A MODEST PROPOSAL:
DIFFERENTIATING DAUGHTERS ARE SACRIFICED DURING STARVATION TO PROTECT STEM CELLS IN THE DROSOPHILA TESTIS

by

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DEDICATION

This thesis is dedicated to my best friend and girlfriend Cailin; to my incredibly talented and rambunctious sister Heidi; and most of all to my parents, Xiaogen and Yaling, for their unconditional love and support. Thank you for teaching me the value of 锲而不舍，只争朝夕.

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LIST OF ABBREVIATIONS

Bam: Bag-of-marbles

BrdU: 5-bromo-2'-deoxyuridine

CC: Cyst cell

Cnn: Centrosomin

CySC: Cyst stem cell

DAPI: 4',6-diamidino-2-phenylindole

DIAP1: Drosophila inhibitor of apoptosis protein 1

Dpp: Decapentaplegic

FasIII: Fasciclin III

GB: Gonialblast

GFP: Green fluorescent protein

GSC: Germline stem cell

Hs-bam: Heat shock-bam

InR: Insulin receptor

JAK-STAT: Janus kinase-signal transducer and activator of transcription

JNK: c-Jun N-terminal kinase

PBS: Phosphate buffered saline

PBS-T: Phosphate buffered saline-Tween

PH3: Phosphohistone H3

Puc: Puckered

RFP: Red fluorescent protein

S-phase: Synthesis-phase

SG: Spermatogonia

Tj: Traffic jam

Upd: Unpaired

YFP: Yellow fluorescent protein

Zfh-1: Zinc finger-homeodomain transcription factor 1

ABSTRACT

Tissue homeostasis, defined as the balanced state between cell production and loss, must change in response to nutrient conditions in order to optimize the efficient use of resources. While it may be advantageous for tissues to robustly generate new cells when nutrients are abundant, proliferation must be scaled down when nutrients are limited or absent. Thus, to cope with the fluctuating availability of food throughout life, tissues often shift between different states of homeostasis. How such “shifting” is accomplished is poorly explored, and little is known about how impairments in this process can irreversibly impact overall tissue organization and functionality. Considering that many human diseases, most notably those associated with aging and cancer, are characterized by dysfunctional tissue homeostasis, it is possible that defective “shifts” in tissue homeostasis might underlie these pathological conditions. An understanding of such mechanisms could thus reveal previously unexplored strategies for therapy.

Because stem cells are responsible for generating new cells within a tissue, much of the attention in understanding tissue homeostasis has been focused on the behavior of stem cells. However, stem cells are likely not the only point of regulation. In many tissues, stem cells produce differentiating daughters that undergo transit-amplifying divisions prior to terminal differentiation, which is thought to be a strategy to protect stem cells from exhaustion. Transit-amplifying cells represent a considerable fraction of cell divisions within many tissues and likely also contribute to shifting tissue homeostasis. While it has been reported that nutrients can affect the behavior of transit-

amplifying cells, it remains unclear how the response of stem cells and transit-amplifying cells are integrated in a concerted manner to allow a tissue to adapt to fluctuating nutrient conditions. The Drosophila testis, which allows unequivocal identification of these cell types, is thus an ideal model system with which to investigate the behavior of stem cells and transit-amplifying cells in response to starvation.

In this thesis, I describe work showing that during protein starvation the Drosophila testis does not drastically alter the behavior of stem cells. Whereas previous studies suggest that both GSC number and cell cycle decrease during protein starvation, my analysis of intermediate time points, multiple cell cycle assays, and comparison with age-matched controls reveals that GSC number is maintained during prolonged starvation, albeit at a slightly reduced number, and that rate of proliferation is unaffected. These results imply that the reduction in germ cell production during starvation in the Drosophila testis is regulated beyond the stem cell compartment. Analysis of the differentiating cells revealed that the reduction in the overall production of germ cells is achieved by the elimination of transit-amplifying SGs, which is triggered by the apoptosis of somatic CCs. I further show that the regulated elimination of SGs is vital to ensuring GSC maintenance during starvation. Inhibition of SG death during protein starvation leads to GSC dysfunction and a collapse in tissue homeostasis, leading to a failure in recovery upon reintroduction of nutrients to the system. Additional observations suggest that the contents of the dying SGs are absorbed by neighboring cells, suggesting that the elimination of SGs not only alleviates the tissue's demand for resources, but also locally recycles nutrients during starvation. Taken together, this work is significant because it introduces the idea that a coordinated response among

multiple cell types within a tissue is essential for successfully shifting tissue homeostasis in response to changes in nutrient availability.

CHAPTER 1:
INTRODUCTION

Many tissues are comprised of a heterogeneous population of cells, each with a different role, fate, and lifespan. To maintain tissue function and organization throughout the life of an organism, the number and activity of these cells must be regulated in a process known as tissue homeostasis, which can be broadly defined as the balance between cell production and loss within each tissue (Guillot and Lecuit, 2013). Because of the numerous interactions among different cell types within the tissue and with the environment, tissue homeostasis is a complex and dynamic process, and we are only just beginning to understand the mechanisms that underlie it.

Studying the response of tissues to changing nutrient availability is an effective method to investigate several fundamental aspects of tissue homeostasis. The rationale is simple: in order to maximize the efficient use of resources and optimize tissue function, the rate of cell turnover within each tissue must be adjusted in response to nutrient conditions (Mihaylova et al., 2014). Whereas new cells can be rapidly generated when nutrients are available, such activity must be scaled down when nutrients are scarce (Fielenbach and Antebi, 2008; Lopes et al., 2004; Tatar and Yin, 2001). The adaptive response of tissues and their cells to nutrient changes is widely conserved, and therefore it presents a powerful experimental paradigm. Moreover, the principle of reversibility gives an additional layer of significance to the nutrient response: to cope with frequent and unpredictable fluctuations in diet, tissues must adapt in a

reversible manner (Angelo and Van Gilst, 2009; McLeod et al., 2010), and parameters such as the ratio of cell populations, tissue organization, and signaling environments must be preserved. The mechanisms that govern the tissue response while integrating such considerations therefore present an intriguing field of inquiry.

1.1 Overview of tissue homeostasis

1.1.1 Tissue homeostasis in human biology

Tissue homeostasis is vital to life; loss of homeostasis can result in a variety of pathologies that decrease tissue function and fitness of an organism. In human health, defects in tissue homeostasis are thought to contribute to deleterious processes resulting in tumorigenesis and aging (Morrison and Kimble, 2006). Understanding how homeostasis is regulated is thus a fundamental step towards understanding the defects that result in the loss of homeostasis.

In many tissues, cells have a finite lifespan before they are lost due to damage, programmed death, or consumption. To maintain constant function and integrity, tissues keep up with such loss by constantly producing cells. In mammalian skin, for example, epithelial cells divide frequently in order to sustain a tissue where cells have an average lifespan of several weeks (Blanpain and Fuchs, 2009). Turnover is more rapid in the intestinal mucosa, where cells are lost on average every 3-4 days (De Mey and Freund, 2013). Cell lifespan in the hematopoietic system can vary depending on type and function, ranging from approximately 120 days for erythrocytes, which accumulate membrane damage and are cleared from the blood stream (Ajmani and Rifkind, 1998), to less than a week for some leukocytes, whose survival in the circulation can fluctuate

depending on the presence of infection. In the case of the germline, cells are created for the sole purpose of being consumed for reproduction. In this case, the constant production of gametes is not critical for host survival but is nevertheless important for maximizing reproductive advantage.

In addition to producing new cells to replace those that are lost, tissues must also account for changing functional demands. To better protect underlying structures in response to abrasive physical stimuli, for example, the epidermis may generate more cells to increase its thickness (Thomas et al., 1985). Female mammary glands undergo dramatic expansion during pregnancy to prepare for lactation (Brisken and O'Malley, 2010), and the testis begins spermatogenesis during puberty and sexual maturation (Haase, 2013). In the hematopoietic system, erythrocyte production can be increased under hypoxic or anemic conditions in order to facilitate oxygen transport (Haase, 2013), and immune cells can rapidly expand in the presence of infection (George, 2012). In each of these cases, shifting the balance towards cell production must be carefully controlled so as to meet tissue needs but without leading to pathologic overproliferation. By extension, when tissue demands decrease, cell production must be accordingly downregulated.

Investigating the regulation of tissue homeostasis has thus focused primarily on how tissues control the rate at which new cells are generated. Whereas failure to produce enough cells to offset cell loss and accommodate functional needs can result in insufficiency, cell production must also be limited to prevent tumorigenesis and malignancy. Many pathways have been discovered that regulate the rate of cell production, both at a constitutive level as well as in response to external or

environmental stimuli. Unsurprisingly, defects in these pathways can lead to a loss of tissue homeostasis, and such defects often underlie many pathological conditions.

Though such investigation has provided valuable insight towards understanding tissue homeostasis, a significant gap in understanding arises regarding the responsibility of different cell types in the process of cell production. In many tissues, only a fraction of cells are mitotically active, but many other cells have the ability to influence this activity. Furthermore, within the mitotically active cell types there often exists a hierarchy in the control of cell division. Much recent work has thus been focused on elucidating the differential roles of these mitotically active cells as well as the surrounding environment that supports and influences their function.

1.1.2 Stem cells lie at the heart of tissue homeostasis

Research over the last few decades has elucidated the fundamental importance of tissue stem cells in tissue homeostasis. Stem cells reside in specialized microenvironments within tissues and often divide asymmetrically to simultaneously self-renew and produce daughter cells that differentiate according to tissue needs (Morrison and Spradling, 2008). In many mammalian stem cell systems most notably in mouse intestinal stem cells (Snippert et al., 2010) and in spermatogonial stem cells (Yoshida et al., 2007), it has been clearly shown that stem cells likely undergo stochastic decisions of division, either symmetrically or asymmetrically, to maintain the stem cell pool as a population. Stem cell divisions can thus be considered the first step in the regulation of tissue homeostasis, as influencing the rate and fidelity of stem cell divisions can have dramatically amplified repercussions.

Asymmetric stem cell division is a simplest strategy to maintain the stem cell number as it can be achieved at single cell level, and in many cases it can be considered the most critical point of regulation (Morrison and Kimble, 2006). Stem cell function can be influenced by modulating the fidelity of asymmetry; increasing the frequency of symmetric divisions can lead to either a depletion of the stem cell pool through symmetric differentiation or an overabundance of stem cells at the expense of producing differentiating cells. Left unchecked, failure to divide asymmetrically eventually leads to a loss of tissue homeostasis. A rapidly growing body of evidence shows that asymmetry can be specified intrinsically or extrinsically, and it can be altered by many factors (Yamashita et al., 2010). Intrinsically, the preferential inheritance of old and new cellular material such as DNA (Yadlapalli and Yamashita, 2013), histones (Tran et al., 2013), polarity and adhesion proteins (Peng and Axelrod, 2012), and organelles (Pelletier and Yamashita, 2012) between the asymmetric daughter cells (stem vs differentiating) has been found to correlate with fate determination. Extrinsically, the plane of stem cell division, which is often specified by a physical attachment or morphogen gradient, can produce positional or environmental asymmetry between the two daughter cells after division (Byrd and Kimble, 2009; Yadlapalli and Yamashita, 2012). Recent investigation of asymmetric stem cell divisions in the Drosophila germline suggest that the determination of intrinsic and extrinsic asymmetry can be interdependent, as perturbations in one aspect can often lead to perturbations in the other (Yadlapalli and Yamashita, 2013).

The rate of stem cell division is another parameter that is essential to tissue homeostasis and thus tightly controlled. For optimal function, each stem cell division

must take into consideration the needs of the tissue as well as the availability of resources. To balance these considerations, many signaling pathways integrate the rate of stem cell division with external inputs. Cytokines and mitogens can be secreted by the tissue to stimulate or suppress stem cell divisions according to tissue needs, and stem cells also possess autonomous mechanisms that can interpret direct environmental cues to influence the cell cycle. Inhibitory mechanisms exist as well to prevent stem cells from aberrant activity such as overproliferation that can predispose a tissue to malignancies.

Additionally, stem cells must also take into account the intrinsic cost of division to the stem cell's integrity, which leads to the increased likelihood of damage and dysfunction over time (Lopez-Otin et al., 2013). The observation that stem cell function gradually declines with age implies that stem cells have a finite lifespan despite their capacity for self-renewal, and it has been proposed that the upper limit is closely related to the number of times a stem cell can replicate its genome (Johnson et al., 1998). However, an explanation for why such a limit exists remains controversial beyond the idea that oxidative stress and DNA damage tend to accumulate during stem cell aging and certain parts of the genome are difficult to repeatedly replicate. Regardless, it is generally agreed upon that the preservation of stem cell function and integrity over many divisions is important for the maintenance of tissue homeostasis (Signer and Morrison, 2013).

1.1.3 Stem cells produce transit-amplifying daughters

It has been proposed that one strategy for stem cells to be protected from

exhaustion is to produce transit-amplifying daughters -- progenitor cells that are ultimately fated for differentiation but continue to mitotically expand prior to terminal differentiation. Found in many tissues -- epithelial, mesenchymal, hematopoietic (Fig. 1.1), and germline, to name a few -- these cells can exponentially increase the number of differentiating stem cell progeny without necessarily increasing the number of stem cell divisions (Diaz-Flores et al., 2006). Therefore, it is thought that this process allows stem cells to remain relatively dormant while their short-lived intermediate progeny adopt the burden of producing the majority of cells for tissue needs.

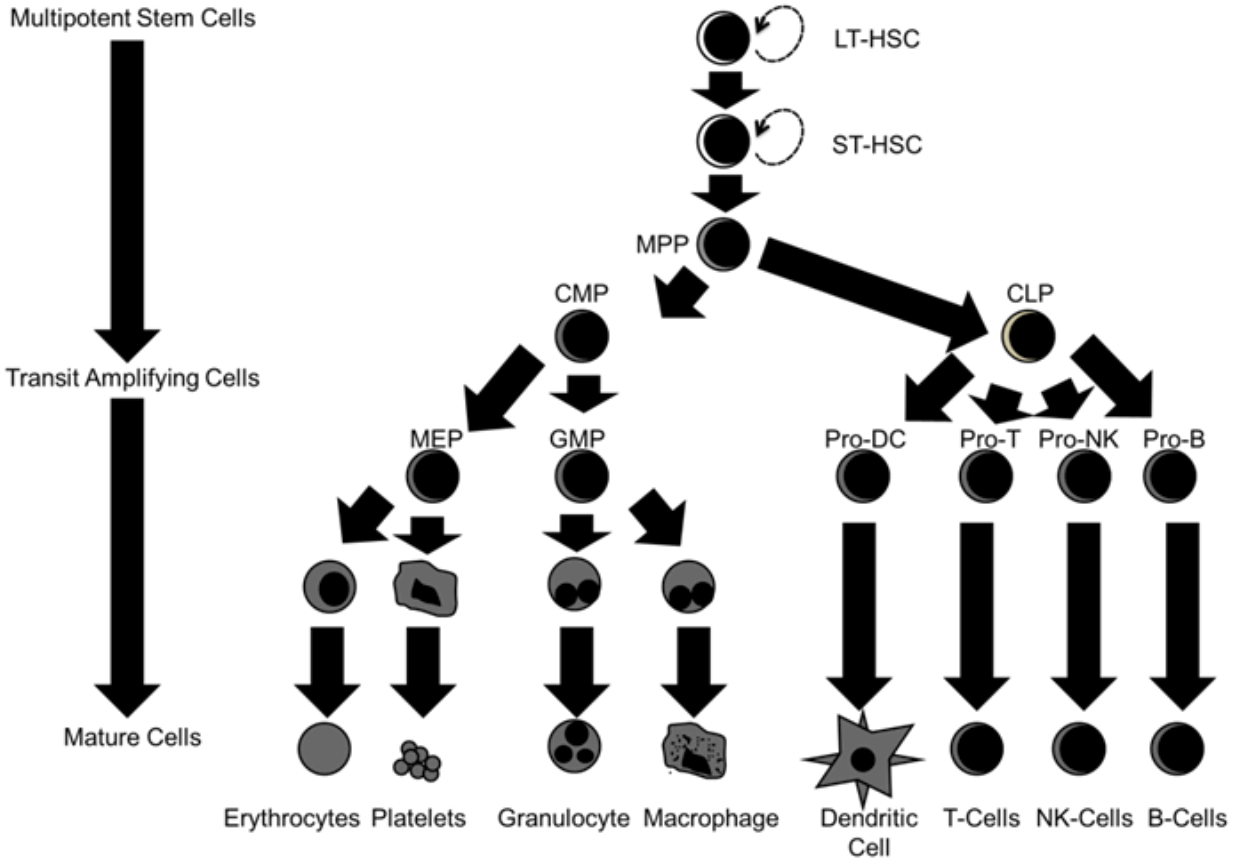

Figure 1.1 Stem cells and transit-amplifying cells in the hematopoietic lineage (Rae and Trobridge, 2013). Multipotent long-term hematopoietic stem cells (LT-HSC) produce short term hematopoietic stem cells, which give rise to committed progenitors that undergo transit-amplification to produce the differentiated cells in the hematopoietic lineage.

Depending on lineage, transit-amplifying cells can be unipotent (germline) or multipotent (hematopoietic), and the ratio of the transit-amplifying population to stem cell population can vary depending on the nature of the tissue. In the mammalian hematopoietic system, for example, it is estimated that HSCs comprise less than 0.01% of the mitotic cells within the bone marrow (Clevers, 2013; Kiel et al., 2005). In intestinal crypts, the ratio is presumed to be much higher, with estimates ranging form 4-16 stem cells that produce 30-40 transit-amplifying cells (Clevers, 2013). In the Drosophila germline, there can be up to 31 transit-amplifying cells per stem cell at any given point (1+2+4+8+16), assuming constant cell cycle rates.

In tissues with large populations of highly active transit-amplifying cells compared to stem cells, one important yet underappreciated consideration is that control of cell production likely also involves regulation of transit-amplifying cell behavior. In other words, if tissue homeostasis were regulated only at the level of the stem cells, it would place a disproportionate amount of importance on a small fraction of cell divisions while ignoring a much larger fraction of cell production in the tissue that occurs downstream of stem cells. It is reasonable to hypothesize that the mechanisms governing transit-amplifying cell behaviors such as rate of division, development, survival, and timing of differentiation can be altered in order to efficiently and rapidly regulate tissue homeostasis in response to tissue needs. Whereas changing the outcome of one stem cell division would have only distant tissue consequence, transit-amplifying cells are developmentally much closer to fully functional differentiated cells, and thus alterations in their behavior would have more immediate effects.

Another corollary regarding the regulation of stem cells vs. the regulation of

transit-amplifying cells that has not been adequately considered is the relative advantage or disadvantage associated with each process. The basis for this idea is that in order to alter the behavior of any cell, some form of perturbation to its biology must occur. These perturbations, regardless of modality, increase the probability that a defect may result, and they often also possess the likelihood of being inherently defective. For instance, accelerating DNA replication to facilitate a faster cell cycle can result in a higher frequency of replication errors (Yue et al., 2003), and increasing oxidative processes to meet metabolic demands can result in the more rapid accumulation of damaged proteins (Venkataraman et al., 2013). Additionally, pre-existing mutations in genes that are not normally used -- such as those involved in signal transduction for the stress response -- may be exposed, particularly when cells receive input from multiple signaling pathways, each of which can require dozens if not hundreds of genes. With regard to stem cells and transit-amplifying cells, the case can thus be made that it may be more advantageous to alter transit-amplifying cell activity than it would be to perturb stem cells due to the idea that irreversible errors that occur in transit-amplifying cells are rarely permanent.

Many currently established behaviors in stem and transit-amplifying populations can be interpreted to indirectly support this idea, but direct evidence has yet to be provided. One limitation to testing such a concept is that stem cells cannot be easily distinguished from transit-amplifying cells in many model systems, thus making the existence of such differential regulation difficult to determine. Another limitation is the relative lack of common stimuli or stressors that can be used experimentally to challenge tissue homeostasis in different tissues and organisms through a conserved,

physiologically relevant manner. For these reasons, investigation of the nutrient response in the Drosophila testis is currently the most definitive paradigm with which to study the behavior of stem cells and transit-amplifying cells in tissue homeostasis.

1.2 Tissues adapt to changing nutrient conditions

Throughout evolution, the fluctuating and unpredictable availability of nutrients has been a universal challenge. All organisms, from prokaryotes to humans, must be able to tolerate periods of starvation in order to survive; as such, many strategies used among disparate organisms have been found to be surprisingly conserved. In the tissues of complex, multicellular organisms, nutrient stress presents a multi-faceted challenge as all components must adapt in concert in order to maximize survival.

1.2.1 Starvation leads to tissue involution

Tissue homeostasis is dynamically regulated according to nutrient conditions in order to balance tissue function with the efficient use of resources. The delicate balance between cell production and cell loss must often shift to a new equilibrium in order to optimally adapt to such limitations. In many tissues, a phenomenon we define as involution, characterized by a reduction in total cell number and decreased flux, can occur in response to starvation in order to conserve resources (Shaw et al., 2012). Starvation-induced involution results in a temporary sacrifice of maximal tissue function in favor of prolonged endurance so that the organism can increase its chances of "waiting it out" until the next reappearance of food.

Involution is likely to have evolved as an active adaptation rather than a passive one, as it must be carefully regulated such that tissue function is not compromised upon the reintroduction of nutrients. The reduction in cell number must occur in a manner that preserves the appropriate ratios of the different cell types that comprise a tissue, and it must furthermore be accomplished in a way that preserves the organized architecture

between the different cell types. It can therefore be postulated that starvation-induced involution is efficient, coordinated, and reversible in order to maximize survival of the organism both in the short and long term.

Signaling pathways such as those involving insulin and TOR help coordinate the activities of the heterogeneous cell populations within each tissue in response to nutrients (Guo, 2014; Jewell and Guan, 2013). Depending on the tissue and cell type, these pathways can exert a variety of effects on cell behavior and can modulate parameters such as proliferation, differentiation, and survival. In adipose tissue, for example, the upregulation of insulin signaling when nutrients are abundant can drive stem cell divisions and stimulate adipocyte differentiation (Wabitsch et al., 1995). In intestinal crypts, calorie restriction acts through mTORC1 in the stem cell niche to promote stem cell self-renewal at the expense of producing differentiating cells (Yilmaz et al., 2012). Many of the disparate effects of nutrient signaling on the heterogeneous cell types within a tissue have been documented, but the importance of coupling these outcomes, particularly in the broader context of tissue homeostasis as a whole, has not been adequately explored.

1.2.2 Nutrient control of stem cells and transit-amplifying cells

The nutrient control of stem cells and transit-amplifying cells is an important aspect of tissue homeostasis. Because of their disparate signaling environments and roles within the tissue, stem cells and transit-amplifying cells may respond differently to nutrient changes (Shim et al., 2013). How such alterations in behavior are coordinated and coupled in order to allow tissue homeostasis to adapt is thus an intriguing question.

The nutrient response is known to be primarily mediated by the insulin pathway

and the target of rapamycin (TOR) (Ables et al., 2012; Kim et al., 2013). The actions of such signaling pathways is highly evolutionarily conserved, and they have been particularly well studied in the tissues of Drosophila (Tatar et al., 2014). In the Drosophila male and female germline, protein restriction leads to decreased insulin signaling, which reduces cell cycle progression (Drummond-Barbosa and Spradling, 2001; McLeod et al., 2010). In Drosophila lymphoid tissues, starvation decreases hematopoietic stem cell maintenance and promotes differentiation (Benmimoun et al., 2012; Dragojlovic-Munther and Martinez-Agosto, 2012). Drosophila neuroblasts can become quiescent or proliferative in response to dietary amino acid content and depends on the activation of PI3Kinase, a downstream effector of the insulin receptor (Chell and Brand, 2010). In the fly intestine, starvation is thought to reversibly decrease stem cell number as well as proliferation (McLeod et al., 2010).

The effect of starvation on differentiating and transit-amplifying populations has not received prominent attention in such studies. Observations regarding such effects are often tangential and perfunctory; moreover, the biological significance of their role in shifting tissue homeostasis has not been tested. The differential response between stem and transit-amplifying cells and the relationship between them thus remains an intriguing mystery. In the mammalian intestinal crypts, for instance, transit-amplifying cells were noted to have decreased cell cycle progression in response to calorie restriction while stem cell self-renewal is increased (Yilmaz et al., 2012). This prompts further exploration into whether these processes are interdependent. In the Drosophila ovaries, amino acid starvation causes transit-amplifying cells to undergo programmed cell death and become engulfed (McPhee and Baehrecke, 2010), which contributes

significantly to the overall decrease in germ cell production, while stem cells persist (Drummond-Barbosa and Spradling, 2001). Could there be a feedback mechanism allowing the engulfment of transit-amplifying cells to promote stem cell function?

Many manipulations have been performed to understand the biological significance of the stem cell nutrient response, but little investigation has been made to determine that of the transit-amplifying cells. What are the consequences to the tissue if their transit-amplifying populations fail to respond to nutrient signaling in an appropriate manner? Is it possible that the transit-amplifying cells' response is closely coupled to that of the stem cells'? Performing such studies to investigate the behavior of both populations in response to nutrients and shifting tissue homeostasis would thus address these questions as well as raise many more additional exciting questions.

1.3 The Drosophila testis as model system for tissue homeostasis

The Drosophila testis is an ideal model system for the study of tissue homeostasis because it is one of few tissues in which stem cells and differentiating cells can be distinguished and studied at the single-cell resolution. Spermatogenesis begins at the apical tip and proceeds spatiotemporally throughout the length of the tissue. This allows convenient identification of all cell types within the tissue at various developmental stages. The stem cell niche is anchored at the apical tip by a somatic structure known as the hub, and stem cells are defined by their attachment to the hub. Many factors that regulate stem cell activity as well as the development of differentiating cells have been identified, providing tools with which to conduct precise genetic manipulations. Furthermore, the robust germ cell production in the testis is highly sensitive to the availability of amino acids. Protein starvation has a pronounced effect on testis morphology and cell behavior, and it has thus been a highly efficient tool with which to study tissue homeostasis in response to changing nutrient conditions.

1.3.1 The Drosophila testis stem cell niche

Each Drosophila testis contains approximately 8 germline stem cells (GSCs) that divide asymmetrically to self-renew and produce germ cells that begin the process of transit amplification (Davies and Fuller, 2008). These GSCs are located in their niche environment at the apical tip, where they are attached to a cluster of 10-12 somatic hub cells (Fig. 1.2). The adhesion and interaction between the GSCs and the hub cells is of vital importance for GSC maintenance. Hub cells produce factors important for stem cell maintenance such as the BMP ligand Dpp and the JAK-STAT ligand Upd. Additionally,

the hub-GSC contact is mediated by adhesion molecules such as E-cadherin that specify GSC polarity (Inaba et al., 2010; Song et al., 2002; Voog et al., 2008). When GSCs divide, the axis of division is always orthogonal to the plane of the hub, and this is accomplished by the interphase positioning of centrosomes (Yamashita et al., 2003). The outcome of division is that one daughter cell remains attached to the hub, thus continuing to receive stem cell-specifying signals, while the other is displaced and begins to differentiate. GSCs have been found to have built-in quality control mechanisms such as the centrosome orientation checkpoint and spindle orientation checkpoint to maintain the polarity of division with respect to the hub (Cheng et al., 2008; Inaba et al., 2010). The relationship between GSC and hub thus forms the basis for defining GSCs, and with immunoflourescent visualization techniques, it allows for unambiguous identification of GSCs as well as the ability to distinguish between GSCs and differentiating cells. The fact that GSCs remain attached to the hub after multiple divisions is also a useful principle for the investigation of GSC aging.

In addition to GSCs, cyst stem cells (CySCs) are another stem cell population that resides at the hub (Leatherman and Dinardo, 2008, 2010). Thought to be the Drosophila analogue to Sertoli cells in the mammalian testis, CySCs are interspersed between the GSCs and have cytoplasmic extensions that contact the hub and envelop germ cells. CySCs receive stem cell signals from the hub as well to specify their identity, but they additionally contribute to the maintenance of GSCs and comprise part of the GSC niche (Flaherty et al., 2010; Leatherman and Dinardo, 2008, 2010; Sinden et al., 2012). Furthermore, CySCs are also responsible for generating cyst cells (CCs), which encapsulate the transit-amplifying spermatogonia (SGs) produced by GSCs and

support their development (Lim and Fuller, 2012; Schulz et al., 2002). The exact number of CySCs surrounding the hub is currently controversial, with estimates ranging from 14 to 30 per testis. Due to their irregular morphology, identifying CySCs by their contact with the hub is less straightforward than identifying GSCs, and thus nuclear markers, such as the transcription factor Zfh1, must be used (Leatherman and Dinardo, 2008). Because downregulation of Zfh1 does not occur immediately following CySC divisions, it is occasionally present in newly produced CCs as well, thus creating ambiguity. Determining the stoichiometry between GSCs and CySCs remains critical for understanding the nature of the dual stem cell niche, and with respect to tissue homeostasis regulation, the behavior of CySCs is an important consideration in order to accurately interpret the behavior of GSCs.

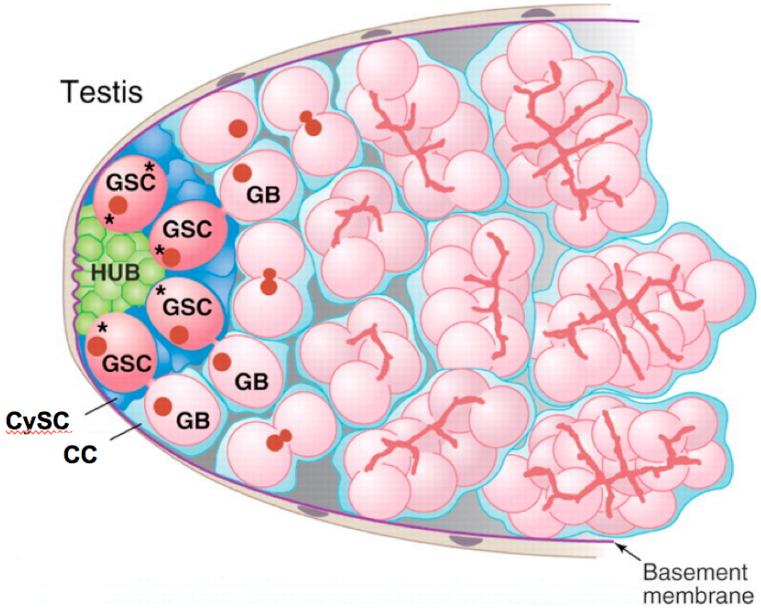

Figure 1.2 The Drosophila testis stem cell niche (Fuller and Spradling, 2007). Germline stem cells (GSCs, dark pink) are attached to the hub (green). Cyst stem cells (CySCs, blue) are also attached to the hub and intercalate between the GSCs. Upon division, GSCs produce gonialblasts (GB, pink), which undergo transit-amplification as 2-, 4-, 8-, and 16-cell SGs connected by a fusome (red). SGs are encapsulated by differentiated cyst cells (CCs, cyan), which are produced by the CySCs.

1.3.2 The development of transit-amplifying spermatogonia (SGs)

Another advantage the Drosophila testis provides as a model system for tissue homeostasis is that transit-amplifying cells can be clearly identified at each stage of development. After a GSC divides asymmetrically, the daughter cell that is displaced from the hub and fated for differentiation is called a gonialblast (GB) or 1-cell spermatogonium (SG) (Davies and Fuller, 2008). Each GB subsequently undergoes four rounds of synchronous mitotic divisions with incomplete cytokinesis to become interconnected 2-cell, 4-cell, 8-cell, and 16-cell SGs (Fig. 1.2). These cells are connected to each other by a reticular structure called the branching fusome, which can be easily visualized by immunofluorescent staining against any of its cytoskeletal components such as adducin and spectrins (de Cuevas and Matunis, 2011; Gonczy et al., 1997). Importantly, fusome morphology allows for the identification of each SG stage within the testis. Upon reaching the 16-cell stage, SGs undergo a pre-meiotic S-phase and become primary spermatocytes. Each cell increases its cytoplasmic volume by approximately 25-fold in preparation for meiosis and spermiogenesis.

The timing of transit-amplification is thought to be governed autonomously by the differentiation factor Bag of marbles (Bam) (Gonczy et al., 1997). Bam is first expressed around the 4-cell stage, and its accumulation over the next two mitotic divisions is believed to ultimately initiate the SG to SC transition. SG development throughout the transit-amplification process also requires the contribution of the encapsulating CCs. After CySC divide, the differentiating CCs begin to ensheathe the SGs (Shields et al., 2014), after which the SGs are completely enveloped within the CCs for the duration of germ cell development until the later stages of sperm individualization. CCs are critical

for successful SG maturation: a lack of CCs results in a failure of SGs to develop beyond the 4-cell stage, and ablation of CCs after encapsulation results in SG death (Lim and Fuller, 2012). Although the specific role of CCs in promoting SG development remains unclear -- whether it is ligand secretion, nutrient provision, or simply environmental insulation -- these observations nevertheless imply the possibility that certain aspects of SG behavior can be regulated by the soma.

The Drosophila testis responds reversibly to amino acid levels

The constant germ cell production in the Drosophila testis is highly sensitive to the availability of dietary amino acids. During protein starvation, spermatogenesis is scaled down, and the tissue as a whole undergoes dramatic involution (McLeod et al., 2010). Importantly, the response is reversible: within days of refeeding, the Drosophila testis can resume a high level of germ cell production and restore its morphology (Fig. 1.3). The response of the Drosophila testis to dietary protein thus makes a simple and powerful tool for investigating the nutrient response of different cell types within the tissue.

Our understanding of how the Drosophila testis involutes during protein starvation is currently incomplete. Previous work suggests that GSCs have a slowed rate of proliferation in response to decreased amino acid availability. Flies that are fed restricted levels of dietary protein have GSCs with highly misoriented centrosomes, which mediates a delay in the cell cycle via the centrosome orientation checkpoint (Roth et al., 2012). This suggests that reduced GSC activity could at least partially account for the downregulation of germ cell production. Importantly, in the case of protein

restriction, the maintenance of GSC number is not affected. Experiments using protein starvation further support a stem cell-centric explanation, showing that GSCs are lost during prolonged protein starvation. In this condition, a reduction in GSC number from 8 to 6 was reported after 15 days of protein starvation, and a reduction in cell cycle is thought to occur as well. However, neither the response of transit-amplifying SGs and somatic cells in response to starvation nor their contribution to tissue involution and recovery has been investigated in either protein restricted or protein starvation condition. This gap in knowledge thus prompts further investigation in order to better understand the regulation of tissue homeostasis in response to changing nutrient conditions.

Figure 1.3 The Drosophila testis responds reversibly to protein starvation (McLeod et al., 2010). Representative images of testes from (A) fed wild type fly after 20 days, (B) wild type fly starved for 20 days (C) starved 15 days (D) starved 15 days, refed for 5 days. Germ cells are stained with vasa (green), and the hub is stained with fasIII (red). Scale bars represent 20 μm.

CHAPTER 2:
DIFFERENTIATING DAUGHTERS ARE SACRIFICED DURING STARVATION TO PROTECT STEM CELLS IN THE DROSOPHILA TESTIS

This chapter presents material that has been submitted for publication as:

Yang H, Yamashita YM. (2014) The regulated elimination of transit-amplifying cells protects stem cells during starvation in the Drosophila testis.

2.1 Summary

How tissues adapt to varying nutrient conditions is fundamental to understanding how robust tissue homeostasis is maintained throughout an organism's lifespan, but the underlying mechanisms are not well characterized. Here we show that Drosophila testis responds to protein starvation by eliminating transit-amplifying spermatogonia (SGs) while maintaining a reduced pool of actively proliferating germline stem cells (GSCs). During protein starvation, SGs die in a manner that is mediated by the apoptosis of somatic cyst cells (CCs) that encapsulate them and regulate their development. Strikingly, GSCs cannot be maintained during protein starvation when CC-mediated SG death is inhibited, leading to an irreversible collapse of tissue homeostasis. SG death is associated with phagocytic processes that may directly promote GSC function via local nutrient recycling. We propose that the regulated elimination of transit-amplifying cells is essential to preserve stem cell function and tissue homeostasis during protein

starvation.

2.2 Introduction

Tissue homeostasis, defined as the balanced state between cell production and loss, must change in response to nutrient conditions in order to optimize the efficient use of resources (Fielenbach and Antebi, 2008; Lopes et al., 2004; Tatar and Yin, 2001). While it may be advantageous for tissues to robustly generate new cells when nutrients are abundant, proliferation must be scaled down when nutrients are limited or absent (Angelo and Van Gilst, 2009; Padilla and Ladage, 2012). Thus, to cope with the fluctuating availability of food throughout life, tissues often shift between different states of homeostasis. How such “shifting” is accomplished is poorly explored, and little is known about how impairments in this process can irreversibly impact overall tissue organization and functionality.

The Drosophila testis is an ideal model system with which to investigate the behavior of stem cells and transit-amplifying cells. The system offers unequivocal identification of these cell types at the single cell resolution, allowing detailed examination of their behavior during different states of tissue homeostasis. In Drosophila male and female germlines, gametogenesis is highly sensitive to the availability of dietary amino acids (Drummond-Barbosa and Spradling, 2001; McLeod et al., 2010; Roth et al., 2012; Wang et al., 2011). In the testis, germ cell production scales down during protein starvation, and the reduction of the germline is reflected by dramatic involution of the tissue. Importantly, the testis can efficiently recover and increase germ cell output when protein is reintroduced into the diet (McLeod et al.,

2010). This system provides a simple yet powerful tool with which to investigate how tissue homeostasis shifts in response to changes in nutrient availability.

2.3 Results

2.3.1 Stem cells are maintained at steady state during prolonged protein starvation.

It was previously reported that in wild type Drosophila testes, average germline stem cell (GSC) number decreases from approximately 8 to 6 per testis after 15 days of protein starvation (McLeod et al., 2010), which we confirmed using similar protein starvation conditions (Fig. 2.1A, see Experimental Procedures). Interestingly, we found that stem cell loss does not proceed linearly. GSC number decreases between day 3 and day 6 of protein starvation (Fig. 2.1A-B), but no further decrease is observed for at least 12 additional days. The fact that approximately 6 GSCs are maintained during prolonged starvation prompted us to investigate the manner in which GSCs are maintained.

To determine whether the 6 remaining GSCs become quiescent or have altered proliferation, we measured the kinetics of BrdU incorporation in GSCs of flies that had been starved for 2, 9, and 18 days. After 48 hours (equivalent to ~3 normal cell cycles) of continuous BrdU feeding, we expected three major possible outcomes: If all GSCs in the starved condition are proliferating similar to those in the age-matched fed controls (Fig. 2.1C, scenario 1), 100% of GSCs will become BrdU+ at the same rate as in fed condition. If all GSCs are proliferating but at a slower rate during starvation, 100% of GSCs will still become BrdU+ but at a later time point than those in fed conditions (Fig.

2.1C, scenario 2). If a subset of GSCs becomes quiescent, the percent of GSCs to become BrdU+ will plateau at less than 100% (Fig 2.1C, scenario 3). The results shown in Fig. 2.1D reveal that all GSCs in flies starved for 9 days and 18 days proliferate at the same rate as those in fed conditions (Fig. 2.1D), suggesting that the cell cycle length of GSCs is unaffected during prolonged protein starvation and no GSCs become quiescent.

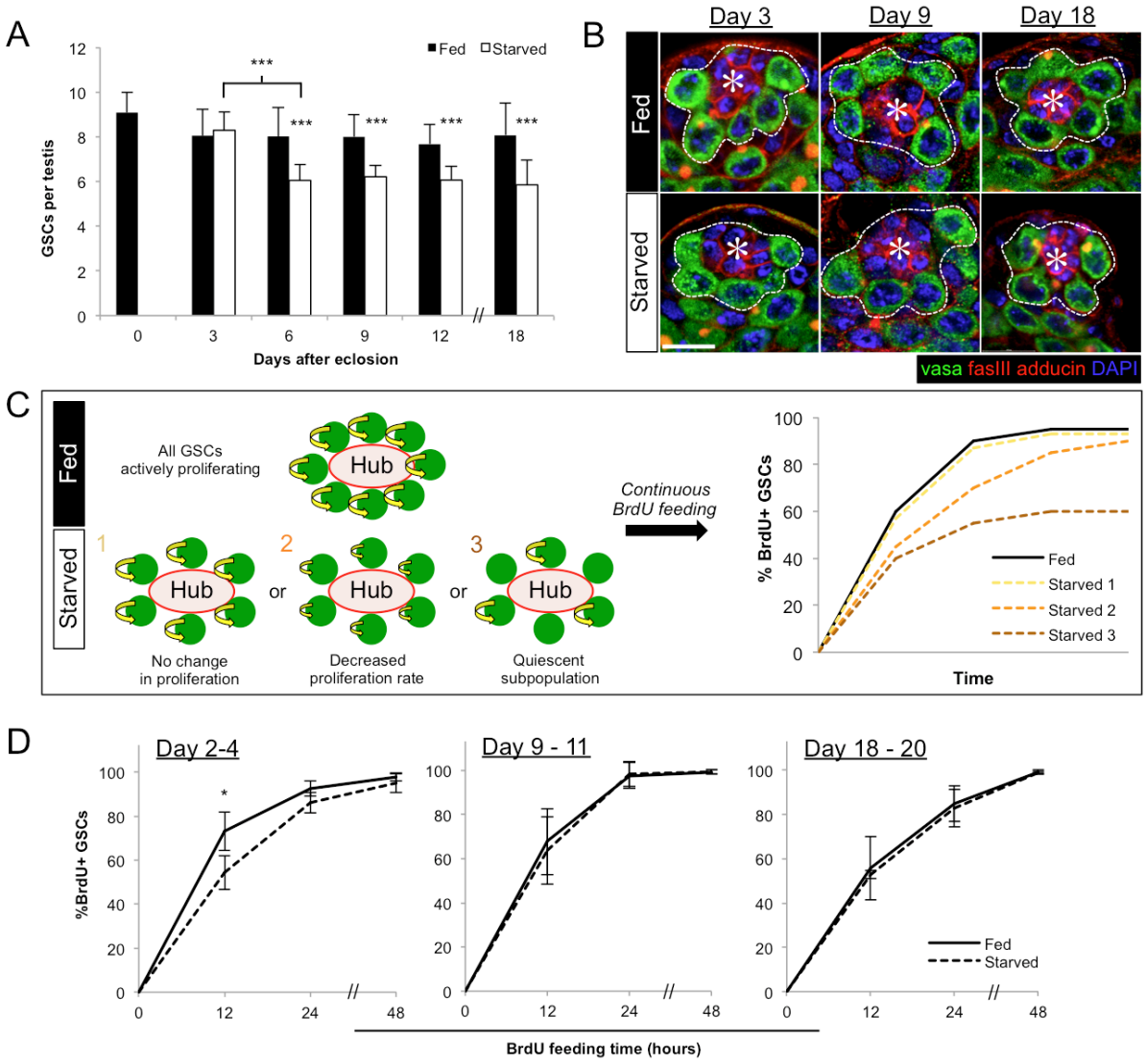

Figure 2.1 GSCs are maintained during starvation and continue to proliferate. Wild type (yw) flies that were allowed to develop to adulthood in a rich protein source were transferred upon eclosion into protein starvation conditions. (A) Average GSC number

per testis over 18 days. Data presented as mean±s.d. N > 30 testes per data point. *** denotes p < 0.0005 (student t-test). (B) Representative images of the hub (asterisk) and attached GSCs. Some GSCs are not visible due to focal plane limitations. (C) Continuous feeding of BrdU during starvation tests three scenarios of GSC behavior during starvation: 1. No change in proliferation, reflected by an equal rate of BrdU incorporation; 2. Decreased overall proliferation, reflected by reduced BrdU incorporation kinetics; 3. A quiescent subpopulation, in which some GSCs will never become positive for BrdU. (D) Kinetics of BrdU incorporation over 48 hours beginning on day 2, 9, and 18. Data presented as mean±s.e. N > 100 GSCs in triplicate per data point. * denotes p < 0.05 (student t-test).

Consistently, the frequency of GSCs in S phase or mitosis was not significantly altered by protein starvation (Fig. 2.2A-D). In both fed and starved conditions, these cell cycle indices decreased after 9 days, likely reflecting an effect of aging rather than starvation (Inaba et al., 2011). Together, these results suggest that the remaining GSCs continue to proliferate during prolonged protein starvation.

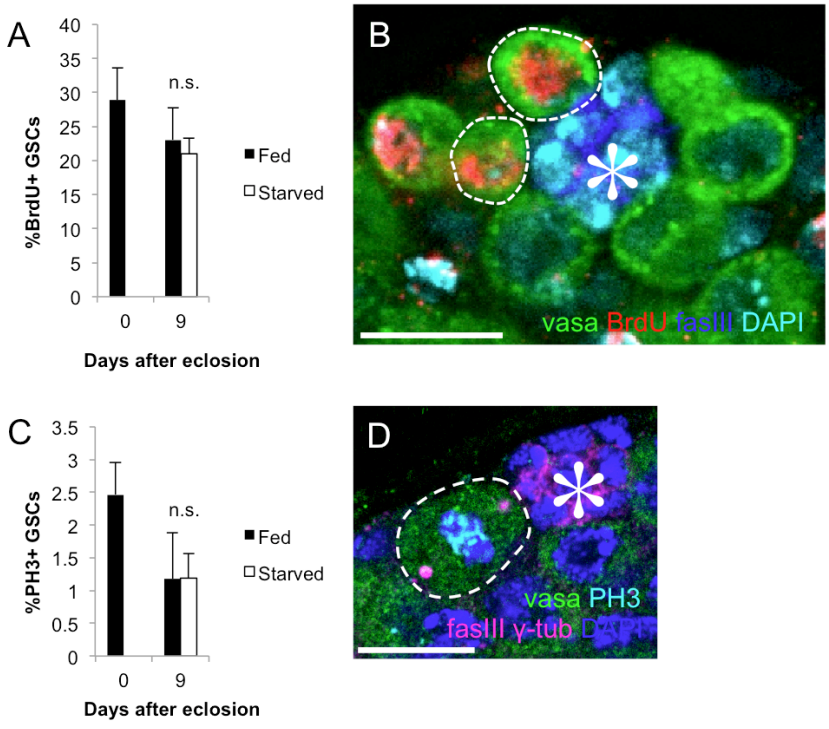

Figure 2.2. S-phase index and mitotic index decrease with age but are not significantly affected by protein starvation. (A) Frequency of GSCs in S-phase at 0 and 9 days of protein starvation. N>100 GSCs per data point in triplicate. (B) Representative image of GSCs in S-phase (dotted outline) with BrdU+ nuclei (red). (C)

Frequency of GSCs in mitosis at 0 and 9 days of starvation. N>500 GSCs per data point in triplicate. (D) Representative image of a GSC in mitosis (dotted outline) with oriented centrosomes (γ-tub, magenta) and chromatin positive for phosphohistone H3 (PH3, cyan). Hub is denoted by asterisk. Scale bars = 10 μm.

We previously reported that flies cultured in “poor” media -- containing low levels of protein as opposed to none in the protein starvation condition described in this study -- have GSCs with high rates of centrosome misorientation with respect to the hub (Roth et al., 2012), which slows cell cycle via the centrosome orientation checkpoint (Cheng et al., 2008). This is seemingly contradictory to our observation here that GSC cell cycle is unchanged during protein starvation. However, GSC number does not decrease in poor media, as opposed to the protein starvation condition in which 25% (2 of 8) of GSCs are lost. Interestingly, on day 3 of protein starvation, which is prior to GSC loss (Fig. 2.1A), we observed a transient increase in centrosome misorientation (Fig. 2.3A-B) concomitant with a decrease in cell cycle progression (Fig. 2.1D, 2.3C). Thus, we speculate that at the initial phase of protein starvation, the systemic environment transitions through a poor nutrient state in which GSC number is maintained but cell cycle is slowed. Upon reaching steady state in protein starvation after 6 days of starvation, 2 of 8 GSCs are lost, and the remaining 6 GSCs have normal cell cycle activity. These results suggest that protein restriction and protein starvation present distinct challenges to which the system responds differently.

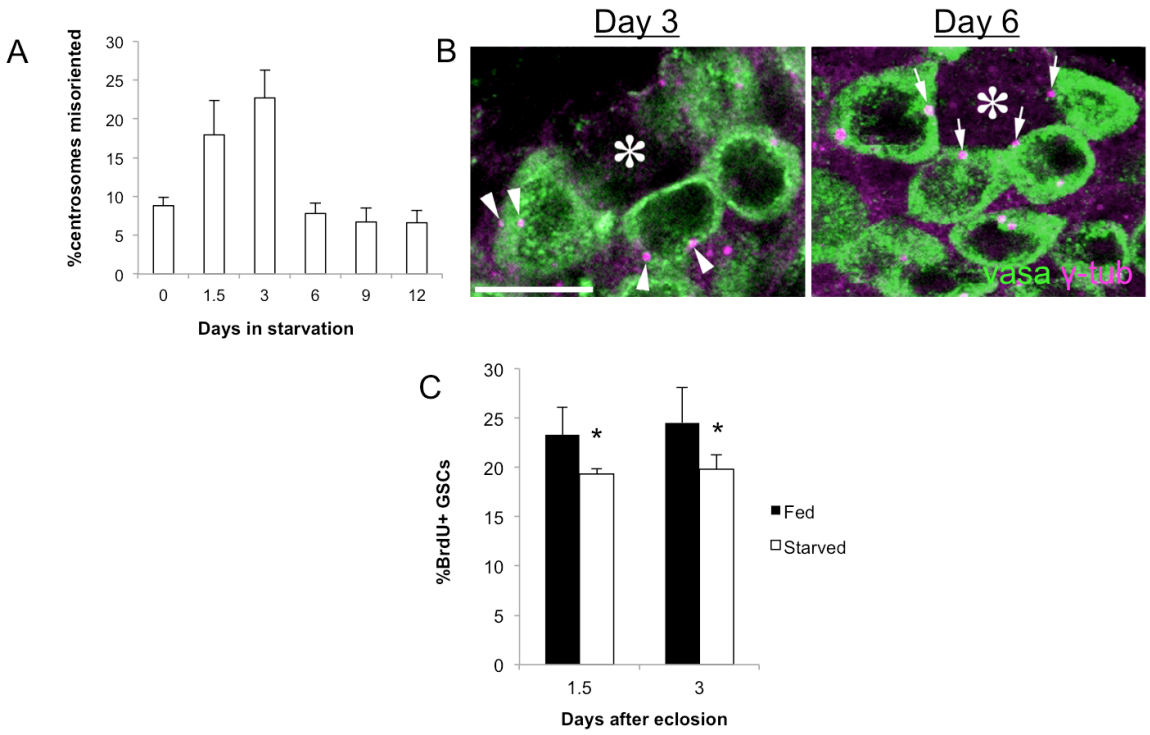

Figure 2.3. Upon protein starvation, GSCs have transient centrosome misorientation and reduced cell cycle progression. (A) Percent of GSCs with misoriented centrosomes over 12 days of protein starvation. (B) Representative images of GSCs with misoriented centrosomes (arrowheads) on day 3 of starvation and oriented centrosomes (arrows) on day 6 of starvation. The hub is denoted by an asterisk (*). (C) Percent of GSCs in S-phase after 1.5 and 3 days of starvation. Scale bar = 10 μm.

Although it is currently unknown how GSCs are lost upon protein starvation, we found that an impairment of dedifferentiation might contribute to the decreased GSC number. Dedifferentiation is a process by which differentiating spermatogonia reacquire GSC identity (Brawley and Matunis, 2004) (Sheng et al., 2009), a phenomenon that gradually increases with age. We found that dedifferentiated GSCs do not accumulate during starvation (Fig. 2.4A), suggesting that the lack of dedifferentiation may contribute to the decreased number of GSCs per testis. Consistently, the process of dedifferentiation itself appears to be inhibited during starvation, as recovery of the niche

after artificial removal of GSCs is significantly delayed once steady state is reached in protein starvation (Fig. 2.4B). The steady upkeep of 6 GSCs per testis in light of impaired dedifferentiation thus suggests that GSCs are remarkably well maintained during protein starvation, and the turnover within the niche associated with constant GSC loss and replacement is minimized.

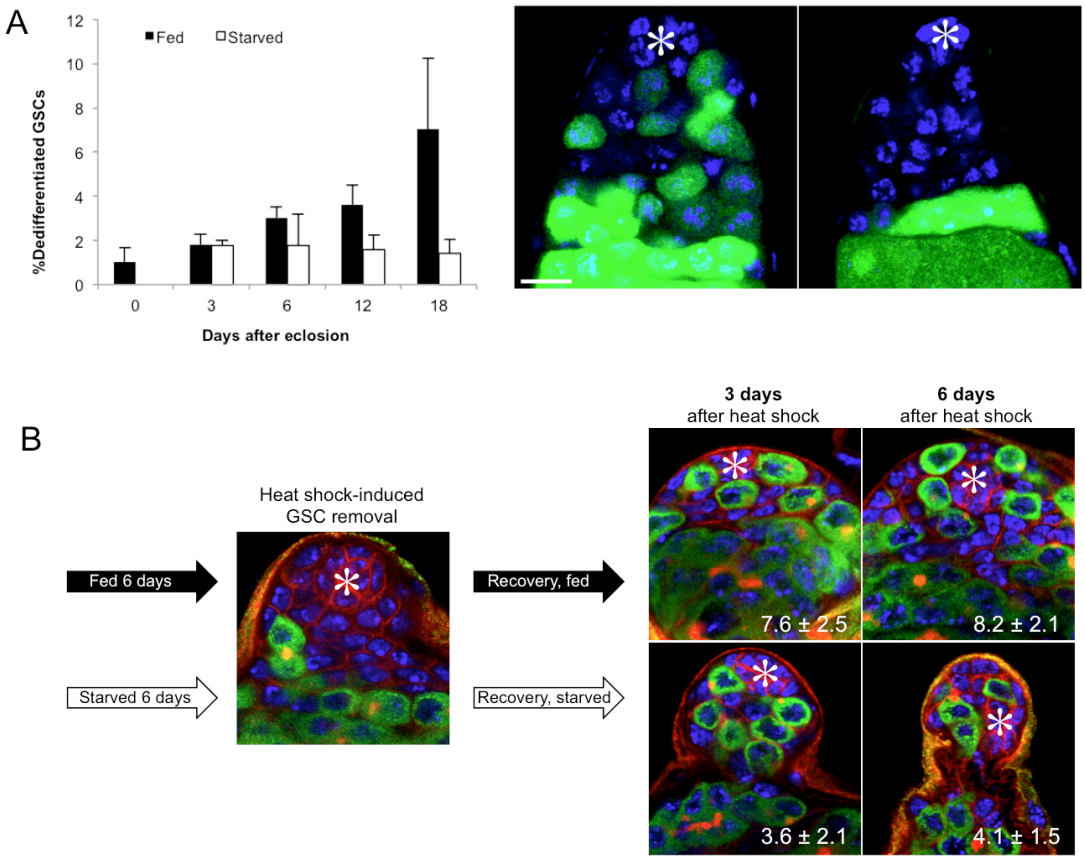

Figure 2.4 Prolonged starvation prevents the dedifferentiated GSCs from accumulating during aging. (A) The percent of GSCs that are dedifferentiated over 18 days of adulthood. Data is shown as mean±s.e. N > 50 testes in triplicate per data point. Dedifferentiated GSCs (green, adjacent to hub, which is marked with an asterisk (*)) are marked by permanently marking SGs at the Bam+ stage with GFP. (B) GSCs are artificially removed from the hub (asterisk) using hs-Bam, after which GSC number is allowed to recover via dedifferentiation. The rate of dedifferentiation is estimated by scoring GSC number after 3 and 6 days of recovery in either fed or starved conditions. Data is shown as mean±s.d. N>30 testes per data point.

2.3.2 Transit-amplifying spermatogonia are eliminated during protein starvation.

The presence of 6 active GSCs throughout prolonged protein starvation prompted us to ask whether a response in other cell types may also contribute to the dramatic involution of the testis during protein starvation (Fig. 2.5). We therefore turned our attention to the transit-amplifying cells, i.e. spermatogonia (SGs) to investigate their response to starvation.

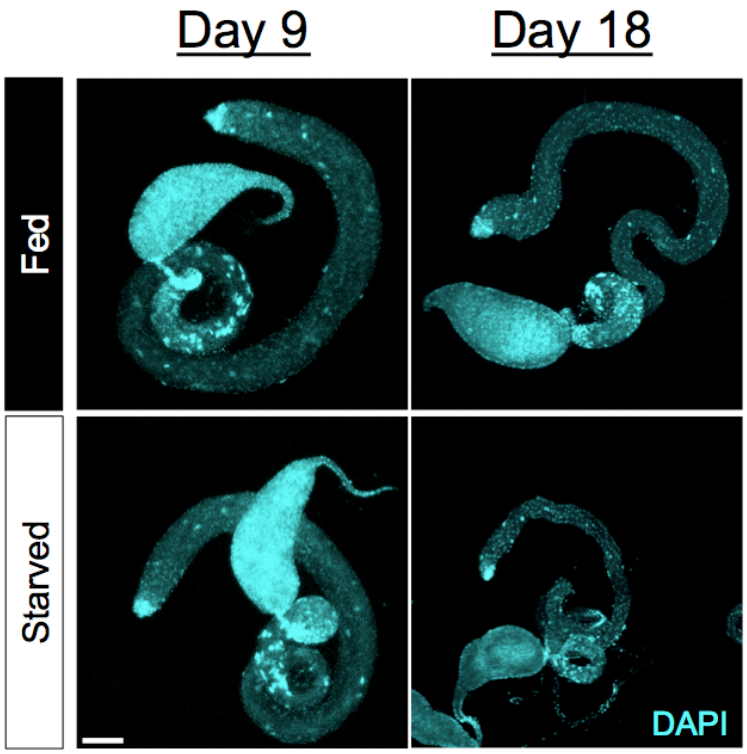

Figure 2.5 The Drosophila testis involutes upon protein starvation. Representative images of fed and starved wild-type testes at day 9 and 18. The previously reported testis involution (see main text) (McLeod et al., 2010) was confirmed in our protein starvation protocol. Scale bar = 100 μm.

After each GSC division, the differentiating daughter, called the gonialblast (GB), undergoes exactly four rounds of transit-amplifying divisions as SGs prior to becoming spermatocytes. As in mammals, SG divisions are characterized by incomplete

cytokinesis, yielding 2-, 4-, 8-, and 16-cell SGs. Each stage can be readily identified by counting the number of SGs that are interconnected by branching fusomes (Fig. 2.6E). When SGs were quantified according to stage, we found a specific decrease in the 4-, 8-, and 16-cell SGs without a significant decrease in the number of GBs and 2-cell SGs under protein starvation conditions (Fig. 2.6A). These results suggest that transit-amplifying divisions are specifically impaired around the 2-cell and 4-cell stage in response to protein starvation.

We investigated the nature of SG reduction during protein starvation and found that SG death, particularly at the 4-cell stage, is increased. As recently reported (Yacobi-Sharon et al., 2013), germ cell death occurs sporadically during normal spermatogenesis in a caspase-3-independent manner. Accordingly, dying germ cells cannot be identified using conventional markers of apoptosis such as anti-cleaved caspase 3 staining or an apoptosis sensor, Apoliner (Bardet et al., 2008) (see below). Instead, we detected dying germ cells using Lysotracker, which stains the acidified compartments associated with dying germ cells (Yacobi-Sharon et al., 2013). Because SGs die synchronously, the additional staining of the nuclear envelope allowed quantification of dying SGs by stage. This scoring method likely underestimates the total amount of cell death as the nuclear envelope disintegrates during cell death (2.7, but for comparative purposes, the results reveal that that SGs die more frequently during protein starvation (Fig. 2.6B, C). Specifically, GBs, 2-cell SGs, and 4-cell SGs begin to die more frequently after 3 days of protein starvation (Fig. 2.6D). At 9 days of starvation, the increase in SG death is most prominent at the 4-cell stage (Fig. 2.6D). The cumulative effect of increased SG death over time likely accounts for the decrease in

late-stage SGs (i.e. 8- and 16-cell SG stages) at day 9 and day 18. Importantly, the death of 8- and 16-cell SGs does not appear to be as dramatically affected by starvation, presumably because late-stage SG death in both conditions is governed primarily by a nutrient-independent mechanism as previously described (Yacobi-Sharon et al., 2013) (Fig. 2.6E).

These data indicate that the survival of SGs in the early stages of transit-amplifying divisions is significantly impaired upon protein starvation. As we were unable to detect a significant change in the rate of SG divisions during starvation, we conclude that SG death is most likely responsible for decreased SG number.

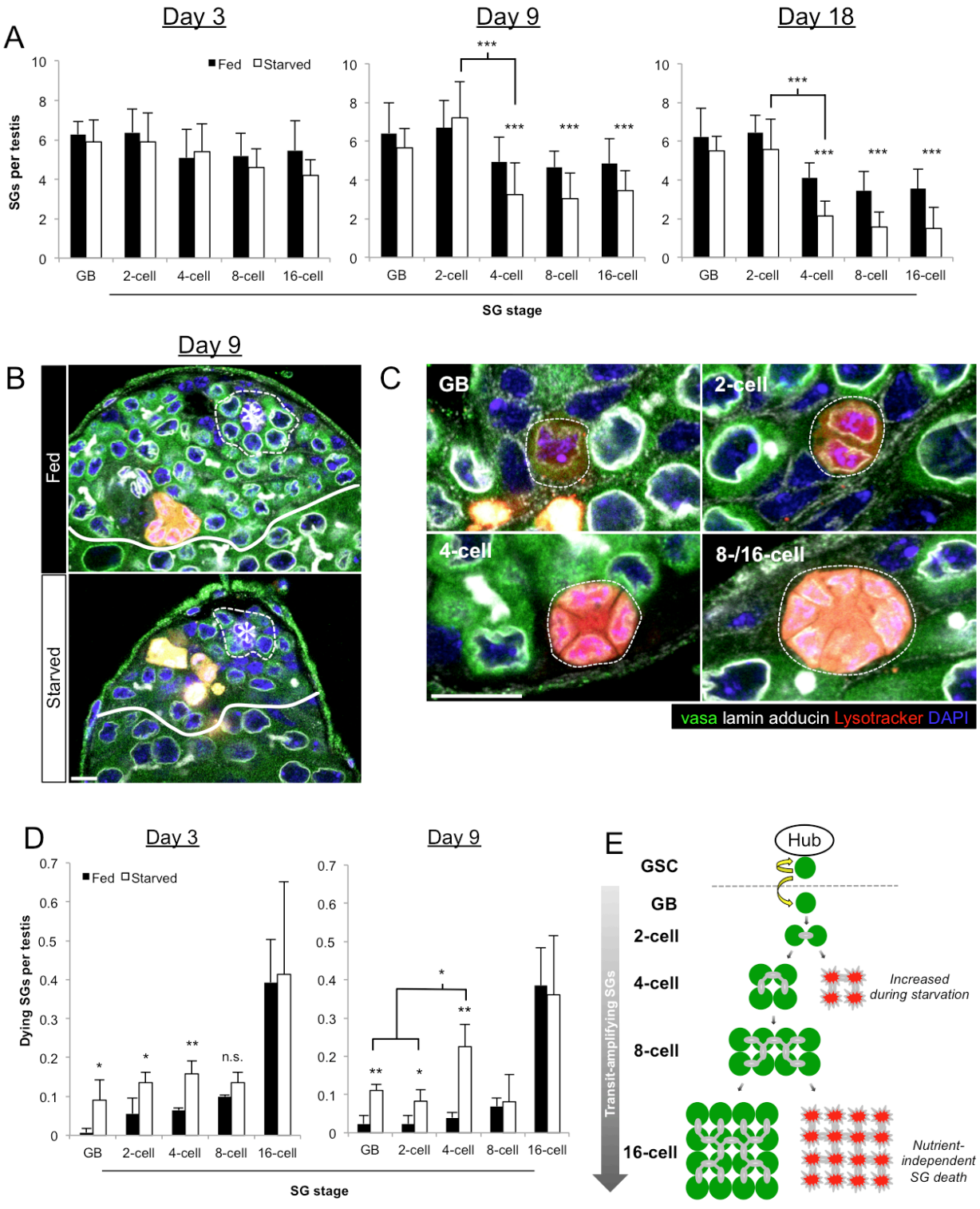

Figure 2.6 Spermatogonia are eliminated between the 2-cell and 4-cell stage during protein starvation. The number of SGs per stage per testis was quantified by scoring the number of SGs connected by a fusome. (A) SG number per stage per testis

at day 3, 9, and 18. Data presented as mean±s.d. N > 20 testes per condition per time point. *** denotes p-value < 0.0005 (student t-test). (B) Representative images of testes stained with Lysotracker to identify dying cells in the mitotic. (C) Representative images of dying SGs (dotted outline) stained for lamin, which allows SG death to be scored according to stage as dying GBs, 2-cell SGs, 4-cell SGs, and 8-/16-cell SGs (some cells are not visible due to focal plane limitations). (D) Quantification of SG death by stage on day 3 and day 9 of starvation. Data presented as mean±s.e. N > 30 testes in triplicate per data point. * denotes p < 0.05; ** denotes p < 0.005 (student t-test). (E) Schematic of SG death. Protein starvation increases SG death near the 2-cell to 4-cell transition, whereas death at the later stages occurs independent of nutrient conditions. Scale bars = 10 μm.

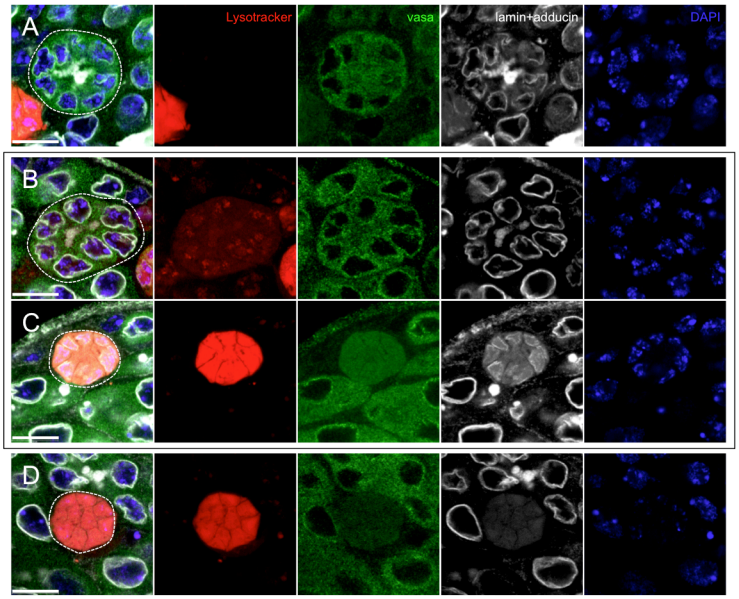

Figure 2.7 Dying SGs can be quantified using Lysotracker and Lamin staining. SGs die synchronously by (A) rounding up, (B) staining positive for Lysotracker, (C) losing cytoplasmic vasa staining, and (D) losing all identifying markers except for Lysotracker. To accurately quantify SG death by stage, only (B) and (C), which are positive for Lysotracker and Lamin, were scored.

2.3.3 Starvation increases the apoptosis of cyst cells that encapsulate SGs.

SGs require supporting somatic cyst cells (CCs) to undergo transit-amplifying divisions and differentiation (de Cuevas and Matunis, 2011; Lim and Fuller, 2012; Schulz et al., 2002). Their progenitors, called cyst stem cells (CySCs), are attached to the hub and provide essential signals to specify GSC identity (Leatherman and Dinardo,

2008, 2010). CySCs divide asymmetrically to produce CCs (Cheng et al., 2011), which encapsulate GBs/SGs to promote their differentiation (Lim and Fuller, 2012; Schulz et al., 2002).

In parallel with the elimination of SGs, the number of CCs marked by Tj (Li et al., 2003), but not cyst stem cells (CySCs) marked by Zfh1 (Leatherman and Dinardo, 2008), is decreased noticeably (Fig. 2.8A-B) during protein starvation. Closer examination revealed that this is due to an increase in CC apoptosis. In contrast to germ cell death, CC death can be detected by anti-cleaved caspase 3 (Fig. 2.9A) and Apoliner (Fig. 2.8C), a fluorescent reporter that yields nuclear localization of nlsGFP in the presence of active caspases (Bardet et al., 2008). The dying CCs were almost always Zfh1- (Fig. 2.8C), suggesting that differentiated CCs but not CySCs undergo apoptosis. By using Apoliner, we determined that the frequency of dying CCs increases two-fold during starvation (Fig. 2.8D). Importantly, the frequency of CySC mitoses does not decrease significantly relative to GSC number per testis, suggesting that the decline in CC number during protein starvation is primarily due to increased apoptosis.

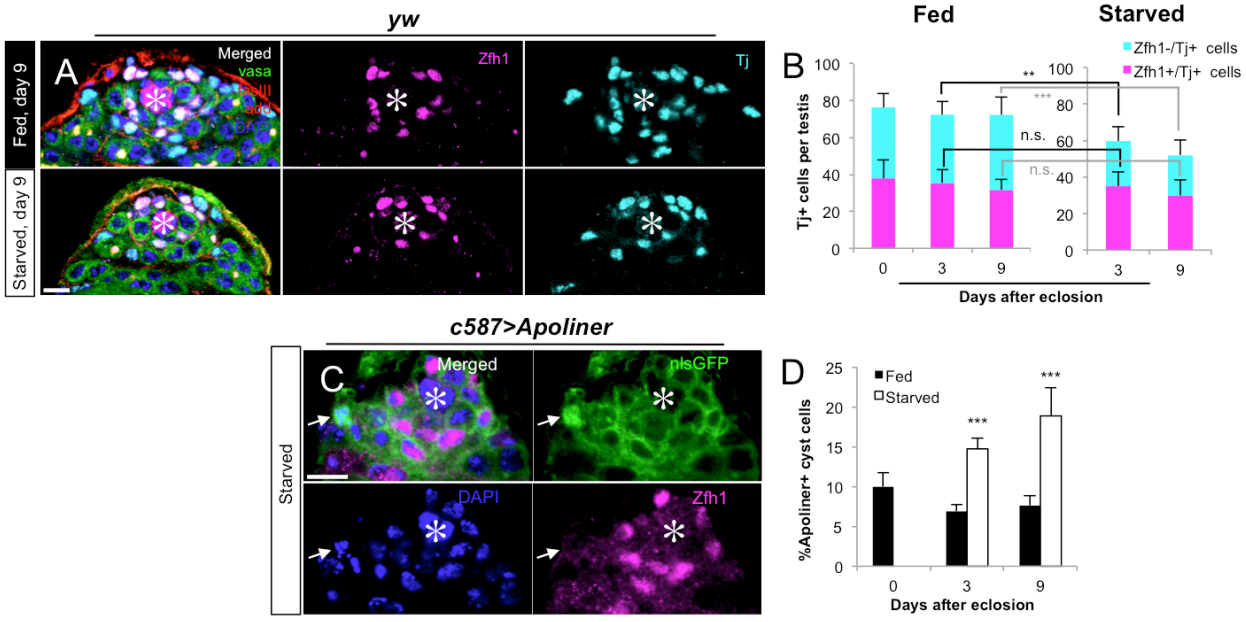

Figure 2.8 Increased apoptosis reduces the number of differentiated cyst cells during protein starvation. (A) Representative images of testes stained with the CySC marker Zfh1 and CySC/CC marker Tj. (B) Quantification of Tj+ and Zfh1+ nuclei on day 0, 3, and 9. N > 30 testes per time point. Data is presented as mean ± s.d. ** denotes $p < 0.005$; *** denotes $p < 0.0005$ (student t-test). (C) Dying CCs can be marked by expressing UAS-Apoliner in the CySC/CC lineage using the c587-gal4 driver. Apoliner+ cells (arrow) almost never occur adjacent to the hub (asterisk) and are rarely observed to be Zfh1+. (D) Frequency of Apoliner+ CCs scored as percent all Tj+/Zfh1- cells. Data is presented as mean ± s.e. N > 20 testes in triplicate per condition per time point. **** denotes $p < 0.0005$ (student t-test). Scale bars = 10 μm.

2.3.4 CC apoptosis initiates SG death during protein starvation.

Because CCs play a critical role during SG development (Lim and Fuller, 2012; Tazuke et al., 2002), we hypothesized that the increase in CC apoptosis during starvation mediates the increase in SG death. To explore this relationship, we first examined whether CC apoptosis was sufficient to trigger SG death. We induced CySC/CC apoptosis using temperature-dependent expression of Grim, a proapoptotic gene, in the CySC/CC lineage (c587-gal4>UAS-Grim, tub-gal80^{ts}) as previously described (Lim and Fuller, 2012). Consistent with the reported observation that expression of Grim in the CySC/CC lineage ablates germ cells as well as CySCs/CCs, we observed a dramatic increase in SG death within 6 hours of Grim induction (Fig. 2.9B) and almost complete elimination by 24 hours. We also noted that all SG death induced by the somatic expression of Grim is associated with Lysotracker staining and has morphological characteristics reminiscent of starvation-induced SG death. These results indicate that CC apoptosis is sufficient to rapidly initiate SG death and suggest that starvation-induced SG death follows a similar mechanism.

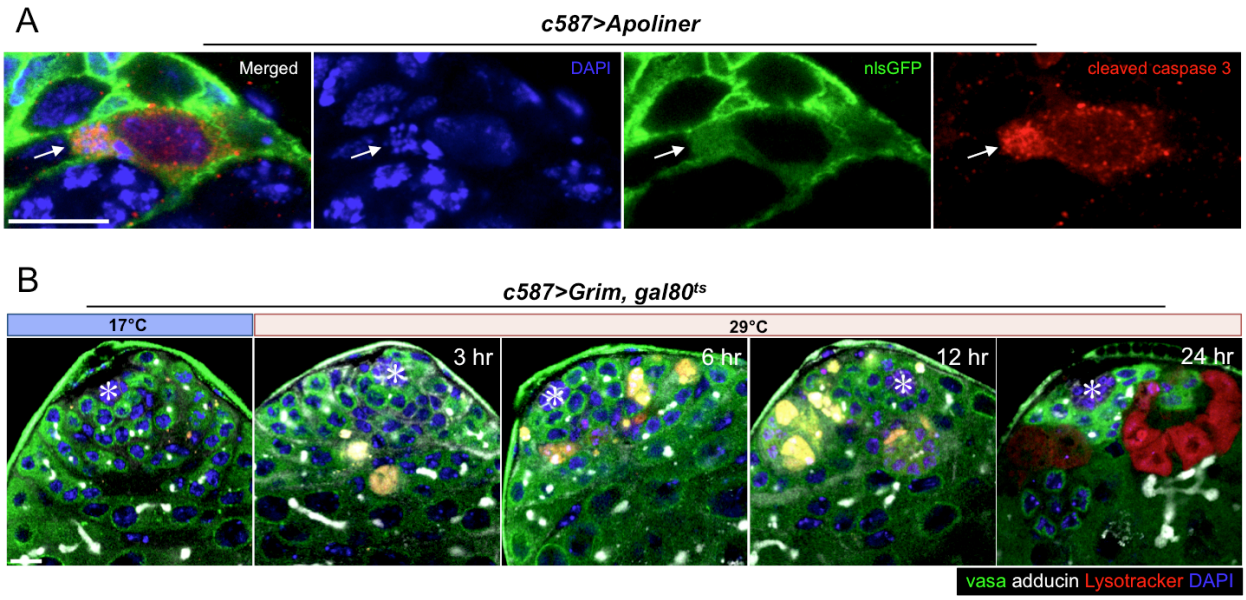

Figure 2.9 CC apoptosis is caspase-dependent and is sufficient to initiate SG death. (A) In c587>Apoliner testes, cleaved caspase-3 (red) can be detected in CCs with dim Apoliner signal (arrow) characteristic of advanced stages of apoptosis (Bardet et al., 2008). (B) Detection of SG death (red) in testes expressing UAS-Grim in the CySC/CC lineage in a temperature-dependent manner. Hub is denoted by asterisk (*). Scale bars = 10 μm.

To further test this idea, we inhibited CC apoptosis during protein starvation using overexpression of Drosophila inhibitor of apoptosis 1 protein (DIAP1) (Orme and Meier, 2009), as well as knockdown of the initiator caspase Dronc with RNAi (Leulier et al., 2006). We found that inhibition of CC apoptosis leads to a considerable decrease in starvation-induced CC apoptosis (Fig. 2.10A), which is accompanied by a significant decrease in SG death at the 2- and 4-cell SG stages (Fig. 2.10B). These data demonstrate that CC apoptosis is necessary and sufficient to induce SG death. Importantly, inhibition of CC apoptosis by DIAP1 expression does not suppress as dramatically the basal level of SG death in the 8- and 16-cell stages. These data also imply the existence of two subclasses of SG death governed by distinct mechanisms: 1) “Early” stage death (2-, 4-cell SGs), which is specifically increased upon protein

starvation and likely induced by CC apoptosis, and 2) “late” stage SG death (8-, 16-cell SGs), which is observed irrespective of nutrient conditions and is less correlated with CC apoptosis. This classification is supported by the observation that CCs associated with “early” stage SGs are more frequently apoptotic during protein starvation than those associated with “late” stage SGs (Fig. 2.10C-D). Consistently, dying “early” SGs are almost always accompanied by apoptotic CCs (Fig. 2.10E), whereas dying “late” SGs are frequently observed without accompanying apoptotic CCs (Fig. 2.10F). These data thus support the model in which starvation specifically increases the apoptosis of CCs associated with 2-cell and 4-cell SGs, which in turn leads to the death of those SGs (Fig. 2.10G).

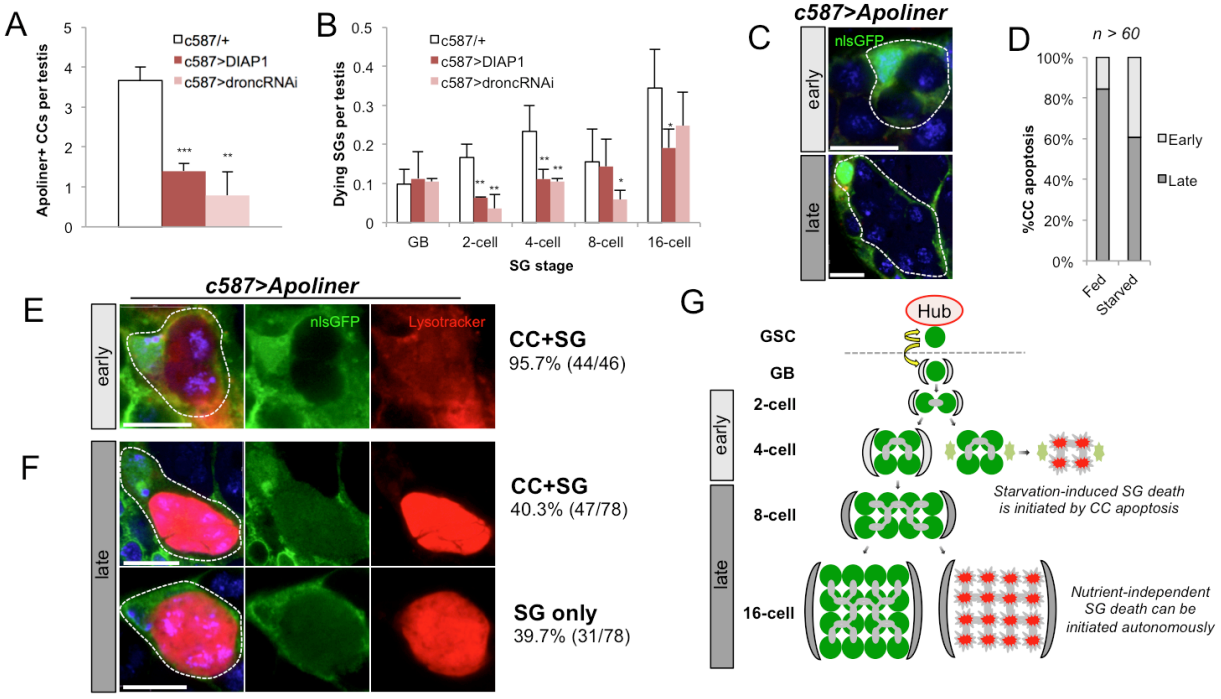

Figure 2.10 Starvation-induced CC apoptosis mediates the death of associated 2- and 4-cell SGs. (A) Quantification of CC apoptosis after 3 days of starvation by combining Apoliner and DIAP1 overexpression or Dronc RNAi knockdown. Data is presented as mean±s.e. * denotes $p < 0.05$; ** denotes $p < 0.005$; *** denotes $p < 0.0005$ (student t-test). N > 15 testes in triplicate per data point. (B) Quantification of SG death per testis after 3 days of starvation. N > 30 testes in triplicate per data point. (C)

Representative images of dying CCs scored according to “early” or “late” based on the number of enveloped SGs. (D) Percentage of “early” and “late” CC death in testes fed or starved 9 days. (E-F) Representative images of dying “early” (E) and “late” (F) SGs and their encapsulating CCs with observed frequencies. (G) Starvation-induced “early” SG death is initiated by CC apoptosis, whereas “late” SG death can be initiated autonomously. Scale bars = 10 μm.

2.3.5 GSC maintenance is impaired when CC-mediated SG death is inhibited during protein starvation.

The highly regulated nature of CC-mediated elimination of SGs compelled us to explore the biological relevance of such a response. To address this question, we began by examining the consequence of inhibiting CC apoptosis during protein starvation. As described above, the expression of DIAP1 in the CySC/CC lineage efficiently blocks starvation-induced CC apoptosis as well as the death of associated “early” SGs (Fig. 2.10B-C).

Strikingly, we found that DIAP1-expressing testes cannot maintain GSCs at steady state during protein starvation. Whereas wild type and control flies maintain 6 actively proliferating GSCs per testis for an extended period of time in protein starvation, flies expressing DIAP1 in CySC/CC lineage (c587>DIAP1) continue to lose GSCs, decreasing to ~3 GSCs per testis at day 12 of protein starvation (Fig. 2.11A). Importantly, DIAP1 expression does not significantly affect GSC number under fed conditions (7.6 ± 1.1 GSCs per testis, day 9, N>30 testes, compared to 8.0±0.5 in wild type, $p>0.05$), suggesting that the blockade of CC apoptosis adversely affects GSC maintenance specifically during protein starvation. Moreover, we found that the rate of GSC cell cycle progression is decreased in DIAP1-expressing flies (Fig. 2.11B), suggesting that not only maintenance but also proliferation of GSCs is impaired when

CC-mediated SG death is blocked during protein starvation.

In addition to the continued decline of GSCs in DIAP1-expressing testes (c587>DIAP1) during protein starvation, the remaining GSCs appear to be qualitatively different from 6 GSCs maintained during starvation in control testes. Although we scored all germ cells attached to the hub cells as GSCs in Figure 5A, such cells in DIAP1-expressing testes are often connected to other germ cells by a fusome and resemble SGs (Fig. 2.11C-E). After 18 days of starvation, only 31% of remaining GSCs appear to be single-cell GSCs (18% 2-cell, 42% 4-cell, 9% 8-cell, N=90 hub-associated germ cells), leading to the calculation of less than one bona fide GSC per hub in starved DIAP1-expressing flies (31% x 2.8 germ cells/hub = 0.9 GSCs). In contrast, SG-like germ cells are much less often observed attached to the hub in wild-type testes after 18 days of starvation (<10%).

The combined loss of GSCs and reduction in GSC cell cycle thus leads to dysregulated tissue involution that can be observed at 9 days of starvation in DIAP1-expressing testis (Fig. 2.11F-G). After 18 days, a small but non-negligible fraction of testes lack GSCs and other germ cells in DIAP1-expressing starved flies (5/105 testes at 18-days of starvation; 6/62 testes after 24 days of starvation) (Fig. 2.11H). These results suggest that CC-mediated SG death is critical for maintaining functional GSCs and preserving tissue homeostasis during protein starvation.

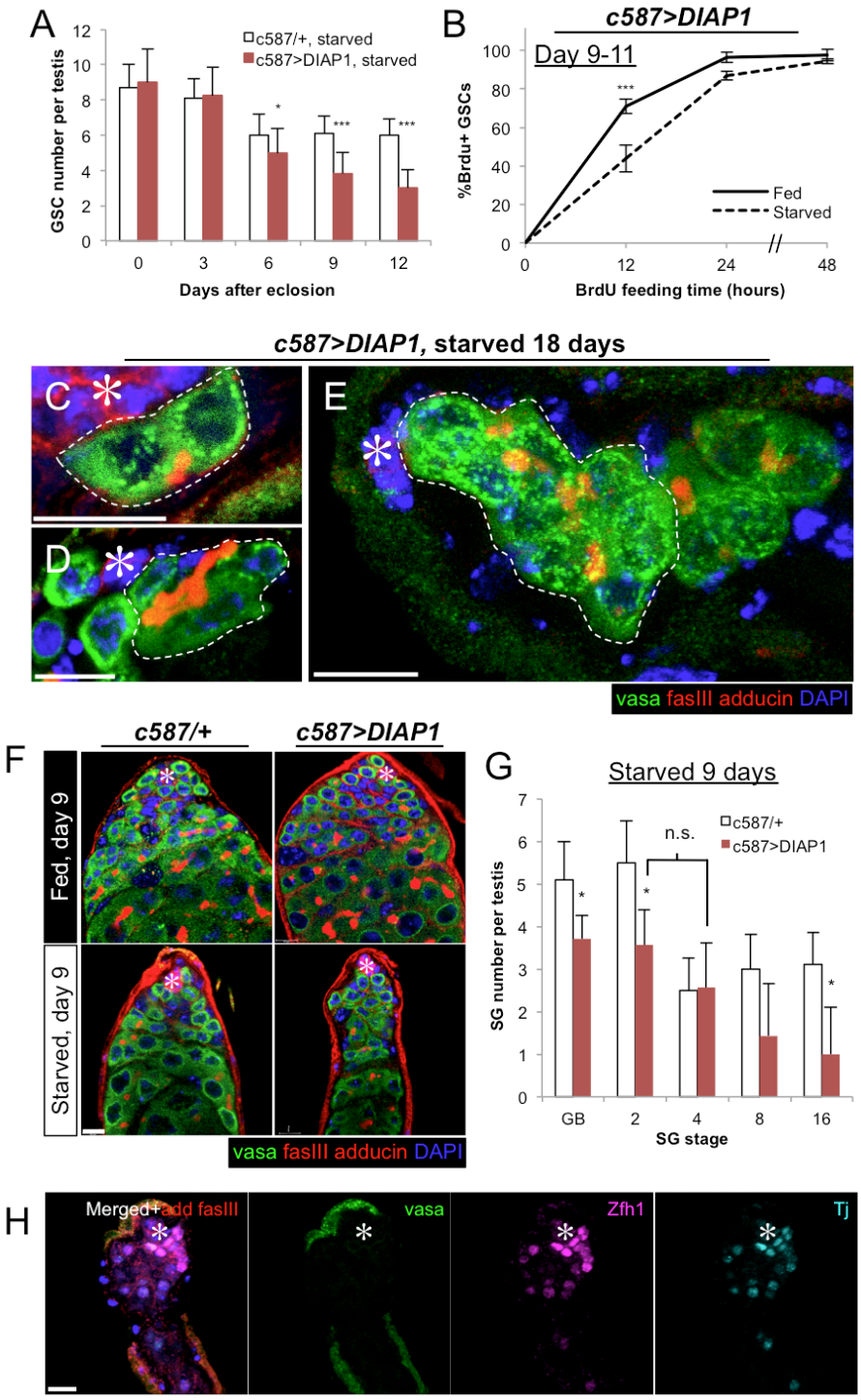

Figure 2.11 GSCs cannot be maintained during protein starvation in the absence of CC-mediated SG death. Starvation-induced CC apoptosis is inhibited by overexpression of DIAP1 using the c587-gal4 driver. (A) Average GSC number per testis over 12 days of starvation DIAP1-expressing testes. Data presented as mean±s.d. N > 30 testes per data point. * denotes $p < 0.05$; *** denotes $p < 0.0005$ (student t-test). (B) BrdU incorporation kinetics in GSCs after 9 days of starvation. Data

presented as mean±s.e. N > 100 GSCs in triplicate per data point. (C-E) Representative images of interconnected 2-cell (C), 4-cell (D), and 8-cell (E) SG-like clusters attached to the hub at 18 days of starvation. (F) Representative images of DIAP1-expressing testes compared to control at day 9 of fed and starved conditions. (G) Quantification of SG number in DIAP1-expressing testes after 9 days of starvation compared to control. Data presented as mean±s.d. * denotes $p < 0.05$ (student t-test). (H) Representative image of DIAP1-expressing testis that contain CySC/CCs but are devoid of germ cells after 18 days of starvation. Scale bars = 10 μm.

2.3.6 Inhibition of CC-mediated SG death during protein starvation leads to impaired germline recovery upon reintroduction of protein.

Because GSC maintenance during protein starvation is compromised in the absence of CC-mediated SG death, we wondered how germline recovery upon reintroduction of dietary protein would be affected in DIAP1-expressing testes. To assess recovery, we reintroduced protein into the diet after flies were starved for 18 days. Consistent with previously reported observations (McLeod et al., 2010), testis involution is reversible, and robust gametogenesis is efficiently reconstituted in all wild type/control testes within 6 days of protein reintroduction (Fig. 2.12, N>100). In DIAP1-expressing testes, however, only 54% of testes are able to recover to this state within 6 days (Fig. 2.13A, N=73). Even after 12 days of recovery, only 60% of DIAP1-expressing testes exhibit full recovery (Fig. 2.13B, N=30).

The remaining fraction of testes (46% of testes after 6 days of recovery, N=73) had severely impaired recovery upon protein reintroduction: We found that 40% of DIAP1-expressing testes contain only SGs, but not SCs or later stage germ cells, apparently because they fail to develop to the spermatocyte stage (Fig. 2.13C). In these cases, no spermatocytes or elongating spermatids are observed, even when recovery is extended to 12 days (Fig. 2.13D). Finally, the remaining 6% of testes contain no germ

cells after 6 days of recovery (Fig. 2.13E), presumably representing testes that had completely lost GSCs during starvation (Fig. 2.11H). Strikingly, in the cases of impaired recovery, aberrantly proliferating cells can often be found. In testes with abnormal SG differentiation, tumor-like SGs, characterized by an abnormally large number of SGs connected by a fusome (Fig. 2.13G) (5/73 after 6 days refeeding; 3/29 after 12 days refeeding) can be found. In the cases where germ cells were depleted, protein reintroduction results in the overproliferation of undifferentiated CySCs (Fig. 2.13H), likely due to a deranged signaling environment in the absence of germ cells (Gonczy and DiNardo, 1996).

These results demonstrate that compromised CC/SG death during protein starvation leads to a collapse in tissue homeostasis and severely impairs the ability of the tissue to recover, and the damaged tissue architecture can also promote the abnormal overproliferation of cells during recovery.

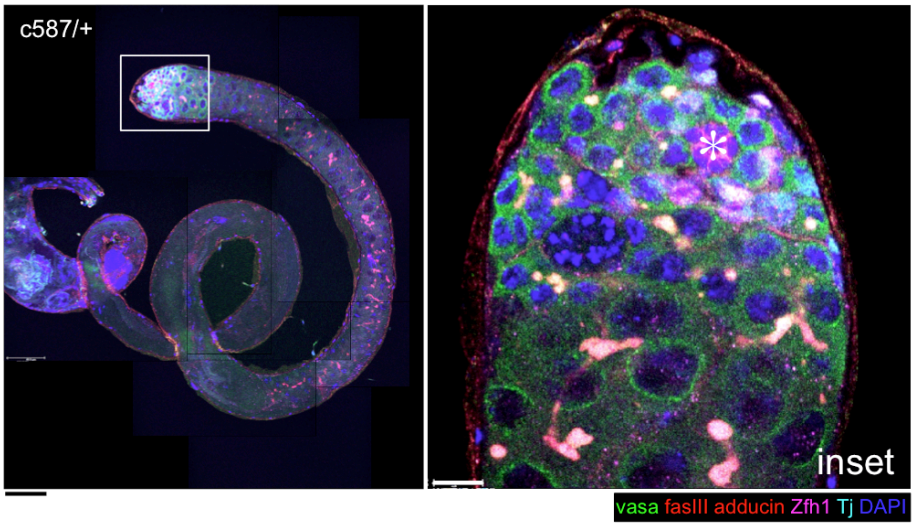

Figure 2.12 Starved control testes can robustly recover upon reintroduction to protein. Representative image of a control testis starved for 18 days and then refed for 6 days. All stages of germ cell development from GSCs to individualizing spermatids are present. Hub is denoted by asterisk (*). Black scale bar = 100 μm. White scale bar = 10 μm.

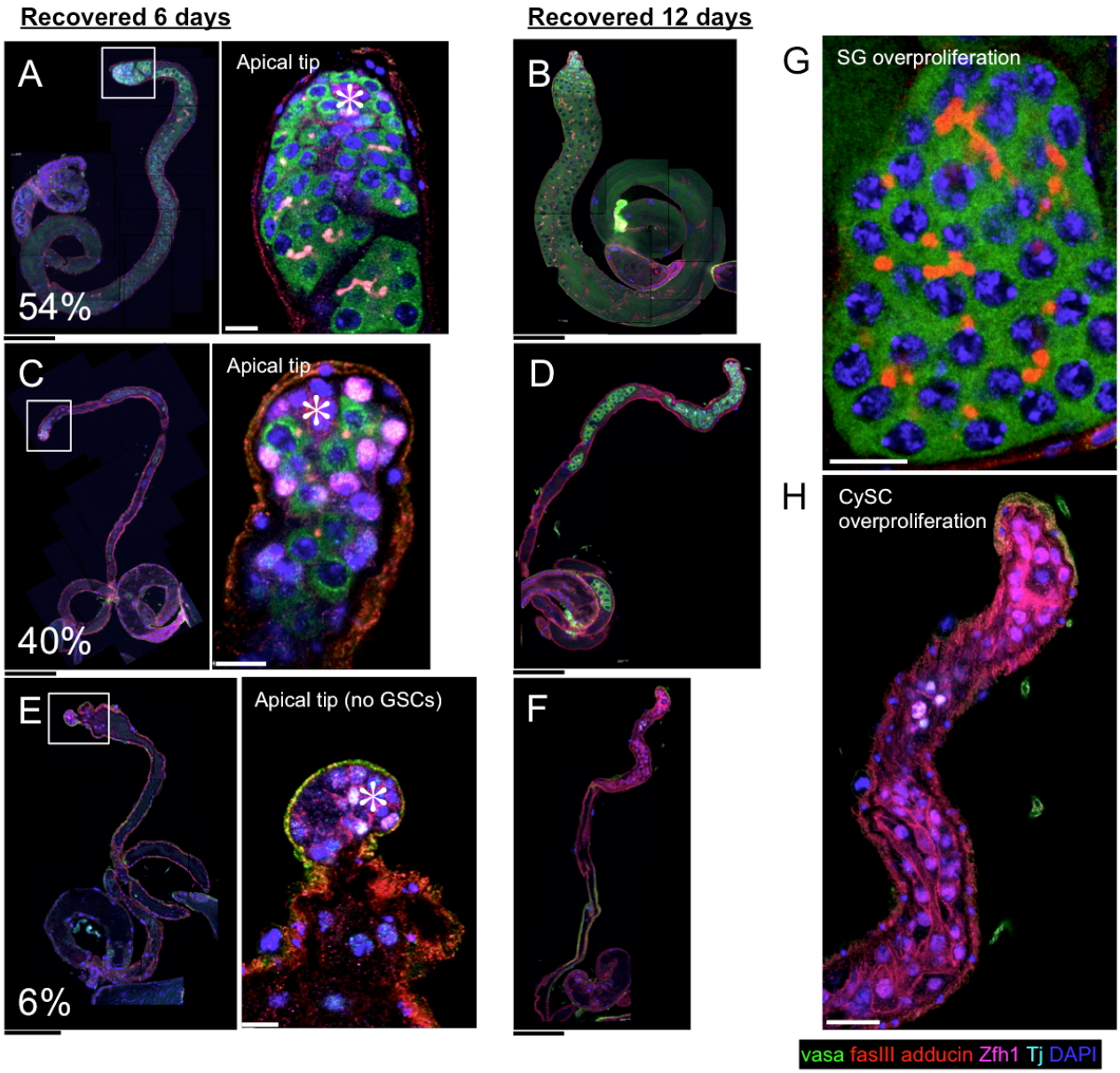

Figure 2.13 Recovery of the germline upon reintroduction to protein is compromised in DIAP1-expressing flies. c587>DIAP1 flies are starved 18 days before protein is introduced to the diet. (A) 54% of c587>DIAP1 testes recover with normal germ cell development after 6 days. Apical tip (inset) shows normal organization. (B) Normal recovery of DIAP1-expressing testes after 12 days. (C) 40% of DIAP1-expressing testes have GSCs and SGs, but SG development is compromised. Apical tip (inset) contains GSCs but few SGs and no SCs. (D) After 12 days of recovery, testes with defective SG development contain no cells beyond the SG stage. (F) 6% of DIAP1-expressing testes have no germ cells upon recovery. (G) Representative image of overproliferating SGs with > 16 interconnected cells after 12 days recovery. (H) Representative image of overproliferating CySCs in the absence of germ cells after 12 days recovery. Black scale bars = 100 μm. White scale bars = 10 μm.

2.3.7 The contents of dying cells are absorbed by neighboring CySC/CCs.

The data presented above suggest that SG death is required to maintain active GSCs during prolonged starvation. Indirectly, the elimination of transit-amplifying cells lowers the nutrient demand of the tissue, which likely contributes to the survival of stem cells. However, considering the fact that the flies are not fed any protein for a prolonged time period in our experimental procedure, it is unlikely that GSCs are able to maintain such an active state solely because of decreased competition for resources within the tissue.

Intriguingly, our observations suggest that the dying SGs are absorbed by surviving cells, possibly to nourish surviving cells. We noticed that dying CCs/SGs are often associated with CySCs/CCs that contain unusually large Lysotracker+ lysosomes (Fig. 2.14A). It should be noted that these lysosome containing-cells are not the CCs that undergo apoptosis together with dying SGs as described in Fig. 2.9A. Instead, they are CySC/CCs that are associated with living germ cells. We call these CCs “surviving CCs” to distinguish them from the CCs that die along with dying SGs. The lysosomes observed in surviving CCs near the dying SGs are much larger and more numerous than those not associated with dying SGs. The engulfment of dying germ cell cytoplasm by somatic cells during starvation has been characterized in the female germline and is reported to require JNK signaling (Etchegaray et al., 2012). Similarly, we found that surviving CCs associated with dying SGs displayed clear JNK pathway activation, visualized by the expression of puc-lacZ (Fig. 2.14B), a well-established reporter for JNK pathway activation. In contrast, CCs that were not associated with dying SGs did not stain for puc-LacZ. These data suggest that surviving CySCs/CCs absorb dying cell

debris during starvation in a manner similar to the engulfment phenomenon previously described in the female germline.

The absorption of cell debris during starvation implies that nutrients from dying SGs are locally recycled via the CySCs/CCs. In further support of this idea, we detected GFP-Atg8a, whose expression is under the control of the endogenous promoter, on lysosomes that are closely associated with dying SGs (Fig. 2.14C-D). It should be noted that chloroquine, which increases lysosomal pH and has been used to visualize GFP fluorescence within lysosomes (Ni et al., 2011), was used to enhance visualization of GFP-Atg8a. Because it is known that LC3, the mammalian homologue of Atg8, functions in phagocytic processes to transport phagosomes to lysosomes (Martinez et al., 2011), these observations thus suggest that Atg8 may play a role in clearance of dying SGs through phagocytic process. Taken together, we propose that the contents of dying SGs are absorbed and recycled locally, possibly to fuel GSC maintenance and activity during protein starvation.

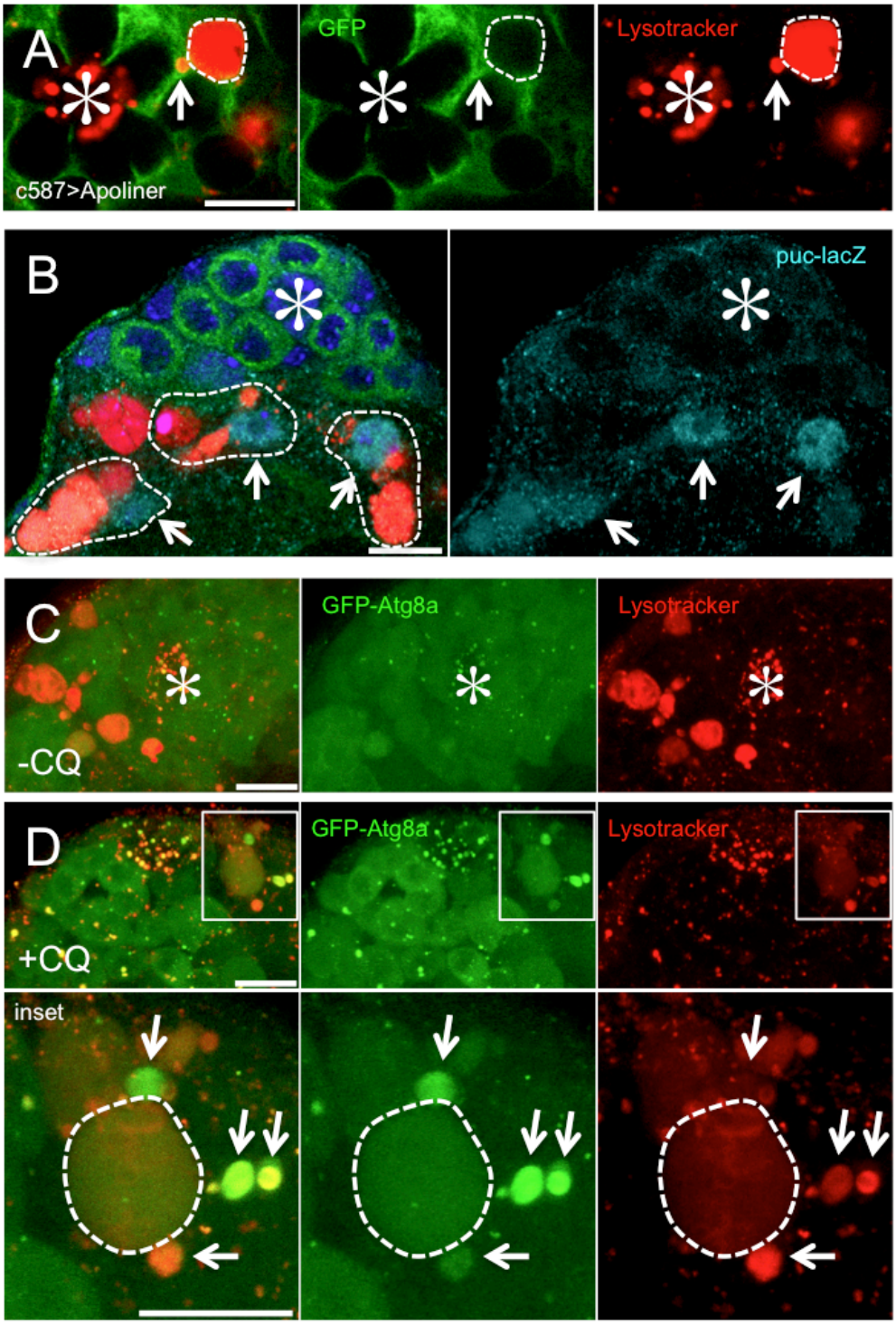

Figure 2.14 SG death is associated with phagocytosis and Atg8a. (A) Apical tip of unfixed testis with c587>Apoliner as CySC/CC marker. A prominent lysosome (arrow) in the CySC/CC is adjacent to a dying SG (dotted outline). (B) Positive puc-lacZ staining in CCs associated with dying SGs (dotted outlines). (C) GFP-Atg8a is expressed in the testis and can be visualized without chloroquine treatment as scattered puncta. (D) Chloroquine treatment enhances visualization of the presence of GFP-Atg8a in

lysosomes (arrows, inset) surrounding dying SGs (dotted outline, inset). Hub is denoted by an asterisk (*). White scale bars = 10 μm.

2.4 Discussion

Tissues alternate between different states of homeostasis in order to cope with ever-changing availability of nutrients, but little is known about how different cell populations respond in a concerted manner to maximize tissue function with limited resources. Our study reveals the importance of the regulated elimination of transit-amplifying cells during protein starvation while stem cells are maintained and continue to proliferate. Considering the proposed role of transit-amplifying cells, i.e. to increase cell production without increasing stem cell divisions, it is fitting that the transit-amplifying cell population is downsized to decrease overall cell output in a tissue without dramatically affecting stem cell behavior. Failure to eliminate transit-amplifying cells during starvation results in a collapse of tissue homeostasis and leads to defects in recovery upon reintroduction of nutrients. Thus, our study reveals that dramatic yet reversible “shifts” in tissue homeostasis, such as the one observed during starvation, must be carried out in a precise manner that involves a coordinated response in many cell types.

Importantly, our results do not argue that stem cells do not respond to starvation. Stem cell proliferation is transiently reduced throughout the first few days of protein starvation, similar to the “poor media” response we described previously. However, upon reaching steady state in starvation, GSC number is maintained at 6 (only 25% decrease from 8 GSCs in fed condition) for a prolonged time period, and these 6 GSCs maintain an active cell cycle. It awaits future investigation to understand why it is advantageous for GSCs to continue proliferating during starvation rather than slow down or become quiescent.

We show that transit-amplifying SGs are eliminated through CC-mediated death. The elimination of transit-amplifying cells can be considered to be sacrificial in nature, as their death allows stem cell activity to be maintained. Furthermore, our observations suggest that contents of dying SGs might be absorbed by surviving CySC/CCs, and it appears that these cells may function as non-professional phagocytes. The presence of Atg8a on lysosomes in surviving CySC/CCs suggest that they use a non-canonical autophagic in the clearance of dying cells (Mehta et al., 2014).

It is interesting to note that defective response to protein starvation due to the blockade of CC/SG cell death by DIAP1 expression leads to a tumor-like overproliferation of cells upon reintroduction of protein. These cells are not inherently tumorigenic in the classical sense, as they likely do not contain mutations in tumor suppressor genes or oncogenes. Instead, their dysregulated behavior appears to be a result of damaged tissue architecture and an imbalance among different but interdependent cell types. When the surviving cells are exposed to nutrients again, uncontrolled proliferation and abnormal differentiation may be the consequence of an inappropriate signaling environment. By extension of this idea, it is tempting to speculate that there may be human tumor cells that have wild type genomes: instead of mutations that cause these cells to be inherently hyper-proliferative, their tumorigenic behavior arises from tissue damage acquired during a defective “shift” in tissue homeostasis.

In summary, our study reveals regulated elimination of transit-amplifying cells as a critical step to protect stem cells in response to starvation. We show that inhibiting this

process can lead to irreversible tissue damage, thus introducing the idea that organisms and their tissues have highly evolved strategies to undergo reversible “shifts” in tissue homeostasis throughout their lifespans.

2.5 Experimental Procedures

Fly Strains and Husbandry

Flies were cultured in standard Bloomington medium at 25°C. For protein starvation experiments, newly eclosed adult flies were transferred within 24 hours (day 0) onto either standard food (fed) or 16% sucrose / 0.7% agar (starved) at a density of 20-40 flies per vial. Flies were transferred to fresh vials every three days. The following fly stocks were used: c587-gal4 (Decotto and Spradling, 2005), nos-gal4 (Van Doren et al., 1998), UAS-Apoliner (Bloomington Stock Center), UAS-DIAP1 (Bloomington Stock Center), UAS-droncRNAi (KK100424) (Vienna Drosophila RNAi Center), UAS-Grim (D. Bennett, University of Liverpool, Liverpool, UK), tub-gal80^{ts} (Bloomington Stock Center), GFP-Atg8a (E. H. Baehricke) (Denton et al., 2009), and LacZ$^{puc\text{-}A251.1}$ (Martin-Blanco et al., 1998).

Immunofluorescence

Immunofluorescence staining of testes was performed as described previously (Cheng et al., 2008). Briefly, testes were dissected in PBS, transferred to 4% formaldehyde in PBS and fixed for 30-60 minutes. The testes were then washed in PBS-T (PBS containing 0.1% Triton-X) for at least 30 minutes, followed by incubation with primary antibody in 3% bovine serum albumin (BSA) in PBS-T at 4°C overnight. Samples were washed for 60 minutes (three 20-minute washes) in PBS-T, incubated with secondary antibody in 3% BSA in PBS-T at 4°C overnight, washed as above, and mounted in VECTASHIELD with DAPI (Vector Labs). The following primary antibodies were used: mouse anti-Adducin-like (hu li tai shao – Fly Base) [1:20; Developmental

Studies Hybridoma Bank (DSHB); developed by H.D. Lipshitz]; rat anti-vasa (1:50; DSHB), rabbit anti-vasa (1:200; d-26; Santa Cruz Biotechnology), mouse anti-fasciclin III (1:200; DSHB; developed by C. Goodman), anti-lamin Dm0 (1:200; DSHB; developed by P. A. Fisher), rabbit anti-Thr3-phsophorylated Histone H3 (PH3) (1:200; Upstate, Millipore, Billerica, CA); rat anti-BrdU [1:100, BU1/75 (ICR1); Abcam], guinea pig anti-Traffic jam (Tj) (1:1000; Dorothea Godt) (Li et al., 2003), rabbit anti-Zfh1 (1:5000; Ruth Lehmann), and rabbit anti-cleaved caspase 3 (1:400; Cell Signal). Images were taken using a Leica TCS SP5 and a Leica TCS SP8 confocal microscope with a 63x oil-immersion objective (NA=1.4) and processed using Adobe Photoshop software.

Cell Cycle Analysis

For BrdU feeding assay, previously fed or starved adult flies were transferred into fresh vials of food or starvation media containing BrdU (1 mg/mL). Methylene blue was also added to the media to monitor feeding behavior. Testes were dissected and analyzed at 12, 24, and 48 hours after initiation of BrdU feeding. For ex vivo S-phase analysis, testes were soaked in PBS with BrdU (0.1 mg/mL) for 45 min. prior to fixation. Testes from both BrdU experiments were then treated with RQ1 DNase in 1x DNase buffer (Promega) for 2 hours and incubated with primary antibody (anti-BrdU and anti-vasa) overnight at 4°C. For mitotic index analysis, testes were dissected and fixed within 5 minutes of anesthesia to minimize effects of elevated CO_2 on mitoses (Inaba et al., 2011). Testes were then stained with anti-PH3 and processed as described above.

Quantification of Cell Death

For detection of germ cell death, testes were stained with Lysotracker Red DND-99 (Life Technologies) in PBS for 30 minutes prior to formaldehyde fixation. Anti-lamin Dm0 staining (described above) was used to outline intact nuclear lamina. Dying SGs were scored according to stage by counting the number of connected Lysotracker+ and lamin+ germ cell nuclei (Fig. 2.7B-C). Dying SGs that lacked lamin staining, likely reflecting nuclear disintegration during later stages of germ cell death, were excluded from our scoring. Occasionally, dying SGs had irregular numbers of countable germ cells (e.g. 3, 6, 12); these were included in our data by rounding up to the nearest power of two (4, 8, 16).

Live Imaging

Unfixed testes were mounted onto slides with PBS and imaged within 10 minutes of dissection to minimize the effects of hypoxia. Lysotracker was used to label lysosomes and dying germ cells, and c587>Apoliner was used as a somatic marker. For visualization of GFP-Atg8a, flies were fed 1 mM Chloroquine (Sigma) for 12 hours prior to dissection. Movies were taken on a Leica SP8 confocal microscope.

2.6 Additional Data and Figures

2.6.1 Drosophila can survive in protein starvation for up to 24 days.

An important consideration underlying our studies is whether flies can survive protein starvation for an extended period of time. If protein starvation as a form of nutrient stress is too harsh and survival is dramatically decreased, it would be difficult to gauge whether changes in testes biology can be attributed to successful compensatory mechanisms. To examine the effect of protein starvation on fly lifespan, we starved flies and measured the rate of survival every three days for 30 days. We found that starved adult flies can survive well past the 18 day period of observation that we used in a majority of our experiments (Fig. 2.15). This suggests that indeed the tissue response to protein starvation is a physiologically relevant adaptation for investigation.

In comparison, adult flies that were subjected to total nutrient deprivation (no protein, no sugar, water only) could survive for no longer than 2-3 days. This implies several intriguing possibilities: 1) the flies' mechanism for coping with nutrient stress is limited to amino acids only; 2) the ability to cope with protein starvation is dependent on the availability of carbohydrates and calories; 3) total nutrient deprivation as a form of nutrient stress is too harsh for flies to adapt to long term. Elucidation of these possibilities awaits further investigation.

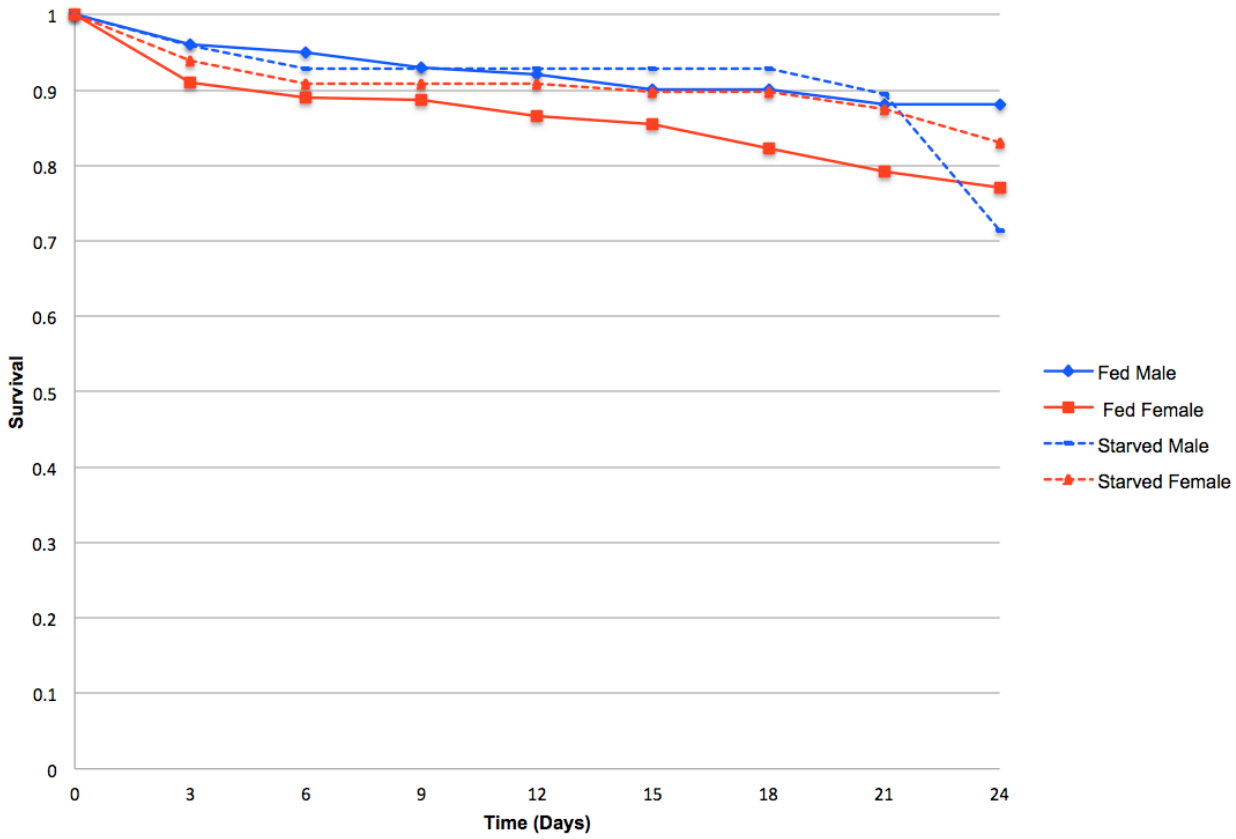

Figure 2.15 Drosophila can survive up to 24 days of protein starvation. The fraction of surviving male and female flies over time in both fed and starved conditions. 100 flies per condition were maintained in their respective vials with an initial density of 10 pairs per vial. Remaining flies were counted every three days, at which point surviving flies were transferred into new vials.

2.6.2 Drosophila testis involution occurs during normal aging between day 3 and day 6 and continues in protein starvation.

While studying the effect of starvation on Drosophila testis morphology, we noticed that involution occurs normally in fed flies as well as in starved flies (Fig. 2.16). Age-related involution in the fed condition occurs approximately between day 3 and day 6 of adulthood, after which tissue size remains relatively constant. In contrast, starved testes continue to involute after day 6. These results imply that the change in tissue size between day 3 and day 6 starved conditions may be partially due to a developmental process unrelated to nutrient conditions. However, the continued involution of starved testes compared to fed controls confirm the dramatic effect of starvation on tissue morphology.

While the reason for involution between day 3 and day 6 is currently unknown, it is reasonable to suspect that it is influenced by a change in the developmental program of spermatogenesis between the pupal stage and adulthood. In GSCs, for instance, it has been previously reported that the cell cycle regulator String (Cdc25) is rapidly diminished upon reaching adulthood, resulting in the maintenance of a slower cell cycle (Inaba et al., 2011), and similar effects have been observed in other model organisms such as C. elegans (unpublished data).

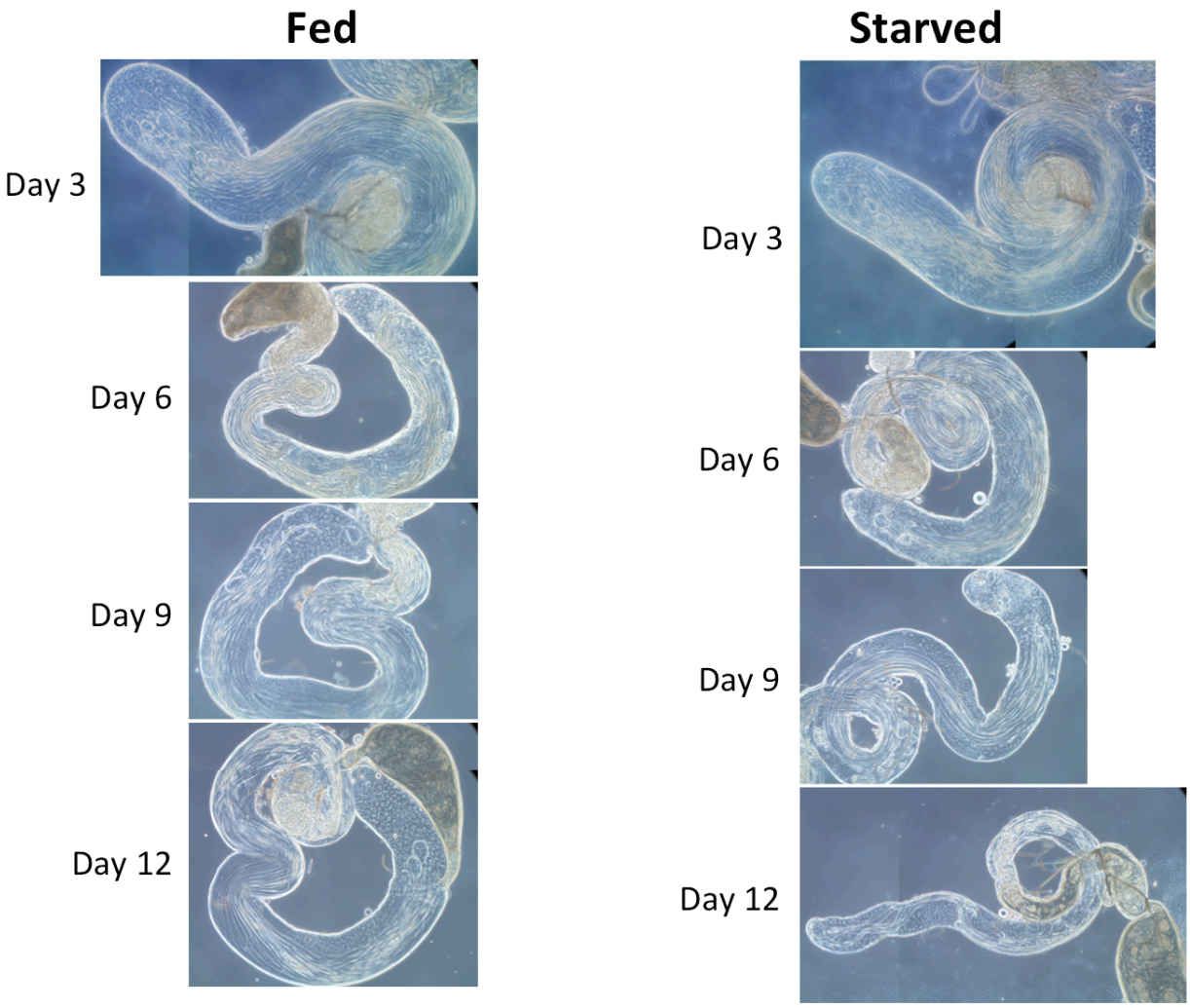

Figure 2.16 Phase contrast microscopy of testes in fed and starved conditions through 12 days after eclosion.

2.6.3 Starvation does not significantly affect hub size or Upd secretion, and GSCs maintain Stat expression.

Given the observation that GSCs are lost after 3 days of starvation and then subsequently maintained, one important question is whether starvation affects any aspects of niche size or function to influence this outcome. To address this, we first quantified hub cell number by scoring the number of hub cell nuclei per testis, which revealed that starvation does not significantly change hub cell number (Fig. 2.17A). To assess hub function, we investigated the production of Unpaired (Upd), a JAK-STAT ligand secreted by the hub that is essential for GSC identity and maintenance. We used a Upd-YFP fusion protein to assess Upd localization in the hub. Surprisingly, we observed for the first time that Upd is localized in discrete, stationary vesicles and confined to the hub-GSC interface, contrary to the previous belief that Upd forms a diffusion gradient emanating from the hub (Fig. 2.17B). These Upd vesicles were visible only in unfixed samples, as they became virtually absent after fixation and treatment with detergent. When we compared the number of Upd vesicles in hub cells between fed and starved conditions, we observed no significant difference, suggesting that neither hub size nor function is affected by starvation. Consistent with this result, when we further quantified transcriptional activity using both qRT-PCR (data not shown) and a Upd promoter fused to a YFP reporter (data not shown), we again detected no significant difference. Finally, we assessed Stat92E expression in GSCs using a GFP fusion protein and confirmed that Stat appears to be intact in starved GSCs even after GSC loss (Fig. 2.17C).

Together, these results suggest that GSC loss from 8 to 6 per testis during

starvation is likely not due to changes in hub size or Upd secretion. Although the hub-GSC interaction involves other signaling pathways that we did not explore and thus cannot rule out, it is reasonable to predict that intrinsic changes in GSCs during starvation are more likely to contribute to the selective maintenance of the remaining 6 GSCs.

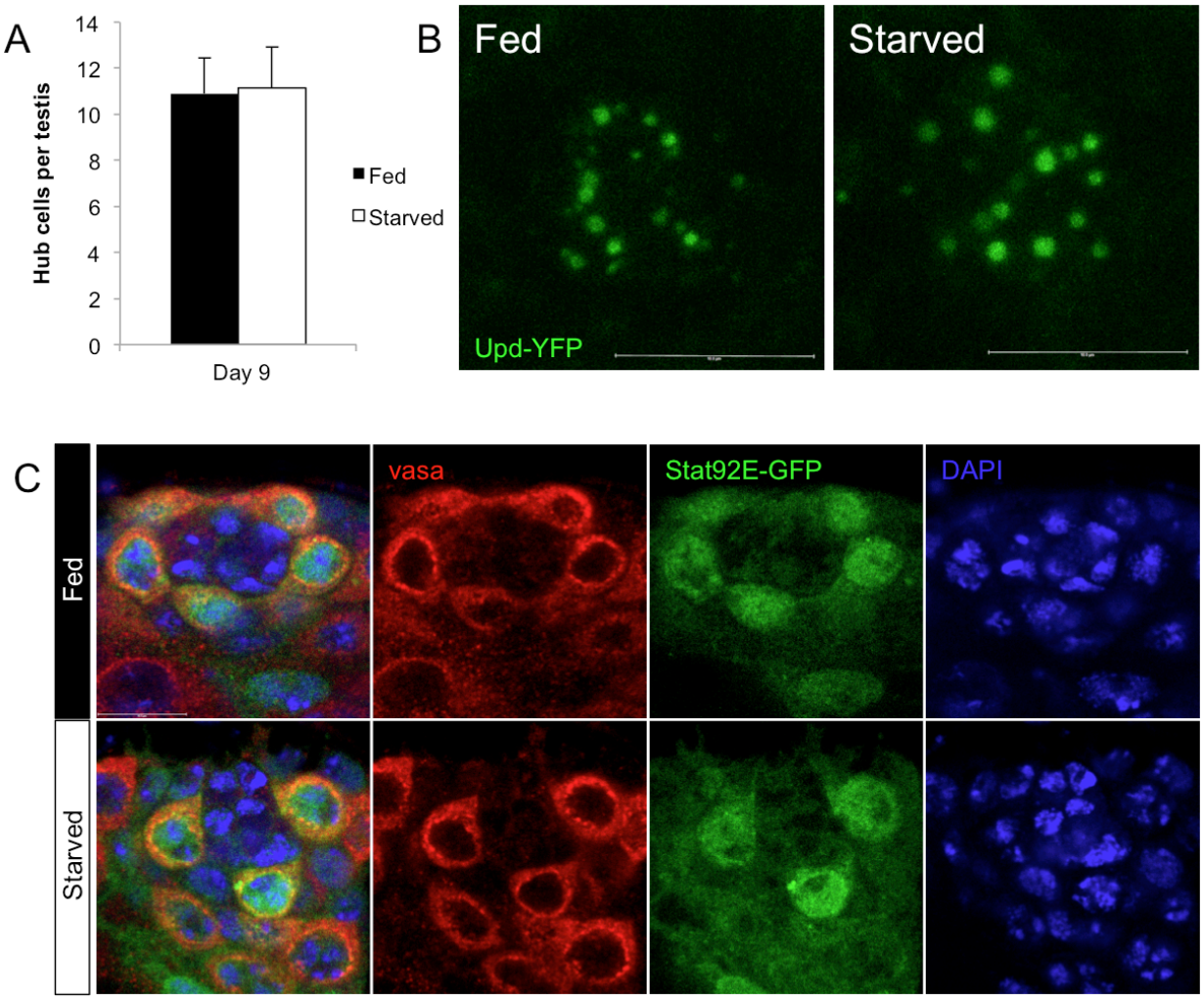

Figure 2.17 Starvation has no significant effect on hub size, Upd secretion, or Stat levels in GSCs. (A) Quantification of hub cell nuclei at 9 days after eclosion. Data presented as mean±s.d. p-value > 0.05. (B) Upd-YFP puncta are located in hub cells. Representative images taken at 9 days after eclosion. (C) Representative images of Stat92E-GFP (green) expression in GSCs at 9 days after eclosion.

2.6.4 Germ cell death does not autonomously require the initiator caspase Dronc.

The molecular mechanism of germ cell death has been elusive. Although it is believed to be a form of programmed cell death, thus far it has not been shown to follow any of the canonical cell death pathways, including caspase-3-dependent apoptosis or autophagic cell death. A recent report characterized germ cell death in the Drosophila testis and proposed a pathway involving lysosomes and the initiator caspase Dronc. When we began our analyis of SG elimination during protein starvation, we first confirmed that SG death does not proceed with effector caspase activation, as Apoliner expression in the germline does not yield positive nlsGFP localization to dying SG nuclei. To our surprise, we also found that the knockdown of Dronc using RNAi in the germline also has no effect on the basal rate of germ cell death at the 16-cell stage (Fig. 2.18A). While this previously validated RNAi construct suppresses apoptosis in CySC/CCs and also SG death during both fed and starved conditions, no significant change was observed in SG death when it was expressed only in the germline. Additionally, starvation-induced SG death in early SGs was unaffected after three days of starvation (Fig. 2.18B). These data suggest that the suppression of SG death observed in Dronc mutant alleles as previously reported reflects a non-autonomous effect as a result of inhibiting CC apoptosis, a possibility that was not considered in the study. This distinction is critical to the pursuit of correctly identifying the molecular pathways involved in programmed germ cell death. While we propose that Dronc may not play an intrinsic role in germ cell death, the idea that CC apoptosis, which does proceed through Dronc, can be responsible for initiating SG death paves the way for further investigation.

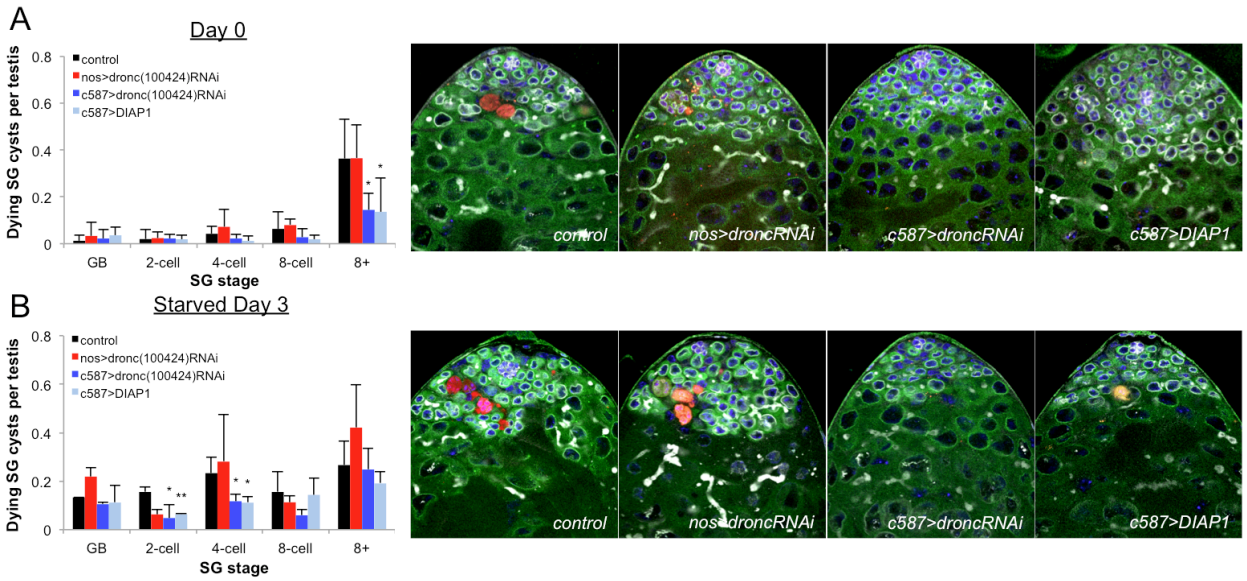

Figure 2.18 Knockdown of dronc in the germline does not affect germ cell death. (A) Quantification of germ cell death in control, nos>Dronc-RNAi, c587>Dronc-RNAi, and c587>DIAP1 upon eclosion. Dying SGs are scored according to stage. Representative images of testes are shown stained with vasa (green), fasIII (white), adducin (white), lamin (white), Lysotracker (red), and DAPI (blue). (B). Quantification of germ cell death after three days of protein starvation.

2.6.5 CySC/CC apoptosis is reversible upon refeeding and is influenced by insulin signaling

We have shown that the Drosophila testis adapts to protein starvation by eliminating SGs via CC apoptosis. To ascertain the reversibility of this mechanism for tissue involution, we measured the frequency of CC apoptosis after flies were starved and then reintroduced to protein. We found that within three days of recovery, the level of CC apoptosis returns to the level observed prior to starvation (2.19A). This result strongly suggests that the survival of CCs is directly influenced by nutrient availability.

To identify the molecular mechanism involved in the control of CC death and survival in response to nutrients, we tested the insulin signaling pathway as the most likely possibility. We manipulated insulin signaling only in the CySC/CC lineage by using the c587-gal4 driver to avoid the documented autonomous effects of insulin signaling in the germline (Roth et al., 2012). Because Drosophila insulin signaling utilizes a single receptor, a consitutively active insulin receptor mutant (InR^{A1325D}) was overexpressed to upregulate insulin signaling; a dominant negative insulin receptor mutant (InR^{K1409A}) was overexpressed to downregulate insulin signaling. We found that InR^{A1325D} overexpression in the CySC/CC lineage results in low levels of CC apoptosis during starvation (Fig. 2.19B), and that InR^{K1409A} results in high levels of CC apoptosis in both fed and starved conditions (data not shown), suggesting that insulin signaling is necessary and sufficient to influence CC survival in response to nutrients.

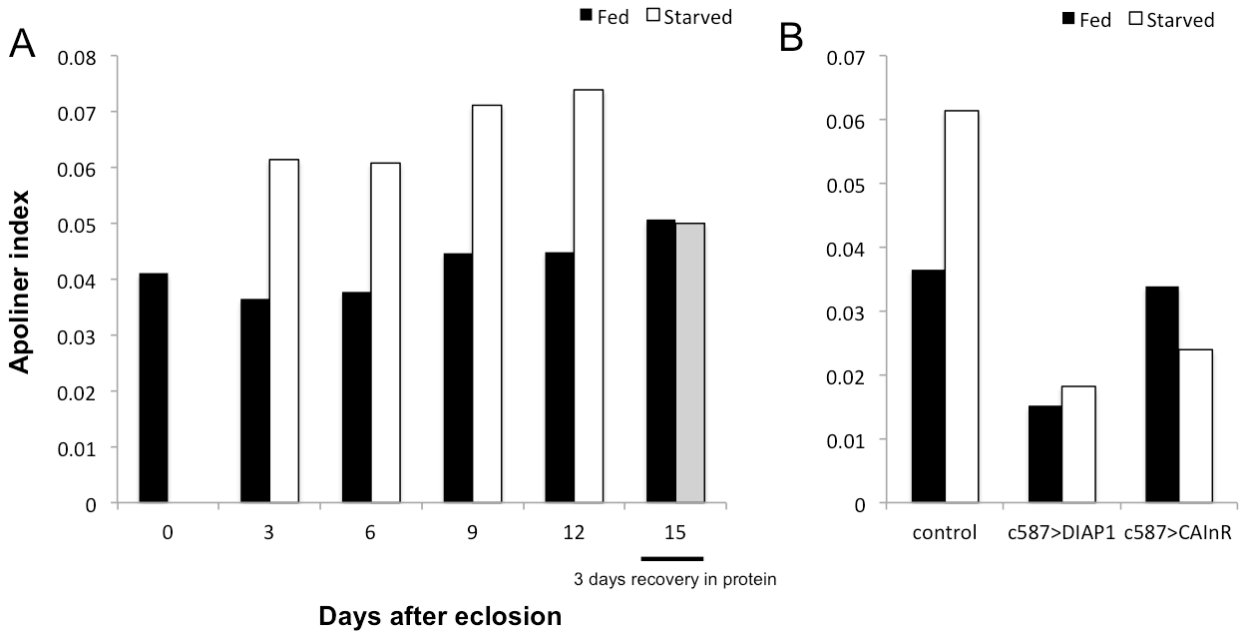

Figure 2.19 CySC/CC apoptosis is reversible and influenced by insulin signaling. (A). Quantification of CC apoptosis over 15 days. Flies were starved until day 12, upon which they were transferred back to a rich source of protein. The gray bar represents apoliner index after 3 days of refeeding. (B) Frequency of apoliner activation in control, DIAP1 overexpression, and constitutively active insulin receptor mutants. Data points were taken after 3 days of fed or starved conditions.

CHAPTER 3:
FUTURE DIRECTIONS

This work provides a strong framework for future investigation. Most significantly, by demonstrating the highly regulated and reversible effect of protein starvation on stem cells and transit-amplifying cells in the Drosophila testis, we introduce a new concept of "shifting" tissue homeostasis that has not previously been identified. Confirmation or elaboration of this phenomenon in other tissue types and model organisms thus beckons. In addition to broader conceptual advancement, however, our work provokes many enticing questions specifically pertaining to stem cells, germ cells, and even the Drosophila testis as a model system. Below I delineate several of the most prominent aims that are suitable for immediate exploration.

3.1 The mechanism of GSC loss during protein starvation

Although one of our primary conclusions is that GSCs are maintained during protein starvation rather than lost, the observation that GSC number drops from 8 to 6 between day 3 and 6 of starvation remains intriguing. What is the mechanism of GSC loss, and why is the nature of this phenomenon such that precisely two GSCs on average are lost per testis?

Our data provide several major clues towards such questions. First, we never detected GSCs to be Lysotracker+, which suggests that GSC loss does not occur via

germ cell death at the hub. The most likely possibility is thus that GSC loss occurs by detachment, after which the displaced cells can either differentiate or undergo cell death. Second, the correlation between GSC loss and the reduction in GSC centrosome misorientation appears to be highly suggestive regarding how GSCs might be selectively detach and become lost. Two major possibilities exist to explain this observation: 1) GSCs that have misoriented centrosomes after three days of starvation are specifically lost, or 2) GSCs are stochastically lost, but centrosome misorientation is reduced only after the GSC loss. We speculate that the first possibility is the more likely of the two due to the importance of centrosome orientation in ensuring the polarity of GSC division as well as cell cycle rate. It has been shown that GSCs divide only when their misoriented centrosomes become re-oriented with respect to the plane of the hub-GSC interface, and there exists a centrosome orientation checkpoint to ensure that this criterion is met prior to entry into mitosis (Cheng et al., 2008; Inaba et al., 2010; Roth et al., 2012). GSCs with misoriented centrosomes thus have slower cell cycles due to the delay prior to mitosis during which centrosome re-orient. Under protein starvation conditions, when only 6 GSCs can be supported at the hub, it is thus reasonable to speculate those with misoriented centrosomes are specifically lost.

Many tools are available to test this hypothesis, and some preliminary work has already been done. One way to determine whether there is GSC selection based on centrosome orientation is to manipulate components required for centrosome orientation as well as those involved in the centrosome orientation checkpoint. The protein Bazooka, for example, has been demonstrated to form a small patch at the hub-GSC interface, and centrosomes are thought to anchor to this structure in order to

become oriented (Inaba et al, unpublished data). Knockdown of Bazooka using RNAi thus results in increased centrosome misorientation. When we knocked down Bazooka in GSCs and studied the long-term effects of starvation, we found that GSCs could not be maintained at the hub in either fed or starved condition, suggesting that Bazooka-mediated centrosome orientation is required for GSC maintenance. Interestingly, when we mutated centrosomin (cnn), which is a critical component of the centrosome orientation checkpoint and is also required for centrosome orientation, we found that GSCs are not lost in either fed or starved conditions despite having highly misoriented centrosomes. These data thus imply that the centrosome orientation checkpoint is an important component of GSC selection while centrosome orientation itself per se is not required. A reasonable extension of this idea is that the centrosome orientation checkpoint induces slowing of the cell cycle when centrosomes are misoriented, thus causes a subset of GSCs to become less competitive at the hub.

To test this possibility, we plan to manipulate centrosome orientation in a subset of GSCs per testis to investigate its effect on competition at the hub during starvation. Bazooka and cnn knockdown can be achieved clonally using a heat-shock induction system, at which point the maintenance of the GSC clones during starvation can be compared to the wild type neighboring cells. If our hypothesis is correct, we predict that Bazooka knockdown will result in rapid loss of clones from the hub, while cnn mutant clones persist much longer compared to wild type GSCs. Cell cycle analyses such as the BrdU feeding assay and measurement of mitotic index should also be performed to verify the correlation between cell cycle progression and GSC maintenance. Furthermore, direct observation of GSC loss can be achieved using long-term (24-hour)

ex vivo live imaging techniques. To supplement these experiments, constitutively active or dominant negative forms of the insulin receptor can also be clonally expressed in GSCs to determine whether insulin signaling, which has a strong direct effect on GSC centrosome orientation and cell cycle, influences selection as well.

Another question that can be alternatively considered is why the hub can support only 6 GSCs per testis during protein starvation. Although GSC centrosome misorientation could potentially explain how such the GSCs are selected, the fact that the GSC pool must be reduced during protein starvation is striking in and of itself. For comparison, GSCs in flies cultured in “poor” media -- food containing low levels of protein as opposed to no protein in the starvation condition -- a significant fraction of GSCs have misoriented centrosomes and slowed cell cycles, but these GSCs can be maintained for at least 10 days. The reduction of GSC number is thus specific to protein starvation and cannot be explained simply by an increase in misoriented centrosomes. Because we have been unable to detect the presence of any significant perturbations to hub cell number or function, it is unlikely that starvation-induced changes to the niche environment can explain the reduction in GSC number.

One interesting possibility that can be inferred from our dedifferentiation and centrosome misorientation data is that protein starvation reveals two distinct pools of GSCs: one group of “hardy” GSCs that are strongly adherent to the hub, and one group of dedifferentiated GSCs that is less likely to be maintained under stressful physiological conditions. The observation that dedifferentiated cells do not accumulate during protein starvation is a significant clue regarding the nature of the remaining GSCs. It is possible that a pre-existing subpopulation of dedifferentiated GSCs is stochastically lost and

replaced during normal aging, but because starvation inhibits dedifferentiation, the equilibrium shifts such that these GSCs, once lost, do not become replaced, thus resulting in a reduced pool of GSCs. If found to be true, this distinction would dramatically alter our understanding of stem cell identity and how stem cell populations are maintained.

To test our hypothesis, we will first investigate whether the average of 6 GSCs maintained per testis during protein starvation can be perturbed by increasing or decreasing the number dedifferentiated GSCs. Increasing the number of dedifferentiated GSCs can be accomplished in two ways: 1) Allowing flies to age for more than 30 days, and 2) artificially removing GSCs from the hub using heat shock driven expression of the differentiating factor Bam (Brawley and Matunis, 2004; Gonczy et al., 1997), then allowing GSC number to recover via dedifferentiation at room temperature. After these flies are subjected to protein starvation, GSC number can be scored to ascertain whether the decrease from 8 to 6 GSCs per testis still occurs. Finally, to inhibit dedifferentiation, SGs that develop to the Bam+ stages can be genetically manipulated to permanently overexpress Bam, thereby preventing their ability to reattach to the hub. GSC maintenance in the absence of dedifferentiation can be assessed by aging these flies in both fed and starvation conditions.

Together, these investigations will provide further understanding of not only starvation-induced GSC loss but also provide insight toward the fundamental nature of GSC identity and maintenance.

3.2 The biological significance of preventing dedifferentiation during protein starvation

Our observation that dedifferentiated GSCs do not accumulate during protein starvation prompts many questions regarding its biological repercussions. The reprogramming of differentiating cells in physiological dedifferentiation, which is distinct from the anaplastic transformations observed in cancer cells, has recently gained significant attention in the field as a mechanism to replace lost stem cells (Sheng and Matunis, 2009). Dedifferentiation was first demonstrated in the Drosophila germline (Brawley and Matunis, 2004; Kai and Spradling, 2004) and has since been shown in other tissues such as the mammalian testis (Yoshida et al., 2007) and respiratory epithelium (Tata et al., 2013).

What makes dedifferentiation an intriguing phenomenon is that it is currently unclear how dedifferentiated stem cells differ from the “original” stem cells. If the two cell types are qualitatively dissimilar, particularly if dedifferentiated stem cells are defective at certain aspects of stem cell function, the ad hoc reconstitution of the stem cell population by dedifferentiating cells can be seen as non-ideal. The accumulation of dedifferentiated cells would potentially explain the decline in overall stem cell function during aging. However, if the two cell types are essentially equivalent, then ideas pertaining to the uniqueness of stem cell identity as well as the importance of “original” stem cells in longevity will become blurred. Currently, scant evidence exists to make either conclusion. In the Drosophila testis, dedifferentiated GSCs were shown to have highly misoriented centrosomes, slowed rates of cell cycle progression (Cheng et al., 2008), and a reduced ability to asymmetrically segregate the X/Y sister chromatids

(Yadlapalli and Yamashita, 2013). However, it is unknown whether these apparent defects have a significant impact on tissue function and fertility. Furthermore, the rare cases of physiological dedifferentiation described in the Drosophila testis occur on a constitutive basis in the mammalian male germline, where connected spermatogonial cells frequently fragment and reattach to the seminiferous tubule membrane, whereupon they restart the spermatogenic process as bona fide stem cells. This suggests that any functional differences within the heterogeneous mammalian GSC pool, if any, are minimal.

Nevertheless, the Drosophila testis remains the model system best equipped to rigorously examine the biology of dedifferentiated stem cells. A simple genetic tool allows permanent labeling of SGs that reach the Bam+ stage around the 2-cell/4-cell stage. These cells can thus be tracked if they do return to the hub and reattach as dedifferentiated GSCs. Furthermore, differentiation can be artificially induced using the heat shock promoter to ectopically express Bam and deplete the GSC niche, thus allowing for dedifferentiated GSCs to repopulate the hub. In addition, our observation that protein starvation prevents such dedifferentiated GSCs from accumulating allows us to examine the biology of specifically the non-dedifferentiated GSCs.

The first question we can ask is at what rate do the hallmarks of germline aging, which is believed to be a direct consequence of GSC aging, occur during protein starvation and after recovery. Such hallmarks include decreased GSC proliferation (Inaba et al., 2011), SG death, which reflects the probability that a GSC division results in functional gametes (Zhao et al., 2008), nucleolar fragmentation (unpublished data) and derepression of stellate, which reflects the ability of germ cells to maintain the

integrity of their genome, and overall fertility, which is a crude but important assessment of tissue function. Many of these aspects are believed to be deleteriously altered in dedifferentiated GSCs but not in non-dedifferentiated GSCs (Cheng et al., 2008; Yadlapalli and Yamashita, 2013), but the existing data are primarily correlative. Using protein starvation would thus be an effective tool to study GSC aging in the absence of dedifferentiation. If currently held beliefs are correct regarding aging and dedifferentiation, this study would shed insight toward how tissue longevity can be extended during protein starvation, a striking phenomenon that was previously described in the C. elegans gonad (Angelo and Van Gilst, 2009).

The second question we can ask is whether the rate of dedifferentiation directly correlates with nutrient availability. Observations in many organisms have suggested that overnutrition accelerates tissue aging (Cornu et al., 2013). If the accumulation of dedifferentiated GSCs can be considered a mechanism for testis aging, we can test this hypothesis by increasing the amount of dietary protein for the flies and measuring the number of dedifferentiated GSCs over time. Our data currently provides the amount of dedifferentiation in starved (no protein) and fed (moderate protein) conditions, but food with intermediate protein levels as well as excessive amounts of protein should be tested to explore a direct correlation. If our hypothesis is correct, not only would it provide a cellular mechanism for the nutrient effect on aging within a stem cell population, it would also present the idea that dedifferentiation, currently believed to be a reparative process, can be a pathologic one as well. Even if an alternative scenario is demonstrated, we would still be able to glean insight about the nature of dedifferentiation and begin to explore the currently unknown molecular mechanisms that

govern it.

3.3 Nutrient recycling in the Drosophila testis stem cell niche

Another observation that presents a ripe opportunity for follow-up investigation is the presence of autophagic and phagocytic processes in the Drosophila testis. These data suggest that nutrient recycling is an active process surrounding the stem cell niche, likely facilitated by the non-professional phagocytic activity of CySC/CCs. Based on our findings, we have proposed that the absorption of cellular debris from cell corpses is potentially a direct mechanism for how GSC activity can be supported by the death of transit-amplifying cells. However, the exact nature of this phenomenon remains to be clarified.

The most important question to be addressed is to what extent does nutrient recycling contribute to the maintenance of GSCs and tissue homeostasis during protein starvation. Because it is alternatively possible that tissue involution in and of itself is sufficient to allow the tissue to adapt to protein by reducing overall nutrient demand, it is unknown whether corpse clearance and absorption is simply an anti-inflammatory process or whether the processed nutrients are required to sustain stem cell activity. We can test these possibilities first by inhibiting autophagic and phagocytic processes during protein starvation and measuring whether the maintenance of active GSCs is affected. Candidate genes to knock down or inhibit are Atg8, Atg5, Atg1, and Basket (Bsk). The frequency of SG death as well as any changes to dying cell morphology or corpse clearance should also be assessed in order to better characterize the requirement of these genes as well as to identify compensatory mechanisms (e.g.

increased SG death/tissue involution) that may be triggered if the targeted machinery is indeed required to support tissue homeostasis during protein starvation.

To further investigate our hypothesis that nutrients -- amino acids in particular -- from dying SGs are locally absorbed and used by the GSCs, a definitive experiment would be to monitor the location of amino acids from dying SGs by selectively tagging proteins or amino acids in only SGs. The method of tagging must be specific such that GSCs or surrounding CySC/CCs are not also marked, and the nature of the tag must be such that it is resistant to lysosomal or proteasomal degradation. Although this experiment presents a great technical challenge that will likely require the development of new genetic and biochemical tools, there are simple approaches that can be tested first.

One method would be to use the Bam-gal4 driver to express a foreign protein such as ß-galactosidase in the SGs. Once the SGs die, we can determine whether any fragments of the ß-galactosidase protein can still be detected using an antibody stain. If it remains detectable, we can monitor the extent to which it becomes distributed in the surrounding CySC/CCs. Although this experiment is not likely to be successful given the amount of lysosomal degradation we suspect is involved in germ cell corpse clearance, a negative result would indicate that proteolytic degradation of the ß-galactosidase protein is in fact occurring within the acidified dying SG compartments. This experiment can be repeated using other proteins as well, particularly those with smaller tags such as His6, myc, or HA, which likely have epitopes that are more resistant to degradation. However, our ability to detect these tags likely faces the same technical limitations.

A potentially more definitive but more technically difficult approach would be to

biochemically tag amino acids in SGs. For example, using the Bam-gal4 driver, we could express an exogenous enzyme in the SGs that performs an irreversible modification to amino acids or even nucleotides. Importantly, such a modification should ideally not interfere with normal cellular processes. One possibility would be to use an enzyme that converts a basic amino acid or nucleotide into one not normally found in Drosophila. During prolonged starvation, testes expressing this enzyme in the SG population can be fractionated into its cellular components, and levels of the modified amino acid or nucleotide in GSCs and CySC/CCs can be subsequently assayed using biochemical methods.

Once established, a strategy to detect nutrient recycling from one cell type to another would be a significant contribution to the field of cell biology. The study of amino acid homeostasis has thus far been limited to intracellular processes, and we are only beginning to understand how the basic building blocks of life can be shared within a tissue. The role that intercellular amino acid recycling plays is potentially a fundamental requirement to tissue homeostasis, and defects in this process could contribute to many diseases. Investigation of this phenomenon in a simple and powerful model system such as the Drosophila testis could thus yield many new and unpredictable insights.

3.4 The molecular mechanism of germ cell death

As described above, much of the intrinsic molecular machinery required for programmed germ cell death remains unidentified. While our work is consistent with the previous report that programmed germ cell death involves lysosomes (Yacobi-Sharon et al., 2013), our data suggest that the initiator caspase Dronc has a non-autonomous effect originating from the soma rather than directly in the germline. Our characterization of germ cell death, particularly with regard to morphology and stage, thus forms a solid basis from which to further explore germ cell death. Most significantly, our work confirms that dying SGs always die together, that dying SGs become acidified, and that under normal circumstances SGs are more likely to die at the 16-cell stage than any other stage.

Interestingly, we have acquired preliminary data that provide more nuanced characterization of SG death. We detected the presence of stable acetylated microtubules in dying SGs, and these structures have a morphology reminiscent of nurse cell death in the female germline (Grieder et al., 2000). These acetylated microtubules appear during the early stages of SG death and disappear during advanced stages. In addition, we noticed that ring canals, marked by Pavarotti-GFP (Minestrini et al., 2002), become aligned when dying SG cells round up, which is also reminiscent of nurse cell death. These observations suggest that male germ cell death may have features similar to programmed nurse cell death in the female germline. Using this connection to our advantage, we can conduct a focused screen to manipulate genes known to be involved in germ cell death within the Drosophila ovary, and we can use the morphological parameters that we have established to determine whether any

of these genes are involved in male germ cell death and at what stage of cell death they may act. This screen can be expanded to include a wide array of genes with either knockdown or overexpression strains available in public stock centers. Importantly, to confine the manipulations to the germline so as to avoid non-autonomous effects, the germline-specific nanos-gal4 driver will be used to drive expression of the mutant alleles.

More intriguingly, we suspect that not all the germ cells within an SG cyst die simultaneously. Observations from our experiments involving BrdU, Lysotracker, and Lamin suggest that particularly at the 16-cell stage, oftentimes one or two cells within the SG cyst are out of synchrony with the others. These cells are either BrdU+ or BrdU- while the other cells display the opposite (Fig. 3.1C), they are slightly different in size than the other cells, and they can also have either relatively delayed or accelerated Lamin disintegration (data not shown). While this unsynchronized behavior in one or two cells within a cyst is reminiscent of oocyte determination and provides yet another parallel between male and female germ cell death, it is also reason to suspect that a hierarchy exists within the interconnected SG cyst. It is tempting to speculate that these one or two cells that are out of synchrony and have oocyte-like behavior are the oldest germ cells in the 16-cell cyst. Many questions thus arise: Do these cells play a role in determining the survival of their interconnected brethren? Can we identify functional differences to distinguish these cells before they die? Also, to further explore the oocyte analogy, is there any transfer of cytoplasm towards these idiosyncratic cells during germ cell death?

After more preliminary data are collected to determine the frequency that one or

two cells out of 16 are behaving differently from the rest, we can perform many exploratory experiments to ascertain the nature of these cells. The question of hierarchy and synchronicity, for example, can be approached in straightforward manner using an established femtosecond laser ablation technique in live Drosophila testes. In this manner, we can employ rapid and powerful laser pulses to enucleate SG cells without damaging surrounding structures and then observe the consequences (Chen et al, unpublished data). Can we perturb the manner in which SGs die by targeting specific cells within the cyst? After laser ablation of a single cell in a 16-cell SG, we anticipate two major possibilities: 1) Only the ablated cell dies, while the rest of the SGs survive and continue to develop, or 2) The entire cyst dies. By visualizing SGs with nuclear markers as well as fluorescent ring canals, we can target cells by their presumed age, i.e. cells with only 1 ring canal are the youngest and cells with 4 ring canals are the oldest. It is possible that the expected outcomes differ based on the age of the enucleated cell, and such a result would demonstrate a clear hierarchy and lead to even more fascinating inquiry -- if there are hierarchical differences between individual SGs, are these differences structural, epigenetic, or genetic in nature, and do they translate to the gametes that they eventually become? If the laser ablation experiment demonstrates that the consequences are equivalent, then the exact nature of the consequence -- whether only one cell dies or the entire cyst dies -- would be highly revealing. If only one cell dies, this would suggest that synchronous SG death is not an autonomously regulated event. If the entire cyst dies, then identifying both the signal and the manner by which programmed germ cell death is communicated between each cell within the cyst would be a logical follow-up.

These investigations would thus have a significant impact on our understanding of germ cell death and reproductive biology. Gametogenesis is a fundamental process in sexually reproducing organisms, yet we still have yet to fully appreciate the complex yet simultaneously elegant mechanisms governing germ cell development that have evolved over millions of years to optimize reproductive advantage.

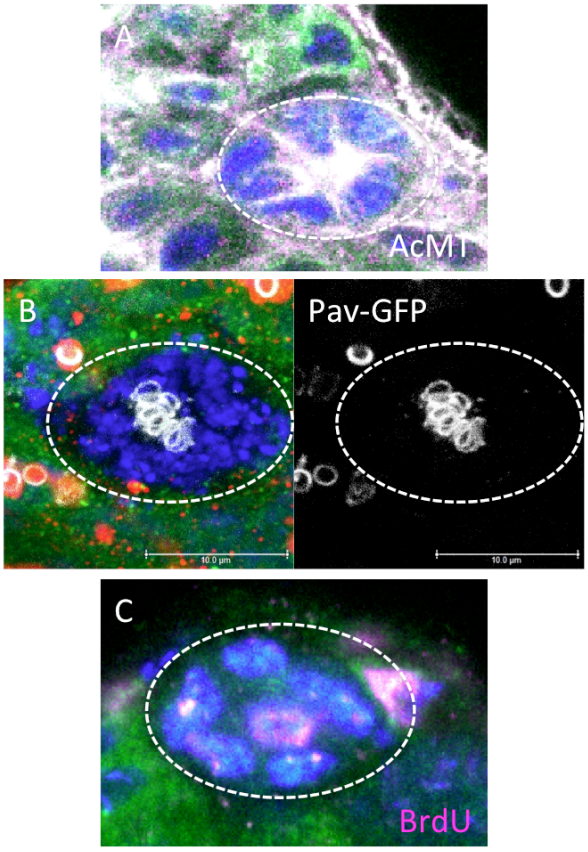

Figure 3.1 Dying SGs have stabilized acetylated microtubules, aligned ring canals, and asynchronous cells. (A) Dying SGs have acetylated microtubules (white) that radiate outwards from the center. (B) Pavarotti-GFP visualization shows clustered and aligned ring canals toward the center of the dying SG cyst. (C) Within a dying cyst, one or two germ cells is BrdU+ while the others are not, signifying asynchronicity among the SGs.

CHAPTER 4: CONCLUSIONS

Stem cells play a critical role in the regulation of tissue homeostasis, but stem cells do not act alone when tissues must adapt to changing nutrient conditions. It is thus important to understand not only the role of stem cells but also that of differentiating and support cells in order to fully appreciate the coordinated mechanisms that govern dynamic tissue homeostasis.

In the work presented in this thesis, I demonstrate that germline stem cells (GSCs) are not significantly perturbed during protein starvation in the Drosophila testis. There is a minor reduction in number from 8 to 6 per testis, but 6 GSCs can be maintained long term without significant turnover (GSC loss and replacement via dedifferentiation). Furthermore, GSC cell cycle activity remains unchanged from that of fed conditions, and niche function remains intact. I provide strong evidence to conclude that active GSCs can be maintained during starvation, which is contrary to previous interpretations based on incomplete data favoring a stem cell-focused mechanism for starvation-induced tissue involution. This new conclusion thus led to further inquiry regarding how spermatogenesis can be downregulated downstream of the GSCs.

I next show that the transit-amplifying spermatogonia (SGs) respond to protein starvation by dying in early developmental stages. Whereas SGs normally die in a nutrient-independent manner at the 16-cell stage prior to becoming spermatocytes, protein starvation causes them to die at the 2- and 4-cell stages. The decrease in early

SG survival over time thus leads to an overall reduction in the number of SGs per testis, particularly in the subsequent stages of development. Because no significant change in the SGs' rate of division could be detected, it can be concluded that that elimination of SGs via starvation-induced death is the mechanism for decreased germ cell production.

I next show that starvation-induced SG death is not initiated autonomously. Instead, it requires the apoptosis of the encapsulating CCs, which is caspase-3-dependent, increases in frequency during starvation, and is likely regulated by the insulin signaling pathway. Inhibition of CC apoptosis using knockdown of initiator caspase Dronc or overexpression of antiapoptotic protein DIAP1 in the CySC/CC lineage is sufficient to block starvation-induced SG death. Although the autonomous mechanism for SG death remains unknown, identifying the requirement of CC apoptosis provides a tool with which to inhibit the effect of starvation on the CCs and SGs and which in turn allows for exploration of its biological significance.

To this end, I demonstrate that CC-mediated SG elimination is required for the maintenance of GSC number and proliferative activity and also for the preservation of tissue homeostasis. Testes lacking the ability to achieve regulated elimination of SGs during protein starvation cannot stably maintain their GSC population. Not only is GSC number reduced, but GSC cell cycle also becomes slower compared to both wild type as well as the fed controls. After prolonged starvation, irreversible tissue damage occurs to a large fraction of testes, and testis function cannot be recovered even after the reintroduction of dietary protein. In these testes, germ cell development appears to be impaired at the spermatocyte stage, and no mature sperm are produced. In a small fraction of testes, the entire germ cell population becomes depleted after prolonged

starvation, and recovery of the germline is no longer possible. Strikingly, the lack of germ cells results in an overproliferation of undifferentiated CySCs, likely arising from an abnormal signaling environment. Taken together, it can be concluded that the regulated elimination of SGs is important in order to reversibly shift tissue homeostasis -- altering the ratios of the cells within the tissue must be achieved in a manner that maintains tissue integrity and allow for tissue function to recover once nutrients are reintroduced (Fig. 4.1).

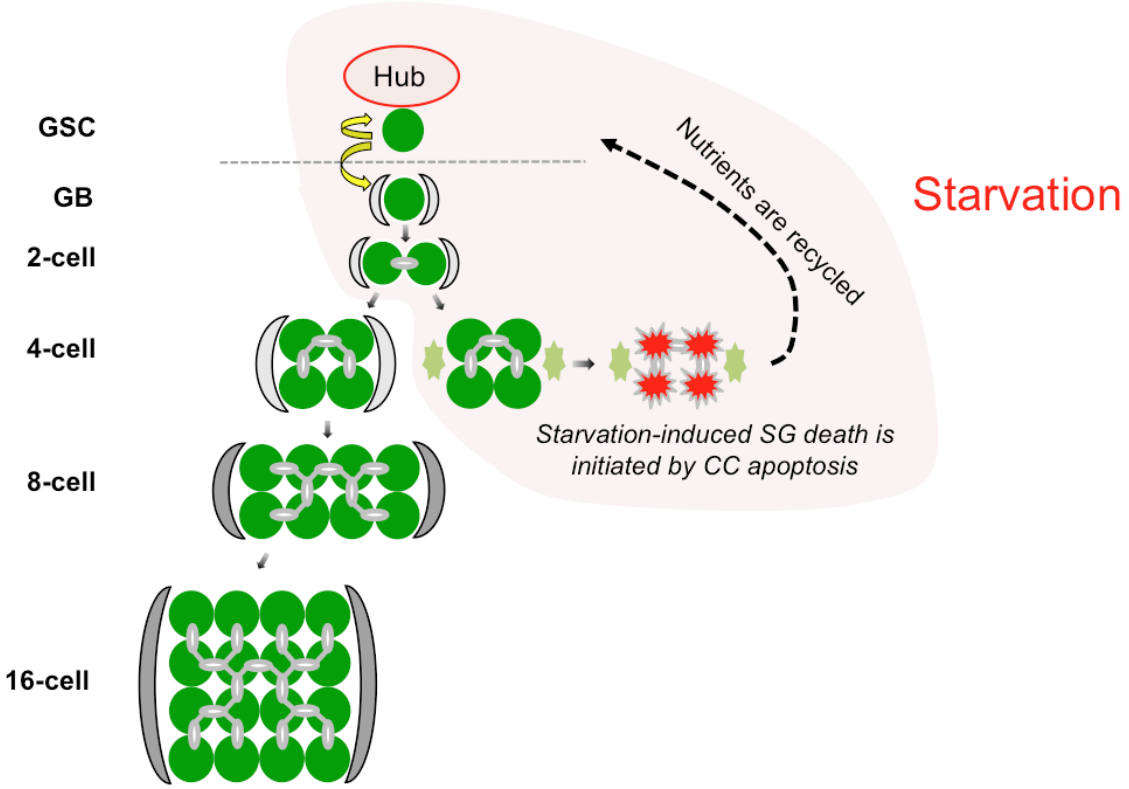

Figure 4.1 The starvation response of the Drosophila testis. GSCs are maintained and continue to divide asymmetrically while SGs die in a CC-mediated manner to decrease germ cell production and provide a local source of nutrients.

Finally, I show the existence of phagocytic and autophagic processes in the testis in association with SG death. These processes have been clearly demonstrated in other

tissues to contribute to nutrient recycling and corpse clearance. The presence of these phenomena in the Drosophila testis thus suggests that SG death may additionally contribute to GSC activity and tissue maintenance by providing a source of nutrients that can be locally reabsorbed and recycled. While more work is required to fully test this idea, the data illuminate several promising directions for future investigation.

Taken together, this thesis highlights several important concepts that may dramatically change our understanding of tissue homeostasis. First, it introduces the previously unexplored idea of “shifting” tissue homeostasis, that different cells within a tissue must undergo coordinated changes in behavior in order to appropriately and reversibly respond to environmental conditions or tissue needs. This idea highlights the need to interpret cell behavior in context of the tissue as a whole in order to fully grasp the biological significance. Second, it convincingly shows an example of a tissue in which the stem cells are not the primary point of regulation in response to nutrient changes. This challenges the field to reconsider the role that stem cells play in the regulation of tissue homeostasis and to potentially reinterpret existing data correlating stem cell behavior with nutrients and longevity. Finally, it demonstrates a case of tumorigenesis in vivo in which the cells likely have a “wild-type” genotype, in which no mutations to oncogenes, tumor suppressors, or other factors important to differentiation are responsible for driving aberrant, overproliferative behavior. This finding enhances our current understanding of tumorigenic processes, and it has numerous exciting implications towards our understanding of cancer biology and approach to therapy.

REFERENCES

Ables, E.T., Laws, K.M., and Drummond-Barbosa, D. (2012). Control of adult stem cells in vivo by a dynamic physiological environment: diet-dependent systemic factors in Drosophila and beyond. Wiley interdisciplinary reviews Developmental biology *1*, 657-674.

Ajmani, R.S., and Rifkind, J.M. (1998). Hemorheological changes during human aging. Gerontology *44*, 111-120.

Angelo, G., and Van Gilst, M.R. (2009). Starvation protects germline stem cells and extends reproductive longevity in C. elegans. Science *326*, 954-958.

Bardet, P.L., Kolahgar, G., Mynett, A., Miguel-Aliaga, I., Briscoe, J., Meier, P., and Vincent, J.P. (2008). A fluorescent reporter of caspase activity for live imaging. Proc Natl Acad Sci U S A *105*, 13901-13905.

Benmimoun, B., Polesello, C., Waltzer, L., and Haenlin, M. (2012). Dual role for Insulin/TOR signaling in the control of hematopoietic progenitor maintenance in Drosophila. Development *139*, 1713-1717.

Blanpain, C., and Fuchs, E. (2009). Epidermal homeostasis: a balancing act of stem cells in the skin. Nature reviews Molecular cell biology *10*, 207-217.

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

Brisken, C., and O'Malley, B. (2010). Hormone action in the mammary gland. Cold Spring Harbor perspectives in biology *2*, a003178.

Byrd, D.T., and Kimble, J. (2009). Scratching the niche that controls Caenorhabditis elegans germline stem cells. Seminars in cell & developmental biology *20*, 1107-1113.

Chell, J.M., and Brand, A.H. (2010). Nutrition-responsive glia control exit of neural stem cells from quiescence. Cell *143*, 1161-1173.

Cheng, J., Tiyaboonchai, A., Yamashita, Y.M., and Hunt, A.J. (2011). Asymmetric division of cyst stem cells in Drosophila testis is ensured by anaphase spindle repositioning. Development *138*, 831-837.

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

Clevers, H. (2013). The intestinal crypt, a prototype stem cell compartment. Cell *154*, 274-284.

Cornu, M., Albert, V., and Hall, M.N. (2013). mTOR in aging, metabolism, and cancer.

Current opinion in genetics & development *23*, 53-62.

Davies, E.L., and Fuller, M.T. (2008). Regulation of self-renewal and differentiation in adult stem cell lineages: lessons from the Drosophila male germ line. Cold Spring Harbor symposia on quantitative biology *73*, 137-145.

de Cuevas, M., and Matunis, E.L. (2011). The stem cell niche: lessons from the Drosophila testis. Development *138*, 2861-2869.

De Mey, J.R., and Freund, J.N. (2013). Understanding epithelial homeostasis in the intestine: An old battlefield of ideas, recent breakthroughs and remaining controversies. Tissue barriers *1*, e24965.

Decotto, E., and Spradling, A.C. (2005). The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals. Dev Cell *9*, 501-510.

Denton, D., Shravage, B., Simin, R., Mills, K., Berry, D.L., Baehrecke, E.H., and Kumar, S. (2009). Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. Curr Biol *19*, 1741-1746.

Diaz-Flores, L., Jr., Madrid, J.F., Gutierrez, R., Varela, H., Valladares, F., Alvarez-Arguelles, H., and Diaz-Flores, L. (2006). Adult stem and transit-amplifying cell location. Histology and histopathology *21*, 995-1027.

Dragojlovic-Munther, M., and Martinez-Agosto, J.A. (2012). Multifaceted roles of PTEN and TSC orchestrate growth and differentiation of Drosophila blood progenitors. Development *139*, 3752-3763.

Drummond-Barbosa, D., and Spradling, A.C. (2001). Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. Dev Biol *231*, 265-278.

Etchegaray, J.I., Timmons, A.K., Klein, A.P., Pritchett, T.L., Welch, E., Meehan, T.L., Li, C., and McCall, K. (2012). Draper acts through the JNK pathway to control synchronous engulfment of dying germline cells by follicular epithelial cells. Development *139*, 4029-4039.

Fielenbach, N., and Antebi, A. (2008). C. elegans dauer formation and the molecular basis of plasticity. Genes & development *22*, 2149-2165.

Flaherty, M.S., Salis, P., Evans, C.J., Ekas, L.A., Marouf, A., Zavadil, J., Banerjee, U., and Bach, E.A. (2010). chinmo is a functional effector of the JAK/STAT pathway that regulates eye development, tumor formation, and stem cell self-renewal in Drosophila. Dev Cell *18*, 556-568.

Fuller, M.T., and Spradling, A.C. (2007). Male and female Drosophila germline stem cells: two versions of immortality. Science *316*, 402-404.

George, T.I. (2012). Malignant or benign leukocytosis. Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program *2012*, 475-484.

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

Gonczy, P., Matunis, E., and DiNardo, S. (1997). bag-of-marbles and benign gonial cell neoplasm act in the germline to restrict proliferation during Drosophila spermatogenesis. Development *124*, 4361-4371.

Grieder, N.C., de Cuevas, M., and Spradling, A.C. (2000). The fusome organizes the microtubule network during oocyte differentiation in Drosophila. Development *127*, 4253-4264.

Guillot, C., and Lecuit, T. (2013). Mechanics of epithelial tissue homeostasis and morphogenesis. Science *340*, 1185-1189.

Guo, S. (2014). Insulin signaling, resistance, and the metabolic syndrome: insights from mouse models into disease mechanisms. The Journal of endocrinology *220*, T1-T23.

Haase, V.H. (2013). Regulation of erythropoiesis by hypoxia-inducible factors. Blood reviews *27*, 41-53.

Inaba, M., Yuan, H., Salzmann, V., Fuller, M.T., and Yamashita, Y.M. (2010). E-cadherin is required for centrosome and spindle orientation in Drosophila male germline stem cells. PloS one *5*, e12473.

Inaba, M., Yuan, H., and Yamashita, Y.M. (2011). String (Cdc25) regulates stem cell maintenance, proliferation and aging in Drosophila testis. Development *138*, 5079-5086.

Jewell, J.L., and Guan, K.L. (2013). Nutrient signaling to mTOR and cell growth. Trends in biochemical sciences *38*, 233-242.

Johnson, F.B., Marciniak, R.A., and Guarente, L. (1998). Telomeres, the nucleolus and aging. Curr Opin Cell Biol *10*, 332-338.

Kai, T., and Spradling, A. (2004). Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. Nature *428*, 564-569.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Yilmaz, O.H., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kim, S.G., Buel, G.R., and Blenis, J. (2013). Nutrient regulation of the mTOR complex 1 signaling pathway. Molecules and cells *35*, 463-473.

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

Leatherman, J.L., and Dinardo, S. (2010). Germline self-renewal requires cyst stem cells and stat regulates niche adhesion in Drosophila testes. Nat Cell Biol *12*, 806-811.

Leulier, F., Ribeiro, P.S., Palmer, E., Tenev, T., Takahashi, K., Robertson, D., Zachariou, A., Pichaud, F., Ueda, R., and Meier, P. (2006). Systematic in vivo RNAi analysis of putative components of the Drosophila cell death machinery. Cell Death Differ *13*, 1663-1674.

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

Lim, J.G., and Fuller, M.T. (2012). Somatic cell lineage is required for differentiation and

not maintenance of germline stem cells in Drosophila testes. Proc Natl Acad Sci U S A *109*, 18477-18481.

Lopes, F.L., Desmarais, J.A., and Murphy, B.D. (2004). Embryonic diapause and its regulation. Reproduction *128*, 669-678.

Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. Cell *153*, 1194-1217.

Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A.M., and Martinez-Arias, A. (1998). puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. Genes & development *12*, 557-570.

Martinez, J., Almendinger, J., Oberst, A., Ness, R., Dillon, C.P., Fitzgerald, P., Hengartner, M.O., and Green, D.R. (2011). Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proc Natl Acad Sci U S A *108*, 17396-17401.

McLeod, C.J., Wang, L., Wong, C., and Jones, D.L. (2010). Stem cell dynamics in response to nutrient availability. Curr Biol *20*, 2100-2105.

McPhee, C.K., and Baehrecke, E.H. (2010). The engulfment receptor Draper is required for autophagy during cell death. Autophagy *6*, 1192-1193.

Mehta, P., Henault, J., Kolbeck, R., and Sanjuan, M.A. (2014). Noncanonical autophagy: one small step for LC3, one giant leap for immunity. Current opinion in immunology *26C*, 69-75.

Mihaylova, M.M., Sabatini, D.M., and Yilmaz, O.H. (2014). Dietary and Metabolic Control of Stem Cell Function in Physiology and Cancer. Cell Stem Cell *14*, 292-305.

Minestrini, G., Mathe, E., and Glover, D.M. (2002). Domains of the Pavarotti kinesin-like protein that direct its subcellular distribution: effects of mislocalisation on the tubulin and actin cytoskeleton during Drosophila oogenesis. Journal of cell science *115*, 725-736.

Morrison, S.J., and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. Nature *441*, 1068-1074.

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

Ni, H.M., Bockus, A., Wozniak, A.L., Jones, K., Weinman, S., Yin, X.M., and Ding, W.X. (2011). Dissecting the dynamic turnover of GFP-LC3 in the autolysosome. Autophagy *7*, 188-204.

Orme, M., and Meier, P. (2009). Inhibitor of apoptosis proteins in Drosophila: gatekeepers of death. Apoptosis *14*, 950-960.

Padilla, P.A., and Ladage, M.L. (2012). Suspended animation, diapause and quiescence: arresting the cell cycle in C. elegans. Cell Cycle *11*, 1672-1679.

Peng, Y., and Axelrod, J.D. (2012). Asymmetric protein localization in planar cell polarity: mechanisms, puzzles, and challenges. Current topics in developmental biology *101*, 33-53.

Roth, T.M., Chiang, C.Y., Inaba, M., Yuan, H., Salzmann, V., Roth, C.E., and Yamashita, Y.M. (2012). Centrosome misorientation mediates slowing of the cell cycle under limited nutrient conditions in Drosophila male germline stem cells. Mol Biol Cell *23*, 1524-1532.

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

Shaw, D., Gohil, K., and Basson, M.D. (2012). Intestinal mucosal atrophy and adaptation. World journal of gastroenterology : WJG *18*, 6357-6375.

Sheng, X.R., Brawley, C.M., and Matunis, E.L. (2009). Dedifferentiating spermatogonia outcompete somatic stem cells for niche occupancy in the Drosophila testis. Cell Stem Cell *5*, 191-203.

Sheng, X.R., and Matunis, E.L. (2009). Make room for dedifferentiation. Fly *3*, 283-285.

Shields, A.R., Spence, A.C., Yamashita, Y.M., Davies, E.L., and Fuller, M.T. (2014). The actin-binding protein profilin is required for germline stem cell maintenance and germ cell enclosure by somatic cyst cells. Development *141*, 73-82.

Shim, J., Gururaja-Rao, S., and Banerjee, U. (2013). Nutritional regulation of stem and progenitor cells in Drosophila. Development *140*, 4647-4656.

Signer, R.A., and Morrison, S.J. (2013). Mechanisms that regulate stem cell aging and life span. Cell Stem Cell *12*, 152-165.

Sinden, D., Badgett, M., Fry, J., Jones, T., Palmen, R., Sheng, X., Simmons, A., Matunis, E., and Wawersik, M. (2012). Jak-STAT regulation of cyst stem cell development in the Drosophila testis. Dev Biol *372*, 5-16.

Snippert, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D.*, et al.* (2010). Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell *143*, 134-144.

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

Tata, P.R., Mou, H., Pardo-Saganta, A., Zhao, R., Prabhu, M., Law, B.M., Vinarsky, V., Cho, J.L., Breton, S., Sahay, A.*, et al.* (2013). Dedifferentiation of committed epithelial cells into stem cells in vivo. Nature *503*, 218-223.

Tatar, M., Post, S., and Yu, K. (2014). Nutrient control of Drosophila longevity. Trends in endocrinology and metabolism: TEM.

Tatar, M., and Yin, C. (2001). Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms. Experimental gerontology *36*, 723-738.

Tazuke, S.I., Schulz, C., Gilboa, L., Fogarty, M., Mahowald, A.P., Guichet, A., Ephrussi, A., Wood, C.G., Lehmann, R., and Fuller, M.T. (2002). A germline-specific gap junction protein required for survival of differentiating early germ cells. Development *129*, 2529-

2539.

Thomas, S.E., Dykes, P.J., and Marks, R. (1985). Plantar hyperkeratosis: a study of callosities and normal plantar skin. The Journal of investigative dermatology *85*, 394-397.

Tran, V., Feng, L., and Chen, X. (2013). Asymmetric distribution of histones during Drosophila male germline stem cell asymmetric divisions. Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology *21*, 255-269.

Van Doren, M., Williamson, A.L., and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. Curr Biol *8*, 243-246.

Venkataraman, K., Khurana, S., and Tai, T.C. (2013). Oxidative stress in aging--matters of the heart and mind. International journal of molecular sciences *14*, 17897-17925.

Voog, J., D'Alterio, C., and Jones, D.L. (2008). Multipotent somatic stem cells contribute to the stem cell niche in the Drosophila testis. Nature *454*, 1132-1136.

Wabitsch, M., Hauner, H., Heinze, E., and Teller, W.M. (1995). The role of growth hormone/insulin-like growth factors in adipocyte differentiation. Metabolism: clinical and experimental *44*, 45-49.

Wang, L., McLeod, C.J., and Jones, D.L. (2011). Regulation of adult stem cell behavior by nutrient signaling. Cell Cycle *10*, 2628-2634.

Yacobi-Sharon, K., Namdar, Y., and Arama, E. (2013). Alternative germ cell death pathway in Drosophila involves HtrA2/Omi, lysosomes, and a caspase-9 counterpart. Dev Cell *25*, 29-42.

Yadlapalli, S., and Yamashita, Y.M. (2012). Spindle positioning in the stem cell niche. Wiley interdisciplinary reviews Developmental biology *1*, 215-230.

Yadlapalli, S., and Yamashita, Y.M. (2013). Chromosome-specific nonrandom sister chromatid segregation during stem-cell division. Nature *498*, 251-254.

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

Yamashita, Y.M., Yuan, H., Cheng, J., and Hunt, A.J. (2010). Polarity in stem cell division: asymmetric stem cell division in tissue homeostasis. Cold Spring Harbor perspectives in biology *2*, a001313.

Yilmaz, O.H., Katajisto, P., Lamming, D.W., Gultekin, Y., Bauer-Rowe, K.E., Sengupta, S., Birsoy, K., Dursun, A., Yilmaz, V.O., Selig, M.*, et al.* (2012). mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. Nature *486*, 490-495.

Yoshida, S., Nabeshima, Y., and Nakagawa, T. (2007). Stem cell heterogeneity: actual and potential stem cell compartments in mouse spermatogenesis. Annals of the New York Academy of Sciences *1120*, 47-58.

Yue, W., Santen, R.J., Wang, J.P., Li, Y., Verderame, M.F., Bocchinfuso, W.P., Korach, K.S., Devanesan, P., Todorovic, R., Rogan, E.G.*, et al.* (2003). Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. The

Journal of steroid biochemistry and molecular biology *86*, 477-486.

Zhao, R., Xuan, Y., Li, X., and Xi, R. (2008). Age-related changes of germline stem cell activity, niche signaling activity and egg production in Drosophila. Aging Cell *7*, 344-354.