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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Consequences of ectopic JAK/STAT pathway activation in the *Drosophila* male germline

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Eric Robert Murtfeldt

Committee in charge:

Professor Leanne Jones, Chair
Professor David Traver, Co-Chair
Professor Amy Kiger

2008

The Thesis of Eric Robert Murtfeldt is approved, and it is acceptable in quality and form for publication on microfilm:

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University of California, San Diego

2008

TABLE OF CONTENTS

Signature Page……………………………………………………………...…... iii

Table of Contents……………………………………………………………….. iv

Acknowledgements...…………………………………………………………… v

Abstract ………………………………………………………………………..… vi

Introduction………………………………………………………………………. 1

Materials and Methods……………………...………………………………..…… 9

Results………..…………………………………..……………………………… 12

Concluding Thoughts…………………………………………………………… 31

Appendix…………....…………………………………………………………… 33

References.……………………………………………………………………… 51

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ABSTRACT OF THE THESIS

Consequences of ectopic JAK/STAT pathway activation in the *Drosophila* male germline

by

Eric Robert Murtfeldt

Master of Science in Biology

University of California, San Diego, 2008

Professor Leanne Jones, Chair

Professor David Traver, Co-Chair

Stem cell maintenance is an essential process that is needed to preserve a delicate balance between self-renewal and differentiation. Excess amounts of self-renewal can lead to tumorigenesis, while too little can lead to stem cell depletion. In the male *Drosophila* gonad, local activation of the JAK/STAT pathway by the ligand unpaired (Upd) is needed to maintain this

stem cell self-renewal. Here it is shown that selective ectopic expression of Upd in certain germline cells is sufficient to induce over-proliferation of both early germline and somatic cells. This over-proliferation can be induced through activation of JAK/STAT signaling in germ cells as late as partially differentiated spermatogonia, suggesting that these cells may possess the ability to de-differentiate. Over-expression of the Upd homologue Upd2 also appears to be sufficient to induce over-proliferation, and may actually have a more pronounced effect on somatic cells and disrupt the transition from spermatogonial cysts to spermatocytes. The response of these cells to ectopic JAK/STAT activation can help further our understanding of how stem cell self-renewal is organized, and the ways in which the surrounding somatic cells support this process.

Introduction

Stem cells are essential because they allow for the maintenance and regeneration of new tissue throughout the lifespan of an organism due to their unique ability to both self-renew and to initiate differentiation along a specific lineage. This allows for the maintenance of cell populations that are highly differentiated but short-lived such as skin, blood, or sperm. The decision between self-renewal and differentiation upon division must be highly regulated. If too many cells differentiate, this could lead to depletion of the stem cell pool. On the other hand, if too many stem cell daughters continue to self-renew, this could lead to an expansion of the stem cell population, leading to tumorigenesis. Understanding how the decision between self-renewal and initiation is regulated is an important part of figuring out how these cells function.

The *Drosophila* germline stem cell (GSC) system

In *Drosophila*, spermatogenesis is maintained by a small population of germ line stem cells (GSCs) that surround a cluster of somatic support cells, called the hub, that is located at the apical tip of the testis (Fig 1A, D). GSCs normally divide such that one daughter cell remains in contact with the hub and retains stem cell identity, while the other cell is displaced away from the hub and initiates differentiation (Fig 1A, B). The hub serves as a critical

component of the stem cell microenvironment, or niche, by expressing the ligand Unpaired (Upd), which activates signaling via the Janus kinase-signal transducer and activators of transcription (JAK/STAT) pathway. Upd acts as a self-renewal signal and ensures that the daughter cell in contact with the hub will maintain stem cell identity, while in the absence of Upd, the other daughter cell initiates differentiation into a gonialblast. The GSCs are also surrounded by a group of somatic stem cells, called cyst progenitor cells (CPCs), which give rise to somatic cyst cells (Fig 1A, B). These cyst cells encapsulate the newly divided gonialblasts and help to guide them down the differentiation pathway. The gonialblasts undergo four rounds of mitotic transit-amplification division with incomplete cytokinesis to give rise to a cluster of 16 interconnected spermatogonia (Fig 1B). During this amplification stage, the cyst cells encapsulate the dividing germ cells and provide support without undergoing additional rounds of division themselves.

The JAK/STAT signal transduction pathway in *Drosophila*

In the canonical model of JAK/STAT signaling, the extracellular ligand Upd binds to the transmembrane receptor Domeless (Dome). This causes a conformational change in Dome that leads to the activation and trans-phosphorylation of the receptor and the attached JAK homolog, Hopscotch (Hop). Once activated, the Dome/JAK complex provides a docking site for the STAT homolog Stat92E, leading to the subsequent phosphorylation and

activation of STAT. Once activated, Stat92E dimerizes, is translocated into the nucleus, and then binds to specific DNA sequences to activate transcription of downstream target genes.

Males carrying viable mutations in *hop* are sterile. Primordial germ cells are established initially in these flies, but over time both the somatic and germline stem cells are lost, giving rise to an adult testis full of only mature sperm (Kiger et al., 2001; Tulina and Matunis, 2001). In the *hop* mutants, the system is unable to maintain a population of either germline or somatic cells, resulting in a complete depletion of the early stages of spermatogenesis.

In addition, Stat92E mutant GSC clones are lost, suggesting that Stat92E activity is required for GSC maintenance (Kiger et al., 2001; Tulina and Matunis, 2001). In both of these examples GSCs lose the ability to self-renew in the absence of JAK/STAT signaling, but the mutant germ cells are able to initiate and complete terminal differentiation. Therefore, JAK/STAT signaling is required to maintain a stable population of stem cells at the apical tip of the testes.

In contrast, when the JAK/STAT pathway is hyper-activated by ectopically expressing Upd in the germline, there is excess self-renewal and tumorigenesis occurs (Kiger et al., 2001). In these tumor-like bodies, both somatic and germ cells respond to the excess self-renewal signal and fail to proceed down the differentiation pathway.

While JAK/STAT signaling has been very well characterized and studied in many different organisms, one component that has received significantly less attention is the different activating ligands. Many different ligands, such as cytokines and growth factors (Boulay et al., 2003), have been identified and implicated in JAK/STAT signaling in vertebrates and other organisms, but the only ligands to be identified in *Drosophila* are the Unpaired-like family of proteins (Harrison et al., 1998). This family of proteins is made up of the three homologous proteins Upd, Upd2, and Upd3. While each ligand has been shown to possess the ability to activate the JAK/STAT pathway, they each have slightly different properties that affect their potential signaling ability.

The most well-known and best-characterized member of the family is Upd. Previous studies have shown that *upd* encodes a glycosylated protein capable of potent JAK/STAT activation and shows strong association with the extracellular matrix (Harrison et al., 1998). Contrary to Upd, very little is known about the other two homologues. A recent study showed that Upd3 appears to play a role in the immune response in the fat body following an injury (Agaisse et al., 2003). Even less is known about third homologue Upd2. Previous studies have shown that *upd2* can be semi-redundant to *upd* during development: in early embryonic pattering, *upd2* is able to partly rescue the phenotypes resulting from loss of JAK/STAT signaling in the absence of *upd* (Gilbert et al, 2005). Analysis of Upd-like proteins revealed that the coding

sequences of Upd and Upd2 are very similar, and both have been shown to possess the ability to activate JAK/STAT signaling in vivo. Tissue culture-based assays have also demonstrated that both Upd and Upd2 are secreted ligands capable of conditioning surrounding media (Hombria et al., 2005). However, visualization of these two homologues using GFP fusion proteins has shown that Upd associates with the extracellular matrix (ECM), while Upd2 is restricted by its N-terminal anchoring sequence, and shows minimal ECM association (Hombria et al., 2005). Although both Upd and Upd2 are capable of potent JAK/STAT pathway activation, they may be secreted through different mechanisms and activate JAK/STAT signaling differently.

Potential tumor formation mechanisms

When the JAK/STAT pathway is hyper-activated in the germline it leads over-proliferation and a tumor-like growth at the apical tip of the testis (Kiger et al., 2001). There are several different mechanisms that could be contributing to this observed phenotype. One possibility is that GSCs continue to divide asymmetrically, but at a much higher rate, creating an abundance of early germ cells around the hub that may disturb the ability of these cells to differentiate. Another possibility is that the increased self-renewal signal may cause GSCs to begin dividing symmetrically, resulting in two new GSCs from every division instead of one GSC and one gonialblast. This would cause an increase in the amount of early germ cells present at the apical tip, and would

disrupt differentiation by decreasing the number of gonialblasts that start down the differentiation pathway. The ability of early germ cells to re-populate the stem cell niche through de-differentiation may also play a role in this observed phenotype (Brawley and Matunis, 2004). Another possibility is that the build-up of early germ cells at the tip of the testis may be the result of a failure of partially differentiated gonialblasts to proceed down the differentiation pathway. Each of these potential mechanisms may play a role in the massive build-up of early germ cells present at the apical tip of the testis in this observed phenotype.

De-differentiation of progenitor cells into functional stem cells

In the *Drosophila* testis, partially differentiated germ cells (spermatogonia) have been shown to possess the ability to re-populate the stem cell microenvironment and replace lost GSCs. Depletion of the GSC population through the conditional manipulation of JAK/STAT signaling is sufficient to cause partially differentiated early germ cells to re-populate the stem cell microenvironment and take on the identity of a GSC (Brawley and Matunis, 2004). This is important because it indicates that even partially differentiated germline cells maintain their ability to respond to the Upd self-renewal signal. Having this capability allows these partially differentiated germ cells to revert to a stem cell fate when placed in close proximity to the hub during either aging or as the result of injury.

Spermatogonial cyst to spermatocyte transition

Ectopic expression of *upd* in early germ cells has previously been shown to bias stem cell divisions towards self-renewal, at the expense of differentiation (Kiger et al., 2001, Tulina and Matunis, 2001). Expansion of the cyst cell pool may compromise the ability of these cells to support and guide germ cells through spermatogenesis. Later stage germ cells initiate expression of the *bag-of-marbles* (*bam*) gene during mitotic amplification divisions. When expression of *bam* is lost, the transition from 16 cell spermatogonial cysts to spermatocytes is disrupted, resulting in continued spermatogonial division and eventually cell death (Gonczy et al., 1997). The changes in the cyst cell population as a result of ectopic *upd* expression in various parts of the germline may also play a large role in the observed phenotypes, and may have an effect on the spermatogonial cyst to spermatocyte transition.

Given these observations, I sought to address the mechanisms by which ectopic JAK-STAT signaling leads to tumorigenesis in the Drosophila male gem line. The Specific Aims of this proposal are:

Specific Aim #1: To characterize the phenotypes resulting from over-expression of *upd* in various cellular locations at the apical tip of the testis.

Specific Aim #2: To assess the ability of the Upd homolog Upd2 to activate the JAK/STAT dependent self-renewal pathway when ectopically expressed in and around the germline in the testis.

Specific Aim #3: To investigate the effects of ectopic JAK/STAT activation on both germline and somatic cells that have already initiated mitotic amplification and are beginning to proceed down the differentiation pathway.

Materials and Methods

Fly husbandry and stocks

Flies were raised at 25°C on standard cornmeal-molasses-agar medium. Fly strains used in this study are Oregon R (OreR), *nanos*GAL4:VP16/MKRS, *bam*GAL4:VP16/TM3, *C587*GAL4, *upd*GAL4, *E132*GAL4, UAS-*EGFP2x*/MKRS, UAS-*upd*-GFP/TM3, UAS-*upd2*-GFP/Cyo, UAS-*upd*/CyO.

Ectopic expression of Upd family proteins

To accomplish the goal of ectopic expression of the Upd ligands, Upd and Upd2 were selectively expressed in the hub cells, cyst cells, germ cells, and 4-8 cell spermatogonial cysts using a collection of GAL4 lines that have been previously characterized. The following lines were used: *nanos*GAL4:VP16/MKRS, *bam*GAL4:VP16/TM3, *C587*GAL4, *upd*GAL4, *E132*GAL4. These GAL4 lines were crossed with a UAS-*upd*-GFP line and a UAS-*upd2*-GFP line that were previously obtained. The ligands in both lines were tagged with GFP for visualization purposes.

Testis squashes, immunofluorescence, and microscopy

Testes were dissected in phosphate-buffered saline (PBS) and examined using phase contrast microscopy. The phase contrast images of squashed testes were obtained using a Leica DM5000 microscope paired with a DC500 camera using Firecam imaging software. Immunofluorescence imaging was performed on squashed testes using one of the following procedures. For the squash method, testes were dissected in PBS solution and immediately squashed with poly-lysine-coated slides/SuperFrost Plus and frozen in N_2(*l*). Following a EtOH wash testes were fixed in 4% Formaldehyde for 7 minutes, rinsed in PBS, and then stained using standard Immunofluorescence staining techniques. For the whole mount method, testes were dissected and fixed in 2% PFA in PLP buffer (0.075 M lysine, 0.01 M sodium phosphate buffer pH7.4) for 1 hour at room temperature, rinsed in PBS, followed by standard Immunofluorescence staining techniques. Images were captured using a Zeiss Axiovert 200 microscope and processed using AxioVision (version 4.5; C. Zeiss) and Adobe Photoshop software (Mountain View, CA).

The guinea pig anti-traffic jam (1:3000) (gift from D. Godt), mouse anti-GFP (1:200) (Molecular Probes), and the rabbit anti-phosphorylated Histone H3 (1:200) (Upstate Biotechnologies) antibodies were obtained and used at the noted concentrations during staining. The mouse anti-α-spectrin (3A9)(1:10), mouse anti-Fasciclin III (7G10)(1:10), rat anti-vasa (1:10), and mouse anti-eyes absent (Eya10H6)(1:10) antibodies were obtained from the

Developmental Studies Hybridoma Bank developed under the sponsorship of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences. All of the secondary antibodies were obtained from the Molecular Probes and used at a 1:100 concentration. Samples were mounted for observation with Vectashield mounting medium with DAPI (Vector Laboratories).

Results

Expression in early germ cells

When *upd* is ectopically expressed at the tip of the testes it activates excess JAK/STAT signaling and causes large amounts of over-proliferation at the apical tip, resulting in predominantly spherically shaped testes (Fig 2B). This phenotype is presumably due an excess amount of the JAK/STAT "self-renewal" signal present in the germline, and results in a tumor-like growth of small and undifferentiated cells.

Ectopic expression of the Upd ligand in the early germ cells was accomplished using a *nanos*-Gal4 driver (Fig 2A). Expression of Upd in this pattern produced viable adults with overgrown testes that have a large bulbous apical tip. When viewed using phase contrast imaging, the body of the testis has been completely overwhelmed by the massive overgrowth at the apical tip and has taken on an almost entirely spherical morphology (Fig 2B). The testis is densely packed of almost entirely of small cells that are the result of increased self-renewal in early germ cells and a decrease in differentiation (Fig 2B).

The small cells that have accumulated in these testes are typical of GSCs and gonialblasts. In wild-type testes, GSCs are normally connected to gonialblasts by a cytoplasmic bridge that is occupied by a small and spherical

fusome. Once these gonialblasts differentiate into spermatogonia their fusome becomes more branched (Fig 2F). Staining with the fusome marker α-spectrin shows that in the testes where Upd is ectopically expressed in early germ cells, the majority of cells contained within the expanded apical tip have spherical fusomes (Fig 2H). Immunofluorescence staining of these testes with the mitotic marker phosphorylated Histone H3 (PHH3) also indicated that many cells in these testes are mitotically active and dividing individually (Fig 2K). These are both indications that the cells present in the expanded apical tip are mostly GSCs or gonialblasts.

Staining with the early cyst cell marker traffic jam (TJ) showed that the number of cyst cells present in these testes was also greatly increased (Fig 3D-F). This suggests that CPCs or early cyst cells are able to proliferate in response to either the Upd signal itself, which is being secreted from germ cells, or through a secondary signal that is activated during the massive expansion of germ cells.

In addition to the cyst cells, the hub morphology was affected in all of the observed testes. It appears that the normal apical hub still exists, but there are also other ectopic regions that express the hub cell marker Fasciclin III (fas III). These ectopic fas III positive regions are often larger than a normal hub and contain cells that are less tightly packed (Fig 3A-C). While these regions are positive for fas III, it has yet to be shown if they retain the functional properties of a wild-type hub. These results indicate that forced

expression of Upd in early germ cells also causes an increased amount of self-renewal and over-proliferation of the somatic cell population. Since Upd is a secreted ligand, this may be due to a direct response to the Upd signal itself, or a secondary effect resulting from the over-proliferation of early germ cells.

The observed over-proliferation phenotype could be caused by several different mechanisms. One possibility is that GSCs continue to divide asymmetrically, but at a much higher rate, creating an abundance of early germ cells around the hub that aggregate before they have a chance to differentiate. Another possibility is that GSCs may begin dividing symmetrically. This would result in two new GSCs from every division instead of one GSC and one gonialblast, causing an increase in the amount of early germ cells present at the apical tip and a disruption in differentiation. Ectopic activation of the JAK/STAT pathway in early germ cells may also be sufficient to cause de-differentiation. As a result, partially differentiated gonialblasts would be able to revert to a GSC instead of proceeding down the differentiation pathway, resulting in excess GSCs at the expense of differentiation. Another possibility is that Upd signaling in early germ cells may simply result in a breakdown of the differentiation pathway and an aggregation of early germ cells that have failed to differentiate. Each of these potential mechanisms may play a role in the observed over-proliferation phenotype and build-up of early germ cells present at the apical tip of the testis.

Expression in hub cells

Ectopic expression of Upd in the hub was accomplished by using both an *upd*-GAL4 and an *E132*-GAL4 driver (Sup Fig 1E). When these drivers were crossed to the UAS-*upd*-GFP line, the apical tip of the testis of a 1-day-old male appears to be the same as a normal wild-type 1-day-old male (Sup Fig 1F). This is the expected result based on the fact that Upd is normally expressed exclusively in the hub. The additional expression of Upd in the hub does not appear to have a profound effect other than continuing to encourage self-renewal of the surrounding stem cells. The normal realm of influence of the Upd ligand appears to be restricted to the hub and first immediate cell layer surrounding it despite the increased amount of expression.

Expression in cyst cells

The expression of Upd in the cyst cells was achieved using a *C587*-GAL4 driver paired with the UAS-*upd*-GFP cell line (Sup Fig 1A). The testes of 1-day old adults produced from this cross appeared to also be the same as the testes of a 1-day old wild-type type fly (Sup Fig 1B). One possible explanation for this is that somatic cells are not able to respond to the Upd signal directly in the same manner as germ cells. Secretion of the Upd ligand may also be compromised in cyst cells preventing activation of the JAK/STAT pathway in the surrounding germ cells. Somatic cell over-proliferation appears to hinge more on the number of germ cells present that need cyst cell support.

Over-expression of Upd in cyst cells alone does not appear to be sufficient to cause an increase in CPC proliferation or to prevent the continued differentiation of early cyst cells along with the germ cells they enclose (Sup Fig 1C,D). Signaling in the somatic cells of the testis may be controlled by a different set of pathways or ligands.

Expression in spermatogonial cells

The effects of ectopic Upd expression at the 4-8 cell spermatogonial cyst stage were assayed using a *bam*-GAL4 driver (Fig 4A). The *bam* gene has previously been indicated in the spermatogonial cyst to spermatocyte transition, and its expression is thought to be restricted to the 4-8 cell spermatogonial cyst stage (Schulz et al., 2004). The expression of the Upd ligand later in the germline will help to determine the ability of partially differentiated germ cells to respond to a self renewal signal.

When Upd is expressed in 4-8 cell spermatogonial cysts, the testes in these flies also appeared to contain an increased amount of cells at the apical tip (Fig 4B). However, the morphology of these testes is largely maintained and the tip expansion is smaller what is observed when Upd is over-expressed in early germ cells. The expanded region at the tip also appears to be separated from the rest of the body of the testis by a refractile region that may be a result of apoptosis (Fig 4B). Immunofluorescence staining with the fusome marker α-spectrin reveals that the cells at the apical tip contain both

spherical and branched fusomes (Fig 4F-H), indicating the presence of both early stage GSCs and gonialblasts along with spermatogonia. Staining with the mitotic marker PHH3 confirmed that there are cells dividing both individually and synchronously in groups present in the apical tip (Fig 4I-K). This again suggests the presence of both early stage GSCs and gonialblasts along with later stage spermatogonia.

Expression of Upd in 4-8 cell spermatogonial cysts also causes a marked increase in the somatic cell population. In wild-type testes, TJ staining of cyst cells is present only in the immediate vicinity of the hub (Fig 3E). However, ectopic *upd* expression in the transit-amplification stage causes a large increase in the TJ positive cyst cell population throughout the apical tip (Fig 5D-F). In addition to the cyst cell expansion, ectopic fas III positive populations similar to what was observed when Upd was driven in the early germ cells were present in 40% (n=25) of the testes observed (Fig 5A-C). While the number of ectopic fas III cell clusters observed is reduced from the earlier expression of *upd* in early germ cells, the size of these fas III positive cell populations often appears to be larger and the cells appear to be less tightly packed together. The location of these ectopic fas III populations can also be variable throughout the over-proliferating region, and is not limited to areas surrounding the initial hub. The expansion of the TJ positive region and presence of ectopic fas III indicates that the somatic cell population at the 4-8 cell spermatogonial cyst stage has the ability to respond either to the increase

in *upd* expression directly, or as a result of germline expansion and over-proliferation.

The observed over-proliferation caused by expression of *upd* in 4-8 cell spermatogonial cysts may be due to several different mechanisms. One possibility is that activation of the JAK/STAT pathway in spermatogonial cyst cells may be sufficient to cause de-differentiation. This would cause spermatogonial cells to revert to either a gonialblast or a GSC identity instead of proceeding down the differentiation pathway. Another possibility is that JAK/STAT pathway activation in spermatogonial cysts may cause a breakdown in differentiation and an aggregation of partially differentiated spermatogonial cysts. The last possibility is that Upd signaling in spermatogonial cysts may disrupt the transition from 16 cell spermatogonial cysts to spermatocytes. This possibility will be explored in more detail in specific aim #3. Each of these potential mechanisms may play a role in the observed over-proliferation phenotype present at the apical tip of the testes.

The ability of Upd to cause over-proliferation in the apical tip of the testes when expressed exclusively in spermatogonial cysts suggests that even partially differentiated germ cells maintain some self-renewal ability. The fact that Upd is a glycosylated ligand that is tightly controlled makes it somewhat unlikely that the ligand would be able to travel across several cell distances to contact early germ cells when expressed exclusively in 4-8 cell spermatogonial cysts. Also, the fact that ectopic *upd* expression caused over-

proliferation of somatic cells suggests that these cells can either respond to Upd signaling themselves, or that they are able to respond to the over-proliferation of germ cells and that there is some communication between germline and somatic cells.

Comparing the effects of Upd and Upd2

Another area of interest is the relationship of the two secreted JAK/STAT homologues Upd and Upd2. Both ligands have been previously shown to be potent JAK/STAT activators and may be semi-redundant in development (Hombria et al., 2005). However, due to an N-terminal anchoring sequence, Upd2 is not able to associate with the ECM in the same manner as Upd. As a result, they may be secreted by slightly different mechanisms and may elicit different responses when ectopically expressed in the testis.

As expected, ectopic expression of UAS-*upd2*-GFP in early germ cells under the control of the *nanos*-GAL4 driver is able to activate JAK/STAT signaling and results in an over-proliferation phenotype (Sup Fig 2B). This phenotype closely resembles the one observed earlier when UAS-*upd*-GFP was ectopically expressed in the early germ cells using the n*anos*-GAL4 driver (Sup Fig 2A). This suggests that Upd2 is also able to activate JAK/STAT signaling and induce over-proliferation when ectopically expressed in early germ cells at the apical tip of the testis.

While the over-proliferation phenotype may be slightly less severe with Upd2 then with Upd, many of the same over-proliferative characteristics were observed between the two phenotypes. Ectopic regions staining positive for the hub cell marker fas III were observed in 85% (n=20) of the Upd2 expressing testes, and appeared to have a similar morphology to the ones

observed earlier as a result of Upd over-expression (Sup Fig 2I, J). Also, the α-spectrin staining indicates that the cells present at the bulbous apical tip are mostly early stage gonialblasts or GSCs due to the presence of predominately spherical fusomes (Sup Fig 2E, F). The mitotic marker PHH3 also shows that a large number of these cells are mitotically active and are dividing individually, indicating that these cells are mostly early germ cells that have yet to enter the transit-amplification stage (Sup Fig 2C, D). Staining with the cyst cell marker TJ shows that the somatic cell population is greatly expanded in both phenotypes as well (Sup Fig 2G, H). These images indicate that Upd2 is able to activate the JAK/STAT signaling pathway ectopically in early germ cells in a similar manner to Upd despite the published differences in cellular localization and possible different secretion mechanisms between the two ligands.

Several interesting phenotypes were observed when comparing the ectopic expression of Upd-GFP and Upd2-GFP in the 4-8 cell cyst stage using the b*am*-GAL4 driver. Ectopic expression of either Upd or Upd2 during the transit-amplification stage resulted in over-proliferation. However, the tumor-like expansion present at the apical tip is noticeably larger when Upd is expressed rather than Upd2 (Fig 6A, B).

Ectopic expression of Upd2 in the 4-8 cell spermatogonial cyst stage also produces very similar fusome morphology to what was observed during Upd expression. Immunofluorescence staining with α-spectrin shows that the

cells inside of the tumor-like growth contain both branched and spherical fusomes, indicating the presence of both early and later stage germ cells (Sup Fig 3C, D). The presence of both individual and groups of PHH3 positive staining cells further confirms that there are both early and later stage germ cells present in the tumor-like growth (Sup Fig 3G, H).

Despite the similarities between the germ cell populations, there were a few differences observed in the somatic cells when comparing the Upd and Upd2 phenotypes. As mentioned earlier, expression of Upd in 4-8 cell spermatogonial cysts produced testes that had at least one ectopic fas III positive site 40% of the time. However, when Upd2 expression was driven in this same cell population, there were no instances of ectopic fas III positive regions observed (Fig 6C-F). This may be a result of the typically smaller amount of over-proliferation observed when expressing Upd2, but also raises the question whether Upd and Upd2 have different effects on the somatic population.

Another difference between Upd and Upd2 was observed when looking at staining with the cyst cell marker TJ. When Upd is driven in 4-8 cell spermatogonial cysts, there is a TJ expansion that spans the entire area of the tumor-like region present at the apical tip (Fig 6G, I). However, when Upd2 is driven in this same region, there is a subtle expansion of TJ at the apical tip, but also a larger expansion of TJ outside of the tumor-like region and distally down the body of the testis (Fig 6H, J). These two TJ positive cell populations

are separated by a noticeable gap that appears immediately after the region of over-proliferation at the apical tip and spans several cell distances before contacting the second TJ positive cell population. This brings up several interesting questions regarding the ability of Upd and Upd2 to cause different somatic cell phenotypes when expressed at the 4-8 cell cyst stage.

Consequences of *upd* expression in partially differentiated germ cells

The most interesting observed phenotypes from the earlier screen were observed while judging the ability of partially differentiated cells to respond to ectopic activation of the JAK/STAT pathway. The earlier results suggest that activation of JAK/STAT at this stage with the ligand Upd2 may be sufficient to cause a drastic expansion in TJ positive cyst cells away from the apical tip of the testis. Also, it also appears that at least early spermatogonial cyst cells do in fact maintain their ability to respond to JAK/STAT signaling and can still initiate self-renewal when exposed to Upd signaling. The ability of later stage spermatogonial cells to respond to JAK/STAT signaling may be decreased, but it appears to at least partially disrupt the transition of spermatogonial cysts into spermatocytes.

The somatic cell population expansion observed is much larger when Upd2 is ectopically expressed in the 4-8 cell spermatogonial cyst stage rather then Upd, and appears to get larger with age. This expansion of TJ positive cells is present in the tumor-like region at the apical tip, and often extends distally down the body of the testis towards the spermatocytes. The TJ positive cells contained within the tumor-like region at the apical tip and those further down the body of the testes are also often separated from each other by a well-defined gap of several cell distances (Fig 7A, B). The morphology of

the nuclei of these TJ positive somatic cells that are outside of the tumor-like region also appears to be changed, and they appear to be more spherical. This may occur because these TJ positive cyst cells are no longer surrounding and providing support for individual germ cells or spermatogonial cysts.

There is also a marked increase in TJ positive cells present in aged testes over-expressing Upd2 in 4-8 cell spermatogonial cysts. The tumor-like region present at the apical tip does not appear to get larger with age, but the amount of TJ positive cells present within the body of the testes is drastically increased in a 20-day old testis (Fig 7B, C). This expansion of TJ positive cells within the body of the testes is again isolated from the apical tip by a well-defined gap that is several cell distances (Fig 7D, E). The TJ positive cells at the apical tip appear to be surrounding spermatogonial cysts and resemble early cyst cells seen in wild-type testes. However, the nuclei of the TJ positive cells further from the tip and in the body of the testis appear to be much more isolated and have taken on a much more rounded morphology. They also do not appear to be supporting any spermatogonial cysts and are found predominately along the inner walls of the lumen of the testis (Fig 7F, G).

Further investigation using the later stage cyst cell marker eyes absent (Eya) shows that there may be separate somatic cell populations present within the testis. In a 1-day old testis that is over-expressing Upd2 in the 4-8 cell cyst stage, there is already the presence of two separate TJ positive populations, with one at the apical tip and one several cell distances away

towards the body of the testis (Fig 8C, D). In addition to these two populations, there are also Eya positive cyst cells present in both populations of TJ positive cells and within the gap separating them. The area immediately surrounding the hub contains only TJ positive somatic cells, indicating the presence of cyst progenitor cells (CPCs) and early cyst cells, and taken alone largely resembles a wild-type apical tip (Fig 8A, B). However, once you start looking several cell distances away from the hub you see somatic cells that stain positively for both TJ and the late cyst cell marker Eya. There is then a section that is void of germ cells and contains sparse amounts of Eya positive and TJ negative cyst cells. However, on the other side of this gap there is another population of TJ positive cyst cells that are Eya negative, few Eya positive cyst cells that are TJ negative, and also cells that are both TJ and Eya positive (Fig 8C, D). While the cellular makeup of these testes appears to be maintained immediately around the hub, only several cell distances away there are dramatic changes in both the somatic and germ cell populations. These changes seem to indicate that the somatic support cells respond either to the Upd signal itself, or a cue from the surrounding self-renewing germ cells. The changes in the morphology of the nuclei of the TJ positive cells further from the hub may again be due to the fact that they are no longer encapsulating neighboring germ cells.

Further investigation with aged flies shows a marked increase in one population of TJ positive cells and a no change in the other. In 20-day old

flies, the TJ and Eya staining around the hub appears to be the same as in a 1-day old fly. However, once you observe the second population of TJ positive cells further away from the hub it becomes apparent that it is greatly increased (Fig 8E, F). The number of TJ positive and Eya negative cells is noticeably increased, while the population of Eya (+) TJ (-) and Eya (+) TJ (+) cells is also either slightly increased or simply maintained. This observation of TJ positive cells distally down the body of the testis is very unusual and much different from a wild-type testis. It is unclear whether cyst cells that have started to differentiate are reverting back to an earlier cyst cell state or if the transition from early to later stage cyst cells is disrupted. This observed phenotype raises several questions about the effects of JAK/STAT signaling on somatic cells.

Another question about JAK/STAT pathway activation in partially differentiated cells is whether or not ectopic expression of *upd* in spermatogonial cells is sufficient to cause de-differentiation. In wild-type flies, the early germ cells with spherical fusomes are found exclusively around the hub, while more differentiated cells with branched fusomes begin a few cell distances away. However, when *upd* is driven in 4-8 cell spermatogonial cysts, the area immediately around the hub containing early germ cells is noticeably increased, and additional individual spherical fusomes can be found even further from the hub mixed in with the population of more differentiated cells (Fig 9A, B). The fact that expression of *upd* in the 4-8 cell cyst stage

cases an increase in the population of both early and later stage germ cells suggests that the spermatogonial cells in the 4-8 cell cyst stage are able to respond to the Upd self-renewal signal. The presence of a significant amount of germ cells with spherical fusomes within the population of more differentiated cells also indicates that these later stage cells may be able to revert to earlier germ cells.

In addition to the presence of early germ cells further from the hub, GFP positive cells, which were presumably part of 4-8 cell cysts expressing *upd*, can occasionally be seen near the hub (Fig 9I-L). This may suggest that the cells in these 4-8 cell cysts have the ability to de-differentiate, and when placed in close proximity to the hub may be able to revert into early germ cells.

However, the majority of cells present in this tumor-like region contain branched fusomes that are characteristic of more differentiated spermatogonial cells undergoing transit-amplification (Fig 9B). This suggests that these cells may not be able to self-renew in response to Upd at the same level as GSCs and gonialblasts, which may be a result of a downgrade in the JAK/STAT signaling pathway that begins once germ cells enter into the transit-amplification stage. Another explanation may be that the supporting somatic cyst cells and germ cell linking fusome prevent all of these Upd expressing spermatogonial cells from fully de-differentiating and reverting to an early germ cell fate.

As observed earlier, the activation of JAK/STAT signaling in 4-8 cell spermatogonial cysts may result in the loss of somatic support by encapsulating cyst cells. This could possibly contribute to a mechanism for how spermatogonial cells are able to de-differentiate and take on the identity of earlier germ cells. The transition from a spermatogonial cell contained within a cyst to an early spermatogonial cell or even a GSC first requires that the cell is able to break away from the group of cells it is connected to within the cyst. Without the support of surrounding cyst cells, these spermatogonia under the influence of the Upd self-renewal signal may be able to induce a breakdown of the fusome, leading to the breaking off of individual germ cells. If this were in fact the case, one would expect that after breaking away from a cyst, it is likely that the cell quickly turns off *bam* transcription to promote self-renewal instead of differentiation.

The changes in the somatic cell population also may play a role in the formation of cysts that contain many more than 16 spermatogonial cells. Ectopic Upd expression in the 4-8 cell spermatogonial cysts seems to cause a disruption in the transition from 16 cell spermatogonial cysts to spermatocytes. In these testes, a number of spermatogonial cysts continue to increase in size past the normal 16 cells, and can contain upwards of 30 or 40 spermatogonial cells (Fig 9C). The spermatogonial cells in these cysts appear to be less tightly packed together, and are usually located just outside of the tumor-like region in the body of the testes. Staining with α-spectrin shows that the

fusomes within these overgrown cysts are still branched, but appear to be showing signs of breakdown. The branches present are much shorter than normally seen at the 16-cell stage, and no longer appear to link together the entire cluster of spermatogonial cells (Fig 9D, E). Further analysis into the division of these cells with the mitotic marker PHH3 shows that occasionally division within these larger cysts is not completely synchronized. Smaller groups of PHH3 positive spermatogonial cells can sometimes be observed within these larger cysts (Fig 9F-H). A possible explanation is that the transition of later stage cysts into spermatocytes is disrupted without the guidance of encapsulating cyst cells, and the presence of Upd causes increased proliferation in within the cluster of spermatogonial cells. If this were the case, it would suggests that cyst cells play an important role in controlling the transition of spermatogonial cysts into spermatocytes, and that later stage spermatogonial cells within 8 or even 16 cell cysts maintain at least a limited ability to respond to Upd signaling.

The activation of JAK/STAT signaling in partially differentiated germ cells causes several different over-proliferation phenotypes, and appears to show that these later stage cells maintain the ability to self-renew under the right signaling conditions. While the results suggest that both germline and somatic cell types are able to respond to JAK/STAT signaling, it is unclear to what degree and how the response leads directly to the observed phenotypes.

Concluding Thoughts

Previous studies have shown that the JAK/STAT pathway activating ligand Upd is both "sufficient and necessary" for germline stem cell self-renewal. It has also been shown that ectopic expression of *upd* in early germ cells causes massive over-proliferation at the tip of the testes of both early germline and somatic support cells. These results were initially confirmed by ectopically expressing *upd*-GFP in early germ cells and observing a similar over-proliferation phenotype. This shows that germline stem cells and gonialblasts are able to self-renew in response to Upd signaling. In addition, ectopic Fas III positive regions were also observed within the enlarged tip of the testes and may suggest the presence of ectopic niches. However, additional experimentation is required to characterize these regions for other hub characteristics.

To test the characteristics of partially differentiated cells further removed from the stem cell niche, Upd-GFP was expressed in 4-8 cell spermatogonial cyst cells. The observed over-proliferation phenotype suggests that these partially differentiated spermatogonial cells retain at least some ability to self-renew in response to Upd signaling. The presence of significant amounts of both early somatic and germline cells within the over-proliferation region also seems to indicate that either the both cell types are able to respond to the Upd signal itself, or that the supporting somatic cells

over-proliferate as a direct response to the over-proliferation of the surrounding germline cells.

While Upd is thought to normally be the only JAK/STAT activating ligand present in the testes, ectopic expression of the homologue Upd2 in the testes appeared to be sufficient for JAK/STAT activation. When expressed in early germ cells, Upd2 caused over-proliferation of both somatic and germline cells, and there were minimal observed differences between Upd and Upd2 expression. However, there were no ectopic Fas III regions present when Upd2 was expressed suggesting that the interaction of Upd2 with the somatic hub cells may be slightly different than with Upd.

A comparison of the over-proliferation phenotypes observed when either Upd or Upd2 was expressed in 4-8 cell spermatogonial cysts seemed again to indicate that there may be slight differences between the ways in which the two *upd* homologues cause self-renewal. The expression of Upd2 seemed to cause a greater expansion of somatic support cells that became more pronounced with age (20 days). It also appeared to disrupt the transition of cyst cells from early to later stage while Upd expression did not. This may indicated that Upd2 has a slightly different mechanism of causing over-proliferation of somatic cells than does Upd.

Appendix

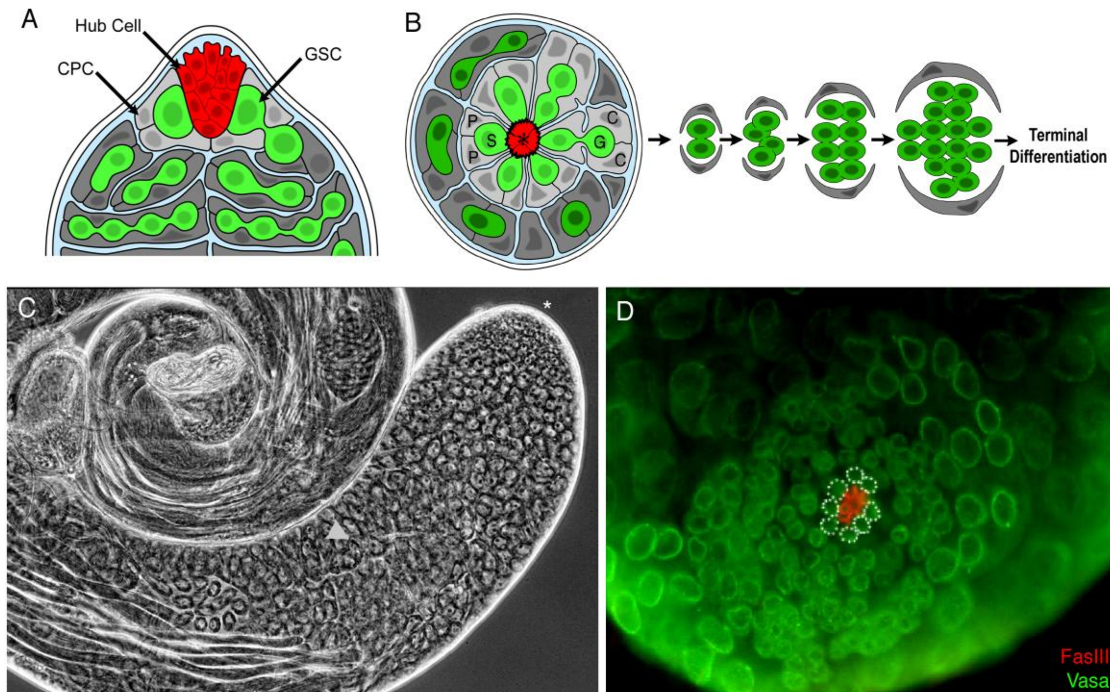


Figure 1: Male *Drosophila* Testis and Early Spermatogenesis

(A) Diagram of the apical tip of the *Drosophila* testis, showing the early stages of spermatogenesis. Both germline stem cells (GSCs) and cyst progenitor cells (CPCs) surround the apical hub (red). **(B)** Diagram of the apical tip of the testis demonstrating the progression of germline differentiation from GSCs [S] and gonialblasts [G] through four rounds of mitotic amplification producing 16 interconnected spermatogonia. CPCs [P] produce somatic cyst cells [C] that encapsulate the gonialblast and do not divide again. **(C)** Phase contrast image of a 1-day old wild-type testis. Notice the progression of differentiated cells from small early germ cells around the hub (asterisk) to spermatocytes

(arrowhead) further down the body of the testis. **(D)** Immunofluorescence image with the cell surface protein Fasciclin III (red) marking the hub and the germ cell specific antigen Vasa (green) marking early germ cells. The 8 present GSCs surrounding the hub are outlined in white for demonstration purposes.

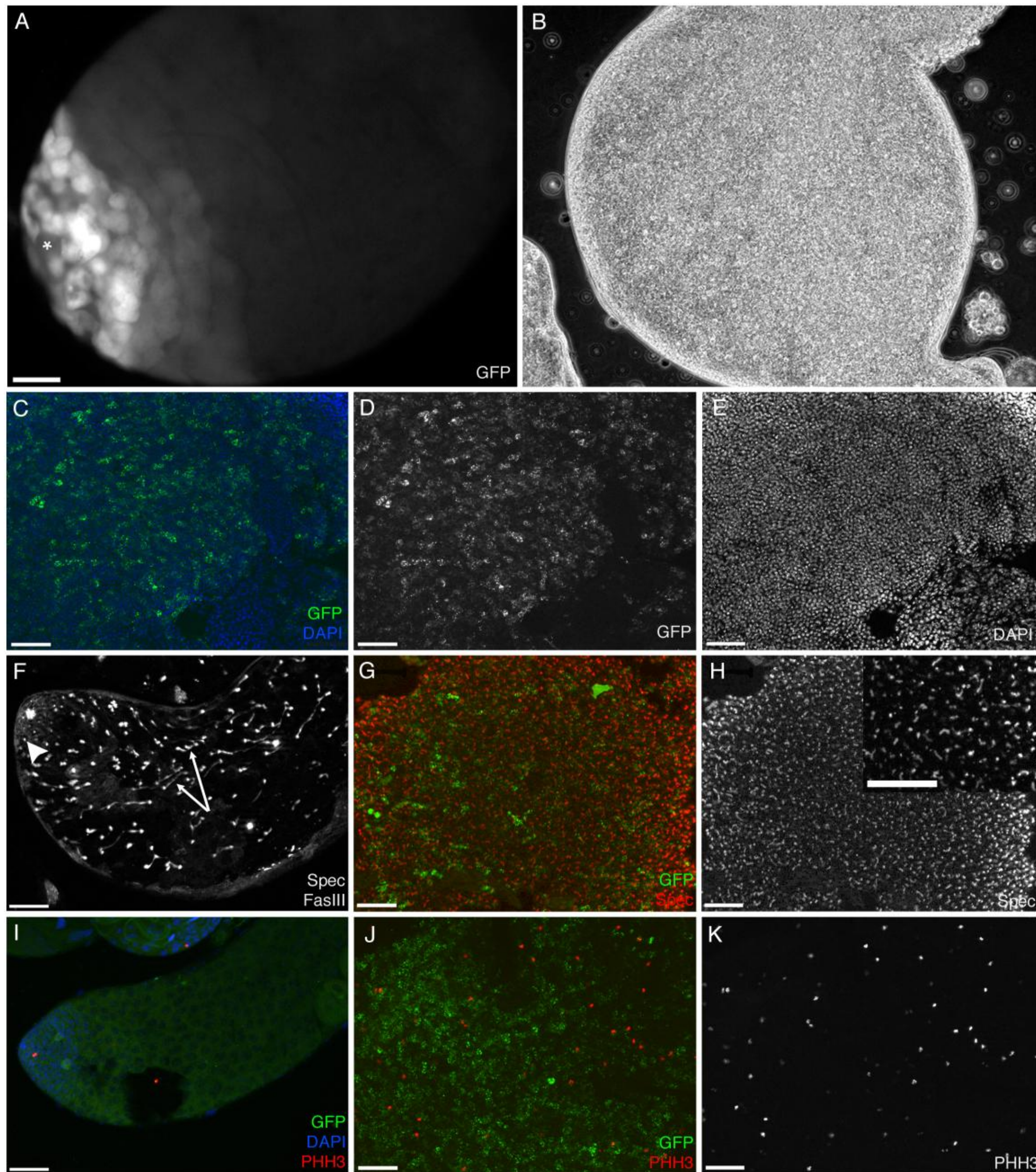


Figure 2: Ectopic expression of *upd* in the germline: Germ Cells

(**A**) 1-day old testis expressing UAS-EGFP under the control of the *nanos*GAL4 driver. Notice the concentration of GFP around the apical hub (Asterisk). **(B)** Phase contrast image of a testis with UAS-*upd*-GFP expression being driven by the *nanos*GAL4 driver. Notice the spherical appearance of the

testis and the high concentration many small cells. **(C, D,** and **E)** Immunostaining for GFP and DAPI in a testis expressing UAS-*upd*-GFP under the control of the *nanos*GAL4 driver further illustrates the over-proliferative phenotype and the abundance of both Upd and many small cells within the testis. **(F, G,** and **H)** Immunostaining for α-spectrin to mark spherical spectrosomes in GSCs and gonialblasts (arrowhead), and branched fusomes in spermatogonia (arrows) in a wild-type testis [F] and a testis expressing UAS-*upd*-GFP under the control of the *nanos*GAL4 driver [G and H]. Compare the predominance of spherical spectrosomes within the testis in G and H to the progression from spherical to branched spectrosomes present in the wild type control F. **(I, J,** and **K)** Immunostaining for phosphorylated histone H3 (pHH3) to label mitotically active cells in a wild-type testis [I] and a testis expressing UAS-*upd*-GFP under the control of the *nanos*GAL4 driver [J and K]. Notice the greater number of mitotically active cells present in J and K compared to the wild type control I, and that all of the pHH3 positive cells are dividing individually.

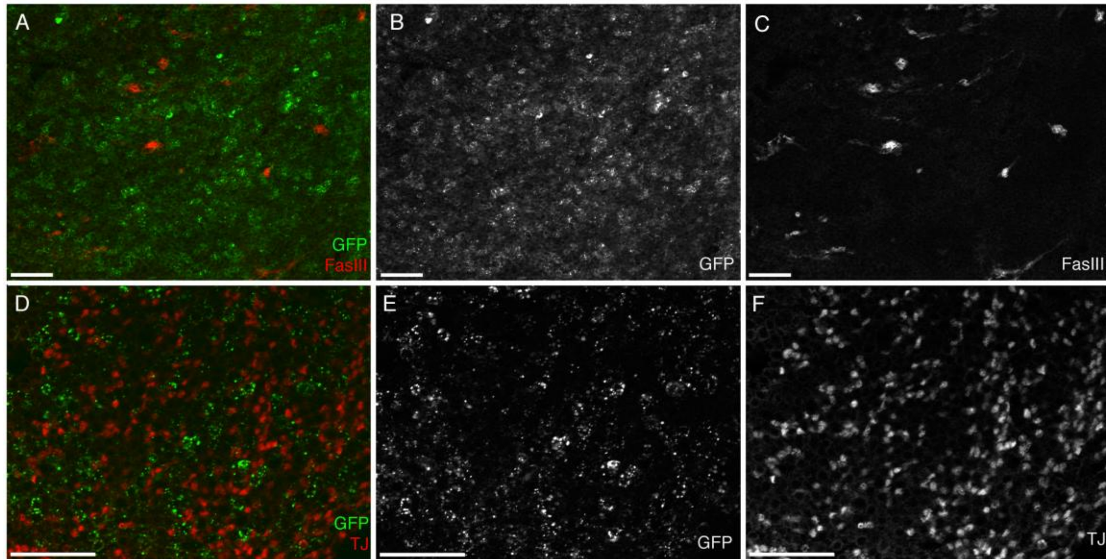


Figure 3: Ectopic expression of *upd* in the germline: Somatic Cells

(A, B, and **C)** Immunostaining for Fas III to mark hub cells in a testis expressing UAS-*upd*-GFP under the control of the *nanos*GAL4 driver. Notice the multiple Fas III positive regions present within the testis that may represent ectopic niches. **(D, E,** and **K)** Immunostaining for the early cyst cell marker Traffic Jam (TJ) in a testis ectopically expressing UAS-*upd*-GFP with the *nanos*GAL4 driver. Notice the expansion of TJ positive cells within the GFP positive region.

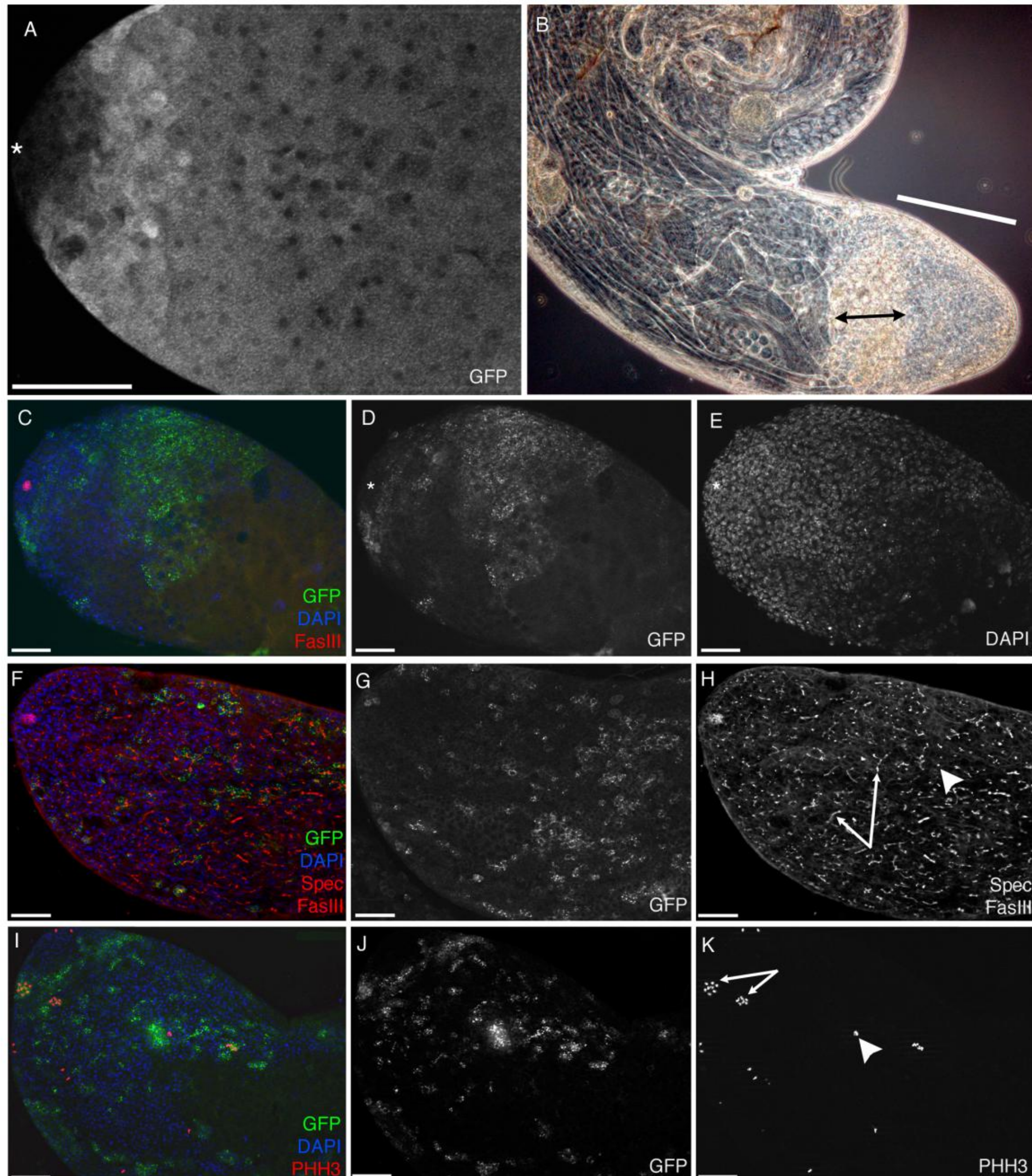


Figure 4: Ectopic expression of *upd* during mitotic amplification: Germ Cells

(A) 1-day old testis expressing UAS-EGFP under the control of the *bam*GAL4 driver. Notice the concentration of GFP several cell distances away from the

apical hub (Asterisk). **(B)** Phase contrast image of a testis expressing UAS-*upd*-GFP under the control of the *bam*GAL4 driver. Notice the build-up of small cells at the apical tip (bar) and the presence of a darker staining region separating the apical tip from the rest of the testis (arrows). **(C-K)** Immunofluorescent images of several testes expressing UAS-*upd*-GFP under the control of the *bam*GAL4 driver. **(C, D,** and **E)** Immunostaining for GFP and DAPI illustrates the over-proliferative phenotype and shows the presence of an increased amount of small cells at the apical tip. **(F, G,** and **H)** Immunostaining for α-spectrin (spec) to mark spherical spectrosomes in GSCs and gonialblasts (arrowhead), and branched fusomes in spermatogonia (arrows). Notice the presence of spherical spectrosomes around the hub and branched fusomes several cell distances away, but within in the region of over-proliferation. **(I, J,** and **K)** Immunostaining for phosphorylated histone H3 (pHH3) to label mitotically active cells. Notice that there are a greater number of mitotically active cells than normal, and those cells can be found both individually (arrowhead) or in groups (arrows).

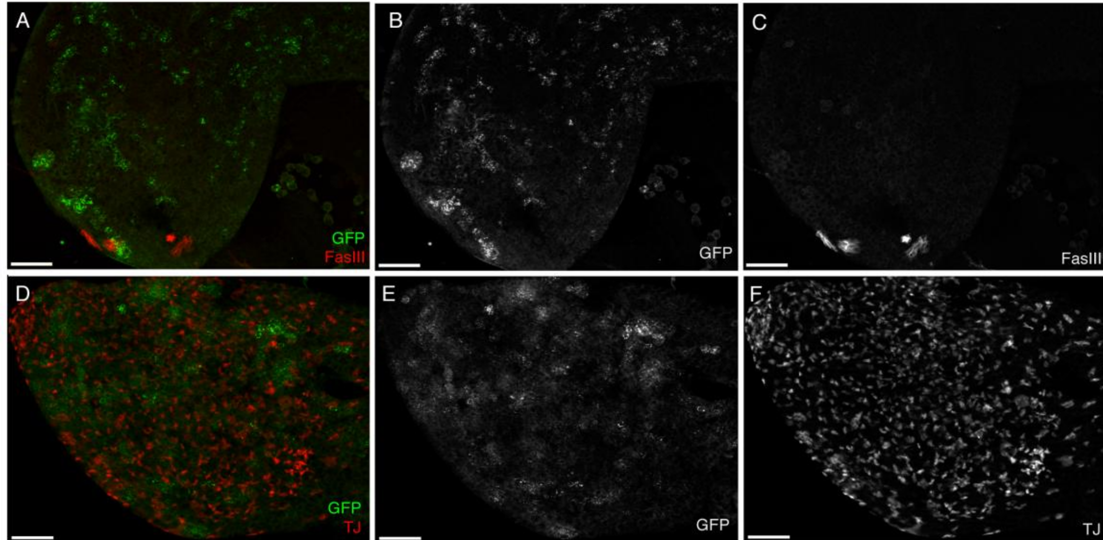


Figure 5: Ectopic expression of *upd* during mitotic amplification: Somatic Cells

(A, B, and **C)** Immunostaining for Fas III to mark hub cells in a testis expressing UAS-*upd*-GFP under the control of the *bam*GAL4 driver. Notice the multiple Fas III positive regions present within the apical tip of the testis that may represent ectopic niches. **(D, E,** and **K)** Immunostaining for the early cyst cell marker Traffic Jam (TJ) in a testis expressing UAS-*upd*-GFP under the control of the *bam*GAL4 driver. Notice the expansion of TJ positive cells throughout the entire expanded apical tip of the testis.

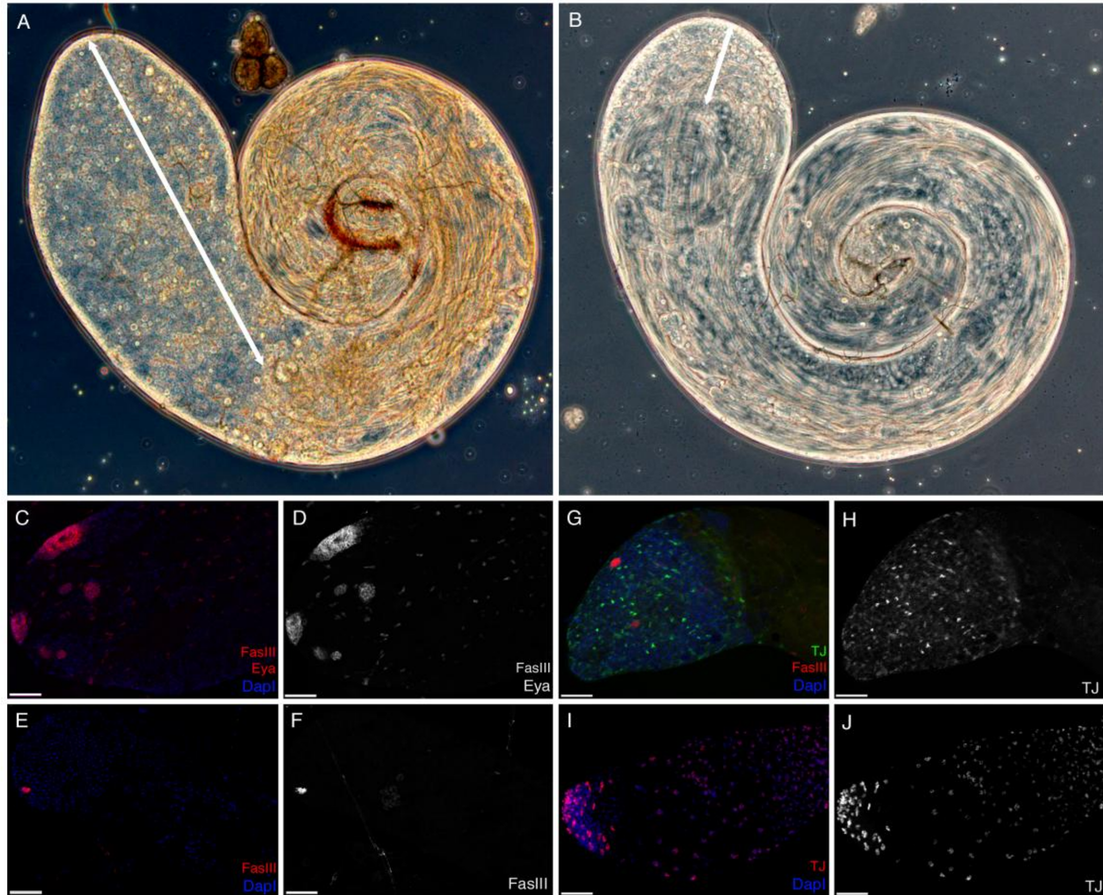


Figure 6: Differences in *upd* and *upd2* expression during mitotic amplification

(A and **B)** Phase contrast images of a *bam*GAL4;UAS*upd*GFP testis [A] and a *bam*GAL4;UAS*upd2*GFP testis [B]. Notice the larger expanded apical tip region in A as compared to B (arrows). **(C-F)** Immunofluorescence images comparing the fas III staining present in a *bam*GAL4;UAS*upd*GFP testis [C, D] and a *bam*GAL4;UAS*upd2*GFP testis [E, F]. Notice the ectopic fas III positive regions present in C and D and the single group of hub cells present in E and F. **(G-J)** Immunofluorescence images comparing TJ staining present in a *bam*GAL4;UAS*upd*GFP testis [G, H] and a *bam*GAL4;UAS*upd2*GFP testis [I,

J]. Notice the larger somatic expansion in I and J, and how it extends further down the body of the testis.

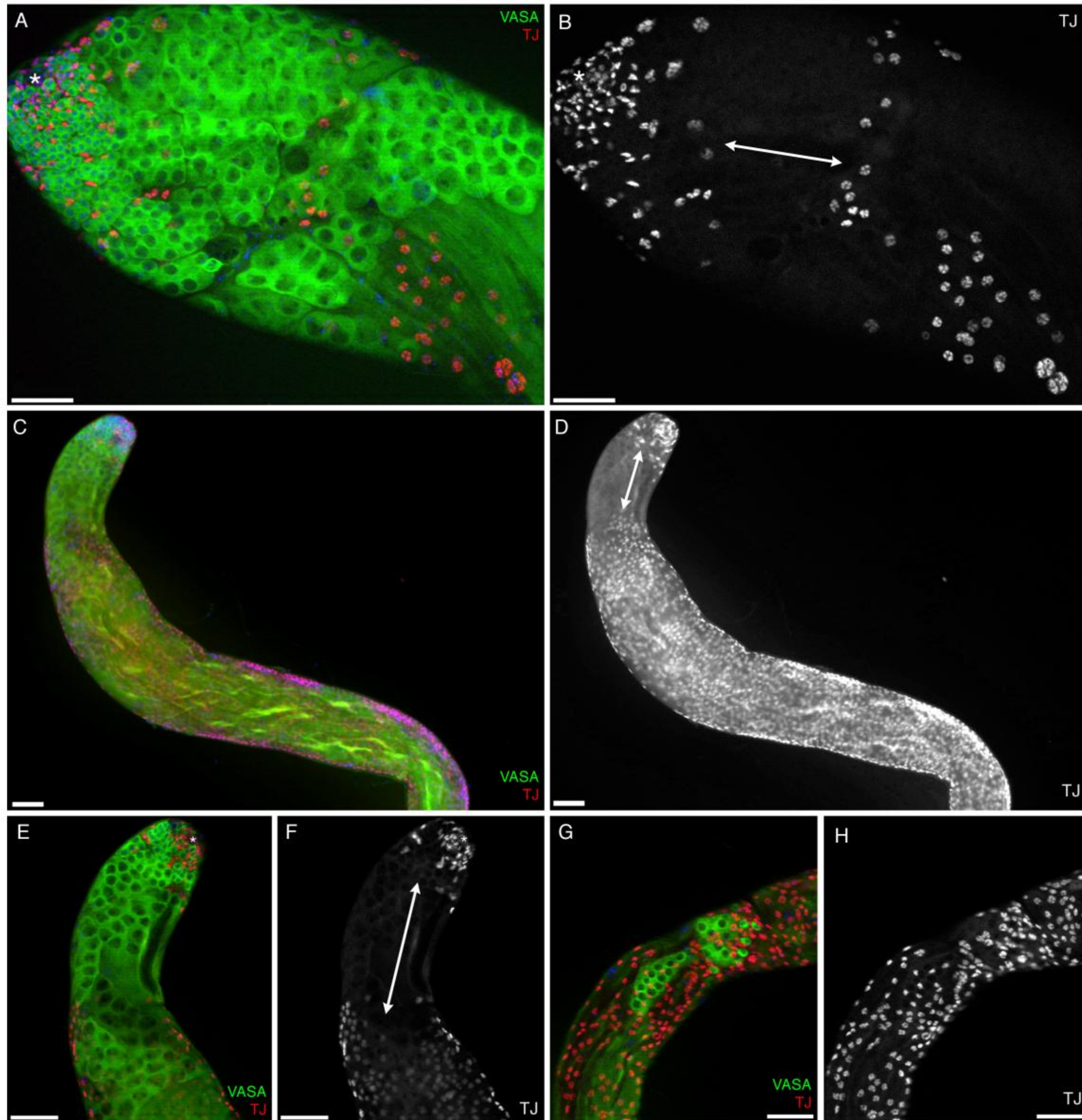


Figure 7: Somatic expansion with *upd2* expression

(A and **B)** Immunostaining of a 1-day old *bam*GAL4;UAS*upd2*GFP testis for early germ cell marker Vasa (green) and cyst cell marker TJ (red). Notice the two separate TJ populations present separated by a well-defined gap (arrows). Also note the differences in morphology between the TJ positive cells around the hub (asterisk) and those further down the body of the testis. **(C-H)** Immunostaining of a 20-day old *bam*GAL4;UAS*upd2*GFP for Vasa (green) and TJ (red). Notice the large expansion of TJ positive cells and absence of germ cells present in the body of the testis in C and D. Also notice the gap between the TJ positive cells around the hub (asterisk) and those further down the body of the testis in E and F. Lastly note the morphology of the TJ positive cells in the body of the testis and how they interact with the cyst present in G and H.

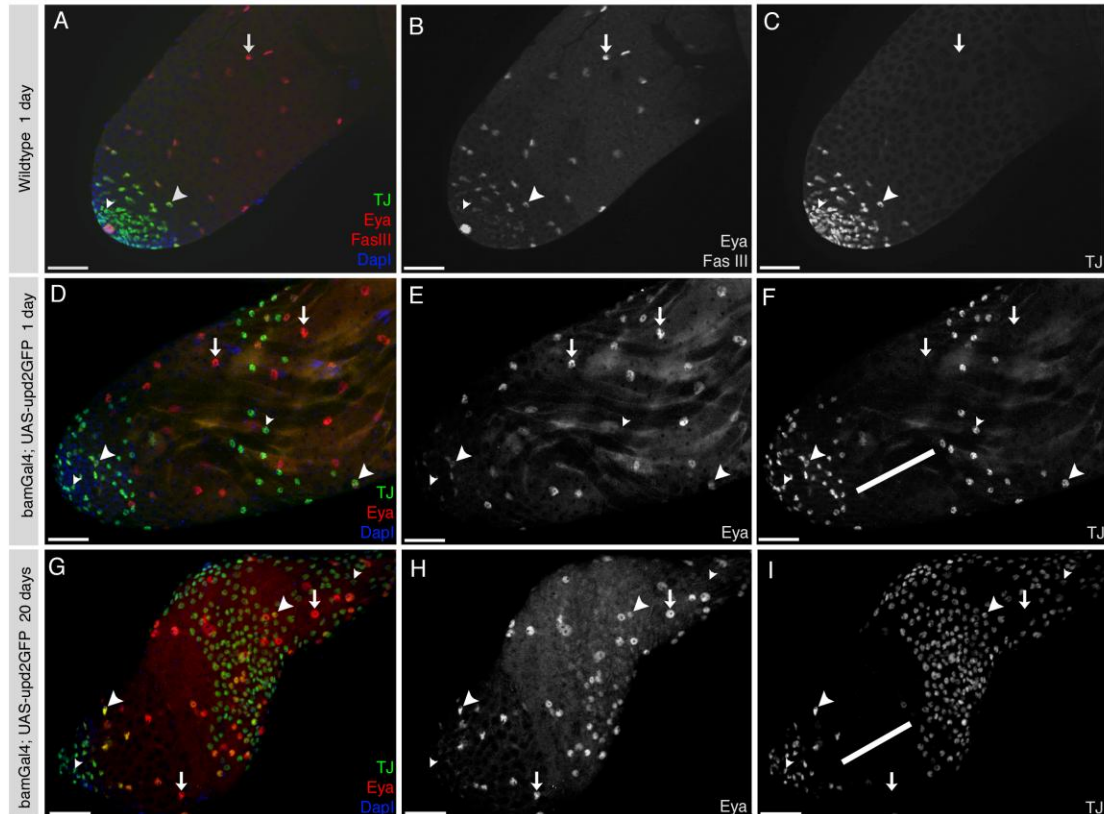


Figure 8: Eya v. TJ

(A-C) Immunostaining of a wild-type 1-day old testis for early cyst cell marker TJ (green), later cyst cell marker Eya (red), and hub marker Fas III (red). TJ positive and Eya negative cells (small arrowhead) are concentrated around the hub, while joint TJ and Eya positive cells (large arrowhead) are found a few cell distances away, and Eya positive and TJ negative (arrow) are found even further down the body of the testis. (**D-F)** Immunostaining of a 1-day old *bam*GAL4;UAS*upd2*GFP testis for TJ (green) and Eya (red). Notice the expansion of the TJ positive and Eya negative cell population (small arrowhead) and the presence of a second population of cells further from the

hub and separated by a well-defined gap (bar). Also note the presence of Eya positive and TJ negative cells (arrows) within this gap between the TJ populations. Lastly, notice the presence of joint Eya and TJ positive cells further down the body of the testis within the new population of TJ cells (large arrowhead). **(G-I)** Immunostaining of a 20-day old *bam*GAL4;UAS*upd2*GFP testis for TJ (green) and Eya (red). Notice the further expansion on the TJ positive and Eya negative population (small arrowhead) away from the hub and the maintenance of the gap between cell populations (bar). Also note the continued presence of Eya positive and TJ negative cells (arrows) within this gap and joint Eya and TJ positive cells (large arrowhead) further down the body of the testis.

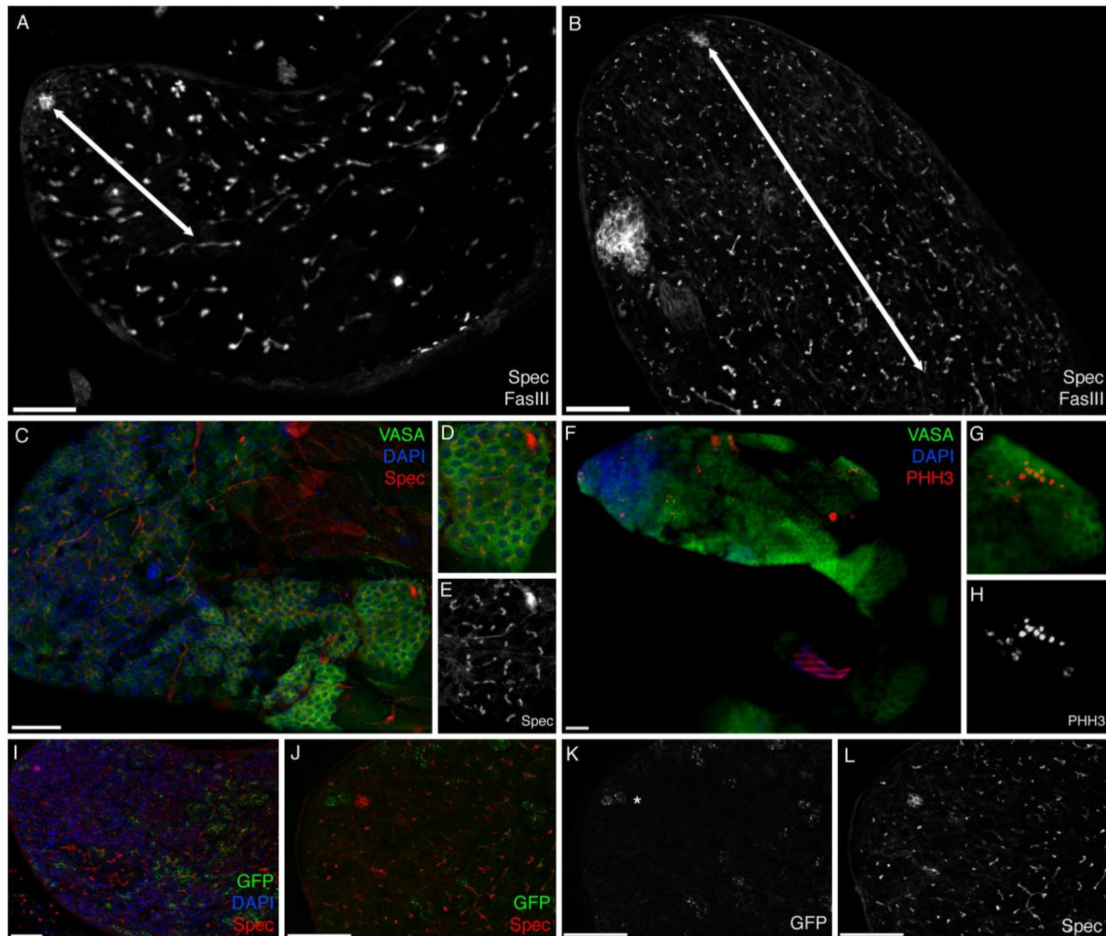
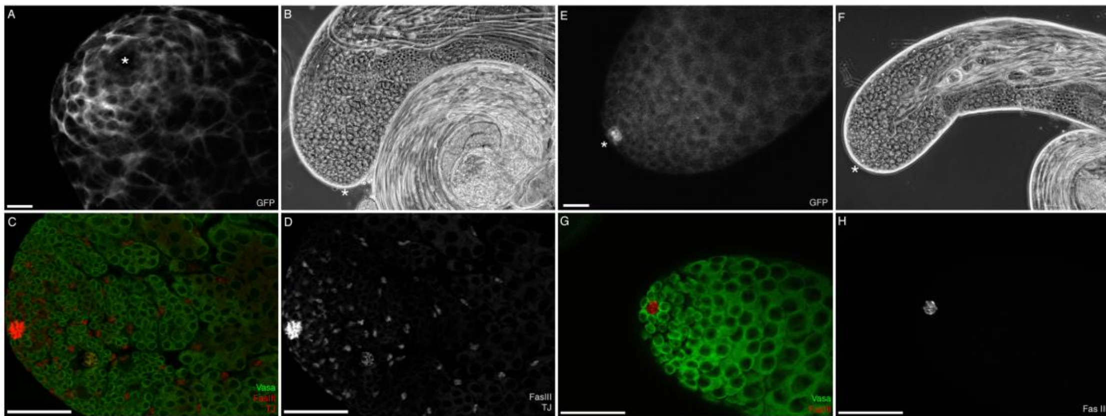


Figure 9: Is de-differentiation a possible explanation?

(A and **B)** Immunostaining of a wild-type [A] and a *bam*GAL4;UAS*upd*GFP testis for α-spectrin and fas III. There is a noticeable increase in the amount of early germ cells present at the apical tip (bar) in B signified by the region with spherical spectrosomes. **(C-E)** Immunofluorescence image of the apical tip of a *bam*GAL4;UAS*upd*GFP testis stained with vasa (green) and α-spectrin (red). The zoomed in image of a cyst [D] contains many more than 16 cells and its fusome appears to be less connected and may be showing signs of

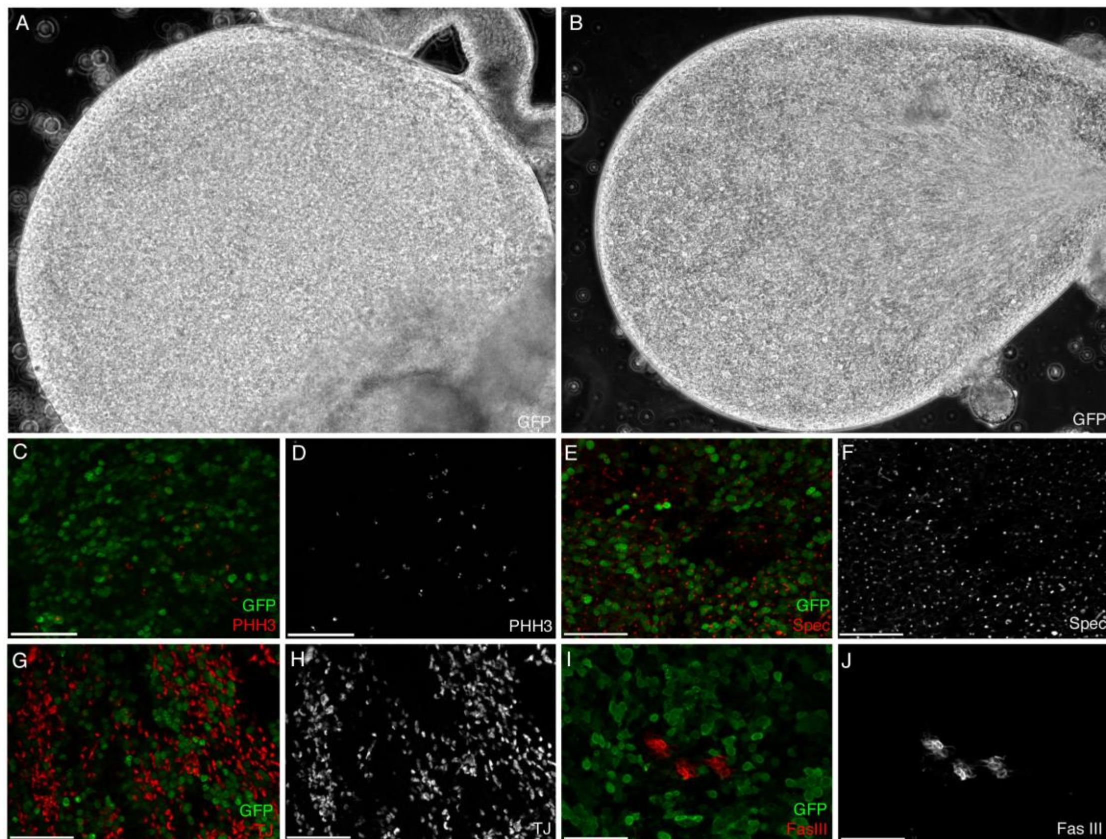
break down [E]. **(F-H)** Immunostaining of a *bam*GAL4;UAS*upd*GFP testis for vasa (green) and pHH3 (red). The cyst in G also appears to contain more than 16 cells and only a subset of cells is pHH3 positive and mitotically active in this image [H]. **(I-L)** Immunostaining of a *bam*GAL4;UAS*upd*GFP testis with a zoomed in view of the apical tip [J-L]. Note the appearance of two GFP positive cells immediately adjacent to the hub [J-L].



Supplementary Figure 1: *C587* and u*pd* GAL4 drivers

(**A**) Immunofluorescence image of a 1-day old testis with UAS-EGFP expression driven by the *c587*GAL4 driver. Notice the concentration of GFP in a spider web-like pattern around the hub (asterisk), indicating expression in the somatic cyst cells. **(B)** Phase contrast image of a 1-day old *c587*GAL4;UAS-*upd* testis. **(C** and **D)** Immunostaining of a *c587*GAL4;UAS*upd*GFP testis for the germ cell marker vasa (green), early cyst cell marker TJ (red), and hub marker fas III (red). **(E)** Immunofluorescence image of a 1-day old testis with UAS-EGFP expression

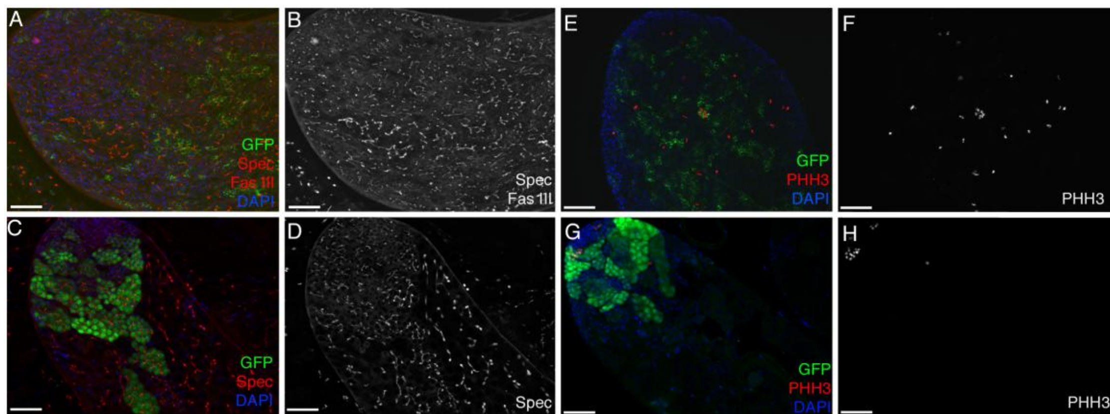
driven by the *upd*GAL4 driver. Notice the concentration of GFP exclusively in the hub (asterisk). **(F)** Phase contrast image of a 1-day old *upd*GAL4;UAS-*upd* testis. **(G** and **H)** Immunostaining of a *upd*GAL4;UAS-*upd* testis for vasa (green) and fas III (red).



Supplementary Figure 2: ***upd2*** **expression in early germ cells:**

(A and **B)** Phase contrast images of a 1-day old *nanos*GAL4;UAS*upd*GFP testis [A] and a 1-day old *nanos*GAL4;UAS*upd2*GFP testis. **(C-J)** Immunostaining of various 1-day old *nanos*GAL4;UAS*upd2*GFP testes. **(C** and

D) Immunostaining for GFP (green) and the mitotic marker pHH3 (red). Note the increased amount of pHH3 positive cells and that all of the cells appear to be dividing individually. **(E** and **F)** Immunostaining for GFP (green) and α-spectrin (red). Notice that all of the stained fusomes are spherical in shape. **(G** and **H)** Immunostaining for GFP (green) and cyst cell marker TJ (red). **(I** and **J)** Immunostaining for GFP (green) and Fas III (red). Note the unusual hub morphology present.



Supplementary Figure 3: *upd2* v. *upd* expression during mitotic amplification: Germ Cells

(A-D) Immunostaining of a 1-day *bam*GAL4;UAS*upd*GFP testis [A and B] and a 1-day old *bam*GAL4;UAS*upd2*GFP testis [C and D] with GFP (green) and α-spectrin (red). Notice that in both B and D the region of over-proliferation contains both spherical and branched fusomes. **(E-H)** Immunostaining of a 1-day *bam*GAL4;UAS*upd*GFP testis [E and F] and a 1-day old

*bam*GAL4;UAS*upd2*GFP testis [G and H] with GFP (green) and pHH3 (red). Notice that there are pHH3 positive cells dividing both individually and in groups in both F and H.

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