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Specification and Morphogenesis of the Drosophila Testis Niche

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Abstract

Adult stem cells have the unique ability to either self-renew or differentiate, thus giving them tremendous therapeutic potential. These tissue-specific stem cells are directed to self-renew by signals from the local microenvironment termed the stem cell niche. While reconstitution assays have demonstrated the existence of stem cell niches in many adult organs, unambiguous identification of the resident stem cells and their niche cells continues to be a challenge. Accordingly, the mechanisms that direct specification and formation of a stem cell niche in vivo remain unclear. The Drosophila testis has emerged as a powerful system in which to study stem cell-niche interactions. The niche cells, called hub cells, form a small aggregate at the apical tip of the testis. Hub cells promote attachment and self-renewal in the germline stem cells and cyst stem cells, which are organized in a radial array around the hub. The signaling pathways that direct the maintenance and differentiation of these lineages have been well characterized; however, the initial specification and organization of the niche is still being elucidated. It was previously shown that Notch activation in a subset of somatic gonadal precursors specifies them as hub cells in the embryonic gonad. Here we use genetic analysis to show that Notch signaling activates a branched pathway for hub cell differentiation. Along one arm of the pathway, the Maf factor Traffic jam is downregulated to allow for niche signaling and adhesion. Along a separate arm, the transcription factor Bowl, promotes the assembly of hub cells at the anterior of the gonad where they recruit and organize stem cells. We also use live imaging to reveal two phases of niche morphogenesis; 1) a sorting and guidance phase in which hub cells are directed to the anterior by an extra-gonadal cue and 2) a compaction phase characterized by the formation of an acto-myosin cable around the compacting hub concomitant with the onset of oriented GSC divisions. These observations suggest a model in which the germ cells shape their own niche by driving hub compaction. These findings greatly advance our understanding of how a stem cell niche develops within a tissue.

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Lindsey Wingert

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ABSTRACT

SPECIFICATION AND MORPHOGENESIS OF THE *DROSOPHILA* TESTIS NICHE

Lindsey Wingert

Steve DiNardo

Adult stem cells have the unique ability to either self-renew or differentiate, thus giving them tremendous therapeutic potential. These tissue-specific stem cells are directed to self-renew by signals from the local microenvironment termed the stem cell niche. While reconstitution assays have demonstrated the existence of stem cell niches in many adult organs, unambiguous identification of the resident stem cells and their niche cells continues to be a challenge. Accordingly, the mechanisms that direct specification and formation of a stem cell niche *in vivo* remain unclear. The *Drosophila* testis has emerged as a powerful system in which to study stem cell-niche interactions. The niche cells, called hub cells, form a small aggregate at the apical tip of the testis. Hub cells promote attachment and self-renewal in the germline stem cells and cyst stem cells, which are organized in a radial array around the hub. The signaling pathways that direct the maintenance and differentiation of these lineages have been well characterized; however, the initial specification and organization of the niche is still being elucidated. It was previously shown that Notch activation in a subset of somatic gonadal precursors specifies them as hub cells in the embryonic gonad. Here we use genetic analysis to show that Notch signaling activates a branched pathway for hub cell differentiation. Along one arm of the pathway, the Maf factor Traffic jam is downregulated to allow for niche signaling and adhesion. Along a separate arm, the transcription factor Bowl, promotes the assembly of hub cells at the anterior of the gonad where they recruit and organize stem cells. We also use live imaging to reveal two

phases of niche morphogenesis; 1) a sorting and guidance phase in which hub cells are

directed to the anterior by an extra-gonadal cue and 2) a compaction phase

characterized by the formation of an acto-myosin cable around the compacting hub

concomitant with the onset of oriented GSC divisions. These observations suggest a

model in which the germ cells shape their own niche by driving hub compaction. These

findings greatly advance our understanding of how a stem cell niche develops within a

tissue.

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CHAPTER 1: General Introduction

During embryonic development, the single-celled zygote gives rise to a complex, multicellular organism through multiple rounds of cell division, cell specification and coordinated morphogenetic events by those cells. The zygote is the only totipotent cell, giving it the unique ability to generate every cell type within the organism. However, the cells derived from the zygote lose that potential as they become fated and more specialized (Figure 1.1). By the time embryonic development is complete, the vast majority of cells within an organism are either post-mitotic or unipotent, meaning they divide and generate only identical daughters. Given the terminal differentiation of most cells in an adult organism, an intriguing question in cell biology is how adult tissues that undergo extensive turnover, such as the intestine or blood, maintain the required number and relative fractions of specialized cell types within the tissue. Furthermore, how are cells rapidly replaced after injury to an organ in a process called regeneration? The solution to the problem of cellular turnover in adult tissues is an undifferentiated subset of cells found in most organs called adult or tissue-specific stem cells[1]. Although their lineage is more restricted than the zygote, adult stem cells have the amazing potential to self-renew or differentiate, thus making them an intriguing therapeutic target for disease and aging.

Adult Stem Cells

Adult stem cells have now been identified in most organs from those highly regenerative tissues like the intestine and blood to those with limited regenerative capacity such as the brain and heart[2–6]. A key focus in the field of stem cell biology is to understand the signaling pathways and mechanisms that regulate the balance between self-renewal and differentiation so that it can be harnessed to treat disease. For example the expansion of the hematopoetic stem cell *in vitro* would be paramount in treating

blood-related diseases and leukemias that result form depletion of certain hematopoietic

lineages and unchecked proliferation of others. For these reasons, it is critical to

understand the mechanisms of stem cell development and maintenance within their

resident tissues to better target these cells and tip the balance in support of self-renewal

or differentiation (Figure 1.2). To do this, you must first be able to unambiguously identify

the stem cells in a given tissues and manipulate them. Fortunately, the extensive use of

animal models has allowed for identification and characterization of many adult stem cell

lineages and the signaling pathways that regulate them are often shared across tissues

and species[7].

Stem cell fate is coordinated by cell intrinsic and extrinsic cues that result in

either symmetric or asymmetric divisions[1] . A prominent example of a stem cell lineage

that is regulated intrinsically is the *Drosophila* neuroblast, which divides asymmetrically

to generate one daughter neuroblast and one daughter ganglion mother cell.

Asymmetric cell fate is achieved through unequal segregation of cytoplasmic

determinants and proper orientation of the mitotic spindle[8,9]. This method of fate

determination has been demonstrated in other *Drosophila* lineages and in *C. elegans*;

however, there are few mammalian examples[1]. Instead, both asymmetric and symmetric

stem cell divisions of most mammalian stem cell lineages are regulated by the local

microenvironment[10,11].

Stem Cell Niche

Adult stem cells reside within close proximity to specific cells types, secreted

proteins and extracellular matrix proteins that direct their self-renewal. Schofeld originally

proposed the existence of a stem cell "niche" in his work on blood cells in 1978;

however, evidence supporting this idea was not uncovered until the turn of the 21st

century when the germline stem cell niche in *Drosophila* ovary was first described[12,13].

Since then, niche signals have been discovered contributing to the regulation of almost every stem cell lineage identified, including the hematopoetic stem cell, the intestinal stem cell and the spermatogonial stem cell, to name a few[2,3,14]. While reconstitution and lineage tracing experiments demonstrate the presence of stem cells as well as the extrinsic requirements for their self-renewal, an ongoing difficulty in stem cell biology is the unambiguous identification of the stem cells and niche cells using definitive markers[4,15].

Drosophilla GSC niche

One particularly well-described niche and the focus of my thesis work is the *Drosophila* testis niche (Figure 1.3)[10]. The testis houses the germline stem cell niche, which maintains spermatogenesis through regulation of the germline stem cell (GSC). The niche is composed of a small aggregate of post-mitotic somatic cells anchored at the apical tip of the testis. These cells, called hub cells, secrete factors that promote attachment and self-renewal in the resident stem cell populations. Arranged in a radial array around the hub are the GSCs and a somatic stem cell population called cyst stem cells (CySCs). The CySC provides a continual supply of daughter cyst cells that are necessary for the differentiation of the germline lineage[16,17]. To this end, two daughter cyst cells ensheath a differentiating GSC daughter, or gonialblast (GB). Although the cyst cells exit the cell cycle, the GB undergoes four rounds of transit amplifying divisions and meiosis to generate sperm. It was more recently demonstrated that the CySC also plays a role in GSC self-renewal through secretion of niche factors along with the hub[18]. Since the niche was first described fifteen years ago, the signaling pathways required for the steady-state maintenance of these stem cell populations have been well

characterized and have also been found to function in maintaining other tissue-specific stem cells.

Stem cell niche signaling

The Jak/STAT pathway is a core signaling pathway used iteratively during development and adult life. In both vertebrates and invertebrates, a receptor is activated by secreted cytokines or growth factors resulting in phosphorylation of the effector molecule STAT by Janus Kinase (Jak), which allows for its nuclear translocation. Within the nucleus, pSTAT is able to bind DNA and activate transcription, thus resulting in changes in gene expression upon pathway activation[19,20]. In addition to its numerous developmental roles, Jak/STAT signaling is also employed in the regulation of several adult stem cell lineages[21–24]. In the Drosophila midgut, Jak/STAT is activated within the intestinal stem cell (ISC) lineage upon ablation of differentiated enterocytes. This functions in regeneration of the gut epithelium by promoting proliferation and differentiation of ISCs[23,25]. Additionally, STATs 3 and 5 have been demonstrated to have a role in hematopoietic stem cell self-renewal and STATs 1,3 and 5 are activated ectopically in various leukemias[26]. Jak/STAT signaling in the ovary maintains follicle stem cells, which are the source for follicle cells that surround the oocyte[27]. Additionally, it non-autonomously directs GSC self-renewal by modulating Bone Morphogenetic Protein (BMP) signaling in somatic support cells[28,29].

Jak/STAT signaling was the first signaling pathway identified to act in stem cell self-renewal in the *Drosophila* testis. The hub cells secrete the ligand Unpaired (Upd), which binds to the receptor Domeless in adjacent stem cells, thus activating the Jak/STAT pathway in those cells[22,24]. Mutations in the pathway lead to depletion by differentiation of both stem cell populations, and ectopic activation of the pathway is

sufficient to generate a tumorous collection of CySCs and GSCs in the testis by preventing their differentiation[22,24]. As the mechanism of Jak/STAT signaling in the testis niche was further investigated, more intricate roles for signaling within each lineage were discovered. In GSCs, for example, pSTAT upregulates E-cadherin, a component of adherens junctions that serves to maintain attachment to the hub rather than directly inducing self-renewal in those cells[18,30]. In CySCs, however, pSTAT does lead to self-renewal by activation of Zfh1, a zinc-finger transcription factor necessary and sufficient for CySC self-renewal[31]. Therefore, as in the female ovary, pSTAT is not required cell autonomously in the GSC for self-renewal but instead maintains attachment of the GSC to niche cells that direct its self-renewal[28,32]. Finally, Jak/STAT signaling in CySCs upregulates Suppressor of Cytokine Signaling E (Socs36E), a negative regulator of the pathway that serves to dampen the response. CySCs mutant for *socs36E*, out compete GSCs for niche access, possibly through upregulation of integrin. In this way, Jak/STAT signaling functions to sustain CySC self-renewal, while at the same time attenuating the pathway through Socs36E to maintain proper niche architecture[33].

The BMP signaling pathway is another core developmental pathway that functions in many adult stem cell lineages[28,34–36]. BMPs are secreted ligands that upon receptor binding activate TGF-β signaling through the phosphorylation and nuclear translocation of Mad (Smad in vertebrates)[37]. BMP signaling has been demonstrated to regulate GSC self-renewal in both the ovary and testis[34,36,38]. In the testis, hub cells secrete the ligands Decapentaplegic (Dpp) and Glass bottom boat (Gbb). These ligands activate the TGF-β pathway in GSCs to repress bag-of-marbles, a factor that induces differentiation in the germline[38,39].

Previously, BMP signaling was thought to have a minor role in GSC self-renewal as ectopic expression of the secreted protein Upd maintained GSC self-renewal distal to the hub[22,24]. However, it was later demonstrated that pSTAT activation specifically within the germline fails to generate ectopic GSCs. Furthermore; CySCs can maintain self-renewal of GSCs depleted for STAT[32]. Given these data, it is clear that Jak/STAT signaling does not regulate self-renewal intrinsically within the germ cell. Instead, BMP signaling is the key self-renewal event in GSCs. CySCs, in addition to hub cells, secrete the BMP ligands Gbb and Dpp, and pMad accumulates robustly in GSCs ectopically maintained by CySCs[32,34,38,39]. Finally, GSCs depleted for STAT (thus unable to adhere to the hub) can no longer be rescued if BMP signaling is attenuated within the testis using a pathway antagonist[32]. Together, these data demonstrate that pSTAT functions cell autonomously to regulate adhesion within GSCs and self-renewal within CySCs. However, if GSCs can no longer adhere to the hub cell niche, CySCs can also direct GSC self-renewal by secreting BMPs and thus, can act as niche cells[18].

Given the vital role for somatic cells in GSC maintenance, the signaling pathways required for CySC self-renewal have also been well characterized. As previously stated, Jak/STAT signaling is necessary and sufficent for CySC self-renewal through upregulation of the transcription factor Zfh1[31]. It is thought that Zfh1 activates transcription of BMP ligands within CySCs to maintain GSC self-renewal away from the hub cell niche[32]. Curiously, ectopic expression of *zfh1* in CySCs is sufficient for CySC and GSC self-renewal, while ectopic activation of BMP receptors in the germline does not generate ectopic GSCs[18,31,34,38]. This suggests that there is another effector of Zfh1 in CySCs that nonautonomously maintains GSCs.

In addition to Jak/STAT signaling, Hedgehog signaling has also been demonstrated to play a role in CySC self-renewal[40,41]. In the absence of ligand binding, the receptor Patched (Ptc) inactivates the signal transducer Smoothened (Smo). Hedgehog secreted from the hub binds Ptc causing it to release Smo which influences the cleavage of the transcription factor Cubitis Interruptus (Ci) from a repressor to an activator[40–42]. CySCs with loss-of-function mutations in Smo or Ci are lost from the niche. Conversely, ectopic activation of the pathway through expression of *hedgehog* or RNAi knockdown of *ptc* results in an increase in Zfh1^{+} CySCs. It was further demonstrated that Jak/STAT signaling and Hedgehog signaling were both required for CySC maintenance and mutations in one pathway could not be rescued by activation of the alternate pathway[40]. While the influence of Hedgehog signaling on GSCs in the testis is controversial, it has been shown to maintain ovarian GSCs non-autonomously by regulating expression of BMPs in escort cells[40,41,43,44].

A final pathway implicated in steady-state maintenance of the testis niche is the Epidermal Growth Factor Receptor (EGFR) signaling pathway. The EGFR pathway is one of several receptor tyrosine kinase (RTK) pathways that result in a kinase cascade called the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway[45]. In contrast to the previously discussed pathways in which ligands secreted by the niche cells (hub or CySCs) ultimately maintain GSC self-renewal, the EGF ligand is secreted by the germline and acts within the cyst lineage to non-autonomously promote germline differentiation[16,17]. EGFR signaling in the cyst lineage results in both transcriptional changes through the small GTPase Raf and in cytoskeletal changes through modulation of the small GTPases Rac and Rho[16,17,46,47]. Both effector pathways of EGFR signaling are required for the switch from GSC to GB fate and the initiation of germline

differentiation. EGFR signaling is similarly required in the ovary for the ensheathment of the differentiating germline by the escort cells[47].

EGFR signaling has also been demonstrated to regulate proliferation in stem cell niches. In the intestinal and gastric stem cell lineages, EGFR acts cell autonomously to promote proliferation and exit from quiescence[23,48–50]. In the Drosophila ovary, EGFR is activated in the soma by the germline. This functions to activate survival in the intermingled somatic cells, which in turn inhibit PGC proliferation[51]. Similarly, in the testis, EGFR functions in the somatic cells to decrease proliferation in the germline as GSCs in EGFR mutants have an increased mitotic index[52]. Clearly the output of EGFR signaling is complex as it acts cell autonomously in some stem cell lineages to increase proliferation and non-cell autonomously in other stem cell lineages to decrease proliferation[23,48–51]. Thus, it is important to identify the effectors of the core signaling pathways in each individual stem cell lineage to identify their functions in self-renewal or differentiation.

Stem cell niche development

While much has been elucidated about the steady-state maintenance of many stem cell niches, including the *Drosophila* GSC niche, less is known about their development. This is partly due to the absence of good stem cell markers in various organs but also due to the reorganization of these niches from embryonic development to adulthood. Still, maintenance of adult stem cells throughout an organism's life requires that the niche cells and stem cells be specified and organized appropriately within the tissue during development. Thus, identifying the signaling mechanisms that specify stem cells and niche cells will inform research into how to culture and expand stem cells both *in vivo* and *ex vivo*.

Hematopoietic stem cells (HSCs) have incredible therapeutic potential making them a major focus in stem cell niche biology. Adult HSCs are derived from the aorta-gonad-mesonephros region during embryogenesis[53]. Several signaling pathways have been implicated in the specification of HSCs from the precursor pool of hemogenic endothelial (HE) cells[54]. Notch and Hedgehog signaling promote development of the HE niche and emergence of HSCs, while Estrogen and Fibroblast Growth Factor (FGF) signaling limit the region of specification[35,55,56]. Once specified, HSCs emerge into circulation and migrate to the fetal liver where they proliferate[54,57]. Following bone formation and vascularization, HSCs migrate to and colonize the bone marrow niche where osteoblastic, perivascular, endothelial, Schwann and neuronal cells regulate their quiescence and activation[58].

Like the HSC niche, the mammalian neurogenic niche is restructured from embryogenesis to adulthood[59]. The neural progenitor cells (NPCs) are derived from neuroepithelial cells (NECs) in the embryonic ventricular/subventricular zone of the mouse cerebral cortex. During embryogenesis, the NECs are more proliferative, thus expanding the lineage (similar to early HSCs in the fetal liver). BMP and Insulin-like growth factor (IGF) signaling from the cerebrospinal fluid promote NPC proliferation and self-renewal during embryogenesis. Additionally, vascular endothelial cells activate Notch and secrete factors to promote NEC contact and self-renewal[59,60]. During development into adulthood, the complexity of the SVZ increases as new cell types and extracellular matrix (ECM) components are introduced. Adult NSC self-renewal is further modulated by ECM stalks called fractones which bind growth factors and ependymal cells, which secrete the BMP antagonist Noggin and PEDGF[59].

While knowledge of the regulation of these mammalian stem cell niches has been gained, there is immense complexity associated with the vast reorganization that these niches undergo during development. Additionally, the array of cell types and secreted cues acting on the stem cells makes it difficult to parse out the individual contributions to self-renewal and differentiation[59]. The *Drosophila* GSC niches are composed of fewer cell types, established early in developmnet within the embryonic and larval gonads, and undergo very little remodeling following their initial specification. Therefore, they have proven to be attractive models for identifying the core signaling pathways required for stem cell niche specification and morphogenesis[51,61–66].

Gonad development

The gonad forms from the coalescence of the somatic gonadal precursors (SGPs), from which the niche cells are derived, and the primordial germ cells (PGCs), from which the GSCs are derived. Prior to their coalescence with the SGPs, the PGCs are specified at the posterior pole of the embryo and remain outside of the embryo proper until after gastrulation and a morphogenetic event in *Drosophila* known as germ band extension. Following germ band extension, the PGCs actively migrate through the endodermal germ layer into the embryo where they associate with SGPs. In bilateral clusters, the PGCs and SGPs migrate on either side of the developing gut, an endodermal derivative, and subsequently settle and compact into spheres in the posterior half of the embryo[67].

Prior to their coalescence with the germ cells, the SGPs are specified from mesoderm under the influence of the transcription factors Tinman, Zfh1, and Eyes absent (Eya)[68,69]. At this time, the embryo has segmental boundaries generated from alternating developmental gene regulatory networks acting along the anterior-posterior

axis[70]. SGPs are specified from the anterior domain of parasegments (PS) 10-12 while the posterior domain gives rise to fat body precursors from which the male gonadal sheath cells will be specified[69,71–74]. Additionally in male embryos, a cluster of SGPs arising from PS13 join and remain attached to the posterior end of the gonad; while in females, these SGPs are specified, but subsequently apoptose[75].

At the time of their association with the germ cells, clusters of SGPs are already distinct in their transcriptional regulation by the homeotic genes. The anterior SGPs, those from PS10 and 11 were specified from the subdomain under Abdominal-A (AbdA) regulation, while those arising from PS12 are also influenced by AbdB[69]. In males, this initial asymmetry sets up their potential to become niche cells for the germline. For example, AbdA is necessary for SGPs to adopt hub cell fate, while ectopic expression of AbdB is sufficient to suppress hub cell fate[63,69]. This led scientists to question whether anterior SGPs were equally competent to become hub cells or whether they too were subdivided and thus, already fated. Interestingly, PS11 SGPs were lineage traced and shown to contribute to both hub and CySC lineages, suggesting that in this subset of SGPs influenced by AbdA, cells are competent to become either lineage[74,76].

At the embryo to larval transition, the niche is established within the male gonad (Figure 1.4). Hub cells can be identified by their enrichment for junctional proteins, and expression of the self-renewal factor *unpaired*. The hub cells, in turn, specify and recruit GSCs and CySCs by Jak/STAT activation within several hours of hatching and continue to act as niche by promoting self-renewal throughout adulthood[63,77,78]. In contrast, the female GSC niche is only established several days later (in the third larval instar) gonad by Notch signaling[61]. In the meantime, hormonal and EGFR signaling promote growth and homeostasis of the PGCs and intermingled somatic cells[51,62]. The early specification

of the niche cells and subsequent recruitment of stem cells make the male gonad an ideal system with which to investigate the specification of a niche cell lineage and will be the main focus of my thesis work.

Niche specification

Given that hub cells cannot be identified early during gonadogenesis, it was widely thought they were specified late during embryogenesis[63]. As in the female, it was demonstrated that Notch signaling is required for niche cell specification although the critical Notch ligand, source of the ligand and timing of Notch activation are somewhat controversial[64,66]. In *Drosophila*, a single Notch receptor is activated by the ligands Delta or Serrate. Ligand binding results in cleavage of the receptor allowing it to translocate to the nucleus and bind DNA and activate transcription with Supressor of Hairless and Mastermind[79,80]. *Notch* mutant embryos specify few to no hub cells using several markers of hub cell fate[64,66]. Okegbe and DiNardo used a transgenic Notch reporter to demonstrate that Notch was activated in a subset of SGPs early during gonadogenesis and that Notch-activated SGPs were found in the hub at the end of embryogenesis. The authors carefully identified the window for Notch requirement by resupplying Notch in a *Notch* mutant background early and late during gonadogenesis. This resulted in a rescue of hub cell specification when Notch was resupplied early but not late. The time of Notch requirement correlates with the coalescence of SGPs and germ cells and their collective migration adjacent to the gut. Therefore, the authors went on to show that Notch is activated in SGPs by Delta delivered from the endodermally-derived posterior midgut at this time (Fig 3.1)[64].

Kitadate and Kobayashi also identified the requirement for Notch in hub cell specification. Using a similar transgenic Notch reporter, they showed all SGPs being

activated for Notch, although it is worth mentioning that only a subset become hub cells.

They additionally showed that Serrate is the activating ligand and is expressed on SGPs

after the stage at which Okegbe and DiNardo identified as the window of Notch

requirement. Kitadate and Kobayashi went on to overexpress an activated form of the

Notch receptor in mesodermal lineages (which include SGPs) and found a small

increase in the number of hub cells specified[66]. Although, there are apparent

discrepancies in the findings, (discussed in Chapter 2), my thesis work supports the

conclusion proposed by Okegbe and DiNardo that hub cell specification by Notch

activation occurs around mid-embryogenesis in a subset of SGPs. Notably, there are

two mechanisms of Notch signaling, inductive signaling and lateral inhibition. Inductive

signaling typically occurs when one cell type expresses the ligand and activates Notch in

an adjacent cell type. In lateral inhibition, Notch signaling is stochastically activated in a

field of equivalent cells. Minor increases in Notch activity are amplified in that cell and

downstream events prevent signaling in the adjacent cell to specify distinct cell fates[81,82].

Therefore, one possible model is that inductive Notch signaling from the endoderm

initiates niche specification and subsequent lateral inhibition within the SGPs refines hub

cell number. Future work investigating the interplay between Notch signaling and RTK

signaling (discussed below) may also elucidate hub cell specification in the gonad[64,66].

Receptor tyrosine kinase (RTK) pathways have also been implicated in hub cell

specification[65,66]. Rather than positively specifying hub cells like Notch activation, RTK

activation in posterior SGPs functions to repress hub cell fate thereby restricting hub

cells to the anterior. In one RTK pathway, Bride of Sevenless (BOSS) is expressed by

male PGCs and activates Sevenless (Sev), which is only displayed on posterior SGPs.

Furthermore, *Abd-B*, which specifies posterior SGP identity, is required for robust *sev*

expression, providing the mechanism by which pathway activation is restricted to the posterior. Although Boss/Sev signaling is required for repression of hub cell fate in posterior SGPs, ectopic activation of Sev in anterior SGPs is not sufficient to repress hub cell fate[65].

EGFR is also required to activate RTK signaling and repress hub cell fate in posterior SGPs. As in the case of Boss/Sev, the PGCs present EGF ligands to the somatic component of the gonad. Interestingly, the EGF receptor is not restricted to posterior SGPs, although transcriptional readouts of pathway activation are only visible in the posterior. Like Sev expression, activation of the EGFR pathway in posterior SGPs requires AbdB function. EGFR signaling appears to be the critical RTK pathway acting to repress hub cell fate as gonads mutant for *egfr* and *sev* do not have a significant increase in hub cells compared to gonads mutant for *egfr* alone. Furthermore, in contrast to activation of Sev, constitutive activation of EGFR represses hub cell fate in the anterior, thus reducing hub cell number[65,66]. Thus, RTK signaling pathways play a crucial role in restricting hub cell number and spatially restricting hub cell fate to the anterior half of the gonad.

The previously identified pathways leave an open question in the field regarding the interplay between Notch and EGFR signaling in hub cell specification. In another stem cell niche, these two pathways have also been shown to act antagonistically to regulate the transition from neural stem cell to neural precursor cell fate[83]. Competition and antagonism between Notch and EGFR signaling can occur through several mechanisms[82]. In the *C.elegans* gonad, EGF expression is limited to the anchor cell (AC) due to Notch activation in the initially equivalent ventral uterine precursor cell. This leads to post-transcriptional downregulation of a transcriptional activator of the EGF

ligand, thus restricting EGFR signaling[84]. EGF ligand expression from the AC then acts on the vulval precursor cells (VPCs) to activate Ras, which can alter the endocytic sorting of LIN-12 (Notch), thus downregulating it[85]. In *Drosophila* photoreceptor cell specification, Notch signaling can repress expression of Atonal, which is a transcriptional activator of Rhomboid, a protein required for processing of the EGF ligand Spitz[82,86]. Additionally, in *Drosophila*, in was shown that EGFR signaling can lead to the phosphorylation and inactivation of Groucho, a global co-repressor that functions in Notch-mediated transcriptional repression[87,88]. Therefore, Notch and EGFR can interact at multiple steps in the pathways. Elucidating the mechanism by which Notch and EGFR signaling interact to specify hub cells will require that effectors of these pathways be identified in SGPs.

Niche morphogenesis

The specification of hub cells results in changes in cell morphology and establishment of niche organization, in addition to activation of niche signaling[63]. During gonad coalescence, SGPs are intermingled with PGCs[89,90]. SGPs extend cytoplasmic extensions to encyst and isolate the germline. This interaction requires Ecadherin (Ecad) and drives gonad compaction[89]. Although hub cells are specified early during gonadogenesis, their morphology is indistinguishable from non-hub SGPs at this time[89]. At the end of embryogenesis, however, hub cells form a compact aggregate at the anterior of the gonad and can be identified using cell biological markers. Differentiated hub cells appear to be epithelial-like since hub-hub interfaces are enriched for epithelial junctional proteins, although it is unclear if they are true epithelial cells with apical-basal polarity[91]. The adherens junction proteins, E-cadherin (Ecad), N-cadherin (Ncad) and β-catenin, are all enriched at hub-hub and hub-germ cell interfaces, while FasciclinIII

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(FasIII), a component of septate junctions (the equivalent of mammalian tight junctions) is found enriched only between hub cells[30,63,92]. Since Ecad is expressed earlier during gonadogenesis, it may become selectively enriched around hub cells at late stages because of accumulation of the protein at interfaces as hub cells aggregate, upregulation of *shotgun (shg)*, the gene that encodes Ecad, in hub-specified cells (see Chapter 2), and upregulation of Ecad in germ cells recruited by Upd-Jak/STAT signaling[18,63,77]. Conversely, FasIII and Ncad are barely detectable in the gonad prior to hub cell aggregation[63].

The enrichment of adhesion proteins in hub cells upon niche formation has led to the hypothesis that hub cells sort together and away from non-hub-specified cells via preferential adhesion with one another[93]. However, no evidence has been found to support a differential adhesion hypothesis despite attempts to modulate adhesive proteins within the gonad (personal communication with M.Van Doren)[94]. Additionally, preferential adhesion alone is not sufficient to explain the final location of the hub at the most anterior region of the gonad, given that hub cells are derived from both PS 10 and, more centrally located, PS 11 SGPs[74,95]. One adhesion protein, however, has been shown to play a role in hub positioning. Integrin is composed of one α and one β subunit and mediates cell adhesion to extracellular matrix (ECM) proteins. Embryos mutant for *myospheroid*, the βPS subunit of integrin, display an internalized hub, completely enveloped by other gonadal cells[94]. Aside from internalization, the hub is normally enriched for Ecad and Ncad, and appears to function normally as evidenced by proper orientation of GSC divisions orthogonal to the hub[94,96]. Therefore, integrin is required to anchor the hub at the periphery of the anterior of the gonad.

A requirement for Integrin suggests that ECM is also important in positioning the niche. Indeed, the ECM components Laminin-A and Nidogen are detected around the gonad at late stages[94]. Furthermore, the intracellular actin-binding proteins Talin and Lasp are required for the hub to remain anchored at the anterior in the adult testis[94,97]. It is yet unclear whether Integrin-mediated attachment to an ECM is required for hub cell movement toward the anterior in addition to its requirement for anchoring the hub. Indeed, it is evident that some hub cells do move anteriorly. The PS 11 cells, from which we know a fraction of hub cells are derived, are located more centrally within the gonad and therefore, must migrate towards the anterior where the established hub is identified[63,74,76]. It is possible that Abd-B has a role in positioning the niche via expression Integrin subunits as *abd-B* mutant gonads have variable niche positioning and Abd-B regulates Integrin in the larval and adult testis[63,98]. ECM components might provide a scaffold for hub cell migration or, perhaps more intriguingly, could regulate signaling that directs hub cell assembly to the anterior. For example, Type IV Collagen has been shown to regulate Dpp signaling in *Drosophila* embryos and the matricellular protein Magu regulates BMP signaling in the testis niche [99,100]. If Integrin and ECM are required for hub cell movement to the anterior, along with hub cell anchoring, it will be important to identify which cells secrete the ECM components crucial for niche formation.

Project Summary

In my thesis work, I sought to further the understanding of stem cell niche biology by investigating the specification and morphogenesis of the GSC niche in the male *Drosophila* gonad. I was able to follow up on previous work on the Notch pathway in our lab and in Chapter 2, I demonstrate that the large Maf factor Traffic jam (Tj) functions

downstream of the Notch pathway in hub cell specification. I was able to show that Traffic jam is downregulated in hub cells and that Notch signaling is required for this downregulation. By examining *tj* mutants alone and in conjunction with a *Notch* mutation, I was able to genetically dissect the pathway downstream of Notch in hub cell specification and identify the role of the transcription factor Bowl, in hub cell assembly. This demonstrates that niche signaling and niche morphogenesis are separable, and Chapter 3 of my thesis aims to characterize and investigate niche morphogenesis. To that end, I developed *in vivo* and *ex vivo* live imaging of gonads and identified two stages of niche morphogenesis; the movement of hub cells anteriorly where they *assemble* as a cap on the periphery, and their subsequent *compaction* into a tight aggregate. Hub cell assembly occurs at a defined position facing two candidate tissues that may function to direct this assembly. Hub cell compaction is characterized by a decrease in hub cell area and is correlated with the appearance of a myosin II-purse string around the hub and a burst of germ cell divisions orthogonal to the hub, suggesting a role for acto-myosin contractility and tension generated by germ cell divisions in hub compaction.

Developmental potential

Totipotent
Zygote

Pluripotent
ICM/ES cells, EG cells,
EC cells, mGS cells
iPS cells

Multipotent
Adult stem cells
(partially
reprogrammed cells?)

Unipotent
Differentiated cell
types

B cell   Macrophage   Fibroblast Muscle
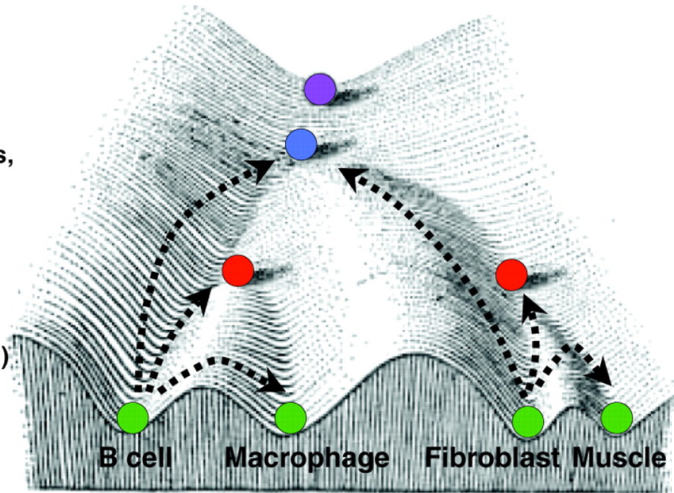
Figure 1.1: **Cell differentiation potential during normal development** (adapted from[101]). Adult stem cells retain some developmental potential and thus, have the ability to either self-renew or differentiate.
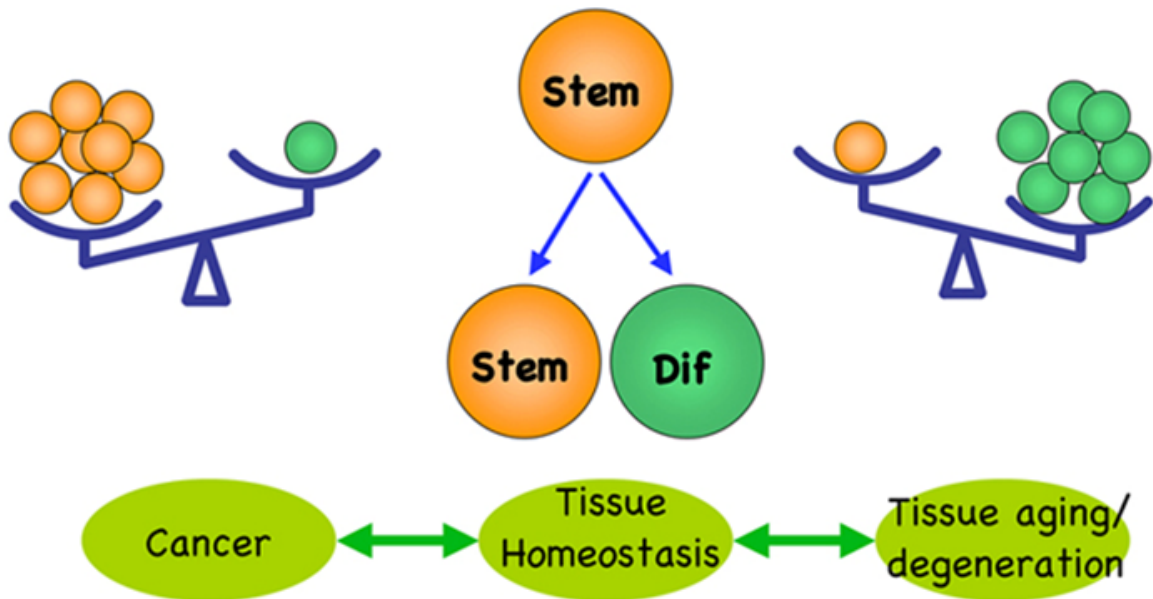
Figure 1.2: Adult stem cells balance self-renewal and differentiation. Misregulation of stem cells can result in a stem cell tumor or depletion of the stem cell lineage. Figure taken from (Yamashita, 2009)
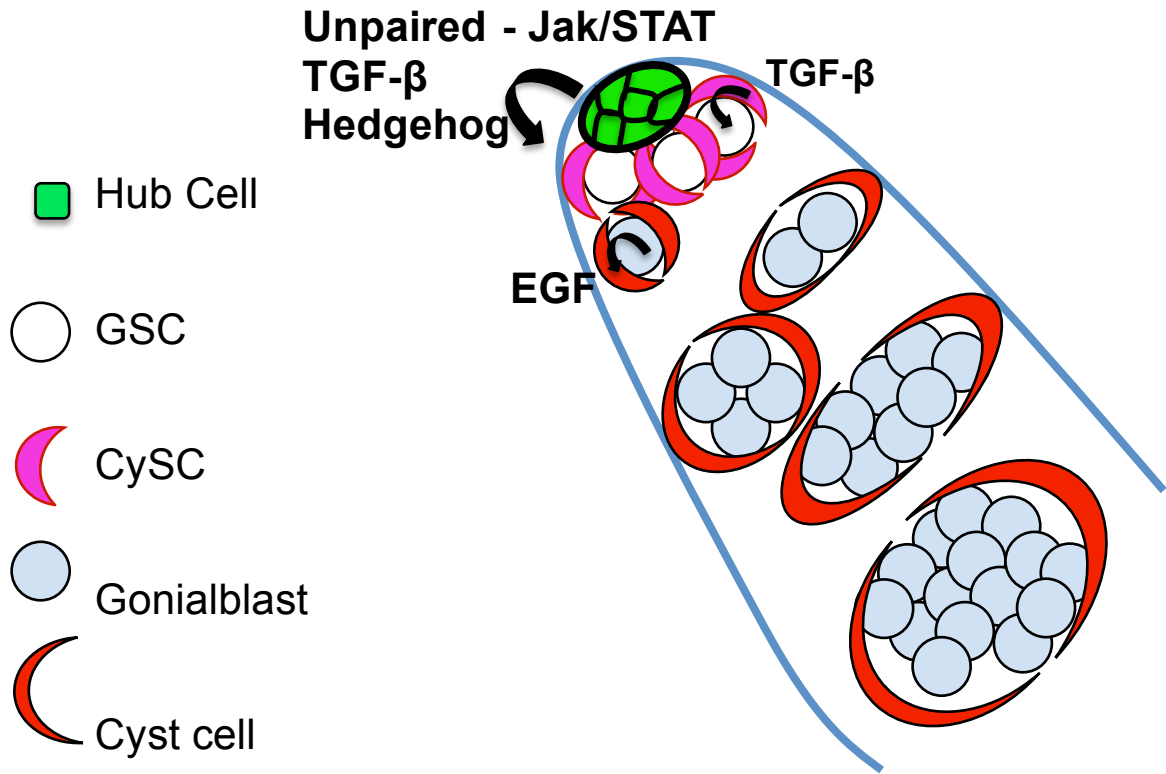
Figure 1.3: The *Drosophila* testis stem cell niche. The hub cells act as niche for the germline stem cells (GSC) and cyst stem cells (CySC) by secreting ligands that activate Jak/STAT, TGF-β and Hedgehog signaling. The CySCs can also function as niche cells for the GSCs by secreting BMPs. The gonialblasts activate EGF signaling in the cyst cells, which aid in differentiation of the germline.
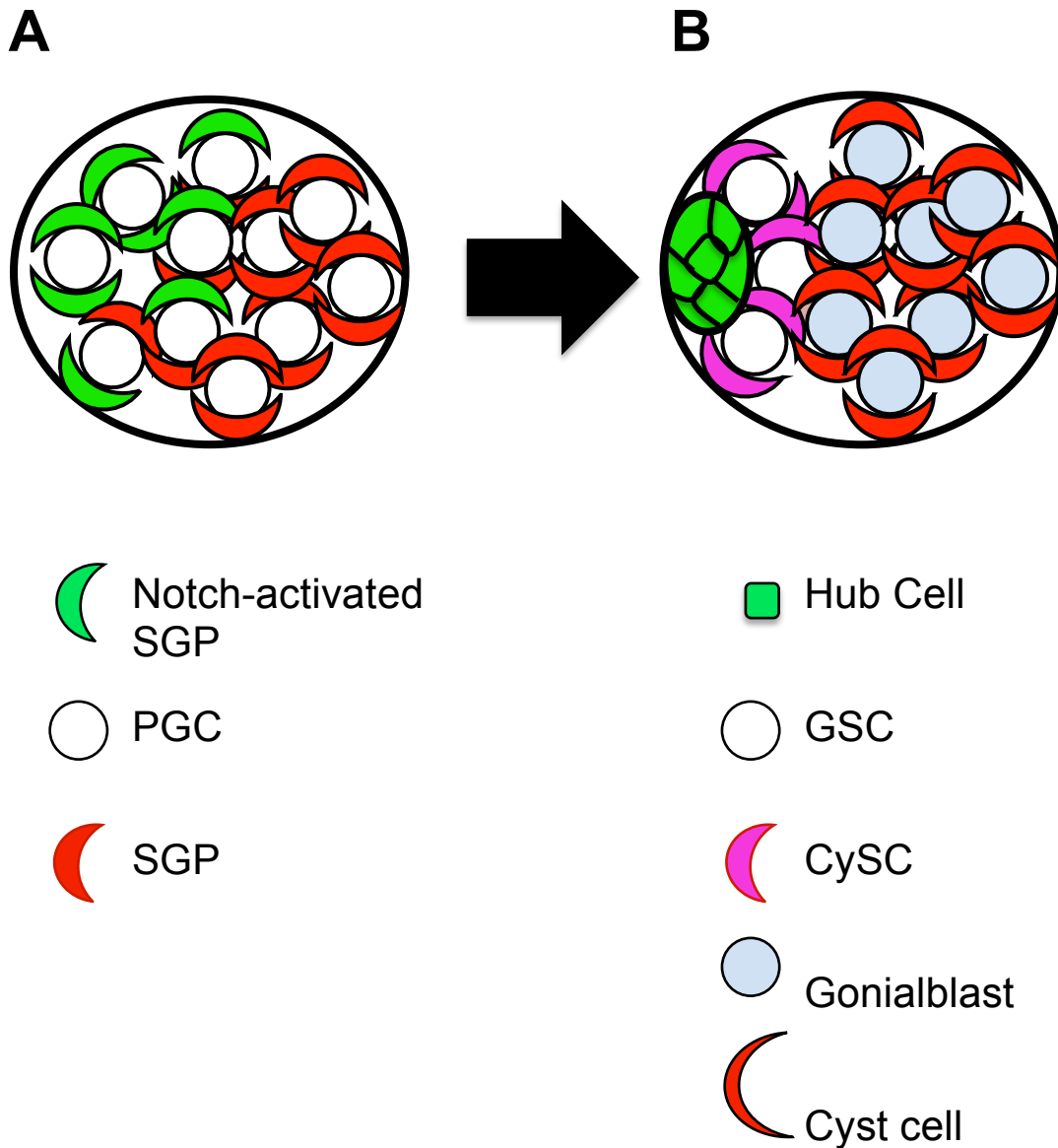
Figure 1.4: Hub cell specification and niche formation in the male *Drosophila* gonad. Shortly after gonad coalescence (A) a subset of somatic gonadal precursors (SGPs) are Notch-activated and thus specified as hub. However, they are indistinguishable from non-hub specified SGPs as they are not enriched for markers of hub differentiation and are intermingled with the germline. When the niche has adopted its final architecture (B) hub cells are identifiable at the anterior of the gonad by their enrichment for adhesion proteins and expression of niche signals. At this time, they recruit germline stem cells (GSCs) and Cyst stem cells (CySCs).

CHAPTER 2:

Traffic jam functions in a branched pathway from Notch activation to niche cell fate*

Summary

The niche directs key behaviors of its resident stem cells, and thus is critical for tissue maintenance, repair and longevity. However, little is known about the genetic pathways that guide niche specification and development. The male germline stem cell (GSC) niche in *Drosophila* houses two stem cell populations and is specified within the embryonic gonad making it an excellent model for studying niche development. The hub cells that comprise the niche are specified early by Notch activation. Over the next few hours, these individual cells then sort together and take up a defined position before expressing markers of hub cell differentiation. This timing suggests that there are other factors yet to be defined for niche development. We have identified a role for the large Maf transcription factor Traffic jam (Tj) in hub cell specification downstream of Notch. Tj downregulation is the first detectable effect of Notch activation in hub cells. Furthermore, Tj depletion is sufficient to generate ectopic hub cells that can recruit stem cells. Surprisingly, ectopic niche cells in *tj* mutants remain dispersed in the absence of Notch activation. This led us to uncover a branched pathway downstream of Notch in which Bowl functions to direct hub cell assembly in parallel to Tj downregulation.

Introduction

Adult stem cells are a crucial component of most organs. Having the unique ability to either self-renew or replace differentiated cells, they function in steady-state homeostasis and can also be activated in response to injury[102]. The instructive cues that guide stem cells to self-renew or differentiate often come extrinsically from the local microenvironment called the stem cell niche[10]. While previous work focused primarily on the identification and steady-state function of the stem cell niche, the mechanisms by which a niche is established during development are only more recently being

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addressed[3,4,103]. Several pathways required for niche cell specification have now been identified including Sonic Hedgehog in the neurogenic niche, hormonal and Notch signaling in the *Drosophila* ovarian niche and Wnt signaling in the *C. elegans* germline niche[61,62,104,105]. Comprehensive analysis of the pathway targets and how they individually direct niche cell fate is limited by the complexity of the system. Fortunately, the *Drosophila* testis GSC niche is well characterized and is established early within the male embryonic gonad. The niche cells can be unambiguously identified with multiple markers as early as the embryo to larval transition and stem cells are recruited shortly thereafter[63,77,78]. For this reason, the male gonad provides an ideal system to identify and dissect the pathways required for niche specification.

The male GSC niche is located at the apical tip of the testis and is composed of 8-10 aggregated somatic cells called hub cells[63,92]. The hub cells secrete BMPs and the cytokine Unpaired to activate self-renewal and adhesion in the GSCs[18,22,24,34,38]. An additional component of the niche, the Cyst Stem Cell (CySC) lineage, is maintained by Unpaired and Hedgehog secreted from the hub[31,32,40,41]. Steady-state function of the niche is established within the larval gonad following hub cell specification and stem cell recruitment[63,77,78].

The specification of hub cells from a pool of somatic gonadal precursors (SGPs) occurs via Notch activation [64,66]. Around mid-embryogenesis, the germ cells travel through the gut where they coalesce with SGPs. At this time, the SGPs are briefly exposed to an endodermally-derived Delta ligand which activates Notch in some SGPs [64]. While Notch signaling is required early, cell surface and gene expression markers of hub cell fate are not detected until many hours later, at the end of embryogenesis. Thus, the hub only becomes identifiable in late stage embryonic gonads after hub cells have

sorted to an anterior position, accumulated significant levels of epithelial adhesion proteins and adopted cobblestone morphology. At this time, gene regulatory changes result in hub-specific expression of *unpaired*[63,106].

The delay in differentiation suggests there are unknown intermediate effectors in hub cell specification downstream of Notch. The transcription factor Bowl and receptor tyrosine kinase (RTK) signaling have also been implicated in hub cell fate determination; however, their relationship to Notch activation has not been elucidated[65,66,76]. This work focuses on the role of the large Maf transcription factor Traffic jam (Tj) during hub cell specification. Previous work has shown that *tj* is expressed in SGPs and is required in gonad morphogenesis[90]. Additionally, Tj can suppress accumulation of the septate junction protein Fasciclin III (FasIII) and its RNA in the somatic cells of the adult testis and ovary[90]. Here we show that 1) Tj represses markers of hub cell fate and niche signaling, 2) Tj is downregulated in a subset of anterior gonadal cells and 3) that Notch is required for this suppression. Finally, we show that Tj acts along one arm of a pathway downstream of Notch activation for hub cell specification, while Bowl acts in a parallel arm to direct hub cell assembly.

Materials and Methods:

Fly stocks

Fly lines used were tj^{e02}, tj^{z4735} (D. Godt), *unpaired*-GAL4 UAS GFP (FBtp0016756), UAS-RedStinger (FBti0040830) (*upd*RFP), 10X-STATGFP (E. Bach), $N^{264.39}$ (FBal0029934), *hedgehog*-LacZ (FBst0005530), GTRACE (FBst0028281), UAS drm (J.A. Lengyel). Heterozygous siblings or w^{1118} were used as controls.

Immunostaining

Embryos were collected and aged 22-24 hours in a humidified chamber to 1st instar larvae or 36-48 hours for Late L1. Hatched larvae were dissected in Ringers and the internal organs 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 20 minutes (to reveal dpERK, tissue was fixed in 8% formaldehyde for 25 minutes), washed in PBS plus Triton-X-100 and blocked for 1 hour in 4% normal serum. Primary antibodies were used overnight at 4°C or 4 hours room temperature. Secondary antibodies were used at 1:400 (Alexa488, Cy3 or Cy5; Molecular Probes; Jackson ImmunoResearch) for 1 hour at room temperature. DNA was stained with Hoechst 33342 (Sigma) at 0.2 µg/ml for 6 minutes.

We used rabbit antibodies against Vasa 1:5000, Zfh1 1:5000 (a gift from R. Lehmann, Skirball Institute, New York, USA), RFP 1:500 (Abcam), STAT 1:1000 (E. Bach), dpERK 1:100 (Cell Signaling); mouse antibodies against Fasciclin III 1:50, Eya 10H6 1: 20, α-Spectrin 1:200, Patched 1:50 (DSHB), βgal 1:10,000 (Promega); rat antibodies against DE-cadherin DCAD2 1: 20, DN-cadherin 1:20 (DSHB); guinea pig anti-Traffic jam 1:10,000 (Dorothea Godt, University of Toronto, Canada); goat anti-Vasa 1:400 (Santa Cruz); and chick anti-GFP 1:1000 (Molecular Probes).

Fluorescence Imaging Quanitifcation

Z-stacks were obtained through the depth of the gonad using a Zeiss Axioplan with an ApoTome. Immunofluorescence signals of Tj were quantified using Metamorph by measuring nuclear pixel intensity.

S-phase labeling

Dissected tissues were incubated for 30 min in 10 µM Edu and then fixed. Edu incorporation was visualized using the Click-iT EdU Alexa Fluor 647 Kit (Invitrogen).

Sex identification and Genotyping

Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985). Male embryos and larvae were identifiable owing to the larger size of the gonad. For other cases, sex was determined by immunostaining male-specific SGPs. Fluorescent balancer chromosomes (P{w+ TM6 Hu ubi-GFP}(FBst0004533) or P{w+ CyO act-GFP} (FBst0004887)) distinguished heterozygous from homozygous mutant larvae. *Notch* mutants were identified by their neurogenic phenotype.

Results:

Tj is downregulated in hub cells

Traffic jam (*tj*) is initially expressed in SGPs upon contact with germ cells around stage 12 of embryogenesis[90]. Following compaction into a spherical gonad, Tj protein accumulated at similar levels in all SGPs while the hub cell marker, FasIII, was not detected (Fig2.1A). Following niche formation, gonads contained an anteriorly-localized aggregate of hub cells identified by FasIII enrichment (Fig 2.1C, asterisk). Interestingly, Tj appeared much lower in hub cells compared to the first tier of somatic cells around the hub (Fig 2.1C). Given that Tj is downregulated in hub cells, we asked whether presumptive hub cells could be identified prior to niche formation by reduced Tj accumulation.

We observed a reduction of Tj protein accumulation among a subset of anterior SGPs prior to niche formation (Fig 2.1B, arrows). Anecdotally, prior lineage tracing

revealed that hub cells are derived from among the anterior half of SGPs[63,76](L.W.

unpublished). We quantified Tj accumulation by averaging signals from the anterior half

of SGPs and comparing that to the posterior half. During stage 15, the anterior to

posterior ratio was 1.0 ± .15 (Std dev) (n=5 gonads) but decreased to 0.7 ± .08 (n=6)

during stage 16, confirming that SGPs in the anterior had reduced Tj signal compared to

those in the posterior (p =.0016 by student's t-test). Some anterior SGPs maintained

high Tj levels (Fig2.1B). This is consistent with the fact that not all anterior SGPs

contribute to hub and suggests that our quantification of anterior vs. posterior enrichment

is a conservative estimate of the downregulation of Tj in hub-specified cells.

Furthermore, prior to assembly of the hub at the anterior, we detected faint puncta of

FasIII around Tj-low SGPs but not around Tj-high SGPs (Fig2.1B). By L1, the ratio of

average pixel intensity in hub cells compared to the first two tiers of somatic cells around

the hub was .3 ± .05 (n=7). These data strongly suggest that Tj downregulation is

evident prior to other markers of hub cell fate including FasIII enrichment. We

hypothesized that Tj repression might be a critical step that instructs hub cell

differentiation.

tj represses hub cell differentiation

If Tj restricts hub cell fate in SGPs, we would expect global loss of Tj to result in

more SGPs adopting hub cell fate. To test this, we examined *tj* mutant gonads for

epithelial and niche-specific gene expression markers. Control gonads accumulated

FasIII at interfaces between hub cells (Fig 2.2A, asterisk). In *tj* mutant gonads, we

always observed an endogenous hub consisting of a cluster of FasIII-enriched cells with

cobblestone morphology assembled at the anterior (Fig 2.2B, asterisk). However, in

addition, there were FasIII-enriched cells that did not assemble at the anterior, but

instead remained dispersed and intermingled with germ cells (Fig 2.2B, arrows). The average number of cells in *tj* mutants that accumulated FasIII on at least one interface was 42 ± 10.7 (n=18) compared to 10.8 ± 3.7 in controls (n=18) while the total number of SGPs was similar (53.9 ± 4.6, n=15; 50.4 ± 5; n=14). We found that E-cadherin, which is enriched in endogenous hub cells, also accumulated ectopically (Fig 2.3).

The ectopic cells also induced the niche gene expression marker, *unpaired* (*upd*RFP) similar to the endogenous hub (Fig 2.2A,B) *Upd*RFP overlapped with FasIII accumulation in almost all cells resulting in *tj* mutant gonads containing an average of 39.3 ±10.4 *upd*RFP-expressing cells compared to 12.7 ± 3.6 in controls. Furthermore, these cells were active in inducing Jak/STAT signaling in response to Unpaired. In control gonads, a somatic cell reporter for STAT induction (STAT-GFP) accumulated in the cytoplasm of the hub cells (Fig 2.2C, asterisk) and in select somatic cells near the hub, presumably recruited as CySCs (Fig 2.2C, arrow). In *tj* mutant gonads, GFP accumulated in most of the somatic cells, including those far from the endogenous hub (Fig 2.2D, arrows), indicating that the ectopic hub cells activate STAT. Thus, *tj* mutants contained more hub cells compared with controls indicating that Tj functions to repress hub cell differentiation in SGPs.

Ectopic stem cells are recruited in *tj* mutant gonads

If the ectopic hub cells in *tj* mutants act as niche, they should recruit and maintain stem cells. GSCs recruited to the hub stabilize STAT within the nucleus which is required for E-cadherin-mediated attachment[18,77,96]. Just as in controls (Fig 2.4A,C), *tj* mutants exhibited STAT accumulation within the GSCs contacting the endogenous hub and E-cadherin enrichment at their shared interface (Fig 2.4B,D, endogenous hub

marked by asterisk). Germ cells contacting ectopic hub cells also accumulated STAT

(Fig 2.4B, arrows) and exhibited E-cadherin enrichment (Fig 2.4D,D' inset, arrows;

N=10/11). On occasion, we detected interfaces between ectopic hub cells and germ

cells that were less enriched for E-cadherin (Fig2.4D"-D"'; N=1/11 gonads). We also

staged germ cells by fusome morphology. In control gonads, GSCs surrounding the hub

contained dot fusomes (Fig 2.5A,C arrowheads), while differentiating germ cells located

farther from the hub accumulated branched structures (Fig 2.5A,C arrows).   *tj* mutant

gonads contained only dot or dumbbell-shaped fusomes at L1 (Fig2.5B, arrowheads)

signifying that cells had not progressed past the two cell stage. In later developmental

stages (L3), some branched fusomes were detected but only among germ cells at some

distance from ectopic hub cells (Fig 2.5D, arrow). Strikingly, germ cells adjacent to

ectopic hub cells often contained dot fusomes (Fig 2.5D, arrowheads). Since the ectopic

cells were several tiers removed from the endogenous hub, these data strongly suggest

that ectopic hub cells in *tj* mutants exhibit niche qualities.

We also examined *tj* mutant gonads for the presence of CySCs. During normal

development, both *Zfh1*and *Eyes absent (eya)* are expressed in SGPs prior to niche

formation[68,69]. Following niche formation and CySC specification, Zfh1 is selectively

enriched in somatic cells near the hub while Eya accumulates in a complimentary

pattern in more distal, differentiating cells (Fig 2.5E,G)[78,107]. In L1 *tj* mutant gonads, Zfh1-

enriched, Eya-depleted somatic cells were detected more than two tiers from the

endogenous hub suggesting the presence of ectopic stem cells (Fig 2.5F, arrowheads).

However, we also detected Eya^{pos} cells adjacent to ectopic hub cells (2.5F arrow). Given

that *eya* was previously expressed in these cells, it is possible that its repression is

delayed in *tj* mutants. Therefore, we examined L3 *tj* mutant gonads, days after CySC

specification would have occurred. Mature *tj* mutant gonads contained Zfh1^{pos} cells both

adjacent to the endogenous hub and several tiers away, near the ectopic hub cells, while

Eya^{pos} cells were only found distal to any hub cells (Fig 2.5H,H' endogenous hub,

asterisk). We conclude that *tj* mutants contain two pools of niche cells, an endogenous

compact hub and dispersed hub cells, both of which can support GSCs and CySCs.

Ectopic niche cells in *tj* mutants exhibit incomplete conversion to hub cell fate

While some of the ectopic cells were cuboidal, resembling endogenous hub cells

(Fig 2.2B, arrows), others were elongate with extensions that partially encysted germ

cells, a quality also exhibited by CySCs (Fig 2.4D", arrow). This suggested that some of

the ectopic cells in tj mutants were of mixed hub-CySC identity. To test this, we

compared the levels of the CySC marker Zfh1 with cell cycle state since only CySCs,

and not hub cells, actively cycle. In L1, Zfh1 was low in Fas3^{pos} hub cells but was easily

detectible in the first two tiers of somatic cells around the hub (Fig 2.6A). Zfh1^{pos} CySCs

also incorporated Edu while FasIII^{pos} hub cells did not (Fig 2.6A, arrow; n=18 gonads). In

tj mutants, we observed FasIII^{pos} cells which accumulated Zfh1 at similar levels to

CySCs (Fig 2.6B, arrowheads). A subset of the FasIII^{pos}Zfh1^{hi} cells incorporated Edu (Fig

2.6C-C''' arrow; n=23 gonads). Spatially, these aberrantly cycling cells were located

either among the outer cells of the endogenous hub or among the ectopic hub cells, but

were never internal among the endogenous hub. This demonstrates that the ectopic hub

cells in *tj* mutants exhibited some CySC behavior.

We next tested if other aspects of hub cell fate differed between the endogenous

and ectopic hub cells. Hedgehog secreted from the hub activates signaling within the

CySC lineage. This is visualized by punctate accumulation of the receptor Patched (Ptc)

representing trafficking intermediates [40,41]. In late L1 controls, Ptc is enriched around the

hub (Fig 2.7A, asterisk) and the first two tiers of Zfh1^{pos} CySCs (Fig 2.7A, arrows). In *tj*

mutant gonads, Ptc was detected within the endogenous hub (Fig 6B asterisk) and

within CySCs adjacent to the endogenous hub. However, within the same *tj* mutant

gonad, Ptc staining was also observed around Zfh1^{pos} cells (Fig 2.7B arrow) several tiers

from the endogenous hub, yet adjacent to ectopic hub cells (Fig 2.7B arrowhead). We

also examined ligand expression using a LacZ enhancer trap for *hedgehog* (*hh*LacZ). As

in controls (Fig 2.7C), the endogenous hub cells in *tj* mutants coexpressed *hedgehog*

with *unpaired* (Fig 2.7D, asterisk); however, the ectopic *unpaired*-expressing cells did

not express *hedgehog* (Fig 2.7D, arrowheads) even though there was ectopic activation

of the pathway. Furthermore, L3 *tj* mutant gonads accumulated *hh*LacZ only within the

endogenous hub and not ectopic hub cells (data not shown). Perhaps the ectopic hub

cells expressed levels of *hh*LacZ below our limits of detection. Alternatively, since

signaling normally occurs in CySCs and not cyst cells, it is possible that the ectopic

CySCs recruited in *tj* mutant gonads are all competent to respond to Hedgehog secreted

from the endogenous hub.

Having identified two pools of hub cells in *tj* mutants, we sought to determine

whether ectopic hub cells maintained hub fate later in development. To this end, we

lineage-traced *unpaired*-expressing cells using GTRACE. This allowed us to examine

late developmental timepoints and distinguish between cells having historically

expressed *upd* (by GFP) in early development from those cells currently expressing *upd*

(by RFP) (Evans et al., 2009). In control and *tj* mutant L3 gonads, the endogenous hub

cells coexpressed RFP and GFP, as expected if cells were committed to hub fate from

early stages (Fig 2.8, asterisk). In addition, all *tj* mutant gonads contained cells removed

from the endogenous hub that were co-labeled with RFP and GFP (Fig 2.8; n=14). Thus

the majority of *unpaired*-expressing cells in *tj* mutants maintained expression from earlier

stages as expected if they were fully committed hub cells. However, we also detected a

novel population of cells initially specified as hub cells that no longer expressed *upd*

(GFP only), and cells that had adopted hub cell fate later during development (RFP only;

Fig 2.8, arrowheads and arrows, respectively). The finding that somatic cells in *tj* mutant

gonads can convert fates, further suggests that *tj* is required for restricting hub cell fate

to a select number of somatic cells.

Tj is downregulated by Notch activation to specify hub cells

Activation of Notch in a subset of SGPs is necessary, though not sufficient, for

the eventual adoption of hub cell fate[64]. Given that our data indicate a role for Tj in

restricting hub cell fate, we speculated that loss of Tj in SGPs might be due to Notch

activation in those cells. Consistent with this, we found that Tj was no longer

downregulated in anterior SGPs in *Notch* mutant gonads (n=7) (Fig 2.9A,B). Indeed, the

ratio of anterior to posterior Tj signal in late stage embryos was 1.0 ± .27 compared to .7

± .08 in controls (p-value = .015). Since the number of SGPs does not vary significantly

between *Notch* mutant gonads and controls [64,66], we conclude that Notch is required for

Tj downregulation in SGPs. The regulation of Tj downstream of Notch could be direct or

indirect and might occur via interaction with the RTK pathway (see discussion, Fig2.10).

If Tj downregulation is a key effector of Notch signaling during hub cell

specification, then removing Tj in *Notch* mutants should restore hub fate. As reported

previously, very few SGPs accumulate FasIII in *Notch* mutant gonads (Fig 2.9B).

Additionally, only 33% of *Notch* mutant gonads exhibited any activation of the STAT-

34

GFP reporter, compared to 100% of controls (n=45 and 22, respectively; Fig 2.9E,F). Strikingly, when *tj* was also removed in *Notch* mutants, FasIII accumulation was restored in many SGPs and STAT was activated in 87% of doubly mutant gonads (n=31; Fig 2.9D). We conclude that Tj downregulation is a critical step in hub cell specification and this is achieved through Notch activation in a subset of anterior SGPs.

Note that gonads singly mutant for *tj* have two pools of hub cells: a compact, cobblestone grouping at the anterior, and a dispersed collection of ectopic cells. In tj mutants, the cells that assembled at the normal position (Fig2.9C, asterisk) were likely those that had experienced, Notch activation at earlier stages. If so, this suggests that Notch activation in the endogenous hub has an additional role in directing hub assembly. The *N;tj* doubly mutant gonads supported this idea since Fas3^{pos} cells failed to form a compact aggregate with a cobblestone morphology, and instead remained dispersed and intermingled with germ cells (Fig 2.9D). Thus, while removing *tj* bypassed some requirements for Notch in specifying several aspects of hub fate, it was not sufficient to bypass the requirement for hub assembly. Thus, Notch-dependent repression of Tj represents only one branch of the pathway from Notch activation to hub cell fate.

Activating Bowl rescues anterior assembly of ectopic hub cells in *tj* mutants

The lack of a compact, anterior hub in *N;tj* double mutants combined with the incomplete conversion of ectopic cells to hub cell fate in *tj* single mutants suggests that a second arm of the pathway downstream of Notch needs to be engaged for complete hub cell specification. In other tissues, the Odd-Skipped related factors, Drumstick and Bowl are known to be downstream of Notch activation [109–111]. Additionally, our lab previously reported that *bowl* mutant gonads contain fewer hub cells and that increased Bowl

activity in CySCs causes them to adopt partial hub cell fate[76]. Thus, we hypothesized that hub cell specification might also require activation of Bowl in parallel to Tj downregulation. If this were true, activating Bowl in the ectopic hub cells in *tj* mutants should allow them to convert more fully to hub cell fate. To test this, we activated Bowl by using *updgal4* to express Drumstick (Drm), a protein that binds and sequesters Lines, preventing its association with Bowl, therefore allowing Bowl to regulate transcription[112]. As expected, the hub was located at the anterior in control gonads as judged by FasIII and N-cadherin (an additional adhesion protein enriched between hub cells) (Fig 2.11A-A"). In *tj* mutant gonads, N-cadherin and FasIII were co-enriched on both endogenous (Fig 2.11B-B" asterisk) and ectopic hub cells (Fig 2.11B-B" arrows). Upon Bowl activation in *tj* mutants, we found a striking increase in the number of hub cells assembled at the anterior resulting in a larger endogenous hub (Fig 2.11C-C"). Qualitatively, Bowl-activated hub cells in *tj* mutants closely resembled endogenous hub cells, appearing more compact and less elongate than the ectopic hub cells of *tj* mutants, and adopting a more cuboidal morphology. We quantified the rescue by counting anteriorly-associated, N-cadherin-enriched hub cells. Control gonads exhibited 13.7±1.3 or 14.3±3.5 anterior hub cells (*upd>uas gfp*; *upd>uas drm,* respectively) and *tj* mutants averaged 15.5 ±4.1 cells. However, *tj* mutants with activated Bowl (*tj*; *upd> drm)* averaged significantly more anteriorly-assembled hub cells (19.5±4.4; p < .004). Additionally, 8 out of 21 *tj* mutants with activated Bowl had greater than 22 anterior hub cells compared to only 2 out of 21 *tj* single mutants (Fig 2.11D). We conclude that Bowl activation is sufficient for proper hub cell aggregation and assembly at the anterior and is likely a Notch target that functions in parallel to Tj downregulation.

Although we observed a rescue of hub assembly, we detected no change in the number of cells expressing *hh*lacZ (*tj; upd>drm* = 16 cells) compared to controls (*upd>drm* = 16.2, *upd>gfp* = 18, *tj* = 18). Lastly, Edu pulse labeling showed ectopic hub cells continued cycling with Bowl activation (data not shown) suggesting the existence of a Notch effector in addition to the two branches described here, or an unknown, parallel input to hub cell fate.

Discussion

Previous genetic and lineage tracing data demonstrated that a subset of SGPs is Notch-activated early during gonadogenesis [64]. Several hours later, these cells assemble into a compact niche that expresses various factors required for stem cell recruitment, attachment and self-renewal[6363](Fig 2.12). Here, we have dissected the pathway downstream of Notch activation in hub cell specification. Specifically, Notch activation downregulates Traffic jam thus relieving repression of *unpaired* and allowing for the accumulation of multiple adhesion proteins in these cells. In a parallel arm, Bowl is activated and regulates anterior assembly of hub cells. It is likely that *hedgehog* is also activated by this parallel arm (Fig 2.12). Therefore, our work is the first to look at the individual inputs into hub cell fate and uncovers a branched pathway downstream of Notch specification of niche fate.

Notch signaling downregulates traffic jam

Previously, the earliest effect of Notch activation detected in SGPs was the induction many hours later of hub-specific *unpaired* expression. Now we have demonstrated that Tj downregulation is visible prior to this time, and loss-of-function data suggests this is controlled by Notch. While some work suggests that Notch is activated

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in all SGPs[66], we observe relatively few cells induced for Notch[64]. This is consistent with down-regulation of Tj in the few hub cells that are specified. Furthermore, forced activation of Notch only moderately increases the number of hub cells[66] and these extra hub cells also exhibited reduced Tj accumulation (L.W. unpublished data). While that might suggest direct control of Tj by Notch, the situation is more complex. The fact that Notch activation is not sufficient for hub cell fate, coupled to the fact that a significant period exists between the requirement for Notch and a detectable reduction of Tj protein (approximately 6 hrs) suggests that Notch repression of Tj likely occurs through intermediate effectors. It was recently shown that robust Tj accumulation in early-stage SGPs requires the gene *midline* which encodes a T-box20 transcription factor[113]. In other tissues, Midline can antagonize Notch signaling and is repressed in Notch-activated cells[114]. Therefore, it would be interesting to determine whether *midline* is regulated by Notch in SGPs.

Notch also acts in specifying the female germline stem cell niche in Drosophila[61]. Since these niche cells also express *traffic jam*, it is worth investigating whether Traffic jam mediates Notch signaling during germarium niche specification. Furthermore, The regulatory relationship we in the male gonad between Notch and the Maf factor Tj might apply in mammals. Interestingly, both c-Maf and MafB are expressed in somatic cells intermingled with the germline in the developing mammalian gonad[115]. Additionally, Notch signaling restricts Leydig cell differentiation within the interstitial compartment at the same developmental timepoint [116]. Our data suggest that it would be reasonable to test whether Notch and Maf factors function together in specifying mammalian gonadal cell types.

Traffic jam represses cytokine signaling

We found that Tj depletion relieves repression of *unpaired* in SGPs allowing them to activate Jak/STAT thus bypassing one role for Notch. Recent work showed that Tj functions in border cell migration where it also modulates the Jak/STAT pathway. In that case, Tj enhances expression of the Jak/STAT pathway antagonist Suppressor of Cytokine Signaling E[117]. While we found that Tj functions by repressing expression of the ligand, both studies support a role in which Tj attenuates STAT signaling. Notably, in T helper cells, a large Maf factor also regulates expression of cell type-specific cytokines that activate STAT signaling[118,119]. In this system, c-Maf activates expression of the ligand rather than repressing it, however large Mafs can activate or repress transcription depending on context [120]. These examples highlight recurring evidence of cooperation between Maf factors and cytokine signaling.

Bowl mediates hub cell assembly

Proteins representing different adhesive complexes accumulate on hub cells, including FasIII, Ecadherin and Ncadherin[63,92]. However, depleting one or subsets of these factors has little effect on hub cell assembly or aggregation at the anterior (personal communications with M.Van Doren). This suggests either significant redundancy in hub cell adhesion or that a yet unidentified factor is responsible for mediating the proper aggregation and assembly of hub cells. In *tj* mutants, ectopic hub cells were enriched for multiple, different epithelial complexes (FasIII and E-cadherin or FasIII and N-cadherin), yet these cells did not exhibit compact, anterior assembly. This suggests a requirement for an unknown factor. Since activating Bowl was sufficient to significantly rescue hub cell assembly, perhaps Bowl regulates this factor. Bowl and Drumstick, both members of the Odd-skipped family of zinc finger proteins, regulate morphological changes in the developing *Drosophila* leg[111]. Overexpression of another

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family member, *Odd*, results in cell autonomous and non-autonomous morphological changes and increases in F-actin[111]. Therefore, examining changes in f-Actin enrichment and identifying actin regulators downstream of Bowl would reveal the mechanism for hub assembly.

We have not clarified the epistatic relationship between *bowl* and *Notch,* which can be complex. In leg development, *bowl* expression is induced by Notch signaling[109,111]. Furthermore, Bowl can repress *delta* in the Notch-activated cell, thus stabilizing a Notch signaling interface at leg segment boundaries [121]. In the wing, however, Bowl modulates Notch signaling by reducing availability of the Notch co-repressor Groucho[110]. In the gonad, mutants for either *Notch* or *bowl* have fewer hub cells, though the Notch phenotype is more severe[64,66,76]. Due to this and to the early requirement for Notch in SGPs, we suspect that Notch functions upstream of Bowl, forming a pathway parallel to Notch and Tj (Fig 2.12). Whether the Notch-Bowl arm results in *hedgehog* expression is unresolved. Similar to the complex relationship between Bowl and Notch, Hedgehog signaling can either promote Bowl accumulation (in the epidermis) or Bowl can induce *hedgehog* expression (in retinogenesis)[112,122]. While we did not detect *hh*-LacZ induction in gonads activated for Bowl, inviability restricted us from examining later times. Previously, we observed hhLacZ induction five days after clonal activation of Bowl in adult CySCs[76]. Given that CySCs are derived from SGPs, we favor the idea that Bowl can induce *hedgehog*.

Our model suggests that Tj and Bowl mediate many aspects of hub cell fate downstream of Notch signaling. As noted above, a fraction of hub cells are still specified in *bowl* mutants, and these appear to assemble anteriorly[76]. This could suggest that only a subset of hub cells require Bowl for proper assembly (perhaps centrally-located SGPs

that need to move anteriorly). Alternatively, there could be an additional, unknown

Notch effector that can compensate for *bowl*. Indeed, there is room for some complexity

as RTK signaling represses hub cell fate in posterior SGPs and its interplay with the

Notch pathway is yet unclear. In this regard, it is intriguing that *tj* mutant SGPs with

forced activation of Bowl continue to cycle, while endogenous hub cells are quiescent

(Fig 2.6D and data not shown). Interestingly, we detected EGFR pathway activity in

some ectopic hub cells (Fig 2.10D). EGF signaling mediates the proliferation of

Drosophila intestinal and gastric stem cells[23,48–50,123]. Perhaps the ectopic hub cells in *tj*

mutants remain cycling due to an inability to repress EGF signaling. With the pathway for

hub cell specification now more clearly delineated (Fig 2.12), future work can address

the intersecting cell biological and gene expression targets of multiple pathways required

for hub cell fate.

Acknowledgements
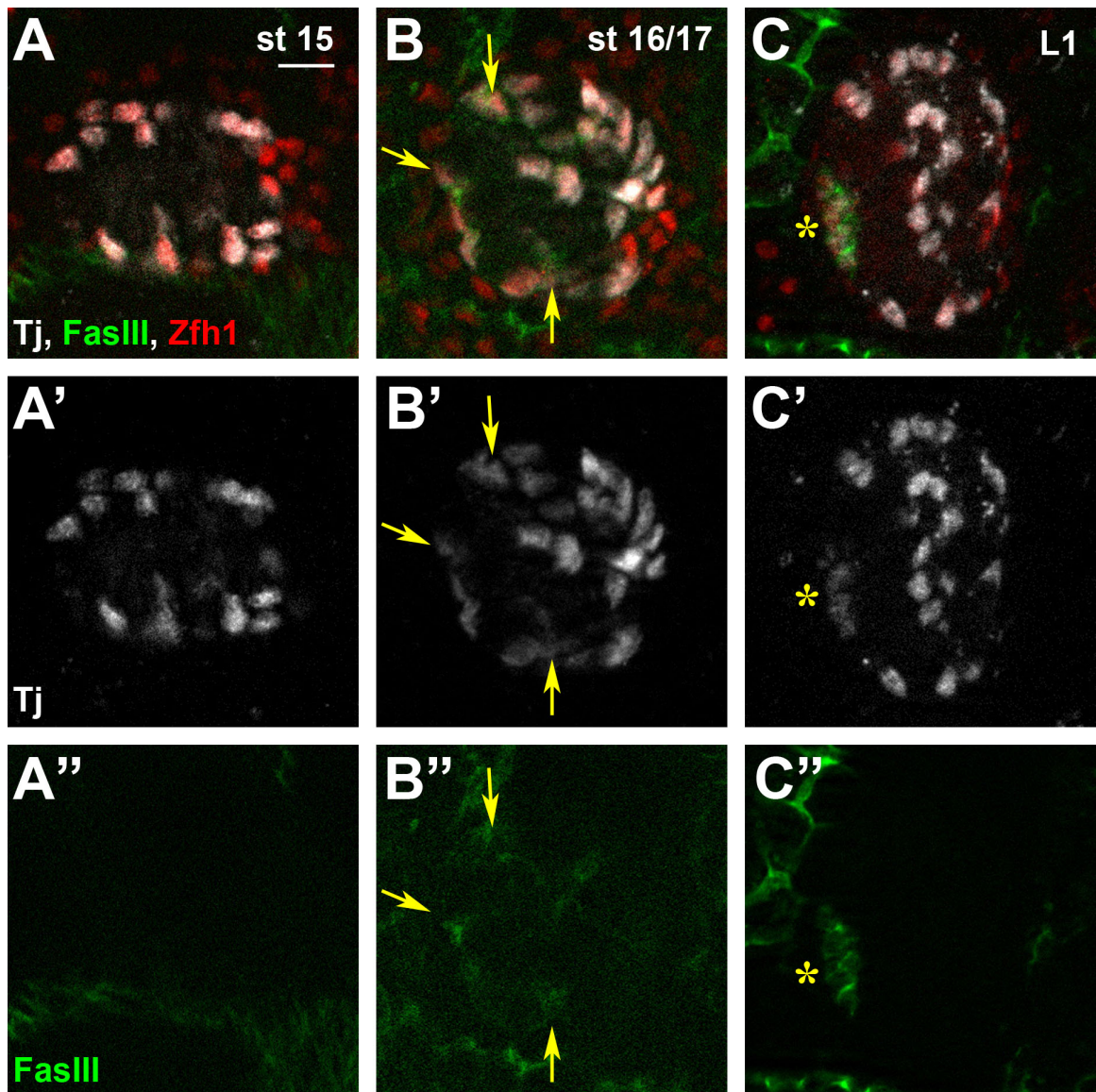
Figure 2.1: Tj is downregulated in hub cells during niche formation. Anterior is left in all panels. Gonads stained with anti-Zfh1 (red) to mark somatic cells, anti-Tj (white) and anti-FasIII (green). Male-specific SGPs at posterior are Zfh1$^+$Tj$^-$. (A) In a stage 15 gonad, Tj accumulates in all SGPs (A') and FasIII is absent (A"). (B-B") At stage 16/17, Tj accumulation is lower in anterior SGPs (B' arrows) in which FasIII puncta are detected (B" arrows). (C-C") At L1, Tj accumulation is low in FasIII$^+$ hub cells (asterisk) compared to FasIII$^-$ SGPs. Scale bar in A for all panels: 10 μm.
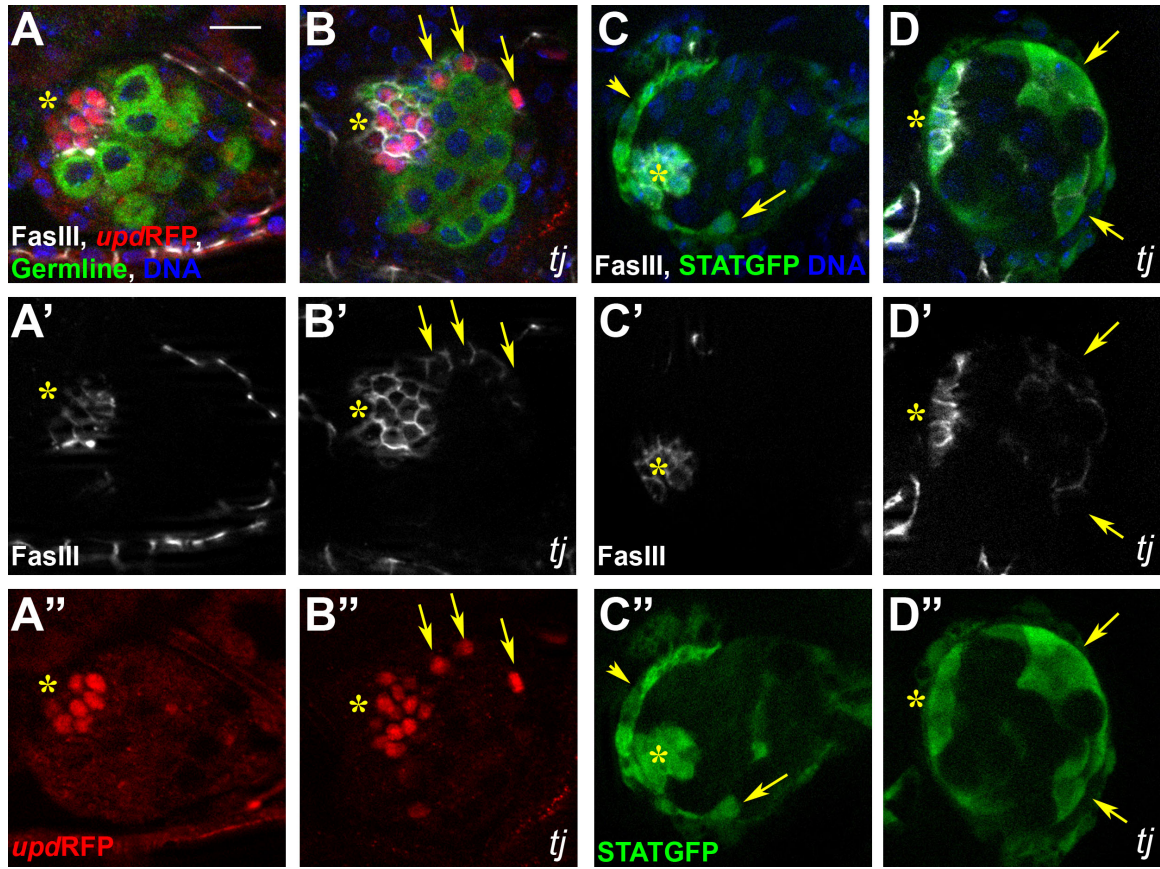
Figure 2.2: *tj* mutant gonads contain an endogenous hub and supernumerary hub cells. (A,B) Control (A) and *tj* (B) L1 gonads stained with anti-Vasa (green) to mark the germline, anti-FasIII (white) and anti-RFP (*upd*RFP). (A-A") Control gonad with FasIII (A') and RFP (A") accumulating only within an anterior cluster. (B-B") In addition to the endogenous hub (B, asterisk), a *tj* mutant gonad contains ectopic accumulation of FasIII (B') and RFP (B" arrows). (C,D) Control (C) and *tj* (D) L1 gonads stained with anti-FasIII (white) and anti-GFP (STAT-GFP). (C-C") In a control gonad, GFP is detected in FasIII$^+$ hub cells (asterisk), somatic cells near the hub (arrow) and the gonadal sheath (arrowhead). (D-D") In a *tj* mutant gonad, GFP is detected within the endogenous hub (asterisk) and also within the ectopic Fas III$^+$ hub cells (arrows). Similar results were obtained using a homozygous combination of *tj*^{e02} and a heteroallelic combination of *tj*^{e02} and *tj*^{Z4537}.
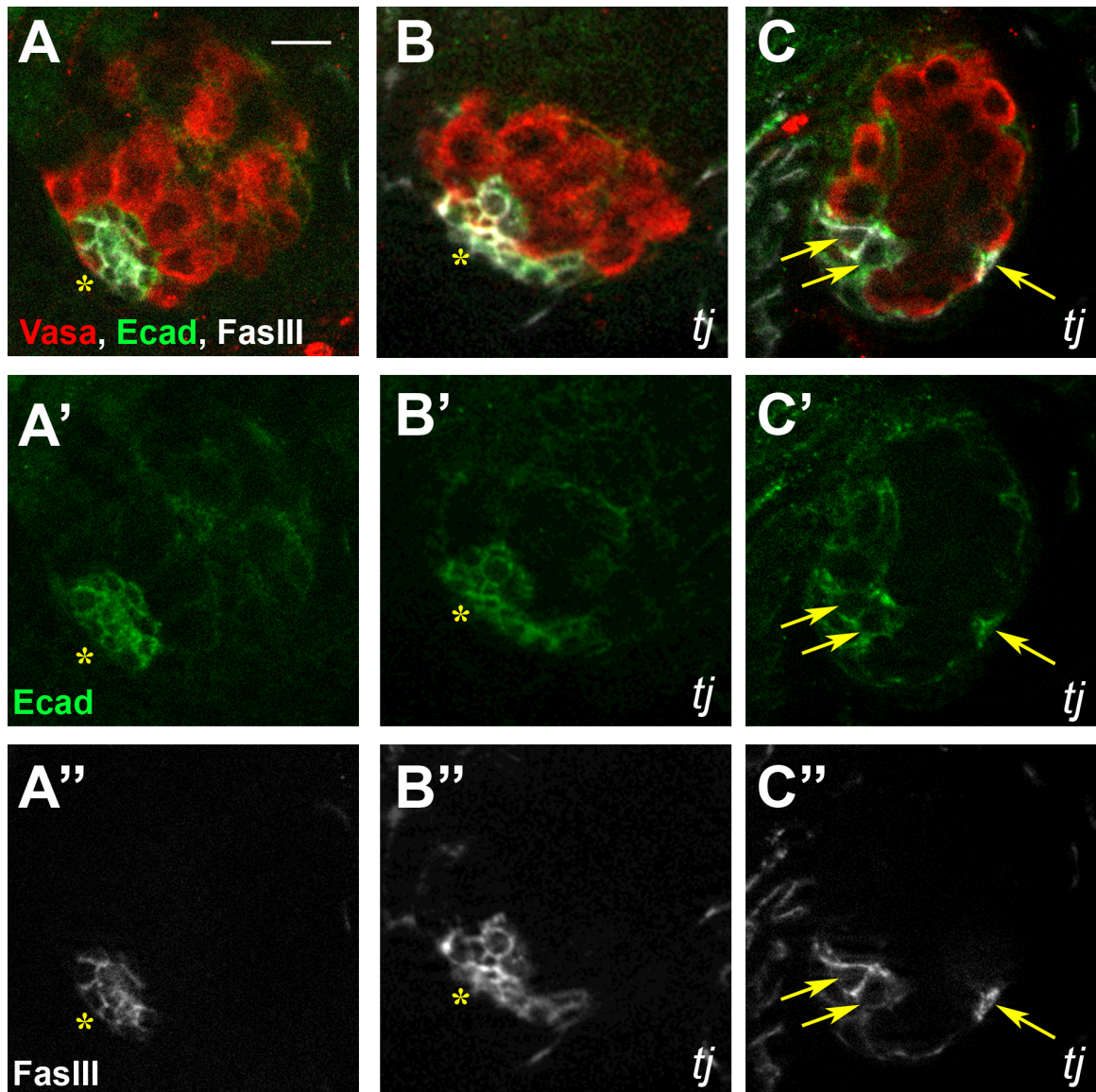
Figure 2.3: Proteins that function in epithelial adhesive complexes are enriched around *tj* mutant SGPs. L1 control (A) and *tj* (B-C) mutant gonads stained for anti-Vasa to mark the germline, anti-E-cadherin (green) as a marker for adherens junctions and anti-FasIII (white) as a marker for septate junctions. (A-A") Control gonad showing E-cadherin enriched around the anterior aggregate of hub cells and accumulating to a lesser extent around other somatic cells of the gonad (A'). FasIII accumulated only around the hub cells in the control (A"). (B-B") *tj* mutant gonad containing an anteriorly-localized endogenous hub enriched for FasIII and E-cadherin. (C-C") In a different focal plane, somatic cells outside of the hub are enriched for both FasIII and E-cadherin in *tj* mutant.
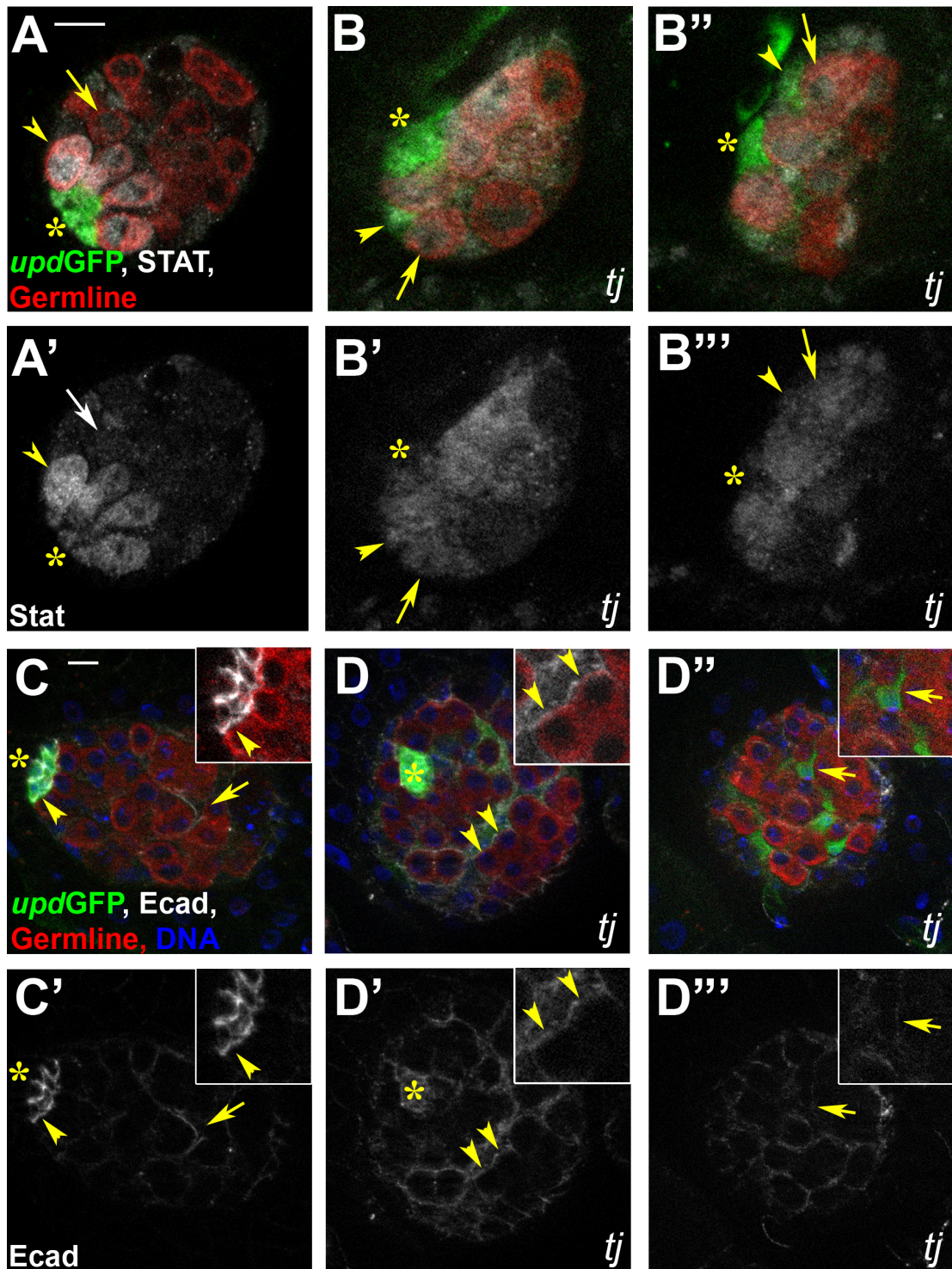
Figure 2.4: Germ cells adjacent to ectopic hub cells accumulate STAT and E-cadherin. (A-B) Control (A) and *tj* (B) late L1 gonads stained with anti-Vasa (red), anti-

GFP (*upd*GFP) and anti-STAT (white). (A,A') In a control gonad, STAT accumulates within GSCs (arrowhead) attached to the hub (asterisk) but not gonialblasts (arrow). (B-B''') In a *tj* mutant gonad, STAT accumulates in germ cells (arrows) adjacent to ectopic hub cells (arrowheads) that are not contacting the endogenous hub (asterisk). (C-D) Control (C) and *tj* (D) late L1 gonads stained with anti-Vasa (red), anti-GFP (*upd*GFP) and anti-E-cadherin (white). (C,C') Control gonad showing E-cadherin enrichment between GSCs and hub cells (arrowhead, inset) and lesser enrichment around a differentiating germline cyst distal to the hub (arrow). (D-D') A *tj* mutant gonad with Ecadherin enriched between GSCs and endogenous hub cells (asterisk) and ectopic hub cells (arrows, inset). (D'',D''') In a different *tj* mutant gonad, a germ cell exhibits little E-cadherin at the interface with an ectopic hub cell (arrow).
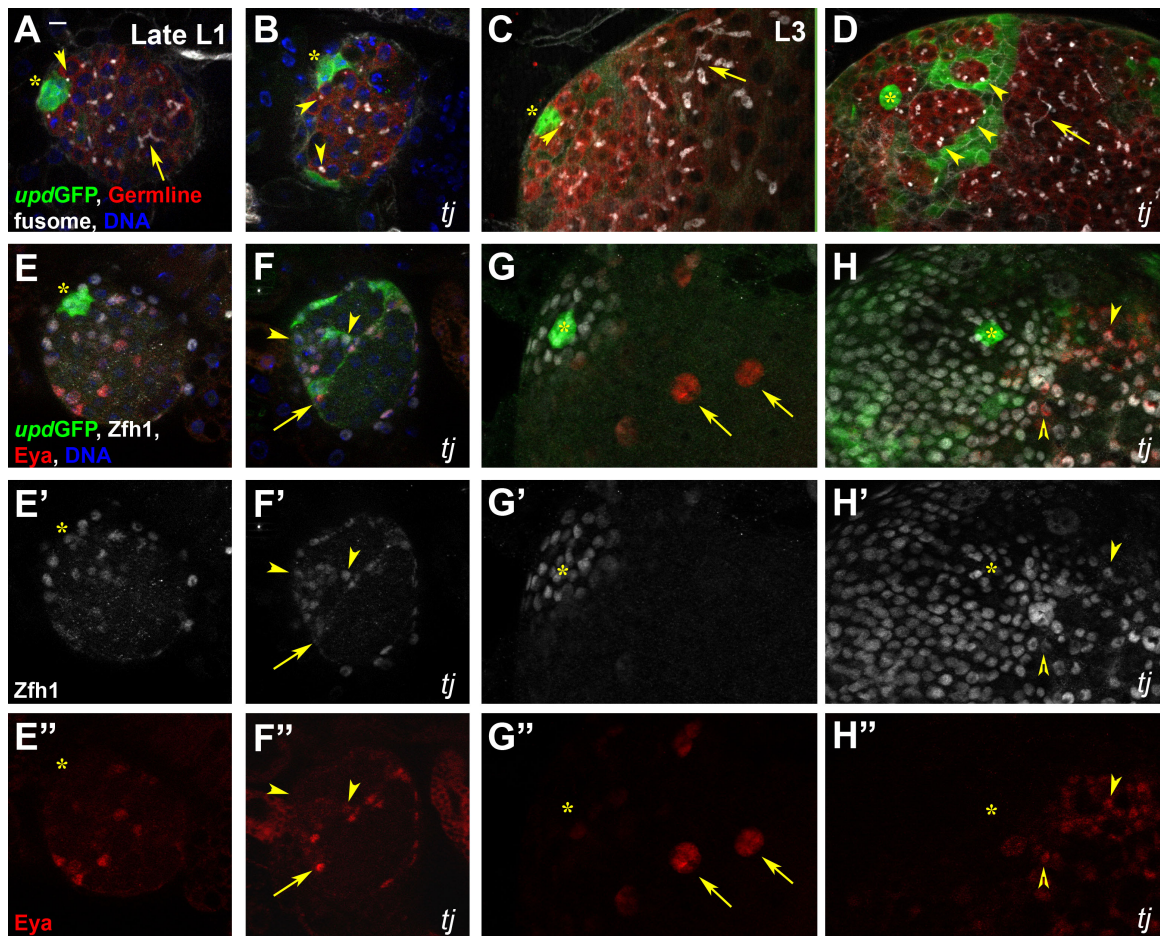
Figure 2.5: Ectopic hub cells maintain germline and cyst stem cells (A-D) Control (A,C) and *tj* (B,D) gonads stained with anti-Vasa (red), anti-GFP (*upd*GFP), and anti-α-spectrin (white) to mark fusomes. L1 (A) and L3 (C) control exhibit dot fusome-containing germ cells (arrowheads) only around the hub (asterisk) while branched fusomes are observed several tiers away (arrows). (B) L1 *tj* mutant gonad exhibits only dot or dumbbell shaped fusomes (B, arrowheads). (D) In an L3 *tj* mutant gonad, dot fusome-containing germ cells (arrowheads) are found several tiers from the endogenous hub (asterisk) yet adjacent to ectopic hub cells (*upd*GFP). Branched fusomes are observed distal to endogenous and ectopic hub cells (arrow). (E-H) Control (E,G) and *tj*

(F,H) gonads stained with anti-GFP (*upd*GFP), anti-Zfh1 (white) and anti-Eya (red). In L1 (E) and L3 (G) control gonads, Zfh1⁺Eya– nuclei are found closest to the hub (asterisk), while Eya accumulates distally. Peripheral Zfh1$^+$ nuclei mark the gonadal sheath. Fully differentiated cyst cells with large Eya$^+$Zfh1$^-$ nuclei are detected farthest from the hub (G, arrows). In L1 (F) and L3 (H) *tj* mutant gonads, Zfh1$^+$Eya$^-$ nuclei (F arrowheads) are found several cell diameters from the endogenous hub (F, not in focal plane, H, asterisk). Eya$^+$ nuclei are also found adjacent to ectopic hub cells in L1 (F, arrow) but farther from hub cells in L3 (H, arrowheads) Fully differentiated cyst cells that accumulate Eya only are not detected.
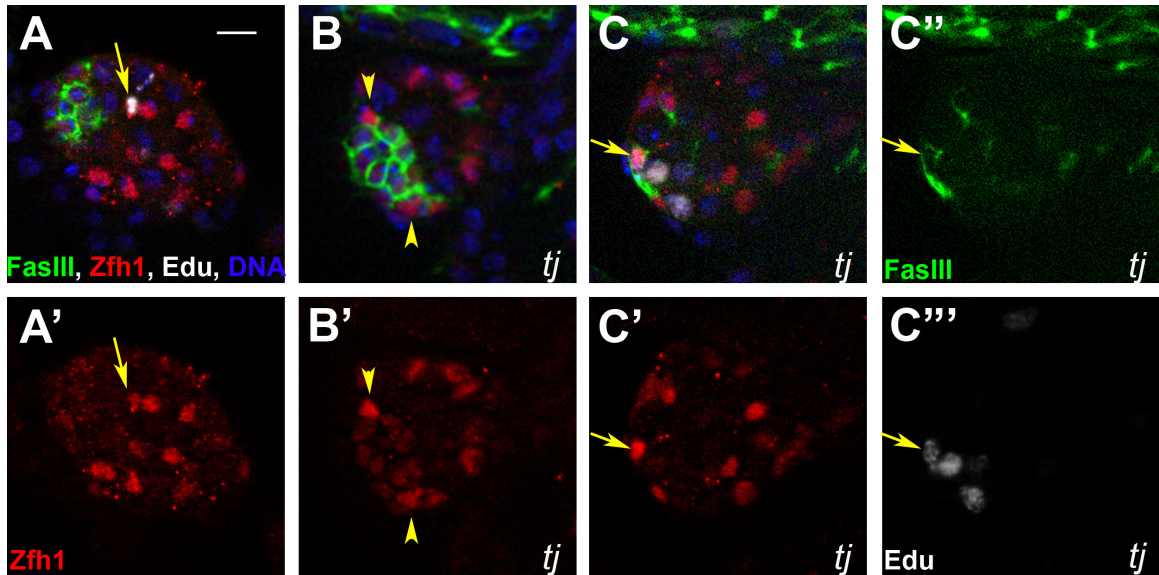
Figure 2.6: Ectopic hub cells in *tj* mutants accumulate Zfh1 and cycle. Late L1 control (A) and *tj* mutant (B-C) gonads stained with anti-FasIII (green), anti-Zfh1 (red) and EdU (white). (A,A') In a control gonad, Zfh1 is low in FasIII^{pos} hub cells but accumulates in nearby CySCs, one of which incorporates EdU (arrow). (B,B') *tj* mutant gonad containing Zfh1^{hi}FasIII$^+$ hub cells (arrowheads). (C-C") In a different focal plane within the same gonad, a Zfh1^{hi} FasIII$^+$ (arrow) incorporates EdU.
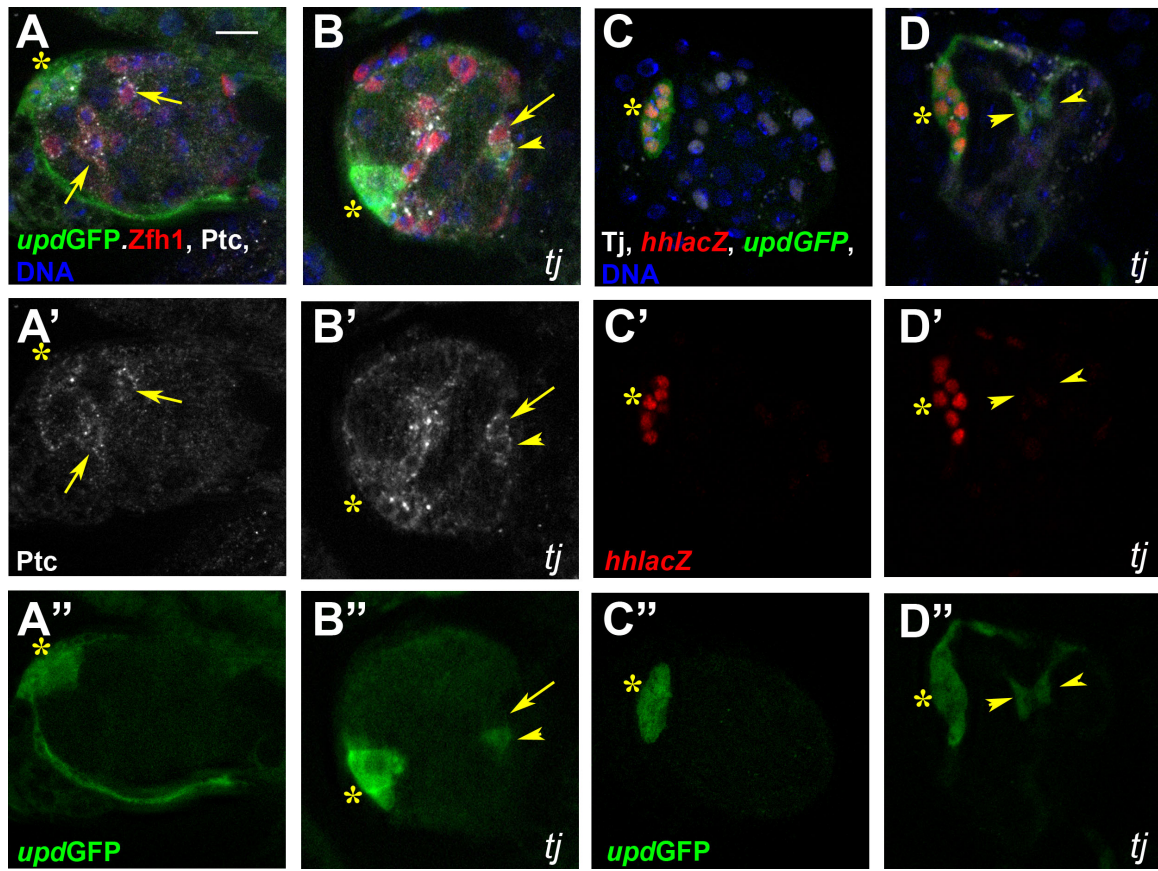
Figure 2.7: Hedgehog signaling range is broader in *tj* mutants. (A-B) Control (A) and *tj* mutant (B) gonads stained with anti-Zfh1 (red), anti-GFP (*upd*GFP), and anti-Patched (Ptc) (white). (A-A") In a control gonad, Ptc is only detected on hub cells (asterisk) and Zfh1^{+} cells near the hub (arrows). (B-B") In a *tj* mutant gonad, Ptc accumulates within the endogenous hub (asterisk) and adjacent somatic cells but also on an ectopic hub cell (arrowhead) and an adjacent somatic cell (arrow) several tiers from the endogenous hub. (C,D) L1 control (C) and *tj* mutant (D) gonads stained with anti-Tj (white), anti-GFP (*upd*GFP) and anti-*lacZ* (*hhlacZ*). (C-C") Control gonad showing *lacZ* co-expressed with GFP in hub cells. (D-D") In a *tj* mutant gonad, *lacZ* is co-expressed with GFP in endogenous hub (asterisk) but is absent in ectopic GFP^{+} cells (arrowheads). *tj*[e02] allele encodes for truncated Tj protein resulting in cytoplasmic staining.
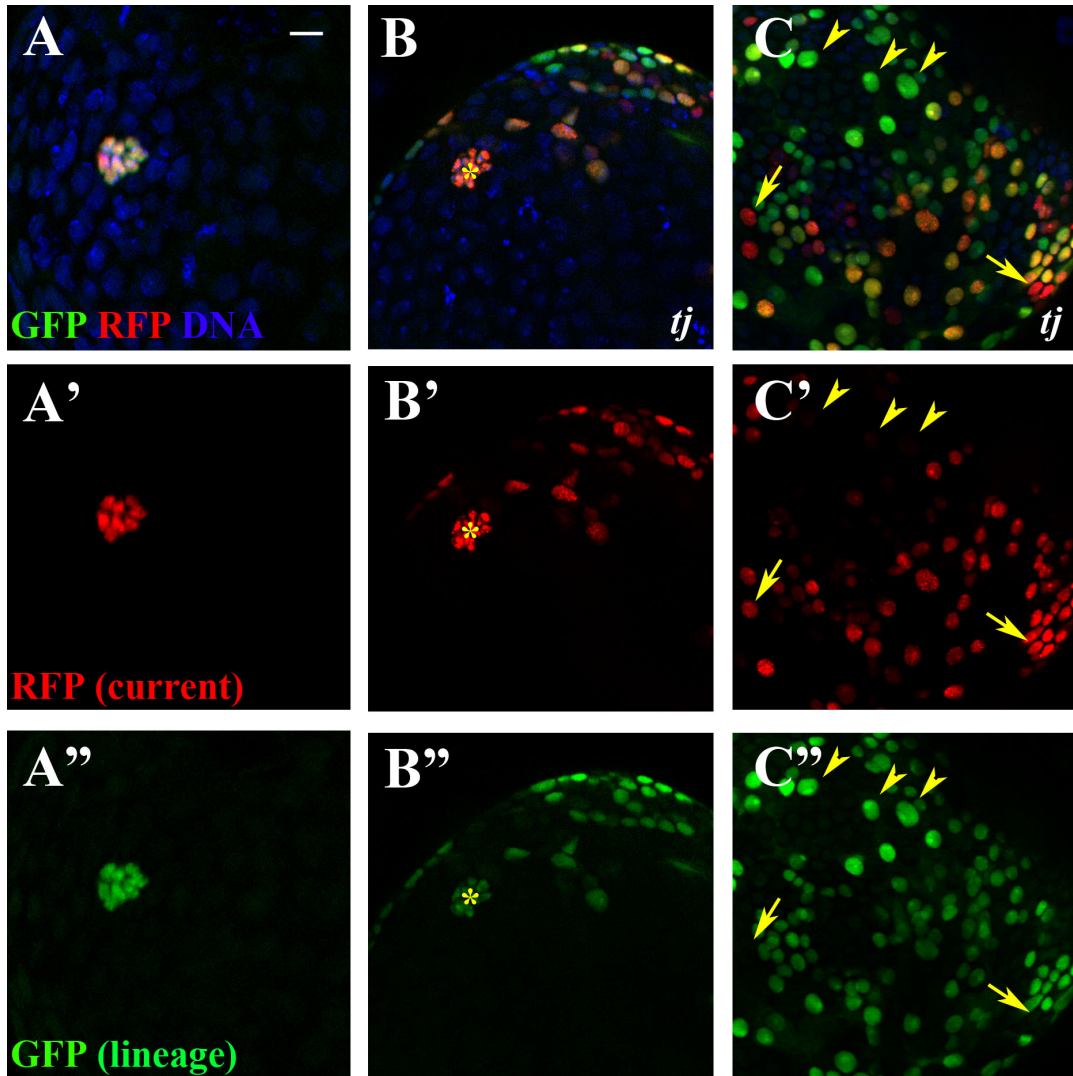
Figure 2.8: Lineage tracing reveals fully committed and uncommitted hub cells during early development of *tj* mutant gonads. GTRACE in control (A) and *tj* (B-C) L3 gonads stained with anti-RFP marking cells currently expressing *unpaired* and anti-GFP marking cells with historic expression of *unpaired*. (A-A") Control gonad with hub cells co-labeled with RFP (A') and GFP (A"). (B-B") tj mutant gonad containing an endogenous hub co-labeled with RFP and GFP (asterisk). (C-C") *tj* mutant showing singly labeled RFP (arrows, C') and singly labeled GFP (arrowheads C") nuclei in addition to co-labeled nuclei outside of the endogenous hub. A fraction of control gonads contained GFP^pos RFP^neg CySCs and cyst cells (data not shown). Previous clonal analysis and a decrease in hub cell number during early development, suggest that a hub cell may leave the niche just after niche establishment (L.W. and S.D., unpublished observations). If the cell adopted CySC fate for a short period its progeny might populate the cyst lineage with GFP^pos cells.
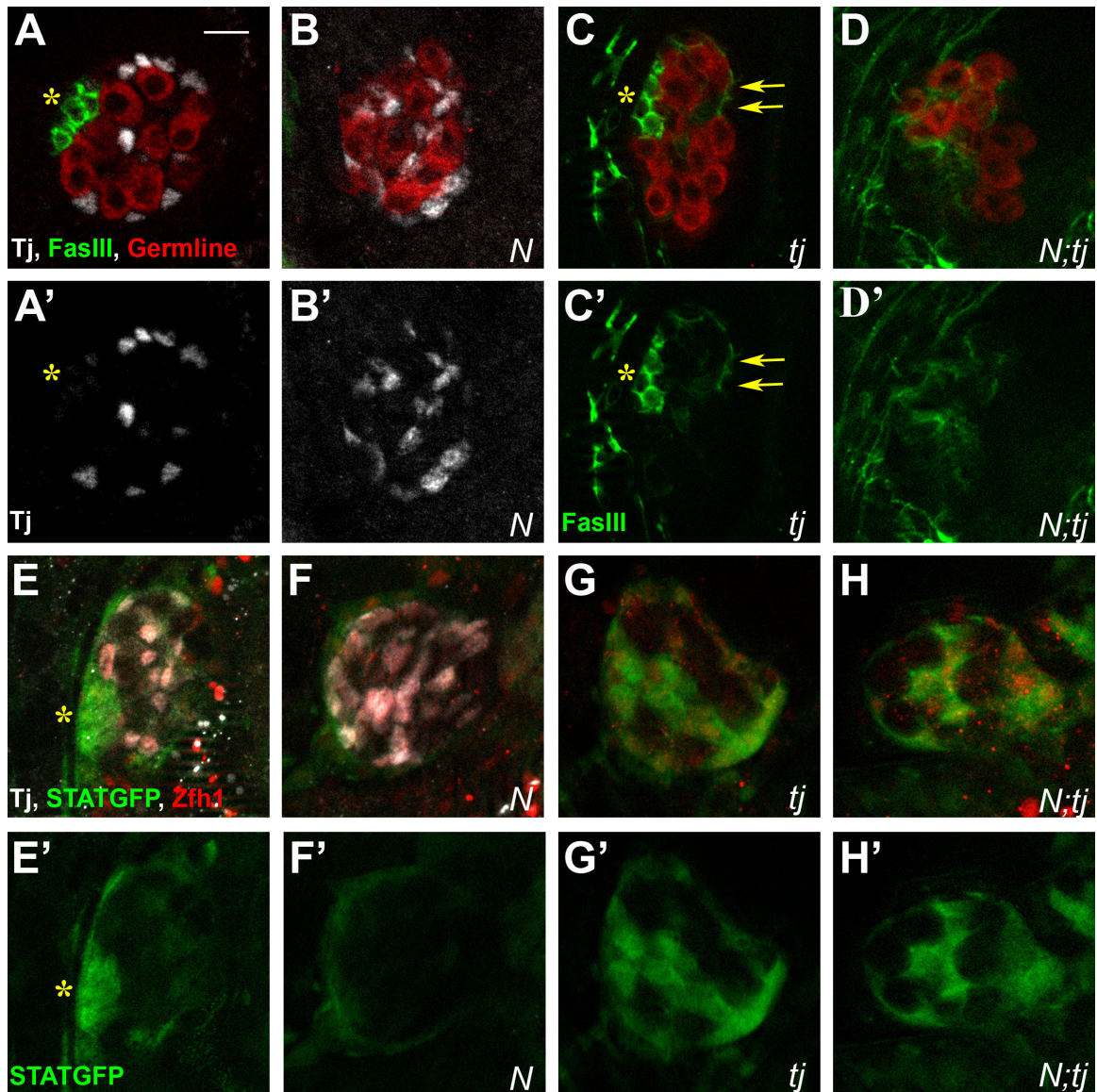
Figure 2.9: Tj functions downstream of Notch activation in a branched pathway to specify hub cells. (A-D) Stage 17/L1control (A), *Notch* (B), *tj* (C) and *N;tj* (D) mutant gonads stained with anti-Vasa (red), anti-FasIII (green) and anti-Tj (white). (A,A') Control gonad containing Fas III⁺Tj^low hub cells (asterisk). (B,B') *Notch* mutant gonad accumulates Tj in all SGPs and lacks FasIII enrichment. (C,C') *tj* mutant gonad contains an endogenous FasIII-enriched hub (asterisk) and ectopic FasIII-enriched cells (arrows). (D,D') In a *N;tj* mutant gonad, SGPs accumulate FasIII but fail to organize as an anterior, compact aggregate. (E-H) Stage 17/L1control (E), *Notch* (F), *tj* (G) and *N;tj* (H) mutant gonads stained with anti-Zfh1 (red), anti-GFP (STAT-GFP) and anti-Tj (white). (E,E') In a control gonad, GFP is detected within the hub (asterisk) and gonadal sheath. (F,F') In a *Notch* mutant gonad, GFP is detected only within the sheath. (G-H') In *tj* (G) and *N; tj* (H) mutant gonads, GFP is detected in most somatic cells (G'-H').
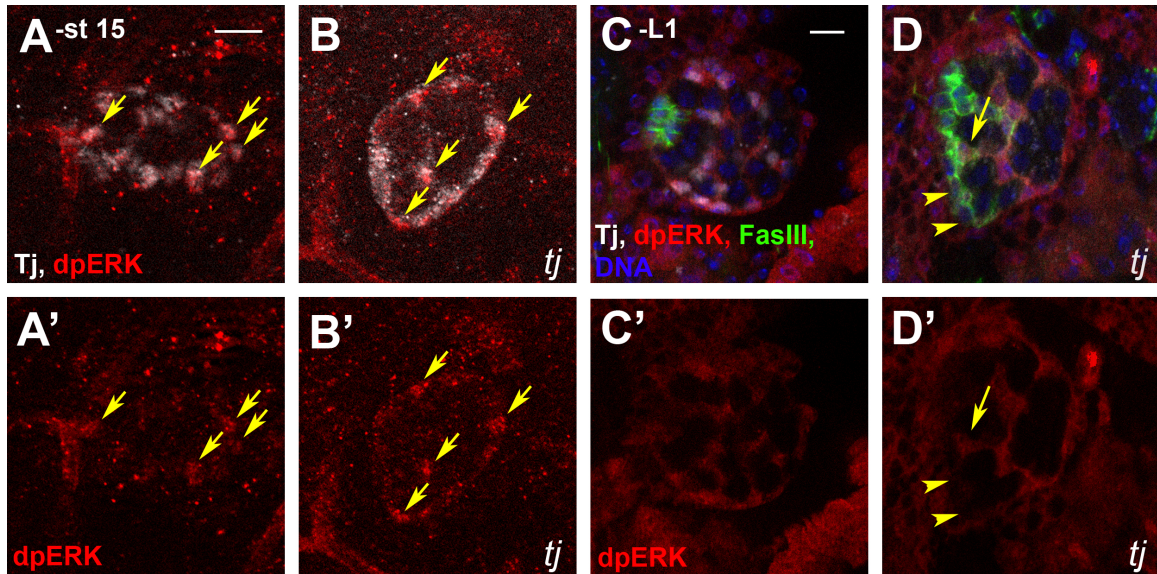
Figure 2.10: dpERK accumulates in SGPs prior to and following niche formation.(A,B) dpERK (red) accumulates in SGPs marked by Tj (white) in control (A) and *tj* (B) mutant gonads in stage 15 embryos. (C,D) dpERK accumulates in somatic cells marked by Tj in control (C) and *tj* (D) gonads following niche formation in L1. Hub cells are marked by Fas III (green). Note that in the *tj* mutant gonad, a few hub cells lack dpERK (arrowheads D'), while an ectopic hub cell (arrow, D') accumulates dpERK.

We found that RTK signaling in tj mutant gonads was normal as seen by diphospho-ERK (dpERK) accumulation in SGPs. We also considered whether RTK signaling which acts in posterior SGPs to repress hub cell fate might function to induce *tj* among posterior SGPs. *tj* is still expressed in SGPs in the absence of germ cells, which are the source of RTK ligand[65,66,90]. However, it is still possible that RTK signaling is required for robust *tj* expression.

Zfh1, FasIII, Ncad

FasIII

Ncad

tj

tj; upd>drm

D
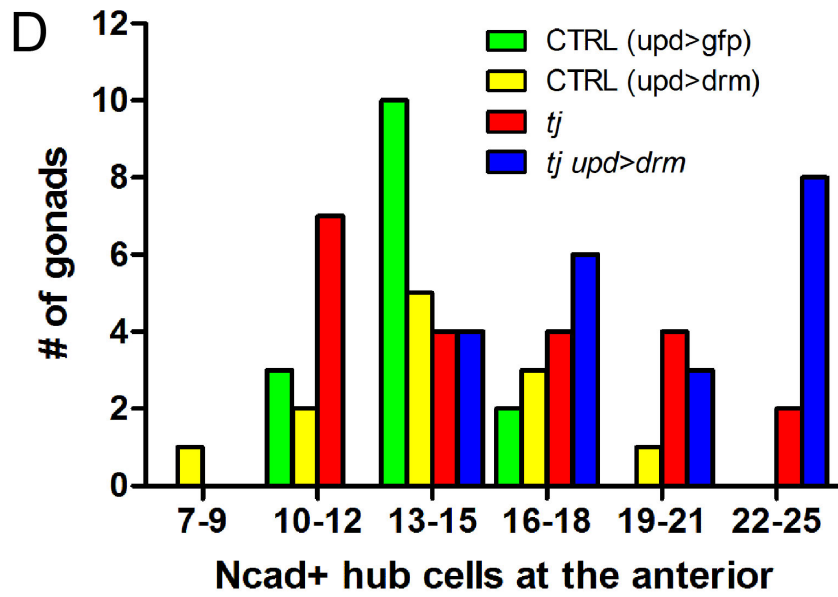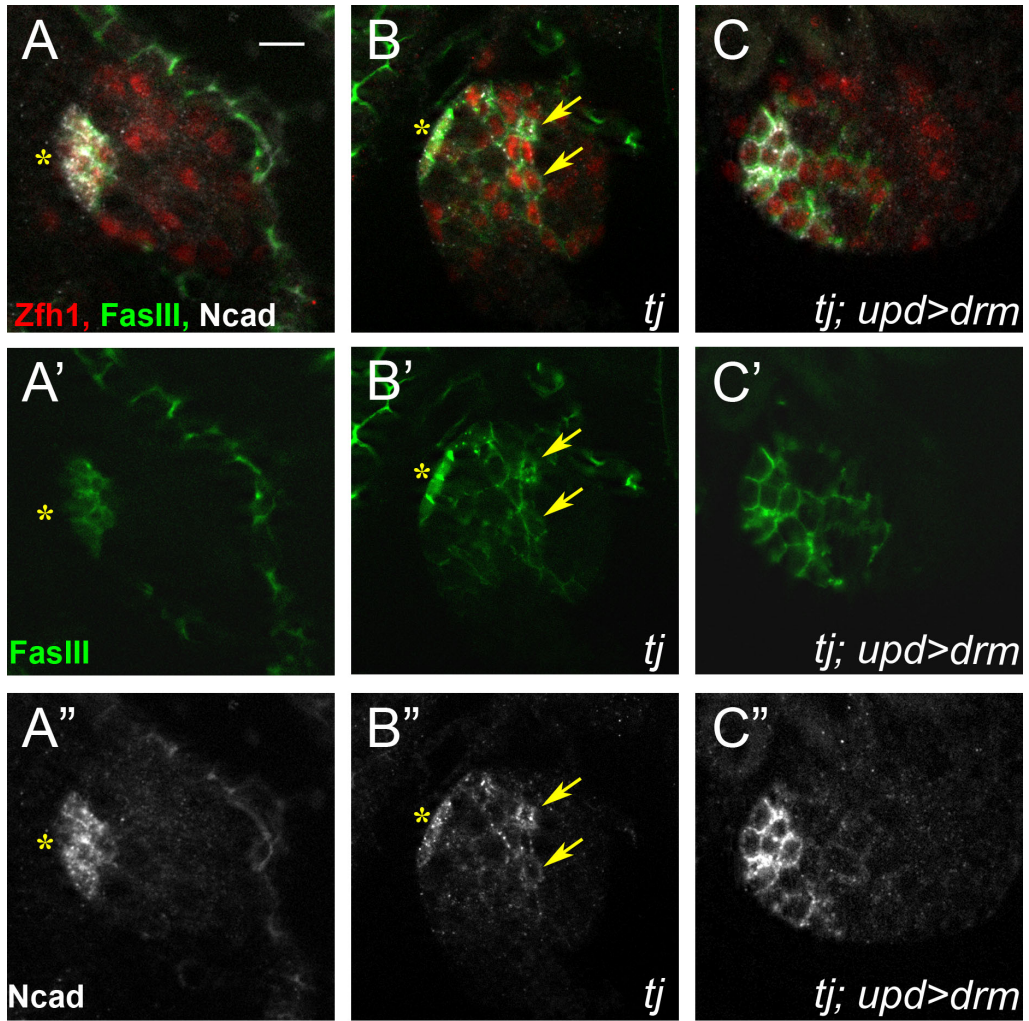
Figure 2.11: Bowl activation rescues anterior assembly of *tj* mutant hub cells.(A-C) L1 control (*upd>drm*) (A), *tj* (B) and *tj; upd>drm (*to activate Bowl) (C) gonads stained with anti-Zfh1 (red), anti-FasIII (green) and anti-Ncadherin (white). (A-A") Control gonad contains an anterior aggregate of hub cells that accumulate FasIII and Ncad (asterisk). (B-B") A *tj* mutant gonad exhibits FasIII and N-cadherin accumulation around the endogenous, anterior hub (asterisk) and ectopic, dispersed hub cells (arrows). (C-C") A *tj* mutant gonad with activated Bowl contains a larger anterior hub enriched for FasIII and N-cadherin with cobblestone morphology. D) Histogram quantifying anteriorly-assembled, N-cad$^+$ hub cells.
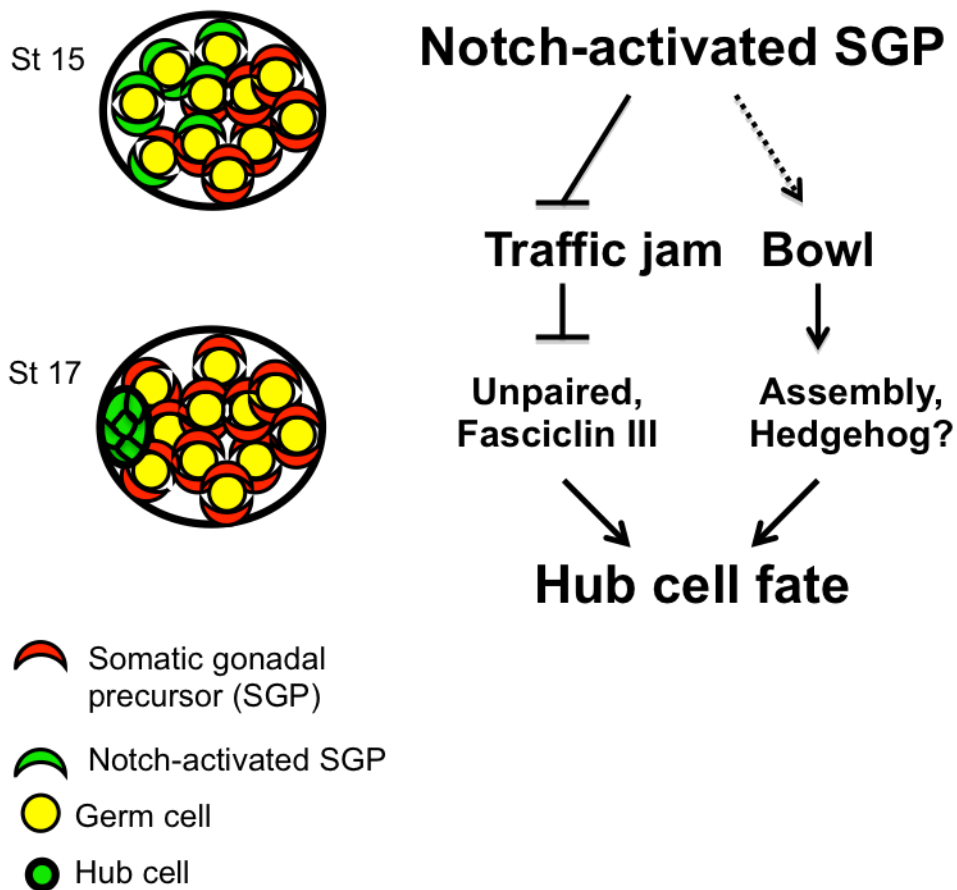
Figure 2.12: Model of branched pathway of hub cell differentiation downstream of Notch activation. During hub cell specification, Notch-activated SGPs (green) downregulate Tj (red) to relieve repression of *unpaired* and *fasciclinIII*. Along a parallel arm, Bowl activation mediates proper assembly and aggregation of hub cells at the anterior possibly through regulation of F-actin. Based on our data in the adult testis, Hedgehog is placed downstream of Bowl activation.

CHAPTER 3:

Live imaging reveals mechanisms for hub cell assembly and compaction during niche morphogenesis

Summary

Adult stem cells are maintained by a local microenvironment termed the niche. Cells of the niche direct self-renewal of resident stem cells and are therefore crucial for both normal homeostasis and tissue regeneration. In many mammalian tissues, niche cells cannot be unambiguously identified and are thus difficult to observe *in vivo*. Fortunately, the *Drosophila* germline stem cell (GSC) niche is well defined, allowing for unambiguous identification of both niche cells and resident stem cells. The testis niche is composed of a small aggregate of cells, called hub cells, which support two stem cell populations, the GSCs and Cyst stem cells (CySCs). During gonadogenesis, hub cells are specified among a larger pool of somatic gonadal precursors by selective activation for Notch. Following specification, hub cells assemble at a defined position within the gonad, become enriched for homotypic adhesions and secrete factors for attachment and self-renewal that serve to organize a rosette of stem cells around the hub. We sought to investigate the mechanisms of niche formation by live imaging and we have identified two stages of hub morphogenesis 1) a sorting and guidance, in which hub cells migrate individually along a basement membrane to assemble at the anterior and 2) a compaction phase in which hub cells reduce their area and to achieve final niche arthitecture. Furthermore, we have identified two tissues as candidates for directing hub cell migration as each tissue adjoins the gonad precisely where the hub assembles. Finally, hub compaction occurs concurrently with a burst in germ cell divisions and the formation of a Myosin II (MyoII) purse string at the hub-germ cell interface. We propose a model in which the MyoII purse string is generated by GSC divisions orthogonal to the hub, thus allowing GSCs to shape their niche.

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Introduction

Stem cell niches function in many adult organs to direct the self-renewal of tissue-specific stem cells[10]. For this reason, the proper specification and development of the niche is critical for adult organ function and regeneration. In mammalian stem cell niches, markers for the stem cells and niche cells are still being identified[4,15,103]. Additionally, these niches are often reorganized during development. This can be due to the introduction of new cell types in adulthood, as is seen in the neurogenic niche, or due to the migration of the stem cells to colonize a new niche as is seen with hematopoietic stem cells[54,59]. This added complexity makes it difficult to parse out the signaling pathways and cell types required for niche development. Furthermore, it is virtually impossible to investigate these processes live in mammals. Instead, our lab uses the *Drosophila* male gonad, the precursor to the testis, as a model to study stem cell niche specification and development. This is because the niche cells and stem cells can be unambiguously identified with well-characterized markers. Also, both the niche cells and stem cells are specified and organized within the gonad during development and are not remodeled during testis maturation. Therefore, the gonad provides an excellent system for studying the mechanisms required to assemble a functional stem cell niche.

The *Drosophila* testis houses the germline stem cell niche, which is composed of a tight aggregate of post-mitotic somatic cells called hub cells. The hub cells secrete signals that promote attachment and self-renewal in the resident stem cell populations[22,24,32]. Surrounding the hub in a radial array are the germline stem cells (GSCs) and cyst stem cells (CySCs). Both stem cell populations undergo asymmetric divisions producing one daughter cell that is directed to self-renew by the niche and one

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daughter cell that exits the niche, allowing it to differentiate. The hub also serves to anchor the stem cells through Integrin-extracellular matrix (ECM) interactions at the apical tip of the testis[94,97]. This promotes tissue polarity in the testis by forcing differentiating daughters away from the closed end and towards the seminal vesicles for sperm production. Importantly, tissue polarity and niche architecture are established within the embryonic gonad and are maintained during testis maturation[63].

The gonad forms from the coalescence of the Primordial germ cells (PGCs) and somatic gonadal precursors (SGPs), which are derived from a mesodermal subdomain in parasegments (PS) 10-12 [69](Fig3.1). In male gonads, an additional cluster of SGPs, so named male-specific SGPs, join at the posterior[124]. Following coalescence, the SGPs and PGCs migrate anteriorly, then arrest in abdominal segment 5 while morphing from an elongated structure to a spherical gonad (Fig 3.1). Previous data suggest that expression of the homeotic gene *abdominalA* (*abdA*) anterior to the gonad functions to arrest migration at abdominal segment 5[69]. Additionally, Ecadherin (Ecad) was demonstrated to function in spherical compaction of the gonad by mediating the interactions between the soma and germline[89]. Live imaging has also revealed roles for the transcription factor Six4 and the actin regulator Enabled in the coalescence and compaction of the gonadal soma[125,126].

While the previous studies characterized interactions between the soma and germline during *gonad* morphogenesis, much less is known about *niche* morphogenesis. Male gonads establish the GSC niche by the end of embryogenesis, while female gonads, do not specify and organize a niche until several days later (in the third larval instar)[61,63,77,78]. In fact, hub cells are specified quite early during male gonadogenesis when SGPs and PGCs coalescence. At this time, the adjacent endodermally-derived gut

activates Notch in a subset of SGPs, thus specifying them as hub cells[64] (Fig3.1) Although the hub cells are specified early, they do not upregulate most markers of differentiation until the end of gonadogenesis[63,64]. Indeed, hub cells can be identified only after they have formed a tight aggregate at the anterior of the gonad; only then can one visualize enrichment in the adhesion proteins Ecad and Fasciclin III (FasIII) and expression of the niche signal *unpaired* (*upd*), as well as a recruited a tier of GSCs[63,77]. Until now, the field has had to rely on end point analysis carried out in fixed tissues. Therefore, there is little data describing the morphological changes of hub cells as they go from being intermingled with the germline and non-hub SGPs to sorting together at the anterior[63].

In this study, we undertook a live imaging approach to investigate niche morphogenesis. We discovered that there are two phases of niche morphogenesis, 1) a sorting and guidance phase in which hub cells move anteriorly and assemble and 2) a subsequent compaction phase in which the assembled hub cells reduce their area, thus forming a smaller aggregate (not to be confused with Ecad-mediated compaction into a spherical gonad). In the sorting and guidance phase, the path of migration and placement of the assembled hub allowed us to identify candidate tissues for directing hub cells to the anterior. The compaction phase, which occurs after the onset of embryonic muscle contractions, cannot be imaged in embryos. Therefore, we developed a protocol for imaging cultured gonad explants in which we observed compaction of the previously assembled hub cells. The *ex vivo* experiments suggested a mechanism for compaction whereby germ cells contribute to shaping their own niche by generating an acto-myosin purse string around the hub.

Materials and Methods:

Fly stocks

Fly lines used were *six4*GFP (D. Finnegan), PrdGal4 (FBst0001947), *mys*[1] (FBst0000059), UAS tdTomato (FBst0036328), TypeIVCollagenGFP (Fly trap 110692), PerlecanGFP (Flytrap), *abdA*Gal4 (M. Samir), *tup*Gal4 (FBst0046960), *tup*AMEmoegfp (M. Frasch), *No*s-GFP-moe (R. Lehmann), *sqh*-mcherry (A. Martin), *tup*[1] FBst0036503, *tup*[ex4] (S. Campuzano). Heterozygous siblings were used as controls.

For *six4*Gal4, the third intron of Six4 was amplified from wildtype genomic DNA and cloned using Gateway recombination methods (Invitrogen) and cloned into a GAL4-vector. Transgenic flies were generated using standard P-element transposition.

Live embryo time-lapse imaging

Embryos were collected overnight on agar plates. The following morning, embryos were dechorionated using 50% bleach and stage 15 embryos were selected according to Campus and Hartenstein. Embryos were mounted with the dorso-lateral side towards the objective using tape adhesive dissolved in heptane and then covered with s700 halocarbon oil. Z-stacks were taken at 5-15 min intervals with 35 1µm z-slices through the gonad.

***Ex Vivo* culture and time-lapse imaging of gonads**

Embryos were collected for 1-2 hrs on agar plates and then aged at room temperature for 17 hrs (unless otherwise noted). Embryos were then dechorionated in 50% bleach, hand-devitellinized in Ringers solution and dissected using tungsten needles. Gonads were gently massaged out onto poly-lysine coated coverslip of a round

imaging dish. Ringers was then removed and imaging media (15% FBS, 0.5X penicillin/streptomycin, 0.2mg/mL insulin in Schneider's insect media) was added. For ROK inhibitor experiments, the imaging media additionally contained 380µM of Y-27632 (Sigma Aldrich; 129830-38-2) or 10µM of the H-1152 (Santa Cruz; sc-203592). Gonads and embryos were imaged on either an inverted Leica spinning disk confocal or an inverted Olympus IX71 spinning disk confocal overnight for up to 6hrs.

Immunostaining

For *tup* mutants, embryos were collected and aged 21-23 hours in a humidified chamber for late stage 17 embryos. Unhatched larvae were dechorionated, hand-devitellinized and dissected in Ringers and the internal organs gently massaged out as above. Tissue was fixed in 4% formaldehyde, Ringers and 0.1% Triton-X-100 for 20 minutes, washed in PBS plus Triton-X-100 and blocked for 1 hour in 4% normal serum. Primary antibodies were used overnight at 4°C or 4 hours room temperature. Secondary antibodies were used at 1:400 (Alexa488, Cy3 or Cy5; Molecular Probes; Jackson ImmunoResearch) for 1 hour at room temperature. DNA was stained with Hoechst 33342 (Sigma) at 0.2 µg/ml for 6 minutes. For gonads dissected and cultured *ex vivo*, immunostaining was performed as above within imaging dish.

We used rabbit antibodies against Vasa 1:5000, (R. Lehmann), RFP 1:500 (Abcam), mouse antibody against Fasciclin III 1:50 (DSHB) and Islet (*Drosophila* Tailup) 1:100 (DSHB); rat antibodies against DE-cadherin DCAD2 1: 20 (DSHB); guinea pig anti-Traffic jam 1:10,000 (D. Godt); and chick anti-GFP 1:1000 (Molecular Probes).

Image and Statistical Analysis

Time lapse images were analyzed, Z projections were generated, and hub cell area was measured using Fiji software. Student T-tests were used for all statistical comparisons. In each analysis, p values less than 0.05 were considered to be statistically significant.

Sex identification and Genotyping

Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985). Male embryos and larvae were identifiable owing to the larger size of the gonad. For other cases, sex was determined by robust *six4*GFP in male-specific SGPs. Fluorescent balancer chromosomes (P{w+ TM6 Hu ubi-GFP}(FBst0004533) or P{w+ CyO act-GFP} (FBst0004887)) distinguished heterozygous from homozygous mutant larvae.

Results

Hub cells are guided along the periphery during anterior assembly

We first wanted to determine whether we could visualize niche formation in embryonic gonads using timelapse imaging. A construct containing the *six4* enhancer fused to GFP was previously used to live image SGP coalescence and compaction into a spherical gonad[125,126]. Since there is no driver to specifically mark hub cells during imaging, and earlier studies were not carried out to late stages when the niche forms, we first sought to determine whether we could image through later stages, including niche formation, and identify a hub using the *six4*GFP construct (which marks all SGPs). Stage 15 embryos (approximately 12 hours after egg lay; 12h AEL) were selected and imaged at five-minute intervals until the onset of embryonic muscle contractions, which occur around stage 17 (17h AEL). At the beginning of imaging, SGP nuclei were dispersed and

intermingled with germ cells (Fig3.2A). However, towards the end of imaging, a subset of SGPs had assembled along the periphery at the anterior (Fig3.2B, outline). Furthermore, there was a tier of negative space between the anteriorly assembled SGPs and the remaining SGPs indicating that germ cells were being recruited to the anterior. Previous analysis of fixed gonads also described an anterior "cap" of E-cadherin-enriched cells marking the prospective hub (pro-hub) visible prior to other markers of hub cell fate[63]. In our imaging, embryonic muscle contractions precluded further imaging shortly after these nuclei assembled at the anterior. Although the hub in late stage dissected gonads appears smaller (Fig3.2C, outline), we speculate the early assembly of anterior cells represents a pro-hub based on their location and recruitment of germ cells.

While the niche forms at the anterior, it includes hub cells that are specified more centrally in the gonad. Previous lineage tracings have demonstrated that a fraction of SGPs derived from PS11 become hub cells suggesting that they must move anteriorly to assemble with PS10-derived hub cells[74,95]. To determine the path of their migration, we expressed cytoplasmic tdTomato in cells derived from odd-numbered parasegments (PS), while simultaneously marking all SGPs with nuclear GFP (*six4*GFP). At the beginning of imaging, tdTomato was visible in centrally-located PS11 SGPs and posterior PS13 male-specific SGPs. Tdtomato+ hub cells (derived from PS11) travelled short distances, less than 10 µm, in order to assemble with the anterior hub (Fig 3.3, arrows). Interestingly, anterior movement always occurred along the periphery of the gonad and never within the internal milieu. This was assessed by taking z-stacks through the entire gonad to determine the location of the cell relative to the gonad. Furthermore, PS11 hub cells could be identified encysting germ cells prior to their assembly at the anterior, confirming that hub cells encyst the germline as has been

described for SGPs. However, hub cells retract their cytoplasmic arms and adopt cobblestone morphology upon assembly at the anterior with other hub cells (Fig 3.3 A'-D', arrowheads).

The peripheral movement of hub cells as they assemble anteriorly suggested that they use a basement membrane (BM) as a substrate for migration rather than other gonadal cells. Therefore, we looked for the presence of BM around the gonad during hub assembly. It was previously demonstrated that the BM proteins Nidogenin and Laminin A were enriched around late stage embryonic gonads[94]. However, the timing and location of enrichment were not characterized. We used timelapse imaging of a GFP protein trap for Type IV-Collagen (ColGFP), along with *six4*GFP to determine when BM is deposited relative to hub cell assembly. At the onset of imaging, ColGFP was not visible (Fig3.4A-B). However, ColGFP was detected along the outside of the gonad at the same time as hub cells move peripherally and anteriorly, indicating it could act as substrate for assembly (Fig 3.4C-E). While accumulation at the anterior first might have suggested a spatial cue for assembly, ColGFP appeared to accumulate evenly around the gonad (Fig3.4D-E, red arrowhead). Similar results were obtained imaging Perlecan-GFP.

Previous data demonstrated that Integrin is required for anchoring the hub at the periphery[94]. However, these experiments were carried out using fixed samples and only analyzed the end point. Therefore, it is still unclear whether hub cells require an Integrin attachment to BM in order to respond to a cue to assemble at the anterior. Considering our finding that BM is deposited around the gonad at this time, we sought to distinguish between a role for Integrin-BM attachments in positioning the hub versus one simply for anchoring. Therefore, we imaged hub assembly using *six4*GFP in *myospheroid* (*mys*[1])

mutants, which lack the βPS subunit required for functional Integrin[127]. As expected from the previous end-point analysis, *mys* mutants, at the end of imaging, contained an aggregation of hub cells internalized within the gonad, rather than anchored at the periphery (Fig3.5F; compare to 3.2C). However, we were interested in determining whether the hub first assembled peripherally and dropped in or whether mutations in Integrin prevented anterior assembly altogether. Live imaging revealed that *mys* mutant gonads failed to form an assembled peripheral cap prior to internalization (Fig3.5F; compare to 3.2B). We did not detect any anterior movement of hub cells. Instead, centrally-located hub cells can be seen joining an already internalized hub (3.5D-F, arrowheads). This could indicate that Integrin is required for PS11 hub cells to migrate or that Integrin is required to transmit a cue to migrate. In addition, PS10 (anterior) hub cells are lost from the periphery (Fig3.5A-F arrows), sometimes in clusters, and continue to aggregate with other hub cells as they are internalized. These data are consistent with a role for Integrin in anchoring hub cells. PS11 hub cells could fail to move anteriorly because Integrin-mediated attachments are required for their migration. However, it is also possible that PS11 hub cells did not receive the cue for migration. *mys* mutant embryos also displayed variability in the position of the gonad within the embryo, likely due to defects in other tissues. Thus, if there is an extragonadal cue guiding hub assembly, it is reasonable that it is no longer nearby. A more precise test would be to knockdown Integrin specifically within SGPs but efficient knockdown of Integrin subunits or its cytoskeletal linker Talin cannot be achieved during gonadogenesis (data not shown)[94].

Hub cells are guided by extra-gonadal cues

Our time lapse imaging of hub assembly indicated that hub cells move peripherally and then towards the anterior where they assemble with additional hub cells. Furthermore, we discovered that hub cell assembly is biased towards the internal organs and the dorsal side of the embryo (data not shown). This bias suggested an extra-gonadal cue to direct hub assembly since random aggregation of hub cells should not result in consistent placement towards particular organs. Therefore, we reasoned that the tissues nearest to where the hub assembles would be good candidates for directing hub assembly. We searched various Gal4 lines (driving GFP or tdTomato) along with *six4*GFP (to mark the gonad) to identify tissues nearest the gonad. Bap3Gal4, expressed in the visceral mesoderm surrounding the gut and BreathlessGAL4, expressed in the dorsal trunk of the trachea confirmed that hub cell placement was biased towards these tissues, rather than towards the lateral body wall muscle or ventral mesodermal derivatives (Fig 3.6A; Bap3gal4 not shown). We were able to visualize all tissues in this segment of the embryo by expressing tdTomato under the homeotic gene *abdA* and were struck by a y-shaped structure just anterior to the gonad (Fig3.6B, outline). When expressing TdTomato with *six4*, we noted a similar structure consisting of a triangular group of cells (Fig3.6C, arrow) just anterior to the assembled hub (Fig 3.6C, outline). *six4* is expressed in broader domain of the mesoderm early during development and then becomes restricted to SGPs and a subset of muscles. Therefore, perdurance of *six4*GFP and expression of *six4*>tdTomato suggests that the cells found just anterior to where the hub assembles are mesodermal or muscle derivatives. Interestingly, there was a tendon-like structure (Fig 3.6C, arrowhead) extending dorsally from the triangular grouping.

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We next searched the literature for mesodermal derivatives in the location of the gonad that might resemble the structure we observed. We found that alary muscles (AMs) are specified in segmental repeats, tracking dorso-ventrally along the boundary of each segment[128]. They extend dorsally to provide structural support for the heart, and ventral-laterally towards the body wall muscles. Recently, it was shown that AMs dip under the dorsal trunk branch of the trachea[129]. We hypothesized that this would place the AM of abdominal segments 4 and 5 (AM 4/5) just anterior to the gonad. To test this, we examined the expression pattern of *tailup* (*tup*), a transcription factor that shares homology with mammalian *islet* and is required for specification of AMs[129]. Accumulation of tdTomato driven by a *tailup* (*tup*) regulatory sequence can be seen in cells ensheathing the gonad (white arrow), the triangular grouping anterior to the gonad (yellow arrow), the 4/5 AM just anterior and the 5/6 AM just posterior to the gonad (Fig 3.6D, arrowheads). Additionally, live imaging of an AM-specific enhancer within *tup* fused to the actin-binding domain of Moesin (*tup*AMEmoeGFP) reveals the close juxtaposition of the AM 4/5 alary muscle prior to and throughout hub assembly (Fig3.6E-E'). At later timepoints, when the hub assembles facing the internal organs, only the region of AM underneath the trachea and can be seen as the rest of the AM is flush with the body wall (Fig3.6E' arrowhead). Thus, our tissue expression analysis reveals a grouping of cells and an AM just anterior to where the hub assembles. Interestingly, AM-like muscles have been shown to anchor internal organs by connecting them to the epidermis. It is reasonable then that the AM could function to anchor the gonad. Furthermore AMs have been demonstrated to provide guidance cues for migration of the renal tubules[129,130]. Therefore, they may play a similar role in hub cell guidance. We hypothesized that the AM plays a role in hub cell guidance during assembly.

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To test a functional role for AMs in guiding hub cell assembly, we analyzed *tup* mutants by fixing and staining late stage gonads for hub cell markers. Heteroalleleic null mutations of *tup* were examined in the background of *six4*GFP transgenic flies. Gonads were dissected out of embryos and immunostained (cuticle deposition otherwise prevents antibody penetration at this state). Since gonads were removed from the embryo, the precise orientation of hub assembly within the embryo could no longer be ascertained. However, in isolated gonads, anterior from posterior can be discerned as the posteriorly located msSGPs express high levels of *six4*GFP. In control gonads, the adhesion protein FasciclinIII (FasIII) accumulates only at hub cell interfaces and can be seen within an anterior aggregate of GFP$^+$Tjl^{ow} cells (Fig 3.7A-A", hub marked by asterisk). Additionally, the hub is located on the opposite side of the gonad from the posterior msSGPs (Fig3.7A arrowhead). Gonads mutant for *tup* often displayed smaller aggregates of FasIII$^+$ cells or dispersed FasIII$^+$ cells (Fig3.7B,B" arrows). Additionally, these cells were found in close proximity to posterior msSGPs (Fig3.7B arrowhead). Similar results were found staining for E-cadherin, another adhesion protein enriched in hub cells (data not shown). These data indicate that *tup* is required for hub assembly, possibly through specification of AMs. However, it should be noted that *tup* mutant embryos exhibit defects in other tissues. Therefore, further experiments must be performed to confirm the specific role of AMs (see discussion).

Hub cells compact after assembling at the anterior

Our *in vivo* imaging concluded with the prospective hub (pro-hub) covering a large area at the anterior. Thus, while hub cell assembly could be imaged in live embryos, we were not able to visualize compaction of the hub into a tight aggregate (Fig3.2C) prior to the onset of embryonic muscle contractions; the contractions made

further *in vivo* imaging impossible. Therefore, we developed a protocol for dissecting out gonads from early stage 17 embryos, then culturing and imaging similar to previously described adult testis explants[131]. Viability and proper development of explanted gonads was confirmed by culturing for up to 7 hours, followed by fixation and immunostaining to assay for various markers of hub cell fate, hub cell adhesion and oriented germ cell divisions. Thus, explant culture afforded the first analysis to what changes occurred to the pro-hub for the niche to adopt its final structure.

We hypothesized that the final architecture (tightly aggregated hub seen at later stages) could result from 1) a subset of the pro-hub cells remaining aggregated while the rest disperse, making room for GSCs or 2) the entire pro-hub area compacting into a smaller space. We used Histone-RFP (HisRFP) to mark nuclei and germline actin revealed by *nanos* driving the actin-binding domain of Moesin fused to GFP (*nos*-Moe-GFP) to visualize this process. We found a striking compaction of the entire pro-hub area into a tight aggregate over a 6hr period of imaging (Fig 3.8). Compaction was evident by the decrease in negative space between hub cell nuclei and tightening of encircling germ cells (Fig3.8E, hub, asterisk). As the hub compacted, cortical f-actin became polarized in the germ cells towards the hub cell interface indicating recruitment of germ cells and establishment of adhesive complexes; those adhesive complexes are known to be critical for stem cell behavior (Fig3.8A'-E' arrowheads).

The compacting hub is surrounded by an acto-myosin cable

The previous data demonstrate the compaction of the pro-hub area and suggest that hub cell shape changes are required for niche formation. To address the mechanism for compaction, we visualized cytoskeletal dynamics. As morphological

changes often utilize contractile f-Actin networks facilitated by Non-muscle Myosin II (MyoII), we examined the localization of the regulatory light chain of MyoII fused to mCherry during compaction using live imaging. We observed an enrichment of MyoII at the hub cell-germ cell interface concurrent with hub compaction (Fig3.9). Prior to compaction, MyoII did not appear enriched along any interface (Fig 3.9B). However, at later time points when germline f-Actin is polarized towards the hub cell interface, MyoII also accumulated along those interfaces (Fig 3.9D, f-Actin enrichment in different z-plane). The co-enrichment of F-actin and MyoII is reminiscent of an acto-myosin purse string, which is used iteratively during development during cell sorting or boundary formation[132,133]. These data suggest that acto-myosin contractility is required for hub cell compaction.

There is a burst of GSC divisions during hub compaction

In addition to acto-myosin contractility, our timelapse imaging revealed additional factors that may play a role in hub compaction. Over the course of many imaging sessions, conducted on various genotypes, I anecdotally noted that there was often a burst of germline divisions during hub compaction. It appeared that these divisions were orthogonal to the hub as is expected for GSCs during steady state. To quantify germ cell divisions more rigorously, we imaged *six4*GFP to mark somatic cells along with HisRFP (Fig 3.10 A-D). Germ cells in mitosis were identified by chromosome condensation and segregation. At the beginning of imaging, GSCs contacting the assembled hub divided orthogonal to it (Fig 3.10B-C, yellow arrow). Often these GSC divisions resulted in displacement of the nearest hub cell away from the GSC and towards the other hub cells (Fig 3.10B-C white arrow). This was fascinating in that it suggested a mechanism whereby an assembled hub orients GSC divisions that then force hub cells closer

together. At the end of compaction, hub cell nuclei were tightly aggregated and germ cells were found in a radial array around the aggregate (Fig 3.10D). We quantified germ cells in mitosis during *ex vivo* imaging of hub compaction and found that on average gonads contain approximately 1 mitotic germ cell per every 30 minutes during compaction (Fig3.10I).

While the previous experiments in explanted gonads suggest a role for germ cell divisions in driving compaction, we wanted to bolster our hypothesis by determining when germ cell divisions begin and when they peak relative to hub formation. PGCs are arrested in the cell cycle during their migration to the gonadal soma but they reenter the cell cycle during gonadogenesis due to Jak/STAT signaling[106,134] Therefore, we returned to imaging gonads in intact embryos to quantify germ cell divisions using HisRFP relative to hub assembly using *six4*GFP. Prior to hub assembly, mitotic germ cells were not observed (Fig3.10E-F). However, 3-4 hours into imaging (approximately 16-17h AEL), mitotic germ cells were visible at every timepoint (10 min intervals) and germ cells contacting the assembled hub divided orthogonal to it (Fig3.10G-H). Quantification of mitotic germ cells *in vivo* demonstrates that germ cells resume dividing concurrently with the end of hub assembly and beginning of hub compaction (Fig3.10J). Taken together, these data suggest a possible role for oriented GSC divisions in driving hub compaction. This is intriguing given that niche signals first recruit the germ cells to the hub, thus specifying them as GSCs. If GSCs functioned in compaction, they would be shaping their own niche.

In order to test the proposed mechanisms of hub compaction, we needed a method to definitively quantify compaction. Qualitatively, hub compaction is apparent due to aggregation of hub cells and recruitment of GSCs, however, absence of

compaction is more ambiguous. Therefore, we fixed and immunostained gonads with an antibody against Ecadherin to outline hub cells prior to and following compaction. We then quantified the area of individual hub cells and averaged hub cell areas from all gonads for a given embryonic stage (Fig 3.11). For gonads processed at 15-17h AEL, the average hub cell area was 10.8µm ± 2.3 standard deviation (n=170 hub cells) (Fig3.11A-A"). For gonads, processed at 19-21 h AEL, the average hub cell area was significantly smaller with an average of 7.4µm ± 1.9 (n=88 hub cells; p-value<.001) (Fig 3.11B-B"). This demonstrates that hub cell area decreases during compaction and can be used as a measurement of compaction.

We next sought to test the requirement for acto-myosin contractility in hub cell compaction by pharmacological inhibition of Rho Kinase. Rho Kinase (ROK) phosphorylates the regulatory light chain of MyoII, which is necessary for MyoII activity[133]. We dissected gonads at 17h AEL, cultured them in the presence or absence of the ROK inhibitor Y-27632 for 4 hours, and then imaged germ cell actin and MyoII (Fig 3.12A-B). As seen previously, control gonads exhibited germline f-actin enrichment at the hub cell interface and tightly encircled the hub (Fig3.12A, arrowheads). Gonads treated with the ROK inhibitor lacked MyoII enrichment and germ cells failed to form a flattened interface enriched for f-Actin with hub cells suggesting that ROK is required for germ cells to properly adhere to hub cells (Fig3.12B arrowheads). As a result, the germ cells appear dispersed, making the hub seem less compact (Fig3.12B). To quantify hub cell area, we repeated the above experiment but fixed and stained with E-cadherin to outline hub cells. Dissected gonads expressing HisRFP and *six4*GFP 17h AEL were cultured for 5h with or without a ROK inhibitor. For this experiment, we used a distinct inhibitor, H-1152, which has been suggested to be a more specific for ROK. Antibodies

against HisRFP revealed bi-nucleated germ cells in H-1152 treated gonads (Fig3.12C-D). This was expected given that cytokinesis requires acto-myosin contractility, and these bi-nuceated cells likely represent dividing germ cells whose cleavage furrows had regressed upon drug addition. While this suggests some level of activity by the ROK inhibitor, quantification of hub cell area by Ecadherin staining revealed no difference in compaction. Controls had an average hub cell area of 10.9 µm ± 2.3 (n= 225) and H-1152-treated gonads had an average of 10.9 µm ± 2.8 (n= 177) (Fig3.12C'-D'). Surprisingly, the average area for the control hub cells in these explanted gonads did not differ from pre-compacted gonads *in vivo* (Fig3.11). This suggested that compaction didn't occur as efficiently in control, explanted gonads. We suspect that compaction in explanted and cultured gonads may take longer than *in vivo*. In retrospect, the cooler incubation temperature during imaging (~22°C) might cause the relatively slower rate of compaction to embryos aged in the incubator at warmer temperatures (~25°C).

Discussion

Timeline of niche development

Our work is the first to establish the stages of niche morphogenesis, which we observed by live imaging both in intact embryos and explanted gonads. Our observations are summarized in a timeline of niche development, in which we have uncovered the steps of hub assembly and hub compaction that occur in between hub cell specification and GSC recruitment (Fig3.13). While previous work demonstrated that Notch activation specifies hub cells around 8-9h AEL, our timelapse imaging indicates that hub cells remain morphologically similar to non-hub SGPs for several hours, as they are found encysting the germline at 12h AEL. From approximately 12-16h AEL, PS11 hub cells move anteriorly at which point they retract their cytoplasmic arms and adopt a

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cuboidal morphology. Interestingly, hub cells move peripherally rather than internally through the gonad, suggesting guidance by an extra-gonadal cue and possible interaction with BM components. Indeed, our functional studies indicate a possible role for the 4/5 AM in hub assembly and positioning. Finally, our timelapse imaging in explanted gonads, revealed the subsequent compaction of the assembled hub cells into a tight aggregate. Concurrent with compaction is a burst of GSC divisions at the onset of niche signaling and generation of an acto-myosin cable at the hub-GSC interface. Our preliminary data suggest we can pharmacologically inhibit GSC divisions and acto-myosin contractility to address the mechanism of compaction. Importantly, this is the first study to characterize niche morphogenesis and propose mechanisms based on those observations.

An extra-gonadal cue for hub assembly

Previous experiments to address the mechanisms of hub formation relied on fixed images and end point analysis[63,94]. The fact that hub cells are enriched for adhesion proteins and appear to sort out from other cell types in the gonad led to the hypothesis that hub cells assemble due to preferential adhesion to one another. However, attempts to modulate adhesion proteins within the gonad have failed to prevent proper hub assembly at the anterior[94,135]. Furthermore, preferential adhesion alone does not explain the consistently biased placement of the hub towards the internal organs that we observed during imaging. In fact, rather than sorting with other hub cells and moving collectively towards the anterior, our imaging revealed individual movement of hub cells along the periphery. These data indicate an attractive cue derived from outside of the gonad rather than adhesion-mediated sorting. Indeed, in the absence of Integrin, the hub is internalized, and our ability to image this process revealed the

aggregation of clustered hub cells as they are enveloped within the gonad. This suggests that hub cells will sort due to preferential adhesion but require Integrin to counteract internal sorting.

Our imaging data also revealed candidate tissues for guiding hub assembly. The AM is an intriguing possibility as it has already been demonstrated to have a role in guiding migration of the *Drosophila* renal tubules[129,130]. In this case, the tip cells of the renal tubules migrate towards and sequentially interact with AMs in different segments, and each transient interaction is required for the tubes to achieve their normal (looped) morphology[130]. In order to get at the mechanism of AM attraction, the authors altered their homeotic (hox) gene identity. The segmentally repeated AMs are specified under different homeotic (hox) gene domains, *ultrabithorax* (*ubx*), *abdominalA* (*abdA*), and *AbdominalB* (*AbdB*) from the anterior to more posterior AM[128]. Although the Hox gene identity of the AMs did not affect renal tubule guidance, we are interested in testing this in hub cell guidance given that the AM anterior to hub cells is specified within the *abdA* domain while the AM posterior to hub cells receives *AbdB* input. Indeed, some support for Hox genes influencing hub cell assembly comes from various manipulations of AbdA and AbdB. First of all, ectopic expression of *abdA* generates more AMs along the body and ectopic expression of *abdA* causes gonadal cells to continue their anterior migration, rather than arresting at abdominal segment 5. Furthermore, mutants for *AbdB* have defects in hub placement[63,69,128]. If the AM were providing the guidance cue, the defect in migration may be due to ectopic chemoattractant. Alternatively, the phenotypes resulting from *abdA* and *AbdB* manipulation might be through their effects on other tissues rather than AMs. While a number of tissues could have been affected in these experiments, we speculate that segmentally distinct AMs may guide anterior hub assembly.

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Alary muscle reporter expression shows a separation between the 4/5 AM and the gonad, while broader mesodermal expression shows the AM projecting and connecting to a triangular grouping just anterior to the hub cells. Therefore, future experiments will address whether the AM actually contacts the gonad. Instead the AM may contact the anterior grouping of cells, which in turn contact the gonad. The anterior mesodermal cells may be a subset of the fat body-derived pigment cells that are recruited to ensheath the gonad or a specific region of the visceral mesoderm surrounding the gut [74,136]. Both possibilities are intriguing as fat body cells can secrete Pericardin, the primary ECM protein used by the AMs to adhere to the *Drosophila* cardiac tube[137,138]. Therefore, the fat body cells could serve to recruit the AM. Alternatively, thoracic-alary related muscles (TARMs) have been shown to make connections with the visceral mesoderm of the midgut, which suggests the AM could also anchor this region of the migut[129]. Our imaging data and preliminary functional experiments suggest AMs have a role in hub cell assembly. We speculate that they interact with fat body or visceral mesoderm just anterior to where the hub assembles and function in guiding hub cells to that location.

Hub compaction and acto-myosin contractility

Our data reveal a striking compaction of the hub following its assembly at the anterior. Until recently, the decrease in hub cell area that occurs during compaction was unknown. We have now developed methods to image this process live and probe the mechanisms required for it. The finding that hub compaction occurs concurrently with the generation of an acto-myosin cable is unsurprising given the numerous roles for these cables in driving cell shape changes and boundary formation[132,133]. Since GSCs undergo a burst of divisions at the time the MyoII purse string first appears, an exciting

hypothesis is that GSCs are responsible for generating this cable through divisions orthogonal to the hub. Hub cells initially recruit germ cells to the niche and orient GSC divisions, thus it is intriguing that niche signaling may function to shape the niche[77,96].

Pharmacological inhibition of ROK disrupts both acto-myosin contractility and germ cell divisions. Our preliminary findings from treatment with the ROK inhibitor H-1152 did not result in a significant decrease in compaction upon drug addition. One consideration is that experiments *in vivo* are performed at warmer temperatures than the experiments allow when using live imaging of explanted gonads allow. We have found significant differences in developmental timing due to changes in temperature in the past and will need to account for this in future experiments. Furthermore, while all H-1152 treated gonads contained at least one bi-nucleated germ cell, we did not observe many bi-nucleated cells, suggesting the concentration of the inhibitor could be increased or the initial burst of divisions occurred prior to drug addition. The peak in germ cell divisions occurs around 17h AEL *in vivo*, indicating we may need to inhibit cycling or acto-myosin contractility prior to this point. Future *in vivo* studies will include expression of a kinase-dead ROK in the soma or germline to test the role for acto-myosin contractility or modulation of the cell cycle in germ cells to test the role of GSC divisions.

Niche morphogenesis and niche function

Critical follow-up experiments will investigate the interplay between niche signaling and niche morphogenesis. Since Upd-Jak/STAT signaling both initiates and orients GSC divisions, we will test the requirement Jak/STAT signaling in hub compaction by expressing a negative regulator of the pathway, *suppressor of cytokine signaling*, in germ cells. Given that the phases of niche morphogenesis are only now

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being described, it is also unclear what effect a failure of assembly or compaction would have on establishment of niche signaling. There are few existing examples of defects in niche organization[94,97,135,139]. On example includes Integrin mutants, which exhibit an internalized, yet aggregated and compact hub. These internalized niches appear to function normally as evidenced by oriented GSC divisions. Unfortunately, these mutants cannot be carried out past embryogenesis due to lethality, limiting niche characterization to the initial oriented divisions but no analysis of self-renewal of differentiation[94]. However, there is reason to believe that niche architecture is important for niche function. Bone Morphogenetic Proteins (BMPs) secreted from the hub direct self-renewal of GSCs and prevent differentiation[34,38]. Like other morphogens, BMP secretion is spatially restricted by a variety of factors to ensure specificity of signaling[99,100]. In the adult testis, it has been suggested that BMPs are localized to the adherens junctions between hub cells and GSCs[140]. Compaction may aid in forming the proper junctions and thus restrict niche signaling to adjacent germ cells. Our current work uncovering the phases and mechanisms of niche morphogenesis has begun to address the requirements for assembling a functional niche during development.

Acknowledgements
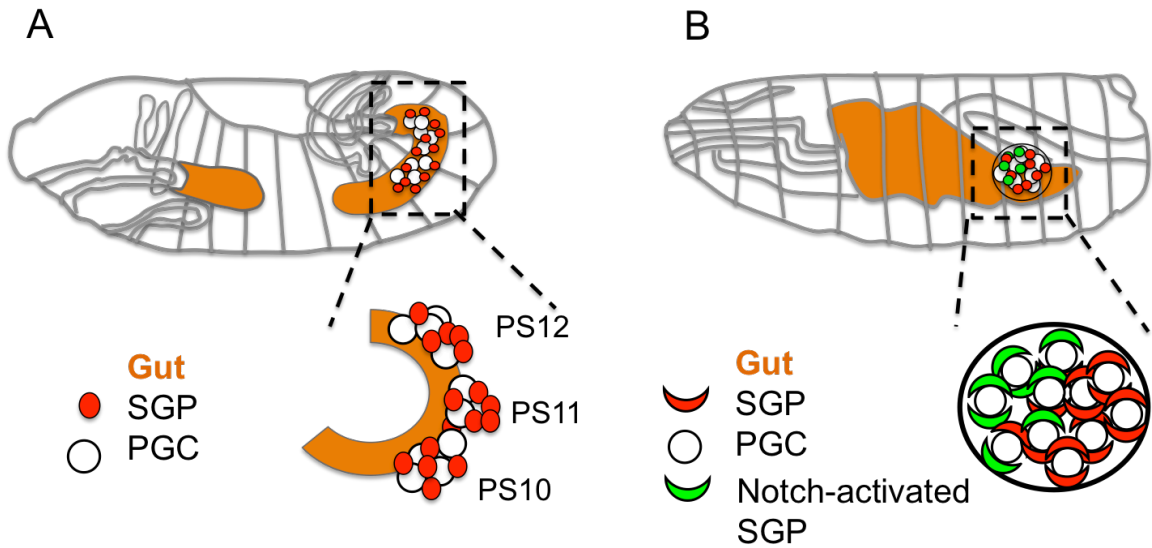
Figure 3.1: Hub cells are specified by Notch activation early during gonadogenesis. Anterior is to the left in all panels. A) Primordial germ cells (PGCs) coalesce with somatic gonadal precursors (SGPs) specified from parasegments 10-12. Delta-derivated from the gut activates Notch in a subset of SGPs at this time, thus specifying them as hub cells. B) SGPs (some of which are Notch-activated) migrate and compact into a sphere with PGCs, finally settling in abdominal segment 5.
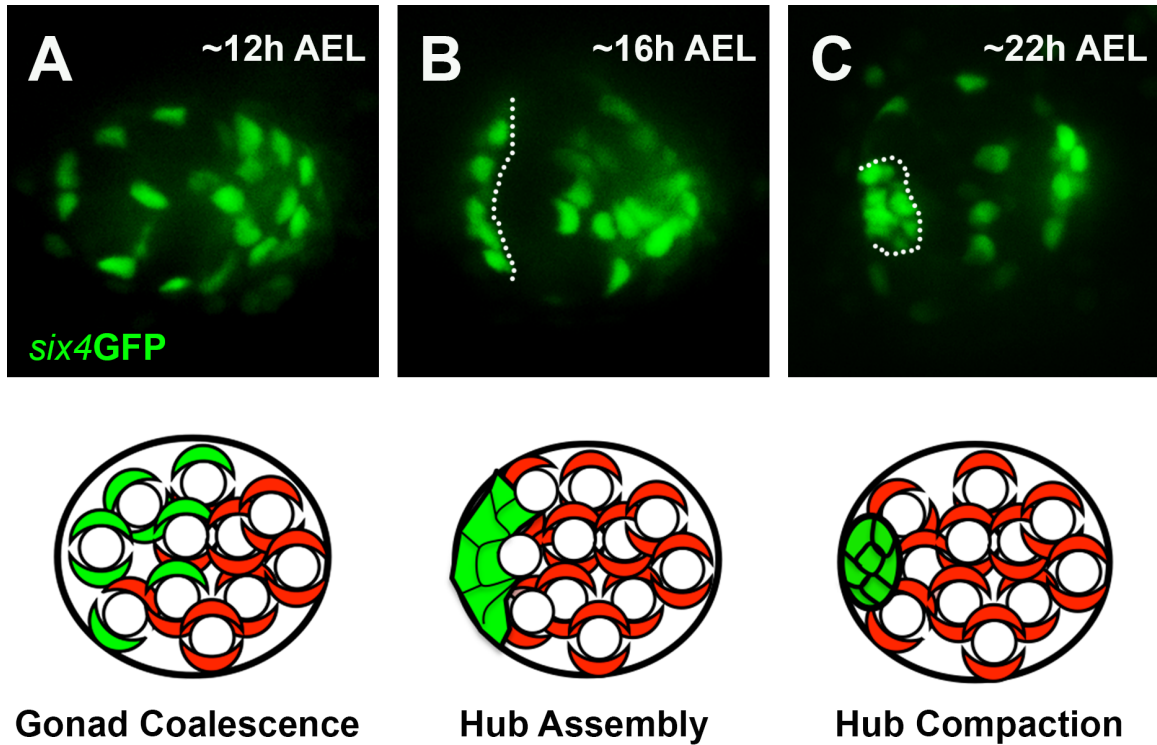
Figure 3.2: Live imaging reveals that prospective hub assembles peripherally at anterior. *six4*GFP imaged in embryos at A) 12h after egg lay (AEL) and B) 16h AEL. C) six4GFP imaged in dissected gonad at 22 h AEL. SGPs initially appear dispersed at 12h AEL (A). Several hours later, a subset of SGPs assemble at the anterior forming the prospective hub (B). In dissected gonads (C), the hub appears smaller and takes up less area than the prospecive hub imaged at earlier time points. Schematics below micrographs represent the stage of imaging.

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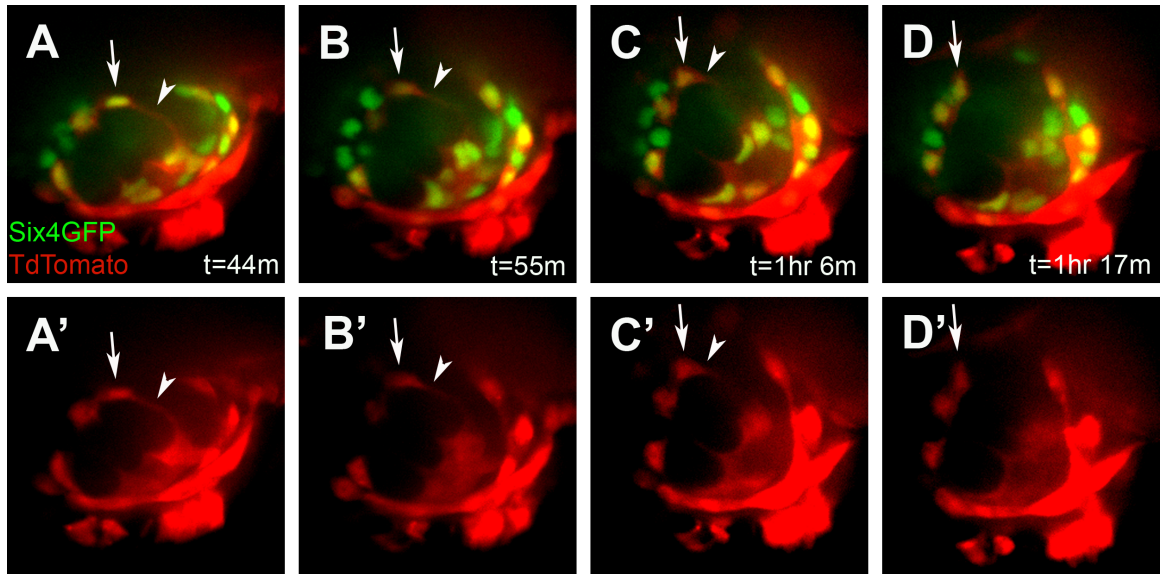
Figure 3.3: PS11 hub cells migrate anteriorly along the periphery Live imaging of
*six4*GFP marking all SGPs and tdTomato expressed in odd parasegments (PS). A PS11
hub cell can be seen moving anteriorly along the periphery (A-D, arrow; periphery
determined by Z-plane). Initially, the hub cell has a cytoplasmic extension around a germ
cell, but it is retracted upon assembly with other hub cells at the anterior (A'-D'
arrowhead).
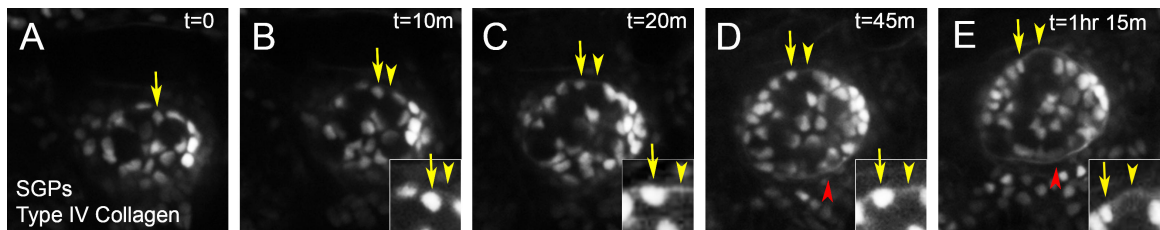
Figure 3.4: Basement membrane is desposited during hub assembly. Live imaging
of gonad expressing six4GFP to mark somatic cells and TypeIV Collagen GFP
(ColGFP). A PS11 hub cell first moves to the periphery (A-C, arrow) then moves
anteriorly (C-E, arrow) as ECM is deposited (arrowheads). ECM is deposited evening
around the gonad (yellow and red arrowheads).
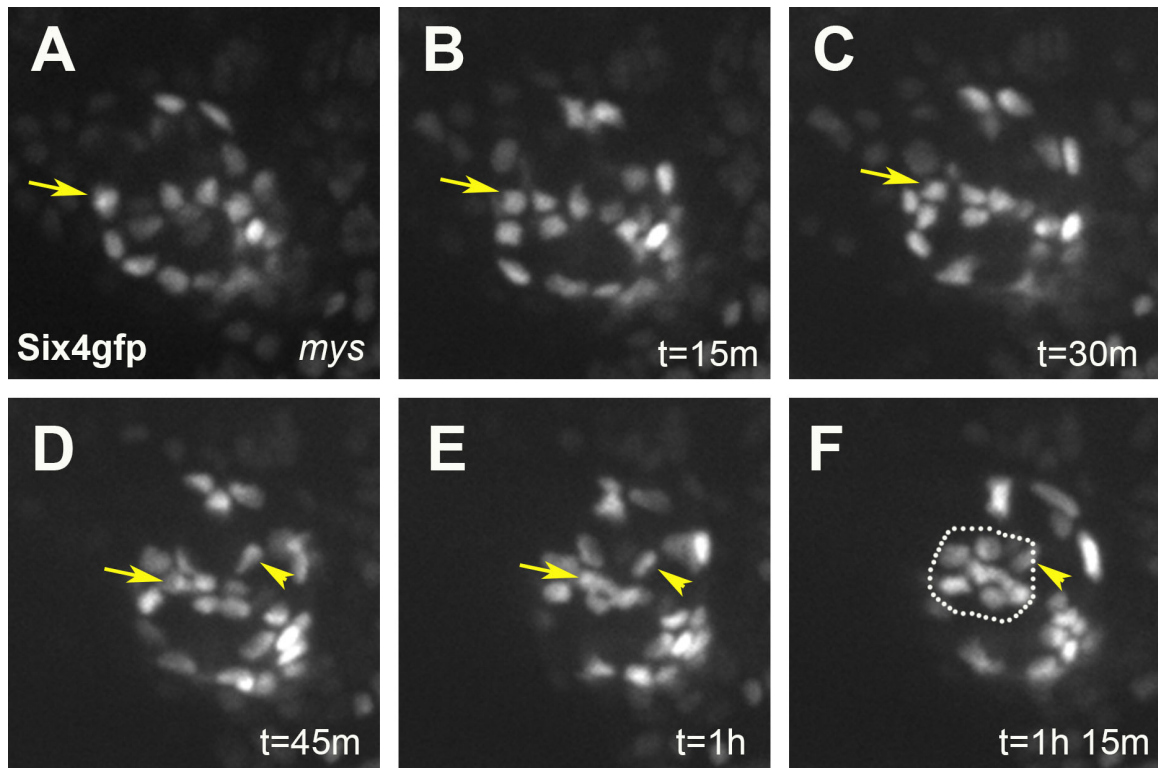
Figure 3.5: Integrin mutants have defects in hub cell anchoring and assembly. A *myospheroid* (*mys*) mutant embryo expressing *six4*GFP to mark somatic cells. PS10 hub cells originating on the periphery at the anterior are drop in from the periphery shortly after imaging beings (A-E arrows). Rather than assembling at the anterior, PS11 hub cells (E,F arrowhead) join the hub cell aggregate as it is being internalized (hub outlined in F).
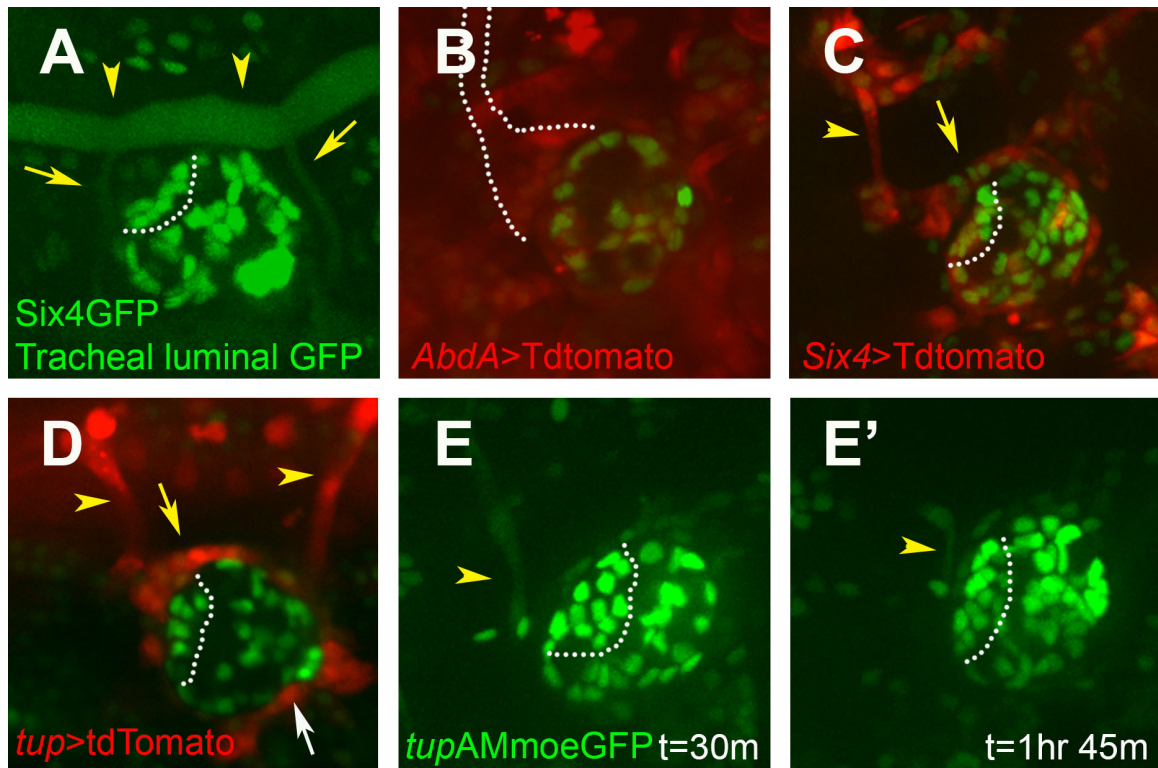
Figure 3.6: Live imaging reveals tissues adjacent to assembling hub. A) *six4*GFP (SGPs) and GFP in the tracheal dorsal trunk reveals assembled hub is biased dorsally. B) *six4*GFP and tdTomato driven by the hox gene *abdA* reveal structure anterior to the hub (B, outline). C) *six4*GFP and *six4*>tdTomato reveal clustered mesodermal cells (arrow) anterior to assembled hub (outline). Extending from the cluster is a string of cells (arrowhead) that project dorsally. D) *six4*GFP and *tailup* (*tup*) driving tdTomato again reveals grouped cells (yellow arrow) just anterior to assembled hub (outline) and also posterior (white arrow). Two alary muscles (AMs) can be seen anterior and posterior to the gonad (arrowheads). E-E') Timelapse of *six4*GFP and *tup*AMEmoeGFP to specifically mark AMs, demonstrates AM (E, arrowhead) is anterior to gonad when hub cells begin to assemble (outline; AM does not appear to contact hub). An hour later, most of the AM is no longer in view with assembled hub (outline) but a small region is still visible (E', arrowhead).
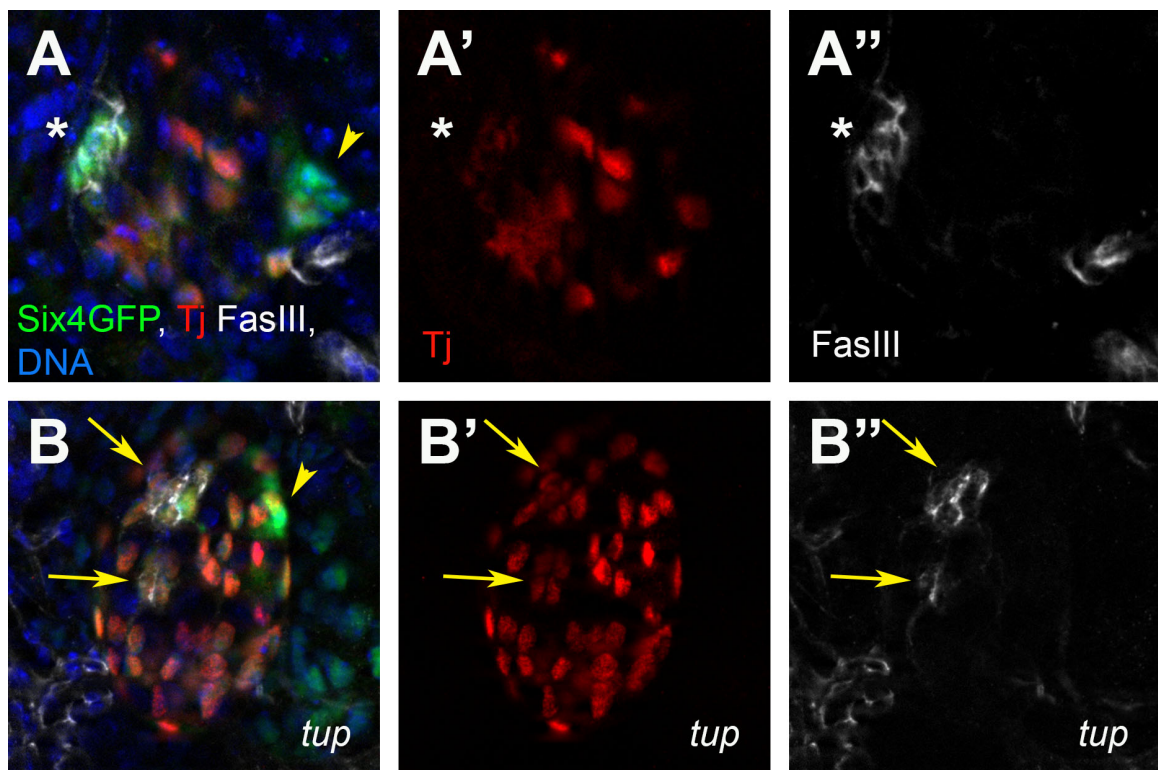
Figure 3.7: *tup* **mutants exhibit defects in hub assembly.** Stage 17 (21-23h AEL) *tup* mutant embryos dissected and stained with anti-GFP (six4GFP), anti-Tj to mark SGPs and anti-FasIII to mark hub cells. A-A") Control gonad contains an anterior aggregate of hub cells enriched for FasIII (asterisk). Anterior is determined relative to posterior msSGPs marked by bright *six4*GFP (arrowhead). B-B") *tup* mutant gonad contains two separate aggregates of FasIII-enriched hub cells (arrows). Additionally, these aggregates do not appear to be assembled at the anterior relative to posterior msSGPs (arrowhead).
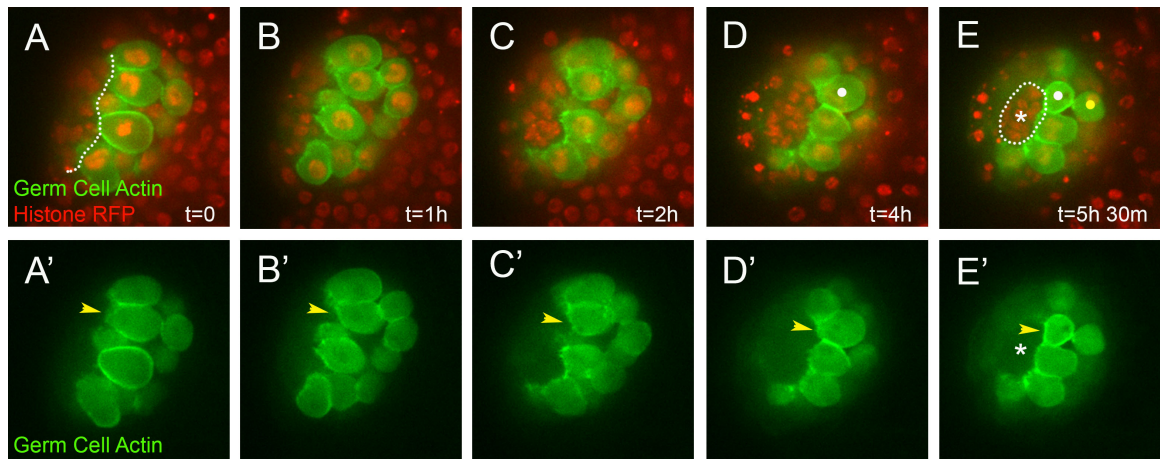
Figure 3.8: Live imaging of explanted gonads reveals prospective hub compacts to achieve final niche architecture. A-D) Timelapse of explanted gonad expressing *nos*-Moe-GFP to mark actin in the germline and HisRFP to mark nuceli. At the beginning of the timelapse (A) the pro-hub (outline) covers a large area at the anterior and germ cells have cortical actin enrichment (A'). By the end of the timelapse (E), the entire pro-hub has compacted into a small aggregate (outline) tightly encircled by germ cells. The germ cells now exhibit actin enrichment at the hub cell interface (E' arrowhead). Additionally, a GSC (D, E, white dot) divides (E, daughter marked by yellow dot) orthogonal to the hub (E, asterisk).
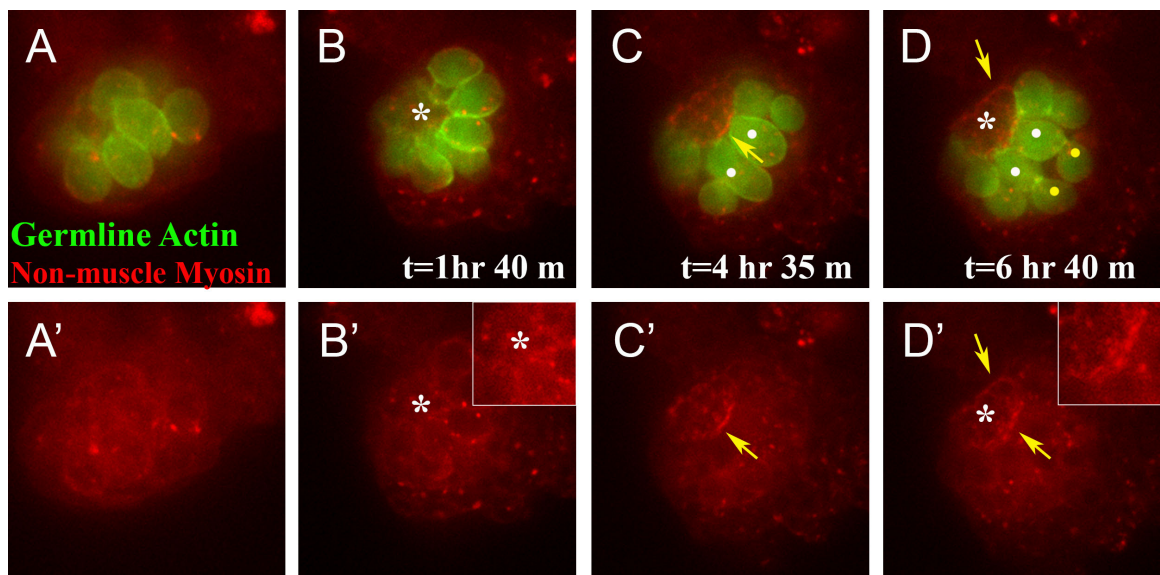
Figure 3.9: A MyoII purse string appears around the compacting hub. Timelapse of explanted gonad expressing *nos*-Moe-GPF to mark germline actin and the regulatory light chain of Non-muscle Myosin (MyoII) fused to mCherry. At the beginning of the timelapse (A-B), MyoII is not enriched along any interface (B', asterisk marks

presumptive hub). As the hub compacts, MyoII is enriched at hub cell-GSC interface (C',
D', arrows). Additionally, two GSCs (white dots) divide orthogonal to the hub between C
and D.
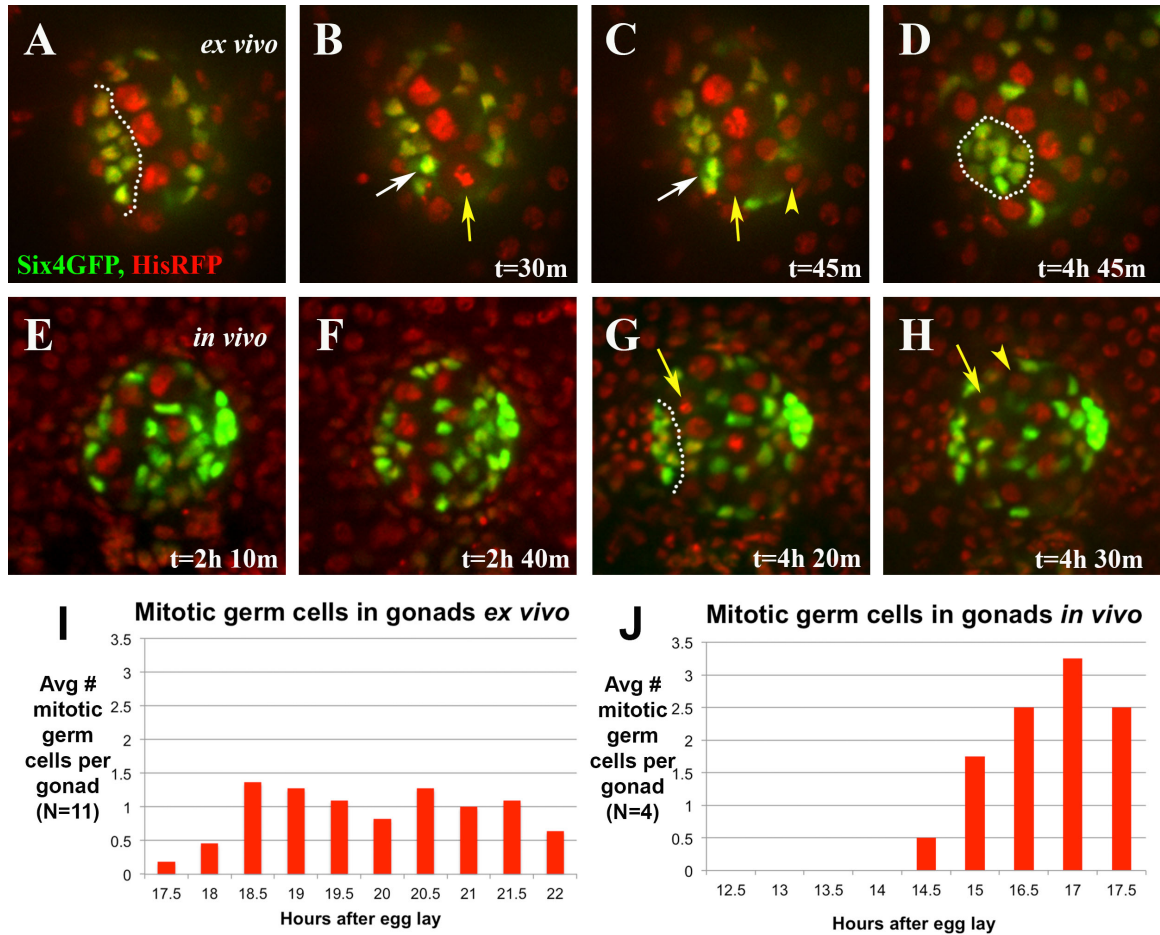
Figure 3.10: There is a burst in germline divisions during hub compaction.

A-D) Timelapse of explanted gonad expressing *six4*GPF and HisRFP. At the beginning
of the timelapse, the pro-hub covers a large area (A, outline) and hub cell nuclei are
separated by negative space. A GSC (B,arrow;C, arrow and arrowhead mark daughters)
divides orthogonal to the hub. The hub cell (B, white arrow) nearest the dividing GSC,
moves closer to the hub cell just below it (compare internuclear distance in B,C). Several
hours later the hub has compacted into a tight aggregate with little negative space in
between hub cells (D, outline). E-H) Timelapse of gonad in embryo prior to hub
assembly (D). After hub assembles (G, outline), a GSC divides orthogonal to the hub
(G,H, daughter cells marked by arrowhead, arrow). I-J) Quantification of germ cells in
mitosis in explanted gonads (I) and in embryos (J).
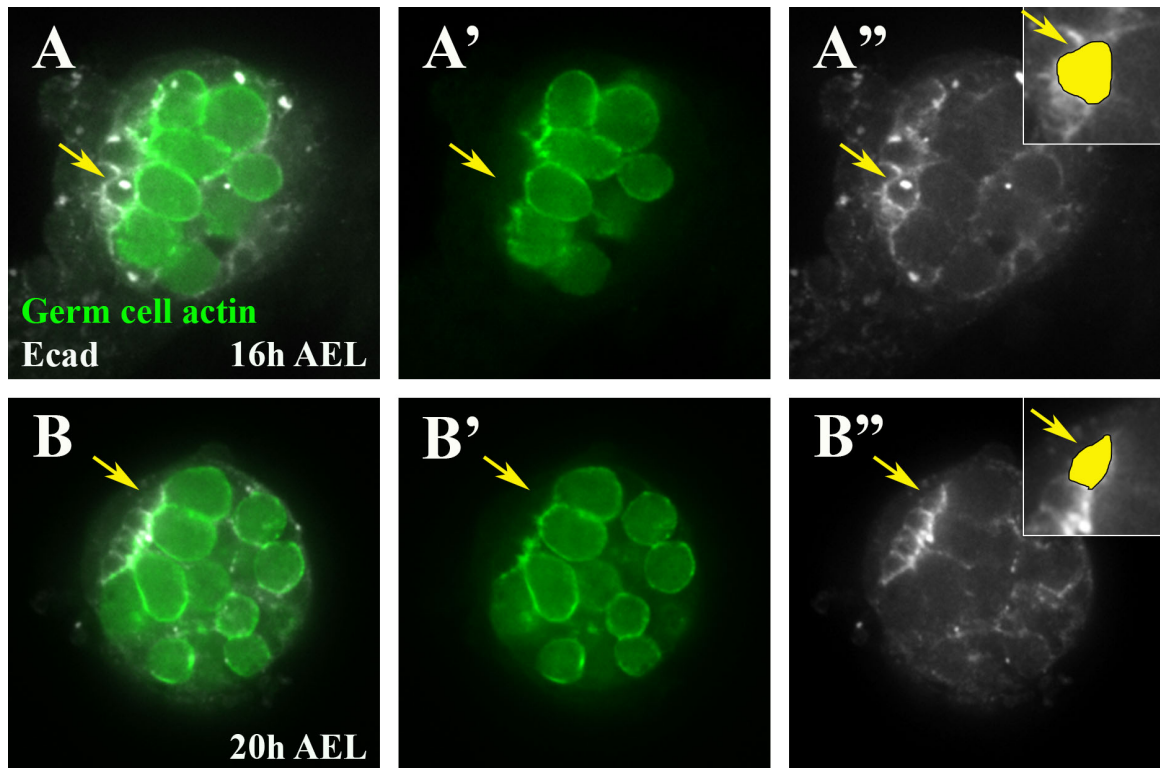
Figure 3.11: Hub compaction can be measured by a decrease in hub cell area.
Gonads raised at 25° C, then dissected, fixed and stained with anti-GFP (Germ cell actin) and anti-Ecadherin to mark hub cells at A) 16h and B) 20h AEL. Hub cell area was measured for each individual hub cell (A",B" inset) and averaged across all gonads for that timepoint.
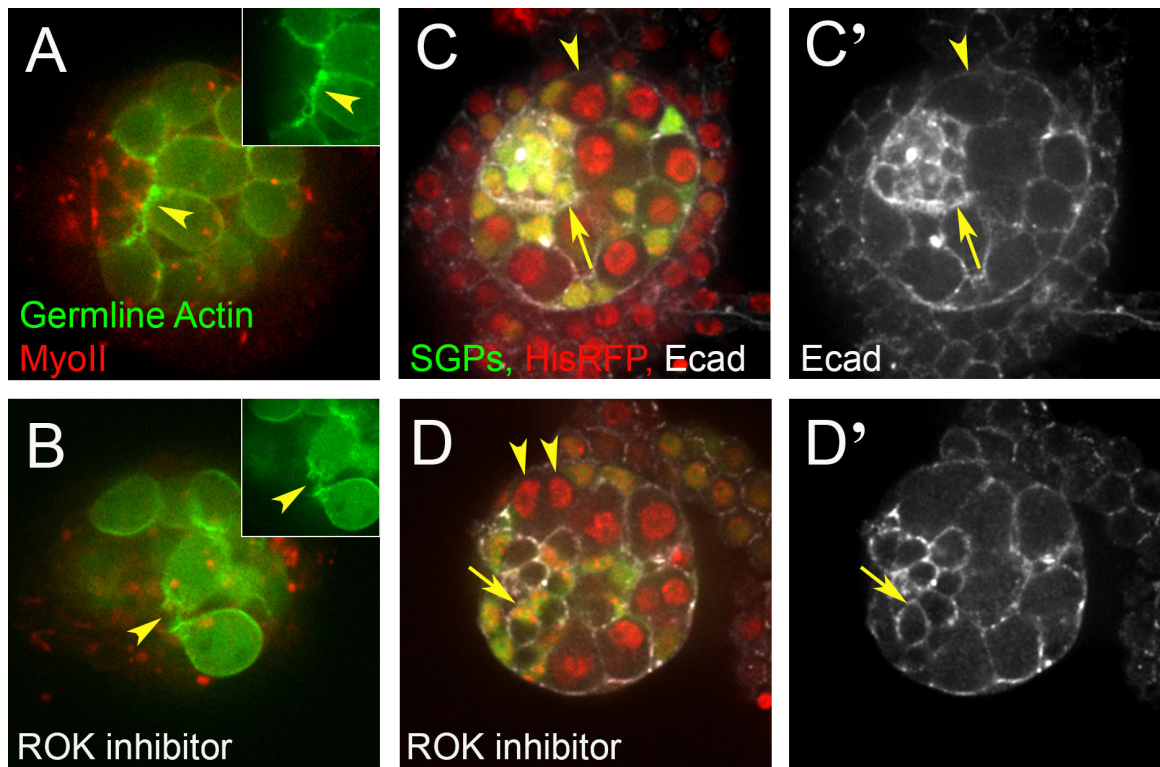
Figure 3.12: Rho kinase inhibitor blocks acto-myosin contractility in explanted gonads. A,B) Explanted control gonad (A) and ROK inhibitor Y-27632-treated gonad (B) cultured expressing *nos*-MOE-GFP to reveal germ cell f-Actin and the regulatory light chain of Non-muscle Myosin II (MyoII) for 4 hrs at approximately 22°C, then imaged live. Germ cells in control gonad polarize actin towards the hub cell interface where MyoII is enriched (A, arrowhead). Germ cells in ROK-treated gonad, exhibit filopodia (B, arrowhead) but fail to form a flattened f-Actin-enriched interface with hub cells. C-D') Explanted control gonad (C-C') and ROK inhibitor H-1152-treated gonad (D-D') cultured for 5 hrs at approximately 22°C, then stained with anti-RFP (HisRFP), anti-GFP (*six4*GFP) to mark SGPs and anti-E-cadherin to mark hub cells. Control gonads contain germ cells with a single nucleus (C, arrowhead) while ROK-treated gonad contains bi-nucleated germ cells (D, arrowheads). Hub cells in control gonads appear more compact
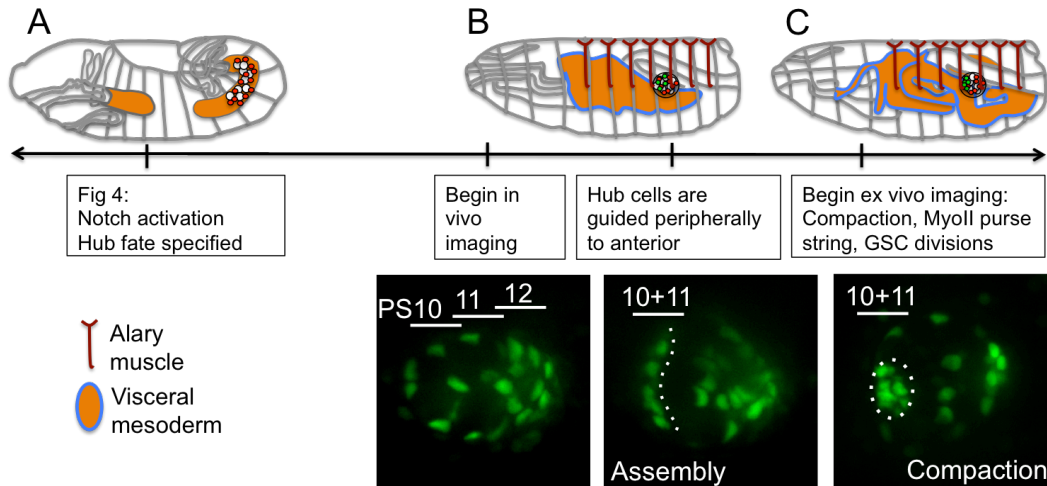
Figure 3.13: Timeline of niche development. Micrographs are of live imaged gonads expressing *six4*GFP to mark SGPs. Anterior is to the left in all panels. A) Primordial germ cells (PGCs) coalesce with somatic gonadal precursors (SGPs) specified from parasegments 10-12. Delta-derivated from the gut activates Notch in a subset of SGPs at this time, thus specifying them as hub cells. B) 12hrs AEL we can image PS11 hub cell migration along the periphery to assemble anteriorly with PS10 hub cells. An alary muscle (AM) is found just anterior to where the hub assembles and visceral mesoderm surrounds the gut just internal to where hub assembles. These tissues may function in guiding assembly. C) After the hub has assembled we can dissect and image explanted gonads approximately 17 hr AEL. At this point the assembled pro-hub compacts to achieve final niche architecture (hub outlined).

CHAPTER 4: General Discussion

Cell fate specification via Notch regulation of Maf factors

The *Drosophila* gonad is a powerful system for studying stem cell niche development, and our experiments here have provided a more complete understanding of niche specification and morphogenesis. Our lab previously demonstrated that Notch is activated in a subset of SGPs during their migration over the developing gut[64]. While it would be ideal to identify targets of Notch signaling in hub cell specification by immunoprecipitating Suppressor of Hairless Su(H) bound chromatin, the limited number of Notch-activated cells in the gonad precludes this type of analysis. Therefore, we have attempted to elucidate the pathway downstream of Notch using a candidate approach and epistasis experiments. Our experiments identified the large Maf factor Traffic jam (Tj) as an effector of Notch signaling in hub cell specification. Interestingly, Tj regulates both adhesion and niche signaling and our subsequent investigation into niche morphogenesis suggests that both are important for niche function[90,117,135].

One remaining question is whether transcriptional activation downstream of Notch signaling directly downregulates Tj. We did not confirm regulation of Tj by Su(H) and in fact, it seems unlikely that *tj* is a direct target given the delay between Notch activation and Tj downregulation (approximately 6 hrs). Instead we postulate one mechanism whereby another transcription factor, Midline, activates *tj* and is repressed by Notch signaling[113,114]. Future experiments will determine the possible interactions between Notch, Midline and Tj in the gonad. It is also reasonable, however, that regulation of Tj is mediated post-transcriptionally. Large mammalian Maf factors have multiple phosphorylation sites regulating their stability and degradation. For example, MafA can be phosphorylated by GSK3-β and p38 kinase, and L-Maf is phosphorylated

by Fibroblast Growth Factor (FGF)/ERK signaling[141–143]. Similar regulation of Tj is supported by our attempts at gain-of-function experiments, in which we overexpressed *tj* in hub cells after niche formation (data not shown). Overexpression failed to result in increased accumulation of Tj within the hub suggesting that *tj* is post-transcriptionally modified to maintain low levels within the hub. Experiments in the ovary suggest that Tj accumulation must be tightly controlled to regulated proper adhesion[90,117] Therefore, it would be interesting to determine whether Tj contains conserved phosphorylation sites with mammalian Mafs. Future experiments might include mutating these sites and assaying for failure to downregulate Tj upon Notch activation. This would suggest that a particular kinase is a target of Notch signaling rather than Tj.

Although development of the mammalian gonad and the *Drosophila* gonad are in part disparate processes, parallels can be drawn. First of all, the PGCs undergo similar migration through the endoderm and from there are delivered to the somatic gonad, called the genital ridge in mammals[67]. Secondly, niche cells are specified from the genital ridge and these niche cells aid in self-renewal of the germline (in males)[144]. In mammals, Sertoli cells act as the niche cells for spermatogonial stem cells through their expression of self-renewal factor *glial-cell derived neurotrophic factor (Gdn*f)[14]. Like hub cells, the Sertoli cells are specified first and then aid in the specification and organization of other cell types within testis cords or future seminiferous tubules[77,78,144]. A role for Notch signaling in Sertoli cell specification has not been demonstrated; however, the Notch pathway is activated in Sertoli cells and non-autonomously influences germ cell differentiation[145]. Interestingly, Notch is also activated in a subset of cells in the interstitial compartment where steroidogenic Leydig progenitor cells are maintained and constitutive Notch signaling blocks their differentiation[116,145]. In a separate study, the

large Maf factors MafB and C-Maf were also shown to accumulate overlapping with Leydig progenitor cell markers[115]. Based on our work in the gonad, future experiments testing the regulation of Maf factors by Notch signaling in the specification of Leydig cells may reveal another conserved process comparing Drosophila and mammalian gonadal development.

PS11 hub cell specification

Our dissection of the pathway downstream of Notch more precisely defined the role for Bowl in hub cell specification. The transcription factor Bowl can activate or repress transcription, but Lines prevents Bowl activity by sequestering it in the cytoplasm[112]. We previously demonstrated that Lines and Bowl mediate the cell fate decision between hub cells and cyst cells during gonadogenesis. The requirement for Bowl is complex given that *bowl* mutants specify fewer hub cells than controls, but more hub cells than *Notch* mutants[76]. This may suggest that Bowl has a minor role in niche cell specification or that Bowl regulates a specific subset of SGPs, perhaps PS11 hub cells. In Chapter 2, we demonstrate that Bowl can rescue anterior assembly and cuboidal morphology of ectopic *tj* mutant hub cells. We can speculate then that PS11 hub cells have a greater reliance on Bowl, as they are the cells that need to migrate anteriorly. It would be useful to determine whether PS11 cells are found in the hubs of *bowl* mutants. If not, we must next ask whether Bowl is required to receive a migratory cue or whether Bowl simply mediates the morphological response to said cue. Work in the leg disc suggests it may be the latter since overexpression of *odd-skipped* family proteins (of which Bowl is a member) drives f-actin enrichment and invagination in the epithelium[111]. Taken together, this suggests a conserved role for the odd-skipped

transcription factors in mediating morphological changes specific to that tissue, resulting in segmentation in the leg and niche assembly in the gonad.

If Bowl indeed regulates hub assembly, specifically in the PS11 cells that need to migrate anteriorly, it would be ideal to identify Bowl targets in these cells. However, we are again limited by the number of gonadal cells and cannot isolate sufficient amounts of DNA to ChIP Bowl target genes. Instead, we have investigated assembly using live imaging. In the future, it would be useful to determine if any f-actin regulators identified by our imaging analysis require Bowl for their activity in the gonad. This would serve to demonstrate that factors important for niche specification also function in proper assembly.

PS11 hub cell migration

The mechanism that allows PS11 cells to assemble with more anterior PS10 cells has been of interest to the field ever since the contribution of PS11 cells was discovered[74]. Several possible mechanisms could facilitate the aggregation of hub cells from both parasegments at the anterior. Based on the enrichment of homotypic adhesions proteins observed in differentiated hub cells, it was speculated that hub cells sort away from other gonadal cell types due to their preferential adhesion with one another. Since hub cell fate is specified in anterior hub cells and repressed in posterior hub cells, this could place the hub in the anterior half of the gonad. However, random sorting would not account for the biased placement of the hub towards the internal organs that we observe in imaging. Finally, modulating adhesion proteins within the gonad, has failed to disrupt hub cell assembly or placement[94] (personal communications with M.Van Doren). Therefore, while adhesion likely plays a role in hub cell aggregation it seems unlikely that it is the driving force for anterior assembly.

Another possible mechanism for hub assembly is that PS10 and PS11 cells coalesce and then collectively migrate to the anterior. The best-studied example of collective cell migration is the border cells in the *Drosophila* ovary. The border cells are specified from the follicular epithelium of the developing egg by Upd-Jak/STAT signaling. These cells then aggregate as they detach from the epithelium and migrate through the nurse cells towards the oocyte. Collective migration and direction sensing are facilitated by local activation of Rac at the leading edge of one cell in the cluster and subsequent communication to the remaining cells via Ecadherin-mediated tension[146,147]. Our time lapse imaging in embryonic gonads, however, demonstrates that PS11 hub cells move individually rather than collectively. In fact, they do not aggregate into a compact grouping until after their assembly at the anterior.

An extra-gonadal hub cell guidance cue

We hypothesized that if PS11 hub cells migrate in response to a secreted cue, we would see dynamic filopodia in migrating cells. Previous live imaging, which did not discriminate between male and female gonads demonstrated that actin-rich filopodia could be observed extending anteriorly in PS10 cells approximately 10 hr AEL when the gonad is still elongated. Within the next hour, anterior protrusions are retracted and filopodia are instead directed internally as SGPs begin to encyst the germline[126]. Mutations in the actin regulator Enabled block these protrusions and disrupt gonad morphogenesis into a sphere. Surprisingly, we did not identify filipodial processes by labeling actin in later stage migratory SGPs. One possible explanation for this is that SGPs have overlapping cytoplasmic arms once they encyst the germline, making identification of filopodia in individual SGPs difficult[148]. However, another reasonable explanation is the resolution with which we could see filopodia. The previous study

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identifying small actin-rich protrusions was performed using multi-photon microscopy. Thus, it would be reasonable to re-examine f-actin in PS11 cells during migration under higher resolution.

We also showed that the extracellular matrix (ECM) protein TypeIV Collagen is enriched around the gonad at the time when hub cells are assembling. ECM can aid in migration by transporting attractive or inhibitory cues and also acts as a scaffold for cell migration via integrin based adhesions[99,149,150]. Furthermore, matrix-modifying enzymes can alter the path of migration through interstitial space[151]. The specific type of ECM constructed around epithelial tissues is called basement membrane and in Drosophila BM separates most internal organs from hemolymph[150]. Often BMs are modified during the development of these tissues in order to allow for growth or migration[152]. Although TypeIV Collagen and Perlecan are deposited evenly around the gonad, it is possible that these ECM components are specifically modified at the anterior to promote hub assembly there. In *C.elegans* gonadogenesis, for example, the BM surrounding the distal tip cells, which constitute the niche, is modified to direct migration and growth of the tubules[152]. Future investigations into the specific ECM components and modifying enzymes enriched around the *Drosophila* male gonad may elucidate their role in hub assembly.

A thorough analysis of the particular ECM components deposited around the gonad may also indicate roles for other tissues in gonadogenesis. We have identified three tissues near the gonad that may function in directing hub cell assembly. One candidate, the alary muscle (AM), uses the ECM component Pericardin to adhere to the cardiac tube[137,138]. Therefore the presence of Pericardin or Tiggrin, another ECM component enriched at muscle attachment sites could further support a role for the AM

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in gonadogenesis[150]. Fat body, the *Drosophila* liver, is also known to develop in close proximity to the gonad, and a subset of fat body cells may constitute the triangular grouping of nuclei we identified adjoining the anterior of the gonad[71,72]. Interestingly, the fat body cells and the hemocytes (*Drosophila* blood cells) are responsible for secretion of ECM proteins during *Drosophila* development[153–155]. Finally, the hub assembles near the visceral mesoderm surrounding the gut. One possible model is that hub cells are attracted to a specific region of visceral mesoderm. There are constrictions in the developing midgut that are controlled by factors secreted from specific regions of the visceral mesoderm. The gonad is located in close proximity to a constriction regulated by the Tbx ortholog, Org-1; therefore, mutations in *org-1* or *biniou*, which specifies visceral mesoderm, would determine whether this tissue functions in hub assembly[156,157]. Once we have identified a requirement for a particular tissue in hub cell assembly, we can begin screening secreted cues by dsRNA knockdown with a tissue-specific Gal4 to identify the attractive cue for hub cells.

A role for germ cells in hub compaction

Our series of experiments in explanted gonads demonstrate that hub cell compaction occurs following hub cell assembly. Compaction of hub cells has not been described previously and we have developed methods to image and quantify the process. Previous experiments have provided anecdotal evidence that germ cells play a role in hub compaction[65,158]. Mutants that fail to specify germ cells, which result in agametic gonads, appear to have less compact hubs. However, these observations are confounded by the fact that germ cells function to restrict hub cell fate to the anterior by activating receptor tyrosine kinase signaling in the posterior. Thus, agametic gonads specify more hub cells and in doing so, generate hubs that cover a larger area[65,66]. Our

data suggest a role for germ cells in regulating hub cell compaction in addition to hub cell fate. There is a growing amount of evidence that stem cells can contribute to their tissue-specific niches. For example, the follicle stem cells in the Drosophila ovary secrete the ECM protein LamininA that serves to anchor them within their niche[159]. Additionally adult hippocampal neural stem cells secrete the neurogenic growth factor VEGF that is important for their survival[160]. Here, we also propose an intriguing mechanism for the germline stem cells in shaping their niche. Based on our observations, we hypothesize that GSCs are recruited from the PGC pool to the hub, and then function in compaction by orienting their divisions orthogonal to the hub. We have designed future experiments to test for a requirement for germ cell divisions in hub compaction and measure tension generated at the GSC-hub interface.

In order to determine whether germ cell divisions are required for hub compaction, we will express the cell cycle regulator *tribbles* in germ cells. Tribbles facilitates the degradation of Cdc25, a phosphatase required for the G2/M transition[161]. PGCs are arrested in G1 of the cell cycle during their migration through the embryo but reentry into the cell cycle occurs during late stage embryogenesis in male gonads due to Jak/STAT signaling from the gonadal soma. Therefore, we suspect overexpression in newly specified PGCs will allow sufficient time for Tribbles to accumulate and promote degradation of Cdc25 to prevent mitosis. An alternative experiment is to decrease Jak/STAT signaling, given its role in cell cycle reentry. We have been unsuccessful using double stranded (ds) RNA to knockdown mRNA in the short time window of gonadogenesis. Instead, we will attenuate Jak/STAT signaling by expressing the STAT inhibitor, *suppressor of cytokine signaling 36e (socs36e)* in germ cells and examine an affect on hub compaction[162].

Future experiments are also planned to measure tension along the hub cell-GSC interface. Our hypothesis is that the mitotic spindle generates force against the cell cortex. Because the spindle is oriented, this force would be directed towards the hub cell interface, thus recruiting f-actin and MyoII within the hub cell. If our hypothesis is true, laser ablation of the mitotic spindle or low concentrations of nocadazole treatment to selectively deplete astral microtubules should prevent recruitment of MyoII and compaction. Additionally, we can measure the relative tension at the hub cell-GSC interface prior to and during compaction by laser cutting and measuring the retraction velocity of released vertices. We would expect the retraction velocity to be higher during compaction when tension is greater[163]. Preliminary attempts at laser cutting hub-GSC interfaces during compaction also resulted in an increase in area of an adjacent hub cell (data not shown). This suggests that severing one part of the acto-myosin cable disrupts compaction in nearby cells.

While we favor a model in which germline divisions function to compact hub cells, an alternative hypothesis is that differential adhesion between the hub cells and germ cells generates the acto-myosin cable. Differential expression of the homotypic adhesion protein Echinoid in a field of cells results in like cells sorting together. Furthermore, the Echinoid-depleted interface between the two cell types generates a smooth border with an acto-myosin contractile network similar to what we observe in hub cell compaction[132]. This mechanism functions in multiple tissues during *Drosophila* development including dorsal closure of the ectoderm over the amnioserosa and generation of the dorsal appendages from the ovarian follicular epithelium. A previous post-doc in the lab (S.Dilks) showed that Echinoid is expressed in hub cells of the adult testis. Therefore, it would be worthwhile to determine whether Echinoid is expressed in hub cells or germ

cells during gonadal development and examine the effects of Echinoid loss-of-function in hub compaction.

Niche architecture and niche function

Finally, we would like to address whether proper niche architecture is required for niche function. In the past, we have been unable to address the consequences of failed niche organization because we had little insight into what was required for the process. My thesis work has contributed to the understanding of niche specification and made breakthroughs in identifying two distinct phases of niche morphogenesis. Future work can address how the initial specification of hub cells regulates their morphogenesis and what effects morphogenesis has on niche function. Given that niche signaling must be spatially restricted to allow for differentiation outside of the niche we expect niche architecture to have critical roles in regulating this signaling. The Drosophila GSC niche is perhaps the best-characterized stem cell niche, and we expect our findings here to have broad implications for building a tissue-specific niche during development.

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