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Characterization of germline stem cell behavior during aging in *Drosophila melanogaster*

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by

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Publications

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ABSTRACT OF THE DISSERTATION

Characterization of germline stem cell behavior during aging in *Drosophila melanogaster*

by

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Professor Leanne Jones

Adult stem cells replenish tissues during normal cellular turnover or injury. A stem cell divides asymmetrically to produce one stem cell (a process called self-renewal), and one daughter cell that initiates differentiation. The mechanisms ensuring the balance of cells are critical for tissue homeostasis. Misregulation of stem cells during disease or aging can lead to impaired tissue maintenance and repair resulting in diminished life quality or death of an individual.

Stem cells are located in a microenvironment, or niche, within a tissue. The niche, composed of other cells, ECM, and/or a basement membrane, provides extrinsic cues to stem cells that regulate self-renewal, maintenance, and survival. Therefore, analyses of stem cells should include the niche, to ensure that an observation is physiologically relevant. While *in vivo* studies of stem cells are critical, complex vertebrate tissues are difficult for 1) identifying stem cells 2) determining niche components 3) deciphering signaling pathways within stem cells, and between stem cells and the niche. Therefore, studies of stem cells within the context of disease or aging would add more hurdles to the research process.

This dissertation presents research using the powerful model organism, *Drosophila melanogaster*, to study the effects of aging on germline stem cell (GSC) behavior in the testis, a well-characterized tissue. Chapter 2 shows that aging results in reduced GSC function due to extrinsic changes to the niche and intrinsic changes to the GSCs. Janus kinase -Signal Transducer and Activator of Transcription, Jak-STAT, signaling that regulates stem cell maintenance, was reduced during aging, and restoration of signaling led to a rescue of GSC number within the niche in aged animals. Male flies are able to replace lost GSCs through reversion (dedifferentiation) of early germline progenitor cells. Chapter 3 shows that GSC numbers are lower during aging possibly due to compromised reversion. Analysis of early cyst cells, one of the components of the niche, shows defects in response to reversion in aged flies. This dissertation summarizes that

interactions between the stem cells and their niche dictate tissue health; changes to the niche and stem cells lead to decreased tissue homeostasis during aging.

Chapter 1: Introduction

An assembly of organ systems makes a higher organism. The tissues and organs are functional subunits, formed and shaped by a myriad of specialized cell types, and are essential for the survival of an individual and the species. How do long-lived organisms sustain these tissues and organs when cells turnover and damage occurs? During aging, the integrity of tissue and organ maintenance and repair diminishes dramatically compared to those from a more youthful age. What are the mechanisms leading to the loss of tissue homeostasis during aging?

1.1 Tissue and Organ Homeostasis and Adult Stem Cells

The concept of a cell that allows for regeneration of tissues began in the late 19th century. The term “stem cell” was coined within the context of developmental biology - a single-cell fertilized egg that could produce all the cell types in the organism (Ramalho-Santos & Willenbring 2007). Biologists took this idea one step back and classified gamete-producing cells as stem cells. It was not until Ernest McCulloch and James Till in the 1963 hallmark study in the hematopoietic system demonstrated that differentiated spleen colonies originated from single cells, now known as hematopoietic stem cells (Becker *et al.* 1963). Since this study, scientists have been identifying and validating the presence of adult stem cells in many tissues such as muscle, brain, skin, and bone.

The modern definition of a stem cell is a cell that is capable of dividing asymmetrically to make one copy of itself in a process termed self-renewal and another daughter that initiates differentiation into specific cell type. A stem cell is functionally defined as a single cell that when serially transplanted to a host tissue lacking stem cells, is able to maintain regeneration of tissues over time (Morrison & Spradling 2008). Stem cells hold promise in cell-based therapies and are potential targets for certain types of cancers. This section describes briefly the current knowledge on adult stem cells in vertebrates and introduces the use of the male gonad of *Drosophila melanogaster* as a simple model for studying the mechanisms regulating adult stem cell behavior.

1.1.1 The stem cell niche

Adult stem cells are not distributed haphazardly within a tissue; they are found in specialized locations called the stem cell niche. The stem cell niche hypothesis was first proposed for hematopoietic stem cells where the microenvironment, or niche, is where stem cells reside and maintain stem cell activity (Schofield 1978). In many situations, when a stem cell is taken out of the niche, the stem cell loses its ability to self-renew.

The niche provides the signals that regulate stem cell self-renewal, maintenance, and survival. Stem cells could be in contact with other cells, an extra cellular matrix, and/or basement membrane (Jones & Wagers 2008). These components have signaling molecules such as growth factors and adhesion molecules that are necessary for proper stem cell function. Thus, the study of

stem cells is incomplete without understanding their interactions with the components of the niche.

Obstacles for identifying stem cells are inherent for complex tissues because adult stem cells are extremely rare, often do not have specific markers for distinguishing them from progenitor cells, and activity is sometimes exhibited only after tissue damage or at specific developmental stages (Voog & Jones 2010). Furthermore, examining stem cells *in vivo* within intact tissue is difficult, so stem cells are purified and examined in primary culture conditions. Therefore, discovering the niche components - whether they are cells, matrices, or membranes, and which signaling pathways are conducted between the stem cell and the niche - is an even more daunting task. Regardless of the difficulties, the understanding of stem cell models in vertebrates has expanded dramatically over the past few years. The benefits of vertebrate models are the ability for single stem cell transplantation studies and investigations of systemic influences on stem cell behavior using the parabiosis paradigm in mice.

The hematopoietic stem cell (HSC) model system was historically one of the firsts to be characterized mainly because of its medical feasibility such as the bone marrow transplant (Ramalho-Santos & Willenbring 2007). HSCs give rise to many cell lineages that make up the blood and must continuously generate cells to maintain the blood due to normal cellular turnover. The HSC system is unique from other stem cell models because HSCs can be found within the bone marrow and also circulating in the blood stream many distances away from the primary niche. The definition of the HSC niche (or possibly niches) is still under debate

but essentially the endosteum, the interface between the bone and the bone marrow, is where HSCs reside (Morrison & Spradling 2008). HSCs have been shown to respond to signaling from the bone, osteoblasts and osteoclasts, for maintenance via Wnt and Notch signaling and also from perivascular and vascular cells via chemokine signaling. The HSC system allows for study of long-range migration or homing of stem cells, which are essential for stem cell replacement therapy.

Muscle stem cells (MSCs) give rise to one type of cell, the myoblast, which eventually fuses with other myoblasts to become a muscle fiber. Unlike HSCs, the demand for myofiber regeneration occurs upon injury, such as after exercise. The current model is that upon injury, the quiescent MSC is activated to proliferate to generate more MSCs which migrate and differentiate into myoblasts (Wagers & Conboy 2005). The MSC niche is still being defined. The basal lamina of an existing myofiber has been proposed to be where Wnt, Notch, and SDF-1a, CXCL12 signaling occur in response to injury (Voog & Jones 2010). MSC studies have allowed the study of the switch between the quiescent and proliferative state of a stem cell.

The spermatogonial stem cells (SSCs) produce spermatogonia that ultimately become mature sperm. The niche is located within each seminiferous tubule of the testis and consists of mainly sertoli cells next to a basement membrane, although other cells in close proximity to these components may also play a role (Oatley & Brinster 2008). SSCs are systemically regulated by hormones, but are regulated directly by the growth factor, GDNF, which has been

shown to be essential for SSC self-renewal. The SSCs produce germ cells that divide with incomplete cytokinesis leading to spermatogonia that are interconnected in the cytoplasm, stereotypical of germ cells from flies to mammals.

1.1.2 Stem cell models in *Drosophila melanogaster*

What we know about stem cell models today has been gleaned from studies in *Drosophila*. In addition to all the benefits that comes with working with a model organism, the tissues in the fly are simple allowing the study of stem cell and niches *in vivo,* and the signaling pathways are rudimentary making it easier to tease apart the interactions between the stem cells and the niche components (Voog & Jones 2010). Like mammals, the fly has tissues that regenerate and repair after damage. The ovaries and the testes have been the classic examples of stem cell niches, but stem cells have been found in the developing CNS, the guts (intestine), the hemolymph (blood), and malpighian tubules (kidney).

The niches in *Drosophila* have unique properties that translate to mammalian systems. The neuroblasts, hematopoietic stem cells, and the germline stem cells are found within niches with a cellular component that offers opportunities to study cell-cell interactions (Fuller & Spradling 2007; Martinez-Agosto *et al.* 2007; Doe 2008). The stem cells in the midgut and hindgut are situated on a basement membrane layer that serves a niche component allowing for study of a cell-free niche (Casali & Batlle 2009). The gonads contain multiple populations of stem cells interacting with each other thus allowing the study of

how the niche can differentially influence two populations of stem cells and how each population talks to each other (Fuller & Spradling 2007). Although the data is preliminary, the malpighian tubules stem cells control their own maintenance through autocrine signaling, provides an example where a fixed niche may not be necessary (Singh *et al.* 2007). In the midgut, the immediate progenitor cells have been shown to serve as a transient niche for stem cell specification, a novel concept that could be applied to other stem cells (Mathur *et al.* 2010).

Signaling pathways that play a role in stem cell self-renewal and maintenance are shared by many of these stem cell models within the fly and are conserved in mammals. Understanding how one or a combination of these pathways – Jak-STAT, BMP, Hedgehog, insulin, Notch, Wingless – regulates stem cell behavior within one model, will shed new light onto another (Morrison & Spradling 2008). However, the fine-tuning of signaling is likely made specific for one stem cell type, and thus may be regulated differently using different co-factors, paralogs, and other signaling pathways.

The stem cell niches within the gonads of *Drosophila* are the better-characterized models. Although this dissertation will focus on the testis as the model, much of the knowledge of the germ line have been discovered in the ovary and applied to the testis and vice versa. Briefly, the ovary contains 2-3 germline stem cells (GSCs) found at the tip of the ovariole (germarium). GSCs are in contact with cap cells and escort stem cells (ESCs) and close to the terminal filament, three of the proposed GSC niche components. BMP signaling from the cap cells activates GSCs self-renewal to prevent differentiation (Fuller &

Spradling 2007) and Jak-STAT signaling regulates ESCs self-renewal and GSC maintenance. The early steps of oogenesis (e.g. the mitotic germ cells) are relatively similar to the testis described below.

1.1.3 Spermatogenesis in *Drosophila*

Drosophila males have a pair of testes; each is a tube composed of muscle and pigment cells with spermatogenesis arranged spatially and temporally within the sheath (Hardy *et al.* 1979). Spermatogenesis begins at the tip of the testis with the GSCs giving rise to daughter gonialblasts that undergo four mitotic cycles to produce a 16-cell cyst of interconnected spermatogonia (Fuller 1998). Terminal differentiation occurs after spermatogonia transition to the primary spermatocytes growth phase followed by meiosis to produce 64 spermatids. The flagella elongate, and the spermatids become individualized into a single mature sperm. The sperm are stored in the seminal vesicle until use.

The different stages of spermatogenesis can be determined by morphology of the cells using phase contrast microscopy. For example, spermatogonia are distinguished from spermatocytes as being much smaller denser than the large primary spermatocytes (Fuller 1998). In addition, markers are available for distinguishing different stages of spermatogenesis – for example, Bag of marbles (Bam) labels late staged spermatogonia and fuzzy onion labels spermatocytes (Hwa *et al.* 2002). The accessibility of this tissue allows for rapid detection of defects in spermatogenesis.

The early germ cells divide with incomplete cytokinesis, resulting in syncytium of germ cells with a shared cytoplasm. The structure that connects each germ cell is the ring canal (Hime *et al.* 1996). Insect germ cells also contain an ER-like organelle called the fusome that penetrates the cytoplasm. The fusome is spherical in shape (spectrosomes) in GSCs and their immediate daughters and becomes progressively branched throughout the mitotic amplification step and through gametogenesis (Lin *et al.* 1994). The early stages of gametogenesis are similar between females and males, and therefore have similar fusome compositions (Lighthouse *et al.* 2008). The fusome has been shown to play a role in coordinating and synchronizing the cysts of germ cells throughout the stages of spermatogenesis. In addition to membrane skeletal components such as alpha/beta spectrin and adducing-like protein hu-li tai shao and a novel protein Bam and cell-cycle regulator cyclin A are also expressed in the fusome (McKearin & Spradling 1990; Lilly *et al.* 2000).

1.1.4 The male germline stem cell niche

The germinal proliferation center is at the apical tip of the testis. The stem cell niche contains two population of stem cells, GSCs and CySCs, that surround and are in contact with a tight cluster of ~11 cells that makes the hub. When a GSC divides asymmetrically, one daughter remains next to the hub and the other daughter is displaced away from the hub and initiates differentiation as a gonialblast. A pair of CySCs encapsulates each GSC to prevent excess GSC self-renewal (Kiger *et al.* 2000). CySCs give rise to cyst cells; a pair of cyst cells

encapsulates each daughter gonialblast to regulate differentiation of the germ line all the way through sperm individualization. CySCs can also give rise to hub cells (Voog *et al.* 2008). With only three types of cells, the GSC niche in the testis is an ideal system for studying cell-cell interactions.

Although there are no specific markers labeling GSCs, GSCs can be identified anatomically by their physical contact with the hub using a germ cell specific marker such as Vasa and a fusome marker to detect spherical fusomes. The other component of the GSC niche is the CySC that is also in contact with the hub. Anatomical studies using electron microscopy showed that roughly a pair of CySCs exists for each GSC (Hardy *et al.* 1979). Hence, CySC number can be estimated by determining the GSC number. However, the lack of markers for specific identification of CySCs makes it difficult to accurately determine exact numbers. A pair of CySCs physically wraps around GSCs. The niche is physically constrained; to contact the hub, the CySCs extend their cytoplasmic arms between GSCs thus leaving the nuclei just below a GSC. Also a recent study proposed that there may even be two populations of somatic cyst cells within the niche based on proliferation markers and anatomical position (Voog *et al.* 2008). Thus the dense tip of the testis may still contain unidentified cell types. The nuclei of CySCs and early cyst cells are triangle-shaped possibly a combination of physical constraints as well as physical contacts with germ cells. When the germ line is lost, the loss of physical contacts and constraints results in round cyst cell nuclei (Kiger *et al.* 2000). GSCs have been found to also have

projections reaching out to maintain contact with hub cells (Boyle unpublished data).

1.1.5 Cell-cell interactions in the *Drosophila* male GSC niche

The coordination of activity between GSCs and CySCs is important for maintaining the correct balance of cells in the niche. A GSC was once hypothesized to divide in synchrony division with a pair of associated CySCs (Hardy *et al.* 1979) with but to date no evidence supports all three cells, or even just two cells dividing together. Furthermore, a recent paper suggested that there are two populations of somatic stem cells in the niche so that extra cells are available to receive the displaced gonailblast (Voog *et al.* 2008). CySCs also receive signaling from GSCs to mediate encapsulation of GSCs. Through epidermal growth factor (Egf) signaling, GSCs secrete the Spitz ligand to the Egf receptor (Egfr) in CySCs to activate genes regulating membrane shape changes (Kiger *et al.* 2000; Schulz *et al.* 2002; Sarkar *et al.* 2007). Encapsulation is critical for restricting GSC self-renewal since defects in Egf signaling lead to uncontrolled GSC proliferation. Furthermore, when the germ line is ablated genetically, the remaining somatic cell population proliferates and exhibits expansion of hub markers suggesting that a signal from the germ line keeps the somatic cyst cells in check (Gonczy & DiNardo 1996). There is also a reciprocal relationship where cyst cells signal to GSCs. Raf, a serine-threonine kinase, required for CySCs maintenance, when deficient leads to uncontrolled GSC proliferation (Tran *et al.* 2000). Thus the two populations of stem cells in the GSC

niche have a homeostatic balance to keep each population functioning properly. Changes in regulation of either of these populations could shift the balance leading to dysfunctional spermatogenesis.

Proper levels of adhesion are important for a functional niche. The hub expresses a variety of markers including factors for cell adhesion; Fasciclin2 and 3, cadherin (E and N), and armadillo, the beta-catenin homolog, which has been implicated to be important for holding the hubs cells in place and/or directing oriented stem cell division (Yamashita *et al.* 2003). The CySCs and GSCs also express the adhesion molecules E and N cadherin, and E-cadherin has been shown to be required for maintenance for both populations (Voog *et al.* 2008). Integrin signaling has also been suggested to be important for mediating cell-adhesion of the stem cells around the hub (Yamashita *et al.* 2003). The hub is in contact with an extra cellular matrix and studies have implicated that integrins play a role in anchoring the hub at the testis tip (Tanentzapf *et al.* 2007). If adhesion decreases, the hub integrity is loss and stem cells may not be retained within the niche, as described in Chapter 2 (Boyle *et al.* 2007). If adhesion is increased, stem cells may be ‘stuck’ within the niche and overproliferate such as in the case where CySCs that outcompeted GSCs for the niche have high integrin levels (Schulz *et al.* 2002; Issigonis *et al.* 2009).

The hub also expresses a variety of other factors that are important for regulating GSC behavior, many which are still not well characterized. The hub secretes the cytokine, Unpaired (Upd), one of the ligands for the Jak-STAT pathway (Kiger *et al.* 2001; Tulina & Matunis 2001) which has been implicated in

the maintenance and self-renewal of the two stem cell populations (see section below). A genetic study showed that bone morphogenetic protein (BMP) signaling from the hub and the early cyst cells are also required for GSC maintenance (Kawase *et al.* 2004). In addition, the hub also expresses the developmental patterning ligand Hedgehog (hh) based on a read out by a promoter reporter (*hh*-lacZ) and preliminary data suggest that Hh is likely involved in regulating early cyst cell differentiation (Forbes *et al.* 1996)(Will Ansari, unpublished data). Relating to these signaling pathways, heparan sulfate proteoglycans (HSPGs) often serve as co-receptors for growth factor signaling. The glypican, Dally-like protein (dlp), is expressed in the hub and plays a role in short-distance relay of ligand signals, BMP ligands such as Decapentaplegic (Dpp) and Glass bottom boat (Gbb) and/or Upd (Guo & Wang 2009; Hayashi *et al.* 2009). Center divider (Cdi), a serine-threonine kinase, is also expressed in the hub (detected by an enhancer trap) but the function Cdi in the hub is not known (Le Bras & Van Doren 2006). Thus, the hub provides many signaling molecules and factors that could possibly be involved in hub function and/or relayed to the adjacent cell populations. The orchestration of this variety of components plays an important role in maintaining tissue homeostasis.

Asymmetric GSC division is critical for maintaining the balance of stem cells within the niche. If GSCs divide symmetrically, there would be too many stem cells or daughters depending on the fate choice. This division is mediated by the centrosomes, the microtubule organization centers. There is one centrosome in the early phase of the cell cycle in GSCs, and in a mechanism that

is poorly understood, the centrosome duplicates when a cell begins to prepare for mitosis (Monk *et al.* 2010). The first 'mother' centrosome is oriented towards the niche so the GSC inherits it while the new centrosome is inherited by the GB illustrating a programmed method to ensure asymmetric division (Yamashita *et al.* 2007). GSC division is oriented perpendicular to the hub likely mediated by E-cadherin and Armadillo, the beta-catenin homolog, to coordinate centrosomes via Adenomatous Polyposis Coli 2 (APC2), a tumor suppressor gene (Yamashita *et al.* 2003). Recent studies have shown by live-imaging that misoriented centrosomes are held at a checkpoint until realignment allows for efficient resumption of the cell cycle in GSCs (Cheng *et al.* 2008). The CySC division orientation is not well documented.

There are cell-intrinsic mechanisms that are critical for GSCs behavior. For example, insulin signaling is mediated by insulin-like peptides that are present systemically as well as locally within the testis (although the local role is yet to be understood). GSC maintenance is disrupted shown by loss-of-function Insulin receptor clones implicating a nutrition-sensing mechanism within GSCs for germ line production (McLeod unpublished data). Held-out-wings (HOW), an RNA binding protein, regulates mitosis via cyclin B, was shown to be required intrinsically for GSC maintenance (Monk *et al.* 2010). Also, within the testis, Yb, a component that is localized in 'Yb bodies' in germ cells, is required for GSCs maintenance. Yb is thought to be involved with PIWI and hh signaling in the testis (Szakmary *et al.* 2009). Thus intrinsic mechanisms are in place within GSCs to ensure proper function.

1.1.6 Jak-STAT Signaling Cascade and Role in GSCs

The most well characterized pathway for GSC maintenance in the testis is the Jak-STAT signaling cascade which is conserved from slime mold to man. There are three ligands from the cytokine family, with no direct mammalian homolog, that activate canonical Jak-STAT signaling, Unpaired (upd), upd2, and upd3 (Rawlings *et al.* 2004). Upd is expressed in the hub and Upd3 is expressed in the hub and cyst cells as detected by RNA *in situ* analysis and by enhancer traps (Kiger *et al.* 2001; Tulina & Matunis 2001)(Jones Lab, unpublished data). The role Upd3 in the testis is still unknown, but upd2 and upd3 may play redundant roles by fine tuning signal activation or responding to different environmental cues such as in immune response (Hombria *et al.* 2005), (Agaisse *et al.* 2003; Jiang *et al.* 2009). Upd activates the relatively straightforward canonical Jak-STAT signaling cascade. Upon Upd binding to the receptor domeless, the receptor gets phosphorylated by the tyrosine kinase Jak(Gregory *et al.* 2008). The one *Drosophila* STAT, Stat92E, in the cytoplasm gets phosphorylated on a tyrosine residue, dimerizes and gets translocated to the nucleus where it activates target genes. This pathway is kept in check by the SOCS (suppressors of cytokine signaling) family of proteins, which Socs36E has been shown to inhibits Jak-STAT signaling in the testis (Karsten *et al.* 2002).

Stat92E is the ancestor of 7 mammalian STAT proteins. STAT proteins recently have been found to exhibit many contrasting and surprising roles outside of the canonical role. Most STAT proteins excite a proliferative response with the exception of STAT1, which induces an inhibitory response to inflammation

(Arbouzova & Zeidler 2006). This property has been observed in *Drosophila* wing disc development; during one phase Stat92E acts in a proliferative manner and in a different phase acts in an inhibitory manner (Mukherjee *et al.* 2005). Thus the diverging STAT function in mammals is preserved in *Drosophila*. Another study showed in vitro that Stat92E binds and may interact with cyclins and cyclin dependent kinases for its stability suggesting that its activation may be governed by a variety of kinases (Chen *et al.* 2003). STAT3 and 5 in mammals have been shown to play a role in the mitochondria where they are involved in oxidative phosphorylation rather than its traditional roles as a transcription factor (Gough *et al.* 2009; Wegrzyn *et al.* 2009). This activity is dependent on phosphorylation of different site, and a mutation in the DNA binding domain of these STATs still allow for function within the mitochondria emphasizing that STAT protein function is modular. Stat92E has putative mitochondrial phosphorylation sites (Wong unpublished data). Lastly, Stat92E may play a role in heterochromatin formation or maintenance as it interacts with HP1 (Shi *et al.* 2008).

Our understanding of how the CySCs and GSCs self-renewal has changed over the past couple of years. Before 2008, it was shown that Jak-STAT signaling was necessary and sufficient for self-renewal of both CySCs and GSCs (Kiger *et al.* 2001; Tulina & Matunis 2001). Stat92E is express in GSCs, immediate daughters, and in CySCs ((Boyle *et al.* 2007; Flaherty *et al.* 2010). However, the activation of Stat92E in the germ line is not sufficient for self-renewal and that activation within early cyst cells results in non-autonomous germ line proliferation (Leatherman & Dinardo 2008). The new model is that Jak-

STAT signaling directed at CySCs, which activates GSCs for self-renewal. The signaling from CySCs to direct GSC self-renewal has yet to be elucidated.

1.1.7 Regeneration of tissues

Many mechanisms are in place to ensure the maintenance of a healthy pool of stem cells for regeneration of tissues. In the case of dramatic tissue loss or damage, could an organism heal from that? After asymmetric division, the differences between a stem cell and its initial daughter progenitor may be little but as the daughter further differentiates, its similarity to the original stem cells decreases. Are these changes are intrinsically hardwired? Can these differentiation hallmarks be reversed so that progenitor cells can become a stem cell again?

One of the explanations to why it is difficult to purify, isolate, and identify stem cells is due to the rare differences between a stem cell and its differentiating daughter cell. Currently, in *Drosophila*, the intestinal stem cells are identified from their daughters based on morphological differences (Casali & Batlle 2009) and in the gonad the stem cell is based on anatomical location next to niche cells (Fuller & Spradling 2007). The rare differences imply that perhaps the intrinsic changes within a daughter progenitor cell is still plastic and takes little effort to change back to stem cells.

1.1.8 Stem cell-based regeneration of tissues

The role of stem cells is to maintain and replace cells within a tissue after damage. Some animals such as the planaria and starfish have a store of

totipotent neoblasts/interstitial cells within the body that is able to regenerate the entire organism (Birnbaum & Sanchez Alvarado 2008). For higher organisms, if stem cells are still in place, mechanisms exist to alert stem cells after tissue damage evident in injury repair and wound response stem cell models. For example, wound repair in the skin leads to contribution of stem cell progeny to the epithelium however along with an influx of secreted factors (Stappenbeck & Miyoshi 2009). Thus, it is not well known what relays the injury signal to stem cells. Using a simpler system, the *Drosophila* midgut, it was discovered that when gut cells are destroyed by induced apoptosis or infection, surrounding cells elicit Jak-STAT signaling to ISCs for stem cell self-renewal to replace lost cells (Jiang *et al.* 2009). There are many exciting and unknowns in the field of wound repair, but they are beyond the scope of this thesis.

1.1.9 Plasticity of differentiated cells and reversion in *Drosophila*

In animals and plants, the ability to regenerate tissues post-injury can be stem cell based as described above, or through a mechanism that induces differentiated cell types to dedifferentiate or transdifferentiate to a stem cell which then can utilize for replacement of different cell types. (Birnbaum & Sanchez Alvarado 2008). In most organisms, an injury occurs in an area with only differentiated cells, such as in salamander limbs where terminally differentiated cells can reacquire stem cell like properties and contribute to different tissue types. Recent studies have shown many common pathways used in development and stem cells are required for regeneration. Understanding the

cues that elicit regeneration and the mechanisms regulating the reversion to a stem cell-like state will aid in the future of regenerative medicine.

Reversion (dedifferentiation) of a non-stem cell back to a stem cell state has been shown using genetic manipulations to occur in the germ line of *Drosophila*. In both, the ovary and testis, inducible expression of bam, a differentiation gene, in the larval and adult gonads lead to the depletion of the GSCs as marked by appearances of germ cells containing branched fusomes (Kai & Spradling 2004; Sheng *et al.* 2009). When flies were allowed to recover from the heat shock, GSCs reappear. In the testis, GSCs were forced to differentiate into spermatogonia using a temperature sensitive mutant allelic combination for Stat92E (Stat92Ets)(Brawley & Matunis 2004). Once allowed to recover at the permissive temperature, GSCs reappear next to the hub. Both of these methods demonstrated evidence that the syncytium of germ cells break down to single GSCs by pinching off the ring canals and fragmentation of the fusome (Brawley & Matunis 2004)(Kai & Spradling 2004; Sheng *et al.* 2009). Although similar to the separatation of a GSC:daughter cell bridge, the mechanism regulating this is unknown. Interestingly, reversion capacity is lost when a germ cell is past the mitotic stage suggesting that plasticity is increasingly limited as a cell differentiates (Brawley & Matunis 2004).

The heat shock bam paradigm forces GSCs to differentiate without affecting cyst cell number or Jak-STAT signaling whereas the Stat92Ets paradigm affects cyst cell number and depends on Stat92E. Comparison of these two reversion paradigms reveal that reversion is not 100% using the Stat mutant

technique suggests that either cyst cells or Jak-STAT signaling is required for efficient reversion. A block in Jak-STAT signaling in the heat shock Bam paradigm by misexpressing Socs36E reduces the efficiency of reversion (Sheng *et al.* 2009). Also, spermatogonia proximal to the hub have high levels of Stat92E (indicative of response to Jak-STAT signaling) and have actin-rich projections towards the hub. These observations suggest that reversion requires the hub, and that Jak-STAT signaling may be important for successful reversion.

Using a method to permanently mark late differentiating spermatogonia, an increasing number of marked GSCs were found in flies that have been irradiated (Cheng *et al.* 2008). Thus, under extreme tissue damage, spermatogonia can revert back to GSCs suggesting that reversion can be used normally to replace lost GSCs. Furthermore, discussed in the following section, an increasing number of marked GSCs were found during the course of normal aging alluding to the idea that lost GSCs are normally replaced by reversion (Cheng *et al.* 2008).

Reversion of germ cells has also been observed in mice suggesting that is process is conserved. Late spermatogonia can be transplanted to testes that are depleted of germ cells and over time, spermatogenesis is resumed, suggesting that reversion has occurred (Barroca *et al.* 2009). *In vitro* culture of spermatogonia can be force to revert to stem cells again by introducing the self-renewal factor GDNF and FGF2 showing that factors regulating stem cell self-renewal or maintenance may be regulating reversion as well. Reversion as a

mechanism to replace cells during tissue damage and during normal aging could be a conserved mechanism.

1.2 Stem Cells and Aging

Aging can be summarized by a gradual decline in tissue and organ function. As stem cells are responsible for normal tissue turnover and repairing after damage, it is possible that changes in stem cell behavior leads to decline in tissue homeostasis during aging.

It is important to dispel the idea that stem cells are immortal. Given that even in the unicellular organism, the budding yeast, the mother yeast would eventually senescence. A stem cell can only go through serial transplantation a finite number before reconstitution efficiency decreases (Sinclair *et al.* 1998). Furthermore, stem cells that have gone through many replicative cycles yield changes due to DNA damage, telomere shortening, and senescence (Sahin & Depinho 2010). In addition to intrinsic changes to stem cells, influences from the niche as well as the systemic environment clearly affects stem cell behavior showing that extrinsic factors may also influence stem cell aging (Drummond-Barbosa 2008).

Higher organisms have tissues with varying stem cell properties; some stem cells are always in the process of producing large number of progenitors such as HSCs while others are quiescent waiting for damage or injury such as in the muscle stem cells (MSCs) (Rando 2006). However, both of these examples when active, regenerate tissues quickly. These highly proliferative stem cells

divide frequently and thus are likely subjected to replicative aging. Furthermore, the mother stem cell will also experience temporal aging so that highly active stem cell models may suffer from both replicative and temporal aging (Rando 2006). There are other tissues however, where tissue turnover is low and that regeneration is inefficient or non-existent such as in the brain and spinal cord. These differing characteristics likely will be reflected during the aging process. Thus, tissues that are mostly post-mitotic such as the brain, would experience mostly temporal aging attributed to the differentiated cells rather than the stem cells (Rando 2006). Since stem cells reside in niches which are normally fixed and post-mitotic, aging will likely be a result of replicative aging as well as dysfunctional interactions between stem cells and the aging niche. This section will focus on the 'stem cell theory of aging' hypothesizing that changes in stem cell function during aging is the cause of the decline in tissue homeostasis over time.

1.2.1 The stem cell niche and systemic signals during aging

Although some cell-intrinsic changes can be elucidated in culture, stem cells cannot merely be studied in isolation as the niche clearly plays a critical role in stem cell behavior. Intrinsic changes to stem cells during aging have been discovered by isolating stem cells from whole animals. HSCs and NSCs from aged animals were found to have elevated levels of p16ink4a tumor suppressor which leads to an arrest in cell cycle and that partially reducing this level allowed stem cells to behave more youthful (Sahin & Depinho 2010). DNA damage have

some implications on stem cell integrity, as irradiation leads to direct differentiation of melanocyte stem cells resulting in gray hairs (Inomata *et al.* 2009). These studies suggest that DNA damage and senescence contributes to a decline of stem cell behavior during aging.

Direct changes within the stem cell niche can dramatically alter stem cell activity during aging. In aged mice, muscle stem cells progenies have altered lineage after injury due to increased Wnt signaling (Brack *et al.* 2007). It has not been determined whether this signaling is from the niche or the systemic environment. Notch signaling has also been implied in regulating muscle stem cell behavior during aging but the identification of the source of Notch is still unknown (Conboy *et al.* 2005). In HSCs, one of the niche components, the osteoblasts, respond to increase of insulin-like proteins during aging resulting in increased HSC numbers (Mayack *et al.* 2010). The complexity of the mammalian tissues and the inability to study the native niches *in vivo* makes it difficult to pinpoint the exact cause of aging.

Systemic signals for regulating stem cell behavior are more nebulous because is difficult to isolate a factor that is everywhere. One benefit of using mice is the ability to share circulation between two different animals using parabiosis. Using a heterochronic pair of mice, young and aged, and an *in vitro* niche and stem cell culturing system, niche cells were restored to young conditions. Thus systemic signals may have a dominant effect on the stem cell niche and subsequently affecting the local stem cells (Mayack *et al.* 2010). Similarly an inhibitory systemic signal has been proposed for muscle stem cells

and liver stem cells using an injury-repair heterochronic system (Conboy *et al.* 2005; Brack *et al.* 2007). However, in these cases it is not known what exact factors are leading to this change in niche behavior.

1.2.2 Aging in *Drosophila*

The ability to study stem cells *in vivo* is ideal for understanding a complex phenomenon like aging. In addition to the well-characterized stem cell models in *Drosophila*, laboratory inbred wild type flies have a median life span of 40 days allowing aging studies to be relatively short.

Before our study described in Chapter 2, the study on the effects of aging on stem cell behavior was not extensive and had not been thoroughly described in *Drosophila*. Decreased spermatogenesis in aged flies has been noted for a while as testes where thinner and contained fewer germ cells (Hardy *et al.* 1979). However, stem cells were not attributed to this observation because the number of GSCs were determined by electron microscopy in young and one aged males was about the same (Hardy *et al.* 1979). Of course, the sample size was low and we know now that the number of GSCs between flies of the same age varies. This question was not revisited until much later, where a paper described that aging results in decreased number of GSCs and lower proliferation of GSCs (Wallenfang *et al.* 2006). Furthermore, that the rate that GSCs are lost is less than the predicted loss of GSCs based on the half-life suggesting that a maintenance mechanism is intact. Also, this was the first to address the niche, where they documented a decreased number of hub cells in aged males.

A parallel study in the *Drosophila* ovary (Pan *et al.* 2007) and our study described in Chapter 2 followed (Boyle *et al.* 2007). Both of our studies added the mechanism by which signaling from the niche directly leads to decreased GSC number and that decreased adhesion in the niche may also play a role in GSC maintenance during aging. Interestingly, overexpressing superoxide dismutase, an enzyme that removes free radicals, in GSCs in the ovary can prevent the loss of GSC number and proliferation overtime suggesting that free radicals/DNA damage may lead to intrinsic changes in GSCs (Pan *et al.* 2007).

Loss of tissue homeostasis has been also documented in the gut (Biteau *et al.* 2008). Unlike the observations in the germ line, aging results in the failure of ISC daughter cells to differentiate properly due to high levels of Notch leading to an accumulation of stem-cell like cells. Aberrant JNK signaling leads to sustained and misexpression of Notch during aging.

Since the two studies in the germ line, a study on the centrosome behavior during aging in the testis demonstrated that aging results in higher number of misoriented centrosomes in GSCs leading to cell cycle arrest complementing our previous observations that aged GSCs have a lower proliferation index (Cheng *et al.* 2008). This study demonstrated that stalled GSCs with misoriented centrosomes resume the cell cycle after the centrosomes are reoriented demonstrating a possible checkpoint to ensure asymmetric division (Cheng *et al.* 2008). The increase of misoriented centrosomes in aged males was correlated with increased reversion of spermatogonia whose centrosomes are normally not oriented. These data indicates that perhaps a

decreased GSC proliferation may not pertain to intrinsic defects in GSCs, and rather is due to the pathways regulating GSCs maintenance. My analysis of reversion as a role for stem cell maintenance during aging is described in Chapter 3.

GSCs are not the only components in the niche that are maintained during aging. The hub has been historically described as a static structure, but recent studies revealed that the hub is maintained by CySCs via signaling from a transcription factor, escargot during aging and also after injury (Voog *et al.* 2008){unpublished). Interestingly esg also seem to control the plasticity of the hub cells – loss of esg causes hub cells to revert back to CySCs and overexpression of esg leads to overproliferation of CySCs {Voog unpublished}. These data support that maintenance of both the niche and the stem cell populations are essential to maintain healthy tissues throughout life.

Recent data in our lab is revealing how signaling in the niche can be misregulated during aging. Chapter 2 showed that Upd RNA levels decrease during aging likely not due to transcriptional activity an Upd reporter line show strong expression in aged animals (Wong unpublished). Post-transcriptional regulation of Upd RNA is mediated by protection from miRNAs with the RNA binding protein, Imp (Katchalski unpublished). However during aging, IMP is targeted by microRNAs thereby exposing Upd mRNA for miRNA targeted degradation. Misregulation of miRNAs during aging has been shown to affect stem cells in mammals (Hammond & Sharpless 2008). Analysis of the

mechanisms regulating stem cell behavior during aging in *Drosophila* can be translated to mammalian systems.

Chapter 2: Decline in Self-Renewal Factors Contributes to Aging of the Stem Cell Niche in the *Drosophila* Testis

2.1 Summary

Aging is characterized by compromised organ and tissue function. A decrease in stem cell number and/or activity could lead to the aging-related decline in tissue homeostasis. We have analyzed how the process of aging affects germ line stem cell (GSC) behavior in the *Drosophila* testis and report that significant changes within the stem cell microenvironment, or niche, also occur that contribute to a decline in stem cell number over time. Specifically, somatic niche cells in testes from older males display reduced expression of the cell adhesion molecule DE-cadherin and a key self-renewal signal unpaired (upd). Loss of upd correlates with an overall decrease in stem cells residing within the niche. Conversely, forced expression of upd within niche cells maintains GSCs in older males. Therefore, our data indicate that age-related changes within stem cell niches may be a significant contributing factor to reduced tissue homeostasis and regeneration in older individuals.

2.2 Introduction

Adult stem cells, also known as tissue stem cells, support tissue homeostasis and repair throughout the life of an individual. However, maintenance and regeneration of tissues such as skin, liver, blood and muscle decrease dramatically with age. Cell intrinsic changes have been proposed to play a role in the observed decrease in stem cell function (reviewed in Rando, 2006). For example, increased expression of the cyclin dependent kinase inhibitor p16INK4a correlates with aging of hematopoietic stem cells (HSCs) and neural progenitor cells, possibly contributing to an age-related decline in HSC repopulating activity and neurogenesis (Janzen et al., 2006; Molofsky et al., 2006). Studies have also demonstrated that cell extrinsic changes contribute to a decline in the ability of aged stem cells to repair damaged tissues (Conboy et al., 2005).

Many stem cell populations lose the capacity for self-renewal when removed from the stem cell niche, suggesting that the local environment plays a major role in controlling stem cell fate (Morrison et al., 1997; Schofield, 1978). Therefore, changes to the niche could contribute to a decline in stem cell number and activity during aging. Transplantation studies suggest that the age-related decline in spermatogenesis observed in 2 year old mice is due to aging of the niche, rather than intrinsic changes within spermatogonial stem cells (Ryu et al., 2006; Zhang et al., 2006). However, the precise molecular changes within the stem cell environment have been difficult to assess, due to the lack of identified niche components in many stem cell models. Consequently, mechanisms

underlying the age-related decline in stem cell activity in many tissues are not well understood.

Drosophila is a well-established model for studying organismal aging and functional senescence, as well as mechanisms that regulate stem cell behavior (Grotewiel et al., 2005; Helfand and Rogina, 2003; Yamashita et al., 2005). *Drosophila* spermatogenesis is maintained by a population of GSCs that lie at the tip of the testis surrounding a cluster of somatic cells called the apical hub (Figure 1A)(Hardy et al., 1979). Hub cells secrete the ligand Unpaired (Upd) which activates the Janus kinase -Signal Transducer and Activator of Transcription (JAK-STAT) signal transduction pathway in adjacent GSCs to specify stem cell self-renewal (Harrison et al., 1998; Kiger et al., 2001; Tulina and Matunis, 2001). Therefore, hub cells are an essential component of the stem cell niche in the *Drosophila* testis (Kiger et al., 2001; Tulina and Matunis, 2001).

We have analyzed the effects of aging on stem cells and the niche in the *Drosophila* testis. Here, we show that levels of upd decline with age and that this loss correlates with a reduction in GSCs residing within the niche (Wallenfang et al., 2006). Conversely, forced expression of upd within hub cells maintains GSCs in older males. Therefore, our data indicate that a decline in self-renewal factors is one mechanism that contributes to decreased niche function with age, leading to reduced tissue homeostasis and impaired regeneration in older individuals.

2.3 Results

2.3.1 Age-related decline in GSCs

Asymmetric division of GSCs begins in the testis by late embryogenesis and maintains spermatogenesis throughout larval development and adulthood. Upon GSC division, the daughter cell that maintains contact with the hub retains stem cell identity, while the displaced daughter cell initiates differentiation as a gonialblast (Figure 1A; Hardy et al., 1979). Gonialblasts undergo 4 mitotic divisions to generate a cyst of 16 spermatogonia that differentiate into spermatocytes and, eventually, 64 mature sperm.

The mean lifespan of D. melanogaster is approximately 40 days (Helfand and Rogina, 2003). Analysis of testes from wild type 30 and 50-day old aged males revealed a marked decrease in spermatogenesis: testes from aged males were thinner than testes from young (1 to 2-day old) males and contained fewer differentiating germ cells (Figure 1B,C). One explanation for the observed decrease in spermatogenesis with age is loss of GSCs. Quantification of GSC number in testes of 1, 30 and 50-day old males revealed a significant decrease from an average of 8.3 GSCs/testis in 1-day to 6.11 GSCs/testis in 30-day and 5.1 GSCs/testis in 50-day old OregonR males ($p<0.05$; Figure 1D,E; Table S1; Figure S1A,B). These data are consistent with studies reporting a 25% decrease in GSCs in 35-day old males (Wallenfang et al., 2006).

Although the number of GSCs decreased in older males, the remaining GSCs continued to express hallmarks of stem cells. GSCs contain a spherical,

cytoplasmic structure called a fusome, which becomes branched as germ cells differentiate as interconnected spermatogonia (Figure 1F; Lin et al., 1994). GSCs in aged testes contained spherical fusomes; however, highly branched fusomes were found closer to the tip of the testis when compared to testes from 1-day old males, indicating fewer differentiating spermatogonial cysts (Figure 1F,G). Furthermore, single germ cells adjacent to the hub of older males showed no overt signs of differentiating prematurely (Figure 1H,I). The bag of marbles (bam) gene is expressed in differentiating spermatogonia within 4-16 cell cysts and is required for limiting the number of mitotic divisions (Gönczy et al., 1997; McKearin and Ohlstein, 1995). Testes stained with antibodies for the cytoplasmic pool of Bam protein (BamC) indicated that dividing spermatogonia within older testes initiate differentiation similar to spermatogonia in 1-day old males.

2.3.2 Decline in GSC divisions in testes from aging males

To determine whether GSCs in testes from older males continue to divide, antibodies to phospho-histone H3, a marker of mitosis, were used to stain testes at 1 and 50-days. At 1-day, an average of 0.9 dividing GSCs/testis were observed (n=20), whereas an average of 0.4 dividing GSCs/testis were observed at 50-days (n=50; Figure 1J,K). The decrease in dividing GSCs was significant ($p<0.05$) and similar to the 40% reduction in total GSCs surrounding the hub (Table S1).

Staining with another marker of cell cycle progression verified that GSCs present in testes from old males continue to divide, albeit less often than GSCs in testes from young males. BrdU labeling, which marks cells in S phase, showed a decrease in the average number of dividing GSCs in aged OregonR testes: 1.6 BrdU+ GSCs/testis in 1-day old males (n=51), in contrast to an average of 0.8 BrdU+ GSCs/testis in 50-day old males (n=59). These data revealed a significant drop in GSC proliferation rate in 50-day old males when compared to 1-day old males (S-phase index= average number of BrdU+ GSCs/average number of GSCs, $p<0.05$; Table S2) and are consistent with studies reporting a decline in GSC activity at earlier times (Hardy et al., 1979; Tran et al., 2000; Wallenfang et al., 2006). We conclude that fewer GSCs, in combination with decreased GSC divisions, contribute to fewer differentiating progenitor cells (spermatogonia and spermatocytes), ultimately leading to the observed decrease in spermatogenesis in aged males.

The average number of GSCs expressing high levels of cyclin E, a regulator of progression through the G1 phase of the cell cycle, increased in testes from older males (Figure S2). Cyclin E levels varied, with a distribution of high and low levels in GSCs from 1-day old males (n=20). However, testes from 50-day old males showed more GSCs expressing high levels of cyclin E (Figure S2C). High cyclin E levels are consistent with an arrest in or extension of G1 and may explain the subtle decrease in the percentage of cells progressing through S phase (Hatfield et al., 2005; Table S2). An age-related accumulation of Dacapo, the *Drosophila* homolog of the CIP/KIP family of cyclin dependent kinase

inhibitors, was not apparent in GSCs from older males (data not shown; de Nooij et al., 1996; Lane et al., 1996). However, upregulation of dacapo expression or protein is not always coupled to cell cycle arrest (Meyer et al., 2002).

A second stem cell population, the somatic cyst progenitor cells (CPCs), is located at the tip of the testis (Figure 1A; Hardy et al., 1979). Self-renewing CPCs contact both GSCs and hub cells and give rise to cyst cells that enclose the differentiating gonialblast. CPCs are distinguished from other somatic cells in the testis based on their mitotic activity and position adjacent to the hub (Hardy et al., 1979). Dividing somatic cells were detected adjacent to the hub in both 1 and 50-day old males using both pHH3 staining and BrdU incorporation (Figure 1K; Table S2). However, an assessment of any aging-related decline in CPC number or division rate was hindered by a lack of specific markers to distinguish CPCs from differentiating cyst cells at early stages.

2.3.3 The apical hub remains intact in testes from aged males

We next examined whether there were detectable changes to a key component of the stem cell niche in the testis: the apical hub. The hub consists of approximately 8-16 cells that express high levels of cell adhesion molecules such as Fasciclin III (FasIII; Gönczy et al., 1997) and the *Drosophila* homologs of E-cadherin, (DE-cadherin or DE-cad) and neural cadherin, DN-cadherin (DN-cad; Le Bras and Van Doren, 2006).

The hub was still detectable in testes from aged males as assayed by four markers (Figure S3). Quantification of hub cell number using DN-cad revealed a

small, but significant drop in the total number of cells from 11.4 ±0.48 at 1-day (n=23) to 9.3 ±0.35 at 50-days (n=23; $p<0.05$), and only 2.0% of testes (3/147) showed complete loss of FasIII staining in OregonR males (± = Standard Error; Figure S3A,B). The small decline in hub cell number is consistent with, but less dramatic than, previous findings (Wallenfang et al., 2006). Although the overall diameter of the hub was normal in the majority of testes from older males, an increase in hub diameter from 10-15 μM to larger than 20 μM was observed in 5.4% (8/147) of the testes examined, as assayed by FasIII staining (Figure S3D-F). An expansion of somatic cells in testes and ovaries often accompanies loss of germ cells, one example being an expansion of FasIII expressing cells in agametic gonads from young flies (Gönczy and DiNardo, 1996; Margolis and Spradling, 1995).

A consistent decrease in DE-cad levels was also observed within the hub in testes from aged males compared to 1-day old testes (Figure 2A'', B"). In contrast, significant changes in DN-cad levels were not observed (Figure 2C, D). The *Drosophila* homolog of β−Catenin, Armadillo, was still expressed and co-localized with DE-cad, suggesting that the remaining DE-cad was capable of binding to core adherens junctions components (Figure 2E). As DE-cad is required for stem cell maintenance in the *Drosophila* gonad, a decrease in expression in either hub cells or GSCs could contribute to loss of GSCs with age (Song and Xie, 2002; Song et al., 2002; Voog and Jones, in preparation).

2.3.4 Age-related decline in stem cell niche function

Previous analyses demonstrated that upd is sufficient for specifying GSC self-renewal in the *Drosophila* testis (Kiger et al., 2001; Tulina and Matunis, 2001). Therefore, we assayed upd expression within hub cells in testes from aging males. Analysis of upd mRNA expression showed a progressive loss in hub cells in testes from aged males (Figure 3A-C). While wild type levels of upd expression in the hub were detected in 99% (103/104) of 1-day old testes, only 30-40% of testes from 30-day (49/119) and 50-day old (25/82) males displayed wild type or nearly wild type levels of upd (Figure 3A-C, I; Figure S4F). In contrast, expression of the transcription factor escargot, which is normally detected in hub cells and surrounding stem cells, was still evident in hub cells in testes from 30 and 50-day old flies, despite a significant decrease in the surrounding stem cells (Figure S4).

Declining upd expression in the testis during aging could be specific to the male GSC niche or reflect a general decline in upd expression levels with age. To distinguish between these possibilities, we examined upd expression in ovaries from aging females. Expression of upd in distinct subsets of cells within the ovary activates JAK-STAT signaling to specify and guide migration of specialized somatic cells (McGregor et al., 2002; Silver and Montell, 2001). In a majority of ovarioles from 50-day old females (114/116), upd expression was still detectable (Figure 3D,E), indicating that aging-related changes in upd levels do not occur in all tissues in which upd is expressed.

Decreased upd expression in hub cells suggested that JAK-STAT signaling might be compromised within the niche. Stat92E is known to regulate its own expression, leading to an increase in Stat92E protein. Therefore, an overall increase in Stat92E protein in combination with Stat92E nuclear localization can be used to assay JAK-STAT pathway activation (Wawersik et al., 2005). The average number of Stat92E positive (Stat+) germ cells was 15 in 1-day (n=40), 9.8 in 30-day (n=50), and 6.9 in 50-day old (n=51) OregonR testes, which corresponded to 7.8 Stat+ GSCs at 1 day (n= 20), 5.5 Stat+ GSCs at 30 days (n= 23), and 4.7 Stat+ GSCs at 50 days (n=22; Figure 3F-H, Table S3, S4). These data demonstrate a significant decrease in early germ cells responding to JAK-STAT signaling in aged males ($p<0.05$).

2.3.5 Constitutive expression of upd in hub cells delays loss of GSCs

To determine whether sustained upd expression could rescue the aging-related decline in GSCs, flies in which upd was constitutively expressed in hub cells were aged (Brand et al., 1994). A modest, yet significant increase in GSCs was observed in testes from these males when compared to age-matched controls (Figure 4A,B; Table S3). Furthermore, upd expression was now easily detectable in the majority of 50-day old testes (Figure S4D-F). When another transgenic line was used to express upd in hub cells, GSC loss was essentially blocked in aging males. The average number of GSCs in testes from 30-day old males remained constant at 7.4 GSCs/testis (n=68), corresponding to an average of 6.0 Stat+ GSCs/testis (n=22; Figure 4; Table S3, S4). In contrast, controls

showed the expected 20-25% decline to 6.4 GSCs/testis (n=38), which corresponded to 4.6 Stat+ GSCs/testis (n=27, $p<0.05$; Figure 4, Table S3, S4). Therefore, not only were GSCs maintained upon constitutive expression of upd, but JAK-STAT activation was also preserved, suggesting that the decrease in upd expression in hub cells is an important contributing factor to the decline in GSCs with age.

Despite the maintenance of JAK-STAT responsive GSCs, testes in which upd was constitutively expressed in hub cells showed a decline in the total number of germ cells responding to the pathway, as few or no Stat+ gonialblasts were observed (Figure 4E, F; compare to Figure 2F; Table S4). One explanation for this could be a decline in the proliferation rate of GSCs, leading to a decrease in the pool of early germ cells available to respond to JAK-STAT signaling. GSC proliferation rates in testes constitutively expressing upd in the hub were assayed using BrdU incorporation, which revealed a significant decrease in GSC proliferation between 1 and 30 days (Figure 4J). However, when compared to 30-day old controls, a further decrease in the rate of GSC proliferation was observed (Figure 4J). Consistent with this observation, cyclin E levels in GSCs in testes in which upd expression was maintained in the hub were also considerably higher than in 30-day controls (Figure S2). Therefore, a decrease in the rate of GSC proliferation upon constitutive expression of upd in hub cells could provide one explanation for the decline in early germ cells responding to the JAK-STAT pathway.

Consistent with a decline in GSC proliferation and loss of Stat+ gonialblasts, spermatogenesis was not restored in testes in which upd is constitutively expressed in hub cells. In fact, testes from 30-day old males were thinner than controls and resembled testes from considerably older males, due to a dramatic decrease in developing germ cells found within the testis lumen (Figure 4H, I; compare to Figure 1 A,B). Furthermore, these flies did not live much beyond 30 days, preventing any analysis at later time points. Based on the accelerated decline in GSC proliferation and spermatogenesis and shortened life span, we hypothesize that flies overexpressing upd at higher than physiological levels, but within the normal domain, may be dying due to acceleration of the aging process.

2.4 Discussion

Our data indicate that aging results in molecular changes within a stem cell niche, including a decrease in expression of a key self-renewal factor, upd. We propose that the reduction in upd reflects a change in niche function, which contributes to loss of GSCs over time. Ultimately, fewer GSCs within the niche leads to a subsequent decrease in the number of progenitor cells that generate mature sperm, resulting in an overall decrease in spermatogenesis in aged males.

In addition to the decline in upd expression, we observed a consistent decrease in DE-cad levels within the hub in testes from aged males (Figure 2). As localized adherens junctions have been demonstrated to be required for

holding stem cells within the niche, close to self-renewal signals, a decrease in DE-cad expression in either hub cells or GSCs could contribute to GSC loss in aged testes (Song and Xie, 2002; Song et al., 2002). Experiments to examine DE-cad function within hub cells and GSCs during aging are ongoing. However, in contrast to upd, our data to date suggest that overexpressing DE-cad in the hub alone does not result in GSC maintenance in older males (Boyle and Jones, unpublished data). In addition, a considerable decrease in upd expression in niche cells was observed at 30 days, which preceded any detectable change in DE-cad expression; therefore, the decrease in upd expression is one of the first molecular hallmarks of aging within the niche.

Clearly, multiple factors are likely to influence GSC number and activity over time. Similar to what has been proposed in mammalian systems, cell autonomous changes may occur that either block cell cycle progression or prevent stem cells from responding to self-renewal cues (Janzen et al., 2006; Molofsky et al., 2006; Ryu et al., 2006; Figure S5). Variation in systemic factors, such as *Drosophila* insulin-like peptides (dILPs), ecdysone, or juvenile hormone (JH), could also act directly or indirectly to affect stem cell activity during aging (Conboy et al., 2005; LaFever and Drummond-Barbosa, 2005). Understanding how local, systemic and cell autonomous changes are integrated to elicit changes in stem cell behavior that occur over time presents an exciting challenge (reviewed in Rando, 2006).

Despite a net decrease in stem cells, the actual decline in GSCs during aging is less than one might predict. A recent study of the dynamics of GSC

behavior in 30-day old males revealed the half-life of GSCs to be 14 days (Wallenfang et al., 2006). However, more than half (65%) of GSCs remain at 50 days, suggesting that mechanisms are in place to maintain a stem cell pool within the niche (Table S1; Wallenfang et al., 2006). Similar findings have been made for GSC maintenance in the *Drosophila* ovary (Margolis and Spradling, 1995; Xie and Spradling, 1998, 2000). Replacement of lost stem cells could occur through symmetric divisions of remaining GSCs, as was shown for female GSCs in *Drosophila* (Xie and Spradling, 2000). Another possibility is that dedifferentiation of spermatogonia into functional GSCs maintains GSCs in older males (Brawley and Matunis, 2004). An extension of the half-life of male GSCs to longer than 14 days or a slowed division rate (> 32 hours) could also delay stem cell exhaustion. The observed increase in cyclin E levels in aged GSCs may indicate an arrest or significant delay in the G1 phase of the cell cycle (Figure S2).

Previous studies have shown that JAK-STAT signaling is necessary and sufficient to specify stem cell self-renewal in the *Drosophila* testis (Kiger et al., 2001; Tulina and Matunis, 2001). Here we show that constitutive expression of upd within the hub maintains GSCs during aging. In addition to specifying self-renewal, Upd may also facilitate, either directly or indirectly, the ability of existing germ cells to replace lost GSCs, thereby maintaining an adequate number of GSCs within the niche. We predict that as upd levels within the hub diminish during aging, mechanisms that serve to maintain GSCs are compromised, leading to loss of GSCs. Mechanisms that replace lost stem cells to maintain an

active stem cell pool must be in constant competition with cell autonomous, local and systemic changes that occur during aging (Figure S5); once stem cell replacement becomes less efficient, the overall balance tips toward loss of active stem cells. It will be interesting to determine whether aging directly or indirectly affects mechanisms contributing to stem cell maintenance once they have been identified.

Our data suggest that aging results in loss of functional stem cell niches. Compromised niche function over time may lead to the selection of stem cells that acquire the ability to self-renew independently of the niche and/or progenitor cells that acquire self-renewal capabilities (Li and Neaves, 2006). Such cells could be the precursors to cancer stem cells that contribute to tumorigenesis in a variety of tissues. In addition to laying the groundwork for investigating how the process of aging can affect tissue homeostasis, these studies also have important implications for stem cell-based therapies. We predict that tissue stem cells transplanted into older individuals may be unable to initiate self-renewing divisions to functionally replace damaged or diseased tissues without co-transplantation of “younger” niche cells or mobilization of endogenous stem cells from functional niches.

2.5 Methods

2.5.1 Fly husbandry and stocks

Flies were raised at 25ºC on standard cornmeal-molasses-agar medium. Flies for aging experiments were supplemented with fresh yeast paste, and vials

were changed every 3 days. Aging flies were obtained by collecting newly eclosed 0-1 day old flies (~20 males and 20 females/vial, and ~30 males/vial when maintained in isolation). The updGAL4, UAS-gfp (or E132GAL4, UAS-gfp) was a gift from E. Bach. Two UAS-upd lines were used (gifts from D. Harrison; Harrison et al., 1998): one transgene is inserted on chromosome II; the other (w-; UAS-upd, TM2) is an insertion on chromosome III and consistently gives a stronger phenotype when overexpressed in early germ cells (Kiger et al., 2001; M. Boyle and L. Jones, unpublished data).

2.5.2 Immunofluorescence and microscopy

Testes were dissected into phosphate buffered saline (PBS) and examined using phase contrast microscopy. Immunofluorescence (IF) experiments on squashed testes were performed as described (Kiger et al., 2001) or as follows: testes were dissected and fixed in 2% PFA in PLP buffer (0.075 M lysine, 0.01 M sodium phosphate buffer pH7.4) for 1 hour at RT, rinsed in PBS, followed by standard IF staining, using antibodies listed in below.

Phase contrast images of squashed testes were obtained using a Leica DM5000 microscope equipped with a DC500 camera using Firecam imaging software (version 1.7.1; Leica Microsystems). All other images were obtained using a Zeiss Axiovert 200 microscope and processed using AxioVision (version 4.5; C. Zeiss) and Adobe Photoshop software (Mountain View, CA), except for the analysis of Armadillo and DE-Cadherin staining which was obtained using a

Leica TCS SP2 AOBS confocal microscope using LCS Lite 2.61.1537 software (Leica Microsystems).

The polyclonal rabbit anti-Vasa (1:2000, gift from P. Lasko), rat anti-BamC (1:50) (gift from D. McKearin), rabbit anti-STAT92E (1:500) (gift from S. Hou), guinea pig anti-traffic jam (1:3000) (gift from D. Godt), guinea pig anti-cyclin E (1:1000) (gift from T. Orr-Weaver), mouse anti-Dacapo (1:4) (gift from C. Lehner), mouse anti-BrdU (1:100) (BD Biosciences), mouse anti-GFP (1:200) (Molecular Probes), and the rabbit anti-phosphorylated Histone H3 (1:200) (Upstate Biotechnologies), were used at indicated concentrations. Mouse anti-a-spectrin (3A9)(1:10), mouse anti-Fasciclin III (7G10)(1:10), rat anti-DE-Cadherin (DCAD2)(1:20), rat anti-DN-Cadherin (Ex#8)(1:20), and mouse anti-Armadillo (N2 7A1)(1:10) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences. Secondary antibodies were obtained from Molecular Probes. Samples were mounted in Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

2.5.3 Quantification of GSCs and hub cells

Germ cells were counted as stem cells only when contacting FasIII+ hub cells. Only those samples with an easily distinguishable hub of normal size (10-15μm) were included in experiments to quantify GSC number. Hub cells were

identified by analyzing serial sections through the tips of testes stained with an antibody to DN-cad and DAPI to stain hub cell nuclei (see Supplemental Data).

2.5.4 BrdU incorporation

BrdU incorporation was performed as described (Gönczy and DiNardo, 1996) with the following modifications: males were starved in empty vials with a wet vial plug inserted inside the vial overnight (14-18 hours). Flies were transferred without CO2 into new vials and fed 100mM BrdU in grape juice for 30 minutes and dissected immediately.

BrdU incorporation was analyzed using IF as follows: primary antibodies, other than anti-BrdU, were applied for 3 hours at 37ºC or overnight at 4ºC and fixed for 7 minutes in 4% formaldehyde. DNA was subsequently denatured by pre-treating slides with 2M HCl for 30 minutes. Samples were washed for 1 hour with PBS before the BrdU antibody was applied, and standard IF was performed.

2.5.5 RNA *in situ* hybridization

RNA *in situ* hybridization was performed as described (Kiger et al., 2001). Probes were generated from linearized plasmid using the Roche Molecular Biochemicals RNA-labeling kit.

2.5.6 Statistical Analyses

Statistical analyses were conducted using a Student's two-tailed t-test assuming equal variance. Alpha values are significant when $p<0.05$. Comparison of S Phase Indices was performed as follows: the S phase index (average number of BrdU positive GSCs / average number of GSCs) for each testis was

transformed using the arcsin of the square root to normalize the data. The transformed data were subsequently analyzed by the Welch t-test, testing means equal and allowing standard deviation not equal, comparing either two age groups or two genotypes using JMP (vers. 5.1.2 SAS Institute Inc., Cary, NC). The probability value of $p<0.05$ was denoted to be statistically significant.

2.5.7 Analysis of hub size and markers

Hub cells were identified by a combination of DN-cadherin and with DAPI to highlight the hub cell nuclei. DN-cadherin was chosen because it is enriched at hub cell-hub cell junctions as well as hub cell-stem cell junctions (Le Bras and Van Doren, 2006), and our analyses indicate that levels of DN-cadherin do not change with age. A significant decrease in the average number of hub cells was observed in OreR males from 1 to 50 days. However, a significant decrease was not observed in testes from UAS-upd, TM2/+ or updGAL4, UAS-gfp; UAS-upd, TM2/+ males when compared at 1 and 30 days.

Analysis to determine hub size/diameter was performed simultaneously with quantification of GSCs, using FasIII as a hub marker. Hub diameter was measured along the longest axis using the Measure tool, which is a component of Axiovision software (ver.4.5; Carl Zeiss). Throughout these studies, we have analyzed hub morphology using the hub markers FasIII, DE-cadherin, DN-cadherin, and Arm in multiple genetic backgrounds, including OregonR, updGAL4; updGAL4, UAS-gfp updGAL4, UAS-gfp; UAS-upd; updGAL4, UAS-gfp; UAS-upd, TM2 and observed similar changes to the hub. However, flies in

which an α-tubulinGFP transgene is overexpressed in germ cells have drastically altered hub morphology at 50 days, which is why this genotype was largely excluded from the present studies.

Intensity of cyclin E in GSCs and DE-cadherin and DN-cadherin staining in hub cells was quantified by mean densitometric analysis of serial Z-sections using Axiovision software (ver.4.5; Carl Zeiss). Equivalent exposure times were used during imaging. The distribution of cyclin E levels was separated into low (mean pixel density between 0-30), medium (30-50) and high (>50). The number of GSCs exhibiting an average mean pixel density of >50 increased significantly between 1 and 50 days ($p < 0.001$) for both genotypes. For DE-cadherin levels, the mean pixel density for each genotype was: 1d OreR, 37.3 (n=24); OreR 50d, 24.4 (n=23); 1d updGAL4, UAS-gfp; UAS-upd, 32.9 (n=32); 50d updGAL4, UAS-gfp; UAS-upd, 25.0 (n=28). The decrease in mean pixel density between 1 and 50 days was statistically significant ($p < 0.001$) for both genotypes. For DN-cadherin the values were: 1d OreR, 10.4 (n=32); OreR 50d, 9.4 (n=34). The decrease from 10.4 to 9.4 was not statistically significant.

2.6 Acknowledgments

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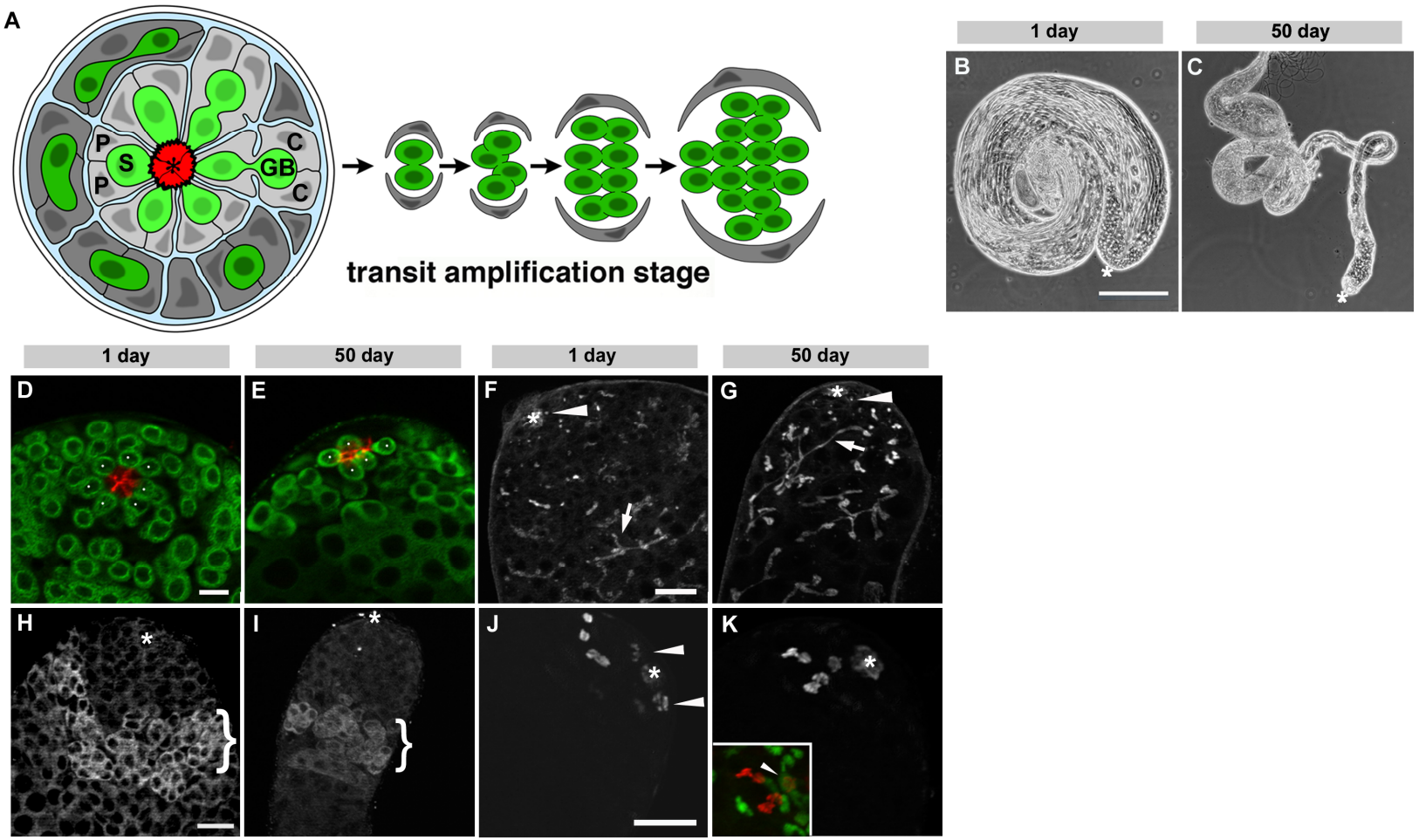
Figure 2.1 Decreased spermatogenesis and GSC loss with age

(A) The apical tip of the *Drosophila* testis illustrating the early stages of spermatogenesis (modified from Hardy et al., 1979). GSCs [S] and somatic cyst progenitor cells, CPCs [P], surround the hub (red). Gonialblasts [GB] are enveloped by cyst cells [C] and undergo 4 mitotic divisions to create a cyst of 16 spermatogonia. (B, C) Phase contrast images of testes from (B) 1 and (C) 50-day old yw; nanosGAL4:VP16,UAS-α−tubulin:gfp (Tubulin:GFP) adults. * marks the testis tip. Note decreased size at 50 days. (D, E) Staining for the germ cell antigen Vasa (green) and the cell surface protein Fasciclin III (FasIII, red) in the hub in (D) 1 and (E) 50-day old males. GSCs are marked by a white dot, with 7 GSCs in D and 5 GSCs in E. (F, G) FasIII (*) staining and α-spectrin, which marks spherical fusomes in GSCs and GB (arrowheads) and branched fusomes in spermatogonia (arrows). (F) One section through a 1-day and (G) projection of 5 through a 50-day old testis, each with fusomes in germ cells near the hub (arrowheads). Note branched fusomes, representing 8-16 cell spermatogonial cysts, closer to the tip in G. (H, I) Staining for BamC, a 4-8 cell spermatogonial cyst marker in (H) 1 and (I) 50-day old Tubulin:GFP testes. Note reduced BamC region (brackets) in I. (J, K) Staining for phospho-histone H3 (pHH3) to label mitotic cells in (J) 1 and (K) 50-day old testes. Note dividing GSCs (arrowheads) and 2 cells of a 4-cell cyst in J and a 2-cell cyst in K. Inset shows a dividing 2-cell cyst labeled by pHH3 (red) and a dividing cell (arrowhead) positive for Traffic Jam (TJ, green), a marker of CPCs and early cyst cells. Dividing GSCs were counted as pHH3+, TJ- cells adjacent to the hub. Scale bars: B, 250 μM; D, 10 μM; F, H, J, 20 μM. Testes are from OregonR flies and all images are single sections unless otherwise indicated. * marks hub.


A
P
S
P
GB
C
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transit amplification stage
1 day
50 day
B
C
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50 day
1 day
50 day
D
E
F
G
H
I
J
K

Figure 2.2 Aging-related changes to the apical hub.

(A-D) View of the hub stained with FasIII (red) and the cell adhesion molecules DE-cadherin (DE-cad, green, A,B) or DN-cadherin (DN-cad, green, C, D) in 1 (A, C) and 50-day old (B, D) testes. DE-cad expression level is decreased in 50-day old testes compared to 1-day (compare A'' to B''). In contrast, DN-cad levels are similar at 1 and 50-days (compare C" to D"). Note change in FasIII localization: at 1-day, FasIII is concentrated along hub cell/hub cell junctions (arrowhead in A'), with little or no staining along the hub periphery. In aged testes, FasIII is often present at the periphery (arrowhead in B'). (E) View of the hub stained with DE-cad (red, E') and Armadillo (green, E'') in 50-day old testis. Germ cells stained for Vasa (blue). * marks GSCs. Scale bars, 10μM.

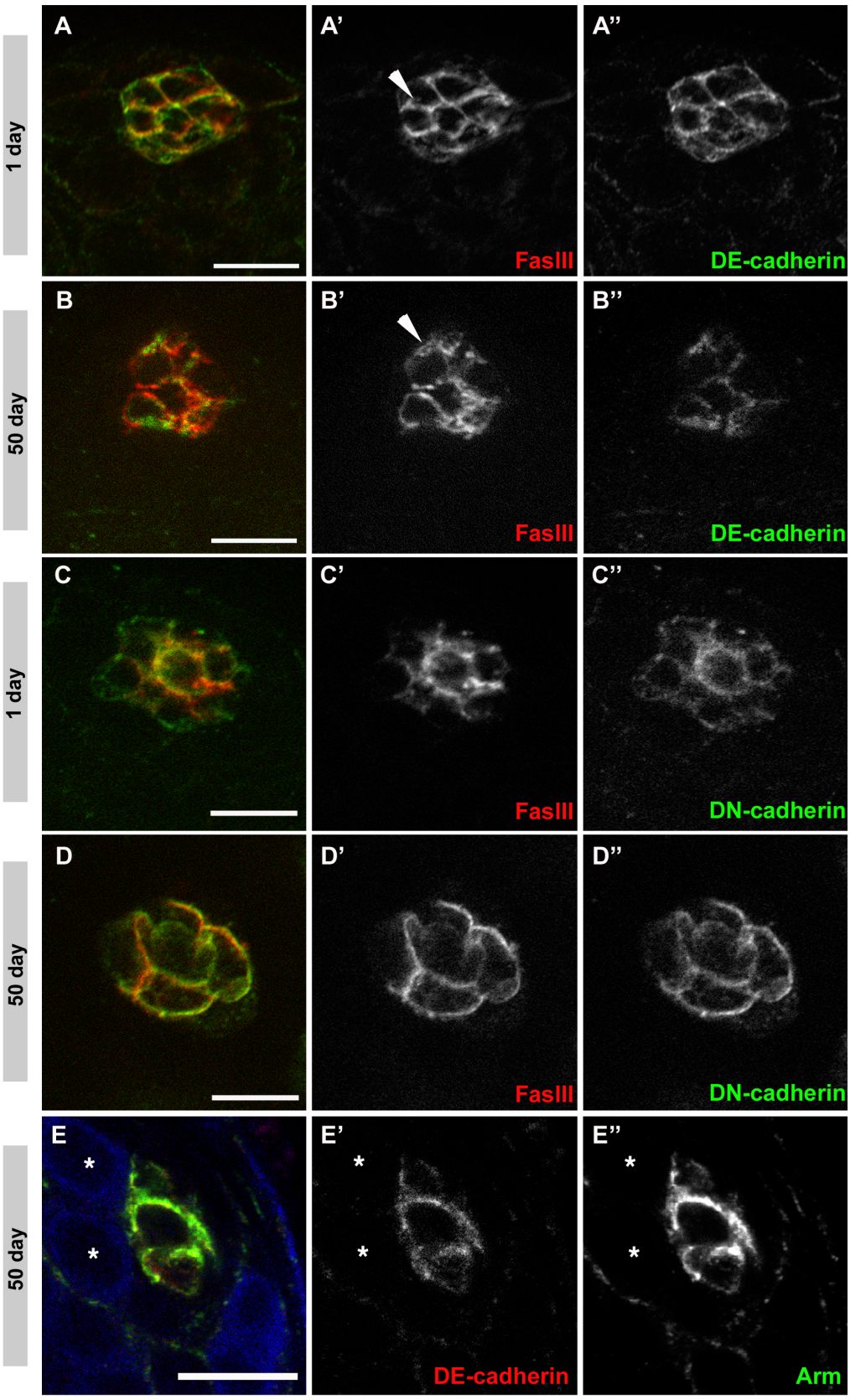

1 day
A
A'
A''
FasIII
DE-cadherin
50 day
B
B'
B''
FasIII
DE-cadherin
1 day
C
C'
C''
FasIII
DN-cadherin
50 day
D
D'
D''
FasIII
DN-cadherin
50 day
E
E'
E''
DE-cadherin
Arm

Figure 2.3 Aging-related changes in JAK-STAT signaling.

(A-C) RNA *in situ* analysis for upd, which encodes a ligand of the JAK-STAT pathway, in the hub in (A) 1 (B) 30 and (C) 50-day old testes. B shows a 50% decrease compared to wild type, with complete loss of expression shown in C. (D, E) upd mRNA expression in polar follicle cells (pfc, arrows) in ovarioles from (D) 2 and (E) 50-day old females. (F-H) Staining for Stat92E, a target of the JAK-STAT pathway, in (F) 1 (G) 30 and (H) 50-day old testes showing fewer Stat92E positive (Stat+) cells with age. * marks hub. (I) Distribution of upd expression levels in testes from 1, 30 and 50-day old flies. Numbers are combined from 4 independent experiments. Scale bars: A, F, 20 μM; D, 50 μM.

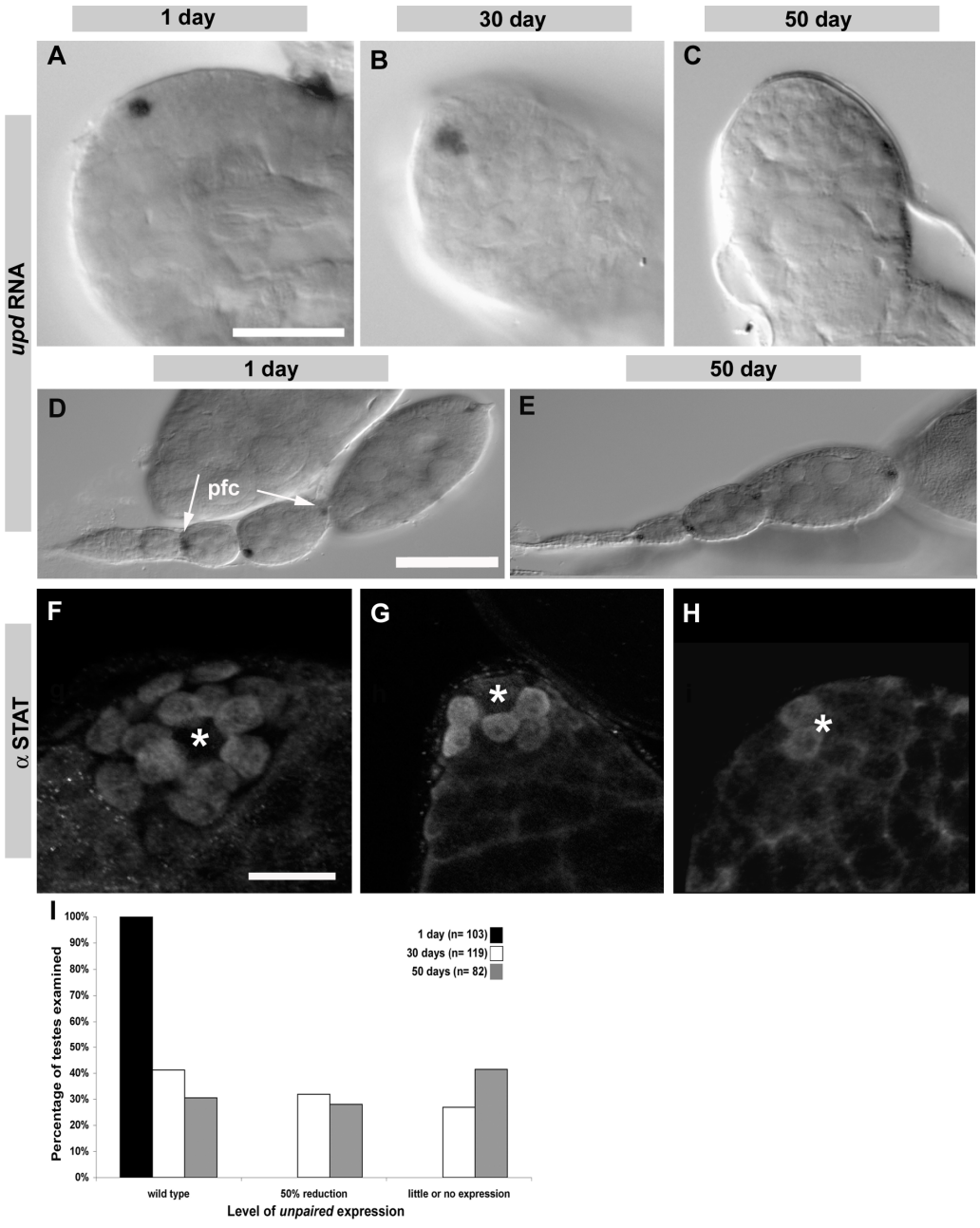
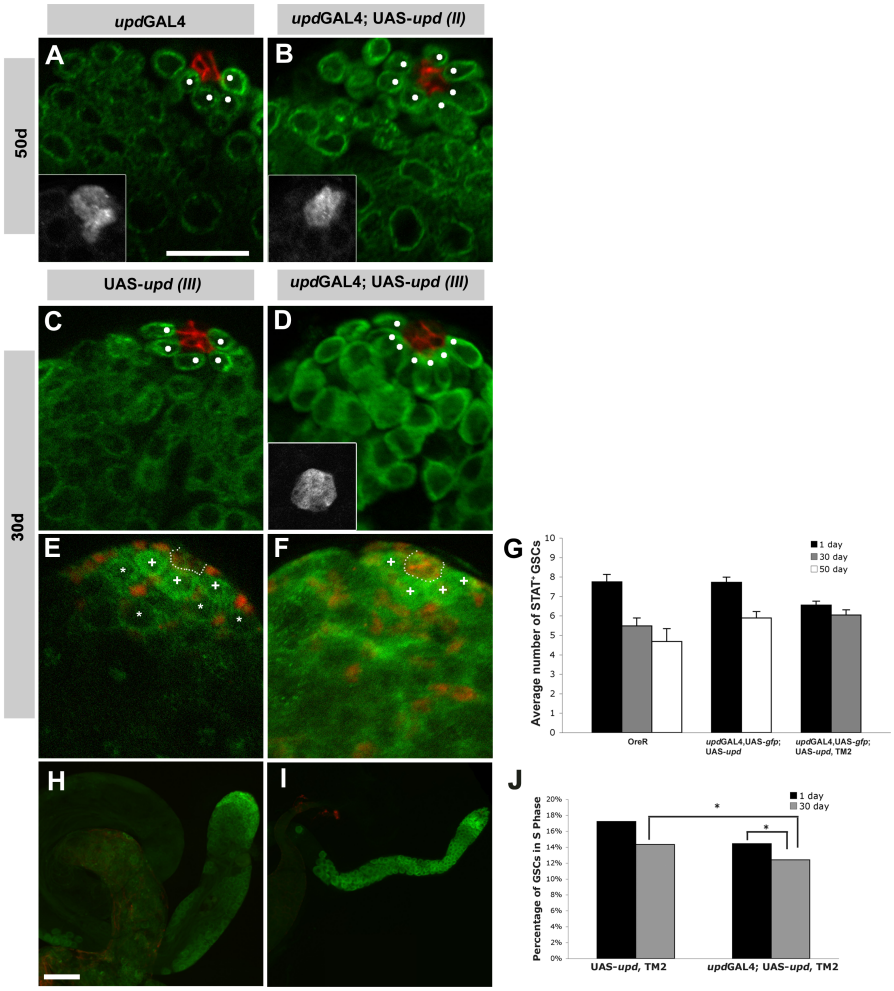

1 day
30 day
50 day
A
B
C
upd RNA
1 day
50 day
D
pfc
E
F
G
H
α STAT
I
1 day (n= 103)
30 days (n= 119)
50 days (n= 82)
Percentage of testes examined
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
wild type
50% reduction
little or no expression
Level of unpaired expression

Figure 2.4 Constitutive expression of upd delays loss of GSCs during aging.

The GAL4-UAS system, which allows temporal and tissue specific gene expression, was used to express upd in hub cells. (A, B) Testes stained for Vasa (green) and FasIII (red) from 50-day old (A) updGAL4,UAS-gfp and (B) updGAL4,UAS-gfp; UAS-upd males. (C, D) Vasa (green) and FasIII (red) staining in 30-day old (C) UAS-upd,TM2/+ and (D) updGAL4,UAS-gfp; UAS-upd,TM2/+ males. Insets: GFP staining in hub cells. Dots mark GSCs. (E, F) Stat92E+ cells in testes overexpressing upd in hub cells. Testes stained for Stat92E (green) and TJ (red) in 30-day old (E) UAS-upd,TM2/+ and (F) updGAL4,UAS-gfp; UAS-upd,TM2/+ males. + marks Stat+ GSCs. * marks Stat+ gonialblasts, GB. (E) 3 of 6 total GSCs are evident in this section, while 4 of 7 GBs are visible. (F) 4 of 7 total GSCs and 0 of 2 total GBs are evident in this section. Note activation of JAK-STAT pathway in the hub (outline) as visualized by Stat92E staining (compare with Fig. 2 F-H). (G) Maintenance of Stat+ GSCs upon constitutive expression of upd in both updGAL4,UAS-gfp; UAS-upd and updGAL4,UAS-gfp; UAS-upd,TM2/+ males as compared to OregonR males during aging. (H, I) Low magnification views of testes from 30-day old (H) UAS-upd,TM2/+ and (I) updGAL4,UAS-gfp; UAS-upd,TM2/+ males. (J) Graph representing drop in proliferation rate upon constitutive expression of upd from 14.4% in 1-day to 12.4% in 30-day old updGAL4,UAS-gfp; UAS-upd,TM2/+ males. * - statistically significant ($p<0.05$). Genotypes: (A) updGAL4,UAS-gfp(X); +/+; +/+. (B) updGAL4, UAS-gfp(X); UAS-upd/+(II); +/+. (C, E, G)+/Y; +/+; UAS-upd,TM2/+(III) (D, F, H) updGAL4, UAS-gfp(X); +/+; UAS-upd,TM2/+(III). Scale bars: A- F, 20 µM; H, I, 100 µM.


updGAL4
updGAL4; UAS-upd (II)
50d
A
B
UAS-upd (III)
updGAL4; UAS-upd (III)
30d
C
D
E
F
H
I
G
Average number of STAT+ GSCs
1 day
30 day
50 day
OreR
updGAL4,UAS-gfp; UAS-upd
updGAL4,UAS-gfp; UAS-upd, TM2
J
Percentage of GSCs in S Phase
1 day
30 day
UAS-upd, TM2
updGAL4; UAS-upd, TM2

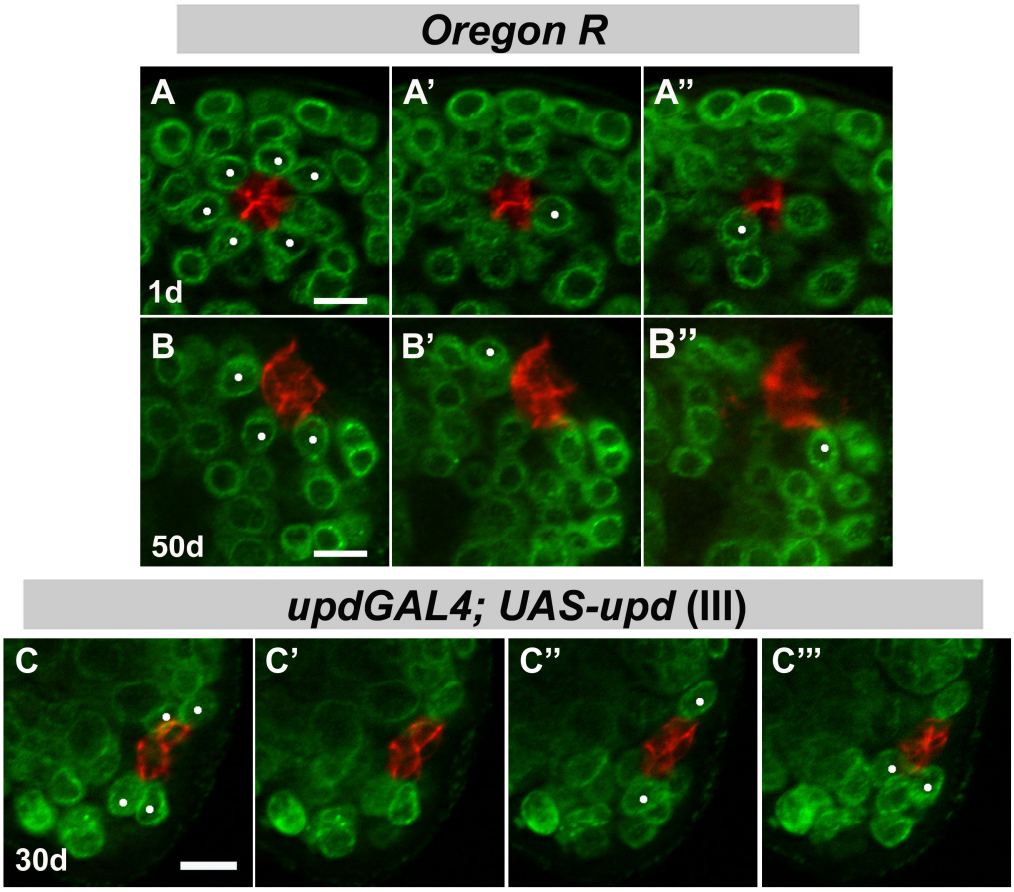
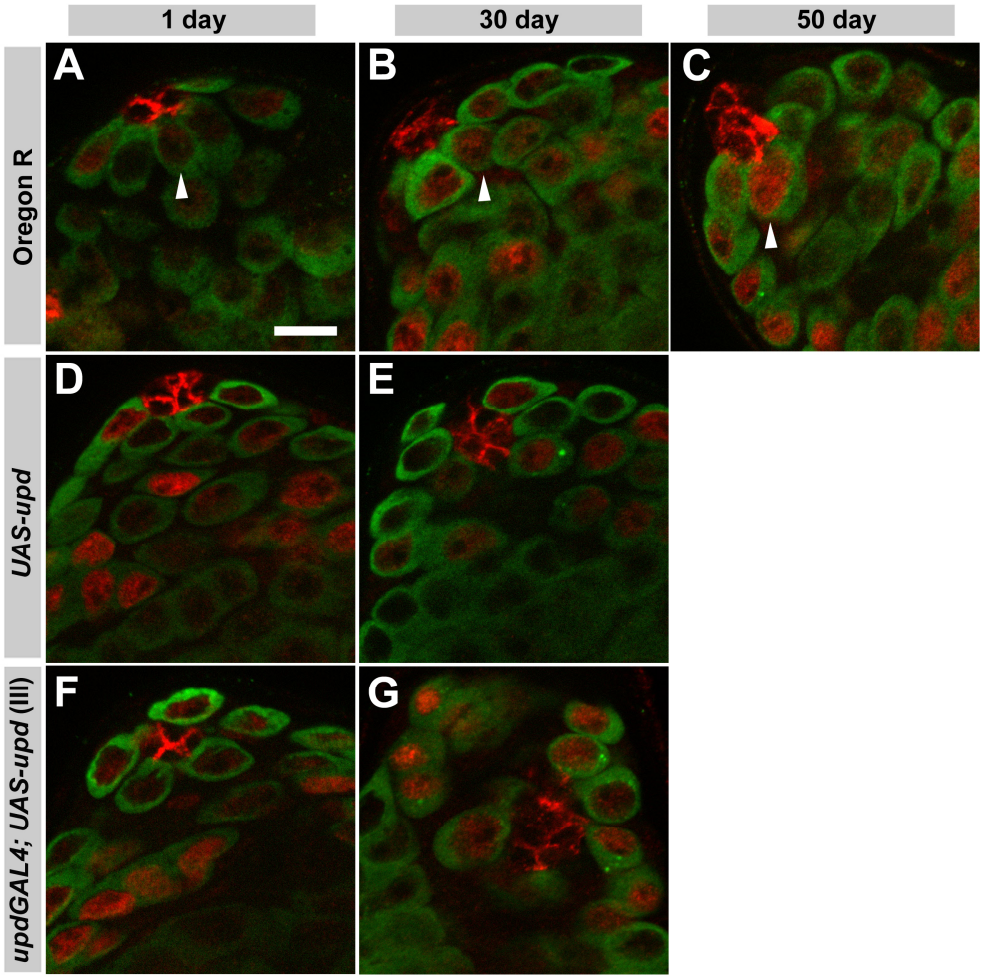


Figure S2.1 Age-related changes to the GSC niche

The apical tips of 1-day (A) and 50-day old (B) OregonR testes stained with the germ cell specific antigen Vasa (green) and cell surface marker FasIII (red) to mark the hub. (A-B) Sequential serial sections; white dots (.) mark the GSCs as they emerge in sections. (A-A") 8 GSCs are identified contacting the hub in a typical 1-day old testis, while 5 GSCs are present in a typical 50-day old testis (B-B"). (C-C"') Serial sections through the tip of a testis from a 30-day old male overexpressing upd in hub cells stained for the germ cell specific antigen Vasa (green) and the cell surface protein Fasciclin III (FasIII, red) to mark the hub. White dots denote new GSCs as they emerge through the sections, here showing 8 GSCs adjacent to the apical hub. Scale bar, 10 μM.

Figure S2.2 Levels of a key regulator of the G1/S transition, Cyclin E, increase in GSCs with age

(A-G) Apical tip of testes labeled with antibodies to FasIII (red) to identify the hub, Vasa (green) to mark germ cells, and cyclin E (red). (A-C) The number of GSCs expressing high levels of cyclin E increases with age in testes from OregonR males; relatively low levels are predominant at 1 day (A, arrowhead), moderate levels after 30 days (B, arrowhead), and increasing numbers of high level expressing GSCs at 50 days (C, arrowhead). (D-E) Testes from control (UAS-upd TM2/+) males at 1 (D) and 30 days (E), showing a similar distribution of cyclin E staining GSCs. (F-G) Testes from males constitutively expressing upd in hub cells (updGal4,UAS-gfp; UAS-upd TM2/+) at 1 day (F) and 30 days (G), showing a dramatic increase in number of GSCs expressing high levels cyclin E. (H) Distribution of cyclin E staining intensity in testes from aging males.



H

Age (day)	% of GSCs			n
Cyclin E Level	High	Medium	Low	
Oregon R				
1	4%	27%	69%	20
30	34%	44%	22%	20
50	38%	42%	20%	20
UAS-*upd, TM2*				
1	22%	31%	47%	20
30	27%	30%	43%	20
*upd*GAL4,UAS-*gfp*; UAS-*upd, TM2*				
1	24%	28%	48%	20
30	44%	47%	9%	20

Figure S2.3 Age-related changes to the GSC niche

The apical tips of 1-day (A,C) and 50-day old (B, D-F) OregonR testes stained with the cell surface marker FasIII (red) to mark the hub and DAPI (green) to mark the hub cell nuclei (A, B) or with the germ cell specific antigen Vasa (green) (C-F). (A-B) Sequential serial sections; white dots (.) mark the hub cells as they emerge in sections. (A-A’’’’) 11 hub cells are identified in a typical 1-day old testis, while 9 hub cells are present in a typical 50-day old testis (B-B’’’’). (C-F) Range of changes in hub morphology in aged testes; insets show high magnification view of FasIII staining of apical hubs. (C) Hub of a 1-day testis, approximately 12μm in diameter. (D) A typical 50-day testis showing similar size hub. (E, F) Example of a (E) small (<10μm) and (F) large hub found in a minority of 50-day old testes. Scale bar, 10 μM.

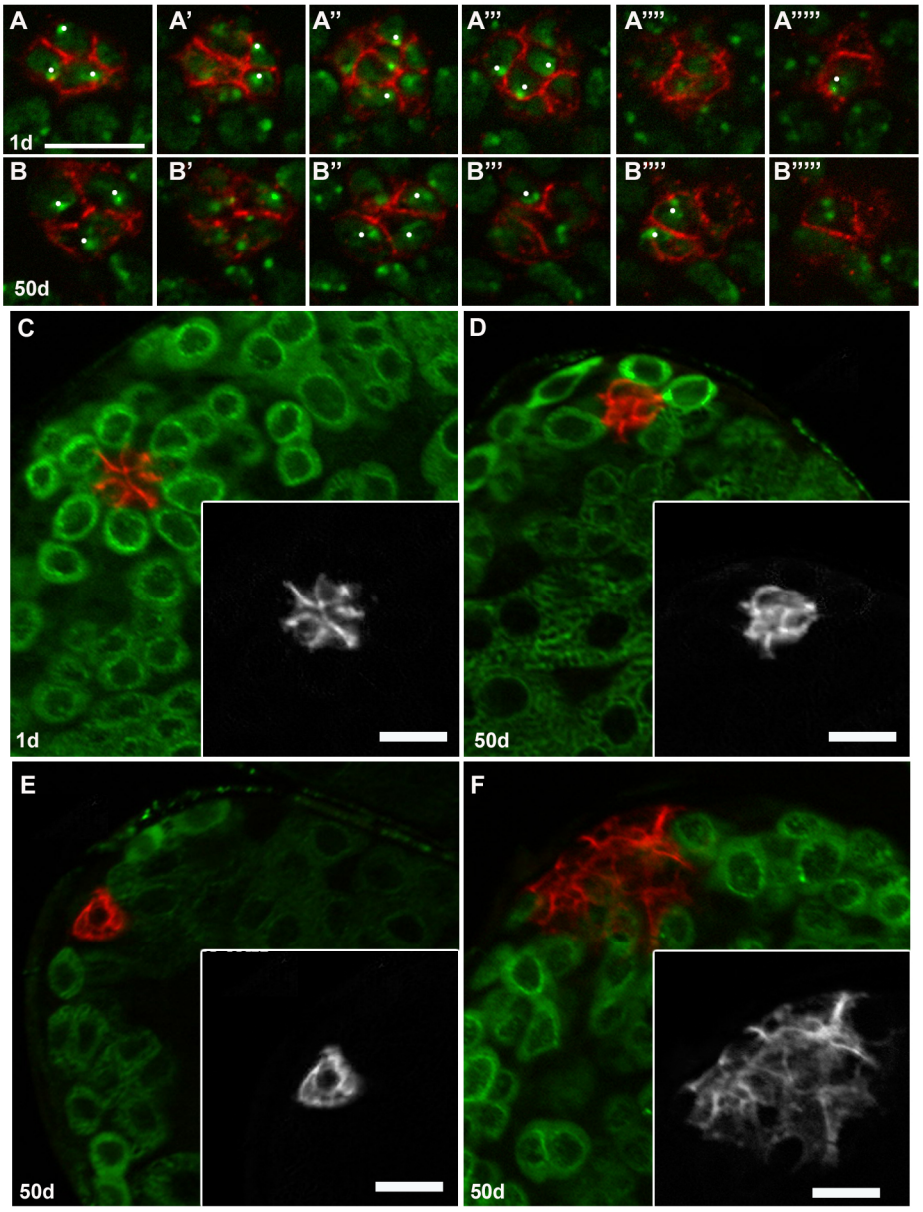

A
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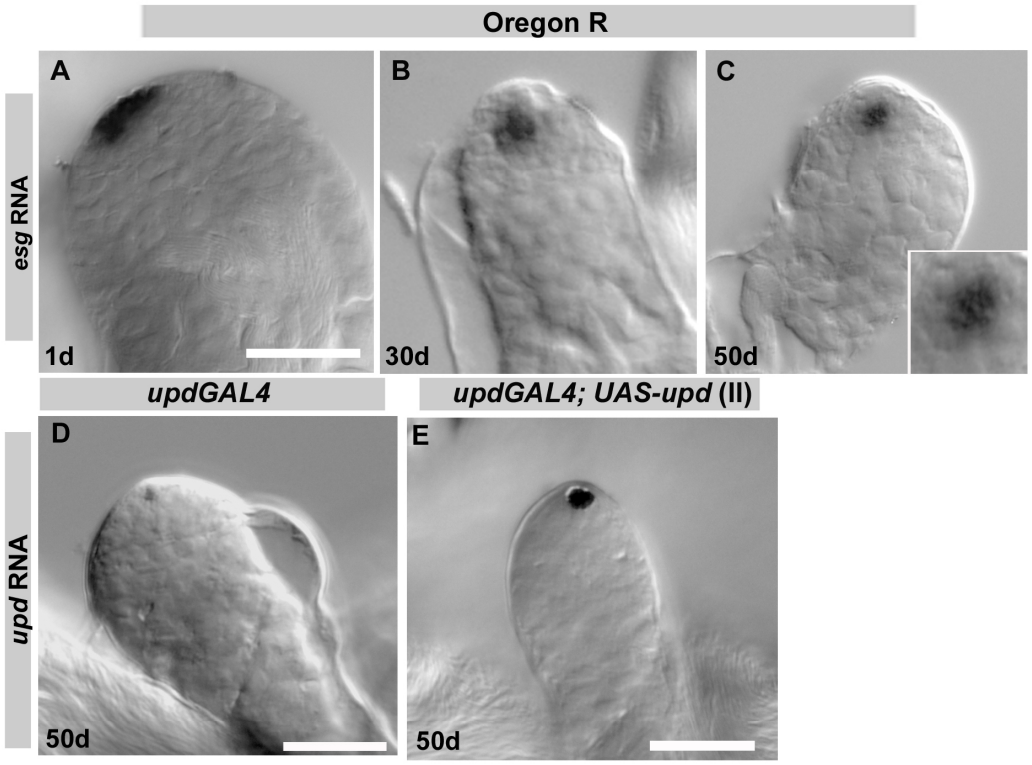
Figure S2.4 upd and esg *in situ*s in aged testes

(RNA *in situ* analysis for expression of the transcription factor esg in (A) 1-day, (B) 30-day, and (C) 50-day old testes shows maintenance of esg expression in the hub with age. Inset in C; high magnification view of hub. (D-E) RNA *in situ* analysis of upd expression of in testes from 50 day old (D) updGal4,UAS-gfp (control) and (E) updGAL4,UAS-gfp; UAS-upd TM2/+ testes. Note high levels of upd RNA in apical hub in E. (F) Hub staining intensity in testes from 50-day old flies.



F

Hub cell staining intensity		wild type	50% reduction	little or no staining	
Genotype	Probe				
OreR	*upd*	30.50%	28.10%	41.50%	n = 82
*upd*GAL4; UAS-*upd* (II)	*upd*	57.40%	31.50%	11.10%	n = 54

Hub cell staining intensity		strong staining	little or no staining	
Genotype	Probe			
OreR	*esg*	82.90%	17.10%	n = 41

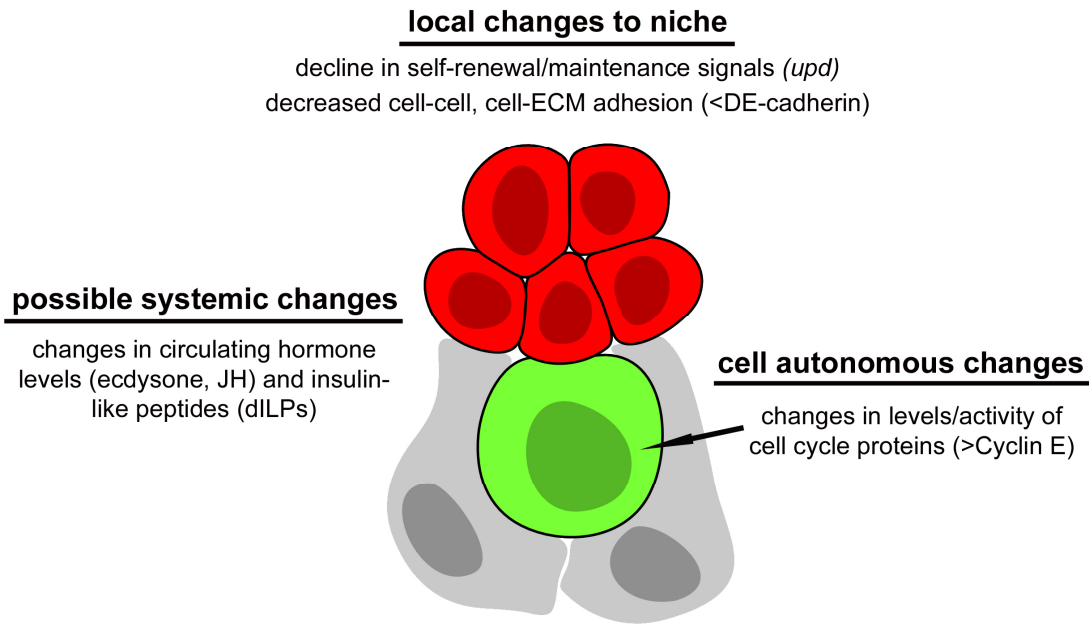


Figure S2.5 Multiple factors contribute to age-related changes in stem cell behavior.

Schematic of the male germline stem cell niche: hub cells (red), GSCs (green), somatic CPCs (grey). Changes to the local stem cell microenvironment lead to a decline in stem cell maintenance, while cell autonomous changes could contribute to altered cell cycle kinetics, leading to a decline in stem cell activation. Other changes in gene expression, such as expression of negative regulators of self-renewal signaling pathways, could result in a decline in stem cell activity. Similar to what has been demonstrated in mammalian systems, changes in circulating growth factors, hormones, and neuropeptides could also contribute to changes in stem cell behavior during aging.

Table 2.1 Quantification of GSCs in young and aged males

Age (day)		GSCs/testis ± SE[#]			% decrease	n
Oregon R						
1	-	8.27	±	0.23	-	66
30	M[1]	5.85	±	0.25	29.30%	66
30	MF[2]	6.07	±	0.24	26.64%	58
30	SM[3]	6.79	±	0.39	17.90%	29
50	M	5.27	±	0.22	36.24%	51
50	MF	5.06	±	0.20	38.83%	66
50	SM	5.08	±	0.44	38.57%	25
50	NY[4]	5.32	±	0.31	35.66%	31
TubulinGFP[5]						
1	-	8.52	±	0.29	-	46
30	M	5.62	±	0.58	34.11%	26
30	MF	6.16	±	0.47	27.74%	19
50	M	6.16	±	0.38	27.72%	44

± SE - Standard Error
1Males kept in isolation.
2Males kept with females
3Males serially mated with virgin females every 2-3 days.
4Males kept with females but without yeast supplement. In performing aging studies, low nutrient conditions can significantly shorten lifespan and/or modify any lifespan extension observed in certain longevity mutants. In addition, nutrient conditions have also been shown to affect *Drosophila* oogenesis. In order to avoid any contribution of low nutrient conditions to changes in GSC number or behavior, flies were maintained in vials supplemented with yeast.
5y,w; nanosGAL4:VP16, UAS-α-tubulin:gfp testes expressing GFP tagged α-tubulin protein in early germ cells
The differences in GSC number between males kept in isolation and males kept with females, serial mated and non-serial mated males, and males without yeast supplement and males supplemented with yeast are not significantly different ($p>0.05$).

Table 2.2 Quantification of BrdU positive GSCs and somatic cells in young and aged OregonR testes

Age (day)	Average # $BrdU^+$ GSCs/testis ± SE			S Phase Index[1]	[2]n
1	1.6	±	0.14	19.40%	51
50	0.8	±	0.12	15.60%	59
Age (day)	Average # $BrdU^+$ somatic cells[3]/testis ± SE				n
1	3.1	±	0.25	-	23
50	0.9	±	0.19	-	23

1 Average number of BrdU positive GSCs/average number of GSCs. 8.27 GSCs in 1-day and 5.1 GSCs in 50-day old flies.
2 Average of two experiments.
3 BrdU positive, Vasa negative nuclei.

Table 2.3 Forced expression of upd in hub cells leads to maintenance of GSCs in aged males

Age (day)	Average # GSCs/testis	±	SE	% decrease	n
*upd*GAL4,UAS-*gfp*					
1	8.32	±	0.28	-	31
30	6.44	±	0.22	22.60%	25
50	5.20	±	0.32	37.52%	30
*upd*GAL4,UAS-*gfp*; UAS-*upd*					
1	8.60	±	0.28	-	45
30	5.83	±	0.18	32.17%	72
50	6.33	±	0.28	26.42%	64
UAS-*upd, TM2*					
1	8.05	±	0.22	-	43
30	6.39	±	0.29	20.53%	38
*upd*GAL4,UAS-*gfp*; UAS-*upd, TM2*					
1	7.79	±	0.16	-	61
30	7.41	±	0.18	4.84%	68

Table 2.4 Forced expression of upd in hub cells leads to maintenance of Stat+ GSCs

Age (day)	Average Stat^{+} GSCs/testis±SE	% decrease	Average Stat^{+} germ cells/testis±SE	% decrease	% of GSCs that are Stat^{+}	% of Stat^{+} germ cells that are GSCs	n
OregonR							
1	7.75 ± 0.38	-	15.00 ± 0.59	-	93.71%	51.67%	20
30	5.48 ± 0.42	29.31%	9.80 ± 0.58	34.67%	93.65%	55.90%	23
50	4.68 ± 0.67	39.59%	6.90 ± 0.72	54.00%	88.84%	67.85%	22
*upd*GAL4,UAS-*gfp*							
1	7.83 ± 0.27	-	15.50 ± 0.72	-	94.15%	50.54%	18
50	5.14 ± 0.25	34.35%	10.36 ± 0.69	34.42%	98.90%	49.66%	14
*upd*GAL4,UAS-*gfp*; UAS-*upd*							
1	7.73 ± 0.26	-	15.18 ± 0.64	-	89.85%	50.90%	22
50	5.89 ± 0.32	23.72%	11.53 ± 0.72	21.38%	93.12%	51.14%	19
UAS-*upd, TM2*							
1	6.63 ± 0.19	-	11.37 ± 0.55	-	82.39%	58.31%	27
30	4.59 ± 0.28	30.73%	9.00 ± 0.65	20.85%	71.82%	51.03%	27
*upd*GAL4,UAS-*gfp*; UAS-*upd, TM2*							
1	6.56 ± 0.20	-	10.44 ± 0.36	-	84.19%	62.79%	27
30	6.05 ± 0.27	7.78%	6.55 ± 0.31	39.51%	81.59%	92.36%	22

Chapter 3: Compromised spermatogonial reversion during aging

Adult stem cells are critical for tissue homeostasis; therefore, the mechanisms utilized to maintain an adequate stem cell pool are important for the survival of an individual. In *Drosophila*, male germline stem cells (GSCs) can be replaced by reversion (de-differentiation) of early progenitor cells throughout life. However, the average number of GSCs decreases in old flies, suggesting that this reversion process is compromised during aging. Using a temperature sensitive allelic combination of Stat92E to control the reversion process, we found that successful germline reversion is reduced in aged males. Interestingly, in the early stage(s) of reversion the proliferation of CySCs is decreased in aged males, indicating that CySCs may facilitate the initiation of germline reversion. Our data suggest that decreased niche function contributes to compromised reversion and GSC maintenance, leading to a loss of tissue homeostasis during aging.

3.1 Introduction

In regenerative tissues, such as skin and blood, adult stem cells support tissue homeostasis by replenishing cells lost due to normal cellular turnover and/or damage throughout life. Stem cells are found in unique locations within a tissue, known as stem cell niches, which support stem cell self-renewal, maintenance, and survival. Stem cell self-renewal provides a means to maintain

a pool of active stem cells; however, in some tissues, the number and/or activity of stem cells decline during aging, suggesting that changes in stem cell behavior likely contribute to reduced tissue homeostasis in older individuals (Drummond-Barbosa 2008).

In the *Drosophila* testis, male germline stem cells (GSCs) and cyst stem cells (CySCs) are located at the apical tip where they are in contact with a cluster of somatic cells called the hub (Figure 1A). Hub cells secrete the ligand Unpaired (Upd), which activates the Janus kinase - Signal Transducer and Activator of Transcription (Jak-STAT) signal transduction pathway within the adjacent stem cells to specify maintenance (Kiger et al. 2001; Tulina & Matunis 2001; Leatherman & Dinardo 2008). In addition to interactions with hub cells, signaling between GSCs and CySCs is required for the coordination of proper function of each cell population to ensure successful spermatogenesi *(*Kiger et al. 2000; Tran et al. 2000; Schulz et al. 2002; Sarkar et al. 2007; Leatherman & Dinardo 2008).

We previously demonstrated that aging results in a decline in spermatogenesis, which could be attributed in part to a significant decrease in the average number of GSC *(*Wallenfang et al. 2006; Boyle et al. 2007). The remaining stem cells appear to progress through the cell cycle more slowly (Boyle et al. 2007). In addition, we observed a striking aging-related decline in expression of upd (Boyle et al. 2007). Constitutive expression of upd in hub cells was sufficient to block the loss of GSCs with age, indicating that a decline in

niche function contributes to changes in stem cell behavior over time (Boyle et al. 2007).

Based on the predicted half-life of GSCs in newly eclosed males, the testis should be devoid of stem cells by 50 day *(*Wallenfang et al. 2006). Therefore, mechanisms must exist to replace lost stem cells over time. For example, stem cells could divide symmetrically to replace lost stem cells and maintain full occupancy of the niche, as was demonstrated in the *Drosophila* ovary, where symmetric division of GSCs was observed to replace neighboring GSCs lost to differentiation (Xie & Spradling 2000). Somatic follicular stem cells (FSC) in the *Drosophila* ovary can also replace lost somatic stem cells in niches several cell distances awa *(*Nystul & Spradling 2007).

In addition, partially differentiated precursor cells can revert (de-differentiate) to a stem cell state and re-acquire the ability to self-renew. Reversion has been shown to occur *in vivo* in the germ line of both *Drosophila* (Brawley & Matunis 2004; Kai & Spradling 2004) and mice after depletion of the endogenous stem cell pool (Barroca et al. 2009).

Using a system to permanently mark differentiating spermatogonia in the *Drosophila* testis, marked GSCs were found in increasing numbers within the niche in response to DNA damage and in aged animals, suggesting that lost stem cells can be replaced by reverting spermatogonia (Cheng et al. 2008). Despite the ability of spermatogonia to revert to replace lost GSCs, the average number of GSCs is reduced in testes of aged males (Boyle et al. 2007; Cheng et

al. 2008). Therefore, mechanisms utilized to maintain and replace GSCs may be compromised in older males.

In this study, we have used the *Drosophila* male germ line to investigate the impact of aging on reversion. The ability of spermatogonia to successfully revert is decreased during aging. In contrast, early cyst cells are adequately replaced during this reversion paradigm. However, during the early reversion process, the S-phase index of early cyst cells is decreased in aged flies compared to young flies. Our data indicate that changes within the early cyst cell population may affect the reversion capacity of spermatogonia during aging.

3.2 Results

3.2.1 Initiation of Reversion is Compromised During Aging

To study the reversion process during aging we used a temperature sensitive allelic combination of Stat92E (Stat92Ets) that permits manipulation of GSC differentiation within the niche (Figure 1A; Materials and Methods)(Brawley & Matunis 2004). Germ cell differentiation can be monitored by the morphology of the fusome, a germ cell-specific organelle that is spherical in GSCs and GBs (spectrosome) and becomes progressively branched in spermatogonia during mitotic amplification (Figure 1A). At the permissive temperature (18℃), all testes from young Stat92Ets flies (0-5 day post-eclosion, dpe) contained at least one germ cell with a spectrosome adjacent to the hub (n=46, Figure 1B-B',H). At the restrictive temperature (29℃), few testes had germ cells with spectrosomes adjacent to the hub (19%, n=42; Figure 1C-C',H), indicating that GSCs

differentiated within the niche. When flies at 29℃ a re shifted back to 18℃, testes containing germ cells with spectrosomes appeared adjacent to the hub, verifying that spermatogonia had reverted into single GSCs (58%, n=101; Figure 1D-D',H). Control flies (Materials and Methods) contained germ cells with spectrosomes at 29℃ in young flies (100%, n=32, data not shown), ind icating that temperature does not affect stem cell maintenance.

We then subjected aged (50-55 dpe) Stat92Ets males to the reversion paradigm. In testes from aged Stat92Ets flies maintained at 18℃, germ cells around the hub contained spectrosomes (97%, n=55; Figure 1E-E',H), while testes from aged Stat92Ets flies at 29℃ contained only few germ cells with spectrosomes (18%, n=39; Figure 1F-F',H), similar to the behavior of germ cells in young flies. However, in contrast to young flies, successful reversion was blocked in a statistically significant majority of the testes examined from aged, Stat92Ets flies that were shifted back to 18℃ (42%, n=1 20; Figure 1G-G',H). Testes assayed after the recovery period either contained GSCs and spermatogonia or only spermatocytes. Interestingly, when the average number of GSCs in testes with successful reversion was assayed, the number after reversion was comparable to the average number present in age-matched flies maintained at 18℃ (Figure 1I). These data suggest th at initiation of reversion, rather than completion, is likely the explanation for the inability of spermatogonia to revert successfully. Similar results were obtained with another temperature sensitive combination of Stat92E alleles (Figure S1A; Materials and Methods).

3.2.2 Early cyst cell behavior during reversion

Communication between germ cells and cyst cells is essential for regulating GSC self-renewal and differentiation of spermatogonia. Therefore, we analyzed the behavior of CySCs and cyst cells during reversion. Jak-STAT signaling specifies self-renewal and maintenance of CySCs (Kiger et al. 2001; Tulina & Matunis 2001; Leatherman & Dinardo 2008); therefore, we hypothesized that the number of CySCs might also decrease upon compromised Stat92E activity. We stained for Zfh-1, a transcription factor expressed in early cyst cells including CySCs, to quantify early cyst cell numbers during the reversion paradigm (Leatherman & Dinardo 2008). In young Stat92Ets males, the average number of Zfh-1+ cells decreased from 30.4 (n=37; Figure 2A;S2A-A') at 18℃ to 22.5 (n=30; Figure 2A;S2B-B')) at 29℃, demonstrating that cyst cells are sensitive to a decline in Stat92E activity. The number of Zfh-1+ cells increased to 25.9 (n=25; Figure 2A) after a 1-day recovery at 18°C and was restored to 29.3 (n=54; Figure 2A;S2C-C') after just 2 days of recovery at 18℃. Although the average number of Zfh-1+ cells was lower, a similar trend was observed during the reversion paradigm in older Stat92Ets males: 18.2 (n=39; Figure 2A) at 18℃, 13.2 (n=37; Figure 2A) at 29℃, 16.0 (n=25; Figure 2A) 1 day of recovery at 18℃, and restoration to 17.6 (n=19; Figure 2A) 2 days of recovery at 18℃. No decrease in the average number of Zfh-1+ cells was detected in young (n=33; Figure 2A) or aged, control flies shifted to 29℃ (n=44; Figure 2A), indicating that high temperature does not affect early cyst cell number. These observations, also observed with another temperature sensitive combination of Stat92E alleles

(Figure S1B), suggest that early cyst cells are rapidly restored in both young and aged flies under the reversion paradigm.

Determining how early cyst cell numbers are efficiently replaced during the reversion paradigm could reveal clues about the early steps of reversion. The restoration of early cyst cell number could be from reversion of early cyst cells, in a manner similar to reversion of spermatogonia. However, due to the lack of specific markers for distinguishing CySCs from early cyst cells, we were not able to address this possibility. Instead, we analyzed whether early cyst cell replacement during reversion could result from proliferation of the remaining CySCs. CySC proliferation was assayed using *ex vivo* incorporation of EdU, a thymidine analog, to label cells in S-phase, and the percentage of Zfh-1+ cells that were also EdU+ was calculated (S-phase index). We determined the S-phase index after one day recovery at 18℃, prior to complete recovery of Zfh-1+ cell number, and found that the S-phase index for early cyst cells in young Stat92Ets males returns to baseline from 9.1% at 29℃ (n=30; Figures 2B) to 13.5% upon the recovery at 18℃ (n=25; Figures 2B). This resumption of proliferation is also observed in young control males in which the S-phase index increases from 11.1% at 29℃ (n=33; Figure 2B) to 16.6 % (n=33; Figure 2B) at 18℃. A decline in S-phase index at 29℃ likely refl ects the decrease in Stat92E activity in both control and Stat92Ets males.

We next analyzed the S-phase index after one day of recovery at 18℃ in aged flies and found a similar response to temperature shifts in aged controls, from 9.7% at 29℃ (n=44, Figure 2B) to 14.3% at 18° C (n=37, Figure 2B,

statistically significant). In contrast, an increase in the S-phase index in aged Stat92Ets males was not observed: 13.3% at 29℃ (n=37, Figure 2B) to 13.9% at 18℃ (n=18, Figure 2B). The lack of resumption of proliferation in aged Stat92Ets males, when compared to aged matched controls, is indicative of an aged-related defect in CySC behavior with respect to this reversion paradigm. Similar results were obtained with another temperature sensitive combination of Stat92E alleles (Figure S1C).

3.3 Discussion

Our data demonstrate that aging results in decreased reversion capacity due to defects during the early stages of the reversion process, perhaps during initiation of reversion. Our analysis of early cyst cell behavior during reversion reveals that CySCs have an altered proliferation profile in aged flies. The Stat92Ets model suggests that decreased number of GSCs during aging could be partially explained by compromised reversion in part due to changes in CySCs activity.

The temperature-sensitive Stat92E reversion paradigm relies upon one functional copy of Stat92EF, described as a temperature sensitive point mutation in the DNA binding domain. A recent study in young flies using a Stat92E-independent paradigm for reversion reported Jak-STAT signaling plays a role in the reversion efficiency (Sheng et al. 2009); therefore, the Stat92Ets reversion paradigm may be compromised. However, given that our studies were performed with proper controls and revealed relative changes in reversion with

age, these data clearly provide insights into how the reversion process is regulated.

In the Stat92Ets background, functional protein levels are theoretically reduced by half at the permissive temperature (Figure S3A-C'). However, the extent to which temperature affects Stat92EF protein function is unknown. At the restrictive temperature, staining for Stat92E shows localization within the nuclei of early cyst cells (Figure S4F-F'), which in combination with the observed changes in CySC proliferation (Figure 2B), suggests that Stat92Ets flies maintain some active Stat92E protein. Therefore, the protein at the restrictive temperature could have residual transcriptional activity, may not be targeted for degradation, or could have a prolonged half-life. In addition, studies have suggested that STAT proteins have non-canonical roles,that are unrelated to it's role as a transcription factor (Chen et al. 2003) (Gough et al. 2009; Wegrzyn et al. 2009).

Our data using the Stat92Ets reversion paradigm indicate that when reversion occurs, in an all-or-nothing manner, even in young flies. Once a critical threshold step is achieved, spermatogonia revert back to a stem cell state and are capable of self-renewal; otherwise, spermatogonia complete differentiation into spermatocytes. A Stat92E-independent reversion paradigm in which only germ cell behavior is modified demonstrated up to 100% efficiency in reversion, suggesting that early cyst cells could play a role in the reversion process (Sheng et al. 2009). Based on the current model that the hub signals to CySCs that then relay self-renewal signals to GSCs (Leatherman & Dinardo 2008), similar coordination and signaling between all three cell types almost certainly is

required for the reversion process. Therefore, decreased Jak-STAT signaling during aging is likely one mechanism that could contribute to decreased reversion in older males.

Reversion as a mechanism for replenishing lost stem cells is a conserved process that is still poorly understood. Future studies in specific stem cell markers, signaling pathways, and technology development will be critical for understanding the physical interplay between germ cells and cyst cells within the context of a shared niche. The mechanisms regulating the reversion of a germ cell to a GSC will be important for understanding the plasticity of differentiated cell types, such as induced-pluripotent stem cells, that will be used for disease modeling and stem cell-based therapies.

3.4 Methods

3.4.1 Fly husbandry and stocks

Flies were raised on standard cornmeal-molasses-agar medium at 18ºC unless otherwise indicated. Newly eclosed 0-5 day old male flies were collected in vials; each vial contained up to 30 males and 10 females. Vials for aging experiments were supplemented with fresh yeast paste and changed every 7 days.

Three Stat92E mutant alleles were used: temperature sensitive mutant allele Stat92EFrankenstein (Stat92EF)(gift from E. Matunis)(Baksa et al. 2002) and the null Stat92E06346 (Bloomington)(Hou et al. 1996) and null Stat92EJ6C8 alleles (gift from N. Perrimon)(Spradling et al. 1999). The temperature sensitive

heteroalleic combinations (‘Stat92Ets’) used were: Stat92EF/ Stat92E06346 and Stat92EF/ Stat92EJ6C8, and specific genotypes are noted in figure legends. ‘Control’ flies are Stat92EF/+ (Stat92EF out-crossed to wildtype OregonR females).

The reversion paradigm is as follows and previously described (Brawley et al., 2004). Flies raised at 18℃, shifted to 29℃ fo r 2 days, shifted back to 18℃ to recover for (a) 5 days for visualizing completely reverted GSCs (b) 2 days for visualizing restoration of early cyst cell number or (c) 1 day for assaying proliferation of CySCs.

3.4.2 Immunofluorescence and Microscopy

Immunofluorescence experiments of testes were performed using the whole mount method as described previously (Boyle et al., 2007).

The polyclonal rabbit anti-Vasa (1:3000, gift from P. Lasko), guinea pig anti-Traffic Jam (1:3000) (gift from D. Godt), rabbit anti-Stat92E (1:800)(gift from D. Montell) and guinea pig anti-zfh1 (1:3000) (gift from C. Doe) were used at indicated concentrations. Mouse anti-α-spectrin (3A9)(1:10), mouse anti-Fasciclin3 (7G10)(1:10), and mouse anti-Eya (EYA10H6)(1:10) were obtained from the Developmental Studies Hybridoma Bank (developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences). Secondary antibodies were obtained from Molecular Probes. Samples were mounted in

Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Images were obtained using a Zeiss Axiovert 200M microscope or a Zeiss AxioObserver, and processed using AxioVision (version 4.8; Carl Zeiss) and Adobe Photoshop software (Mountain View, CA).

3.4.3 Quantification of average number of GSCs and the percentage of testes containing spectrosomes

GSCs are defined as a germ cell (Vasa+) that contacts Fasciclin3+ hub cells. Only those samples with an easily distinguishable hub within the sizes of 10-18μm were included in the quantification. Testes were counted as containing a spectrosome when at least one GSC contained a spectrosome marked by anti-α-spectrin. A Chi-squared test was performed to evaluate statistical significance. unless otherwise stated in the text. For quantification of GSCs after a successful reversion, GSCs were counted only in those testes that contained at least one spectrosome, and a Student's two tailed t-test was performed to evaluate statistical significance. Above experiments were performed at least 2 times with a total n>= 40 testes for percentage of spherical fusomes or n>=30 for average number of GSCs.

3.4.4 *Ex vivo* EdU incorporation

EdU incorporation was performed and analyzed using the Click-iT EdU Imaging Kit (Invitrogen), with the following modifications. All procedures were performed at room temperature with minimal exposure to light. Crude dissection

of testes was performed in 1X Ringer's buffer and then transferred immediately to 1X Ringer's buffer in a glass embryo dish for no more than 10 minutes. Testes were subsequently transferred to 30μM EdU diluted in 1X Ringer's buffer for 30'. After incorporation, testes were fixed for 20' in 4% paraformaldehyde diluted in 1X PBS, followed by two washes with 1X PBST (0.5% Triton-X100) and blocked with 3% BSA in 1X PBS. Testes were bathed in the Click-iT reaction cocktail for 30 minutes. IF was performed as indicated above.

3.4.5 Quantification of average number of Zfh-1+ early cyst cells and Percentage of EdU+ cells

All Zfh-1+ early cyst cells around the hub were counted, unless they exceeded 30μm away from the hub. A Student's two tailed t-test was performed to evaluate statistical significance ($p<0.05$). An EdU+ cell was assayed as positive when the majority of the cell was labeled and EdU+ co-localized with Zfh-1. The S-phase index was determined as the number of EdU+ cells/Zfh-1+ cell X 100. A Tukey-Kramer HSD test was used to determine statistical significance, (positive value was considered significant). Experiments were performed at least 2 times with a total n>= 30 testes, unless otherwise stated in text.

3.5 Acknowledgements

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Figure 3.1 Initiation of reversion is compromised during aging in the *Drosophila* testis.

(A) Schematic of early spermatogenesis and the Stat92Ets reversion paradigm. The germline stem cell (GSC) niche includes GSCs (light green) and cyst stem cells (CySCs, light gray) that surround the hub (red). A pair of CySCs envelopes each GSC. A pair of cyst cells (dark gray) envelope each gonialblast which undergoes 4 rounds of mitotic divisions to create an interconnected cyst of 16-spermatogonia (dark green). Germ cells contain an organelle called the fusome (red). Jak-STAT signaling is required for stem cell maintenance. The reversion paradigm uses a heteroallelic mutant of Stat92E (inset), where at the restrictive condition (29℃) GSCs differentiate next to the hub, and upon recovery at the permissive temperature (18℃), spermatogonia revert to GSCs. (B-G') Immunofluorescence images of testes stained for the hub (Fasciclin3 [Fas3]; red), fusomes (α-Spectrin; red), and germ cells (Vasa; green) throughout the reversion paradigm. (B-B',E-E') At 18℃, young and aged flies have spectrosomes (arrowheads) within GSCs and branched fusomes (arrows) within spermatogonia. (C-C',F-F') When young and aged flies were shifted to 29℃ for 2 days, branched fusomes within spermatogonia are next to the hub (arrows). (D-D') Once shifted back to 18℃ for 5 days, young flies have spectrosomes within spermatogonia (arrowheads) right next to the hub. (G-G') Example of a testis from aged flies with branched fusomes after the shift back. (H) Graph of the percentage of testes containing at least one spectrosomes in young and aged flies throughout the reversion paradigm at 18℃, 29℃ , and recovery at 18℃. (I) Graph of the average number of GSCs in testes that contained GSCs in young and aged flies raised at 18℃ and after the recovery at 18℃. Bracket with * shows statistically significant changes, $p<0.05$. Scale bar: 10μm. Genotype: Stat92EF/Stat92E06346.

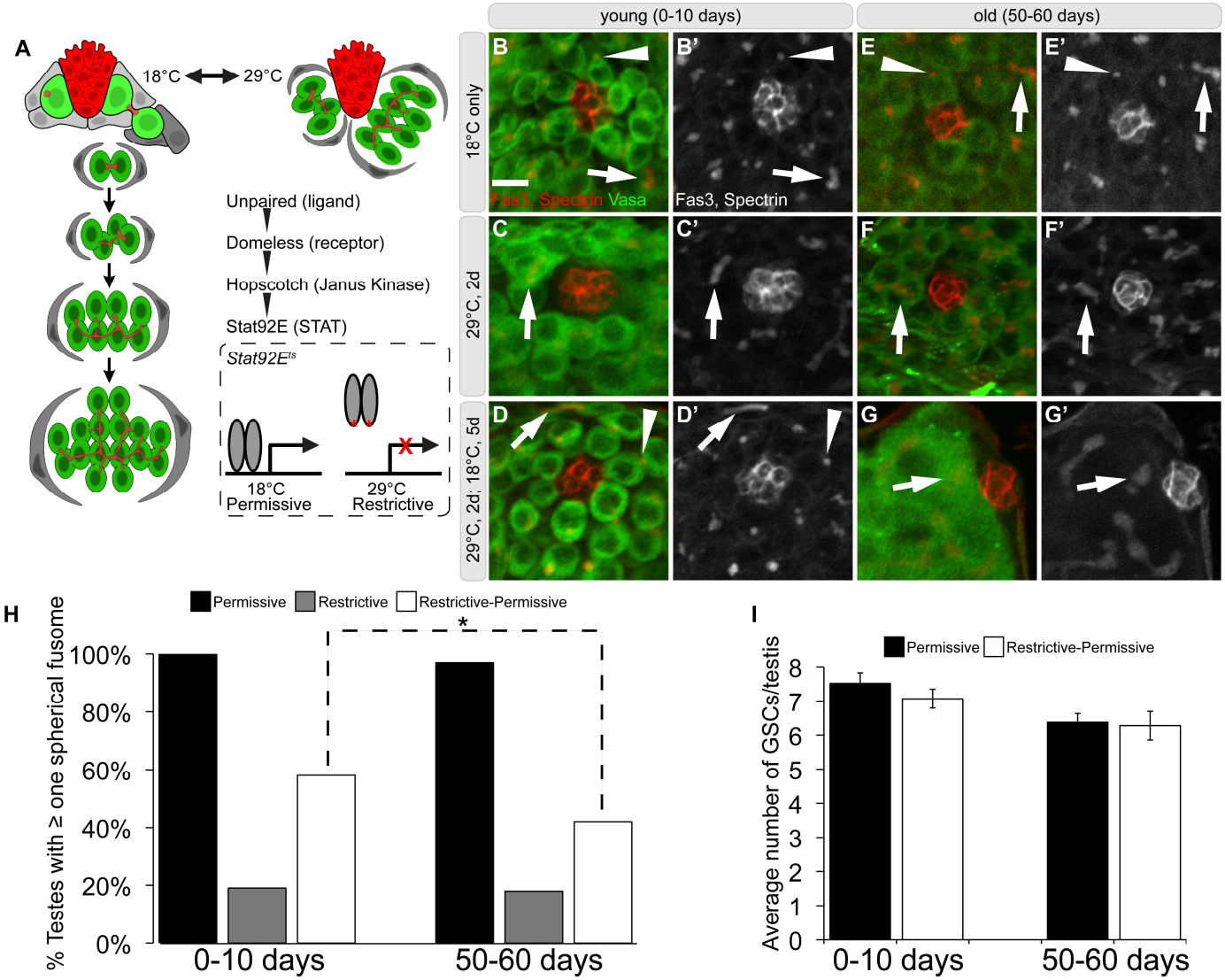

A
18°C
29°C
Unpaired (ligand)
Domeless (receptor)
Hopscotch (Janus Kinase)
Stat92E (STAT)
Stat92E^ts
18°C
Permissive
29°C
Restrictive
young (0-10 days)
old (50-60 days)
18°C only
29°C, 2d
29°C, 2d; 18°C, 5d
B
B'
E
E'
C
C'
F
F'
D
D'
G
G'
Fas3, Spectrin Vasa
Fas3, Spectrin
H
Permissive
Restrictive
Restrictive-Permissive
% Testes with ≥ one spherical fusome
100%
80%
60%
40%
20%
0%
*
0-10 days
50-60 days
I
Permissive
Restrictive-Permissive
Average number of GSCs/testis
8
7
6
5
4
3
2
1
0
0-10 days
50-60 days

Figure 3.2 Early cyst cells behavior during the reversion paradigm.

(A) Graph of the average number of Zfh-1+ cells throughout the reversion paradigm in young and aged, control and Stat92Ets flies (B) Graph of the percentage of Zfh-1+ cells in S-phase labeled by EdU in young and aged control and Stat92Ets flies throughout the reversion paradigm. Bracket with * shows statistically significant changes. Genotype: Stat92EF/+ and Stat92EF/Stat92E06346.

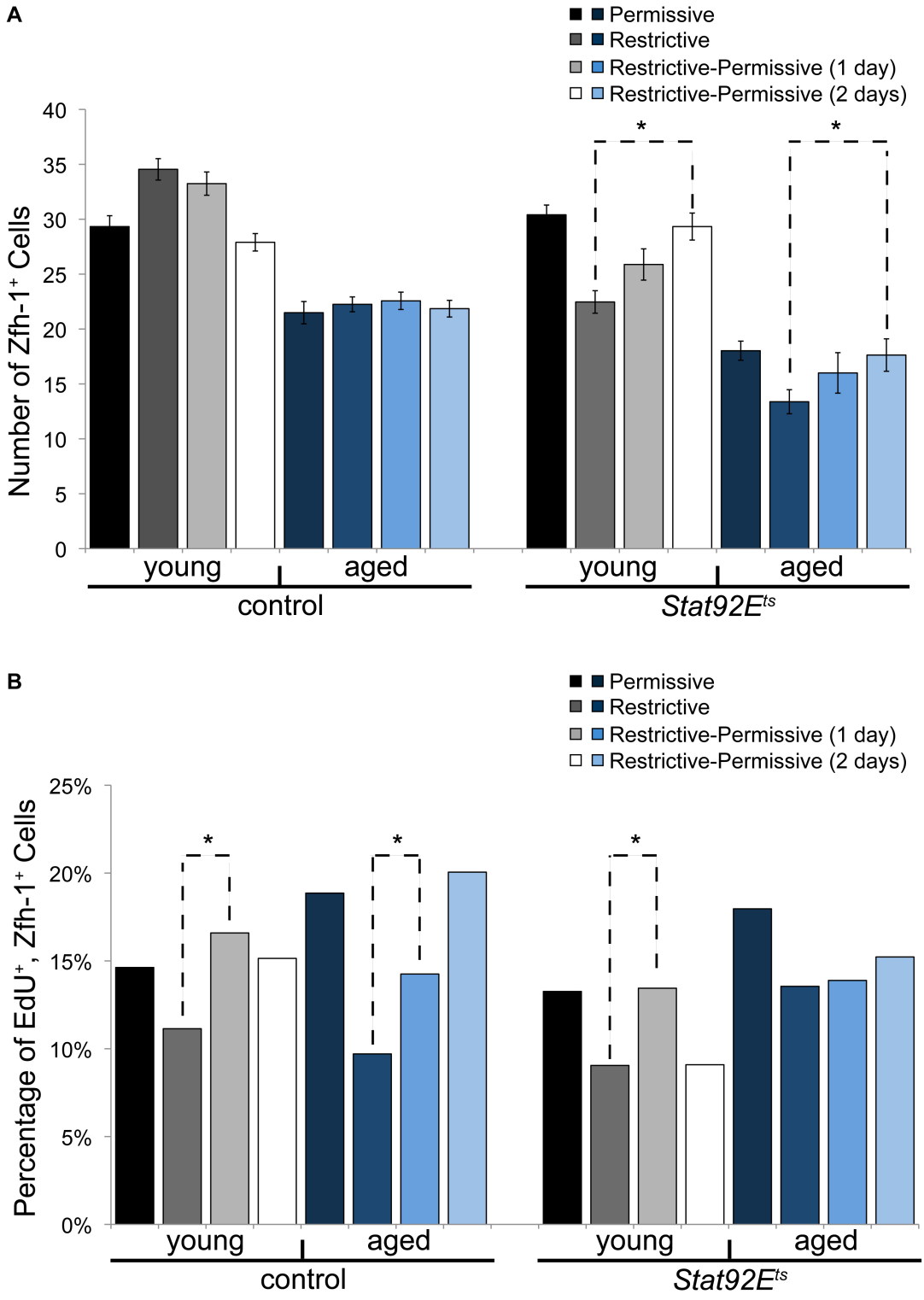
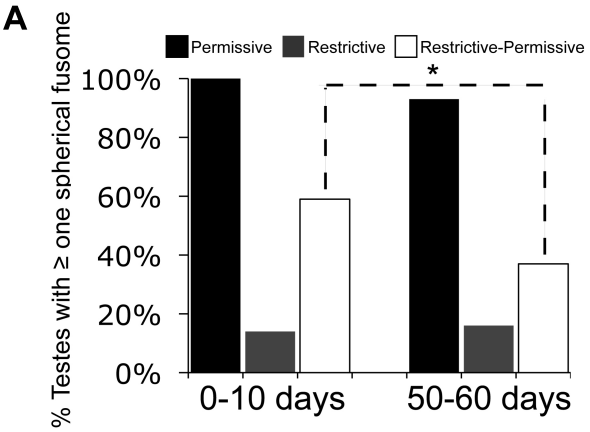
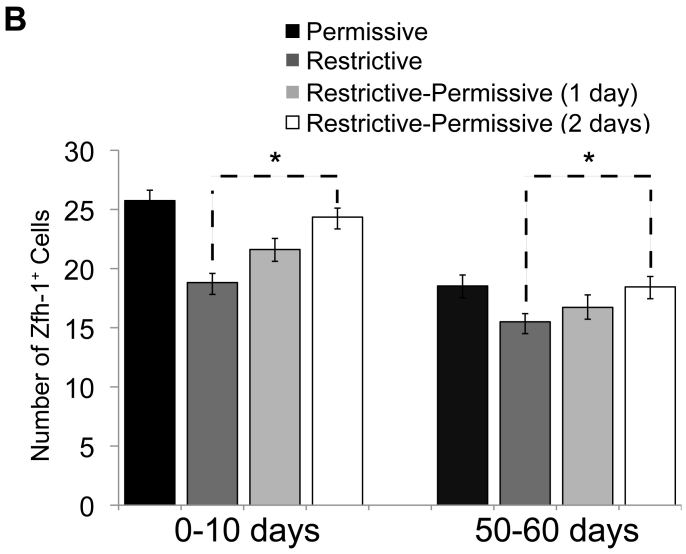

A
Permissive
Restrictive
Restrictive-Permissive (1 day)
Restrictive-Permissive (2 days)
Number of Zfh-1+ Cells
40
35
30
25
20
15
10
5
0
*
*
young
aged
young
aged
control
Stat92Ets
B
Permissive
Restrictive
Restrictive-Permissive (1 day)
Restrictive-Permissive (2 days)
Percentage of EdU+, Zfh-1+ Cells
25%
20%
15%
10%
5%
0%
*
*
*
young
aged
young
aged
control
Stat92Ets

Figure S3.1 Reversion is compromised during aging and early cyst cell behavior during aging: Stat92EF/Stat92EJ6C8

(A) Graph of the percentage of testes containing at least one spectrosome in young and aged flies throughout the reversion paradigm at 18℃, 29℃ for 2 days, and shift back to 18℃ for 5 days. (B) Graph of th e average number of Zfh-1+ cells throughout the reversion paradigm and (C) Graph of the percentage of Zfh-1+ cells in S-phase labeled by EdU in young and aged flies throughout the reversion paradigm. Bracket with * shows statistically significant changes. Genotype: Stat92EF/ Stat92EJ6C8


A
Permissive
Restrictive
Restrictive-Permissive
% Testes with ≥ one spherical fusome
100%
80%
60%
40%
20%
0%
*
0-10 days
50-60 days


B
Permissive
Restrictive
Restrictive-Permissive (1 day)
Restrictive-Permissive (2 days)
Number of Zfh-1+ Cells
30
25
20
15
10
5
0
*
*
0-10 days
50-60 days

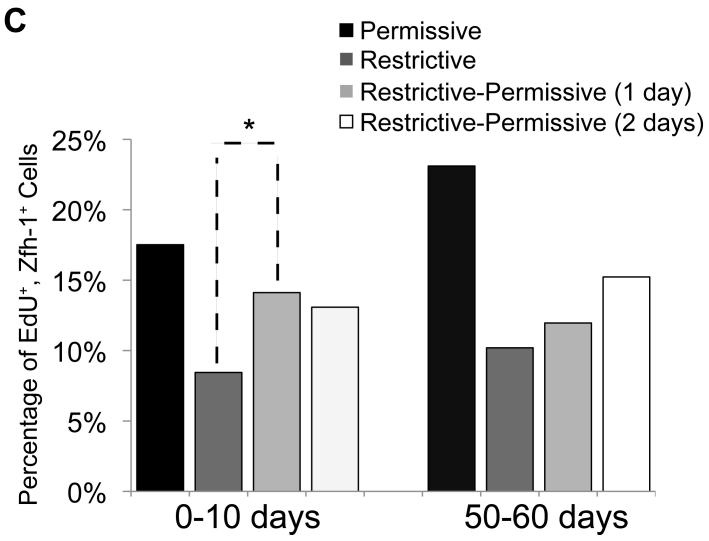

C
Permissive
Restrictive
Restrictive-Permissive (1 day)
Restrictive-Permissive (2 days)
Percentage of EdU+, Zfh-1+ Cells
25%
20%
15%
10%
5%
0%
*
0-10 days
50-60 days

Figure S3.2 Early cyst cell behavior during the reversion paradigm.

(A-C') Immunofluorescence images of testes from young flies throughout the reversion paradigm stained for the hub (Fas3; red, outline), late cyst cells (Eyes Absent [EyA]; red, arrowhead), and early cyst cells (Zfh-1; green). (A-A') at 18℃ (B-B') at 29℃, and (C-C') recovery at 18℃ for 2 da ys. Number of merged 1μm z-slices to represent majority of Zfh-1+ cells for (A-A') z=4 (B-B') z=1 (C-C') z=3. Note morphological changes and density of Zfh-1 cells. Scale bars: 10μm. Genotype: Stat92EF/Stat92E06346.

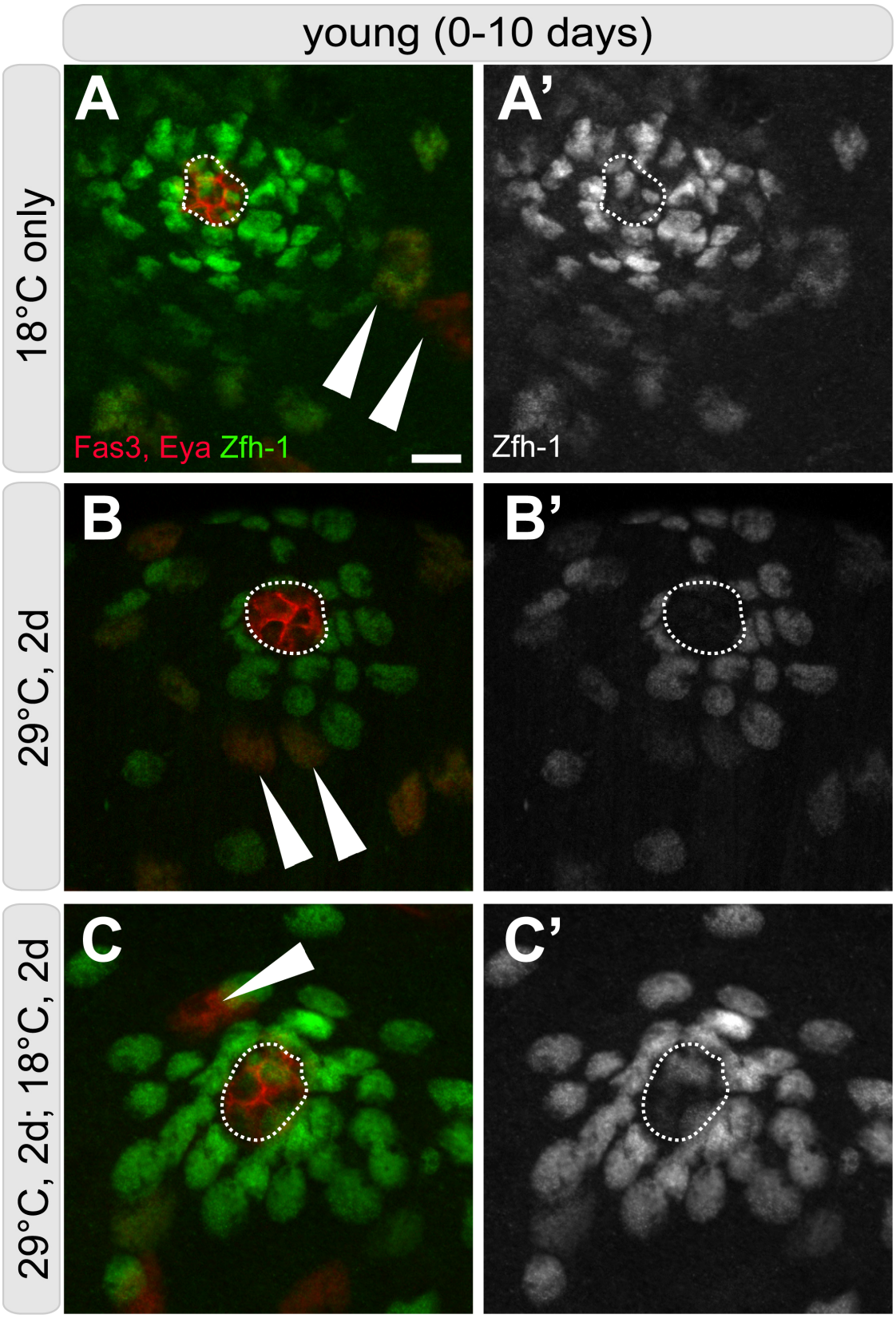
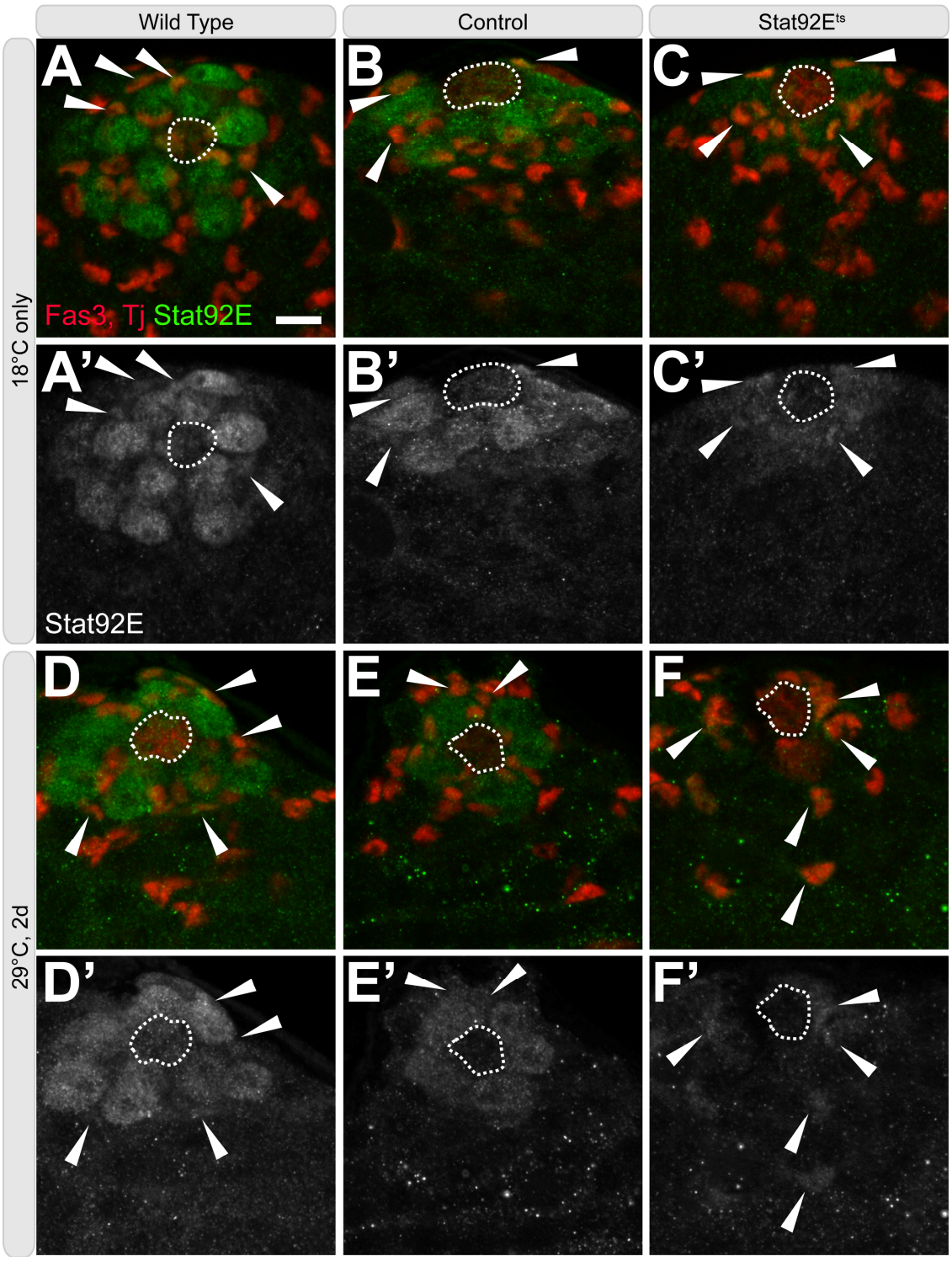

young (0-10 days)
18°C only
29°C, 2d
29°C, 2d; 18°C, 2d
A
A'
B
B'
C
C'
Fas3, Eya Zfh-1
Zfh-1

Figure S3.3 Stat92E localization in young flies during the reversion paradigm.

(A-F') Immunofluorescence images of testes from young wild type, control, and Stat92Ets flies stained for the hub (Fas3, red, outline), early cyst cells (Traffic Jam [TJ], red), and Stat92E (green). (A-C') at 18℃ (D-F') at 29℃. Arrowheads point to Stat92E+TJ+ cells. Scale bars: 10μm. Genotype: (A-A',D-D') OregonR; (B-B',E-E') Stat92EF/+; (C-C') Stat92EF/ Stat92E06346 ; (F-F') Stat92EF/ Stat92EJ6C8


Wild Type
Control
Stat92E^ts
18°C only
29°C, 2d
A
B
C
Fas3, Tj Stat92E
A'
B'
C'
Stat92E
D
E
F
D'
E'
F'

Chapter 4: Conclusions and Future Directions

4.1 Conclusions

The critical roles that adult stem cells play in our bodies have been acknowledged for about a century (Ramalho-Santos & Willenbring 2007). Work in the past 15 years have advanced our knowledge about the intrinsic self-renewing properties of stem cells and how stem cells interact and work together with important extrinsic niche components to maintain tissue homeostasis (Morrison & Spradling 2008). At the beginning of this thesis research, stem cell niches were just being characterized in a variety of tissues, with the *Drosophila* gonads as the leading models showing stem cell and niche interactions *in vivo* (Xie & Spradling 2000; Kiger *et al.* 2001). The idea that aging could be attributed in part to decreased stem cell behavior had been outlined in studies mainly addressing intrinsic changes to stem cells rather than changes within the niche (Van Zant & Liang 2003). Using the *Drosophila* testis, this work was able to use the variety of markers, reagents, and genetic tools to characterize the stem cell niche during aging. This analysis led to the exciting results that in addition to cell-autonomous changes, the environment surrounding the stem cells are altered during aging. This work was one of the firsts to provide the mechanism that decreased signaling from the niche leads to stem cell loss and that restoration of this signal can prevent this loss (Boyle *et al.* 2007).

Initially, we thought (hoped!) that restoration of Upd signal in the hub would not only restore the number of GSCs around the hub, but the maintained GSCs will now behave like young GSCs. However, this was not the case; and in fact, the restored GSCs in aged flies were in S-phase less and the testes looked even more atrophied than those from normal aged-matched males despite having high levels of Stat92E. Of course, ‘fixing’ up just one part of the niche will not result in a healthy home for stem cells. Thus, understanding the regulation of other affected areas such as adhesion within the niche is also critical. The more severe effects observed in the rescued animals suggests that Upd activation of Jak-STAT signaling must be tightly controlled. Artificially introducing Upd in the hub likely interrupts the intricate balance of signaling within the niche among all three cell types, the extra cellular matrix, and systemic signals leading to altered stem cell behavior.

The observation that some old organisms still maintain some, albeit less, functional stem cells likely is the reason why stem cells was historically thought to be immortal. However, we know now that stem cells have a limited life-span and maintenance mechanisms ensure long-lived organisms still have stem cells towards the end of life. One mechanism discussed in this work, reversion (dedifferentiation) of early progenitors, may be utilized for stem cell replacement, and changes in this process during aging could possibly lead to decline stem cell pool over time. This analysis of reversion was the first to use the Stat92Ets reversion paradigm to reveal that reversion is compromised during aging possibly from the inability of early cyst cells to respond to the cues for reversion.

The *Drosophila* testis is a unique system for studying stem cells because the niche contains two populations of stem cells that are dependent on each other. The analysis using the Stat92Ets reversion paradigm revealed different behavior of GSCs and CySCs during aging. The analysis of cyst cell behavior during this paradigm reveals that unlike GSCs, these cyst cells are efficiently replaced regardless of age. CySCs are also more proliferative in aged flies compared to young flies, unlike GSCs where the opposite is true. These data emphasize that distinctive stem cells respond differently to cues despite residing in the same niche. Changes in the niche during can have different effects on various cell types.

Although reversion has been reported over 6 years ago, the process of reversion is still not very well understood. This work revealed that regardless of age, if reversion goes to completion, the original number of stem cells is restored. Thus the reversion process requires the niche, which has a “memory” where it dictates how many GSCs and CySCs it can accommodate. This work also illustrates a possible threshold step that a spermatogonial cell must overcome to become a stem cell; a novel concept that has not been addressed. Understanding how reversion is used for stem cell maintenance and how the stem cell niche affects this process is critical for understanding the decline of tissue homeostasis during aging.

4.2 Future Directions

This dissertation has laid the foundation to understanding how stem cell behavior is affected during the universal process of aging. Studies described in Chapter 2 begun to address some fundamental questions about how aging manifests in multiple phenotypes within one tissue. The basis of the loss of tissue homeostasis is a change of stem cell behavior due in part to the altered niche. This concept has been observed and applied to other stem cell models. As research advances, more information is revealed about the complex nature (signaling pathways, structural components) of the testis stem cell niche. Thus, analysis of these new discoveries in the context of aging will reveal a more comprehensive knowledge of the tissue.

However, merely correcting one signaling pathway in an aged individual will not completely reverse the tissue to what it once was. The specific targeted signaling pathway could be downstream of the 'aging origin'. Furthermore, since not all stem cell systems within an individual use the same mechanisms for maintaining stem cells, correcting one aged tissue (e.g. germ line) would leave another tissue (e.g. intestine) aged. Studies of systemic regulation of aging in mice suggest that there are dominant 'aging' factors within the circulation of an animal that affects stem cell niche integrity and subsequently influence stem cell behavior. This aging element has yet to be found. With advances in systems biology, high throughput analyses of young and aged animal systemic fluids may reveal a grocery list of molecules specific to aged animals. A follow up of these

molecules could reveal a true systemic aging factor. Thus finding the panacea for aging will require looking at the organism as a whole.

Reversion (dedifferentiation) has been shown to be a mechanism used by *Drosophila* to replenished lost stem cells over time. Chapter 3 provides evidence that the reversion process as a whole is affected during the aging process possibly contributed by changes to the stem cell niche. However, physical process of reversion is still a black box. Using the benefits of the different reversion paradigms and advances in live-imaging of germ line and somatic cells of intact tissues will be critical to understand how the three cell types complete the reversion process. One part of this process is how and when a future GSC becomes re-encapsulated by a pair of CySCs. Egf signaling from the GSC to the CySCs mediates the encapsulation process (Kiger *et al.* 2000; Schulz *et al.* 2002) and could be the same signal for encapsulation during reversion. Recent studies have shown using a different reversion paradigm that reverting germ cells possess actin-rich protrusions towards the hub (Sheng *et al.* 2009). This gives evidence that cell motility could be an important aspect for a GSC to re-establish its place within the niche. Furthermore, long-distance migration could be critical because during normal aging, reverted GSCs have been suggested to come from more differentiated spermatogonia theoretically 3 cell distances away in a space-restricted terrain (Cheng *et al.* 2008).

It is unknown if and how the niche selects from the pool of spermatogonia for the future GSC. In a wild type situation, there are many germ cells to select from and from an efficiency point of view, the daughter cell gonialblast closest to

the hub would theoretically be the one chosen. This would eliminate the energy required to breakdown cysts, travel, and reversal of transcriptional and epigenetic programs. However, we know this may not be the case since reverted GSCs may have originated from spermatogonia much further away (Cheng *et al.* 2008). Although Jak-STAT signaling from the niche has been shown to play a role in reversion (Sheng *et al.* 2009), the conventional model for Upd signal in the testis is that it is a short-range local signal (Harrison *et al.* 1998). Thus, the selection of cells for reversion in the niche could be regulated by other long distance signaling pathways. Yet, signaling from the niche may not be responsible for recruiting potential GSCs. For example, the GSCs that are more competitive for the niche compared to wildtype GSCs requires E-cadherin not the self-renewal signal in the Drosohila ovary (Jin *et al.* 2008). Similarly, competition for the niche between CySCs and GSCs is mediated by intrinsic regulatioin in CySCs (Issigonis *et al.* 2009) suggesting that there can be some cell-intrinsic mechanism for cells to be attracted to the niche. Thus understanding cell competition and signaling regulating the selection process could reveal some interesting changes to reversion during aging.

Presumably only a few of the GSCs around the niche are lost at a time during aging. When one GSC within a cyst has been selected to revert, the destiny of the rest of the cyst has not been discovered. During the Stat92Ets reversion paradigm, I showed in Chapter 3 that the number of GSCs gained back after reversion is the same as what it started with (around 8 for young and 6 for aged) suggesting that not all of the germ cells in an 8 or 16 cell cyst become a

stem cell. Studies using both of the reversion paradigms demonstrated that there was no elevated number of apoptotic events during the reversion process suggesting that the unused cyst may merely differentiate (Brawley & Matunis 2004; Kai & Spradling 2004). So far one has looked carefully enough to observe odd number of cells within a cyst after reversion or in aged animals.

Progenitor germ cells must go through extreme physical changes to become a GSC. Spermatogonia and the equivalent in ovaries (cystocytes) are packets of up to 16 cells with a shared cytoplasm and a fusome interspersed in all the germ cell cytoplasm. Previous report using the heat shock bam reversion paradigm in the ovary showed fusomes being pinched off by ring canal closure (Kai & Spradling 2004). As a result of reversion, fragments of fusomes and ring canals are often seen in reverted GSCs (Brawley & Matunis 2004; Kai & Spradling 2004; Sheng *et al.* 2009). This pinching is like what happens when a GSC divides and produces a differentiating daughter cell. Interestingly, an observation (though made with only one aged testis) showed many GSC-GB bridges in aged flies (Hardy *et al.* 1979). Thus, exploring whether the same signaling pathways that regulate ring canal closure of GSC-GB division is used dedifferentiation could explain the observed compromised reversion during aging. Since mammalian germ cells also are in cysts, understanding this process would also reveal how other higher organisms complete the reversion process.

One possible explanation for decreased GSC function during aging could be that reverted GSCs may not behave exactly like original GSCs. First do reverted GSCs reset their age as a stem cell? Reverted GSCs correlated with

misoriented centrosomes suggesting that it takes time for a progenitor cell to become a functional stem cell (Yamashita *et al.* 2007). Also, after reversion, GSCs contain fragmented fusomes and whether these remnants compromises cell function is unknown. Furthermore, transcriptional changes must occur as well for example, in late spermatogonia, Bam must turn off to become a GSC. Furthermore, epigenetic changes occur when stem cells differentiate, although perhaps early progenitor cells may not have full epigenetic stability compared to terminally differentiated cells (Skora & Spradling 2010). Another question is whether serial reversion can be sustained – for example, how many times can a GSC go through reversion? Thus, compromised reversion during aging could be a consequence of too many reversion and differentiation cycles.

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