GENETIC AND ENVIRONMENTAL REGULATION OF STEM CELLS AND THEIR PROGENY IN THE *DROSOPHILA MELANOGASTER* TESTIS

by

BENJAMIN B. PARROTT

(Under the Direction of Cordula Schulz)

ABSTRACT

Stem cells are required in adult animals to replenish certain tissues throughout the life of an individual. Exploring the fundamental principles that regulate stem cell behavior in animals is crucial to both realizing their therapeutic potential and understanding the pathological roles they may play in cancer etiology. The work reported in this thesis has employed the population of germline stem cells (GSCs) residing in the testis of the genetically tractable *Drosophila melanogaster* as a model to gain general insights into how stem cells function to create tissue homeostasis, how stem cells interact with their cellular microenvironment, and how the differentiation of their daughters is regulated. In Chapter 2, we report that the mitotic activity of GSCs is increased in males upon exposure to females, uncovering a pathway linking the demand for sperm to the frequency of GSC divisions. We show that while the GSC response to females requires physical contact, it appears to be regulated independently of pathways previously reported to affect the frequency of stem cell divisions. In Chapter 3, we report that communication between GSCs and the surrounding soma via the Epidermal Growth Factor (EGF) signaling pathway is required to repress the frequency of GSC divisions. This role for

EGF signaling in repressing the frequency of GSC divisions was both genetically and developmentally distinct from the previously reported role of EGF in promoting germ cell differentiation. Thus, these findings represent a novel role for the stem cell microenvironment in repressing stem cell divisions. In Chapter 4, studies on a mutation in a conserved nucleoporin locus reveal a role for nucleoporins in the transit amplification divisions of stem cell daughters. The findings reported in this thesis contribute to our understanding of the fundamental principles that govern stem cell biology.

INDEX WORDS: *Drosophila,* Stem Cell, EGF, Germline Stem Cell, nucleoporin

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DEDICATION

This thesis is dedicated to my lovely wife, Amber Leigh – For her steadfastness, grace, and general beauty endured in this great adventure.

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CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Stem cells in adult animals are required to maintain tissues throughout the life of an individual. For example, tissue-specific stem cells give rise to daughters that replenish cells comprising the epidermis, cornea, blood, lung, intestine, and other tissues. Although stem cells are often thought to primarily replenish tissues consisting of short-lived cells, it is also known that stem cell progeny can contribute to longer-lived cellular populations, including neurons and their glial counterparts (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993). In all of these cases, stem cells undergo asymmetric divisions; one daughter cell will undergo self-renewal and retain stem cell identity, whereas the second daughter cell will undergo a developmental program ultimately resulting in a specialized and terminally differentiated cell. This unique ability of stem cells to maintain a proliferative potential while also contributing to tissue replenishment provides an ideal opportunity to study questions central to developmental biology including: How are different fates assigned to cells resulting from the same division? How do undifferentiated stem cell daughters become a specialized, terminally differentiated cell? And how is the production of cells coordinated with tissue loss?

Background

Recent advances in the general field of stem cell biology

Today, stem cell biology is arguably the fastest advancing field in all of the life sciences. Many of the major advances occurring in the last two decades have resulted primarily from in vitro studies directed towards the regenerative potential of stem cell therapeutics. In 1998, James Thompson and his colleagues reported on their ability to isolate and maintain cells from the inner cell mass of blastocyst stage human embryos (Thompson et al, 1998). This work ushered in an exciting wave of studies aimed at treating degenerative diseases previously thought to have little promise for therapeutic treatments (Wichterle et al, 2002; Bjorklund et al, 2002; Keirstead et al, 2005). Although these studies highlighted the diverse capabilities of embryonic stem cells, left exposed were obstacles for their practical use. Whereas some of these limitations arose from societal and political pressures, other technical limitations, including how a patient's immune system might tolerate these foreign cells, also provided a barrier to the clinic. However, in 2007, the Yamanaka Lab and the Thompson Lab, working independently, demonstrated that somatic cells in adult animals could be coerced in culture to dedifferentiate into cells that closely resembled embryonic stem cells (Yu et al., 2007; Takahashi et al., 2007). These cells, termed induced Pluipotent Stem Cells (iPSCs), provided an uncontroversial means to create patient-specific stem cells that can be used for therapeutic purposes (see Hannah et al, 2007 and Raya et al, 2009 for proof-of-principle). In addition, iPSCs can be generated from patients with poorly understood diseases, and used as a model to understand their molecular underpinnings (Liu et al, 2011). While the therapeutic potential of iPSCs is hard to overstate, there are still significant challenges in translating these findings into the clinic. For example, recent studies have shown

that iPSCs retain aberrant epigenetic signatures that in some cases can be traced back to the tissue of their origin (Kim et al, 2010; Lister et al, 2011).

Stem cell function in living animals

Although both exciting and promising, these recent advances in the stem cell field have focused largely on the *in vitro* manipulation of stem cells. As a consequence, our understanding of how stem cells function in living animals has not kept pace. In many cases, definitive stem cell populations have yet to be indentified. While in other cases where stem cell populations have been identified and defined based on *ex vivo* functionality, elucidating the fundamental mechanisms that govern their behavior *in vivo* has been limited by the complexity and heterogeneity of the given system (da Silva Meirelles et al, 2008; Houbracken and Bouwens, 2010). Despite these challenges, studies are increasingly demonstrating roles for stem cells in cancer, ageing, and even cognitive functions.

Commonalities amongst varied cellular populations maintained by stem cells

Adult stem cells are responsible for tissue replenishment in many different species and tissues. Using a comparative approach, it becomes apparent there are a few characteristics present in many of these cellular populations. The most common and definitive characteristic of stem cells is their ability to undergo asymmetric divisions. Another common characteristic of stem cell-based replenishment is that differentiating stem cell daughters undergo a series of mitotic amplification divisions (Blanpain and Fuchs, 2009; Casali and Batlle, 2009; Suh et al, 2009). These transit amplification divisions serve to expand the pool of precursor cells while minimizing the introduction of mutations into stem cells due to replication. More recently, as

more stem cell populations are better characterized, the cellular environment surrounding stem cells and their progeny is becoming increasingly realized as playing a prevalent role in stem cell function (Shaker and Rubin, 2010; Xie et al, 2009, Sarkar et al, 2007). Thus, these fundamental properties consisting of self-renewal, transit-amplifications, and a critical role for the surrounding cellular environment creates a central paradigm of stem cell-based replenishment.

Cancer & Stem Cells

The cancer stem cell hypothesis was introduced to account for the similarities between cancer cells and stem cell populations. The hypothesis consists of two independent conjectures: The first is that long-lived adult stem cells may be primary tumor-initiating cells in some cancers, and the second is that the heterogeneous composition of tumors is organized into a hierarchy-- with only certain cells, acting as cancer stem cells, possessing the ability to independently establish additional tumors (Reya et al, 2001). One of the first lines of evidence supporting the cancer stem cell hypothesis was the observation that only a small subset of Acute Myeloid Leukemia (AML) cells was able to initiate disease when transplanted into immunocompromised mice (Lapidot et al, 1994). Furthermore, this subset of cells expressed cell surface markers characteristic of normal hematopoietic stem cells (HSCs), but not progenitors committed to differentiation (Bonnet and Dick, 1997). Although the majority of our current knowledge about cancer stem cell populations and the hierarchal organization of cancers has been gained through studies on hematopoiesis and AML, it is clear that cancer stem cells also exist in solid tumors (Al-Hajj et al, 2003; Singh et al, 2004; Li et al, 2007; O'Brien et al, 2007; Ricci-Vitiani et al, 2007). Interestingly, just as in normal stem cell function, there seems to be a crucial role for the cancer stem cell microenvironment (Reviewed in Kenny et al, 2007).

These findings reveal a new layer of complexity in cancer biology, and highlight the similarities between the organization of cellular populations maintaining normal tissues and those maintaining tumors. In addition, these studies provide a strong rationale for further investigating the basic biology of stem cell function in animals affected by disease.

Stem cells, the niche, & the microenvironment

Stem cell behavior is influenced by cell intrinsic and cell extrinsic factors (Broadus and Doe, 1997; Waters and Rienke, 2011; Yamashita et al, 2007). The cellular environment that surrounds stem cells and their progeny has been shown to exert profound effects on stem cell self-renewal and stem cell daughter differentiation (Sarkar et al, 2007; Kiger et al, 2001; Tulina and Matunis, 2001). In many stem cell populations, factors secreted by stromal cells in contact with or in proximity to stem cells are required for their self-renewal. This anatomical location that functions to maintain stem cell identity is commonly referred to as the stem cell niche (Scadden, 2006, Schofield, 1978).

Spermatogenesis in Drosophila as a model for basic stem cell biology

In mammalian tissues, poorly defined stem cell populations and complex cellular architectures have limited the study of stem cell behavior *in vivo*. Obviating some of these obstacles, investigators have turned to simple and well-defined cellular populations within model organisms (Joshi et al, 2010; Pellettieri and Sanchez Alvarado, 2007). In particular, studies on spermtogenesis in *Drosophila melanogaster* have yielded fundamental insights into our current understanding of stem cell biology (See Wong et al, 2005 for a review). Sperm production consists of a stem cell-based mode of replenishment, and contains those transcending properties

of stem cell populations found in different tissues amongst different species. These characteristics include an anatomically and functionally defined niche, a cellular microenvironment required for the development of stem cell progeny, and a population of transit-amplifying cells.

Spermatogenesis in *Drosophila* takes place in a coiled tube that contains stem cells residing at the tip and mature sperm at the base (Fig1.1A). The testis is approximately 2mm long and its exterior is sheathed in a layer of muscle and pigment cells. In each testis, there are approximately nine to twelve Germline stem cells (GSCs) organized in a rosette around a group of stromal cells, termed the hub. The hub secretes signals that are required for GSC self-renewal, and thus in part, forms the stem cell niche. A GSC division results in two daughter cells; one will self-renew by maintaining contact with the hub, and the other will be displaced away from the hub and begin to differentiate (Fig1.1B). This displaced cell, termed a gonialblast, will undergo a well-coordinated differentiation program that begins with four transit amplification divisions (Fuller, 1993). These four mitotic events are characterized by an incomplete cytokinesis resulting in 16 interconnected spermatogonia. At the conclusion of transit-amplification divisions, spermatogonia undergo an extensive growth phase in which their cytoplasmic volume increases approximately 25-fold. They then enter into meiosis as they transition into spermatocytes. Subsequent to meiotic divisions, dramatic cellular rearrangements including elongation take place eventually resulting in 64 mature spermatids. As this process unfolds, germ cells move basally. This developmental process takes place along the length of the testis and provides a spatio-temporal axis that facilitates experimental observations.

The germ cell microenvironment

Germ cells are intimately associated with somatic cyst cells during spermatogenesis (Hardy et al, 1979). Each GSC is in contact with the hub, and also surrounded by two cyst stem cells (CySCs) that send cytoplasmic extensions around the GSCs and into the hub (Fig1.1B). Thus, the combination of CySCs and the hub comprise the stem cell niche. A CySC division results in self-renewal and the production of a post-mitotic cyst cell. Two cyst cells completely encase every gonialblast that is produced from a GSC division. As the gonialblast undergoes transit-amplification divisions and subsequent development, the two cyst cells associated with it grow in size and continue to encapsulate the germ cells (Fig1.1C). The encapsulation of germ cells by cyst cells is required for germ cell development and comprises the cellular microenvironment. This association between the two cyst cells and germ cells represents the basic unit of spermatogenesis, and is often referred to simply as a cyst.

EGF signaling in the microenvironment

Germ cells communicate with cyst cells via the Epidermal Growth Factor (EGF) signaling pathway (Fig 1.2). The EGF ligand, *spitz (spi)*, is expressed in germ cells and is cleaved from its inactive form to a secreted form by the germ cell expressed protease, *stet* (Urban et al., 2002, Schulz et al., 2002). Germ cell secreted Spi is received by the *Drosophila* EGF Receptor (dEGFR) expressed on the membrane of cyst cells (Kiger et al, 2000). In testes from animals harboring mutations in either *spi*, *stet,* or the *dEGFR*, cyst cells fail to properly encase germ cells. This failure of the microenvironment to form results in the accumulation of germ cells resembling GSCs and early-stage spermatogonia. This accumulation of under-developed germ cells takes place at the expense of more mature germ cells, and resembles a germ cell

tumor. Thus the communication between germ cells and cyst cells is required for germ cell development. Although cyst cells are post mitotic, they do undergo a developmental program consisting of the sequential expression of a series of transcription factors that is dependent upon the developmental stage of their associated germ cells (Leatherman and DiNardo, 2008; Fabrizio et al, 2003).

JaK-STAT signaling and self-renewal

The molecular pathways that govern the self-renewal of GSCs in the *Drosophila* testis have been relatively well studied. In 2001, the expression of the Janus Kinase-Signal Transducer and Activator of Transcription (JaK-STAT) ligand, *unpaired (upd),* in the hub was reported to be required for GSC divisions (Kiger et al, 2001; Tulina and Matunis, 2001). GSCs lacking cell autonomous *stat* expression failed to self-renew, and it was assumed that Upd secreted from the hub signaled directly to GSCs to induce stem cell identity (Tulina and Matunis, 2001). However, an observation that was less emphasized in these studies was that ectopic expression of *upd* resulted in not only a dramatic increase in the number of GSCs, but also CySCs. In 2008, the transcriptional regulator, Zfh-1, was found to be a target of JaK-STAT signaling in CySCs (Leatherman and DiNardo, 2008). Expression of *zfh-1* in CySCs was necessary and sufficient to induce CySC identity. Furthermore, expression of *zfh-1* or a constitutively active *stat* specifically in CySCs was sufficient to induce an expansion in the population of GSCs. Interestingly, *stat* activation in GSCs was not sufficient to induce the expansion in GSCs that results from the ectopic expression of *upd* (Leatherman and DiNardo, 2008). In 2010, the story became clearer as it was shown that *stat* was required in GSCs for their attachment to the hub, but not for self-renewal (Leatherman and DiNardo, 2010). In summary, Upd secretion from the

hub acts to induce CySC identity which is required for GSCs identity. Thus, the hub acts to induce CySC identity and to bring GSCs in contact with CySCs.

Summary and questions to be investigated

A better understanding of adult stem cell function in living animals is critical to realizing the full therapeutic potential of stem cells and the etiological role they play in cancer biology. *Drosophila melanogaster* has emerged as a leading model to investigate the fundamental principles that govern stem cell behavior. In Chapter 2 of this thesis, GSCs in *Drosophila* males were utilized as model to study how stem cells respond to environmental conditions in which the demand for the terminally differentiated cell that the stem cells replenish is altered. In Chapter 3, a genetic approach was used to identify the signaling pathways that regulate the frequency of GSC divisions. A role for the EGF signaling pathway in the GSC microenvironment is tested. In Chapter 4, a mutation severely affecting the early stages of spermatogenesis is identified and characterized. A role for a locus encoding a component of the nuclear pore is identified. The studies in this thesis utilize the anatomical simplicity and the genetic tractability of the *Drosophila* testis to uncover fundamental aspects of stem cell behavior.

Figure 1.1. The *Drosophila* spermatogenesis is maintained by germline stem cells. (A) A phase contrast image of a testis showing the region containing transit amplifying spermatogonia, marked with an *a*, the region containing meiotic spermatocytes, marked with a *b*, and bundles of 64 elongated spermatids marked with arrows. The asterisks indicates the apical tip. (B) A schematic depicting the architecture of the GSC niche and spermatogonial cellular microenvironment. (C) The apical tip of a testis stained with antibodies labeling the cytoplasmic extensions of cyst cells. The arrows indicate cyst cell extensions encasing germ cells. The Asterisks indicates the hub.

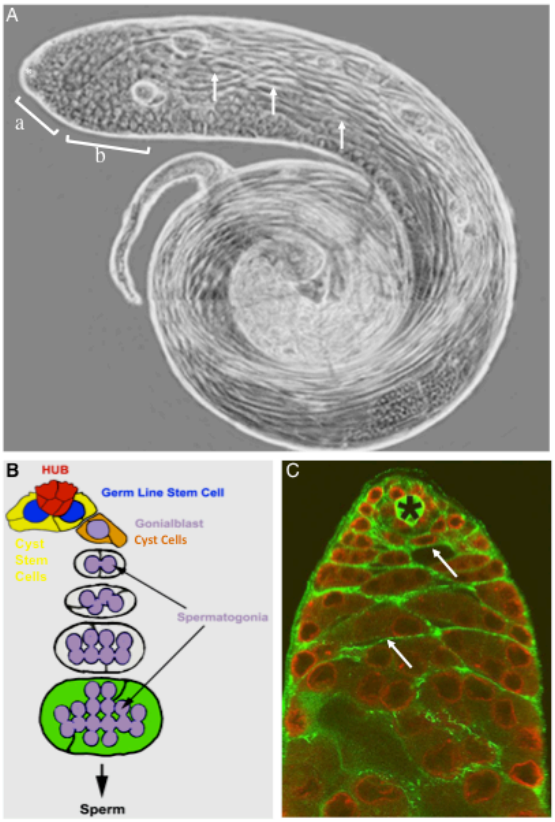
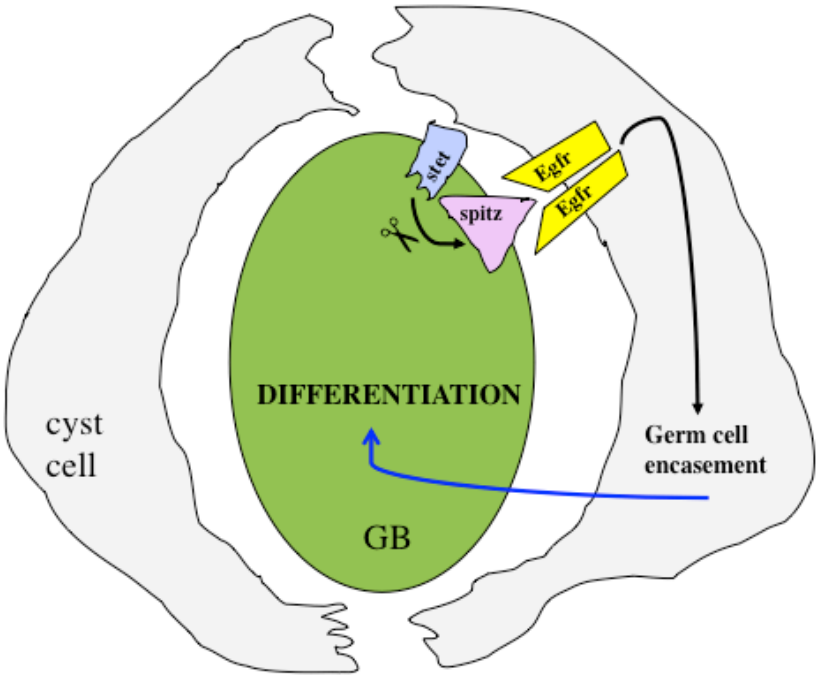

A
a
b
B
HUB
Germ Line Stem Cell
Gonialblast
Cyst Cells
Cyst
Stem
Cells
Spermatogonia
Sperm
C

Figure 1.2. A schematic of EGF signaling in the germ cell microenvironment. A gonialblast (Green), is encapsulated by two cyst cells (Grey). The EGF ligand, Spitz, is cleaved by the Rhomboid-like protease, Stet, to produce a secreted ligand. The secreted signal is received by the EGFR expressed on the surface of cyst cells. This communication is required for cyst cells to properly encase germ cells, and for germ cells to subsequently differentiate.


stet
spitz
Egfr
Egfr
DIFFERENTIATION
cyst
cell
GB
Germ cell
encasement

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CHAPTER 2

MATE EXPOSURE INCREASES THE FREQUENCY OF GERMLINE STEM CELL DIVISIONS THROUGH A CONTACT-DEPENDENT MECHANISM[1]

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Abstract

Understanding the mechanisms that coordinate tissue replenishment with tissue loss is of fundamental importance. Spermatogenesis in *Drosophila* provides an attractive model to study these dynamics because the production of mature gametes depends on a stereotypical process beginning with a well-defined population of stem cells. We found a substantial increase in the frequency of male germline stem cell divisions in environmental conditions where mate availability was high compared to conditions where mate availability was low. We show that physical contact is required for the female-induced increase in the frequency of germline stem cell division, indicating that volatile female pheromones are not sufficient to induce the stem cell response. Furthermore, the genetic pathway regulating the frequency of germline stem cell divisions in response to mate availability appears to be independent of Insulin and EGF signaling, two pathways previously shown to influence the mitotic activity of germline stem cells. Our results provide evidence that stem cells respond to the demand for the terminally differentiated cell that they replenish by altering their mitotic activity.

Introduction

Throughout the life of an individual, tissues are constantly lost and replenished. This balance is critical as generating more cells than are lost can result in hyperplasia, and not producing enough cells may result in hypotrophy. Adult stem cell divisions produce a pool of cells that after differentiating will replace those cells that are lost. Thus, the proliferation of adult stem cells and the differentiation of their progeny must be tightly coordinated to tissue loss.

Many of the tissues maintained by adult stem cells are directly affected by an organism's external environment. For example, in humans, high levels of exposure to UV radiation have

substantial consequences on the tissue replacement dynamics of the epidermis (Walsh, 1995; Clydesdale et al, 2001; Van Laethem et al, 2005). Furthermore, limited metabolic resources may be more efficiently utilized in specific tissues in response to fluctuations in environmental demands. While it is clear that environmental factors may influence the rate of tissue loss, the mechanisms by which tissue replenishment is coordinated with this loss is not well understood.

The testis of *Drosophila melanogaster* is a coiled tube with stem cells required to maintain gametogenesis found at the apical tip and mature sperm at the base. Spermatogenesis in *Drosophila* is similar to mammalian spermatogenesis, and in addition, shares many of the fundamental characteristics of other adult stem cell populations in other tissues. Approximately nine to twelve germline stem cells (GSCs) residing at the apical tip are organized in a rosette around a group of post-mitotic stromal cells, termed the hub. The hub secretes factors that are required for stem cell identity, and thus represents a component of the stem cell niche. When a GSC divides, one daughter undergoes self-renewal by maintaing contact with the hub, whereas a second daughter is physically displaced away from the hub (Yamashita et al, 2003). This displaced daughter cell, termed a gonialblast, will begin to migrate basally as it undergoes a largely invariant and stereotypical differentiation program. This program consists of exactly four rounds of synchronous transit-amplifying mitotic divisions that are characterized by incomplete cytokinesis. These transit-amplification divisions produce an interconnected cluster of 16 spermatogonia that will then transition into spermatocytes, grow in size, undergo meiotic divisions, and ultimately differentiate into 64 spermatids. Mature sperm are seen at the base of the testis, where they exit through the terminal epithelium into the reproductive tract and eventually aid in reproduction (Hardy et al, 1979; Fuller, 1993).

Germ cells are closely associated with somatic cyst cells during *Drosophila* spermatogenesis. Each GSC is associated with two Cyst Stem Cells (CySCs) that form cytoplasmic extensions around the GSCs and into the hub, and together with the hub form the GSC niche. CySCs divide asymmetrically to give rise to a renewed CySC and a post-mitotic cyst cell. Two cyst cells will enclose a newly formed gonialblast produced from a GSC division and this association is maintained until the final stages of spermatogenesis. Cyst cells form the cellular microenvironment for differentiating germ cells and send signals to the enclosed germ cells that are crucial for their differentiation (Goency et al, 1996; Matunis et al, 1997; Kiger et al, 2000; Schulz et al, 2002).

Whereas our understanding of the molecular pathways that regulate the self-renewal of GSCs and the differentiation of their daughters in the *Drosophila* gonad has increased considerably in recent years (Yamashita et al, 2005; Leatherman and DiNardo, 2010), relatively little is known about the fundamental mechanisms that coordinate sperm production with sperm loss. Each gonialblast produced by a GSC division can potentially produce 64 mature sperm, and thus, small changes in the frequency of GSC divisions can have dramatic effects on the number of sperm produced. It is possible that GSCs divide at frequencies largely governed by nutrient availability, and unused sperm in males that have not recently mated is either reabsorbed or discharged. However, such models in which the frequencies of GSC divisions are regulated independently of demand may be metabolically inefficient under certain conditions. Natural populations of *Drosophila* are likely to experience a broad range in population density and mate availability, and a simple model in which stem cell divisions are increased during conditions in which the demand for sperm is high and decreased in conditions in which the demand for sperm

is low would be energetically advantageous. Here we investigate the frequency of GSC divisions in conditions with altered levels of mate availability and demand for sperm.

Relatively few signaling pathways have been shown to influence the mitotic activity of individual GSCs in *Drosophila*. The Insulin signaling pathway in response to environmental nutrient availability alters the rate at GSCs divisions in *Drosophila* males and females (Drummond-Barbosa and Spradling, 2001; Hsu et al., 2008; McLeod, 2010). GSCs in flies fed a diet rich in protein divide at higher frequencies than those GSCs in flies fed a protein-poor diet. Expression of the *Drosophila insulin receptor* (*dinr*) in GSCs is required for the higher rates of GSC division observed in flies fed a protein rich diet (LaFever and Drummond-Barbosa, 2005). However, it is not clear whether Insulin signaling mediates the response of GSCs to other environmental inputs such as mate availability.

In addition to Insulin signaling, the EGF signaling pathway in the stem cell microenvironment has also been shown to influence the frequency of GSC divisions in *Drosophila* males (Chapter 3). The EGF ligand, *spitz* (*spi*), is expressed in GSCs and this signal is received by the *Drosophila* EGF Receptor (*dEGFR)* expressed on the surface of CySCs (Schulz et al, 2002; Chapter 3). When EGF signaling is attenuated, GSCs divide at frequencies approximately two-fold higher than normal, suggesting that EGF functions to repress the frequency of GSC divisions. Interestingly, EGF signaling is developmentally regulated. Whereas EGF signals are required in sexually mature adult males to repress the frequency of GSC divisions, EGF reduction in sexually immature larvae has no effect. It is possible that EGF is required specifically in adult males to repress the frequency of GSC divisions in environmental conditions where mate availability and the subsequent demand for sperm is low.

In this study, we investigate the influence of mate availability on the mitotic activity of GSCs in *Drosophila* males. We find that GSCs in males exposed to females divide at higher frequencies than those GSCs in males not exposed to females, uncovering a fundamental mechanism linking sperm production to sperm demand. In addition, we demonstrate that physical contact is required for the female-induced increase in GSC division frequency. Furthermore, we show that the increase in the frequency of GSC divisions in those males exposed to females is independent of the previously reported pathways affecting the frequency of GSC divisions.

Results

In an effort to determine if mate availability and the associated demand for sperm affect the frequency of GSC divisions in *Drosophila* males, we developed an assay enabling the comparison of the mitotic indices of GSCs between either males exposed to females or males not exposed to females. Males were collected as virgins soon after eclosing from pupae to limit their exposure to females, and then placed into one of two groups. In the first group, males were placed into food bottles containing virgin females, and in the second group, males were placed into food bottles that contained only other virgin males. Females that have recently mated are less receptive to subsequent male courtship (Manning, 1962; Chapman, 2002). To increase the potential number of mating events for each male, females used in the assay were replaced after each day with new virgin females. On the fourth day, testes were dissected from males in both groups (see Figure 2.1 for a schematic of the assay). Testes were then stained with antibodies raised against the germ cell marker, Vasa, the hub marker, Fasiclin III (FasIII), and a maker for cells in mitosis, phosphorylated-Histone H3 (pHH3). GSCs were identified as those $VASA^{+}$ cells in contact with the hub, and the mitotic index was calculated by dividing the number of

pHH3^{+} GSCs by the total number of GSCs. GSCs from males exposed to females had a higher mitotic index of (12.2%, n= 950, p= 0.0002) when compared to the mitotic index of (7.3%, n= 1028) observed in males without exposure to females (Fig. 2.1B). This finding suggests that GSCs likely respond to altering demands for mature gametes in their environment by adjusting the frequency of their divisions.

The increase in the mitotic index of GSC in males exposed to females could be due to the increased number of mating events experienced by males. Alternatively, it is known that neuronal cues in response to female-specific pheromones are sufficient to trigger courtship behavior in males (Sturtevant, 1915; Shorey and Bartell, 1970). Based on these findings, we reasoned that female-dependent neuronal cues acting independently of direct physical contact could also be sufficient to increase the frequency of GSC divisions in males exposed to females. To test if physical contact is necessary for the female-induced increase in GSC divisions, we designed an assay in which males were separated from females by a screen barrier (Fig. 2.2). Whereas the screen barrier prevents most physical contact and all copulation, as virgin females separated from males never laid fertilized eggs (data not shown), it is probable that volatile pheromones and other gender-specific stimuli are able to transverse the screen. After performing an experimental regimen similar to that of the aforementioned assay, we found that the mitotic index of GSCs from males separated from females (11%, n= 541) by a screen barrier was nearly identical to that of males separated from other males (12%, n= 433). Serving as a positive control, the mitotic index of GSCs males exposed to females in the absence of a barrier (22%, n= 419, p= 0.0001) was higher than either cohort in which the screen barrier was present. These data suggest that although volatile pheromones or other contact-independent sensory cues may have an indirect role in initiating courtship, they likely do not play a direct role in mediating the

increased frequency of GSC divisions observed in males exposed to females. Instead, our data demonstrate that physical contact is required to induce the increase in mitotic activity of GSCs in males exposed to females.

In our previous work we discovered that EGF signaling from GSCs to the surrounding cyst cells is required to repress the frequency of GSC divisions (Chapter 3). GSCs from males homozygous for a mutation in the EGF ligand, $spi^{77\text{-}20}$, have a roughly two-fold increase in their mitotic index. Based on this finding, we hypothesized that in wild-type males with high levels of mate exposure, a possible mechanism for increasing the frequency of GSC divisions could be to down regulate EGF signaling (Fig. 3.3). If this were the case, we would expect the mitotic index of GSCs in $spi^{77\text{-}20}$ mutant males to be similar to the mitotic index of wild-type males exposed to females. Furthermore, one might expect that the mechanism that increases the mitotic index of GSCs in response to mate exposure is the down regulation of *spi* then the mitotic index of GSCs in $spi^{77\text{-}20}$ mutant males would be independent of exposure to females. To test this hypothesis, we utilized our mate exposure assay to measure the mitotic index of GSCs in $spi^{77\text{-}20}$ mutant males. The mitotic index of GSCs in $spi^{77\text{-}20}$ mutant males exposed to females (24.8%, n= 330) was higher than that of GSCs in $spi^{77\text{-}20}$ mutant males not exposed to females (17.8%, n= 411, p= 0.02). Furthermore, the mitotic index of GSCs in $spi^{77\text{-}20}$ mutant males not exposed to females was higher, albeit not statistically significantly higher, than that of GSCs in w^{1118} males exposed to females (13.8%, n= 378, p= 0.14). Taken together, these data suggest that the genetic pathway mediating the increase in GSC divisions in those males exposed to females acts independently of the EGF pathway.

The Insulin signaling pathway has also been reported to influence the frequency of GSC divisions in males and females (Drummond-Barbosa and Spradling, 2001; McLeod et al, 2010).

The *Drosophila insulin receptor* (*dinr*) acting cell autonomously in GSCs has been shown to be required for the increased frequency of GSC divisions in flies fed a protein-rich diet (LaFever and Drummond-Barbosa, 2005). To test the possibility that the *dinr* acting in GSCs is also required for the increased mitotic index of GSCs in males exposed to females, we performed a mate exposure assay in animals in which the germline was depleted of *dinr*. To knock down the *dinr*, we employed the binary UAS/Gal4 expression system to express a transgenic RNAi construct targeting the *dinr* (UAS-$dinr^{RNAi\ GL00139}$) in GSCs using the *nanos*-Gal4 (Brand and Perrimon, 1993; Phelps and Brand, 1998; Van Doren et al., 1998). We observed that in both the female exposed cohort and the cohort not exposed to females, the mitotic indices of GSCs were lower in males with germline-depleted *dinr* (*nos*-Gal4>UAS-$dinr^{RNAi\ GL00139}$) compare to those males harboring either the *nos*-Gal4 or the UAS-$dinr^{RNAi\ GL00139}$ alone (Fig. 4.4). These results are consistent with previously reported mitogenic roles for Insulin signaling, and suggest that the UAS-$dinr^{RNAi\ GL00139}$ construct is functional. However, we found that in males with germline-depleted *dinr* (*nos*-Gal4>UAS-$dinr^{RNAi\ GL00139}$), the mitotic index of GSCs in males exposed to females (9.2%, n= 457) was higher than those not exposed to females (4.8%, n= 455, p= 0.01) (Fig. 4.4). Taken together, these data suggest that the increase in the mitotic index of GSCs in males exposed to females acts independently of direct insulin signaling.

Experimental Procedures

Drosophila Stocks and Genetics

All fly stocks were raised and maintained on standard cornmeal molasses agar unless otherwise noted. Fly stocks that were used in experiments were raised at 26.5 °C. Fly stocks used in this study include: w^{1118} (Bloomington Stock Center), $spi^{77\text{-}20}$ (Sarkar et al, 2007), *nanos-gal4-VP16* (Van Doren et al., 1998), and UAS-$dinr^{RNAi\ GL001394}$ (TRiP at Harvard Medical School).

Mate Exposure Assays

All mate exposure assays were performed at 26.5 °C using w^{1118} females. Males were collected as virgins and aged in vials for approximately 2-4 days post eclosion. Males were then placed in a bottle containing either other virgin males or virgin females at a 1:4 ratio. Flies were fed an apple juice agar plate with a dollop of yeast paste. After 24 hours, flies were anesthetized with CO_2 and females were sorted out and replaced with fresh virgin females. To control for the possible effect of anesthesia, the time taking to sort and replace females was recorded, and the male only cohort was anesthetized for an identical period of time. This process was repeated four times every 24 hours. At the conclusion of the fifth day, Testes were dissected and placed in Testis Isolation Buffer (10 mM Tris-HCl, ph 6.8, 180 mM KCl) on ice.

Immunohistochemistry

Testes were subsequently fixed in 4% formaldehyde in PBT for 30 min. Primary antibody incubation took place overnight at 4°C, and secondary antibody incubation took place for 2 hrs at room temperature. Testes were mounted onto slides using Vectashield mounting media with DAPI. Tissues were observed using a Zeiss Axiophot microscope in fluorescent microscopy. Images were taken with a CCD camera using an Apotome and Axiovision Rel Software. Antibodies and dilutions used were as follows: goat anti-Vasa (1:500, Santa Cruz Biotechnology Inc.), rabbit anti-phospho-histone H3 Ser10 (1:750, Millipore), and anti-Fasiciclin III 7G10 (1:10, obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242: developed by C. Goodman). Alexa-488-, Cy3-, and Cy5-conjugated secondary antibodies were used at 1:500 (Invitrogen).

Discussion

The Drosophila testis as a model for studying the dynamics of tissue replenishment

Our studies show that the mitotic activity of stem cells is dependent on the demand for the terminally differentiated cell that they replenish. We found that males placed in environments where demand for sperm is high contain GSCs dividing at higher frequencies than when demand for sperm is low. We also show that physical contact is required to induce the mate-induced increase in GSC mitotic activity. Finally we demonstrate that the increased GSC mitotic index in response to female exposure is independent of signaling pathways previously shown to regulate the frequency of GSC divisions.

While our analysis demonstrates that the altered mitotic activity of stem cells is a mechanism by which animals respond to changing environmental factors, it does not rule out the contribution of other modes of regulation. In fact, our finding that male GSCs still divide in the absence of females suggests that the frequency of stem cell divisions fluctuates only within a given range in response to mate availability. There is likely a lower limit of GSC division frequency that results in decreased fitness when crossed. Therefore, it is likely that some form of cellular turnover downstream of gonialblast production, such as apoptosis or mating-independent emission, occurs during conditions of low mate availability. Our studies on the *Drosophila* testis provide a model in which the influence of environmental demands on stem cell-based renewal can be experimentally approached.

Pheromones, Courtship, and Mating

Drosophila pheromones play critical roles in aggregation, mate recognition, and in the initiation of male courtship (Bartlet et al, 1985, Jallon, 1985; Greenspan and Ferveur, 2000).

Whereas nonvolatile hydrocarbons on the female cuticle are sensed by gustatory receptors and are likely to require physical contact to illicit male courtship, volatile pheromones are sensed by olfactory receptors and have also been shown to increase the frequency of male courtship behavior (reviewed by Amrein, 2004). Female odors in the physical absence of females have been reported to increase male courtship (Sturtevant, 1915; Shorey and Bartell, 1970). However, we observed that females separated from males by a screen barrier are not sufficient to induce the female-induced increase in the mitotic index of male GSCs, demonstrating a requirement for physical contact. Based on this requirement for physical contact, it is likely that either nonvolatile pheromones or actual copulation are required to induce the observed increase in male GSC divisions upon exposure to females.

There are a couple of experimental approaches that may aid in delineating between the requirement of nonvolatile pheromones and copulation. Cuticular hydrocarbons can be easily extracted from female and male flies with washes in hexane (Ferveur, 1991). Testing the sufficiency of extracts from males, females, or hexane alone to elicit the increased mitotic activity of GSCs in males lacking mate exposure may uncover a role for non-volatile female pheromones. On another note, decapitated flies do not immediately die, but instead will remain viable until their energy stores are depleted or they desiccate (Deither, 1962). Furthermore it was noticed that *Drosophila* males actively court decapitated females, yet only rarely copulate (Speith, 1966). Future studies utilizing decapitated females in which males contact and court, but do not copulate may aid in distinguishing specific cue(s) that lead to the female-induced GSC response.

Regulation of GSC divisions in response to mate-exposure

We found that the EGF and Insulin signaling pathways, both previously shown to influence the frequency of GSC divisions, do not appear to be required for the increase in GSC divisions observed in males exposed to females. These findings suggest a novel pathway regulating the frequency of GSC divisions is responsible for the fluctuations in the mitotic index of GSCs in response to mate availability. The *dinr* has been shown to act cell autonomously in GSCs to regulate their divisions. Yet, GSCs in males in which the *dinr* was knocked-down in the germline still had an increased mitotic index when males were exposed to females. However, although it appears that direct Insulin signaling to GSCs is not required, we cannot rule out the possibility that insulin signaling plays an indirect role in mediating the frequency of GSC divisions in response to mate availability. Another possibility is that the frequency of GSC divisions in response to mating is dependent on hormonal signaling. Accessory glands produce the non-gametic components of the seminal fluid, and are a critical component of the reproductive system in males. Shorty after copulation, a marked increase in protein synthesis can be observed in the accessory glands (Hihara, 1981; Schmidt et al, 1985). In non-mated males, the ectopic treatment of Ecdysone or juvenile hormone to the male cuticle was sufficient to elicit the increase in protein synthesis observed in mated males (Herndon et al, 1997). Therefore, it is not unlikely that the same or a similar pathway may be involved in regulating the frequency of GSC divisions in response to mate-exposure. In future studies aimed at uncovering the pathways mediating the GSC response to mate availability, it will be important to ensure that the frequency of courtship and subsequent copulations of the males under investigation are not affected.

Figure 2. 1. Mate exposure increases the mitotic index of male GSCs. (**A**) A flow chart depicts the experimental design of the mate-exposure assay. (**B**) The mitotic indices (percentage of $pHH3^{+}$ GSCs) of GSCs from w^{1118} males are plotted. The p-value was determined by a two-tailed Fisher's exact test.

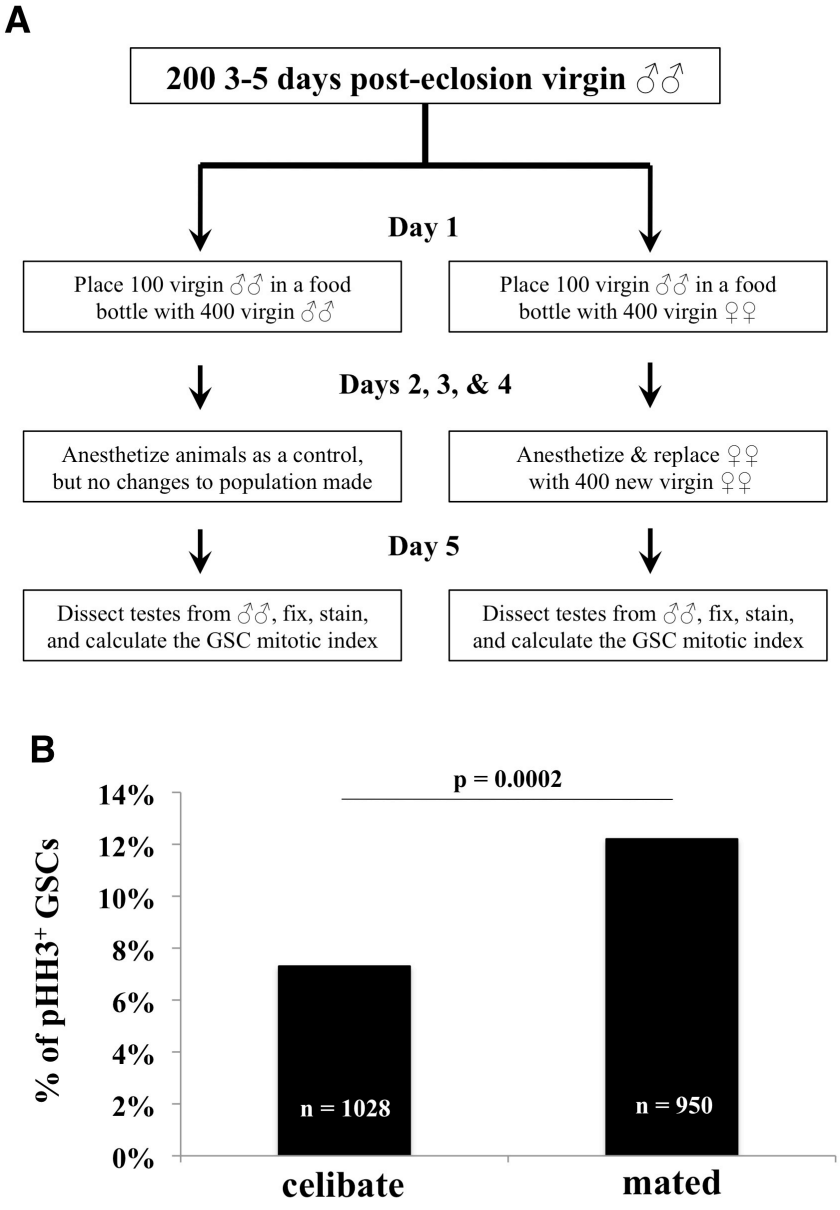

A
200 3-5 days post-eclosion virgin ♂♂
Day 1
Place 100 virgin ♂♂ in a food bottle with 400 virgin ♂♂
Place 100 virgin ♂♂ in a food bottle with 400 virgin ♀♀
Days 2, 3, & 4
Anesthetize animals as a control, but no changes to population made
Anesthetize & replace ♀♀ with 400 new virgin ♀♀
Day 5
Dissect testes from ♂♂, fix, stain, and calculate the GSC mitotic index
Dissect testes from ♂♂, fix, stain, and calculate the GSC mitotic index
B
p = 0.0002
% of pHH3+ GSCs
14%
12%
10%
8%
6%
4%
2%
0%
n = 1028
n = 950
celibate
mated

Figure 2. 2. Physical contact is required for the increase in the mitotic index of GSCs in males exposed to females. (**A**) Schematic of the experimental design, and pictures (**B, C**) of the apparatus showing (**B**) the screen barrier with and apple juice plate with yeast, and (**C**) the lateral side with ventilation holes. (**D**) The mitotic index of GSCs from w^{1118} males undergoing the indicated conditions. The p-value was determined by a two-tailed Fisher's exact test.

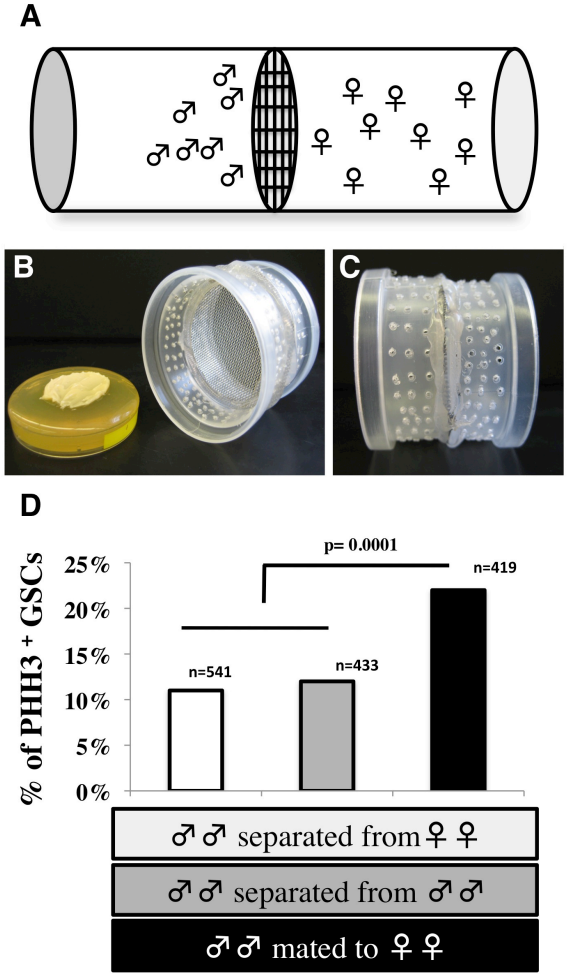

A
B
C
D
% of PHH3 + GSCs
25%
20%
15%
10%
5%
0%
p= 0.0001
n=541
n=433
n=419
♂♂ separated from ♀♀
♂♂ separated from ♂♂
♂♂ mated to ♀♀

Figure 2. 3. *spi* is not required for the mate-induced increase in the mitotic index of male GSCs. The mitotic index of GSCs is plotted. Genotypes are as indicated. p-values were determined by a two-tailed Fisher's exact test.

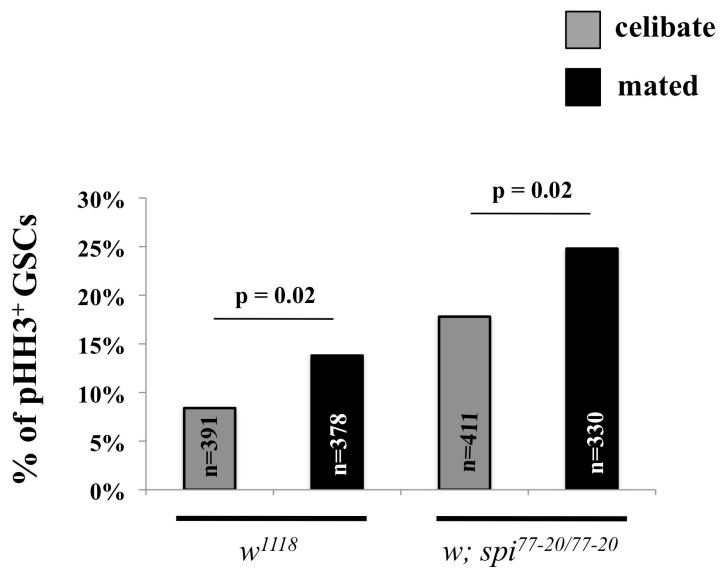
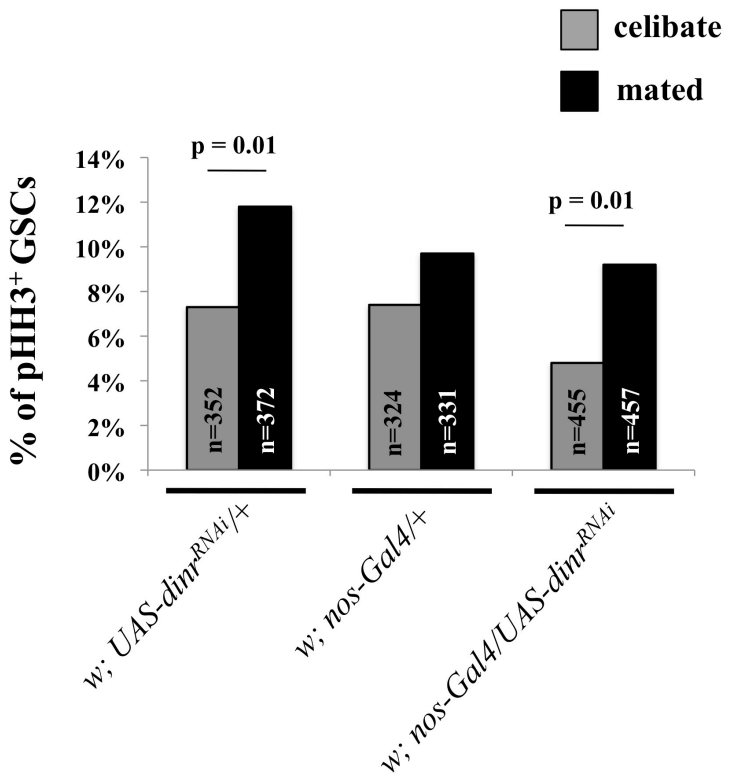

celibate
mated
% of pHH3+ GSCs
30%
25%
20%
15%
10%
5%
0%
p = 0.02
p = 0.02
n=391
n=378
n=411
n=330
w1118
w; spi77-20/77-20

Figure 2. 4. The RNAi-mediated knock-down of the *dinr* in the male germline does diminish the mate-induced increase of the GSC mitotic index. The mitotic indices of GSCs from animals harboring either the UAS-*dinr*$^{RNAi\ GL00139}$ construct alone, the *nos*-Gal4 construct alone, or both constructs in tandem is plotted. The p-values were determined by a two-tailed Fisher's exact test.


celibate
mated
% of pHH3+ GSCs
14%
12%
10%
8%
6%
4%
2%
0%
p = 0.01
p = 0.01
n=352
n=372
n=324
n=331
n=455
n=457
w; UAS-dinrRNAi/+
w; nos-Gal4/+
w; nos-Gal4/UAS-dinrRNAi

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CHAPTER 3

EGF SIGNALING IN THE STEM CELL MICROENVIRONMENT REPRESSES THE FREQUENCY OF GERMLINE STEM CELL DIVISIONS INDEPENDENTLY OF PROMOTING GERM CELL DIFFERENTIATION[2]

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Abstract

Exploring adult stem cell behavior in normal and disease states is crucial to both better realizing their therapeutic potential and understanding the pathological roles they may play in cancer etiology. To begin to address these issues, we studied the dynamics of *Drosophila* Germline Stem Cells (GSCs) in testes containing germ cell tumors resulting from the perturbation of conserved signaling pathways. Here we report a novel role for Epidermal Growth Factor (EGF) signaling in repressing the frequency of GSC divisions. When EGF signaling was attenuated, GSCs divided at frequencies approximately two-fold higher than those of wild-type animals. The increased mitotic activity of individual stem cells in EGF mutants could be rescued by restoring signaling specifically within the stem cell microenvironment. Interestingly, GSCs in testes with tumors resulting from the perturbation of other conserved signaling pathways divided at normal frequencies, indicating a specific role for the EGF pathway. These results suggest that although the accumulation of undifferentiated cells may be a general hallmark of hyperplastic growth, the increased mitotic activity of individual stem cells may play a role in only a subset of tumors. Finally, by exploiting genetic modifier experiments and investigating the defects at larval and adult stages, we show that EGF acts through distinct genetic pathways and at different developmental time points to regulate germ cell differentiation and GSC division frequency. These data advance our understanding concerning the role of the stem cell microenvironment in regulating stem cell dynamics, and provides a model of the behavior of individual stem cells in a tumor model.

Introduction

Adult stem cells self-renew and give rise to differentiating daughters that maintain tissues throughout the life of an individual. The therapeutic potential of stem cells and the etiological role they may play in cancer biology make understanding the behavior of these cells in living animals crucial to our long-term ability to both treat and prevent disease (Weissman, 2000; Reya et al., 2001). Over the past two decades, our understanding of how stem cells contribute to tissue homeostasis has increased considerably. Specifically, the developmental pathways regulating cell fate decisions that stem cells and their daughters undergo to either self-renew or to reach a terminally differentiated state have been heavily studied in several model organisms. Likewise, the physical nature of the microenvironment that stem cells and their daughters require to properly undergo these developmental processes have been identified (Yamashita et al, 2005; Palasz and Kaminski, 2009).

However, less is known about how the mitotic activity of individual stem cells is regulated *in vivo*. This understudied aspect of stem cell biology is crucial as small changes in the frequency of stem cell divisions can dramatically alter the number of terminally differentiated cells. In mammalian tissues, stem cells are generally thought to be long-lived and slow cycling (Cheng and Scadden, 2006; Fuchs, 2009). Yet, it is not well understood how the unique cell cycle of stem cells is regulated to ensure that a critical balance between self-renewal and differentiation is maintained. In addition to their role in tissue homeostasis, stem cells have been postulated to play a crucial role in tumor initiation and progression (Reya et al., 2001). However, we have yet to gain a full understanding of stem cell behavior in tissues containing tumors. Hence, insights into the stem cell dynamics within tumor bearing tissues may shed light on their oncogenic properties.

Stem cell populations residing in the *Drosophila* gonad are remarkably similar to those found in vertebrates, and studies in this model have revealed fundamental insights into current stem cell biology. The *Drosophila* testis is a coiled, tubular structure that contains nine to twelve germline stem cells (GSCs) at the apical tip, that are organized around a group of stromal cells, termed the hub (Figure 3.1A) (Hardy et al, 1979; Fuller, 1993). When a GSC divides, one of the daughter cells will maintain contact with the hub and retain stem cell identity. The other daughter cell will be physically displaced away from the hub and initiate a highly coordinated cascade of differentiation steps. This well-defined population of GSCs coupled with the genetic tractability of *Drosophila* provides an ideal model to investigate the mechanisms by which stem cell divisions are regulated.

Similar to mammalian tissues, the differentiation program of a GSC daughter, the gonialblast, begins with transit amplification divisions. In *Drosophila* males, gonialblasts will undergo precisely four rounds of transit amplification divisions with incomplete cytokinesis that give rise to exactly 16 interconnected spermatogonia. These cells then transition into spermatocytes, grow in size, undergo meiotic divisions, and ultimately differentiate into spermatids. This differentiation program of germ cells in the *Drosophila* testis occurs in a spatio-temporal order along the apical to basal axis, with GSCs at the apical tip and differentiating germ cells in more basal positions. Mature sperm are seen at the base of the testis, where they exit through the terminal epithelium into the reproductive tract (Figure 3.1B, Hardy et al, 1979; Fuller, 1993).

In the *Drosophila* testis, germ cells are intimately associated with somatic cells that comprise their cellular microenvironment. Each GSC is associated with two Cyst Stem Cells (CySCs) that form cytoplasmic extensions around the GSCs and into the hub (Figure 3.1A), and

together with the hub form the GSC niche. A tight localization of cell adhesion molecules at the interface of the hub and the GSCs assures physical contact that is important for the maintenance of the GSC population (Yamashita, 2010; Inaba et al, 2010). Furthermore, GSCs receive signals from the niche for their identity (Kiger et al, 2001; Tulina and Matunis, 2001; Shivdasani and Ingham, 2003; Kawase et al, 2004; Leatherman and DiNardo, 2008). CySCs divide asymmetrically to give rise to a renewed CySC and a post-mitotic cyst cell. Two cyst cells will enclose a newly formed gonialblast produced from a GSC division, and this association is maintained until final stages of spermatogenesis. Cyst cells form the cellular microenvironment for differentiating germ cells and send signals to the enclosed germ cells that are crucial for their differentiation (Goency et al, 1996; Matunis et al, 1997; Kiger et al, 2000; Schulz et al, 2002).

Several signaling pathways have been identified that are necessary for the proper function of the GSCs, and perturbations in these pathways result in germ cell tumors. EGF signaling between germ cells and their somatic counterparts is required for the enclosure of the germ cells by the cyst cells. The EGF ligand, Spitz (Spi), is expressed in germ cells, and is cleaved by the protease Stet, producing a secreted ligand (Urban et al., 2002; Schulz et al., 2002). Secreted Spi signals are received by the *Drosophila* EGF Receptor (dEGFR) on the surface of cyst cells, which induce the cellular rearrangements associated with the formation of the germ cell microenvironment. Germ cells in testes from animals harboring mutations in either *spi, stet,* or the *dEGFR*, do not associate with cyst cells and fail to differentiate. Instead the germ cells accumulate as GSCs, gonialblasts, and early stage spermatogonia, resembling germ cell tumors (Schulz et al, 2002; Sarkar et al, 2007). Several lines of evidence suggest that EGF signaling, in addition to its role in germ cell enclosure, may also directly induce cyst cells to send differentiation signals to the enclosed germ cells (Kiger et al., 2000).

Other germ cell tumors result from the hyper-activation of either the Janus Kinase-Signal Transducer and Activator of Transcription (JaK-STAT) or the Transforming Growth Factor-β (TGF-β) signaling pathways. The JaK-STAT ligand, Unpaired (Upd), is normally secreted by hub cells, and induces stem cell identity in the neighboring CySCs. CySCs then relay a signal conferring stem cell identity to the encased GSCs, possibly via stimulating TGF-β signaling from CySCs to the GSCs (Leatherman and DiNardo, 2010). TGF-β signals are critical for GSC maintenance, and animals mutant for the TGF-β ligands have a strong reduction in the number of GSCs (Shivdasani and Ingham, 2003; Kawase et al, 2004). Conversely, over-expression of either *upd* or the TGF-β ligand, *decapentaplegic (dpp)*, in the germline cells results in the accumulation of early stage germline cells. Whereas *upd* over-expression leads to testes filled with single germ cells that resemble GSCs (Kiger et al, 2001), *dpp* over-expression leads to clusters of supernumerary spermatogonia (Schulz et al, 2004; Bunt and Hime, 2004). Although the germ cell tumor phenotypes arising from EGF attenuation, JaK-STAT hyper-activation, or TGF-β hyper-activation are unique in many regards, they can all be classified as over-proliferation phenotypes. A unifying theme amongst these overproliferation phenotypes is the failure of germ cells to differentiate past spermatogonial stages.

Here, we report on the division dynamics of GSCs. We discovered that GSCs in testes with germ cell tumors resulting from attenuated EGF signaling displayed increased rates of cell division, whereas GSCs in testes with germ cell tumors resulting from the hyper-activation of either the TGF-β or the JaK-STAT signaling pathways divided normally. These data show that subsets of hyperplasias are not only characterized by an increase in the number of cycling cells, but also by an increased mitotic activity of individual stem cells. Confirming the role for EGF signaling in regulating the frequency of GSC divisions, germline-specific expression of EGF

rescued the hyper-proliferation of GSCs in EGF mutant animals, and RNAi-mediated knockdown of the *dEGFR* in cyst cells recapitulated the increased mitotic activity of individual GSCs. Our data demonstrate a novel and specific role for EGF signaling in the stem cell microenvironment, the repression of GSC division frequency.

This novel role of EGF is developmentally and genetically independent of its previously reported role in promoting germline cell differentiation. We show that EGF is required to repress the frequency of GSC divisions specifically in adult animals but not during larval stages. In contrast to this developmentally regulated role, previous reports showed that EGF is required during both larval and adult spermatogenesis for the differentiation of germ cells (Kiger et al, 2000; Schulz et al, 2002; Sarkar et al, 2007). Furthermore, different genetic pathways appear to regulate GSC division frequency and germ cell differentiation. We performed a genetic modifier analysis that uncovered two suppressors of EGF signaling, the orphan nuclear receptor, *seven-up* (*svp*), and the homeodomain containing transcription factor, *homothorax* (*hth*). Attenuation of either of these genes in the EGF mutant background suppressed the accumulation of early stage germ cells, but surprisingly did not have any effect on the increased mitotic activity of GSCs in EGF mutants. Taken together, these findings reveal a surprising and substantial bifurcation of EGF function in maintaining the critical balance between GSC division and germ cell differentiation.

Results

EGF signaling prevents GSC hyper-proliferation

To better understand the in vivo behavior of stem cells in tissues containing tumors, we quantified the frequency of GSC divisions in testes from wild-type animals and mutant animals

that contained germ cell tumors. GSC division frequency was investigated by labeling the hub (Fasciclin III), the germ cells (Vasa), and cells in mitosis phospho-Histone H3 (pHH3), and subsequently counting the percentage of GSCs next to the hub in mitosis (Figure 3.1C). To address the role of EGF signaling in regulating the frequency of GSC divisions, we first investigated testes from animals harboring the temperature sensitive EGF allele, $spi^{77\text{-}20}$, grown at a restrictive temperature. These testes are small and filled with early stage germline cells (Figure 3.1D). The percentage of GSCs positive for pHH3 was approximately two-fold higher in testes from $spi^{77\text{-}20}$ mutants (15.6%, n=854) than the percentage calculated for GSCs in w^{1118} control testes (7.7%, n=1158) (Figure 3.1E). We next tested if the observed increase in the percentage of $pHH3^+$ GSCs in $spi^{77\text{-}20}$ mutants reflected an increase in GSC divisions, or if mitosis in EGF mutants occupied a larger proportion of the cell cycle compared to controls. For this, we quantified the percentage of GSCs is S-phase (S-phase index) by measuring the ex vivo incorporation of the thymidine analog, BrdU. The S-phase index of GSCs from $spi^{77\text{-}20}$ homozygotes (25.3%, n=843) was significantly higher than the S-phase index calculated for GSCs from w^{1118} controls (17.1%, n=521) (Figure 3.1F). The above data provide strong evidence that GSCs in testes from $spi^{77\text{-}20}$ mutants divide at higher frequencies than GSCs in w^{1118} control animals. This implies that EGF acts to repress the frequency of GSC divisions in normal animals.

We next investigated if EGF signaling is also required to repress the frequency of CySC divisions. We reasoned that GSC and CySC divisions may be coordinated to ensure that two cyst cells are produced for each gonialblast. CySCs and their daughters associated with early stage spermatogonia express the transcription factor, Traffic Jam, in their nuclei. We noticed that the only Traffic Jam-positive cells that were also $pHH3^+$ were found within one cell

diameter from the hub, indicating CySC identity. Therefore, by measuring the percentage of Traffic Jam-positive cells located one cell diameter from the hub that were pHH3^{+}, we were able to calculate the mitotic index of CySCs. Interestingly, the mitotic index of CySCs in *spi*$^{77\text{-}20}$ mutants (3.3%, n=602) was similar to that of *w*^{1118} controls (2.4%, n=1120), suggesting that different pathways regulate the divisions of the two stem cell populations (Figure 3.1G).

GSCs divide at normal frequencies in testes with tumors resulting the hyper-activation of either JaK-STAT or TGF-β

We next addressed if germ cell tumors resulting from perturbations to JaK-STAT or TGF-β signaling also contained hyper-proliferative GSCs. Overexpression of *dpp* in germ cells using the UAS/Gal4 binary expression system (Brand and Perrimon, 1993; Phelps and Brand, 1998) resulted in excessive spermatogonial transit amplification divisions leading to an accumulation of early stage germ cells (Figure 3.1H). Similarly, over-expression of *upd* in germ cells resulted in the expected tumor-like phenotype. In this case, testes filled with single germ cells that resemble GSCs (Figure 3.1I). Although the expected tumorous germ cell phenotypes were present, the mitotic indices of GSCs from animals over-expressing *dpp* (*nanos*-Gal4 > UAS-*dpp*) or *upd* (*nanos*-Gal4 > UAS-*upd*) within their germ cells were similar to control animals harboring either the *nanos*-Gal4, the UAS-*dpp*, or the UAS-*upd* alone (Figure 3.1E). Thus, the mitotic hyperactivity of individual GSCs does not appear to be a hallmark of all hyperplastic phenotypes, but is specifically associated with the loss of EGF signaling.

EGF signaling in the stem cell microenvironment acts to represses GSC divisions

Expression of a secreted form of EGF, *s-spi,* (Tsruya, 2002) specifically in the germ cells rescues the germ cell enclosure and differentiation defects in $spi^{77\text{-}20}$ mutant testes (Sarkar et al, 2007). As expected, the mitotic indices of GSCs in testes from $spi^{77\text{-}20}$ homozygotes harboring either the *nanos*-Gal4 (15.7%, n=1060) or the UAS-*s-spi* (16.7%, n=1157) alone had mitotic indices similar to $spi^{77\text{-}20}$ alone and approximately two-fold higher than those calculated from w^{1118} testes (6.7%, n=669) (Figure 3.2A). In contrast, GSCs from $spi^{77\text{-}20}$ homozygotes carrying both the *nanos-Gal4* and the UAS-*s-spi* constructs together had a reduced mitotic index (7.2%, n=833) similar to that observed in w^{1118} controls (Figure 3.2A). These data clearly demonstrate that the hyper-proliferation of GSCs observed in $spi^{77\text{-}20}$ mutants is due to the reduction of EGF specifically within germ cells.

We next investigated if the EGF Receptor expressed in cyst cells is required to repress the frequency of GSC divisions. To test this, we expressed a transgenic RNAi construct targeted specifically against the *dEGFR* (UAS-$dEGFR^{JF02384}$) in CySCs and cyst cells. Just as in testes from $spi^{77\text{-}20}$ animals, testes from animals with cyst cell-depleted dEGFR (*eyaA3*-Gal4 > UAS-$dEGFR^{JF02384}$) displayed germ cell tumor phenotypes. In addition GSCs within these testes had a higher M-phase index (17.4%, n=1222) compared to the GSC mitotic indices calculated from control animals harboring either the *eyaA3*-Gal4 (10.8%, n=944) or the UAS-$dEgfr^{JF02384}$ (8.7%, n=620) alone (Fig. 3.2B). These data strongly suggests that GSC-secreted EGF is received via the dEGFR on CySCs, and that this signaling event in the stem cell microenvironment in turn represses the frequency of GSC divisions.

spi repression of GSC division frequency is developmentally regulated

To gain insights into how stem cell behavior is governed during development, we investigated the division dynamics of GSCs in larvae and adults. The testes of *Drosophila* third instar larvae are round discs that have yet to undergo the morphogenetic events that result in a coiled tube connected to the reproductive tract and genitalia. Although *Drosophila* males do not reach sexual maturity until after eclosion, spermatogenesis begins during the 1st instar of larval development. By the end of the 3rd larval instar, testes contain germ cells in most stages of spermatogenesis, occasionally including elongated spermatids (Figure 3.3A). Similar to the phenotype of adult testes from $spi^{77\text{-}20}$ mutants, $spi^{77\text{-}20}$ larval testes are filled with early stage germ cells at the expense of more mature germ cells (Figure 3.3B). We reasoned that if *spi* is required for germ cells to adopt late stage cell fates in larval testes that GSCs in *spi* mutants might also hyper-proliferate during this stage. However, the percentage of $pHH3^{+}$ GSCs lying next to the hub in $spi^{77\text{-}20}$ (8.4%, n=733) larval testes was similar to that of w^{1118} larval testes (7.5%, n=702) (Figure 3.3C), suggesting that EGF signaling is dispensable in regulating the frequency of GSC divisions during larval stages.

To rule out the possibility that low levels of persisting Spi activity were sufficient to repress the frequency of GSC divisions in $spi^{77\text{-}20}$ mutant larvae, we measured the mitotic activity of GSCs in larvae harboring strong loss-of-function alleles of the ligand-activating protease, *stet*. The mitotic index of GSCs in larval testes from $stet^{1}/stet^{2}$ mutant animals (5.8, n= 495) was similar to the mitotic index of GSCs in testes from w^{1118} larvae. We conclude that although an active EGF ligand is required for the proper differentiation of germ cells in larval testes, it is dispensable in larvae for the regulation of GSC division frequency. Thus, EGF signaling appears to have two differentially regulated functions in *Drosophila* spermatogenesis: to promote germ

cell differentiation in both larvae and adults, and to repress the frequency of GSC divisions in adults, but not larvae.

spi acts in distinct genetic pathways

spi expression in the germ cells of adult *Drosophila* testes is required for the differentiation of early stage germ cells and for repressing the frequency of GSC divisions. However, the genetic pathways that *spi* interacts with to regulate these two aspects of spermatogenesis have yet to be identified. *spi* may coordinate these dual functions by acting through a single genetic pathway or by acting through multiple distinct genetic pathways.

In an ongoing effort to identify novel genes that regulate GSCs and GSC daughter differentiation, we employed genetic interaction studies. A pilot screen (C.S. unpublished data) revealed two suppressors of *spi*, *homothorax (hth)* and *seven-up (svp)*. Testes from wild-type animals contain small transit-amplifying spermatogonia, large meiotic spermatocytes, and bundles of elongated spermatids (Figure 3.4A). In contrast, the phenotypes of testes from $spi^{77\text{-}20}$ homozygous animals raised at the semi-restrictive temperature of 26.5 °C have variable degrees of severity. $spi^{77\text{-}20}$ mutant testes can be classified into groups according to their phenotypes as containing: only early-stage germ cells that resemble spermatogonia (Figure 3.1C), containing both spermatogonia and large spermatocytes (Figure 3.4B), or by containing all stages of germ cells found in wild-type testes, including elongated spermatids (Figure 3.4C). Under these sensitizing conditions, the attenuation of additional genes can be scored for either their enhancing or suppressing activity. We discovered that single copies of strong loss-of-function alleles of *svp* or *hth* in the $spi^{77\text{-}20}$ homozygous background resulted in a dramatic reduction in the proportion of testes displaying severe phenotypes (Fig. 3.4D). Whereas 48% of testes (n=129)

from *spi*$^{77\text{-}20}$ mutants animals contained only early stage germ cells, only 18% of testes (n=124) from *spi*$^{77\text{-}20}$ animals heterozygous for the *svp*^{e22} allele were scored in this category. Likewise, only 16% of testes (n=90) from *spi*$^{77\text{-}20}$ animals heterozygous for the *hth*^{05745} allele contained only early stage germ cells. The observation that attenuation of either *svp* or *hth* suppressed the *spi*$^{77\text{-}20}$ phenotype indicates that *spi* acts in opposition to *svp* and *hth* in a genetic pathway that regulates the fates of early stage germ cells.

We next asked whether reducing the dose of *svp* and *hth* also suppressed the increased frequency of GSC divisions associated with *spi* mutants. If *spi* acts in the same genetic pathway to both regulate the cell fates of early stage germ cells and repress the division frequency of GSCs, then attenuation of *svp* or *hth* should also repress the hyper-proliferation of GSCs in *spi* mutants. To address this, the mitotic indices were calculated for GSCs within testes from *spi*$^{77\text{-}20}$ mutant animals with the same heterozygous alleles of *svp* and *hth,* and grown under the same conditions as those scored for their suppressing activity. As in preceding experiments, GSCs from *spi*$^{77\text{-}20}$ homozygotes had a much higher mitotic index of (15.4%, n=595) when compared to *w*^{1118} controls (6.4%, n=471). Surprisingly, the mitotic indices of GSCs from *spi*$^{77\text{-}20}$ homozygotes with single copies of either the *svp*^{e22} (18.3%, n=208) or the *hth*^{05745} (16%, n=363) allele were not significantly reduced when compared to those of *spi*$^{77\text{-}20}$ homozygotes alone (Figure 3.4E). These data demonstrate that *spi* acts in one genetic pathway with *svp* and *hth* to balance the cell fates of early stage germ cells, and in a distinct genetic pathway to regulate the frequency of GSC divisions.

Experimental Procedures

Drosophila Genetics

All fly stocks were raised and maintained on standard cornmeal molasses agar unless otherwise noted. Fly stocks that were used in experiments were raised at 26.5 °C. Most genetic mutations and transgenic elements are described at http://flybase.bio.indiana.edu or in the appropriate references provided below. Fly stocks used in this study include: w^{1118}, UAS-*upd,* UAS-*dpp,* (Bloomington Stock Center), $spi^{77\text{-}20}$ (Sarkar et al., 2007), svp^{e22} (Hiromi et al., 1993), svp^{07842} (Mlodzik et al., 1990), hth^{05745} (Rieckhof et al., 1997), $stet^{1}$ and $stet^{2}$ (Schulz et al, 2002) the germ cell driver *nanos-gal4-VP16* (Van Doren et al., 1998), the cyst cell driver *eyaA3*-Gal4 (Leatherman et al., 2008), UAS-*s-spi* (Tsruya et al., 2002), and UAS-$dEgfr^{JF02384}$ (TRiP at Harvard Medical School).

Immunohistochemistry, BrdU labeling, and fluorescence microscopy

Testes were dissected and placed in Testis Isolation Buffer (10 mM Tris-HCl, ph 6.8, 180 mM KCl) on ice. Testes were subsequently fixed in 4% formaldehyde in PBT for 30 min. Primary antibody incubation took place overnight at 4°C, and secondary antibody incubation took place for 2 hrs at room temperature. Testes were mounted onto slides using Vectashield mounting media with DAPI. Tissues were observed using a Zeiss Axiophot microscope. Images were taken with a CCD camera using an Apotome and Axiovision Rel Software. Antibodies and dilutions used were as follows: goat anti-Vasa (1:500, Santa Cruz Biotechnology Inc.), rabbit anti-phospho-histone H3 Ser10 (1:750, Millipore), mouse anti-BrdU (1:20, Upstate), and anti-Fasiciclin III 7G10 (1:10, obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa,

Department of Biological Sciences, Iowa City, IA 52242: developed by C. Goodman). Alexa-488-, Cy3-, and Cy5-conjugated secondary antibodies were used at 1:500 (Invitrogen).

BrdU ex vivo labeling of GSCs was performed as described by Wallenfang et al, 2006 with minor differences. Testes were dissected into 10 μM BrdU in Testes Isolation Buffer on ice. Testes were then shifted to 26.5 °C on a rotating platform for 30 min before being fixed in 4% fomaldehyde in PBT for 30 min.

Cell cycle analysis

The S-phase and M-phase indices were calculated by dividing either the number of $Brdu^+$ (S-phase) or $pHH3^+$ (M-phase) GSCs by the total number of GSCs scored. Optical sections were taken, using an apotome in conjunction with Axiovision Software, of the focal plane in which the middle of the hub was detected. GSCs were defined as $Vasa^+$ cells in direct contact with the hub. We detected approximately three GSCs in the focal plane scored for each testis. All indices represent the cumulative total of three independent experiments. All p-values were calculated using a two-tailed Fisher's exact test.

Discussion

A model for EGF signaling in Drosophila spermatogenesis

Our data clearly demonstrate that EGF is required for the regulation of GSC division frequency. Small changes in the frequency of stem cell divisions can dramatically alter the number of terminally differentiated cells that are produced. Hence, it is not surprising that mechanisms have evolved to regulate this aspect of stem cell biology. The EGF-dependent repression of stem cell division may be important for maintaining a balance between sperm

production and the demand for sperm, for reducing the frequency of unnecessary cell divisions that increase the chance of mutations being introduced into the germline, or simply for increasing the duration of fitness by mobilizing energy away from sperm production.

The role for EGF signaling in regulating GSC division frequency appears to be regulated independently of the role for EGF in promoting the differentiation of early stage spermatogonia. Based on our findings in developmental timeline and genetic interaction experiments, we propose a model that demonstrates the bifurcation of the Egf pathway (Figure 3.5). EGF acts in the stem cell microenvironment in one genetic pathway to regulate GSC division frequency, and in another pathway together with *svp* and *hth* to promote germ cell differentiation. This model advances our understanding regarding the core functions of the stem microenvironment, and how conserved signaling pathways function to perform distinct roles within the same tissue.

Although *upd* and *dpp* over-expression led to an accumulation of early stage germ cells at the expense of more mature cells, it did not alter the mitotic indices of GSCs. These findings cast doubt on a homeostatic model in which the repression of GSC divisions is dependent on retrograde signaling from more mature germ cells. Instead, we propose that signaling within the stem cell microenvironment is crucial in regulating GSC division frequencies.

Bifurcation of EGF function

The developmental and genetic bifurcation of EGF function during *Drosophila* spermatogenesis reveals a fundamental uncoupling between stem cell proliferation and stem cell daughter differentiation. We show that loss of EGF signaling does not alter GSC divisions during larval development, but is specifically required in the adult. This stage-specific requirement may reflect the different functions of GSCs in immature and mature tissues. The

initial function of GSCs may be to quickly populate the immature testis with germ cells, while GSCs in adult testes need to replenish differentiated cells dependent on demand.

Our study is the first report of a stage-specific impact of a signaling pathway on the activity of GSCs, and suggests that this developmental switch in GSC activity between larval and adult stages requires the activities of additional stage-specific pathways. On a molecular level, redundant signaling pathways may be active during larval stages that counteract the increased division frequency observed in adult GSCs upon loss of EGF. In larval testes, nutrient availability and cell growth may be the primary factors governing the frequency of stem cell divisions. Conversely, soon after eclosion, *Drosophila* males reach sexual maturity, and spermatogenesis may then rely on additional signaling pathways to acutely regulate stem cell divisions.

The mitotic activity of GSCs in adult male and female animals is also affected by fluctuations in insulin signaling (Drummond-Barbosa and Spradling, 2001; Hsu et al., 2008; McLeod, 2010). Animals fed a protein rich diet contain faster cycling GSCs than those fed a diet lacking protein. The mitogenic effect of systemic Insulin levels is mediated directly by cell autonomous Insulin Receptor activity in the germline. Yet, it is unclear how insulin signals navigate through the stem cell microenvironment, which consists of the complete encasement of GSCs by hub cells and CySCs. A simple and attractive model is that the increased mitotic activity of GSCs in *spi* mutants may be due to the disruption of this physical barrier, which results in a hyper-sensitivity of GSCs to Insulin signaling. Our data add a new role for the microenvironment. In addition to serving as a source of instructive signals, the microenvironment may act as a guardian shield to prevent the exposure of GSCs from excessive

mitogenic signals, or as a direct mediator of systemic factors that influence the enclosed germ cells.

We show that *spitz* genetically interacts with *svp* and *hth*. Multiple roles for *Drosophila svp* and *hth* have been reported in developing animals. For example, loss of *hth* results in the transformation of antennae to leg (Rieckof et al, 1997; Casares and Mann, 1998). Svp acts in the developing eye, heart, and the malpigian tubules (Moldzik et al, 1990; Kerber et al, 1998; Lo and Frasch, 2001). In the developing malpigian tubules, *svp* acts downstream of EGF to promote proliferation (Kerber et al, 1998). Here we report that attenuation of either *hth* or *svp* partially rescues tumor-like phenotypes in testes. Specifically, *svp* and *hth* act in opposition to EGF signaling as reduction of either gene promoted the differentiation of germ cells in EGF mutant testes. Future studies into the cellular mechanisms of the modifying activity of *svp* and *hth* attenuation on the *spi* phenotype may reveal further insights into how these two genes influence germ cell differentiation. However, reduction in *svp* or *hth* did not restore the normal GSC division frequency in $spi^{77\text{-}20}$ mutant testes. This implies that EGF acts in distinct genetic pathways to regulate these two aspects of spermatogenesis. EGF signaling may induce CyCSs to secrete signals regulating frequency of divisions to the enclosed GSCs, and induce cyst cells to secrete signals for differentiation to the enclosed stem cell daughters. CySCs associated with GSCs serve different functions and express different genes than the cyst cells associated with differentiating germ cells. This is reflected in the expression of molecular markers within the cyst cell lineage. For example, CySCs express high levels of the transcription factor Zfh-1, while cyst cells express decreasingly lower levels of Zfh-1 (Leatherman and DiNardo, 2008). CySCs and cyst cells may interpret germline-secreted EGF differently due to this differential

gene expression. It is probable that these differences in gene expression between CySCs and cyst cells result in a different EGF-mediated dialogue with their germline counterparts.

Stem cells and tumorogenesis.

Our findings demonstrate two distinct modes of proliferation in tumors. On one hand, cells continue to proliferate instead of undergoing differentiation. On the other hand, single cells divide at higher frequencies. Although it appears that the failure of cells to differentiate is a general characteristic of germ cell tumors, the mitotic hyperactivity of individual stem cells may be specific to a subset of tumors. Among the genetic backgrounds we tested, only the attenuation of EGF signaling led to an increase in the frequency of GSC divisions. This increase in the frequency of GSC divisions may lead not only to the production of more proliferating cells, thereby increasing tumor size, but also may lead to an increased risk of stem cells accumulating transforming mutations. Tumors that contain hyper-proliferating stem cells in addition to a blockade in differentiation may be more aggressive than tumors consisting primarily of partially differentiated cells. Our comparison between these tumor types strengthens the underlying rationale for alternative treatment options for different tumors.

Outlook

In summary, we show an unprecedented role for the EGF pathway in regulating the frequency of stem cell divisions. Our data are consistent with a model in which stem cell microenvironments play critical roles in regulating how stem cells divide. This may also apply to mammalian stem cells, especially those characterized by a lengthened cell cycle.

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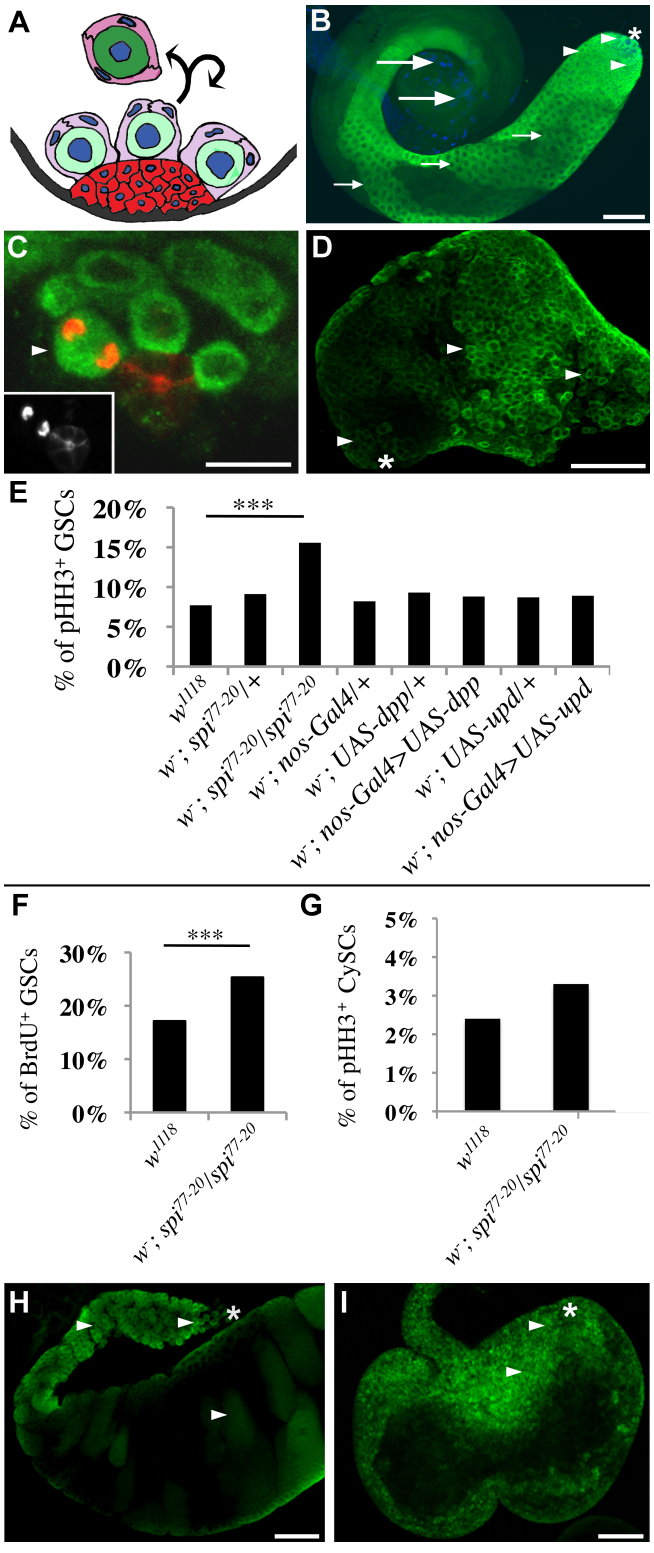
Figure 3. 1. EGF is required to repress the division frequency of GSCs**.** (**A**) Cartoon showing the architecture of the stem cell and germ cell niches. GSCs (Light Green) are organized around the hub (Red). CySCs (Light Pink) encase GSCs and are also in contact with the hub. The gonialblast (Dark Green) is displaced away from the hub and encased by two cyst cells (Dark Pink). **(B)** Immunoflourescent image of a control w^{1118} testis stained with the germ cell marker Vasa (Green) and the DNA stain, DAPI (Blue). . (**C**) A $pHH3^{+}$ GSC at the apical tip of a testis from a w^{1118} animal stained with antibodies labeling the germ line cells (anti-Vasa, green), hub cells (anti-Fasciclin III, red), and mitotic DNA (anti-phospho-Histone H3, red). Arrowhead indicates a GSC in anaphase. Scale bar indicates 10um. **(D)** Testes containing germ cell tumors resulting from *spi* attenuation stained for anti-Vasa. (**E**) The percentage of $pHH3^{+}$ GSCs (mitotic index) from control testes and testes containing germ cell tumors. Genotypes as indicated. >500 GSCs were scored for each genotype, ***p-value < 0.0001. (**F**) The S-phase index of GSCs in w^{1118} testes and $spi^{77\text{-}20}$ homozygotes mutant testes, ***p-value = 0.0004. (**G**) The percentage of $pHH3^{+}$ CySCs is plotted. A significant difference between the mitotic indices from w^{1118} controls and *spi* mutants was not detected. p = 0.28. (**H, I**) Anti-Vasa staining to testes with germ cell-intrinsic overexpression of **(G)** *dpp* shows excessive clusters of the small early germ cells, and **(H)** *upd* is filled with small germ cells. Asterisks: apical tips of testes, arrowheads: spermatogonia, short arrows: spermatocytes, long arrows: elongated spermatids. Scale bars indicate 50um unless otherwise noted.


A
B
C
D
E
% of pHH3+ GSCs
20%
15%
10%
5%
0%
***
w1118
w-; spi77-20/+
w-; spi77-20/spi77-20
w-; nos-Gal4/+
w-; UAS-dpp/+
w-; nos-Gal4>UAS-dpp
w-; UAS-upd/+
w-; nos-Gal4>UAS-upd
F
% of BrdU+ GSCs
30%
20%
10%
0%
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w1118
w-; spi77-20/spi77-20
G
% of pHH3+ CySCs
5%
4%
3%
2%
1%
0%
w1118
w-; spi77-20/spi77-20
H
I

Figure 3. 2. Egf Signaling in the Germ Cell Microenvironment Represses GSC Mitotic Activity**.** (**A-B**) The percentage of $pHH3^{+}$ GSCs for each indicated genotype was plotted. (**A**) The expression of a transgenic cDNA encoding the secreted form of *spitz, s-spi,* in germ cells rescues the hyper-proliferation of GSCs in $spi^{77\text{-}20}$ mutants. (**B**) *dEGFR* RNAi-mediated knock-down in SCCs results in a higher GSC mitotic index, $^{***}p \leq 0.0001$.

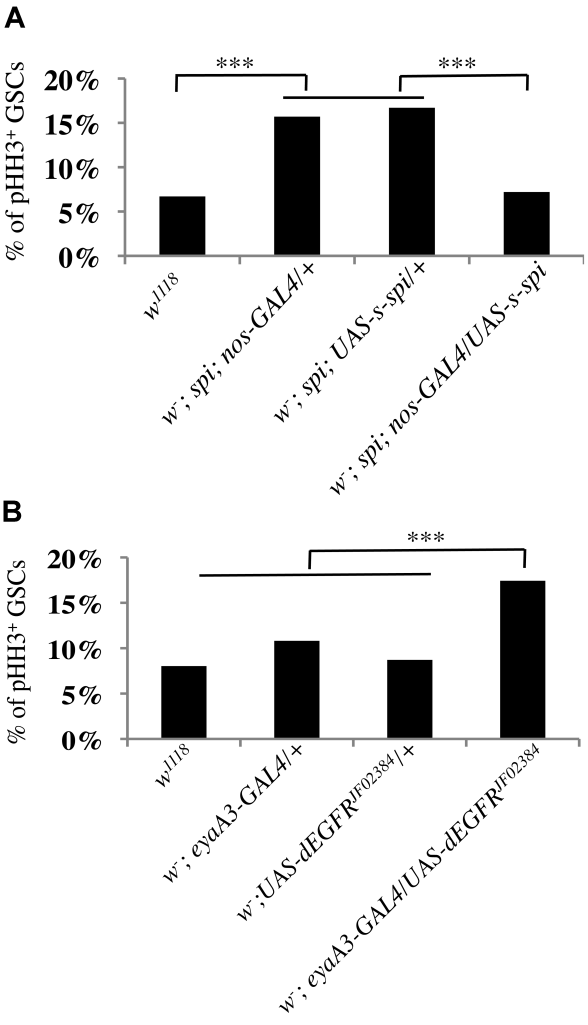

A
% of pHH3+ GSCs
20%
15%
10%
5%
0%
***
***
w1118
w-; spi; nos-GAL4/+
w-; spi; UAS-s-spi/+
w-; spi; nos-GAL4/UAS-s-spi
B
% of pHH3+ GSCs
20%
15%
10%
5%
0%
***
w1118
w-; eyaA3-GAL4/+
w-;UAS-dEGFRJF02384/+
w-; eyaA3-GAL4/UAS-dEGFRJF02384

Figure 3. 3. The EGF repression of GSC divisions is developmentally regulated. (**A, B**) Testes from either (**A**) w^{1118} or (**B**) w^{-}; $spi^{77\text{-}20}/spi^{77\text{-}20}$ 3rd instar larvae stained with anti-Vasa (green) and DAPI (blue). Arrows: spermatocytes, arrowheads: spermatogonia, scale bars: 50um. (**C**) The mitotic index (percentage of $pHH3^{+}$ GSCs) for each indicated genotype was plotted. (**D**) A model depicting the requirement of EGF signaling in both larvae and adults for the differentiation of early stage spermatogonia to later stage germ cells. In contrast, *spi* is not required in larvae for the repression of GSC divisions, demonstrating a developmental uncoupling of EGF function.

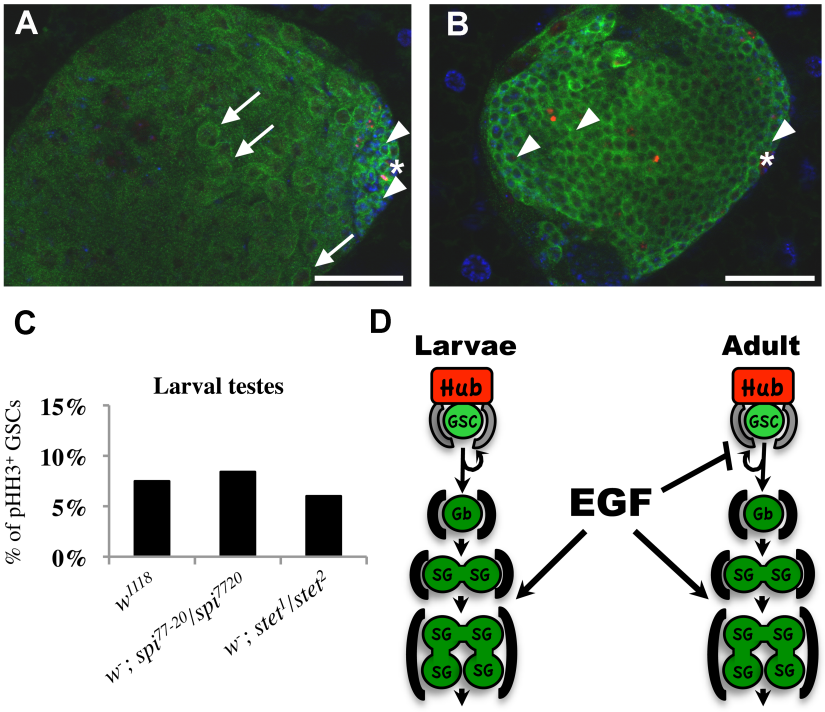

A
B
C
Larval testes
% of pHH3+ GSCs
15%
10%
5%
0%
w1118
w-; spi77-20/spi7720
w-; stet1/stet2
D
Larvae
Adult
Hub
GSC
Gb
SG
EGF

Figure 3. 4. EGF interacts through distinct genetic pathways to repress GSC divisions and promote germ cell differentiation. (**A-C**) Testes from animals stained with anti-Vasa (green), anti-Armadillo (red), and DAPI. **A)** Testis from a w^{1118} animal containing spermatogonia, spermatocytes, and elongated spermatids. (**B, C**) Testes from w^{-}; $spi^{77\text{-}20}/spi^{77\text{-}20}$ mutants raised at the semi-restrictive temperature of 26.5 °C contain **(B)** spermatogonia and spermatocytes, but not elongated spermatids, or (**C**) all stages of spermatogenesis. Arrowheads: spermatogonia, small arrows: spermatocytes, large arrows: bundles of elongated spermatids, asterisks: apical tips of testes, scale bars: 50um. (**D**) The percentage of testes scored according to the stages of germ cells they contain is plotted, ***$p < 0.0001$. (**E**) The mitotic indices of GSCs within testes from animals with the specified genotypes are plotted, **$p < 0.001$.

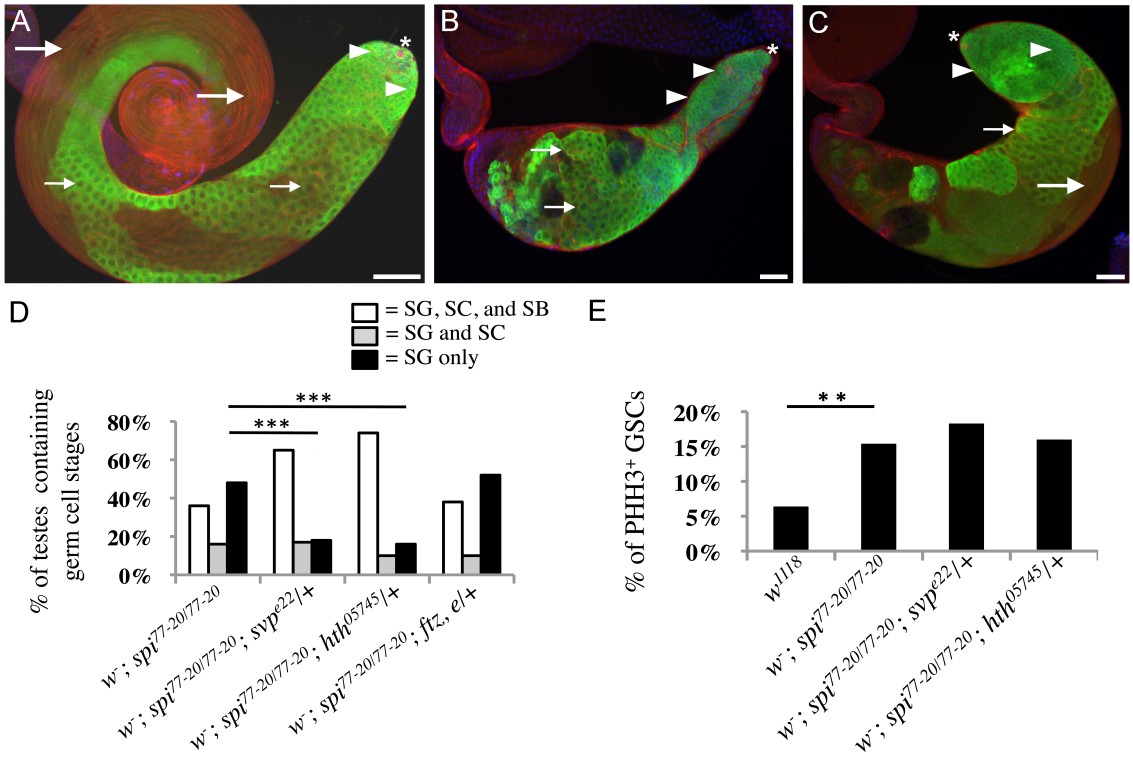
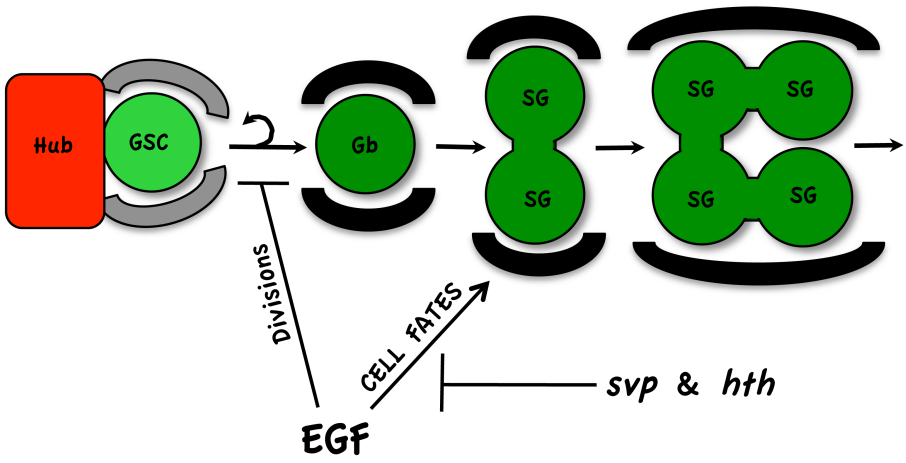

A
B
C
D
= SG, SC, and SB
= SG and SC
= SG only
% of testes containing germ cell stages
80%
60%
40%
20%
0%
***
***
w-; spi77-20/77-20
w-; spi77-20/77-20; svpe22/+
w-; spi77-20/77-20; hthl05745/+
w-; spi77-20/77-20; ftz, el/+
E
% of PHH3+ GSCs
20%
15%
10%
5%
0%
**
w1118
w-; spi77-20/77-20
w-; spi77-20/77-20; svpe22/+
w-; spi77-20/77-20; hthl05745/+

Figure 3. 5. A model for EGF signaling in adult spermatogenesis**.** EGF signaling both represses the frequency of GSC divisions and promotes the differentiation of spermatogonia. *spitz* acts in opposition to *svp* and *hth* in a genetic pathway that regulates the cell fates of spermatagonia. Yet, *spi* acts in a distinct genetic pathway to repress the frequency of GSC divisions.


Hub
GSC
Gb
SG
SG
SG
SG
SG
SG
Divisions
CELL FATES
EGF
svp & hth

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CHAPTER 4

NUCLEOPORIN98-96 FUNCTION IS REQUIRED FOR TRANSIT AMPLIFICATION DIVISIONS IN THE GERM LINE OF *DROSOPHILA MELANOGASTER*[3]

[3] Parrott, B.B., Chiang, Y., Hudson, A., Sarkar, A., Guichett, A., and Schulz, C. Submitted to *PLoS,* 6/12/2011.

Abstract

Production of specialized cells from precursors depends on a tightly regulated sequence of proliferation and differentiation steps. In the gonad of *Drosophila melanogaster*, the daughters of germ line stem cells (GSC) go through precisely four rounds of transit amplification divisions to produce clusters of 16 interconnected germ line cells before entering a stereotypic differentiation cascade. Here we show that animals harboring a transposon insertion in the center of the complex *nucleoporin98-96* (*nup98-96*) locus had severe defects in the early steps of this developmental program, ultimately leading to germ cell loss and sterility. A phenotypic analysis indicated that flies carrying the transposon insertion, designated $nup98\text{-}96^{2288}$, had dramatically reduced numbers of germ line cells. In contrast to controls, mutant testes contained many solitary germ line cells that had committed to differentiation as well as abnormally small clusters of two, four or eight differentiating germ line cells. This indicates that mutant GSCs and GSC daughters had either completely skipped transit amplification divisions or initiated the differentiation cascade after less than four rounds of amplification divisions. This phenotype remained unaffected by hyper-activation of signalling pathways that normally result in excessive proliferation of GSCs and their daughters. Expression of wild-type *nup98-96* specifically in the germ line cells of mutant animals fully restored development of the GSC lineage, demonstrating that the effect of the mutation is cell-autonomous. Nucleoporins are the structural components of the nucleopore and have also been implicated in transcriptional regulation of specific target genes. The nuclear envelopes of germ cells and general nucleocytoplasmic transport in *nup98-96* mutant animals appeared normal, leading us to propose that *Drosophila nup98-96* mediates the transport or transcription of targets required for the developmental timing between amplification and differentiation.

Introduction

In development and tissue homeostasis, the proliferation of precursor cells and the initiation of terminal differentiation are temporally separated. For example, regeneration of organs typically involves proliferation of de-differentiated or pre-existing pluripotent cells followed by coordinated differentiation. Tissue homeostasis from self-renewing populations of stem cells follows a similar two-step process. First, stem cell daughters exiting the stem cell fate multiply by transit amplification divisions to create a pool of precursor cells. Then these precursors develop into specialized cell types through a precisely coordinated cascade of differentiation events (Loeffler and Potten, 1997; Gilbert, 2006). The *Drosophila* gonad has served as a highly successful model for elucidating many of the signaling pathways that regulate the cell fate, amplification, and differentiation of the GSC lineage (Jones et al, 2004; Gilboa and Lehmann, 2004). However, comparatively little is known about the molecules and mechanisms that coordinate developmental timing and, specifically, the timing between amplification and differentiation of stem cell daughters.

Here, we show that a normal balance between transit amplification divisions and terminal differentiation depends on the complex *nucleoporin98-96* (*nup98-96*) locus. Nucleoporins are structural components of the nuclear pore and have well-established functions in nucleo-cytoplasmic transport as well as the breakdown and re-assembly of the nuclear envelope during mitosis (Adam, 2001; Rout and Aitchison, 2001; Suntharalingham and Wente, 2003). More recently, it has become clear that members of this protein family also contribute to the regulation of developmental processes via their effect on gene transcription. Specifically, *Drosophila* Nup98 was found to associate with actively transcribed chromatin in salivary glands of 3rd instar wild-type larvae in a manner dependent on Ecdysone, a steroid hormone and key regulator of

molting and metamorphosis. Transcriptional up-regulation in response to Ecdysone is correlated with increased chromatin occupancy of Nup98 while down-regulation correlated with a decrease in Nup98 chromatin binding. Transcriptional profiling of *Drosophila* S2 cells further established that Nup98 and a second nuleoporin, Sec13, control the transcription of specific target genes regulating developmental transitions and the cell cycle (Capelson et al, 2010; Kalverda et al, 2010).

The highly conserved *nup98-96* locus is complex and gives rise to two distinct proteins, Nup98 and Nup96. Alternative splicing generates two major transcripts: a short mRNA containing an open reading frame for only Nup98, and a long mRNA with an open reading frame for a Nup98-Nup96 poly-protein. Processing by autocatalytic cleavage subsequently separates the two functional units, Nup98 and Nup96. In *Drosophila*, *nup98-96* transcripts were detected at all stages of development (Wente and Blobel, 1994; Fontoura et al, 1999). Mutations harboring a stop codon in Nup98, and thus presumably eliminating both Nup98 and Nup96 function, are associated with lethality prior to metamorphosis, possibly reflecting the role of Nup98 in Ecdyson-dependent gene transcription (Presgraves et al, 2003).

Here, we investigate the role of the *nup98-96* locus in the germ line stem cell lineage. In a screen for mutations effecting the development of germ line cells, we identified a transposon-insertion in the center of the *nup98-96* locus. In *Drosophila* wild-type males, the daughters of GSCs amplify by exactly four rounds of mitosis with incomplete cytokinesis to produce clusters of 16 spermatogonia that remain interconnected by cytoplasmic bridges. After mitosis, the spermatogonia become spermatocytes, which enter the terminal differentiation cascade. The first step of terminal differentiation is an extreme increase in germ cell size accompanied by the expression of most of the genes that mediate subsequent differentiation steps. Subsequently, the

spermatocytes undergo meiosis and develop into spermatids (Hardy et al, 1979; Fuller, 1993). In the gonads of males homozygous for the *nup98-96*^{2288} mutation, or harboring *nup98-96*^{2288} in trans to a deficiency that uncovers the locus (*Df(3R)mbc-R1*), GSCs and their daughters appeared to differentiate into spermatocytes either directly or after less than four rounds of transit amplification divisions. These defects were fully complemented by expression of *nup98-96* specifically in the germ line, revealing a cell autonomous mode of action. Manipulations of signalling pathways that result in the over-proliferation of germ line cells in otherwise wild-type testes did not attenuate the *nup98-96*^{2288} phenotype. As the nuclear pore of mutant animals showed no obvious defects, we propose that the defects in *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant animals are due to the lack of either nucleocytoplasmic transport or transcription of as yet unidentified factors required for timing the transition between amplification and terminal differentiation.

Results

The nup98-96 locus is required for maintaining germ line cells in an undifferentiated state.

Animals carrying the *nup98-96*^{2288} mutation were first identified in a genetic screen for sterile animals with abnormally small gonads. We subsequently observed the same gonad phenotype in animals trans-heterozygous for *nup98-96*^{2288} and *Df(3R)mbc-R1*, suggesting that the *nup98-96*^{2288} allele acts as a strong allele with respect to the gonad phenotype. No other morphological abnormalities were obvious in these animals, implying that *nup98-96*^{2288} is a mutation with a specific effect on gametogenesis.

In testes from *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant animals (hence forth referred to as *nup98-96*^{2288}*/Df(3R)mbc-R1* testes), the germ line cells were progressively lost with increasing age of the animal. Normally, the germ line cells are arranged in a spatio-temporal gradient along

the apical to basal axis of the testis (Figure 4.1A). GSCs are confined to the apical tip and surround a group of somatic cells, called the hub (red in Figure 4.1A). Their immediate daughters (gonialblasts) and clusters of between two and 16 interconnected cells in the process of transit amplification divisions (spermatogonia) become displaced basally and are found a short distance from the hub. Large spermatocytes that have initiated the differentiation cascade and mature spermatids occupy more basal positions within the testis. At all stages of development, GSCs and their progeny are fully enclosed by somatic support cells (black circles in Figure 4.1A). This germ cell microenvironment, or niche, provides external cues that regulate stem cell self-renewal, stem cell daughter amplification, and germ line differentiation (Matunis et al, 1997; Kiger et al, 2000).

In situ hybridization with a gonad-specific probe (*piwi*-RNA) revealed that the gonads from *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant embryos were similar in size to gonads from control animals (in the following referred to as control testes, compare Figure 4.1C to 4.1B, n>50). However, by the 3rd instar larval stage, *nup98-96*^{2288}*/Df(3R)mbc-R1* testes were noticeably smaller than control testes (compare Figure 4.1E to 4.1D, n>100), and did not contain any early stage germ line cells (GSCs, gonialblasts, or spermatogonia). In preparations stained with 4',6-diamidino-2-phenylindole (DAPI), the nuclei of early stage germ line cells appear as characteristic small bright signals (Figure 4.1D, arrows) due to the small size of their nuclei, whereas spermatocytes that have initiated the differentiation cascade have larger, less brightly staining nuclei (Figure 4.1D, arrowhead). In contrast to control testes, the apical region of *nup98-96*^{2288}*/Df(3R)mbc-R1* testes did not contain many small, brightly stained nuclei, suggesting that early stage germ line cells were depleted. However, larger and less brightly staining nuclei

characteristic of spermatocytes were present in *nup98-96^{2288}/Df(3R)mbc-R1* testes (Figure 4.1E, arrowheads).

Labelling with an antibody against a-spectrin confirmed that 3rd instar larval *nup98-96^{2288}/Df(3R)mbc-R1* testes lacked transit amplifying spermatogonia, but contained germ line cells at the spermatocyte stage. In germ line cells, a-spectrin labels a sub-cellular structure called the fusome, and the shape and the size of fusomes is indicative of the germ line cell's developmental stage (Lin et al, 1994). The GSCs and the gonialblasts contain round fusomes, commonly referred to as spectrosomes. The spectrosomes containing GSCs (Figure 4.1F, G arrowheads) are found next to the hub (red and marked with an asterisk in Figure 1F-1L). Clusters of interconnected germ line cells contain branched fusomes that reach through their intercellular bridges. a-spectrin-staining reveals that spermatogonia in transit amplifying divisions have small, branched fusomes and are located relatively close to the hub while the spermatocytes have large, branched fusomes and are found more basally (Figure 4.1F, small and large arrows, respectively).

3rd instar *nup98-96^{2288}/Df(3R)mbc-R1* testes did not contain cells with a-spectrin-positive spectrosomes located next to the hub (Figure 4.1H, n>50). Cells with a-spectrin-positive structures were mostly found toward the middle and basal region of mutant testes (Figure 4.1I). Germ line cells with round spectrosomes characteristic of GSCs were detected. However, these cells were larger than GSCs (Figure 4.1I, arrowheads) and contained large and less brightly DAPI-stained nuclei typical of spermatocytes (Figure 4.1I, inset). We propose that these solitary differentiating germ line cells originated from GSCs and gonialblasts that failed to undergo amplification divisions. In addition, we detected many wide fusomes (Figure 4.1J, arrows) that connected large germ line cells with large nuclei and less brightly staining DNA (compare Figure

4.1J-4.1E), similar to wild-type spermatocytes. In contrast to the fusomes in 16 cell stage spermatocytes in control testes, the fusomes in the *nup98-96^{2288}/Df(3R)mbc-R1* testes consistently had fewer than 16 branches and appeared to connect only two, four or eight spermatocytes. This implies that most of the germ line cells of *nup98-96^{2288}/Df(3R)mbc-R1* testes only went through one, two or three instead of the stereotypical four rounds of amplification divisions. In support of this conclusion, the *nup98-96^{2288}/Df(3R)mbc-R1* testes contained far fewer germ line cells than control testes: on average, only 34 a-spectrin-positive cells were found per mutant testis (s.d. 20, range: 0-54, n=40) compared to several hundred a-spectrin-positive cells found in control testes (n>50).

Adult *nup98-96^{2288}/Df(3R)mbc-R1* testes completely lacked early stage germ line cells and, on the basis of immuno-labelling with the germ line cell markers Vasa and a-spectrin, rarely contained late stage germ line cells. In control testes, Vasa-positive GSCs (arrowheads in Figure 4.1K) form a rosette around the apical hub (n>100). Vasa-positive spermatogonia are found at a distance from the tip in the apical region of the testes (Figure 4.1K, small arrows), whereas large Vasa-positive spermatocytes are located more toward the base (Figure 4.1K, large arrows). Double-staining with Vasa- and a-spectrin-antisera revealed that 98% of adult *nup98-96^{2288}/Df(3R)mbc-R1* testes did not contain any germ line cells (Figure 4.1L, n>100). The remaining 2% of the mutant testes contained either two or four large, Vasa-positive spermatocytes located in the testis coil, or a few immature sperm bundles (data not shown). We conclude that the failure to undergo the normal numbers of amplification divisions completely exhausted the germ line of *nup98-96^{2288}/Df(3R)mbc-R1* males.

nup98-96^{2288} plays a parallel role in the female gonad.

Similar to the testis, the gonad of female flies is organized in an apical-to-basal differentiation gradient of germ line cells. GSCs lie at the apical tip of the germarium. The stem cell daughters (cystoblasts), and their transit amplifying progeny (cystocytes) become progressively displaced away from the tip toward the base (Figure 4.2A) (Spradling, 1993). GSCs and cystoblasts are characterized by the presence of a a-spectrin-positive spectrosome (Figure 4.2B, arrowheads) whereas the interconnected cystocytes contain branched fusomes (Figure 4.2B, arrows). Labelling with a-spectrin antibodies revealed the absence of both spectrosomes and fusomes in most germaria from *nup98-96*^{2288} homozygous (data not shown) and *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant females (Figure 4.2C, 70%, n>100).

We next investigated if the loss of early stage germ line cells observed in *nup98-96*^{2288}*/Df(3R)mbc-R1* germaria was age-dependent. Ovaries from wild-type control and *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant flies were collected at three and ten days post-eclosion, immuno-labelled with the germ line marker anti-Vasa, and the number of Vasa-positive germ line cells in each germarium was quantified (Figure 4.2D). We found that the number of germ line cells in control animals did not change significantly between three and ten days post-eclosion. Control germaria contained on average 35 Vasa-positive cells three days post-eclosion (n=100; s.d. 16, range: 21 – >40; blue bars in Figure 4.2D) and 32 Vasa-positive cells 10 days post-eclosion (n=100; s.d. 16; range: 16 – >40; green bars in Figure 4.2D). Control germaria without Vasa-positive cells were never observed. In contrast, *nup98-96*^{2288}*/Df(3R)mbc-R1* germaria showed a dramatic loss of germ line cells. Three days post-eclosion, mutant germaria contained an average of 13 Vasa positive cells (n=100; s.d. 27), but this number varied widely (from 0 to 40; yellow bars in Figure 4.2D). Ten days post-eclosion, mutant germaria, on average, contained only 2 Vasa-positive cells (n=100; s.d.13; range: 0 – 15; red bars in Figure 4.2D). Notably, 75% of the

nup98-96^{2288}*/Df(3R)mbc-R1* germaria had no detectable germ line cells. We conclude that the *nup98-96*^{2288} mutation has similar effects on both male and female early stage germ line cells: in both sexes, the GSC lineage is rapidly depleted with increasing age, presumably due to premature differentiation.

The defects in the nup98-96^{2288}*/Df(3R)mbc-R1 mutant gonads are due to disruption of a nuclear pore locus.*

We mapped the *nup98-96*^{2288} mutation to chromosomal region 95A5 to C10. Complementation tests using mutations in genes along this region revealed that the defects in the mutant animals were due to a disruption of the *nup98-96* locus. Sequencing of genomic DNA from *nup98-96*^{2288} mutant animals revealed two changes to the published gene sequence of the *nup98-96* locus. Mutant animals harbored a point mutation that results in an amino acid exchange of the Nup98 coding sequence (CAA to CGA, Glutamine^{860} to Arginine). However, the same amino acid exchange is found in *nup98-96* alleles of *Drosophila pseudoobscura* (Flybase Consortium, 2003), strongly suggesting it is a natural variant and does not cause the defects associated with the *nup98-96*^{2288} allele. In addition, mutant animals carried a *Pogo*-element insertion in the fourth intron of the *nup98-96* locus (Figure 4.3A, indicated as 2288). This insertion is predicted to disrupt the splicing of exon 4 to exon 5 (encoding the N-terminal portion of Nup96) and thus should specifically prevent the formation of Nup96.

Expression of a rescue construct in the gonads of mutant animals confirmed that the *nup98-96*^{2288} mutant phenotype was due to lesions in the *nup98-96* locus. We generated flies carrying a full-length cDNA encoding *nup98* and *nup96* under control of Yeast Upstream Activating Sequence (UAST-*nup98-96*, Figure 4.3A). Flies carrying UAST-*nup98-96* were

crossed to flies carrying *gal4*-transactivators to induce tissue specific expression (Phelps and Brand, 1998). Expression of UAST-*nup98-96* in germ line cells of *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant males using the germ cell specific driver *nanos-gal4-VP16* (*nos-gal4*) (Van Doren, 1998) restored spermatogenesis.

An adult wild-type testis is a coiled, tubular organ that is, on average, 2 mm long (n>100) and contains germ line cells at all stages of spermatogenesis, including sperm bundles (Figure 4.3B, arrow). Adult *nup98-96*^{2288}*/Df(3R)mbc-R1* testes were much shorter (Figure 4.3C) than control testes, measuring only 100-500 μm in length (n>100). In addition, mutant testes contained very few, if any, germ line cells (see above). *nup98-96*^{2288}*/Df(3R)mbc-R1* testes with germ line specific expression of UAST-*nup98-96* were of normal size and contained germ line cells at all stages of spermatogenesis, including mature sperm (Figure 4.3D, arrow, n>100). Expression of the UAST-*nup98-96* construct in the somatic cells of the gonad did not restore spermatogenesis (n>50, data not shown), demonstrating that the defects were due specifically to loss of *nup98-96* from the germ line cells.

Surprisingly, expression of a cDNA (UAST-*nup98*) which only encoded *nup98* (Figure 4.3A) within the germ line cells from *nup98-96*^{2288}*/Df(3R)mbc-R1* testes did not restore spermatogenesis and testes remained small (Figure 4.3E, n>50). Western blot analysis using protein extracts from control and mutant animals revealed that both antibodies (raised against either Nup98 or Nup96) failed to detect significant levels of either protein in the mutant animals (Figure 4.3F). We conclude that the defects in the *nup98-96*^{2288}*/Df(3R)mbc-R1* gonads are due to a strong reduction in both proteins, Nup98 and Nup96.

Confirming the role of the *nup98-96* locus in the GSC lineage, expression of two independent RNA-interference lines targeted against *nup98-96* (Figure 4.3A, indicated as RNAi)

in the germ line cells of otherwise wild-type animals also resulted in progressive loss of early stage germ line cells and the appearance of single cell spermatocytes (Figure 4.3G, arrow, n>50). Conversely, expression of UAST-*nup98* or UAST-*nup98-96* (Figure 4.3H) in germ line cells of otherwise wild-type animals did not cause any defects in spermatogenesis (n>50) suggesting that the *nup98-96* locus plays a permissive role in germ line development.

In nup98-96^{2288}/Df(3R)mbc-R1 animals, the nuclear envelope and general nucleocytoplasmic transport appear normal.

The molecular nature of *nup98-96* suggests that the proteins play a structural role in germ line cells. We therefore used the nuclear envelope marker LaminC and the nuclear pore marker mAB414 to determine if *nup98-96^{2288}* caused any visible alterations in nuclear envelope morphology. This analysis was performed using ovaries from young wild-type and *nup98-96^{2288}/Df(3R)mbc-R1* females since the female germ line cells are larger than male germ line cells and thus enable imaging with better sub-cellular resolution. In *nup98-96^{2288}/Df(3R)mbc-R1* gonads, LaminC (Figure 4.4B, 4.4G), and mAB414 (Figure 4.4D, 4.4E, 4.4I) localization to the nuclear envelopes and nuclear pores of the germ line cells appeared normal compared to the controls (Figure 4.4A, 4.4C, 4.4F, 4.4H).

Next, we surveyed the effect of the *nup98-96^{2288}* mutation on nucleocytoplasmic transport by determining the localization of selected proteins normally found either in the nucleus or in the cytoplasm. The following proteins were assayed: the transcription factors Groucho (Figure 4.5A, 4.5B, arrows) and phosphorylated Jun-kinase (Figure 4.5C, 4.5D, arrows), the cytoplasmic proteins Vasa (green in Figure 4.5A-4.5D and 4.5G-4.5H) and Sex-lethal (Figure 4.5E, 4.5F, arrows), and the nuclear protein phosphorylated Histone-H3 (Figure 4.5G, 4.5H, arrows). As in

the controls, Sex-lethal and Vasa were localized to the cytoplasm of *nup98-96^{2288}/Df(3R)mbc-R1* germ line cells, indicating that general mRNA export was not disrupted. Likewise, Groucho, phosphorylated Jun-Kinase, and phosphorylated Histone-H3 were localized to the nuclei of both control and *nup98-96^{2288}/Df(3R)mbc-R1* germ line cells, showing that the mutant germ line cells are capable of importing these proteins into their nuclei.

Finally on the basis of phosphorylated Histone-H3 and Bromodeoxyuridine (BRDU) labeling, *nup98-96^{2288}/Df(3R)mbc-R1* ovaries did not contain germ line cells that appeared to be blocked in S-phase or M-phase of the cell cycle (compare Figure 4.5H, 4.5J to Figure 4.5G, 4.5I). Thus, our analysis failed to reveal any support for the view that the *nup98-96^{2288}* mutation has a noticeable effect on either the structure of the nuclear pore or its function in the general transport of mRNAs and proteins to and from the nucleus.

The nup98-96^{2288} mutation causes differentiation of the germ line cells, even in the presence of proliferation-promoting factors.

To further explore the role of *nup98-96* in the germ line cells, we tested the genetic interaction of *nup98-96^{2288}* with perturbations in signaling pathways that regulate early stage germ line cells. In wild-type ovaries, somatic cap cells signal via the Transforming Growth Factor-β (TGF-β) pathway to the adjacent GSCs to regulate their decision between stem cell and cystoblast fate. Upon receptor activation, the TGF-β signal transducers, Mad and Medea, translocate into the nucleus and silence transcription of the differentiation factor *bag of marbles* (*bam*) in the GSCs. Therefore, Bam is normally not found in the cytoplasm of GSCs but is found in the cytoplasm of cystoblasts and cystocytes. Overexpression of *bam* within the germ line cells

of otherwise wild-type ovaries causes the germ line cells to be lost, first from the GSC position and then from the entire germaria (Ohlstein and Mckearin, 1997; Song et al, 2004).

The expression pattern of Bam is an excellent tool for determining whether a defect in TGF-β signalling exists in mutant ovaries. In ovaries from freshly eclosed *nup98-96^{2288}/Df(3R)mbc-R1* females, Bam was expressed in a pattern similar to that seen in wild-type ovaries. As in wild-type germaria (Figure 4.6A, arrowhead), the *nup98-96* mutant GSCs located next to the apical tip did not contain cytoplasmic Bam (Figure 4.6B, arrowhead). In *nup98-96^{2288}/Df(3R)mbc-R1* germaria, GSC daughters displaced away from the GSC position showed normal cytoplasmic Bam expression (Figure 4.6B, arrow), as seen in wild-type germaria (Figure 4.6A, arrow). We conclude that TGF-β signalling from the soma to the GSCs was not disrupted in *nup98-96^{2288}/Df(3R)mbc-R1* mutant germaria.

This conclusion is consistent with the differences between the gonad phenotypes observed in flies with mutations in TGF-β signalling pathway and *nup98-96^{2288}/Df(3R)mbc-R1* mutant animals. In both genders, loss of TGF-β signalling specifically causes GSC loss. However, upon loss of TGF-β signalling, the GSC daughters undergo normal numbers of amplification divisions (Schulz et al, 2004; Bunt and Hime, 2004). In contrast, *nup98-96^{2288}/Df(3R)mbc-R1* mutant GSCs and their daughters differentiated either directly or after undergoing fewer than the normal four rounds of mitosis.

Loss of signalling via the Epidermal Growth Factor Receptor (EGFR) has an opposite effect on germ line cells than the *nup98-96^{2288}* mutation does. EGFR-dependent signalling promotes the growth of the somatic support cells that surround the germ line cells and form their microenvironment (black circles in Figure 4.1A). Depletion of EGFR signalling, for example via loss of Stet, an enzyme required for processing the EGFR ligand, results in a loss of this

regulatory microenvironment. As a consequence, the germ line cells over-proliferate and produce hundreds of early stage germ line cells, which populate the entire testis (Schulz et al, 2002; Sarkar et al, 2007).

We investigated the genetic relationships between *stet* and *nup98-96* in the male germ line by creating double mutant animals. Early stage germ line cells that stain brightly with the nuclear dye DAPI were confined to the tip of wild-type testes (Figure 4.6C, arrow) but filled the testes of animals homozygous for the strong $stet^{1}$ allele (Figure 4.6D, arrows) (Schulz et al, 2004). In contrast, testes from $stet^{1}$; $nup98\text{-}96^{2288}$ double-homozygous animals were much smaller than $stet^{1}$-testes (compare Figure 4.6E to 4.6D) and contained few brightly stained, small nuclei (Figure 6E). Immuno-labelling with antibodies against a-spectrin revealed few germ line cells in $stet^{1}$; $nup98\text{-}96^{2288}$ double-mutant testes, all of which were large spermatocytes (Figure 4.6F, arrowhead) and had a spectrosome (Figure 4.6F, arrow) similar to the spermatocytes in $nup98\text{-}96^{2288}$/*Df(3R)mbc-R1* testes. These results reveal that $nup98\text{-}96^{2288}$ can suppress the germ line defects associated with loss of EGFR signalling.

We next determined whether the $nup98\text{-}96^{2288}$ allele also suppressed the overproliferation phenotypes resulting from the hyper-activation of signalling pathways. Hyper-activation of the TGF-β pathway in otherwise wild-type testes forces spermatogonia to proliferate beyond the normal four rounds of amplification division, producing clusters of 64, 128, or more spermatagonia that ultimately die (Schulz et al, 2004; Bunt and Hime, 2004). Hyperactivation of the Janus Kinase/Signal Transducer and Activator of Transcription (JaK/STAT) pathway in otherwise wild-type animals results in testes that are filled with thousands of cells resembling GSCs and gonialblasts (Kiger et al, 2001; Tulina and Matunis, 2001) Both phenotypes could be reproduced by either over-expression of the TGF-β ligand *decapentaplegic (dpp)* (Figure 4.6G,

n>50), or the JaK/STAT ligand *unpaired (upd)* (Figure 4.6H, n>50), in the germ line cells of otherwise wild-type animals. In contrast, over-expression of either ligand in the germ line cells of *nup98-96*^{2288} animals failed to increase the number of early stage germ line cells. Instead, the testes were depleted of early stage germ line cells and, occasionally, a spermatocyte was observed (Figure 4.6I, arrow, n>50). The interaction of *nup98-96*^{2288} with EGFR, TGF-β, and JaK/STAT signaling strongly argue that *nup98-96* function is an essential prerequisite for maintaining germ line cells in an undifferentiated state.

Experimental procedures

Fly strains

Flies were raised on standard cornmeal molasses agar medium. The *nup98-96*^{2288} mutation was identified by Antoine Guichet and Anne Ephrussi in a screen for flies with small gonads. Flies expressing RNAi-constructs for *nup98-96* (UAS-*nup98-96*^{RNAi}, VDRC lines 31198 and 31199) were obtained from the Vienna *Drosophila* Resource Center (VDRC). The *stet*^{1} allele is described in (Schulz et al, 2002). *Df(3R)mbc-R1*, *nup98-96*^{339}, UAS-*dpp*, UAS-*upd*, , *nos-gal4:VP-16, C784-gal4*, *w*^{1118}, Oregon R, balancer chromosomes, the 3rd chromosome Deficiency kit, and mutants that mapped to the chromosomal area 95A-C are as described in (The Flybase Consortium, 2003) and were obtained from the Bloomington Stock Center.

Mapping

The *nup98-96*^{2288} mutation was mapped using Deficiencies spanning the 3rd chromosome. Deficiencies Df(3R)mbcR1 (95A5-7 to 95D6-11) and Df(3R)mbc-30 (95A5-7 to 95D10-11) produced a germ line cell loss phenotype when in trans to *nup98-96*^{2288} whereas deletions

surrounding the area did not. Fly stocks carrying mutations in genes mapping to the 95A-C chromosomal region were tested for complementation. The *nup98-96*^{339} mutation failed to complement *nup98-96*^{2288} while all other mutants in the area complemented *nup98-96*^{2288}.

Molecular techniques

Generation of genomic DNA, sequencing, SDS-page, and Western blotting were performed following standard procedures (Sambrook, 1989). Protein extracts were made from whole 1st instar larvae. For western blots, chicken anti-Nup96-serum was used at 1:20,000, rabbit anti-Nup98-serum was used at 1:5,000, and mouse-anti-LaminC was used at 1:70. Horseradish peroxidase coupled secondary antibodies for Western blotting were obtained from Promega (anti-chicken, 1:5,000) and GE-Healthcare (anti-rabbit, 1:20,000, anti-mouse 1:10,000).

Generation of UAST-nup98-96-constructs

Testis cDNA clones for *nup98-96* were obtained from the *Drosophila* Genomics Resource Center. The clone AT20377 contained 380 base pairs of the *nup98-96* 5' prime sequence that contains a TATA box for polymerase binding and the coding region for *nup98* (nucleotides 1 to 2250). The clone AT01311 contained the coding region for *nup96* and 500 base pairs of the *nup98-96* 3' prime sequence, which contains consensus sequences for polyadenylation to assure transcript stability (nucleotides 2496 to 6522). A full-length clone of *nup98-96* was generated by standard molecular cloning techniques. A 1 kb fragment spanning the end of *nup98*, the auto-cleavage site, and the beginning of *nup96* was generated by PCR from genomic wild-type DNA and cloned directionally into the EcoR1 and EcoN1 restriction sites of AT01311, resulting in plasmid POT-CS-96. Subsequently, the *nup98-96* 5' prime sequence and

nup98-coding region were directionally cloned using EcoR1 and BspH1 into POT-CS-96 resulting in POT-*nup98-96*. The cDNA was cloned into a UAST-vector to generate UAST-*nup98-96*. To generate a *nup98*-cDNA, UAST-*nup98-96* was cut with Mlu1 and Nco1 to remove the *nup96* coding sequences, the Klenow enzyme filled the ends, and the vector was re-ligated. This resulted in a *nup98*-construct (UAST-*nup98*) that contains the *nup98-96* 5' prime sequence, the nup98 coding region, the auto-cleavage site, a STOP codon, and the *nup98-96* 3' prime sequence. The Best Gene, Inc, injected the constructs into flies. Fly stocks were established from the injected animals and several independent lines were used for our experiments. All lines yielded the same results.

UAS-Gal4 expression studies

All crosses for cell-type specific expression (using the germ line cell nos-*gal4:VP-16* and the somatic cell *C784-gal4* transgene drivers) were set up and the subsequent F_1 progeny raised at 29°C.

Immunofluorescence and histochemistry

Tissues were dissected in testis buffer (10 mM Tris-HCl, ph 6.8, 180 mM KCl). Immunofluorescence was performed following standard procedures (Ashburner, 1989). Tissues were observed using a Zeiss Axiophot microscope in brightfield and fluorescent microscopy. Images were taken with a CCD camera using an Apotome and Axiovision Rel Software. The following hybridoma/monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242: mouse anti-a-

spectrin 3A9 (1:10) developed by D. Branton and R. Dubreuil; mouse anti-LaminC (1:10) developed by P. A. Fisher; mouse anti-Sex lethal M18 (1:10) developed by P. Schedl; mouse anti-Groucho (1:5) developed by C. Delidakis; and mouse anti-FasciclinIII 7G10 (1:10) developed by C. Goodman. Goat anti-Vasa (1:1000) and mouse anti-phosphorylated Jun-Kinase (1:50) were obtained from Santa Cruz Biotechnology. Covance supplied the mouse anti-mAB414. Rabbit anti-phosphorylated Histone-H3 (1:500) and mouse anti-Bromodioxyuridine (1:200) were obtained from Millipore. Mouse-anti-Bam antibody (1:2,000) was kindly provided by Dennis McKearin. Fluorescence-coupled secondary antibodies (Molecular Probes) were used at 1:1,000. Tissues were embedded in Vectashield (Vector Laboratories) either with or without DAPI, or Slow Fade Gold (Molecular Probes).

In situ hybridization

In situ hybridization was performed as previously described (Ohlstein and McKearin, 1997). A full-length *piwi*-DNA in a pBST-vector for generation of the RNA-probes using the SP6 and T7 polymerase start sites was kindly provided by Dan Cox.

Discussion

The *nup98-96*^{2288} mutation disrupts the normal progression of germ line cells through gametogenesis in both male and female flies. Zero or very few germ line cells were found in adult animals. A developmental analysis revealed that the loss of early stage germ line cells was due to the premature differentiation of GSCs and their daughters. *nup98-96*^{2288}*/Df(3R)mbc-R1* testes contained only late stage germ line cells that were similar to wild-type spermatocytes. Spermatocytes normally develop in clusters of 16 cells that are derived from a single gonialblast undergoing four rounds of transit amplification divisions with incomplete cytokinesis. In

contrast, the late stage germ line cells of *nup98-96*^{2288}*/Df(3R)mbc-R1* testes were either solitary, with a single large spectrosome, or part of small clusters of cells with wide, branched fusomes that connected only two to eight cells. This finding implies that GSCs and gonialblasts initiated the differentiation cascade either without or after a reduced number of transit amplification divisions.

The *nup98-96* gene products are structural components of the nuclear pores and it seems possible that the defects seen in the *nup98-96*^{2288}*/Df(3R)mbc-R1* gonads may have been caused by generic defects in the nuclear pores or nuclear envelope. However, the nuclear envelopes of *nup98-96*^{2288} germ line cells did not exhibit defects apparent by immuno-fluorescence experiments, and the localization of several nuclear markers as well as cytoplasmic markers was unaffected in *nup98-96*^{2288}*/Df(3R)mbc-R1* gonads. Furthermore, a reduction in the numbers of amplification divisions has not been reported in animals harboring mutations in other Nucleoporins. However, it has been shown that localization of a Lamin, Otefin (Ote), to the nuclear envelope of GSCs is required for stem cell maintenance in female flies. Ote physically interacts with Medea to silence Bam in the GSCs. Over-expression of *ote* in the germ line cells increased the number of GSCs, implying that it is instructive for stem cell identity (Jiang et al, 2008). In contrast, *bam* expression at the tip of *nup98-96*^{2288}*/Df(3R)mbc-R1* germaria did not extend into the GSC position, and overexpression of *nup98-96* had no effect on GSC number. These findings argue against the view that Nup98-96 acts in a common pathway with Ote. With all of the above observations taken together, it seems unlikely that the defects in maintaining early stage germ line cells in the *nup98-96*^{2288}*/Df(3R)mbc-R1* mutants is due to a generic defect in nuclear pore or nuclear envelope structure, or general nucleocytoplasmic transport. Instead, it

is likely that *nup98-96* function plays a specific role in the developmental timing between amplification and differentiation.

Recent studies have implicated the *nup98-96* gene products in a variety of specific functions in metazoans that appear to go beyond its role at the nuclear pore: *Arabidopsis thaliana* Nup96 was found to be required for basal immune-responses and constitutive resistance to non-host pathogens (Zhang and Li, 2005); mouse Nup96 regulates the nuclear export of Interferon regulated mRNAs in immune responses (Faria et al, 2006); and, finally, *Drosophila* Nup98 mediates gene transcription in response to the molting hormone Ecdysone (Capelson et al, 2010; Kalverda et al, 2010). By analogy, we propose that the *nup98-96*^{2288} allele eliminates a specific aspect of *nup98-96* function that is required for maintaining early stage germ line cells in an undifferentiated state. On a mechanistic level, this function could be mediated by transcriptional regulation or selective nucleocytoplasmic transport of factors required for timing the transition between amplification and terminal differentiation. While no such timing mechanisms have been identified in *Drosophila*, nuclear exclusion of the transcription factor Oct4, a master regulator of differentiation, is a prerequisite for maintaining mammalian tissue stem cells in an undifferentiated state (Pan, 2004).

In support of this view, the germ line cells in the *nup98*-96^{2288}/Df*(3R)mbc-R1* mutant gonads were not responsive to the external cues that tightly control and influence the early stage germ line cells. EGFR-dependent signaling from the somatic support cells to the germ line cells promotes differentiation whereas activation of the TGF-β and JaK/STAT pathways promotes proliferation. A genetic analysis revealed that these pathways were not able to modify the number of amplification divisions in *nup98-96*^{2288} gonads. *nup98-96*^{2288} suppressed the effect of loss-of-function mutations in the EGFR pathway, which normally lead to dramatic over-

proliferation of early germ line cells. Similarly, over-expression of TGF-β or JaK/STAT ligands in the germ line of *nup98-96*^{2288}*/Df(3R)mbc-R1* animals remained without effect on germ line cell amplification. Furthermore, the expression pattern of *bam*, the main target of TGF-β signalling in the ovary, appeared normal in *nup98*-96^{2288}/Df*(3R)mbc-R1* ovaries, suggesting that TGF-β signals act independently of *nup98-96*.

As targeted expression of a wild-type *nup98-96* cDNA in the germ line cells rescued the gonadal defects in *nup98-96*^{2288} mutants, the function of Nup98-96 in maintaining an undifferentiated state reflects a germ line-intrinsic mechanism. Instead of regulating differentiation factors, *nup98-96* could be required cell-intrinsically for germ cell proliferation and a failure of the germ line cells to proliferate could trigger a cell-intrinsic differentiation response. The *nup98-96* locus in mice and *Drosophila* has been implicated in regulating proliferation. T-cells from Nup96+/- mice hyper-proliferate (Kalverda et al, 2010; Chakraborty, 2008), and overexpression of Nup98 in *Drosophila* embryonic S2-cells results in increased expression levels of cell cycle genes. In contrast, the germ line cells in *nup98-96*^{2288}*/Df(3R)mbc-R1* testes displayed an opposite response: reduced proliferation upon the loss of *nup98-96*. Overexpression of *nup98-96* in germ line cells, by way of UAST-*nup98-96,* did not increase the number of germ line cells in gonads from otherwise wild-type animals. These results demonstrate that the *Drosophila nup98-96* locus regulates a distinct response from the *nup98-96* locus in mouse.

The germ cell phenotype of *nup98*-96^{2288}/Df*(3R)mbc-R1* animals adds to the current view of germ cell development and possibly to the development of other specialized cells from precursors. In testes, GSC daughters express gradually increasing levels of Bam, peaking at the eight-cell stage, and the expression of Bam regulates the number of mitotic amplification

divisions. Reduction in *bam* expression causes the proliferation of spermatogonia beyond the 16-cell stage (McKearin and Spradling, 1990) while over-expression of Bam causes premature differentiation at the 8-cell stage (Insco et al, 2009). This phenotype is significantly different from the *nup98*-96^{2288}/Df*(3R)mbc-R1* phenotype. IN *nup98*-96^{2288}/Df*(3R)mbc-R1*testes, GSCs, gonialblasts, as well as spermatogonia enter the spermatocyte stage prematurely. Our findings suggest that the switch between transit amplification divisions and terminal differentiation has to be controlled at multiple levels. *nup98-96* is required for GSCs and GSC daughter amplification whereas Bam plays an instructive role in exiting the mitotic program. Further understanding of *nup98-96* function in the germ line cells awaits the identification of regulatory factors that terminate transit amplification divisions and initiate the terminal differentiation program.

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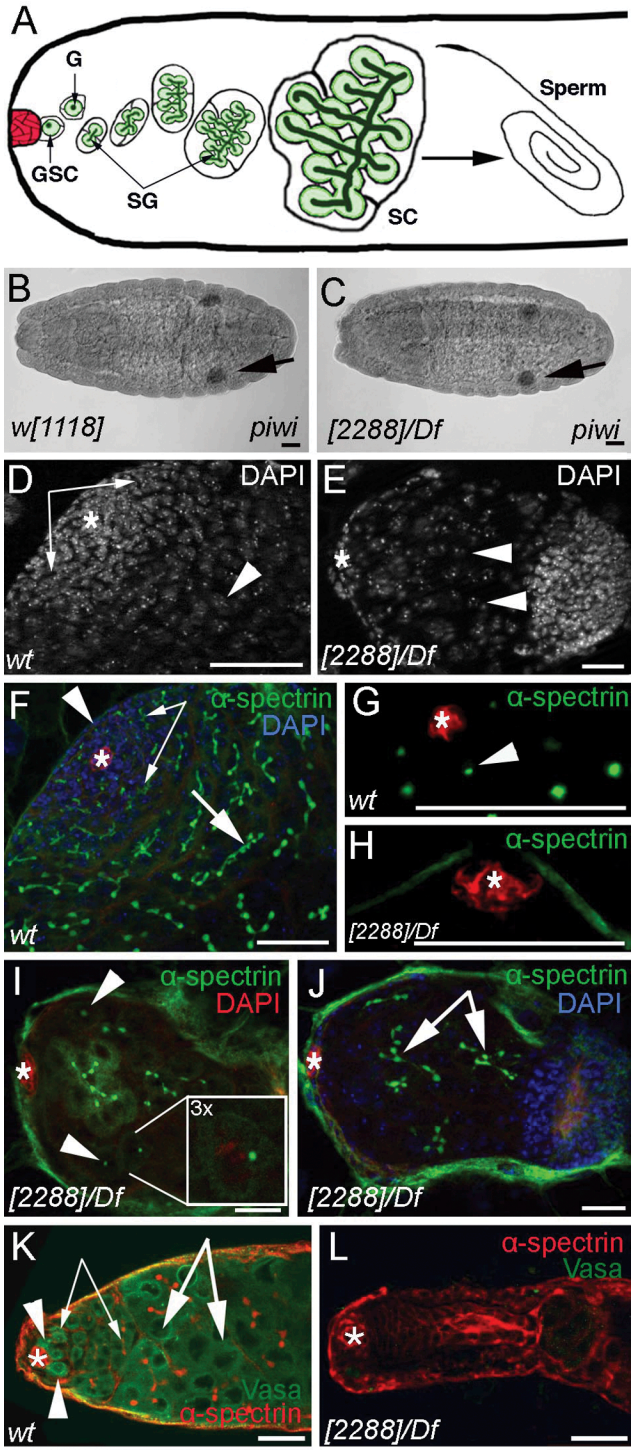
Figure 4. 1. Germ line cells differentiate prematurely in *nup98-96^{2288}/Df(3R)mbc-R1* mutant testes. **(A)** Drawing showing the stages of germ line cell differentiation in testes. GSC: germ line stem cell, G: gonialblast, SG: spermatogonia, SC: spermatocytes. Black circles: somatic support cells enclosing the germ line cells. **(B, C)** *In situ* hybridization with a *piwi*-RNA-probe to **(B)** *w^{1118}* and **(C)** *nup98-96^{2288}/Df(3R)mbc-R1* mutant embryos. Anterior to the left. **(D-J)** 3rd instar larval testes. **(D, E)** DNA in (**D**) the apical region of a wild-type (wt), and **(E)** a whole *nup98-96^{2288}/Df(3R)mbc-R1* testis. Arrows point to early stage germ line cell nuclei, arrowheads point to spermatocyte nuclei. **(F-L)** FasIII and asterisks label the hub. Arrowheads point to spectrosomes, small arrow points to the small, branched fusomes as normally seen in the spermatogonia, large arrows point to the wide, long, branched fusomes as normally seen in the spermatocytes. **(F)** Apical region of a wild-type testis. **(G, H)** High magnification of apical tips of testes from **(G)** wild-type and **(H)** *nup98-96^{2288}/Df(3R)mbc-R1*. **(I, J)** Whole *nup98-96^{2288}/Df(3R)mbc-R1* testes. Note the inset in **(I)** showing the spectrosome (green) and DNA (red) in a single, large germ line cell. **(K, L)** Apical regions of adult testes from **(K)** wild-type and **(L)** *nup98-96^{2288}/Df(3R)mbc-R1*. Scale bars: 30 μm


A
G
GSC
SG
SC
Sperm
B
w[1118]
piwi
C
[2288]/Df
piwi
D
DAPI
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DAPI
[2288]/Df
F
α-spectrin
DAPI
wt
G
α-spectrin
wt
H
α-spectrin
[2288]/Df
I
α-spectrin
DAPI
3x
[2288]/Df
J
α-spectrin
DAPI
[2288]/Df
K
Vasa
α-spectrin
wt
L
α-spectrin
Vasa
[2288]/Df

Figure 4. 2. Germ line cell loss in *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant germaria.

(A) Drawing showing the organization of GSCs and their daughters in a wild-type germarium. GSC: germ line stem cell, CB: cystoblast, CC: cystocytes, EC: egg chamber, FC: follicle cells, black marking in germ line cells: spectrosomes and branched fusomes. **(B, C)** Germaria. Asterisks mark the apical tips, arrowheads point to spectrosomes, arrows point to branched fusomes, scale bars: 50 μm. **(D)** Histogram depicting the number of Vasa-positive germ line cells in the germaria at different time points post-eclosion. Genotypes are as indicated.

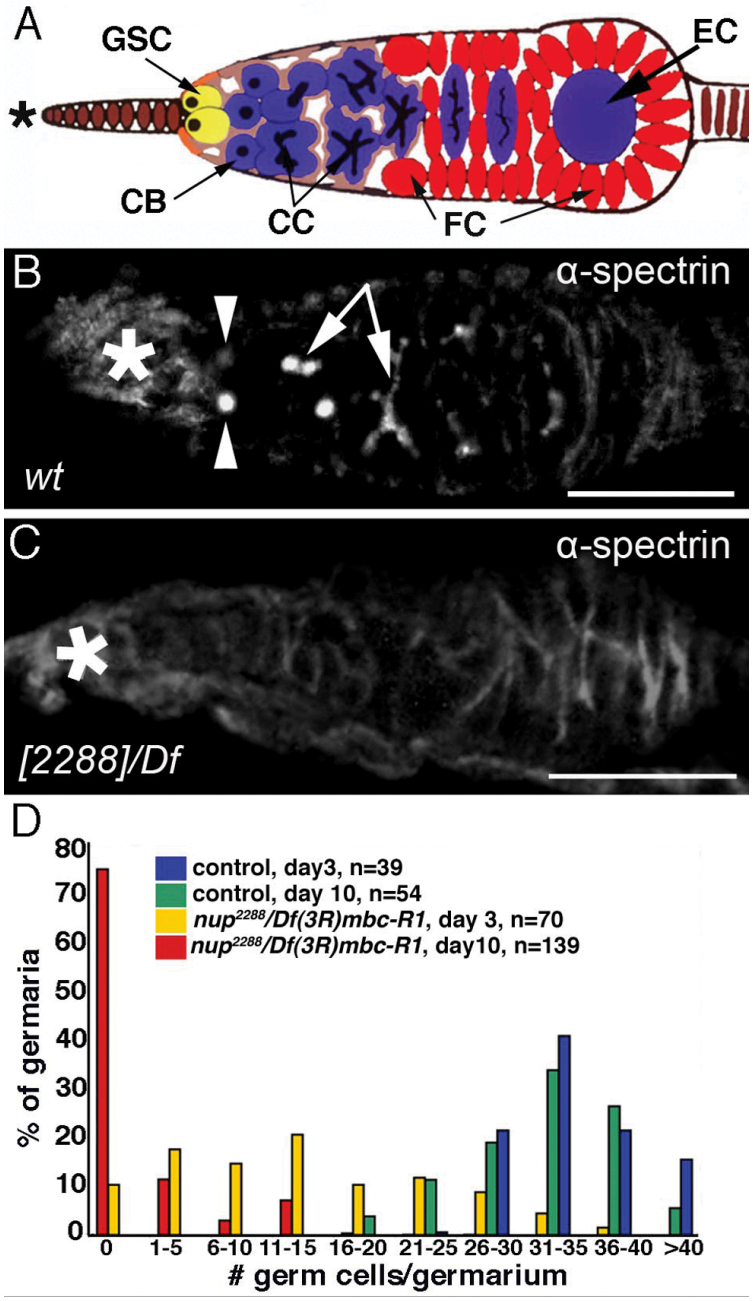

A
GSC
EC
CB
CC
FC
B
α-spectrin
wt
C
α-spectrin
[2288]/Df
D
control, day3, n=39
control, day 10, n=54
nup2288/Df(3R)mbc-R1, day 3, n=70
nup2288/Df(3R)mbc-R1, day10, n=139
% of germaria
0
10
20
30
40
50
60
70
80
0
1-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
>40
germ cells/germarium

Figure 4. 3. Mutations in *nup98-96* disrupt gametogenesis. **(A)** Top: Intron-exon structure of the *nup98-96* locus. Coding region in dark grey. Gene products as indicated. Bottom: Rescue constructs containing the whole transcription unit for *nup98-96* or *nup98* only. Arrowhead: Pogo-insertion in *nup98-96*^{2288}, arrow: RNAi-sequences.

(B-E) Bright field images of whole testes. Genotypes as indicated. Arrows point to sperm. Asterisks mark the apical tips. Scale bars: 100 μm. **(F)** Western blot analysis. Genotypes and antibodies as indicated. The Nup96 antibody does not detect a 95 kd protein in extracts from mutant animals. Instead, the Nup96 antibody detects a high molecular weight bands that may be abnormal Nup96 protein or Nup98-Nup96 polyprotein. The Nup98 antibody detects extremely low levels a 95 kd protein in the mutant compared to the control. **(G, H)** Apical tips of adult testes with germ line expression of **(G)** UAST-*nup98-96*-RNAi (arrow points to a single spermatocyte), and (H) UAST-*nup98-96*. Asterisks mark the apical tips. Scale bars: 30 μm.

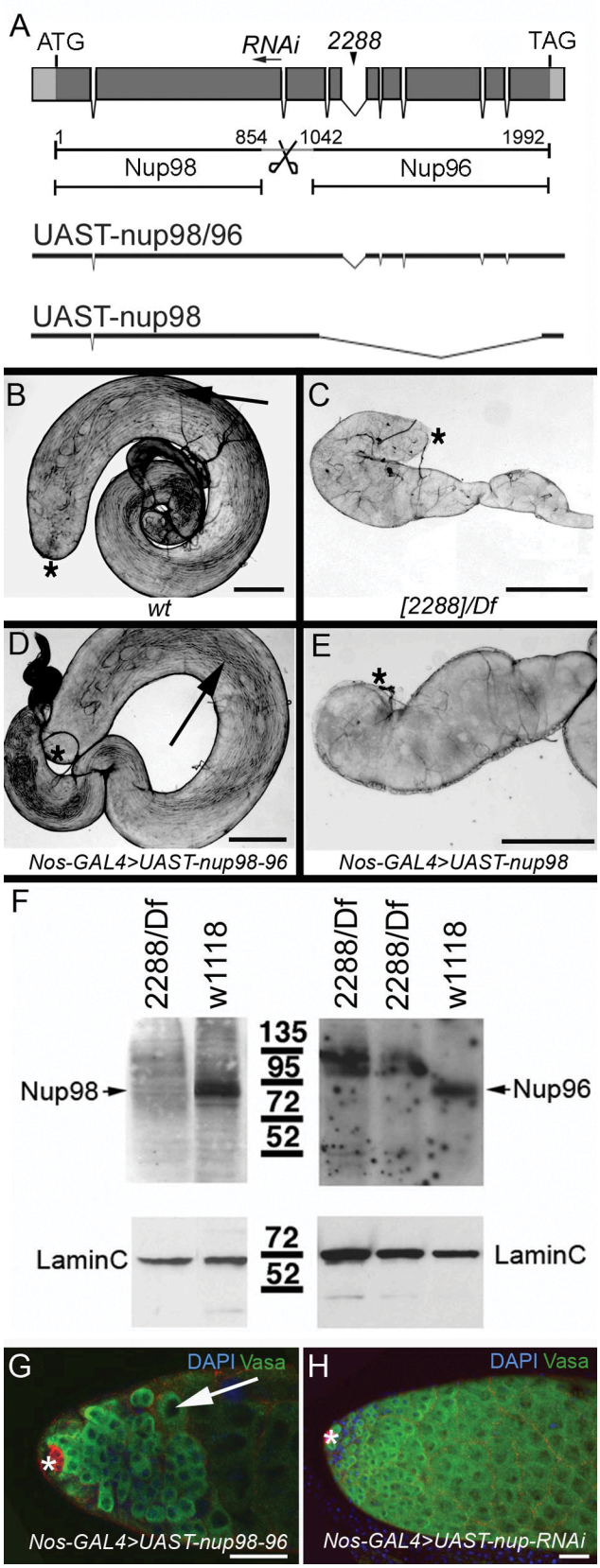

A
ATG
RNAi
2288
TAG
1
854
1042
1992
Nup98
Nup96
UAST-nup98/96
UAST-nup98
B
wt
C
[2288]/Df
D
Nos-GAL4>UAST-nup98-96
E
Nos-GAL4>UAST-nup98
F
2288/Df
w1118
2288/Df
2288/Df
w1118
135
95
72
52
Nup98
Nup96
LaminC
72
52
LaminC
G
DAPI Vasa
Nos-GAL4>UAST-nup98-96
H
DAPI Vasa
Nos-GAL4>UAST-nup-RNAi

Figure 4. 4. Germ cells from *nup98-96*^{2288}*/Df(3R)mbc-R1* animals have normal nuclear envelopes**. (A-E)** Small arrows point to early stage germ cell nuclei in the germaria and large arrows point to nurse cell nuclei in egg chambers. Immunofluorescence-labelling as indicated. **(A, B)** Apical region of **(A)** a wild-type and **(B)** a *nup98-96*^{2288}*/Df(3R)mbc-R1* ovariole. **(C)** The apical region of a wild-type ovariole.

(D) The germarium and **(E)** an egg chamber of a *nup98-96*^{2288}*/Df(3R)mbc-R1* ovariole.

(F-I) Immuno-labelling of single nurse cell nuclei, antibodies and genotypes as indicated. Asterisks: apical tips of germaria, scale bars: 20 μm.

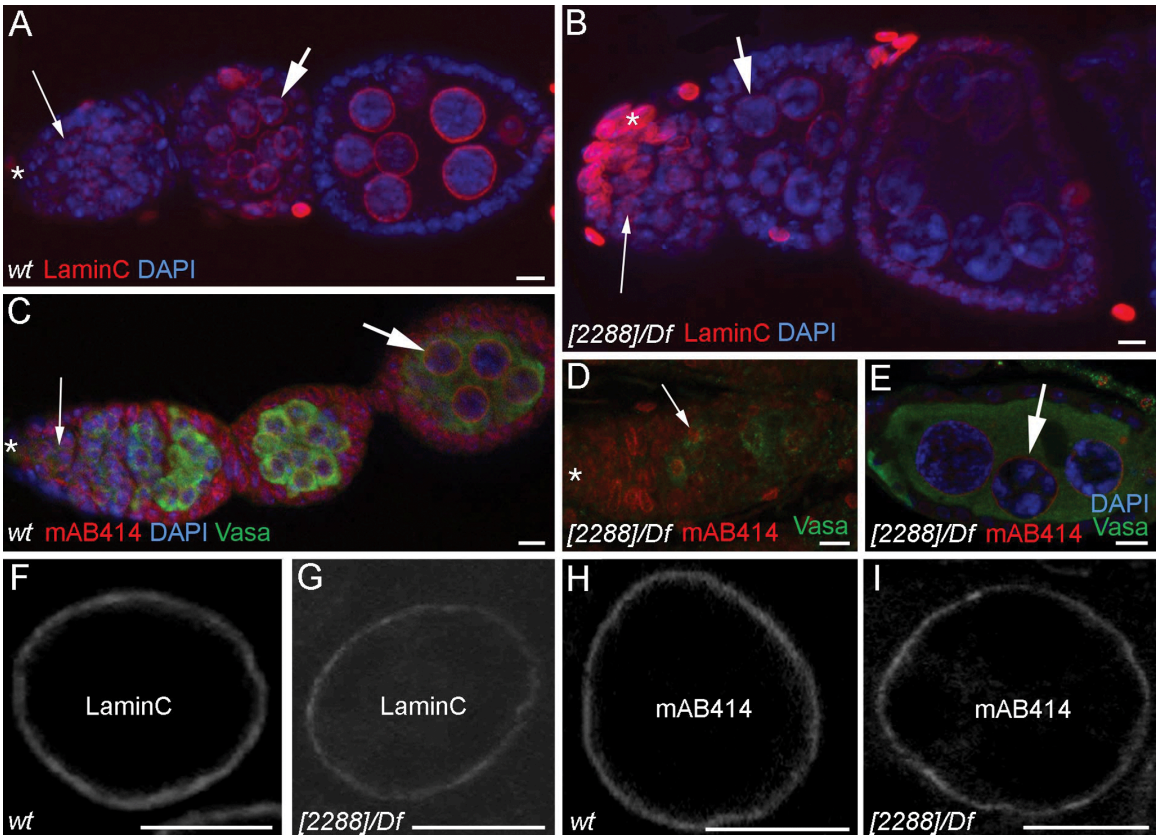

A
*
wt LaminC DAPI
B
*
[2288]/Df LaminC DAPI
C
*
wt mAB414 DAPI Vasa
D
*
[2288]/Df mAB414 Vasa
E
DAPI
[2288]/Df mAB414 Vasa
F
LaminC
wt
G
LaminC
[2288]/Df
H
mAB414
wt
I
mAB414
[2288]/Df

Figure 4. 5. Germ line cells from *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant animals have normal protein localization patterns**.** Immuno-labelling of germaria, antibodies and genotypes as indicated: **(A, B)** nuclear Groucho (red) and cytoplasmic Vasa (green); **(C, D)** nuclear phosphorylated Jun-Kinase (red) and cytoplasmic Vasa (green); note that some germaria (arrowheads) are empty in the *nup98-96*^{2288}*/Df(3R)mbc-R1* mutant ovaries; **(E, F)** cytoplasmic Sex-lethal in GSCs and gonialblasts; **(G, H)** nuclear phosphorylated Histone-H3 (red) and cytoplasmic Vasa (green); **(I, J)** Anti-BRDU (green) and DAPI (red). Asterisks: apical tips, arrows point to intra-cellular protein localizations, scale bars: 50 μm.

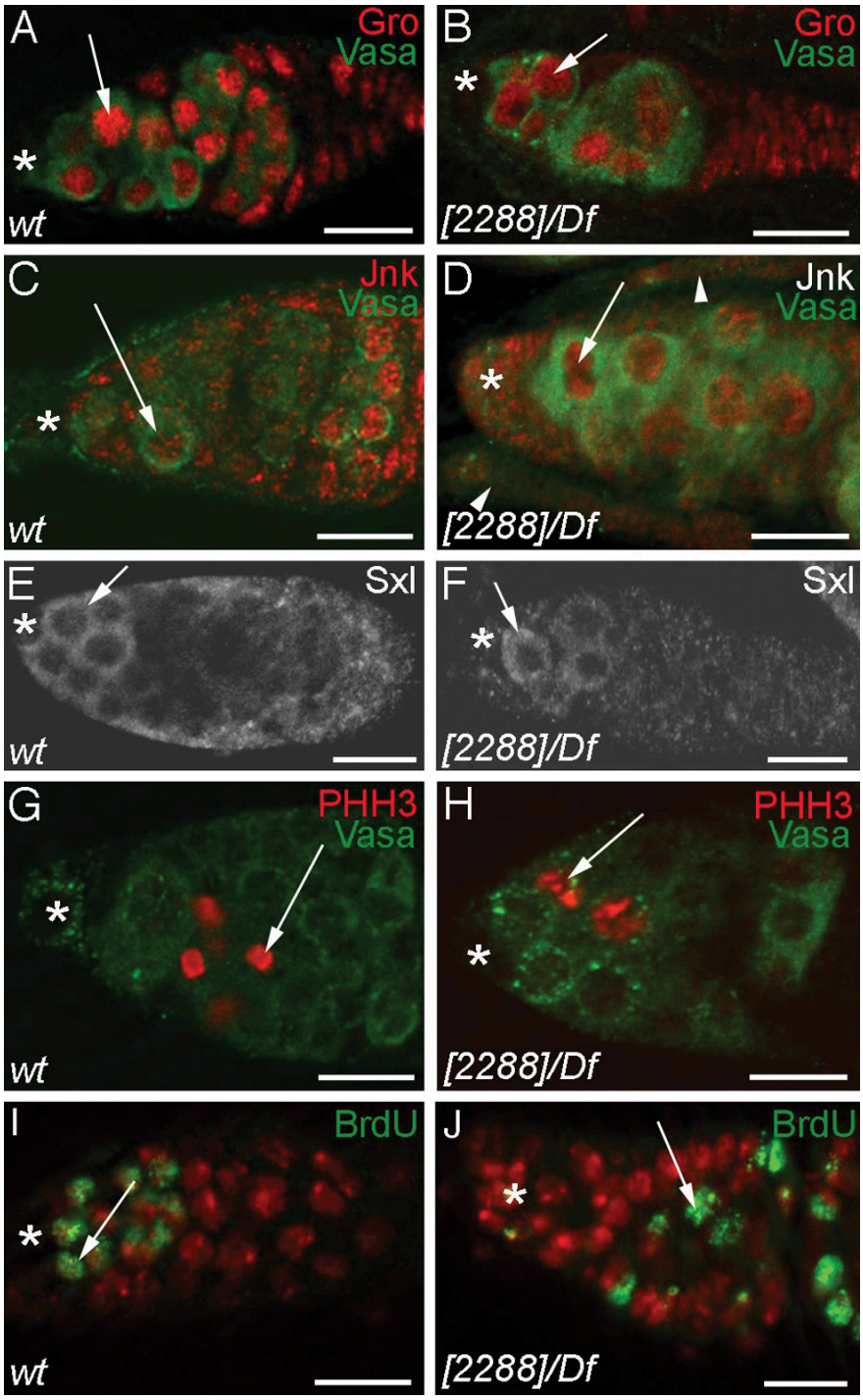
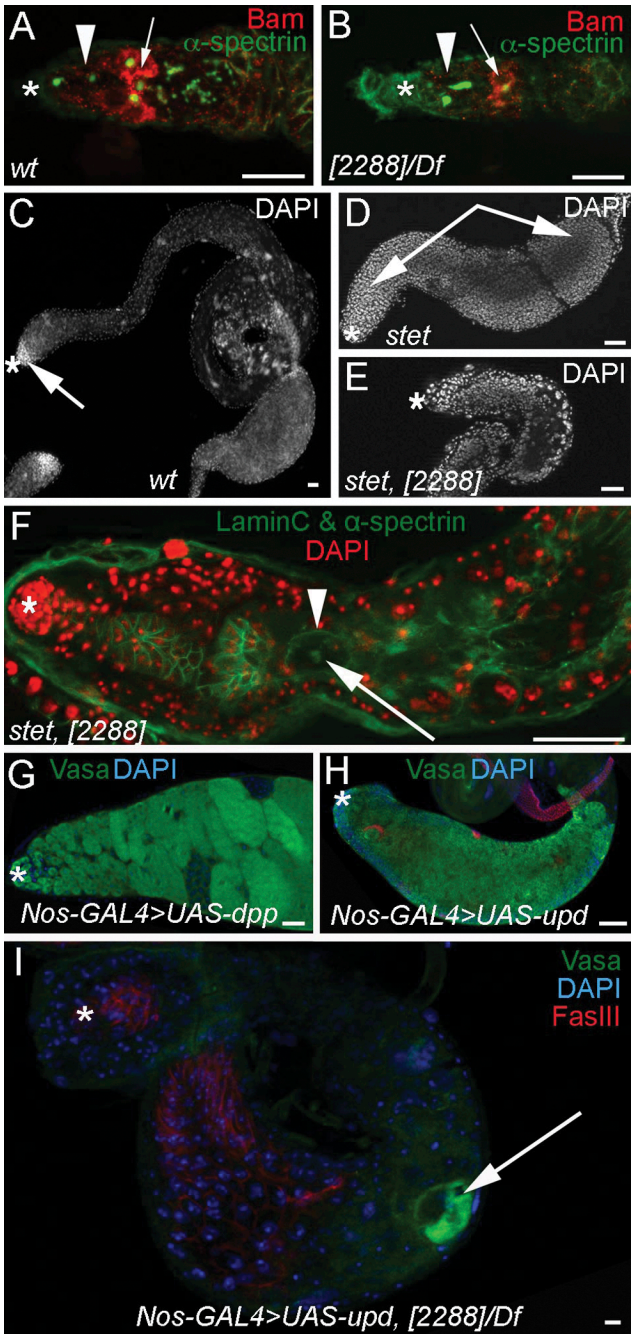

A
Gro
Vasa
wt
B
Gro
Vasa
[2288]/Df
C
Jnk
Vasa
wt
D
Jnk
Vasa
[2288]/Df
E
Sxl
wt
F
Sxl
[2288]/Df
G
PHH3
Vasa
wt
H
PHH3
Vasa
[2288]/Df
I
BrdU
wt
J
BrdU
[2288]/Df

Figure 4. 6. Nup98-96 acts upstream of signalling pathways regulating early stage germ line cells. **(A, B)** Immuno-labelling of germaria from **(A)** wild-type and **(B)** *nup98-96^{2288}/Df(3R)mbc-R1* mutant females. Arrowheads point to GSCs, arrows point to Bam-positive cells, scale bars: 50 μm. **(C-I)** Adult testes, genotypes as indicated. **(C-E)** DNA-labelling (DAPI), arrows point to early stage germ line cells. **(F)** Whole *stet^{1}; nup98-96^{2288}* testis showing a single spermatocyte (arrowhead) with a spectrosome (arrow). **(G, H)** Hyper-activation of signalling pathways results in accumulation of early stage germ line cells. **(G)** Apical region of a testis with germ line expression of *dpp*, and **(H)** whole testis with germ line expression of *upd*. **(I)** Whole *nup98-96^{2288}/Df(3R)mbc-R1* testis with germ line expression of *upd*. Arrow points to single spermatocyte.

Asterisks mark the testes tips, scale bars: 30 μm.


A
Bam
α-spectrin
wt
B
Bam
α-spectrin
[2288]/Df
C
DAPI
wt
D
DAPI
stet
E
DAPI
stet, [2288]
F
LaminC & α-spectrin
DAPI
stet, [2288]
G
Vasa DAPI
Nos-GAL4>UAS-dpp
H
Vasa DAPI
Nos-GAL4>UAS-upd
I
Vasa
DAPI
FasIII
Nos-GAL4>UAS-upd, [2288]/Df

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CHAPTER 5

CONCLUSION

Understanding stem cell behavior in living animals is necessary to realizing their full therapeutic potential and elucidating their role in the etiology of cancer biology. In addition, stem cell function lies at the center of basic biological questions. For example, how does an undifferentiated cell develop into a specialized cell with very specific functions? How is this process controlled to ensure that the correct number of cells is made? While many advances in the field have occurred in the 50 years since hematopoietic stem cells (HSCs) were identified based on their ability to repopulate an irradiated host, we still lack complete answers to many of these questions (Till and McCulloch, 1961). Due to the relative complexity and heterogeneity of mammalian systems, investigators have turned to model organisms with clearly defined stem cell populations and distinct genetic advantages.

Studies on the stem cells residing in the testes of *Drosophila melanogaster* have been particularly useful in linking the cellular anatomy of the stem cell niche to its specific functional roles in influencing processes such as self-renewal and dedifferentiation (See Li and Xie, 2005 for a review). For example, the anatomical center of the niche, the hub, was linked to its function by the finding that JaK-STAT signals secreted from the hub was required for the self-renewal of the surrounding CySCs and GSCs (Kiger et al, 2001; Tulina and Matunis, 2001). It was generally thought that JaK-STAT signals emanating from the hub acted directly on GSCs. However, recent studies have shown that JaK-STAT signals secreted from the hub directly act on

CySCs, and CySCs relay a cue instructing GSC identity (Leatherman and DiNardo, 2008; Leatherman and DiNardo, 2010). These findings demonstrating the relationship between the architecture of the niche and the signaling pathways that act in them highlight the usefulness of the *Drosophila* model in establishing a foundation upon which directed studies can be performed in more complex systems.

The majority of our current understanding of stem cell behavior is a result of studies directed towards uncovering the mechanisms of the asymmetric inheritance of cell fate in stem cell daughters and understanding the pathways that regulate the differentiation of stem cell progeny. However, studies on how the mitotic activity of individual stem cells is regulated are less common. Many of the putative stem cell populations in mammals have been indentified based on their long-term retention of labeled nucleotide analogs (Fuchs, 2009). These cells, termed label-retaining cells (LRCs), are thought to undergo infrequent divisions and in some cases exist in a state of quiescence (Cheshier et al, 1999; Majo et al, 2008; Cotsarelis, 1990). Yet, little is understood how this unique cell cycle is regulated.

In a *Drosophila* model, we show a pathway linking the mitotic activity of stem cells to the demand for the terminally differentiated cell that they replenish. The mitotic activity of GSCs within males exposed to females was higher than those GSCs within males exposed to other males. Although physical contact is required for this response, the cue that triggers the mitotic activity of GSCs downstream of physical contact awaits further investigation. It is possible that the GSC response is dependent on non-volatile pheromones expressed on the female cuticle. It is also possible that the response is dependent upon copulation. In either case, these findings represent an opportunity to further uncover the specific tissues and genetic pathways that influence stem cell divisions.

We also reported on a new role for the stem cell microenvironment in regulating the frequency of stem cell divisions. In mammals, the cellular microenvironment surrounding adult stem cells has recently garnered increased attention because of the pathological role it may play in the formation and maintenance of tumors. Illustrating this point, Calabrese et al, 2007 reported that brain tumor stem cells were found to preferentially localize to the perivascular niche of normal neural stem cells. We found that EGF signaling between GSCs and the surrounding CySCs is required to repress GSC divisions. A role for EGF signaling in promoting the development of GSC progeny has previously been reported (Kiger et al, 2000; Sarkar et al, 2007). In the current work, we show that these two functions of EGF are genetically and developmentally uncoupled. Moving forward, it will be interesting to find out if the principle findings reported in this work have parallels in other species and in other tissues. Do pathways that influence the differentiation of stem cell progeny in other species also affect the frequency of stem cell divisions? Does the stem cell microenvironment influence the frequency of stem cell divisions in mammals?

Another question raised in this work is what the mechanism is by which *svp* and *hth* attenuation suppress the *spi* phenotype. In Appendix A of this thesis, we report that *svp* expression is not detected in the germ cells, cyst cells, or the hub. However, *svp* expression was detected in the adipose tissue, corporum allatum, and the pigment cells lining the testis exterior of the testis. These observations suggest that tissues outside of the testis may influence the development and differentiation of germ cells. Further investigating the role of *svp* in these tissues may reveal insights into how these tissues affect spermatogenesis.

The maintenance of certain tissues relies on a balance between proliferation and cell turnover. The work in this thesis addresses the role of stem cells and the transit-amplification of

their daughters in mediating this balance. We establish a link connecting the mitotic activity of GSCs to the demand for sperm. Using this model, further studies aimed at identifying the environmental triggers and signaling pathways that transmit the demand for sperm to GSCs are needed. We also uncover a novel role for the cellular microenvironment that surrounds GSCs plays in regulating the frequency of their divisions. In a separate set of experiments, we describe a role for a locus that encodes two conserved nucleoporin genes in the transit-amplification divisions of stem cell progeny. Thus, the contributions of this thesis result from utilizing the genetic tractability of *Drosophila* coupled with the anatomical simplicity of the testis to gain insights into the fundamental aspects of stem cell behavior.

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APPENDIX A

INVESTIGATIONS INTO THE FUCNTIONS OF *SEVEN-UP* AND *HOMOTHORAX*, TWO GENETIC SUPPRESSORS OF *SPITZ*

Abstract

During *Drosophia* spermatogenesis, germ cell proliferation must be balanced with germ cell differentiation. In testes with attenuated EGF signaling, early stage germ cells accumulate at the expense of more mature germ cells. Our previous work reported that the attenuation of two genes, *svp* and *hth*, in EGF mutants suppressed this germ cell over-proliferation phenotype. Here we investigate the possible mechanisms responsible for the observed genetic interactions. In doing so, we further characterize the phenotypes arising from mutations in the EGF ligand, *spitz (spi),* and the germ cell division dynamics in wild-type testes. We found a two-fold increase in the mitotic index of spermatogonia in *spi* mutants. In addition, we found that testes from $spi^{77\text{-}20}$ mutants have decreased numbers of cyst cells, and that this decrease results from a deficiency specifically in mature cyst cells. In both cases, *svp* and *hth* attenuation in *spi* mutants partially rescued these phenotypes. Through mosaic analysis, we show that germ cell morphology and development appear to be independent of cell autonomous *svp* and *hth* function. In addition, *svp* function does not appear to be required cell autonomously for cyst cell function. Furthermore, we find that whereas *svp* expression is not detected in germ cells or cyst cells, *svp* expression was detected in pigment cells surrounding the testis and in tissues associated with hormonal signaling. In all the tissues, *svp* expression appeared to be independent of mutations in *spi*. Our studies suggest that *svp* functions outside of the testis, possibly in a hormonal pathway, that influences spermatogenesis in *spi* mutants.

Introduction

Drosophila melanogaster spermatogensis provides a simple stem cell-based model to investigate complex genetic interactions. The testis of *Drosophila* is a long coiled tube that contains germline stem cells (GSCs) residing at the apical tip and mature sperm residing at the base. GSCs are organized in a rosette around a cluster of stromal cells termed the hub (Hardy, 1979). When a GSC divides, one daughter will maintain stem cell identity through its continued association with the hub, and the other daughter will be physically displaced away from the hub. This displaced daughter, called a gonialblast, will begin a stereotypical differentiation program (Fuller, 1993). The gonialblast will undergo exactly four rounds of mitosis with incomplete cytokinesis resulting in 16 interconnected spermatogonia. These spermatogonia then undergo an extensive growth phase, whereby the cytoplasmic volume undergoes a 25-fold increase before transitioning into spermatocytes and beginning meiosis. After meiosis, germ cells undergo complex morphological changes, including elongation. As this process takes place, germline cells gallivant basally creating a spatio-temporal axis that facilitates experimental observations.

Germ cells are intimately associated with somatic cyst cells during spermatogenesis. GSCs are surrounded by two cyst stem cells (CySCs) that are also in contact with the hub. CySCs give rise to post-mitotic cyst cells, two of which will encase each gonialblast produced from a GSC division. These two cyst cells will remain associated with the gonialblast and its progeny until the late stages of spermatogenesis. Although cyst cells are post-mitotic, they express a series of transcription factors corresponding to the developmental stage of the germ cells they are associated with. Whereas both CySCs and those cyst cells encapsulating spermatogonia express Traffic-jam (Tj), only CySCs express the transcription factor, Zfh-1 (Li et al, 2003; Leatherman and DiNardo, 2008). Furthermore, as spermatogonia transition into

spermatocytes, their associated cyst cells down-regulate Tj expression and begin to express Eyes Absent (Eya) (Fabrizio et al, 2003).

The encasement of germline cells by cyst cells forms a cellular microenvironment that is critical for germ cell development (Matunis et al, 1997; Kiger et al, 2000). Epidermal Growth Factor (EGF) signaling from germ cells to cyst cells is required for cyst cells to properly encapsulate germ cells (Schulz et al, 2002). Cyst cells fail to send out cytoplasmic extensions around germ cells in animals harboring a hypomorphic mutation in the EGF ligand, *spitz*$^{77\text{-}20}$ *(spi*$^{77\text{-}20}$*)* (Sarkar et al, 2007). In these testes, germ cells resembling GSCs and early-stage spermatogonia accumulate and fill the testes at the expense of more mature germ cells. In addition to this accumulation of early-stage germ cells in the testes, we discovered that GSCs in *spi*$^{77\text{-}20}$ mutants divide at frequencies approximately two-fold higher than GSCs in normal animals (Chapter 3). EGF signaling in the microenvironment also mediates this repression of GSC division frequency.

Although both germ cell differentiation and GSC division frequency are mediated by *spi* signaling in the cellular microenvironment, these two functions of EGF signaling appear to be developmentally and genetically distinct (Chapter 3). The attenuation of two genes, *seven-up (svp)* and *homothorax* (*hth),* in the *spi*$^{77\text{-}20}$ mutant background resulted in a higher proportion of testes that contained differentiated germ cells. However, the attenuation of either *svp* or *hth* in *spi*$^{77\text{-}20}$ mutants had no effect on the frequency of GSC divisions. While roles for both of these genes have been described in the context of developing animals, their function in adult animals in unknown.

seven-up and *homothorax* are both highly conserved genes with clear homologues present in higher vertebrates (Mlodzik et al, 1990; Rieckhof et al, 1997). *seven-up* is a orphan hormone

receptor, with no known ligand, that contains both a DNA-binding domain and a ligand domain. Svp is remarkably conserved to its human homolog, COUP-TF, as 75% of their amino acid sequences are identical. A role for *svp* was first reported in regulating cell fates in the developing eye disc (Mlodzik, 1990). Subsequently, *svp* has been implicated in processes such as neurogenesis, heart development, and regulating cell proliferation in the larval malpighian tubules (Doe, 1992; Kerber et al, 1998; Molina and Cripps, 2001; Lo and Frasch, 2001). Homothorax is a homeo-domain containing protein whose mammalian homolog, Meis I, has been described as a proto-oncogene (Rieckhof et al, 1997). Homothorax binds Extradenticle, a HOX cofactor, and this interaction is required for Extradenticle to translocate into the nucleus. *hth* has been implicated in specifying antennae versus leg development in developing larvae. Animals lacking either *hth* or *exd* function develop ectopic legs at the expense of antennae (Casares and Mann, 1998). In addition, roles have been reported for *hth* in the proximal-distal patterning of limbs and eye development (Pichaud and Casares, 2000; Dong et al, 2001). These developmental roles for *svp* and *hth* in the embryos and larvae reflect the previous strengths and limitations of the *Drosophila* model. However, as our knowledge base has grown and our techniques have improved, it is important to ask what roles these "development" genes play in adult animals.

In this appendix, we attempt to better understand the underlying mechanisms by which *svp* and *hth* attenuation suppress the testis phenotype of $spi^{77\text{-}20}$ mutants. We found that spermatogonia and GSCs have different mitotic indices in wild-type animals, indicating that there are fundamental differences in the cell cycle of either cellular population. Our previous work reports that the mitotic index of GSCs is increased in $spi^{77\text{-}20}$ mutants. Here, we find that the mitotic index of spermatogonia is also increased. Interestingly, whereas the attenuation of either

svp or *hth* had no affect on the increased mitotic index of GSCs in $spi^{77\text{-}20}$ mutants, the increased mitotic index of spermatogonia was dramatically reduced. In addition, we observed that testes from $spi^{77\text{-}20}$ mutants contain fewer cyst cells. Upon a more detailed analysis, this decrease in cyst cell number was primarily found to be due to a reduction in the number of mature cyst cells expressing Eya. Similar to the effect on germ cells, *svp* and *hth* attenuation partially rescued this deficiency of cyst cells in $spi^{77\text{-}20}$ mutants. We also investigated what, if any, roles *svp* and *hth* function had in germ cells and cyst cells. Neither *svp* nor *hth* appear to be required cell autonomously for germ cell or cyst cell development, as mosaic animals with germ cell and cyst cells lacking *svp* or *hth* function were morphologically indistinguishable from those with *svp* function. However, although germ cell morphology was independent of *svp* function, our data suggest that GSCs lacking *svp* divide at decreased rates when compared to controls. These observations led us to investigate the expression patterns of *svp*. We found that *svp* is expressed in the pigment cells that surround the exterior of the testes, but not in germ cells or cyst cells. In addition, *svp* was expressed in the fatbody that surrounds the larval testis and also the corpora allatum, the primary source of juvenile hormone synthesis. In all tissues, *svp* expression did not appear to be altered in *spi* mutants. While these data are somewhat inconclusive, they point to a role for *svp* acting in a hormonal pathway that influences spermatogenesis.

Results

svp and hth attenuation reduce the mitotic index of spermatogonia in spi mutants

In an effort to better understand the mechanisms by which *svp* and *hth* genetically interact with EGF signaling to suppress the defects in spermatogenesis associated with $spi^{77\text{-}20}$ mutants (described in Chapter 3), we sought to better characterize the dynamics of germ cell divisions

during spermatogenesis. We noticed that in wild-type animals, the mitotic index of GSCs (7.6%, n= 657) was higher than the mitotic index of spermatogonia (4.85%, n= 3568, $p < 0.005$), suggesting that there may be fundamental differences in the division dynamics between these two germ cell populations (Figure 1A). We also observed that the mitotic index of spermatogonia in $spi^{77\text{-}20}$ homozygous mutants (11.3%, n=1788) was higher than that of spermatogonia in $spi^{77\text{-}20}$ heterozygous control animals (6.6%, n=1071, $p < 0.0001$) (Figure 1B). We reasoned that although the attenuation of either *svp* or *hth* had no effect on the increased mitotic index of GSCs in $spi^{77\text{-}20}$ mutants (Chapter 2), their attenuation could reduce the mitotic index of spermatogonia in $spi^{77\text{-}20}$ homozygotes. To test this possibility, we calculated the mitotic indices of spermatogonia either in $spi^{77\text{-}20}$ heterozygous animals, $spi^{77\text{-}20}$ homozygous animals, and $spi^{77\text{-}20}$ homozygous animals that also contained heterozygous loss-of-function alleles of either *hth* (hth^{05745}) or *svp* (svp^{e22}) (references). We found that in introducing one copy of either the svp^{e22} or the hth^{05745} allele into $spi^{77\text{-}20}$ mutants reduced the mitotic index of spermatogonia to those observed in $spi^{77\text{-}20}$ heterozygous animals (Figure 1B, 1C). On the surface, these data indicate that *svp* and *hth* may interact with EGF signaling to regulate the frequency of spermatogonial divisions.

hth attenuation rescues the decrease in cyst cell number associated with spi mutants

We next analyzed the somatic cyst cell populations in testes from $spi^{77\text{-}20}$ mutants. We stained testes with antibodies raised against cyst cell markers. Traffic Jam (Tj) is expressed in the nuclei of CySCs and cyst cells associated with spermatogonia, whereas Eyes Absent (Eya) is expressed in the nuclei of cyst cells associated with spermatocytes (Figure 2A, 2B). By counting the total number of cells expressing somatic cell markers, we found that testes from $spi^{77\text{-}20}$

mutants contained fewer cyst cells (80, n=10) than w^{1118} controls (99, n=10, p= 0.009) (Figure 2E). We next asked if the decreased number of cyst cells observed in $spi^{77\text{-}20}$ mutants was rescued in testes suppressed by *svp* and *hth* attenuation. Although a slight increase was observed, the introduction of the svp^{e22} allele into the $spi^{77\text{-}20}$ mutant background had little effect on the total number of cells expressing cyst cell markers (89, n= 18) (Figure 2E). However, when the hth^{05745} allele was introduced into the $spi^{77\text{-}20}$ background, the average number of cells expressing cyst cell markers exceeded those observed in w^{1118} control animals (106, n=19, p= 0.0006) (Figure 2E). These data suggest that attenuated EGF signaling leads to a reduction in the total number of cyst cells within each testis. In addition, whereas *svp* attenuation does not appear to significantly increase the deficiency of cyst cells in testes from spi^{7720} mutant, *hth* attenuation rescues this aspect of the $spi^{77\text{-}20}$ mutant phenotype.

svp and hth attenuation increases the number of late stage cyst cells

Upon further investigation, it appeared that decrease in cyst cell number in testes from $spi^{77\text{-}20}$ mutants was not due to decreased numbers of Tj^+ cells, but instead due to fewer numbers of Eya^+ cells (compare Figure 2A to 2B). This observation was confirmed by comparing the average number of Eya^+ cyst cells in w^{1118} testes (59, n=10) to the average number of Eya^+ cells in spi^{7720} mutants (7, n=10, $p < 0.0001$) (Figure 2F). This reduction in the number of Eya^+ cells in testes from $spi^{77\text{-}20}$ mutants appeared to be partially rescued in testes from $spi^{77\text{-}20}$ mutants in which either *svp* or *hth* was attenuated (Figure 2B-2D). This observation was confirmed as the number of Eya^+ cyst cells was increased in $spi^{77\text{-}20}$ mutants that harbored a single copy of either the svp^{e22} allele (32, n=18, p=0.004) or the hth^{05745} allele (44, n=19, $p < 0.0001$) (Figure 2F). These data indicate that the decreased numbers of cyst cells in testes from $spi^{77\text{-}20}$ mutants is due

to the reduction in cyst cells expressing Eya. Furthermore, these data suggest that the attenuation of *svp* or *hth* may suppress the testis phenotype associated with $spi^{77\text{-}20}$ mutants by acting during the spermatogonia to spermatocyte transition.

Ectopic svp expression promotes early stage cell fates of cyst cells and germ cells.

The attenuation of *svp* in $spi^{77\text{-}20}$ mutants suppressed the accumulation of early stage germ cells and increased the number of cyst cells expressing late stage genes, suggesting that *svp* may function in normal animals to oppose the differentiation-promoting activities of EGF signaling (Chapter 3). We reasoned that if this were the case, the over-expression of *svp* in normal testes may result in the accumulation of early stage germ cells. To over-express *svp*, we employed the bipartite Gal4/UAS expression system (Brand and Perrimon, 1993; Phelps and Brand, 1998). Flies harboring the coding sequence of the Gal4 transactivator under the control of the *heat-shock* promoter (*hs*-Gal4) were crossed to flies harboring the coding sequence of *svp* directly downstream of UAS Gal4 binding sequences (UAS-*svp*). Flies were then heat-shocked on three consecutive days to induce ubiquitous Gal4 expression, and then labeled with cyst cell and germ cell markers. Flies harboring both the *hs*-Gal4 and the UAS-*svp* in combination contained supernumerary Tj^+ cyst cells at the tip of the testes when compared with testes from flies harboring the UAS-*svp* alone (Figure 3B, 3D). Furthermore, when compared to testes from control animals harboring the UAS-*svp* alone, Tj^+ cyst cells were observed further away from the apical tip in testes from flies ectopically expressing *svp* (Figure 3A, 3C). In addition, testes from animals over-expressing *svp* labeled with the germ cell marker, Vasa, appeared skinny and contained fewer and more immature germ cells when compared to controls (Figure 3E, 3F). Distinct from hyper-proliferative phenotypes, the germ cells in testes from animals over-

expressing *svp* appeared to be blocked from differentiating. However, these phenotypes were not completely penetrant as both the increased number of Tj^+ cyst cells and the apparent block in germ cell differentiation were observed in only one-third of testes (n=33). These data suggest that *svp*, when expressed ectopically, is sufficient to either increase the proliferation or block the differentiation of both early stage germ cells and cyst cells.

We next investigated if *svp* expression in germ cells or cyst cells could recapitulate the phenotypes resulting from ubiquitous *svp* expression. A Gal4 under the control of the *nanos* promoter (*nos*-Gal4) was used to drive germline-specific expression of *svp*. However, the phenotypes of testes from those animals harboring both the UAS-*svp* and the *nos*-Gal4 were indistinguishable from those harboring the UAS-*svp* alone (Figure 3G, 3H). In contrast, when svp expression was driven in cyst cells using a *tj*-Gal4, testes contained many more Tj^+ cyst cells when compared to testes from animals with the UAS-*svp* alone (Figure 3I, 3J). Thus, whereas our data support a hypothesis in which *svp* expression in cyst cells is sufficient to induce cyst cell proliferation. However, the germ cell phenotype resulting from ubiquitous *svp* expression was not recapitulated when *svp* expression was driven by the *tj*-Gal4, indicating that *svp* expression in cells other than germ and cyst cells may be sufficient to affect germ cell differentiation.

Germ cell development is independent of cell autonomous svp or hth expression

We next investigated if *svp* expression was required in germ cells for their proper development. To avoid the embryonic lethality associated with strong alleles of *svp*, we utilized flip recombinase (Flp)-mediated recombination to create germ cell clones that were homozygous for the svp^{e22} allele. To generate germ cell clones, males harboring a *flp* transgene under the control of the *heat-shock* promoter (*hs-flp*), and transheterozygous for either a ubiquitously

expressed GFP transgene or the svp^{e22} allele directly downstream of flip recombinase target (FRT) sites were heat-shocked to induce recombination in dividing cells (Golic and Lindquist, 1989). Flp-mediated recombination in mitotic cells resulted in one daughter homozygous for the GFP transgene, and one daughter homozygous for svp^{e22}. Therefore, germ cells homozygous for the svp^{e22} allele could be indentified by their lack of GFP expression. Males were aged for 7 days after heat-shocking to allow for germ cells not produced from GSC clones to clear the testis. Testes were then dissected and labeled with antibodies raised against GFP, the germ cell marker, Vasa, and a marker for the membranes of cyst cells, Armadillo (ARM). GFP^{neg} and $Vasa^{+}$ germ cell clones were identified (Figure 4A-4C). However, excluding the absence of GFP expression, svp^{e22} homozygous germ cell clones were morphologically indistinguishable from other GFP^{+} germ cells. Using this same technique, we also made germ cell clones that were homozygous for a strong allele of *hth,* hth^{P2}. In embryos harboring the hth^{P2} allele, *hth* expression was abolished (refs). Similar to those germ cells lacking *svp* function, germ cells homozygous for the hth^{P2} allele were morphologically indistinguishable from controls (data not shown), suggesting that germ cell development is independent of cell autonomous *svp* and *hth* function. Furthermore, svp^{e22} or hth^{P2} homozygous germ cell cysts appeared to be properly encased by cyst cells, suggesting that neither *svp* or *hth* is required in germ cells for their association with cyst cells.

The rate of GSC divisions is dependent on cell autonomous svp expression

Although germ cells homozygous for the svp^{e22} allele showed no morphologic defects, we wondered if svp^{e22} homozygous GSCs divided at the same frequencies as those control GSCs homozygous for a mutant allele of *rosey (ry),* a gene involved in eye pigmentation (references).

Males were heat-shocked for three consecutive days and then placed in a vial for seven days to allow for all germ cell clones not derived from GSC clones to clear the testes. After seven days, testes were dissected and the number of GFP^{neg} ry^{506} and svp^{e22} spermatocyte clones was scored. The average number of svp^{e22} clones per testis was significantly lower (2.5, n=111, $p < 0.001$) than the average number of ry^{506} spermatocyte clones per testis (4.3, n=122), suggesting that the GSCs homozygous for the svp^{e22} allele divide at slower rates than control GSCs (Figure 4E). The number of GSCs in svp^{e22} heterozygous animals before heat-shock (3.7, n=51) was similar to the number of GSCs in ry^{506} heterozygotes (3.3, n=58), suggesting that the decreased number of clones observed in svp^{e22} were not due to fewer GSCs within svp^{e22} heterozygotes (Figure 4F). In addition, the mitotic index of GSCs from svp^{e22} heterozygous males before heatshock (10.8%, n=186) was similar to ry^{506} heterozygous males (12.7%, n=189, p= 0.63), suggesting that differences in the percentage of dividing GSCs at the time of heat-shock were not responsible for the decreased number of *svp* mutant clones. Instead, taken together, these data suggest that *svp* is required cell autonomously for GSCs to divide at normal frequencies.

Cyst cell function appears to be independent of cell autonomous svp and hth expression

In addition to generating germ cell clones that lacked *svp* function, we also investigated if *svp* acting in cyst cells was required for their ability to properly encase germ cells. Utilizing the MARCM system (Mosaic Analysis with a Repressible Cell Marker), we were able to make svp^{e22} homozygous cyst cells that expressed a membrane-associated GFP (mCD8:GFP) (Lee and Luo, 1999). svp^{e22} homozygous cyst cells were recovered and appeared to properly send out cytoplasmic extensions around their associated germ cell cyst (Figure 4D). In addition, the germ cells that mutant cyst cells appeared to be encasing were indistinguishable from those surrounded

by GFP^{neg} cyst cells (Figure 4D). These data show that *svp* function is not required for cyst cells to encase germ cells, and imply that *svp* deficient cyst cells are still able to aid in germ cell development.

svp is expressed in pigment cells surrounding the testis.

The modifying activity of *svp* attenuation on the testis phenotype resulting from mutations in *spi* led us to investigate in what cell types *svp* is expressed. Two transgenic enhancer trap lines harboring p-elements containing a lacZ reporter in the *svp* promoter have previously been shown to accurately report endogenous *svp* transcription (Mlodzik et al, 1990; Strutt and Mlodzik, 1995). These two insertions result in two alleles of *svp*, svp^{07842} and svp^{3}. Staining testes from svp^{07842} heterozygotes with antibodies raised against β-Galactosidase (β-Gal) revealed, that although no β-Gal expression was not detected in germ cells or cyst cells, strong β-Gal staining was observed in the pigment cells lining the exterior of the testis shealth (Figure 5A, 5B) (Fuller, M., 1990). Confirming the localization of *svp* transcriptional activity, β-Gal expression was observed in the pigment cells of svp^{3} heterozygotes (data not shown). In addition, staining wild-type testes with an antibody raised against the mammalian homolog of Svp, COUP-TFII, revealed a clear signal in pigment cells (data not shown).

svp transcription is regulated independently of spitz

We next investigated if *svp* expression is altered in *spi* mutants. We reasoned that because *svp* attenuation in *spi* mutants suppresses the testis phenotype of *spi* mutants, that *spi* may normally repress *svp* expression in certain cell types. If this were the case, we might expect to see the expression domain of *svp* expanded in *spi* mutants. To test this hypothesis, we stained

testes from *spi*$^{77\text{-}20}$ mutants that also harbored the *svp*^{07842} enhancer trap with antibodies raised against β-Gal. However, β-Gal expression in testes from *spi*$^{77\text{-}20}$ mutants was similar to that of testes harboring the *svp*^{07842} allele alone, suggesting that *svp* transcriptional activity in the testes is regulated independently of *spi* (Figure 5C, 5D).

svp is expressed in tissues associated with hormonal signaling

Our observations that *svp* does not appear to be expressed in either germ cells or cyst cells and that loss of *svp* function in either germ cells or cyst cells had no detectable effect on cellular morphology suggested that *svp* may function outside of the testis to influence spermatogenesis. To investigate other tissues where *svp* activity might affect spermatogenesis, we examined the expression of β-Gal in *svp*^{07842} and *svp*^{3} heterozygous larvae. We observed strong staining in the fat body, the adipose tissue of the fly, including the portion of the fatbody surrounding the larval testis (Figure 6A, 6B). In addition, we also noticed a cluster of cells in the portion of the ring gland comprising the corpora allatum (Figure 6C-6E). The ring gland lies anterior to the optic lobes of the CNS and is composed of the prothoracic gland, the corpora cardiaca, and the corpora allatum. The corpora allatum is the primary site for the synthesis of juvenile hormone, a molecule controlling aspects larval development and acting as a gonadotrophic hormone in adults (Feyereisen, 1985; Koeppe et al, 1985; Kelly et al, 1987). We also looked to see if β-Gal expression resulting from the *svp*^{07842} and *svp*^{3} alleles was altered in larvae that were homozygous for *spi*$^{77\text{-}20}$. However, in all cases, β-Gal expression appeared to be independent of mutations in *spi*. These data suggest that *svp* may act in hormonal pathways that influence spermatogenesis in *Drosophila*.

Experimental Procedures

Drosophila Genetics and Stocks

All fly stocks were raised and maintained on standard cornmeal molasses agar unless otherwise noted. Fly stocks that were used in experiments were raised at 26.5 °C. Most genetic mutations and transgenic elements are described at http://flybase.bio.indiana.edu or in the appropriate references provided below. Fly stocks used in this study include: w^{1118}, *FRT82B-GFP.NLS, FRT82B-*svp^{e22}, (Bloomington Stock Center), *FRT82B-*hth^{P2} (gift from Claude Desplan), $spi^{77\text{-}20}$ (Sarkar et al., 2007), svp^{e22} (Hiromi et al., 1993), svp^{07842} and svp^{3} (Mlodzik et al., 1990), hth^{05745} (Rieckhof et al., 1997), the germ cell driver *nanos-gal4-VP16* (Van Doren et al., 1998), and the cyst cell driver *tj*-Gal4 (Kyoto Stock Center, DGRC #104055). Homozygous cells were labeled by the MARCM technique (Lee and Luo, 1999) with *act-Gal4 UAS-mCD8-GFP* (cell bodies) (A gift from Tory Herman).

Immunohistochemistry, X-gal staining, and fluorescence microscopy

Testes were dissected and placed in Testis Isolation Buffer (10 mM Tris-HCl, ph 6.8, 180 mM KCl) on ice. Testes were subsequently fixed in 4% formaldehyde in PBT for 30 min. Primary antibody incubation took place overnight at 4°C, and secondary antibody incubation took place for 2 hrs at room temperature. Testes were mounted onto slides using Vectashield mounting media with DAPI. Tissues were observed using a Zeiss Axiophot microscope. Images were taken with a CCD camera using an Apotome and Axiovision Rel Software. Antibodies and dilutions used were as follows: goat anti-Vasa (1:500, Santa Cruz Biotechnology Inc.), rabbit anit-Armadillo (1:500, Santa Cruz Biotechnology Inc.), rabbit anti-β-Galactosidase (1:250, Cappel), rabbit anti-phospho-histone H3 Ser10 (1:750, Millipore), mouse anti-Fasiciclin

III 7G10 (1:10, obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242: developed by C. Goodman), mouse anti-Fasiciclin II (1:10 Developmental Studies Hybridoma Bank), mouse anti-Eya (1:20 Developmental Studies Hybridoma Bank), and guinea pig anti-Tj (1:5000, Dorothea Godt). Alexa-488-, Cy3-, and Cy5-conjugated secondary antibodies were used at 1:500 (Invitrogen). X-gal staining was performed as previously reported (Gonczy et al, 1992).

Cell cycle analysis

The M-phase indices were calculated by dividing the number of $pHH3^+$ (M-phase) spermatogonia by the total number of spermatogonia scored. Optical sections were taken, using an apotome in conjunction with Axiovision Software, of the focal plane in which the middle of the hub was detected. In $spi^{77\text{-}20}$ mutant testes filled with early stage cells, spermatogonia were defined as small $Vasa^+$ cells lying within a 100um square one cell diameter away from the hub. GSCs were defined as those $Vasa^+$ cells in contact with the hub. All indices represent the cumulative total of three independent experiments. All p-values were calculated using a two-tailed Fisher's exact test.

Discussion

During *Drosophila* spermatogenesis, germ cells undergo a well-coordinated program of both proliferation and differentiation. In our previous work, we showed that EGF signaling between germ cells and their surrounding cyst cells promotes germ cell differentiation and

represses the frequency of GSC divisions. We showed that two genes, *svp* and *hth*, act in a genetic pathway in opposition to *spi* signaling. In this appendix, we investigate the underlying mechanisms responsible for the observed genetic interactions. During the course of performing these studies, we better characterized the phenotype of $spi^{77\text{-}20}$ mutants, and also uncovered a fundamental difference between the divisions of GSC and spermatogonia.

We found that GSCs have a higher mitotic index than spermatogonia. These results indicate that either a difference in the rate of divisions or a difference in the proportion of the cell cycle that mitosis comprises exists between these two cellular populations. Future experiments measuring the proportion of cells in the other phases of the cell cycle will aid in delineating between these two possibilities. Furthermore, our study measured the combined mitotic index of spermatogonia consisting of one-, two, four-, eight-, and 16-germ cell cysts. A more detailed and finely tuned experiment, quantifying the mitotic index of GSCs and gonialblasts may reveal deeper insights into the dynamics and regulation of cell division in germ cells during specific stages.

In our previous work, we observed that the increased mitotic index of GSCs in $spi^{77\text{-}20}$ mutants was not affected by the attenuation of either *svp* or *hth*. In contrast, we show here that the increased mitotic index of spermatogonia in $spi^{77\text{-}20}$ mutants was partially rescued upon attenuation of either *svp* or *hth*. On the surface, these results would seem to imply that spermatogonia divide more frequently in $spi^{77\text{-}20}$ mutants and that this increased rate of division is abated upon *svp* or *hth* attenuation. Alternatively, it is possible that the increased mitotic index of spermatogonia in $spi^{77\text{-}20}$ mutants is due to a high proportion of germ cells that, although displaced away from the hub, are stuck in the GSC fate. If this were the case, because GSCs have a higher mitotic index than spermatogonia, the increased mitotic index of spermatogonia in

spi$^{77\text{-}20}$ mutants may be due to a higher proportion of cells dividing as GSCs. Thus, the attenuation of *svp* or *hth* may not affect the frequency of spermatognial divisions, but instead result in fewer germ cells displaced away from hub in the stem cell fate. This added layer of complexity makes drawing strong conclusions about the exact mechanisms responsible for the increased mitotic activity of spermatogonia in *spi*$^{77\text{-}20}$ mutants difficult.

Testes from *spi*$^{77\text{-}20}$ mutant contain a dramatic reduction in the number of cyst cells expressing late stage markers. This observation is consistent with the reduction of more mature germ cells in testes from *spi*$^{77\text{-}20}$ mutants. These parallels between altered germ cell and cyst cells development in *spi*$^{77\text{-}20}$ mutants leaves one with a "chicken-or-the-egg" dilemma: are there fewer late-stage germ cells because of the lack of late-stage cyst cells, or are there fewer late-stage cyst cells because of the lack of late-stage germ cells? Recently, we have observed an increase in cyst cell apoptosis in testes from animals in which EGF signaling has been attenuated, suggesting that germ cell-secreted *spi* may be required not only the encasement of germ cells by cyst cells, but also for cyst cell survival (Cordula Schulz and Alicia Hudson, unpublished data). In addition, when cyst cells are genetically ablated, germ cells accumulate at early stages at the expense of more mature germ cells, resembling the phenotypes seen in those testes from *spi*$^{77\text{-}20}$ mutants (Parrott, unpublished). Taken together, these data suggest that the deficiency of late stage cyst cells in *spi*$^{77\text{-}20}$ mutants is due to cyst cell death, and as a consequence germ cells are not able to properly differentiate. The attenuation of either *svp* or *hth* partially rescued the cyst cell deficiency associated with *spi*$^{77\text{-}20}$ mutants. Future experiments in which the affect of *svp* or *hth* attenuation on testes with genetically ablated cyst cells may aid in distinguishing as to if the suppression acts on the level of cyst cells or germ cells. If the testes phenotypes associated with

testes lacking cyst cells are suppressed by the attenuation of *svp* or *hth*, it would imply that the mechanism of suppression is central to germ cell function rather than cyst cell function.

The morphology and general development of germ cells and cyst cells appears to be independent of cell autonomous *svp* function. In addition germ cells associated with cyst cells lacking *svp* function appeared normal. Furthermore, our analysis of *svp* expression in normal and $spi^{77\text{-}20}$ mutant testes revealed no detectable signal in germ cells or cyst cells. Instead we detect *svp* expression in the pigment cells that line the exterior of the testis. In *wnt-2* null mutants, males completely lack the pigment cells surrounding the testis (Kozopas et al, 1998). Whereas testes from *wnt-2* mutants were small, abnormally shaped, and lacked pigment, both germ cell and cyst cell populations appeared to be unaffected. Thus, this report suggests that pigment cells are not required for spermatogenesis. However, *svp* attenuation was found to suppress the testis phenotype resulting from $spi^{77\text{-}20}$ mutants. Therefore, it would be interesting to see if the testis phenotypes of $spi^{77\text{-}20}$ mutants are affected by loss of *wnt-2*.

svp expression was also detected in the fat body and corpora allatum, two tissues associated with hormonal signaling (Deutsch et al, 1989; Gruntenko et al, 2010). Testes are embedded in the fat body during larval development and remain associated with cells of the fat body in adults. Due to this proximity, it is possible that signaling from the fat body might influence certain aspects of spermatogenesis. The corpora allatum is the primary site of *de novo* juvenile hormone synthesis. In males, mating induces a marked increase of protein synthesis in the accessory glands, a tissue responsible for producing non-gametic components of the seminal fluid (Hihara, 1981; Schmidt et al, 1985). This post-copulatory increase in protein synthesis is regulated by juvenile hormone (Herndon et al, 1997). Thus it is plausible that *svp* acting in the corpora allatum may act to influence certain aspects of spermatogenesis. Future experiments in

which the effect of tissue-specific attenuation of *svp* in *spi*$^{77\text{-}20}$ mutants will aid in determining in what tissue *svp* acts to influence spermatogenesis.

Figure A. 1. The mitotic index of germ cells within the testis is altered depending on germ cell stage and genetic background. (**A**) The mitotic indices of GSC and spermatogonia are plotted. (**B, C**) The mitotic indices of spermatogonia are plotted. Genotypes are as indicated. **p-value < 0.01, ***p-value < 0.001.

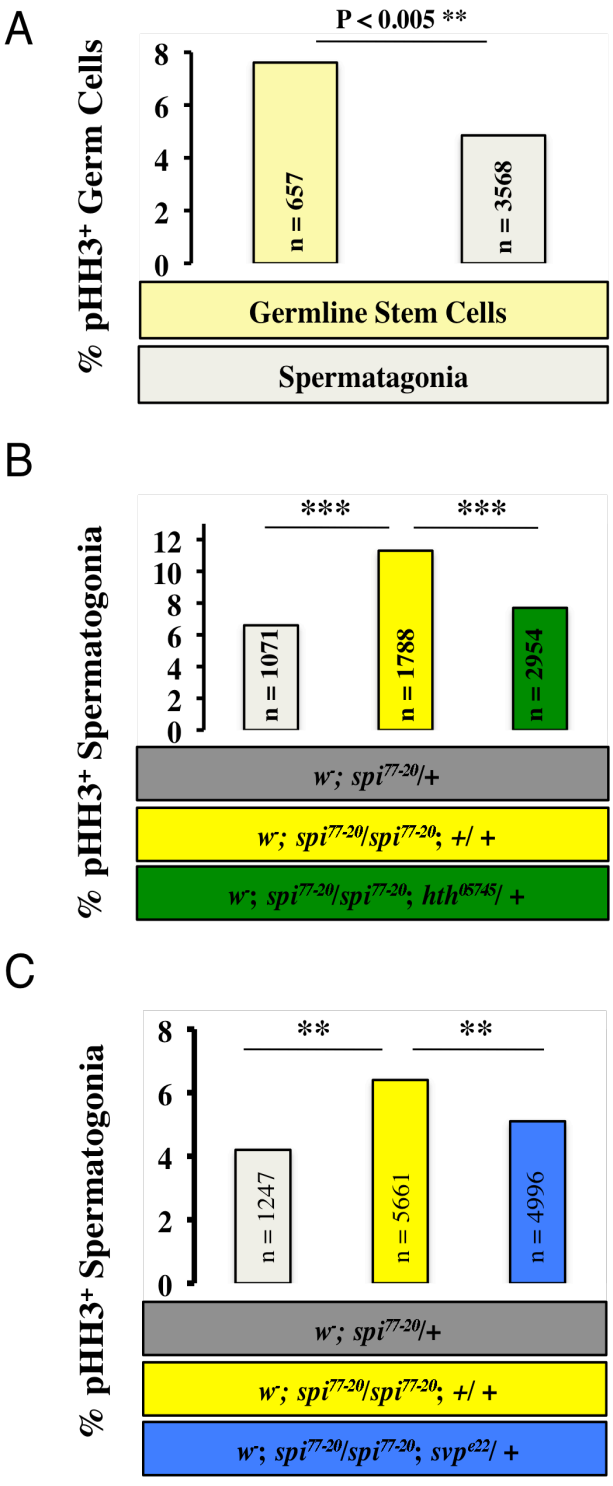

A
P < 0.005 **
% pHH3+ Germ Cells
8
6
4
2
0
n = 657
n = 3568
Germline Stem Cells
Spermatagonia
B
***
***
% pHH3+ Spermatogonia
12
10
8
6
4
2
0
n = 1071
n = 1788
n = 2954
w-; spi77-20/+
w-; spi77-20/spi77-20; +/ +
w-; spi77-20/spi77-20; hth05745/ +
C
**
**
% pHH3+ Spermatogonia
8
6
4
2
0
n = 1247
n = 5661
n = 4996
w-; spi77-20/+
w-; spi77-20/spi77-20; +/ +
w-; spi77-20/spi77-20; svpe22/ +

Figure A. 2. The attenuation of *svp* or *hth* partially rescues the deficiency of cyst cells expressing late stage genes in testes from $spi^{77\text{-}20}$ mutants**.** (**A-D**) Testes stained with DAPI (Blue) and antibodies raised against either Tj (Green) or Eya (red). Genotypes are as indicated. Asterisks indicate the apical tip of testes. Scale bars represent 50um. (**E**) The average number of total cyst cells per testis for the indicated genotypes. Error bars depict the standard deviation. Attenuation of *svp* did not result in a statistically significant difference when compared to homozygous $spi^{77\text{-}20}$ mutants. (**F**) The number of cyst cells expressing Eya is plotted for each indicated genotype. All p-values were derived from two-tailed *t*-tests.

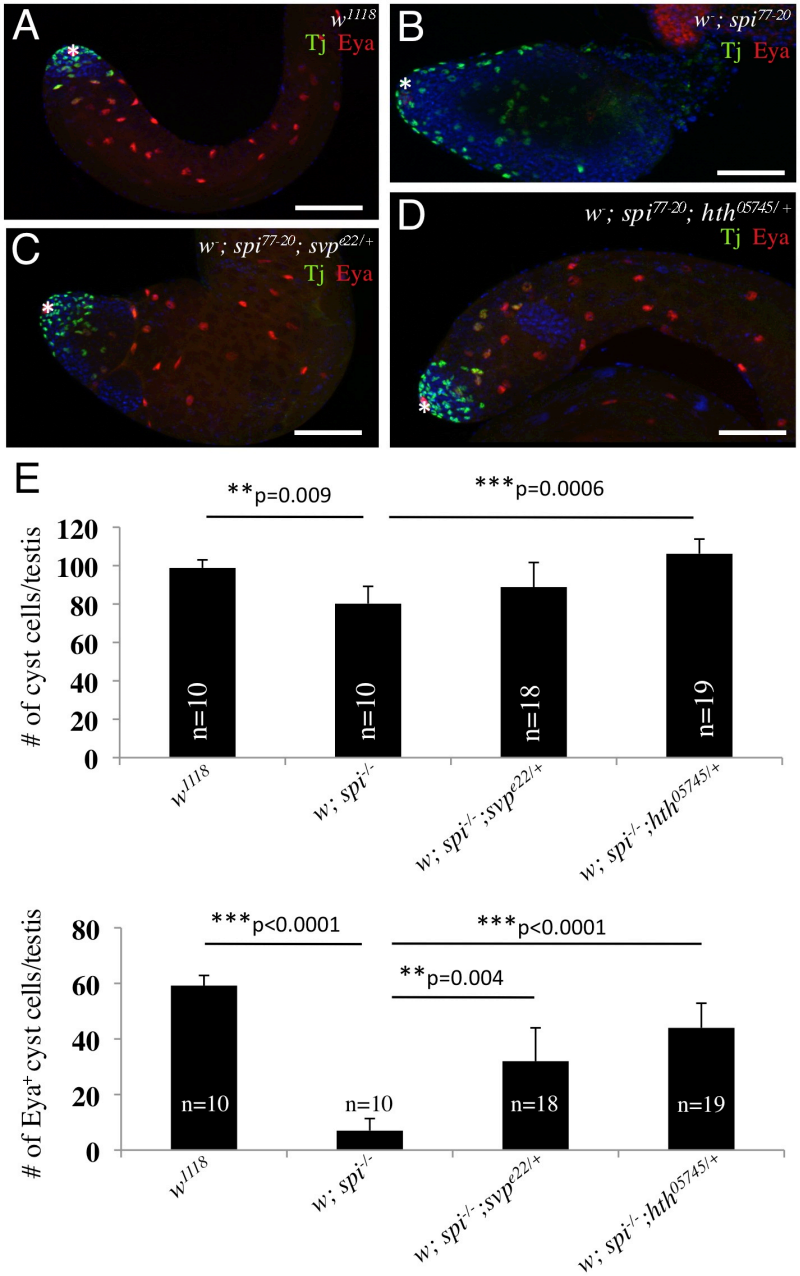

A
w1118
Tj Eya
B
w-; spi77-20
Tj Eya
C
w-; spi77-20; svpe22/+
Tj Eya
D
w-; spi77-20; hth05745/+
Tj Eya
E
**p=0.009
***p=0.0006
# of cyst cells/testis
120
100
80
60
40
20
0
n=10
n=10
n=18
n=19
w1118
w; spi-/-
w; spi-/-;svpe22/+
w; spi-/-;hth05745/+
***p<0.0001
***p<0.0001
**p=0.004
# of Eya+ cyst cells/testis
80
60
40
20
0
n=10
n=10
n=18
n=19
w1118
w; spi-/-
w; spi-/-;svpe22/+
w; spi-/-;hth05745/+

Figure A. 3. Ectopic expression of *svp* results in excessive early-stage cyst cells and disrupts germ cell development**.** (**A-F**) Testes from animals undergoing heat-shocks on three consecutive days. (**A-D**) Testes stained with antibodies recognizing the nuclei of early-stage cyst cells, Tj (Green), and cyst cell membranes, Armadillo (Arm) (Red). (**A, B**) Testes from animals containing the UAS-*svp* alone or (**C, D**) in combination with the *hs*-Gal4. Large arrows point to Tj^+ cyst cell nuclei, and small arrows point to the cytoplasmic extensions of cyst cells. (**E, F**) Testes stained with the germ cell marker,Vasa. Testes from animals harboring the (**E**) UAS-*svp* alone or (**F**) in combination with the *hs*-Gal4. (**G, H**) Testes stained with Tj (purple) and Arm (Red) from animals (**G**) over-expressing *svp* in germ cells or (**H**) harboring the UAS-*svp* alone. (**I, J**) Testes stained with DAPI and antibodies raised against Tj (Green) and FasIII (Red). Testes are from animals harboring (**I**) the UAS-*svp* alone or (**J**) in combination with the *tj*-Gal4. Asterisks indicate the hub.

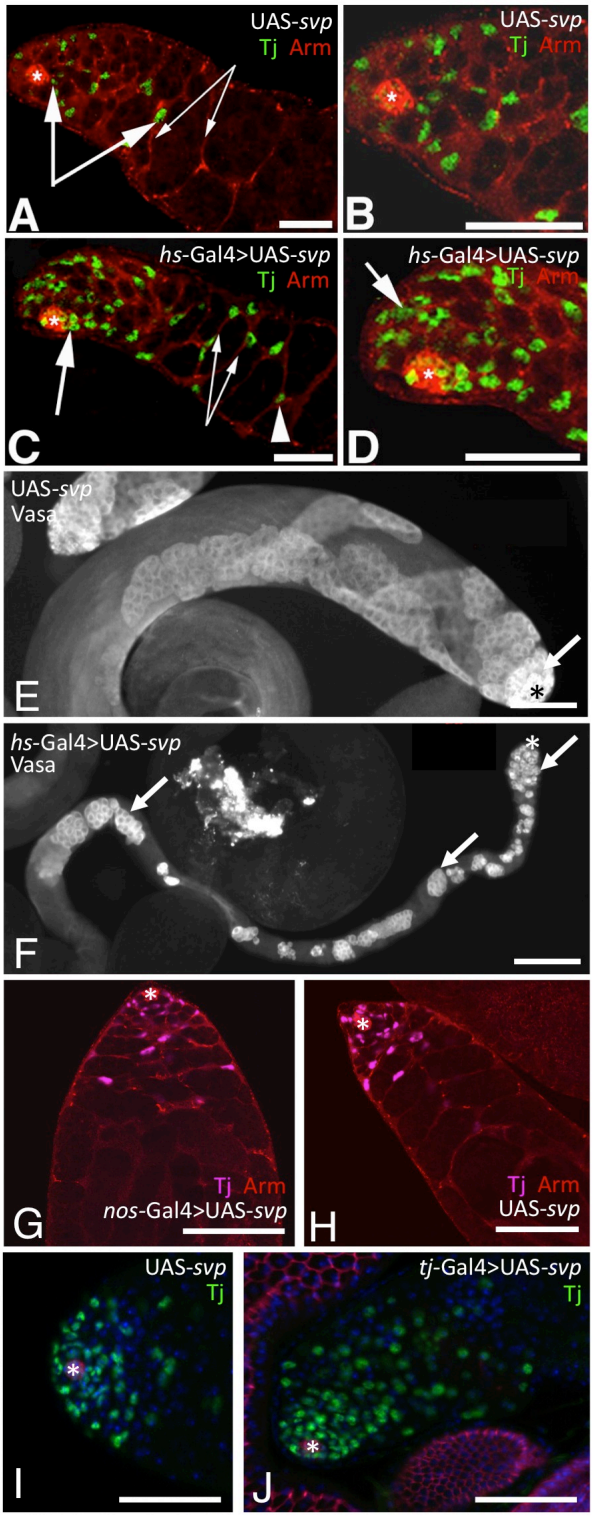

UAS-svp
Tj Arm
A
UAS-svp
Tj Arm
B
hs-Gal4>UAS-svp
Tj Arm
C
hs-Gal4>UAS-svp
Tj Arm
D
UAS-svp
Vasa
E
hs-Gal4>UAS-svp
Vasa
F
Tj Arm
nos-Gal4>UAS-svp
G
Tj Arm
UAS-svp
H
UAS-svp
Tj
I
tj-Gal4>UAS-svp
Tj
J

Figure A. 4. Loss of cell autonomous *svp* function does not affect cellular morphology, but does result in fewer clones produced. (**A-D**) Testes stained with antibodies raised against GFP (Green), Vasa (Red), and Arm (purple). (**A-C**) A representative testis showing GFP^{neg} germ cells homozygous for svp^{e22}. Asterisks indicate the hub. (**D**) A representative testis showing a GFP^{pos} cyst cell homozygous for svp^{e22} generated using the MARCM system. The cyst cell and the germ cells it is associated with appear normal. Scale bars represent 50um. (**E**) The number of spermatocyte clones per testis is plotted for each indicated genotype. Flies were heat-shocked for 3 consecutive days then aged 7 days before scoring. ry^{506} clones served as a control. The p-value was determined by a two-tailed *t*-test, **p-value < 0.001. (**F**) The number of GSCs per testis is plotted for each indicated genotype before heat-shock treatment. (**G**) The mitotic index is plotted for each indicated genotype before heat-shock treatment. A fisher's exact test was used to determine a p-value, p-value = 0.63.

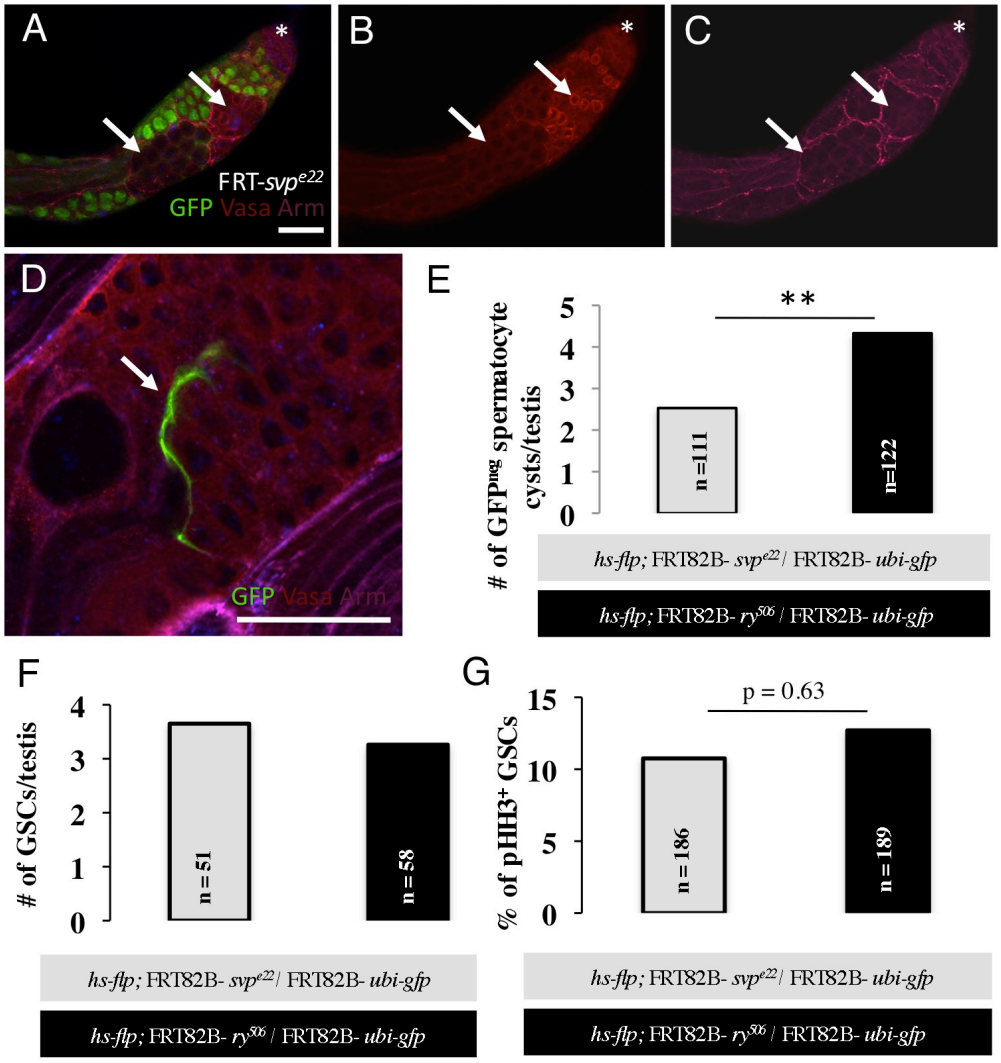

A
B
C
D
E
F
G
FRT-svp^e22
GFP Vasa Arm
GFP Vasa Arm
# of GFP^neg spermatocyte cysts/testis
**
n=111
n=122
hs-flp; FRT82B- svp^e22/ FRT82B- ubi-gfp
hs-flp; FRT82B- ry^506 / FRT82B- ubi-gfp
# of GSCs/testis
n = 51
n = 58
hs-flp; FRT82B- svp^e22/ FRT82B- ubi-gfp
hs-flp; FRT82B- ry^506 / FRT82B- ubi-gfp
% of pHH3+ GSCs
p = 0.63
n = 186
n = 189
hs-flp; FRT82B- svp^e22/ FRT82B- ubi-gfp
hs-flp; FRT82B- ry^506 / FRT82B- ubi-gfp

Figure A. 5. *svp* is expressed in the pigment cells lining the exterior of the testis**.** (**A-D**) Testes from svp^{07842} heterozygotes in an otherwise wild-type background (**A, B**) or in the $spi^{77\text{-}20}$ mutant background (**C, D**). (**A, C**) Testes stained with antibodies raised against β-Gal. (**B, D**) The same testes shown in A and C with β-Gal (Red), but also showing staining with antibodies against Vasa (Green), and the DNA stain, DAPI (Blue). β-Gal is detected in the nucleus of pigment cells (arrows). Asterisks indicate the apical tip of the testis. Scale bars represent 50um.

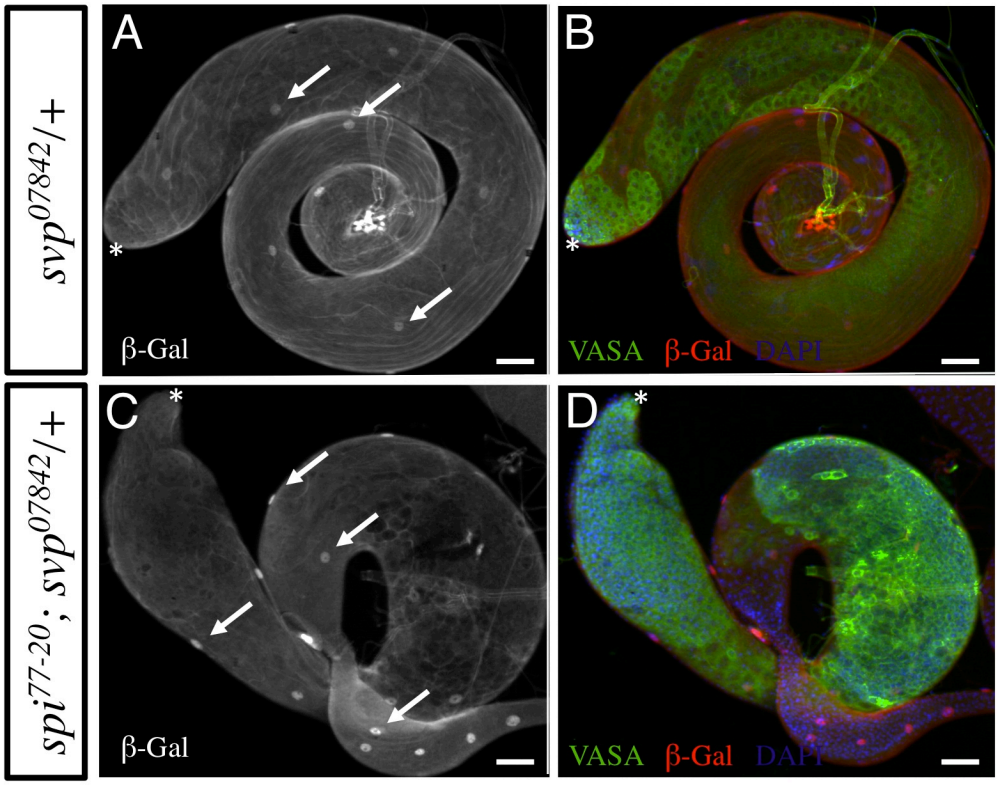
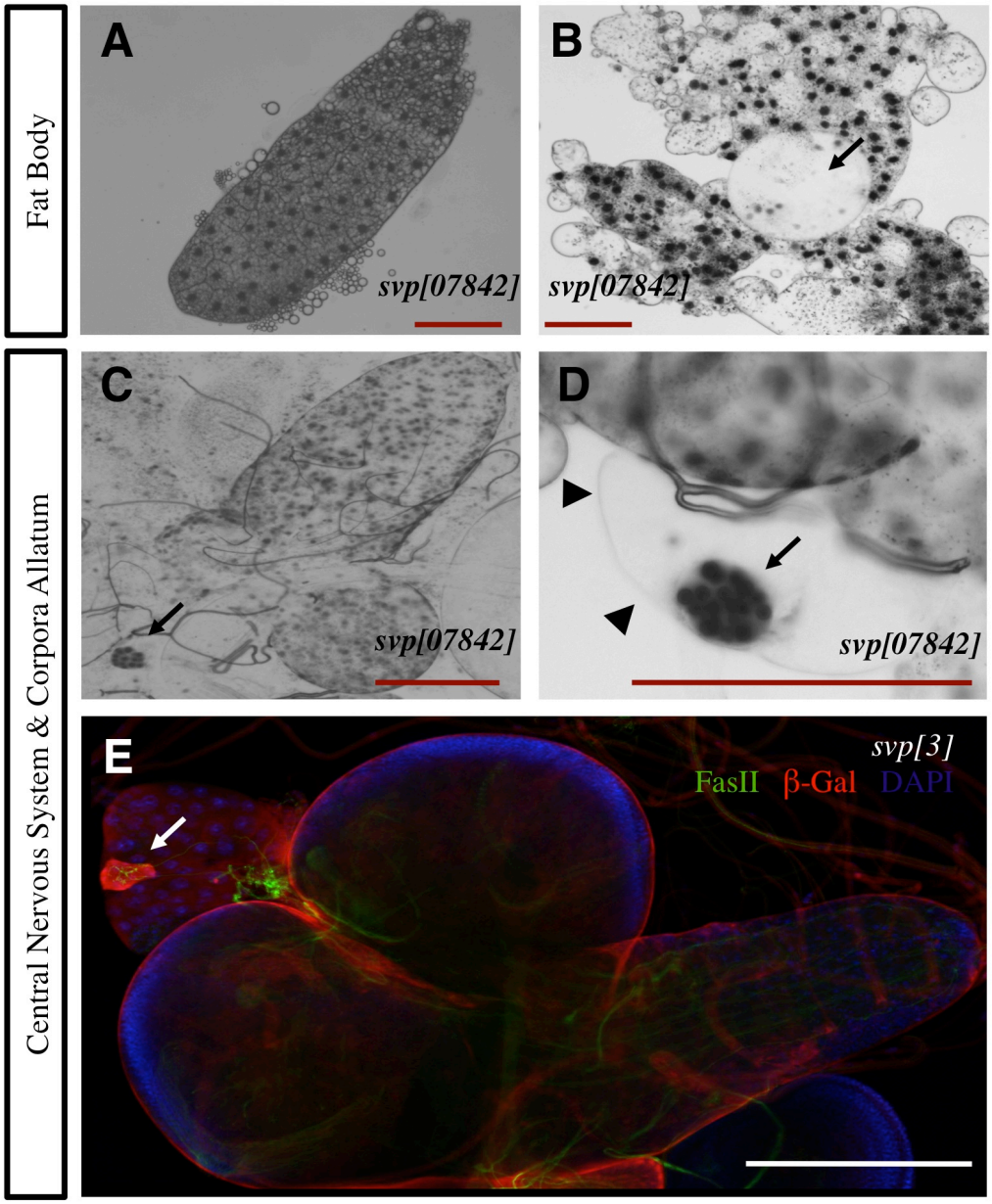

svp07842/+
spi77-20; svp07842/+
A
B
C
D
β-Gal
VASA β-Gal DAPI
β-Gal
VASA β-Gal DAPI

Figure A. 6. *svp* is expressed is tissues associated with hormonal signaling centers**.** (**A-D**) Tissues from *svp*^{07842} heterozygote 3rd instar larvae stained with x-gal. (**A**) larval adipose tissue, (**B**) A larval testis (arrow) embedded in fat body. (**C-E**) *svp* expression in the corpora allatum (arrows). (**C, E**) The corpora allatum is seen in relation to the central nervous system. In (**D**), the outline of the ring gland can be seen (arrowheads). (**E**) CNS and ring gland stained with antibodies against FasII (Green) which labels the neurites that innervate the corpora allatum. Scale bars represent 50um.


Fat Body
Central Nervous System & Corpora Allatum
A
svp[07842]
B
svp[07842]
C
svp[07842]
D
svp[07842]
E
svp[3]
FasII β-Gal DAPI

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