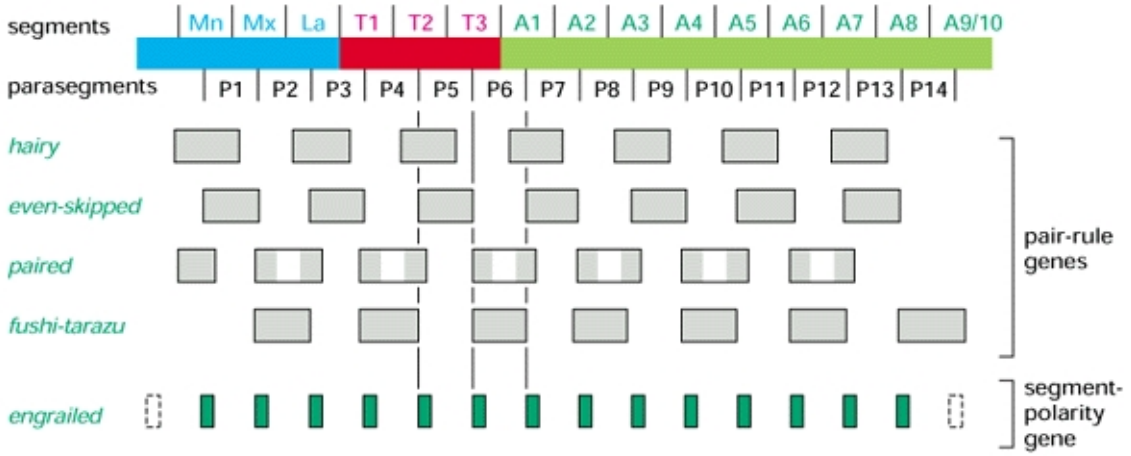


Figure 4.6: Pair-rule gene expression defines segments in the *Drosophila* embryo

Subdivisions of segments and parasegments are illustrated across the top of the diagram. The diagram shows the pattern of transcription for four of eight known pair-ruled genes, which are required in alternating parasegments for proper embryo segmentation. The shaded grey regions illustrate where transcription of these genes occur. The combination of pair-rule gene activity regulates segment polarity genes, such as *engrailed* (green), which allows for finer patterning of individual segments. [Figure taken from (6)]

Addendum:

TOWARDS UNDERSTANDING HUB CELL GENE EXPRESSION IN THE *DROSOPHILA* TESTIS

Summary

We have shown that both the Notch and *bowl* pathways influence hub cell specification in the *Drosophila* gonad. However, it still remains unclear how the hub cell gene expression program is initiated in newly specified hub cells. In an attempt to understand hub cell gene regulation, we have identified an ~1.5kb region of the *hedgehog (hh)* promoter, which drives selective hub expression. Within this stretch of DNA exist four evolutionarily conserved regions, which could control *hh* expression in the hub. To narrow down the DNA regions critical for selective hub expression, we have made a series of transgenic fly strains, individually deleting each conserved region. This analysis will hopefully allow us to define smaller DNA regions responsible for *hh* hub expression, which could ultimately implicate regulatory transcription factors.

In a complementary approach we sought to identify candidate transcription factors that may bind to and regulate hub-expressed genes. One such transcription factor that could regulate hub cell gene expression is *bowl.* Two hub-expressed genes, *unpaired (upd)* and *hh,* are regulated by *bowl* in other contexts during *Drosophila* development. Moreover, preliminary data suggests that *bowl* regulates the expression of *upd* in hub cells. In the future, it will be of interest to further define the role of *bowl* and other transcription factors in regulating the hub cell gene expression program.

Introduction

Niche cells play a critically important role in regulating stem cell behavior in all stem cell-niche systems [Reviewed in Morrison (126)]. These cells produce various signals that activate pathways in neighboring stem cells allowing them to self-renew and to be maintained within the niche. Although these niche cells have a fundamental supporting role for stem cells, we are just beginning to understand how these cells are specified and develop. I have identified two signaling pathways that are necessary for proper niche cell specification in the *Drosophila* testis: *Notch* and *bowl.* However, it remains unclear how the hub cell gene expression program is initiated once these niche cells are specified.

To date, we have identified three genes that appear to be selectively expressed in hub cells in the male germline niche: *pentagone (pent), unpaired (upd),* and *hedgehog (hh)* (48, 54, 93, 172, 197). *Pent* encodes a putative secreted protein that regulates cell-matrix interactions (179). It acts as a modulator of the BMP pathway to control GSC maintenance, such that *pent* mutant testes exhibit reduced GSC number (197). Although the exact mechanism of *pent* action in the testis niche is unknown, the role of *upd* in this system is well understood.

The chemokine, *upd*, is secreted from hub cells and activates Jak-STAT signaling in neighboring germline and somatic cells, allowing them to be maintained as germline stem cells (GSCs) and cyst stem cells (CySCs), respectively (93, 153, 172). STAT activation in stem cells is necessary to ensure proper adhesion to the hub and in instances where it is lost, stem cells differentiate (79, 111). Given this important role in activating

the Jak-STAT pathway, functional hub cells have historically been defined by *upd* expression [Reviewed in (44)].

Hh protein also accumulates in hub cells, however its role in the male germline niche has remained elusive (48, 54). Recently, the Schulz lab began studying the role of *hh* in the *Drosophila* hematopoietic niche (170). In an effort to identify and characterize the transcriptional enhancer that controls *hh* expression in the hematopoietic stem cell (HSC) niche, the authors developed a series of transgenic reporter fly lines encompassing DNA that spanned the entire *hh* gene as well as intragenic and upstream sequence. We obtained these fly lines from the Schulz lab and similarly tested each for selective hub expression. Intriguingly, we identified a hub-specific enhancer. The enhancer appears to lie within two overlapping regions of DNA that drive *hh* robustly within the hub. Although *upd* has a functionally important role in this system, we have not yet tried to narrow down an *upd* transcriptional enhancer region specific for hub cells. Therefore, we have chosen to explore the regulatory region of *hh* in an effort to identify the regions of DNA that might be necessary for selective hub cell expression. This approach would potentially allow us to determine which transcription factors bind and regulate *hh* gene expression. This knowledge could be extended to other hub-expressed genes since genes expressed in the same tissue are usually regulated through similar mechanisms (115).

To complement the aforementioned approach, we have also decided to take a candidate approach. Since *bowl* is a known transcription factor and is expressed in hub cells, we wondered whether *bowl* could regulate a suite of hub-specific genes. It is important to note that this would be a distinct role for *bowl*, separate from its requirement in specifying hub cells. Interestingly, *bowl* regulates the spatially localized expression of

upd during *Drosophila* gut morphogenesis and it controls the proper activation of *hh* protein during retinogenesis, though direct regulation has yet to be shown (24, 81, 85). Given the regulatory role *bowl* possesses over these genes in other developmental contexts, we wondered whether *bowl* also regulates the expression of these genes within the hub. Here, we show preliminary genetic data that suggests that *bowl* does indeed regulate *upd* expression. Although it still remains unclear if this regulation it direct, this provides a first step towards understanding the initiation of hub cell gene expression in the testis and provides promising insight into niche cell biology.

Results

Narrowing down the *hh* hub-specific enhancer region

Since we knew from previous work that *hh* accumulates in hub cells (48, 54), we scanned transgenic reporter fly stocks that encompass 21kb of *hh* upstream and intragenic sequence, in an attempt to define a hub-specific transcriptional enhancer element (170). The 21kb region was split into seven 3kb intervals and each DNA fragment was fused to a GFP reporter (Figure A.1 A) (170). To determine if there was hub-specific *hh* expression in any of the reporter fly lines, we stained adult testes with Vasa to label germ cells and an antibody against GFP to detect reporter expression. We identified two overlapping regions of DNA that drove selective expression in the hub cells of adult testes, *hhF5* and *hhF6* (Figure A.1 B). These two 3kb regions lie within *hh* intragenic sequence between exon one and two and overlap by 1531 nucleotides (nt) precisely. I will refer to this overlapping region as simply *hh1.5* for the remainder of the narrative.

We hypothesized that a hub-specific enhancer existed within the *hh1.5* fragment of DNA and decided to further define the enhancer element by comparing this sequence to other *Drosophilid* species using BLAST (Basic Local Alignment Search Tool). The genomes of twelve *Drosophila* species, ranging from ~2 to almost 40 million years in divergence, have been sequenced. It has been shown that conservation of particular regions of DNA among *Drosophilids* tend to be functionally important in controlling tissue-specific gene expression (61). In general, we expect to observe sequence conservation among closely related *Drosophila* species, while nucleotide conservation decreases as you scan more divergent *Drosophilids.* Typically however, conserved regions of DNA remain, even in divergent species, hinting that these sequences are constrained from diverging and thus functionally important. We therefore searched for blocks of DNA conservation in the *hh1.5* fragment BLAST report that would imply that a region serves an important regulatory role in controlling hub-specific *hh* gene expression. We found that four regions were highly conserved among the *Drosophila* species (Figure A.2). The regions were 33 nt, 42 nt, 54 nt and 39 nt in length, respectively.

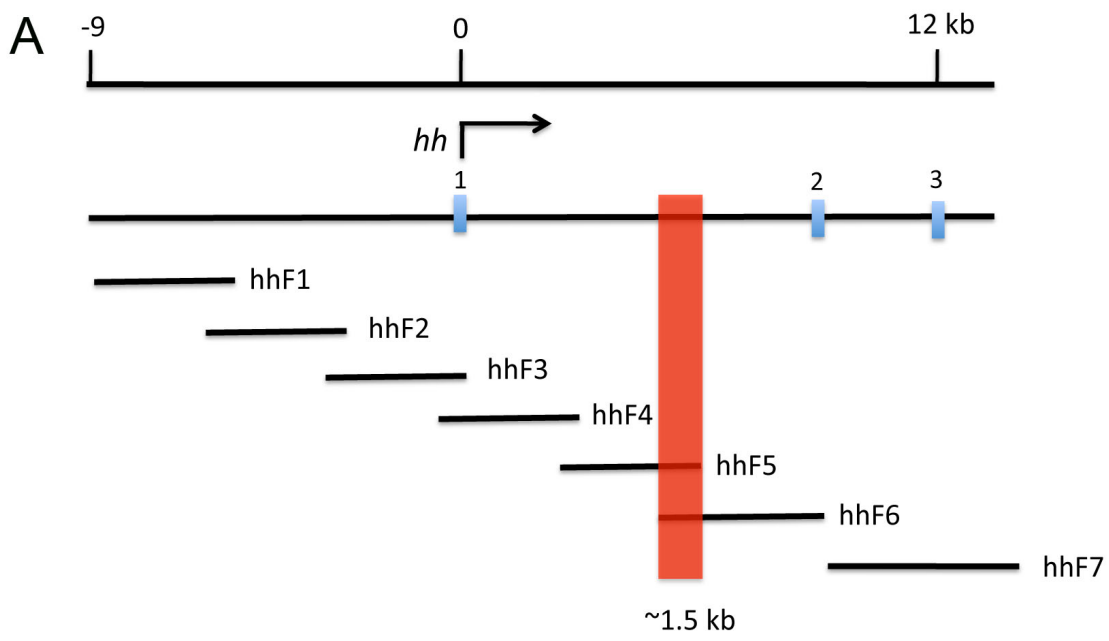
To further narrow down the DNA region responsible for hub-specific *hh* expression, we decided to make a series of transgenic GFP-reporter flies (Figure A.3). To first confirm that we could recapitulate hub Hh reporter expression as seen in the fly lines from the Schulz lab, we made a *hhF6*-GFP transgenic fly. Next, to determine if the *hh1.5* fragment was sufficient to drive selective hub expression, we also made a *hh1.5*-GFP transgenic fly. Within this *hh1.5* fragment, we then constructed a series of deletions constructs by individually deleting each of the four conserved regions. We made the transgenic DNA constructs and these were sent off for injection to establish fly lines.

In the future, it will be of interest to test all of these lines and to determine which conserved regions within *hh1.5*, if any, control selective *hh* hub cell gene expression.

bowl **regulation of a hub-expressed gene**

Given that *bowl* is a known transcriptional regulator and functions to regulate the expression of *upd* and *hh* in other developmental contexts, we decided to determine if *bowl* plays a role in regulating *upd* expression in the hub. We assayed the expression of *upd* using a reporter construct in larval gonads mutant for *bowl* compared to heterozygous sibling controls. We used an enhancer trap at the *upd* locus, *upd*-Gal4 Uas-GFP, and stained larval gonads with Vasa to recognize germ cells, Filamin to recognize hub cells and an antibody against GFP. We then quantitated *upd* reporter expression by measuring the average pixel intensity of hub cells (see Materials and Methods). We found that there was a statistically significant decrease in *upd* hub expression in *bowl* mutant gonads compared to controls (Figure A.4). This data suggests that *bowl* regulates the expression of *upd* in hub cells during gonadogenesis. However, it still remains unclear if this regulation is direct. This role appears similar to *bowl* regulation of *upd* during gut development. Similar analysis with a *hh* reporter will be undertaken in the near future.

Figure A.1:


A
-9
0
12 kb
hh
1
2
3
hhF1
hhF2
hhF3
hhF4
hhF5
hhF6
hhF7
~1.5 kb

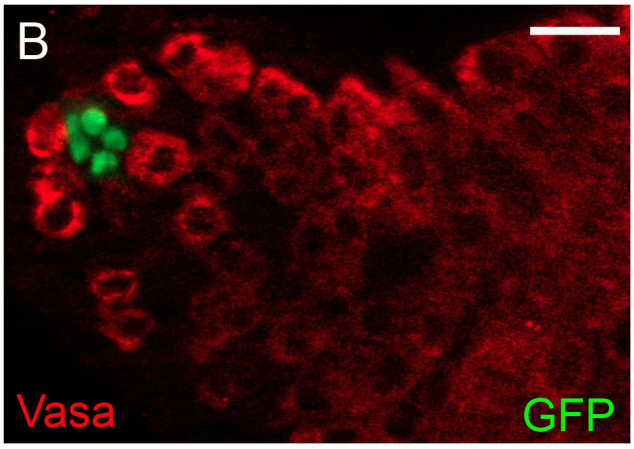

B
Vasa
GFP

Figure A.1: A *hh* transcriptional enhancer drives selective hub expression

(A) Location of overlapping *hh*-GFP test DNAs. Blue boxes represent exons and the arrow denotes the transcription start site. The red rectangle indicates the ~1.5kb overlapping region that likely drives selective hub cell expression. (B) Anterior is to the left. An adult testis (*hhF6* #135) stained with Vasa (red, germ cells) shows selective GFP reporter expression (green) in hub cells. Scale bar is 100 μm.

Figure A.2:

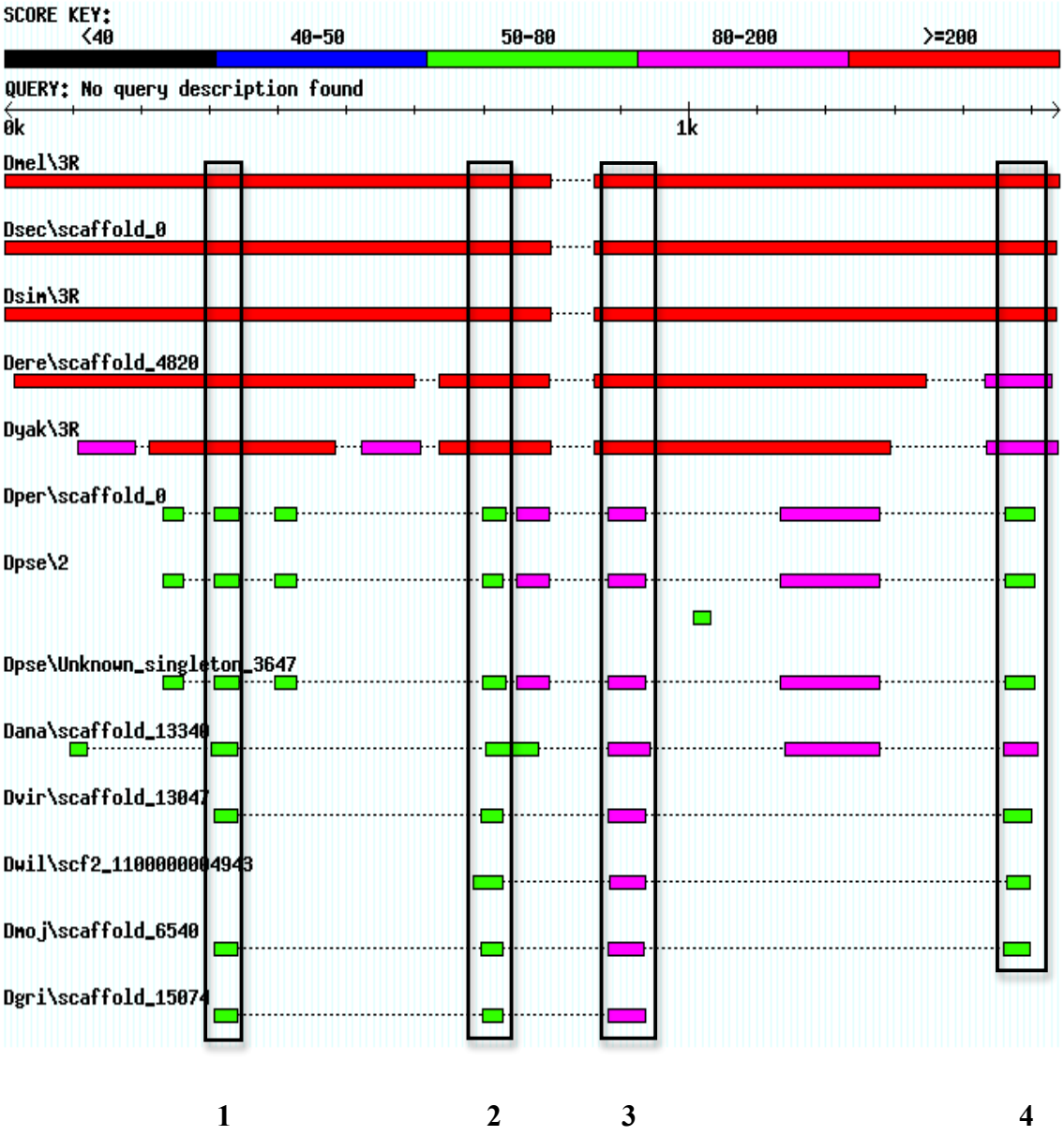


Figure A.2: The *hh1.5* DNA fragment harbors four evolutionarily conserved regions

The BLAST report shows that there are four regions evolutionarily conserved among *Drosophilids* (species names appear in abbreviations on the left). The four regions are each highlighted by a black rectangle and are numbered accordingly. The regions vary in nucleotide length and are as follows from region 1-4: 33 nt, 42 nt, 54 nt and 39 nt.

Figure A.3:

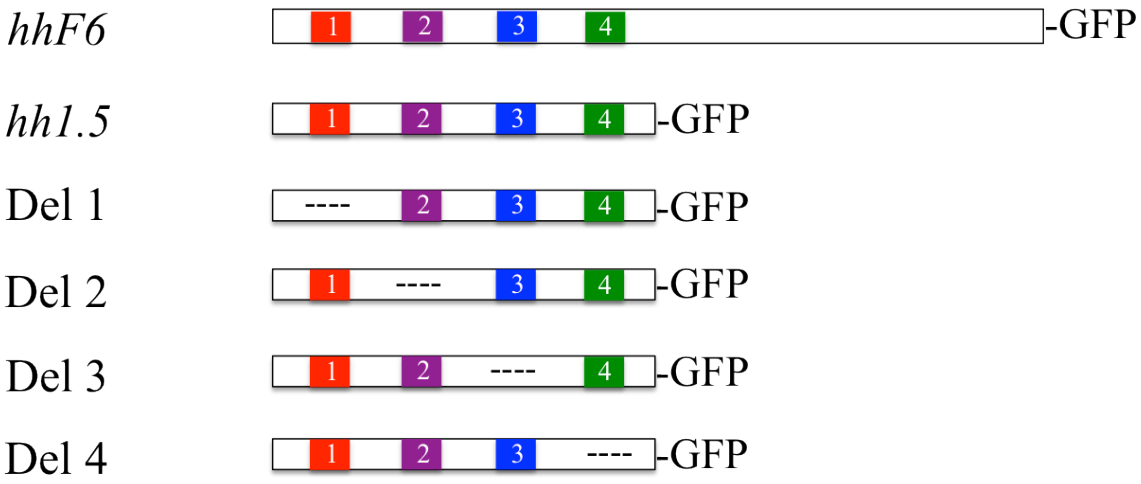


Figure A.3: Schematic of *hh*-DNA-GFP constructs

Schematics of the *hh*-DNA-GFP constructs are shown for *hhF6, hh1.5* and deletions 1-4. Colored boxes with numbers represent the conserved regions found within the ~1.5kb DNA fragment.

Figure A.4:

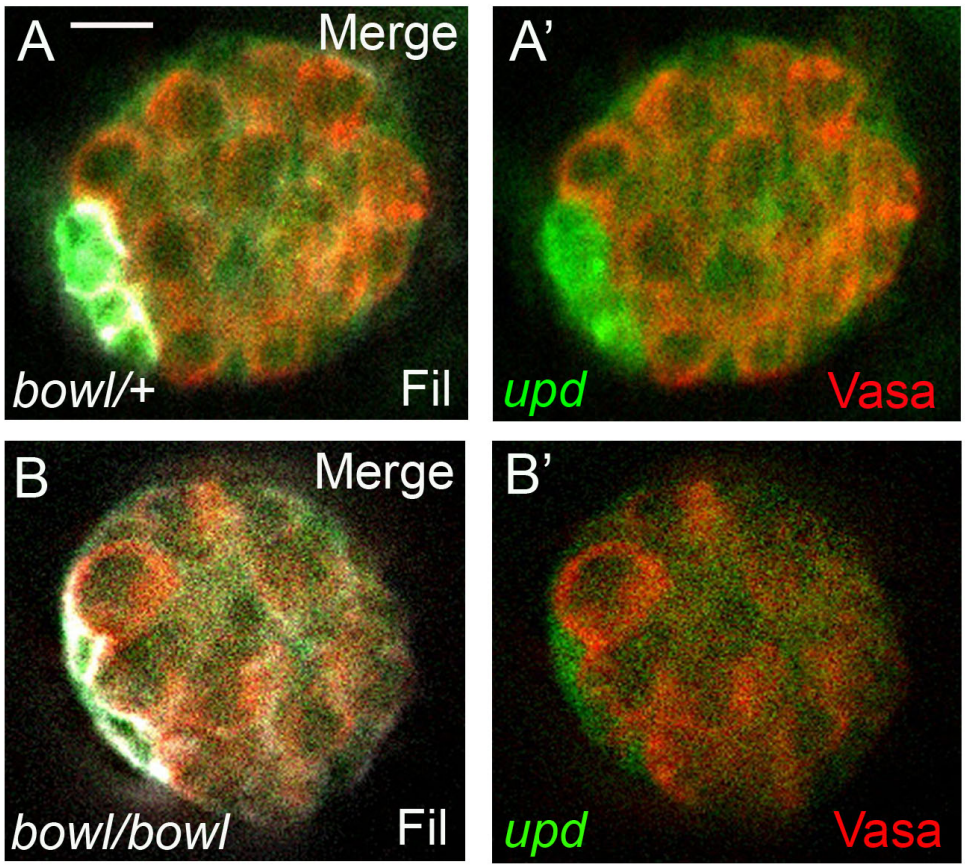


Figure A.4: *bowl* regulates *upd* hub expression

Anterior is to the left in each panel. (A-B) 1st larval instar male gonads from control (A; *bowl/+*) and *bowl* mutants (B) were stained with Vasa (red, germ cells), Filamin (white, hub cells) and an antibody against GFP to detect *upd* reporter expression (green). Note that in addition to a reduction in hub cell number, there is a significant decrease in reporter expression detected in *bowl* mutants (B) compared to heterozygous controls (A). Scale bar is 10 μm.

Discussion

With this work, we have sought to further define niche cells in the *Drosophila* testis by understanding how the hub cell gene expression program is initiated. Here, we have potentially identified a hub-specific transcriptional enhancer element that lies within ~1.5kb of *hh* intragenic sequence. Furthermore, we identified four evolutionarily conserved regions within *hh1.5* and have made a series of transgenic flies harboring individual deletions to determine the necessity of each in driving selective hub expression. Additionally, in a complementary approach, we identified *bowl* as a candidate transcription factor that could control some hub cell gene expression. In fact, it appears to regulate the expression of at least one hub-specific gene, *upd.* With these combined approaches, we hope to identify additional transcriptional regulators that control hub cell gene expression and by extension, stem cell self-renewal.

Identification of a transcriptional enhancer controlling *hh* expression in hub cells

There are several potential outcomes from this work. First, given that evolutionary conservation of particular DNA sequences typically correlates with an important regulatory role (61), we believe that at least one of the conserved regions will be necessary for selective hub expression. Therefore, we would expect to identify at least one deletion line that completely abrogates *hh* hub expression. This would suggest that a hub-specific cis-regulatory element lies with that region of DNA. However, if we find that hub expression is maintained in all four individual deletion lines, it is possible that two or more of the regions function redundantly and that multiple regions can initiate hub gene expression. If that is indeed the case, we could make a series of deletions in tandem,

for example, deleting regions one and two, one and three and so on. Hopefully, this would allow us to identify the regions of DNA that drive hub expression.

Furthermore, identifying DNA regions that drive *hh* would potentially allow us to identify the transcription factors that bind to these sequences and regulate hub gene expression. Several computational programs exist to identify putative transcription factor binding sites within cis-sequences, such as PROMO or TRANSFAC (121, 123). Any putative transcription factor binding sites, and thus transcription factors, could be tested for functionality by either mutating the binding sites within the *hh* regulatory region and assaying reporter expression or examining *hh* hub gene expression in a background mutant for the particular transcription factor. If the transcription factor positively controls *hh* hub expression we would expect to observe a complete loss of hub expression. These analyses should prove fruitful in our attempt to understand *hh* hub expression. Lastly, although it is presently unclear what functional role *hh* plays in this system, it would still be interesting to narrow down potential downstream targets genes in the hub.

Additionally, these analyses could be extended to other hub-expressed genes, since tissue-specific genes are usually regulated in a similar manner. By scanning the regulatory regions of other hub-expressed genes and determining if similar cis-sequences exist, this work could also shed light on the transcription factors that regulate the expression of *upd* and *pent*.

bowl **regulates the expression of a hub-expressed gene**

Our preliminary genetic data reveal that *bowl* regulates the expression of one hub-expressed gene, *upd.* This role for *bowl* is distinct from its role in properly specifying hub

cells that I have previously identified. We find that in scoring individual hub cells, *upd* reporter expression decreases significantly in *bowl* mutants compared to sibling controls (Figure A.4). This is interesting since *upd* activates the Jak-STAT signaling pathway, which is necessary and sufficient for stem cell self-renewal in the testis (93, 172). It thus appears that *bowl* has two critical roles in this system: it influences initial hub cell specification and also initiates the expression of the ligand *upd* within some hub cells, and by extension ultimately regulates the specification of stem cells.

This is a key finding as it may shed light onto general hub regulation of gene expression. It is highly possibly that *bowl* will also regulate the expression of *hh* in the hub, as it does during *Drosophila* retinogenesis (24). To assess this, similar analysis will be undertaken by examining *hh* reporter expression in *bowl* mutants compared to sibling controls. Furthermore, *bowl* regulation could also be extended to a third hub-expressed gene, *pent.* It is important to note though, that our evidence for *bowl* regulation of *upd* is genetic in nature, and therefore indirect. It is useful to look for hints from other tissues, but to date, direct regulation of *hh* and *upd* by *bowl* has not been tested in any context (24, 81, 85).

Since *bowl* typically functions as a transcriptional repressor, it is likely that *bowl* regulation of hub-expressed genes is indirect (60). This may mean that *bowl* activity represses the expression of a hub-gene repressor, ultimately allowing the accumulation of a positive-acting transcription factor and thus the expression of *upd, hh* and *pent. Bowl* is negatively regulated by an upstream antagonist, *lines* (73). While *bowl* promotes hub cell specification, we have shown that *lines* instead promotes cyst cell fate (48). Therefore, we would expect that in instances where *bowl* is inactive, due to repression by *lines*, the

hub cell gene expression program would not be initiated and SGPs would instead develop as cyst cells. By combining a candidate approach with cis-regulatory element analyses, we should be able to identify the transcriptional network necessary for hub cell gene expression.

Materials and Methods

Fly stocks

Heterozygous siblings were used as controls as appropriate. We analyzed gonads and testes from the following mutants, or involving these transgenic lines: *bowl*^{1} (Fbal0051737), *hhF5*-GFP (lines #228 and #211, A gift from Robert Schulz, University of Notre Dame), *hhF6*-GFP (lines #135 and #230, Robert Schulz), *bowl*^{1} *hhF6*-GFP #135, *upd*-Gal4 Uas-GFP (Erika Bach, NYU), *bowl*^{1} *upd*-Gal4 Uas-GFP. Stocks were balanced over CyO P[w+ Ubi-GFP].

Immunostaining

Embryos were collected on apple agar plates and aged 22-24 hours in a humidified chamber to 1st instar larvae. Hatched larvae were dissected in half with tungsten needles in Ringers solution and the internal organs were gently massaged out. Unhatched larvae were dechorionated, hand-devitellinized and dissected as above. Tissue was fixed in 4% formaldehyde, Ringers and 0.1% Triton-X-100 for 15 minutes, washed in PBTX and blocked one hour at room temperature in 2% normal donkey serum/normal goat serum. Primary antibodies were used overnight at 4°C. Secondary antibodies were used at 1:400 (Alexa488, Cy3 or Cy5; Molecular Probes; Jackson Immuno Research) for

1 hour at room temperature. DNA was stained with Hoechst 33342 (Sigma) at 0.2 μg/ml for 2 minutes.

Immunostaining for testes was performed as previously described except 1X PBS was substituted for Buffer B (169). The following primary antibodies and concentrations were used: rabbit anti-Vasa 1:5000 (Ruth Lehmann, Skirball Institute), guinea pig anti-Traffic Jam 1:10,000 (Dorothea Godt, University of Toronto), mouse anti-βgal 1:10000 (Promega), chick anti-GFP 1:1000 (Aves Labs), and rat anti-Filamin-C terminal 1:1000 (Lynn Cooley, Yale University; recognizes C-terminal isoform).

Sex identification, genotyping and staging of embryos

Male embryos were unambiguous due to larger size of the gonad. Balancer chromosomes containing a GFP-transgene P[w+ Ubi-GFP] were used to distinguish between heterozygous and homozygous mutant larvae. Embryos were staged according to Campos-Ortega and Hartenstein (32).

Identification of evolutionarily conserved regions

The ~1.5kb region of *hh* that we believe drives selective hub expression was BLASTed against the genomes of other sequenced *Drosophilids.* By this method, we found three regions to be conserved among all 13 species, while the fourth region was conserved among 12 of the 13 species.

Quantifying pixel intensity

All images were exposure matched, with an exposure of ~100 ms. Pixel intensity was quantified using the Axiovision software by measuring the intensity of gene

expression in approximately 5 hub cells per gonad. These measurements were averaged for each genotype and a p-value was calculated by Student's t-test.

Generation of transgenic *Drosophila* strains

To generate the *hhF6*-GFP and *hh1.5*-GFP constructs, genomic DNA was PCR amplified from a pH stinger clone (A gift from Robert Schulz) using Phusion polymerase (Finnzymes), with the addition of NotI and XbaI restriction sites on the forward and reverse primers, respectively. The fragments were TOPO cloned using the TOPO TA Cloning Kit Dual Promoter (Invitrogen), digested using the aforementioned restriction sites, gel purified (Geneclean II Kit) and directionally cloned into the pEGFP.attB (A gift from Konrad Basler) vector which was similarly linearized by NotI and XbaI. Fly lines were then established (BestGene, Inc.) after germline transformation of the constructs. The following oligonucleotide sequences were used for PCR amplification:

NotI-*hhF6*-GFP For 5' ATAAGAATGCGGCCGCGCGATACAGCACCCTTAATC 3' (forward primer used to clone both *hhF6* and *hh1.5*);

XbaI-*hhF6*-GFP Rev 5' ATGCTCTAGATGCAAAAGAGGGCAGAGAAC 3';

XbaI-*hhF5*-GFP Rev 5' ATGCTCTAGATTATACCCATAGCCATAGCC 3' (reverse primer used to clone *hh1.5*)

Deletion constructs

hh1.5-GFP deletion constructs were generated by outward directed PCR amplification, using Phusion polymerase, from the *hh1.5*-GFP-TOPO clone. Forward and reverse primers were made to flank each of the four conserved regions. PCR products were DpnI-treated (NEB) to eliminate template DNA and PNK-treated (NEB) to phosyphorylate

product ends. PCR products were then ligated, yielding a *hh1.5*-GFP-TOPO clone minus the deleted conserved region (*hh1.5*-GFP-TOPOΔ 1-4). Each of the four *hh1.5*-GFP-TOPOΔ clones was digested with NotI and XbaI to cut out the deletion fragments. Deletion fragments were gel purified and then directionally cloned into the pEGFP.attB vector, which was similarly linearized, by NotI and XbaI. Fly lines were then established after germline transformation of the constructs. The following oligonucleotide sequences were used for PCR amplification:

Del 1 For 5' GATCCAGCTGGAGCTGCGGATTGGCATTGC 3';

Del 1 Rev 5' CATCGCTTCATTAGAATTAGCGGCGGTCTTTGATT 3';

Del 2 For 5' TGCGATCTCAATCAGTGCCGGGAATCAAAG 3';

Del 2 Rev 5' GTCGAAAAAATACGAGTTGAAACTCTGAAGAAATCACG 3';

Del 3 For 5' TATAAAAAAAGGGGTGACTCCCCTGGCAGC 3';

Del 3 Rev 5' GCTGCCAGGGGAGTCACCCCTTTTTTTATA 3';

Del 4 For 5' CGCCTTTTTCGGGGTAATGGCTGAAGAAAA 3';

Del 4 Rev 5' TTTTCTTCAGCCATTACCCCGAAAAAGGCG 3'

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