A novel mechanism of spermatogonia death in *Drosophila* that contributes to tissue homeostasis during starvation

By

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On graduate school and tissue homeostasis:

逝者如斯（水），而未嘗往也。盈虛者如彼（月），而卒莫消長也。蓋將自其變者而觀之，則天地曾不能以一瞬。自其不變者而觀之，則物與我皆無盡也。

蘇軾*.前赤壁賦*

The stream flows away, but never dries up. The moon may appear full or crescent-shaped, but it never changes its size. From the viewpoint of change, the world cannot remain the same for longer than a blink. From the viewpoint of constancy, everything including us will last forever.

- Su Shi*, Ode to the Red Cliff* (1082)

"'Who are YOU?' said the Caterpillar.

Alice replied, rather shyly, 'I-I hardly know, sir, just at present-- at least I know who I WAS when I got up this morning, but I think I must have been changed several times since then.'"

- Lewis Carroll, Alice in Wonderland (1865)

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Table of Contents

Acknowledgements.....ii

List of Figures.....ix

List of Abbreviations.....xi

Abstract.....xiii

Chapter 1: Introduction.....1

1.1 Tissue homeostasis as one aspect of life.....1

1.1.1 How tissue homeostasis can be achieved.....2

1.1.2 Components that contributes to tissue homeostasis.....3

1.1.2a Cell death.....3

1.1.2b Stem cell division governs tissue homeostasis.....5

1.1.2c Transit-amplifying cell proliferation.....8

1.1.2d Regeneration in non-stem cell tissues.....10

1.1.2e Differentiation.....11

1.2 Drosophila spermatogenesis as a model system to study tissue

homeostasis……11

1.2.1 The stem cells in the Drosophila testis……..…..12

1.2.1a JAK/STAT signaling regulates CySC and GSC maintenance……..…13

1.2.1b BMP signaling is required for GSC maintenance……..13

1.2.1c GSC and CySC asymmetric division…….....……...14

1.2.2 CCs regulate germline differentiation and survival…..…..15

1.2.3 Interdependence of germline and soma……..……17

1.2.3a Germline regulates cyst cell morphology…….17

1.2.3b Regulation of cell division between CySC and GSC……..18

1.3 Modulation of tissue homeostasis in response to functional demand and environmental change……..……19

1.3.1 Morphological changes in tissues facing starvation induced stress.....20

1.3.2 Responses to nutrient deprivation in the germline……...21

1.3.3 Nutrient availability regulates tissue homeostasis in the Drosophila testis…….22

1.4 Figure. …….....25

1.5 References……...29

Chapter 2: *spict*, a cyst cell-specific gene, regulates starvation-induced spermatogonial cell death in the *Drosophila* testis…….....35

2.1 Abstract……...….35

2.2 Introduction…….....……35

2.3 Results…….....37

2.3.1 spictis expressed in differentiating cyst cells….....…37

2.3.2 *spict* expression can be used to better identify the CySC population in combination with Zfh-1…….....39

2.3.3 spict-expressing CCs can become CySCs…….....…39

2.3.4 spict does not regulate BMP signaling in the *Drosophila* testis.....…41

2.3.5 *spict* is required for SG death and maintenance of the GSC pool upon starvation…….....42

2.3.6 Spict protein is expressed in CCs associated with dying SGs and is transferred from CCs to dying SGs…….....….43

2.3.7 Spict localizes to the late endosome/phagosome, during engulfment of dying SGs by CCs……..46

2.3.8 *spict* is required for normal progression of SG death…….....47

2.4 Discussion…….....48

2.5 Materials and Methods……50

2.5.1 Fly strains and husbandry…….....50

2.5.2 Generation of *spict*^{65} allele…….....51

2.5.3 Immunofluorescent staining and microscopy…….....51

2.5.4 Lineage tracing of *spict*-expressing cells…….....52

2.5.5 Pulse-chase experiment to track SG death progression…..…53

2.6 Acknowledgement……..……53

2.7 Figure…….....……..……..54

2.8 References……....….68

Chapter 3: Discussion and future directions…….....……71

3.1 Distinguishing the CySCs and CCs in *Drosophila* testis.....……...71

3.1.1 Spict is a novel marker for differentiating CCs……...72

3.1.2 What regulates *spict* expression pattern?.....……....73

3.2 Spict regulates the progression of SG death…….....76

3.2.1 Does Spict regulate magnesium concentration in the engulfing CC?.....77

3.2.2 Does Spict regulate fatty acid metabolism in the engulfing CC?.....79

3.3 Recycling of nutrients from dead SG to the Drosophila stem cell niche?.....81

3.3.1 Monitoring the movement of nutrient…….....81

3.3.1a Monitoring protein transportation…….....82

3.3.1b Monitoring amino acid transportation…….....….82

3.3.2 Investigating the functional role of nutrient movement...……..84

3.3.2a Investigating amino acid absorption from dying SG.....…....84

3.3.2b Investigating amino acid export from the CC……..85

3.4 Assisted programmed cell death……...85

3.4.1 Similarities between Drosophila male and female germ cell death….....87

3.4.2 Does phagocytosis or autophagy in CC facilitate SG death progression?……..88

3.4.3 What is the role of soma in the germ cell death?.....89

3.5 Similarity between mammalian and fly testis…….....……90

3.6 Figures…….....93

3.7 References…….…….....95

List of Figures

1.1 Mechanisms of stem cell divisions that generates differentiated cells.....25

1.2 The *Drosophila* testis architecture…….....27

1.3 Signaling between somatic cells and germ cells.....28

2.1 *spict* is expressed in differentiating cyst cells…….....54

2.2 Lineage tracing of *spict*-expressing cells in the testis reveals the frequent conversion of *spict*-positive cells into CySCs……...56

2.3 pMad level is not obviously changed in the *spict* mutant testis……..…58

2.4 *spict* is required for SG death and GSC maintenance during protein starvation…….....…..59

2.5 Spict localizes to CCs associated with dying SG and transferred dying SGs……...……60

2.6 Progression of SG death characterized by Lysotracker, Vasa, LaminDm0, Add and DAPI staining.……...……62

2.7 Identification of dying CC and surviving CC associated with SG death …….....……64

2.8 Spict localizes to Rab7-positive phagosomes that encapsulate dying SGs.……..…65

2.9 *spict* regulates the progression of SG death. …….....……67

3.1 Multiple alignment of *Drosophila* and human homologs [adapted from (Wang et al., 2007)] …….....……93

3.2 Translocalization of Spict-mRFP from SG…….....……94

List of Abbreviations

Bam: Bag-of-marbles

BrDU: 5-bromo-2'-deoxyuridine

CC: Cyst cell

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: Heat shock

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

PH3: Phosphohistone H3

Puc: Puckered

mRFP: Red fluorescent protein

SG: Spermatogonia

SC: Spermatocyte

Tj: Traffic jam

Upd: Unpaired

YFP: Yellow fluorescent protein

Zfh-1: Zinc finger-homeodomain transcription factor 1

Abstract

Tissues are maintained in a homeostatic state by balancing the constant loss of old cells with the continued production of new cells. Importantly, dysfunction of tissue homeostasis can lead to tumors or aging. Tissue homeostasis is a highly regulated and constantly shifting process as it has to cope with environmental stress while maintaining the integrity and functionality of the tissue. For example, tissues often slow down their turnover and/or scale down tissue size to conserve energy when nutrient availability is limited. This shift of tissue homeostasis needs to be quickly reversed once the environmental stress is removed.

Because they are responsible for producing differentiated cells, the role and behavior of resident stem cells in response to changes in the external environment have been heavily studied. However, stem cell division only constitutes a small fraction of total cell proliferation. Of equal importance, transit-amplifying cells, produced by stem cell divisions, mitotically amplify prior to terminal differentiation and thus lessen replication stress on their parental stem cells. Despite the fact that the transit-amplifying cells account for the majority of proliferation, the contribution of these cells to shifting tissue homeostasis has not been thoroughly investigated.

The *Drosophila melanogaster* testis serves as an ideal model system to study the behavior of stem cells and transit-amplifying cells, as a result of its well-defined anatomy and the wide array of genetic tools available to manipulate gene function in a cell type-specific manner. Recently, we demonstrated that elimination of transit-amplifying cells (i.e. spermatogonia) plays a critical role in maintaining the stem cell population during protein starvation in the *Drosophila*

testis. Inhibition of starvation-induced spermatogonial death leads to a loss of stem cells, impairment of tissue homeostasis, and failure to recover from starvation when nutrients are reintroduced. Regulation of transit-amplifying cells in the face of an environmental challenge is thus an essential process; however, it remains unclear how the death of spermatogonia leads to stem cell survival during protein starvation.

In this dissertation, we identified a gene, *spict*, which is specifically expressed in differentiating somatic cells, and the corresponding Spict protein, which is stabilized in cyst cells surrounding the dying germ cells. We found that starvation-induced spermatogonial death was decreased in the *spict* mutant, resulting in a failure to maintain germline stem cells during prolonged protein starvation. We further demonstrated that dying spermatogonia are phagocytosed by neighboring somatic cyst cells, in which Spict protein is stabilized. Taken together, we propose that phagocytosis of dead spermatogonia, which is promoted by Spict, contributes to nutrient recycling and subsequent stem cell maintenance during prolonged protein starvation.

Chapter 1:

Introduction

1.1 Tissue homeostasis as one aspect of life

Although the adult human body maintains a seemingly static appearance, most tissues are actually continually and dynamically changing at the cellular level. In fact, billions of cells in the human body die every day, and new cells are generated to replace them. To preserve proper function and architecture of each tissue throughout the lifespan of the organism, the balance between cellular proliferation and elimination must be achieved. This balance is known as tissue homeostasis.

Tissues are often composed of various cell types, and each cell type may have a function and life span that differs from its surrounding cell types. Attaining tissue homeostasis in such complex tissues requires interaction and coordination amongst these different cell types. Furthermore, to live in an ever-changing environment, the tissue has to fine-tune this homeostasis in response to the organismal needs, the underlying mechanisms of which we have just started to understand.

One of the most common challenges to tissue homeostasis that an organism encounters is a constant fluctuation in nutrient availability. As a critical response to starvation conditions, an organism has to reduce its proliferative capacity to achieve a new state of tissue homeostasis. This is important for the organism to

optimize the usage of limited resources without compromising basic physiological needs. This ability to adapt to a new state of tissue homeostasis can help the organism survive until nutrient availability is re-established Importantly, this temporary sacrifice must not disrupt the overall tissue architecture and has to be reversible. Studying tissue homeostasis in organisms subjected to sub-optimal conditions, such as starvation, is a great experimental model to help further understand the mechanisms that coordinate different cell types to achieve tissue homeostasis.

1.1.1 Cell turnover as an aspect of life

Maintaining tissue homeostasis is crucial for survival, and loss of tissue homeostasis can lead to several pathologies. For instance, uncontrolled proliferation of cells can result in tumorigenesis while reduced regeneration results in tissue degeneration, a hallmark of aging (Morrison and Kimble, 2006).

Most cells have a limited lifespan and can be lost due to programmed cell death or physiological damage. For instance, the gut epithelium in the intestinal mucosa has been shown to turnover rapidly: it is estimated that 10^9 cells are produced and die every five days (Wong et al., 1999). Skin epithelial cells, on the other hand, have a slightly longer lifespan of 10-30 days (Blanpain and Fuchs, 2009). In the hematopoietic lineage, cells can have a broad range of lifespans: erythrocytes survive for around four months while leukocytes only survive for a week (Ajmani and Rifkind, 1998). These cells are replaced by new cells in order to maintain constant function and integrity of the tissue.

Conceptually, tissue turnover can be represented by a simple equation: the sum of cell death and production. Typically, new cells are generated by adult stem cells or transit-amplifying cells. Failure to produce sufficient cells to compensate for the cells lost can lead to tissue degeneration, whereas proliferation has to be restricted in order to prevent tissue hyperplasia/tumorigenesis. Thus, to replace cells that are lost due to either

steady-state loss or tissue damage, the system must be able to sense how many cells need to be produced.

Multiple environmental factors contribute to the rate of cell production as well as to the rate of cell loss, and thus the production and loss of cells must be coordinated to achieve tissue homeostasis. Correspondingly, issue homeostasis is not a set-in-stone static state, but a balance between proliferation and cell loss. In this section, the mechanisms that govern tissue homeostasis are summarized with an emphasis on how these mechanisms are tightly regulated.

1.1.2 Components that contribute to tissue homeostasis

1.1.2a Cell death

Cell death plays a pivotal role in both tissue formation during development and adult tissue homeostasis. Over the last couple of decades, there has been increased recognition of multiple forms of cell death, including apoptosis, autophagy-induced death, and necrosis. While apoptosis and autophagy are considered cell-autonomous programmed cell death, necrosis is often triggered by unregulated environmental insults and leads to a loss of membrane integrity and uncontrolled release of cell debris into the extracellular space. Irrespective of the method of cell death, the corpses of the dying cells are often removed by phagocytosis.

Apoptosis is widely recognized as the primary cause of cellular elimination during adult tissue homeostasis. Apoptosis can be induced either extrinsically by activation of death receptors or intrinsically by release of apoptogenic factors from the mitochondria. For the extrinsic pathway, the extracellular ligand Fas-L binds to the death receptor Fas and sequentially activates pro-caspase8 and its downstream caspases, which act together with morphological changes, such as rupture of the mitochondrial membrane, to execute the apoptotic process (Green and Llambi, 2015; Ouyang et al., 2012). For the intrinsic pathway, the death is caused by stress-induced permeabilization of the mitochondrial outer membrane

that causes the release of the apoptogenic factor cytochrome c into the cytosol, which sequentially activates Caspase9 and its downstream effector caspases (Czabotar et al., 2014; Green and Llambi, 2015). Both pathways later converge on activation of effector caspases that cause morphological changes, such as condensation of nuclei, fragmentation of genomic DNA, and shrinkage of cytoplasm, which lead to cell death.

Autophagy-induced cell death is a type of cell death that is characterized by enhanced levels of autophagosomes, a feature of autophagy. Autophagy is a cellular mechanism that is often induced by starvation or ER stress to self-degrade and recycle cytoplasmic material. This process initiates through elongation of small membranes to form an autophagosome, a double-membrane vesicle that sequesters material from the cytoplasm. Later, mature autophagosome fuses with lysosome for degradation.

Originally, the autophagy-induced programmed cell death was discovered to occur during development, such as salivary gland destruction and midgut degeneration during the pupae stage in insects (Baehrecke, 2003). Later, autophagy-induced programmed cell death was also found to occur during development of other various organisms including humans. Autophagy-induced cell death can also be induced in mammalian cells and contributes to the regulation of cell death in the absence apoptosis (Shimizu et al., 2004; Shimizu et al., 2010). And, since autophagy itself also functions as a cellular mechanism to degrade material to cope with starvation or stress and promote survival. The detailed mechanism of how autophagy induces cell death, instead of survival, is still largely unknown (Liu and Levine, 2015; Yonekawa and Thorburn, 2013).

Finally, there is a passive form of cell death called necrosis that proceeds without complex regulatory mechanisms. It is believed that injury and infection cause necrotic cell death, which results in dilation of organelles, loss of cell membrane integrity and uncontrolled release of cellular material into the extracellular space. However, the exact cause and mechanism of necrotic cell

death are still largely unknown (Vanden Berghe et al., 2014; Ziegler and Groscurth, 2004).

Regardless of the precise mechanism that triggers cell death, failure to remove the materials released from the dying cell induces inflammation, triggers an autoimmune response, and is potentially deleterious for the tissue (Elliott and Ravichandran, 2010; Ravichandran and Lorenz, 2007). Therefore, the dying cell often releases the “eat-me” signal such as phosphatidylserine (PS), which causes the dead cell to be phagocytosed and digested by a phagocyte or a neighboring cell. The phagocytic pathway not only eliminates the dead material; recent studies suggest that it also induces the local production of growth factors, potentially coupling regeneration and cell death to sustain tissue homeostasis coordinately (Golpon et al., 2004; Koh and DiPietro, 2011; Morimoto et al., 2001).

1.1.2b Stem cell division governs tissue homeostasis

The majority of cell types in an adult organism are post-mitotic cells with a limited life span. Those fully differentiated, post-mitotic cells are constantly lost and are unable to regenerate by themselves. Stem cells, in contrast, are cells that have the ability to self-renew (generating more stem cells) as well as to produce differentiated cells (Fig. 1.1A). Researchers have provided evidence showing that stem cells are the fundamental source of the new cells that sustain tissue homeostasis in the blood, germline, skin, and muscle, (Ajmani and Rifkind, 1998; Almeida et al., 2016; Barker, 2014; Blanpain and Fuchs, 2009).

As stem cells have the potential to yield both new stem cells and differentiated cells upon cell division, stem cell division has three possible outcomes: 1) an asymmetric cell division that generates one stem cell and one differentiating daughter, 2) a symmetric division that generates two new stem cells, or 3) a symmetric division that generates two differentiating cells (Fig. 1.1B). While the stem cell number remains the same after asymmetric stem cell division, the stem cell population can either increase or decrease after symmetric division.

Thus, stem cell division can serve as a critical regulatory step for controlling stem cell number and, consequently, tissue homeostasis.

The most straightforward way to maintain a constant stem cell number is through asymmetric stem cell division, as this type of division can regulate stem cell number at the single cell level (Morrison and Kimble, 2006). Two major pathways, one cell-intrinsic and one cell-extrinsic, have been attributed to the regulation of asymmetric cell division.

In brief, the intrinsic mechanism that regulates asymmetric cell division often involves the asymmetric distribution of cell fate determinants between the two daughter cells (Fig. 1.1C). The specific distribution of cell fate determinants can be governed by the asymmetric localization of polarity proteins at the cell cortex, which also determines the mitotic spindle orientation during cell division. This mechanism is evolutionarily well conserved from nematodes to humans (Doe and Bowerman, 2001; Izumi and Kaneko, 2012). Recently, additional asymmetries during asymmetric stem cell division have been identified. For instance, certain organelles (Derivery et al., 2015; Pelletier and Yamashita, 2012), protein aggregates (Coelho et al., 2014), histones (Tran et al., 2013; Xie et al., 2015), and sister chromatids (Yadlapalli and Yamashita, 2013; Yamashita, 2013) can be asymmetrically segregated to the two daughter cells following stem cell division. Furthermore, that the asymmetric segregation of these materials is correlated with daughter cell fate (Xie et al., 2015; Yadlapalli and Yamashita, 2013). Although the contribution of these asymmetrically inherited materials to cell fate remains unknown, it is possible that they carry certain information that confers an asymmetric fate to the daughter cells, which suggests that the collection of fate determinants is far more diverse than previously believed.

Other well-established strategies for controlling asymmetric cell division are through regulation by cell-extrinsic mechanisms. Stem cells often reside in a specialized microenvironment called the niche, which helps to define asymmetric daughter cell fate (Fig. 1.1C). The niche provides short-range signals that specify

the stem cell fate. Consequently, cells that are unable to receive the limited-range niche factors will undergo differentiation. In this manner, the number of stem cells can be controlled by the availability of niche factors (Chen et al., 2016).

One example of asymmetric stem cell division governed by both cell intrinsic and cell extrinsic mechanisms is the *Drosophila* germline stem cells. In this system, stem cells and differentiated cells can be identified at single cell resolution owing to the well-defined anatomy of the tissue. Stem cells of both the male and female *Drosophila* germline are anchored to relatively simple niches via adherens junctions. The niche secretes signaling ligands to activate the BMP and JAK/STAT signaling pathways, and the secretion of niche factors is restricted in an extremely organized way as to only induce self-renewal within the proximity of the niche (see below) (Losick et al., 2011). Male germline stem cells utilize stereotypical centrosome orientation that is specified by the niche-stem cell interface (Yamashita et al., 2003). This faithful orientation of the centrosomes in the germline stem cells leads to assembly of the mitotic spindle in such a way that division generates one daughter that remains within the proximity of the niche and one daughter that is displaced from the niche and begins to differentiate (Chen et al., 2016; Morrison and Kimble, 2006).

During development, many stem cell lineages expand through symmetric division. Similarly, symmetric stem cell division is also utilized in adult tissues to increase stem cell number. For example, *Drosophila* female germline stem cells can be induced to divide symmetrically by ablation of one of the stem cells, despite the fact that they mostly undergo stereotypical asymmetric cell division under normal conditions (Xie and Spradling, 2000). By controlling the proportion of symmetric versus asymmetric divisions, the population of stem cells can be tightly regulated.

Moreover, the stem cell number is not the only parameter that contributes to the biology of stem cell-regulated tissue homeostasis. Regulation of the stem cell division rate can also play a role in regulating tissue homeostasis. An

example of tissue homeostasis being regulated by cell cycle rate is found in mammalian hematopoietic stem cells (HSCs). Whereas almost all of the fetal HSCs appear to be actively cycling at a high rate during development, the adult HSCs divide much less frequently. HSC populations with two different cell cycle lengths are found in adult mammals. While active HSCs are reported to be primarily in S/G2/M phase and are estimated to divide every 57 days on average, the dormant HSC population divides approximately once every 150 days. The quiescent nature of the dormant population is proposed to be a mechanism to protect the HSC population. In fact, HSC division can be induced upon injury or application of external stimuli (Hao et al., 2016; Zhao et al., 2014). Therefore, the regulation of stem cell division frequency could be one parameter important for the regulation of tissue homeostasis. Indeed, disruption of HSC quiescence results in HSCs failing to self-renew and, eventually, in HSC exhaustion.

1.1.2c Transit-amplifying cell proliferation

Despite the fact that hematopoietic stem cells (HSCs) only constitute a small fraction of the bone marrow, approximately 10^{12} blood cells that are generated each day to replenish the total 27×10^{12} blood cell present in adult body originate from HSCs (Ogawa, 1993; Sender et al., 2016). However, HSCs have been shown to only divide once every few months, as described above (Diaz-Flores et al., 2006), implying that an intermediate cell population must be present and actively proliferating on account of the rapid generation of blood cells observed. Indeed, HSCs have been shown to produce an intermediate population of progenitor cells, collectively known as transit-amplifying cells. These transit-amplifying cells have low self-renewal capacity but high proliferative capacity which can further amplify the number of differentiated cells without relying on constitutive stem cell divisions (Fig. 1.1A).

In fact, most mammalian self-renewing tissues utilize transit-amplifying intermediates. First, stem cells generate transit-amplifying daughters that, as opposed to stem cells, have a strictly limited proliferation capacity and are

already committed to differentiation. Transit-amplifying cells can mitotically amplify, although with a limited number of divisions. This intermediate amplification magnifies the number of progeny without solely relying on stem cell divisions. For example, during *Drosophila* spermatogenesis, one transit-amplifying cell that is produced from one asymmetric stem cell division generates 64 mature sperms owing to 4 transit-amplifying divisions (and 2 meiotic divisions). In mammals, less than 0.01% of the mitotic cells that generate blood cells are estimated to be the hematopoietic stem cell whereas 99.99% of the mitotic cells are transient-amplifying cells (Kiel et al., 2005). Similarly, 300 million new intestinal epithelial cells must be produced by a small population of stem cells and a large population of transit-amplifying cells in order to sustain the rapid turnover of the intestinal epithelium (Barker, 2014).

Transit-amplification is believed to protect the stem cells from exhaustion by lessening the burden on stem cells to produce a sufficient number of differentiated cells. Furthermore, the gradual decline of stem cell function, which is a hallmark of aging, is proposed to be closely related to the exhaustion of the replicative capability as manifested in telomere shortening(Johnson et al., 1998; Lopez-Otin et al., 2013). Additionally, DNA replication errors accumulate through multiple cell divisions (Burhans and Weinberger, 2012; Yue et al., 2003). Oxidative stress is also generated through the continued metabolic activity associated with those divisions (Venkataraman et al., 2013). Both of these processes account for the cellular damages that limit stem cell division. Indeed, activation of quiescent HSCs results in the early onset of stem cell exhaustion. Also, artificially accelerated stem cell divisions disrupts the differentiation program in aged *Drosophila* testes (Inaba et al., 2011). By using a transit-amplification mechanism, these replication-induced burdens on stem cells can be reduced and the integrity of stem cell function can be protected.

Research on tissue homeostasis has largely centered on stem cells, and only several studies to date have focused on transit-amplifying cells despite the fact that transit-amplifying cells account for the majority of mitotic activity in a

given tissue. Whereas there are more transit-amplifying cells and they often have a rapid cell cycle, stem cells only constitute a small fraction of the proliferating cells, have longer cell cycles, and divide at much lower frequencies. Therefore, it can be logically deduced that the transit-amplifying cells can, in theory, react faster to modulate the number of differentiated cells and reach a new state of tissue homeostasis after encountering an environmental change that alters the needs of the organism. However, only a few studies have focused on the behavior of transit-amplifying cells in shifting of tissue homeostasis.

1.1.2d Regeneration in non-stem cell tissues

While adult stem cells are responsible for sustaining tissue homeostasis in many tissues, some tissues do not have a stem cell population. Similar to stem cell-based regeneration, new cells are produced by progenitor cells in order to replace the cells within the tissue that have died. The main difference is that the progenitor cells originate from differentiated cells that have reverted or dedifferentiated in order to re-enter the cell cycle.

This has recently been shown to occur during the extensive tissue regeneration seen in newts and zebrafish following amputation of a limb or fin, respectively (Sehring et al., 2016). In both cases, the cells around the damaged area change morphologically and become blastema. The blastema is capable of regenerating the whole limb/fin, arranging the various cell types appropriately despite their complex structure, polarity, and body plan (Satoh et al., 2015). Interestingly, despite the fact that blastema derived from different tissues appears uniform, the differentiation potential of each blastema cell is often limited depending on the cell of origin (Kragl et al., 2009). Similarly, in mammals, many tissues, such as the kidney (Kusaba et al., 2014) and liver (Font-Burgada et al., 2015) regenerate without the presence of stem cells, suggesting that maintaining tissue homeostasis by de-differentiation of differentiated cells into progenitor cells is a widely utilized mechanism.

1.1.2e Differentiation

Many stem cell types, including hematopoietic stem cells, are “multipotent”, being capable of producing a vast array of distinct cell types. Hematopoietic stem cells generate the full spectrum of blood cells, each of which has a distinct function, such as erythrocytes that carry oxygen and lymphocytes that are responsible for the adaptive immune response. Similarly, the intestinal stem cells are responsible for producing differentiated intestinal epithelial cells, enterocytes, and secretory cells (Shaw et al., 2012). As each cell type within a tissue is interdependent on the other cell types and contributes together to the integrity and function of that tissue, the choice between the differentiated cell fates must be tightly instructed through signaling pathways in order to maintain tissue homeostasis (Apidianakis and Rahme, 2011). Furthermore, this choice between the differentiated cell fates must also be modulated as necessary in order to ensure continued tissue function and safeguard organismal survival.

1.2 *Drosophila* spermatogenesis as a model system to study tissue homeostasis

In this work, we chose to use the *Drosophila* testis as a model system to study how tissue homeostasis changes in response to starvation because of its simple and well-defined architecture. The various differentiation stages are arranged spatiotemporally along the testis with the stem cells and mature sperm residing at opposite ends of the testis (Fig. 1.2A). The simple construction along with a plethora of cellular markers (see below) that allow for accurate identification of different cell populations with single-cell resolution make the *Drosophila* testis an excellent model system to study differentiation and tissue homeostasis. Furthermore, spermatogenesis in flies and mammals share striking similarities, therefore, germline tissue homeostasis in flies may closely reflect germline tissue homeostasis in humans, enforcing the value of using the *Drosophila* testis as a model (see discussion). Here, I briefly describe the tissue

architecture and the signaling pathways that regulate spermatogenesis in *Drosophila*.

Drosophila spermatogenesis proceeds spatiotemporally with the less differentiated cells, including the stem cells, residing in the apical region of the testis and mature sperm residing in the basal region. As revealed by electron-microscopy, there are two types of stem cells, germline stem cells (GSCs) and somatic cyst stem cells (CySCs). Both GSCs and CySCs attach to a group of 10-12 post-mitotic somatic cells called hub cells at the apical tip of the testis that serve as a major component of the stem cell niche (Fig. 1.2B) (Davies and Fuller, 2008; Hardy et al., 1979). Within the stem cell niche, each GSC is enveloped by two CySCs that extend cytoplasmic processes toward the hub. The GSC and the encapsulating CySCs both divide asymmetrically to generate differentiating daughter cells. The GSC divides to produce a gonialblast (GB) that is encapsulated by two cyst cells (CCs), which are produced by CySC division. (Cheng et al., 2011; Yamashita et al., 2003). The proposed model for determining which daughter cell differentiates and which self-renews is simple: after an asymmetric division, one daughter cell is displaced away from the hub due to stereotypical orientation of the mitotic spindle and thus receives fewer hub-derived signals and therefore differentiates. The gonialblast then undergoes four rounds of transit-amplification with incomplete cytokinesis to generate interconnected 2, 4, 8 or 16 cell spermatogonia (SG). 16 cell SGs become spermatocytes (SCs), meiotic cells that will undergo the reductional and equational meiotic divisions, and then complete the morphological changes of spermiogenesis to form 64 individual sperm (Fig. 1.2B). During all stages of germline differentiation, the pair of CCs remains associated with and co-differentiates with the encapsulated germ cells. However, CCs will not divide again and instead enlarge tremendously to encompass and co-differentiate with the growing germ cell cyst.

1.2.1 The stem cells in the *Drosophila* testis

A series of genetic experiments provided insights into how both GSCs and CySCs are maintained by niche signals provided by the hub. The hub secretes niche factors to activate signaling pathways in the stem cells that are important for their self-renewal. The range of these factors is thought to be strictly restricted to a small region so that only the cells in immediate proximity to the hub are maintained as stem cells. Therefore, hub attachment is crucial for both GSC and CySC maintenance within the stem cell niche (Fig. 1.3A). This idea is supported by studies that knocked down the adhesion molecules responsible for hub attachment and noted that this leads to stem cell lost (Issigonis et al., 2009; Voog et al., 2008).

1.2.1a JAK/STAT signaling regulates CySC and GSC maintenance

One of the well-studied niche signals secreted from the hub is Upd, the ligand that activates the Janus Kinase and Signaling Transducer and Activator of Transcription (JAK/STAT) signaling cascade in both GSCs and CySCs (Fig. 1.3A). In either *jak* or *stat* temperature sensitive mutant testes, both CySCs and GSCs are lost progressively when flies are cultured at the restrictive temperature (Tulina and Matunis, 2001). Activation of STAT in the germ cells does not autonomously promote self-renewal of germ cells (Leatherman and Dinardo, 2008). Additionally, the function of the JAK/STAT pathway in GSCs was later shown to regulate the GSC-hub cell adhesion and, consequently, GSC maintenance (Leatherman and Dinardo, 2010). In contrast, activation of JAK/STAT pathway in CCs regulates its target Zfh-1 that was shown to be sufficient to cause overproliferation of CySCs and GSCs (Leatherman and Dinardo, 2008). These findings indicate that the ligand Upd secreted from the hub activates JAK/STAT signaling in CySCs to maintain CySC identity while STAT activity in GSCs promotes their hub adhesion (Fig. 1.3A). These findings also suggest that additional signaling is required for GSC self-renewal.

1.2.1b BMP signaling is required for GSC maintenance

In addition to the JAK/STAT pathway, the BMP (Transforming Growth Factor-β, TGF-β) pathway has been shown to regulate GSC maintenance. Flies mutant for the BMP ligands, *dpp* and *gbb*, or the BMP receptors, *punt* and *tkv*, show a GSC loss phenotype (Kawase et al., 2004). The BMP ligand is reported to be expressed in the hub cells (Inaba et al., 2015; Kawase et al., 2004). Further genetic experiments showed that the JAK/STAT signaling cascade activates the expression of BMP ligand in the CySCs. The activation of BMP activity in the GSC then regulates GSC self-renewal and suppresses germ cell differentiation (Kiger et al., 2001; Leatherman and Dinardo, 2010; Tulina and Matunis, 2001) (Fig. 1.3A). Although BMP and JAK/STAT signaling in the GSC are necessary for GSC maintenance, activation of neither the BMP nor the JAK/STAT signaling pathways in the GSC is sufficient for GSC self-renewal. This is in stark contrast to the overexpression of JAK/STAT signaling in the CySC alone, which leads to both GSC and CySC overproliferation. Therefore, additional, unknown factors provided by the CySC might be required to regulate GSC self-renewal.

An interesting remaining question is how the self-renewal signal secreted from the niche is restricted to the stem cell compartment leaving the differentiating daughter cells untouched despite them being only one cell diameter away. Several pieces of evidence regarding BMP signaling in the testis have revealed multiple mechanisms by which this strict regulation might be achieved.

Recently, several extracellular matrix (ECM) components, such as Magu and Dally-like, have been found to modulate the concentration of BMP ligand Dpp to the stem cell niche (Hayashi et al., 2009; Zheng et al., 2011). Furthermore, a recent study showed that the interaction between the BMP ligand Dpp and BMP receptor Tkv is predominantly conducted on GSC protrusions called MT-nanotubes, which extend from the GSC into the hub cells, in order to restrict the receptor-ligand interaction within a short range from the niche (Inaba et al., 2015). Together, these studies highlight the importance of niche restriction and the

mechanisms by which stem cells interact with the diffusion-limited signaling components within the restricted radius of the niche.

1.2.1c GSC and CySC asymmetric division

Although GSCs and CySCs both divide asymmetrically, the mechanism and outcome are rather different. The GSC almost always divides asymmetrically to generate a self-renewing and a differentiating daughter cell. The GSC asymmetric division is preceded by stereotypical centrosome positioning that is set up during interphase such that the mitotic spindle is aligned perpendicular to the GSC/ hub interface (Salzmann et al., 2013; Sheng and Matunis, 2011; Yamashita et al., 2003). In this way, the daughter cell destined to differentiate (the GB) is displaced one cell diameter away from the hub while the daughter cell destined to self-renew remains in contact with the hub.

On the other hand, CySCs specifically and transiently orient their mitotic spindle during anaphase (Cheng et al., 2011) and do not align the plane of division prior to the onset of mitosis as seen in GSCs. The result of CySC division is similar to that of GSCs: one daughter is displaced from the hub (the CC) and destined to differentiate while one daughter remains in contact with the hub to self-renew. This anaphase spindle repositioning requires functional centrosome and can be perturbed by overexpression of the actin-membrane linker Moe, in the CySC, which results in increase in symmetric CySC division and leads to increase of CySC number (Cheng et al., 2011). However, the fate of both daughters after asymmetric CySC division appear to be rather fluid. As shown by clonal analysis, two days after clone induction, significant percentages of single CySC clones are either lost (both daughter cells differentiate) or expanded (both daughter cells result in self-renewal) instead of maintaining consistent outcome of asymmetric CySCs divisions (Amoyel et al., 2014). It remains unclear why GSC and CySC division differ so greatly when these cell types act cooperatively throughout spermatogenesis, and the exact mechanism of CySC division is not completely understood yet. Nonetheless, asymmetric

division of CySCs is required to maintain stem cell homeostasis in the testis (Cheng et al., 2011).

1.2.2 CCs regulate germline differentiation and survival

After the GSC asymmetric division, the differentiating daughter cell, the GB, undergoes four rounds of synchronous transit-amplifying division to generate 2, 4, 8 and 16-cell SG. Cytokinesis during SG division is known to be incomplete and results in SGs that are interconnected by a structure called the fusome. 16-cell SG then become spermatocytes (SCs), meiotic cells that dramatically increase in size in preparation for the meiotic divisions. These morphological changes allow us to identify each stage of germ cell development during spermatogenesis.

Additionally, the differentiation of SG is regulated by the differentiation factor Bag-of-Marbles (Bam). Bam expression typically starts in 4-cell SGs, and the accumulation of Bam is believed to induce the SG to SC transition (Fig. 1.2B). Ectopic expression of Bam in GSCs causes GSC loss via differentiation.

The somatic CC lineage can be visualized due to the spatiotemporal expression of three transcription factors, Zinc Finger Homeodomain-1 (Zfh-1), Traffic jam (Tj), and Eyes absent (Eya), during cyst cell differentiation (Fig. 1.2B). Zfh-1 is expressed in CySCs and their immediate daughters and functions as part of the CySC self-renewal machinery (Leatherman and Dinardo, 2008) (Fig. 1.3A). Tj, a large Maf transcription factor, is expressed in early CCs that are boarder than Zfh-1 expressing cells, to facilitate SG differentiation (Li et al., 2003) (Fig. 1.2B and 1.3B). Eya is expressed at a low level in the early CCs, and its expression is highly upregulated in the CCs that associate with SCs, and its function has been shown to be required for SC development (Fig. 1.2B and 1.3B) (Fabrizio et al., 2003). This suggests that germ cell differentiation is highly regulated by the encapsulating CCs. Indeed, germ cells in testes that have had all of their CCs ablated frequently fail to differentiate further than 2-cell SG (Lim

and Fuller, 2012). However, this phenotype was only observed in approximately half of examined testes: the remaining half were devoid of germ cells. Since CC death is known to induce the death of germ cells encapsulated by the dying CC (Hetie et al., 2014; Yang and Yamashita, 2015), the drastic ablation of CCs could account for the complete loss of germ cells. Moreover, germ cells that escaped the CC death-induced germ cell death can repopulate and stay undifferentiated in the absence of the cyst cell suggesting that cyst cells regulate germ cell differentiation (Lim and Fuller, 2012). Recent studies suggest that the regulation of germ cell differentiation could at least partly contribute by proper encapsulation by cyst cells (Fairchild et al., 2015; Lim and Fuller, 2012; Schulz et al., 2002; Shields et al., 2014). In summary, the somatic cells in the testis play a crucial role in regulating many aspects of germline physiology, including self-renewal, survival, and differentiation.

1.2.3 Interdependence of germline and soma

The above shows that germ cell differentiation and self-renewal are tightly regulated by somatic cells. Moreover, it is likely that somatic cells are regulated by germ cells that they encapsulate. It was shown that, in the absence of germ cells, somatic cells overproliferate to form a tumor-like growth (Gonczy and DiNardo, 1996), suggesting that the reciprocal regulation underlies tissue homeostasis of the *Drosophila* testis (and likely in mammalian testis, see Chapter 3 discussion).

1.2.3a Germline also regulates cyst cell morphology

An early experiment showed that testes lacking germ cells retain a CC population and these CCs behave normally when the testes of young flies are examined. However, the number of early CCs starts to increase after five days, and the CCs that are distant to the hub start to incorporate BrdU in these older flies, which is typically only observed in CySCs, as CCs do not divide. This suggests that the germ cells might play a suppressive role, preventing

differentiated CCs from re-entering the cell cycle (Gonczy and DiNardo, 1996). Furthermore, Epidermal Growth Factor (EGF) is secreted by the germ cells and is received by the CCs where it induces the CC to encapsulate the germ cells, which promotes SG differentiation through an unknown factor (Fig. 1.3B) (Schulz et al., 2002). These experiments indicate that germ cells can also regulate CC fate and proliferation. Taken together, germ cells and cyst cells co-differentiate via their mutual regulation.

1.2.3b Regulation of cell division between CySC and GSC

Cell division during spermatogenesis is tightly regulated: CySCs are the only mitotically active somatic cells in the testis while germ cells undergo one round of asymmetric division followed by exactly four mitotic divisions. During spermatogenesis, each germ cell cyst contains between 1 and 16 germ cells as well as a pair of encapsulating CCs, which are generated concurrently by the division of both a GSC and two CySCs. To maintain the architecture of the testis, the ratio of cell divisions between GSCs and CySCs has to be proportional; it would disrupt tissue homeostasis if there were significantly more germ cells than CCs or vice versa. Indeed, the ratio of GSC to CySC mitoses has been shown to be 1:2 (Inaba et al., 2011).

In addition to the niche factors provided by the hub, CySCs also regulate GSC division. This was shown by overexpression of the cell cycle regulator Cdc25 (String) in either somatic or germ cells in the testis. While overexpression of String in germ cells does not result in an increase in the mitotic index of either CySCs or GSCs, CySC mitotic index can be significantly increased upon overexpression of String using the pan-CC (including CySC and differentiating CC) driver c587-Gal4. This indicates that CySC division does not depend on GSC division, but GSC division is likely under some non-autonomous regulation (Inaba et al., 2011). Indeed, when String is overexpressed concurrently in both the germline and soma, both GSC and CySC mitotic indexes increase drastically, however, they still maintain a 1:2 ratio (Inaba et al., 2011). These experiments

suggest that the CySC division plays a permissive role instead of an instructive role in GSC division. Together, these studies reveal the complex communication that exists between germ cells and somatic cells that allows them to cooperate as a functional unit.

In summary, there are two cell lineages residing in the *Drosophila* testis, a germ cell lineage and a somatic cell lineage, and they are able to arrange systematically and co-differentiate in a spatial-temporal manner. Along with those signaling molecules secreted by the stem cell niche as well as signals that allow for communication between the germ cells and somatic cells (Smendziuk et al., 2015), the testis homeostasis can be regulated through systematic signaling, such as insulin (see below) and steroid hormone ecdysone (Li et al., 2014). Therefore, the *Drosophila* testis provides a simple yet powerful experimental model to investigate how a complex tissue responds to environmental changes in order to maintain tissue homeostasis.

1.3 Modulation of tissue homeostasis in response to functional demand and environmental change

Although tissue homeostasis can be seen as achieving a balance in cell turnover, the homeostatic state is dynamic and is often actively and continually regulated. In fact, organisms are surrounded by ever-changing environments, such as fluctuation of food supply, and the state of tissue homeostasis must adapt to these changes to help ensure optimal organismal survival. Thus, tissue homeostasis is not a set-in-stone static state, but an adjustable balance between proliferation and cell loss.

For example, to facilitate oxygen uptake and transport, an increased number of erythrocytes are generated in response to hypoxic or anemic conditions (Haase, 2013). Upregulation of lymphocyte production occurs in the presence of pathogens (George, 2012). Epidermal proliferation is induced by

repetitive physical stimuli to form callosity to ensure the integrity of the barrier that shields most tissues from the environment (Thomas et al., 1985).

Additionally, tissue homeostasis can also be fine-tuned in response to physiological demands imposed by the organism on itself. For instance, during the menstrual cycle, the uterus undergoes drastic proliferation to prepare for pregnancy and shrinkage during menstruation (Lucas et al., 2013). During pregnancy, female mammary glands expand significantly to prepare for lactation (Brisken and O'Malley, 2010).

As described above, tissues have to respond to internal physiological demands and reach a new state of tissue homeostasis in order to best cope with these demands. When the physiological demand no longer exists, the tissue must adjust again to match the current organismal need efficiently.

1.3.1 Morphological changes in tissues facing starvation induced stress

Maintaining fitness while facing unpredictable nutrient availability is a major challenge for all organisms. Extensive research has focused on how cells proliferate to meet the demands of the organism, as well as how tissue homeostasis adjusts to limited resources. To optimize the use of available resources efficiently in response to nutrient deprivation, many tissues undergo involution, which reduces the total cell number to reduce energy demands by the tissue. For instance, it is not surprising that adipocyte differentiation is repressed during glycogen deprivation (Hwang et al., 1997). Also, to conserve energy, muscle cells and the epithelial cells of the intestine undergo drastic involution upon starvation (Ohanna et al., 2005; Shaw et al., 2012). Hypothetically, the starvation-induced reduction can be a temporary compromise between maximal tissue performance and stamina; and this may allow the organism to survive until more favorable environmental conditions return.

An organism is comprised of trillions of cells, and each cell can only sense its own energy/nutrient level. For example, at the cellular level, Glucokinase

senses the intracellular glucose in addition to its function in catalyzing glucose to glucose-6-phosphate (Pirkmajer and Chibalin, 2011). Additionally, amino acid scarcity can be indirectly measured from uncharged tRNAs that are normally loaded with amino acid (Boer et al., 2010). These intracellular nutrient level can then regulates cell proliferation through the mediator mTOR (Hietakangas and Cohen, 2009). Moreover, a cell must not only detect its internal condition and immediate surroundings, but it must be able to interpret the signals secreted from distant cells which may be able to better assess conditions such as nutrient availability and respond in a way that benefits the organism as a whole. For instance, at the organismal level, lipid binding by the CD36 receptor in taste buds induces calcium release, propagating a signal that travels through the nervous system to indirectly/remotely upregulate enterocyte lipid uptake (Martin et al., 2011). Last but not least, endocrine signals, such as neural-derived *Drosophila* insulin-like peptides (DILPs), can be released from the insulin-producing cells in the brain to instruct germ cell proliferation from a distance (LaFever and Drummond-Barbosa, 2005).

The TOR pathway is used by cells to interpret multiple converging signals and to determine the appropriate response based on a summation of various extracellular/environmental conditions. Depending on the tissue or cell type, the TOR pathway modulates cellular responses, including proliferation, differentiation, and survival. Nutrient information can synergistically regulate autonomous or non-autonomous cellular behavior to manipulate tissue homeostasis. For instance, amino acid starvation induces quiescence in *Drosophila* neuroblasts by suppression of PI3K, which acts downstream of the insulin receptor (Chell and Brand, 2010). These intricate mechanisms reveal how each tissue can coordinate its response to nutrient fluctuations in order to optimize organismal survival.

1.3.2 Responses to nutrient deprivation in the germline

Most tissues in an adult organism are necessary for maintaining overall organismal survival/physiology. The germline, however, is dispensable for survival and is only essential for reproduction. In fact, the lifespan of *C. elegans* increases significantly upon removal of the germline, which suggests that fertility is an energy consuming process that the organism selects to undertake at the expense of survival (Arantes-Oliveira et al., 2002). Therefore, when faced with starvation conditions, temporarily compromising the germline would have little impact on the organism overall as long as the germline maintains its potential to repopulate quickly to maximize fertility when nutrient conditions improve. Indeed, the germline undergoes drastic involution during starvation. For example, starvation induces drastic germ cell death in *C. elegans* (Salinas et al., 2006). Nutrient-dependent germ cell proliferation has been shown to be cell autonomous and acts through the insulin pathway *(Michaelson et al., 2010).* In *Drosophila*, fertility is highly correlated with nutrient availability. Recent studies showed that during oogenesis, the germline, as well as the soma, adjust their proliferation rate in response to nutrient availability, which results in an almost 60 fold of changes in the rate of egg production (Drummond-Barbosa and Spradling, 2001). Later, this dietary regulation of oogenesis was shown to occur in an insulin-dependent manner (Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005). In worm and fly, the nutrients required for spermatogenesis and oogenesis are mostly provided by the adult animal. Moreover, although the underlying mechanism is less clear, fertility is drastically reduced under starvation conditions in both adult male and female mammals. In males, starvation greatly affects spermatogenesis, and germ cell death during spermatogenesis drastically increases (Guan et al., 2015). Together, these studies indicate that the drastic response of germline in reaction to nutrient availability is conserved between organisms. Therefore, understanding how germline homeostasis shifts during starvation in order to conserve energy yet maintain its overall function can be a critical aspect of understanding how organisms cope with an unfavorable environment.

1.3.3 Nutrient availability regulates tissue homeostasis in the *Drosophila* testis

Testes from flies that are shifted from a standard diet to a protein-poor diet show that germ cell production is reduced and dramatic involution of the testis occurs. Interestingly, within days after being switched back to a standard diet, the testis morphology is robustly restored. This indicates that tissue involution upon starvation is a temporary side effect of the animal trying to conserve energy during a period of limited nutrients, and such a condition can be reversed when nutrient conditions are restored (McLeod et al., 2010).

Previous studies on the effect of decreased amino acid availability on GSC proliferation rate suggest that GSC proliferation is modulated by delaying their cell cycle through activation of the centrosome orientation checkpoint, which can be rescued by overexpression of constitutively-active insulin (Roth et al., 2012). This suggests that the downregulation of germ cell production in response to starvation could be partly caused by reduced GSC proliferation. Additionally, under a more stringent experimental setup, the GSC number was reduced from 8 to 6 upon 15 days of prolonged starvation in which protein was completely absent from the fly's diet (McLeod et al., 2010). Recently, the decrease in GSC number upon protein starvation was shown to take place abruptly after 3 to 6 days rather than gradually over the period of prolonged starvation (Yang and Yamashita, 2015). This suggests that the remaining 6 GSCs are actively maintained after the initial GSC loss. Meanwhile, in addition to changes in the stem cell compartment, an increase in CC death induced germ cell death was shown to greatly increase the elimination of transit-amplifying SGs. This reduction in the number of transit-amplifying SGs is responsible for the testis involution upon starvation. The removal of excess transit-amplifying SGs is essential for the maintenance of the remaining 6 GSCs upon prolonged protein starvation: suppression of SG death leads to gradual collapse of the GSC population (Yang and Yamashita, 2015).

Taken together, these studies highlight multiple facets of regulation that act to impact testis homeostasis upon starvation: the decrease in GSC number, the alteration of the GSC cell cycle, and soma-induced elimination of transit-amplifying cells. However, it remains unclear what role the CCs exactly play in regulating SG death, and how CC death contributes to stem cell number upon protein starvation.

In this dissertation, I showed that neighboring CCs phagocytose dead SGs, in particular upon protein starvation. We identified *spichthyn (spict)* as a gene that is expressed in differentiating CCs and Spict protein colocalizes with late endosome and lysosome compartment during SG death, suggesting a potential role in facilitating SG death. Indeed, Spict is required for proper progression of SG death, and is required to maintain GSCs during prolonged protein starvation. Taken together, my results show that Spict is a critical regulator of the starvation response in the *Drosophila* testis via its ability to promote SG death, thereby contributing to GSC maintenance during protein starvation.

1.4 Figure

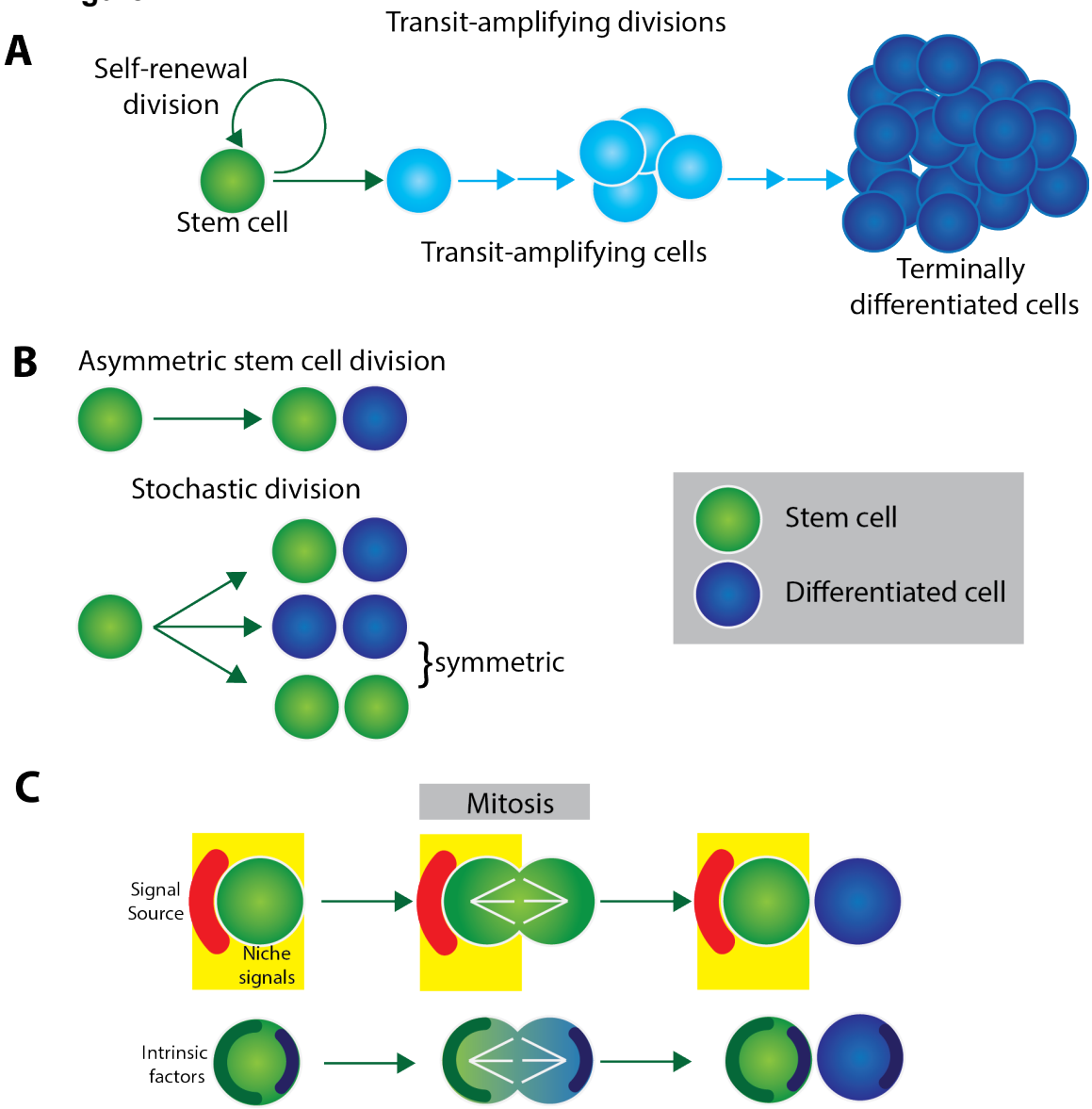

Fig. 1.1 Mechanisms of stem cell divisions that generates differentiated cells

(A) Stem cell generates daughters that self-renew or differentiate. Most adult stem cells (green) generate transit-amplifying cells (light blue) which are able to further divide through limited rounds to generate terminally differentiated cells (dark blue).

(B) Stem cell division can be asymmetric which generates one stem cell and one differentiated cell or stochastic in which each stem cell division can result in two stem cells, two differentiating daughters or one of each type.

(C) The mechanisms of asymmetric cell division. Asymmetric cell division can be regulated by extrinsic signals such as niche signals (yellow) that are provided by

the stem cell niche (signal source, red). The cell division results in one cell staying within the radius of niche signals and not differentiating (green) while the other cell is replaced and no longer receives niche signals which leads to another cell fate (blue). Asymmetric cell division can also be regulated by intrinsic factors. The intrinsic fate determinants (dark green and blue) are polarized during interphase and are segregated asymmetrically into daughter cells, which determines the fate of each daughter.

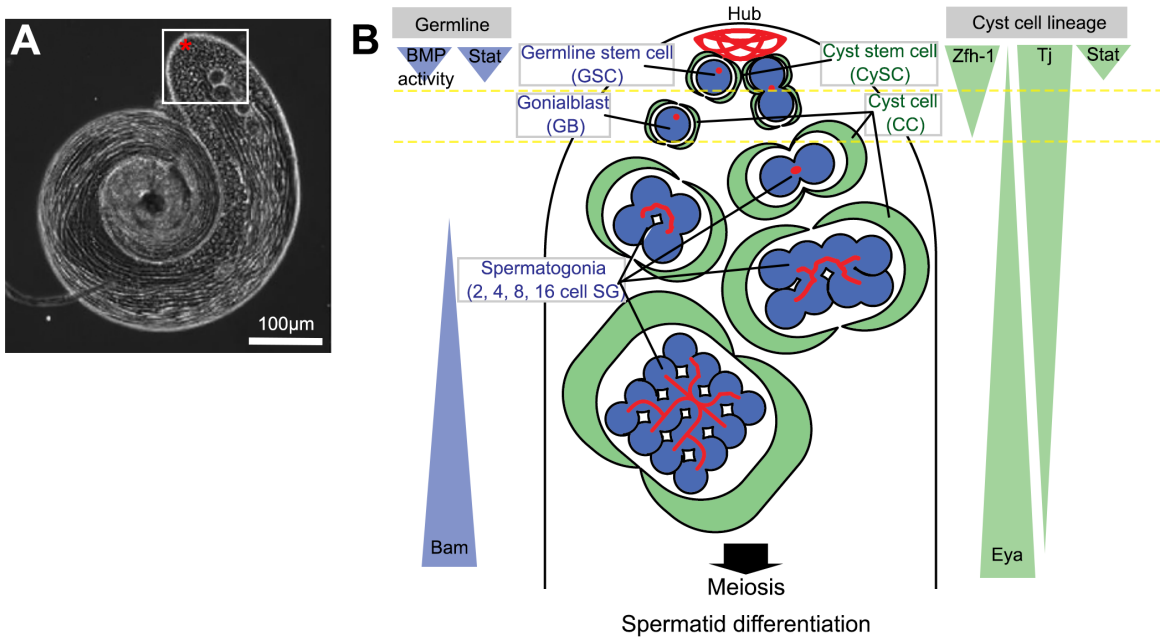

Fig. 1.2 The *Drosophila* testis architecture

(A) Phase contrast image of a wild type testis (Moon et al., 2011). The stem cells and early differentiating cells reside in the apical tip of the testis (box). The hub is indicated by the asterisk.

(B) A group of post-mitotic somatic cells forms the stem cell niche (hub) at the apical tip of the testis. GSCs (blue) and CySCs (green) lie in a rosette surrounding the hub. The asymmetric division of GSCs and CySCs result in self-renewal of a daughter that stays within the niche and a differentiating daughter that is displaced away from the niche. The differentiating daughter of a GSC, called a gonialblast, then becomes spermatogonia that undergo 4 rounds of mitotic divisionbefore entering meiosis and spermatid differentiation. Cyst cells, the differentiating daughters of CySCs, encapsulate and co-differentiate with the developing spermatogonia. Molecular markers over various differentiation stages for the germline are shown on the left side and molecular markers for the cyst cell lineage are shown on the right side.

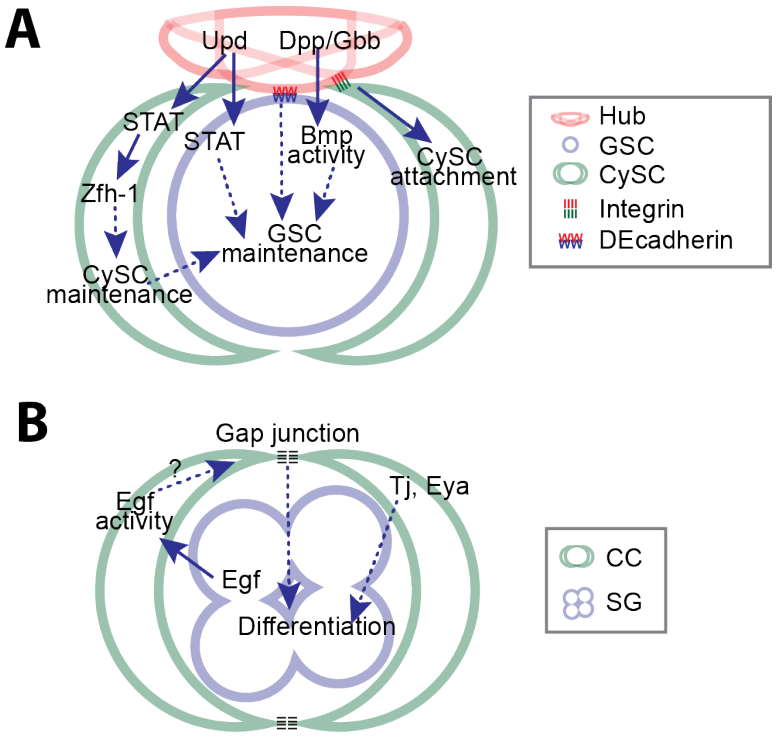

Fig. 1.3 Signaling between somatic cells and germ cells

(A) Signaling from the hub and between GSCs and CySCs. The CySC and GSC are attached to the hub cells through adhesion molecules, Integrin and DEcadherin, respectively. Ligands of JAK/STAT and BMP pathways are secreted from the hub activating signaling pathways in the stem cells.

(B) Signaling between SG and CCs. Transcription factors, Tj and Eya, are required for CC differentiation which regulate SG differentiation. On the other hand, Egf secreted from SG activates Egf signaling pathway in CCs which maintains the Gap junction between the pair of CCs and facilitates proper germ cell differentiation.

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Chapter 2:

spict, a cyst cell-specific gene, regulates starvation-induced spermatogonial cell death in the *Drosophila* testis

2.1 Abstract

Tissues are maintained in a homeostatic state by balancing the constant loss of old cells with the continued production of new cells. Tissue homeostasis can shift between high and low turnover states to cope with environmental changes such as nutrient availability. Recently, we uncovered that elimination of transit-amplifying cells plays a critical role in maintaining the stem cell population during protein starvation in the *Drosophila* testis. Here, we identify *spict*, a gene expressed specifically in differentiating cyst cells, as a regulator of spermatogonial death. Spict is upregulated in cyst cells, which phagocytose dying spermatogonia. We propose that phagocytosis and subsequent clearance of dead spermatogonia, which is partly promoted by Spict, contribute to stem cell maintenance during prolonged protein starvation.

2.2 Introduction

Tissue homeostasis can shift between high and low turnover rates, depending on the needs demanded by the external environment. For example, tissues can slow down their turnover and/or scale down their overall size when the amount of available nutrients is limited (Angelo and Van Gilst, 2009; McLeod et al., 2010; Padilla and Ladage, 2012). Stem cells are widely considered the

master regulators of tissue homeostasis, and how stem cells respond to changes in their external environment has been heavily studied (Mihaylova et al., 2014; Nakada et al., 2011). However, the majority of cell proliferation in tissues occurs in transit-amplifying cells (stem cell progeny that mitotically amplify prior to terminal differentiation, thereby lessening the burden on stem cells) (Davies and Fuller, 2008; Hsu et al., 2014; Lui et al., 2011; van der Flier and Clevers, 2009; Watt, 1998). Despite this, the response of transit-amplifying cells during shifting tissue homeostasis is poorly explored.

The *Drosophila* testis is an excellent model system to study the behavior of stem cells and transit-amplifying cells owing to the well-defined anatomy of the tissue and the ample genetic tools available for manipulating gene function in a cell type-specific manner. At the apical tip of the *Drosophila* testis, two stem cell populations, germline stem cells (GSCs) and somatic cyst stem cells (CySCs), are attached to the hub cells, which organize the stem cell niche for both stem cell populations (Fig. 2.1A) (Hardy et al., 1979; Losick et al., 2011). In addition, CySCs encapsulate GSCs and together with the hub cells they function as the GSC niche by contributing to the critical signaling environment (Leatherman and Dinardo, 2008, 2010). Upon stem cell division, GSCs produce gonialblasts (GBs), whereas CySCs produce cyst cells (CCs). GBs then undergo four rounds of transit-amplifying divisions as spermatogonia (SGs). As cytokinesis of these divisions is incomplete, transit-amplifying divisions yield a cluster of 16 interconnected spermatogonia (SGs), which then undergo meiotic divisions and spermiogenesis. Connectivity of SGs (2-cell, 4-cell, 8-cell, 16-cell SGs) serves as a reliable marker for their differentiation stage (Fig. 2.1A). A pair of CCs encapsulates the differentiating SGs to regulate their differentiation. CCs are critical for the survival and differentiation of SGs beyond the 2-cell SG stage (Fig. 2.1A) (Lim and Fuller, 2012).

Recently, we reported that SG death dramatically increases in response to protein starvation (Yang and Yamashita, 2015). The GSC population, however, is relatively well-maintained even during a prolonged period of protein starvation.

After an initial drop in GSC number from ~8/testis to ~6/testis upon 3-6 days of starvation (McLeod et al., 2010), the remaining ~6 GSCs were steadily maintained for an additional ~20 days (Yang and Yamashita, 2015). Moreover, these remaining ~6 GSCs proliferated at an unchanged rate compared to fed conditions. This argues that transit-amplifying cells, but not stem cells, may be a major point of regulation in response to changes in nutrient conditions. We have shown that starvation-induced SG death is triggered by apoptosis of CCs (Yang and Yamashita, 2015). When CC death is blocked by inhibiting apoptosis, starvation-induced SG death was also blocked. Concomitantly, testes failed to maintain their GSC population, leading to collapsed tissue homeostasis and compromised ability to recover upon reintroduction of nutrients (Yang and Yamashita, 2015). These results led us to propose that SG death upon protein starvation serves as a mechanism to protect GSCs. First, SG death would reduce the need for nutrients, thereby indirectly saving nutrients for GSCs. Second, we speculated that nutrients from dead SGs may be recycled to feed GSCs. However, the underlying mechanisms to recycle nutrients from dead SG to support GSC survival and proliferation remain elusive.

Here, we report our characterization of *spichthyn (spict)*, a gene that is expressed in differentiating CCs. We found that Spict is specifically upregulated at a post-transcriptional level in CCs near dying SGs. We show that CCs phagocytose dead SGs, and that Spict is associated with lysosomes during phagocytosis of SGs, suggesting that *spict* might be involved in the process of SG phagocytosis or in the clearance of dead SGs. Finally, *spict* mutants failed to maintain the GSC population during protein starvation. Taken together, we propose that SG death, facilitated by the function of *spict*, plays an important role in protecting the GSC population during protein starvation, possibly via recycling of nutrients from dead SGs.

2.3 Results

2.3.1 *spict* is expressed in differentiating cyst cells

In a small-scale screen to identify genes expressed in the *Drosophila* testis, we identified a *gal4* enhancer trap of *spichthyin (spict*), which was originally identified as the *Drosophila* homolog of the human *NIPA1* and *ichthyin* genes (Wang et al., 2007). When the expression pattern of *spict* was visualized by expressing *UAS-nlsGFP* (nuclear localization signal-containing GFP) with the *spict-gal4* driver, we found that GFP was specifically observed in the nuclei of differentiating CCs. Notably, nlsGFP was absent from the nuclei of the somatic cells that are in close contact with the hub cells, which likely represent the CySCs. In contrast, the well-established CC driver *c587-gal4* drove the expression of nlsGFP in all early somatic cells at the apical tip, including the CySCs (Fig. 2.1B, C).

The lack of nlsGFP expression in CCs near the hub indicates that *spict-gal4* expression might be excluded from CySCs. To test this idea, we examined the relationship of *spict*-expressing cells with two characteristics of CySCs: 1) attachment to the hub cells and 2) the ability to undergo mitosis. To examine the attachment to the hub cells, UAS-mCD8-GFP (a plasma membrane marker) was expressed using either *c587-gal4* or *spict-gal4*. Consistent with *c587-gal4* being expressed in all early CCs including the CySCs, we observed mCD8-GFP-labelled cell processes attached to the hub cells (Fig. 2.1D) (Cheng et al., 2011; Hardy et al., 1979) and 100% of testes contained multiple mCD8-GFP-positive processes attached to the hub cells (N= 19). In contrast, when the expression of UAS-mCD8-GFP was driven by *spict-gal4,* mCD8-GFP-positive processes were rarely associated with the hub (only <5% of testes contained hub-touching processes, N= 87). These results demonstrate that most of the *spict*-expressing cells do not contact the hub, suggesting that *spict-gal4*-expressing cells are not CySCs (Fig. 2.1E).

Next, we examined whether *spict-gal4*-expressing cells can undergo mitosis. In the *Drosophila* testis, CySCs are the only somatic cell population that undergoes mitosis(Cheng et al., 2011), and all other somatic cells are post-mitotic. To examine whether *spict-gal4*-expressing cells undergo mitoses, we

labeled mitotic cells with anti-phosphorylated histone H3 (PH3) antibody. When PH3 staining was combined with *c587-gal4>mCD8-GFP*, 100% of mitotic somatic cells were mCD8-GFP-positive (N=19), consistent with the notion that *c587-gal4* is expressed in CySCs. In contrast, when *spict-gal4>mCD8-GFP* was combined with PH3 staining, only 2.5 % of total PH3-positive cells were also positive for mCD8-GFP (N=119), supporting the idea that *spict-gal4*-expressing cells are rarely dividing. Taken together, these results strongly argue that *spict-gal4* is excluded from CySCs, and that *spict* expression marks differentiating CCs.

2.3.2 *spict* expression can be used to better identify the CySC population in combination with Zfh-1.

The best marker for labeling CySCs identified to date is Zfh-1(Leatherman and Dinardo, 2008). Zfh-1 is a transcriptional repressor, whose function is critical for the maintenance of CySC identity. However, Zfh-1 not only marks CySCs but also their immediate daughters that have been displaced away from the hub and have initiated differentiation as CCs. Accordingly, the number of Zfh-1-positive cells is higher than the expected number of CySCs (Hardy et al., 1979; Leatherman and Dinardo, 2008). We reasoned that *spict-gal4* could be used to negatively mark CySCs and to better identify the CySC population when combined with Zfh-1. Indeed, we found that the Zfh-1-positive population can be subdivided into *spict*-negative (*spict*$^{-}$) vs. *spict*-positive (*spict*$^{+}$) populations (Fig. 2.1F). We scored the number of 'Zfh-1^{+} *spict*$^{-}$' and 'Zfh-1^{+} *spict*$^{+}$' somatic cells. We observed that there were 33.6±10.3 Zfh-1^{+} cells in the wild type flies used in this study (Fig 2.1G). Among the Zfh-1^{+} cells, 16.1±4.6 were *spict*$^{-}$, whereas 17.5±11.0 were *spict*$^{+}$ (N=13 testes). Given that essentially all CySC characteristics (attachment to the hub and the ability to divide) are confined within 'Zfh-1^{+} *spict*$^{-}$' cells, we conclude that this population (~16 cells/testis) represents the population with the highest CySC concentration identified to date.

2.3.3 *spict*-expressing CCs can become CySCs

It is well established that GSCs divide asymmetrically by orienting their mitotic spindle perpendicular to the hub cells and that this spindle orientation is prepared by stereotypical centrosome positioning throughout the cell cycle (Yamashita et al., 2003). In contrast, CySCs do not have consistent centrosome positioning in interphase, and enter mitosis with randomly oriented spindles, but reposition their spindles during anaphase such that one daughter of the CySC division remains attached to the hub, whereas the other daughter is displaced away from the hub and initiates differentiation (Cheng et al., 2011). Based on these observations, we proposed that CySC divisions are also stereotypically asymmetric. However, a recent lineage tracing study (Amoyel et al., 2014) found that CySCs likely undergo stochastic self-renewal and differentiation and suggested that there is no stereotypical asymmetric stem cell division in the CySC population.

We reasoned that *spict-gal4*'s ability to specifically mark differentiating CCs might help to reconcile these differences. We used *spict-gal4* to drive the expression of FLP recombinase to permanently label CCs that were *spict*-positive at one point (*spict-gal4, UAS-FLP, act>stop>gal4, UAS-GFP, tubP-gal80^{ts}*) and followed their fates. The flies were raised at 18°C to repress *spict-gal4* expression until adulthood, and the young adult flies of this genotype were shifted to 29°C after eclosion to allow the lineage tracing of *spict*-positive cells. Before temperature shift, there was no *gal4* activity, thus the entire testis was GFP-negative (Fig. 2.2A). 6 hours after temperature shift, GFP$^+$, Zfh-1$^+$ cells were apparent (Fig. 2.2B). At this point, none of GFP-positive, Zfh-1-positive cells were observed adjacent to the hub cells, consistent with the above results that *spict*-positive cells are not CySCs. By 24 hours after temperature shift, the number of GFP-positive Zfh-1-positive cells had dramatically increased, and they started to appear adjacent to the hub (Fig. 2.2C, D). Eventually, almost all Zfh-1-positive cells became GFP-positive (Fig. 2.2D). This suggests that *spict*-positive CCs frequently revert back to *spict*-negative, CySC state. This likely explains the neutral competition model proposed by Amoyel et al. (Amoyel et al., 2014). If

CySCs divide symmetrically without ever expressing *spict*, our *spict-gal4*-mediated lineage tracing would not have resulted in an increase of GFP-positive CySCs. Combined with our previous report that CySCs consistently divide asymmetrically with regard to the attachment to the hub cells (i.e. 96% of CySC division yielded one daughter cell attaching to the hub, the other detaching from the hub (Cheng et al., 2011)), we propose that a CySC divides asymmetrically with respect to *spict* expression (and attachment to the hub cells), generating a *spict*-negative CySC and a *spict*-positive differentiating CC, and that *spict*-expressing CCs frequently dedifferentiate to reacquire CySC identity. As a result of frequent dedifferentiation events, the CySC population follows a neutral competition model as demonstrated by Amoyel et al. (Amoyel et al., 2014).

2.3.4 *spict* does not regulate BMP signaling in the *Drosophila* testis

It was shown that *spict* facilitates BMP receptor endocytosis to negatively regulate BMP activity in the *Drosophila* neuromuscular junction (Wang et al., 2007). Spict bears homology to mammalian NIPA1, 2 and Ichthyin proteins, which are predicted to encode transmembrane proteins (Chai et al., 2003; Dahlqvist et al., 2012; Lefevre et al., 2004). NIPA1 was also shown to be required for downregulating BMP signaling in the cultured neuron (Goytain et al., 2007; Goytain et al., 2008). Therefore, we sought to test whether *spict* may be required for BMP signaling in the *Drosophila* testis.

Upon BMP ligand-receptor binding, Mad (mother against Dpp) is phosphorylated (pMad) and translocates to the nucleus for downstream transcriptional regulation (Massague and Wotton, 2000; Raftery and Sutherland, 1999; Sekelsky et al., 1995). By using pMad as a readout of BMP activity, we investigated whether a *spict* mutant may have altered BMP signaling activity in the testis. In wild type testes, it is well known that GSCs are positive for pMad, as BMP signaling functions in GSC self-renewal (Inaba et al., 2015; Kawase et al., 2004; Leatherman and Dinardo, 2010). In addition, it has been observed that differentiating CCs far from the hub also show pMad signal, which was shown to

regulate spermatocyte differentiation (Chang et al., 2013; Matunis et al., 1997). We observed no detectable difference in pMad levels between control and *spict* mutant testes, either in the GSC population or in the late CC population (Fig. 2.3). These results suggest that *spict* might not play a role in regulating BMP activity during spermatogenesis.

2.3.5 *spict* is required for SG death and maintenance of the GSC pool upon starvation.

In order to explore the function of *spict* in *Drosophila* spermatogenesis, we examined Spict protein localization using *UAS-spict-mRFP* expressed under the control of *spict-gal4*. We noticed that Spict-mRFP shows a similar pattern to Lysotracker staining (Fig. 2.4A, B, see below for detailed Spict localization), which we have previously shown to mark dying SGs (Yang and Yamashita, 2015). Indeed, we confirmed that Spict-mRFP co-localized with Lysotracker, a dye that detects low pH compartments, such as lysosomes (Fig. 2.4A, B). These observations prompted us to ask whether *spict* plays a role in SG death.

In our previous work, we showed that SG death is significantly upregulated upon protein starvation (Yang and Yamashita, 2015). SG death is triggered by the apoptosis of an encapsulating CC, which, in turn, initiates SG death, likely due to the CCs' essential role in SG survival (Lim and Fuller, 2012). We further showed that inhibition of SG death by blocking CC apoptosis led to a continuous decline in GSC number and an eventual collapse in tissue homeostasis under protein starvation conditions, suggesting that sacrificing SGs plays a critical role in maintaining the GSC population during starvation (Yang and Yamashita, 2015). Spict-mRFP was observed in dying SGs irrespective of nutrient conditions (Fig. 2.4A, B), but more Spict-mRFP was observed under starvation conditions, as SG death increases under starvation conditions. We found that SG death was significantly decreased in *spict* mutants ($spict^{65}/spict^{41}$) or upon RNAi-mediated knockdown of *spict* (*tj-gal4>*$spict^{RNAi}$) compared to control (Fig. 2.4C). Consistent with our previous study, which showed that SG

death is required for maintaining the GSC pool (Yang and Yamashita, 2015), *spict* mutants failed to maintain GSC number during prolonged protein starvation (Fig. 2.4D, E, F). *spict* mutants maintained similar numbers of GSCs compared to control under fed conditions (data not shown), suggesting that *spict*'s requirement is more profound during protein starvation.

Taken together, these results suggest that *spict* is required to promote starvation-induced CC/SG death, and consequently for the maintenance of the GSC pool in response to protein starvation.

2.3.6 Spict protein is expressed in CCs associated with dying SGs and is transferred from CCs to dying SGs.

To gain further insights into the role of *spict* in SG death, we expanded our analysis of Spict localization. Spict protein was highly upregulated in the CCs near dying SGs and appeared punctate in those cells (Fig. 2.5A). Spict-mRFP accumulation near the dying SG is likely due to increased mRNA translation or protein accumulation as the *spict-gal4* reporter (*spict-gal4>UAS-GFP*) described above (Fig. 2.1) shows ubiquitous expression of GFP in early CCs (excluding CySCs). In support of this idea, when a CC clone expressing *UAS-spict-mRFP* was generated using a constitutive *actin* promoter (*hs-Flp, Act>stop>gal4, UAS-GFP, UAS-spict-mRFP*), Spict-mRFP was observed specifically when the CC clone was adjacent to dying SGs (Fig. 2.5B-D). Accumulation of Spict-mRFP was observed in 77% of the CC clones that are associated with dying SGs, whereas Spict-mRFP accumulation was observed only in 14% of the CC clones that are not adjacent to dying SGs (Fig. 2.5E).

In addition, Spict-mRFP was observed in the dying SGs themselves (Fig. 2.5A). Since *spict-gal4*, which is expressed in CCs but not in germ cells (Fig. 2.1), was used to drive *UAS-spict-mRFP* expression, Spict-mRFP is likely transferred from the CC to the dying SG. A similar result was obtained using a well-established CC driver, *tj-gal4* (data not shown), suggesting that the Spict-

mRFP observed in dying SGs is not due to upregulation of *spict* expression in dying SGs, but due to the transfer of Spict protein from CCs to dying SGs. Taken together, these results suggest that Spict protein is stabilized in CCs near the dying SGs, then transferred to the dying SGs.

To better understand Spict-mRFP's localization during the progression of SG death, we first conducted a detailed characterization of the SG death process by combining various markers (Vasa, LaminDm0, DAPI and Lysotracker, Fig. 2.6), extending our previous characterization (Yang and Yamashita, 2015). Based on these markers, we now divided SG death into 4 phases. During phase 1, as SGs initiate cell death, the level of Vasa (a germ cell marker) decreases compared to neighboring SGs that are not dying, coinciding with the appearance of low levels of Lysotracker in the dying SG. Lysotracker intensity increased significantly during phase 2, indicating acidification of the germ cell cyst and thus progression of the cell death process. This phase was also characterized by the complete disappearance of Vasa staining, whereas a nuclear envelope marker, LaminDm0, and DAPI staining remained at this phase, suggesting that the nuclear compartment is still relatively intact. This was followed by phase 3, when LaminDm0 staining disappeared while DAPI staining remained, indicating that digestion of the nuclear compartment is progressing. Finally during phase 4, all Vasa, LaminDm0 and DAPI staining disappeared, and the remnants of dead SGs were only visible with Lysotracker, which sometimes was weaker than phases 2 and 3. The temporal order of SG death was confirmed by inducing synchronized SG death through expression of the proapoptotic gene *grim* in CCs, as described previously (Lim and Fuller, 2012). Indeed, after induction of *grim* expression, phases 1 through 4 appeared in the expected order, suggesting that phase 1-4 characterization is accurate (Fig. 2.6).

We adapted these criteria to further characterize Spict localization in detail during SG death (Fig. 2.5F-J). Because the patterns of Vasa, LaminDm0 and DAPI during SG death were fairly consistent, cell death phases can be determined even without the use of Lysotracker. Thus, we combined Spict-mRFP

with Vasa, LaminDm0, and DAPI to follow the localization of Spict-mRFP during SG death. We found that dying SGs are not yet associated with Spict-mRFP during phase 1 (Fig. 2.5F). During phase 2, Spict-mRFP started to accumulate near dying SG (Fig. 2.5G), followed by phase 3, when Spict-mRFP was highly upregulated in the CCs near dying SGs and it was also observed in dying SGs (Fig. 4H). During phase 4, Spict-mRFP in the CC was mostly gone, and it was mainly observed in dying SGs (Fig. 2.5I).

The above results suggest that Spict-mRFP protein accumulates in CCs and dying SGs after the initiation of SG death, i.e. in phases 2 through 4. This raised a question as to in which CCs Spict-mRFP might be upregulated. Our previous study showed that SG death is triggered by the apoptosis of CCs (Yang and Yamashita, 2015), indicating that CC apoptosis must occur before/during phase 1. If this is the case, Spict-mRFP is not expressed in the CCs that apoptose to trigger SG death. We suspected that only one of two CCs that encapsulate the SG cyst die to trigger SG death, and the other CC might survive, which is the CC that upregulates Spict protein. To resolve the temporal order of CC death and Spict expression, we labeled CC nuclei with histone H1-YFP (*c587-gal4>UAS-histone H1-YFP*) (Fig. 2.7). With this method, we found that the CC death that triggers SG death does indeed occur during phase 1 of SG death: during phase 1 of SG death, histone H1-YFP-positive and Lysotracker-positive cells were identified near the dying SG, indicating that these are the CCs whose death triggers SG death. However, during phase 2, no histone H1-YFP positive, Lysotracker positive cells were observed near the dying SGs, suggesting that CC death is complete by this time. However, interestingly, during phase 3, we again observed Lysotracker-positive, histone H1-YFP-positive CCs near the dying SGs. Because the death of CCs is complete by phase 2, the histone H1-YFP-positive, Lysotracker-positive CCs observed during phase 3 is likely the surviving CCs (one of the two CCs that encapsulate the SGs as a pair, Fig. 2.1A). The surviving CCs become Lysotracker-positive, not because they are dying, but because they are likely absorbing the contents of dead SGs (see below).

Taken together, these results suggest that Spict-mRFP is upregulated in surviving CCs after the initiation of SG death. Moreover, our observations described here indicate that the death of SGs is triggered by apoptosis of one of the CCs within the pair that encapsulate the SGs, and that Spict is subsequently upregulated in the second CC of the pair (Fig. 2.5K).

2.3.7 Spict localizes to the late endosome/phagosome, during engulfment of dying SGs by CCs.

Previously, it was shown that Spict colocalizes with Rab5-positive early endosomes (Wang et al., 2007). Likewise, it was also shown that a mammalian homologue of Spict, NIPA1, colocalizes with various endosomal compartments (Tsang et al., 2009). Their localization to endosomal compartments was linked to their role in the regulation of BMP receptors. Although our results indicate that *spict* likely does not regulate BMP signaling in the *Drosophila* testis (Fig. 2.3), colocalization of Spict-mRFP with Lystotracker (Fig. 2.4, 2.5) indicates that Spict is involved in the endocytic pathway. Thus, we examined the potential colocalization of Spict-mRFP with various endosomal compartments by expressing Spict-mRFP together with EYFP-tagged endogenous Rab small GTPases (Dunst et al., 2015) or UAS-GFP tagged Rab GTPases (Entchev et al., 2000; Zhang et al., 2007). We did not observe obvious colocalization of Spict-mRFP with early endosomal markers, such as Rab5, or with recycling endosome markers, such as Rab4, Rab11 and Rab35 in the testis (Fig. 2.8A and not shown) (Grant and Donaldson, 2009). However, we found that Spict often colocalizes with Rab7, a marker for the late endosome (Fig. 2.8B). Given that Spict also colocalizes with Lysosomes, it appears that Spict is associated with the late endosome/lysosome compartment in the *Drosophila* testis. We noticed that Spict-positive dying SGs were frequently contained within a large vesicle positive for Rab7-EYFP (Fig. 2.8B). Such Rab7-positive ‘vesicles’ could reach almost ~20 μm in diameter, which is the size of the entire cyst of dying SGs containing up to 16 cells. Since Rab7 is known to be required for phagosome maturation (Rupper et al., 2001; Shandala et al., 2013), we suspected that the

entire dying SG might be phagocytosed by the neighboring cells (i.e. CCs). Indeed, when a single CC clone expressing GFP and a membrane marker (mCD4-tdTomato) (*hs-FLP; act>stop>gal4, UAS-mCD4-tdTomato*) was induced, the plasma membrane of the clone marked by mCD4-tdTomato continuously wrapped around the Lysotracker-positive dying SGs (Fig. 2.8C). This CC is most likely the surviving CC of the pair, which did not undergo apoptosis at the beginning of the death process. Moreover, staining with another membrane dye, FM4-64, corroborated that the Rab7-EYFP-positive vesicle was contained within a single CC (Fig. 2.8D). Taken together, these data suggest that dying SGs are encapsulated by a single CC, likely via phagocytosis (Fig. 2.8E). The data also indicate that the CC that phagocytoses the dying SGs is the surviving CC, in which Spict is upregulated.

2.3.8 *spict* is required for normal progression of SG death

Based on our observation that Spict is highly upregulated in the CCs that phagocytose dying SGs, we speculated that *spict* might be required for processing/clearing the dead SGs. However, we did not see obvious changes in the degree of acidification in dying SG or the frequency of Rab7-posistive phagosome formation in *spict* mutants (data not shown). The SG cell death process from initiation to complete clearance of dead SG appears to take a long time (>24 hours, based on indirect inference from our observations), making it difficult to assess the cell death process via live observation. Therefore, to study the process of SG death progression, we developed an *ex vivo* testis culture system. First, dissected testes were stained with Lysotracker for 30 minutes to label SGs that were already dying. Testes were then transferred to Lysotracker-free medium and cultured for 8 hours (Fig. 2.9A). At the end of the culture period, testes were fixed and stained for Vasa, LamDm0, and DAPI to identify dying SGs as described above (Fig. 2.6). With this method, SGs that were already dying before the culture period can be identified as Lysotracker-positive dying SGs, whereas SGs that initiated the cell death process during the culture period can be identified as Lysotracker-negative dying SGs (Fig. 2.9B). We restricted our

analysis to the Lysotracker-negative dying SGs, because these allowed us to precisely follow how SGs initiated the death process and progressed through the cell death stages during the 8 hour chase period.

Using this approach, we compared control and *spict* mutant testes to see whether *spict* mutants might show any defects in the process of SG death progression. After the 8 hour chase period, the control testes had 0.77±0.15 Lysotracker-negative dying SGs/testis (N=119) whereas *spict* mutant testes had 0.55±0.16 dying SGs/testis (N=123 testes, p=0.028). This result suggests that *spict* mutants are defective in initiating SG death, which is consistent with the results described in Fig. 2.4. In addition, 38% of dying SGs in control testes were observed to be in phase 2 and 3, indicating that some dying SGs in control testes progressed through the SG death phases after initiation during the 8 hour culture period (Fig. 2.9C). In contrast, in *spict* mutant testes, only 21% of Lysotracker negative dying SGs progressed beyond phase 1 (p=0.032). These results indicate that *spict* mutants are also defective in progressing through the SG death phases after the initiation of death (Fig. 2.9C). This further indicates that it takes longer for SGs to complete the cell death process in *spict* mutants. If it takes longer for each dying SG to complete the death process in *spict* mutants, the frequency of SG death in *spict* mutants is likely an overestimate, when estimated based on the number of dying SGs in a fixed sample (Fig 2.4C). Thus, the decrease in SG death in *spict* mutants described above is likely more profound than is shown in Fig 2.4C. In summary, we conclude that *spict* is required for initiation and progression of SG death in the *Drosophila* testis.

2.4 Discussion

In this study, we identified *spict* as a gene that is specifically expressed in the differentiating cyst cells (CCs) of the *Drosophila* testis and that it can serve as a novel marker to better identify cyst stem cells (CySCs). We showed that Spict protein is specifically stabilized in the CCs that envelope dying SGs, and that *spict* is required for the normal progression of SG death. Our study indicates that

CC-mediated SG death plays a critical role in allowing the tissue to cope with protein starvation in order to maintain the germline stem cell (GSC) pool.

Our data showed that dying SGs are encapsulated entirely by Rab7-positive vesicles. Moreover, our data showed that such large, Rab7-positive vesicles were contained within a single CC. Thus, we propose that dying SGs are phagocytosed by neighboring CCs, which clear the corpses of SGs. Our earlier study showed that the apoptosis of a CC is required to trigger SG death (Yang and Yamashita, 2015). Now, our study shows that the remaining CC encapsulates the dying SGs, further elucidating how SG death occurs in the *Drosophila* testis. We propose the following scenario of CC-mediated SG death. First, one of the two CCs that encapsulate the SGs apoptoses in response to certain stimuli, such as protein starvation. Death of one CC breaks the 'blood-testis-barrier' generated by the pair of CCs (Fairchild et al., 2015), leading to SG death (Lim and Fuller, 2012). The remaining CC ('surviving CC') now engulfs the dying CC and the SGs and clears the dead cells via phagocytosis. Spict protein was specifically upregulated in surviving CCs and apparently transferred to dying SGs. Considering that the progression of SG death is slower in *spict* mutants compared to the control, we speculate that *spict* may be required for the progression of phagocytosis, the subsequent digestion of engulfed dead cells, and/or the recycling of digested SG material in the surviving CCs. However, we did not observe any differences in the frequency of large, Rab7-positive phagosome formation between control and *spict* mutant testes (not shown). In addition, we did not detect any differences in the degree of lysosome/phagosome acidification in control vs. *spict* mutant testes (not shown). Therefore, it remains unclear exactly how *spict* may promote the progression of SG death.

Programmed cell death can be induced in a cell-intrinsic (suicide) or -extrinsic (murder) manner (Green and Llambi, 2015). Recently, engulfment genes were shown to be required for the developmentally programmed death of the B.alapaav cell in *C. elegans* by assisting with cell death processes *(Johnsen and Horvitz, 2016)*. In addition, engulfment genes were also shown to promote

the unequal segregation of apoptotic potential to induce NSMsc cell death in *C. elegans* (Chakraborty et al., 2015). Likewise, it was shown that in *Drosophila* oogenesis, the phagocytic machinery of follicle cells is required for developmentally programmed death and removal of nurse cells (Timmons et al., 2016). Starvation-induced SG death in the *Drosophila* testis described in this and our previous study (Yang and Yamashita, 2015) holds a striking similarity with these cell death processes in that the cell death precedes via a complex interaction of multiple cell types. SG death depends on the apoptosis of a CC, and later, its removal/clearance depends on the phagocytic activity of the remaining CC. Our data suggest that *spict* is likely required for promoting the process of SG removal after phagocytoses by the remaining CC.

In summary, our study identified *spict* as a gene expressed in the differentiating CCs in the *Drosophila* testis. *spict* is required for the proper progression of SG death and for maintaining the GSC population during protein starvation. Through a detailed characterization of Spict localization, we revealed a highly regulated process of SG death that involves CC death and phagocytosis by the surviving CC, and our data suggest that *spict* may be involved in this process. We propose that carefully regulating the death of transit-amplifying cells during starvation is a critical mechanism to preserve the stem cell population and that the transit-amplifying cell population serves as a major point of regulation in shifting tissue homeostasis.

2.5 Materials and Methods

2.5.1 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-spict-mRFP, spict*^{41} were generous gifts of Cahir O'Kane (Wang et al., 2007), *UASt-H1-YFP* was a kind gift of Alexei Tulin, Fox Chase cancer center (Pinnola et al., 2007), *UAS-mCD4-tdTomato, tj-gal4, UAS-spict-RNAi* ^{GLC01402}, *UAS-spict-RNAi*^{HMS01647}, *UAS-Rab4-GFP, Rab5-EYFP, Rab7-EYFP, UAS-Rab7-GFP, tub-gal80*^{ts} were obtained from the Bloomington Stock Center, *spict-gal4*^{NP112900} was obtained from the Kyoto Stock Center, *UAS-grim* was a kind gift from Margaret T. Fuller (Wing et al., 1999).

2.5.2 Generation of *spict*^{65} allele

Two target sequences (AACAGAGC|AAGTGAGTCATA AGG and GCAAGGGA|TGTAACTAGACC TGG) for CRISPR-mediated knockout were selected to delete the whole second exon of *spict*. These constructs were co-injected into *vas-Cas9*$^{ZH-2A}$ flies. The genotype of potential mutants was determined by PCR and sequenced to confirm the deletion. *spict*^{65} contains the deletion with imprecise repair at the junction resulting in a short, in-frame insertion (underlined, CAGAAC-AGAAACAGA-ACA) and truncation of *spict*.

2.5.3 Immunofluorescent staining and microscopy

Immunofluorescent 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; developed by A.

Spradling), rabbit anti-vasa (1:200; d-26; Santa Cruz Biotechnology), mouse anti-Fasciclin III (1:200; DSHB; developed by C. Goodman), anti-LaminDm0 (1:200; DSHB; developed by P. A. Fisher), rabbit anti-Thr3-phsophorylated Histone H3 (PH3) (1:200; Upstate, Millipore, Billerica, CA);, guinea pig anti-Traffic jam (Tj) (1: 400; a kind gift of Dorothea Godt) (Li et al., 2003), rabbit anti-Zfh-1 (1:5000; a kind gift of Ruth Lehmann). Images were taken using Leica TCS SP5 and TCS SP8 confocal microscopes with 63x oil-immersion objectives (NA=1.4) and processed using Adobe Photoshop software. For detection of germ cell death, testes were stained with Lysotracker in PBS (1:1000) for 30 minutes prior to formaldehyde fixation. Cell death phases were identified by using anti-Vasa, anti-LaminDm0, DAPI staining and Lysotracker.

For observation of unfixed testes, testes were dissected directly into PBS and incubated in the dark with the desired dye(s) for 5 minutes, mounted on slides with PBS and imaged within 10 minutes of dissection. The dyes used in live imaging are: Lysotracker Red DND-99 (1:1000), Lysotracker Blue DND-22 or Lysotracker Green DND-26 (1:200) (Thermo Fisher Scientific), Hoechst 33342 (1:1000), and the FM4-64FX in PBS (1:200) (Thermo Fisher Scientific).

2.5.4 Lineage tracing of *spict*-expressing cells

To lineage-label cells that once expressed *spict*, *spict-gal4* was used to drive *UAS-FLP*, which removes the stop codon between the *actin* promoter and *gal4* (*spict-gal4, UAS-FLP, Act>stop>gal4, UAS-GFP, tubP-gal80^{ts}*). Cells that once expressed *spict* will be labeled with GFP. *spict-gal4* activity was repressed by *tub-PGal80^{ts}* when flies were cultured at 18°C, ensuring that no labeling occurred before adulthood. Newly eclosed flies were shifted to 29°C to activate *spict-gal4* expression.

2.5.5 Pulse-chase experiment to track SG death progression

Freshly eclosed control (*spict^{65}/ CyO* or *spict^{41}/ CyO*) and *spict* mutant (*spict^{64}/spict^{41}*) flies were starved for three days. Testes were dissected directly

into Schneider's *Drosophila* medium (Thermo Fisher Scientific) on a glass dissection dish and soaked in Lysotracker Red (1:1000 in Schneider's media) in the dark for 30 minutes (tubes were rotated to ensure even staining). The tissues were then rinsed with media three times prior to the chase period to avoid carry over of extra Lysotracker. <5 testis were transferred into 20µl-droplets of media on a 35mm petri dish. To prevent evaporation, extra media droplets were added on the dish near the tissue-containing droplets, and the dish was sealed with parafilm. The samples were then kept in the dark at room temperature for 8 hours (chase period) prior to fixation and staining.

2.6 Acknowledgements

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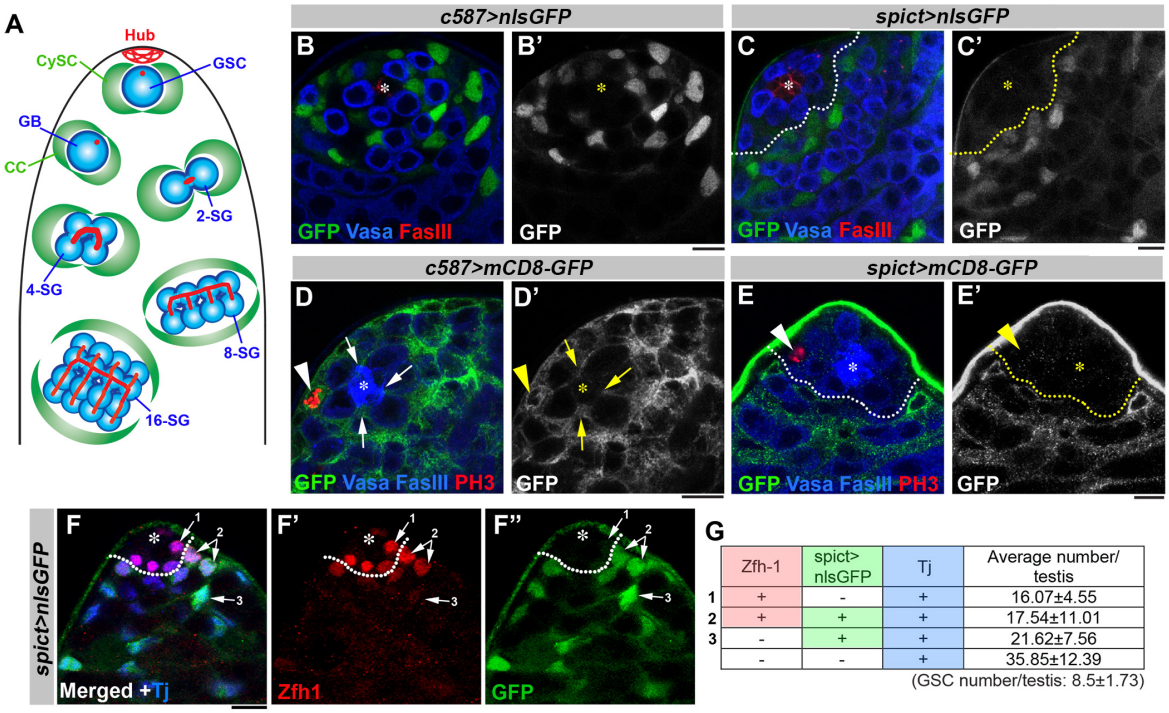
2.7 Figures



	Zfh-1	spict> nlsGFP	Tj	Average number/ testis
1	+	-	+	16.07±4.55
2	+	+	+	17.54±11.01
3	-	+	+	21.62±7.56
	-	-	+	35.85±12.39

(GSC number/testis: 8.5±1.73)

Fig. 2.1 *spict* is expressed in differentiating cyst cells.

(A) Diagram of early spermatogenesis at the apical tip of the *Drosophila* testis. Germline stem cells (GSCs), gonialblast (GB), 2,4,8,16-cell spermatogonia (SGs), cyst stem cells (CySCs), cyst cells (CCs). GSCs and CySCs are attached to the stem cell niche component hub cells. CySCs encapsulate GSCs. GSCs produce GBs by asymmetric division. GBs are encapsulated by CCs, which promote differentiation of germ cells as SGs.

(B, C) Expression of UAS-nlsGFP under the control of the *c587-gal4* driver (B) or the *spict-gal4* driver (C). nlsGFP illuminates the nuclei of gal4-expressing cells.

(D, E) Expression of UAS-mCD8-GFP under the control of the *c587-gal4* driver (D) or the *spict-gal4* driver (E). mCD8-GFP outlines the cell surfaces of gal4-expressing cells. Processes of cyst cells are outlined by expression of membrane-bound UAS-mCD8-GFP with the pan-cyst cell driver *c587-gal4* (D) or *spict-gal4* (E). Mitotic cells are labeled with PH3 (arrowhead). CySC processes that touch the hub are indicated by arrows (D, D').

(F) Apical tip of a testis showing nlsGFP expression under control of the *spict-gal4* driver and co-stained with Zfh-1 (red) and Tj (blue).

(G) Quantification of somatic cells based on the expression of Zfh-1, Tj and *spict>nls-GFP*. Asterisk indicates the hub; the dotted line indicates the boundary of *spict* expression. Bar: 5μm.

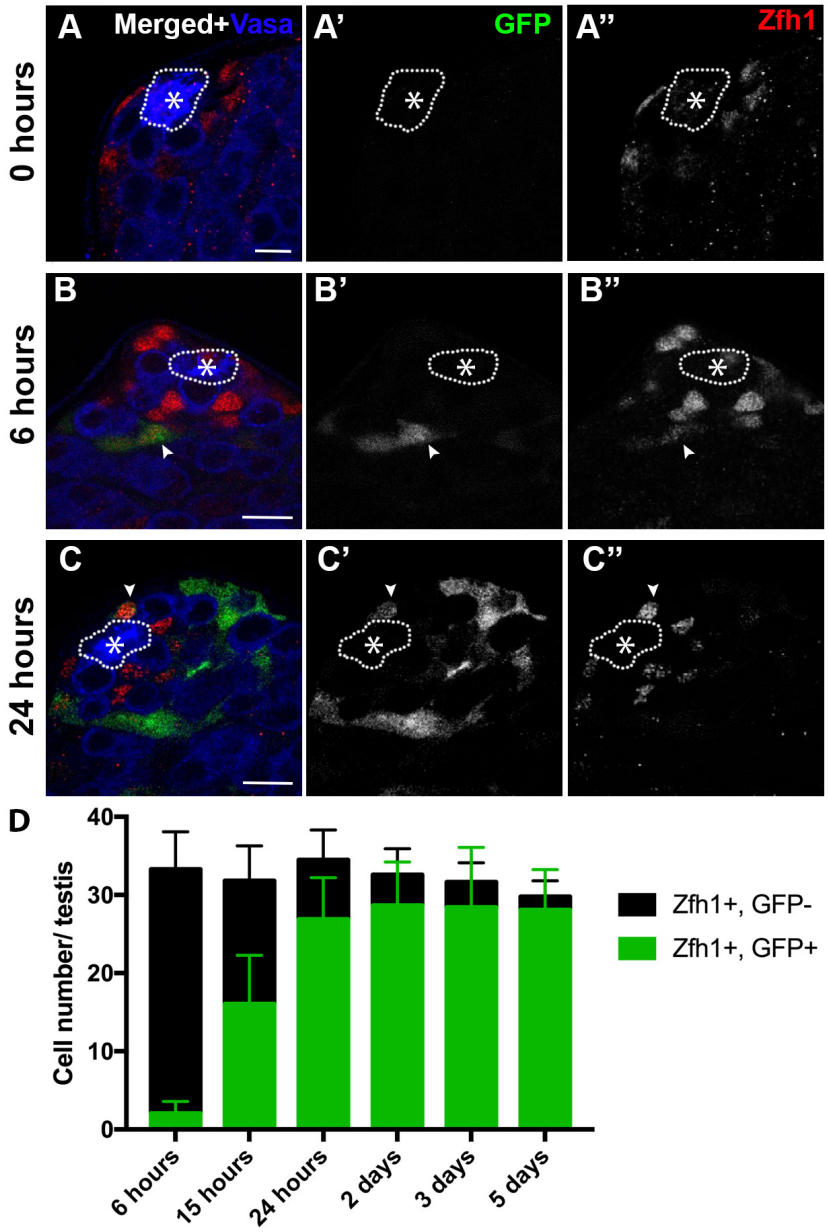


Fig. 2.2 Lineage tracing of *spict*-expressing cells in the testis reveals the frequent conversion of *spict*-positive cells into CySCs.

(A-C) *spict-gal4; UAS-FLP* flies were crossed with *Act>stop>gal4, UAS-EGFP; tubP-gal80^{ts}* at the permissive temperature (18˚C) until eclosion (A, 0 hours). Upon eclosion, adult flies were transferred to the non-permissive temperature (29˚C) for 6 hours (B), and 24 hours (C). Zfh-1-positive, GFP-positive CCs (arrowheads) appear after 6 hours, and Zfh-1-positive, GFP-positive CySCs appear after 24 hours (C). Bar: 10μm. The hub is indicated by an asterisk and outlined by a dotted line.

(D) Quantification of Zfh-1-positive cells based on GFP expression.

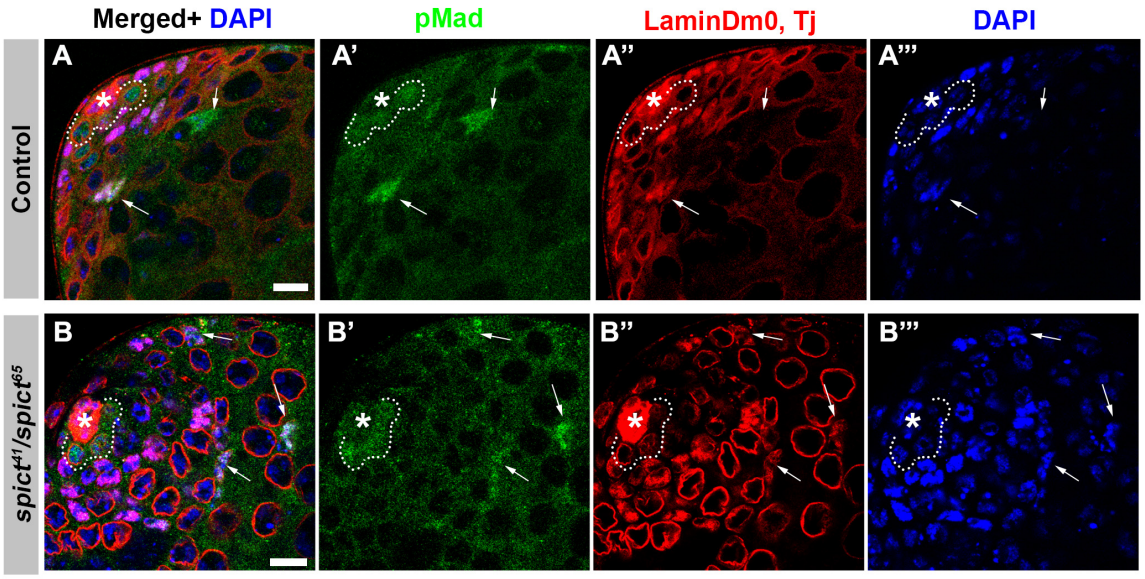


Fig. 2.3 pMad level is not obviously changed in the *spict* mutant testis.

(A, B) Testes from control (*spict*^{41}*/Cyo,* A) or mutant (*spict*^{65}*/ spict*^{41}, B) testes were starved for three days upon eclosion and stained for pMad (green), LaminDm0/Tj (red) and DAPI (blue). In both genotypes, pMad was detected in the GSC nuclei (encircled by a dotted line) around the hub (asterisk) as well as late differentiating CCs (arrows) associated with spermatocytes. Bar: 10μm.

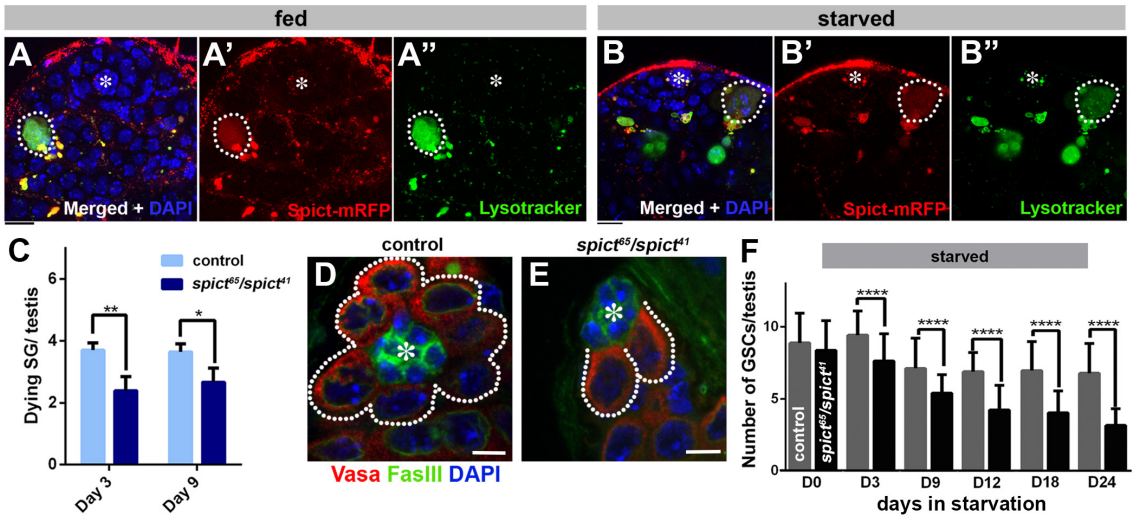


Fig. 2.4 *spict* is required for SG death and GSC maintenance during protein starvation.

(A, B) Localization of Spict-mRFP (*spict-gal4>UAS-spict-mRFP*) in an adult testis under fed (A) and protein-starved conditions (B). A dotted line encircles dying SG; Bar: 10μm.

(C) Quantification of SG death in control (*spict*^{41}*/ CyO*) and mutant (*spict*^{65}*/ spict*^{41}) testes upon 3 and 9 days of protein starvation.

(D, E) Examples of the apical tip after 24 days of protein starvation in control (D) and *spict*^{65}*/ spict*^{41} mutant (E) testes. GSCs are indicated by dotted lines. The hub is indicated by asterisks. Bar: 5μm.

(F) GSC number in control (gray) and *spict*^{65}*/ spict*^{41} mutant (black bar) testes during protein starvation (*: P<0.05, **: P<0.005, ****: P<0.00005).

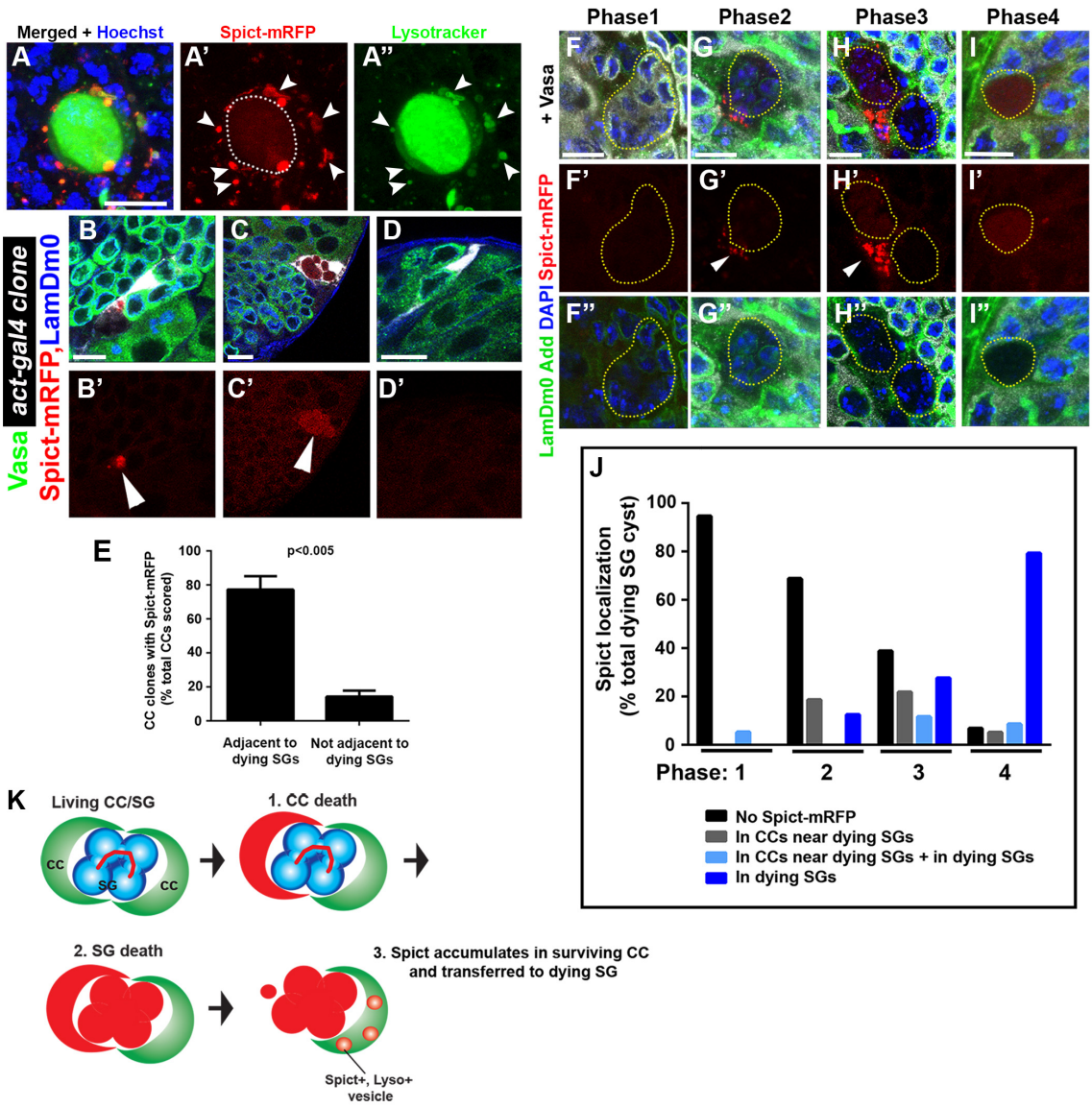


Fig. 2.5 Spict localizes to CCs associated with dying SG and transferred to zdying SGs.

(A) An example of dying SGs positive for Lysotracker (green) and Spict-mRFP (red) in a *spict-gal4>UAS-spict-mRFP* testis. Bar: 10μm.

(B-D) Examples of CC clones that express Spict-mRFP in the presence (G, H) or absence (I) of neighboring dying SGs. Even though all CC clones (*act-gal4*-positive, white) activate *UAS-spict-mRFP* expression, Spict-mRFP protein was visible only when the clones were adjacent to dying SGs. Arrowheads indicate dying SGs (G', H'). Bar: 10μm.

(E) Quantification of the percentage of Spict-mRFP-positive CC clones in the presence or absence of neighboring dying SGs.

(F-I) Representative images of Spict-mRFP localization during the course of SG death. Phase 1(B), phase 2 (C), phase 3 (D) and phase 4 (E). Yellow dotted lines encircle the dying SGs. Arrowheads indicate CCs with upregulated Spict-mRFP near the dying SGs (C', D'). Bar: 10μm.

(J) Distribution of Spict-mRFP localization during the course of SG death.

(K) Model of the SG death process: the living SGs are encapsulated by a pair of CCs. The death of one CC (1) triggers SG death (2) and the Spict protein accumulates in the surviving CC and is transferred to the dying SGs (3).

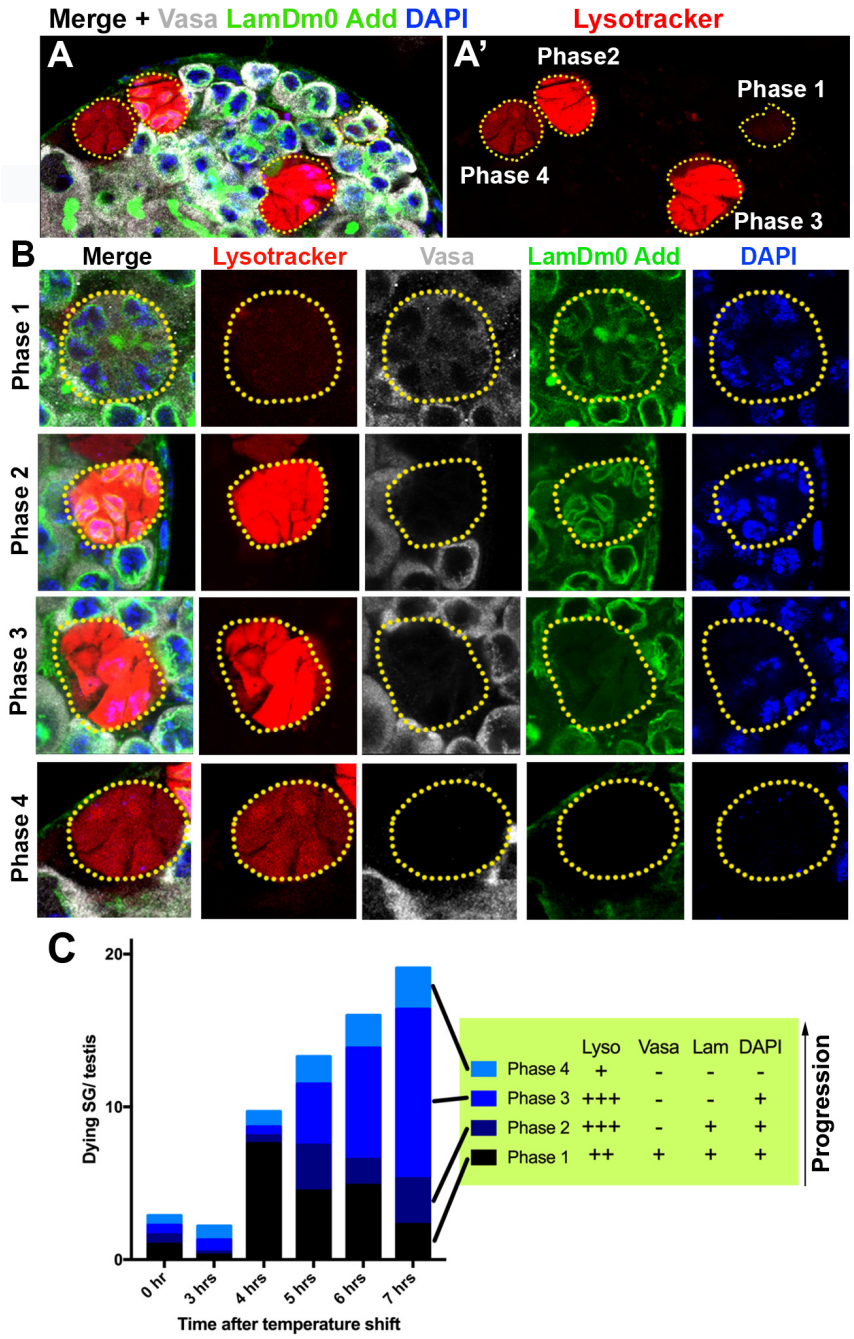


Fig. 2.6 Progression of SG death characterized by Lysotracker, Vasa, LaminDm0, Add and DAPI staining.

(A) An example of a testis apical tip containing multiple phases of SG death.

(B) SG death is divided into 4 phases based on Vasa, LaminDm0, Add, DAPI and Lysotracker staining. In phase 1, SGs become weakly Lysotracker-positive, whereas Vasa staining becomes slightly weaker. LaminDm0, Add, and DAPI staining still remains. In phase 2, Lysotracker staining becomes stronger and

Vasa staining becomes undetectable, leaving LaminDm0, Add and DAPI staining. In phase 3, Lysotracker staining remains strong, Vasa and LaminDm0 staining become undetectable, DAPI staining remains. In phase 4, all but Lysotracker staining are gone.

(C) Quantification of SG death phases upon synchronized induction of SG death by expression of Grim in CCs (*tj-gal4; tubP-gal80^{ts}, UAS-Grim*), confirming the order of the 4 phases of SG death.

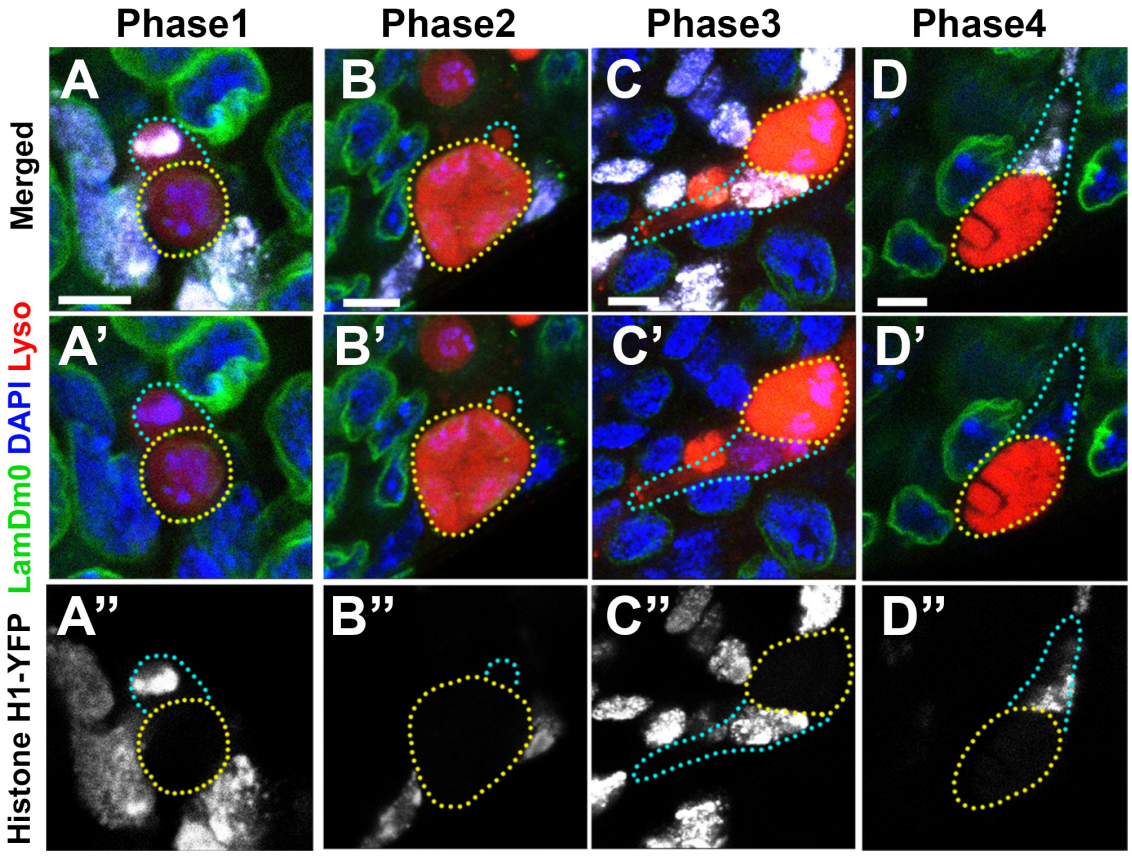


Fig. 2.7 Identification of dying CC and surviving CC associated with SG death

(A-D) Testes expressing histone H1-YFP in CCs (*c587-gal4>UAS-histone H1-YFP*) were stained for LaminDm0, Lysotracker and DAPI. During phase 1 of SG death (A), a CC near the dying SG becomes Lysotracker positive, indicating its death. At this point, the nuclei of the dying CC is positive for histone H1-YFP, indicating that its nucleus is still intact. During phase 2 (B), the dying CC associated with the dying SG becomes histone H1-YFP negative, indicative of completion of its death. During phase 3 (C) and phase 4 (D), however, Lysotracker-positive, histone H1-YFP-posistive CCs reappear associated with the dying SGs. These CCs are likely the ones of the encapsulating pair that did not die (see Fig. 2.5K and 2.8F for the model that one CC out of a pair of CCs that encapsulate SGs, one dies to trigger SG death and the other survives to phagocytose dying SGs). Dying SGs are indicated by yellow dotted lines, and associated CCs are indicated by cyan dotted lines. Bar: 5μm.

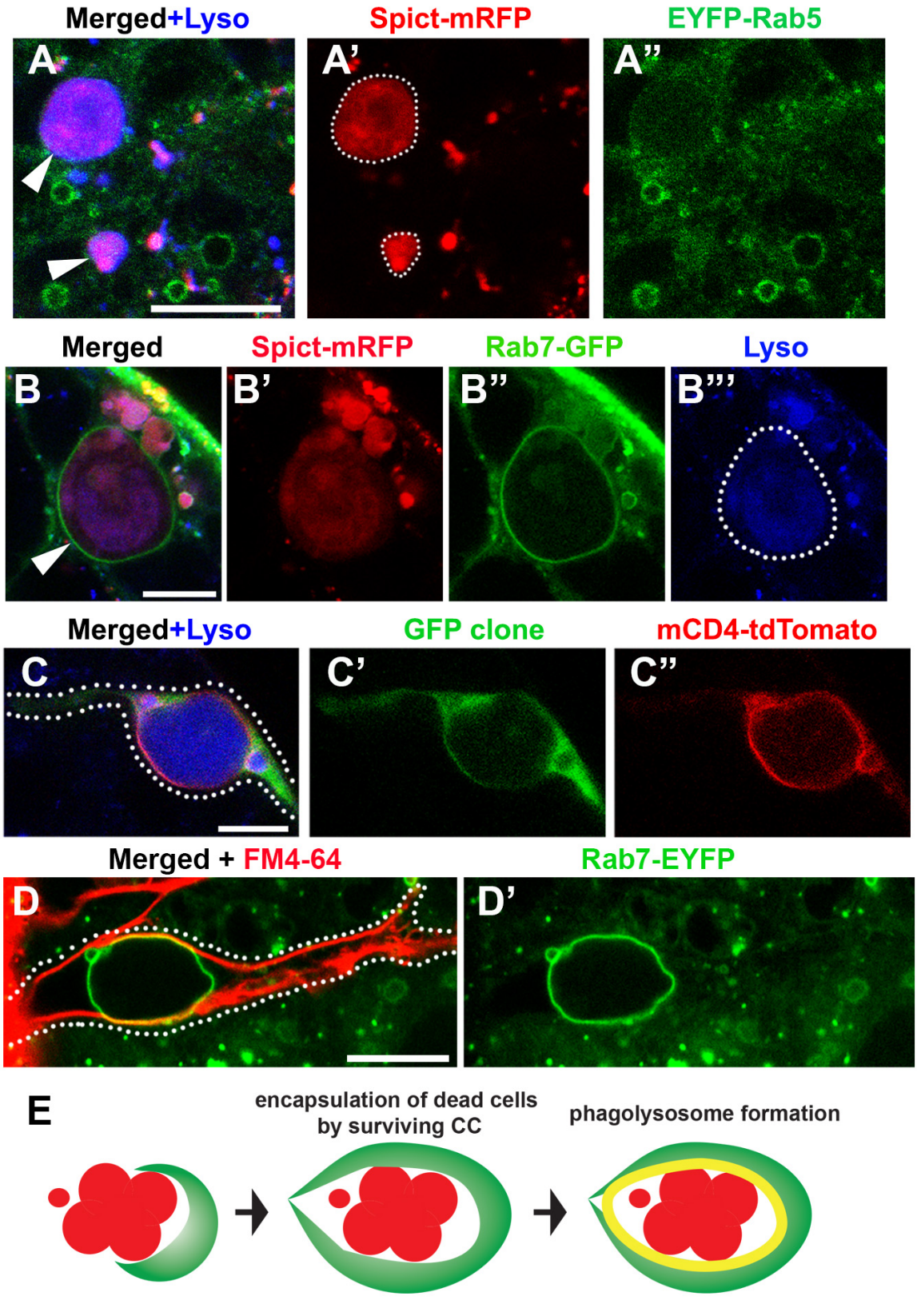

Merged+Lyso
Spict-mRFP
EYFP-Rab5
A
A'
A"
Merged
Spict-mRFP
Rab7-GFP
Lyso
B
B'
B"
B"'
Merged+Lyso
GFP clone
mCD4-tdTomato
C
C'
C"
Merged + FM4-64
Rab7-EYFP
D
D'
E
encapsulation of dead cells
by surviving CC
phagolysosome formation

Fig. 2.8 Spict localizes to Rab7-positive phagosomes that encapsulate dying SGs.

(A) Spict-mRFP expressed in CCs (*spict-gal4>UAS-spict-mRFP*) does not colocalize with the early endosome marker Rab5. Dying SGs are indicated by arrowheads. Bar: 10μm.

(B) The late endosome marker Rab7 colocalizes with Spict-mRFP and forms a large vesicle encapsulating dying SGs (arrowhead). Dying SGs are encircled by the dotted line in B'''. Bar: 5μm.

(C) An example of a single CC clone expressing GFP and mCD4-tdTomato (*hs-FLP, act>stop>gal4, UAS-GFP, UAS-mCD4-tdTomato*) encapsulating dying SGs entirely. A single CC clone is indicated by the dotted line. Bar: 10μm.

(D) Rab7-EYFP testis stained for the membrane dye FM4-64 to label the CC plasma membrane, demonstrating that the Rab7-positive vesicle is entirely encapsulated within a single CC. CC boundary is indicated the by dotted line. Bar: 10μm.

(E) Model of SG phagocytosis by the surviving CC.

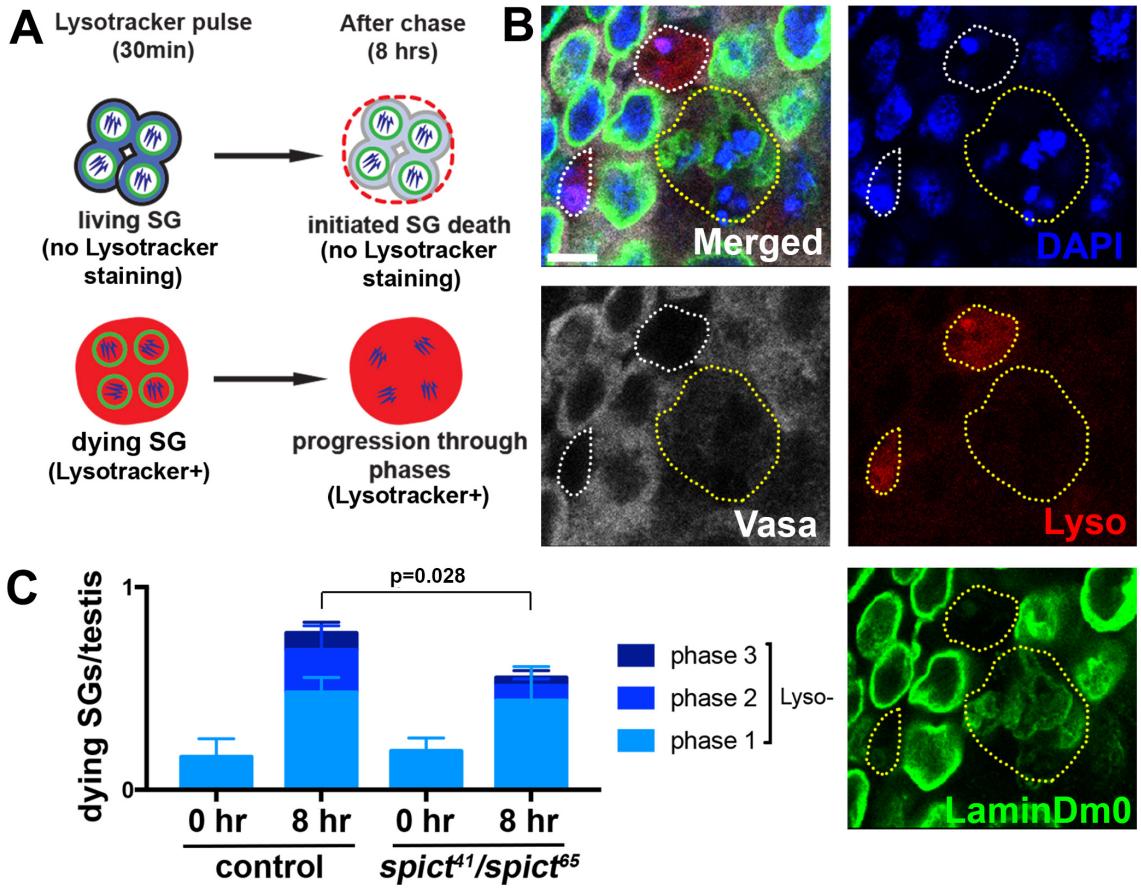


Fig. 2.9 *spict* regulates the progression of SG death.

(A) Schematic of the Lysotracker pulse-chase experiment. The dissected testes were stained with Lysotracker for 30 minutes and cultured in lysotracker-free media for 8 hours. SGs that initiated death during the 8 hour culture period are Lysotracker-negative, whereas SGs that were already dying at the beginning of culture are Lysotracker-positive. Dying SGs were identified by DAPI, Vasa and LaminDm0 staining.

(B) Example of SGs that initiated the death process during the chase period (Lysotracker-negative, yellow dotted line) and those that were already dying during pulse period (Lysotracker-positive, white dotted lines). Bar: 5μm.

(C) Quantification of cell death phase for SGs that committed to death during the chase period (lysotracker-negative) ($P<0.05$).

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Chapter 3:

Discussion and Future Directions

In this dissertation, I identified Spict as a protein that is specifically upregulated in the differentiating cyst cells (CCs) of the *Drosophila* testis. When CCs are in close proximity to dying spermatogonia (SG), Spict protein is stabilized and aggregated, suggesting a possible role of Spict in SG death. The dying SGs often reside in the Rab7-positive compartment, suggesting that they are engulfed by neighboring CCs. I further demonstrated that *spict* is required for the progression of SG death and, consequently, the maintenance of germline stem cells (GSCs) during protein starvation. Taken together, this work provides new insights into the maintenance of tissue homeostasis under starvation conditions, through the elimination of transit-amplifying cells.

In this section, I will summarize my findings and place them in the context of current questions in the fields of 1) distinguishing CySC/CC and 2) how engulfing cells facilitates tissue homeostasis during starvation.

3.1 Distinguishing the CySCs and CCs in *Drosophila* testis

Drosophila germline is a well-established model for asymmetric stem cell division. The spatial-temporal arrangement of its cellular architecture and morphology provides excellent resolution to pinpoint the developmental stage of each germ cell. However, unlike the GSC that can be identified at a single cell

resolution, due to its round nature, the physical character of CySC does not allow precise identification. To date, three transcription factors are used to distinguish differentiation stages of the somatic cell lineage. Zfh-1 marks CySCs and their immediate daughter cells, and is used as a proxy for CySCs (Leatherman and Dinardo, 2008). Tj labels a slightly broader population of early CCs, which includes Zfh-1-positive cells (Li et al., 2003). And finally, Eya is upregulated in late CCs associated with 8-cell SG or later spermatogonial cysts (Fabrizio et al., 2003). Hence, to further understand the nature of CySC, a better marker that distinguishes bona fide CySC from the Zfh1^{+} cell pool is needed.

3.1.1 Spict is a novel marker for differentiating CCs

In this dissertation, I identified *spict* as a gene that is specifically upregulated in differentiating CCs, a population previously indistinguishable from CySCs using Zfh-1 as a marker. Spict-expressing cells that also express Zfh-1 are mitotically inactive and do not project to the hub, thus representing the non-CySC population. This leaves a group of ~16 cells that are Zfh-1^{+} and Spict^{-}, and we propose that this population of CCs is highly enriched in CySCs.

Our observed number of ~16 Zfh-1^{+}, *spict*$^{-}$ CySC matches the previously proposed GSC:CySC ratio of 1:2 (Hardy et al., 1979). However, a recent study by Amoyel et al. estimated the GSC:CySC ratio to be 1:1 by analyzing the percentage of Zfh-1^{+} clones with a hub projection (Amoyel et al., 2014). The discrepancy may result from the methods used in each study to estimate the number of CySCs. Hardy et al. directly observed the architecture of CySCs from electron microscopy images while we used Zfh-1 and Spict expression as a way to estimate the number of cells that qualify as CySCs (Zfh-1^{+}, Spict^{-}, mitotically active, and attached to the hub). Amoyel et al., on the other hand, estimated CySC number based on the frequency of induced somatic clones with hub cell attachment. Because 30% of somatic clones displayed hub cell attachment, a characteristic that is unique to CySCs, they reasoned that each testis contains 30% CySCs (13 out of a total of 43 Zfh-1^{+} cells/testis). However, Amoyel et al.

examined the hub cell attachment of CySC clones two days post-heat shock. Considering that CySCs undergo stochastic loss, with a replacement rate of 0.84 cells/day (Amoyel et al., 2014), it is possible that CySC clones lost attachment after heat shock, resulting in an underestimation of CySC number. An alternative explanation is that the number of GSCs and CySCs may merely reflect genetic background, and that the key CySC function is to generate two CCs per gonialblast (GB) (Inaba et al., 2011). The latter may be achieved by adjustment of CySC and GSC proliferation rate.

One direct way to test whether only half of the Spict^{-} cells are CySCs [8 (equal to GSC number) out of 16 Zfh-1^{+} *spict*$^{-}$ cells], the fly can be fed with BrdU to label cells when they go through S phase. Theoretically, if the *spict*$^{-}$ population contains CCs, it shouldn't be BrdU positive after 1 CySC cell cycle time. In contrast, if most of Spict^{-} cells can self-renew, very few Spict^{-} cells will be left unlabeled after one CySC cell cycle time. The disadvantage for this particular method is that 1) the rate of BrdU uptake through feeding could be inconsistent; 2) CySC division can result in symmetric self-renewal during the feeding period, which can lead to over-estimation of CySC; 3) it is possible that the differentiating CC undergo endoreplication, which incorporates BrdU into CC's chromosome without undergoing mitosis.

Ultimately, the bona fide CySC number may be tested by live observation of single Spict^{-} cells and follow their mitosis over time (ideally, microinjected with florescent dye). However, this live experiment is limited by the length of time that the testis can be cultured ex-vivo (<24 hours, while CySC cell cycle length is around 12~16 hours) assuming the ex-vivo culturing condition does not perturb CySC physiology.

3.1.2 What regulates *spict* expression pattern?

Previously, the CySC population was shown to undergo stochastic replenishment or loss; therefore single CySC clones rapidly turned over (Amoyel

et al., 2014). In contrast, the CySCs often re-orient their spindles during anaphase and divide asymmetrically (Cheng et al., 2011). Disruption of the CySC anaphase spindle repositioning leads to expansion of CySCs (Cheng et al., 2011), indicating that asymmetric cell division is essential in maintaining CySC homeostasis. It is interesting how CySC divides asymmetrically yet undergoes stochastic replenishment or loss. A likely explanation is that a daughter of CySC division that is displaced away from the hub (thus initiated differentiation) often undergoes dedifferentiation, and re-acquires CySC identity. Lineage tracing method employed by Amoyel et al does not provide a cellular mechanism by which CySCs self-renew stochastically. If CCs frequently dedifferentiate, such events would be detected as stochastic self-renewal. As discussed in Chapter 2, we propose that asymmetric CySC division combined with frequent dedifferentiation is the likely scenario to reconcile the conclusions of the two papers (Amoyel et al., 2014; Cheng et al., 2011).

In the dissertation, I identified *spict-gal4* as an insertion that specifically drives expression in the differentiating CCs excluding the CySC population, and can be utilized as a negative marker to refine the current CySC population. Although lineage tracing of spict-expressing cells shows rapid dedifferentiation of CCs that supports the proposed neutral drift model (Amoyel et al., 2014), the fact that the Spict^{+} cells are rarely observed around the hub region suggests that Spict expression is quickly turned off once CCs become CySCs. Therefore, *spict*$^{+}$ and *spict*$^{-}$ populations can serve as markers to investigate fate regulation of the CySC and CCs.

In this section, I propose to examine the number/ratio of Spict^{+} and Spict^{-} cells to investigate the factors that regulate *spict* translation.

CySC identity is known to be regulated by JAK/STAT pathway; ectopic activation of JAK/STAT pathway results in CySC tumors while suppression of JAK/STAT activity leads to CySC loss (Leatherman and Dinardo, 2008). Therefore, manipulating JAK/STAT activity can be a great experimental paradigm

to test whether spict transcription is directly under the control of CySCs. If the spict reporter can be suppressed when cells acquire CySC fate, spict expression should also be absent during ectopic activation of JAK/STAT pathway and spict expression should be apparent in somatic cells around the hub when JAK/STAT pathway is inhibited.

One of the well-established characteristics of CySCs includes the processes that project toward the hub cell (Fig. 2.1D). The hub attachment through DE-cadherin is essential for the CySC maintenance (Voog et al., 2008). It is possible that the attachment distinguishes CySC from its immediate daughter, the Zfh-1^{+} *spict*$^{-}$ early CC. If losing attachment is one of the components that triggers differentiation, overexpression of DE-cadherin would have an increased Spict^{-}/Spict^{+} ratio. Alternatively, knockdown of DE-cadherin should lead to the decrease of *spict*$^{-}$*/spict*$^{+}$ ratio. Since the attachment is essential for CySC maintenance, knockdown will have to be temporally controlled to catch the time point when adhesion is weaker yet the cell is still attached to the hub.

Another possible difference between CySC and CC is that they encapsulate GSC and GB respectively. Since it is well established that germline and soma communicate and co-differentiate together, it is possible that spict expression is regulated by associated GB and/ or repressed when associated with GSC. To test this hypothesis, Spict^{-}/Spict^{+} cells will be investigated in testis with different germ cell compositions. First, if signals from the germline are necessary to regulate spict expression, composition of *spict*$^{-}$*/spict*$^{+}$ cells in the maternally contributed *oskar* (*osk)* mutant progeny (Gonczy and DiNardo, 1996) carrying germ cell-less testis will either have decreased Spict^{-} cell (if spict translation is suppressed by the presence of GSC) or increased Spict^{-} cell (if spict translation is induced by the presence of GB). It is noteworthy that somatic cells gradually increase in the agametic testis, and the dynamic change of *spict*$^{-}$*/spict*$^{+}$ cells can also be investigated temporally (Gonczy and DiNardo, 1996). Secondly, forced differentiation of GSCs by ectopically expressing differentiation factor Bam

can be used to investigate whether the signals from GB is required to induce spict translation.

Lastly, CySCs often undergo asymmetric cell division which results in one cell replaced away from the hub proximity. It is possible that spict expression requires CySC asymmetric cell division. To test this idea, spict expression can be investigated when the asymmetric cell division is perturbed by overexpression of an ERM family protein Moe (Cheng et al., 2011; Salzmann et al., 2013).

One disadvantage for these experiments is that *spict*$^{+}$ cells are marked with the expression of a visible marker driven by the presence of Gal4 under the regulation of the *spict* promoter. Thus, the additional genetic modifications that require overexpression or knockdown will need another inducible regulatory system, for instance, LexA/LexAo (Pfeiffer et al., 2010), that regulates gene expression independent of Gal4/ UAS. Nonetheless, spict transcriptional reporter allows further investigation of CySC and CC physiology.

3.2 Spict regulates the progression of SG death

A previous study demonstrated that the SG death is upregulated upon starvation, which is required to maintain the integrity of the stem cell compartment during prolonged protein deprivation (Yang and Yamashita, 2015). CC death is both necessary and sufficient to induce this SG death since overexpression of DIAP (inhibitor of apoptosis) in the CC suppresses the SG death while overexpression of pro-apoptotic gene Grim leads to both CC and SG death (Lim and Fuller, 2012; Yang and Yamashita, 2015). However, the mechanistic role of the CC in SG death it is still unclear.

In the current study, I showed that dying SG clusters are entirely encapsulated by Rab7^{+} vesicles. These vesicles are contained within a single CC, suggesting that dying SG are phagocytosed by neighboring CCs. Taken together, we propose a novel mechanism for CC-mediated SG death. First, protein starvation induces apoptosis in one cell of the CC pair encapsulating a

SG, resulting in loss of the blood-testis barrier and triggering the death of the SG that is encapsulated by the apoptotic CC (Fairchild et al., 2015; Lim and Fuller, 2012). The dying SG are then phagocytosed by the surviving CC and eventually degraded.

During the progression of SG death, I observed that Spict protein was specifically upregulated and stabilized in surviving CCs, and likely transferred from the CCs to the dying SG. Considering that only the progression of SG death is slower in the *spict* deletion mutant, we speculated that *spict* is required for efficient phagocytosis and/or subsequent degradation of the engulfed cells. Therefore, several pilot experiments were performed to test the function of CC-expressed *spict* in SG death.

First, $Rab7^{+}$ phagosomes present in the dying SG can form in both controls and *spict* mutants (data not shown), suggesting that Spict does not directly regulate phagosome formation. Furthermore, the level of phagosome/lysosome acidification, as measured in live tissue by the pH-sensitive fluorescent protein pHMA (Fishilevich et al., 2010) and LysoSensor, was similar in controls and *spict* mutants (data not shown). Therefore, it appears that phagosome and phagolysosome acidification are unaffected in *spict* mutants. At present, we are unable to detect defects in phagosome/lysosome maturation because our reagents (such as LysoTracker) primarily detect acidified compartments to mark the dying SG. Our data suggests the need for the development of acidification-independent parameters of lysosome maturation.

Thus, the exact molecular function of CC-expressed Spict to promote progression of SG death remains unclear. In **Chapter 2**, we demonstrated that another possible Spict function, suppression of BMP activity, was not disrupted in the *spict* mutant testis. Here, we propose to investigate alternative molecular functions of Spict in the CC.

3.2.1 Does Spict regulate magnesium concentration in the engulfing CC?

Spict protein shows high homology to mammalian NIPA1 and NIPA2 (Wang et al., 2007), magnesium transporter proteins whose mutation is correlated with hereditary spastic paraplegia, a neurodegenerative disease (Goytain et al., 2007; Goytain et al., 2008; Rainier et al., 2003). Plasma membrane localization of NIPA1 is upregulated in response to extracellular Mg^{2+}, and expression of NIPA1 increases cytosolic Mg^{2+} concentration, suggesting that NIPA1 regulates Mg^{2+} uptake (Goytain et al., 2007). Recently, mutation of a Mg^{2+} transporter in *Dictyostelium* was shown to affect phagosome protease activity, suggesting that Mg^{2+} homeostasis is required for phagosome function (Lelong et al., 2011). It is thus possible that Spict regulates SG death through control of intraphagosomal or intralysosomal Mg^{2+} homeostasis.

If Spict functions on the lysosomal membrane in a similar manner to NIPA1 on the plasma membrane, it could act as a transporter to pump Mg^{2+} out of the phagosome. Therefore, to test this hypothesis, the levels of intracellular/lysosomal Mg^{2+} in the dying SG and neighboring CC would be probed using Mg^{2+} specific dye. However, the Mg^{2+} indicators are often sensitive to other bivalent ions especially calcium (Trapani et al., 2012). In fact, the Mg^{2+} dyes are more sensitive to Ca^{2+} than Mg^{2+}, which in tandem with Ca^{2+} abundance in the cell, makes it impossible to reliably investigate the Mg^{2+} concentration in situ (Trapani et al., 2012). Recently a new Mg^{2+} indicator with higher specificity has been reported, and this reagent may allow the investigation of local Mg^{2+} distribution (Yu et al., 2016). If our hypothesis is correct, in the *spict* deletion mutant, the dying SG would have abnormally accumulated Mg^{2+} in the Lysotracker^{+} compartment compared to the control while the dying SG neighboring CC would have decreased level of cytosolic Mg^{2+}.

Additionally, to test whether the concentration of Mg^{2+} affects the process of SG death, the phagosome function would be tested under different Mg^{2+} concentration. The testis would be cultured *ex-vivo* in a Mg^{2+} excessive condition as a proxy to mimic the condition of having high concentration of Mg^{2+} in the lysosome. On the contrary, the *spict* deletion mutant can be cultured in a Mg^{2+} -

deprived condition as a rescue experiment. The phagosome function will be investigated by measuring the progression of SG death in both conditions. If Spict pumps Mg^{2+} out of the phagosome to facilitate SG death, excess Mg^{2+} in the culture medium should suppress the SG death progression while spict mutant phenotype should be rescued in the Mg^{2+} deprived culture condition.

3.2.2 Does Spict regulate fatty acid metabolism in the engulfing CC?

Another mammalian protein that shares homology with Spict is Ichthyin (Wang et al., 2007). Loss of function mutation in *Ichthyin* results in abnormal skin desquamation and epidermal water barrier disruption. Of interest, Ichthyin is known to interact with FATP4, a very long-chain fatty acid transporter with acyl-CoA synthetase activities, and also to regulate epidermal lipid metabolism (Dahlqvist et al., 2012; Hall et al., 2005; Li et al., 2013).

The lysosome serves as one of the major organelles where lipid degradation occurs (Schulze and Sandhoff, 2011). Degradation of membrane lipids starts from the intraluminal vesicles of multivesicular bodies, where cholesterol is first exported (Kolter and Sandhoff, 2010). Complex lipids require lipid hydrolases and carbohydrases for further degradation in the lysosome (Schulze et al., 2009). Impairment of complex lipid digestion often results in the accumulation of undigested lipids and subsequent lysosomal storage disease (Schulze and Sandhoff, 2011). Furthermore, steroid hormone that can be generated by lipid metabolism is shown to regulate CySC and GSC maintenance (Li et al., 2014). Taken together, it is possible that Spict facilitates the metabolism or degradation of phagosomal lipids and thus affects the SG death machinery and the integrity of the stem cell compartment in the testis.

To test whether lipid uptake is perturbed in *spict* mutants, the testis was stained with Nile Red, a general lipid indicator. We didn't observe any significant difference between mutant and control testis (not shown). This can be because Nile Red stains neutral and polar lipids in general and lacks resolution to mark

specific types of lipids. Considering FATP4 affects very long chain fatty acid metabolism, Nile Red might not be able to detect differences in the metabolism of specific lipid subtypes in the *spict* mutant.

Thus, we propose to investigate composition of specific lipid subtypes (especially fatty acids) in control and *spict* mutant testes. The fatty acid transporter activity can be measured by a pulse-chase experiment using live imaging of a fluorescent fatty acid analog (Viktorova et al., 2014). If Spict specifically affects metabolism of very long chain fatty acids in lysosomes, we would see prolonged accumulation of very long chain fatty acid in the phagosome/lysosome in *spict* mutants. On the other hand, the delay of SG degradation phenotype in spict mutation might be rescued by overexpression of *Drosophila* homologs of FATP4. Furthermore, since the lipid breakdown in the lysosome requires a series of enzymatic digestions, the requirement of lysosomal lipid degradation for starvation-induced testis homeostasis can be further investigated by impairment of the cholesterol transporter *NPC1* or lysosomal lipases *lip1, lip2,* and *lip3* (Pistillo et al., 1998).

The other possible reason why Nile Red does not detect noticeable differences between control and *spict* mutant testis can be the experimental condition: while both starvation and standard medium contain sugar, protein content (amino acid source) and lipids are absent from the starvation medium. Considering that sugar can be metabolized into a lipid source, the lipid is not the absolute limited resource under both starvation and fed conditions. Therefore, the *spict* mutant phenotype may be weaker under current culture conditions and could not be detected when lipids in the cell are abundant. This can be easily tested by challenging the system with low sugar in addition to protein starvation to investigate the function of spict in lipid metabolism.

Taken together, based on the function of mammalian *spict* homologs, we speculate that Spict may regulate progression of SG death through two possible mechanisms: regulating Mg^{2+} concentration or fatty acid metabolism inside

phagosomes. If Spict affects phagosome/lysosome function through either Mg^{2+} uptake or fatty acid metabolism, the morphological difference in SG death is unlikely able to be detected by Lysotracker which primarily probes acidified compartments.

3.3 Recycling of nutrients from dead SG to the *Drosophila* stem cell niche

Previous work has shown that the autophagy pathway is highly upregulated in CCs during SG engulfment, and that the surviving CC phagocytoses the SG. Interestingly, the surviving CC associated with the phase 3 dying SG often shows strong cytosolic LysoTracker (Fig. 2.7C), suggesting that the CC may actively absorb materials from the dying SG (Supplemental Fig. S3). Furthermore, prevention of SG death causes GSC loss during starvation. Based on these findings, we propose that stem cell maintenance is supported by CC-mediated recycling of nutrients from dead SG. However, there is currently no direct evidence to support this hypothesis. Here, I propose to investigate 1) whether nutrients are absorbed by the CC and then transferred to the stem cell compartment, and 2) if these recycled nutrients are required to support the integrity of the stem cell compartment.

3.3.1 Monitoring the movement of nutrients

Our current hypothesis is that broken-down nutrients from dead SG can be taken up by a neighboring CC and ultimately transported to support stem cells. A direct way to test this hypothesis is to detect the presence of SG-generated nutrients in the stem cell compartment. Furthermore, the degree of nutrient recycling should be affected by manipulation of CC apoptosis-induced SG death. However, we face a number of technical challenges, including the need for an extensive combination of genetic tools. For our approach to succeed, the labeling method 1) must not disrupt normal physiology, 2) must survive lysosomal or proteasomal degradation, and 3) must be incorporated into the stem cell compartment in a detectable manner. Because previous studies indicate that the

starvation response is largely due to a lack of amino acids, this discussion will focus on detection of protein/amino acid recycling; nonetheless, a similar idea can be applied to other nutrients, such as lipids.

3.3.1a Monitoring protein transportation

A straightforward approach to monitor transportation to the stem cell compartment is through ectopic expression of a foreign protein using the differentiating, germline-specific driver *bam-gal4*. However, the technical limitation of this method is that foreign peptides are not likely to be resistant to degradation from the acidification and digestion of the dying SG. Interestingly, *spict-gal4*-driven Spict-RFP in the CC can be constantly observed in the hub, despite the lack of spict-gal4 expression in the hub (Fig. 2.4A, B, see asterisk). Furthermore, our preliminary data demonstrate that when Spict-RFP is driven by *bam-gal4* in differentiating germ cells, Spict-RFP signals in the hub are significantly higher than background autofluorescence (Fig. 3.2). This suggests there is constant molecule transportation from the differentiated region toward the hub cells and some fluorescent proteins might partially survive lysosome degradation. Additional fluorescent proteins, as well as reporter enzymes and small tagging peptides (Myc, HA, Flag, His, etc.), can be used to further affirm this observation.

3.3.1b Monitoring amino acid transportation

As mentioned above, proteins and peptides are typically subjected to degradation once phagocytosed. An alternative approach to trace materials from dying SG is to directly modify amino acid residues. Recently, a method known as BioID was established in culture cells to detect protein-protein associations (Roux et al., 2012). BioID requires the introduction of a promiscuous biotin ligase into a target cell, which subsequently biotinylates proteins on the lysine residue, allowing detection using avidin (Kim et al., 2014; Roux et al., 2012). This method can be used in the *Drosophila* testis to specifically modify proteins on the lysine

residue in differentiating SG. However, during the normal biotin protein cycle, the biotin modification will be removed by biotinidase. Thus, the activity of *Drosophila* biotinidase needs to be knocked down in CCs, assuming the deficiency of biotinidase does not lead to a testis-specific phenotype (Smith et al., 2007). Additionally, endogenous biotin increases the 'noise' associated with this experiment and can obscure the recycling of biotinylated lysines specifically from differentiated SGs. Therefore, the combination of this complex experimental procedure and the complicated genetics make this method unfavorable.

A similar concept can be adapted to locally produce an unnatural amino acid (Charbon et al., 2011; Krzycki, 2013). For example, the enzyme required for pyrrolysine synthesis, PylB, can be specifically expressed in differentiating SG using the *Bam* promoter. To monitor transportation of pyrrolysine into hub cells, the mRNA of a reporter that carries a premature amber stop codon (UAG) can be generated under control of the hub-specific promoter, *Upd*. The fully functional reporter will not be translated unless the presence of a pyrrolysine pyrrolysine/$tRNA_{CTA}$ pair which allows the reporter to be synthesized through the amber codon (Bianco et al., 2012). Therefore, reporter activity can be used to investigate the levels of an unnatural amino acid that originates from differentiated SG. This method is not limited to detection of amino acid transportation in hub cells; by placing the reporter under regulation of different promoter regions, other cell types can be investigated. For example, the *nanos* promoter regulates mRNA transcription specifically within the GSC (unpublished data from Zsolt Venkei). By attaching a degradation sequence to the 3'UTR region of the reporter, the reporter mRNA rapidly decays and is not carried over to SG, thus allowing detection specifically in the GSC.

Unlike BioID, this method 1) requires the presence of the amino acid inside the cell of interest in order to activate the reporter and 2) can only detect the end result of transportation.

Although the aforementioned approaches require sophisticated genetic manipulation with experimental difficulty, these strategies may allow us to visualize absorption, recycling, and transportation between cells. The proposed methods can be further used to monitor whether intracellular nutrient transportation has directionality or is simply the result of free diffusion.

3.3.2 Investigating the functional role of nutrient movement

3.3.2a Investigating amino acid absorption from dying SG

In the section above, I proposed strategies to monitor nutrient transportation from dying SG to the stem cell compartment. However, it is still unclear whether nutrient absorption and recycling from the dying SG are required to sustain stem cell integrity. To directly investigate the requirement of amino acid recycling, amino acid uptake in the lysosome can be disrupted by interrupting lysosomal amino acid transporter function in somatic cells. It is known that defects in lysosomal amino acid transport cause lysosomal storage diseases, such as cystinosis (Kalatzis et al., 2001; Platt et al., 2012); thus, constant suppression of the lysosomal amino acid transporter may lead to tissue damage. Therefore, the interruption of transporter function has to be temporally controlled to maintain the overall integrity of the testis. A *LacZ* enhancer trap of *Puckered* (*Puc*), which is downstream of the engulfment pathway, displays specific expression in the CCs that neighbor dying SG (Etchegaray et al., 2012; Yang and Yamashita, 2015), and can be used to transiently knock down lysosomal amino acid transporters. The requirement of amino acid recycling can be examined by measuring GSC loss upon protein starvation in controls and amino acid transporter mutants. The current knowledge regarding lysosomal amino acid transporters, however, is limited. Only a small number of eukaryotic lysosomal amino acid transporters have been identified. A mammalian member of the amino acid/auxin permease family, *LYAAT-1*, is known to be a lysosomal neutral amino acid transporter (Jezegou et al., 2012; Sagne et al., 2001). There are seven genes in *Drosophila* (*CG16700*, *CG3424*, *CG13384*, *CG6327*,

CG1139, *CG7888*, and *CG8785*) with 36-46% identity to *LYAAT-1* (Sagne et al., 2001) which represent potential candidates for our experiments.

3.3.2b Investigating amino acid export from the CC

In addition to amino acid absorption from the lysosome, transfer of digested amino acids from dying SG also requires exocytosis of the absorbed materials from the engulfing CC. There are two extensively investigated models of amino acid export: amino acid neurotransmitter release in neurons and amino acid transport across the cell in the intestines/renal epithelia. In neurons, amino acid neurotransmitters such as glutamate, aspartate, glycine, and cysteine are released from the presynaptic dendrite to activate or suppress the postsynaptic neuron. The amino acids are first transferred from the cytosol into synaptic vesicles via vesicular transmitter transporters, and then released into the synaptic cleft after membrane depolarization (Munster-Wandowski et al., 2016; Nadler, 2011). In the intestine, amino acids absorbed by enterocytes are released into the blood by amino acid transporters. In the kidney, reabsorbed amino acids are transported back to the blood to avoid wastage (Broer, 2008). The amino acid export machinery is highly conserved: in *Drosophila*, amino acid transporters responsible for transport out of cells include *LAT1*, *TAT1*, and *EEAT2* (Besson et al., 2005; Hyde et al., 2007; Reynolds et al., 2009). It will be interesting to test whether knockdown/mutation of these transporters disrupts maintenance of the stem cell compartment upon starvation. This experiment will also provide further insights into the physiology of amino acid redistribution within a tissue.

3.4 Assisted programmed cell death

Programmed cell death can be induced in a cell-intrinsic (suicide) or cell-extrinsic (murder) manner to activate the downstream pathway in the dying cell to control its own progression of death (Green and Llambi, 2015). Recently, increasing evidence indicates that although apoptosis itself is typically viewed as

a cell-autonomous process after the program is induced in the dying cell, neighboring cells can send out regulatory signals to modulate progression and completion of cell death (see below).

For instance, despite the fact that engulfment is traditionally thought as the last stage of apoptosis to prevent uncontrolled release of the corpse without playing a role in the actual death process, recently, engulfment genes were shown to assist programmed cell death processes in *C. elegans* (Chakraborty et al., 2015; Hoeppner et al., 2001; Johnsen and Horvitz, 2016; Reddien et al., 2001). Asymmetrically expressed engulfment genes in the surrounding cells were shown to instruct the asymmetric inheritance of apoptotic potential into NSMsc cell that is fated to die after mitosis (Chakraborty et al., 2015). This suggests that neighboring cells can induce cell death through not only the expression of the death factor but also through regulation of apoptotic potential. Additionally, B.alapaav cell death in engulfment mutants shows an intermediate morphology in between live and dead cells in *C. elegans* (Johnsen and Horvitz, 2016). This also reveals the role of engulfment in facilitating apoptosis. Furthermore, the engulfment cells are shown to facilitate apoptosis during DNA degradation. In mammals, macrophage secreted DNAseII promotes degradation of DNA from engulfed cells to complete their apoptosis in a non-autonomous fashion (Kawane et al., 2003). Taken together, these studies indicate that some forms of apoptosis are not completely cell-autonomous and engulfing cells are required to promote or facilitate this process.

In this dissertation, I provided evidence showing that SG death progression is regulated by neighboring engulfing CCs in *Drosophila* testis upon starvation. In contrast to the engulfing cell facilitating apoptosis in *C. elegans* (Johnsen and Horvitz, 2016), SG death in *spict* mutants does not persist in the intermediate condition between live and dead cells suggesting there are various levels of involvement of regulating cell death by the engulfing cells. Instead, Spict regulates the progression of phases of SG death indicating that the dynamics of SG death is a highly regulated process and Spict is involved in the early phases

of SG death. Considering that phagosome formation is required for clearance of cell corpse in *C. elegans* but individual inactivation of most lysosomal proteases does not drastically block clearance of dead cells (except cathepsin L (Wang and Yang, 2016; Xu et al., 2014)) suggests that lysosomal hydrolases could be redundant. Furthermore, inactivation of individual hydrolases could affect the progression of clearance of apoptotic cells although it does not completely block the clearance of cell corpses.

3.4.1 Similarities between *Drosophila* male and female germ cell death

In previous studies, engulfment genes in *Drosophila* testis have been shown to be required for SG death (Kawane et al., 2003; Yang and Yamashita, 2015). But the CC's role in engulfment and progression of SG death was still unclear. In this dissertation, I demonstrated that the CC engulfs, and regulates the process of SG death. This process is similar to the cell death that occurs during *Drosophila* oogenesis. During the nutrient-sensitive mid-stage oocyte death, overexpression of engulfment genes in somatic cells can trigger the germ cell death while mutations in *Atg1* and *7* reduces DNA fragmentation of dying germ cells (Hou et al., 2008; Jenkins et al., 2013). This suggests that at least part of the mid-stage cell death can be regulated by autophagy in the somatic cell. Additionally, during late oogenesis, some germ cells are developmentally programmed to die after transporting cytoplasm materials to the future oocyte. It is known that apoptosis and autophagy contribute only marginally to late stage nurse cell death (Jenkins et al., 2013), whereas the phagocytic machinery in soma is essential for the morphological changes in late-stage female germ cell death including nuclear rupture, DNA fragmentation, and acidification (Timmons et al., 2016). These similarities reveal the shared nature of germ cell death between male and female *Drosophila*. Analogy to the female can, therefore, provide great insight into the molecular mechanism of male germ cell death. For example, *atg1* or *7* mutant females reduce DNA fragmentation during mid-stage oocyte death suggesting that autophagy regulates part of oocyte death (Hou et

al., 2008). These connections can be used to conduct a small-scale screen to further investigate molecular mechanisms that are involved in SG death.

3.4.2 Does phagocytosis or autophagy in CC facilitate SG death progression?

Our previous study demonstrated that the phagocytic and autophagic pathway (shown by a core autophagy molecular, Atg8) is upregulated within the engulfing CC. In our current research, we demonstrated that the dying SG is immediately phagocytosed by the neighboring, surviving CCs. Together, these data suggest the phagocytotic pathway and/or autophagy in the CCs might facilitate SG death.

In *Drosophila*, phagocytosis starts with induction of engulfment receptors such as Draper, and then the plasma membrane re-organizes to engulf foreign particles. After phagosome formation, the maturation of phagosome requires small GTPases that also function in endosome maturation such as Rab5 (early endosome) and Rab7 (late endosome) and ultimately fuses with a lysosome for degradation. These candidate genes can be knocked-down specifically in the Puc-expressing cell (see above, 3.3.2a) to test the requirement of phagocytotic pathway in SG death progression under starvation.

Our previous study showed that Atg8 protein is highly upregulated in the CC neighboring dying SG. However, it is still unclear whether autophagy is required for initiation of SG death and facilitating SG death progression. The role of autophagy pathways in SG death initiation or progression can be tested by inhibiting (loss-of-function) or overexpressing (gain-of-function) the key regulators such as *Atg5 and Atg8* in the engulfing CC during protein starvation.

In addition, whether the upregulation of Atg8 is merely a starvation response or specifically upregulated in the engulfing CC to facilitate SG degradation is currently unclear. This question can be addressed by investigating Atg8-GFP reporter in the phagocytosis mutant. If phagocytosis of SG in the CC is

not required for initiation of autophagy, Atg8 should remain upregulated in the absence of phagocytosis during starvation. Furthermore, recent studies demonstrated that LC3 (Atg8 in *Drosophila*) is associated with phagosomal membranes to induce clearance of *Aspergillus fumigatus*, a fungal pathogen in mammals. Rubicon is specifically required for LC3 associated phagosome but not autophagy (Martinez et al., 2015). It is possible that engulfing CCs regulate SG death through LC3-associated phagosomes. This can be tested by knocking down of CG12722, the *Drosophila* homologue of Rubicon, in the engulfing CC.

3.4.3 What is the role of soma in germ cell death?

Previously, loss of CC or loss of the tight junction that seals the SG between encapsulating CCs has been shown to induce SG death (Fairchild et al., 2015; Lim and Fuller, 2012). Additionally, one interesting observation in Chapter 2 is that the SG death progresses rapidly (progress from phase 1 to 3 occurs in 3 hours, Supplemental Fig. S2) when the SG death is induced by global expression of apoptosis activator, grim, in the soma. In contrast, the dynamics of the SG death observed in the pulse-chase experiment suggests a much slower process (around 8 hours in stage 1, Fig. 2.9). Therefore, although the sequential morphological order of the SG death is similar, it is still unclear how the SG death caused from these two methods is different from each other.

The obvious difference between these two experiments is that during normal SG death, the dying SG is always engulfed by a CC, while killing all CCs by overexpression of Grim would result in an inability to engulf SGs. The latter may be a drastic assault for the remaining germ cells compared to the SG death in normal physiology. Although the underlying differences is still unclear, it is possible that the engulfment of SG by CC occurs to regulate the dying process; in contrast, the germ cells may die rapidly in a less regulated manner when the testis cannot cope with the drastic insult.

The germ cell death morphology has been reported to share features from both apoptosis and necrosis: similar to necrosis, the dying SG acidifies the cytoplasm while the dying SG shrinks and condenses its chromatin similar to apoptotic morphology (Yacobi-Sharon et al., 2013). Previously, Yang and Yamashita have shown that the starvation induced SG death primarily affects early SG (from GB to 4-cell SG) while the late SG (16-cell SG) death does not depend on nutrient condition (Yang and Yamashita, 2015). Of note, the starvation induced SG death in the early SG can be regulated by CC death (Yang and Yamashita, 2015). In contrast, repression of the CC death only marginally suppresses 16-cell SG death while the early SG death is largely affected (Yang and Yamashita, 2015). A recent study showed that 16-cell SG is upregulated by DNA damage (preliminary data from Kevin Lu) and the 16-cell SG death can be induced by inhibition of DIAP in the germ cell (Yacobi-Sharon et al., 2013), suggesting that 16-cell SG death might be cell-autonomous, rather than being regulated by soma. Moreover, revealed by EM, unlike the nutrient dependent early SG death exhibiting swollen and deformed mitochondria, the 16-cell SG death shows upregulation of ROS activity (Yacobi-Sharon et al., 2013). This suggests that there are underlying differences between these two types of SG death. At present, the differences in fundamental cause, morphology, and dynamics of these two SG deaths are still unclear, and it is possible that the differences between these two types of death can be the cause for the discrepancy of the rate in SG death progression. Nonetheless, these studies suggest that there are at least two major mechanisms that govern SG death under different situations: CC death-induced early SG death during starvation and CC death-independent late SG death in response to DNA damage in the germ cell, and CCs may participate and regulate these processes differently.

3.5 Similarity between mammalian and fly testis

Spermatogenesis is a highly similar process in the animal kingdom, and a great number of features are shared from fly to human. As in the fly testis, mammalian spermatogenesis can be roughly distinguished into three major

stages: proliferation/ differentiation stage, meiotic stage, and spermiogenesis stage. These three stages of germ cells are arranged spatially in seminiferous tubules, where the proliferation happens in the basal compartment of the seminal vesicle and sperm is generated in the luminal region to be released into the lumen. While *Drosophila* GSCs are strictly maintained by anchoring to the hub cells and can be identified at the single cell resolution, only a subset of the A_{single} cells are considered to be mammalian spermatogonial stem cells (SSCs). Furthermore, molecular markers for SSCs have only been suggested recently by lineage tracing experiments, although the exact characters of SSC are still unclear (Aloisio et al., 2014; Chan et al., 2014; Sun et al., 2015). Similar to mitosis in *Drosophila* spermatogonia, the mammalian germ cells undergo incomplete cytokinesis and generate interconnected germ cells: A_{paired} and $A_{aligned}$. Interestingly, the interconnected germ cells constantly fragment into single cells and acquire stem cell identity (Hara et al., 2014; Nakagawa et al., 2010; Oatley et al., 2011). The $A_{aligned}$ spermatogonia then differentiate into spermatocytes that undergo meiotic division.

In mammalian seminiferous tubules, Sertoli cells have been viewed as the counterpart of the *Drosophila* cyst cells. They also function to provide nutrients and growth factors to support spermatogenesis. Interestingly, while the soma associates with the same spermatogonia cysts and co-differentiate together in the fly testis, approximately 30-50 developing germ cells at various differentiation stages associate and interact with a single Sertoli cell (Weber et al., 1983). How a single Sertoli cell provides factors that regulate various stages of germ cell differentiation at the same time is currently unclear.

Additionally, CCs in fly and Sertoli cells in mammals both function in providing the blood-testis-barrier (BTB) to protect germ cells from environmental assault and are therefore required for germ cell survival (Fairchild et al., 2015; Murphy and Richburg, 2014). Furthermore, the BTB organization can be modulated by the energy level, such as ATP level, or mTOR activity in Sertoli cells to control spermatocyte survival and differentiation (Li and Cheng, 2016;

Rato et al., 2012). Germ cell death is mediated by signals from Sertoli cells (Print and Loveland, 2000), and materials from the dying mammalian germ cell can be phagocytosed and used by Sertoli cells (Gillot et al., 2005; Xiong et al., 2009).

Mammalian fertility is often affected by nutrient availability, as nutrient deprivation and obesity are deleterious for reproductive function. Recent studies regarding metabolism and male fertility has shown that metabolism-associated hormones regulate reproduction (Bertoldo et al., 2015; Hill et al., 2008) while sex steroid hormones control energy metabolism (Crown et al., 2007; Silva et al., 2001), suggesting that the regulation of nutrients and reproduction are heavily intertwined. However, current research has focused on how hormones regulate reproductive function and lack the resolution of cellular interactions. *Drosophila* shares a striking similarity with mammalian spermatogenesis, and thus provides a great experimental paradigm to investigate the homeostasis of reproduction system during starvation.

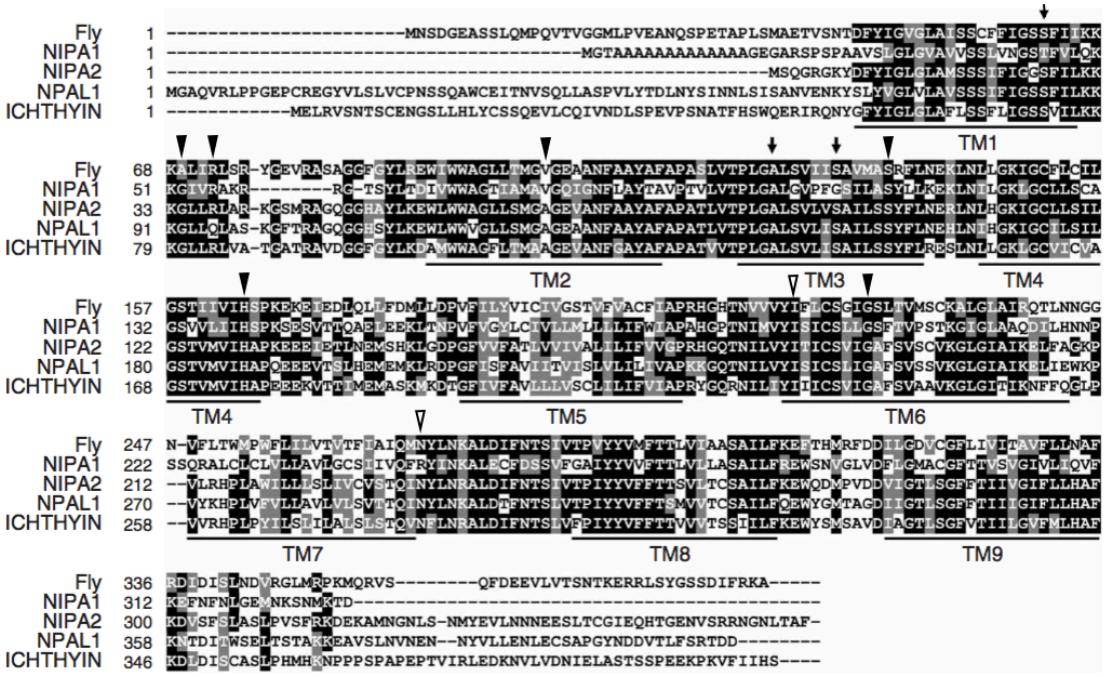

Fig. 3.1 Multiple alignment of *Drosophila* and human homologs [adapted from (Wang et al., 2007)]

Spict is predicted to be a protein with 7-9 transmembrane domains (underline, TM1-9). The alignment between Spict and 3 NIPA1 family proteins was generated through ClustalW. The letters with black background represent identical amino acid residue while letters with gray background represent similar amino acid. The arrows indicate the mutation sites (T45R, A100T, and G106R, respectively) identified in human with hereditary spastic paraplegia (HSP) (Beetz et al., 2008). The empty arrowheads indicate mutations (I178F, and N244S respectively) of NIPA2 found in childhood absence epilepsy patients (Jiang et al., 2012; Xie et al., 2014). The arrowheads indicate mutations in Ichthyin (R83X, G80V, A114N, S146F, H175N, and G235R) from congenital ichthyosis patients (Dahlqvist et al., 2012; Lefevre et al., 2004).

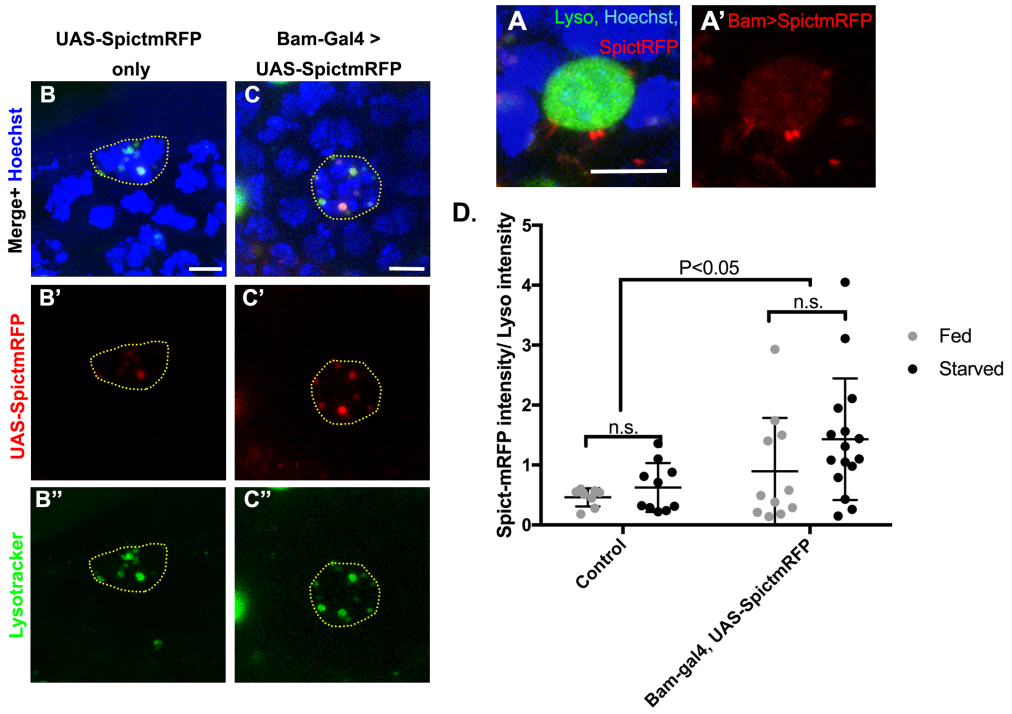

Fig. 3.2 Translocalization of Spict-mRFP from SG

(A) Representative image of phase 3 dying SG when Spict-mRFP is ectopically expressed in the differentiating germ cells (Bam-Gal4 driven UAS-SpictmRFP). Spict-mRFP localizes to dying SG (Lysotracker^{+}) and forms an aggregate around the dying SG similar to Spict-Gal4 driven UAS-Spict-mRFP. This suggests that Spict-mRFP in dying SG can be re-absorbed by CC regardless of its origin (expressed from CC in Fig. 2, and from SG in this figure).

(B) Representative image of the autoflourescence of mRFP excitation wavelength in the hub cells (yellow dotted circle) after 3 days of starvation.

(C) Representative image of the Spict-mRFP wavelength in the hub cells (yellow dotted circle) after 3 days of starvation.

(D) Quantitation of the relative intensity per hub in fed or starved condition. To avoid the ambiguity of intensity measured from different Z-plane, Lysotracker and SpictmRFP signals were measured in proton counting mode.

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