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Cyst Stem Cell Development During Drosophila Testis Morphogenesis

Matthew Badgett
College of William and Mary

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Abstract:

Stem cell specification and maintenance is necessary for proper organ development and tissue homeostasis. The testes of the fruit fly *Drosophila melanogaster* is one of the most thoroughly characterized systems for studying this process. The two populations of stem cells in the *Drosophila* testes, the germline stem cells (GSCs) and a somatic population of cyst stem cells (CySCs), are both localized to the anterior of the testes in a stem cell niche. The niche proper (also called the hub), GSCs, and CySCs communicate with each other to balance stem cell maintenance and differentiation. In order to determine how such a system forms, recent experiments have investigated GSC development and found that functional, asymmetrically dividing GSCs are first established from primordial germ cells (PGCs) around 23 hours after egg laying at the embryo to larval transition (Sheng et al, 2009). This occurs at the same time that the cells that comprise the stem cell niche coalesce to form the hub. Here, I examine CySC establishment and what mechanism regulates CySC maintenance and specification during embryonic and early larval testes development. I found that functional, asymmetrically dividing CySCs are present at the time of GSC establishment and that the Jak-STAT signaling pathway is both necessary and sufficient for CySC maintenance shortly thereafter. Jak-STAT signaling, however, does not appear to be necessary for early stages of CySC specification during embryogenesis. In a separate set of experiments, when Jak-STAT signaling is inhibited in the soma after stem cell establishment, cells are lost in the posterior testes, possibly due to cell death or loss of adhesion. I found that this is not the result of defects in CySC establishment or the initial differentiation of cyst cells. Together, these findings show that the CySCs are established soon after GSCs during morphogenesis and require Jak-Stat signaling to be maintained soon thereafter. Additionally, Jak-Stat signaling has a previously uncharacterized function later in differentiation that is suspected to involve adhesion or viability. Continued research should further our understanding of what factors are involved in CySC establishment and when precisely it occurs as well as the precise function of the Jak-STAT pathway in the posterior testes.

Introduction:

Adult stem cells are necessary to replace dead or dying cell populations in their respective organs (Slack, 2008). They are capable of undergoing asymmetric cell divisions that results in the creation of one cell that maintains the stem cell characteristics and another that is capable of differentiation into at least one other cell type. Through this mechanism, stem cells can continuously produce functional cells within an organ (Lajtha, 1979). Although varying greatly in composition, all stem cells are located within niches, microenvironments composed of different cells that assist in maintaining stem cells (Schofield, 1978). Understanding the function of the niche is necessary for manipulating stem cells for potential therapy. Despite this, little research has been done looking at how the niche and its stem cells are specified during organogenesis. Such research may provide information on how to apply this process to tissue regeneration in adult organisms. To do this, I am studying the process of stem cell development in developing *Drosophila testes*. Specifically, I have been examining the development of somatic cyst stem cells (CySCs) that regulate gametogenesis within the male *Drosophila testes* stem cell niche. Data presented here examines the timing and regulation of CySC behavior in developing testes. In the following introduction, I will: (1) provide further background on our current understanding of stem cell development during organogenesis, (2) provide a general overview of *Drosophila* as a model organism for studying development, (3) discuss *Drosophila testes* as a model system for studying stem cell behavior, and (4) describe what is known about stem cell development in *Drosophila testes*."

Stem cell development during organogenesis:

During early embryonic development, regardless of organism, there are no stem cells in the traditional sense (Slack, 2008). Rather than dividing asymmetrically, most precursor cells divide symmetrically to produce additional precursor cells so there are a sufficient number of cells for development to proceed. As development progresses, however, cells adopt different identities. This proceeds hierarchically as embryonic progenitors differentiate first into one of the three germ layers: ectoderm, mesoderm, and endoderm and then, depending on their eventual fate, into progressively more highly specified cell types. During organ and tissue formation, stem cell

populations capable of asymmetric cell division must be specified and maintained so that functional cell types required for organ function and tissue homeostasis are produced. This likely requires the coordinated development of a stem cell niche which promotes asymmetric stem cell division, but the mechanisms by which stem cell specification and establishment occur are not well understood.

The process of stem cell development has been examined in a number of mammalian systems such as hematopoietic, neural, and epithelial development (reviewed in Chia et al. 2008; Fuchs and Nowak, 2008; Slack, 2008). Certain similarities and unique mechanisms have emerged. In general, factors required for stem cell specification are also required for stem cell maintenance once the stem cell niche has formed. However, others factors are for either specification or maintenance. During hematopoietic stem cell (HSC) development, murine blood cells are initially derived from precursor cells within the yolk sac, and the first definitive HSCs are observed in the aorta-gonad-mesonephros (AGM), major vitilline and umbilical vessels and placenta. As development progresses, HSCs are mobilized to new tissues, including the fetal liver, until they eventually engraft stem cell niches in the adult bone marrow and thymus. During this process, the transcription factor complex stem cell leukemia/T-cell acute lymphocytic leukemia (*SCL/tal1*) is expressed in stem cell progenitors that arise within the yolk sac and develop into functional HSCs as they mobilize to other tissues, but *SCL/tal1* expression has not been detected in adult HSCs. On the other hand, mixed lineage-leukemia (MLL) and runt-related transcription factor (*Runx1*) are necessary for maintenance of early HSCs in the aorta-gonad mesonephros during development and are also believed to be expressed in HSCs that mobilize to other tissues later in development and in the adult. Other proteins, such as PU.1, growth factor independent 1 (*Gfi-1*), and CCAAT/enhancer-binding protein α (*C/EBP α*), function in the HSCs as well as some of their differentiated lineages (Orkin and Zon, 2008). The variety of transcriptional pathways and cell population involved in hematopoiesis demonstrates the complexity of stem cell development during embryogenesis.

In other organ systems, stem cells can be derived from multiple sources. Both the liver and pancreas cells are each derived from two different progenitors in the endodermal epithelium of the embryonic foregut (Tremblay and Zaret, 2005; Field et al, 2003). Although it is

known that both progenitors contribute to regenerative liver buds and the endocrine and exocrine cells in the pancreas, respectively; there may be other epigenetic differences that affect more specific aspects of functionality between the original cell populations (Zaret and Grompe, 2008). In at least one instance, the first differentiated cells have a distinct origin from the adult stem cells that eventually populate that organ: first generation anterior pituitary cells are derived separately from the nestin-positive adult stem cells and express a different transcription profile during development. This shows that stem cells can mature through a different development pathway than that of the first generation of differentiated cells do (Gleiberman et al, 2008). All of these system show that stem cells can be specified through a number of characteristically different pathways and be maintained with similar or radically different factors. A stronger understanding of the functions of different factors that act during stem cell specification and/or maintenance is necessary for isolation and maintenance of stem cells. This may be applied to novel therapeutic treatments and will provide a better understanding of organogenesis and defects that may arise during organ formation.

Drosophila as a model organism:

First propagated in a lab in 1901, the fruit fly, *Drosophila melanogaster* is one of the oldest and most thoroughly studied model organisms (Reeve, 2001). Populations of *Drosophila* are easy to maintain, genders are easily distinguishable, and females can lay ~ 100 eggs per day at their peak and up to 2000 eggs during their lifetime. It takes 11-12 days for the embryo to mature into adulthood under optimal conditions and a fly can live for up to 60 to 70 days (Figure 1). The ease and speed of growing fruit flies, along with low space requirements and low costs, make them easy to work with in the lab.

Thomas Hunt Morgan's pioneering work with *Drosophila* genetics has enabled scientists to utilize fruit flies for genetic analyses. Thanks to their simple set of 4 chromosomes, tracking where mutations occur in the *Drosophila* genome is relatively easy. Early studies tracking phenotypic changes associated with gene mutations led to the earliest chromosomal maps that have helped us to understand chromosome structure. This was also made easier by the polytene chromosomes located in the salivary glands that were much larger and easier to

examine than those in other cells. Furthermore, balancer chromosomes, first used by Miller in 1918, allow fly stocks to be maintained through self-crossing. This occurs because balancer chromosomes contain multiple inversions that prevent normal chromosomal alignment required for meiotic recombination; if cross over occurs, the organism dies because it is missing some genes while having two copies of others. Many balancer chromosomes also carry dominant, homozygous lethal gene mutations that allow maintenance of fly lines that are heterozygous mutant for a gene of interest, while enabling scientists to study the impact of homozygous mutants on behavior and development (Greenspan, 2004: Fly Pushing). In 2000, the *Drosophila melanogaster* genome was sequenced, allowing for a stronger understanding of the genetic components at work in the organism (Reeve, 2001: The Encyclopedia of genetics).

Adaptation of the *Gal4/UAS* gene expression system has also enabled *Drosophila* to become an important system for studying development. This system allows for tissue-specific expression of genes and/or gene inhibitors (Figure 2). *Gal4*, a yeast transcription factor, binds to a yeast enhancer element, termed the upstream activation sequence (*UAS*), resulting in transcription of a transgene regulated by the *UAS* element. By crossing fly lines that express *Gal4* in certain tissues with a target gene under *UAS* regulation, the target gene can be induced in tissue-specific manner at a given time. This basic strategy can be used to examine gene expression and/or cell morphology using *UAS-GFP* or *UAS-lacZ* transgenes. It can also be used to overexpress proteins and suppress expression through overexpression of known inhibitors. This allows study of whether a given gene is necessary and sufficient to promote specific aspects of development (Greenspan, 2004: Fly Pushing). Because of these, and other genetic manipulations now possible in flies, *Drosophila melanogaster* has become a very powerful model organism in biology.

Drosophila testes as a model stem cell system:

Spermatogenesis, specifically in *D. melanogaster*, is a good system to examine stem cell maintenance because germline stem cells (GSCs) can be examined functionally and the development of primordial germ cells (PGCs) is remarkably similar in vertebrates and invertebrates (Orkin and Zon, 2008). The basic process of spermatogonial differentiation is also

similar, with asymmetric division of GSCs resulting in production of spermatogonia that continue to divide with incomplete cytokinesis to produce spermatocytes that initiate meiosis and develop into functional sperm (Fuller, 1993; Gilbert, 2010; De Cuevas and Matunis 2011). The process of spermatogonial differentiation is also nurtured by somatic cells that communicate with spermatogonia as they mature; Sertoli cells in mice and cyst cells in *Drosophila*. There are, however, some key morphological differences between flies and mice (Figure 3). Notably, mouse GSCs reside at vascular branch points found at seemingly random locations along the periphery of the basal membrane throughout seminiferous tubules (Figure 3A). Spermatogonia differentiate away from these multiple niches so that functional sperm are released into the center of the seminiferous tubules which then feed into the vas deferens (Gilbert, 2010). In contrast, *Drosophila* testes form a single coiled tube with one blind end where the germline stem cell (GSC) niche is localized, and the other end connected to the genital tract (Figure 3B). The relative simplicity of this system makes *Drosophila* testes ideal for studying basic aspects of stem cell biology and organ formation.

Additional research has uncovered considerable detail about the morphology and regulation of GSCs in adult *Drosophila* testes. Indeed, two populations of stem cells reside within the male GSC niche: GSCs that produce and cyst stem cells (CySCs) that help regulate GSC maintenance and whose progeny nurture spermatogenic differentiation (Figure 3B). Five to nine GSCs are anchored to a cluster of non-dividing somatic cells, called the hub, found at the tip of the testes. The CySCs are also arrayed around the hub, but only thin membrane projections that interdigitate between GSCs directly contact the hub, so that the main body of each CySC is displaced from the hub. Spermatogenesis begins with the asymmetric division of a GSC, such that one daughter cell remains anchored to the hub and remains a GSC, while the other daughter cell is displaced from the hub and takes on gonialblast's fate. The gonialblast divides four more times with incomplete cytokinesis to form a 16-cell spermatogonia which undergoes meiosis, individualizes, and differentiates into functional sperm. At the same time as GSC division, each CySC divides into two cells, one which retains stem cell identity and its association with the hub, and another becomes a mitotically inactive cyst cell that promotes

spermatogonial development; regulating the rate of spermatogonial cell division and growing in size to encompass the spermatogonia as they differentiate (De Cuevas & Matunis, 2011).

The function of a number of protein pathways in regulation of GSC and CySC maintenance in adult testes has been examined (Figure 4; reviewed in Jemc, 2011). One key pathway is the Janus kinase-signal transducer and activator of transcription (Jak-STAT) signaling pathway (reviewed in De Cuevas & Matunis, 2011). In the testes stem cell niche, the Jak-STAT activating ligand, unpaired (Upd), is expressed and secreted from the hub and binds to the Domeless (Dome) receptor, located on both CySCs and GSCs. This activates Hopscotch (Hop), a Janus kinase, which phosphorylates Signal Transducer and Activator of Transcription 92E (STAT92E), leading to expression of STAT-responsive genes such as *suppressor of cytokine signaling at 36E (Socs36E)*, *zinc finger homeodimer-1 (zfh-1)*, *gbb (glass bottom boat)*, and *decapentaplegic (dpp)*. In a classic negative feedback loop, SOCS36E suppresses Jak by preventing Jak-activation of the Domeless receptor through phosphorylation. Zfh-1, GBB and DPP play a role in regulating stem cell maintenance. Specifically, Zfh-1 induction appears specific to CySCs, and is required for CySC maintenance (Leatherman & DiNardo, 2008). Similarly, upregulation of *dpp* and *gbb* in CySCs, which encode activating ligands for the transcription growth factor- β (TGF- β) signaling pathway, appears to be responsible for repressing differentiation of GSCs that reside at the hub (Kawase et al., 2004). While the direct targets of STAT in GSCs are not yet clear, Jak-STAT signaling is required for the formation of Cadherin-based adhesions between GSCs and hub cells, that are believe to orient asymmetric GSC divisions away from the hub. Thus, the Jak-STAT signaling pathway is a major regulator of stem cell maintenance in the adult *Drosophila* testes.

Stem cell development in Drosophila testes:

Development of the gonad during morphogenesis has been examined in some depth. Testes development begins during embryogenesis ~10 hours after egg laying (AEL) and onset of spermatogenesis is observed by mid larval 1st instar (30-36 hrs AEL) (Sheng et al, 2009)(Figure 5). Two main cell types are essential for formation of the testes: primordial germ cells (PGCs) and somatic gonadal precursors (SGPs). PGCs arise from the germ plasm at the posterior of the

embryo and are first observed ~2-3 hrs after egg laying (AEL) (stage 4-5 of embryogenesis) (Hartenstein, 1993). At ~3.5 hrs AEL (embryonic stage 7), PGCs migrate into the embryo as the posterior moves anteriorly and into the midgut region. Meanwhile, SGP are specified with bilateral symmetry in parasegments (PS) 10-13 of the mesoderm at ~5-7 hrs AEL (embryonic stage 11) so that migrating PGCs intermingle with the SGP at either of these two locations by ~7-10 hrs AEL (embryonic stage 12). Shortly thereafter, SGP and PGCs coalesce into a tight cluster, with SGP fully ensheathing the PGCs by ~10 hrs AEL (embryonic stage 13). By this time, other somatic cells located in the posterior, called male-specific SGP (msSGP), are also associated with the embryonic testes. The msSGP eventually give rise to the terminal epithelium in the adult testis (DeFalco et al, 2008). As embryogenesis progresses, hub cells are specified within a sub-population of SGP in the anterior of the testes (Le Bras and Van Doren, 2006; Okegbe and DiNardo, 2011), and these cells coalesce into a tight cluster that behaves similar to adult hub cells with functional, asymmetrically dividing GSC arrayed around the hub (Sheng et al, 2009; Figure 4). This process is completed by the end of embryogenesis, and GSC differentiation reaches a steady state for production of gonialblast cells by mid- to late- L1, indicating that a fully functional male GSC niche is formed by the embryo to larval transition (~23 hrs AEL).

The presence of a functional hub and differentiating GSCs at this time implies that CySCs are established during embryogenesis and before L1. Recent evidence indicates that CySCs are partially specified within PS11 and may be controlled in part by the *lines/bowl* regulatory pathways, since hub cells are also specified in PS11 and are affected by *lines/ bowl* expression (DiNardo et al, 2011). However, little work to date has examined CySCs in the developing testes or what mechanisms regulate CySCs within the early niche.

By examining known reporters of adult CySCs and adult cyst cells, I have shown here (1) that functional CySCs are established at the time of hub formation and GSC establishment, (2) that Jak-STAT signaling is necessary and sufficient for CySC maintenance after hub formation, and (3) that Jak-STAT signaling has a novel role in differentiation of somatic cells in the posterior region of the developing testes.

Methods

Fly Stocks:

y , w^{1118} and w^{1118} flies were used as the controls. *c587-Gal4* (A. Spradling) was used to drive expression of *UAS-Hop^{TumL}* (D. Harrison) and *UAS-mCD8::GFP* in the somatic gonad. *c587-Gal4* (A. Spradling), *tj-Gal4/CyoKrGFP* (M. Van Doren), *dsx-Gal4* (C. Goodwin), *Six4-Gal4* (M. Van Doren), and *upd-Gal4* (M. Van Doren) were all used to drive expression of *UAS-Socs36e* in the soma. *mwh red e stat92E^{Frankenstein}* or *stat92E^{TS}* (C. Dearolf) and *stat92E⁰⁶³⁴⁶* were balanced over *Tm3, Sb, KrGFP* for genotype selection of larvae. The enhancer trap line *Socs36E-PZ* (A. Spradling) was also used. Fly stocks were obtained from the Bloomington Stock Center (<http://flystocks.bio.indiana.edu/>), unless otherwise specified.

Collection of embryos, larvae & adult testes:

Wild type embryos and larvae were collected at times ranging from 0-24 hours (embryos/L1e), 24-48 (L1) or 48-72 (L2) hours after egg laying (AEL) at 24°C. Samples were then fixed with 4% formaldehyde and immunostained as described (Sheng et al, 2009). Embryos were staged according to morphology (Hartenstein, 1993) with late stage 17/early L1 testes confirmed by FasIII or N-Cad staining in the hub. Wild type larvae were sorted by age according to larval size compared with larval age standards at 24-30, 30-42, 42-48 and 48-55 hrs AEL. To obtain *stat92E* mutants, either *stat92E⁰⁶³⁴⁶/TM3,Sb,KrGFP* or *stat92E^{TS}/stat92E⁰⁶³⁴⁶* virgin females reared at 18°C were mated to *stat92E⁰⁶³⁴⁶/TM3,Sb,KrGFP* males and embryos collected for 6-12 h AEL at 18°C followed by incubating for 24-30 hours at 29°C. To obtain *os(df)* mutants, *os(df)* was crossed with itself and embryos were collected 0-24hours AEL at 24°C. To obtain larvae with ectopic *hop^{TumL}* expression, *c5867-Gal4* virgins were crossed with *UAS-hop^{TumL}* males, embryos collected 0-7 h AEL at 24°C, then incubated for 41 (late L1), 48 (early L2) or 55 (mid L2) hours at 29°C. To obtain larvae with somatic *Socs36e* expression, virgins from one of the Gal4 lines, or w^{1118} virgins as a control, were crossed with males carrying *UAS-Socs36e* homozygous on chromosome II or *UAS-socs36e* on chromosome III. These embryos

were then collected for 0-24 hours AEL at 24°C, incubated for 48 hours (L2), and then fixed for immunostaining. All adult testes were isolated 5-10 days after eclosion.

Antibodies and Immunostaining:

Immunostaining of embryos, larvae, and adult testes was performed as described (Matunis et al, 1997; Sheng et al, 2009). The following primary antibodies were used: chick anti-Vasa at 1:2000 (K. Howard); rabbit anti-ZFH1 at 1:5000 (R. Lehmann); rabbit anti-GFP at 1:2500 (Torrey Pines); rabbit anti- β -gal at 1:5000 (Cappel); mouse anti- β -gal at 1:5000 (REF); mouse anti-Fascilin III at 1:10 (Developmental Studies Hybridoma Bank [DSHB]); mouse anti-Eya at 1:25 (DSHB); mouse anti-1B1 at 1:4 (DSHB); rat anti-N-Cadherin at 1:20 (DSHB). Secondary antibodies (Molecular Probes) used were: goat anti-chick 546, goat anti-chick 633, goat anti-mouse 488, goat anti-mouse 633, goat anti-rabbit 488, goat anti-rabbit 633, goat anti-rat 546, goat anti-rat 633. All secondary antibodies were used at 1:500. Nuclei were stained using DAPI at 1 μ g/mL (Roche) for 3 minutes. In order to best stain embryos and larvae with Zfh-1 and Eya, unique methods were developed. Embryos and larvae were washed with either rabbit anti-Zfh-1 or mouse anti-Eya solutions alone for a full day. Following that, the antibodies were rinsed off and the samples were washed with secondary antibodies, either goat anti-rabbit 488 (or 633) or goat anti-mouse 488, respectively, for another day. In the case of the c587-Gal4 UAS-hop^{tuml}, Zfh-1 and Eya as well as their secondary washes were all given one full day of washing. After this process, samples were washed with all primary antibodies overnight followed by washing with all secondary antibodies overnight. Samples were then stained with DAPI before preservation in DAPCO.

Confocal microscopy:

Embryos, larvae and testes were mounted in DABCO (REF) supplemented with *p*-phenylenediamine anti-fade agent at a final concentration of 0.2 mg/ml. Slides were viewed with an Olympus BX51 microscope equipped with a DSU spinning disc confocal system and Q-imaging RETIGA-SRV CCD camera. Images were captured and analyzed with Slidebook 5.0 software by 3I.

Results:

CySCs reporters are present in mid-L1 testes:

Previous research in the Wawersik lab has shown that germline stem cells are established in the *Drosophila* testes at the embryo to larval transition (Sheng et al, 2009). This occurs at the same time as coalescence of the hub in developing testes (Gonczy et al., 1996; Le Bras et al., 2006; Sheng et al, 2009). Maintenance and differentiation of GSCs depends on cyst stem cells and their progeny, termed cyst cells, in the adult testes (Leatherman and DiNardo, 2008, 2010). As such, I sought to examine the development and differentiation of CySCs during testes morphogenesis. I first looked at expression of adult CySC markers (Broihier et al., 1998; Issigonis et al., 2009; Singh et al., 2010) in mid-1st instar larval testes (~36 AEL) shortly after GSC establishment has occurred. Specifically, I examined expression of Zinc finger homeodomain protein-1 (Zfh-1). Expression of another adult CySC reporter, SOCS36E-PZ, was examined by Daniel Sinden, a fellow lab member. To look at CySC differentiation, I also examined expression of the cyst cell marker, Eyes Absent (EYA) (Fabrizio et al., 2003) at the same time point.

As observed in adult testes (Fig 6A), Zfh-1 expression is also enhanced in somatic nuclei located proximal to the hub in mid-L1 (Fig 6B). Low-level Zfh-1 expression was also observed in the hub and cells encasing the testes at both times (Fig 6A&B). Expression of the SOCS36E-PZ was similarly detected in the somatic cells near the hub and in the hub in both adult (Fig 6C) and mid-L1 (Fig 6D). These data suggests that by mid-1st instar, CySCs are present surrounding the hub.

We next characterized EYA expression in mid-L1 and the adult testes. At this time, EYA is only detected in the posterior half of the testes but not seen in the hub or proximal cells, where the CySC and immediate progeny would be expected to reside (Fig 6F). Similarly, in the adult testes, EYA is not expressed in or proximal to the hub but begins to be expressed in differentiating cyst cells that surround spermatogonia after the 4-cell stage (Fig 6E). Thus, both CySCs and cyst cells appear to be present in the newly formed testes by mid- larval 1st instar.

CySC establishment correlates with GSC niche formation:

Hub cells are specified in developing testes by ~7.5 hrs AEL (Okegbe et al, 2011; Kitadate & Kobayashi, 2010), while formation of a tightly coalesced hub as well as GSC establishment are complete by the embryo to larval transition (~23 hrs AEL) (Gonczy et al; Le Bras and Van Doren, 2006; Sheng et al., 2009). We, therefore, examined CySC development and differentiation during earlier stages of gonad morphogenesis using expression of Zfh-1, SOC36E-PZ, and EYA.

Before hub formation in late embryogenesis, the adult CySC marker Zfh-1 is detected in somatic nuclei throughout the testes (Fig 7A, A'). After hub coalescence, Zfh-1 is still expressed ubiquitously throughout the developing testes (Fig 7B). However, hub proximal somatic nuclei were displaced from the hub similar to CySCs in adult testes (Fig 7B'; compare with Figure 6A). Shortly thereafter, in early-L1 testes (~28 hours AEL), Zfh-1 expression is restricted to hub proximal somatic cells (Fig 7C), and expression remains restricted during later stages of development (Fig 7D).

Analysis of SOCS36E-PZ expression, conducted by Daniel Sinden, shows that this additional CySC reporter is initially expressed in hub precursors cells prior to hub formation (~18 hours AEL; Fig 7E). As development proceeds, however, expression in the hub becomes stronger and extends into somatic nuclei proximal to the hub (Fig 7 F-H). The induction of SOCS36E-PZ in hub proximal cells shortly after hub coalescence suggests a new transcriptional program is initiated in these cells. Taken together, the re-positioning of hub-proximal somatic cells and the changes in Zfh-1 expression suggest that CySCs form at the embryo to larval transition in a process that is coordinated with hub formation and GSC establishment.

To assess when cyst differentiation begins, EYA expression was examined across the embryo to larvae transition. Prior to hub coalescence, EYA is detected exclusively in male-specific somatic gonadal precursors (msSGPs), which arise in the mesoderm from parasegment 13 and associate with the posterior of the gonad before the hub coalesces (Fig 7I; DeFalco et al., 2003; DeFalco et al., 2008). I find that this expression pattern persists through larval 1st instar (Fig 7 J, K) until mid-L1. At which point, EYA is also detected in somatic nuclei throughout the posterior half of the gonad (Fig 7L). This correlates with onset of spermatogonial differentiation as characterized by expression of the spermatogonial marker, Bag-of-marbles

(Sheng et al, 2009). Another lab member, Amanda Simmons, has also shown that somatic cells in the posterior half of the gonad ensheath the multicellular spermatogonial cysts at this time-point (Sinden and Badgett et al, in preparation). Additionally, experiments conducted by Ron Palmén in the Wawersik lab (Sinden and Badgett et al, in preparation) show that hub-proximal cells divide and orient their mitotic spindle away from the hub in a manner characteristic of CySC undergoing asymmetric cell division. Taken together, these data suggest that functional cyst cells that support spermatogonial differentiation have formed by mid-L1, and that these cells are most likely the product of asymmetrically dividing CySCs that first become functional at the time of hub formation and GSC establishment. This interpretation is supported by previous analyses indicating that GSCs reach a steady state for gonialblast differentiation shortly after GSC establishment (Sheng et al, 2009).

Jak-STAT signaling is necessary and sufficient for early CySC maintenance:

Previous research has shown that Jak-STAT signaling regulates CySC maintenance in adult *Drosophila* testes (Leatherman and DiNardo, 2008, 2010). We, therefore, sought to examine the impact of altered Jak-STAT signaling on CySC development and whether it is necessary or sufficient for maintenance early in development. As discussed previously, the Jak-STAT signaling pathway is initiated by binding of the secreted ligand, unpaired (Upd), to its cell surface receptor. This results in the activation of the receptor associated Janus-kinase (Jak), Hopscotch (Hop), which then phosphorylates and activates the transcription factor, STAT92E.

To assess whether Jak-STAT signaling is sufficient to promote GSC maintenance in in developing testes, Daniel Sinden (Sinden, Badgett et al, *in preparation*) examined the impact of over-expressing a heat-activated Jak allele (*Jak^{heat-activated}*) specifically in all cyst cells using the Gal4/UAS system using *UAS-Jak^{heat-activated}* and *c587-Gal4*, driving Gal4 in cyst cells. This resulted in expression of CySC marker, Zfh-1, throughout late L1 testes (Fig 8A'; n=21), while sibling control showed the normal pattern of restricted Zfh-1 to hub-proximal nuclei (Fig 8B; n=20). Absence of the cyst differentiation marker, EYA, after *Jak* hyper-activation further indicates that CySC population is expanded and differentiation of cyst cells is inhibited (Fig 8A, B; n=16). This interpretation is supported by subsequent experiments conducted in the Wawersik lab by Jake

Fry (Sinden & Badgett, *in preparation*) that show repressed spermatogonial differentiation in the germ cells when somatic *Jak* hyperactivation occurs, since functional CySC in adult testes also promote GSC maintenance. This indicates that *Jak* expression is sufficient for CySC maintenance.

To examine whether *Jak*-*STAT* signaling is necessary for CySC maintenance in the newly formed stem cell niche, I examined whether CySCs expressing *Zfh-1* are present in mid-L1 testes lacking both maternal and zygotic *stat* gene function. Specifically, I examined *STAT*^{TS} larvae that are maternally trans-heterozygous for two different mutant alleles of *STAT92E* (*stat*^F/*stat*^{null}) and homozygous mutant for zygotically expressed *STAT92E* (*stat*^{null}/*stat*^{null}) as well. *stat*^F, or *stat*^{Frankenstein}, is a temperature sensitive allele of *STAT92E* that operates normally at lower temperatures (18°C) but when raised to 29°C, ceases to operate (Baksa et al, 2002). *Stat*^{null} is a loss of function allele caused by the insertion of a transposable element in an intron (Hou et. al, 1996)). By using these mutant alleles together, *stat*^F/*stat*^{null} mothers can be reared at low temperature with no impact on viability, while growth of *STAT*^{TS} progeny derived by mating with *stat*^{null}/*stat*^{wild-type} fathers result in loss of all *STAT92E* function at higher temperatures. My analysis of *STAT*^{TS} testes reveals that *Zfh-1* is absent or reduced in all testes examined. ~87% of testes (n=32) lack *Zfh-1* cells in hub proximal cells (Fig 9A) and all other testes have only one *Zfh-1* positive hub proximal cell. In contrast, sibling controls showed normal *Zfh-1* expression was normal, showing multiple (approximately 10-14) *Zfh-1* positive cells proximal to the hub (Fig 9B, n=20). This suggests that *STAT92E* is necessary for CySC maintenance at this time point.

To assess the contribution of maternal *stat92E* to CySC maintenance in larval testes, we also examined whether CySCs are maintained in the newly developed niche if they lack only zygotic *stat* gene function. My analysis of mid-L1 testes homozygous mutant for zygotic *stat92E* (*stat*^{null}/*stat*^{null}; n=22) are similar to *STAT*^{TS} analysis, with 86% of testes examined lacking *Zfh-1* hub proximal cells, and all other testes with one *Zfh-1* hub proximal cells (Fig 9C; n=22). In contrast, *Zfh-1* expression was normal in sibling controls (Fig 9D; n= 18). Thus, it appears that maternal *STAT92E* is dispensable for CySC maintenance in larval testes. Taken together, these data indicate that the *Jak*-*STAT* signaling pathway is both necessary and sufficient for early CySC establishment during formation of the GSC niche.

Zygotic STAT is not necessary for specification of CySC progenitors:

We next sought to assess the impact of Jak-STAT signaling on CySC specification. As loss of zygotic *stat92E* gene function is required for CySC maintenance at mid-L1, we assessed for presence of CySCs at the embryo to larval transition. Specifically, we examined expression of the cyst stem cell marker, *Zfh-1*, earlier in the developing testes. This assay is limited in late-stage embryos because *Zfh-1* is broadly expressed throughout the somatic gonad, at this time. As such, *Zfh-1* expression is not an accurate marker for CySC until after hub coalescence (~24hrs AE). However, some CySCs are known to arise from progenitors within PS11 of the embryonic gonad (DiNardo et al., 2011), and one might expect to see reduction or absence of *Zfh-1* expression in portions of the somatic gonad if CySCs are not specified properly. In all late-stage embryonic testes examined (16-24 AEL) (Fig 10A; n=5), *Zfh-1* expression was seen throughout the testes, in a pattern similar to wild type control testes (Fig 7B). Within limits of our assay, this suggests that the Jak-STAT pathway is not necessary to specify CySC progenitors within the SGP population. To further examine the role of Jak-STAT signaling in CySC specification, we also examined *Zfh-1* expression in early 1st instar larval testes (24-32 hours AEL). In this time-frame, asymmetrically dividing CySCs appear to have been established (previous results), but *Zfh-1* is not always fully restricted to the CySCs. Of early L1 testes examined (n=7), 57% had no *Zfh-1* expression within the testes while 43% had two *Zfh-1* positive cells displaced from the hub (Fig 10B). Counter to our analysis of late embryonic testes, these data could be interpreted to suggest that Jak-STAT signaling plays a role in CySC specification. However, because CySCs appear to be functional at this time, it is more likely that loss of *Zfh-1* expression in some testes is the result of defects in CySC maintenance rather than CySC specification. Thus, additional assays must be developed in order for any studies on CySC specification to be conclusive.

Over-expression of the Jak-STAT inhibitor, SOCS36E, in the soma alters testes morphology:

To examine the role of Jak-STAT signaling in CySC maintenance, we also over-expressed the Jak inhibitor, Suppressor of Cytokine Signaling 36E (SOCS36E), in somatic cells throughout the gonad. Specifically, *UAS-Socs36E* was ectopically expressed using the *c587- and traffic-jam-*

Gal4 drivers (see introduction on Gal4/UAS system) (Duffy, 2002) While no phenotype was observed in 1st instar larval testes over-expressing SOCS36E, 2nd instar larval testes (48-72 hrs AEL) displayed a dramatic defect in posterior gonad morphology. In particular, testes showed a void between germ cells and the gonad sheath at the testes posterior. (Fig 11A, 11B; compare to control in Fig 11D). This was observed upon over-expression of UAS-*Socs36E* using two cyst cell specific drivers of Gal4: *traffic jam-Gal4* (*tj-Gal4*, n=29) (Fig 11A) and *c587-Gal4* (Fig 11B, n=27; Fig 11C, n=18) drivers. Additionally, this phenotype was seen with two different UAS-*Socs36E* transgenes inserted at different sites in the genome (UAS-*Socs36E* on chromosome II and UAS-*Socs36E* on chromosome 3; Fig 11B and 11C, respectively), but never in control testes lacking a Gal4 transgene (Fig 11D, n=14). Indeed, this phenotype is observed in 38% (n=29) of larvae from parents homozygous for UAS-*Socs36E* on chromosome II and heterozygous for *tj-Gal4*; slightly lower than the Mendelian ratio for inheritance of both the Gal4 driver and the UAS-*Socs36E* transgene. Similarly, 39% (n=18) of larvae from parents heterozygous for UAS-*Socs36E* on chromosome III and homozygous for *c587-Gal4*, while 78% (n=27) of larvae from parents homozygous for both UAS-*Socs36E* on chromosome II and *c587-Gal4* displayed the posterior phenotype. In all testes with ectopic SOCS36E, normal expression of the CySC reporter, *Zfh-1* was observed. Thus, while Jak-STAT signaling is required for CySC maintenance in larval testes, over-expression of the Jak inhibitor SOCS36E throughout CySC and cyst cells results in a posterior phenotype in some, but not all testes. Furthermore, this phenotype does not appear to arise from a defect in CySC maintenance.

To further examine the phenotype caused by ectopic SOCS36E in the soma, we also sought to over-express SOCS36E (UAS-*Socs36E* on chromosome II) using additional Gal4 constructs that result in expression of UAS- transgenes with slightly different spatial or temporal patterns. The *c587-Gal4* and *tj-Gal4* drivers are expressed in somatic cells throughout testes just prior to GSC and CySC establishment (~20 hrs AEL; unpublished observations from the Wawersik lab). Thus, we used a *Six4-Gal4* drivers that is active in SGPs from the time of gonad formation (~5 hrs AEL;REF), a *Double-sex* Gal4 driver (*dsx-Gal4*) that is active in male SGPs after gonad coalescences (~11 hrs AEL; Oliver, personal communication), and an *unpaired* Gal4 (*upd-Gal4*) driver that has been shown to be active in hub cells at the time of GSC/CySC

establishment (~23 hrs AEL; Le Bras et al). We find that the posterior phenotype is observed in 72% (n=29, Fig 12A) of testes from parents homozygous for both *Six4-Gal4* and *UAS-Socs36E* on chromosome II, and in 78% (n=23, Fig 12B) of larvae from parents homozygous for both *dsx-Gal4* and *UAS-Socs36E* on chromosome II.

Unexpectedly, in 100% (n=6, Fig 12C) of larvae from adults homozygous for *Upd-Gal4* and *UAS-Socs36E* on chromosome II, the posterior phenotype was also observed. While observations made using the *upd-Gal4* driver are confounding because it was previously characterized as a hub cell driver, all other observations conducted, including those using multiple somatic Gal4 drivers and using different *UAS-Socs36E* transgenes are consistent with the interpretation that ectopic expression of SOCS36E in the soma results in a change in the posterior morphology but does not alter CySC maintenance. As we have previously shown (see data above) that Jak-STAT signaling regulates CySC behavior in the anterior gonad, this suggests another, previously uncharacterized, role for Jak-STAT signaling in development of cells in the posterior portion of the gonad.

STAT is activated in the posterior region of the gonad:

The SOCS36E over-expression phenotype described above suggests that Jak-STAT signaling is active in the posterior gonad, as well as in CySCs and GSCs (see Figure 16). To confirm this, we examined activation of the *10X-stat92E-GFP* transgene that is a read-out for Jak-STAT activation in somatic cells (Bach et al, 2006). In early 2nd instar larvae (~55hours AEL), we find that GFP expression is strongest in the hub, with strong expression also detected in the CySCs and weaker expression observed in cyst cells in the testis posterior (Fig 13, n=12). Some CySCs have increased levels of GFP expression, suggesting that STAT92E levels fluctuate through the cell cycle (see arrows in Fig 13 A', B, and D). Although dim, GFP expression is seen in the posterior of the testes in the cyst cells that surround differentiating spermatogonia. These observations supports our hypothesis that Jak-STAT signaling is, indeed, activated in posterior somatic cells, and suggests that inhibition of the low levels of STAT92E transcription seen in the posterior may be the cause of posterior morphogenesis defects observed after SOCS36E over-expression.

Cell number but not initial cyst cell differentiation is altered after over-expression of the Jak-STAT inhibitor, SOCS36E:

To further examine posterior gonad morphogenesis defects observed after ectopic SOCS36E expression, we assessed for defects in cyst cell differentiation and for presence/absence of cells in the posterior gonad. To examine cyst cell differentiation, we analyzed EYA expression in 2nd instar larval testes (48-72hrs AEL) over-expressing SOCS36E using the *tj-Gal4* driver. In all phenotypic testes (n=7), we find that EYA-positive cyst cells are observed away from hub, but none are seen within the altered posterior region (Fig 14). This indicates that SOCS36E over-expression does not impact the initial differentiation of cyst cells, or the maintenance of CySCs that produce them.

To determine if cells are even present in the Vasa-negative void between germ cells and the posterior gonad sheath, we examined for presence of DAPI stained nuclei in phenotypic testes (*tj-Gal4; UAS-Socs36E on chromosome II*). Of these testes (n=15), 40% showed regionalized loss of DAPI staining in the Vasa-negative void (Fig 15A), 47% showed a complete loss of DAPI staining in this region (Fig 15B), while 13% showed DAPI staining throughout this region (Fig 15C for representative images). When DAPI stain was present in this region, there were no obvious signs of nuclear fragmentation, as might be expected in cells undergoing apoptosis or autophagy. Taken together, these data show that ectopic expression of SOCS36E in the somatic gonad results in loss of cells in the posterior of testes during 2nd larval instar. Further studies are required to determine exactly when this phenotype arises, how cell are lost, as well as what cell types are present in the posterior region. However, these data indicate that Jak-STAT signaling is active in cells of the posterior testis, suggesting a novel role for Jak-STAT signaling during testes morphogenesis that is independent of its role in CySC maintenance in the testes anterior.

Discussion:

My data indicate that functional CySCs have developed in the *Drosophila* testes niche by the embryo to larval transition (~23 hours) at the same time as hub coalescence. CySC that

express adult CySC markers are organized around the newly formed hub in a manner similar to the adult testes, and adult cyst cell markers are seen in the posterior testis soon afterward. Additionally, Jak-STAT signaling is necessary and sufficient for maintenance of CySC in the larval testis niche. Thus, within 16 hours of gonad formation, *Drosophila* testes undergo coordinated development to form a fully functional stem cell niche with asymmetrically dividing CySCs that behave similar to adult CySCs (Figure 16). While this work helps us understand the timing of CySC establishment in late embryonic/early larval testes, as well as the regulation of CySC maintenance once stem cell establishment has occurred, the mechanism of CySC specification remains elusive. Preliminary evidence indicates that zygotic *stat92E* is not likely to be required for specification CySC specification in embryonic gonads. However, further assays must be developed and performed. Interestingly, we also find that over-expression of the Jak- inhibitor, SOCS36E, in somatic testes results in absence of germ cells and cyst cells, as well as a reduction in the number of nuclei, in the posterior testis at late L1 and early L2. As our data also indicate that Jak-STAT signaling is active in the posterior gonad during late L1, this implies that Jak-STAT signaling plays an additionally role in germ cell differentiation and/or posterior gonad development that has not previously been characterized.

CySC establishment and the potential for direct cyst cell differentiation:

My data indicate that CySC are established shortly after hub coalescence, at the embryo to larval transition (~23 hrs AEL, Figure 7). However, based on data shown in this thesis, it is formally possible that CySCs are specified at this time, but they have not yet initiated asymmetric stem cell divisions. While presence of dividing cyst cells in the posterior testis at mid-L1 suggests the establishment of functional, asymmetrically dividing CySCs at earlier stages of testis development, an alternative mechanism is that a sub-population of embryonic SGPs directly differentiates into cyst cells without taking on CySC fate in order to support early spermatogenic differentiation in the posterior gonad. Indeed, while direct differentiation of SGPs into cyst cells has never been studied, a subset of posterior PGCs have been shown to differentiate directly into sperm-producing gonialblasts cells without ever behaving as a GSCs (Asaoka and Lin, 2004). That said, additional data collected by Ron Palmen in the Wawersik lab

shows that Zfh-1 positive cells adjacent to the hub divide regularly and with the characteristic orientation seen for adult CySCs (Sinden, Badgett et al, *manuscript in preparation*). Furthermore, while the number of differentiating spermatogonia increases throughout larval development, the number of sperm-producing gonialblasts cells is constant by mid-L1, indicating that the constant production of gonialblasts cells via asymmetric GSC division is balanced by differentiation of gonialblasts cells into spermatogonia. As Ron rarely observes cell division in somatic cells localized in the posterior region of L1 testes, cyst cells required for increased spermatogenesis are most likely produced by the CySC that reside adjacent to the hub. Thus, while a small sub-population of cyst cells may arise from SGPs that never take on CySC fate, the majority of cyst cells must be produced via asymmetric division of hub-proximal CySCs that appear to be established as functional, asymmetrically dividing stem cells at or around the embryo to larval transition (~23 hrs AEL).

Development of SGPs and CySC specification:

Jak-STAT signaling is necessary and sufficient for CySC maintenance (see Figures 8 and 9, respectively). However, little is known about CySC specification. Cell lineage tracing experiments indicate that at least some CySCs arise from SGPs from PS11 (DiNardo et al., 2011). However, not all CySC arise from PS11 and not all PS11 SGPs become CySC, as some take on a hub fate (Le Bras and Van Doren, 2006). A number of pathways (the Notch, Lines/Bowl, Epidermal Growth Factor Receptor (EGFR), and Boss/Sevenless regulatory pathways; reviewed in De Cuevas & Matunis, 2011) have been shown to regulate SGP differentiation in the embryonic gonad. Specifically, Notch and Bowl have been shown to promote hub cell fate in anterior SGPs (DiNardo et al, 2011, Kitadate & Kobayashi, 2010) , while Lines, EGFR, and Sevenless repress hub formation and promote posterior cell fate (DiNardo et al., 2011; Kitadate & Kobayashi, 2007).

As STAT is activated in testes at the time of gonad formation (Wawersik et al, 2005), Jak-STAT signaling may also have a role in CySC specification. Our preliminary data suggest that zygotic *stat92e* is not required to specify CySCs from SGPs, but it is necessary for CySC establishment and maintenance (Figure 10). However, it is possible that maternally deposited

STAT plays a role in CySC specification (Wawersik et al, 2005). In order to conclusively determine if zygotic STAT is necessary for specification of CySC from their immediate progeny in the testes, additional studies must be conducted with greater sample size and in a more focused time-frame. For any of these studies, it will also be important to develop a more conclusive assay for CySC specification. One such assay is activation of signaling from CySC progenitors to PGCs through the bone morphogenic protein signaling pathway (BMP). Work in adult testes shows that repression of GSC differentiation in the niche is controlled, at least in part, by BMP signaling from CySCs to neighboring GSCs. Additional work by Ashley Fidler has shown that BMP signaling is activated in a small subset of PGCs within embryonic testes prior to hub formation and GSC establishment. We hypothesize that CySCs are specified from SGPs adjacent to germ cells showing BMP activation. If this proves to be the case, changes in BMP activation in the germline, or altered expression of BMP activating ligands in SGPs, could be used as a readout for CySC identity when examining the role of STAT and other cell signaling pathways in the specification of CySCs.

The fate of SGPs that do not become hub or CySCs is also not clear. The collective data from the Wawersik indicate that CySC are dividing into maturing cyst cells (Sinden and Badgett et al, *manuscript in preparation*). As discussed above, it is possible that some early cyst cells are derived through direct differentiation of SPGs that never take on CySC fate. If this is the case, where might these cells reside? Since a subset of posterior PGCs are known to directly differentiate into spermatogonia (Asaoka and Lin, 2004), posterior SGPs would assist in the maturation of spermatogonial cells. As such, it is feasible that cyst cells differentiate directly from SGPs. Additionally, EGFR signaling, required for ensheathment of gonialblasts by cyst cells in the adult, is detected in the posterior SGPs after gonad formation (Kitadate & Kobayashi, 2010). However, *Eya* expression is not seen till mid L1 (~36hrs AEL), ~12 hours after hub coalescence and *zfh-1* restriction, implying that the CySC give rise to the *Eya* positive cells, although *Eya* expression could lag behind cyst cell specification and not be observed until cyst cells are established. In order to test where posterior cyst cells are coming from in the L1 testes, additionally analyses must be performed to determine what cell population the earliest cyst cells are derived from.

Jak-STAT signaling during testis development:

Jak-STAT signaling has been known to play an important role in maturation of GSCs and CySCs in the developing testes. Previous studies have shown that the Jak-STAT activating ligand, Upd, is expressed in the soma at the anterior half of the testes after gonad formation (Wawersik et al., 2005). During hub morphogenesis, Upd expression restricts to the hub cells that are coalescing at the testes apex (Le Bras and Van Doren, 2006; Sheng et al., 2009). At this time, high-level Jak-STAT activation is restricted to GSCs and CySC established adjacent to the hub (Sheng et al, 2009; Figure 10)

Our results also suggest another function of the Jak-STAT pathway in the testes. Indeed, analysis of 10X-*stat92E-GFP* testes (Figure 13) indicates that Jak-STAT signaling is activated in posterior cyst cells. Defects in posterior gonad development observed upon over-expression of the Jak inhibitor, SOCS36E (Figures 11 and 12) are consistent with a role for Jak-STAT signaling in posterior testes morphogenesis. But what could this role be? One hypothesis is that Jak-STAT signaling is required for maturation of cyst cells. Since *Zfh-1* and *Eya* expression is observed in the testes with this defect (Figures 11 & 13), CySCs appear to be differentiating normally, giving rise to cyst cells. However, *Eya* expression is not observed in regions without *Vasa* expression. This suggests a defect in further cyst cell maturation is responsible for the observed phenotype.

We postulate that down-regulation of the Jak-STAT pathway via SOCS36E, prevents continued differentiation of cyst cells, leading to absence of both identifiable cyst cells and germ cells. Although the specific mechanisms of Jak-STAT function in the posterior is unknown, similarities between the SOCS36E over-expression phenotype I observe and data from Papagiannouli and Mechler (Papagiannouli and Mechler, 2009) suggest that the tumor suppressor gene, *discs large (dlg)*, may be involved. Indeed, this work shows that *dlg* mutant testes are small due to loss of differentiating spermatogonia in the posterior. As *dlg* encodes a protein found in separate junctions, it is hypothesized that testes development defects due to altered *dlg* activity result from reduced adhesion between cyst cells and differentiating spermatogonia. Subsequent work will, therefore, examine the potential link between Jak-STAT signaling, *dlg*, and soma-germ cell adhesion.

Future Directions:

A number of studies must still be done to better confirm some of my data and answer questions raised by the new data. The effect of maternal *stat92e* on CySC specification during hub coalescence must be tested. This can be done with temperature sensitive *stat92e* allele, or using *upd* mutants because there is no maternally deposited *upd* mRNA. While analysis of *upd* mutants was already attempted, my initial study proved inconclusive due to the poor condition of the embryos (see Supplementary Figure 1). Similar studies must, therefore, be repeated.

We must also develop a better assay to analyze CySC specification. As discussed above (section on *CySC specification*), the fate of cells expressing BMP ligands should be tracked in SGPs, as should BMP activation in neighboring PGCs. Alterations in expression of these proteins in this pathway could be used to elucidate the pathway's function as well as a better understanding of the identity of the SGPs in the developing testes.

To assess whether direct differentiation of SGPs into cyst cells occurs, lineage tracing and live cell imaging experiments should be performed to follow the fate of SGPs that arise within different regions of the embryonic testes. Similar assays may also be used to determine which SGPs become CySCs, and to examine cell movement and morphological changes that may occur during the process of testes morphogenesis.

Finally, studying the new function of the Jak-STAT pathway in posterior testes will be difficult since its function in stem cell maintenance will prevent normal differentiation. However, some studies can still be done. One of the first should be to examine *upd* expression in the posterior testis to see whether this initiator of Jak-STAT is expressed in the differentiating regions of the testes. Presence of *upd* expression in this area would be consistent with our observation that Jak-STAT signaling at this time and location, and would help explain observation of a posterior phenotype after UAS-*Socs36E* over-expression from the *upd-Gal4* line that was previously characterized to show only hub cell expression in larval testes. Using additional Gal4 lines that are active in the posterior soma, such as *eya-Gal4*, we could also alter Jak-STAT signaling in just the posterior without interfering with stem cell maintenance. By examining changes in Dlg expression and the formation of septate junctions in these testes, it

should be possible to examine potential links between Jak-STAT signaling, *dlg*, and germline-soma adhesion. Together, through these and other studies, we hope to draw parallels between *Drosophila* testes development and other stem cells systems and systems of development; shedding new light onto mechanisms of organogenesis, and specifically regulation of stem cell specification and establishment during niche formation.

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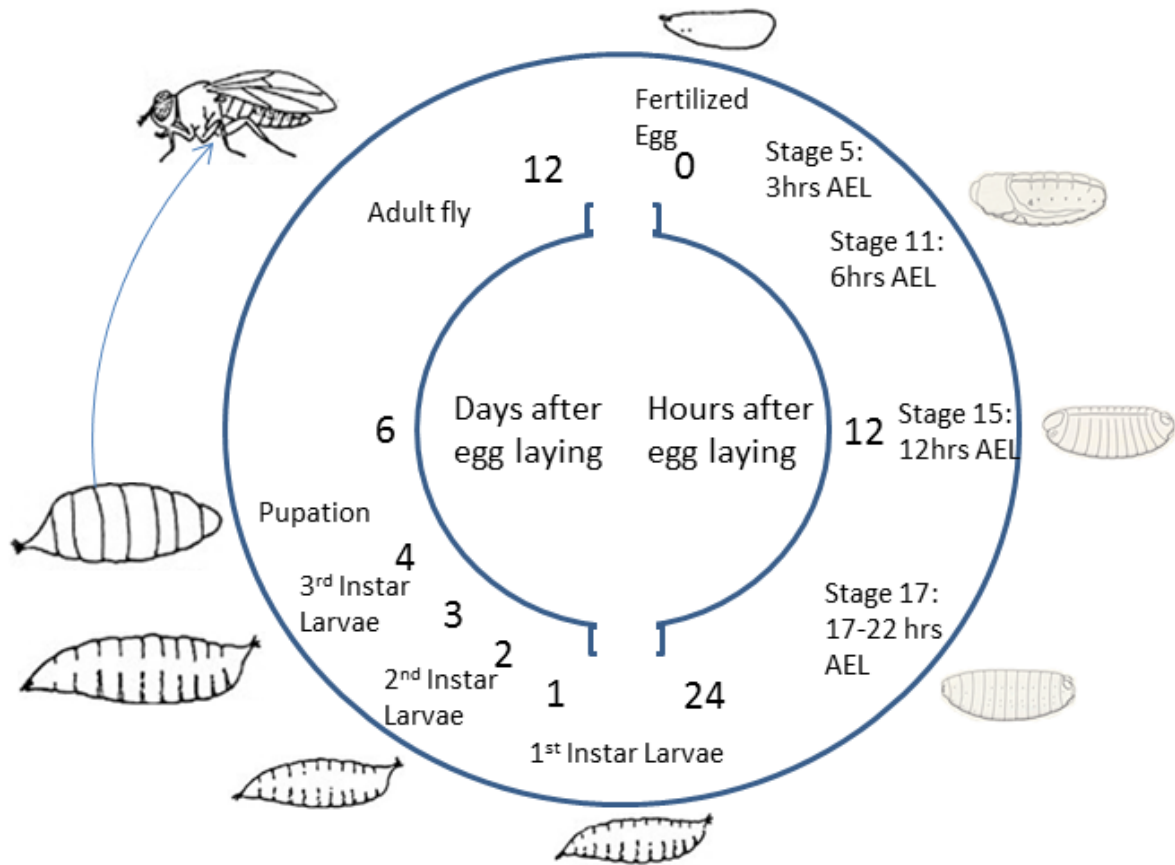


Figure 1 – *Drosophila* life cycle:

After egg laying (AEL), embryo develops over ~24 hours. About 1 day AEL, larvae hatches and that larval development is divided into three stages, separated by molting: instar L1 (24-48hrs AEL), L2 (48-72hrs AEL), L3 (72-96hrs AEL). Pupation, lasting 4 days, results in most of the larval tissues being destroyed, replaced by adult tissues. Times are given for the life cycle at 25°C. Adapted from Wolpert et al. (1998).

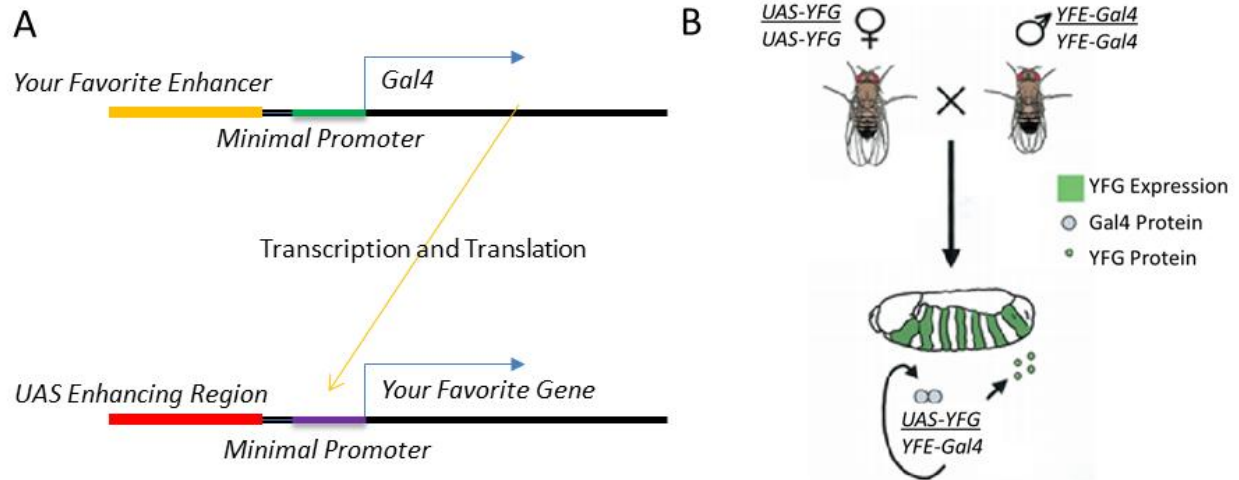


Figure 2 – UAS/Gal4 System:

On the genetic level, when the appropriate transcription factor binds to “Your Favorite Enhancer” (YFE), *Gal4* expression is driven and *Gal4* binds to the UAS sequence upstream of the *hp70* Minimal promoter to induce expression of a “Your Favorite Gene” (YFG) (A). By crossing a female fly with the *UAS-YFG* transgene and a male with the *YFP-Gal4*, resulting offspring will express tissue specific YFG (B).

Figure 2B is adapted from Duffy, 2002.

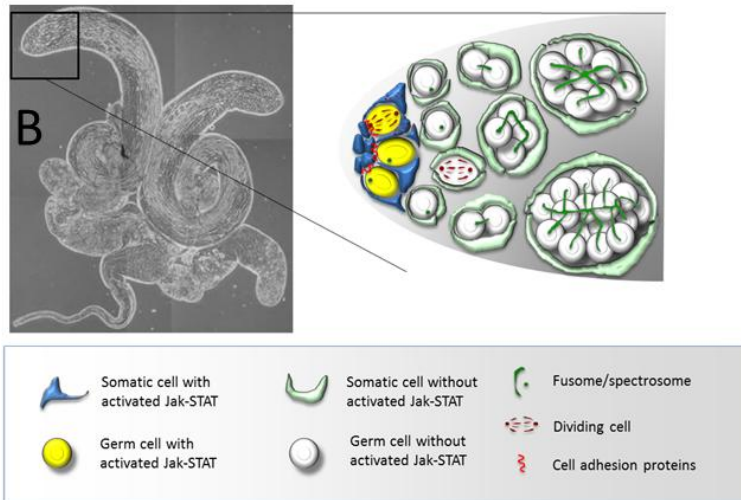
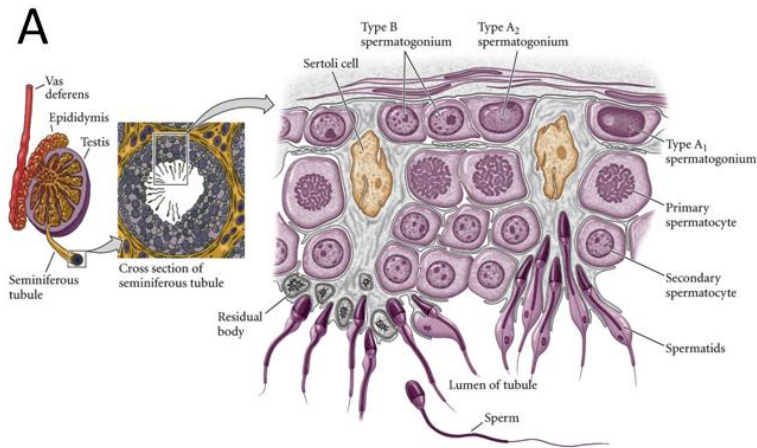


Figure 3 – Adult Testes Structure:

In mammalian testes (A), germline stem cells (spermatogonium) are located on the periphery of the seminiferous tubules. Through asymmetric division, spermatogonium produce primary spermatocytes, which undergo meiosis to eventually produce sperm. Sertoli cells, large somatic cells, guide sperm development at all stages. In the fruit fly testes (B), germ stem cells are found at the apex of the testes. Hub cells maintain the germ stem cells and the cyst stem cells, all of which have activated Jak-STAT. Cells that divide away from the hub during division no longer have activated Jak-STAT and begin to differentiate, resulting in branched fusomes, an organelle found in germ cells. After 16 germ cell clusters form, the cells undergo meiosis, resulting in 64 sperm per cluster.

Mammalian testes taken with permission from Developmental Bio 9th ed by Scott Gilbert, 2009.

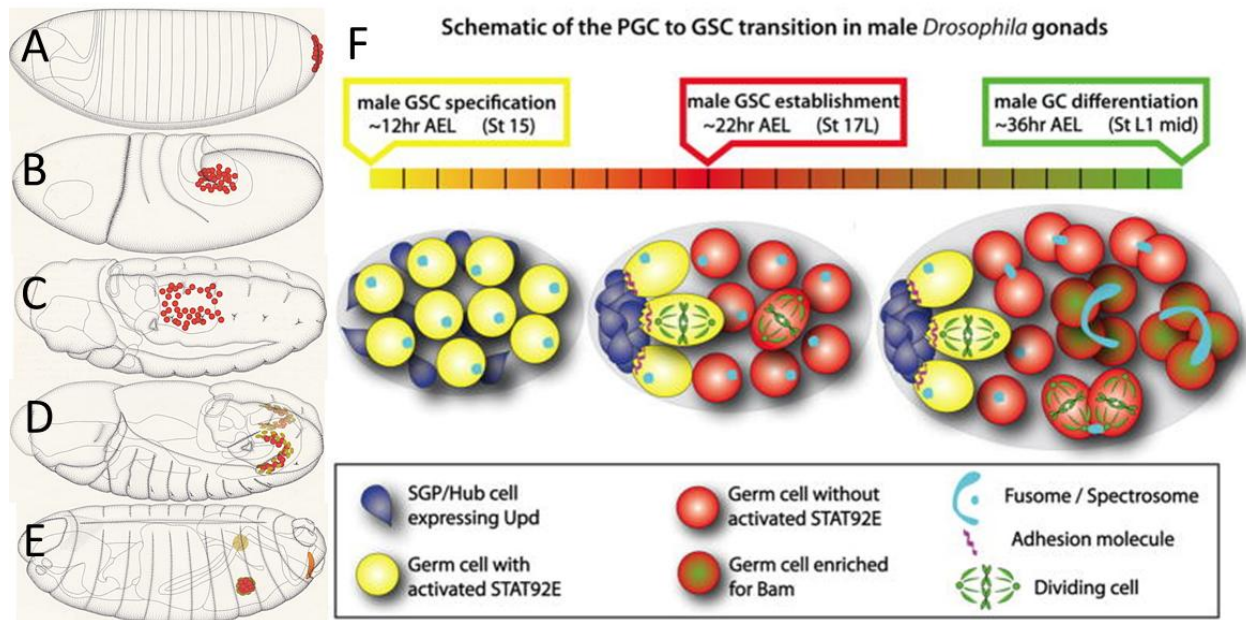


Figure 4 – Embryonic and Larval *Drosophila* Testes:

During stage 5 of embryogenesis, primordial germ cells (red cells, PGCs) form at the posterior of the embryo (A). PGCs are moved to the interior of the embryo when posterior somat is pulled anteriorly into the midgut by stage 8 (B). By stage 11, PGCs migrate out of the midgut pocket in two bilateral clusters toward parasegments 10-12 within the mesoderm where somatic gonadal precursor cells (SGPs) reside (C; SGPs shown in green). PGCs and SGPs begin to intermingle at stage 12 (D), and two testes coalesce into tight clusters, containing PGCs and SGPs, during stage 13 (E). After gonad coalescence, the male gonads activate the Jak-STAT signaling pathway in both PGCs and SGPs by stage 15. However, by the end of embryogenesis, activation of the Jak-STAT signaling pathway is known to be restricted to hub cells in the anterior and germline stem cells that adhere to the hub. By mid L1, differentiation of germ cells is observed in the posterior (F).

Figures 4A-E taken from FlyBase at:

http://flybase.org/static_pages/imagebrowser/imagebrowser10.html

Figure 4F from Sheng et al (2009).

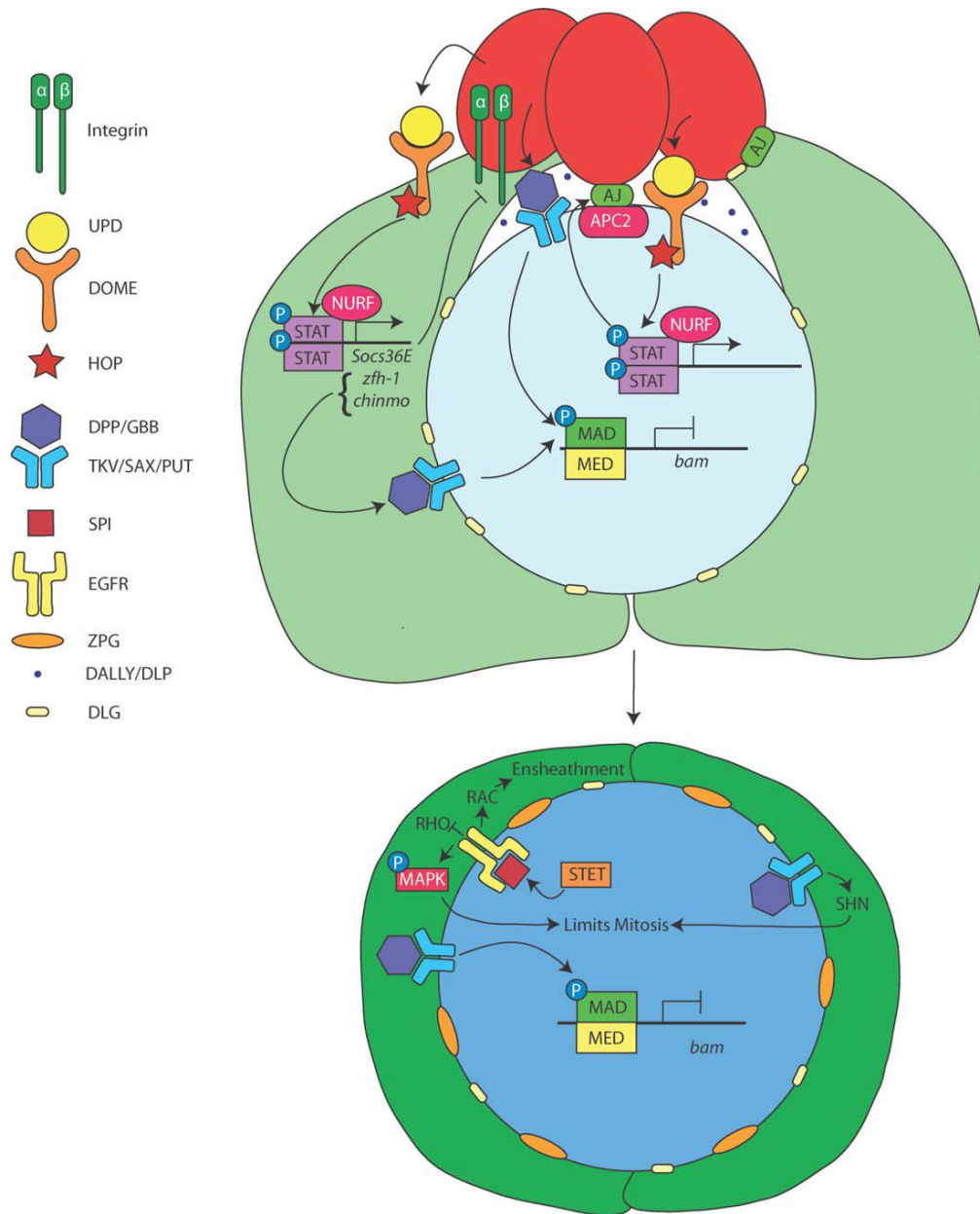


Figure 5 –Regulation of Stem Cell Maintenance and Differentiation in Adult Testes:

Unpaired (UPD) is secreted from the hub and binds to the Domeless (DOME) receptor on both CySCs and GSCs, activating the Jak protein, Hopscotch (HOP), which leads to the phosphorylation, dimerization, and activation of STAT. The STAT dimer activates numerous pathways, eventually resulting in the expression of Decapentaplegic (DPP), Glass Bottom Boat (GBB), and Suppressor of Cytokine Signaling 36E (SOCS36E), which suppresses the Jak-STAT pathway.

Image taken with permission from Jemc et al., 2011.

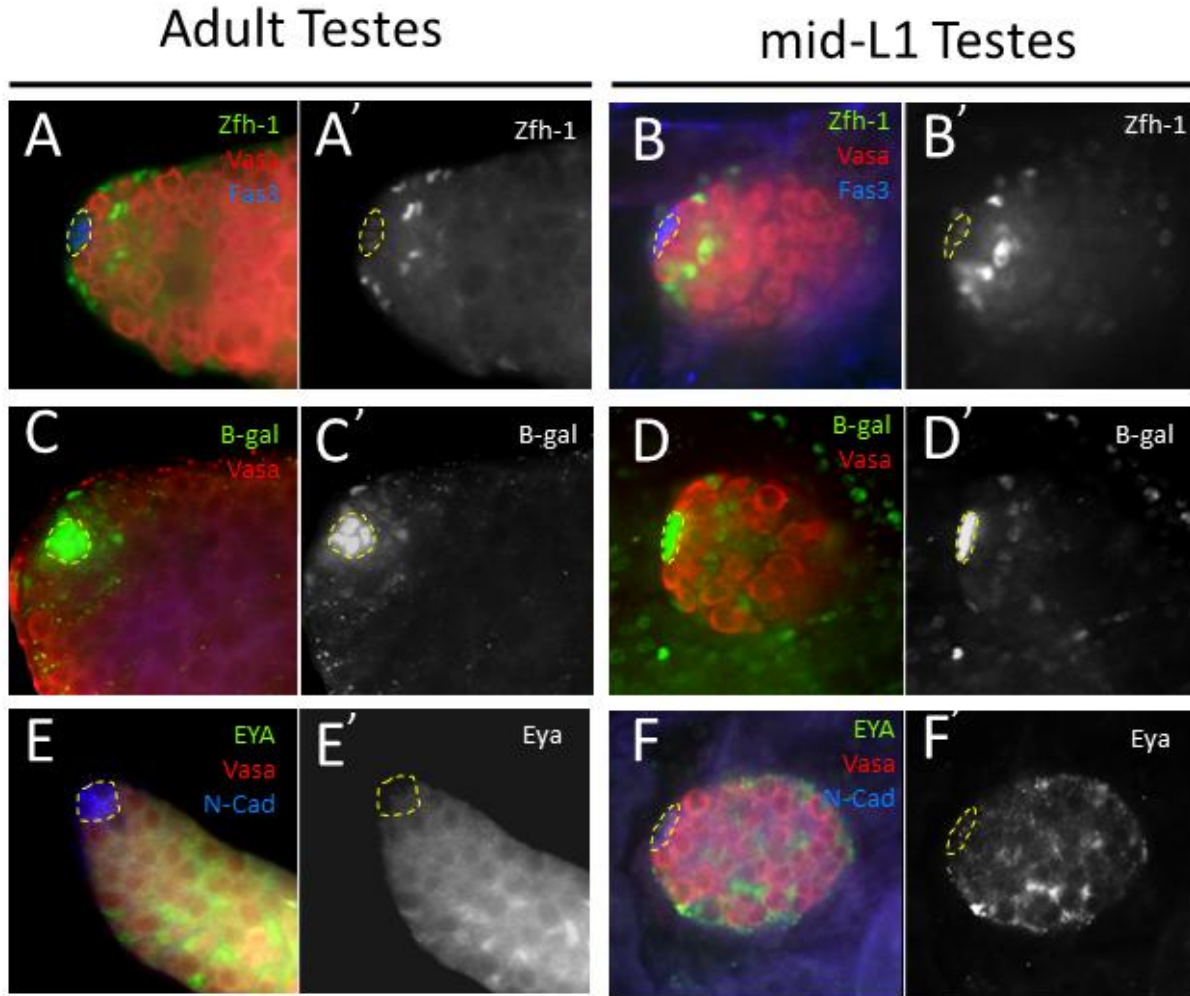


Figure 6 – Cyst Stem Cells are present by larval first instar:

Mid-first instar larval or adult testes immunostained with anti-Vasa (red) to detect germ cells, anti-FasciclinIII (A-D & G-H, blue) or anti-N-Cadherin (E-F, blue) to detect the GSC niche, and either anti-Zfh-1 (A, B, green), anti- β -galactosidase to reveal expression of a *Soc36E-LacZ* gene reporter (C, D. green), anti-Eya (E, F, green) or anti-GFP (G, H, green). The GSC niche at the anterior testis is outlined in all] panels (dotted lines). [A, B] Expression of the CySC marker, Zfh-1, is restricted to nuclei of somatic cells immediately adjacent to the GSC niche in adult (A, A') and mid-L1 (B, B') testes. [C, D] *Socs36e-LacZ*, detected in the GSC niche and CySCs in the adult, shows similar expression in adult (C, C') and mid-L1 (D, D') testes. [E, F] Eya expression,

reflecting presence of differentiating cyst cells, is detected in somatic cells away from the hub in both adult (E, E') and mid-L1 (F, F') testes.

Early-mid 17 ~19 hrs AEL Late 17/L1e ~22 hrs AEL Early L1 ~26 hrs AEL Mid L1 ~36 hrs AEL

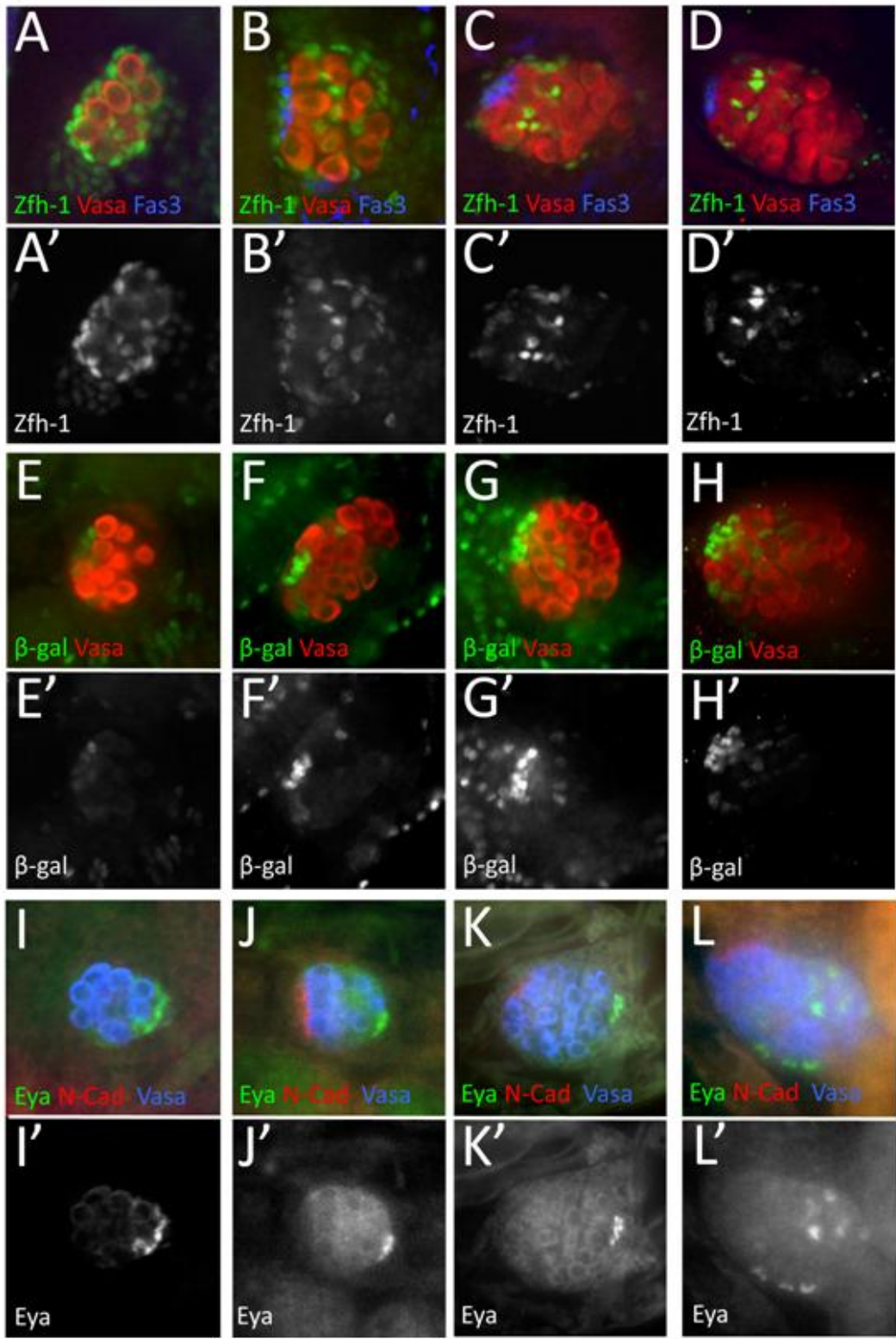


Figure 7– Time course of CySC development and differentiation:

Late-stage embryonic and first instar larval testes immunostained with anti-Vasa (A-H, red; I-L, blue), anti-FasIII (A-D, blue) or anti-N-Cadherin (I-L, red) to reveal location of the GSC niche, and either anti-Zfh-1 (A-D, green, A'-D' alone), anti- β -galactosidase (E-H, green; E'-H' alone) to reveal *Socs36e-LacZ* expression in the developing hub and CySCs, or anti-Eya (I-L, green; I'-L' alone) to examine onset of cyst cell differentiation. Testes are from early-mid stage 17 embryos (~19 hrs AEL; A, E, I), late- stage 17 embryos (~22 hrs AEL; B, F, J), early- 1st instar larvae (~26 hrs AEL; C, G, K) or mid- 1st instar larvae (~36 hrs AEL). All images with anterior oriented left. [A-D] Zfh-1 is expressed broadly in somatic cells of embryonic testes (A, B) with hub proximal nuclei displaced from the hub by GSC at late stage 17 (b), while expression becomes restricted to the anterior half of the gonad and the gonad periphery by early L1 (C). By mid-L1 (D), robust Zfh-1 expression is further restricted to hub proximal somatic cells. [E-H] SOCS36E-LacZ expression in anterior somatic nuclei that form the hub in late- stage embryonic and 1st instar larval testes (E-H). SOCS36E-LacZ is first detected in somatic cells immediately adjacent to the hub in late- stage 17 embryos (F) and this expression pattern is maintained as development proceeds (G, H). [I-L] Eya expression is detected in male-specific somatic gonadal precursors (msSGPs) at the posterior testes (with cross-reactivity in the germline) in early-mid stage 17 embryos (I) and remains restricted to msSGPs at the testes posterior until early-L1 (J, K), after which somatic EYA is also detected in the posterior half of the gonad where spermatogonia are known to form.

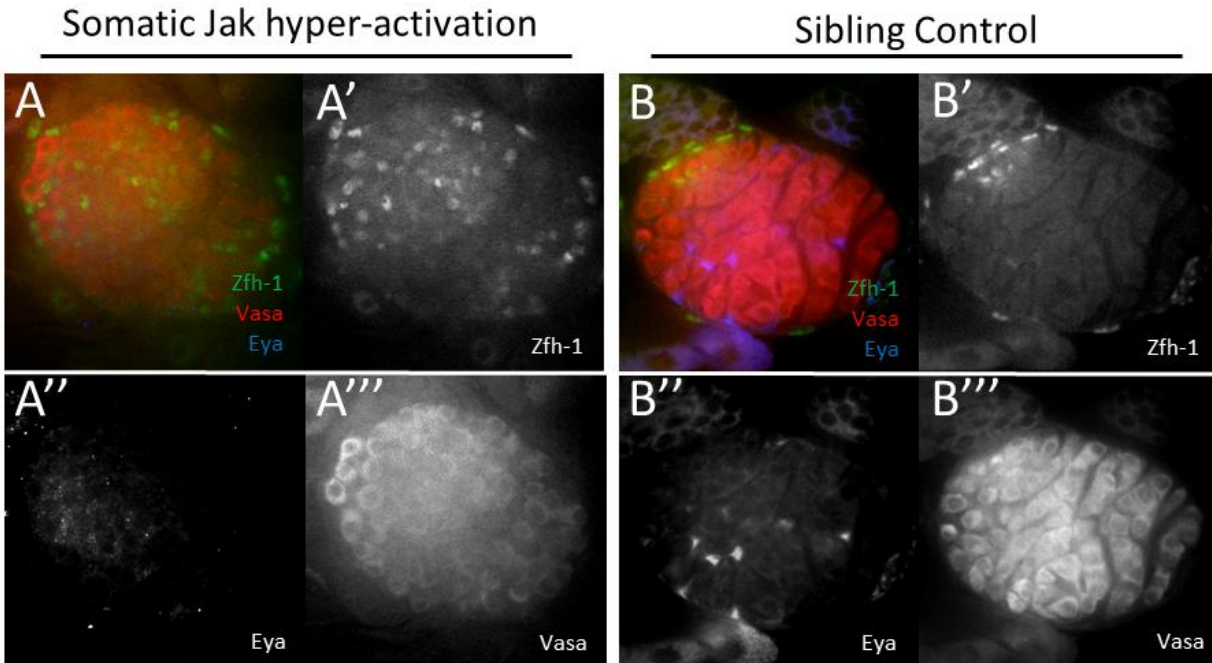


Figure 8 – Jak-STAT signaling is sufficient for CySC maintenance:

Late-larval 1st instar testes immunostained with anti-Vasa (A-B red; A''' & B''' alone) to reveal germ cells, anti-Zfh-1 (A-B, green; A' & B' alone) to reveal CySCs, anti-Eya (A&B, blue; A''& B'' alone) to reveal cyst daughter cells. Testes with Jak hyper-activated in somatic cells (A; c587-Gal4; UAS-hop^{TumL}) show expanded expression of Zfh-1 and loss of Eya expression (A) as compared to sibling controls (B).

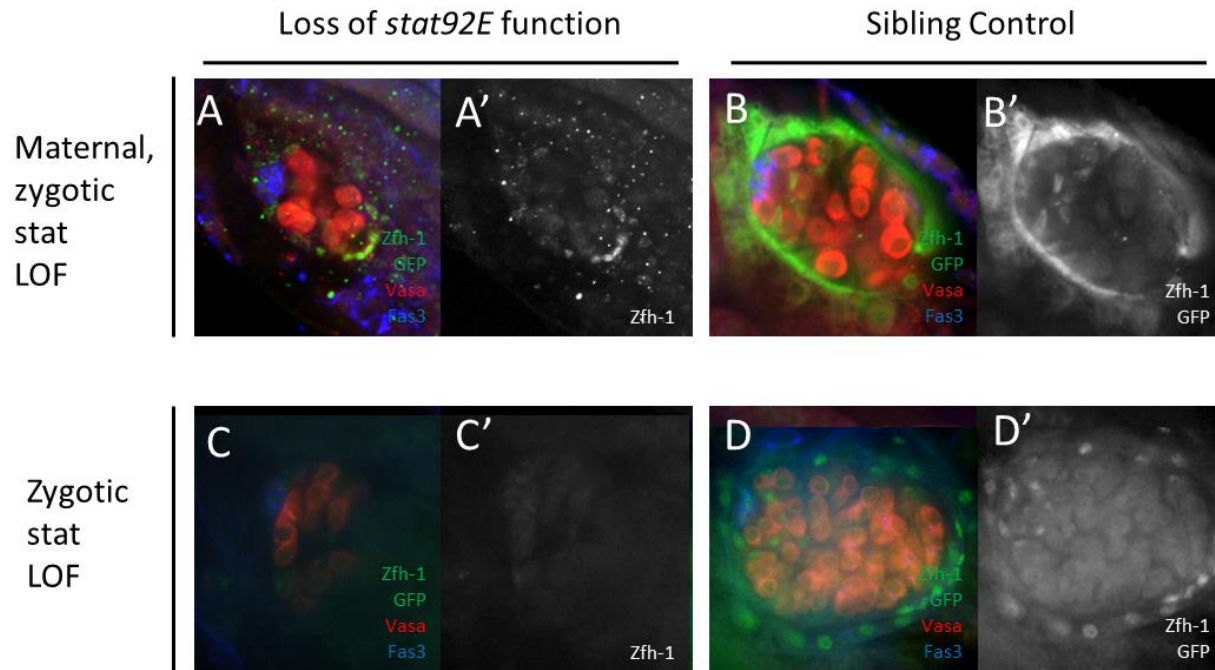


Figure 9 – *Jak-STAT* signaling is necessary for CySC maintenance:

Mid-larval 1st instar testes immunostained with anti-Vasa (A-D, red) to reveal germ cells, anti-Zfh-1 (A-D, green; A'-D', alone) to reveal CySCs, and anti-GFP (A-D, green; A'-D', alone) to determine genotype of *Stat*^{TS} mutants. All images with anterior testes oriented left. *Stat*^{TS} testes mutant for both maternal and zygotic *stat* at high temperatures (29°C) lacks Zfh-1 expression in hub-proximal somatic nuclei (A) while sibling controls are normal (B). *Stat*-null mutant for zygotic *stat* lacks Zfh-1 expression in hub-proximal somatic nuclei (C) while sibling controls are normal (D).

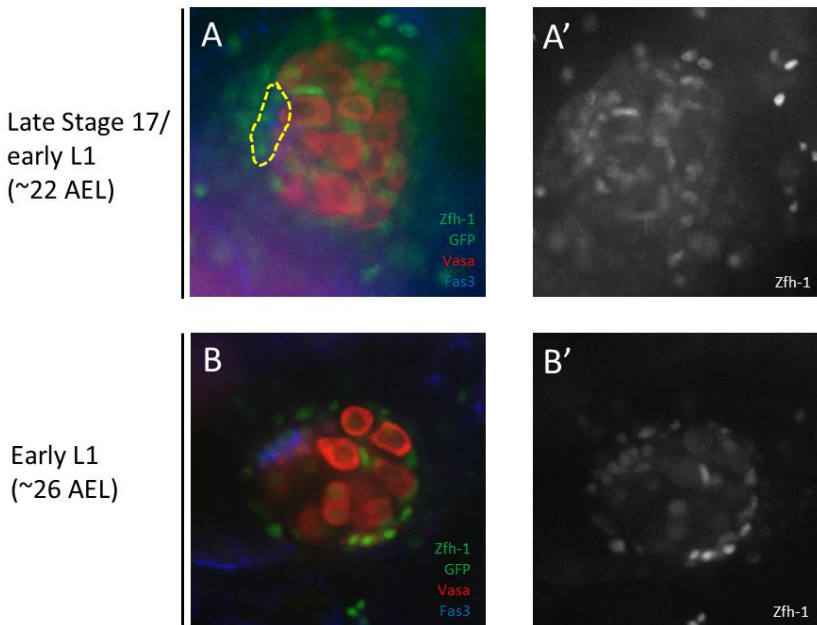


Figure 10 – zygotic *Jak-STAT* signaling is not necessary for initial CySC specification:

Late-stage embryonic and first instar larval testes immunostained with anti-Vasa (A&B, red) to reveal germ cells, anti-FasIII (A&B, blue) to reveal location of the GSC niche, and anti-Zfh-1 (A&B, green, A'-B' alone) to reveal CySCs. *Stat-null* mutants for zygotic *stat* during late-stage 17 (A) have broad expression of Zfh-1 positive cells (A', compare to wild type in Figure 7A,B). In early first instar larval testes, *Stat-null* mutants for zygotic *stat* (B) have moderate restriction of Zfh-1 positive cells adjacent the hub, but the number of Zfh-1 expressing cells in this location appears to be reduced (B', compare to wild type in Figure 7C).

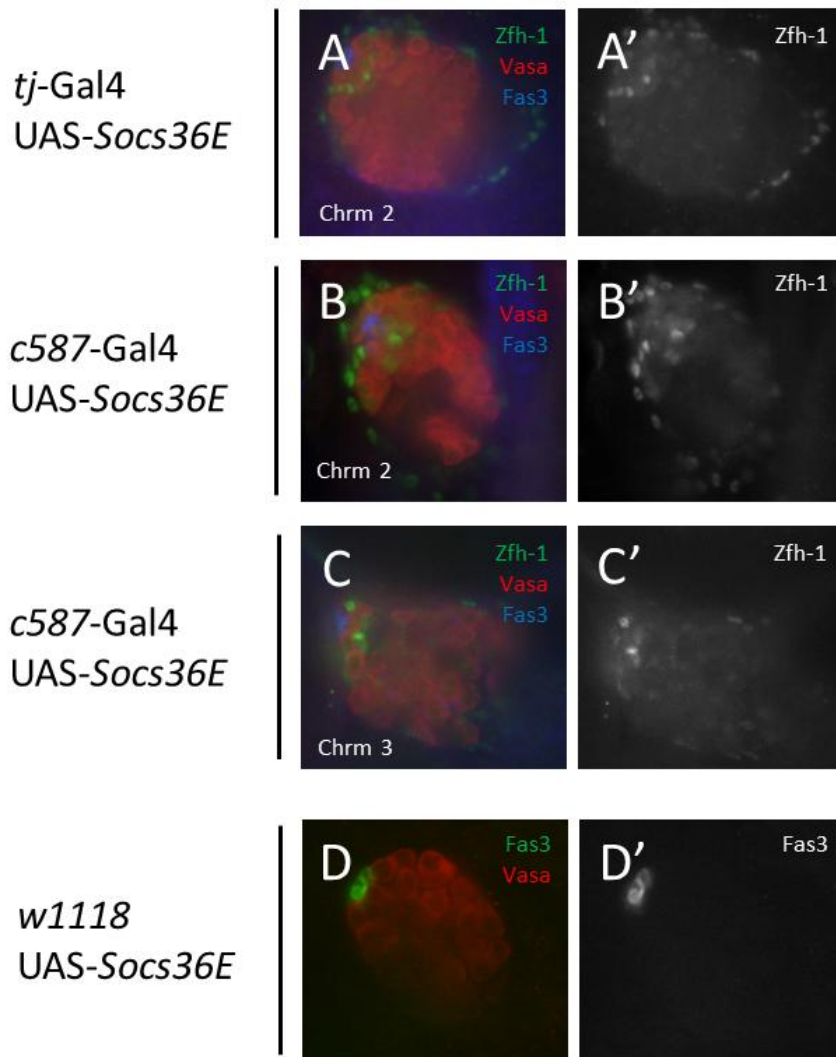


Figure 11 – UAS-Socs36e driven in the cyst cells results in cell absence in the posterior:

Second instar larval testes immunostained with anti-Vasa (A-D, red,) to reveal germ cells, anti-FasIII (A-D, blue; D', alone) to reveal location of the GSC niche, and anti-Zfh-1 (A-D, green; A'-C' alone) to reveal CySCs. In instar stage L2, testes with *Socs36E* hyper-expressed in the cyst cells (either with *C587-Gal4 UAS-Socs36E* (A), or *tj-Gal4 UAS-Socs36E* (B)) have normal Zfh-1 expression (A' & B') but lack the presence of Vasa-positive germ cells in the posterior (A&B). When a different *UAS-Socs36E* construct is used with *C587-Gal4*, the same cell absence phenotype (C) is observed. When *UAS-Socs36E* is crossed to wild type line *W1118*, posterior is present (D).

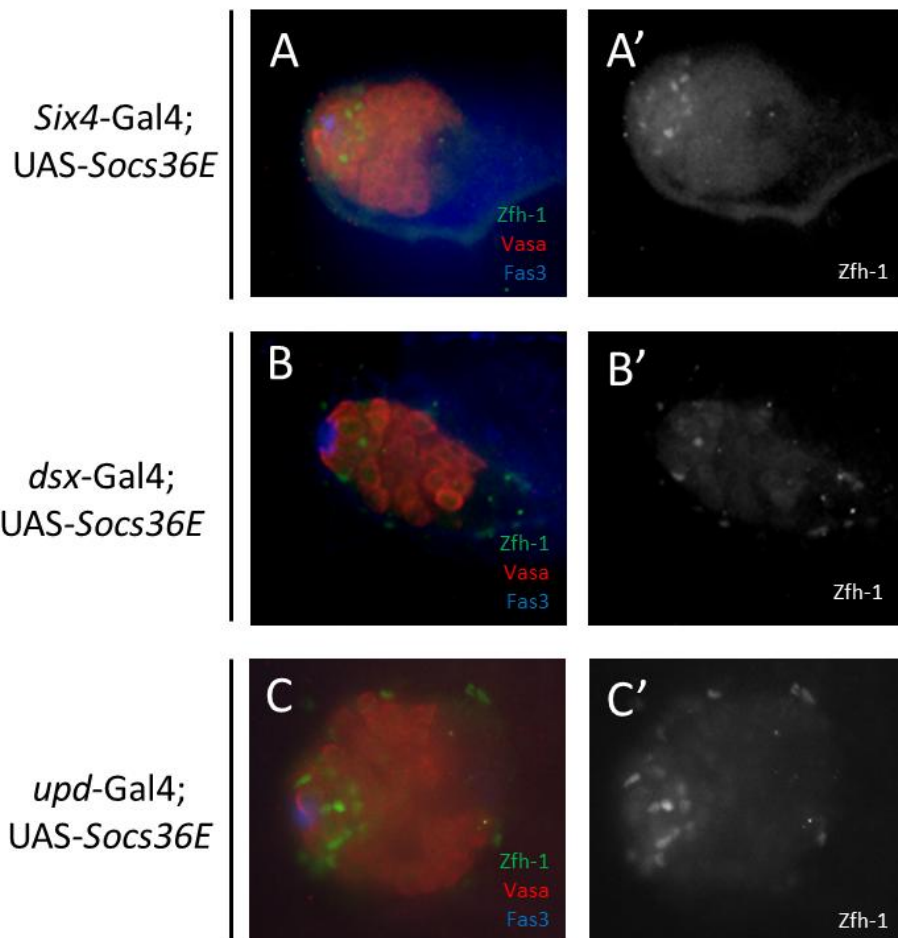


Figure 12 – UAS-Socs36E driven in the soma results in a morphogenesis defect at the testis posterior:

Second instar larval testes immunostained with anti-Vasa (A-C, red,) to reveal germ cells, anti-FasIII (A-C, blue) to reveal location of the GSC niche, and anti-Zfh-1 (A-C, green; A'-C' alone) to reveal CySCs. Testes with SOCS36E hyper-expressed in the soma via *Six4-Gal4* (A), *Dsx-Gal4* (B), and *upd-Gal4*(C) with *UAS-Socs36E* shows the same cell absence phenotype seen when *UAS-Socs36E* was expressed with *C587-Gal4* or *tj-Gal4* (Figure 6).

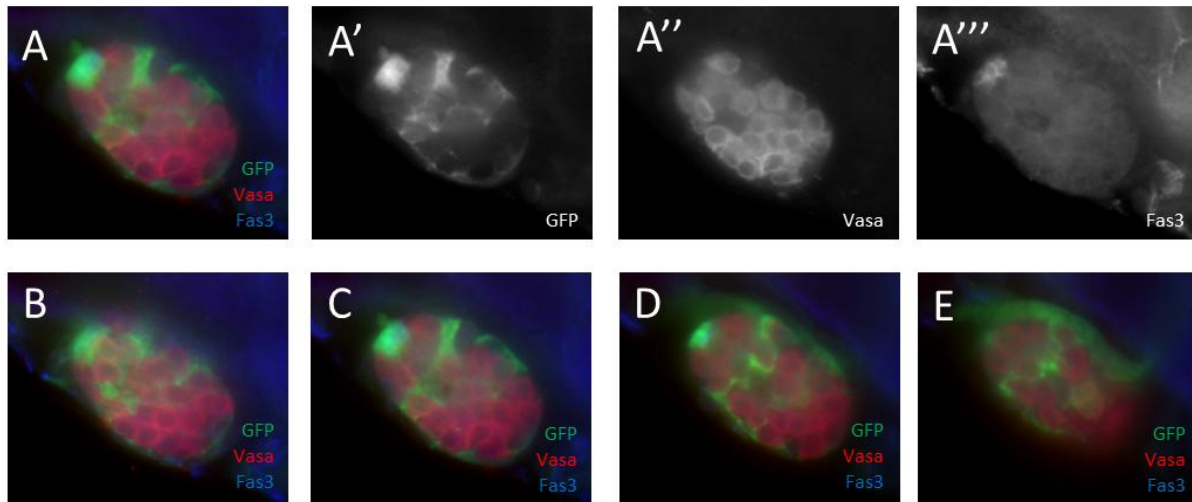


Figure 13 – *Stat* promoter is active in both the anterior and posterior region regions of the testis:

Early 2nd instar testes immunostained with anti-Vasa (A-E, red; A'' alone) to reveal germ cells, anti-GFP (A-E, green; A' alone) to areas of *Stat* promotion, and anti-FasIII (A-E blue, A''' alone) to reveal location of the GSC niche. *Stat* promotion, shown via the 10X-*stat92E-GFP* construct, is brightest in the hub, but expressed weakly throughout the testes. Looking at just GFP (A') and slides through the same testes (B-E), it can be seen that expression is extremely strong in hub as well as in the cyst stem cells. One stem cell, seen in A and C, has even stronger GFP expression. Weaker expression is seen in the posterior, but still present, especially in the most posterior region (A').

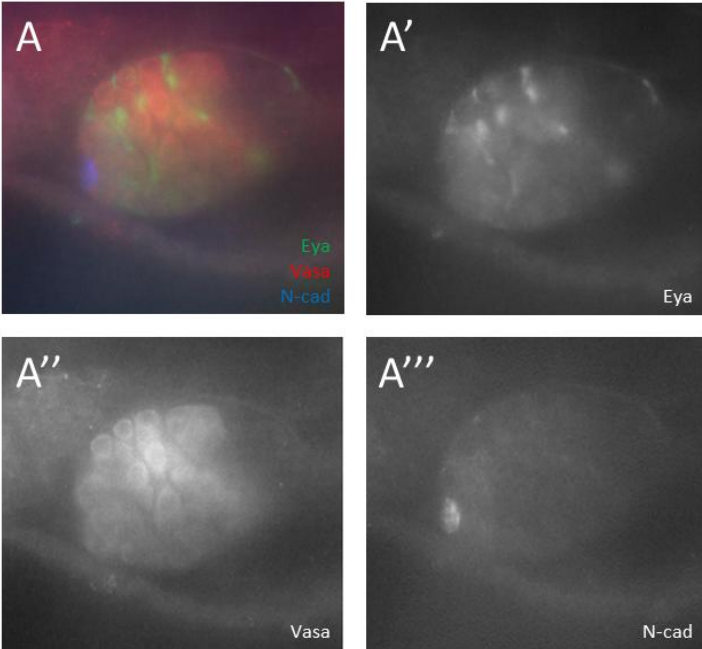


Figure 14 – Cyst cell differentiation is initiated normally after *SOCS36E* over-expressed in the soma:

Second instar larval testes immunostained with anti-Vasa (A red; A'' alone) to reveal germ cells, anti-N-cadherin (A, blue; A', alone) to reveal location of the GSC niche, and anti-Eya (A, green; A', alone) to reveal cyst cells. Cyst cells in *tj-Gal4 UAS-Socs36E*, present after first instar larva in wild type testes, are present (A, A') where Vasa is also expressed (A''), but is not present in the missing posterior region.

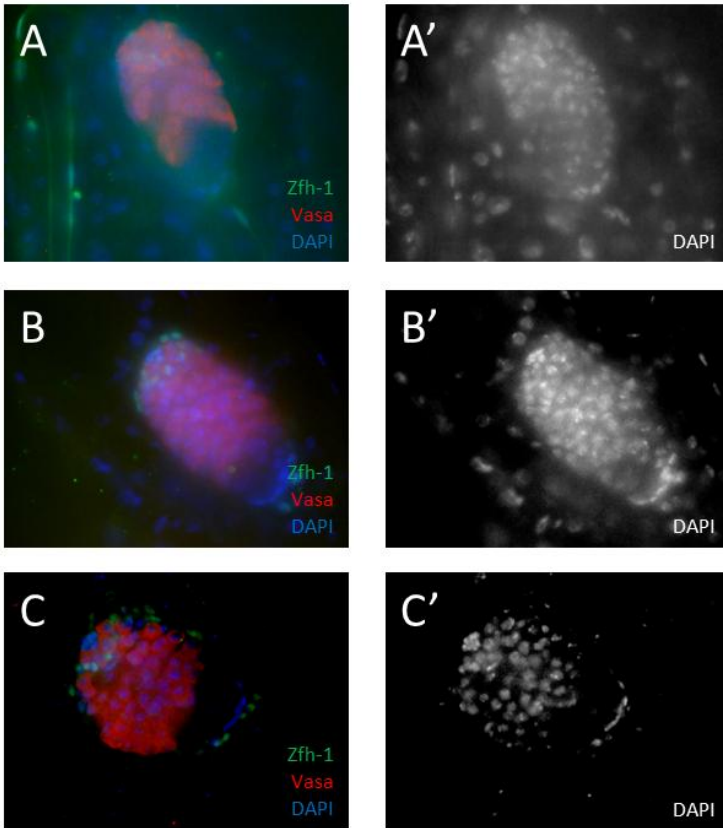


Figure 15 – DAPI staining is reduced in the posterior testis:

Second instar larval testes immunostained with anti-Vasa (A-C, red) to reveal germ cells, to reveal location of the GSC niche, and anti-Zfh-1 (A-C, green) to reveal CySCs, and DAPI stain (A-C, blue; A'-C' alone) to reveal location of DNA. DAPI expression is seen in the Vasa positive region in *tj-Gal4 UAS-Socs36E* (A-C). In the posterior region where germ cells are not seen, DAPI staining is reduced (A'), absent in some areas (B'), or absent entirely (C').

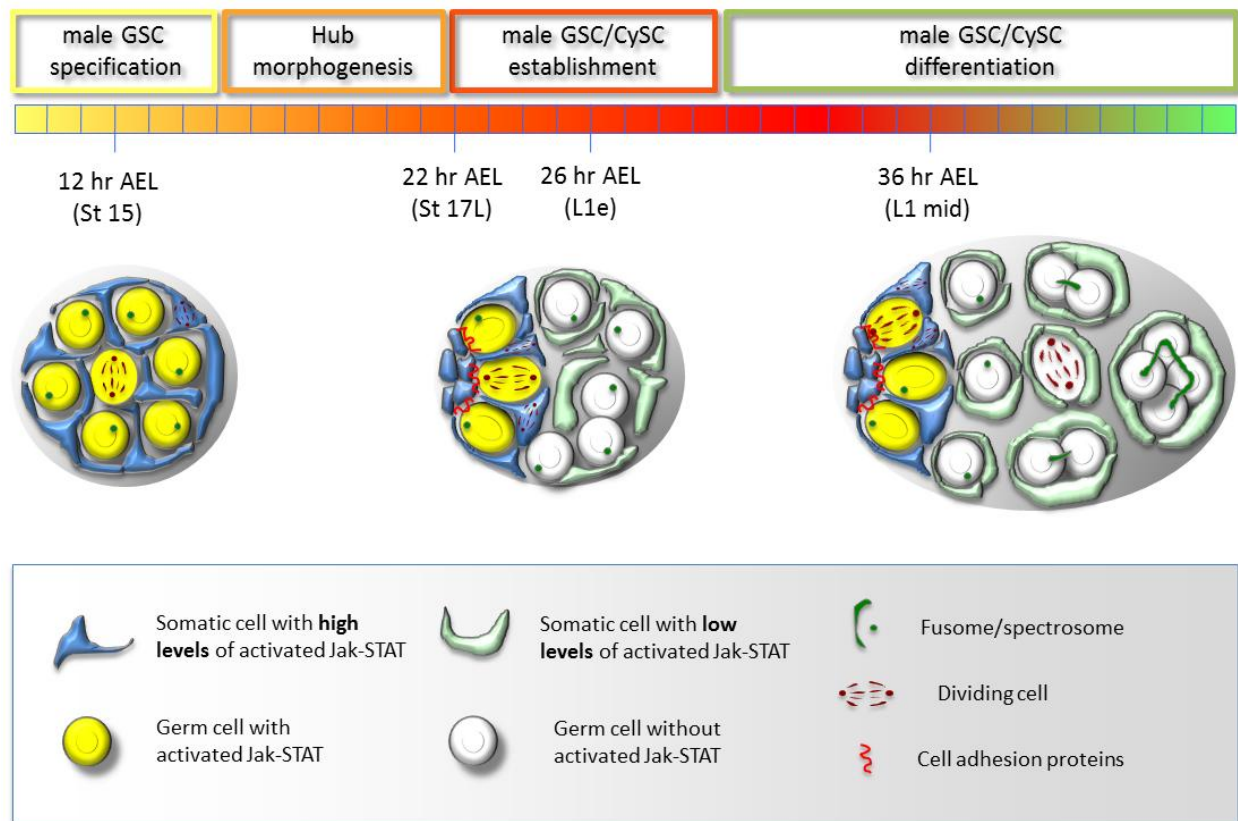
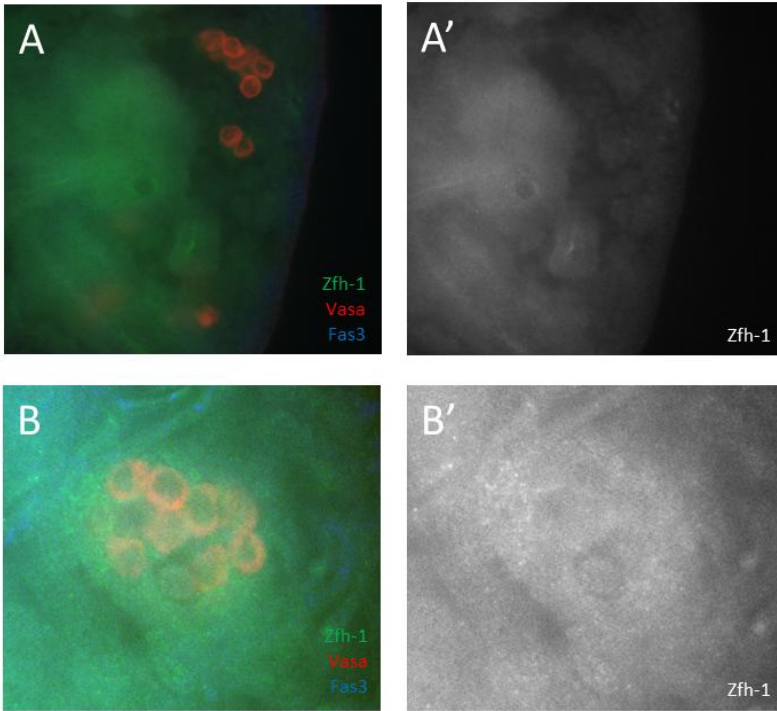


Figure 16 - Current Model of stem cell establishment during testis morphogenesis:

Soon after testes formation (~12 hrs AEL), Jak-STAT is expressed ubiquitously in both SGPs and PGCs. After hub coalescence (~22 hrs AEL), the high-level Jak-STAT activation is restricted to the hub and hub adjacent cells. At this time, functional, asymmetrically dividing GSCs with polarized adhesion and cell division are first established (Sheng et al, 2009). Work by myself, and others in the Wawersik, suggests that asymmetrically dividing CySCs are initially established at this same time, and that restricted activation of high-level Jak-SAT signaling to newly established CySCs promotes maintenance of these cells at the hub by repressing somatic differentiation, whereas somatic cells in the posterior gonad are only exposed to low-level Jak-STAT activation such that cyst cell differentiation is permitted. The differentiation of cyst cells then promotes the subsequent differentiation of gonialblast progeny produced by asymmetric GSC divisions; resulting in the development of spermatogonia by mid larval 1st instar (~36 hrs AEL).



Supplementary Figure 1 – Outstretched deficiency and poor Zfh-1 stains:

Late-stage embryonic testes immunostained with anti-Vasa (A&B, red) to reveal germ cells, anti-FasIII (A&B, blue) to reveal location of the GSC niche, and anti-Zfh-1 (A&B, green, A'-B' alone) to reveal CySC. *Outstretched deficiency* mutants, lacking the protein Unpaired, lack Zfh-1 positive cells (A, A'). However, stains for sibling control also lack Zfh-1 positive cells (B, B').