THE IDENTIFICATION OF NEW MEDIATORS OF SOMA-GERMLINE INTERACTIONS IN THE *DROSOPHILA MELANOGASTER* TESTIS

by

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Abstract

Animal gonads contain two types of cells, the soma and the germline. Interactions between these two tissues regulate cell proliferation, differentiation, patterning, and homeostasis in the gonad during development and throughout the life of the organism. In particular, interactions between the soma and germline in the gonads regulate the behavior of germline stem cells (GSCs). Disruption of germline-soma interactions has severe consequences for gametogenesis and especially on GSC function, resulting in sterility, formation of somatic and germline tumors, and defective sexual determination of the germline. The *Drosophila melanogaster* gonads provide a powerful, established, model system to study how soma-germline interactions regulate GSC function as well as spermatogenesis. Soma-germline interactions are initiated at the stem cell niche where both germline and somatic stem cells are housed. Upon exit from the stem cell niche, the soma encapsulates the germline. The association and communication between the soma and germline ensures proper differentiation of the germline into mature sperm. Signaling events between the germline and soma regulate germline stem cell self-renewal, displacement from the niche, and encystment of germ cells. The cell biological mechanisms that set up the milieu in which these signaling events take place are poorly understood. In order to better understand the regulation of soma-germline interactions, we are performing a genetic screen using tissue-specific RNAi and fertility assays to identify genes involved in this process. We have identified over 200 genes necessary in the soma for spermatogenesis. Here I describe the characterization and classification of genes identified in the screen, as well as initial attempts to understand their role in the fly testis. The identification and characterization of the novel genes that

mediate soma-germline interactions will provide crucial insight into the basic mechanisms that regulate spermatogenesis.

Preface

All work conducted in this thesis is original, unpublished work completed in the laboratory of Dr. Guy Tanentzapf at the University of British Columbia.

Guy Tanentzapf and Michael Fairchild conceived and designed the RNAi screen and initial experiments. Over the course of this work I have directly supervised a number of undergraduate students who helped conduct or are currently assisting with the ongoing RNAi screen in the lab: Martha Smith, Lulu Yang, Lilian Shi, Shannon Percival-Smith, Rachel Webster, and Chad Brown.

Guy Tanentzapf, Michael Fairchild, and I designed the candidate classification scheme. I classified candidates from the screen, with help from Michael Fairchild, as well as Martha Smith and Lulu Yang, who were under my supervision.

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List of Abbreviations

Bone Morphogenetic Protein	BMP
Cyst stem cells	CySCs
Epidermal Growth Factor	EGF
Germline stem cells	GSCs
Individualization complex	IC
Janus kinase and Signal Transducer and Activator of Transcription	JAK/STAT
Male-specific somatic gonadal precursors	msSGPs
Mitogen-activated protein kinase	MAPK
Primordial germ cells	PGCs
Somatic gonadal precursor cells	SGPs
Somatic stem cells	SSCs
Transforming Growth-Factor-β	TGFβ

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To my family and friends, for your love, support and faith in me. You are my pillar of strength. I am blessed to have you by my side, every day and always.

Chapter 1: Introduction

Sexual reproduction requires animals to be able to transfer genetic information to their offspring. This transfer is accomplished by using specialized cells called gametes. Gametes are produced from germ cells. Many aspects of germline development such as the determination of germ cell fates, maintenance of germ cell identity, migration of germ cells to somatic gonadal primordia, and proliferation of germ cells are conserved throughout the animal kingdom (Saffman and Lasko, 1999). The soma has an important and conserved role in regulating germ cell development to ensure the production of functional gametes (Jemc, 2011). Understanding how the soma interacts with and communicates with the germline to control gonadogenesis and gametogenesis is an important open question in developmental biology.

In this thesis, I focus on soma-germline interactions during spermatogenesis, the process by which germ cells give rise to sperm. By identifying the molecular mechanisms that mediate soma-germline interactions, the goal of this work is to understand how the soma and the germline communicate.

1.1 Soma-germline interactions are conserved in metazoans

1.1.1 The germline

The origin and nature of the germline was first described by Owen in 1849, who suggested that certain cells in an animal are set aside to provide the material and instructions to initiate the development of another individual (Saffman and Lasko, 1999). The germline gives rise to gametes and the soma forms all other tissues that support and maintain gamete function (Gilboa & Lehmanm, 2006; Korta et al., 2010; Wistuba et al.,

2007). When an organism dies the somatic cells cease to exist (Saffman and Lasko, 1999), while the germline ensures the continuity of life by forming successive generations (Gilbert, 2010).

In many animals, germ cells are characterized by a nuage. The nuage is a dense, fibrous organelle, unbounded by a membrane, consisting of RNA and protein, associated with clusters of mitochondria, and located in the perinuclear cytoplasm. The transfer of germ cells to successive generations depends on the nuage, which is widely believed to carry the determinants of the germline (Saffman and Lasko, 1999). In animals like *Drosophila, Caenorhabditis elegans,* and *Xenopus,* germ cell differentiation relies on the germ plasm, which is similar in morphology and ultrastructure to a nuage. The germ plasm is a specialized region of cytoplasm in the embryo, comprised of germline-specific factors that are deposited in the egg during oogenesis. Its specific location in the embryo determines where the primordial germ cells (PGCs) will form. The PGCs give rise to all gametes (Gilbert, 2010). Although the nuage is well characterized in many mammalian species, mammals do not possess a germ plasm. The germline determinants in mammals do not rely on inherited factors. Instead, germ cell fate is thought to be dependent on positional information in the embryo. Additionally, germline segregation occurs during gastrulation, in contrast to invertebrates where it occurs early in embryogenesis (Saffman and Lasko, 1999).

In order to form reproductive cells for the next generation, germ cells must remain unresponsive to factors promoting somatic differentiation in other embryonic cells. Germ cells migrate through the embryo to the developing gonad, where they stop migrating and undergo characteristic changes in shape, morphology, and gene expression.

Gametogenesis takes place in the gonad, an organ that produces gametes. Depending on the sex of the gonad, the germ cells form eggs or sperm. Complex mechanisms of gene regulation control germ cell differentiation. Transcriptional and translational regulation in the germline ensures restriction of gene activity and proper expression of germline factors, many which are conserved throughout evolution. The gene *vasa* is a unifying feature of the germline (Saffman and Lasko, 1999), essential for germ cell differentiation and meiosis, in vertebrates and invertebrates (Gilbert, 2010; Johnson et al., 2004). Although the germline is indispensible for the continuity of life, it requires the support of the soma throughout gonadogenesis and gametogenesis.

1.1.2 The soma

The soma forms the body of an organism. It has specialized functions in gonadogenesis and gametogenesis. In the gonad, specialized somatic cells forms the stem cell niche, which physically anchors the stem cells (McCarter et al., 1997; Tanentzapf et al., 2007; Vogl et al., 2008) regulates the behavior of germline stem cells (GSCs) provides signals to regulate germline differentiation (Govindan et al., 2009; Kiger et al., 2000; McCarter et al., 1997; Tran et al., 2000), and acts a source for vital nutrients (Boussouar and Benahmed, 2004; McCarter et al., 1997) as the germline develops. Disruption or loss of somatic cells severely affects and usually stops gamete production resulting in sterility (Griswold, 1998; Korta and Hubbard, 2010; Zoller and Schulz, 2012).

Different types of somatic cells perform different functions. Three well-characterized examples of somatic cells are the Sertoli cells of mammals, the cyst cells of

Drosophila, and the sheath cells of *C. elegans*. In the *Drosophila* ovaries, a tubular structure called the germarium contains the stem cell niche. The germarium is composed of three differentiated somatic cell types: the terminal filament, cap cells, and inner sheath cells, which surround the GSCs. The formation, maintenance, and regulation of the GSC niche depend on the cap cells. The terminal filament and cap cells are able to contact 2-3 GSCs and provide the signals necessary to regulate them (Xie and Spradling, 2000). In mammals, the germline is isolated by somatic Sertoli cells, which mediate endocrine, and transcriptional and translational control to support and maintain germ cells (Ehmcke et al., 2006). The sheath and spermathecal cells in *C. elegans* guide migration and proliferation of germ cells (Killian and Hubbard, 2005; McCarter et al., 1997). In addition, the expulsion of the oocyte during ovulation in *C. elegans* requires contraction of the proximal sheath cells, exemplifying the role of the soma in structural and functional support of the germline (McCarter et al., 1997).

1.1.3 Overview of soma-germline interactions

Several studies in model organisms have underscored the importance of soma-germline interactions in coordinating gonad morphogenesis gametogenesis (Buccione et al., 1990; Hall et al., 1994; Jemc, 2011; Killian and Hubbard, 2005). In the absence of the soma, the germline develops abnormally (Yamashita et al., 2005). In the absence of the germline, the development of the soma is disrupted (Gonczy and DiNardo, 1996). Soma-germline interactions are mediated by cell adhesion molecules, ligand/receptor interactions, or by diffusion of secondary messengers via gap junctions (Hall et al., 1999; McCarter et al., 1997).

Soma-germline interactions control and regulate the amplification, maintenance, and differentiation of germ cells in various organisms (Byrd and Kimble, 2009; Hall et al., 1999; Killian and Hubbard, 2005). Three important biological processes that require soma-germline interactions is the formation of the gonad during embryogenesis, establishment of the stem cell niche, and function of the soma-germline barrier.

Gonad formation requires the cooperation of the soma and germline in various organisms (Pazdernik and Schedl, 2013; Santos and Lehmann, 2004; Wistuba et al., 2007). Furthermore, in *Drosophila*, interactions between the soma and germline determines the sex of the gonad, and regulates differentiation into sperm or eggs (Santos & Lehmann, 2004). In the *Drosophila* testes and ovaries, it is critical to establish an environment where germ cells are continuously produced so they can enter gametogenesis. The establishment of the GSC niche structure depends on communication and interactions between the germline and soma (Yamashita et al., 2005). A soma-germline barrier is a conserved feature found in mammals (Dym and Fawcett, 1970) and insects (Szollosi and Marcaillou, 1977). The main function of the soma-germline barrier in vertebrates, is to promote germline survival by isolating and protecting it from cytotoxic materials (Su et al., 2011), and from the immune system (Kaur et al., 2014). In the *Drosophila* testes, septate junctions have a similar function, acting as a barrier that prevents the diffusion of molecules, and that performs an essential function required for germ cell development (Papagiannouli and Mechler, 2009). This diffusion barrier regulates the transport of metabolites across the germline, gives architectural structural support for the germline (McCarter et al., 1997), and regulates the signaling environment around the germline as it differentiates (Cheng and Mruk, 2012; Franca et al., 2012; Lui

et al., 2003).

Misregulation of soma-germline interactions can give rise to multiple pathologies. The misplacement and overproliferation of primordial germ cells can lead to germ cell tumors, whereas premature differentiation and failure to maintain primoridial germ cells can result in sterility (Killian and Hubbard, 2004). Studying soma-germline interactions has broad implications in medicine for the treatment of infertility as well as testicular and ovarian cancers.

1.1.4 Overview of spermatogenesis in metazoans

Spermatogenesis is the process by which undifferentiated germ cells develop into male gametes. Spermatogenesis is a conserved process across metazoa. The basic process of spermatogenesis: amplification of germ cells, growth and meiosis of spermatocytes, and differentiation of spermatids are similar among animals (Pazdernik and Schedl, 2013; Wistuba et al., 2007). The soma plays crucial roles in spermatogenesis: the soma controls germline proliferation, entry into meiosis, and differentiation of spermatids into mature sperm by establishing and maintaining the spatial organization of germ cells (Kimble and White, 1981; Wistuba et al., 2007; Zoller and Schulz, 2012).

In mammals, the early phases of spermatogenesis occurs at the basal layer, and later phases can be found in the apical compartment of the testis. Similarly, in *Drosophila*, the germ cells are arranged in spatio-temporal order along the axis of the testis, however, the early stages of spermatogenesis occur in the apical region while later spermatid stages take place in the basal region (Figure 1.1). The *Drosophila* germline develops in a cyst, created by somatic divisions, whereas each cluster of mammalian

Figure 1.1 Anatomy of the *Drosophila melanogaster* testis

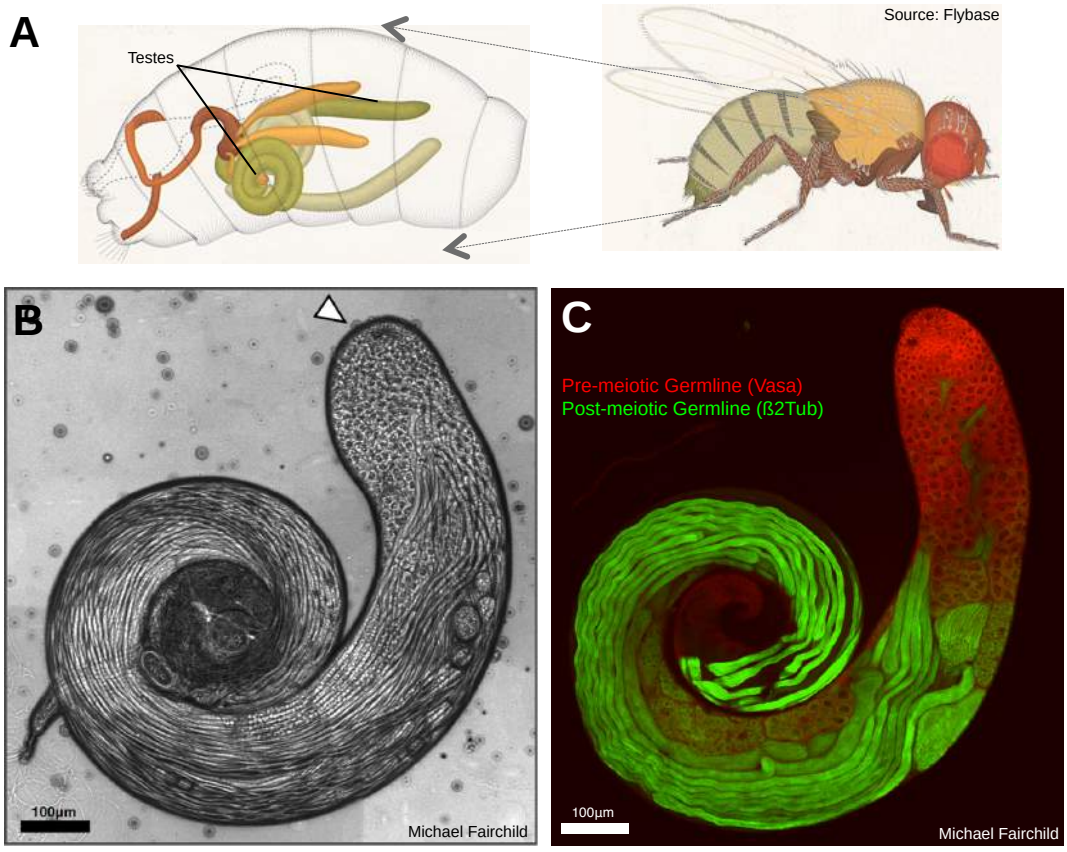


Figure 1.1 Anatomy of the *Drosophila melanogaster* testis. Diagrammatic and microscope representations of the *Drosophila* testis. (A) Drawing of a whole fly and enlarged schematic of the abdomen showing the location of the testes (www.flybase.org). (B) Differential intereference contrast (DIC) image of a *Drosophila* testis, a coiled tube with the stem cell niche at the apical tip (arrowhead) and elongated spermatids in the basal region. (C) Confocal image of a *Drosophila* testis. Vasa marks pre-meiotic germline and β2-Tubulin marks post-meiotic germline (Images B & C, courtesy of Michael Fairchild).

germline resides in a compartment within the Sertoli cell. The Sertoli cells forms a physical barrier, provides a source for nutrients and regulatory molecules, nourishes and protects developing germ cells (Gilbert, 2010). In both cases, the germ cells proliferate while they are completely surrounded by the somatic cells, and undergo transit-amplifying divisions as spermatogonia, which differentiate into spermatocytes. In *C. elegans*, the proliferation of germ cells is also controlled by two somatic cells, known as the distal tip cells in the gonad (Kimble and White 1981). Furthermore, the adult germline in *C. elegans* contains a 'mitotic region' in the distal region, and a 'transition zone' at the proximal end. Germ cells in the mitotic region are stem cells, whereas germ cells in the transition region enter the early stages of meiosis (Kimble and Crittenden, 2005). In many animals, the amplification of germ cells results in a stem cell population or proliferation zone (Killian and Hubbard, 2004), which ensures the constant production of germline stem cells.

In mammals, *Drosophila*, and *C. elegans*, spermatogonia develop into primary spermatocytes and undergo meiosis I to form secondary spermatocytes with reduced genomic content. The secondary spermatocytes undergo meiosis II to split the sister chromatids and form spermatids. This marks the start of spermiogenesis, the process by which spermatids differentiate into mature sperm.

In *Drosophila*, mammals, and *C. elegans*, spermiogenesis is typically characterized by changes in spermatid morphology, chromatin condensation, and removal of cytoplasm, which dramatically reduces cell size (Fabian and Brill, 2012; L'Hernault, 2006; Wistuba et al., 2007; Zoller and Schulz, 2012). In mammals, the apical side of the cell contains the nucleus and a spermatid-specific organelle, the acrosome. The acrosome

supplies metabolic energy and contains digestive enzymes required to fertilize eggs (Fabian and Brill, 2012; Wistuba et al., 2007; Zoller and Schulz, 2012). The basal or posterior side of the cell contains the flagellum, which propels the spermatid through the female reproductive tract (Wistuba et al., 2007)). The spermatozoa of *C. elegans*, however, lack the acrosome and flagella. Instead, they use a single pseudopod to crawl (L'Hernault, 2006).

Continuous sperm production throughout the life of an animal is achieved by maintaining a population of germline and somatic stem cells, which ensures the balance between germline self-renewal and differentiation (Santos and Lehmann, 2004).

1.1.5 Introduction to stem cells – germline and soma

Stem cells are undifferentiated cells that are defined by their ability to self-renew and capacity to differentiate into specialized cells that maintain adult tissues (Xie and Spradling, 2000). They regulate the growth, homeostasis, and repair of many tissues. Disrupting the balance between stem cell self-renewal and differentiation can contribute to tissue aging and tumor formation (Spradling et al., 2011). In 1978 Schofield developed the stem cell niche hypothesis, which proposed that stem cells reside within a discrete compartment, or niche, within a tissue that promotes the maintenance of stem cell properties (Jones and Wagers, 2008).

Stem cells can play important roles in regenerative medicine because they can be used to replace damaged or dying cells in various adult tissues throughout the lifetime of an organism (Jones and Wagers, 2008). In response to physiological changes or damage, stem cells can give rise to tissues that comprise breast, lung, skeletal muscle, and prostate

tissue (Davies and Fuller, 2008). Model organisms, such as *C. elegans* and *Drosophila*, have been extensively used to perform elegant genetic experiments that provided important insight into stem cell regulation. These studies provided some of the first visualizations of stem cell niches in living, intact organisms, and have validated their use in the study of stem cells. In addition, studies in mammals have demonstrated that the key features of stem cell niches are conserved across metazoans (Jones and Wagers, 2008).

Spermatogenesis is a stem-cell mediated process. In *Drosophila*, it involves special stem cell populations: the somatic stem cells (SSCs) and the GSCs, which give rise to sperm. The asymmetric division of the male GSC marks the start of spermatogenesis. Upon asymmetric division, one GSC retains stem cell identity while the other daughter, known as the gonialblast, undergoes transit amplifying cell divisions and differentiates to form mature sperm. The establishment of the stem cell niche and onset and completion of spermatogenesis requires the cooperation of the soma and germline in many animals (Yamashita et al., 2005). One of the key questions in stem cell biology is what are the molecular mechanisms that regulate stem cell self-renewal, maintenance and differentiation. Studying how soma-germline interactions regulate stem cell populations in the testes will contribute important insights into stem cell biology.

1.1.6 Gonad morphogenesis in *Drosophila*

The coordinated development of the soma and germline is required to form the *D. melanogaster* gonad. Interestingly, the pole cells, which are the cellular precursors of the germline are set aside at the posterior end of the embryo, representing one of the earliest determination events in embryogenesis (Fuller, 1993; Santos & Lehmann, 2004). The

embryonic gonad is characterized by 10-15 pole cells intermingled with 25-35 mesodermal cells (Boyle and DiNardo, 1995). Lineage tracing experiments show that *D. melanogaster* somatic cyst cells originate from somatic gonadal precursor cells (SGPs) during embryogenesis, where they are specified from the mesoderm in parasegments 10-12. During gastrulation, the PGCs migrate towards the position of the future gonad, in parasegment 10. As the PGCs move through parasegments 12 and 11, they associate with SGPs and migrate together towards the anterior to join the SGPs in parasegment 10 (Zoller and Schulz, 2012). Here migration of gonadal precursors arrest, and the PGCs and SGPs coalesce to form the gonad. The gonad becomes compact and spherical as a result of cellular projections extended from the SGPs. The sex of the gonad is specified at the time of gonad coalescence from interactions between PGCs and SGPs (Papagiannouli, 2012; Santos & Lehmann, 2004). The SGPs are specified into various somatic cell types of the testes and the ovaries that support, and guide germ cell differentiation into mature gametes (Boyle and DiNardo, 1995; Zoller and Schulz, 2012). Pigment cells join the gonad from the fat body after gonad formation (DeFalco et al., 2008).

The genital imaginal disc gives rise to the seminal vesicle, and accessory glands (Fuller, 1993; Nanda et al., 2009). The terminal epithelium arises from the male-specific somatic gonadal precursors (msSGPs) in the embryonic gonad (Nanda et al., 2009; Whitworth et al., 2012). The terminal epithelium anchors spermatid nuclei during coiling, the seminal vesicle stores mature sperm, and the accessory glands secrete signals that enhance male fertility by supporting sperm storage or maintenance of sperm function in the female (Fuller, 1993). The outer layer of the testis sheath is comprised of pigment cells and a thin layer of muscle cells, separated by a basal lamina. The muscle layer is

separated from the testis lumen by a second basal lamina. Upon joining the gonads during metamorphosis, the pigment cells migrate from the outer covering of the testis to surround the seminal vesicle, and gives rise to the characteristic yellow color of the testis and seminal vesicle (Fuller, 1993).

1.2 Overview of spermatogenesis in *D. melanogaster*

D. melanogaster testes are blind-ended, coiled, elongated tubes, approximately 2 mm in length and 0.1 mm in width. All stages of spermatogenesis can be easily dissected in the adult testis (Figure 1.2): early spermatogonial stages can be found at the apical tip and later spermatid stages are located at the basal end (Hardy et al., 1979). The entire process of spermatogenesis is continuous and occurs over approximately 10 days.

The stem cell niche is located at the apical region, and is formed by a group of cells known as the hub (Hardy et al., 1979). The hub is composed of 8-15 mitotically quiescent somatic cells, which directly contact and maintain the identity of GSCs and SSCs (Davies and Fuller, 2008). The hub regulates stem cell function via cell-cell signaling, changes in cell-cell adhesion, and influencing the orientation of stem cell division. Cell-adhesion mediated by adherens junctions is required for maintaining stem cells within the niche, in proximity to maintenance signals, and inaccessible to differentiation cues (Yamashita et al., 2005). Furthermore, integrin-mediated adhesion is required to maintain the hub, and indirectly the GSC. Stem cell divisions occur erratically when the hub is mispositioned as GSC fate is not maintained, and this results in rapid differentiation into sperm (Tanentzapf et al., 2007). Thus, disrupting soma morphogenesis in the testes leads to the loss of stem cell identity.

6-12 GSCs are arrayed in a rosette around hub (Spradling et al., 2011). GSCs possess a spectrosome, which is a spherical organelle rich in cytoskeletal proteins. This includes proteins like spectrin and ankyrin. Spectrosomes are an identifying feature of GSCs and early gonialblasts. The centrosome is associated with the hub contact site, and replicates before one centrosome moves to the opposite side of the germ cell prior to division. This ensures that division takes place along a plane perpendicular to the hub-cell contact site, with one daughter cell retaining stem cell identity and the other daughter cell being displaced from the niche and beginning differentiation. This process ensures a balance between differentiation and self-renewal in the GSC population.

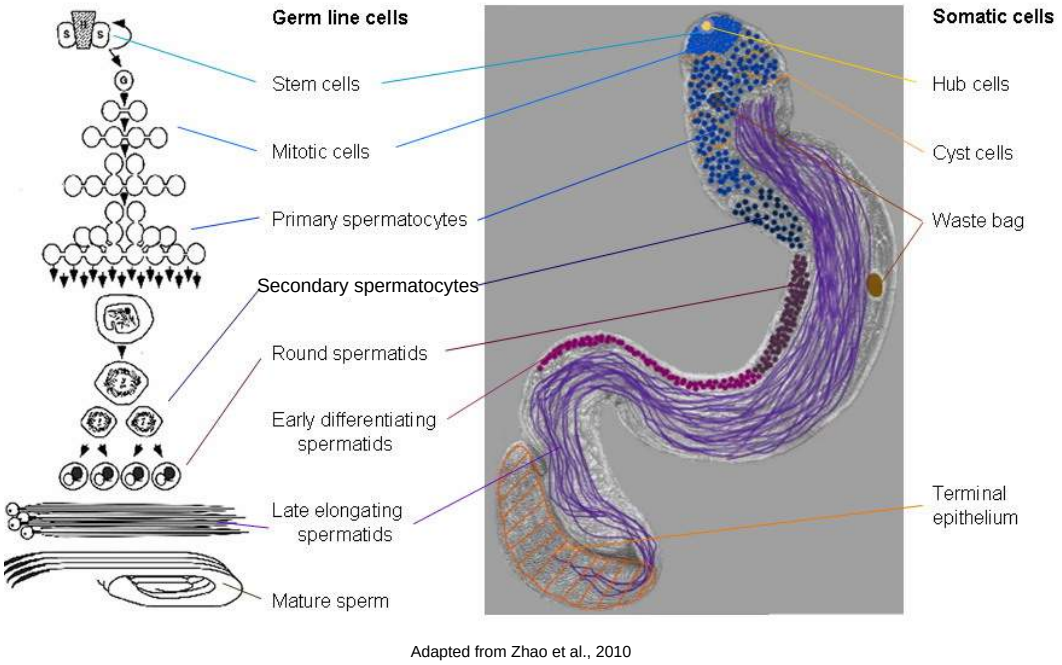
The germline differentiation program is highly coordinated. The germ cell undergoes 4 rounds of transit amplifying mitotic divisions, with incomplete cytokinesis to form 16 spermatogonia, interconnected by cytoplasmic bridges known as ring canals, which mediate the transfer of material between cells, and a branched fusome known to coordinate mitotic germ cell divisions (Hime et al., 1996). Once spermatogonia have completed their mitotic divisions, they are termed primary spermatocytes, and undergo a period of growth, changes in gene expression, and preparation for meiosis. During the growth period, primary spermatocytes undergo a 25-fold increase in volume as they migrate towards the base of the testis (Hardy et al., 1979). Meiosis takes place mid-way through the testis. After Meiosis I, the germ cells are termed secondary spermatocytes. Upon completing meiosis II they are spermatids. The completion of the meiotic stage results in 64 interconnected, haploid, round spermatids.

Spermiogenesis, the final stage of spermatogenesis, involves the maturation of spermatids into functional, motile sperm. It begins when the 64 spermatids undergo vast

morphological changes required to form mature sperm. The acrosome forms on the side of the nucleus opposite the basal body. It is a membrane bound, Golgi-derived organelle that is required for fertilization. The formation of the acroblast requires many Golgi and endosomal trafficking regulators, which are involved in spermatocyte cytokinesis. The key processes of spermiogenesis include elongation, individualization, coiling, and extrusion of mature sperm into the seminal vesicle for storage and/or fertilization (Zoller and Schulz, 2012).

The elongation of spermatids takes place within the syncytial cyst. Spermatids extend in length by 150-fold. In the early stages of elongation, the cyst becomes polarized because all the germline nuclei localize to one end and the growing sperm tails are found on the opposite end. Although spermatid cyst polarization can occur independently of surrounding somatic cyst cells, the upstream signals which establish spermatid cyst polarity are not well understood (Zoller and Schulz, 2012). During elongation, the somatic cells also polarize, forming a head cyst cell, which surrounds the germline nuclei, and a tail cyst cell, which surrounds the growing axoneme. The head cyst cell forms an F-actin rich cap which grows around the spermatid head which may aid in anchoring the spermatid heads in preparation for extrusion into the seminal vesicle. The tail cyst cell surrounds the axonemes and coordinates degradation of excess germline cytoplasm from individualizing spermatids. The spermatids elongate to about 1.8 mm (Hardy et al., 1979), which is accompanied by remodeling of nuclear morphology and chromatin condensation.

Figure 1.2 An explanatory diagram illustrating stages of *Drosophila* spermatogenesis



Adapted from Zhao et al., 2010

Figure 1.2 An explanatory diagram illustrating stages of *Drosophila* spermatogenesis. This diagram shows the 3 key stages of fly spermatogenesis. The germline stem cells asymmetrically divide, producing daughter cells, which undergo 4 rounds of mitotic divisions as spermatogonia. This creates 16 post-mitotic spermatocytes, which grow and undergo meiosis, mid-way down the testis, creating 64 post-meiotic spermatids. These spermatids elongate and are extruded into the seminal vesicle. The soma surrounds and supports germline development throughout spermatogenesis (Schematic adapted from Zhao et al., 2010).

During elongation, the nuclei become thin and the chromatin condenses such that nuclei are needle-shaped and approximately 10 μm in length (Zoller and Schulz 2012). Throughout elongation, secretory trafficking is indispensible.

Individualization of spermatids and coiling of mature sperm follows the elongation stage (Hardy et al., 1979; Zoller and Schulz 2012). An individualization complex (IC) is required during individualization. The IC is composed of 64 actin cones that surround the 64 needle shaped nuclei in a mature spermatid cyst. The spermatid nuclei act as a scaffold to facilitate the assembly of the IC (Fabrizio et al., 1998). The IC moves down the length of the cyst to remove dispensable organelles and cytoplasm, forming a cystic bulge that is known as the waste bag when it reaches the sperm tails and becomes detached. The IC also resolves intercellular bridges, such that the each sperm cell possesses its own plasma membrane. The signals that initiate the process of individualization are not well understood (Fabian and Brill, 2012).

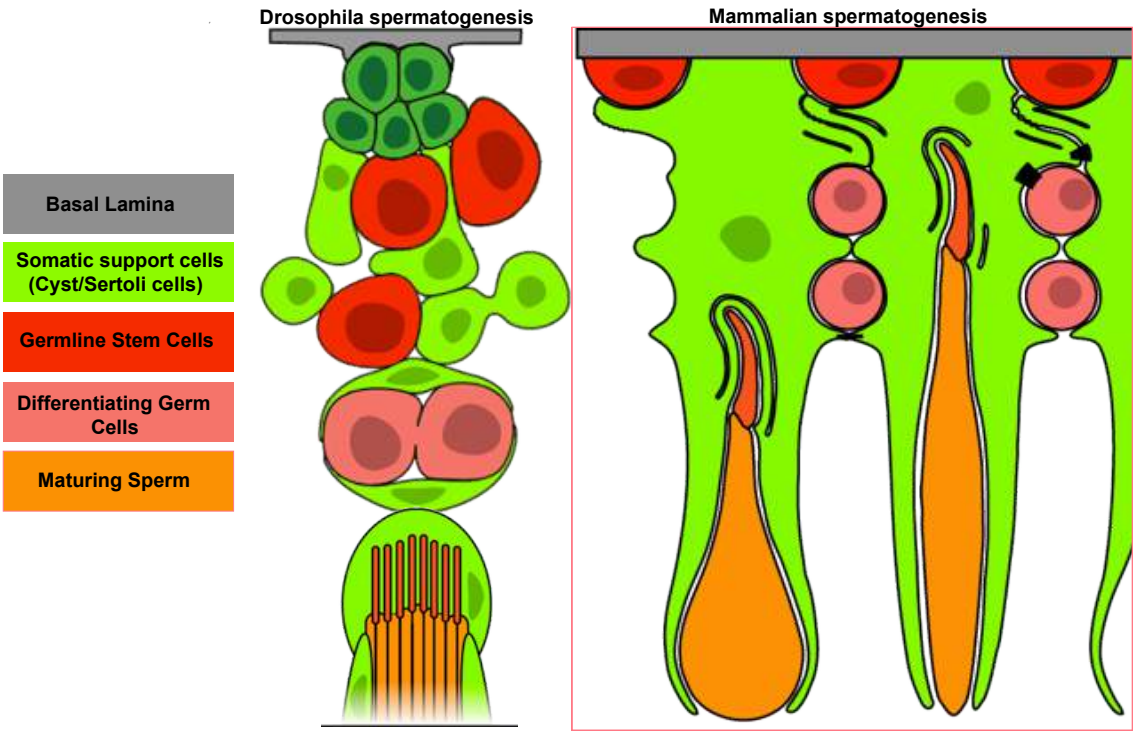
Following individualization, the mature sperm is coiled into the base of the testis and their tails extend towards the apical tip. Upon coiling, the sperm are released in the testis terminal epithelium, and then passed to the seminal vesicle, where mature sperm is stored until mating (Fuller, 1993; Hardy et al., 1979; Zoller and Schulz 2012). The terminal epithelium engulfs and degrades the contents of the waste bag, and the head cyst cells upon liberation of individualized sperm. The seminal vesicle is derived from the genital disk, not the gonad, and is attached to the basal end of the testes. A signal from the seminal vesicle controls shape changes of the testis and enables the formation of a long, coiled tube (Fuller, 1993).

A failure in spermatid individualization results in abnormal spermatids, which are deposited into the waste bag during the coiling process. It has been hypothesized that the individualized sperm are actively passed to the seminal vesicle. During copulation, mature sperm is transferred to the seminal receptacle and spermatheca of the female reproductive tract, where sperm is stored until use (Fuller, 1993).

The coordination of many molecular and morphogenetic processes during spermatogenesis governs the formation of mature, functional sperm (Wakimoto et al., 2004). This includes continuous and rapid changes in mitochondrial morphology as well as chromatin remodeling (Fabian and Brill, 2012; Verver et al., 2013). Chromatin structure and dynamics is vital to drive the mitotic and meiotic cell cycle programs, guide replication and transcription, and protect genomic integrity. The inability to maintain the spatio-temporal organization of chromatin can result in genomic instability. This can lead to the apoptosis of early spermatogonia or meiotic arrest, and consequently infertility due to aberrant chromatin alignment (Verver et al., 2013). Furthermore, the proper localization of signaling proteins for fertility is crucial in spermiogenesis. Sperm-egg interactions require glycosylation of proteins at the plasma membrane for sperm binding and fertilization.

Drosophila spermatogenesis is a good model for mammalian spermatogenesis because in both cases, germline stem cells give rise to daughter cells that undergo a very specific program of differentiation. This program of differentiation, in both cases, is

Figure 1.3 *Drosophila* and mammalian spermatogenesis are similar



Adapted from Voog & Jones, 2010 and Vogl et al., 2008

Figure 1.3 *Drosophila* and mammalian spermatogenesis are similar. In both Drosophila and mammals, undifferentiated germ cells (red) are exposed to the general humoural environment, while differentiating germ cells (pink) are surrounded by somatic cells (green) that insulate the germline. Meiotic and post meiotic sperm forming stages (orange) are thus isolated in a lumen formed by the soma. Schematic adapted from Voog & Jones 2010, and Vogl et al., 2008, courtesy of Michael Fairchild.

dependent upon a microenvironment, formed by surrounding somatic cells (Figure 1.3). Since many stages of spermatogenesis are conserved across species, what we learn from *Drosophila* spermatogenesis will have implications for understanding human male infertility, and therapeutic interventions for testicular and ovarian cancers.

1.3. Drosophila as a model to study stem cell behavior in gametogenesis

1.3.1 Importance of stem cells in gametogenesis

Adult stem cells are responsible for long-term maintenance and repair of tissues in response to physical changes or damage. Some of the defining features of adult stem cells include their long-term ability to proliferate, ability to produce new stem cells (self-renew) and differentiating progeny (Spradling et al., 2011). The maintenance of germline and somatic stem cells is key to ensure gamete production throughout the life of an organism. GSCs are usually found in a somatic microenvironment that restricts their differentiation. They undergo asymmetric division to give rise to daughter cells that proliferate through several rounds of divisions and enter meiosis. GSCs gives rise to sperm in males, and eggs in females (Spradling et al., 2011).

The *Drosophila* testis contains a discrete morphological and well-characterized stem cell niche (Spradling et al., 2011). Two types of stem cells populate the niche: germline and somatic cells (Figure 1.4). *Drosophila* GSCs are one of the best-understood adult stem cell types (Spradling et al., 2011). Cyst stem cells (CySCs) are functional stem cells because they self-renew and give rise to daughters that differentiate into somatic cyst cells (Gonczy and DiNardo, 1996). They form the hub, which maintains GSCs to orchestrate spermatogenesis. The somatic cyst cells surround and form the

microenvironment of the GSCs as they proliferate and differentiate into sperm. CySCs can convert to hub cells and vice versa, which suggests a novel mechanism for maintaining the hub (Gonczy and DiNardo, 1996; Voog et al., 2008). Cyst cell fate might be influenced by the presence of germ cells because hub cells expand and changes morphology in agametic testes (Gonczy and DiNardo, 1996; Voog et al., 2008). Thus, GSCs play a key role in maintaining CySCs population (Gonczy and DiNardo, 1996). Furthermore, the close association of the hub and SSC with the GSC, in addition to their similar gene expression patterns, facilitates studies since there are a wide variety of markers available (Voog et al., 2008).

1.3.2 Overview of signaling events that mediate soma-germline interactions in D. melanogaster

Stem cell behavior is regulated by changes in cell adhesion and signaling (de Cuevas and Matunis, 2011). The number of stem cells must be regulated and coordinated throughout spermatogenesis. Hub-derived signals maintain soma and germline fate (Kiger et al., 2001; Tulina and Matunis 2001; Xie and Spradling 1998). There are two main signaling pathways by which the hub regulates GSCs: Janus kinase and Signal Transducer and Activator of Transcription (JAK/STAT) ligands, encoded by the gene *unpaired* (*upd*), are produced in the hub and activate JAK/STAT receptors in adjacent GSC promoting their self-renewal (Zoller and Schulz, 2012); Bone Morphogenetic Protein (BMP) ligands Glass Bottomed Boat (*gbb*) and Decapentaplegic (*dpp*) induce GSC self-renewal by activating the Transforming Growth Factor-β (TGFβ) signaling pathway in the GSC. Additionally, BMP signals repress the expression of *bag-of-marbles* (*bam*), essential for germline differentiation. This ensures that GSCs maintain their stem

cell fate.

Somatic CySCs are maintained by the same cytokine-like signal that maintains GSC fate. CySC self-renewal is also specified by Upd, which activates JAK-STAT. If *upd* is ectopically expressed in early germ cells or early cyst cells, the CySCs overproliferate similarly to GSCs (Davies and Fuller, 2008). *stat* is required cell autonomously for CySC maintenance, as somatic CySCs homozygous mutant for *stat* fail to maintain stem cell identity, resulting in commitment to differentiation. Additionally, there are two proteins that act as downstream targets to JAK/STAT to regulate CySC fate. The transcriptional repressor *zinc finger-homeodomain transcription factor 1* (*zfh-1*) and Chronologically inappropriate morphogenesis (Chinmo), which may play a role in protein degradation, act downstream to JAK/STAT. CySCs lacking these proteins fail to self-renew. Thus the activities of Zfh-1 and Chinmo rely on JAK/STAT signaling by linking JAK/STAT signaling events to transcriptional regulation of target genes.

Encapsulation, the process by which two newly born somatic cells surround the gonialblast is not well understood. While the gonialblast divides to form 64 meiotic spermatids, the cyst does not divide, but undergoes significant growth to accommodate the growing cyst. This requires significant remodeling and shape change of somatic cells to stretch and surround the expanding germ cells. It is known that Epidermal Growth Factor (EGF) ligand, Spitz, released by germ cells activate the EGF receptor in the soma via the guanine nucleotide exchange factor Vav and GTPase Rac 1, to induce cyst cells to encapsulate the germline. The reorganization of the cyst cell cytoskeleton is mediated by the Mitogen-activated protein kinase (MAPK) pathway, which modulates actin cytoskeleton regulators Rac- or Rho-.

Figure 1.4 The *Drosophila* testis stem cell niche

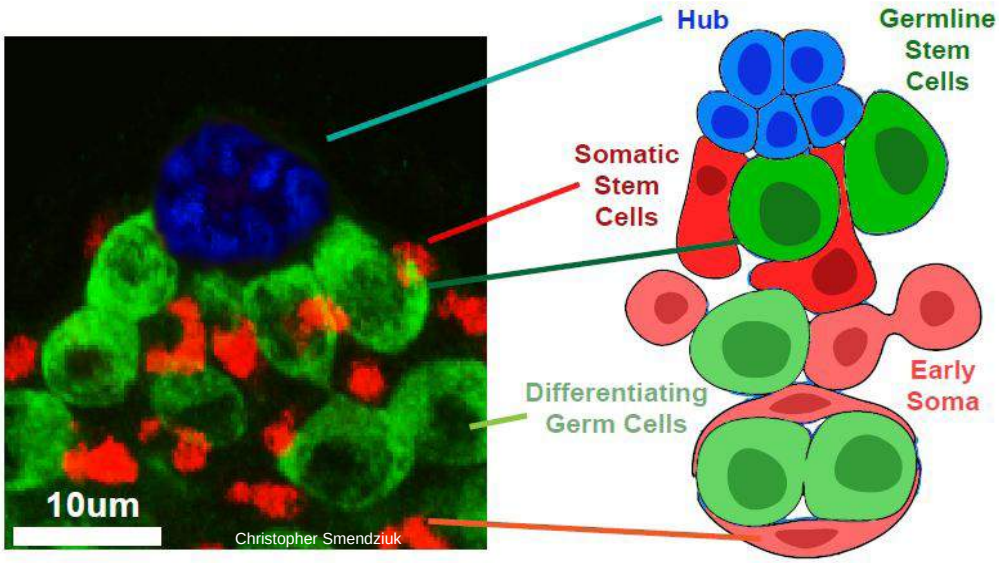


Figure 1.4 The *Drosophila* testis stem cell niche. Confocal image (left) showing the *Drosophila* stem cell niche (Confocal image, courtesy of Christopher Smendziuk). The germ cells are marked by vasa (green), and somatic cells are marked by traffic-jam (red). The hub cells are also marked by traffic-jam (but are false-colour labeled in blue). The schematic (right), adapted from Voog and Jones, 2010, shows that as germline stem cells (dark green) divide, one daughter cell remains anchored to the stem cell niche, while the other daughter cell (light green) undergoes a specific program of differentiation to form mature sperm. The somatic stem cells adhered to the hub (dark red) also undergo asymmetric divisions, forming cyst cells (light red), two of which wrap around the germline prior to its differentiation.

This leads to the growth of the cyst cell around the germline (Kiger et al., 2000; Sarkar et al., 2007; Schulz et al., 2002; Tran et al., 2000). In mutant testes, lacking EGF signaling, GSCs divides at frequencies 2-3 times higher than GSCs of control testes, however the CySCs division frequencies remains unaffected (Zoller and Schulz 2012). This shows that EGF signaling normally downregulates GSC divisions. (Sarkar et al., 2007; Schulz et al., 2002; Tran et al., 2000). This effect, however, remains indirect since EGF emanates from the germline and is received by the CySCs, which respond with a return signal to the GSCs. Thus, the frequency of divisions relies on both systemic factors and cross-talk between the soma and germline.

Signaling mediated by the Notch, the EGF, and the Sevenless signaling pathways determine the specification of the SGPs as CySCs or hub cells. In the embryonic gonad, Notch signaling between the SGPs is sufficient to specify hub fate. However, Notch signaling is inhibited by EGF and Sevenless signaling from the germline to the SGPs throughout the embryonic gonad, except for the most anterior region. In the anterior region of the testis, this leads to the specification of SGPs as hub cells, and determination of SGP fate as CySCs in the rest of the gonad (Zoller and Schulz, 2012).

1.3.3 Implications of stem cells in disease

The control, regulation, and maintenance of stem cell behavior are fundamental to tissue homeostasis (Kawase et al., 2004). Ovarian, testicular and other cancers arise in adult stem cell lineages due to defects in mechanisms that regulate stem cell self-renewal, proliferation, and differentiation. Studying the mechanisms that underlie these defects will provide key insights to tumorogenesis and uncover the potential for stem cells in

regenerative medicine. Thus, dissecting how the local microenvironment of the stem cell niche regulates self-renewal and differentiation of germline stem cells will have important implications on the mechanisms by which the soma and germline maintain gamete production throughout the life of an organism (Spradling et al., 2011).

1.4 Research goals, hypothesis and rationale

To gain insight into the morphogenetic events that regulate somatic tissue in the gonad, and how genes in the soma act to establish and maintain interactions with the germline, I designed a research project based on the following hypothesis and rationale:

1.4.1 Research objective

The main objective of this thesis was to classify a subset of candidate genes uncovered in an ongoing genetic screen in my lab for somatic mediators of germline development. I wanted to determine whether reduced fertility was associated with a visible phenotype in the testes. In addition, I wanted to elucidate the precise role of novel mediators of soma-germline interactions at key stages of spermatogenesis.

1.4.2 Overall hypothesis

There is a specific set of somatic genes that mediate soma-germline interactions.

1.4.3 Rationale

Germline transplantation experiments revealed that the soma controls spermatogenesis by regulating germ line development (Fuller, 1993). These studies

suggested that the soma produces signals that direct the germline to complete meiosis and initiate spermatid differentiation (Fuller, 1993). Therefore, understanding spermatogenesis requires a comprehensive understanding of how the soma regulates the germline.

The *Drosophila* testis is an excellent model system to dissect the cell biological mechanisms by which the soma regulates germline development. In *Drosophila*, all stages of germline development are visible and easy to identify (Fuller, 1993). Additionally, the *Drosophila* testes are very well characterized, and amenable to genetic manipulation (White-Cooper, 2012). There are a wide variety of molecular markers available to visualize all stages of spermatogenesis.

Tissue-specific expression in *Drosophila* has been shown to be a powerful tool to investigate gene function in many tissues and cell types (Dietzl et al., 2007). Combining RNA interference (RNAi) with tissue specific expression allows soma specific gene knockdown. Our lab is currently performing a genome wide RNAi screen to identify new mediators of soma-germline interactions. So far, the screen has identified a large number of candidate genes. The identification and classification of candidate mediators of soma-germline interactions is the basis of the work I carried out in my thesis.

Chapter 2: Materials and methods

2.1 Fly stocks and genetics

The RNAi library was obtained from a variety of sources: the Bloomington *Drosophila* Stock Centre (Indiana, USA), Vienna *Drosophila* Rnai Centre, National Institute of Genetics (NIG-Fly, Kyoto, Japan) as well as the Kyoto *Drosphila* Genetic Resource Centre (DGRC, Kyoto, Japan). The pilot screen targeted cytoskeletal proteins in the fly and was later expanded to the whole genome to gain a comprehensive understanding of soma-germline interactions. The spatio-temporal expression of RNAi transgenes was attained using the UAS/GAL4 system, with the *tj*-GAL4 line as a 'driver' to direct expression to the somatic cells –specifically the hub cells, the Somatic Stem Cells (SSCs) and all somatic tissues during the early spermatocyte stages. The endogenous expression pattern of the traffic jam gene drives strong and uniform expression of RNAi transgenes in somatic tissues in the adult testes. When RNAi lines are crossed to *tj*-GAL4 driver, almost all progeny are viable enabling the analysis of adult testes .

2.2 Fertility assays

All RNAi transgenes were expressed in the soma early on from embryogenesis. Single male progeny, 1-5 days post eclosion, that carry both the driver (*tj*-Gal4, UAS-mCD8:GFP), with the presence of UAS-Dicer2 and the UAS-RNAi, are crossed to wildtype (w^{1118}) virgin females, and allowed to breed. Dicer2 is an enzyme that increases the efficiency of RNAi expression. Progeny are raised at 25°C and after 14 days the cross

is analyzed, and assigned a fecundity score –fertile, low fertility, or sterile based on the number of pupae counted per vial. The presence of zero pupae and absence of larvae indicates sterile candidates, whereas 1-40 pupae indicates low fertility candidates, and >40 pupae indicates fertility.

2.3 Immunohistochemistry and confocal microscopy

All stained flies were fixed using 4% paraformaldehyde in phosphate buffer saline (PBS) and washed and incubated in PBS plus 0.3% Triton-X and 0.5% BSA. Primary antibodies used were: rat anti-Vasa [Developmental Studies Hybridoma Bank (DSHB); 1:20), rabbit anti-GFP (Invitrogen, A6455; 1:1000), mouse anti-Fasciclin3 (DSHB); 1:1000], rat anti-N-Cadherin (DHSB; 1:50) and rabbit anti-Boule (S. Wasserman, University of California, San Diego, CA, USA; 1:1000). Secondary antibodies used were: Rabbit anti-GFP was conjugated to Alexa Fluor 488 (Invitrogen; 1:500), rat anti-Vasa was conjugated to Cy3 (Invitrogen; 1:500), and mouse anti-Fasciclin3 was conjugated to Cy5 (Invitrogen; 1:500). Samples were mounted in Vectashield mounting media with DAPI (Vector Laboratories, H-1200). Confocal images were acquired using an Olympus IX81/FV1000 microscope with 20x and 40x objectives. Images were processed with Adobe Photoshop and Adobe Illustrator.

2.4. Candidate classification scheme

Candidates were classified based on the stage of arrest during germline development. The ‘No soma and No germline’ class is characterized by the absence of somatic and germ cells. The ‘No soma’ class is classified by the absence of somatic cells,

and presence of germ cells. The ‘Arrest after spermatogonia’ class is characterized by the presence mitotic spermatogonia, and absence of spermatocytes and spermatids. The ‘Arrest after spermatocyte’ class is characterized by the presence of spermatocytes, but absence of spermatids. The ‘Extrusion’ class is characterized by the presence of elongated spermatids that fail to enter the seminal vesicle. In all cases, the soma is visualized by the GFP antibody which stains the membrane of somatic cells (mcD8::GFP). The germline is visualized with the Vasa antibody, and the hub, terminal epithelium, and seminal vesicle is visualized with the Fasciclin3 antibody. DAPI (4'6-diamidino-2-phenylindole) is used to identify gross defects in morphology and germline tumors.

2.5 Bioinformatic analysis

The database for annotation, visualization and integrated discovery (DAVID: http://david.abcc.ncifcrf.gov/) was used to perform GO enrichment analysis for the sterile candidates classified. The sterile candidates were grouped into the highest stringency clusters by using GO cluster functional annotation under default settings for gene_ontology. This produced a list of GO terms associated with molecular function, biological processes, or cellular component that varied in GO enrichment when compared to the total genes screened.

Chapter 3: The phenotypic classification of genes required for spermatogenesis

3.1 Introduction to the somatic RNAi screen

An RNAi screen was designed by my lab to identify genes in the soma that regulate germline development. Earlier requirement for genes that act in the soma development program reduces the likelihood that novel mediators will be identified through standard genetic screens. Large gaps exist in our understanding of soma development as this process likely involves many genes that play key roles in earlier developmental events, and are likely required in the germline. Furthermore, although the *Drosophila* testis is a useful system for the study of spermatogenesis, it has for various reasons been relatively underutilized (White-Cooper 2012). To date, there are no comprehensive genetic screens analyzing the role of the soma in *Drosophila* spermatogenesis.

3.1.1 Pilot screen strategy

Our screen used RNAi-mediated knockdown specifically in the somatic cells of the testis, starting at embryogenesis. The feasibility of the screen was initially demonstrated using a large pilot screen. To specifically target RNAi knockdown in the somatic cells of the testis, the somatic cell specific promoter, *traffic jam,* was used to express Gal4 to activate UAS-containing RNAi transgenes (Figure 3.1). This enabled the knockdown of a library of genes in the soma during spermatogenesis. Almost all RNAi lines crossed to *tj*-GAL4 gave rise to viable progeny, thus adult testes could be analyzed. Single male fertility assays were used to identify genes in the soma that are essential for spermatogenesis. Males with somatic knockdown of specific genes were crossed to virgin

females, and their fertility was scored by counting the number of pupae. A ‘hit’ was identified when the majority of assays showed a sterile or low fertility phenotype. Hits were rescreened by up to 10 additional fertility assays. When it was possible, some of these hits were re-screened with multiple RNAi lines. A final candidate listing was created upon re-screening hits. Candidates are genes that continue to display reduced fertility when re-screened. Candidates were chosen for phenotypic classification. *Tj-Gal4>w1118* flies were used as controls in the screen and classification of candidates (Figure 3.1.1).

The pilot screen focused on cytoskeletal genes because they are good candidates for mediating the sort of morphogenetic processes that the soma undergoes over the course of spermatogenesis. dsRNA-mediated interference (RNAi) transgenic lines were obtained from numerous sources. This included the Vienna Drosophila Resource Centre (VDRC), the TRiP collection (Bloomington, Indiana), and the National Institute of Genetics (Kyoto, Japan). Lines known to give effective gene knockdown in other screens were obtained whenever possible. The pilot screen compiled 533 RNAi lines representing 349 genes. Although the majority of these were cytoskeletal genes, 30 transcription factors and 59 regulators of cell adhesion were also screened. The majority of testes with reduced fecundity gave rise to visible defects in the testes. The success of the pilot screen resulted in the expansion of the screen.

Figure 3.1 Tissue specific knockdown method

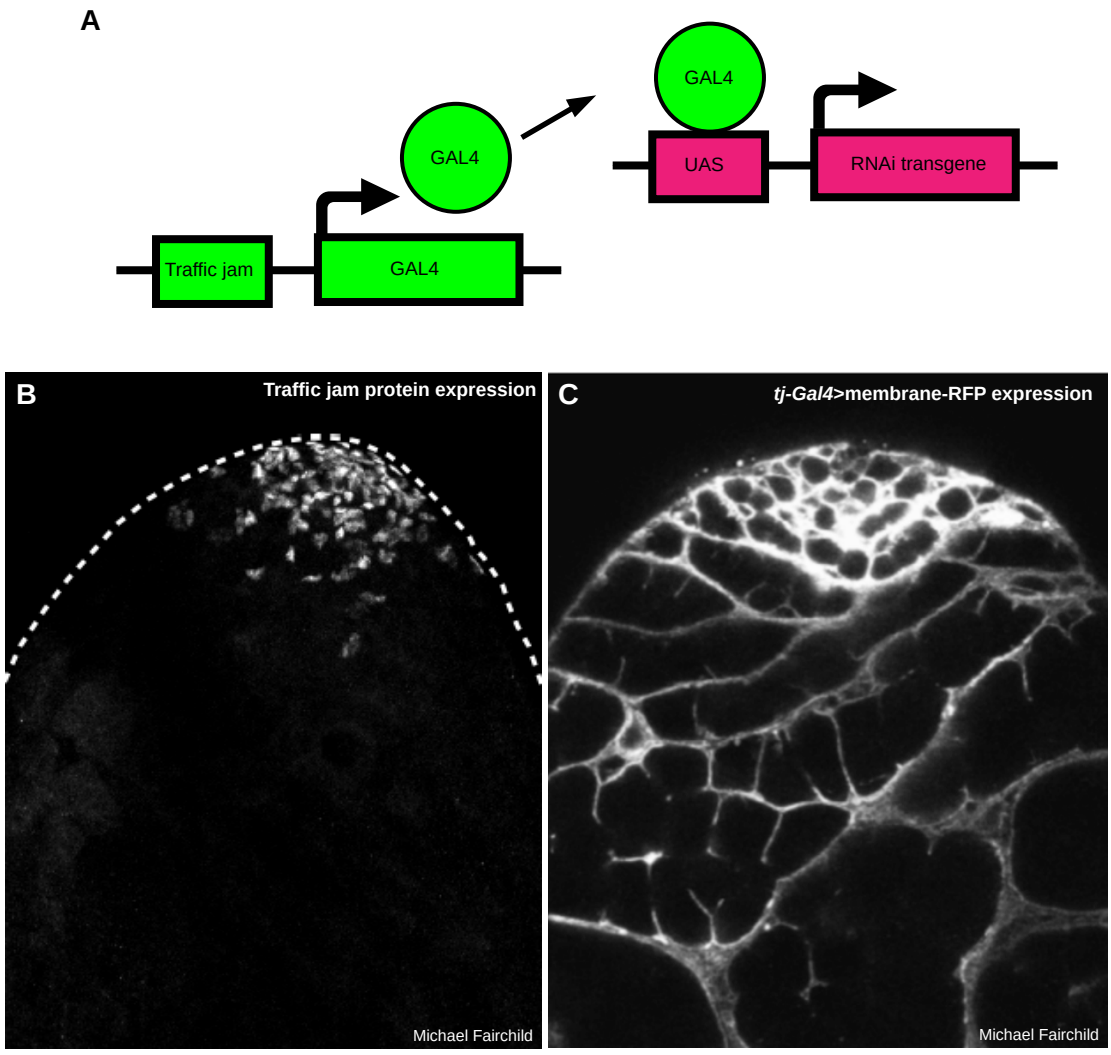


Figure 3.1 Tissue specific knockdown method. (A) We performed tissue-specific RNAi-mediated knockdown in the somatic cells of the testes. GAL4 was expressed under the control of a somatic specific promoter of the gene *traffic jam*, to drive a library of UAS-RNAi lines in the soma. (B) The apical tip of the *Drosophila* testis showing traffic jam protein expression and (C) *tj-Gal4*>membrane-RFP expression in the soma. Scale bar is 30 μ m for (B) and (C) (Images B & C, courtesy of Michael Fairchild)

Figure 3.1.1 Flowchart of RNAi screen and classification scheme

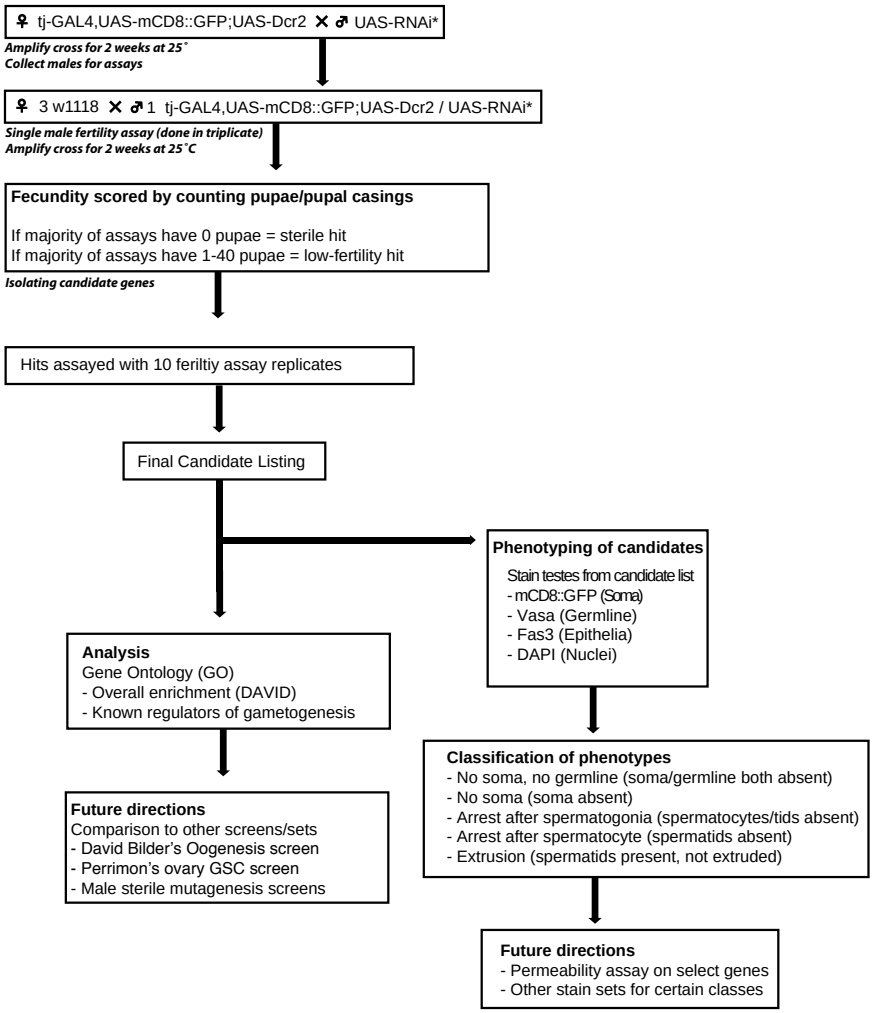


Figure 3.1.1 Flow chart of RNAi screen and classification scheme. This diagram shows the genotype and number of flies used to generate the flies with somatic knockdown of specific genes. In the first pass screen, the fertility assays are set up in triplicate, and hits are identified as sterile if the majority of assays have 0 pupae, or as semi-sterile if the majority of assays have 1-40 pupae. These hits are re-screened in a second pass screen with 10 fertility assay replicates. A final candidate listing is made and composed of hits that exhibit sterility upon re-screening. Phenotyping of candidates is the next stage of analysis. Candidates are classified into the following classes: no soma & no germline, no soma, arrest after spermatogonia, arrest after spermatocyte, or extrusion. Candidates can be further classified by exploring gene ontology terms or performing other types of phenotypic analysis on select genes.

3.1.2 Expansion of the screen

To gain a comprehensive understanding of soma-germline interactions, the RNAi screen was expanded to the whole genome. Although the pilot screen uncovered new mediators involved in the morphogenetic events that underlie the soma development program, it did not address other aspects of this process like differentiation and signaling. In order to expand the screen, several subsets of plasma membrane channels, and adhesion proteins were also investigated, as they would be likely involved in setting up the somatic microenvironment around the germline. In addition, our lab is currently screening a range of signaling molecules, putative stem cell regulatory genes, and a range of genes identified by other groups we collaborated with. One such collaboration was with Dr. Bilder's lab at the University of California, Berekely, which was performing a similar screen to ours, using the *tj*-GAL4 driver, in combination with Dicer2 to increase the efficiency of gene knockdown (Dietzl et al., 2007) in the somatic cells of the *Drosophila* ovary. Since Dr. Bilder is only interested in the ovaries, a large number of genes screened were obtained from their RNAi library, so that a direct comparison of genes required for soma-germline interactions in the ovaries and testis could be done. Recently, a screen for genes required for germline stem cell self-renewal in the *Drosophila* ovaries was published by the Perrimon lab (Howard Hughes Medical Institute, Harvard Medical School, Boston, USA), from where we obtained a set of matching RNAi lines to investigate their requirement in the soma. Off-target effects of RNAi hairpin constructs were controlled by obtaining RNAi lines from various Drosophila RNAi libraries, targeting different RNA sequences in the gene of interest. In addition, my lab has shown that somatic knockdown of genes can be confirmed using

qPCR.

To date, we have screened 2164 RNAi lines representing 1658 genes (about 11.9% of the *Drosophila* genome). Approximately 10, 766 fertility assays were performed to screen the total genes to date. 25.6% of the genes screened were identified as hits. Of these, 10% were sterile hits and 15.5% were low-fertility hits**.** This attests to the reliability of our screen and validates our approach in uncovering new mediators of soma-germline interactions.

3.2 Introduction to the classification scheme

To determine whether sterility was associated with an observable defect in the testes during spermatogenesis, I established a classification scheme based on arrest in germline development during spermatogenesis. I classified candidates into 5 groups: No soma & No germline, No soma, Arrest after spermatogonia, Arrest after spermatocyte, Extrusion, and No overt phenotype. These phenotypic classes represented different stages where germline development was defective, with the exception of the No overt phenotype category where testes appeared wild-type. I was able to de-lineate the role of candidate genes in early (pre-meiotic) and/or late (post-meiotic) stages of germline development. In each case, the germline was visualized using vasa (RNA helicase). The soma was visualized using GFP, to amplify the signal of the *tj-Gal4>mcD8::GFP*. The hub and terminal epithelium were visualized using Fasciclin3 (cell adhesion protein).

The majority of candidates classified represent sterile candidates, while others were low-fertility candidates. Some candidates exhibited an array of defects belonging to

more than one phenotypic class. In these cases, they were classified based on the class of defects exhibited by the majority of testes.

3.3 Classification of candidate genes from the screen

197 candidates were classified into the following categories: No soma & No germline, No soma, Arrest after spermatogonia, Arrest after spermatocyte, Extrusion, and No overt phenotype. 129 sterile candidates were classified. 95.35% of the sterile candidates exhibited defects in germline development, while only 4.65% displayed no overt phenotype. 68 low-fertility candidates were classified. 25% of the low-fertility candidates exhibited defects in germline development, while 75% displayed no overt phenotype. The breakdown of phenotypic classes for sterile and low-fertility candidates is shown in Figure 3.3. The list and number of genes that were assigned to a phenotypic class are summarized in Table 1. The following sub-sections describe the results and features of each phenotypic class. In addition, the database for annotation, visualization and integrated discovery (DAVID) was used to perform gene ontology (GO) enrichment analysis for sterile candidates, to gain insight on their possible molecular functions and involvement in specific biological processes (see Appendix A). A subset of the genes identified is described in 3.3.1-3.3.6. For brevity, only some of the GO terms with enrichment values >1 and significance of $P<0.05$ are mentioned for each phenotypic class.

Figure 3.3 Breakdown of phenotypic classes from screen candidates

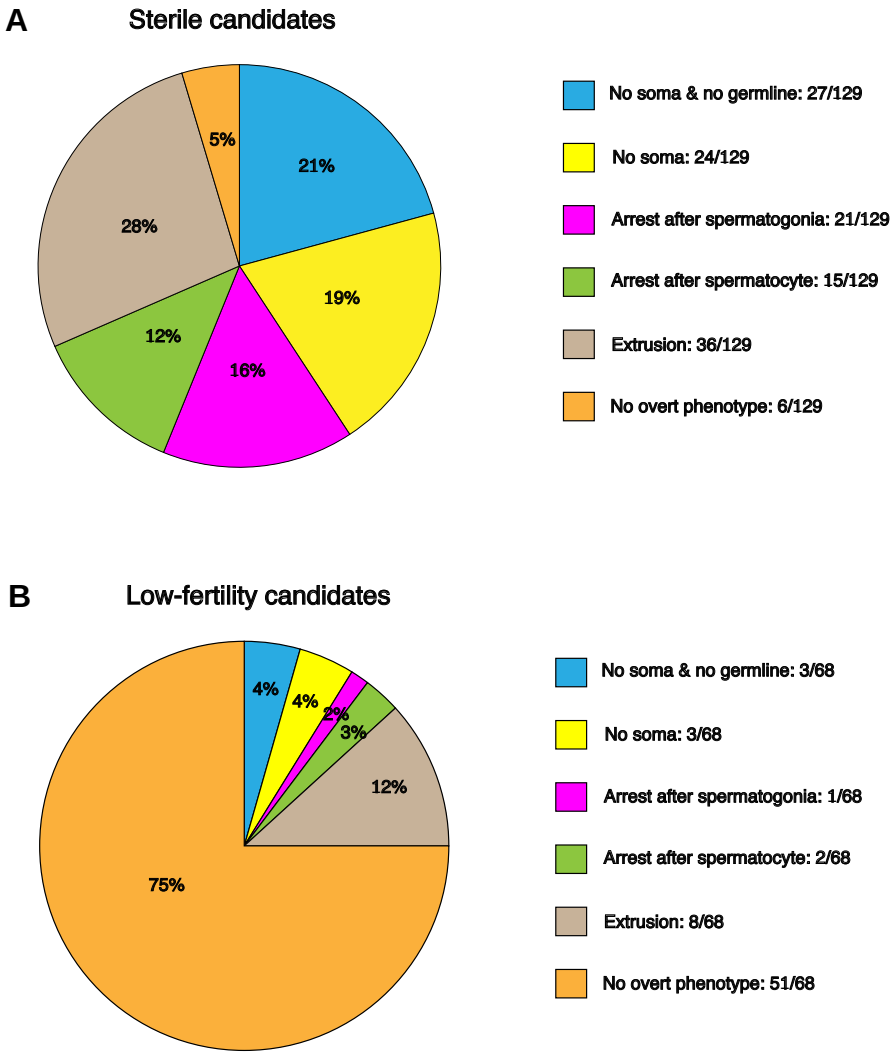


Figure 3.3 Breakdown of phenotypic classes from screen candidates (A) 129 sterile candidates were classified. 21% are in the no soma & no germline class, 19% are in the no soma class, 16% are in the arrest after spermatogonia class, 12% are in the arrest after spermatocyte class, 28% are in the extrusion class, and 5% did not exhibit any visible phenotypes. (B) 68 low-fertility candidates were classified. 4% were in the no soma & no germline class, 4% were in the no soma class, 2% were in the arrest after spermatogonia class, 3% were in the arrest after spermatocyte class, 12% were in the extrusion class and 75% did not exhibit any visible phenotypes.

Table 1 Candidate classification summary

No soma and No germline						
CG	Gene	Summary of GO terms	Sterile or Low-fertility?	Fertility Assays	# RNAi lines screened	Known role in male gamete generation
CG4254	twinstar	actin binding	Low-fertility	10	1	
CG9553	chickadee	actin binding	Sterile	16	3	X
CG1782	ubiquitin activating enzyme 1	ATP binding	Sterile	29	2	
CG33123	-	ATP binding	Sterile	13	1	
CG7528	Smt3 activating enzyme 2	ATP binding	Sterile	16	2	
CG12225	-	chromatin binding	Low-fertility	10	1	
CG10212	SMC2	chromatin binding	Sterile	9	1	
CG11397	gluon	chromatin binding	Sterile	9	1	
CG8416	Rho1	GTPase activity	Sterile	13	2	
CG10414	ATAC complex component 2	H2A histone acetyltransferase activity	Low-fertility	21	2	
CG8749	small ribonucleoprotein particle U1 subunit 70K	mRNA binding	Sterile	10	1	
CG10687	Asparaginyl-tRNA synthetase	nucleic acid binding	Sterile	9	1	
CG32364	-	nucleic acid binding	Sterile	10	1	
CG7935	moleskin	protein binding	Sterile	19	2	
CG5363	cdc2	protein kinase activity	Sterile	22	4	X
CG11556	Rabphilin	Rab GTPase binding	Sterile	13	1	
CG3911	-	Rab guanyl-nucleotide exchange factor activity	Sterile	18	1	
CG2023	-	SNAP receptor activity	Sterile	17	1	
CG13626	-	SNARE binding	Sterile	10	1	
CG5178	Actin 88F	structural constituten of cytoskeleton	Sterile	10	2	
CG8053	Eukaryotic initiation factor 1A	translation intiation factor activity	Sterile	10	1	
CG11526	-	unknown	Sterile	10	1	
CG12050	-	unknown	Sterile	15	2	
CG1430	-	unknown	Sterile	18	1	
CG1639	lethal (1) 10Bb	unknown	Sterile	9	1	
CG2469	-	unknown	Sterile	13	1	
CG6066	-	unknown	Sterile	9	1	
CG9710	nudC	unknown	Sterile	10	1	
CG10042	MBD-R2	zinc ion binding	Sterile	13	1	
CG1250	sec23	zinc ion binding	Sterile	16	1	

No soma						
CG	Gene	Summary of GO terms	Sterile or Low-fertility?	Fertility Assays	# RNAi lines screened	Known role in male gamete generation
CG11949	coracle	actin binding	Sterile	84	3	
CG18102	shibire	actin binding	Sterile	19	2	X
CG2198	Amalgam	antigen binding	Sterile	12	1	
CG9802	Chromosome-associated protein	ATP binding	Low-fertility	22	2	
CG12019	Cdc37	chaperone binding	Sterile	12	2	
CG10149	Regulatory particle non-ATPase 6	endopeptidase activity	Sterile	6	1	
CG10426	-	inositol triphosphate phosphatase activity	Sterile	16	1	
CG8566	unc-104 ortholog (C. elegans)	microtubule binding	Sterile	22	3	
CG2671	lethal (2) giant larvae	myosin binding	Sterile	25	4	
CG4817	Structure specific recognition protein	nucleosomal DNA binding	Sterile	9	1	
CG8827	Angiotensin converting enzyme	peptidyl-dipeptidase activity	Sterile	9	1	X
CG9854	hiiragi	polynucleotide adenylyltransferase activity	Sterile	8	2	
CG34407	Not1	protein binding	Sterile	12	2	
CG4257	Signal-transducer and activator of transcription	protein binding	Sterile	11	1	
CG8264	Bx42	protein binding	Sterile	9	1	
CG6474	enhancer of yellow 1	protein heterodimerization activity	Sterile	10	1	
CG4006	-	protein kinase activity	Sterile	10	1	
CG15224	Casein kinase II beta subunit	protein kinase regulator activity	Sterile	25	3	
CG1740	Nuclear transport factor-2	protein transmembrane transporter activity	Sterile	12	2	
CG10811	-	RNA binding	Low-fertility	10	1	
CG7035	cap binding protein 80	RNA cap binding	Sterile	9	1	
CG7073	-	scaffold protein binding	Sterile	10	1	
CG5546	Mediator complex subunit 19	transcription factor binding	Sterile	13	2	
CG16792	Small ribonucleoprotein particle protein SmF	unknown	Low-fertility	9	1	
CG11920	-	unknown	Sterile	11	1	
CG11985	-	unknown	Sterile	9	1	
CG8427	Small ribonucleoprotein particle protein SmD3	unknown	Sterile	19	3	

Arrest after spermatogonia						
CG	Gene	Summary of GO terms	Sterile or Low-fertility?	Fertility Assays	# RNAi lines screened	Known role in male gamete generation
CG7558	Actin-related protein 3	actin binding	Sterile	21	3	
CG7776	Enhancer of Polycomb	chromatin organization	Sterile	9	2	
CG12530	Cdc42	GTPase activity	Sterile	25	4	X
CG1725	discs large 1	gunaylate kinase activity	Sterile	26	4	X
CG6121	Tip60	histone acetyltransferase activity	Sterile	13	2	
CG7610	ATP synthase, γ subunit	hydrogen-exporting ATPase activity, phosphory	Sterile	13	2	
CG8189	ATP synthase, subunit b	hydrogen-exporting ATPase activity, phosphory	Sterile	10	1	
CG5686	chico	insulin receptor binding	Low-fertility	22	3	X
CG9609	-	metal ion binding	Sterile	9	1	
CG12157	Translocase of outer membrane 40	P-P-bond-hydrolysis-driven protein transmemb	Sterile	13	1	
CG7109	microtubule star	phosphatase regulator activity	Sterile	13	2	
CG43398	scribbled	protein binding	Sterile	12	3	
CG5166	Ataxin-2	protein binding	Sterile	27	1	
CG5684	Pop2	protein binding	Sterile	9	2	
CG9012	Clathrin heavy chain	protein binding	Sterile	12	2	X
CG12532	Beta Adaptin	protein transporter activity	Sterile	19	1	
CG9998	U2 small nuclear riboprotein auxiliary factor 50	RNA binding	Sterile	16	1	
CG31136	Syntaxin 1A	SNARE binding	Sterile	11	1	
CG6827	Neurexin IV	transmembrane signaling receptor activity	Sterile	101	5	
CG10418	-	unknown	Sterile	13	1	
CG13277	-	unknown	Sterile	12	2	
CG31132	BRWD3	unknown	Sterile	9	1	

Arrest after spermatocyte						
CG	Gene	Summary of GO terms	Sterile or Low-fertility?	Fertility Assays	# RNAi lines screened	Known role in male gamete generation
CG9985	skittles	1-phosphatidylinositol-4-phosphate 5-kinase ac	Sterile	13	2	X
CG6174	Actin-related protein 1	actin binding	Sterile	13	2	
CG3612	bellwether	ATP binding	Sterile	9	1	X
CG5670	Na pump alpha subunit	cation transmembrane transporter activity	Low-fertility	30	4	
CG13194	Pyramus	fibroblast growth factor receptor binding	Sterile	22	2	
CG1873	Elongation factor 1alpha100E	GTP binding	Sterile	6	1	
CG3320	Rab1	GTP binding	Sterile	26	2	
CG9476	alpha-Tubulin at 85E	GTP binding	Sterile	12	2	X
CG5771	Rab11	GTPase activity	Sterile	30	3	X
CG8269	Dynamitin	mitotic spindle organization	Sterile	9	2	
CG9834	Endophilin B	phospholipid binding	Low-fertility	18	3	
CG3725	Calcium ATPase at 60A	protein binding	Sterile	13	1	
CG5081	Syntaxin 7	protein binding	Sterile	13	2	
CG8201	par-1	protein kinase activity	Sterile	13	1	
CG7057	AP-50	protein transporter activity	Sterile	19	2	
CG1242	Heat shock protein 83	unfolded protein binding	Sterile	10	1	X
CG8843	sec5	unknown	Sterile	19	2	

Extrusion						
CG	Gene	Summary of GO terms	Sterile or Low-fertility?	Fertility Assays	# RNAi lines screened	Known role in male gamete generation
CG10260	Phosphatidylinositol 4-kinase III alpha	1-phosphatidylinositol 4-kinase activity	Low-fertility	24	3	
CG9901	Actin-related protein 2	actin binding	Sterile	22	2	
CG8978	Actin-related protein 2/3 complex, subunit 1	actin binding	Sterile	19	2	
CG1634	Neuroglian	calcium ion binding	Low-fertility	42	5	
CG9579	Annexin X	calcium-dependent phospholipid binding	Sterile	27	3	
CG12737	Calmodulin-binding protein related to a Rab3 G	calmodulin binding	Sterile	13	1	
CG14028	cyclope	cytochrome-c oxidase activity	Sterile	42	1	
CG14724	Cytochrome c oxidase subunit Va	cytochrome-c oxidase activity	Sterile	10	1	
CG9206	Glued	dynein binding	Sterile	10	1	X
CG18000	short wing	dynein light chain binding	Sterile	9	1	X
CG1913	α-Tubulin at 84B	GTP binding	Sterile	15	3	
CG3401	beta-Tubulin at 60D	GTP binding	Sterile	18	3	
CG4869	beta-Tubulin at 97EF	GTP binding	Low-fertility	9	2	
CG5915	Rab7	GTP binding	Sterile	34	2	
CG7507	Dynein heavy chain 64C	microtubule motor activity	Sterile	21	3	
CG1938	Dynein light intermediate chain	motor activity	Sterile	9	2	
CG42783	atypical protein kinase C	myosin binding	Low-fertility	15	2	
CG15792	zipper	myosin light chain binding	Sterile	19	2	
CG11027	ADP ribosylation factor 102F	NAD(P)+-protein-arginine ADP-ribosyltransfera	Sterile	10	1	
CG3944	NADH:ubiquinone reductase 23kD subunit prec	NADH dehydrogenase activity	Sterile	19	2	
CG5548	NADH dehydrogenase (ubiquinone) B18 subuni	NADH dehydrogenase activity	Sterile	18	1	
CG6343	NADH dehydrogenase (ubiquinone) 42 kDa sub	nucleoside kinase activity	Sterile	17	2	
CG5671	Pten	phosphoprotein phosphatase activity	Low-fertility	21	4	
CG5748	Heat shock factor	protein binding	Low-fertility	19	2	
CG9401	mago nashi	protein binding	Sterile	3	1	
CG2331	TER94	protein binding	Sterile	13	2	
CG5102	daughterless	protein heterodimerization activity	Sterile	6	2	X
CG42281	bunched	protein homodimerization activity	Sterile	10	1	
CG2028	Casein kinase Ialpha	protein kinase activity	Sterile	16	2	
CG11427	ruby	protein transporter activity	Low-fertility	12	2	
CG8432	Rab escort protein	Rab GTPase binding	Sterile	11	2	
CG7398	Transportin	Ran GTPase binding	Sterile	19	2	
CG2595	Rac GTPase activating protein at 84C	Rho GTPase binding	Sterile	6	1	
CG4916	maternal expression at 31B	RNA binding	Sterile	19	2	
CG11173	ubisnap	SNARE binding	Sterile	19	3	
CG3576	schlank	sphingosine N-acyltransferase activity	Sterile	9	1	
CG4027	Actin 5C	structural constituent of cytoskeleton	Sterile	20	3	
CG4247	mitochondrial ribosomal protein S10	structural constituent of ribosome	Sterile	13	1	
CG14750	Vacuolar protein sorting 25	structural molecule activity	Sterile	16	2	
CG4005	yorkie	transcription factor binding	Sterile	12	2	
CG5000	mini spindles	tubulin binding	Sterile	13	3	
CG5505	scrawny	ubiquitin-specific protease activity	Low-fertility	9	2	
CG10846	dynactin-subunit-p25	unknown	Sterile	25	3	X
CG2021	-	unknown	Sterile	11	1	

No overt phenotype						
CG	Gene	Summary of GO terms	Sterile or Low-fertility	Fertility Assays	# RNAi lines screened	Known role in male gamete generation
CG6236	slingshot	actin binding	Sterile	12	2	
CG8224	baboon	activin binding	Low-fertility	11	2	
CG6871	Catalase	antioxidant activity	Low-fertility	9	2	
CG1489	Pros45	ATP binding	Low-fertility	9	2	
CG34120	-	ATP binding	Low-fertility	21	2	
CG8472	Calmodulin	calcium ion binding	Low-fertility	6	2	
CG18660	Nckx30C	calcium, potassium:sodium antiporter activity	Low-fertility	25	4	
CG1709	Vacuolar H[+]ATPase subunit 100-1	calmodulin binding	Low-fertility	9	1	
CG4154	Guanylyl cyclase at 88E	carbon monoxide binding	Low-fertility	6	1	X
CG1429	Myocyte enhancer factor 2	DNA binding	Low-fertility	17	2	
CG5069	croodile	DNA binding	Low-fertility	13	4	
CG8171	double parked	DNA binding	Low-fertility	6	1	
CG5911	ETHR	ecdysis-triggering hormone receptor activity	Low-fertility	19	2	
CG2286	NADH:ubiquinone reductase 75kD subunit prec	electron carrier activity	Sterile	15	2	
CG13551	-	enzyme inhibitor activity	Low-fertility	9	1	
CG7446	Glycine receptor	GABA-A receptor activity	Low-fertility	13	1	
CG9359	beta-Tubulin at 85D	GTP binding	Sterile	29	2	X
CG1403	Septin 1	GTPase activity	Low-fertility	18	3	
CG34418	still life	guanyl-nucleotide exchange factor activity	Low-fertility	10	1	
CG44159	inwardly rectifying potassium channel 1	inward rectifier potassium channel activity	Low-fertility	10	1	
CG7717	Mekk1	MAP kinase kinase kinase activity	Low-fertility	19	2	
CG10959	-	metal ion binding	Low-fertility	16	1	
CG12299	-	metal ion binding	Sterile	16	1	
CG13906	nervous fingers 1	metal ion binding	Low-fertility	6	1	
CG5433	Kinesin light chain	microtubule motor activity	Low-fertility	6	2	
CG14718	-	mRNA binding	Low-fertility	10	2	
CG3201	Myosin light chain cytoplasmic	myosin binding	Low-fertility	9	2	
CG7399	Henna	phenylalanine 4-monooxygenase activity	Low-fertility	6	1	
CG6757	SH3PX1	phosphatidylinositol-3,4,5-trisphosphate bindin	Low-fertility	12	1	
CG5076	eag-like K[+] channel	phosphorelay sensor kinase activity	Low-fertility	9	2	
CG4643	F-box synaptic protein	protein binding	Low-fertility	9	1	
CG17158	capping protein beta	protein heterodimerization activity	Low-fertility	9	2	
CG12306	polo	protein kinase activity	Low-fertility	12	2	X
CG2621	shaggy	protein kinase activity	Low-fertility	9	2	
CG11624	Ubiquitin-63E	protein tag	Low-fertility	9	1	X
CG8318	Neurofibromin 1	Ras GTPase activator activity	Low-fertility	9	2	
CG4755	RhoGAP92B	Rho GTPase activator activity	Low-fertility	26	1	
CG16941	-	RNA binding	Low-fertility	17	1	
CG31762	arrest	RNA binding	Low-fertility	15	2	X
CG10052	Retinal Homeobox	sequence-specific DNA binding	Low-fertility	9	1	
CG3871	Stx4	sequence-specific DNA binding	Sterile	6	1	
CG9415	X box binding protein-1	sequence-specific DNA binding	Low-fertility	6	1	
CG1056	Serotonin receptor 2	serotonin receptor activity	Low-fertility	16	1	
CG12876	ALG-2 interacting protein X	signal transducer activity	Low-fertility	19	1	
CG6625	Soluble NSF attachment protein	SNARE binding	Low-fertility	9	2	
CG15438	Major Facilitator Superfamily Transporter 18	sodium-dependent phosphate transmembrane	Low-fertility	16	1	
CG9261	nervana 2	sodium:potassium-exchanging ATPase activity	Low-fertility	20	2	
CG5657	Sarcoglycan beta	structural constituent of muscle	Low-fertility	6	2	
CG7919	farinelli	structural molecule activity	Low-fertility	9	1	X
CG9543	epsilonCOP	structural molecule activity	Low-fertility	10	1	
CG5366	Cullin-associated and neddylation-dissociated	transcription factor binding	Low-fertility	30	3	
CG10289	-	unknown	Low-fertility	6	2	
CG3443	pecanex	unknown	Low-fertility	9	1	
CG14563	-	unknown	Low-fertility	16	1	
CG10174	Nuclear transport factor-2-related	unknown	Sterile	19	2	
CG43368	cacophony	voltage-gated calcium channel activity	Low-fertility	19	1	
CG9139	Rabex-5	zinc ion binding	Low-fertility	28	2	

Table 1 Candidate classification summary. Candidate genes classified in the following classes: No soma & No germline, No soma, Arrest after spermatogonia, Arrest after spermatocyte class, Extrusion, and genes that displayed No overt phenotype. The tables list the genes classified, a summary of gene ontology (GO) terms (Sourced from www.flybase.org), whether the genes were sterile or low-fertility, the total number of fertility assays that were performed, the number of RNAi lines that were screened, in addition to whether the gene has a known role in male gamete generation. An ‘X’ indicates the gene has a known role in male gamete generation.

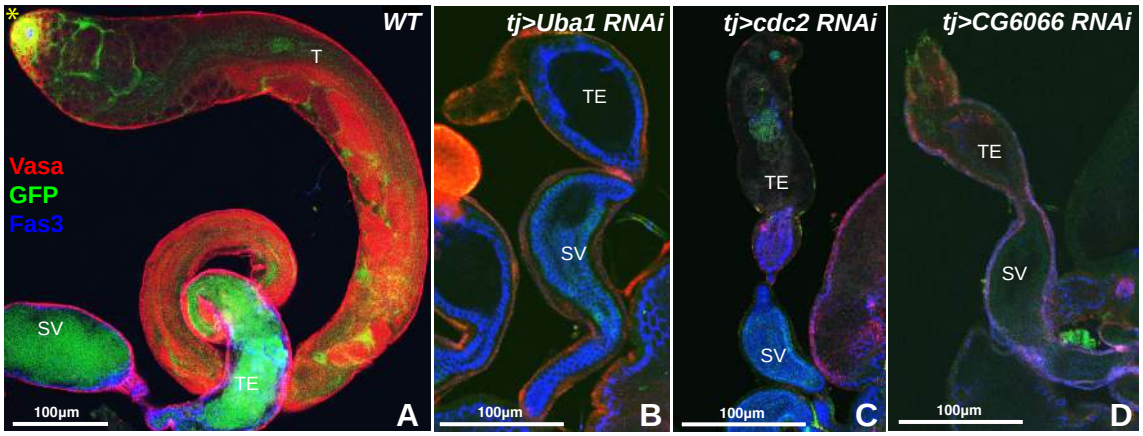
3.3.1 Class 1: No soma & No germline

The ‘No soma & No germline’ class represented candidates that lacked somatic and germ cells. Only genital structures, the terminal epithelium and seminal vesicle remained. The knockdown of *ubiquitin activating enzyme 1 (Uba1)*, *cdc2* and *CG6066* are representative examples of genes in this class that resulted in no soma & no germline (Figure 3.3.1). Approximately 15.2% (30/197) of the genes classified were identified in this class.

Gene ontology enrichment analysis produced 9 clusters of GO terms associated with the sterile genes in this class. Some of the GO terms identified include ATP binding and mitotic spindle organization. The genes associated with ATP binding were: *gluon, SMC2, Smt3 activating enzyme 2, ubiquitin activating enzyme1, cdc2, actin88F*, and *asparaginyl-tRNA synthetase*. The genes associated with mitotic spindle organization were: *small ribonucleoprotein particle U1 subunit 70K, gluon, SMC2*, and *eukaryotic initiation factor 1A*.

Figures 3.3.1-3.3.5 Illustration of phenotypic classes

Figure 3.3.1 Class 1: No soma & no germline



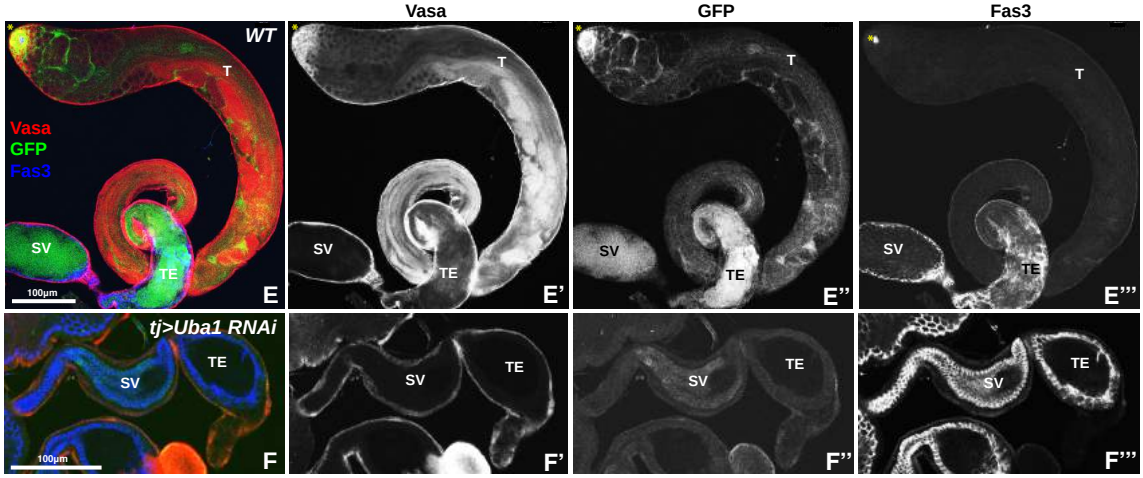


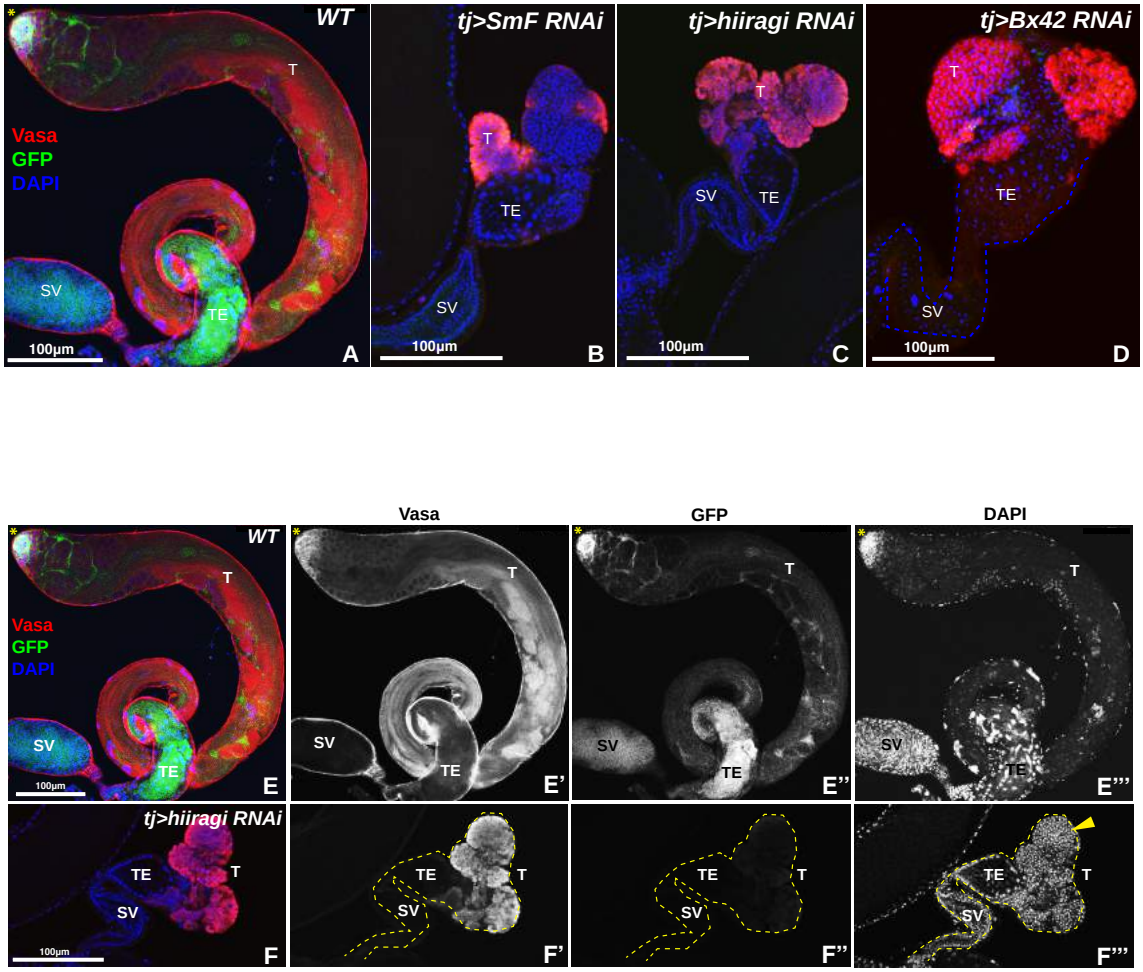
Figure 3.3.1 Class 1: No soma & no germline. (A) Wild-type testis (T) showing the terminal epithelium (TE), which is connected to the seminal vesicle (SV). The hub is denoted by an asterisk. Vasa labels the germ cells. GFP labels the somatic cells. Fas3 labels the hub, TE, and SV. (B) *tj>Uba1 RNAi*, (C) *tj>cdc2 RNAi*, and (D) *tj>CG6066 RNAi* testes are undeveloped and lack T. (E & F) shows every channel for testes shown in (A-D), but the channels are split individually. (E) Wild-type testis contains germ cells (E'), somatic cells (E''), and hub (denoted by an asterisk), TE, and SV (E'''). (F) *tj>Uba1 RNAi* testis lacks germ cells (F') and somatic cells (F''), but retain the TE and SV (F''').

3.3.2 Class 2: No soma

The ‘No soma’ class is characterized by testes that lacked somatic cells, but possessed germ cells and genital structures. The knockdown of *small ribonucleoprotein particle protein SmF*, *hiiragi* and *Bx42* are representative examples of genes in this class that resulted in no soma (Figure 3.3.2). Approximately 13.7% (27/197) of the genes classified were identified in this class. In some candidates, one testis of the pair developed no germline and no soma, while the other testis possessed germ cells, but lacked somatic cells. All testes in this class contained DAPI dense germ cell nuclei which appeared to be tumorous.

Gene ontology enrichment analysis produced 13 clusters of GO terms associated with the sterile genes in this class. Some of the GO terms identified include mRNA processing and RNA splicing. The genes associated with mRNA processing include: *CG11985, Bx42, hiiragi, cap binding protein 80,* and *small ribonucleoprotein particle protein SmD3*. The genes associated with RNA splicing include: *CG11985, Bx42, cap binding protein 80,* and *small ribonucleoprotein particle protein SmD3.*

Figure 3.3.2 Class 2: No soma



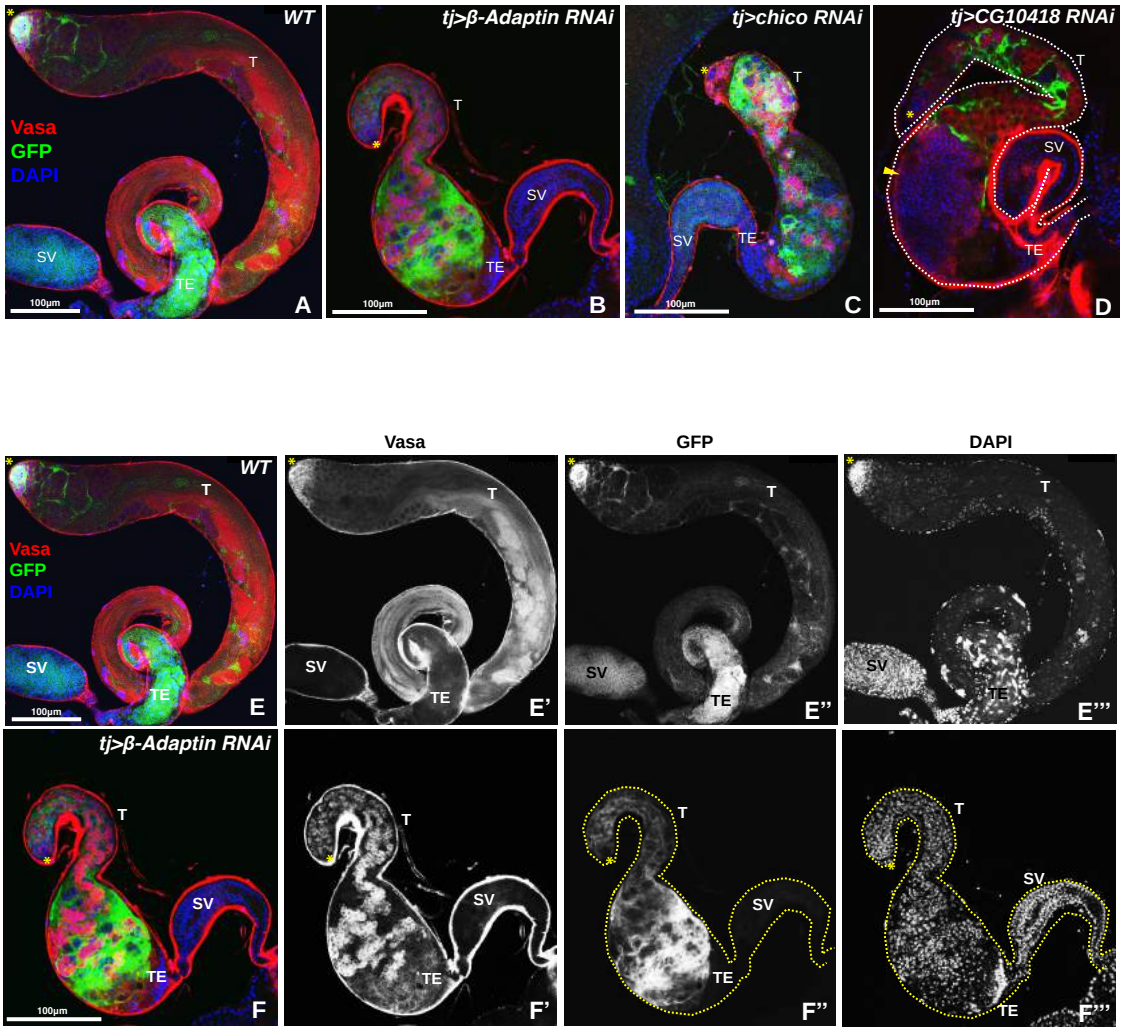
3.3.2 Class 2: No soma. (A) Wild-type testis (T) showing the terminal epithelium (TE), which is connected to the seminal vesicle (SV). The hub is denoted by an asterisk. Vasa labels the germ cells. GFP labels the somatic cells. DAPI labels the nuclei. (B) *tj>smf RNAi*, (C) *tj>hiiragi RNAi*, and (D) *tj>Bx42 RNAi* testes exhibit no soma, but possess germ cells. (E & F) shows every channel for testes shown in (A-D), but the channels are split individually. (E) Wild-type testis contains germ cells (E'), somatic cells (E''), and nuclei (E'''). (F) *tj>hiiragi RNAi* testis possess germ cells (F'), but lack somatic cells (F''). Germ cell proliferation appears to be misregulated, shown by the arrowhead (F''').

3.3.3 Class 3: Arrest after spermatogonia

The arrest after spermatogonia class represented testes that retained only mitotic spermatogonia, and did not contain post-mitotic spermatocytes. The knockdown of *β-Adaptin*, *chico* and *CG10418* are representative examples of genes in this class that resulted in arrest after spermatogonia (Figure 3.3.3). The spermatogonia are generally visible as small, round cells in the apical region of the testis (Hudson et al., 2013). Approximately 11.1% (22/197) of the genes classified were identified in this class. Wild-type testes contained decondensed nuclei while knockdown testes did not, indicating that the germline had not progressed to the meiotic divisions of the spermatocyte stage. Many testes also contained germ cells that were not encapsulated by somatic cells. Some germ cells appeared to be misregulated, and tumorous.

Gene ontology enrichment analysis produced 7 clusters of GO terms associated with the sterile genes in this class. Some of the GO terms identified include synaptic vesicle transport and intracellular protein transport. The genes associated with synaptic vesicle transport include: *neurexin-IV, β-adaptin* ,and *clathrin heavy chain, syntaxin 1A.* The genes associated with intracellular protein transport include: *β-adaptin, clathrin heavy chain, syntaxin 1A,* and *translocase of outer membrane 40.*

Figure 3.3.3 Class 3: Arrest after spermatogonia



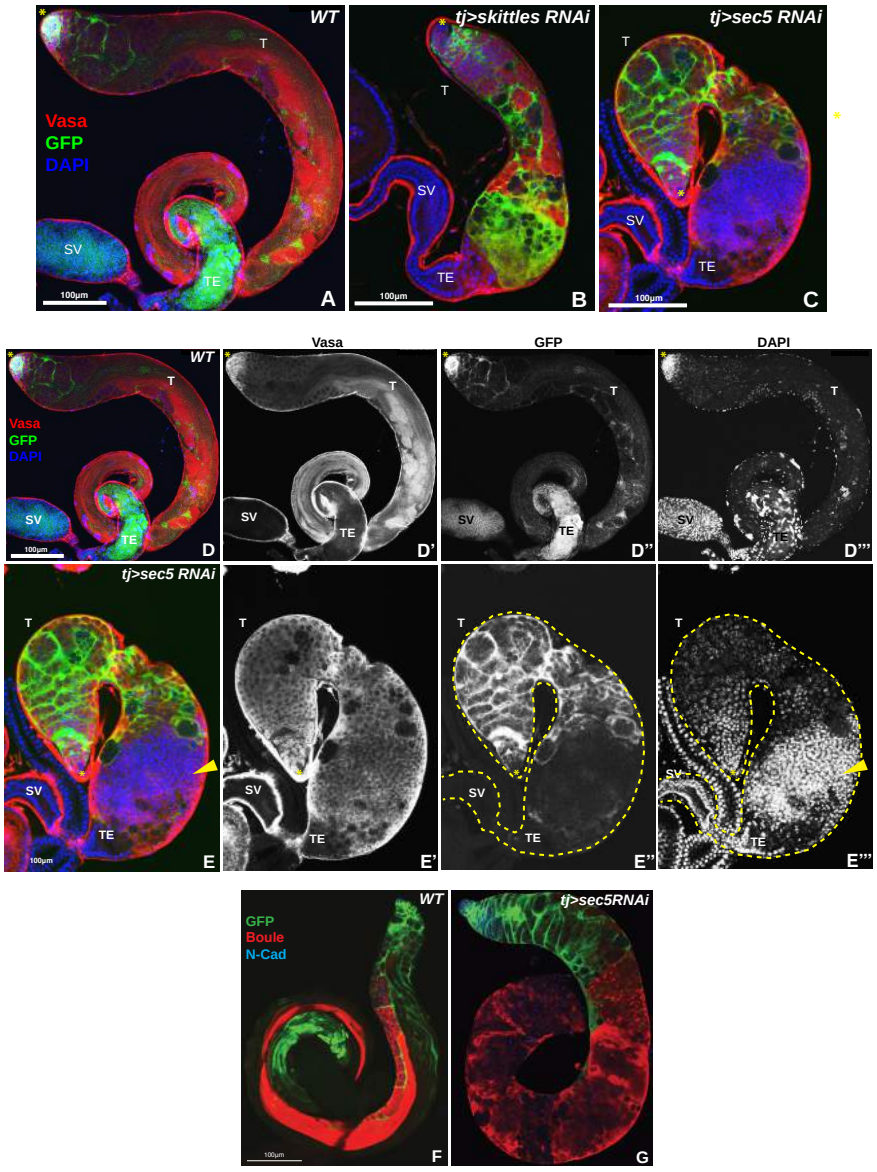
3.3.3 Class 3: Arrest after spermatogonia. (A) Wild-type testis (T) showing the terminal epithelium (TE), which is connected to the seminal vesicle (SV). The hub is denoted by an asterisk.Vasa labels the germ cells. GFP labels the somatic cells. DAPI labels the nuclei. (B) *tj>β-adaptin RNAi*, (C) *tj>chico RNAi*, and (D) *tj>CG10418 RNAi* testes do not progress past spermatogonial stages. In some cases, large-scale spermatogonial tumors are present, shown by the arrowhead (D). (E & F) shows every channel for testes shown in (A-D), but the channels are split individually. (E) Wild-type testis contains germ cells (E'), somatic cells (E''), and nuclei (E'''). (F) *tj>β-adaptin RNAi* testis possess only mitotic spermatogonia (F'), and spermatogonia appear to be encapsulated by somatic cells (F'').

3.3.4 Class 4: Arrest after spermatocyte

In the arrest after spermatocyte class, the testes contained spermatocytes, but the majority of testes lacked spermatids or elongated spermatids. Spermatocytes are found mid-way down the testes and are significantly larger than spermatogonia (Hudson et al., 2013). The knockdown of *skittles* and *sec5* are representative examples of genes in this class that resulted in arrest after spermatocyte. To confirm that some of these candidates contained spermatocytes, I used Boule. Boule is a molecular marker of meiosis (Figure 3.3.4). In addition, the genes in this class were identified when the testes lacked arrowhead nuclei at the base of the testis, which is characteristic of the spermatid stage. Approximately 8.6% (17/197) genes were identified in this class. Some testes contained misregulated germ cells

Genome ontology enrichment analysis produced 8 clusters of GO terms associated with the sterile genes in this class. Some of the GO terms identified include purine ribonucleotide binding, GTPase activity, and microtubule cytoskeleton. The genes associated with purine ribonucleotide binding include: *rab11, elongation factor 1alpha100E, bellwether, α-tubulin at 85E, actin-related protein 87C, rab1, par-1,* and *heat shock protein 83*. The genes associated with GTPase activity include: *rab11, elongation factor 1alpha100E, α-tubulin at 85E, rab1*. The genes associated with microtubule cytoskeleton include: *rab11, α-tubulin at 85E, actin-related protein 87C, dynamitin,* and *heat shock protein 83*.

Figure 3.3.4 Class 4: Arrest after spermatocyte



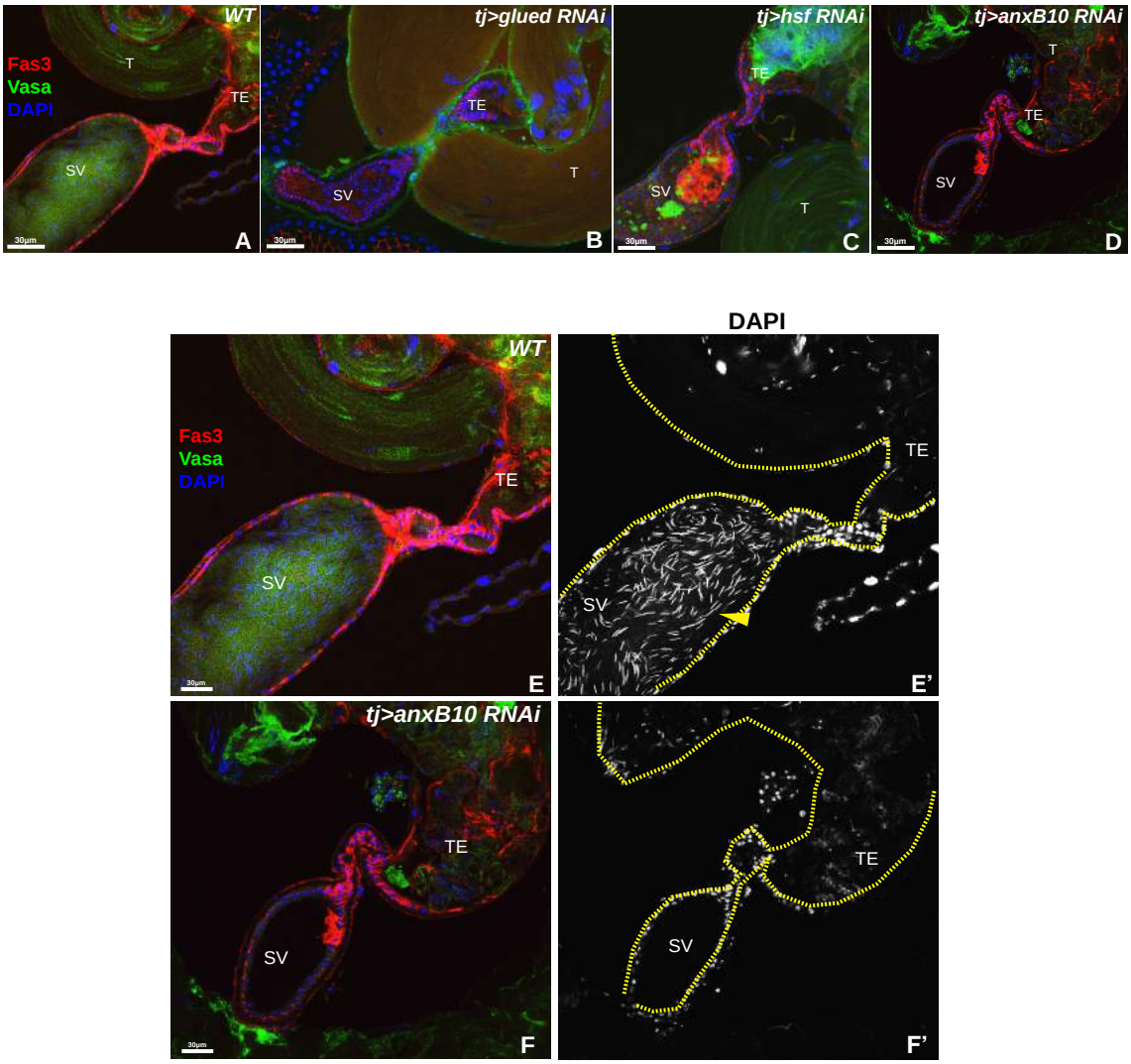
3.3.4 Class 4: Arrest after spermatocyte. (A) Wild-type testis (T) showing the terminal epithelium (TE), which is connected to the seminal vesicle (SV). The hub is denoted by an asterisk Vasa labels the germ cells. GFP labels the somatic cells. DAPI labels the nuclei. (B) *tj>skittles RNAi*, and (C) *tj>sec5 RNAi* testes do not progress past spermatocyte stages. (D & E) shows every channel for testes shown in (A-C), but the channels are split individually. (D) Wild-type testis contains germ cells (D'), somatic cells (D''), and nuclei (D'''). (E) *tj>sec5 RNAi* testis possess only spermatocytes and lack spermatids (E'). The soma encapsulates the germline (E''), with the exception of mitotic spermatogonia, which appears to be misregulated, shown by the arrowhead (E & E'''). (F) Wild-type testis and (G) *tj>sec5 RNAi* testis contain spermatocytes. Boule labels meiotic spermatocytes, GFP labels somatic cells, and N-Cadherin labels the hub.

3.3.5 Class 5: Extrusion

In the extrusion class, the testes contained elongated spermatids and mature sperm, but were not extruded in the seminal vesicle. The knockdown of *glued, heat shock factor* and *annexinB10* are representative examples of genes in this class that resulted in extrusion defects (Figure 3.3.5). Approximately 22.3% (44/197) of the genes classified were identified in this class. The sperm in a wild-type seminal vesicle can be visualized by DAPI stained long, needle like nuclei, but knockdown testes completely lacked, or contained very few needle-like sperm nuclei in their seminal vesicle. Some candidates appeared to posses germ cell tumors.

Gene ontology enrichment analysis produced 17 clusters of GO terms associated with the sterile genes in this class. Some of the GO terms identified include mitochondrial ATP synthesis coupled electron transport and gamete generation. The genes associated with mitochondrial ATP synthesis coupled electron transport include: *CG5548, NADH: ubiquinone reductase 42kD, cyclope, NADH: ubiquinone reductase 23kD,* and *cytochrome c oxidase subunit Va*.

Figure 3.3.5 Class 5: Extrusion



3.3.5 Class 5: Extrusion. (A) A close up image of the terminal epithelium (TE) and seminal vesicle (SV) of a wild-type testis (T). Vasa labels the germ cells. DAPI labels the nuclei. Fas3 labels the hub, TE, and SV (B) *tj>glued RNAi*, (C) *tj>hsf RNAi*, and (D) *tj>anxB10 RNAi* testes lack mature sperm in the SV. (E' & F') shows a split channel of DAPI. (E) Wild-type testis shows the SV contains needle-like sperm nuclei, shown by arrowhead (E'), but *tj>anxB10 RNAi* testis fail to extrude sperm in the SV (F').

3.3.6 Class 6: No overt phenotype

Approximately 28.9% (57/197) of the genes classified displayed no overt phenotype. All the testes exhibited wild-type morphology. Although the majority of candidates in this category were low-fertility, a few sterile candidates also fell into this class.

Chapter 4: Discussion and conclusion

We have been performing a somatic RNAi screen to identify genes that are essential in the soma for germline development during *Drosophila* spermatogenesis. To date, this screen has identified over 200 candidate genes with potential roles in soma-germline interactions during *Drosophila* spermatogenesis. For this thesis, I performed a detailed characterization of the phenotypes of these candidate genes and classified them into phenotypic classes.

Many of the underlying regulatory processes governing each step of germline development are not well characterized. The objective of my thesis has been to identify genes in the soma that are required for male gamete generation. By doing this, not only have I uncovered novel and important regulators of soma-germline interactions, but I also provided insight into the general cellular mechanisms in the soma that influence germline development at key stages of spermatogenesis. Throughout this discussion, I will highlight genes that represent phenotypes of particular interest. Furthermore, I will discuss the reliability of the somatic RNAi screen and the classification scheme. Lastly, I will summarize the major conclusions, and comment on possible future directions that take advantage of the findings of this study.

4.1 Analysis of genes in the 'No soma & No germline' class

The majority of genes identified in the 'No soma & No germline' class exhibited testes that lacked the gonad. Such a phenotype could result from a knockdown in genes that are essential for somatic cell viability. This could have led to an inability to guide

germline development, preventing the formation or maintenance of gonadal tissues during development.

One of the particularly interesting candidates identified in this class is *cdc2*. This gene is of particular interest because *cdc2* had previously been shown to be essential in the germline for meiotic divisions during *Drosophila* spermatogenesis (Sigrist et al., 1995), but its requirement in the soma had not been reported. Cdc2 is cylin dependent kinase 1 that controls the G2/M transition in mitosis and meiosis. Twine/cdc25 phosphatase activates Cdc2 kinase during meiosis (Fuller, 1998; Sigrist et al., 1995). The phosphorylation and de-phosphorylation of the Cdc2 kinase subunits of the G2-Cyclin/cdc2 kinase complex modulates its activity. It is hypothesized that *cdc2* is required for mitosis in the germline and the stem cell and gonialblast cell divisions (Sigrist et al., 1995). Furthermore, males carrying a temperature sensitive allele of *cdc2* grown at restrictive temperatures contain aberrant spermatocytes that are unable to assemble spindles, segregate chromosomes, or undergo cytokinesis (Fuller, 1998).

Another interesting candidate in this class is *twinstar*, which encodes a cofilin/actin depolymerizing factor (ADF) homologue. In *Drosophila*, *twinstar* loss-of-function mutations exhibit defects in centrosome migration during the meiotic divisions of spermatocytes. In addition, animals die between the larval and pupal stages due to a failure in completing cytokinesis during mitosis and meiosis (Gunsalus et al., 1995).

cdc2 and *twinstar* may be required in the soma for cell viability. Knockdown of these genes could have caused defects in cell division arising from failures in spindle organization, centrosome migration, or inability to undergo cytokinesis, and thus prevented somatic cell proliferation. The absence of somatic cells could disrupt the

formation of the embryonic gonad, as pole cells may require cues from the soma to guide their migration to the correct location in the embryo and are required to establish primordial germ cells as germline stem cells (Santos and Lehmann, 2004; Starz-Gaiano and Lehmann, 2001). Thus, I postulate that the lack of the germline is likely a secondary defect resulting from the early death of the soma.

4.2 Analysis of genes in the 'No soma' class

In the 'No soma' class, the unifying phenotype was the absence of somatic cells and the presence of tumorous germ cells. The hub could not be detected in the majority of testes, suggesting defects in hub cell maintenance in adult testes. The presence of germ cells, however, indicates a less severe effect on the germline, in comparison to the 'No soma & No germline' class. It has been previously reported that germline stem cells (GSCs) can be maintained without cyst stem cells (CySCs) (Lim & Fuller, 2012). These results suggest that the candidate genes in this class might be required for the maintenance of somatic cells.

In this class, two Notch signaling effectors were identified: *Bx42* and *hiiragi*. In *Drosophila,* Notch and Egfr signaling act antagonistically to regulate hub differentiation in the male embryonic gonad. The ligand Serrate is expressed in somatic gonadal precursors (SGPs) and activates Notch signaling, whereas the Spitz ligand in primordial germ cells (PGCs) activates Egfr signaling. PGCs signal to the SGPs in a manner that informs SGPs how many PGCs there are. This consequently modulates niche size. For example, a reduction in the number of PGCs leads to ectopic hub formation,

consequently enhancing the recruitment of PGCs as GSCs (Kitadate and Kobayashi, 2010).

Notch is a transmembrane receptor. Upon ligand engagement, an intracellular domain known as NotchIC is cleaved off and translocates to the nucleus to regulate gene expression where it can interact with CBF1. CBF1 is a DNA binding protein that mediates transcriptional repression via recruitment of SMRT –histone deacetylase-containing corepressor complex (SMRT-HDAC). This is converted to an activator complex by interaction of SKIP (Ski-interacting protein) with CBF1; SKIP acts as a tether protein to orchestrate the conversion of CBF1 from an SMRT-HDAC bound transcriptional repressor to a NotchIC-bound activation complex. Contact with SKIP is essential for the biological activity of NotchIC (Zhou et al., 2000).

The precise role of *Bx42* in the Notch pathway remains to be determined in *Drosophila. Bx42* is found associated with chromatin in the salivary glands, and has been hypothesized to play a role in the regulation of chromatin remodeling (Dahl et al., 1998). More recently, *Bx42* and its human homologue, *skip*, were discovered to interact with components of the Notch signal transduction pathway to regulate gene activation vs. repression (Negeri et al., 2002). The RNAi-mediated knockdown of *Bx42* in various tissues at different developmental times has shown that it is required for the formation of numerous tissues in the embryonic, larval, as well as adult stages of development. Some of the defects observed were similar to the phenotypes obtained upon altering the protein levels of Notch signaling pathway components (Negeri et al., 2002).

hiiragi encodes a poly(A) polymerase (PAP) in *Drosophila*. It functions in the polyadenylation of mRNA precursors in the nucleus, which can influence many aspects

of mRNA metabolism, such as stability, translation efficiency, transport, and steps in mRNA synthesis. Mutants of *hiiragi* resulted in a notched wing phenotype, which is characteristic of Notch pathway mutants. Therefore, *hiiragi* plays an important role in the development of the wing in *Drosophila* (Murata et al., 2001), and as may be expected by the wing phenotype, *hiiragi* has been previously shown to be involved in the Notch signaling cascade (Murata et al., 1996).

The involvement of *Bx42* and *hiiragi* in the Notch signal transduction pathway might be required to establish and maintain CySCs during gonadogenesis and spermatogenesis. In the 'No soma' phenotypic category, the fact that the gonad formed suggests that the soma was likely present to guide the migration of pole cells in the embryo, but I conclude that a failure to maintain the somatic cells eventually led to early death of the soma.

4.3 Analysis of genes in the 'Arrest after spermatogonia' class

In the 'Arrest after spermatogonia' class, the testes were more fully developed than in the above classes, and contained soma and germ cells. The germ cells were able to undergo amplifying rounds of mitotic divisions, but failed to exit spermatogonial stages. A common defect in this phenotypic class was the inability of the soma to properly envelop the germline, which likely resulted in germ cell tumors observed in some of the testes (Fairchild et al., 2015). This suggests that the signal required for the transition of germ cells from the mitotic to meiotic program is disrupted.

In this class, we obtained phenotypes by knockding down the genes *scribbled* and *discs large-1*. Scribbled (Scrib) forms a polarity complex with Discs large (Dlg) and

Lethal giant larvae (Lgl), which are associated with the cytoskeletal matrix or septate junctions (Papagiannouli and Mechler, 2010). In mammals, Scrib is expressed in Sertoli cells and functions in the Scrib/Dlg/Lgl complex to regulate blood-testis-barrier (BTB) dynamics (Su et al., 2012). Scrib, Dlg, and Lgl behave as tumor suppressors. Mutations in any of these proteins result in tumorous overgrowth and loss of polarity in tissues (Dow et al., 2007; Qin et al., 2005).

The internal structures of the *Drosophila* embryonic gonad requires Scrib/Dlg/Lgl activity in the soma. Dlg in the soma is essential for the viability, growth, and expansion of spermatocytes and spermatogonia, as well as maintaining the cyst's microenvironment (Papagiannouli, 2013). It has been hypothesized that Dlg functions with the cytoskeleton of the somatic cells to regulate cell-shape changes that form cytoplasmic extensions over spermatogonial and spermatocyte cysts (Papagiannouli and Mechler, 2010). Scrib has been previously reported to act in the soma to regulate testis architecture, differentiation and development (Papagiannouli, 2013). Consistent with previous reports, the knockdown of Dlg and Scrib in the soma resulted in undeveloped testes with defects in transit amplifying spermatogonia. Many testes contained germ cells that were not encapsulated by the soma, preventing their progression past spermatogonial stages.

Another candidate in this class is *clathrin heavy chain* (*chc*), which constitutes the main structural polypeptide of the cytoplasmic surface of clathrin-coated pits and vesicles. Clathrin is essential for protein trafficking, specifically internalization, sorting, processing, and secretion of molecules necessary for development, as well as the differentiation of transport machinery in specific cell types. In *Drosophila*, hemizygous chc^4 mutants results in infertility in males. This could be due to a disruption in the

individualization of spermatids, as the individualization complex appears to be reduced or defective (Bazinet et al., 1993; Fabrizio et al., 1998). Additionally, clonal analysis in the female germline showed that *chc* is required for germ cell viability and development in the ovaries (Bazinet et al., 1993). The requirement for *chc* in the soma has not been previously studied.

Related to my findings regarding *chc*, it is interesting that *β-Adaptin/AP-2 β* is a candidate in this phenotypic class. *β-Adaptin/AP-2 β* belongs to the adaptin protein (AP2) coat assembly protein complex, which plays key roles in clathrin-mediated endocytosis. AP2 links endocytic cargo to the clathrin coat, where internalization of cargo occurs through forming clathrin-coated vesicles. Clathrin-mediated endocytosis is crucial for many cellular processes such as the internalization of activated growth factor receptors, regulation of small molecule receptors, channels and transporters in the plasma membrane and recycling of synaptic vesicles (Zhang et al., 2005). Furthermore, *β-Adaptin/AP-2 β* is implicated in the regulation of germ cell development during spermatogenesis. The role of *β-Adaptin/AP-2 β* in the soma is currently not known.

My observations suggest a novel role for *chc* and *β-Adaptin/AP-2 β* in the soma during spermatogenesis. The knockdown of *chc* and *β-Adaptin/AP-2 β* in the soma could lead to defects in cellular trafficking, which results in an inability of the soma to transport signals required for the spermatogonial to spermatocyte stage transition or disrupted the ability of the cyst cells to coordinate the complex cell shape changes needed to accommodate the differentiating germline cyst. I postulate that this likely causes the dysregulation of germ cell proliferation and the large-scale spermatogonial tumors that I observed.

4.4 Analysis of genes in the 'Arrest after spermatocyte' class

In the ‘Arrest after spermatocyte’ class, the germline was unable to progress from the spermatocyte to spermatid stages. This failure prevented the formation of elongated spermatids. In most cases, I found that the germline was encapsulated by the soma, except in some testes, which appeared to exhibit germ cell tumors.

An interesting gene in this category, *Hsp83*, encodes Hsp90, a heat shock protein involved in protein trafficking, DNA replication, protein synthesis, and regulation of the activities of a large number of signal transducers. Studies *in vitro* have shown that Hsp90 is a molecular chaperone, which prevents the aggregation of unstable or denatured proteins. *In vivo, Hsp83* is only essential when proteins are destabilized by heat, or are unable to acquire stable conformations at normal temperatures. However, the role of *Hsp83* in animals is not well understood (Yue et al., 1999).

Although Hsp90 is involved in several morphogenetic processes, spermatogenesis appears to be the most sensitive process affected by Hsp90 function in *Drosophila***.** Homozygous point mutants of *Hsp83* are lethal, while heteroallelic zygotes give rise to viable adults, but are sterile or have low fertility. Defects in spermatogenesis are typically first observed during the spermatocyte stage, and result in either the absence of sperm or presence of disorganized, non-motile and very fragile sperm in the seminal vesicle (Yue et al., 1999). Thus, I postulate that Hsp90 may have a role in the regulation of microtubule-based processes. This suggests that Hsp90 is required for the structural stability of the axoneme. Further, I postulate that spermatogenesis is the first process affected when Hsp90 function is compromised probably because of the unusual length of *Drosophila* sperm, which contains more microtubules compared to other organisms (Yue

et al., 1999). Although a role for Hsp90 in the germline previously been reported, its role in the soma is less clear.

Another interesting candidate in this class is *sec5*, which is a central component of the exocyst complex. The exocyst complex is involved in many forms of exocytosis, and guides vesicles through the secretory pathway (Murthy et al., 2010). The exocyst complex seems to be important for polarized secretory events. In the *Drosophila* female germline, the plasma membrane localization of Sec5 is predominantly in clathrin-coated pits and vesicles. In germline clones, a null allele of *sec5* causes early arrest of germline cyst development. It is possible that Sec5 is recruited to the coated pits to mediate and facilitate membrane traffic events after endocytic internalization (Sommer et al., 2005). In the *Drosophila* embryo, Sec5 is required for cellularization, the process by which a syncytium of nuclei is subdivided into distinct cells. Here, Sec 5 might be important for directing the polarized addition of new membranes during cytokinesis (Murthy et al., 2010).

My observations have determined potential novel roles for Hsp90 and Sec5 in the soma to direct the transition from the spermatocyte to spermatid stages. The inability of Hsp90 knockdown testes to progress from the spermatocyte to spermatid stages could be the result of dysregulated microtubule dynamics in the soma, which disrupted somatic differentiation. Alternatively, the knockdown of Sec5 in the soma may affect the secretory pathway or the polarized addition of new membrane necessary to encyst the growing spermatocyte, resulting in a failure to exit the spermatocyte stage. This suggests that the regulation of soma differentiation is necessary to drive the progression of germline development from the spermatocyte to spermatid stages.

4.5 Analysis of genes in the 'Extrusion' class

In the ‘Extrusion’ class, the majority of the testes contained elongated spermatids, but lacked sperm in the seminal vesicle. This could arise from defects in spermatid elongation, individualization, coiling, polarity, or extrusion, any of which could prevent spermatids from entering the seminal vesicle. I did not assay for which of these processes may have been disrupted,

One of the more interesting genes identified in this class is *Annexin B10*. One of the biochemical hallmarks of Annexins is their ability to reversibly bind Ca^{2+} and lipid-binding proteins. This enables them to dock onto negatively charged membrane surfaces and regulate the organization of membrane domains and/or cytoskeletal linkages, exocytic and endocytic transport steps, as well as ion fluxes across membranes. The characteristic ability of Annexins to bind certain membrane phospholipids in a Ca^{2+} dependent manner provides a link between Ca^{2+} signaling and membrane functions. Furthermore, Annexins may function as Ca^{2+} channels because they display ion-channel like properties (Gerke et al., 2005). Based on published observations of Annexins, it is possible that *Annexin B10* is required in the soma for Ca^{2+} signaling. If *Annexin B10* can function as a Ca^{2+} channel, it is possible that Ca^{2+} signaling is required for spermatid extrusion. This could explain the absence of mature sperm in the seminal vesicle in *Annexin B10* knockdown testes.

In this class, an effector of BMP signaling was identified: *TER94*. BMPs are a larg subgroup of the transforming growth factor (TGF)- β family that are involved in cell proliferation, differentiation, cell-fate determination, apoptosis, and morphogenesis.

In *Drosophila*, BMP signals regulate a wide range of developmental processes such as dorsal-ventral patterning of the early embryo, oogenesis, wing vein formation, and synapse function at the neuromuscular junction (Zeng et al., 2014). Furthermore, BMP signals are critical for the maintenance of GSCs (Kawase et al., 2004). Interestingly, another candidate, *moleskin*, which was identified in our screen, but belongs to the more severe, 'No soma & No germline' class is also a potential regulator of BMP signaling. Therefore, *moleskin* may be required in the soma during earlier stages of spermatogenesis.

In a recent study, RNAi-based screening in *Drosophila* S2 cells identified Ter94 as a novel component of the BMP signaling pathway. Some mutant alleles of *Ter94* exhibit incomplete oogenesis in germline clones (Zeng et al., 2014). Ter94, an orthologue of the mammalian valosin-containing protein (VCP), is a member of the family of AAA ATPases. They control the level of ubiquitination of bound substrates. VCP regulates BMP signaling in mammalian cells (Zeng et al., 2014) and may be involved in mediating BMP signaling during rat spermatogenesis (Cayli et al., 2012). The knockdown of Ter94 resulted in testes that contained spermatids, which began to elongate, but failed to complete elongation. This suggests a potential novel role for Ter94 in the soma. Ter94 may regulate BMP signaling in the soma. It is possible that the spermatid elongation is disrupted due to a defect in BMP signaling.

There is little known about the mechanisms underlying spermatid elongation (White-Cooper, 2012). In addition, there are no comprehensive reports analyzing the mechanisms, which control spermatid extrusion. *Ter94* and *Annexin B10* may play novel roles in the soma during spermiogenesis. In both cases, the soma might mediate

important signals required for elongation and extrusion of spermatids. The knockdown of these genes highlights the multiple biological mechanisms by which the soma can regulate the development of spermatids into mature sperm.

4.6 Analysis of genes that did not exhibit visible phenotypes

A subset of the candidates classified gave rise to no visible phenotypic defects in the testes. Although these candidates had reduced fertility, the testes contained mature sperm in the seminal vesicle and exhibited an overall wild-type morphology. There are numerous possibilities that could explain the observation of no overt phenotype including false positive candidates, the presence of non-visible defects in spermatogenesis, secondary defects indirectly related to spermatogenesis, inability to detect subtle phenotypes, or experimental error.

It is possible that some of the candidates classified were false-positives. This could have been due to the age of the males used in the fertility assays. Older males are less fertile than younger males due to a reduced number of stem cells (Hardy et al., 1979). Conversely, in some males, the onset of spermatogenesis may have been delayed. These genes can be re-screened by performing additional fertility assays to determine whether they have reduced fertility.

The wild-type morphology of testes could be due to non-visible defects in spermatogenesis. The presence of mature sperm does not mean the fly is fertile. It is possible that the sperm in the seminal vesicle may be non-motile (Haynes et al., 1997; Wakimoto et al., 2004). To investigate this, the seminal vesicle of these testes can be opened during dissections to free sperm and investigate motility. In addition, there could

have been a defect in the transfer of sperm, its storage in females, or in fertilization (Wakimoto et al., 2004). To explore these possibilities, the sperm storage organs in the female reproductive tract can be opened to determine whether sperm was transferred. The absence of sperm could indicate a problem with the transfer or survival of sperm. The presence of sperm might indicate defects in sperm storage in females or post-copulation fertilization.

Secondary defects, indirectly related to spermatogenesis could have led to the observation of no visible phenotype in the testes of these candidates. Since *traffic jam* is also expressed in the nervous system, it is possible that Tj-GAL4 can drive the RNAi transgene in these tissues, which might have altered the mating behavior of these flies, preventing them from breeding. To further assess this, mating behavior can be assayed. If the flies are unable to breed, it could explain their reduced fertility and the wild-type morphology of their testes. It is also possible that the sperm of these flies entered the egg, but were unable to support embryogenesis (Wakimoto et al., 2004), resulting in an absence of progeny, and consequently sterile or low-fertility flies.

There could have been defects in spermatogenesis that the antibodies did not detect. For example, there could have been defects in cyst cell differentiation, which resulted in candidates that exhibited arrest after spermatocyte, or defects in extrusion. The mechanisms that underlie soma differentiation and their morphogenesis are not well understood (White-Cooper, 2012). To investigate this, I can use other antibodies and transgenic markers, such as *Elfless*-GAL4, which can direct the expression of transgenes in tail cyst cells.

Furthermore, experimental error could explain the observation of no overt phenotypes in a few of these candidates. For example, some of these candidates were classified with RNAi lines that may not have strongly knocked down gene expression. This could allow for sufficient knockdown of the target to result in infertility, but with no clear phenotype. To resolve this, these candidates will be classified with the stronger RNAi line.

4.7 Summary of GO enrichment analysis

GO analysis of enriched terms associated with the sterile candidate genes in the different phenotypic classes was by itself not extremely informative. Genes in the different classes generally failed to cluster around specific GO terms. Some very general GO terms like purine ribonucleotide binding were common across different classes. One notable exception was the 'Arrest after spermatogonia' class, where many genes that were identified had GO terms associated with the regulation of membrane trafficking, suggesting this process was particularly important for spermatogenesis to proceed past these stages. Additionally a few of the GO terms that would be predicted to be enriched, namely genes associated with microtubule cytoskeletal organization, mRNA processing, and mitochondrial electron transport, were enriched in some of the phenotypic classes. These are all processes that serve key roles in various stages of spermatogenesis (Fabian and Brill, 2012).

4.8 Analysis of sterile vs. low fertility genes

Based on the genes I classified, there is a much greater likelihood of observing morphological defects in sterile candidates, in comparison to those that are low-fertility. 95% of sterile candidates exhibited defects, while only 4.6% did not. On the other hand, only 25% of low-fertility candidates exhibited defects, and 75% had no overt phenotype. Thus, analyzing sterile candidates will likely provide more insight into their role in spermatogenesis, since sterile candidates are more likely to be associated with observable defects in the testis.

It would be interesting, however, to investigate candidate genes that are low-fertility, since 25% of these exhibited defects in germline development. The majority of candidates in the extrusion class were low-fertility, which suggests that their role in fertility may generally be more important in later stages of spermatogenesis. By definition, spermatogenesis in low-fertility candidates progress to completion, but there are likely subtle defects in the regulation, onset, or rates of spermatogenesis. Detailed examination of low-fertility candidates will expose such possibilities. For example, other somatic-specific drivers, such as *c587-*, *eya-Gal4,* or a combination of *traffic-jam* & *eya* can be used to drive the knockdown of candidate genes during different stages of spermatogenesis. This will facilitate the observation of more subtle defects exhibited by low-fertility candidates.

4.9 Analysis of the classification scheme

The criteria I used to determine whether candidate genes exhibited defects enabled me to target the critical stages of germline development, that were disrupted

upon knock down of specific genes in the soma. It facilitated the observation of similarities in phenotypic defects of candidates from broadly different gene classes, as well as their unifying role in mediating soma-germline interactions.

In some cases, it was challenging to classify candidates that exhibited a range of phenotypes within a class. This could be due to the hypomorphic nature of RNAi or the age of testes examined. The varying perdurance and penetrance of different RNAi lines resulted in a broader phenotypic range exhibited by some candidates. To ameliorate this issue, different ages of testes, and multiple RNAi lines for these candidates can be examined. This will provide better insight, and help deduce the overall phenotypic effects exhibited by candidates with reduced fertility.

One of the limitations of this classification scheme is that it does not provide insight into gene functions. It can only determine the requirement for specific genes in the soma for germline development. However, this can be addressed by performing further phenotypic analyses using different antibodies, comparison to other screens, as well as more comprehensive bioinformatic analysis. The combination of such analyses will contribute to a comprehensive understanding of the precise role of candidates in mediating soma-germline interactions.

In short, the classification scheme I used was advantageous because I was able to identify gross morphological defects using widely available and inexpensive antibodies. I determined that males with reduced fertility generally give rise to visible phenotypic defects in the testis. This analysis has created a great platform that will facilitate future research focused on specific genes.

4.10 Analysis of the RNAi screen

The somatic RNAi screen demonstrated that reduced fertility is a highly reliable predictor of candidates that exhibit defects in spermatogenesis. Fecundity is a phenotype that can be assayed rapidly, and is a sufficiently rigorous selection criterion for identifying novel mediators of soma-germline interactions.

Although the RNAi screen has proven largely successful in identifying new mediators of soma-germline interactions, there are some caveats. In some cases, there are inconsistencies in the results of fertility assays obtained by genes screened with different RNAi lines. This is likely due to the varying efficiency of RNAi in mediating the knockdown of genes in the soma. To address this, re-screening sterile and low-fertility hits by performing additional fertility assays will confirm, and validate sterile and low-fertility candidates from the initial screen with high confidence.

Overall this screening method is reliable because it has identified over 200 novel mediators of soma-germline interactions. In addition, known mediators of soma-germline interactions exhibited reduced fertility, which validated our approach. Consistent with existing literature and previous studies, some of these candidates also exhibited a visible phenotype in the testis.

4.11 Conclusions

This is the first screen to our knowledge designed to identify new mediators of soma-germline interactions in *Drosophila*. The majority of the genes classified have not been previously shown to have roles in spermatogenesis. The requirement of novel mediators in the soma at specific stages of germline development was determined. Two

broad categories of candidates were identified: candidates that contained somatic cells and candidates that did not. Based on analysis of a subset of the genes identified in these categories, candidates that exhibited no soma are likely to be required for somatic cell viability or long-term maintenance of the soma. On the other hand, candidates that did contain somatic cells might be involved in the differentiation of the soma. In both cases, the defects observed in germline development are a secondary consequence, arising from defects in the soma development program. Mining bioinformatics databases, performing additional experiments, and analyzing this data further can confirm the involvement of candidates in these processes.

The genes in the 'No soma & No germline' or 'No soma' classes can be further analyzed by expressing *UAS-p35* in the soma. P35 is a baculovirus protein, which inhibits apoptosis of cells in many animals (Xue and Horvitz, 1995). In *Drosophila*, P35 terminates cell death in the developing embryo and eye (Hay et al., 1994). If this rescues the somatic cell death phenotype induced in candidate knockdown testes, this would indicate that the candidate gene is required for somatic cell viability. To determine whether the germline over proliferates in the 'No soma' class or confirm the presence of germ cell tumors in the 'Arrest after spermatogonial' and 'Arrest after spermatocyte' classes, cell proliferation assays can be performed using 5-ethynyl-2'-deoxyuridine (EdU) labeling. EdU is a synthetic analog of the nucleoside thymidine, which is incorporated into DNA during DNA synthesis, and can be readily detected using a covalently-linked fluorophore (Salic and Mitchison, 2008). Alternatively, the mitotic marker phospho-histone H3 can be used to label mitotic cells. Germ cells positive for EdU and/or pH3 would indicate the presence of proliferating germ cells and can be used

to confirm the presence of germ cell tumors based on the aberrant patterns of proliferation.

To assess whether defects in the germline exhibited by candidates in the ‘Arrest after spermatogonia’ class is due to defects in somatic encapsulation around the germline, or the inability to form a permeability barrier, the genes in this class can be further studied by performing dextran assays (Fairchild et al., 2015). In addition, forced differentiation of the germline, by ectopically expressing the essential germline differentiation gene, *bag of marbles* (*bam*), might reveal whether the candidates in this class are required to transport the signals required for the soma to permit germline differentiation.

To distinguish which candidates in the ‘Arrest after spermatocyte’ class fail to complete meiosis, the meiotic marker Boule can be used. The absence of elongated spermatids would indicate a failure to complete meiosis. This could determine whether the candidates are required to control the onset and completion of meiosis in the germline essential for progression to the spermatid stage.

To investigate whether spermatid elongation, individualization, polarity, or coiling was disrupted by somatic knockdown of genes in the ‘Extrusion’ class, different molecular markers can be used. For example, antibodies that label components of the individualization complex can be used to determine if spermatid individualization is disrupted.

Conditional temperature-sensitive repressors like *ts-Gal80* can be used in combination with *tj-Gal4* to control the spatial and temporal expression of candidate genes at specific stages of germline development. This could reveal the roles of candidate

genes in mediating soma-germline interactions post-gonadogenesis, and reveal the development of a phenotype later in spermatogenesis. This would be helpful in investigating the role of genes that function at multiple stages of spermatogenesis.

Future work in the lab will complete the characterization and classification of all candidates from the screen. We are particularly interested in genes involved in morphogenesis and differentiation that mediate soma-germline interactions, thus we will focus on genes that fit all or the most of the following criteria: i) they have no known role in the soma, ii) they have vertebrate orthologues, iii) they are not generally required for cell viability but have specific roles in the soma, iv) they are required for either soma differentiation, v) are required for germline encapsulation, and/or vi) maintenance of germline/somatic stem cells. For candidate genes where mutations have been identified, the available alleles will be obtained and tested for defects in spermatogenesis. Furthermore, clonal analysis can be performed to de-lineate the precise role of interesting candidate genes in spermatogenesis.

The analysis of novel somatic regulators will improve our understanding of soma-germline interactions. This study is a starting point for exploring the multiple, independent, biological mechanisms by which the soma controls male fertility in *Drosophila* and other organisms.

In humans, 8-10% of healthy males exhibit subfertility. Although the principle causes of subfertility are attributed to environmental factors and infectious diseases, there is supporting evidence for the genetic basis of impaired spermatogenesis. Despite this, the cause of impaired spermatogenesis remains unknown in 40% of the cases reported by male infertility clinics (Hochstenbach and Hackstein, 2000). Many of the genes identified

in this study have human homologs, thus future studies can explore their role in human spermatogenesis, and their impact on the pathogenesis of disease.

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Appendices

Appendix A – Gene ontology (GO) enrichment analysis of sterile candidates in the 5 phenotypic classes

DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) was used to perform GO enrichment analysis for the sterile genes classified. The list of all GO terms involved in biological process (GOTERM_BP_FAT), molecular function (GOTERM_MF_FAT) and cellular component (GOTERM_CC_FAT) for the sterile candidates in the five phenotypic classes are organized as clusters of related GO terms based on their enrichment values. Each phenotypic class is found on separate sheets in the excel document. The length of the table is impractical to be included here. Instead, the relevant document, 'Appendix A-GO enrichment analysis_sterile candidates' can be found online: https://www.dropbox.com/s/7rul2l3adugnsst/Appendix%20A-GO%20enrichment%20analysis_sterile%20candidates.xlsx?dl=0.

Appendices B-E:

Gap junctions are important candidate regulators of soma-germline interactions (Hall et al., 1999; McCarter et al., 1997). Gap junctions enable the passage of ions and small molecules, such as cAMP, IP_3, and Ca^{2+}, which permit rapid signaling between cells (Bauer et al., 2005; Phelan, 2005). *zpg* encodes an invertebrate gap junction protein, Innexin 4. In *Drosophila*, mutants of *zpg* possess rudimentary gonads, which contain some early germ cells, but lack differentiated germ cells (Tazuke et al., 2002). In addition, the somatic cells have an abnormal arrangement and fail to encapsulate germ cells. Zpg has been proposed to form gap junctions with the soma to mediate germline-soma communication (Gilboa et al., 2003; Tazuke et al., 2002).

Our lab has identified Innexin 2 as a candidate in the soma that may form gap junctions with Zpg in the germline. The nature of the signals moving through the gap junctions remains unknown (Gilboa et al., 2003; Tazuke et al., 2002). To understand how Zpg and Inx2 interact to mediate germline-soma communication during spermatogenesis and oogenesis, I expressed biosensors in the soma and germline of *Drosophila* testes to detect cAMP and Ca^{2+}, characterized Zpg plaques, and analyzed the role of Inx2 in the *Drosophila* ovaries.

Appendix B – Epac-FRET biosensor does not detect changes in cAMP levels in the *Drosophila* testis

I worked on a project investigating the role of gap junction mediated signaling between the soma and germline in the *Drosophila* testis. I used an Förster resonance energy transfer (FRET) sensor fused to Epac, an exchange protein directly activated by cAMP (Courtesy of Todd Nystul, UCSF). This sensor can detect intracellular cAMP levels by analyzing changes in CFP and YFP fluorescence, or a decrease in FRET (Ponsioen et al., 2004). I expressed the Epac FRET sensor in the germline and soma of wild-type *Drosophila* testis and used the adenylate cyclase activator forskolin to alter intracellular cAMP in dose response experiments. I expected to observe a decrease in FRET (a decrease in YFP fluorescence) upon increasing forskolin concentrations (Ponsioen et al., 2004). I was not able to observe a decrease in FRET. This implies that there were no changes in cAMP levels or that I could not detect changes in cAMP levels. It is possible that the pilot experiments did not validate the Epac-FRET biosensor due to its low signal to noise ratio and challenge in analyzing fluorescence intensity changes. In

Figure A.1 Epac-FRET biosensor does not detect changes in cAMP levels in the *Drosophila* testis

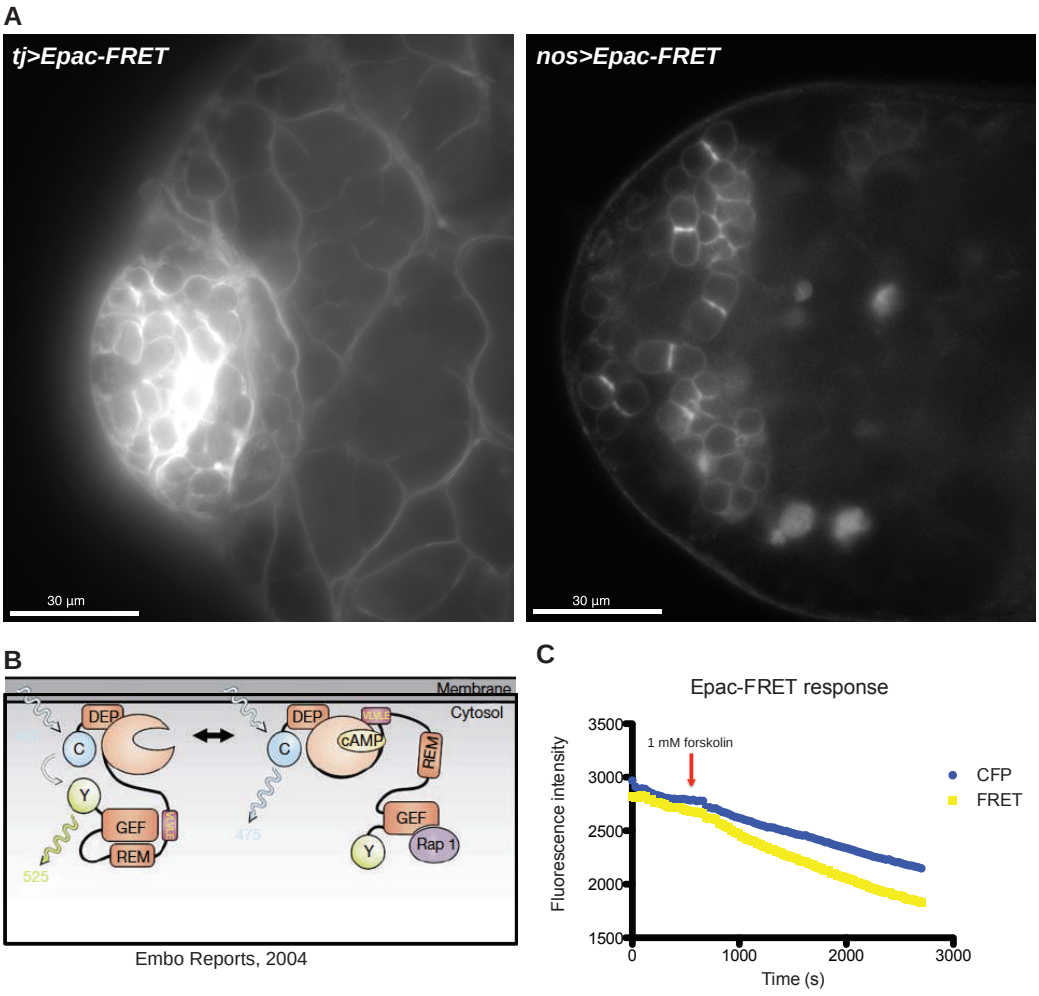

Figure A.1 Epac-FRET biosensor does not detect changes in cAMP levels in the *Drosophila* testis. (A) Epac-FRET expression in the soma (*tj>Epac-FRET*) and germline (*nos>Epac-FRET*). **(**B) Schematic of Epac-FRET sensor (Schematic reproduced from Ponsioen et al., 2004 [pending permission]). **(**C) Epac-FRET response in the soma.

addition, the Epac FRET biosensor has not been previously used in the *Drosophila* testes (Figure A.1).

Appendix C - GCaMP biosensor can be used to visualize Ca^{2+} levels in the *Drosophila* testes

I investigated the role of Ca2+, another signaling molecule that is known to be transported through gap junctions, which may mediate soma-germline communication (Bauer et al., 2005; Phelan, 2005). To detect changes in Ca^{2+} signaling I used a genetically encoded calcium sensor, GCaMP 3.0. It is composed of a fusion of calmodulin, GFP, and M13 (a domain of myosin light chain kinase), and undergoes a conformational change upon binding calcium. Thus, the presence of calcium can be visualized by a change in fluorescence intensity. GCaMP 3.0 has been previously used in *Drosophila* to analyze neuronal activity (Tian et al., 2009).

I expressed GCaMP 3.0 in the soma and germline of wild-type *Drosophila* testis, and performed live-imaging experiments where I recorded 10-20 minute movies to analyze changes in fluorescence intensity of the GCaMP sensor. The pilot experiments were challenging as confocal live-imaging settings had to be optimized for each experiment. The expression of GCaMP 3.0 sensor in the germline was noisy and difficult to visualize. On the other hand, the expression of GCaMP 3.0 in the soma was easy to detect, but only 1 out of 20 movies recorded showed changes in fluorescence intensity, as observed by the pulses of fluorescent intensity in the somatic cells at the apical tip of the testis. The movie file, 'Appendix C- GCaMP in the soma' can be found online: https://www.dropbox.com/s/sbzwx7ygsijrsqm/Appendix%20C-GCaMP%20in%20the%20soma.avi?dl=0.

Appendix D –Characterization of gap junctions in the *Drosophila* testes and ovaries

Gap junction channels form clusters, called plaques, composed of few to thousands of units (Goodenough and Paul, 2009). Gap-junction plaques exhibit punctate membrane labeling (Bohrmann and Zimmermann, 2008). To determine the stages of oogenesis where Zpg and Inx2 plaques form, I dissected and stained egg chambers of various stages. Zpg and Inx2 appear to co-localize in stage 6-7. This suggests that Zpg and Inx2 mediated soma-germline interactions are important in stage 6-7 of oogenesis.

Gap junction plaques are known to exhibit turnover by the addition of subunits at the edges and removal of subunits from the center of the plaques (Goodenough and Paul, 2009). To determine if Zpg exhibits turnover in the *Drosophila* testes and ovaries, I used Fluorescence Recovery After Photobleaching (FRAP) to investigate the turn over of Zpg. The turnover of proteins can be measured by their mobile fraction.

I analyzed the turnover of fluorescently tagged Zpg (Zpg::GFP) in various stages of wild-type *Drosophila* egg chambers and testes. I found the turnover of Zpg varied slightly between different stages of egg chambers, and also between ovaries and testes. There is a general decrease in Zpg::GFP turnover from egg chamber stages 6 to 9. It is possible that Zpg plaques form, and are relatively stable until late stages of oogenesis where complex somatic cell movements likely necessitate rapid remodeling of Zpg plaques (Figure A.2).

Figure A.2 Characterization of gap junctions in the *Drosophila* tests and ovaries

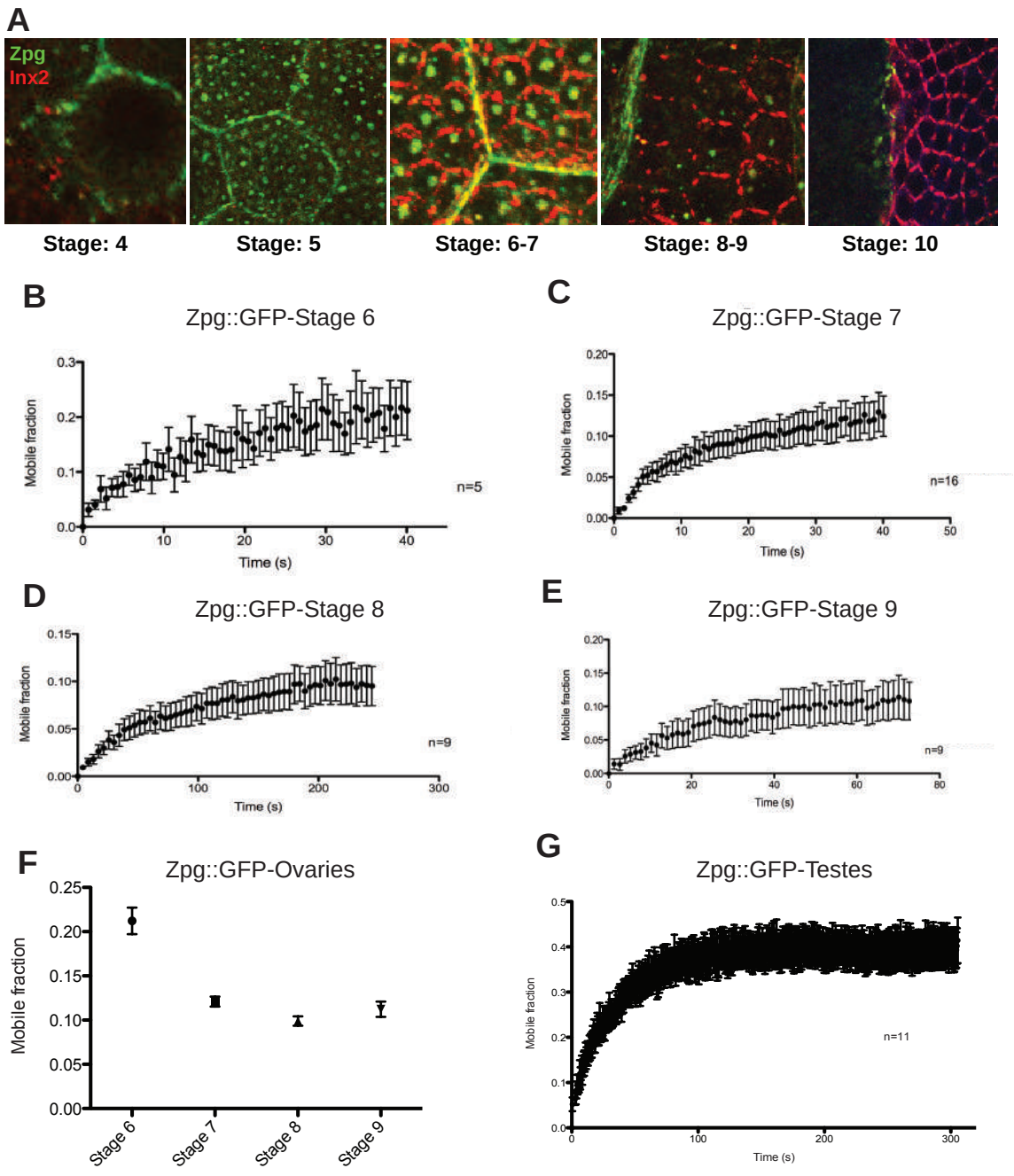

Figure A.2 Characterization of gap junctions in the *Drosophila* testes and ovaries. (A) Zpg (green) and Innexin 2 (red) plaques are not visible in stage 5 egg chambers. Only Zpg plaques are present in stage 5 egg chambers. Zpg and Inx2 appear to co-localize in stage 6-7 egg chambers. Fewer Zpg plaques are observed in stage 8 egg chambers, and Inx2 plaques are absent. Zpg and Inx2 plaques are absent in stage 10 egg chambers. (B-E) Zpg::GFP plaques turnover in ovaries of stage 6, 7, 8 & 9 egg chambers. (F) Mobile fractions of Zpg::GFP plaques in ovaries of stage 6, 7, 8 & 9. Zpg::GFP turnover decreases from stage 6 to stage 7 egg chambers, and appears to stabilize in stage 8 and 9 egg chambers. (G) Zpg::GFP turnover in testes.

Appendix E –Analysis of Innexin 2 in the *Drosophila* ovaries

I analyzed the role of Innexin 2 by inducing RNAi mediated knockdown of Innexin 2 in the soma of adult *Drosophila* ovaries. RNAi-mediated knockdown of Innexin 2 results in degradation of egg chambers after stage 6. This suggested that Inx2 is required for the maintenance of egg chambers in the *Drosophila* ovaries. To investigate this further, I performed clonal analysis of Inx2 by inducing Inx2 RNAi clones in the soma of adult ovaries. This resulted in the knockdown of Inx2, but egg chambers did not degrade (Figure A.3).

Figure A.3 Analysis of Innexin 2 in the *Drosophila* ovaries

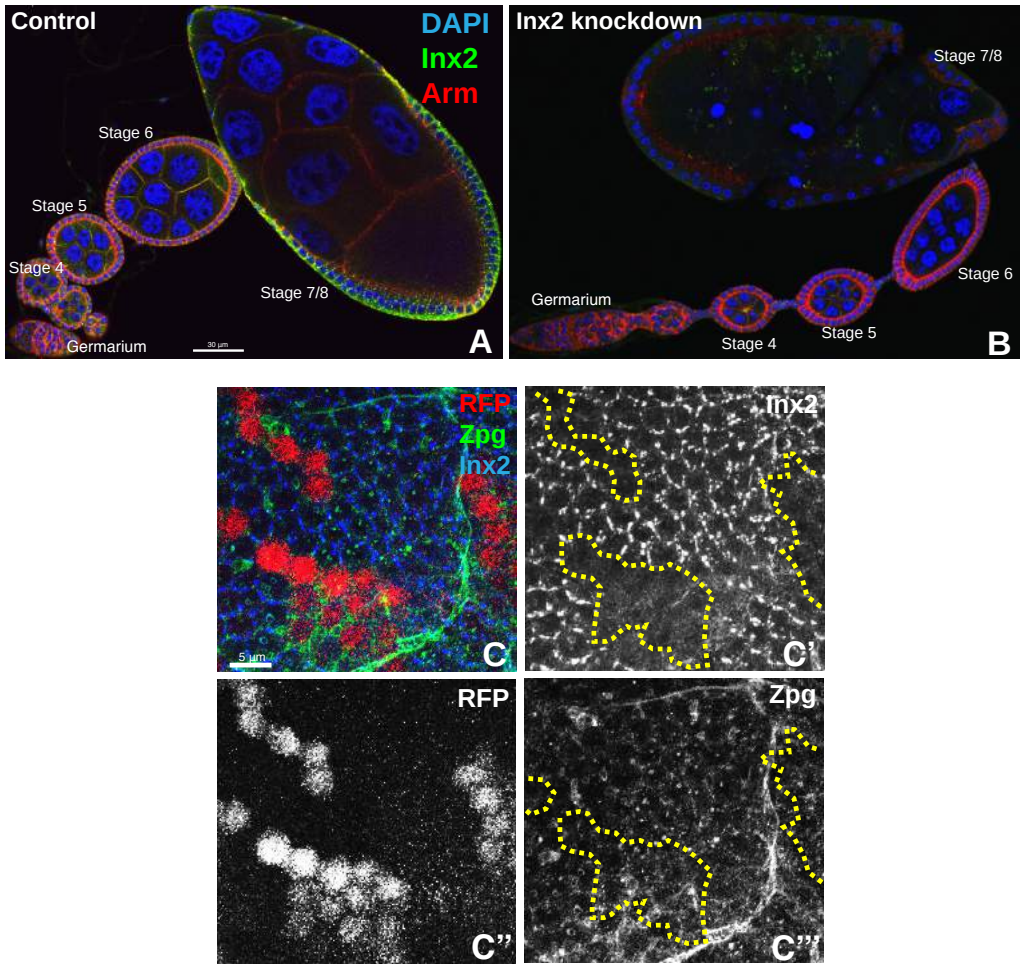

Figure A.3 Analysis of Innexin 2 in the *Drosophila* ovaries. (A & B) Egg chambers stained with DAPI (blue), Innexin 2 (green), and Armadillo (red). (A) Control egg chambers (Germarium-stage 7/8). (B) Inx2 knockdown in the soma shows that egg chambers begin to degrade after stage 6. (C) Clonal induction of Inx2 RNAi in the soma of ovaries. RFP (red) marks somatic clones lacking Inx2 (blue). Zpg (green) plaques appear to be absent in Inx2 somatic clones.