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Defining a genetic framework for stem cell niche maintenance in the *Drosophila* testis

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ABSTRACT OF THE DISSERTATION

Defining a genetic framework for stem cell niche maintenance in the *Drosophila* testis

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Adult stem cells reside in specialized microenvironments, or niches, that have an important role in regulating stem cell behavior. Therefore, tight control of niche number, size, and function is necessary to ensure the proper balance between stem cells and progenitor cells available for tissue homeostasis and wound repair. Identification of genetic networks that regulate

stem cell niches are of particular interest for their potential roles in regenerative medicine. This dissertation work utilizes the *Drosophila* testis as a model for understanding how genetic factors regulate stem cell niche maintenance and regeneration.

In *Drosophila* males, the germline stem cell niche is located at the tip of the testis where germline and somatic cyst stem cells surround the apical hub, a cluster of approximately 10–15 somatic cells that are required for stem cell self-renewal.

Findings described in Chapter 2 led to the hypothesis that somatic cyst stem cells contribute to the apical hub and identified an important role for the Snail family transcription factor *escargot* within the stem cell niche. This work also identified adhesion to the hub is required for both germline and somatic cyst stem cell maintenance. Work presented in Chapter 3 demonstrates the cell type specific requirements of *escargot* within hub cells (maintenance) and somatic cyst stem cells (maintenance and regulation of proliferation). In addition, Chapter 3 provides direct evidence that the hub is absolutely required for germline and somatic cyst stem cell maintenance. Using both genetic and genomic approaches, we demonstrate that an interaction between *escargot* and the transcripton factor *Stat92E* coordinately act in regulating hub cell maintenance and somatic cyst stem cell activity. These data build the framework for findings described in Chapter 4 that provide preliminary evidence that the AP-4 transcription factor *cropped,* a putative transcriptional

target of *escargot*, is required for hub maintenance. Futhermore, these data suggest that the hub is capable of regeneration during adulthood.

This dissertation highlights the dynamic relationship between stem cells and niche support cells and provides insight into genetic programs that regulate niche size and function to support normal tissue homeostasis and organ regeneration during development and adulthood.

Chapter 1: Introduction

1.1 Stem Cell Niches

1.1.1 Stem cell niche function: Niches in tissue maintenance, regeneration, and disease

Adult stem cells are defined by the ability to self-renew and to generate daughter cells that contribute to the maintenance and repair of tissues in various organs. Many adult stem cells lose the abilty to self-renew when removed from their *in vivo* tissues suggesting that the local microenvironmental is necessary for stem cell identity. First hypothesized in respect to the hematopoietic system and verified at single cell resolution in invertebrate models, the microenvironment (stem cell niche) is a major determinant of stem cell quiescence, maintenance, and proliferation.

Stem cell niches are discrete and dynamic functional sites that influence stem cell behavior within specific tissues. Putative stem cell niches have been identified within mammalian tissues including the brain, bone, blood, muscle, skin, intestine, and gonads. Understanding the regulatory capacity of the stem cell niche is essential to the study of tissue physiology. Mechanistic concepts of how the stem cell niche balances stem cell self-renewal and differentiation are emerging and will aid in the understanding of

aging, disease, and regeneration. General themes emerge with the establishment, components, maintenance, and regulation of stem cell niches however the underlying principle is universal—tissue homeostasis under diverse physiological (development and aging) and pathological conditions (injury and disease). This introduction will cover topics including: the functional definitions of stem cells and stem cell niches, recent advances in monitoring stem cell interactions within their *in vivo* niche, models of stem cell niche and tissue regeneration, cancer stem cells and niche involvement in cancer progression and metastasis, and background information on the *Drosophila* male germline stem cell niche.

The experimental data and interpretations presented in this dissertation are based upon studies conducted in the *Drosophila* testis, a model system permitting single cell resolution of the stem cell niche. This work provides the first steps in generating a model to understand the molecular framework of stem cell niche dynamics, maintenance, and regeneration.

1.1.2 A functional definition for stem cells

Adult (or tissue) stem cells are defined by their function to 1) self-renew and 2) generate progeny that contribute to a specific tissue. Stem cells undergo self-renewing divisions, a characteristic that permits their functional identification using lineage tracing strategies. In *Drosophila,* lineage tracing strategies and label retaining methods have identified adult stem cell populations within the gonads (Xie and Spradling, 2000) (Kiger et al., 2001)

(Tulina and Matunis, 2001) and regions of the the digestive system (Ohlstein and Spradling, 2006) (Micchelli and Perrimon, 2006) (Singh et al., 2007) (Takashima et al., 2008). Mammalian lineage tracing strategies have capitalized on the use of Cre recombinase to track stem/progenitor cell fate in multiple tissues and organs including the testis (Nakagawa et al., 2007), skin (Clayton et al., 2007), intestine (Barker et al., 2007) (Sangiorgi and Capecchi, 2008), muscle (Collins et al., 2005), and brain (Merkle et al., 2007) (Young et al., 2007). Lineage analysis is instrumental to understanding stem cell function and fate. Additionally, lineage analysis aids in the confirmation of stem/progenitor cell marker expression and anatomic location of stem cell populations. However, the specificity and fidelity of lineage tracing and label retention strategies are often not sensitive enough to discrimintate at the single cell level between a stem cell and its immediate daughter (Kiel et al., 2008).

Stem cell self-renewal can occur via asymmetric division, symmetric division, or through daughter cell reversion (Kimble and Morrison, 2006). Regulation of asymmetric division is due to both cell-intrinic and extrinsic factors, details of which are best understood in invertebrate models. Asymmetric division is regulated by at least two distinct mechanisms: 1) the segregation of cell fate determinants specifying stem cell self-renewal or daughter cell differentiation and 2) mitotic spindle orientation. Asymmetric localization of cell fate determinants is present in *Drosophila* neuroblasts, as

upon division daughter cells inherit Numb (Spana and Doe, 1996), a negative regulator of Notch signaling, whose function is conserved in mammalian neural and muscle stem cell populations (Zhong et al., 1997) (Conboy and Rando, 2002).

Proper orientation of the mitotic spindle to ensure asymmetric division is orchestrated by the concerted efforts of the conserved PAR-aPKC complex as demonstrated in *C.elegans* embryos (Strome and Wood, 1983) and *Drosophila* neuroblasts (Doe and Bowerman, 2001). Cadherins and intergrins are required for PAR-aPKC localization within mammalian stem/progenitor cell populations in the epidermis (Lechler and Fuchs, 2005), suggesting that extrinsic signals may govern asymmetric cell fate. Oriented mitotic spindles are a prerequisite for asymmetric division of *Drosophila* male germline stem cells (GSCs) (Yamashita et al., 2003). GSC spindle orientation is in reference to positioning relative to the hub, a cluster of support cells that regulates GSC maintenace via short-range JAK-Stat (Janus kinase and signal transducer and activator of transcription) signaling and E-cadherin mediated physical attachment (Kiger et al., 2001) (Tulina and Matunis, 2001) (Voog et al., 2008). As a result of oriented spindle orientation perpendicular to the hub, GSC division results in the generation of a daughter cell (gonialblast) that has limited access to the the JAK-Stat ligand Unpaired. As a consequence of this physical displacement from the hub, the gonialblast undergoes differentiation.

Direct observation of symmetric divisions is present in the *Drosophila* ovary (Xie and Spradling, 2000) and the *C. elegans* gonad (Criddenden et al., 2006). Symmetric division likely functions as a means to increase mammalian stem cell pools during development (Morrison et al., 1995) or in response to acute need due to injury (Bodine et al., 1996).

Reversion (de-differentiation) of daughter cells is a mechanism allowing re-acquistion of stem cell identity. Within the *Drosophila* gonads, reversion occurs as differentiating germline cysts (two- to sixteen-cell clusters) located in regions far removed from the niche, break apart, physically re-occupy the niche, regain GSC markers, reacquire self-renew potential and are capable of generating functional gametes (Kai and Spradling, 2004) (Brawley and Matunis, 2004). This cellular flexiblity has long been a proposed mechanism of self-renewal in mammalian testis (de Rooij and Russell, 2000) and has recently been experimentally verified in this system (Barroca et al., 2008) (Nakagawa et al., 2007).

Thus multiple conserved self-renewal mechanisms (asymmetric division, symmetric division, reversion) are present to ensure that stem cell populations are maintained. However, while the ability to self-renew is complicit with stem cell identity, functionally stem cells must be able to generate daughter cells capable of contributing towards tissue maintenance. Lineage tracing strategies and prospective stem cell transplantation assays

can verify the ability of a stem/progenitor cell to generate differentiated functional cell types.

The best characterized mammalian stem cell is the hematopoietic stem cell (HSC). HSCs are defined by the ability to sustain long term multi-lineage reconstitution of the entire hematopoietic system in an irradiated mouse (Morrison et al., 1995). Enriched populations of hematopoietic cells containing HSCs can be isolated by fluorescent-activated cell sorting (FACS) based upon expression of cell surface marker combinations including: LKS (Lin-, Sca1+, c-Kit+) (Weissman et al., 2001) and SLAM (CD34-, Flk2-, LKS, CD150+, CD48-) (Kiel et al., 2005).

Within solid tissues, stem cell studies are often hindered by non-specific stem cell markers and difficulties in the isolation of putative stem cells (Morrison and Spradling, 2008). However, recent studies in epithelial tissues such as the intestinal epithelium, mammary gland, and prostate have successfully isolated single stem cell capable of tissue regeneration. Elegant work in establishing the identity and function of *Lgr5*+ intestinal crypt stem cell cells was accomplished using lineage analysis and *in vitro* culturing techniques (Barker et al., 2007) (Sato et al., 2009). *Lgr5*+ cells are intercalated between Paneth cells and in direct contact with the basement membrane. Experimenal data demonstrate that entire crypt lineages descend from *Lgr5*+ crypt cells that were capable of long term (>12 month) self-renewal. In addition, single dissociated *Lgr5*+ crypt cells cultured *in vitro*

generated crypt-villus organoid structures resembling intestinal epithelium and containing the appropriate differentiated cell types (Sato et al., 2009). Historical work using label retention suggested that intestinal stem cells are located at the +4 position and a recent study using a *Bmi1* lineage tracing strategy supports this as a putative position for stem cells (Sangiorgi and Capecchi, 2008). Thus overlapping stem cell and niche support cell populations may exist in the intestine.

Similar prospective single cell isolation based on marker expression of mammary stem cells (Lin-, CD29hi, CD24+) (Shackleton et al., 2006) (Stingl et al., 2006) and prostate stem cells (Lin-, Sca-1+, CD133+, CD44+, CD117+) (Leong et al., 2008) capable of generating structurally mature tissues suggest that single stem cells may be able to regenerate normally functioning mature tissue types capable of transplantion. In addition, these studies suggest that functional stem cell niches may also be generated *de novo* in these transplanted tissues. Thus optimized techniques in the isolation, culturing, and assaying of these putative stem cell populations demonstrate that mature cell types and tissue architecture can be generated from a single stem cell.

1.1.3 The stem cell niche balances stem cell self-renewal and differentiation

The defining characteristic of the stem cell niche is the ability to balance stem cell self-renewal and daughter cell differentiation. Thus confident identification of stem cell position and monitoring of stem cell behavior is

paramount to the verification of cell types near stem cells that may influence their behavior.

Using these criteria, at least two general classes of niches appear when classifying stem cell location and behavior in respect to anatomical position: stromal niches and epithelial niches (Morrison and Spradling, 2008). Stromal niches are discrete anatomical sites containing niche support cells that physically contact adjacent stem cells such as in the *C.elegans* and *Drosophila* gonads. These stromal niches influence stem cell behavior via close range signaling often within a single cell distance. Experimentally, the evidence that stromal niches exist is verified by loss of stem cell function upon niche support cell ablation or disrupting niche support cell function.

The best molecularly defined stem cell niche resides in the root tip of *A. thaliana*, where multipotent stem cells surround a cluster of support niche cells necessary for stem cell maintenance that can be experimentally verified surgically and genetically (van den Berg et al., 1997) (Sabatini et al., 2003) (Xu et al., 2006).

Stromal niches exist in invertebrate gonads as a distinct set of niche support cells physically interact and maintain stem cell populations (Morrison and Spradling, 2008)(Fuller and Spradling, 2007). Laser ablation of the distal tip cell (DTC) in the *C. elegans* gonad results in a loss of germline stem cell maintenance demonstrating that the DTC is necessary for niche identity (Kimble and White, 1981). Expression of the Notch ligand Lag-2 in the DTC

regulates regulates germline behavior (Henderson et al., 1994). In the *Drosophila* ovary, a cluster of support cells called cap cells express the BMP ligands Dpp/Gbb that suppress GSC differentiation (Xie and Spradling, 1998). In addition, stem cell anchoring to niche support cells is required for GSC maintenance in the *Drosophila* gonad niche support cells (cap cells and hub cells) mediated in part by the adhesion molecule DE-cadherin (Song and Xie, 2002) (Song et al., 2002) (Voog et al., 2008). The identication of niche cell types and factors regulating stem cell behavior in these invertebrate models is aided by the well defined location of stem cells and genetic tools available.

In contrast to the cellularly defined stromal niche, epithelial niches are defined by the absence of known niche support cells and are often are composed of stem cells in contact with basement membrane. Examples of epithelial niches include the *Drosophila* follicle stem cell (FSC), and mammalian muscle stem cell/satellite cells and basal keratinocytes of the epidermis. Epithelial niches may provide instructive signaling or act as passive/permissive substrates that enable stem cells to act as their own niche.

Drosophila FSCs are present in regions distant from the cap cells of ovarian germline stem cell niche and are in direct contact with the basement membrane. FSC behavior is unique in that these cells undergo long-distance coordinated replacement as a potential means of self-renewal (Nystul and Spradling, 2007). No localized signaling moieties have been identified for

regulating FSC behavior, although FSCs are in a defined location with respect to other cell types.

Muscle stem cells/satellite cells can be prospectively isolated based on marker and morphological expression and function (Sacco et al., 2008). Satellite cells (~5% of muscle nuclei) are mononuclear cells that reside between the muscle fiber and are enclosed by basal lamina. In response to myofiber ablation, satellite cells proliferate suggesting that myofibers may act as a local niche to normally suppress proliferation (Brack and Rando, 2007). Recent work has demonstrated that single cell transplants of muscle stem cell/satellite cell isolated based upon marker and morphological markers are capable of tissue regeneration (Sacco et al., 2008) (Cerletti et al., 2008) Muscle stem cell/satellite cells express the Wnt receptor Fzd7 and during injury induced regeneration Wnt7a expression promotes activation of these cells (Le Grand et al., 2009).

Epidermal skin stem cells are postulated to reside in close proximity to the basembent membrane of the epidermis. The keratin proteins *K5/K14* are used as basal stem/progenitor markers whose expression is localized near the basement membrane. These putative basal epidermal stem cells are influenced by Notch signaling and upon differentiation move suprabasally away from the basement membrane (Blainpain and Fuchs, 2009). Epidermal stem cells have also been identified in regions with high *p63* expression near the basal layer of the epidermis (Koster et al., 2004). These data suggest

epidermal stem/progenitor cell proliferation is regulated by the concerted efforts of *p63* and *p53*, although *p53* may independently regulate suprabasal cell differentiation (Truong and Khavari, 2007). Transcriptional networks regulated by chromatin remodeling factors that influence stem fate within the niche have been identified in epidermal keratinocytes (Sen et al., 2008) and the embryonic epidermis (Ezhkova et al., 2009). However a distinct functional definition of epidermal stem cells and its niche has proved difficult as progenitor cells may share equal self-renewing potential (Clayton et al., 2007).

Interactions between different niches (stromal and epithelial niches) is likely to be a developing theme as a host of factors including collagens, integrins, and the basement membrane are key in regulating stem cell activity and ultimately tissue maintenance. Within the *Drosophila* ovary, stromal (GSCs) and epithelial (FSCs) niches are present and the coordinated interaction between these stem cell niches is required for proper tissue function (egg production). Interestingly Type IV collagen attenuates the self-renewal signal Dpp gradient and may act to sequester this ligand within the ovarian niche (Wang et al., 2008). Similary within the *Drosophila* testis, integrin expression is essential for proper placement of the hub during development and required for germline stem cell niche maintenance (Tanentzapf et al., 2007).

Mammalian stromal and epithelial stem cell niches are less well understood, due largely to lack of definitive stem cell markers. The functional

identities of many niche support cells is based on strong correlative evidence in observing effects on the number and/or behavior of stem/progenitor cell populations. Within the HSC niche, increased numbers of osteoblastic cells due to conditional inactivation of BMP receptor type IA (Zhang et al., 2003) or activated PTH/PTHrP result in an increase in the number of HSCs (Calvi et al., 2003). In addition multiple factors and cell types have been identified that regulate HSC quiescence (Angiopoetin 1-Tie2) (Arai et al., 2004), self-renewal (Wnt) (Fleming et al., 2008) (Reya et al., 2003), maintenance (CXCL12) (Sugiyama et al., 2006) (Calcium receptor)(Adams et al., 2006), engraftment (Galpha(s)) (Adams et al., 2009), and location (adipocytes)(Naveiras et al., 2009). Using SLAM immunohistochemical markers for HSCs (CD34-, Flk2-, LKS, CD150+, CD48-), demonstrated that approximately two-thirds of HSCs are near vascular sinusoids, suggesting that a vascular niche may be present (Kiel et al., 2005). However, HSCs migrate frequently through the vasculature and may encounter multiple niches within different tissues (Laird et al., 2008). Thus HSC function is likely influenced by multiple environmental signals. No definitive conclusions can be made as to the complete nature of the HSC niche until a full characterization using loss of function analysis for putative niche support cells (osteoblasts, endothelial cells, megakaryocytes, perviascular cells, adipocytes) and factors is complete.

Regardless of niche type, paramount to niche discovery and characterization are sensitive and specific tools to perturb and monitor stem

cell and niche cell function to determine causal relationships. Experimental gain and loss of function studies in cell types (stem cell and niche support cell) likely to influence stem cell behavior are necessary to define the stem cell niche. Thus, advances in genetic tools and imaging modalities are essential to defining stem cell and niche support cell identity and function.

1.1.4 Live imaging of stem cell interactions within the niche

The ability to continously observe and analyze stem cell behavior rather than at specific time points in fixed specimens is necessary to a better understanding of stem cell function *in vivo* (Schroeder, 2008). Advanced imaging modalities, enhanced fluorescent probes, and increased data analysis methods have given unprecedented access in observing the derivation of hematopoietic progenitor populations (Eilken et al., 2009), behavior of germline stem cells in *Drosophila* (Cheng et al., 2008), and identifying stem/progenitor cells interactions within the niche in the murine hematopoietic system (Lo Celso et al., 2009) (Xie et al., 2009) and testis (Yoshida et al., 2007).

Recent advances in the developmental origins of blood progenitor cells have been made possible through the use of continous live imaging of explanted embryonic endothelial cells and embryonic stem cell derived endothelial cells at the single cell level. These studies demonstrate using simultaneous detection of morphological and molecular marker expression that blood progenitors can arise from endothelial cells (Eilken et al., 2009).

The fluid nature of the hematopoietic system is ideal for isolation of putative HSC populations via fluorescence activated cell sorting technologies and subsequent transplantation assays to confirm stem cell identity and function. Recent evidence suggests that HSC niches may be endosteal or vascular in nature. However technical limitations and the migratory nature of HSCs may confound conclusions in determining the exact location of the HSC niche when observing fixed specimens.

To address this issue, advances in understanding the components of the adult HSC niche have been aided by intravital microscopy using two-photon video imaging and high resolution confocal optics. Recent studies have observed HSC/progenitor cell behavior within the mouse calvarium of live animals (Lo Celso et al., 2009) or *ex vivo* preparations of the femur (Xie et al., 2009). Live imaging within the mouse calvarium suggest that HSC/progenitor cells reside within perivascular sites near osteoblasts in close contact with endothelial vasculature. In these studies, HSC/progenitor cells were observed to reside significantly closer to osteoblasts within the calvarium that constituitively express the PPR (parathyroid hormone/parathyroid hormone related peptide receptor), demonstrating that non-HSC/progenitor factors do regulate HSC/progenitor behavior, complementing earlier work (Calvi et al., 2003).

Similar insights into identifying stem cell niches have been realized using time lapse imaging and three-dimension reconstructions within the

mouse testis. Specific markers for spermatogenic stem cells do not currently exist, although a subpopulation of stem cells is present in undifferentiated spermatogonia (A_{undiff}) that can be functionally tested via transplantation or clony forming assays (Brinster, 2002) (Nakagawa et al., 2007). Spermatogonia lie at the basement membrane of the seminiferous tubules and are in close contact with Sertoli cells. However a distinct niche has not been identified for spermatogentic stem cells (Tegelenbosch and de Rooij, 1993). In recent work, *Neurogenin3-GFP* A_{undiff} spermatogonia were observed to preferentially localize near vasculature and upon differentiation A_{undiff} migrated away from localized regions of branching vessels. The localized regions of vasculature where A_{undiff} were clustered may include inputs from interstitial cells that influence the niche (Yoshida et al., 2007). These findings also suggest that niche location and function is flexible as vasculature alterations due to surgical transplantation resulted in a relocalization of A_{undiff} and presumably their niche.

Within the well defined stem cell niche of the *Drosophila* gonad, germline stem cells (GSCs) physically contact hub cells (testis) or cap cells (ovary). Time lapse imaging in explanted *Drosophila* testis have complemented observations that the orientation of the GSC mitotic spindle is essential for asymmetric division of male GSCs and that this behavior is centrosome dependent (Yamashita et al., 2007) (Cheng et al., 2008). The asymmetic localization of the small nucleolar ribonucleoprotein *wicked* in

female GSCs and neuroblasts was also recently confirmed using live-imaging techniques. *Drosophila* female GSCs asymmetrically segregate *wicked,* a factor required for their maintenance (Fichelson et al., 2009).

Live-imaging technologies have provided new insights into the description of factors regulating stem cell self-renewal via cell-intrinsic determinants and from non-cell-autonomous niche support cell derived signals. The ability to observe *in vivo* interactions between stem cells and their niche in real time is key to advancing stem cell niche studies.

1.1.5 Stem cell niche dynamics during development

HSCs inhabit multiple tissues that harbor and influence their behavior throughout embryonic development suggesting that multiple unique niches are established during this critical period. Within the mouse the yolk sac, aorto-gonadomesonephros (AGM), placenta and fetal liver participate in embryonic and definitive hematopoiesis in temporal sequence (Zon, 2008). Blood islands composed of nucleated erthrocytes designate the first sign of hematopoiesis in the yolk sac, however these cells are not capable of long term reconstitution in an adult. Definitive HSCs are present in the yolk sac shortly after blood island formation and are also present within the precursor AGM. Debate between HSC migration and *de novo* HSC generation within distinct tissues, is supplied by recent evidence suggesting that coincident HSC development is present in the placenta and not due to HSC migration from the AGM. In *Ncx1* mutant embryos that lack a heartbeat, HSCs were found in the placenta suggesting

that the placenta is a blood forming organ (Rhodes et al., 2008). However, blood flow appears to be a major stimulant for HSC formation in the AGM (North et al., 2009). After initial HSC specification, HSCs migrate from the AGM and/or placenta and expand in the fetal liver. Subsequently, HSCs migrate as hematopoiesis transitions from the fetal liver to the adult bone marrow. Factors regulating engraftment and transition into the bone marrow niche include the Calcium Receptor (CaR) (Adams et al., 2006) and chemokine CXCL12 (Sugiyama et al., 2006). Mutant mice for these factors have normal production of fetal liver HSCs, but fail to transition to hematopoiesis in the marrow space (Adams et al., 2006).

HSCs inhabit sites near the bone marrow and vasulature during adulthood, although the HSC population is in constant flux and HSCs continually migrate throughout the body. Under steady state conditions approximately 6% of HSCs undergo division per day and are in continuous circulation (Kiel et al., 2007). Understanding how the unique and diverse niches are established and regulate HSC behavior throughout development will be accomplished by strictly defining the involvement of specific cell types and factors. The involvement of niche support cells in the expansion and maturation of HSCs may lead to enhanced therapeutic strategies for tissue replacement and regeneration.

1.1.6 Models of stem cell niche regeneration and niche involvement in tissue repair

A key feature of the stem cell niche is the ability to appropriately respond to normal tissue demands of maintenance during the aging process and in acute settings in response to tissue injury or disease. During aging, stem cell activity and stem cell niche function decline. Loss of tissue intergrity and reduced ability to repair injured tissue are hallmarks of aging, and likely a result of diminished stem cell niche function. Studies in *Drosophila* gonads demonstrate that the activity and number of germline stem cells decline with age and that this loss correlates with decreased niche factor production (Boyle et al., 2007) (Pan et al., 2007). These data suggest that niche support cells are a major contributor to stem cell (and tissue) maintenance.

Within mammalian muscle, muscle stem cell/satellite cell number and function decline with age and as these cells are more suspectible to apoptosis.

In response to injury, muscle stem cell/satellite cell proliferation is influenced by Notch signaling from neighboring myofibers (Conboy et al., 2003). However in aged animals, reduced levels of the Notch ligand Delta limit muscle regeneration while muscle stem cells/satellite cells still express normal levels of Notch. Interestingly, muscle stem cells/satellite cells in aged animals can respond to injury when exposed to a young environment via parabiosis (Conboy et al., 2005). These data suggest that aged muscle stem

cells/satellite cells are not unable to adequetely respond, but rather local niche support cell or systemic factors are lost during aging.

How the recruitment and activation of stem cells and their niches is coordinated during tissue regeneration is unclear, but is of great interest towards the development of regenerative therapies. Epidermal tissue damage of the skin is repaired by a concerted effort of multiple distinct stem cell populations that normally do not contribute to epidermal homeostasis (Blainpain and Fuchs, 2009). Follicle stem cells, necessary for hair follicle regeneration, reside in the bulge and do not normally contribute to the epidermis homeostasis. Follicle stem cells can be identified using lineage tracing strategies using *Krt15* or *Lgr5* expression. However, follicle stem cells are recruited in response to epidermal injury and appear to act as transit-amplifying cells that are eliminated in the first stages of wound repair. Similarly during wound repair, *de novo* hair follicle regeneration has been observed suggesting that new stem cell niches are established during adulthood. This work suggests that early developmental processes involving Wnt signaling may be recapitulated to permit tissue and niche regeneration (Ito et al., 2007).

In response to intestinal injury, *Drosophila* intestinal stem cell (ISC) division rate increases and the numbers of ISC progeny increase as a means to repair epithelial disruption. ISCs are multipotent and can be identified with relatively high specificity using the Notch ligand Delta (Ohlstein and Spradling,

2006) (Micchelli and Perrimon , 2006). Insulin receptor signaling regulates the proliferative potential of these damage induced ISCs and their progenitor cells (Amcheslavsky et al., 2009).

Direct experimental evidence demonstrates that the stem cell niche is capable of regeneration in *A. thaliana* (Xu et al., 2006) and additional stem cell niches are likely generated during injury induced repair of the liver (Stanger et al., 2007)(Duncan et al., 2009) and skin (Ito et al., 2007). How the size and number of stem cell niches in particular tissues is determined is not known and aberrant regulation of the activity, size, or number of stem cell niches may confer suspectibility to cancer initiation or progression.

1.1.7 Cancer stem cell niche

The heterogenous nature of tumors has been appreciated for decades (Bruce et al., 1963) but the differing cancer-inducing capacity of distinct cell populations was not demonstrated until the observation that a single acute myelocytic leukemia initating cell (CD34+, CD38-) can recapitulate leukemia in serial transplants in immune-deficient hosts (Lapidot et al., 1994). These data suggest that much like the hierarchal blood system where stem cells generate daughters with limited lineage and self-renewal potential, that cancer may be propagated by cancer stem cells and tumor load by the cancer stem cell's progeny. Thus experimental standards to rigorously demonstrate the identity of a cancer stem cell include the ability of a putative cancer stem cell to reconstitute a cancer phenotype in a serial transplantation assay. From a

therapeutic standpoint these results suggest to eradicate specific types of cancer, treatment must be focused upon ablation of the cancer stem cell population.

Cancer stem cells have been identified in multiple cancer models including: breast (Al-Hajj et al., 2003), colon (Dalerba et al., 2007), prostate (Collins et al., 2005) (Richardson et al., 2004), and brain (Singh et al., 2004). These cancer stem cells have been hypothesized to share similar characteristices to normal adult stem cells (epigenetic states, response to self-renewal signals, resistance to apoptosis). Recent work has demonstrated that hematopoietic stem cells (Ito et al., 2004)(Totozova et al., 2007) and neural stem cells contain lower levels of reactive oxygen species, a feature present in some epithelial tumor initiating cells (Diehn et al., 2009) and glioblastoma initiating cells (Bao et al., 2006). These shared stem cell characteristics confer radiation and chemotherapy resistance in mouse models of medullablastoma (Hambardzumyan et al., 2008) and breast cancer (Diehn et al., 2009). Cancer stem cells may also originate from the normal stem cell population and co-opt niche environments for disease progression. Understanding the origin and factors influencing these tumor intiating cells is paramount to therapeutic intervention.

Recent findings suggest that the microenvironment can regulate cancer cell behavior providing evidence that cancer stem cell niches exist. In stromal tissues surrounding basal cell carcinoma, upregulated expression of the BMP

antagonist *Gremlin1* is observed and may contribute to cancer cell activity (Sneddon et al., 2007). These and other studies suggest that surrounding tumor stroma may influence cancer cell behavior in skin or in the breast (Karnoub et al., 2007). These data are consistent with studies demostrating that myeloproliferative disease can occur with mutations that effect the bone marrow microenvironment and not cancer initiating hematopoietic cells (Walkley et al., 2007).

It is not inherently clear in these cancer stem cell niche models if the surrounding microenvironment is induced by cancer cells, recruits cancer cells, or if the niche is necessary for cancer progression. Permissive sites of metastasis, such as first described in Paget's seed and soil hypothesis, suggest that cancer progression occurs in sites hospitable to tumor initiating cells. Recent work has demonstrated that within the vasculature, tumor metastasis is dependent upon pre-metastatic niche colonization from hematopoetic progenitor cells expressing Vascular endothelial growth factor receptor 1 (VEGFR1) that subsequently are sites of metastasis (Kaplan et al., 2005). Isolated glioblastoma cells can induce vascular remodeling, generating a distinict microenvironment, that is necessary for tumor growth (Bao et al., 2006)(Calabrese et al., 2007). Steps in metastatic niche maturation have been proposed (Psalia and Lyden, 2009) as metastasis likely involves both the establishment of cancer niches and gained competence of cancer initiating cells (Nguyen et al., 2009). The specificity of metastasis to distinct organs has

been recognized for years and recent work suggests that transcriptional network profiles may promote metastasis to specific tissues (Bos et al., 2009).

The origin or involvement of cancer stem cells is unclear in many cancer models. Evidence exists suggesting that cancer initiating cells that are originally derived from progenitor cell populations may re-acquire stem cell characteristics (Rossi et al., 2008). In addition, the frequency of cancer stem cell/tumor initiating cells can be increased several orders of magnitute dependent upon the mouse model used for transplantation (Quintana et al., 2008). This work suggests that cancer stem cells may not be as rare as previously thought and demonstrates that accounting for the genetic background of transplanted animals is key to the interpretation of certain cancer models.

Identification and distinct targeting of cancer stem cell/tumor initiating cells is a great interest and promising results suggest that cancer stem cells can be specifically targeted in AML that do not effect normal hematopoietic stem cells (Guzman et al 2002) (Guzman et al 2007). Thus much work is ahead in the characterization of cancer stem cells and the factors that regulate their behavior.

1.2 The *Drosophila* testis as a model for niche studies

The adult *Drosophila* testis is a coiled transparent blind-ended tube that contains maturing germline cells in a spatial-temporal pattern down its length. At the apical tip of the testis early germ cells are in direct physical contact with somatic cells that influence their behavior. Genetic tools available for lineage tracing, transgene expression, mutant analysis, and immunohistochemistry to identify cell types make the *Drosophila* testis an ideal system to study the stem cell niche (Gonzcy et al, 1992) (Fuller and Spradling, 2007).

1.2.1 Identification and markers of the stem cell niche

Germline (GSC) and somatic stem cells (SSC/CySC) directly contact and receive short range signaling necessary for self-renewal from a cluster of somatic cells called the hub. Hub cells can be identified using immunohistochemical markers and reporter genes that mark the expression of the cell adhesion molecules Fasciclin III, DE-Cadherin, and DN-cadherin; the signaling ligands Unpaired and Hedgehog; and the serine-threonine kinase Center-divider (Le Bras and van Doren, 2006).

GSC and SSC/CySCs have been characterized using lineage tracing strategies, and can be identified based upon marker expression and physical location. GSCs share markers with their immediate daughter cell (gonialblast) that include dot-shaped spectrosomes and Stat92E expression. In addition, *escargot* and *wingless* are expressed in early germline cells are often used as

evidence for the presence of GSCs (Terry et al., 2006). SSC/CySCs are in physical contact with the hub and express the transcription factors Zfh-1, Traffic Jam, Stat92E, and Escargot (Leatherman and Dinardo, 2007) (Li et al., 2003).

1.2.2 Gonad coalescence and embryonic hub formation

The embryonic gonad (composed of somatic and germline cells) is formed in early embryonic development as germ cells positioned at the posterior of the early embryo migrate through the mesoderm and interact with somatic gonadal precursors (SGP) (Boyle and Dinardo, 1995). This interaction occurs by stage 12 of embryogenesis in abdominal parasegments 10 to 13. Early germ cells can be identified by expression of the germ cell specific antigen Vasa (Lasko and Ashburner, 1988). Anterior SGP identity is specified by abdominal-A (Abd-A) while posterior male-specific SGP identity is specified by Abd-B (Defalco et al., 2004). By stage 14, SGPs have ensheathed early germ cells and the gonad is round in structure.

Sexual identity is specified under the coordinated control of *transformer* and *doublesex* by stage 15/16, and expression of *upd* and *esg* confirms male identity (Wawersik et al., 2005)(Casper and Van Doren, 2006). Early germline cells and somatic cells are in contact with each other, and hub specification occurs beginning at stage 16. Abd-A and Abd-B work in concert to specify hub identity (Boyle and Dinardo, 1995). In addition, germ cell expression of the ligand Boss (Bride of Sevenless) activates its cognate tyrosine receptor kinase

Sevenless expressed in somatic cells at the posterior end of the gonad to restrict embryonic hub cell number (Kitadate et al., 2007). Embryonic hub cells express adult hub cell markers in a temporal fashion as *upd* and *esg* are expressed during stage 15/16, and FasIII, DE-cadherin, and DN-cadherin at stage 17 (Le Bras and Van Doren, 2006). A rosette of early germ cells encircle the hub at this stage suggesting that the stem cell niche has formed.

Proper placement of the hub during embryonic development is integrin dependent as hubs in integrin mutant embryos fail to attach to the extracellular matrix of the gonad sheath (Tatenzapf et al., 2007). Instructions for hub placement at the anterior end of the testis are not known, but these data demonstrate that the extracellular matrix is necessary for proper niche position and function.

1.2.3 Hub interactions with germline and somatic cyst stem cells

The hub is a cluster of post-mitotic cells that are in direct contact with two stem cell populations: GSCs and SSC/CySCs (Hardy et al., 1979). Hub cells secrete the Janus Kinase-Stat (JAK-Stat) ligand Unpaired, a molecule necessary and sufficient for GSC and SSC/CySC self-renewal (Kiger et al., 2001) (Tulina and Matunis, 2001). Ectopic expression of *upd* in germ cells results in an expansion of early germline and somatic cells. Conversely, clonal analysis demonstates that *Stat92E* is required for GSC and SSC/CySC maintenance. In addition mutant flies for the JAK homologue, *hop*, exhibit premature GSC and SSC/CySC loss. GSCs and SSC/CySCs also express

high levels of the adhesion molecule DE-Cadherin which is necessary for their maintenance most likely by promoting adhesion to the hub (Voog et al., 2008). Mutant backgrounds of the Rap GTPase PDZ guanine nucleotide exchange factor (RAP-GEF) homologue *Gef26* which regulate DE-cadherin expression result in GSC and SSC/CySC loss (Wang et al., 2006). GSC anchoring to the hub in the *Gef26* mutant background can be rescued by germline expression of DE-cadherin, suggesting that intimate contact with the hub is necessary for stem cell maintenance (Wang et al., 2006).

SSC/CySCs can contribute to the hub during adulthood and are necessary for hub maintenance as conditional ablation of SSC/CySCs leads to hub cell loss (Voog et al., 2008). Evidence that SSC/CySCs had the potentilal to generate hub cells was first observed in agametic mutant backgrounds. Here SSC/CySCs underwent excessive proliferation and adopted hub cell characteristics suggesting that the germline restricts SSC/CySCs from acquiring hub cell fate (Gonczy and Dinardo, 1996). Hub cells autonomously require *esg* and *Stat92E* expression for their maintenance, and hub cell loss results in differentation of GSCs and SSC/CySCs (Voog et al., in preparation). Thus, coordinated interactions between hub cells, GSCs, and SSC/CySCs is essential for proper function of the stem cell niche in the *Drosophila* testis.

1.2.4 Factors regulating hub size and maintenance

Ultrastructural analysis of the *Drosophila* testis using electron microscopy identified the hub as a cluster of 10-15 structurally distinct non-

mitotic cells positioned at apical tip of the testis (Hardy et al., 1979). Hub cell function and number decline during aging (Boyle et al., 2007), however factors regulating hub size have not been extensively studied. Work presented in this disseration will focus upon genes that the maintenance and number of hub cells during adulthood.

1.2.5 Germline stem cell self-renewal and reversion

GSC self-renewal is dependent upon JAK-Stat signaling from adjacent hub cell release of Upd. Germ line loss of function *stat92E* clones demostrate that a cell-autonomous requirement for GSC maintenance. Interestingly, gain of function expression of the JAK orthologue hop^{TumL} within somatic but not germline cells results in both somatic and germline cell proliferation. These data suggest that self-renewal and proliferation is coordinated between GSCs and SSC/CySCs (Leatherman and Dinardo, 2008). Conditional manipulation of JAK-Stat signaling with use of a temperature sensitive loss of function *Stat92E* allele demonstrates that transit-amplying cells (gonialblast to 16-cell cyst) are capable of dedifferentiating and regaining stem cell identity (Brawley and Matunis, 2004). The ability of early germline cells to dedifferenciate declines during aging, however newly reverted GSCs still remain under strict mitotic control to ensure asymmetric cell division (Cheng et al., 2008). Thus GSC self-renewal is influenced by at least two niche support cell types (hub cells and SSC/CySCs) and GSCs use at least two distinct mechanisms

(asymmetric division and reversion) to ensure proper germline stem cell number under homeostatic conditions.

1.2.6 Asymmetric cell division of germline stem cells

Drosophila male GSCs normally undergo asymmetric division to ensure a single GSC and single daughter cell are generated upon mitosis. In this model, GSC spindle orientation is perpendicular to the hub such that upon division the GSC remains in contact with the hub, while the daughter gonialblast is displaced and begins to differentiate (Yamashita et al., 2003). Centrosome position, which dictates spindle orientation in male GSCs, is coordinated during interphase as one centrosome remains adjacent to the hub-GSC interface while the second centrosome moves away from the hub (Yamashita et al., 2003). Using a heat shock induced pulse-lineage tracing strategy, position and inheritance of centrosomes was observed and demonstrated that a 'mother' centrosome is retained within the GSC, while a 'daughter' centrosome is acquired by the gonialblast (Yamashita et al., 2007). During aging, misoriented centrosomes increase in frequency and act to suppress GSC division (Cheng et al., 2008). The factors that regulate this cell cycle checkpoint are not known, but upon centrosome re-orientation GSCs undergo mitosis. The origin of misoriented centrosomes appears to be due, in part, from contributing dedifferentiating early germ cells that contact the hub and re-acquire stem cell identity. The process that recruits and permits germline dedifferentiation is currently unknown. The *Drosophila* male GSC

has emerged as a powerful cell type to study asymmetric division, and appears that many aspects of this process are cell-autonomous. Identification of the involvement of adhesion molecules or other niche derived factors that may influence this highly coordinated process are of interest to the stem cell field in general.

1.2.7 Somatic cyst stem cells

During adulthood, roughly ~15 SSC/CySCs are in close contact with the hub and can be identified based upon position and expression of the transcription facdtors Zfh-1, Traffic Jam, Stat92E, and *escargot* (Leatherman and Dinardo, 2008) (Li et al., 2003). SSC/CySCs are multipotent and can generate hub cells and early cyst cells during adulthood (Voog et al., 2008). SSC/CySC maintenance requires the cell adhesion molecule DE-Cadherin (Voog et al., 2008), Escargot (Voog et al., in preparation), Zfh-1 and C-terminal binding protein CtBP (Leatherman and Dinardo, 2008). SSC/CySC proliferation is under the regulation of JAK-Stat signaling (Kiger et al., 2001) (Tulina and Matunis, 2001), expression of Zfh-1 (Leatherman and Dinardo, 2008), and Escargot (Voog et al., in preparation). The embryonic precursors of SSC/CySCs are not known, although an attractive hypothesis for their identity are anterior SGPs that lose *esg* expression (Le Bras and Van Doren, 2006). A major focus of this dissertation is the characterization of SSC/CySC behavior and factors that regulate SSC/CySC activity.

1.2.8 Early somatic cells regulate germline proliferation and differentiation

Somatic cyst cells (the descendants of SSC/CySCs) encapsulate and are necessary for the proper differentiation of germline cysts. Early (Zfh-1-, TJ+) and late (Zfh-1-, TJ-, Eyes Absent+) somatic cyst cells can be identified based on immunohistochemical marker expression. Somatic cyst cells encapsulate early germline cells through activation of the Epidermal growth factor receptor (EGFR)(Kiger et al., 2000). Germline cells express the EGFR ligand Spitz, and somatic cyst cells express markers of EGFR activity including phospho-MAP-Kinase and *vein-lacZ* (Kiger et al., 2000). Clonal analysis demonstrates that EGFR function is required in somatic but not germline cells, as germline EGFR mutant clones behave normally while somatic EGFR mutant clones do not survive (Kiger et al., 2000). In addition, proper EGFR function within somatic cells is required to restrict early germline cell proliferation. Consistent with these results, mutations in the serine/threonine kinase Raf produces a similar early germ cell accumulation phenotype and Raf appears to be required for somatic cell survival (Tran et al., 2000).

Extending these results, additional components of the EGFR pathway including *stet*, a homologue of the *rhomboid* protease, and the guanine nucleotide exchange factor Vav that balances Rac1 and Rho1 activity are

required for normal encapsulation of early germ cells (Schulz et al., 2002) (Sarkar et al., 2007).

In addition to ensuring normal germline differenation through encapsulation, SSC/CySCs have the capacity to induce germline proliferation with the overexpression of JAK-Stat components or a presumptive target of JAK-Stat signaling Zfh-1 (Leatherman and DiNardo, 2008). Stat92E expression is not detected in proliferating early somatic and germ cells in Zfh-1 overexpression experiments, suggesting that Zfh-1 is downstream or parallel to Stat92E activity. Understanding how SSC/CySC and GSC populations interact and co-regulate each other is of great interest and may provide insight into other stem cell systems.

1.2.9 Acknowledgements

Chapter 1, in part, is currently being prepared for submission for publication of the material. The dissertation author was the primary investigator and author of this paper.

Chapter 2: Multipotent somatic stem cells contribute to the stem cell niche in the *Drosophila* testis

2.1 Summary

Adult stem cells reside in specialized microenvironments, or niches, that have an important role in regulating stem cell behaviour. Therefore, tight control of niche number, size and function is necessary to ensure the proper balance between stem cells and progenitor cells available for tissue homeostasis and wound repair. The stem cell niche in the Drosophila male gonad is located at the tip of the testis where germline and somatic stem cells surround the apical hub, a cluster of approximately 10-15 somatic cells that is required for stem cell self-renewal and maintenance. Here we show that somatic stem cells in the Drosophila testis contribute to both the apical hub and the somatic cyst cell lineage. The Drosophila orthologue of epithelial cadherin (DE-cadherin) is required for somatic stem cell maintenance and, consequently, the apical hub. Furthermore, our data indicate that the transcriptional repressor escargot regulates the ability of somatic cells to assume and/or maintain hub cell identity. These data highlight the dynamic relationship between stem cells and the niche and provide insight into genetic programmes that regulate niche size and function to support normal tissue homeostasis and organ regeneration throughout life.

2.2 Introduction

Many stem cell niches include support cells that influence stem cell behaviour through secretion of diffusible molecules. Physical contact between stem cells and support cells and/or the extracellular matrix holds stem cells within the niche and close to self-renewal signals. Furthermore, niches provide spatial and mechanical cues that influence the fate of stem cell daughters. Therefore, the stem cell niche has an important role in regulating stem cell maintenance, self-renewal and survival (Jones and Wagers, 2008). However, little is known about the factors that regulate niche maintenance or size.

Approximately ten somatic cells, called the hub, are found at the apical tip of the Drosophila testis (Figure 2.1A) (Hardy et al., 1979). Germline stem cells (GSCs) and somatic stem cells (SSCs) surround and are in contact with hub cells. Whereas GSCs sustain spermatogenesis, SSCs produce cyst cells that encapsulate the maturing germ cells and ensure differentiation (Kiger et al., 2000) (Tran et al., 2000). Hub cells secrete the growth factor Unpaired (Upd) (Kiger et al., 2001) (Tulina and Matunis, 2001), which activates the JAK-STAT signal transduction pathway in adjacent stem cells. JAK-STAT signalling is necessary for stem cell maintenance and is sufficient to specify self-renewal of both GSCs and SSCs in the testis (Kiger et al., 2001) (Tulina and Matunis, 2001) (Harrison et al., 1998).

The apical hub is typically described as a post-mitotic, static structure. However, in agametic flies, SSCs proliferate and express hub markers,

leading to an apparent expansion of the apical hub (Gonczy and DiNardo, 1992). Furthermore, recent studies have noted that hub cell number and function decrease with age (Wallenfang et al., 2006) (Boyle et al., 2007), indicating that the stem cell niche in the testis is dynamic. Hub cells and SSCs share numerous features, including similar gene expression patterns and close association with GSCs and each other (Hardy et al., 1979) (Boyle and DiNardo, 1995); however, the precise relationship between SSCs and hub cells has not been explored.

2.3 Results

2.3.1 Somatic stem cell progeny contribute to the hub

We proposed that SSCs may serve as a source of cells that contribute to the apical hub and, consequently, the stem cell niche. To address whether SSCs give rise to hub cells, positively marked beta-galactosidase-expressing (beta-gal+) SSCs were generated using mitotic recombination, a technique typically used for lineage tracing analyses. Labelled SSCs were generated by heat-shocking flies of the appropriate genotype to initiate FLP-mediated recombination, resulting in reconstitution of the alpha-tubulin promoter upstream of the lacZ gene (Harrison and Perrimon, 1993). Hub cells were identified by immunolabelling with antibodies to Fasciclin III (Figure 2.1B) or DE-cadherin (Figure 2.1C) (Yamashita, et al., 2003), cell-surface proteins concentrated at hub cell junctions. SSCs and early cyst cells were identified by

immunolabelling with antibodies to Traffic Jam (TJ), a transcription factor that is strongly expressed in early cyst cell nuclei (Li et al., 2003) (Figure 2.1B) and weakly expressed in hub cells (Figure 2.1B).

Wild-type SSC clones were identified as beta-gal+ cells adjacent to the apical hub and surrounding germ cells (Figure 2.1C and Supplementary Table S2.1). A series of heat shocks after eclosion (hatching) led to at least one beta-gal+ SSC in 57% (n = 47) of testes from 3-day-old males analysed 1 day after heat shock. At 5 days after heat shock, 28% (n = 74) of testes contained at least one beta-gal+ SSC, and this frequency decreased to 10% (n = 183), 10% (n = 225) and 4% (n = 142) at 10, 15 and 30 days after heat shock, respectively (Supplementary Table S2.1).

In addition to beta-gal+ SSCs, beta-gal+ hub cells were also observed that co-labelled with DE-cadherin and FasIII. In fact, beta-gal+ cells were found within the hub in 34% (n = 47), 47% (n = 74) and 60% (n = 225) of testes from males at 1, 5 and 15 days after heat shock, respectively (Figure 2.1E, F and Supplementary Table S2.2). No beta-gal+ cells were observed in flies not exposed to the heat-shock protocol (Supplementary Table S2.2).

2.3.2 BrdU-labelled cells become incorporated into the apical hub

Previous reports concluded that hub cells are post-mitotic (Hardy et al., 1979) (Gonczy and DiNardo, 1996); however, it is possible that hub cells undergo rare divisions to become marked during recombination. To test whether hub cells are mitotic, dividing cells in the testis were labelled with 5'-

bromo-2-deoxyuridine (BrdU), which is incorporated into newly synthesized DNA during S phase. Flies were fed ('pulsed') BrdU for 30 min and aged ('chased') for up to 15 days. Subsequently, labelled testes were co-stained with antibodies to BrdU, as well as to the hub marker FasIII. No BrdU-positive (BrdU+) hub cells were detected after a 1-day chase (n = 82), although cells adjacent to the hub were clearly labelled (Figure 2.2A).

However, BrdU+ hub cells were observed 3–10 days after labelling (Figure 2.2B,D). BrdU+ hub cells were present in 4% (n = 143), 8% (n = 103) and 3% (n = 96) of testes assayed at 5, 7–8 and 10 days after labelling (Supplementary Table S2.3). Moreover, these BrdU+ cells co-expressed FasIII (Figure 2.2B) and an *upd* reporter, indicating that these cells function as hub cells (Figure 2.2D). These data are consistent with previous results indicating that hub cells are post-mitotic (Hardy et al., 1979) (Gonczy and DiNardo, 1996) and support the hypothesis that mitotically active SSCs act as a source of cells that can contribute to the apical hub.

2.3.3 Two populations of mitotically active somatic cells are present near the hub

SSCs are reported to be the only dividing somatic cells in the testis; however, we observed two distinct populations of somatic cells dividing near the testis tip. One group, which constituted 69% (20 out of 29) of phospho-histone-H3-positive somatic cells (n = 453 testes), appeared to be immediately adjacent to the hub, similar to GSCs (Figure 2.3A, B). We also observed

somatic cells that were dividing 1–2 cell distances away from the hub (9 out of 29) (Figure 2.3C, D). Several scenarios could explain these observations: there are two SSC populations, one which gives rise to hub cells and another that sustains the cyst cell lineage, or there are SSCs that produce both hub cells and a transient amplifying somatic cell population.

To distinguish between these possibilities, we adjusted the heat-shock regime to label, on average, only one beta-gal+ somatic cell and analysed the clones derived from these marked cells (Supplementary Figure S2.1). Thirteen per cent of testes examined contained marked somatic cells adjacent to the hub at 1 day after heat shock (n = 185), which decreased to 9.5% (n = 190), 3.3% (n = 212) and 2.8% (n = 72) of testes at 5, 10 and 15 days after heat shock, indicating a half-life for SSCs between 5–10 days. Single, marked somatic cells displaced away from the hub were found initially in 11.3% of testes examined at 1 day after heat shock, but this number decreased to 0.5% by 5 days after heat shock, which is consistent with these cells being transient non-stem-cell clones (Figure 2.3E, F and Supplementary Figure S2.1). In contrast, the number of testes that contained marked hub cells increased from 11.9% at 1 day after heat shock to 25.8%, 24.5% and 18.1% at 5, 10 and 15 days after heat shock, respectively. Clones containing all three cell types were observed in 22% (n = 18) and 14% (n = 7) of testes that contained marked SSCs at 5 and 10 days after heat shock, respectively (Figure 2.3G–I). From these data we conclude that multipotent SSCs self-renew and contribute to

both hub and cyst cell lineages, whereas dividing cyst cells, which we call cyst progenitor cells (CPCs), expand the pool of cells capable of encapsulating newly divided gonialblasts and maturing spermatogonia to ensure terminal differentiation (Kiger et al., 2000) (Tran et al., 2000) (Schulz et al., 2002) (Supplementary Figure S2.1).

2.3.4 Factors required for SSC maintenance and the SSC-hub cell transition

To identify factors required for incorporation of cells into the apical hub, SSCs were generated that were mutant for genes expressed in both cell types: DE-cadherin, which is encoded by the *shotgun* (*shg*) gene, and the transcriptional repressor Escargot (Esg). DE-cadherin is expressed in cyst cells and is strongly enriched in the hub (Figure 2.1C, D). SSC clones were generated that were homozygous mutant for either the loss-of-function shg^{IG29} or amorphic shg^{IH} allele. SSC maintenance and frequency of marked hub cells were assayed at various time points. In this experiment, marked cells and their progeny subsequently become permanently labelled by ubiquitous green fluorescent protein (GFP) expression (Figure 2.4).

Heat-shocked wild-type testes possessed GFP+ GSCs and SSCs, as well as GFP+ hub cells that co-stained with FasIII and DE-cadherin (Figure 2.4 A–C, E). In contrast to wild type, *shg* mutant GSC and SSC clones were not maintained (Figure 2.4D, Supplementary Figure S2.2 and Supplementary Table S2.1), indicating that DE-cadherin has a role in stem cell maintenance in

the testis, similar to its role in the ovary (Song et al., 2002) (Song and Xie, 2002) presumably by holding stem cells within the niche and close to self-renewal signals.

Marked hub cells were observed in 14% (n = 144), 35% (n = 114) and 65% (n = 110) of wild-type testes examined at 5, 10 and 15 days after heat shock, respectively (Figure 2.4E and Supplementary Table S2.2). Notably, progeny of DE-cadherin mutant SSCs contributed to the apical hub at a frequency similar to progeny from wild-type SSCs (Figure 2.4F and Supplementary Table S2.2). These data indicate that although DE-cadherin is required for SSC maintenance, it is not absolutely required for mediating the contribution of SSC progeny to the hub.

To confirm that *shg* is not required in hub cells for maintaining the apical hub, we used RNAi-mediated knockdown of *shg* expression in hub cells. A FasIII+ apical hub was detected in 100% of testes from 1-day-old (n = 12), 10-day-old (n = 15) and 20-day-old males (n = 28), despite a reduction in DE-cadherin expression in hub cells (Supplementary Figure S2.3). Testes collected at 20 days also displayed normal expression of a *upd* reporter (98%, n = 115) and contained TJ+ (100%, n = 25) cells near the apical tip (Supplementary Figure S2.3). These data support our findings that DE-cadherin is not absolutely required in hub cells to maintain a functional stem cell niche.

However, *shg* is required in SSCs and early cyst cells for maintaining the apical hub. Knockdown of *shg* in all SSCs and early cyst cells resulted in a decrease in the number of TJ+ cells in 1-day-old males, consistent with a role for *shg* in SSC maintenance (Figure 2.4G, H and Supplementary Figure S2.4). Surprisingly, decreased levels of DE-cadherin were also observed in hub cells (Supplementary Figure S2.4). In 29% (n = 42) of 15-day-old and 44% (n = 36) of 20-day-old males, the apical hub was severely diminished or lost, as determined by FasIII expression (Figure 2.4I, J and Supplementary Figure S2.4). These data support our model that SSCs act as a source of cells to maintain the apical hub.

The transcriptional repressor Escargot is expressed in many tissues, including GSCs, early cyst cells and hub cells in the testis (Streit et al., 2002) (Le Bras and van Doren, 2006). Males carrying a viable, hypomorphic allele of *esg*, called *shutoff*, exhibit loss of apical hub cells during development. Therefore, we hypothesized that *esg* may be required for regulating the contribution of SSCs to the hub. Mutant labelled SSCs were generated using two amorphic *esg* alleles, as described above.

Unlike progeny from *shg* mutant SSCs, progeny from *esg* mutant SSCs did not contribute to the hub at the same frequency as wild-type controls: esg^{L2} mutant GFP+ hub cells were observed in 5% (n = 38), 3% (n = 40), 0% (n = 36) and 4% (n = 52) of testes examined at 1, 5, 10 and 15 days after heat shock, respectively (Supplementary Table S2.2). In instances when *esg*

mutant GFP+ hub cells were observed, normal hub morphology was often severely disrupted (Figure 2.4K, L). These data suggest that *esg* regulates either the contribution of SSC progeny to the hub, perhaps by facilitating the cell fate transition between SSC and hub cell, or maintenance of hub cell fate.

To explore Esg function further, we used the agametic *oskar* (*osk*) mutant phenotype, in which SSCs proliferate and express hub markers, resulting in an apparent expansion of the apical hub (Gonczy and DiNardo, 1996). If Esg is required for mediating the transition of somatic cyst cells to the apical hub, we predicted that the expansion of FasIII+ cells would be blocked in an *esg;osk* double mutant background. In contrast to the expansion of FasIII+ cells in 82% (n = 16) of *osk* mutant testes, only 22% (n = 63) of testes from esg^{shof}*;osk* mutant males showed expansion of FasIII, despite there being clearly more TJ+ somatic cyst cells (Figure 2.4M, N). These data support our previous results and indicate that *esg* is required for the ability of somatic cells to assume and/or maintain hub cell fate.

2.4 Discussion

2.4.1 Somatic stem cell contribution to the hub

Our findings demonstrating that SSCs can adopt a hub cell fate highlight the dynamic nature of the stem cell–niche relationship and provide a mechanism to regulate the size and function of the stem cell niche in the Drosophila testis. In our model, as somatic cells are displaced from the hub,

there is a decline in self-renewal and proliferation potential, which could be reinforced by encapsulation of differentiating germ cells (Supplementary Figure S2.1). Interestingly, expansion of the somatic cyst cells as a consequence of germline loss suggests that germ cells exert an anti-proliferative influence that must be overcome in SSCs.

A better understanding of how stem cell niches are established and regulated in mammalian systems could facilitate modulation of the niche to enhance transplantation of stem cells in regenerative medicine (Conboy, et al., 2005) (Adams et al., 2007). Conversely, if an expanded or modified niche accompanies tumour progression or metastasis, then blocking niche maintenance programmes (niche ablation) could be used as an important anti-cancer therapeutic (Kaplan et al., 2005) (Sneddon et al., 2006).

2.5 Methods

2.5.1 Fly husbandry and stocks

Flies were raised at 25°C on standard cornmeal-molasses media. The *esg*^{shof} allele was isolated in the Fuller laboratory. The UAS-*shgRNAi* line was obtained from the Vienna *Drosophila* Resource Center (VDRC). The *upd*GAL4-UAS-*gfp* line was a gift from E. Bach. The following stocks were used; more information can be found at Flybase (http://flybase.bio.indiana.edu): *hs-FLP*, *X-15-29*, *X-15-33*, *OregonR*, *hs-FLP-122*, α*-tubulin*GAL80, α*-tubulin*GAL4, *2x*-UAS-e*GFP*, *FRT40A*, *FRTG13*,

osk^{CE4}, *osk*^{301}, *shg*^{IG29}, *shg*^{IH}, *esg*^{G66}, and *esg*^{L2}. Flies of the genotype *esg*^{shof}*; osk* were generated by crossing *esg*^{shof}*/CyO; osk*^{CE4}*/osk*^{301} females to *esg*^{shof}*/CyO; osk*^{CE4}*/TM6b, Hu* males. Controls were progeny from *esg*^{shof}*/CyO; osk*^{CE4}*/TM6b, Hu* or *esg*^{shof}*/CyO; osk*^{301}*/TM6b, Hu* females crossed to *esg*^{shof}*/CyO; osk*^{CE4}*/TM6b, Hu* males.

2.5.2 Lineage Tracing analyses

Adult flies or embyos (*y,w,hs-FLP/+; X-15-29/X-15-33)* were heat shocked for 2 h on 2 consecutive days (maximal clone induction) or for 1 h on 1 day (moderate clone induction) (Nystul and Spradling, 2007).

2.5.3 Mosaic Analysis with a Repressible Cell Marker (MARCM)

For MARCM experiments, late pupae or early larvae were heat shocked at 37°C for 2 h on 2 consecutive days. Flies were collected at various time points after the heat shock, immunostained and analyzed (Lee and Luo, 1999). MARCM wild type control genotypes used were *y,w, hsflp*^{122}; *FRT40A/FRT40A, tub*GAL80; *tub*GAL4/*2x*-UAS-*eGFP*. Mutant genotypes used were: *esg*^{G66} (*y,w, hsflp*^{122}; *FRT40A esg*^{G66}*/FRT40A tub*GAL80; *tub*GAL4/*2x*-UAS-*eGFP*), *esg*^{L2} (*y,w, hsflp*^{122}; *FRT40A esg*^{L2} */FRT40A tub*GAL80; *tub*GAL4/*2x*-UAS-*eGFP*), *shg*^{IG29} (*y,w, hsflp*^{122}; *FRTG13 shg*^{IG29}*/FRTG13 tub*GAL80; *tub*GAL4/*2x*-UAS-*eGFP), shg*^{IH} (*y,w, hsflp*^{122}; *FRTG13 shg*^{IH} */FRTG13 tub*GAL80; *tub*GAL4/*2x*-UAS-*eGFP).*

2.5.4 BrdU labelling

OregonR or *upd*GAL4-UAS-*gfp* 1-day-old males were starved in wet-plugged vials overnight (~16 h). Subsequently, starved flies were moved to vials containing 100 μl of 100mM BrdU dissolved in grape juice for 30 min and then moved to agar-molasses vials and aged. Testes were stained as described previously (Boyle et al., 2007).

2.5.5 Immunostaining and microscopy

Testes were dissected and fixed in 2% PFA in PLP buffer (0.075 lysine, 0.01 M sodium phosphate buffer pH7.4) for 60 min at room temperature, rinsed for 30 min in PBS containing 0.1% Triton X-100 and 0.3% bovine serum albumin, and immunostained with appropriated antibodies overnight at 4°C. Testes were then washed for 60 min at room temperature for 4 h. Testes were analyzed with a Zeiss Axiovert 200 microscope and processed, and Axiovision (version 4.5; C. Zeizz) and Adobe Photoshop software. Analysis of FasIII-, TJ-, and β-galactosidase-labelled testes was performed using a Leica TCS SP2 AOBS confocal microscope and LCS Lite 2.61.1537 software (Leica Microsystems.

2.5.6 Antibodies

Testes were stained using the following: rabbit anti-β-galactosidase (1:2,000) (Cappel), guinea-pig anti-TJ (1:3,000) (gift from D. Godt), mouse anti-BrdU (1:100) (BD Biosciences), rat anti-BrdU (1:100) (Accurate

Chemicals), rabbit anti-GFP (1:5,000) (Molecular Probes), and rabbit anti-phosphorylated histone H3 (1:200) (Upstate Biotechnologies). Mouse anti-Fasciclin III (7G10) (1:10) and rat anti-DE-cadherin (DCAD2) (1:20) were obtained from the Developmental Studies Hybridoma Bank. Secondary antibodies were diluted 1:500 (Molecular Probes). Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories).

2.6 Acknowledgments

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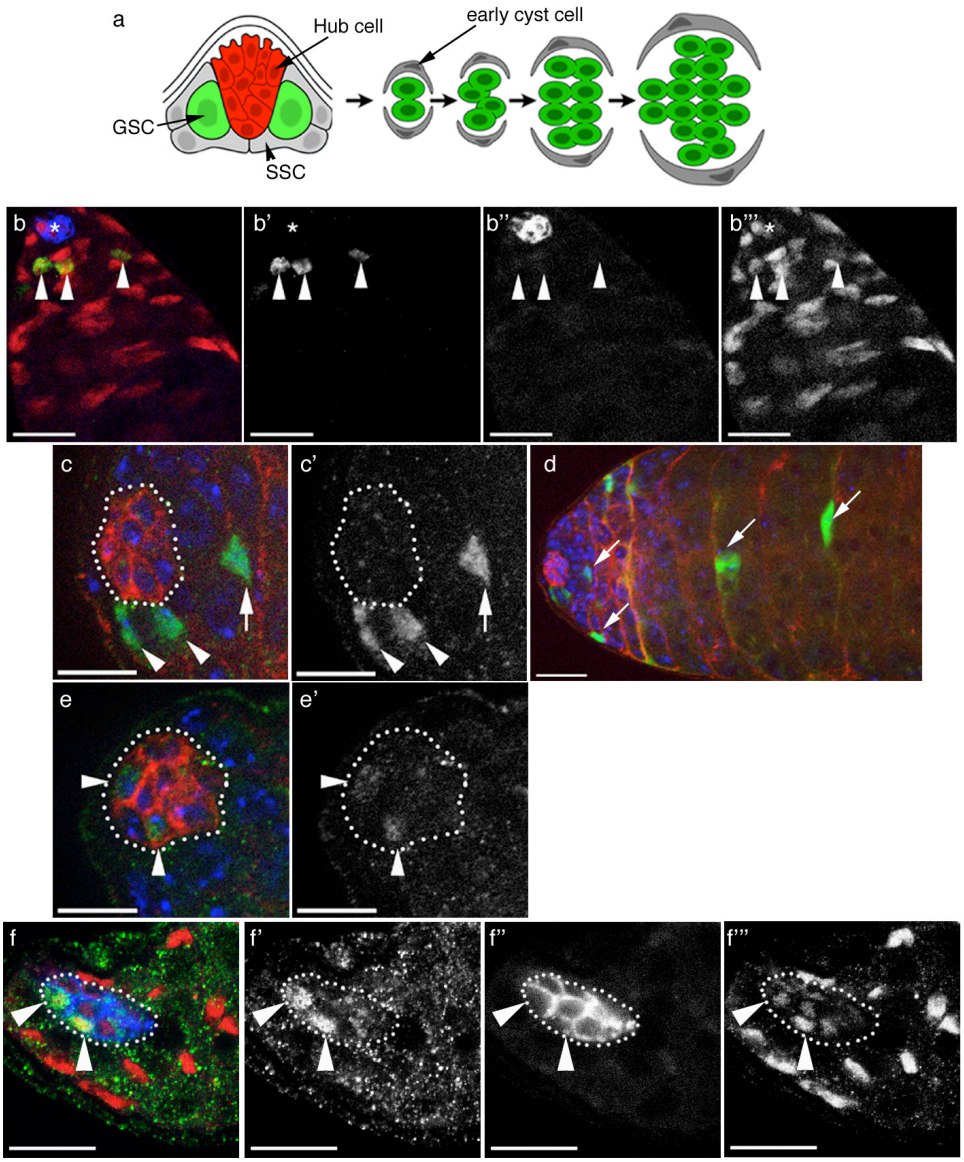


Figure 2.1 Somatic stem cell progeny contribute to the hub

a, Schema of the apical tip of the testis. Hub cells (red) contact somatic stem cells (SSC, light grey) and germline stem cells (GSC, green). SSCs produce cyst cells (dark grey) that envelop developing germline cells (green). **b-f,** Immunofluorescence images of the apical tip of the adult testis from flies heat shocked to generate labeled dividing cells expressing nuclear β-galactosidase (β-gal). Testes stained with antibodies to DE-cadherin (DEC) or Fasciclin III (FasIII), β-gal, and Traffic Jam (TJ) or DAPI. **b,** One day post heat shock (DPHS), three β-gal^{+}(arrowheads, green, **b'**) TJ^{+} somatic cells(red, **b'''**) are near the FasIII^{+}(blue, **b''**) hub (asterisk). **c,** Five DPHS two β-gal^{+} SSCs (arrowheads, green, **c'**) contact hub cells (outline). A β-gal^{+} SSC daughter cell (arrow) is also present. **d,** Lower magnification view of **c,** β-gal^{+} somatic cells (arrows, green) are present along the length of the testis. **e,** Five DPHS, the hub (outline, DEC, red) contains two β-gal^{+} cells (arrowheads, green, **e'**). **f**, Fifteen DPHS two β-gal^{+} hub cells (green, arrows, **f'**), co-stain with FasIII (blue, outline, **f''**), and Traffic Jam (red, **f'''**). Scale bars 10 μm in **c, e, f**, 20 μm in **b, d.**

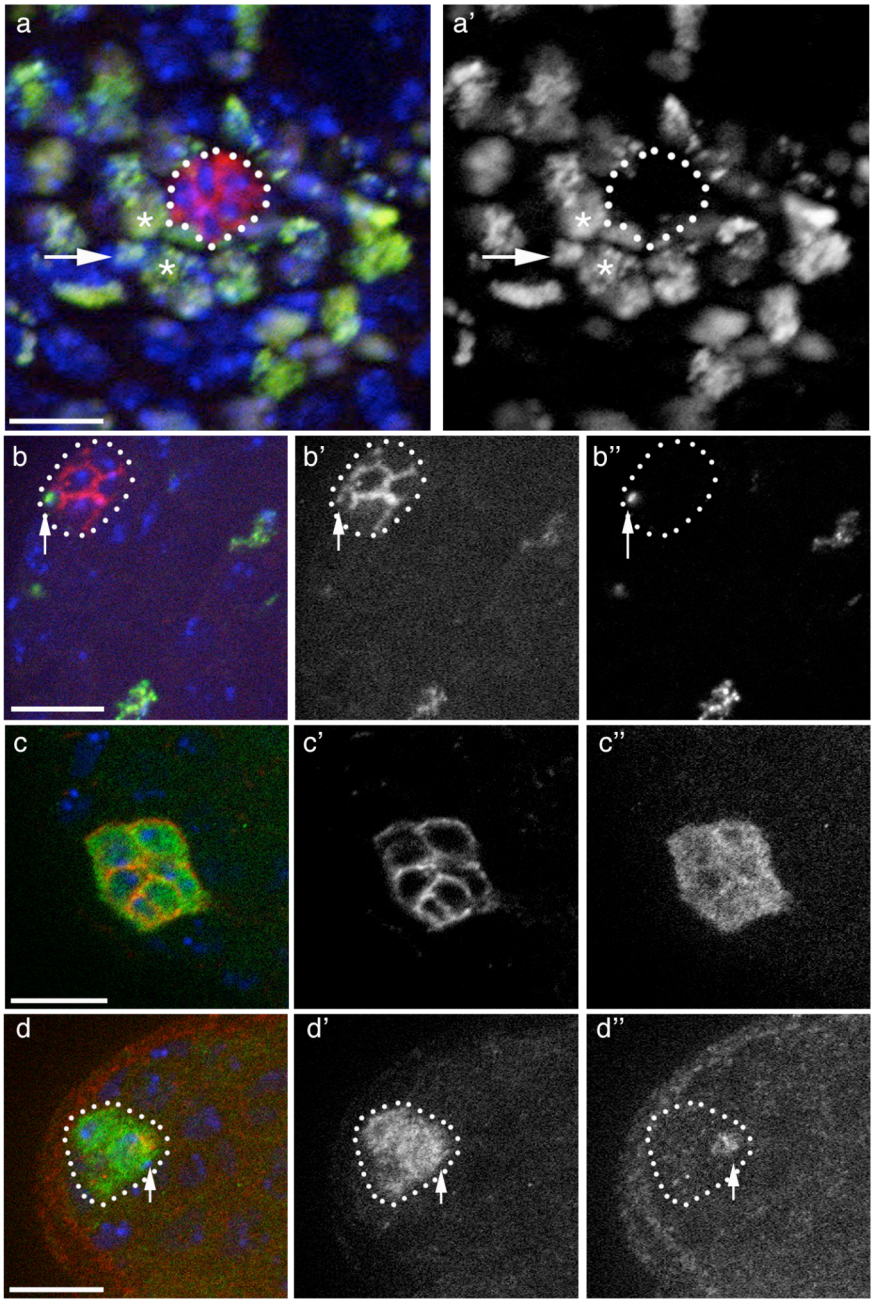


Figure 2.2 BrdU labeled cells become incorporated into the apical hub.
a, b, d, Immunofluorescence images of testes from wild-type (*OregonR)* (**a, b**) or *upd*GAL4-UAS-*gfp* (**d**) adult flies fed BrdU. **a,** BrdU^{+} cells (green) surround the FasIII^{+} hub (red, outline). Two BrdU^{+} GSCs (asterisks) and a BrdU^{+} somatic cell (arrow) are adjacent to the apical hub (outline) after a one day chase. **a',** No BrdU^{+} hub cells are detected. **b,** A BrdU^{+} hub cell (arrow) is present after a three day chase. **b',** FasIII channel **b''**, BrdU channel. **c**, Hub cells stained with DE-cadherin (red, **c'**) and GFP (green, **c''**) in *upd*GAL4-UAS-*gfp* testis. **d**, A BrdU^{+} hub cell (arrow) is detected after a seven day chase. **d'**, GFP channel. **d''**, BrdU channel. Scale bars 10μm.

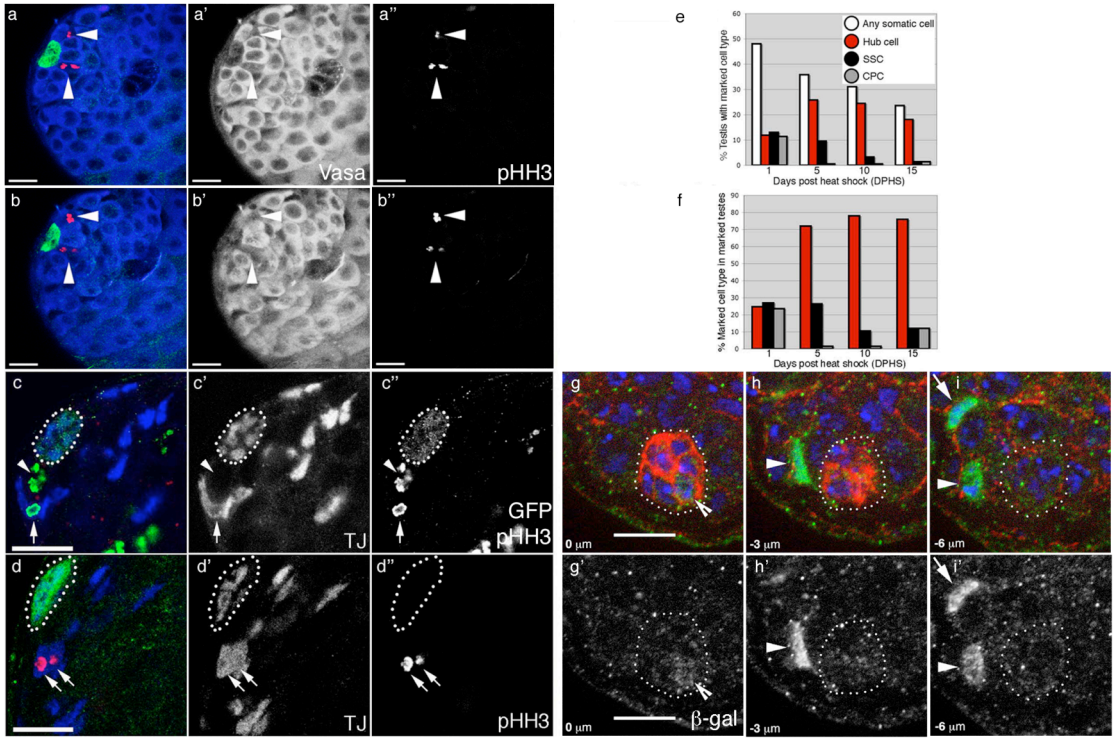


Figure 2.3 Two populations of mitotically active somatic cells are present near the hub

a-d'', Confocal images of *upd*GAL4-UAS-*gfp* testes. **a-b''** Testes stained for Vasa (blue, **a', b'**), phospho-histone-H3 (red, **a''**, **b''**), and GFP (green). pHH3+ SSCs (arrows) are observed near the hub . **c,** Testes stained for TJ (blue, **c'**), phospho-histone-H3 (green, **c''**), and GFP (green, **c''**). The hub is outlined. A mitotic CPC (arrow) surrounds a mitotic GSC (arrowhead). **d**, Testes stained with TJ (blue, **d'**), pHH3 (red, **d''**), and GFP (green). A mitotic CPC (arrows) one-cell distance from the hub (outline). **e**, Graph depicting marked somatic cell type frequency from all testes assayed at various timepoints. **f,** Graph depicting marked somatic cell type frequency from testes containing marked somatic cells at various time points. **g-i,** A testis ten DPHS stained for, β-gal^{+} (green, **g'-l'**), DE-cadherin (red), and DAPI (blue). A β-gal^{+} hub cell (**g,** indented arrowhead), β-gal^{+} SSC (**h,i**, arrowhead), and β-gal^{+} CPC **(l**, arrow**).** Z-section images (1mm) are denoted by depth in lower left corner. Scale bars 10 μm**.**

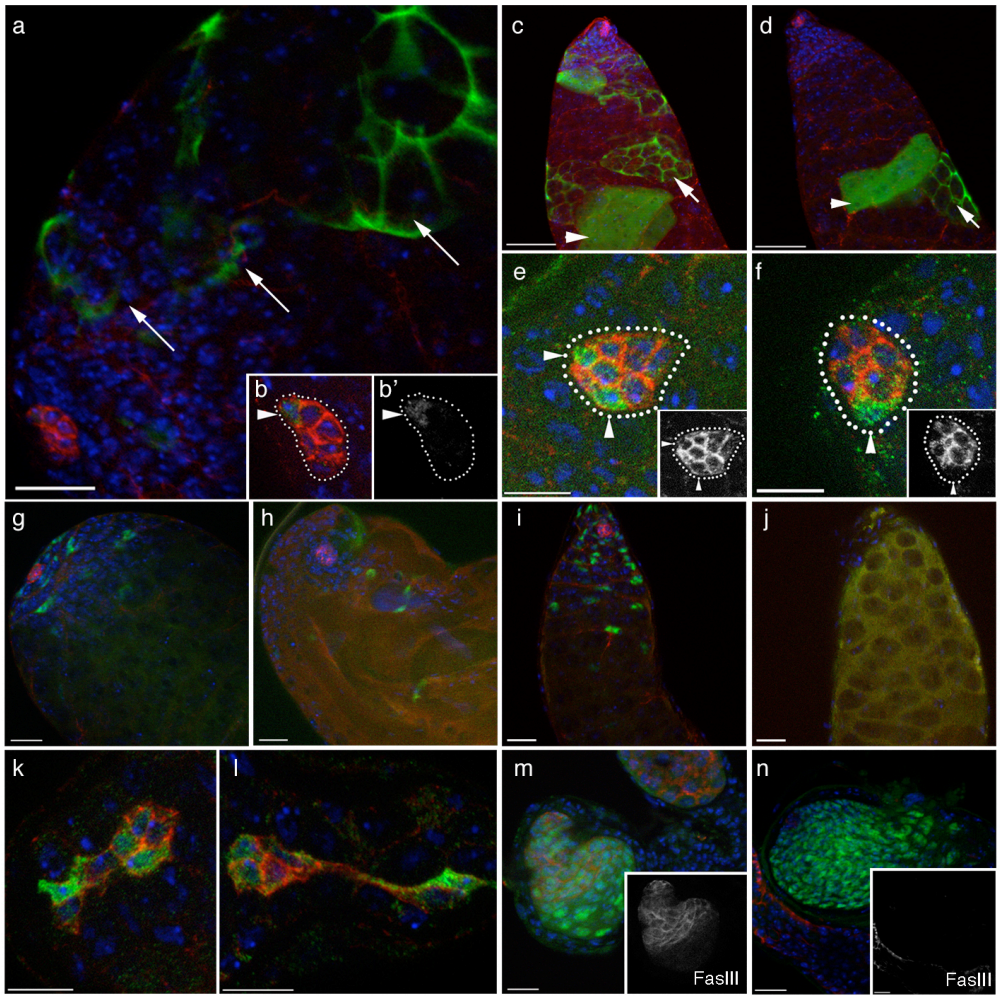
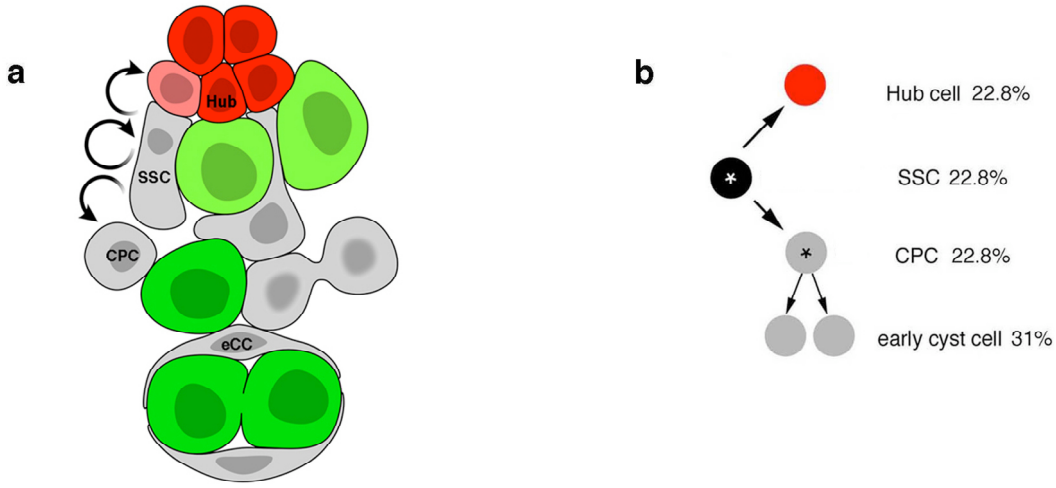


Figure 2.4 Factors required for SSC maintenance and the SSC-hub cell transition

a, Ten days post heat shock (DPHS) a series of GFP$^+$ late cyst cells (green, arrows) and a single labeled hub cell are present (arrowhead in **b**). GFP (green), DE-cadherin (red), and DAPI (blue). **b,** Magnification of the hub (outline, DEC, red) with a GFP$^+$ hub cell (arrowhead, **b')** **c,** Five DPHS GFP$^+$ germline and somatic cells are maintained near the DEC$^+$ hub (red). Late GFP$^+$ germline (arrowheads) and somatic cell (arrows) clones are present basally. **d** Five DPHS GFP$^+$*shg^{IG29}* mutant late somatic cells are present (arrow), but no GFP$^+$ clones are present near the DEC$^+$ hub (red) at apical tip. **e, f** Fifteen DPHS GFP$^+$ hub cells are present from wild-type (**e**) and *shg^{IG29}* (**f**) somatic clones (arrowheads). GFP$^+$ *shg^{IG29}* hub cells display loss of DE-cadherin (red) at hub interface (inset, DE-cadherin channel). **g, h,** One day old *c587*GAL4 (**g**) and *c587*GAL4 / UAS-*shgRNAi* (**h**) testes stained with DE-cadherin (red) and TJ (green). **i, j,** Fifteen day old *c587*GAL4 (**i**) and *c587*GAL4 / UAS-*shgRNAi* (**j**). Note loss of DEC (red) and TJ (green) stain in **j**. **k, l,** Fifteen DPHS distorted GFP$^+$ hub cells are present from *esg^{G66}* (**k**) and *esg^{L2}* (**l**) mutant somatic clones (compare to **e**). Hub marked with DEC (red). **m, n,** *esg^{shof}/CyO; osk/osk* (**m**) and *esg^{shof}/esg^{shof}; osk/osk* (**n**) testes stained for FasIII (red, insets) and TJ (green). Scale bars 20 μm in **a, c, d,** 10 μm in **e-n.**



Supplementary Figure S2.1 (A-B) Model of SSC and CPC divisions

a, Somatic stem cells (SSC) reside adjacent to the hub (red), self-renew and produce both new hub cells and cyst cells. Cyst progenitor cells (CPC) are further away from the hub and divide to produce cyst cells that will encapsulate the dividing germ cells.**b**, Prediction of labeled somatic cells types. SSCs and CPCs are mitotically active cells (asterisk) whose daughters can be labeled during mitotic recombination. Predicted frequencies were calculated using experimental data of mitotic somatic cells (see text). SSCs (69% of mitotic somatic cells) can generate SSCs, hub cells, and CPCs. Assuming an equal probability of labeling a SSC (33%), hub cell (33%), or CPC (33%) during mitotic recombination, the frequency of labeled cells resulting from SSC division can be calculated: 69% x 33% = 22.8%. The frequency of labeled early cyst cells, the result of a CPC division (31% of mitotic somatic cells), can also be calculated: 31% x 100%= 31%. Predictions are similar to observed frequencies at 1-day post heat shock from testes containing clones (SSCs, 26.9%; hub cell, 24.7%, CPC, 23.6%, early cyst cells, 39.3%).

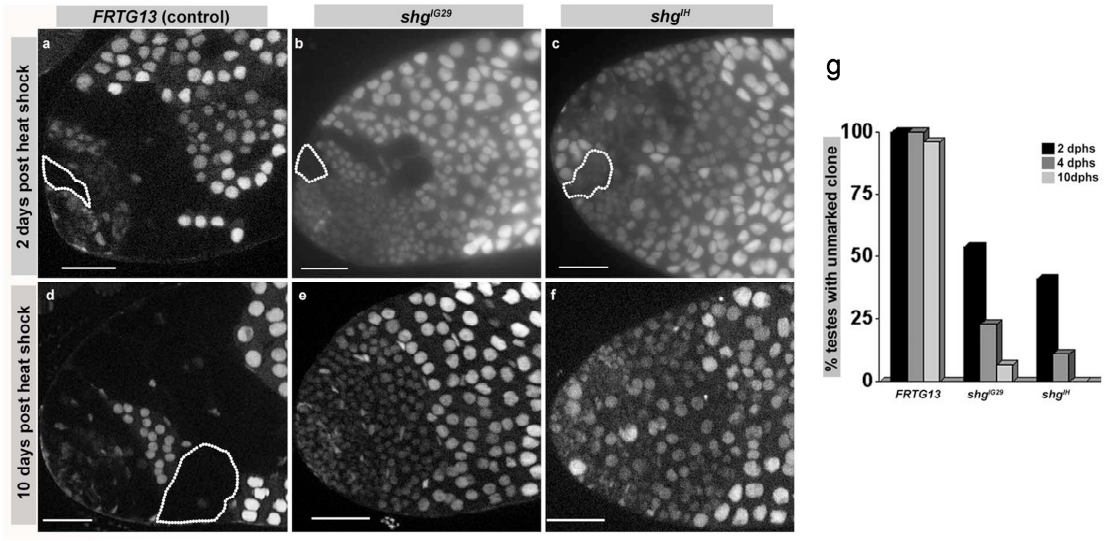
C

1 DPHS		
Testis with any marked somatic cell (HC, SSC, CPC, ECC)[1]	48.1%	(89/185)
% testis with marked SSC in marked testis (no HC present)	22.5%	(20/89)
% testis with marked CPC in marked testis (no HC or SSC present)	20.2%	(18/89)
% testis with marked ECC in marked testis (no HC, SSC, or CPC present)	32.6%	(29/89)
% testis with marked HC only in marked testis	10.1%	(9/89)
% testis with marked HC and ECC in marked testis (no SSC or CPC present)	6.7%	(6/89)
Average number marked SSCs in testis containing marked SSCs	1.25	(30/24)
Average number marked hub cells in marked hubs	1.13	(26/23)
5 DPHS		
Testis with any marked somatic cell (HC, SSC, CPC, ECC)	35.8%	(68/190)
% testis with marked SSC in marked testis (no HC present)	10.3%	(7/68)
% testis with marked CPC in marked testis (no HC or SSC present)	1.5%	(1/68)
% testis with marked ECC in marked testis (no HC, SSC, or CPC present)	11.8%	(8/68)
% testis with marked HC only in marked testis	54.4%	(37/68)
% testis with marked HC and ECC in marked testis (no SSC or CPC present)	4.4%	(3/68)
Average number marked SSCs in testis containing marked SSCs	1.17	(21/18)
Average number marked hub cells in marked hubs	1.24	(61/49)
10 DPHS		
Testis with any marked somatic cell (HC, SSC, CPC, ECC)	31.1%	(66/212)
% testis with marked SSC in marked testis (no HC present)	4.5%	(3/66)
% testis with marked CPC in marked testis (no HC or SSC present)	1.5%	(1/66)
% testis with marked ECC in marked testis (no HC, SSC, or CPC present)	12.1%	(8/66)
% testis with marked HC only in marked testis	71.2%	(47/66)
% testis with marked HC and ECC in marked testis (no SSC or CPC present)	1.5%	(1/66)
Average number marked SSCs in testis containing marked SSCs	1.43	(10/7)
Average number marked hub cells in marked hubs	1.36	(71/52)
15 DPHS		
Testis with any marked somatic cell (HC, SSC, CPC, ECC)	23.6%	(17/72)
% testis with marked SSC in marked testis (no HC present)	5.9%	(1/17)
% testis with marked CPC in marked testis (no HC or SSC present)	11.8%	(2/17)
% testis with marked ECC in marked testis (no HC, SSC, or CPC present)	5.9%	(1/17)
% testis with marked HC only in marked testis	76.5%	(13/17)
% testis with marked HC and ECC in marked testis (no SSC or CPC present)	0%	(0/17)
Average number marked SSCs in testis containing marked SSCs	1.50	(3/2)
Average number marked hub cells in marked hubs	1.30	(17/13)
50 DPHS		
Testis with any marked somatic cell (HC, SSC, CPC, ECC)	0%	(0/24)

[1] HC, hub cell; SSC, somatic stem cell; CPC, cyst progenitor cell; ECC, early cyst cell

Supplementary Figure S2.1 (C) Model of SSC and CPC division

c, Table with frequencies of marked (β-gal+) somatic cell types in testis containing a marked (β-gal+) cell at 1, 5, 10, 15 or 50 days post heat shock (DPHS). Sample numbers are provided in parentheses.

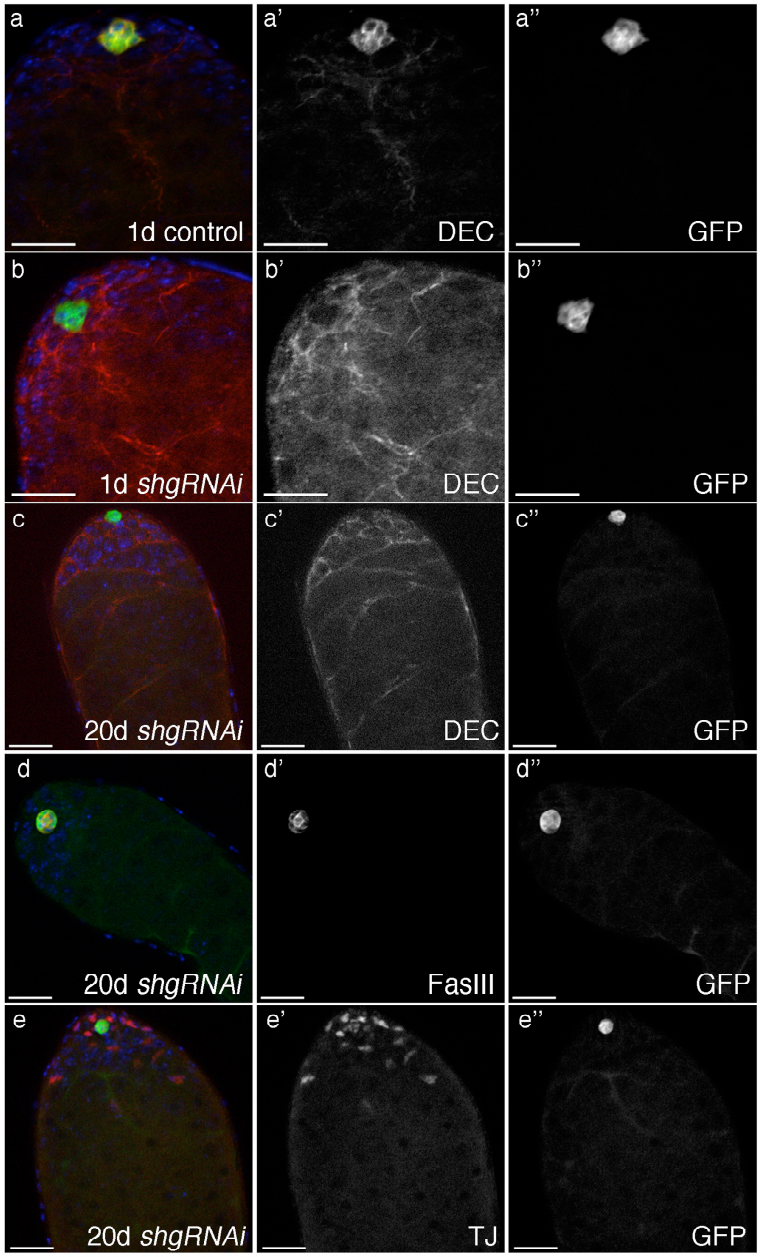


h

Genotype	2 dphs	4 dphs	10 dphs
y,w, hsflp^{122}; FRTG13/FRTG13,GFP3-13-7	100% (n=83) (E)	100% (n=35) (E) 100% (n=35) (L)	96% (n=102) (E) 96% (n=102) (L)
y,w, hsflp^{122}; FRTG13 shg^{IG29}/FRTG13,GFP3-13-7	54% (n=39) (E)	23% (n=40) (E) 46% (n=40) (L)	7% (n=86) (E) 17% (n=86) (L)
y,w, hsflp^{122}; FRTG13 shg^{IH}/FRTG13,GFP3-13-7	41% (n=51) (E)	11% (n=80) (E) 31% (n=80) (L)	0% (n=80) (E) 6% (n=80) (L)

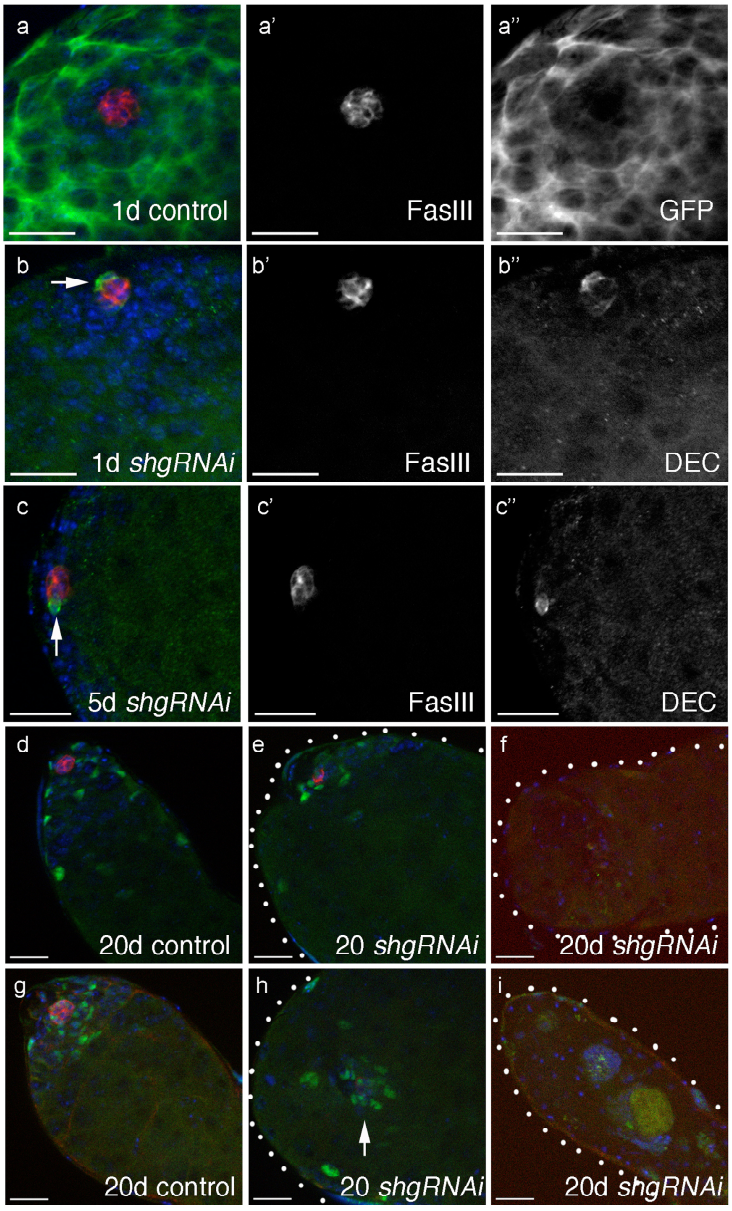
Supplementary Figure S2.2 *shotgun (shg)* function is required for maintenance of male GSCs

a-f, Fluorescence images of Drosophila testes. **a, d,** GFP negative, GSC control clones are evident by two days post heat shock (DPHS) and maintained at ten DPHS in (outlined) in *y,w, hsflp*^{122}; *FRTG13/FRTG13,GFP3-13-7* males. GFP negative *shg* mutant GSC clones are generated using (**b**) *shg*^{IG29} and (**c**) *shg*^{IH} loss of function alleles that are lost by 10 DPHS (**e, f**). **g**, Graphical representation of early (E) clone frequency from **h**. **h,** Testes analyzed for presence and position of GFP negative clones at two, four, and ten DPHS. Clones were scored as Early (E) as outlined in **a-c** or Late (L) as in **d** if observed beyond the early mitotic amplification zone. Scale bars 20 μm.



Supplementary Figure S2.3 Hub cells do not require *shotgun* for hub maintenance

a-e, Immunofluorescence images of 1-day old *upd*GAL4, UAS-*gfp* (**a**) or one (**b**) or twenty day old (**c-e**) *upd*GAL4, UAS-*gfp*; UAS-*shgRNAi* testes. **a-a'',** The hub is labeled with DE-cadherin (DEC, red, **a'**) and GFP (green, **a''**) in *upd*GAL4, UAS-*gfp* testis. **b-b'',** In *upd*GAL4, UAS-*gfp*; UAS-*shgRNAi* testes, DEC stain in the hub is lost (red, **b'**), but GFP labels the hub (green, **b''**). **c-e,** In twenty day old *upd*GAL4, UAS-*gfp*; UAS-*shgRNAi* testes, DEC stain in the hub is lost (red in **c'**), but GFP labels the hub (green, **c'', d'', e''**), FasIII labels the hub (red in **d, d'**), and TJ^{+} cells remain (red in **e, e'**). Scale bars 20 μm.



Supplementary Figure S2.4 SSC depletion by *shotgun* RNAi results in hub loss.

a, *c587*GAL4 drives expression of UAS-*eGFP* (green) in somatic cells (**a''**) but not in the FasIII labeled hub (red, **a'**). Asterisk in a" represents location of the hub. **b, c,** *c587*GAL4/UAS-*shgRNAi* testes at 1-day (**b**) and 5-days (**c**) stained for FasIII (red, **b'**, **c'**) and DE-cadherin (green, **b''**, **c''**). FasIII expression is normal , while DE-cadherin expression is significantly reduced within the hub (arrows). **d-f,** Control (**d**) and *c587*GAL4/UAS-*shgRNAi* twenty-day old testis (**e**, **f**) stained for FasIII (red) and Tj (green). FasIII and Tj stain is reduced (**e**) or absent (**f**). **g-i**, Control (**g**) and *c587*GAL4/UAS-*shgRNAi* 20-day old testis stained for DE-Cadherin (red) and Tj (green). DE-Cadherin and Tj stain is reduced (**h**) or absent **(i**). Scale bars 20 μm.

Supplementary Table S2.1 Somatic stem cell clonal analysis

Supplementary Table S1. SSC clonal analysis

		# Testes with labeled SSC at DPHS[a]				
Brief Genotype	**Heat shock protocol**	**1 DPHS**	**5 DPHS**	**10 DPHS**	**15 DPHS**	**30 DPHS**
tubulin-lacZ [1]	2hr @ E1, E2[b]	11/49 (22%)	ND[e]	ND	ND	ND
	2 hr @ A1,A2[c]	27/47 (57%)	21/74 (28%)	19/183 (10%)	23/225 (10%)	6/142 (4%)
FRTG13 [2]	2 hr @ E1, E2	10/26 (38%)	ND	ND	ND	ND
	2hr @ P9, P10[d]	14/48 (29%)	13/78 (17%)	8/29 (28%)	4/49 (8%)	ND
FRTG13 shg^{IG29} [3]	2 hr @ E1, E2	0/5 (0%)	ND	ND	ND	ND
	2hr @ P9, P10	11/38 (29%)	2/78 (3%)	0/53 (0%)	1/45 (2%)	ND
FRTG13 shg^{IN} [4]	2 hr @ E1, E2	0/14 (0%)	ND	ND	ND	ND
	2hr @ P9, P10	7/59 (12%)	7/89 (8%)	1/49 (2%)	3/50 (6%)	ND

[a] Days Post Heat Shock (DPHS)
[b] Embryos/early larvae were heat shocked for two hours on two consecutive days at days one and two after egg laying
[c] Adult flies were collected and heat shocked for two hours on two consectutive at days one and two after eclosion
[d] Late stage pupae were heat shocked for two hours on two consecutive days at days nine and ten after egg laying
[e] No data
[1] *hsflp-12; X1529/X1533*
[2] *y,w, hsflp^{122}; FRTG13 α-tubulin*GAL80*/FRTG13;* 2x-UAS-*eGFP/α-tubulin*GAL4
[3] *y,w, hsflp^{122}; shg^{IG29}FRTG13 α-tubulin*GAL80*/FRTG13;* 2x-UAS-*eGFP/α-tubulin*GAL4
[4] *y,w, hsflp^{122}; shg^{IN}FRTG13 α-tubulin*GAL80*/FRTG13;* 2x-UAS-*eGFP/α-tubulin*GAL4

Supplementary Table S2.2 Frequency of SSC contribution to the apical hub

Supplementary Table S2: Frequency of SSC contribution to the apical hub

		# Testes with labeled hub cells at DPHS[a]				
Genotype	**Heat shock protocol**	**1 DPHS**	**5 DPHS**	**10 DPHS**	**15 DPHS**	**30 DPHS**
tubulin-lacZ [1]	2hr @ E1, E2[b]	0/49 (0%)				
	2hr @ A1, A2[c]	16/47 (34%)	35/74 (47%)	104/183 (57%)	136/225 (60%)	88/142 (61%)
	no heat shock	0/52 (0%)	0/34 (0%)	0/19 (0%)	0/21 (0%)	ND[e]
Wild type [2]	2 hr @ E1, E2	0/56	ND	ND	ND	
	2hr @ P9, P10[d]	6/102 (6%)	20/144 (14%)	49/114 (35%)	72/110 (65%)	
	no heat shock	6/41 (15%)	4/38 (11%)	4/44 (9%)	0/6 (0%)	
FRT40A [3]	2 hr @ E1, E2	0/30 (0%)	ND	ND	ND	
	2hr @ P9, P10[d]	6/54 (11%)	13/66 (19%)	41/85 (48%)	41/61 (67%)	
	no heat shock	6/41 (15%)	1/7 (14%)	1/15 (7%)	ND	
FRT40A esg^{G66} [4]	2 hr @ E1, E2	ND	ND	ND	ND	
	2hr @ P9, P10	4/43 (9%)	4/43 (9%)	2/16 (13%)	8/27 (29%)	
	no heat shcok	ND	2/14 (14%)	ND	ND	
FRT40A esg^{L2} [5]	2 hr @ E1, E2	0/23 (0%)	ND	ND	ND	
	2hr @ P9, P10	2/38 (5%)	1/40 (3%)	0/36 (0%)	2/52 (4%)	
	no heat shock	0/25 (0%)	0/24 (0%)	0/5 (0%)	0/9 (0%)	
FRTG13 [6]	2 hr @ E1, E2	0/26 (0%)	ND	ND	ND	
	2hr @ P9, P10	0/48 (0%)	7/78 (9%)	8/29 (28%)	31/49 (63%)	
	no heat shock	ND	3/31 (10%)	3/29 (10%)	0/6 (0%)	
FRTG13 shg^{IG29} [7]	2 hr @ E1, E2	0/5 (0%)	ND	ND	ND	
	2hr @ P9, P10	4/38 (11%)	7/78 (9%)	18/53 (34%)	26/45 (58%)	
	no heat shock	ND	0/9 (0%)	1/6 (17%)	ND	
FRTG13 shg^{IH} [8]	2 hr @ E1, E2	4/14 (29%)	ND	ND	ND	
	2hr @ P9, P10	4/59 (7%)	4/89 (4%)	18/49 (37%)	25/50 (50%)	
	no heat shock	ND	3/25 (12%)	1/9 (11%)	ND	

[a] Days Post Heat Shock (DPHS)
[b] Embryos/early larvae were heat shocked for two hours on two consecutive days at days one and two after egg laying
[c] Adult flies were collected and heat shocked for two hours on two consectutive at days one and two after eclosion
[d] Late stage pupae were heat shocked for two hours on two consecutive days at days nine and ten after egg laying
[e] ND
[1] *hsflp-12; X1529/X1533*
[2] Data pooled from *FRT40A* and *FRTG13*
[3] *y,w, hsflp^{122}; FRT40A α-tubulin*GAL80/*FRT40A;* 2x-UAS-*eGFP/α-tubulin*GAL4
[4] *y,w, hsflp^{122}; esg^{G66}FRT40A α-tubulin*GAL80/*FRT40A;* 2x-UAS-*eGFP/α-tubulin*GAL4
[5] *y,w, hsflp^{122}; esg^{L2}FRT40A α-tubulin*GAL80/*FRT40A;* 2x-UAS-*eGFP/α-tubulin*GAL4
[6] *y,w, hsflp^{122}; FRTG13 α-tubulin*GAL80/*FRTG13;* 2x-UAS-*eGFP/α-tubulin*GAL4
[7] *y,w, hsflp^{122}; shg^{IG29}FRTG13 α-tubulin*GAL80/*FRTG13;* 2x-UAS-*eGFP/α-tubulin*GAL4
[8] y,w, hsflp^{122}; *shg^{IH}FRTG13 α-tubulin*GAL80/*FRTG13*; 2x-UAS-*eGFP/α-tubulin*GAL4

Supplementary Table S2.3 Frequency of SSC contribution to the apical hub

Supplementary Table S3: Frequency of BrdU and pHH3 marked hub cells

		# Testes with BrdU+ Hub cells					
Genotype	**Pulse[a]**	**1 d chase**	**3 d chase**	**5 d chase**	**7-8 d chase**	**10d chase**	**15d chase**
Oregon R	1 d adult	0/82 (0%)	ND[b]	6/143 (4%)	8/103 (8%)	3/96 (3%)	0/18 (0%)
	5 d adult	ND	2/52 (4%)	ND	ND	ND	ND
	10 d adult	ND	0/47 (0%)	ND	ND	ND	ND
	15 d adult	ND	1/57 (2%)	ND	ND	ND	ND
			Testes with pHH3 marked hub cells				
	Heat shock	**1 DPHS**	**5 DPHS**	**10 DPHS**	**15 DPHS**		
upd*GAL4, UAS-*gfp	no heat shock	0/31 (0%)	ND	0/31 (0%)	ND		
	2 hrs @ 1d A[c]	0/128 (0%)	0/37 (0%)	ND	0/57 (0%)		

[a] Flies allowed to feed upon 100mM BrdU dissolved in grape juice for 30 minutes
[b] No data
[c] Adult flies were collected and heat shocked for two hours at 37° C

Chapter 3: A transcriptional network regulating stem cell niche maintenance and stem cell activity in the *Drosophila* testis

3.1 Summary

Adult stem cells reside within specialized microenvironments, known as niches, which balance stem cell self-renewal and differentiation to maintain tissue homeostasis (Jones and Wagers, 2008). In the *Drosophila* testis, hub cells secrete the JAK-Stat ligand Upd*,* a molecule necessary and sufficient for germline (GSC) and somatic cyst stem cell (CySC) self-renewal. CySCs are capable of generating hub cells during adulthood, suggesting a means of regulating hub maintenance and size. However, cell type specific factors regulating niche maintenance and CySC activity are incompletely understood. Here we demonstrate that the transcription factor Escargot (Esg) act autonomously to maintain the hub and CySCs during adulthood. Conversely, overexpression of *esg* in somatic cells results in expansion of both germline and somatic lineages. Loss of Stat92E blocks Esg-mediated overproliferation, and Esg binds to the Stat92E promoter to regulate expression, indicating that these two transcription factors are tightly linked to coordinately regulate maintenance of a functional stem cell niche. Furthermore, our studies indicate that formation and maintenance of the stem cell niche can be uncoupled. The

identification and characterization of pathways that are involved in regulating the size and function of stem cell niches could lead to new strategies for stem cell based therapies and anti-cancer therapeutics.

3.2 Introduction

In the *Drosophila* testis, hub cells secrete Upd*,* a ligand that activates the JAK-STAT pathway to specify germline (GSC) and cyst stem cell (CySC) self-renewal (Figure 3.1A) (Kiger et al., 2001) (Tulina and Matunis, 2001). GSCs and CySCs directly contact hub cells via adherens junctions composed of homodimers of the *Drosophila* ortholog of E-cadherin (E-cad), an adhesion molecule necessary for GSC and CySC maintenance (Song et al., 2000) (Voog et al., 2008). During aging, *upd* and E-Cad expression are diminished and GSC number declines, suggesting that the hub is not a static structure and must be maintained to adequately support stem cells (Boyle et al., 2007). CySCs can contribute to the hub providing a potential mechanism to maintain hub size and function (Voog et al., 2008). However no direct experimental evidence has demonstrated that hub cells are absolutely required for GSC and CySC maintenance.

3.3 Results

3.3.1 Germline and somatic stem cells are lost in *esg^{shof}* adult testis

The Snail family transcription factor Escargot is expressed in a number of *Drosophila* stem and progenitor cell populations (Ashraf, et al., 1999) (Ashraf and Ip, 2001) (Kiger et al., 2000) (Micchelli and Perrimon, 2006) (Singh et al., 2007); however its role in regulating adult stem cell behavior is not known. In the testis, Esg is expressed in hub cells and early somatic and germline lineages (Figure 3.1B) (Kiger et al., 2000). During the course of a genetic screen, a loss of function allele of *esg (esg^{shof})* was recovered that resulted in a complete loss of both GSCs and CySCs by early adulthood (Figure 3.1C-J, Supplementary Figure S3.1). Early germ cells can be identified by restricted expression of b-galactosidase (b-gal) in the *S3-46lacZ* enhancer trap line (Figure 3.1E, I), and early somatic cells are identified by expression of the transcription factors Zfh-1 and Traffic Jam (TJ) (Figure 3.1F, J) (Leatherman and Dinardo, 2008) (Li et al., 2003). In testes from *esg^{shof}* adults, progressive loss of GSCs and CySC is due to differentiation, rather than excessive apoptosis during development (Figure 3.1G, H; Supplementary Figure S3.2E). An alleleic complementation series with other loss of function *esg* alleles exhibited identical phenotypes (Supplementary Figure S3.1). In addition to loss of stem cells, the apical hub is also lost in *esg^{shof}* testes. Expression of hub markers Fasciclin III (FasIII), E-Cad, and *center divider (cdi-*

lacZ) was markedly reduced or absent in ~90% of one-day old flies (n=18) (Figure 3.2A, B). Additional hub markers such as *upd* (Supplementary Figure S3.2G) and *Drosophila* N-cadherin are also lost by early adulthood.

3.3.2 Hub and stem cell maintenance is dependent upon hub specific *esg* expression

To determine whether *esg* is required for initial hub formation, we examined stage 16 esg^{shof} embryos for the presence of *upd*. Hub specification and formation occurs during embryonic stages 16 and 17 with expression of *esg* and *upd* as the earliest known sexually dimorphic markers (Streit et al., 2002) (Le Bras and van Doren, 2006). Restricted expression of *upd* to the anterior end of the gonad was detected in roughly 50% of control (24/44), and esg^{shof} (12/27) embryos, suggesting that the hub is specified normally in esg^{shof} embryonic gonads (Figure 3.2C, D). Additional hub markers (FasIII, E-cad, *cdi-lacZ*) are present during embryonic and early larval development in testes from esg^{shof} animals, and early germ cells encircle the embryonic hub (Supplementary Figure S3.2B-D). In contrast, while *esg* expression was highly enriched at the anterior end of ~50% (53/93) of control embryonic gonads, no *esg* expression was detected in ~90% (61/70) of esg^{shof} mutant gonads (Figure 3.2E, F). These data are consistent with earlier reports that *esg* is not absolutely required for initial hub formation (Le Bras and van Doren, 2006) and suggest that *esg* is likely required for hub maintenance into adulthood.

Loss of hub cells and stem cells in *esg*^{shof} could be due to cell intrinsic requirements for *esg* within all of three cell types (hub, CySC, GSC) or a requirement for *esg* within a subset of these cells. Our data indicate that *esg* is required autonomously in hub cells for maintenance of the hub and stem cells. To directly test whether *esg* acts within hub cells to regulate maintenance of the hub, we used the temporally and spatially restricted GAL4-UAS system to drive expression of an *esg-RNAi* transgene specifically within hub cells during adulthood (Dietzl et al., 2007). Flies of the appropriate genotype were raised at 18°C to inhibit transgene activity, and upon eclosion (hatching) flies were shifted to 29°C to induce *esg-RNAi* expression within hub cells. At eclosion, hub cell number was significantly reduced as assayed by expression of FasIII^{+} cells (3.8±2.0, n=16) and E-Cad^{+} cells (4.5±1.6, n=11) (Figure 2G, H), when compared to controls, likely a result of background expression of the *esg-RNAi* transgene. However, hub cell number and structure was stable throughout adulthood in flies kept at 18°C for 10 [FasIII^{+} cells (3.8±2.2, n=22); E-Cad^{+} cells (3.1±2.2, n=12)] or 20 days [FasIII^{+} cells (4.1±2.7, n=30); E-Cad^{+} cells (4.1±1.9, n=29)] (Figure 3.2G).

In contrast, the hub was completely lost in *upd*GAL4, UAS-*gfp*; UAS-*esg-RNAi* flies shifted to 29°C for 10 [FasIII^{+} cells (0.9±1.1, n=13) and E-Cad^{+} cells (0, n=14)] or 20 days [FasIII^{+} cells (0.2±0.6, n=12) and E-Cad^{+} cells (0, n=7)] (Figure 3.2G, K). Hub loss in *upd*GAL4, UAS-*gfp;* UAS-*esg-RNAi* flies closely resembled the *esg*^{shof} phenotype, as surrounding early germ and

somatic cells were absent (Figure 3.2I, J, L, M). These data indicate that *esg* is required autonomously in hub cells for maintenance and that the hub is absolutely required for maintenance of both GSC and CySC populations.

3.3.3 *esg* regulates somatic stem cell maintenance and proliferation

Furthermore, *esg* is also required autonomously for maintenance of CySCs. Positively marked, *esg* mutant CySCs were generated using FRT-mediated mitotic recombination, and clones were scored at various time points post heat shock (PHS) (Lee and Luo, 1999). At 1-day PHS 53% of control (n=54), 60% of esg^{G66} (n=43), and 26% of esg^{L2} (n=38) CySC clones were recovered. Positively marked, control clones were observed in 39% (n=66), 33% (n=85), and 30% (n=61) of testes examine at 5, 10, and 15 days PHS, indicating that the CySCs are maintained (Figure 3.3A, B, D). In contrast esg^{G66} (16%, n=43) and esg^{L2} (5%, n=40) mutant CySC clones were quickly lost by 5 days PHS, and no *esg* mutant clones were observed ten (esg^{G66}, n= 16) (esg^{L2}, n=36) or fifteen (esg^{G66}, n= 27) (esg^{L2}, n=52) days PHS (Figure 3.3C, D). Mutant cyst cells expressed differentiation markers and encapsulated germline cysts, and no increase in CySC apoptosis was detected, indicating that *esg* is not absolutely required for CySC differentiation or survival (data not shown).

Conditional expression of an *esg-RNAi* transgene within CySCs resulted in loss of early Zfh-1^{+} and TJ^{+} somatic cells, providing additional

evidence that *esg* is required for maintenance of CySCs (Supplementary Figure S3.3A-D). Consistent with previous observations, loss of the hub was also observed upon conditional ablation of the CySC population (Supplementary Figure S3.3C, D) (Voog et al., 2008). In contrast, no effect on GSC maintenance was observed using the loss of function esg^{G66} or esg^{L2} alleles (Supplementary Table S3.1), confirming that the requirement of *esg* for GSC maintenance is non-cell autonomous (Streit et al., 2002).

In addition, overexpression of *esg* specifically in somatic cells was sufficient to promote self-renewal of CySCs, providing another indication that *esg* is a key regulator of somatic cell behavior in the *Drosophila* testis. The GAL4-UAS system was used to drive *esg* specifically in CySCs and early somatic cells (Hekmat-Scafe et al., 2005*)*. Overexpression of *esg* was controlled by raising the flies at 18°C and shifting the flies to 29°C upon eclosion, and expression was confirmed by RNA *in situ* analysis (Supplementary Figure S3.4A, B). In flies shifted to 29°C to induce *esg* overexpression, testes were filled with small brightly staining $DAPI^{+}$ cells, indicative of an excess of early somatic and germline cells (Figure 3.3E', F'). Single Zfh-1^{+}/TJ^{+} somatic cells expressed the mitotic marker phosho-histone H3 (pHH3) in regions far removed from the apical hub (Figure 3.3F; Supplementary Figure S3.3E-H). In addition, mitotic germ cells containing spherical spectrosomes, characteristic of GSCs and gonialblasts, were also present along the length of the testis (Figure 3.3I). In contrast, control testes

contained TJ^+ cells only at the apical tip, and germ cells displayed normal branched fusomes in regions far from the hub (Figure 3.3G). In testes in which *esg* was overexpressed, somatic cell clusters containing tens to hundreds of $FasIII^+/E\text{-}Cad^+$ cells were also observed (Supplementary Figure S3.4C); however, ectopic $FasIII^+/E\text{-}Cad^+$ clusters did not express *upd* and thus do not appear to be *bona fide* hub cells (Supplementary Figure S3.4D, E). These data demonstrate that *esg* is capable of inducing excess early somatic cell proliferation with concomitant expansion of GSCs and early germ cells.

Ectopic proliferation of early somatic and germline cells is observed when the JAK-STAT pathway is activated in germline or somatic cells or when somatic cells overexpress *zfh-1* (Kiger et al., 2001) (Tulina and Matunis, 2001) (Leatherman and DiNardo, 2008). Increased levels of Stat were observed in ectopic GSCs and TJ^+ cells (Figure 3.3H, J); however, *esg*-mediated overproliferation of early germline and somatic cells was not a consequence of ectopic *upd* expression, as *in situ* analysis revealed restricted expression of *upd* to the apical tip in both control and *esg* overexpression testes (Supplementary Figure S3.4D, E). These data suggested that *esg* might act downstream of or in parallel with *upd* to specify somatic and germ cell proliferation. However, *upd* overexpression in the germline is capable of inducing germline and somatic proliferation in esg^{shof} mutants, suggesting that *esg* activity is not required downstream of *upd* (Supplementary Figure S3.5).

3.3.4 An *esg-Stat92E* interaction regulates hub cell and somatic stem cell behavior

Given that STAT activation was observed in ectopic GSCs and TJ^{+} cells, we predicted that *Stat92E* would be necessary for *esg*-induced somatic and germline proliferation. To test this hypothesis, *esg* was overexpressed in early somatic cells with simultaneous expression of a *Stat92E-RNAi* transgene. As predicted, 5 days after induction of *esg* and *Stat92E-RNAi* expression, 83% (n=12) of testes were devoid of both somatic and germline cells (Figure 3.4A-D), indicating that a reduction in *Stat92E* expression blocks *esg*-mediated somatic and germ line proliferation. Therefore, consistent with our previous observations, *Stat92E* is required downstream of *esg* or acts in a parallel pathway in CySCs to promote stem cell self-renewal.

Similar to the role *esg* plays in maintenance of hub cells, the JAK-STAT pathway also acts autonomously within hub cells to regulate hub cell behavior. Because of the apparent interaction between *Stat92E* and *esg* in directly regulating CySC behavior, we hypothesized that *Stat92E* may also serve a role within hub cells. Stat92E is highly expressed in GSCs and CySCs, and phosphorylated Stat92E is present in low levels within the hub (Figure 3.4E). While *Stat92E* is required for GSC and CySC maintenance (Kiger et al., 2001) (Tulina and Matunis, 2001) (Leatherman and DiNardo, 2008), little is known about *Stat92E* function in hub cells. To address whether *Stat92E* is required

autonomously for hub maintenance, a *Stat92E-RNAi* transgene was expressed in hub cells during adulthood. Hubs in testes from *upd*GAL4, UAS-*GFP*; UAS-*stat92E-RNAi* adults kept at 18°C were compact in size and maintained normal hub cell number at 1-day (Figure 3.4F) [FasIII$^+$ cells (6.1±2.2, n=31); E-Cad$^+$ cells (5.9±2.1, n=15)], 10 days [FasIII$^+$ cells (5.4±2.3, n=27) and E-Cad$^+$ cells (5.4±1.9, n=25)], and 20 days [FasIII$^+$ cells (5.2±2.8, n=38) and E-Cad$^+$ cells (4.6±2.4, n=35)]. However, hub cell number sharply dropped after 10 days [FasIII$^+$ cells (2.8±2.3, n=40) and E-Cad$^+$ cells (2.6±2.7, n=31)] and 20 days [FasIII$^+$ cells (0.6±1.6, n=21) and E-Cad$^+$ cells (1.4±1.8, n=14)] in flies shifted to 29°C (Figure 3.4G, H), indicating that *Stat92E* is required autonomously in hub cells for maintenance.

Stat92E is a direct target of the JAK kinase Hopscotch (Hop) (Zeidler et al., 2000). To verify that JAK-Stat signaling is required for adult hub maintenance, testes from males carrying the viable, male-sterile *hop^{25}* allele were assayed for hub markers in L3 larvae and early adults. Larval *hop^{25}* mutants exhibited normal expression of FasIII (100%, n=29) and E-Cad (100%, n=29), although fewer early germline (21%, n=29) and TJ$^+$ somatic cells (20%, n=20) were present compared to controls (compare Figure 3.4I, K). By early adulthood, all testes from *hop^{25}* mutants lacked FasIII (n=27) and E-Cad (n=13) expressing cells, and TJ$^+$ somatic cells (n=15) were also absent

(compare Figure 3.4J, L). We conclude that like *esg, hop* and *Stat92E* are required for hub maintenance during adulthood.

To determine whether the JAK-STAT pathway acts downstream of *esg* in regulating hub maintenance, we assayed if hub marker expression could be restored in adult esg^{shof} males by expressing *upd* within the hub (*upd*GAL4, UAS-*GFP;* esg^{shof} ; UAS-*upd*). In 1-3 day old flies, a modest increase in the number of testes expressing hub markers FasIII (24.7%, n=162) and E-Cad (23.8% n=21) was observed, with a concomitant increase in early somatic TJ^{+} cells (31%, n=58) (Figure 3.4 M-P). These data indicate that activation of the JAK-STAT pathway in hub cells is able to delay hub loss in esg^{shof} males and suggest that the JAK-STAT pathway acts downstream of *esg* or in a parallel pathway to promote maintenance of the apical hub, similar to the relationship of these pathways in CySCs.

To determine whether Esg could directly regulate *Stat92E* in the testis, we used the tissue specific Dam-ID strategy to identify potential transcriptional targets of Esg (see Methods). Dam-ID has been successfully used to identify transcriptional targets in *Drosophila*, and Dam-ID targets closely correlate with targets identified using chromatin immunoprecipitation (ChIP) *(*Choksi et al., 2006*).* Our results indicate that Esg is bound upstream of the Stat92E transcription start site; therefore, *Stat92E* is a transcriptional target of Esg (Figure 3.4Q). These experiments support our genetic analysis which indicates

that *Stat92E* acts downstream of *esg* to regulate maintenance of stem cell populations and the hub.

3.4 Discussion

3.4.1 Transcriptional networks regulating stem cell niche activity

Our data strongly suggest that Esg and Stat92E are core components of a transcriptional network regulating both niche support cell and stem cell behavior (Figure 3.4S). Cell type specific requirements for *esg* within hub cells and CySCs suggest that unique transcriptional targets may be present in these cell types or that cell type specific co-factors regulate Esg activity. Genome wide analysis of Esg binding sites revealed a number of targets that are upregulated in hub cells (*cdi*) and/or CySCs (*zfh-1*) (Figure 3.4R). Characterizing how Esg differentially regulates hub and CySC specific targets will be an important step in understanding how Esg ultimately coordinates size and maintenance of the stem cell niche.

As both Esg and Stat92E share a number of direct targets, including *zfh-1, Stat92E, and Socs36E*, these two transcription factors could act in concert to maintain stem cell niche activity by influencing hub cell and CySC behavior. Interestingly, recent data indicate that Stat92E can also bind to regulatory sequences surrounding the *esg* locus (Flaherty et al., 2009); therefore, Esg and Stat92E may co-regulate expression of each other to fine tune gene expression within the male germline stem cell niche. Identifying how

transcriptional specificity is regulated in closely related cell lineages and its effects on cell fate are important emerging themes in stem cell biology. Transcriptional networks regulating stem cell niche maintenance and activity are likely to be present in within mammalian systems, and our data suggest that therapeutic targeting of a single transcription factor within niche cells could promote expansion or ablation of the niche as a therapeutic approach to facilitate regenerative medicine and cancer therapies.

3.5 Methods

3.5.1 Fly husbandry and stocks

Flies were raised at 25 °C on standard cornmeal-molasses media unless otherwise noted. The *esg*^{shof} allele was isolated in the Fuller laboratory. The following stocks were used and more information can be found in Flybase (http://flybase.bio.indiana.edu): *esg*^{G66}, *esg*^{L2}, *FRT40A*, *hs-flp*^{122}, *tub*GAL4, *tub*GAL80, UAS-*2x-eGFP*, UAS-*esgRNAi* (VDRC), Esg-GFP (FlyTrap), *upd*GAL4-UAS-*GFP* was a gift from E. Bach, *cdi-lacZ* was a gift from M. van Doren, *c587*GAL4 was a gift from T. Xie, *S3-46lacZ* was a gift from S. DiNardo.

3.5.2 Mapping of *esg*^{shof}

Local transposition of homozygous viable *esg*^{P2} generated fifteen P-element alleles that failed to complement *shof*. All were homozygous lethal

and all failed to complement *esg*^{VS2}. From this set of P-element insertion alleles eight were mapped molecularly and identified to be independent insertions. From the mapped insertions, five fell between the insertion point of *esg*^{P2} and the transcriptional start site of *esg*. The other three insertions lines mapped to the transcription unit of *esg*.

3.5.3 Sequence analysis of the *esg*^{shof} mutation

Primers (available upon request) were designed using the Primer 3 program (http://frodo.wi.mit.edu/) to regions spanning 3584bp upstream of the *esg* start site to 6236bp downstream of the *esg* start site. Genomic DNA from *OreR* and *S346; esg*^{shof}*/esg*^{shof} males was obtained using the Qiagen Tissue Easy Kit. PCR fragments were generated using standard reaction conditions and samples sequenced by Eton Biosciences Inc. Sequences were analyzed using Chromas Pro software. Sequences from *OreR* and *S346; esg*^{shof}*/esg*^{shof} were identical between 3584bp upstream and 5228bp downstream of the *esg* locus. Beyond this region PCR products from *S346; esg*^{shof}*/esg*^{shof} genomic DNA were unable to be amplified. A ~18kb insert was found in *S346; esg*^{shof}*/esg*^{shof} between sites 5228bp and 5840bp downstream of the *esg* coding region.

3.5.4 Germline and somatic cell clonal analysis

Adult flies were heat shocked at 37 °C for two hours on two consecutive days. Flies were collected at various time points after the last heat shock,

immunostained and analysed. MARCM wild-type control genotypes used were: *y,w, hsflp*^{122}; *FRT40A/FRT40A, tub*GAL80; *tub*GAL4/2*x*-UAS-e*GFP*. Mutant genotypes used were: *esg*^{G66} (*y,w, hsflp*^{122}; *FRT40A esg*^{G66}*/FRT40A tub*GAL80; *tub*GAL4/2*x*-UAS-e*GFP*), and *esg*^{L2} (*y,w, hsflp*^{122}; *FRT40A esg*^{L2} */FRT40A tub*GAL80; *tub*GAL4/2*x*-UAS-e*GFP*). Germline clones were induced by FLP/FRT mediated recombination (Xu and Harrison, 1994). Control genotypes used were: *y,w,hsflp*^{122}; FRT40A/FRT40A ubi-GFP. Mutant genotypes used were: *esg*^{G66} (*y,w,hsflp*^{122}; FRT40A*esg*^{G66}/FRT40A GFP), and *esg*^{L2} (*y,w,hsflp*^{122}; FRT40A*esg*^{L2}/FRT40A GFP). Late pupae were heat shocked in vials in a 37°C water bath for two hours on two consecutive days. Testes dissected into PBS at designated time points after last heat shock were observed by fluorescence microscopy.

3.5.5 In-situ hybridization, immunostaining, and microscopy

Performed as described in (Voog et al., 2008) (Boyle et al., 2007).

3.5.6 Antibodies

Testes were stained with: rabbit anti-Vasa (1:2,000) (P.Lasko), rabbit anti-Zfh-1 (1:5,000) (R.Lehmann), rabbit anti-b-galactosidase (1:2,000) (Cappel), guinea-pig anti-TJ (1:3,000) (D. Godt), mouse anti-phospho histone H3 (1:5,000), rabbit anti-Stat92E (M. Wawersik), and rabbit anti-GFP (1:5,000) (Molecular Probes). Mouse anti-Fasciclin III (7G10) (1:10), mouse anti a-spectrin (1:10), mouse anti-Eyes Absent (1:10) and rat anti-DE-cadherin

(DCAD2) (1:20) were obtained from the Developmental Studies Hybridoma Bank. Secondary antibodies were diluted 1:500 (Molecular Probes). Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories).

3.5.7 DAM-ID

Tissue from *esg-Dam* and *Dam* control adult testis (~12,000 flies) was collected and genomic DNA isolated and amplified. The *esg* CDS was amplified using the primers: 5'-GTACGCGGCCGCTCATGCATACCGTGGAAGACATGTTG-3' and 5'-GGTCTAGATTACGGCTCGGCATAGCCGGCGTAG-3' from a 4-17 hr embryonic cDNA library and cloned into pUASTNDam (Choksi et al., 2006) using NotI and XbaI. Transgenic lines were generated as previously described (Choksi et al., 2006). Stage 10-11 embryos (4-7 hr AEL) were collected from UAS-Dam and UAS-Dam-Esg. DNA isolation, processing and amplification were performed as previously described (Choksi et al., 2006). Samples were labelled and hybridised to a whole genome 2.1 million feature tiling array, with 50-75 mer oligonucleotides spaced at approximately 55 bp intervals (Nimblegen systems). Arrays were scanned and intensities extracted (Nimblegen Systems). Three biological replicates (with one dye-swap) were performed. Log2 mean ratios of each spot were median normalised.

A peak finding algorithm with false discovery rate (FDR) analysis (PERL script available on request) was developed to identify significant binding sites. All peaks spanning 8 or more consecutive probes (> ~900 bp) over a 1.6 log2 fold ratio value were analysed and assigned a FDR value (using 100 iterations). This analysis was performed for each chromosome arm. Genes were defined as targets where a binding event (with a FDR of 0%) occurred within up to 5 kbp of the gene structure (depending on the proximity of adjacent genes).

3.6 Acknowledgements

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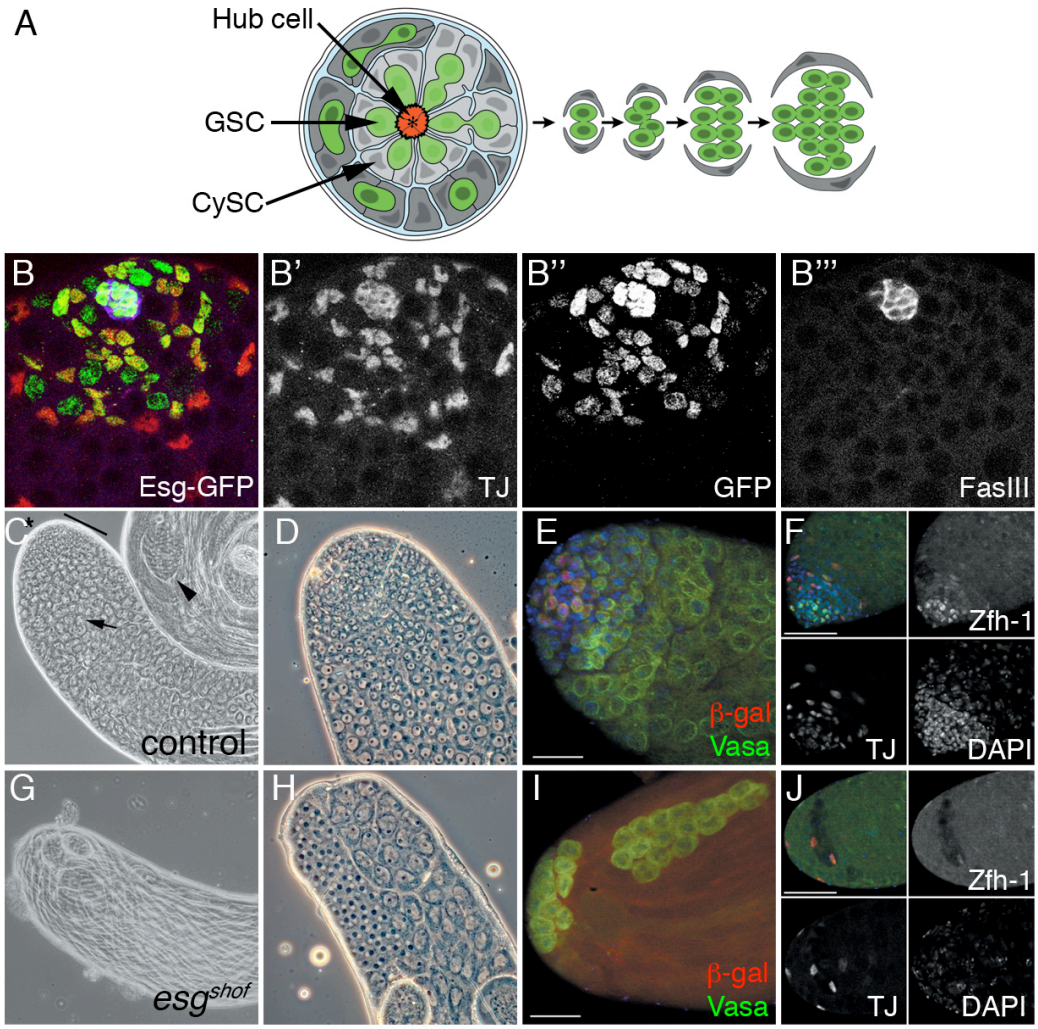
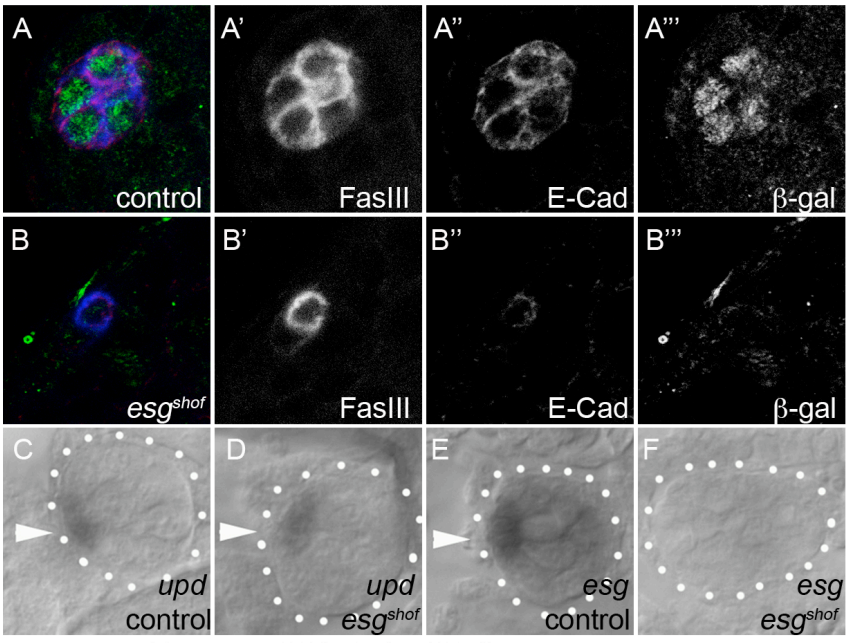


Figure 3.1 Germline and somatic cyst stem cells are lost by adulthood in *esg^{shof}*

(**A**) Schematic illustration of spermatogenesis. A germline stem cell (GSC, light green) contacts hub cells (red) and somatic cyst stem cells (CySC, light grey). Developing germline cysts (green) are surrounded by early cyst cells (dark grey). (**B**) Esg-GFP enhancer trap adult testis stained for Traffic Jam (**B'**, TJ, red), GFP (**B''**, green), and FasciclinIII (**B'''**, FasIII, blue). (**C, D**) Phase contrast images of one-day-old control testes. Asterisk denotes apical tip, bar denotes mitotic amplification zone, arrow denotes early cyst, arrowhead denotes sperm bundle. (**E**) Vasa (green) labels germ cells. The *S3-46* enhancer line labels GSCs and early germ cells (red). (**F**) Early somatic cell markers Zfh-1 (green) and TJ (red) are restricted to the tip of the testis. (**G, H**) Phase contrast of 1-day old *esg^{shof}* mutant testis with (**G**) mature sperm or (**H**) late stage cysts. (**I**) *esg^{shof}* testis lack *S3-46* expression and contain late stage Vasa positive germ cells (green). (**J**) *esg^{shof}* testis lack Zfh-1 (green) and TJ (red) early somatic cells.



G

Temperature Shift Protocol
Avg+S.D. (*n*)

Hub marker	1d at 18°C	10d at 18°C	10d at 29°C	20d at 18°C	20d at 29°C
FasIII	3.8+2.0 (16)	3.8+2.2 (22)	0.9+1.1 (13)	4.1+2.7 (30)	0.2+0.6 (12)
DE-Cadherin	4.5+1.6 (11)	3.1+2.2 (12)	0.0+0.0 (14)	4.1+1.9 (29)	0.0+0.0 (7)

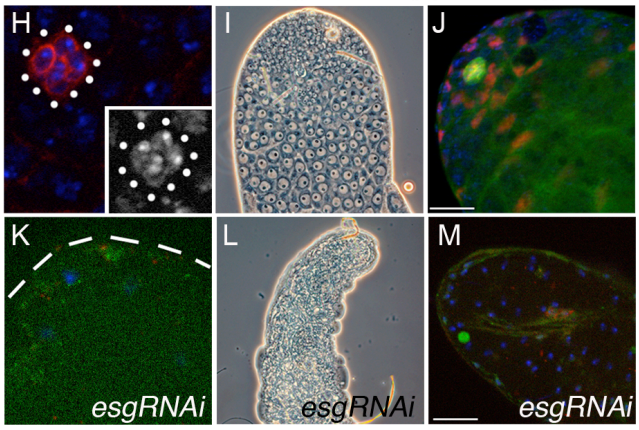


Figure 3.2 Hub and stem cell maintenance is dependent upon hub specific *esg* expression

(**A**) Adult *cdi-lacZ* control testis stained for FasIII (**A'**,blue), DE-cadherin (**A''**, E-Cad, red) and *cdi-lacZ* (**A'''**, green). (**B**) *esg*^{shof}*; cdi-lacZ* adult testis stained for FasIII (**B'**, blue), E-Cad (**B''**, red) and *cdi-lacZ* (**B'''**, green). (**C-F**) *In-situ* analysis of stage 16 embryonic gonads (outline). *upd* is expressed in control (**C,** arrowhead) and *esg*^{shof} (**D**, arrowhead) embryonic hubs. *esg* is expressed in control (**E**) but not *esg*^{shof} (**F**) embryonic gonads. (**G**) Table of hub cell counts using FasIII and E-cad at various time points. (**H, K**) Immunofluorescence stains for E-Cad (red, outline), GFP (green), and DAPI (blue, inset) in *upd*GAL4, UAS-*gfp*; UAS-*esgRNAi* flies at (**H**) 1 day at 18°C and (**K**) 20 days at 29°C. (**I**) Phase contrast image of control and (**L**) *upd*GAL4, UAS-*gfp*; UAS-*esgRNAi* testes at 29°C. (**J**) Control and (**M**) *upd*GAL4, UAS-*gfp*; UAS-*esgRNAi* testes stained for GFP (green), TJ (red), and DAPI (blue).

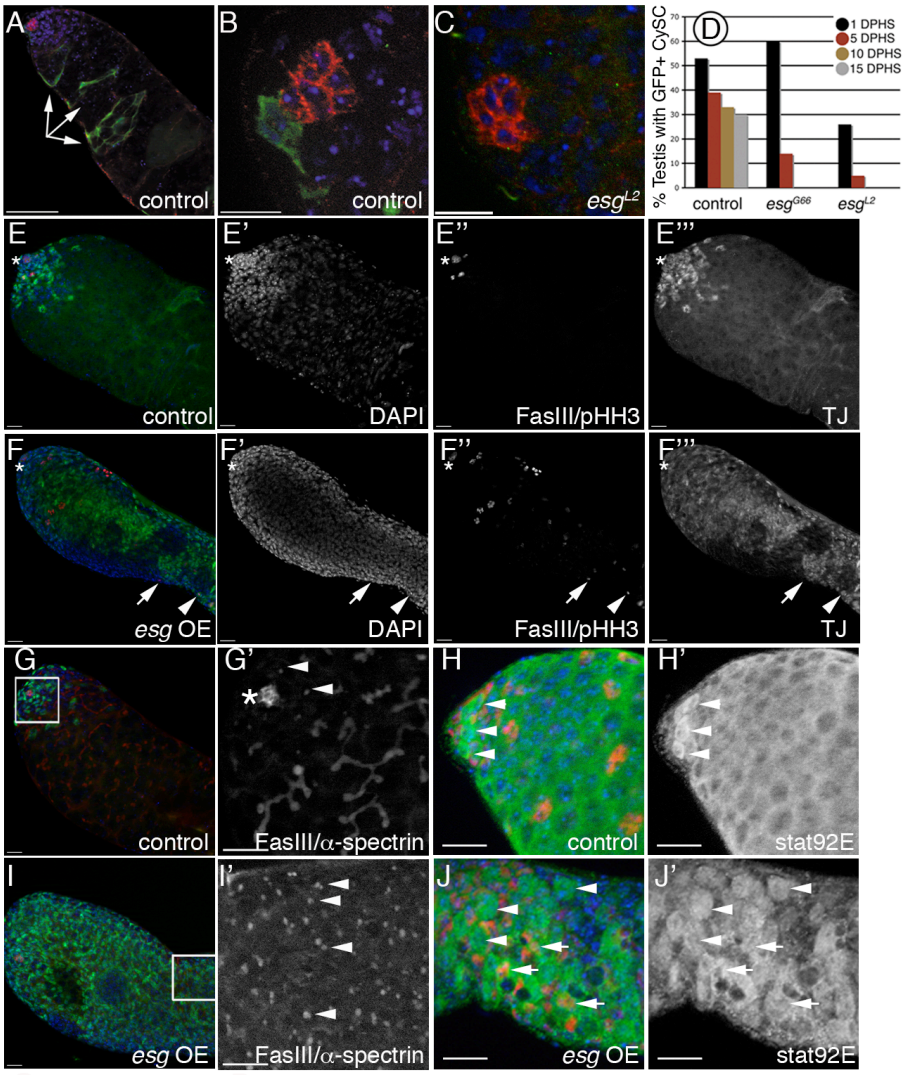


Figure 3.3 *esg* is required for CySC maintenance and is capable of inducing CySC/GSC proliferation

(**A**) Immunofluorescence image of control GFP+ CySC clones (arrows, green) 5 days post heat shock (PHS). (**B**) 63x zoom of **A** showing a single CySC (green) in contact with the E-cad+ (red) hub. (**C**) 10 days PHS *esg*^{L2} mutant CySC clones are not present near the E-cad+ (red) hub. (**D**) Graph depicting GFP+ CySC clonal analysis at 1, 5, 10, and 15 days PHS. (**E, F**) *esg EP2009; c587*GAL4 adult testis raised for 5 days at (**E**) 18°C or (**F**) 29°C and stained for DAPI (**E'**, **F'**, blue), FasIII (**E''**, **F''**, red, asterisk), p-Histone H3 (**E''**, **F''**, red), and TJ (**E'''**, **F'''**, green). (**F**) Arrowhead denotes TJ+/pHH3+ cell, Arrow denotes TJ-/pHH3+ cell. (**G**) Control testis stained for TJ (green), α-spectrin (red), and FasIII (red). (**G'**) 40x zoom of outline in **G,** dot spectrosomes (arrowheads) are present only adjacent to hub (asterisk). (**I**) *esg EP2009; c587*GAL4 testis raised for 5 days at 29°C stained for TJ (green), α-spectrin (red), and FasIII (red). (**I'**) Dot spectrosomes (arrowheads) present in regions distant from the hub (outline in **I**). (**H, J**) *esg EP2009; c587*GAL4 adult testis raised for 5 days at (**H**) 18°C or (**J**) 29°C and stained for TJ (red), Stat92E (**H'**, **J'**, green), and DAPI (blue). Early germ cells (arrowheads) and TJ+ cells (arrows) express Stat92E.

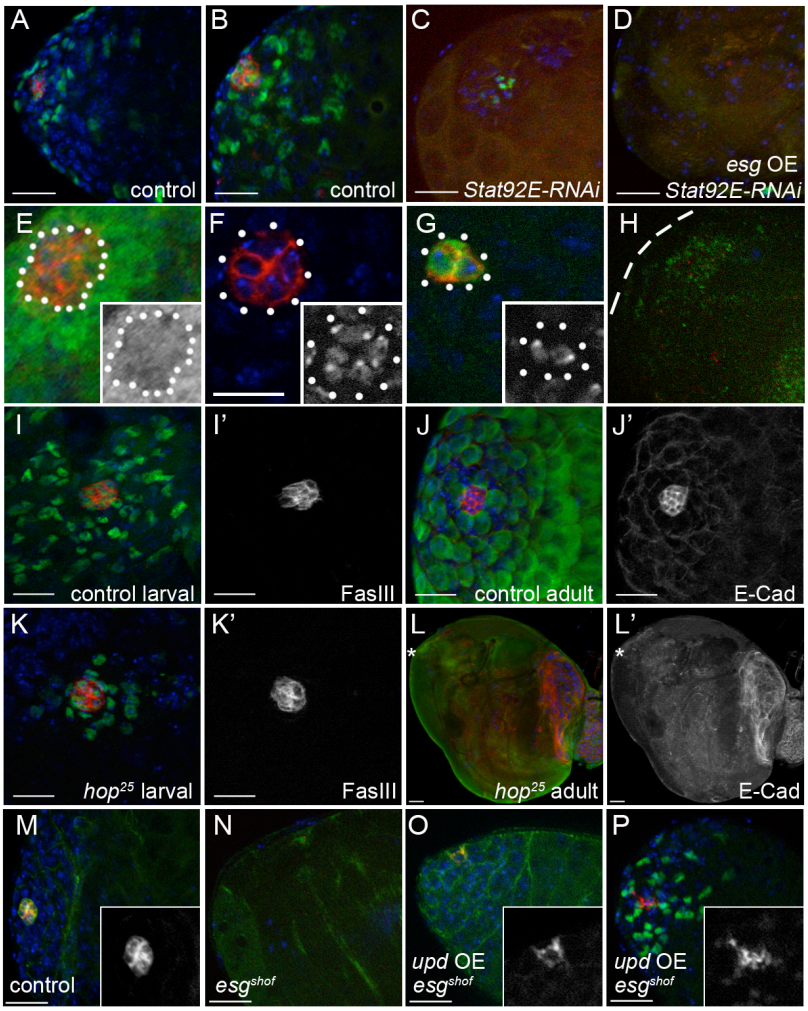
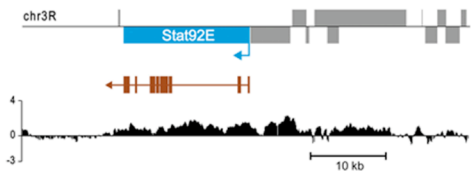


Figure 3.4 (A-P) An Esg-Stat92E interaction regulates CySC and hub cell behavior.

(**A-D)** *esg EP2009/statRNAi* epistasis experiments. (**A**) Control testis raised for 5 days at 29°C stained for FasIII (red), TJ (green), and DAPI (blue). (**B**) *esg EP2009*/UAS-*statRNAi; c587*GAL4 testis raised for 5 days at 18°C, (**C**) UAS-*statRNAi; c587*GAL4 testis raised 5 days at 29°C, (**D**) *esg EP2009*/UAS-*statRNAi; c587*GAL4 testis raised for 5 days at 29°C. (**E**) Control testis stained for p-Stat92E (green, inset) and E-cad (red, outline). (**F-H**) *upd*GAL4, UAS-*GFP*; UAS-*statRNAi* testes stained for E-cad (red, outline), GFP (green), and DAPI (blue, inset) in flies raised for (**F**) 1 day at 18°C, (**G**) 10 days at 29°C or (**H**) 20 days at 29°C. (**I**) Control L3 larval gonads stained for FasIII (**I'**, red), TJ (green), and DAPI (blue). (**J**) Control (*FM7; nanos*GAL4, UAS-*GFP*) adult testis stained for E-cad (**J'**, red), GFP (green), and DAPI (blue). (**K**) hop^{25} L3 larval gonads stained for FasIII (**K'**, red), TJ (green), and DAPI (blue). (**L**) Adult hop^{25}; *nanos*GAL4, UAS-*GFP* testes stained for E-cad (**L'**, red), GFP (green), and DAPI (blue). Asterisk denotes testis tip in **L**. (**M**) Control testis stained for FasIII (red, inset), E-cad (green), and DAPI (blue). (**N**) *upd*GAL4, UAS-*GFP*; esg^{shof} testis stained for FasIII (red), TJ (green), and DAPI (blue). (**O, P**) *upd*GAL4, UAS-*GFP*; esg^{shof}; UAS-*upd* testes stained for (**O**) FasIII (red, inset), E-cad (green), and DAPI (blue) or (**P**) FasIII (red, inset), TJ (green), and DAPI (blue).

Q



R

Top ten genes

Gene	Highest associated peak value (log2)
Adar	4.44
CG10962	4.35
rdgA	4.35
CG33960	4.32
CG11192	4.31
sgg	4.24
CG15279	4.06
Appl	3.93
CG4293	3.93
CG18375	3.8

Hub cell factors

Gene	Highest associated peak value (log2)
Fas3	2.85
Cdi	2.21
Stat92E	1.95
Esg	1.41
Unpaired	1.39

CySC factors

Gene	Highest associated peak value (log2)
Zfh-1	2.27
Dome	2.24
Socs36E	1.95
Stat92E	1.95
Esg	1.41

S

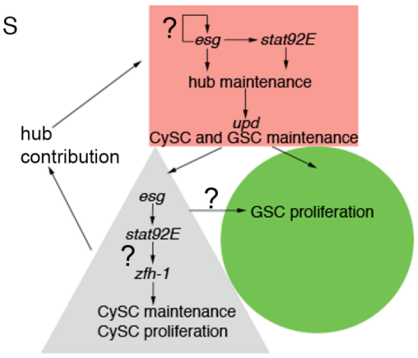


Figure 3.4 (Q-S) An Esg-Stat92E interaction regulates CySC and hub cell behavior.

(**Q**) Binding of Escargot at the *Stat92E* locus. Grey and blue boxes represent genes. Brown bars represent exons. Bar heights are proportional to the average of normalised log2-transformed ratio of intensities from DamID in vivo binding site experiments. (**R**) Genes identified in Esg Dam-ID analysis. (**S**) Genetic model of *esg* and *stat92E* interactions in maintenance of hub cells (red), CySCs (grey), and GSCs (green).

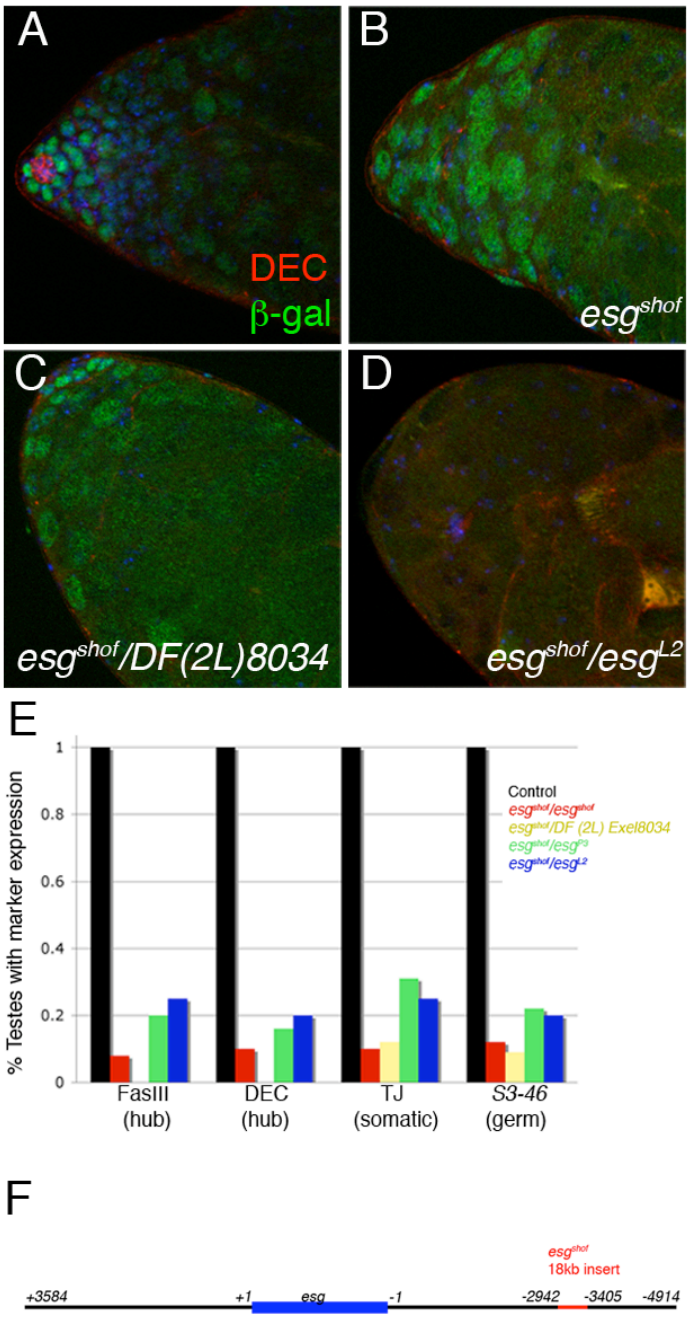


Figure S3.1 Germline and somatic stem cells are lost in *esg^{shof}* adult testis

(**A-D**) Allelic series of *esg^{shof}* adult testis stained for *S3-46* enhancer line (green) and DE-Cadherin (red). (**A**) Control, (**B**) *esg^{shof}* (**C**) *esg^{shof}/DF (2L) Exel8034,* (**D**) *esg^{shof}/ esg^{L2}*. (**E**) Hub, early somatic, and early germline marker expression in allelic series. (**F**) Insertion is approximately 5.2kb from the start site of *esg^{shof}*.

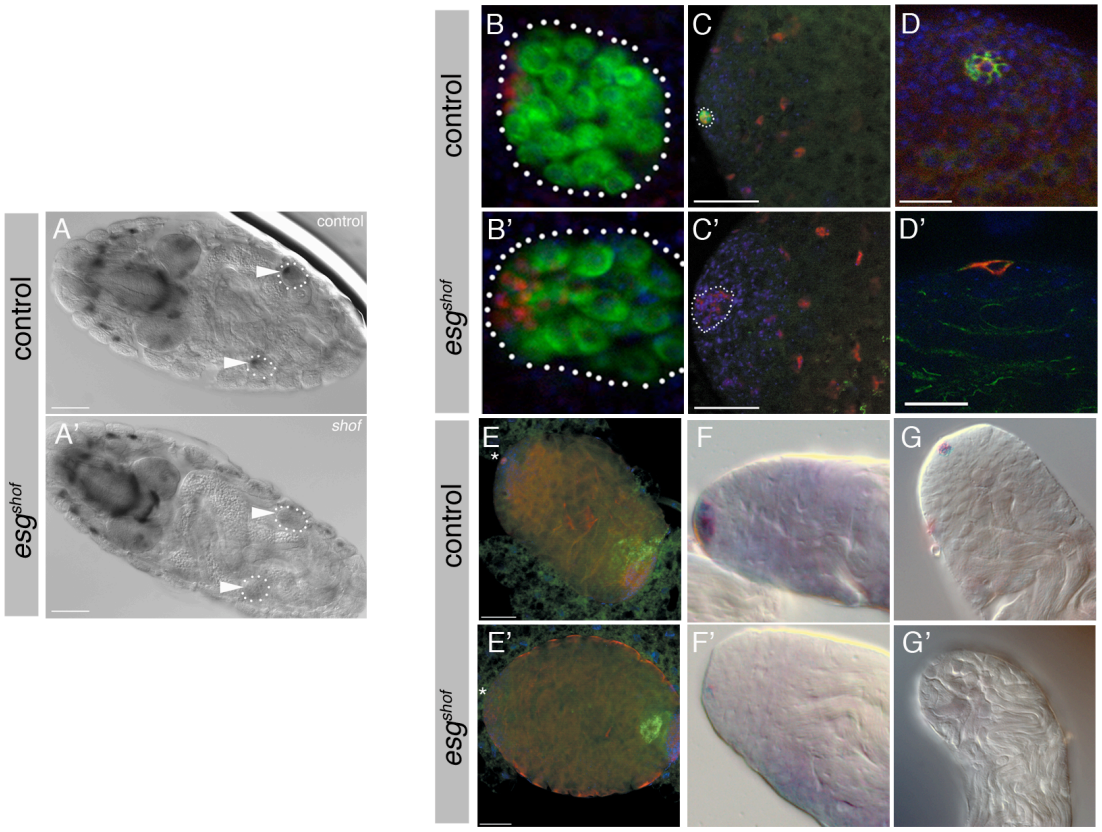


Figure S3.2 Hub marker expression during development in *esg*^{shof}
(**A-A'**) DIC images of in situ analysis for *esg* in stage 16 embryonic gonads (outlined, arrowheads) in (**A**) control and (**A'**) *esg*^{shof}. (**B**) Stage 17 control and (**B'**) *esg*^{shof} gonads (outlined) stained for Vasa (green), *cdi-lacZ* (red), and DAPI (blue). (**C**) *upd*GAL4, UAS-*gfp* and (**C'**) *upd*GAL4, UAS-*gfp; esg*^{shof} larval L2 gonad stained for FasIII (red, outline), Eyes Absent (red), GFP (green), and DAPI (blue). (**D**) Control and (**D'**) *esg*^{shof} larval L3 gonads stained for FasIII (red), E-cad (green), and DAPI (blue). (**E**) *upd*GAL4, UAS-*gfp* and (**E'**) *upd*GAL4, UAS-*gfp; esg*^{shof} L3 gonads stained for DEC (red, asterisk), TUNEL positive cells (green), and DAPI (blue). (**F, F'**) *esg* expression in (**F**) control and (**F'**) *esg*^{shof} adult testis. (**G, G'**) *upd* expression in (**G**) control and (**G'**) *esg*^{shof} adult testis.

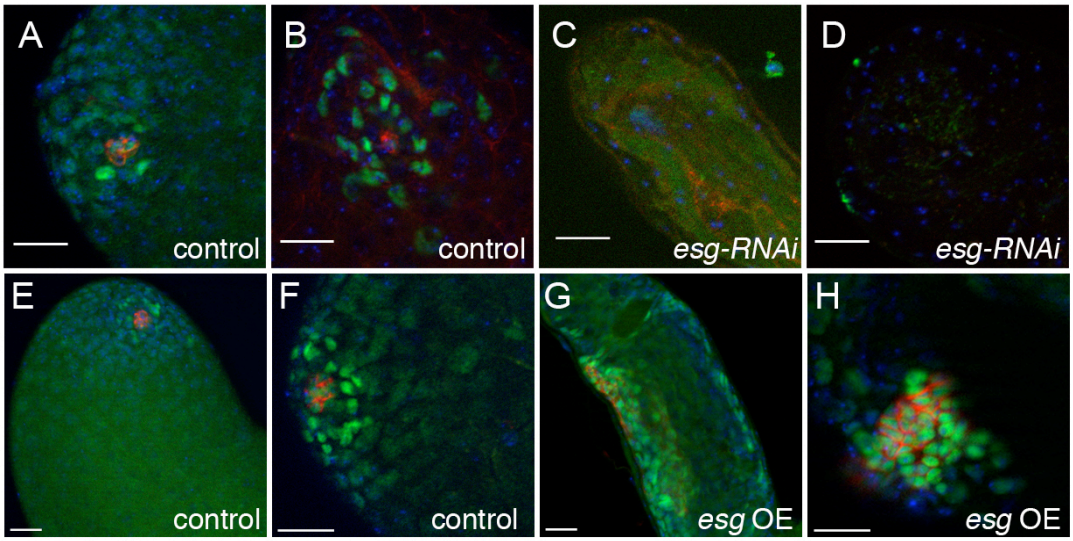


Figure S3.3 *esg* is required for CySC maintenance and is capable of inducing early somatic cell proliferation

(**A-D**) Immunofluorescence stains in *c587*GAL4/UAS-*esgRNAi* testis at one day at 18°C for (**A**) FasIII (red), Zfh-1 (green), and DAPI (blue) or (**B**) E-cad (red), TJ (green) and DAPI (blue). Hub and somatic cell markers are lost by ten days at 29°C for (**C**) FasIII (red), Zfh-1 (green) and DAPI (blue) or (**D**) E-cad (red), TJ (green) and DAPI (blue). (**E-H**) Testes stained for FasIII (red), Zfh-1 (green), and DAPI (blue). (**E**, **F**) In control testis FasIII and Zfh-1 expression is restricted to apical tip. (**G**, **H**) In *esg EP2009; c587*GAL4 adult testis raised for five days at 29°C, FasIII+ and Zfh-1+ cells are found in regions distant from the normal hub.

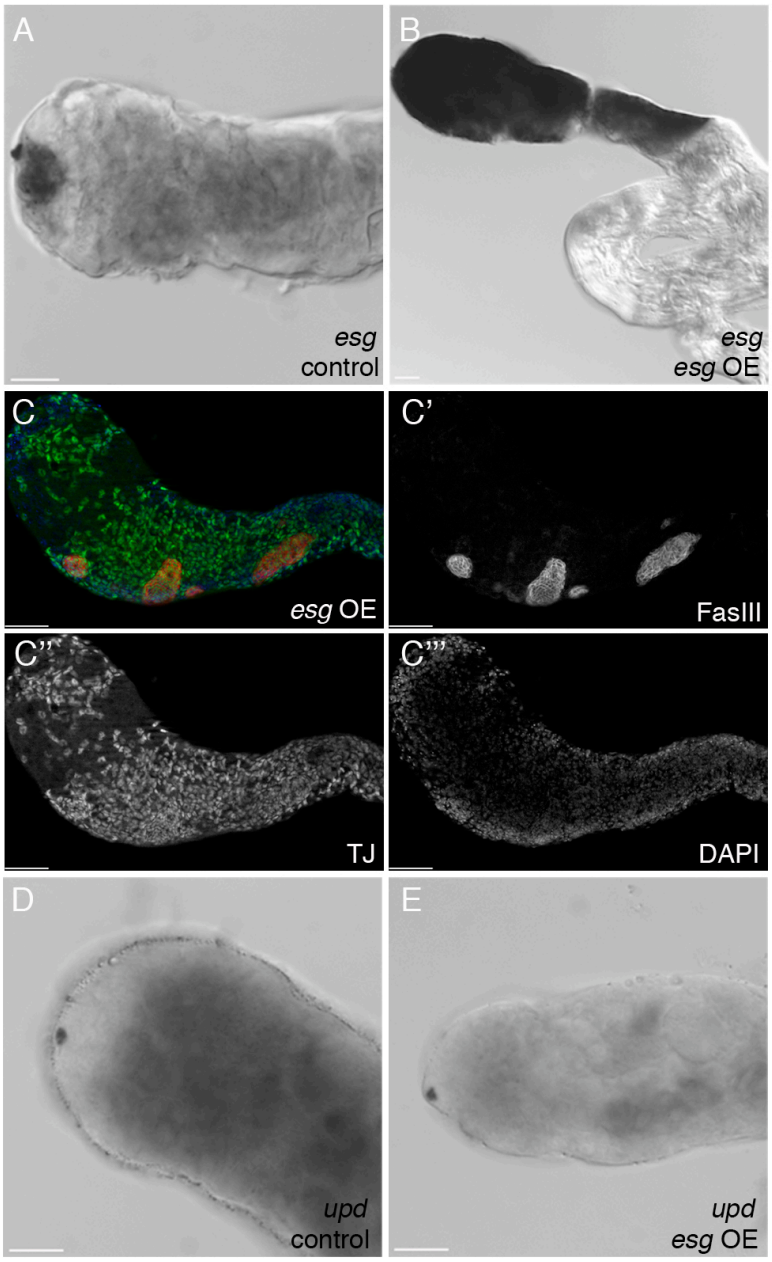


Figure S3.4 Somatic specific overexpression of *esg* results in excess early germ and somatic cells and ectopic hub markers

(**A-B**) In-situ analysis of adult testes for *esg* in *esg EP2009; c587*GAL4 testis raised for five days at (**A**) 18°C or (**B**) 29°C. (**C**) *esgEP2009; c587*GAL4 testis shifted to 29°C for five days stained for FasIII (**C'**, red), TJ (**C''**, green) and DAPI (**C'''**, blue). (**D-E**) In situ analysis of adult testis for *upd* in *esg EP2009; c587*GAL4 testis raised for five days at (**D**) 18°C or (**E**) 29°C.

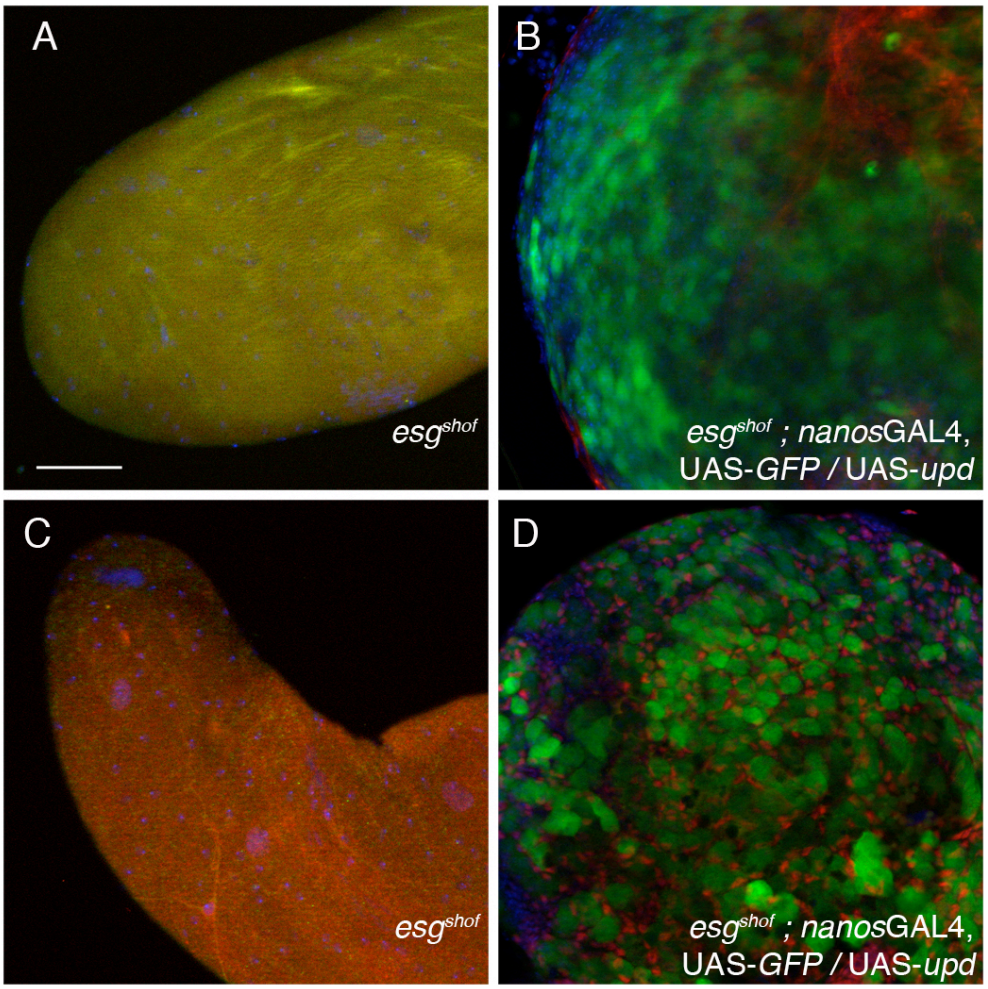


Figure S3.5 *upd* can induce somatic and germline proliferation in *esg*^{shof}
(**A-B**) Immunofluorescence stain for FasIII (red) and GFP (green) in (**A**) *esg*^{shof} ; *nanos*GAL4, UAS-*gfp* and (**B**) *esg*^{shof} ; *nanos*GAL4, UAS-*gfp*/UAS-*upd* testis. (**C-D**) Immunofluorescence stain for TJ (red) and GFP (green) in (**C**) *esg*^{shof} ; *nanos*GAL4, UAS-*gfp* and (**D**) *esg*^{shof} ; *nanos*GAL4, UAS-*gfp*/UAS-*upd* testis.

Supplementary Table S3.1 Effects of *esg* mutations on GSC self-renewal

Genotype	# testes with GFP negative clones (3days)[a]	# testes with GFP negative clones (5 days)	# testes with GFP negative clones (7 days)
Control (FRT40A)	46/77 (60%)	17/38 (45%-E) 30/38 (79%-L)	32/50 (64%-E) 38/50 (76%-L)
esg^{L2}	29/47 (62%)	4/8 (50%-E) 6/8 (75%-L)	27/52 (52%-E) 36/52 (70%-L)
esg^{G66}	21/30 (70%)	2/11(18%-E) 7/11 (68%-L)	23/61(38%-E) 41/61 (67%-L)

(a) Number indicates testes with at least one clone *n* days after clone induction. E indicates early clones within the mitotic amplification zone. L indicates “late” clones of spermatogonial and spermatocyte cysts.

Chapter 4: Regeneration of hub cells in the stem cell niche of the adult *Drosophila* testis

4.1 Summary

Tissue homeostasis in multiple organs is dependent upon replenishment from stem cell populations. Stem cell niches coordinate control of stem cell behavior, yet factors that regulate stem cell niche maintenance and activity are not well defined. Here we demonstrate that *cropped*, an AP-4 transcription factor, is necessary for hub maintenance. In addition, we find the adult hub possesses regenerative capacity which requires *unpaired* expression. This study suggests that a 'set-point' is present for maintaining hub size and as a result stem cell niche size. Understanding the regulatory mechanisms that determine stem cell niche size and homeostasis is critical to the advancement of regenerative medicine.

Adult stem cells reside in distinct microenvironments (niches) that balance stem cell self-renewal and differentiation to maintain tissue architecture (Scadden, 2005) (Jones and Wagers, 2008). Stem cell niches have been identified in a number of tissue types, however factors regulating niche size, maintenance and activity are not well defined. Genetic

characterization of such factors may lead to the development of regenerative medical therapies.

4.2 Introduction

At the tip of the *Drosophila* testis resides the hub, a signaling center composed of approximately ten cells (Hardy et al., 1979). Hub cell production of *unpaired* (*upd)* specifies JAK-STAT pathway activity that mediates self-renewal of germline (GSC) and somatic (SSC/CySC) cyst stem cell populations (Kiger et al., 2001) (Tulina and Matunis, 2001). Within the niche, the hub can be identified by expression of *upd* and the cell adhesion molecules Fasciclin III (FasIII) and DE-Cadherin (DEC) (Brower et al., 1981)(Le Bras and van Doren, 2006). To identify genes that control hub cell number during adulthood a temporally controlled candidate RNAi screen was conducted utilizing the temperature dependent *GAL4-UAS* system (Brand and Perrimon, 1994) (Dielz et al., 2008). To ensure establishment of the hub during development, flies were raised at 18°C to restrict *GAL4-UAS* driven RNAi transgene expression. Upon eclosion, adult flies were shifted to a permissive temperature (29°C) to induce hub specific RNAi directed against candidate genes. Previously we have used this method to identify the transcription factors *esg* and *Stat92E* are required for hub maintenance (Voog et al., in preparation). Here we report that *cropped,* a putative target of *esg,* is required for hub cell maintenance during adulthood. These studies may

provide insight into the regenerative capacity of a well-defined and genetically tractable stem cell niche.

4.3 Results

4.3.1 Cropped is required for hub cell maintenance in adulthood

Cropped is expressed within hub cells, early somatic cells, and early germ cells (Figure 4.1A). The dAP-4 transcription factor, *cropped (crp),* is proposed to regulate the expression of the adhesion molecule *Sgs-4* within the salivary gland (King-Jones et al., 1999). Expression of *crp* is upregulated in response to *upd* in the testis (L. Jones, unpublished), and the promoter region of *crp* is occupied by *escargot* (Voog et al., in preparation), however its requirement within the testis is not known. To test if *cropped* is required for hub cell maintenance during adulthood, *upd*GAL4, UAS-*GFP*; UAS-*crpRNAi* flies were shifted to 29°C for 10 or 20 days after eclosion and FasIII+ or DEC+ hub cells counted. At 1 day of age *upd*GAL4, UAS-*GFP*; UAS-*crpRNAi* hubs contained 8.0 ± 2.0 cells (n=29) (Figure 4.1B). By 10 and 20 days, FasIII+ hub cell number was reduced to 4.9 ± 2.2 (n=51) and 2.6 ± 1.7 (n=66) respectively (Figure 4.1C, D). These data demonstrate the *cropped* is required for hub maintenance during adulthood.

4.3.2 The hub is capable of regeneration

Factors regulating hub cell specification and number during early development have been identified (Kitadate et al., 2007) and hub cell number appears to be under strict control. However how hub size is 'set' and maintained is poorly understood. To probe if the hub is capable of regeneration during adulthood *upd*GAL4, UAS-*GFP*; UAS- *crp-RNAi* flies were raised throughout development at 18°C, shifted to 29°C for 10 days to induce hub cell loss, and subsequently shifted to 18°C for 10 days. Hub cell number was then scored. The number of FasIII+ and DEC+ hub cells was restored, suggesting the hub is capable of regeneration during adulthood (Figure 4.1E) (Table 4.1). No mitotic *upd*+ or FasIII+ hub cells were observed during recovery periods suggesting that that new hub cells are the result of SSC contribution to the hub (Voog et al., 2008).

We did not observe regeneration using the same protocol for other factors regulating hub maintenance (*esg* and *Stat92E*). Driving expression of either *esg-RNAi* or *Stat92E-RNAi* results in a more dramatic loss of hub cells. These data suggest that hub cells (and *upd*) are required for restoration of hub size, and that a hub cannot be generated *de novo* during adulthood.

4.3.3 Proposed method to calculate hub cell half-life

Future experiments will attempt to address the half-life of a hub cell using the Bruintrace lineage tracing strategy (Cory Evans, personal

communication). This strategy permits hub cells to be permanently labeled during embryonic development using a Flp dependent recombination event that drives expression of GFP within a subset of embryonic hub cells. During early development the hub is specified from somatic cells originating in parasegments 10, 11, and 12 (Boyle and Dinardo, 1995). To permanently label a subset of hub cells the *paired*GAL4 line that expresses in a subset of embryonic hub cells will be used (Le Bras and van Doren, 2006). Hub cell turnover will be calculated in Bruintrace flies under *paired*GAL4 control (*paired*GAL4; UAS-*DsRed*, UAS-*flp, Ubi>>GFP)* under the assumption that GFP labeled hub cells will be lost throughout the course of adulthood.

4.3.4 Cropped is required for somatic stem cell maintenance

To address if *crp* is required for somatic stem cell self-renewal, MARCM clonal analysis was performed. Here flies exposed to heat-shock initiate FRT-mediated recombination resulting in generation of labeled (GFP+) homozygous mutant cells in a heterozygous background (Lee and Luo, 1999). GFP+ mutant SSC/CySCs were generated using the loss of function *crp* allele (crp^{Fd2}). At 1-day post heat shock (PHS) 45% (n=20) of crp^{Fd2} testes were observed to have at least one marked SSC/CySC. At 5 days PHS control SSC/CySC clones were present in 39% of control testes examined, while 0% (n=8) of testes contained crp^{Fd2} mutant SSC/CySCs. At 10 and 15 days DPHS, no labeled *crp* mutant SSC/CySCs clones were observed. Thus *crp* is

required for SSC/CySCs maintenance, as reported for *esg* and *stat92E* (Voog et al., in preparation)(Leatherman and Dinardo, 2008).

Complementing clonal analysis findings, a *crpRNAi* transgene expressed using the early somatic cell specific *c587*GAL4 line results in loss of early somatic cells and the hub. Flies were grown at 18°C, and upon eclosion were shifted to 29°C for ten or twenty days to induce RNAi expression. The presence of early somatic cell markers Zfh-1 and Traffic Jam and hub markers Fasciclin III and DE-Cadherin were assayed. Hub and early somatic cells were initially present in 1-day old *c587*GAL4>UAS-*crpRNAi* testes, but early somatic cell markers and hub markers were lost by shifting flies for 10 days to 29°C (Figure 4.2). These data complement evidence that SSCs are required for maintenance of the hub during adulthood (Voog et al., 2008).

4.4 Discussion

4.4.1 Mechanisms of stem cell niche regeneration

Our previous work suggests that the somatic stem cell contribution to the hub is responsible for hub maintenance. Here we identity a role for the AP-4 transcription factor *cropped* using RNAi transgenic approaches and clonal analysis. A requirement of *cropped* for maintenance of hub and somatic stem cells is presented. Interestingly, *cropped* is a putative transcriptional target of *esg* (Voog et al., in preparation), suggesting that coordinated transcription factor activity may be required for regulating hub size. Future

work will focus upon identifying and characterizing the molecular pathways that determine how size of the stem cell niche is set.

4.5 Methods

4.5.1 Fly husbandry and stocks

Flies were raised at 25°C on standard cornmeal-molasses media. The UAS-*crpRNAi* line was obtained from the Vienna *Drosophila* Resource Center (VDRC). The *upd*GAL4-UAS-*gfp* line was a gift from E. Bach. Bruintrace lines were a gift from C. Evans and U. Banerjee. The following stocks were used and more information can be found at Flybase (http://flybase.bio.indiana.edu): *hs-FLP-122*, α-*tubulin*GAL80, α-*tubulin*GAL4, *2x*-UAS-*eGFP*, *FRT40A*, *FRT40A*, and crp^{Fd2}.

4.5.2 Immunohistochemistry and microscopy

Testes were dissected and fixed in 2% PFA in PLP buffer (0.075 lysine, 0.01 M sodium phosphate buffer pH7.4) for 60 min at room temperature, rinsed for 30 min in PBS containing 0.1% Triton X-100 and 0.3% bovine serum albumin, and immunostained with appropriated antibodies overnight at 4°C. Testes were then washed for 60 min at room temperature for 4 h. Testes were analyzed with a Zeiss Axiovert 200 microscope and processed, and Axiovision (version 4.5; C. Zeizz) and Adobe Photoshop software.

4.5.3 Antibodies

Testes were stained using the following: guinea-pig anti-TJ (1:3,000) (gift from D. Godt), rabbit anti-cropped (1:1,000)(M.Lehmann), rabbitt anti-Zfh-1 (R. Lehmann) and rabbit anti-GFP (1:5,000) (Molecular Probes). Mouse anti-Fasciclin III (7G10) (1:10) and rat anti-DE-cadherin (DCAD2) (1:20) were obtained from the Developmental Studies Hybridoma Bank. Secondary antibodies were diluted 1:500 (Molecular Probes). Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories).

4.5.4 MARCM clonal analysis

For MARCM experiments, late pupae or early larvae were heat shocked at 37°C for 2 h on 2 consecutive days. Flies were collected at various time points after the heat shock, immunostained and analyzed (Lee and Luo, 1999). MARCM wild type control genotypes used were *y,w, hsflp*^{122}; *FRT40A/FRT40A, tub*GAL80; *tub*GAL4/*2x*-UAS-*eGFP*. Mutant genotypes used were: *crp*^{Fd2} (*y,w, hsflp*^{122}; *FRT40A crp*^{Fd2}*/FRT40A tub*GAL80; *tub*GAL4/*2x*-UAS-*eGFP*).

4.6 Acknowledgements

We thank E. Bach, D. Godt, R. Lehmann, N. Perrimon, and M. Lehmann for reagents and fly stocks, and Jones laboratory members for discussions and comments on this work.

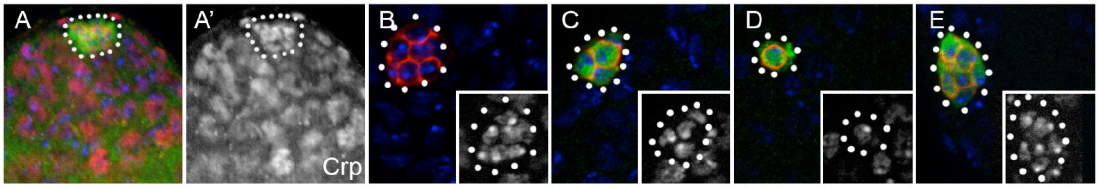


Figure 4.1 Cropped is required for hub cell maintenance and the hub is capable of regeneration.

A) Immunostain one day old *upd*GAL4, UAS-*GFP* testis for Cropped (red, A'), GFP (green), and DAPI (blue). The hub is outlined. (B-E) *upd*GAL4, UAS-GFP; UAS-*crpRNAi* testis stained for DE-Cadherin (red, outline), GFP (green), and DAPI (blue, inset) at (B) one day at 18°C, (C) ten days at 29°C, (D) twenty days at 29°C, and (E) ten days at 29°C and then shifted for ten days at 18°C.

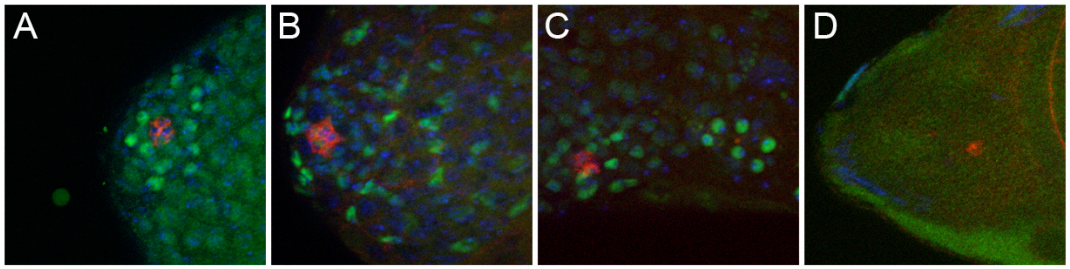


Figure 4.2 Cropped is required for somatic stem cell maintenance.
(A) Immunostain for Fasciclin III (red), Zfh-1 (green), and DAPI (blue) in *c587*GAL4>UAS-*crpRNAi* testes raised for one day at 18°C. (B) Immunostain for DE-cadherin (red), Traffic Jam (green), and DAPI (blue) in *c587*GAL4>UAS-*crpRNAi* testes raised for one day at 18°C. (C) Immunostain for Fasciclin III (red), Zfh-1 (green), and DAPI (blue) in *c587*GAL4>UAS-*crpRNAi* testes raised for ten days at 29°C. (D) Immunostain for DE-Cadherin (red), Traffic Jam (green), and DAPI (blue) in *c587*GAL4>UAS-*crpRNAi* testes raised for ten days at 29°C.

Table 4.1 Cropped is required for hub maintenance and the hub is capable of regeneration

Table 1. Factors regulating hub cell maintenance and regeneration

		Temperature Shift Protocol Avg+S.D. (*n*)					
Genotype	Hub marker	1d at 18°C	10d at 18°C	10d at 29°C	20d at 18°C	20d at 29°C	10d at 29°C / 10d at 18° C
*upd*GAL4, UAS-*GFP*; UAS-*crpRNAi*	FasIII	8.0+2.0 (29)	7.6+1.6 (33)	4.9+2.2 (51)	7.7+1.9 (44)	2.6+1.7 (66)	6.6+2.2 (97)
	DE-Cadherin	7.9+1.4 (34)	7.5+1.2 (10)	2.9+2.0 (13)	6.3+2.1 (38)	2.3+1.6 (40)	6.5+2.3 (48)

Hub cell counts for Fasciclin III and DE-Cadherin for *upd*GAL4, UAS-*GFP*; UAS-*crpRNAi* flies at various time points during temperatures to inhibit (18°C) or enhance (29°C) *RNAi* transgene expression.

Chapter 5: Conclusions and Future Directions

5.1 Conclusions

The field of stem cell biology has direct applications toward the development of regenerative therapies. However factors regulating stem cell behavior are incompletely understood. Future work in characterizing the identity and function of adult stem cells and their niches will provide insight into the processes of aging, disease, and wound repair.

The interpretations of experimental data described in this dissertation are based upon lineage analysis, genetic approaches, and global genomic transcriptional identification assays to characterize factors regulating the maintenance and regeneration of the stem cell niche in the *Drosophila* testis.

Historically, hub cells in the *Drosophila* testis were described to be static in number as hub cells are post-mitotic. However data acquired using lineage tracing strategies suggested that new hub cells are generated during adulthood that is presented in Chapter 2 (Voog et al., 2008). Subsequent experimental approaches demonstrated that somatic stem cells are capable of contributing to the hub. This finding is important, as the ability of somatic cyst stem cells to contribute to the hub suggests that the size, maintenance, and number of niches is under strict genetic control. Identifying factors that regulate somatic cyst stem cell and hub cell behavior is necessary to model stem cell niche behavior. Clonal analysis demonstated that somatic cyst stem

cell maintenance is dependent upon adhesion to the hub via DE-cadherin and ablation of the somatic cyst stem cell population leads to subsequent hub loss. These results complement other work demonstrating that DE-cadherin is required for germline stem cell maintenance (Song et al., 2002).

In addition, our data also provide evidence that DE-cadherin is not required for somatic cyst stem cell contribution to the hub and that hub cells do not require DE-cadherin for their maintenance. Hub cells are enriched in additional cell adhesion molecules including Fasciclin III and DN-Cadherin. It is likely that these cell adhesion molecules act redundantly to ensure that hub position and maintenance.

Clonal analysis identified that Snail family transcription factor *escargot* is necessary for regulating somatic cyst stem cell maintenance. In addition, *escargot* mutant somatic cyst stem cells did not contribute to the hub at the same frequency as wild type somatic cyst stem cells. These findings support observations presented in Chapter 3 demonstrating that *esg* is required for hub cell maintenance (Voog et al., in preparation).

Chapter 3 provides the first direct genetic evidence that the hub is absolutely required for both somatic cyst stem cell and germline stem cell maintenance (Voog et al., in preparation). In this work, loss of function genetic mutants (*esg* and *hop*) and RNAi hub specific knockdown of *esg* and *Stat92E* result in a loss of the hub during adulthood. Hub loss results in subsequent differentiation of both germline and somatic cyst stem cell populations. These

data demonstate that hub cells autonomously require both *esg* and *Stat92E* activity for hub cell and stem cell niche maintenance.

Somatic overexpression of the zinc finger transcription factor *zfh-1* or germline and somatic overexpression of *unpaired* results in excessive stem cyst cell self-renewal (Leatherman and Dinardo, 2008) (Kiger et al., 2001) (Tulina and Matunis, 2001). To address the role of *esg* within somatic cyst stem cells, *esg* was conditional overexpressed using a somatic cell specific GAL4 driver that resulted in a dramatic induction of proliferation in both somatic cyst and germline stem cell populations. This autonomous somatic and non-autonomous germline induced proliferation is *Stat92E* dependent, suggesting that *escargot* may regulate *Stat92E* activity within somatic cyst stem cells.

To identify putative transcriptional targets of *esg* we utilized DamID, a global genomic transcriptional profiling assay described in Chapter 3, in collaboration with the Brand lab at Cambridge University (Choksi et al., 2006). This technique permits the physical location of a Esg-Dam fusion protein to be mapped for the entire genome from genomic DNA isolated from adult *Drosophila* testis tissue. A number of hub specific and somatic cyst stem cell enriched target genes were identified using this approach including *Stat92E*. Our genetic and genomic analysis suggests that a transcriptional interaction between *esg* and *Stat92E* is required for somatic cyst stem cell proliferation. In addition, we find that Stat92E expression is upregulated in *esg*

overexpressing testis providing evidence that *esg* may regulate *Stat92E* expression. Interestingly, while *zfh-1* can induce proliferation of early somatic and germline cells, Stat92E expression is not induced. Future experiments will test the hypothesis that *esg* regulates both *zfh-1* expression and activity.

Based upon the observation that *esg* can regulate *Stat92E* expression in somatic cyst stem cells, we hypothesized an *esg* – *Stat92E* interaction may be present within hub cells. To test this possibility, *upd* expression was driven using the *GAL4-UAS* system specifically in hub cells in *escargot*^{shutoff} flies. Expression of *upd* in this *esg* mutant background resulted in an increase of hub markers, suggesting that activation of *Stat92E* can partially rescue hub cell loss and may be downstream of *esg*.

Taken collectively, these results suggest that an *esg-stat92E* interaction regulates hub cell and somatic cyst stem cell behavior that is necessary for proper stem cell niche activity. Identifying the distinct transcriptional networks that are present within hub cells and somatic cyst stem cells that are required for cell type specific identity and maintenance is of great interest and a priority for future studies.

In Chapter 4, preliminary evidence is presented suggesting that the hub is capable of regeneration during adulthood. These results indicate that conditional knock-down of the *Drosophila* AP-4 transcription factor, *cropped,* specifically within hub cells leads to hub cell loss. Upon re-expression of *cropped,* hub cells are replaced as an increase in hub cell number is

observed. We find that a 'set-point' of hub size is present and suggest that hub cells must be present for hub regeneration. Interestingly, *cropped* is a putative transcriptional target of *esg* based upon DamID analysis, suggesting that coordinated transcription factor activity may be required for regulating hub size. How hub size is set is and hub regeneration occurs is unclear at this point, but is a priority for future studies.

5.2 Future Directions

5.2.1 Potential regulation of *escargot* activity

The mammalian homologue of *escargot* is Snail, a zinc-finger transcription factor implicated in cell migration and down regulation of E-cadherin (Nieto, 2002). Glycogen Synthase Kinase (GSK)-3beta binds and phosphorylates Snail at two distinct sites: one site regulates Snail's ubiquitination and degradation, while the second phosphorylation site regulates Snail's localization within the nucleus or cytoplasm (Zhou et al., 2004). Interestingly, the *Drosophila* orthologue of GSK-3b, *shaggy*, is a potential transcriptional target of *esg* within the testis identified using DamID. Verifying the the interaction between *esg* and *shaggy* will encompass combined biochemical and genetic approaches.

Factors that physically interact with *escargot* have been identified using yeast two-hybrid screens (Giot et al., 2003), however the functional significance of these interactions in regulating *esg* within the testis is not

known. C-terminal binding protein (CtBP), a protein identified from this study (Giot et al., 2003), is known to regulate *Drosophila* Snail through a conserved protein domain present in *esg* and several vertebrate Snail family members (Nieto, 2002). In addition, Zfh-1 can interact with CtBP in the testis, suggesting that transciptional coordination is present within the germline stem cell niche (Leatherman and Dinardo, 2008).

Our data suggest that *esg* genetically and directly interacts with *Stat92E*, likely as an upstream mediator of *Stat92E* activity. Microarray data demonstrates that *esg* is upregulated in response to *upd* expression within the testis (Terry et al., 2006) and eye (Flaherty et al., 2009). A putative Stat92E transcripional binding site is present in the 3' region downstream of *esg* (Flaherty et al., 2009), a site that may be disrupted in the *escargot*^{shutoff} mutant background. An interesting possiblity is that *esg* and *Stat92E* may regulate each other's expression and activity.

Future work in the identification of proteins physically interacting with tagged versions of Escargot using mass spectrometry are currently in process. In addition, validation of DamID candidates is essential to a better understanding of how *esg* regulates stem cell niche activity in the *Drosophila* testis.

5.2.2 Potential mechanisms controlling hub cell and somatic stem cell identity and behavior

In agametic flies the expression of hub markers expands and early somatic cyst cells undergo ectopic proliferation (Gonczy and DiNardo, 1996). These data suggest that the germline normally suppresses somatic cyst stem cell proliferation and hub marker expression. The molecular determinants for this activity are not known, but preliminary data suggests that Notch signaling may regulate somatic cell fate in the testis (Voog, unpublished) (S. DiNardo personal communication). In the *Drosophila* ovary, ectopic expression of the Notch ligand Delta in germline cells results in an increase in the number of niche support cells (cap cells), increase in expression of the self-renewal factor BMP, and a corresponding increase in germline stem cell number (Ward et al., 2006)(Song et al., 2007).

Preliminary studies suggest that overexpression of the Notch ligand Serrate in early germ cells can induce excess hub cells during development and adulthood. An attractive hypothesis is that hub size is dictated in part by germline suppression of somatic cyst stem cell contribution to the hub. A potential mechansim for hub cell fate repression is that JAK-Stat signaling in GSCs represses expression of Serrate (Flaherty et al., 2009) within early germ cells.

5.2.2 Transcriptional networks regulating stem cell niche activity

The genome wide analysis of putative transcriptional targets of *escargot* has provided a framework to investigate and functionally test targets important in the regulation of hub cell maintenance, somatic cyst stem cell maintenance, somatic cyst stem cell proliferation, and stem cell niche behavior in general.

Statistical analyses to determine GO annotation (Ashburner et al., 2000) enrichment on DamID candidates can be accomplished using a web-based set of tools including GOToolbox (Martin et al., 2004). Subsets of candidate genes are enriched in specific cell types including hub cells (Fasciclin III, Center-divider, Unpaired) and somatic stem cells (Zfh-1). How *esg* coordinately regulates expression of these and other genes is presently unknown, but a focus of future experiments.

This work and other studies have prompted many hypotheses to be experimentally tested including: Does *escargot* work in combination with other transcription factors to regulate hub cell or somatic cyst stem cell fate? Is *escargot* regulated by itself or *Stat92E* in an auto-regulatory loop? Does *esg* work in concert with *zfh-1* to regulate somatic cyst stem cell fate? Are similar genetic programs present in the formation, maintenance, and regeneration of the hub involving *esg*? Can hub and niche formation occur *de novo* during adulthood? What factors are present in germ cells that regulate somatic cyst cell and hub cell behavior? Are there competing transcriptional programs for tissue replacement in the contexts of wound healing, aging, and regeneration?

The hypotheses tested and conclusions reached in this dissertation provide insight into factors regulating the function of a stem cell niche. This work has provoked a series of testable hypotheses and generated a model from which to build future studies in this discipline.

References

Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. (2006) Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature *439*, 599–603.

Adams, G.B., Martin, R.P., Alley, I.R., Chabner, K.T., Cohen, K.S,, Calvi, L.M., Kronenberg, H.M., and Scadden, D.T. Therapeutic targeting of a stem cell niche. (2007) Nat Biotechnology *25*, 238-43.

Adams, G.B., Alley, I.R., Chung, U.I., Chabner, K.T., Jeanson, N.T., Lo Celso, C., Marsters, E.S., Chen, M., Weinstein, L.S., Lin, C.P., Kronenberg, H.M., and Scadden, D.T. Haematopoietic stem cells depend on Galpha(s)-mediated signalling to engraft bone marrow. (2009). Nature *459*, 103-7.

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. Prospective identification of tumorigenic breast cancer cells. (2003) Proc Natl Acad Sci USA *100*, 3983–3988.

Amcheslavsky, A., Jiang, J., and Ip, Y.T. Tissue damage-induced intestinal stem cell division in Drosophila. (2009) Cell Stem Cell *4*, 49-61.

Arai, R., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. (2004) Cell *118*, 149–161.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., and Sherlock, G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. (2000) Nat Genet *25*, 25–29.

Ashraf, S.I., Hu, X., Roote, J., and Ip, Y.T. The mesoderm determinant snail collaborates with related zinc-finger proteins to control Drosophila neurogenesis. (1999) EMBO J *18,* 6426-38.

Ashraf, S.I., and Ip, Y.T. The Snail protein family regulates neuroblast expression of inscuteable and string, genes involved in asymmetry and cell division in Drosophila. (2001) Development *128*, 4757-67.

Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., Dewhirst, M.W., Bigner, D.D., Rich, J.N. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. (2006) Nature *444*, 756–760.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., and Clevers, J. (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003–1007.

Barroca, V., Lassalle, B., Coureuil, M., Louis, J.P., Le Page, F., Testart, J., Allemand, I., Riou, L., and Fouchet, P. Mouse differentiating spermatogonia can generate germinal stem cells in vivo. (2009). Nat Cell Biol 11(*2*), 190-6.

Blainpain, C., and Fuchs, E. Epidermal homeostasis: a balancing act of stem cells in the skin. (2009) Nat Rev Mol Cell Biol *10*, 207-17.

Bodine, D., Seidel, N. E. & Orlic, D. Bone marrow collected 14 days after in vivo administration of granulocyte colony-stimulating factor and stem cell factor to mice has 10-fold more repopulating ability than untreated bone marrow. (1996) Blood *88*, 89–97.

Bos, P.D., Zhang, X.H., Nadal, C., Shu, W., Gomis, R.R., Nguyen, D.X., Minn, A.J., van de Vijver, M.J., Gerald, W.L., Foekens, J.A., and Massagué, J. Genes that mediate breast cancer metastasis to the brain. (2009) Nature doi:10.1038/nature08021

Boyle, M., and DiNardo, S. Specification, migration and assembly of the somatic cells of the Drosophila gonad. (1995) Development *121*, 1815–1825.

Boyle, M., Wong, C., Rocha,M., and Jones, D.L. Decline in self-renewal factors contributes to aging of the stem cell niche in the Drosophila testis. (2007) Cell Stem Cell 1 *(4)*, 470-8.

Brack, A.S., and Rando, T.A. Intrinsic changes and extrinsic influences of myogenic stem cell function during aging. (2007) Stem Cell Rev *3*, 226-37.

Brand AH, Manoukian AS, Perrimon N. Ectopic expression in Drosophila. (1994) Methods Cell Biol *44*, 635-54.

Brawley, C. & Matunis, E. Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. (2004) Science *304*, 1331–1334.

Brinster, R.L. Germline stem cell transplantation and transgenesis. (2002) Science *296*, 2174-6.

Brower, D.L., Smith, R.J., and Wilcox, M. Differentiation within the gonads of Drosophila revealed by immunofluorescence. (1981) J Embryol Exp Morphol *63*, 233-42.

Bruce, W.R., and Van Der Gaag, H.A quantitative assay for the number of murine lymphoma cells capable of proliferation in vivo. (1963) Nature *199*, 79–80.

Calabrese, C., Poppleton, H., Kocak, M., Hogg, T.L., Fuller, C., Hamner, B., Oh, E.Y., Gaber, M.W., Finklestein, D., Allen, M., Frank, A., Bayazitov, I.T., Zakharenko, S.S., Gajjar, A., Davidoff, A., and Gilbertson R.J. A perivascular niche for brain tumor stem cells. (2007) Cancer Cell *11*, 69-82.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A.,

Kronenberg, H.M., and Scadden, D.T. Osteoblastic cells regulate the haematopoietic stem cell niche. (2003) Nature *425*, 841-6.

Casper, A., and Van Doren, M. The control of sexual identity in the Drosophila germline. (2006) Development *133*, 2783-91.

Cerletti, M., Jurga, S., Witczak, C.A., Hirshman, M.F., Shadrach, J.L., Goodyear, L.J., and Wagers, A.J. Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. (2008) Cell *134*, 37-47.

Cheng, J., Turkel, N., Hemati, N., Fuller, M.T., Hunt, A.J., and Yamashita, Y.M. Centrosome misorientation reduces stem cell division during ageing. (2008) Nature *456*, 599-604.

Choksi SP, Southall TD, Bossing T, Edoff K, de Wit E, Fischer BE, van Steensel B, Micklem G, Brand AH. Prospero acts as a binary switch between self-renewal and differentiation in Drosophila neural stem cells. (2006) Dev Cell *11*, 775-89.

Collins, A.T., Berry, P.A., Hyde, C., Stower, M.J., and Maitland, N.J. Prospective identification of tumorigenic prostate cancer stem cells. (2005) Cancer Res *65*, 10946–10951.

Collins, C.A., Olsen, I., Zammit, P.S., Heslop, L., Petrie, A., Partridge, T.A., and Morgan, J.E. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. (2005) Cell *122*, 289–301.

Conboy, I.M., and Rando, T.A. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. (2002) Dev Cell 3*(3*), 297-409.

Conboy, I.M., Conboy, M.J., Smythe, G.M., and Rando, T.A. Notch-mediated restoration of regenerative potential to aged muscle. (2003) Science *302*, 1575-7.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L., and Rando, T.A. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. (2005) Nature *433*, 760-4.

Clayton, E., Doupe, D.P., Klein, A.M., Winton, D.J., Simons, B.D., and Jones, P.H. (2007) A single type of progenitor cell maintains normal epidermis. Nature *446*, 185–189.

Crittenden, S.L., Leonhard, K.A., Byrd, D.T., and Kimble, J. Cellular analyses of the mitotic region in the Caenorhabditis elegans adult germ line. (2006) Mol Biol Cell *17*, 3051-3061.

Dalerba, P., Dylla, S.J., Park, I.K., Liu, R., Wang., X, Cho, R.W., Hoey, T., Gurney, A., Huang, E.H., Simeone, D.M., Shelton, A.A., Parmiani, G., Castelli, C., and Clarke, M.F. Phenotypic characterization of human colorectal cancer stem cells. (2007) Proc Natl Acad Sci USA *104*, 10158–10163.

DeFalco, T., Le Bras, S., and Van Doren, M. Abdominal-B is essential for proper sexually dimorphic development of the Drosophila gonad. (2003) Mech Dev *121*, 1323-33.

de Rooij, D.G., and Russell, L.D. All you wanted to know about spermatogonia but were afraid to ask. (2000) J Androl 21(*6*), 776-98.

Diehn, M., Cho, R.W., Lobo, N.A., Kalisky, T., Dorie, M.J., Kulp, A.N., Qian, D., Lam, J.S., Ailles, L.E., Wong, M., Joshua, B., Kaplan, M.J., Wapnir, I., Dirbas, F.M., Somlo, G., Garberoglio, C., Paz, B., Shen, J., Lau, S.K., Quake, S.R., Brown, J.M., Weissman, I.L., and Clarke, M.F. Association of reactive oxygen species levels and radioresistance in cancer stem cells. (2009) Nature *458*, 780-3.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., Couto, A., Marra, V., Keleman, K., and Dickson, B.J. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. (2007) Nature *448*, 151-6.

Doe, C.Q., and Bowerman, B. Asymmetric cell division: fly neuroblast meets worm zygote. (2001). Curr Opin Cell Biol 13 (*1*), 68-75.

Duncan, A.W., Dorrell, C., and Grompe, M. Stem cells and liver regeneration. (2009) Gastroenterology doi:10.1053/j.gastro.2009.05.044

Eilken, H.M., Nishikawa, S., and Schroder, T. Continuous single-cell imaging of blood generation from haemogenic endohelium. (2009) Nature *457*, 896-900.

Ezhkova, E., Pasolli, H.A., Parker, J.S., Stokes, N., Su, I.H., Hannon, G., Tarakhovsky, A., and Fuchs, E. Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. (2009) Cell *136*, 1122-35.

Fichelson, P., Moch, C., Ivanovitch, K., Martin, C., Sidor, C.M., Lepesant, J.A., Bellaiche, Y., and Huynh, J.R. Live-imaging of single stem cells within their niche reveals that a U3snoRNP component segregates asymmetrically and is required for self-renewal in Drosophila. (2009) Nat Cell Biol 11(*6*), 685-693.

Flaherty, M.S., Zavadil, J., Ekas, L.A., and Bach, E.A. Genome-wide expression profiling in the Drosophila eye reveals unexpected repression of notch signaling by the JAK/STAT pathway. (2009) Dev Dyn doi 10.1002/dvdy.21989.

Fleming, H.E., Janzen, V., Lo Celso, C., Guo, J., Leahy, K.M., Kronenberg, H.M., and Scadden, D.T. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessaryto preserve self-renewal in vivo. (2008) Cell Stem Cell 2(*3*), 274-83.

Fuller, M.T., and Spradling, A.C. Male and female Drosophila germline stem cells: two versions of immortality. (2007) Science *316*, 402-4.

Giot, L., Bader, J.S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y.L., Ooi, C.E., Godwin, B., Vitols, E., Vijayadamodar, G., Pochart, P., Machineni, H., Welsh, M., Kong, Y., Zerhusen, B., Malcolm, R., Varrone, Z., Collis, A., Minto, M., Burgess, S., McDaniel, L., Stimpson, E., Spriggs, F., Williams, J., Neurath, K., Ioime, N., Agee, M., Voss, E., Furtak, K., Renzulli, R., Aanensen, N., Carrolla, S., Bickelhaupt, E., Lazovatsky, Y., DaSilva, A., Zhong, J., Stanyon, C.A., Finley, R.L., White, K.P., Braverman, M., Jarvie, T., Gold, S., Leach, M., Knight, J., Shimkets, R.A., McKenna, M.P., Chant, J., and Rothberg, J.M. A protein interaction map of Drosophila melanogaster. (2003) Science *302*, 1727-36.

Gönczy, P., Viswanathan, S., and DiNardo, S. Probing spermatogenesis in Drosophila with P-element enhancer detectors. (1992) Development *114*, 89–98.

Gönczy, P., and DiNardo, S. The germ line regulates somatic cyst cell proliferation and fate during Drosophila spermatogenesis. (1996) Development *122*, 2437–2447.

Guzman, M.L., Swiderski, C.F., Howard, D.S., Grimes, B.A., Rossi, R.M., Szilvassy, S.J., and Jordan, C.T. Preferential induction of apoptosis for primary human leukemic stem cells. (2002) Proc Natl Acad Sci USA *99*, 16220–16225.

Guzman, M.L., Li, X., Corbett, C.A., Rossi, R.M., Bushnell, T., Liesveld, J.L., Hébert, J., Young, F., and Jordan, C.T. Rapid and selective death of leukemia stem and progenitor cells induced by the compound 4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione (TDZD-8). (2007) Blood *110*, 4436–4444.

Hambardzumyan, D., Becher, O.J., Rosenblum, M.K., Pandolfi, P.P., Manova-Todorova, K., and Holland, E.C. PI3K pathway regulates survival of cancer stem cells residing in the perivascular niche following radiation in medulloblastoma in vivo. (2008) Genes Dev *22*, 436-48.

Hardy, R. W., Tokuyasu, K. T., Lindsley, D. L., and Garavito, M. The germinal proliferation center in the testis of Drosophila melanogaster. (1979) J. Ultrastruct. Res. *69*, 180–190.

Harrison, D. A., and Perrimon, N. Simple and efficient generation of marked clones in Drosophila. (1993) Curr. Biol. *3*, 424–433.

Harrison, D. A., McCoon, P. E., Binari, R., Gilman, M., and Perrimon, N. Drosophila unpaired encodes a secreted protein that activates the JAK signaling pathway. (1998) Genes Dev *12*, 3252–3263.

Hekmat-Scafe, D.S., Dang, K.N., and Tanouye, M.A. Seizure suppression by gain-of-function escargot mutations. (2005) Genetics *169*, 1477-93.

Henderson, S.T., Gao, D., Lambie, E.J., and Kimble, J. lag-2 may encode a signaling ligand for the GLP-1 and LIN-12 receptors of C. Elegans. (1994) Development *120*, 2913-24.

Ito, M., Yang, Z., Andl, T., Cui, C., Kim, N., Millar, S.E., and Cotsarelis, G. Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. (2007) Nature *447*, 316-20.

Jones, D. L., and Wagers, A. J. No place like home: anatomy and function of the stem cell niche. (2008) Nature Rev. Mol. Cell. Biol. *9*, 11–21.

Kai T., and Spradling, A. Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. Nature *428*, 564–569.

Kaplan, R.N., Riba, R.D., Zacharoulis, S., Bramley, A.H., Vincent, L., Costa, C., MacDonald, D.D., Jin, D.K., Shido, K., Kerns, S.A., Zhu, Z., Hicklin, D., Wu, Y., Port, J.L., Altorki, N., Port, E.R., Ruggero, D., Shmelkov, S.V., Jensen,

K.K., Rafii, S., and Lyden, D. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. (2005) Nature *438*, 820-7.

Karnoub, A.E., Dash, A.B., Vo, A.P., Sullivan, A., Brooks, M.W., Bell, G.W., Richardson, A.L., Polyak, K., Tubo, R., and Weinberg, R.A. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. (2007) Nature *449*, 57-63.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. SLAM family receptors distinguish hematpoietic stem and progenitor cells and reveal endothelial niches for stem cells. (2005). Cell, 121 *(7*), 1109-21.

Kiel, M.J., He, S., Ashkenazi, R., Gentry, S.N., Teta, M., Kushner, J.A., Jackson, T.L., and Morrison, S.J. Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. (2007) Nature *449*, 238–242.

Kiger, A. A., White-Cooper, H., and Fuller, M. T. Somatic support cells restrict germline stem cell self-renewal and promote differentiation. (2000) Nature *407*, 750–754.

Kiger, A.A., Jones, D.L., Schulz, C., Rogersm, M.B., and Fuller, M.T. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. (2001) Science *294*, 2542–2545.

Kimble, J.E., and White, J.G. (1981) On the control of germ cell development in Caenorhabditis elegans. Dev Biol *81*, 208-19.

King-Jones, K., Korge, G., Lehmann, M. The helix-loop-helix proteins dAP-4 and daughterless bind both in vitro and in vivo to SEBP3 sites required for transcriptional activation of the Drosophila gene Sgs-4. (1999) J Molec Biol *291*, 71-82.

Kitadate, Y., Shigenobu, S., Arita, K., and Kobayashi, S. Boss/Sev signaling from germline to soma restricts germline-stem-cell-niche formation in the anterior region of Drosophila male gonads. (2007) Dev Cell *13*, 151-9.

Koster, M.I., Kim, S., Mills, A.A., DeMayo, F.J., Roop, D.R. p63 is the molecular switch for initiation of an epithelial stratification program. (2004) Genes Dev *18*, 126-31.

Laird D. J., von Andrian, U.H., and Wagers, A.J. Stem cell trafficking in tissue development, growth, and disease. (2008) Cell *132*, 612-30.

Lapidot, T, Sirard, C, Vormoor, J, Murdoch, B, Hoang, T, Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. (1994) Nature *367*, 645–648.

Lasko, P.F., and Ashburner, M. The product of the Drosophila gene vasa is very similar to the eukaryotic initiation factor-4A. (1988) Nature *335*, 611-7.

Leatherman JL, Dinardo S. Zfh-1 controls somatic stem cell self-renewal in the Drosophila testis and nonautonomously influences germline stem cell self-renewal. (2008) Cell Stem Cell *3*, 44-54.

Le Bras, S., and Van Doren, M. Development of the male germline stem cell niche in Drosophila. (2006) Dev Biol *294*, 92–103.

Lechler, T., and Fuchs, E. Aymmetric cell divisions promote stratification and differentiation of mammalian skin. (2005) Nature *437*, 275-80.

Lee, T., and Luo, L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. (1999) Neuron *22*, 451–461.

Le Grand, F., Jones, A.E., Seale, V., Scime, A., and Rudnicki, M.A. Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. (2009) Cell Stem Cell *4*, 535-47.

Leong, K.G., Wang, B.E., Johnson, L., and Gao, W.Q. Generation of a prostate from a single adult stem cell. (2008) Nature *456*, 804-8.

Li, M.A., Alls, J.D., Avancini, R.M., Koo, K., and Godt, D. The large Maf factor Traffic Jam controls gonad morphogenesis in Drosophila. (2003) Nat Cell Biol *5*, 994–1000.

Lo Celso, C., Fleming H.E., Wu, J.W., Zhao, C.X., Miake-Lye, S., Fujisaki, J., Cote, D., Rowe, D.W., Lin, C.P., and Scadden, D.T. (2009) Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. (2009) Nature *457*, 92-6.

Martin, D., Brun, C., Remy, E., Mouren, P., Thieffry, D., and Jacq, B. GOToolBox: functional analysis of gene datasets based on Gene Ontology. (2004) *5*, R101.

Merkle, F.T., Mirzadeh, Z., and Alvarez-Buylla, A. Mosaic organization of neural stem cells in the adult brain. (2007) Science *317*, 381–384.

Micchelli, C.A., and Perrimon, N. Evidence that stem cells reside in the Drosophila midgut epithelium. Nature *439*, 465-479.

Morrison, S. J., Hemmati, H. D., Wandycz, A. M. & Weissman, I. L. The purification and characterization of fetal liver hematopoietic stem cells. (1995) Proc. Natl Acad. Sci. USA *92*, 10302–10306.

Morrison, S.J., Uchida, N., and Weissman, I.L. The biology of hematopoietic stem cells. (1995) Annu Rev Cell Dev Biol *11*, 35-71.

Morrison, S.J., and Kimble, J. Asymmetric and symmetric stem-cell divisions in development and cancer. (2006) Nature, *441* 1068-74.

Morrison, S.J., and Spradling, A.C. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. (2008) Cell *132*, 598-611.

Nakagawa, T., Nabeshima, Y., and Yoshida, S. Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. (2007) Dev. Cell *12*, 195–206.

Naveiras, O., Nardi, V., Wenzel, P.L., Hauschka, P.V., Fahey, F., and Daley, G.Q. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. (2009) Nature doi 10.1038/nature08099

Nguyen, D.X., Bos, P.D., and Massagué, J. Metastasis: from dissemintation to organ-specific colonization. (2009) Nat Rev Cancer *9*, 274-84.

Nieto, M.A. The snail superfamily of zinc-finger transcription factors. (2002) Nat Rev Mol Cell Biol *3*, 155-66.

North, T.E., Goessling, W., Peeters, M., Li, P., Ceol, C., Lord, A.M., Weber, G.J., Harris, J., Cutting, C.C., Huang, P., Dzierzak, E., and Zon, L.I. Hematopoietic stem cell development is dependent on blood flow. (2009) Cell *137*, 736-48.

Nystul, T., and Spradling, A.C. An epithelial niche in the Drosophila ovary undergoes long range stem cell replacement. (2007) Cell Stem Cell *1*, 277–285.

Ohlstein, B., and Spradling, A. Multipotent Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling. (2007) Science *315*, 988–992.

Pan, L., Chen, S., Weng, C., Call, G., Zhu, D., Tang, H., Zhang, N., and Xie, T. Stem cell aging is controlled both intrinsically and extrinsically in the Drosophila ovary. (2007) Cell Stem Cell 1*(4)*, 458-69.

Psaila, B., and Lyden, D. The metastatic niche: adapting the foreign soil. (2009) Nat Rev Cancer *9*, 285-93.

Quintana, E., Shackleton, M., Sabel, M.S., Fullen, D.R., Johnson, T.M., and Morrison S.J. Efficient tumour formation by single human melanoma cells. (2008) Nature *456*, 593-8.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. A role for Wnt signalling in self-renewal of haematopoietic stem cells. (2003) Nature *423,* 409–414.

Rhodes, K.E., Gekas, C., Wang, Y., Lux, C.T., Francis, C.S., Chan, D.N., Conway, S., Orkin, S.H., Yoder, M.C., and Mikkola, H.K. The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. (2008) Cell Stem Cell *2*, 252-63.

Richardson, G.D., Robson, C.N., Lang, S.H., Neal, D.E., Maitland, N.J., and Collins, A.T. CD133, a novel marker for human prostatic epithelial stem cells. (2004) J Cell Sci *117*, 3539–3545.

Rossi, D.J., Jamieson, C.H., and Weissman, I.L. Stem cells and the pathways to aging and cancer. (2008) Cell *132*, 681-96.

Sabatini, S., Heidstra, R., Wildwater, M., and Scheres, B. SCARECROW is involved in positioning the stem cell niche in the Arabidopsis root meristem. (2003) Genes Dev *17*, 354-8.

Sacco, A., Doyonnas, R., Kraft, P., Vitorovic, S., and Blau, H.M. Self-renewal and expansion of single transplanted muscle stem cells. (2008) Nature *456*, 502-6.

Sangiorgi, E. and Capecchi, M.R., Bmi1 is expressed in vivo in intestinal stem cells. (2008) Nature Genetics *40*, 915-920.

Sarkar, A., Parikh, N., Hearn, S.A., Fuller, M.T., Tazuke, S.I., and Schulz, C. Antagonistic roles of Rac and Rho in organizing the germ cell microenvironment. (2007) Curr Biol *17*, 1253-8.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., and Clevers, H. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. (2009) Nature *459*, 262-5.

Scadden, D.T. The stem-cell niche as an entity of action. (2006) Nature *441*, 1075-9.

Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. (1978) Blood Cells *4*, 7–25.

Schroeder, T. Imaging stem-cell-driven regeneration in mammals. (2008) Nature *453*, 345-51.

Schulz, C., Wood, C. G., Jones, D. L., Tazuke, S. I., and Fuller, M. T. Signaling from germ cells mediated by the rhomboid homolog stet organizes encapsulation by somatic support cells. (2002) Development *129*, 4523–4534.

Sen, G.L., Webster, D.E., Barragan, D.I., Chang, H.Y., and Khavari, P.A. Control of differentiation in a self-renewing mammalian tissue by the histone demethylase JMJD3. (2008) Genes Dev *22*, 1865-70.

Shackelton, M., Vaillant, F., Simpson, K.J., Stingl, J., Smyth, G.K., Asselin-Labat, M.L., Wu, L., Lindeman, G.J., Visvader, J.E. Generation of a functional mammary gland from a single stem cell. (2006) Nature *439*, 84-8.

Singh, S.K., Clarke, I.D., Terasaki, M., Bonn, V.E., Hawkins, C., Squire, J., and Dirks, P.B. Identification of a cancer stem cell in human brain tumors. (2003) Cancer Res *63*, 5821–5828.

Singh, S.R., Liu, W., and Hou, S.X. The adult Drosophila malpighian tubules are maintained by multipotent stem cells. (2007) Cell Stem Cell *2*, 191-203.

Sneddon, J.B., Zhen, H.H., Montgomery, K., van de Rijn, M., Tward, A.D., West, R., Gladstone, H., Chang, H.Y., Morganroth, G.S., Oro, A.E., and

Brown, P.O. Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation. (2006) Proc Natl Acad Sci USA *103*, 14842-7.

Song, X., and Xie, T. DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the Drosophila ovary. (2002) Proc. Natl. Acad. Sci. USA *99*, 14813–14818.

Song, X., Zhu, C.H., Doan, C., and Xie, T. Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. (2002) Science *296*, 1855–1857.

Song, X., Call, G.B., Kirilly, D., and Xie, T. Notch signaling controls germline stem cell niche formation in the Drosophila ovary. (2007) Development *134*, 1071-80.

Spana, E.P., and Doe, C.Q. Numb antagonizes Notch signaling to specify sibling neuron cell fates. (1996) Neuron *1*, 21-26.

Stanger, B.Z., Tanaka, A.J., and Melton, D.A. Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. (2007) Nature *445*, 886-91.

Stingl, J., Eirew, P., Ricketson, I., Shackelton, M., Vaillant, F., Choi, D., Li, H.I., Eaves, C.J. Purification and unique properties of mammary epithelial stem cells. (2006) Nature *439*, 993-7.

Stome S., and Wood, W.B., Generation of aymmetry and segregation of germ-line granules in early C. elegans embryos. (1983) 35(*1*), 15-25.

Streit, A., Bernasconi, L., Sergeev, P., Cruz, A., and Steinmann-Zwicky, M. mgm 1, the earliest sex-specific germline marker in Drosophila, reflects expression of the gene esg in male stem cells. (2002) Int J Dev Biol *46*, 159–166.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. (2006) Immunity *25*, 977–988.

Takashima, S., Mkrtchyan, M., Younossi-Hartenstein, A., Merriam, J.R., and Hartenstein, V., The behaviour of Drosophila adult hindgut stem cells is controlled by Wnt and Hh signalling. (2008) Nature *454,* 651-5.

Tanentzapf, G., Devenport, D., Godt, D., and Brown, N.H. Integrin-dependent anchoring of a stem-cell niche. (2007) Nat Cell Biol *9*, 1413-8.

Tegelenbosch, R.A., and de Rooij D.G. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. (1993). Mutant Res *290*, 193-200.

Terry, N.A., Tulina, N., Matunis, E., and DiNardo, S. Novel regulators revealed by profiling Drosophila testis stem cells within their niche. (2006) Dev Biol *294*, 246-57.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., Armstrong, S.A., Passegué, E., DePinho, R.A., and Gilliland, D.G. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. (2007) Cell *128*, 325-39.

Tran, J., Brenner, T. J., and DiNardo, S. Somatic control over the germline stem cell lineage during Drosophila spermatogenesis. (2000) Nature *407*, 754–757.

Truong, A.B., Kretz, M., Ridky, T.W., Kimmel, R., Khavari, P.A. p63 regulates proliferation and differentiation of developmentally mature keratinocytes. (2006) Genes Dev *20*, 3185-97.

Tulina and Matunis, 2001 N. Tulina and E. Matunis, Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling, Science 294 (2001), pp. 2546–2549.

van den Berg, C., Willemsen, V., Hendriks, G., Weisbeek, P., and Scheres, B. Short-range control of cell differentiation in the Arabidopsis root meristem. (1997) Nature *390*, 287-9.

Voog, J., D'Alterio, C., and Jones, D.L. Multipotent somatic stem cells contribute to the stem cell niche in the Drosophila testis. (2008) Nature *454*, 1132-6.

Walkley et al., 2007 C.R. Walkley, G.H. Olsen, S. Dworkin, S.A. Fabb, J. Swann, G.A. McArthur, S.V. Westmoreland, P. Chambon, D.T. Scadden and L.E. Purton, A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. Cell 129 (2007), pp. 1097–1110.

Wallenfang, M. R., Nayak, R., and DiNardo, S. Dynamics of the male germline stem cell population during aging of Drosophila melanogaster. (2006) Aging Cell *5*, 297–304.

Wang, H., Singh, S.R., Zheng, Z., Oh, S.W., Chen, X., Edwards, K., and Hou, S.X. Rap-GEF signaling controls stem cell anchoring to their niche through regulating DE-cadherin-mediated cell adhesion in the Drosophila testis. (2006) Dev Cell *10*, 117-26.

Wang, X., Harris, R.E., Bayston, L.J., and Ashe, H.L. Type IV collagens regulate BMP signalling in Drosophila. (2008) Nature *454*, 72-7.

Ward, E.J., Shcherbata, H.R., Reynolds, S.H., Fischer, K.A., Hatfield, S.D., and Ruohola-Baker, H. Stem cells signal to the niche through the Notch pathway in the Drosophila ovary. (2006) Curr Biol *16*, 2352-8.

Wawersik, M., Milutinovich, A,, Casper, A.L., Matunis, E., Williams, B., and Van Doren, M. Somatic control of germline sexual development is mediated by the JAK/STAT pathway. (2005) Nature *436*, 563-7.

Weissman, I.L., Anderson, D.J., and Gage, F. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. (2001) Annu Rev Cell Dev Biol *17*, 387-403.

Xie and Spradling, 1998 T. Xie and A.C. Spradling, decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary, Cell 94 (1998), pp. 251–260.

Xie and Spradling, 2000 T. Xie and A.C. Spradling, A niche maintaining germ line stem cells in the Drosophila ovary, Science 290 (2000), pp. 328–330.

Xie, Y., Yin, T., Wiegraebe, W., He, X.C., Miller, D., Stark, D., Perko, K., Alexander, R., Schwartz, J., Grindley, J.C., Park, J., Haug, J.S., Wunderlich, J.P., Li, H., Zhang, S., Johnson, T., Feldman, R.A., Li, L. Detection of functional haematopoietic stem cell niche using real-time imaging. (2009) Nature *457*, 97-101.

Xu, T., and Harrison, S.D. Mosaic analysis using FLP recombinase. (1994) Methods Cell Biol *44*, 655-81.

Xu, J., Hofhuis, H., Heidstra, R., Sauer, M., Friml, J., and Scheres, B. A molecular framework for plant regeneration. (2006) Science *311*, 385-8.

Yamashita, Y.M., Jones, D.L., and Fuller, M.T. Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. (2003) Science *301*, 1547–1550.

Yamashita, Y.M., Mahowald, A.P., Perlin, J.R., and Fuller, M.T. Asymmetric inheritance of mother versus daughter centrosome in stem cell division. (2007) Science *315*, 518–521.

Yoshida, S., Sukeno, M., and Nabeshima, Y. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. (2007) Science *317*, 1722-6.

Young, K.M., Fogarty, M., Kessaris, N., and Richardson, W.D. Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. (2007) J. Neurosci. *27*, 8286–8296.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y., and Li., L. Identification of the haematopoietic stem cell niche and control of the niche size. (2003) Nature *425*, 836–841.

Zhong, W., Jiang, M.M., Weinmaster, G., Jan L.Y., and Jan. Y.N. Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. (1997) Development *124*, 1887-97.

Zhou, B.P., Deng, J., Xia, W., Xu, J., Li, Y.M., Gunduz, M., and Hung, M.C. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. (2004) Nat Cell Biol *6*, 931-40.

Zeidler MP, Bach EA, Perrimon N. The roles of the Drosophila JAK/STAT pathway. (2000) Oncogene *19*, 2598-606.

Zon, L. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. (2008) Nature *453*, 306-13.