BI-DIRECTIONAL GAP JUNCTION-MEDIATED SOMA-GERMLINE COMMUNICATION
DURING *DROSOPHILA* SPERMATOGENESIS

by

CHRISTOPHER MICHAEL SMENDZIUK

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Abstract

Soma-germline interactions play conserved essential roles in regulating cell proliferation, differentiation, patterning, and homeostasis in the gonad. In the *Drosophila* testis, the JAK-STAT, Hedgehog, BMP and EGF pathways are used to mediate soma-germline communication via paracrine signalling. In this thesis, I aim to shed light on the role of juxtacrine signalling, mediated by gap junction proteins, during early spermatogenesis in the fly testis.

My analysis demonstrates that gap junctions also mediate direct, bi-directional signalling between the soma and germline. When gap junctions between the soma and germline are disrupted, germline differentiation is blocked and germline stem cells are not maintained. In the soma, gap junctions are required to regulate proliferation and differentiation. Gap junctions are present between germline and somatic stem cells, as well as between differentiating cells by ultrastructural analysis. Localization and RNAi-mediated knockdown studies reveal that gap junctions in the fly testis are heterotypic channels containing Zpg (Inx4) and Inx2 in the germline and in the soma, respectively. Preliminary structure-function analysis of Zpg reveals that the C-terminus of the protein is essential for its function. Furthermore, gap junctions in the testis may be important for mediating calcium signalling. Overall, my results show that bi-directional gap junction-mediated signalling is essential to coordinate the soma and germline to ensure proper spermatogenesis in *Drosophila*. Moreover, I show that stem cell maintenance and differentiation in the testis are directed by gap junction-derived cues.

Preface

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For this publication, I performed all experiments and analyzed all data, with the exception of: the molecular biology, which was performed by Anat Messenberg; the *tj*>RNAi, *nos*>RNAi, and *bam*>RNAi fertility assays, which were performed by Jacelyn Shu and Alis Qinyuan Xu; and the electron microscopy, which was performed by A. Wayne Vogl. I acquired all confocal images contained in this work and performed all the data analyses. Guy Tanentzapf and I designed experiments and co-wrote the paper.

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List of Abbreviations

Arm   Armadillo

α-spec   α-spectrin

Bam   Bag of marbles

BMP   Bone morphogenic protein

CaM   Calmodulin

cAMP   Cyclic adenosine monophosphate

Cher   Cheerio

Chic   Chickadee

Chinmo   Chronologically inappropriate morphogenesis

Ci   Cubitus interruptus

Cora   Coracle

Cx   Connexin

CySC   Cyst stem cell

DAPI   4',6-diamidino-2-phenylindole

Diap1   Death-associated inhibitor of apoptosis

DNA   Deoxyribonucleic acid

Dpci	Days post-clone induction
DPE	Days post-eclosion
Dpp	Decapentaplegic
E-Cad	Epithelial cadherin
EdU	5-ethynyl-2'-deoxyuridine
EGFR	Epidermal growth factor receptor
Gbb	Glass bottom boat
GCaMP	High-affinity calcium probe composed of GFP
GFP	Green fluorescent protein
GSC	Germline stem cell
gcl	Germ cell less
Hh	Hedgehog
His2A	Histone 2A
Hop	Hopscotch
IGF	Insulin growth factor
InR	Insulin receptor
Inx/INX	Innexin
IP_3	Inositol triphosphate

JAK	Janus kinase
Mad	Mothers against decapentaplegic
MAP	Mitogen-activated protein
mCD8-GFP	Membrane-bound and GFP-tagged CD8 protein
mRNA	Messenger ribonucleic acid
N-Cad	Neural cadherin
nls	Nuclear localization signal
nos	Nanos
Ogre	Optic ganglion reduced
PGC	Primordial germ cell
Ptc	Patched
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAi	RNA interference
Sax	Saxophone
SGP	Somatic gonadal precursor
s.e.m.	Standard error of the mean
Shak-B	Shaking-B (lethal)

Smo	Smoothened
Smox	Smad on the X
Spi	Spitz
STAT	Signal transducer and activator of transcription
Tj	Traffic jam
Tkv	Thick veins
Tud	Tudor
UAS	Upstream activating sequence
Upd	Unpaired
Wg	Wingless
Zpg	Zero population growth
Zfh-1	Zinc finger homeodomain-1

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For Jackie Vogel,

"We spend so much time trying to study what disease is, but we don't even understand what
normal is, we don't even know how a cell works." … "So you gather data… data, data, and data.
And you hope to God that at some point it means something and helps someone. Because that's
all you can really do to make sense of it."

Chapter One: Introduction

1.1. Soma-germline introduction

1.1.1. The germline is immortal

Animals are composed to two basic tissue types: the germline, which will form the sperm or eggs necessary for reproduction, and the soma, which forms all other tissues in the animal. The sole function of the germline is to transmit the genetic information of the parent to its offspring [1, 2]. While the soma contributes to every single tissue and cell type in an organism, save for the germline, the soma's most essential role is to nurture and protect germ cells to ensure the success of sexual reproduction [3]. Cooperation between the soma and germline is essential for gametogenesis.

Over one hundred and fifty years ago, the English biologist Sir Richard Owen proposed the existence of a set of cells that are set aside in an organism to contribute to the development of another organism [4, 5]. This idea led to proposal of the germ plasm theory by August Weismann in the late 19th century, which posits that multicellular organisms are composed of germ cells that hold and transmit the material basis of heredity, and somatic which carry out physiological functions [6]. From this, Weismann proposed that heritable information only passes from the germline to the soma, as each successive germline gives rise to new somatic tissues in addition to a germline [6]. Gametogenesis is the process by which the initially undifferentiated germline is guided by the soma to eventually give rise to terminally differentiated gametes, eggs in females or sperm in males [7]. Through this process, the germline condenses and packages its DNA for the purpose of ensuring its DNA is passed onto offspring, leaving the somatic tissues behind [6, 7]. In this sense, the germline passes eternally from parent to offspring, parent to offspring; this perpetual passage of the

germline between generations lends itself to the description of the germline as being immortal [1, 2, 8].

1.1.2. Conservation across metazoan lineages

While the germline may be eternal, passing from one generation to the next, it does not do this on its own. Regardless of the animal, somatic cells assist germ cells in their development. The soma can facilitate germ cell development in a multitude of ways, such as regulating cell adhesion, migration, proliferation, and differentiation [3]. The somatic Sertoli cells in mammalian and teleost testes supply differentiating germ cells with essential nutrients, molecular signals, and protection from the immune system [9, 10]. In nematodes, the somatic cells are required for the proliferation of germ cells early in development, and later form a niche that maintains germ cells in mitotic state before they are ready for meiosis [11]. Soma-germline interactions in the fruit fly *Drosophila melanogaster* begin before the primordial germ cells have begun migrating to the embryonic gonads, and continue throughout the entire life-span of the fly to ensure its reproductive success [3]. Despite the differences across different metazoan lineages, soma-germline interactions are crucial for the production of gametes.

In many species, the germline is among the first lineages specified during development. Primordial germ cells (PGCs) are essentially formed by one of two processes: preformation or epigenesis [12]. Preformation is the process by which germ plasm deposited by the mother is inherited by cells which determines the germline, and occurs in fruit flies, nematodes, frogs, and many fish [12]. In *Drosophila*, for instance, RNAs and proteins synthesized in the germline are transported to the posterior pole of a developing oocyte; this represents the germ plasm [12]. Prior to gastrulation, precocious cellularization at the

posterior of the embryo forms a small number of pole cells, containing the germ plasm, which eventually give rise to the germline [12]. In *C. elegans*, electron-dense granules are scattered throughout the embryo until they move to one side of the embryo after fertilization and segregate asymmetrically [12]. After several rounds of cell division in the early embryo, the granules remain segregated to a single cell, which will become a PGC [12]. And in *Danio*, *vasa* mRNA synthesized during oogenesis is segregated into four cells by the 32-cell stage of embryogenesis, forming the PGCs [12]. Vasa, as it turns out, is one of the germline determinants found in nearly all species examined [12]. Epigenesis, in contrast, is the process by which germ cells are induced to develop in a subpopulation of cells which express germline competence genes [12]. Germ cell specification in mice, humans, and some amphibians occurs via epigenesis [12, 13]. In mice, the extraembryonic ectoderm and visceral endoderm specify a subset of epiblast cells as PGC-competent at the beginning of gastrulation, which then migrate to the posterior proximal region where they are specified [14]. Whether the germ cell lineage is determined by preformation or epigenesist, its specification is the most important step in the initiation of gametogenesis.

1.1.3. Stem cells play important roles in gametogenesis

In order to produce gametes throughout an organism's lifespan, a population of progenitor cells that can continually contribute to gamete formation is essential. Such progenitor cells are referred to as stem cells. Stem cells are cells which are not terminally-differentiated and can divide to generate two daughter cells with asymmetric outcomes, where one cell can differentiate to contribute to a tissue and the other cell can be maintained as a stem cell [15]. A balance between stem cell self-renewal/maintenance and differentiation is essential; excess self-renewal can lead to tumour formation, whereas increased

differentiation can lead to premature tissue aging and degeneration [15]. Stem cells are often maintained in an undifferentiated state within a tissue in a discrete location, termed a stem cell niche [15]. The concept of the stem cell niche was first proposed by Schofield nearly forty years ago [16]. Signals from the niche to stem cells are important for regulation of stem cell self-renewal, adhesion, and differentiation [15]. Damage to a stem cell niche often results in a loss of a tissue's regenerative capacity, by compromising the ability of the niche to maintain stem cell [17]. Stem cells and their niche thus function together to ensure that a pool of progenitor cells is maintained to contribute to tissues during an animal's lifespan.

Germ cells are often maintained within a stem cell niche in the gonad in many organisms [18]. In *C. elegans*, the somatic distal tip cell is essential for maintaining GSCs in a mitotic state; laser ablation of the distal tip cell results in a loss of all mitotic germ cells as they enter meiosis and differentiate [19]. In *Drosophila*, transplantation and laser ablation experiments were critical to identifying the location of germline stem cells (GSCs) within the ovary [20]. Lin & Spradling found that dissected individual fly ovarioles could continue the process of oogenesis upon transplantation into the abdomen of a host fly [20]. Laser ablation of presumptive GSCs at the tip of an ovariole prevented the continuation of oogenesis after transplantation into a host [20]. Subsequent experiments using loss-of-function mutations suggested that several different somatic cells at the tip of the ovary form a niche for GSCs, which is critical for their maintenance [21]. In the fly testis, a population of somatic cells at the apical tip of the testis, termed the hub, was thought to maintain GSCs [22]. Later work demonstrated that the hub secreted cytokines that were required for GSC maintenance [23, 24]. The identification of a stem cell niche for GSCs in mammalian testes has been more difficult. Undifferentiated germ cells reside along the basement membrane of seminiferous

tubles in the mouse testis [18, 25]. GSCs receive essential signals from three different types of somatic cells — Leydig cells, myoid cells, and Sertoli cells— which may together form the niche for GSCs in mammalian testes [25]. Identifying the specific niche for GSCs and factors essential for stem cell maintenance in mammals has been difficult due to the complexity of the testis [18, 25]. Nonetheless, research in worms, flies, and mammals has demonstrated that GSCs are maintained within tissues to ensure continued fertility of each organism.

1.1.4. Germ cells are supported by the soma

From the earliest stages of germ cell formation, somatic cells play important roles in regulating their behaviour [3]. Somatic cells can induce the formation of primordial germ cells, guide their migration to the gonad when necessary, nurture and maintain stem cells within a niche, and guide the differentiation of germ cells into fully formed gametes [3, 12, 18]. Disrupted soma-germline interactions, such as decreased cell adhesion or increased self-renewal signals within the niche, or even loss of the somatic support cells, can result in infertility, tumorigenesis, or both [26, 27]. The interactions between the soma and germline are thus essential for gametogenesis.

1.2. *Drosophila* as a model for studying soma-germline interactions

1.2.1. Soma-germline interactions are difficult to study in humans

Understanding the causes of infertility in humans is important for developing treatments and therapies for affected individuals [26]. While factors such as DNA damage, environmental toxins, infectious diseases, and endocrine disorders can contribute to infertility in men and women, the genetic bases of infertility and sub-fertility are not well-understood [26]. Genes required for male fertility that are located on the Y chromosome are difficult to

study, as they often arise *de novo* in an individual, are not carried by females, and cannot be passed on to the next generation [26]. As X chromosome-associated genes can be carried by females and transmitted to their male offspring — providing female fertility is not affected — and autosomal recessive genes can be passed on by both mothers and fathers, many genes required for fertility are not easy to identify [26]. Genetic model organisms are thus very useful for identifying previously uncharacterized or poorly understood genes required for fertility.

Spermatogenesis is a highly conserved process across different species. Many aspects of spermatogenesis, such as the condensation of haploid DNA, rearrangement of mitochondria to fuel the energy-intensive motility of sperm, exclusion of large portions of the cytoplasm of each germ cell, and the formation of the microtubule-based propulsive machinery of sperm, the axoneme, are found throughout the animal kingdom [26]. The end result of these over-simplified events remains the same: the production of motile packages of DNA that can fertilize an egg and initiate embryogenesis. Through the use of model organisms over years of research, incalculable insight has been provided into human illnesses and mechanisms of tissue regulation, particularly with respect to infertility and spermatogenesis [26, 27].

As an alternative to the arduous goal of studying spermatogenesis in humans, organisms like mice and rats provide attractive systems to address important questions. However, the study of soma-germline interactions is not a simple task. First, spermatogenesis in mammals involves many different somatic cells which interact with the germline during the development and maturation of germ cells, as well as interacting with other somatic cells [9, 25]. The process of investigating specific cell-cell interactions thus becomes quite

difficult in this context. Second, while the genomes of many mammalian species have been sequenced, investigating the role of specific genes via mutant analysis is difficult without identified genetic lesions, alleles of varying severity, and tissue-specific conditional knock-outs of genes after the formation of the gonad [26]. In addition, the roles of essential genes can often be difficult to assess due to pleiotropic effects, whereby one gene may be important for many different processes [26]. Third, the financial and practical limitations of performing large-scale genetic screens to identify new genes and alleles required for fertility are considerable [26]. While evolution has resulted in drastic changes in metazoan species over time, central processes and tenets of cellular life hold true whether they are occurring in humans, mice, nematodes, or fruit flies. In *Drosophila*, many genes required for fertility have been identified which have conserved roles in mice and humans [27]. Mutations in genes that are important for germ cell proliferation, meiosis, spermatid differentiation, sperm motility, and sperm fertilization in mammals have also been shown to be essential in flies and vice versa [27]. Alternative model organisms can thus be used to investigate questions that have important consequences for human health.

Spermatogenesis is a complex, multi-step, tightly regulated process. Understanding the genetic and molecular mechanisms that govern spermatogenesis is a significant undertaking. Studies from the mouse testis underline the difficulty in investigating soma-germline interactions in a mammalian system, regardless of practical and financial limitations [25, 26]. Turning to an invertebrate system, such as *C. elegans*, would provide a much simpler system to study the intricate interactions between the soma and germline; however, there are significant differences between spermatogenesis in worms as compared to other organisms [11, 28]. Interactions between the soma and germline are important for regulating

the entry of germ cells into meiosis and assisting germ cells in transiting through the gonad [11, 28]. In contrast to spermatogenesis in other systems, however, differentiating germ cells in the worm are not intimately encysted by the soma, which is a defining feature of spermatogenesis in other systems [11, 28]. One of the few signalling pathways implicated in the adult gonad for soma-germline interactions in *C. elegans* has been Notch/GLP-1 signalling, which prevents germ cells from entering meiosis prematurely [11, 28]. While sperm-specific differentiation programs have important and generally conserved roles in worms, many sperm-specific genes from flies and mammals have no clear homologue in nematodes [11, 28]. Furthermore, terminal differentiation of sperm in *C. elegans* produces an amoeboid sperm that uses a pseudopod for movement; this is in contrast to the long flagella used for locomotion in sperm in other systems [10, 27-29]. *Drosophila* spermatogenesis, in contrast, involves many of the same signalling pathways, similar interactions between the soma and the germline, and produces long flagellar sperm, as is the case in mammalian spermatogenesis [26, 27, 29, 30]. Thus, the fly is an excellent model system in which to study soma-germline interactions.

1.2.2. The genetic bases of infertility in humans can be investigated in flies

A plethora of studies in *Drosophila* have demonstrated the utility of the fly testis as a model to study spermatogenesis. Due to the similarities between fly and mammalian spermatogenesis, the simplicity of the fly testis, the powerful genetic tools available in *Drosophila*, the relatively short generation time, and low costs of fly husbandry, fruit flies are superbly suited to address basic questions of soma-germline signalling. In brief, the fly testis is relatively simple compared to mammalian testes, while general processes are still conserved. In *Drosophila*, a well-characterized stem cell niche resides at the apical tip of the

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testis, composed of one somatic cell type, hub cells [30]. The niche functions as a signalling centre to maintain undifferentiated somatic cyst stem cells (CySCs) and GSCs (**Fig. 1.1.**) [30, 31]. In the mammalian testis, in contrast, several different types of somatic cells are employed to maintain germ cells in an undifferentiated state and to regulate germ cell self-renewal. [25]. In flies, each differentiating germ cell becomes encysted or wrapped by two somatic cells shortly after exiting the niche [22, 30, 31]. The germline cyst forms the basic unit of spermatogenesis, where the two somatic cyst cells will guide the differentiation of the germline as it divides (**Fig. 1.1.**) [30]. This closely resembles the early events of mammalian spermatogenesis where somatic tissues regulate GSC maintenance, and two Sertoli cells guide germ cells through their development [26]. In both flies and mammals, the germline develops as a syncytium, with intercellular bridges connecting germ cells within a cyst [26, 30]. Before a germline cyst enters meiosis in the fly, a diffusion barrier forms around the cyst, isolating the germ cells from the environment [32, 33]. Upon the completion of meiosis, spermatids undergo complex changes in cell shape, condense their genetic material, and drastically alter the structure of their organelles; this process is known as spermiogenesis (**Fig. 1.1.**) [26, 30]. Only after terminal differentiation does the germline end its relationship with the cyst or Sertoli cells [26, 30]. The similarities between fly and mammalian spermatogenesis and reduced complexity of soma-germline and soma-soma interactions make the *Drosophila* testis a particularly attractive model system.

Powerful genetic tools available in *Drosophila* greatly facilitate the study of soma-germline interactions. Genes required for spermatogenesis can be identified in simple sterility screens, yielding a wealth of information of necessary germline-specific genes, many of which have vertebrate homologs [26, 27, 29]. Indeed, over 60% of identified genes in

Drosophila are conserved in humans, and more than 50% of genes associated with diseases in humans have fly homologs [34-36]. Screening for mutations which cause male sterility can identify previously unknown genes required for spermatogenesis, as well as new alleles of characterized genes, such as gain-of-function or hypomorphic alleles [26, 29, 37]. Many genes required for spermatogenesis are required in other tissues and in other processes, often for viability; therefore, it is often difficult to obtain homozygous-mutant, viable flies for those genes [26]. To circumvent this, an array of tools available in *Drosophila* can be used. The bi-partite UAS-GAL4 expression system, borrowed from yeast and developed by Brand & Perrimon, allows for the tissue-specific expression of transgenes carrying Upstream Activating Sequences (UAS) [38]. GAL4 protein, expressed under the control of a tissue-specific promoter, binds to and activates transcription of UAS-regulated transgenes [38]. This can be used to express double-stranded RNA which will knockdown target mRNA levels, thus indirectly disrupting gene function in a tissue-specific manner, such as in the soma versus in the germline [37]. By coupling this system with the expression of a temperature-sensitive GAL80 inhibitory protein, which represses GAL4 transcription at permissive temperatures, transgene expression can be temporally regulated, depending on growth conditions [39]. Several groups have generated hundreds of transgenic lines that can target nearly every gene in the fly genome, including the Vienna *Drosophila* Resource Center (VDRC) and the Transgenic RNAi Project (TRiP) at Harvard Medical School. To investigate the role of a gene required for viability during spermatogenesis, FRT-mediated recombination can be used to generate mutant cells or clones of cells in an otherwise heterozygous testis [40]. This can be coupled with the ubiquitous expression of GFP, which is lost in mutant cells, to negatively-label mutant clones, or with mosaic analysis with a

repressible cell marker (MARCM) to positively-label mutant cells [37, 41]. The persistence

of mutant clones in a tissue can be used to demonstrate whether the soma, germline, or both

require a specific gene for stem cell regulation or differentiation [37]. In addition to using

MARCM for loss-of-function studies, MARCM can also be utilized for gain-of-function and

rescue experiments within a tissue to shed provide further insight into a gene's role [41]. By

combining the above approaches, genes essential for fertility can be readily identified and

characterized.

Finally, *Drosophila* is attractive as a model organism for purely practical reasons.

Flies have relatively short generation times, developing from a fertilized embryo to an adult

in approximately ten days [26]. Minimal space and modest financial investments are required

for a fly husbandry setup and its maintenance [29]. And large-scale genetic screens can be

conducted relatively quickly and easily [29]. Selecting a single research question to

investigate is often more difficult than designing experiments to address that question.

1.2.3.   Overview of spermatogenesis in flies

Spermatogenesis is a well-characterized and orderly process in *Drosophila*

melanogaster. At the apical tip of the testis, a GSC begins its journey to give rise to sixty-

four sperm (see **Fig. 1.1.**). GSCs undergo an oriented mitosis to produce two daughter cells,

one of which remains in contact with the hub and proximal to niche-derived maintenance

signals, and another cell which is displaced from the niche [30, 31]. CySCs also divide to

produce two daughter cells, one of which is stochastically maintained as a stem cell while the

other can become a cyst cell which begins to differentiate with the displaced germ cell [42].

The differentiating germ cell, a gonialblast, is encapsulated by two cyst cells to form a

germline cyst; the cyst cells no longer divide following the initial CySC division [30]. Next,

a series of rapid, transit-amplifying divisions with incomplete cytokinesis occur where the gonialblast divides to give rise to a 2-cell stage spermatogonia, followed by 4-, 8-, and finally a 16-cell stage cyst [29, 30]. Throughout these stages, a cytoskeletal-rich structure termed the fusome extends throughout the syncytial spermatogonia via the intercellular bridges remaining from the previous cell divisions [30]. Following the final round of mitosis, the germ cell cyst is referred to as a primary spermatocyte and begins to grow in size, increasing its cell volume 25-fold, as it transcribes massive amounts of mRNA prior to meiosis [30]. The first meiotic division generates a 32-cell secondary spermatocyte; the germ cells remain connected by intercellular bridges [30]. Following the second meiotic division, the mitochondria in each cell accumulate to form two large mitochondria, which then interleave to form a structure termed a Nebenkern [30]. Upon completing meiosis, 64 spermatids are present and begin to elongate in synchrony [30]. The somatic cyst cells begin to behave differently, as head and tail cyst cells; the head cyst cell adheres near the base of the testis, to the terminal epithelium, while the tail cyst cell changes drastically in shape as the spermatids elongate [30]. Individualization then occurs, where each spermatid separates from its siblings within the cyst, via an actin-based structure, the investment cone [30]. As the investment cone progresses along each spermatid tail, excess cytoplasm is removed into a waste bag, which is eventually deposited into the testis lumen [30]. Individualized sperm are then extruded from the testis into the seminal vesicle, where they are coiled and stored until mating [30].

1.2.4. Parallels between fly and mammalian spermatogenesis

The general mechanisms of spermatogenesis in mammals, particularly in rodents and humans are conserved with flies. Within the mammalian testis, the somatic Sertoli cells

nurture germ cells in the mammalian testis and contribute to germ cell regulation [9, 43]. Sertoli cells are housed in seminiferous tubules in the testis and fundamentally form an epithelial layer which germ cells transit through during spermatogenesis [43]. Undifferentiated spermatogonia reside between the basal lamina and Sertoli cells [43]. As a spermatogonia divides, it gives rise to one spermatogonia which will remain undifferentiated and another which will undergo limited rounds of mitosis to generate primary spermatocytes [26, 43]. While Sertoli cells interact with the spermatogonia throughout germline development, one of the most crucial interactions occurs when the Sertoli cells remodel their tight junctional complexes to form a diffusion barrier around the differentiating spermatogonia [9, 43, 44]. The diffusion barrier, known as the blood-testis barrier, serves several important roles: it forms an immunological barrier for the germ cells, it induces cell polarity, and it creates an isolated signalling environment around the germline [45]. Following the completion of meiosis, the spermatids individualize to form flagellated sperm, and are released into the lumen of the seminiferous tubules [43]. The entire process requires tightly regulated signalling, the remodelling of cell-cell junctions, and the translocation of germ cells through the tissue, each of which is dependent on soma-germline interactions [9, 43, 44].

Spermatogenesis in flies largely resembles the process in mammals. Undifferentiated germ cells give rise to a daughter cell that remains a stem cell and a daughter that differentiates [30, 31]. Interactions with the soma begin before differentiation begins, such as the somatic control of GSC maintenance and self-renewal [31]. The developing germline cyst is encysted by the somatic support cells, which eventually form a barrier around the germline that isolates the cyst from niche-derived signals [31, 32]. Following meiosis, the spermatids

elongate, individualize to form flagellated sperm, and are extruded from the testis, thereby ending their relationship with the support cells [30]. Thus, spermatogenesis in *Drosophila* follows the same basic principles of mammalian spermatogenesis.

Sperm formation is a complex process, however, and there are significant differences between flies and mammals. In flies, spermatogenesis is cystic, rather than non-cystic as observed in mammals [10, 26]. In cystic spermatogenesis, germ cells travel through the testis with their support cells, as a functional unit, rather than migrating through a seminiferous epithelium [30, 46]. Furthermore, only two somatic cells interact with each germline cyst, whereas two Sertoli cells can interact with many spermatogonia at a time in non-cystic spermatogenesis [10, 46]. Sertoli cells are post-mitotic by the time spermatogenesis begins in earnest in mammals, while CySCs are continually active in the fly testis, and the somatic support cells undergo turnover in cystic spermatogenesis [10, 46]. Mammalian systems also utilize a greater number of signals, both local and systemic, for spermatogenesis, compared to flies [26]. Despite these differences, *Drosophila* spermatogenesis can provide important insight into the mechanisms of soma-germline communication.

The simplicity of the spermatogenic unit in flies, one germline cyst for every two somatic support cells, reduces the difficulty of investigating somatic control of germline development [31]. The CySC population in the fly testis enables the study of somatic stem cell regulation as well as proliferative control. Misregulation of Sertoli cell quiescence in a mammalian testis can disrupt spermatogenesis for numerous germline cysts, particularly given the longevity of the soma in mammals, whereas each pair of cyst cells in the fly testis undergoes apoptosis upon completing spermatogenesis [10]. While the complexity of signalling within the mammalian testis increases the difficulty of addressing basic biological

questions, simpler signalling mechanisms in *Drosophila* enable a researcher to gain mechanistic insight into soma-germline signalling [26]. The essential mechanisms of terminal differentiation can be elucidated more easily for the same reason. Finally, the process of spermatogenesis, in addition to generation time, is more rapid in flies, increasing the experimental potential of the fruit fly.

1.2.5. Role of stem cells in spermatogenesis

In order to maintain the lifelong production of gametes, an animal must often maintain a population of stem cells that can be directed to differentiate and form gametes. Stem cells are cells that are maintained in an undifferentiated state within a tissue, but can self-renew by dividing to generate a daughter cell that will remain a stem cell and a second cell that can then undergo differentiation. The behaviour of stem cells must be tightly regulated to prevent premature differentiation. The microenvironment of a tissue which maintains stem cells and regulates their behaviour is termed the stem cell niche [16]. The niche can contribute to the regulation of stem cells through the secretion of small signalling molecules which prevent precocious differentiation, increase the adhesiveness of stem cells for the niche to ensure they can receive self-renewal signals, and control the asymmetric division of stem cells [7]. Laser ablation experiments that abolished a putative niche, transplantation experiments which demonstrated that transplanted stem cells could find and migrate to a presumptive niche, and mutations that disrupted stem cell self-renewal were instrumental for identifying stem cell niches in a variety of tissues [47]. Ablation of cells at the apical tip of the fly ovariole, as discussed above, resulted in the inability of the ovariole to generate egg chambers [20]. And over a decade of studies in the fly testis has demonstrated that the hub activates multiple signalling pathways to prevent GSC and CySC

differentiation within the niche, such as by activating BMP signalling or up-regulating E-Cadherin-mediated cell-cell adhesion [31]. The stem cell niche plays an essential role in tissues for ensuring the regeneration of tissues after tissue damage, during aging, or in the case of the testis, maintaining production of sperm throughout the reproductive life of the organism.

The stem cell niche is not a static structure; the niche can both respond to tissue-level signals and often actively maintains its discrete location. The hub cells that form the testis stem cell niche in flies are mitotically-quiescent and function to maintain GSC and CySCs [30, 31]. In a study by Gónczy & DiNardo, it was demonstrated that mutations that disrupt the germline led to the disruption of the hub cell population as well [48]. The hub itself adheres to a dense layer of extracellular matrix (ECM) proteins in the basal lamina of the testis [22]. Experiments by Tanentzapf *et al*. have revealed that the attachment of the hub is dependent on cell-ECM adhesion [49]. Mutations that disrupt the cell-ECM adhesion proteins Integrin or Talin also disrupt the formation of the hub during embryonic development [49]. Furthermore, RNAi-mediated knockdown of Talin in adult testes results in detachment of the hub from the basal lamina and loss of the stem cell niche [49]. In a screen to identify regulators of hub cell maintenance, Resende *et al*. identified a role for Headcase in preventing apoptosis in the hub [50]. RNAi-mediated knockdown of Headcase resulted in a hub cells undergoing apoptosis, stem cell loss, and eventual degeneration of the niche [50]. The transcription factor Escargot has been identified as playing a role in both hub maintenance and quiescence. Voog *et al*. demonstrated that the depletion of Escargot from hub cells leads to hub cells acquiring CySC characteristics and differentiating as cyst cells [51]. A study by Hétié *et al*. has shown that the genetic ablation of CySCs additionally causes

hub cells to re-enter the cell cycle to repopulate the original CySC population [52]. Ectopic expression of the cell cycle regulator Cyclin D-Cdk4 in the hub can also induce the hub cells to re-enter mitosis and convert to a CySC state [52]. Together, these studies demonstrate that the stem cell niche itself must be as carefully regulated as the stem cells that reside within it.

1.2.6. Origin of the primordial germ cells, migration to the embryonic gonad, and the establishment of germline stem cells

During development in many organisms, the germline is one of the first cell types which is specified and formed. In *Drosophila*, the embryo begins development by undergoing a series of synchronous nuclear divisions without cellularization [53]. Following the 10th round of nuclear divisions, 8-10 nuclei move closer toward the posterior pole of the syncytial embryo, undergo cellularization, and asynchronously divide to form approximately 40 cells [54]. These are the first cells to form in the embryo and are termed pole cells or primordial germ cells (PGCs). Following their formation, the PGCs mitotically arrest, while the somatic cells of the embryo undergo several additional synchronous nuclear divisions before migrating to the cell membrane, where they undergo cellularization [55].

Before the PGCs have even formed, they are committed to their germ line fate, as a result of their cytoplasmic inheritance of the maternally-contributed pole plasm [53]. The pole plasm is composed of complexes of RNA and proteins, which form electron-dense aggregates known as polar granules when viewed by electron microscopy, [53]. Polar granules contain a number of conserved RNA-binding proteins, such as Oskar, Vasa, Tudor, and Nanos [56]. The *oskar* gene is necessary for pole plasm assembly, upstream of all other polar granule components, and *oskar* mRNA is sufficient to ectopically induce germ cell formation [57, 58]. Vasa, a highly conserved RNA helicase required for pole plasm assembly

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downstream of Oskar, has a conserved role in germ cell formation in mice [59-61]. *tudor* was one of the first genes identified for polar granule and pole cell formation [62]. Subsequent analysis revealed that Tudor is highly conserved and provides docking platforms for polar granule assembly [63, 64]. After germ cell formation, transcription and translation in PGCs is repressed to maintain germ cell fate [56]. *nanos* mRNA is maternally deposited and enriched in the pole plasm, where Nanos functions as a transcriptional and translational repressor; embryos with *nos* mutant germ cells display precocious germline transcription, divide too early, have defects in PGC migration, and often die before the end of embryogenesis [56, 65]. Together, these polar granule components are required for PGC formation. However, the PGCs do not remain at the posterior pole of the embryo following their formation, but must passively and actively migrate to the presumptive gonad.

By Stage 6 of *Drosophila* embryogenesis, approximately 3 hours after fertilization, the somatic cells have completed cellularization and gastrulation has occurred [56]. The PGCs tightly adhere to the presumptive mid-gut, before they undergo a series of movements and migrations across the embryo to eventually reach the embryonic gonad [56]. The first of these movements occurs during germ-band extension, whereby the embryo lengthens along its anterior-posterior axis and narrows along its dorsal-ventral axis [56]. This event passively translocates the PGCs from the posterior pole of the embryo to a dorsal position [56]. As the midgut primordium invaginates, the PGCs are pulled into the embryo [66]. Following invagination, the active migration phase begins, where the PGCs migrate through the mid-gut epithelia to eventually reach the gonadal mesoderm [66]. Several genes have been implicated in the active migration process. *trapped in endoderm 1* encodes a novel G-protein coupled receptor that is required in the migrating germ cells for migration through the endoderm [67].

PGC migration through the endoderm takes advantage of endoderm remodelling; precocious remodelling of the endoderm results in precocious germ cell migration, whereas delays in remodelling correspond to delays in PGC migration [68]. Once the PGCs migrate through the midgut, *wunen* and *wunen-2* — which encode lipid phosphatases — are required in the soma to repel the germ cells and drive them into the mesoderm [69, 70]. The mesoderm provides an attractive cue to the PGCs, dependent upon the gene encoding 3-Hydroxy 3-Methylglutaryl Coenzyme A [56, 71]. Upon reaching the gonadal mesoderm, PGCs coalesce with somatic gonadal precursors (SGPs), and compact to form the gonad [71]. The zinc transporter encoded by *fear of intimacy* and the *Drosophila* E-cadherin homolog *shotgun* work together to regulate the cell sorting and adhesion required for gonad coalescence and compaction [72, 73]. Once the PGCs have arrived in the bilateral gonadal mesoderm, a subset of PGCs eventually become established as GSCs, and play roles in promoting hub formation at the anterior of the gonad, and repressing hub formation at the posterior. Before the PGCs migrate through the midgut epithelia, however, the SGPs must also be specified, before intermingling with the PGCs and eventually forming two gonads.

1.2.7. Specification of the hub stem cell niche and cyst stem cells

The SGPs are essential for forming the somatic tissues of the eventual gonad, including the hub cells and the CySCs. Without formation of the somatic gonad, the PGCs will not be able to establish themselves as GSCs, form gametes, and reproduce [3]. During Stage 10 of embryogenesis, SGPs are specified from the dorsolateral mesoderm in parasegments 10, 11, and 12 as bilateral clusters; a fourth cluster, the male-specific SGPs, is specified in parasegment 13 [3, 74, 75]. Among the many genes required for SGP cell fate, the expression of two homeotic genes, Abdominal A and Abdominal B, are required for the

specification of the SGPs, where Abdominal A specifies the anterior SGP fates and Abdominal A and B together specify posterior fates [74]. By Stage 12, the PGCs have reached the gonadal mesoderm and begun to intermingle with the SGPs, as the SGP clusters migrate and coalesce [3, 74]. The SGPs begin to wrap the PGCs at Stage 13 such that the PGCs become separated from one another [3, 73]. The large Maf transcription factor Traffic jam (Tj) is required for this ensheathment, possibly through the regulation of cell-cell adhesion [76]. After the PGCs and SGPs coalesce, they condense in parasegment 10 to form two round, compact gonads, with the male-specific SGPs at the posterior end of the gonad, where they will eventually give rise to the terminal epithelium [3, 74, 75]. The SLIT/ROBO pathway, which is important for axon guidance, has been implicated in the process of gonad compaction [3, 77]. *slit* and *robo2* mutants both show defects in the fusion of SGP clusters and do not complete gonad compaction [77]. It is not well understood, however, how the SLIT/ROBO guidance system contributes to gonad coalescence and compaction [3]. Following this process, the GSC, CySC, and hub cell fate is specified.

Several different signalling pathways play important roles in the formation of the hub from SGPs, the establishment of GSCs from PGCs, and CySCs from SGPs. Notch, epidermal growth factor receptor (EGFR), and Boss/Sev signalling have demonstrated roles in specifying hub cells by Stage 17 of embryogenesis. One study revealed that the endoderm expresses the ligand Delta, while another study suggested that SGPs themselves express the Serrate ligand; both ligands function to activate Notch signalling in nearby SGPs [78, 79]. Conversely, receptor tyrosine kinase signalling mediated by the Bride of sevenless (Boss) ligand and the EGFR ligand Spitz are expressed in posterior PGCs to prevent hub formation in the posterior of the gonad [79, 80]. The establishment of GSCs from a subset of PGCs

requires JAK/STAT signalling, which is activated in PGCs beginning at Stage 13, but becomes restricted only to the most anterior PGCs by Stage 17 [81]. This results in an up-regulation of E-Cad and the establishment of the most anterior PGCs as GSCs [81]. A study by DiNardo *et al*. identified a requirement for the segment polarity protein Lines in SGPs for specifying CySC fate and the transcription factor Bowl in promoting hub cell fate [82]. *lines* mutant SGPs were observed to take on a hub-like fate, whereas loss-of-function mutations in *bowl* resulted in fewer hub cells forming [82]. Subsequent work by Wingert & DiNardo identified a specific role for Bowl in mediating hub assembly, downstream of Notch signalling [83]. Taken together, these studies have provided insight into the specification of gonadal cell types during embryogenesis, but many questions remain to be studied.

1.3. Soma-germline signalling during early spermatogenesis

Throughout formation of the embryonic gonad, cell-cell signalling plays an essential role. Once the germline ends its migration in the gonad, soma-germline interactions in the testis can begin in earnest. For instance, signals are required from the hub cells to PGCs to ensure the establishment of GSCs, the establishment of CySCs, and to prevent the differentiation of either stem cell within the niche (see **Fig. 1.2.** for a summary of signalling within the stem cell niche). The hub also contributes to the self-renewal of the CySCs [31]. As a GSC divides and gives rise to two daughter cells, the daughter cell which is displaced from the niche begins to differentiate. Differentiation occurs as the germ cell is no longer able to receive high levels of BMP ligands that prevent germline differentiation, upon exit from the niche. [31, 84, 85] This differentiating germ cell also signals to cyst cells, which have also exited the niche, to encapsulate the germ cell and begin to isolate it [32, 86]. The germline cyst undergoes a series of transit-amplifying divisions to form a 16-cell

interconnected spermatocyte, which then grows and prepares for meiosis. Soma-germline signalling is essential during these divisions to ensure the germline cyst divides exactly four times; fewer divisions produce fewer eventual spermatids, whereas a greater number of divisions pose size problems during the remainder of spermatogenesis [30]. The soma and germline must continue to communicate during and after meiosis, until the spermatids terminally differentiate and are extruded from the testis. Signalling missteps at any point between the stem cell niche and terminal differentiation have the potential to lead to infertility, and thus the failure of the germ line to ensure its immortality. Many studies have provided insight into the cell-cell signalling that the soma and germline utilize during spermatogenesis, which are reviewed below.

1.3.1. JAK/STAT signalling

The hub cells form a signalling centre in the testis, secreting factors required for regulating the self-renewal of both GSCs and CySCs, as well as maintaining these stem cells in an undifferentiated state within the stem cell niche. One of the first signalling pathways shown to be important for stem cell regulation in the fly testis was Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signalling [23, 24]. The JAK/STAT signalling pathway represents one of the most direct routes from transducing an extracellular signal into transcriptional cell outcomes. The secreted cytokine Unpaired, an IL-6 family member, binds to a receptor tyrosine kinase, causing dimerization of receptors via phosphorylation of the receptors, JAKs, which then recruit and phosphorylate the cytoplasmic STAT protein, which dimerizes and then translocates into the nucleus to activate transcription of target genes [7, 87]. Thus, JAK/STAT signalling essentially involves a ligand, a receptor, a kinase, and a transcriptional activator to alter cell-cell signalling.

In attempts to identify signalling pathways that were important for hub-mediated stem cell maintenance, two groups investigated JAK/STAT signalling in the *Drosophila* testis [23, 24]. In flies, there are three JAK/STAT ligands, Unpaired (Upd), Unpaired-2, and Unpaired-3, a single receptor, Domeless, a single JAK, Hopscotch (Hop), and a single STAT transcription factor (Stat92E) [87]. In two separate studies, Tulina *et al*. and Kiger *et al*. demonstrated flies carrying loss-of-function mutations in either *hop* or *Stat92E* possessed a hub but lacked stem cells and spermatogonia [23, 24]. Clonal analysis was used to show that *hop* and *Stat92E* are required in GSCs for GSC maintenance; GSCs lacking either component were displaced from the niche and lost to differentiation [23, 24]. Furthermore, hyper-activation of *Stat92E* in wild-type testes expanded the populations of both CySCs and GSCs [23, 24]. This was attributed to a requirement for JAK/STAT signalling for stem cell self-renewal. Finally, both groups demonstrated that *upd* mRNA could be detected within the hub cells, strongly indicating that the hub was the source of the JAK/STAT ligand that then acted on GSCs and the soma [23, 24]. These studies formed the basis of additional experiments to gain insight into JAK/STAT signalling during spermatogenesis.

During embryonic development, JAK/STAT signalling is employed for the establishment of PGCs as GSCs. A study by Sheng *et al*. demonstrated that PGCs begin to accumulate Stat92E in the newly formed gonad, around Stage 13 [81]. By Stage 17, Stat92E accumulation becomes restricted to anterior PGCs, located nearest to the hub [81]. The homophilic cell-adhesion protein E-Cadherin (E-Cad) also becomes enriched at the hub-germ cell interface at this time [81]. E-Cad-mediated adhesion of GSCs to the hub has since been shown to be necessary for GSC maintenance [88]. Similarly, SGPs also show activation of the JAK/STAT signalling pathway during Stage 17 of embryogenesis and loss of Stat92E

prevents the establishment of CySCs [89]. JAK/STAT signalling thus plays important early roles in the *Drosophila* testis.

Additional analysis using a temperature-sensitive, hetero-allelic combination of *Stat92E* alleles demonstrated that differentiating spermatogonia could de-differentiate and repopulate a depleted stem cell niche. Upon shifting flies to the restrictive temperature, GSCs began to show signs of differentiation and eventually were lost from the niche [90]. If flies were returned to the permissive temperature, GSCs could gradually repopulate the stem cell niche [90]. This effect was dependent on the presence of 2-, 4-, and 8-cell stage spermatogonia (but not 16-cell stage spermatogonia), as the effect was not observed if spermatogonia had differentiated to spermatocytes before the return to the permissive temperature [90]. This demonstrated a requirement for JAK/STAT signalling for germline cysts to de-differentiate to repopulate a depleted stem cell niche. A similar mechanism may also occur in mammalian testes based on observations of 3-, 5-, and 11-cell spermatogonia, which may indicate fragmented cysts [91].

Uncovering the targets of JAK/STAT signalling in the testes has been an important goal for researchers interested in *Drosophila* spermatogenesis [92]. Zinc finger homeodomain-1 (Zfh-1) was identified as a potential downstream target of JAK/STAT signalling in CySCs [92]. A study of Zfh-1 demonstrated that it is required for CySC self-renewal and its gradual degradation is required for the differentiation of the soma and, non-autonomously, of the germline [93]. Furthermore, Leatherman & DiNardo were able to determine that Stat92E activity in the soma is required for GSC maintenance [93]. Subsequently, Leatherman & DiNardo demonstrated that JAK/STAT signalling was required in the germline primarily for regulating GSC adhesion to the hub, but not self-renewal [94].

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Because GSCs could be maintained away from the hub by CySCs, it became evident that CySCs produce GSC self-renewal factors [94]. This finding shifted the paradigm of how JAK/STAT signalling contributes to stem cell behaviour in the testis and revealed that the hub and CySCs form an extended niche for GSCs.

Other JAK/STAT signalling targets, including *chronologically inappropriate morphogenesis* (*chinmo*), *chickadee* (*chic*), and *Drosophila inhibitor of apoptosis 1* (*diap1*) have also been demonstrated to play important roles in spermatogenesis [95-97]. Loss-of-function analysis of *chinmo* demonstrated that, similar to *Stat92E*, it is present in both GSCs and CySCs, but its function is only required for CySC maintenance [95]. Over-expression of *chinmo* also resulted in an expansion of early somatic and germ cell populations, resembling the phenotype of *Stat92E* hyper-activation [95]. Despite resembling *zfh-1* phenotypes, *chinmo* does not act through *zfh-1* in CySCs. A mutant screen by another group identified a partial loss-of-function allele of *chinmo* which resulted in a feminization of the somatic cells of the testis, resembling soma development in the fly ovary [98]. *chinmo* was shown to act through the canonical *Drosophila* sex determination pathway to maintain the male identity of the soma [98]. A study of *chic*, which encodes the fly homologue of the mammalian actin-binding protein Profilin, found that Stat92E binds near the *chic* promoter [96]. Additionally, *chic* was shown to be required in GSCs for E-Cad localization to the hub-GSC interface, enrichment of actin at the hub-GSC interface, and subsequently for GSC maintenance [96]. To elucidate the role of cell death pathways in stem cell maintenance, Hasan *et al*. demonstrated that altering Stat92E levels led to a corresponding increase or decrease in DIAP1 protein levels [97]. *diap1* was also found to be required for stem cell survival, while reduced Stat92E made stem cells more susceptible to stress-induced cell death [97]. While

chinmo, *chic*, and *diap1* are all targets of JAK/STAT signalling in the testis, they each play a very different role and highlight the importance of understanding the role of cell-cell signalling in regulating cell behaviour.

1.3.2.   BMP signalling

In addition to regulating cell-adhesion properties and stem cell self-renewal, stem cell niches often actively maintain stem cells in an undifferentiated state. Thus, it would be expected that the hub might secrete additional signals that either promote "stemness" or repress differentiation in adjacent cells. Indeed, studies investigating the Bone Morphogenic Protein (BMP) signalling pathway have provided important insight into the mechanisms of stem cell maintenance in the testis [8]. The BMP family belongs to the larger TGFβ superfamily of secreted dimeric proteins [7]. BMP signalling is implemented throughout metazoan lineages to regulate development and differentiation [99]. Two related families of receptors function together to transduce BMP signals [7, 99]. Type II receptors are constitutively-active serine-threonine kinases which binds to BMP ligands, and phosphorylate Type I receptors, which also binds to the ligand [99]. An active receptor complex includes two of each receptor type, likely due to the dimeric nature of the BMP ligand [99]. Activated Type I receptors bind to and phosphorylate Smad regulatory proteins (R-Smads), which associates with common mediator Smads (Co-Smads) and translocate into the nucleus to directly regulate gene expression [7, 99, 100]. In *Drosophila*, there are three main BMP ligands, Decapentaplegic (Dpp), Glass bottom boat (Gbb), and Screw; Dpp is a homolog of vertebrate BMP2/BMP4 and Gbb and Screw are homologs of BMP5, BMP6, BMP7, and BMP8 [99, 100]. Type I receptors in flies include Thick veins (Tkv), Saxophone (Sax), and Baboon, whereas Punt and Wishful thinking compose the Type II receptors [99,

100]. Flies possess four Smads: Mothers Against Decapentaplegic (Mad) is the main R-Smad in flies; Smad on the X (Smox) also acts as an R-Smad; the co-Smad Medea (Med); and the antagonistic Smad Daughters Against Decapentaplegic (Dad), which is expressed in response to BMP signalling as a negative-feedback [99, 100]. Analysis of the BMP signalling pathway in the testis has provided significant insight into stem cell self-renewal and control of differentiation.

Early analysis of BMP signalling implicated the pathway in regulating the germline [101]. Matunis *et al*. performed a screen to identify genes required for regulation of germline proliferation and identified a requirement for the transcription factor Schnurri, that was known to be required for responding to Dpp signals [101]. Their analysis indicated that Schnurri was required in the soma to prevent over-proliferation of differentiating germ cells in a subset of testes [101]. Previously, only *bag of marbles* (*bam*) and *benign gonial cell neoplasm* were implicated in regulating germline proliferation, as loss of either resulted in over-proliferation and an inability of the germline to differentiate [102]. A subsequent study by Shivdasani & Ingham demonstrated that the germline responds to somatic Gbb ligands to activate BMP signalling, which is required for GSC maintenance [85]. Inability to transduce BMP signalling resulted in premature differentiation of the germline [85]. This was likely due to the fact that BMP signalling in the germline repressed Bam expression [85]. Additional analysis by Kawase *et al*. revealed that in addition to Gbb, Dpp is also required for GSC maintenance, and clonal analysis showed that *tkv*, *put*, *sax*, *mad*, and *med* were all essential for this maintenance [84]. BMP signalling was required to repress *bam* expression in GSCs and forced *bam* expression, via a heat shock-inducible transgene, drove the differentiation of GSCs [84]. The source of the Gbb and Dpp ligands was demonstrated to be

the hub cells and CySCs, based on mRNA expression analysis [84]. In the soma, Li *et al*.

identified a requirement for Sax and Smox, a Type I receptor and R-Smad, respectively

[103]. Loss of Sax could be partially rescued by over-expression of Smox in the somatic

lineage [103]. Both proteins were required non-cell-autonomously for the regulation of

germline proliferation, by restricting the expression of *bam* [103]. Together, these studies

shed light on the role of BMP signalling in stem cell self-renewal and regulation of

differentiation.

Control of BMP signalling also plays a role during the transit-amplifying divisions of

spermatogonia. Smurf, an E3 ubiquitin ligase shown to be important for regulating

proteolysis of BMP receptors in the fly ovary, was shown to be required for the

developmental regulation of BMP signalling in larval testes and for ensuring germ cells only

generated 16-cell cysts prior to meiosis [104, 105]. During development, phosphorylated

Mad (pMad) accumulates in GSCs and early germ cells, only to become restricted to GSCs

immediately next to the niche; this restriction of pMad was shown by Chang *et al*. to be

dependent on Smurf [105]. Failure to down-regulate pMad in *smurf* mutant testes resulted in

extra transit-amplifying divisions, leading to the formation of cysts with 128 spermatids

versus the usual 64 spermatids [105]. Increased division during transit amplifying stages

often disrupts the terminal spermatid differentiation [105]. In a study by Fairchild *et al*.,, it

was demonstrated that isolation of the germline by the somatic cyst cells is required to

prevent the differentiating germline from accessing niche-derived BMP ligands [32].

Disruption of this physical barrier, formed by the fly homologs of occluding junctions,

extended the range of BMP signalling, prevented the timely expression of Bam, and resulted

in sterility [32]. These studies demonstrate important mechanisms by which BMP signalling can be regulated in the testis.

1.3.3. Hh signalling

Hedgehog (Hh) signalling plays important roles in embryonic patterning and the regulation of cell proliferation in many contexts [7]. Body segment patterning in *Drosophila*, regulation of intestinal stem cells in the gut of vertebrates and flies, and patterning of the endoderm in vertebrates require Hh signals [106]. The components of the Hh pathway were characterized in *Drosophila*, beginning with the discovery of the *hedgehog* gene required for larval bristle patterning; *hh* mutant larvae are covered in disorganized bristles, resembling hedgehogs, rather than the orderly pattern found in wild-type larvae [7, 107]. Secreted Hh binds to iHog and the 12-pass transmembrane receptor Patched (Ptc) on the cell surface [7, 106]. In the absence of Hh ligand, Ptc prevents the 7-pass transmembrane protein Smoothened (Smo) from reaching the cell surface; when Hh binds to Ptc, Ptc can no longer sequester Smo [7, 106]. Smo then becomes phosphorylated and moves to the cell surface where it can mediate downstream signalling, while Ptc is endocytosed and degraded [7, 106]. Without Hh signalling, the downstream regulatory protein Cubitus interruptus (Ci) is normally bound to a degradation complex which cleaves it to produce a protein fragment that acts as a transcriptional repressor of Hh-responsive genes; with Hh signalling, Smo prevents the cleavage of Ci, where it can enter the nucleus and activate Hh target genes [7, 106]. In the context of gametogenesis, Hh has been demonstrated to be required in the ovary for regulating follicle stem cells proliferation and oocyte patterning [108-110]. While it has been known for nearly two decades that hub cells express *hh*, little else was known about Hh signalling in the *Drosophila* testis [108].

Several groups have since investigated the roles of Hh signalling during early spermatogenesis. An initial study by Michel *et al*. demonstrated through clonal analysis that *smo* is required in CySCs for stem cell maintenance, whereas *ptc* is required in CySCs to prevent over-proliferation [111]. GSCs, in contrast, did not require Hh signalling for their maintenance [111]. *smo* mutant cells cannot respond to Hh ligands, whereas *ptc* mutant cells experience constitutive pathway activation [111]. Excessive Hh signalling has been implicated in the formation of some cancers in mammalian systems, such as basal cell carcinoma and medulloblastoma [107].

Work by another group, Amoyel *et al*., demonstrated that Hh signalling in CySCs promotes self-renewal independently of JAK/STAT signalling [112]. Furthermore, while constitutive activation of JAK/STAT signaling resulted in stem cell tumour formation, loss of Hh signalling caused these tumours to lose stem cell markers [112]. Subsequent experiments by Amoyel *et al*. demonstrated that *ptc* mutant CySCs, which have constitutive Hh signalling, can outcompete wild-type CySCs for niche occupancy, and eventually displace GSCs [113]. They also found that *ptc* mutant CySCs proliferated faster than controls and this increased proliferation was both necessary and sufficient for outcompeting wild-type CySCs [113]. In the testis, preventing CySCs from outcompeting GSCs is crucial as failure to do so would eventually lead to infertility. These studies highlight the importance of tightly regulating a signalling pathway to promote stem cell self-renewal and how misregulation of a single signalling mechanism can lead to sterility.

1.3.4. EGFR signalling

Epidermal Growth Factor Receptor (EGFR) signalling is an important signalling pathway implicated in many different types of cancer [7, 114]. The study of EGFR signalling

is complicated by the fact that a multitude of ligands, receptors, and downstream signalling components exist in vertebrates; in flies, however, there are four identified ligands and a single receptor which has simplified research into pathway [114]. EGFR is a receptor tyrosine kinase that can be activated by four ligands, Spitz, Gürken, Vein, and Keren, and is inhibited by Argos [115]. Spitz is the most widely-expressed EGFR ligand in *Drosophila* and its secretion is regulated by two transmembrane proteins, Rhomboid and Star [116]. Star translocates Spitz from the endoplasmic reticulum to the Golgi apparatus, where Rhomboid is enriched and functions as a protease to cleave Spitz into an active form [116]. Spitz can then be secreted by the cell and bind to the EGFR on the surface of nearby cells [114]. Once bound to EGFR, the receptors dimerize and phosphorylate one another [7, 117]. Proteins which can bind to phosphorylated tyrosine sites dock onto the active receptor and go on to activate Ras, a monomeric GTPase [7, 117]. Ras can then activate the Mitogen Activated Protein (MAP) Kinase signalling cascade, where activated Ras phosphorylates Raf/MAP kinase kinase kinase, which phosphorylates Mek/MAP kinase kinase, which in turn activates Erk/MAP kinase, leading to further changes in the activity of protein or transcription targets [7, 117]. MAP kinase signalling is used in several different signalling pathways but it is meticulously regulated to control signalling outputs [7, 117].

EGFR signalling was one of the first pathways implicated in stem cell regulation in the fly testis. Kiger *et al*. used a temperature-sensitive loss-of-function allele of *egfr* to demonstrate that EGFR signalling is required during spermatogenesis to restrict GSCs self-renewal and promote their differentiation [118]. Clonal analysis revealed that *egfr* is required in the soma but not the germline, and may be responding to the germ cell-provided ligand Spitz [118]. This was supported by evidence that MAP kinase was activated in somatic cells

near the niche and spermatocyte-stage somatic cells, but not in germ cells [118]. An accompanying study by Tran *et al*. demonstrated that loss of the fly homologue of MAP kinase kinase kinase, *raf*, phenocopied loss of EGFR signalling and the *raf* gene was required specifically in the soma [119]. Analysis by Schulz *et al*. identified a germline requirement for *stet*, encoding a protease related to Rhomboid that may also cleave Spitz [120]. Germline loss of *stet* greatly resembled the EGFR loss-of-function phenotype identified by Kiger *et al*. [120]. Using cytoplasmic somatic markers, Schulz *et al*. revealed that *stet* was required in the germline for somatic encapsulation [120]. The identification of a temperature-sensitive *spitz* allele enabled a study by Sarkar *et al*. that demonstrated that Spitz is required in the germline for encapsulation by the soma [121]. Furthermore, Sarkar *et al*. were able to show that EGFR binds the downstream effector protein Vav, which activates the small GTPase Rac1, which in turn mediates the cell-shape changes necessary for enclosure of the germline [121]. Together, these studies demonstrated how a signal from the germline, Spitz, causes the soma to encapsulate the early germ cells, thereby promoting germline differentiation, upon exit from the stem cell niche.

Study of hub formation during embryonic development also revealed a role for EGFR signalling. A study by Kitadate & Kobayashi found that PGCs activate EGFR signalling in the SGPs via Spitz [79]. Failure to activate EGFR signalling in the posterior SGPs resulted in ectopic hub formation, whereas activation of the EGFR pathway in anterior SGPs was sufficient to repress hub formation [79]. This demonstrated a much earlier requirement for EGFR in the male gonad than previous studies.

A spate of recent studies have implicated EGFR signalling in regulating GSC division frequency, indicated that the level of EGFR signalling determines cell fate, and suggested

that nuclear lamins control EGFR responses in the soma [122-124]. Parrott *et al*. analyzed the division frequency of GSCs in control and *spitz* mutant backgrounds and found that GSCs divide more often when EGFR signalling is compromised [122]. It was suggested that this effect was because the soma directly regulates GSC division frequency and depends on EGFR signalling to do so [122]. This effect was not found upon disruptions of JAK/STAT or BMP signalling, indicating this effect was unique to EGFR signalling [122]. A study by Hudson *et al*. modulated levels of EGFR signalling after encapsulation and found that the levels of signalling must be tightly regulated [123]. Decreased levels of EGFR ligands post-encapsulation disrupt the coordinated differentiation of germ cells within a cyst, whereas high levels of EGFR signalling promote precocious differentiation of the soma and caused the germ line to prematurely initiate terminal differentiation [123]. Finally, a study by Chen *et al*. revealed that the structural nuclear protein lamin-B (Lam) was required for spermatogenesis in *Drosophila*, specifically in CySCs [124]. Loss-of-function analyses showed that the *lam* phenotype greatly resembled EGFR loss-of-function phenotypes [124]. Consistent with this, staining for diphosphorylated ERK (dpERK), indicative of EGFR pathway activation, revealed that dpERK failed to translocate from the cytoplasm to the nucleus in *lam* mutant testes [124]. Expression of a transgenic form of ERK containing a nuclear localization signal in testes depleted of *lam* partially suppressed the *lam* phenotype, supporting this idea [124]. Taken together, these studies illustrate the use of one signalling pathway to regulate stem cell self-renewal, cell shape changes, and cell fate.

1.4. Mechanisms of cell-cell signalling

Multicellular organisms employ essentially four basic mechanisms of cell-cell signalling: autocrine, paracrine, endocrine, and juxtacrine signalling [7]. Autocrine signalling

occurs where the cell that secretes a signal is the target cell that responds to that signal. Paracrine signalling is the release of signals to the extracellular space which act on nearby cells [7]. The release of neurotransmitters by neurons is a classic example of paracrine signalling [7]. Endocrine signalling involves the release of long-distance signals, which travel through the blood or hemolymph of an organism to reach targets far from the secreting cells [7]. Examples of endocrine signals include the sex hormones testosterone and estrogen, or metabolic hormones such as insulin [7]. Juxtacrine signalling, in contrast, requires contact between the communicating cells [7]. Lipids or proteins in the plasma membranes of adjacent cells may signal via contact-dependent interactions [7]. The requirement for direct contact between cells allows for the precise control of important signalling events during development [7]. For example, cells must be physically in contact to initiate the Notch signalling cascade [7]. Additionally, gap junctions can permit cells to use small molecules and ions to communicate, effectively linking the cytoplasm of neighbouring cells [7]. The use of gap junctions as a signalling mechanism allows for the rapid transmittance of signals across an entire tissue, such as electrical coupling in cardiac pacemaker cells [7]. Together, these four mechanisms allow for tightly regulated cell-cell signalling in multicellular organisms.

1.4.1. Paracrine signalling

Within the context of the *Drosophila* testis, many forms of paracrine signalling are employed to ensure stem cells are maintained within the stem cell niche and to provide differentiation cues such that spermatogenesis progresses in an orderly fashion. The hub is required to secrete the paracrine JAK/STAT ligand Upd in order for nearby primordial germ cells to become established as GSCs [81]. Upd continues to be required after embryonic

development for the maintenance of GSCs within the niche, by regulating the adhesiveness of GSCs for the niche [23, 24, 94]. BMP signalling ligands secreted by the hub and CySCs mediate the self-renewal of both GSCs and CySCs [84, 103]. The hub also secretes Hh signals that are required for the maintenance and self-renewal of the CySC lineage [111, 112]. EGFR signals from the germline to the soma ensure somatic encapsulation of the germline [118-121]. This signalling is essential to prevent the formation of germline tumours in the testis and promote differentiation of the germline [118-121]. Each of these paracrine signalling pathways provides important cues for spermatogenesis.

1.4.2. Endocrine signalling

Long-range endocrine signals also play an essential role in regulating the stem cell niche over time. The use of dietary restriction has been shown to result in extended lifespan in flies and other organisms, likely due to changes in insulin growth factor (IGF) signalling [125]. Dietary restriction in flies, by means of protein starvation, has shown that there is an age-dependent loss of GSCs [126]. Local activation of the insulin/IGF signalling pathway in GSCs and their support cells suppresses this loss in response to protein starvation [126]. Mutations in the *Drosophila* insulin receptor gene *InR* or the downstream adaptor protein *chico* (homologous to vertebrate Insulin Receptor Substrates) result in flies with testes containing fewer GSCs and fewer differentiating germline cysts [127]. Insulin signalling also promotes GSC cell cycle progression and the growth of spermatocytes [127]. These studies demonstrate the importance of insulin-mediated endocrine signalling on stem cell behaviour and differentiation of the germline.

Additional work has revealed that the germ line itself may affect the lifespan of the fly, representing another mechanism of long-range signalling, but from the germline to the

organism. The gene *germ cell-less* (*gcl*) is required early in embryogenesis for transcriptional quiescence; *gcl* loss-of-function mutations prevent the formation of pole cells and eventual germ cells[128] . Work by Flatt *et al.* (2008) demonstrated that *gcl* mutant flies, which lack a germline, live longer and exhibit altered IGF signalling, suggesting that germline-derived signals may regulate both lifespan and insulin sensitivity [55]. Interestingly, the removal or ablation of GSCs has been show to increase lifespan in worms [129, 130]. And in mice, neuronal-specific insulin receptor knockout results in defective spermatogenesis and ovarian follicle maturation, while ovariectomy leads to an increase level of circulating IGF receptor ligands [131, 132]. Thus, in addition to long-range somatic signals regulating GSC maintenance and self-renewal, the germline can in turn regulate the soma, in an evolutionarily conserved manner.

1.4.3. Autocrine signalling

The mechanism of autocrine signalling, whereby a cell responds to its own secreted signals, is not well-studied in the fly testis; however, autocrine signalling has been implicated in development of the germline. After the formation of the PGCs during the earliest stages of *Drosophila* embryogenesis, Wingless/Wnt (Wg) signalling is required for the initiation of mitosis in PGCs [133]. Experiments which increase of decrease the level of Wg in either the soma or PGCs result in a corresponding increase or decrease in PGC number [133]. *wg* mRNA is present in PGCs prior to the initiation of mitosis, suggesting that Wg functions as an autocrine signal in this context [133]. Similarly, it has been suggested that autocrine BMP signalling is required in PGCs for maintaining germline specification; loss of these signals results in a loss of PGC markers, such as the germline-specific protein Vasa [134]. Future

studies may demonstrate additional autocrine signalling mechanisms utilized after embryonic development of the gonad.

1.4.4. Juxtacrine signalling

Finally, juxtacrine signals play essential roles in the formation of the gonad during fly embryogenesis and during spermatogenesis. The very formation of the stem cell niche during embryonic development requires juxtacrine signalling via Notch-Delta to specify the hub cells, receiving signals from the endoderm [78, 79]. Gap junctional signalling has been implicated in regulating spermatogenesis based on the study of a putative germline-specific gap junction protein, Zero Population Growth (Zpg) [135]. While it is unknown if Zpg mediates juxtacrine signalling between adjacent germ cells or between the soma and germline, loss-of-function mutations in the *zpg* locus result in infertility in both males and females [135]. Juxtacrine signalling allows for exquisite control over cell fate decisions during development, as is often the case when Notch signalling is employed, but it can also be utilized for rapid cell-cell signalling and the transfer of small molecules and metabolites between neighbouring cells, in the case of gap junction-mediated signalling [7].

1.4.5. Gap junctions mediate juxtacrine cell-cell signalling

One of the quickest and easiest ways for cells to signal to one another can be via juxtacrine signalling. Specifically, gap junctions can permit rapid signalling from a cell to its neighbour via small molecules and metabolites, like cyclic adenosine monophosphate (cAMP) and inositol triphosphate (IP_3), or even via ions, such as Ca^{2+} [7]. This allows neighbouring cells to be coupled both electrically and metabolically [7]. The first hints of a means for rapid signalling between adjacent cells were found in studies of crayfish neurons over fifty years ago [136]. Furshpan & Potter demonstrated that action potentials passed

directly between interneurons and motor neurons in the crayfish nerve cord [137, 138]. Analysis of electron micrographs from a variety of vertebrate and invertebrate tissues suggested that these signals pass through a site of closely apposed cell membranes separated by a gap, termed the nexus [139]. From this, it was proposed that these sites may be the regions of the cell which permit electrical coupling from one cell to another [139]. The cell junction where the nexus was observed was then termed a gap junction [136].

While gap junctions were first found in invertebrates, the genes encoding gap junction proteins were first isolated in rat liver cells [140, 141]. These proteins were named connexins from their identification within the nexus and their role in functionally connecting cells [136]. More than twenty connexin genes have been identified in mice and humans [142]. Connexins are four-pass transmembrane proteins, with a cytoplasmic N-terminus, two extracellular domains, a central cytoplasmic domain, and a cytoplasmic C-terminus (**Fig. 1.3.**) [7]. A connexin hexamerizes in the endoplasmic reticulum with other connexins, before trafficking to the plasma membrane as a hemichannel (**Fig. 1.4.**) [7]. Two hemichannels on apposing cell surfaces can then dock to form an intercellular channel [7]. A group of many of these channels in a discrete region of the plasma membrane, a "plaque," is what generates the characteristic gap of gap junctions [7]. Invertebrate gap junction proteins, termed innexins, function in the same way as connexins to form gap junctions in flies, worms, and even hydra, although they lack sequence homology to connexins [143]. Twenty five genes encoding innexins have been found in *C. elegans* and eight have been identified in *Drosophila* [143]. Gap junctions are better understood in vertebrate systems owing to the earlier discovery of the genes encoding connexins.

The functions of gap junction proteins within species are diverse. Mutations which affect connexins have been implicated in neuropathies, cardiac problems, and infertility, among a host of other ailments [144]. Connexin (Cx) 32 mutations can lead to the progressive degeneration of peripheral nerves in humans [145]. Mice with a complete knockout of Cx32 in the liver suffer from increased frequency of tumours [146]. This supports a long-held view that intercellular channels may transmit inhibitory signals which govern growth control, where gap junctions act as tumour suppressors [147]. Cx40 was found to be required for the conduction of cardiac impulses in mice [148]. In the mouse ovary, Cx37 has been shown to be required for fertility [149]. Cx37 knockout mice oocytes arrest in their growth and are not meiotically competent [149]. Cx43, one of the most widely-expressed connexins, is found in the somatic granulosa cells during oogenesis and forms gap junctions with the oocyte via Cx37 [144, 149]. In each of these contexts, gap junction proteins can mediate the rapid communication of signals across a large tissue, such as the heart, liver, or gonad. Given that gap junctions function in a variety of context to regulate important physiological processes, understanding how they are used to regulate neighbouring cells is crucial.

In vertebrates and invertebrates, gap junctions can differ in their permeability to signals depending on the composition of the junction and post-translational modifications. A hemichannel composed of one type of protein is said to homomeric, or heteromeric when formed by two or more gap junction proteins (**Fig. 1.4.**) [7, 136]. Paired hemichannels containing a single connexin or innexin type are termed homotypic, whereas the presence of at least one different protein in either or both hemichannels is described as heterotypic [7, 136]. Different gap junction proteins can alter the permeability of the intercellular channel

and confer distinct properties to the channel, such as only permitting the passage of signals in one direction [7, 136]. The presence of functional gap junctions can be determined by injection of a fluorescent dye, such as Lucifer yellow, and observing its diffusion from the cytoplasm of a cell to its neighbours [7]. The ability of the dye to diffuse between neighbouring cells is dependent on gap junction intercellular channels [7]. As only fluorescent molecules that are small enough to pass through the channel are able to diffuse across cells, the size of the channel can be determined with additional dye diffusion assays [7]. Changes in the concentration of Ca^{2+} can result in opening or closing of the channel [150, 151]. Similarly, gap junctions are often voltage-gated, rapidly shifting from an open to closed state [152]. And there is evidence that suggests each hemichannel can respond to changes in junctional voltage independently [153]. Cysteine residues on the extracellular loops of connexions and innexins are sites of di-sulfide bridge formation, which are important for hemichannel coupling, and possibly for intramolecular stabilization of the protein [136, 154]. Post-translational modifications, such as phosphorylation of the C-terminal tail, are common among connexins [155]. A family of proteins that exhibit homology to invertebrate innexins, the pannexin family, do not form intercellular channels owing to the addition carbohydrate groups on extracellular loops of pannexins; as a result, pannexins function essentially as hemichannels (**Fig. 1.3.**) [136, 155]. Importantly, hemichannels can permit the passage of small molecules between the cell and the intercellular space, rather than between cells, and often permit the passage of different cargos than intercellular channels [156]. Cx43 can undergo proteolytic-cleavage of its C-terminus, in addition to ubiquitination, which can promote its internalization and degradation [155,

157]. The utility of gap junction proteins in rapid signalling during development or physiological behaviours is enhanced by the modifications that alter their activities.

Most studies of gap junctions in mammalian systems are performed in tissue and cell culture, rather than in a whole organism. This allows for detailed analysis of specific mechanisms of gap junction regulation, such as determining what signalling cargo may move through a connexin channel or the gating voltage of specific hemichannels. It is more difficult, however, to investigate the role of gap junctions in an *in vivo* context. While there is no sequence similarity between the connexion and innexin protein families, the molecular mechanisms by which they regulate cell-cell signalling are homologous. As a result, studies from worms and flies have provided important insight into the roles of gap junctions in intact organisms.

The genes encoding gap junctions in the nematode *C. elegans* began to be identified less than twenty years ago [158]. Starich *et al*. characterized the roles of *eat-5* genes in coordinating action potentials among pharyngeal muscles [158]. Additionally, dye coupling was disrupted between pharyngeal muscles in *eat-5* mutant worms [158]. Bioinformatics and topology modeling suggested that the gene products of *eat-5* and a related gene, *unc-7*, were structurally similar to connexin, and shared significant sequence similarity to two previously identified fly genes, *shak-B* and *ogre* [158]. *shak-B* and *ogre* were later found to encode innexin proteins, representing Innexin8 and Innexin1 [143]. The study of *eat-5* and *unc-7* by Starich *et al*. led to the eventual identification of a total of twenty five innexin genes in worms [158, 159].

Subsequent studies in *C. elegans* have demonstrated many roles for gap junction signalling [159]. The first innexin shown to form intercellular channels was INX-3, which

was shown to be required throughout embryonic development for survival [160]. Like connexions, INX-3 forms plaque-like structures at cell-cell junctions and INX-3 channels have nearly identical channel gating properties [160]. INX-16 is required in intestinal cells for the propagation of waves of calcium signalling that necessary for intestinal muscle contractions [161]. During gametogenesis, INX-14 and INX-22 are expressed in the somatic gonadal sheath cells, which form gap junctions with one another and with differentiating germ cells, to negatively regulate oocyte maturation via cAMP signalling [162-164]. INX-14/INX-22 gap junctions were also identified in germ cells and shown to be important for regulating germline proliferation [164]. Further analysis found that INX-8 and INX-9 function in the soma to mediate gap junctional signalling to the germline [165]. While these studies have demonstrated the importance of gap junction-mediated signalling in an intact organism, fewer than ten of the twenty five worm innexins have been studied to date [159]. Thus *Drosophila*, which only has eight innexin genes, provide a more accessible system to study the mechanisms of gap junction-mediated signalling *in vivo*.

While gap junction signalling had been characterized in *Drosophila* over thirty years ago, another decade passed before the identification and recognition of the gene family which encodes invertebrate gap junction proteins [166]. Shaking-B(lethal) was the first fly protein shown to form gap junctions when expressed in *Xenopus* oocytes [167]. Based upon similarities among Shaking-B(lethal) and three related, putative gap junction proteins identified in *C. elegans*, the innexin family of proteins (invertebrate analogues of the connexins) was proposed to represent invertebrate gap junction proteins [167, 168]. Eight innexins were found to be encoded by the *Drosophila* genome: Innexin1/Optic Ganglion Reduced (Ogre), Innexin2 (Inx2), Inx3, Inx4/Zero population growth (Zpg), Inx5, Inx6, Inx7,

and Inx8/Shaking-B (lethal) (Shak-B) [169]. To determine which tissues expressed which innexins, *in situ* hybridization experiments were performed [169]. Expression of each innexin was detected in the central nervous system, with the exception of *zpg* which was only ever detected in the germline [169]. *inx2* transcripts were also detected in the germline during oogenesis, as well as in the soma, with low levels of *ogre*, *inx3*, and *inx7* also detected [169]. Ogre, Inx2, and Inx3 were also expressed throughout many somatic tissues during development [169]. The function of innexins during development next began to be characterized.

Innexins have been demonstrated to be important in the fly nervous system, epidermis, foregut development, and in soma-germline signalling. Development of the embryonic nervous system requires both Ogre and Inx2 in glial cells [170]. Inx2 is required for gap junction-mediated calcium signalling to communicate metabolic signals between glia and neural stem cells [171]. Glial cells additionally need Inx2 for the long-distance recycling of neurotransmitters in the visual system [172]. Inx7 has been shown to be essential for axon guidance during embryonic development of the nervous system [173]. Inx2 has also been implicated in epithelial morphogenesis and Inx2 and Inx3 form functional heteromeric channels [174, 175]. Inx3 has been demonstrated to be required in the amnioserosa, an extraembryonic tissue, where it may mediate the stability of gap junctions and adherens junctions during development [176]. Work by Bauer *et al.* demonstrated that the *inx2* gene was a downstream target of Wg and Hh signalling, required for foregut development, and mediated gap junction signalling in the embryonic foregut [177, 178]. Zpg, a putative germline-specific innexin, has been shown to be required for fertility in males and females, although it is unclear what specific role it was playing [135]. Together, these studies reveal

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that innexins are required for diverse processes throughout the organism, each of which requires gap junction-mediated juxtacrine signalling.

1.4.6.   Gap junctions as a mechanism of regulating soma-germline signalling

Because innexins can mediate a range of signals, such as calcium ion and metabolite exchange, they represent a promising signalling mechanism for mediating soma-germline signalling. In addition to identifying a role for Zpg in fertility, Zpg was found to be required in the ovary for germ cell differentiation [135, 179]. Subsequent analysis has suggested that Zpg may function in the germline to form gap junctions with Inx2 in the soma during oogenesis [180]. In support of this idea, analysis with a female-specific hypomorphic allele of *inx2* has shown that Inx2 is required in the soma during oogenesis; *inx2* mutant ovaries have somatic cyst cell defects and germ cell development is disrupted [181]. Inx2 was proposed to mediate the transfer of signalling molecules or nutrients from the soma to the germline [181]. Krüger & Bohrmann used a variety of fluorescent indicators, inhibitors to show that the somatic cells surrounding developing oocytes have stage-specific differences in membrane potential, ion transport, and intracellular pH [182]. Inx3 was revealed to have a role in regulating these differences across the somatic follicle cells [180]. The ability of gap junctions to mediate soma-germline signalling and development during oogenesis suggests that gap junctions may function in a similar manner during spermatogenesis.

To date, very little is known about the role of gap junctions in the *Drosophila* testis. The initial characterization of Zpg in the testis indicated that Zpg may be required for germline differentiation; however, many questions about Zpg remained unanswered [135]. Does Zpg function as a hemichannel or form intercellular channels with other innexins? What other innexins are required in the testis, and which ones interact with Zpg if any? At

what stage of spermatogenesis are gap junctions required for signalling? What specific molecular signals are mediated by Zpg-containing gap junctions? Does Zpg function in germline-germline signalling, soma-germline signalling, or both? How is Zpg regulated? Answers to these questions could provide important insight into basic mechanisms of soma-germline signalling, stem cell self-renewal, and gap junction-mediated regulation of differentiation.

Work in the mammalian testis has suggested gap junctions mediate essential soma-germline signals. Indeed, gap junctions have been described from electron micrographs in the rodent testis [183]. Connexin43, widely expressed in the mouse, has been shown to be required in the testis for fertility [183]. Conditional knockouts of Cx43 in the germline and the somatic Sertoli cells showed that Cx43 functions specifically in the soma [184, 185]. Sriharan *et al*. demonstrated that loss of Cx43 in adult Sertoli cells led to cell cycle re-entry of the post-mitotic cells and delayed differentiation [184]. A similar study by Brehm *et al*. revealed that loss of Cx43 resulted in a decrease in spermatogonial cells and an increase in Sertoli cells [186]. While Cx31 transcripts have been detected in the germline of the rat testis but not in Sertoli cells, Cx31 does not form gap junctions with the other connexins that are expressed in the gonad [187, 188]. Freeze-fracture techniques have been successfully used to identify germ cell-germ cell gap junctions in the mink testis, but it is still unclear which connexins function in the germline [189]. It is unknown what specific signals are mediated by Cx43-containing gap junctions in Sertoli cells, but it is possible that metabolic signals that are transmitted by soma-germline gap junctions following the formation of the blood-testis-barrier [183].

Gap junction proteins play an important role in the mammalian testis, and are of interest to clinicians studying the regulation of fertility [45]. Exposure to environmental toxins, such as pesticides, herbicides, heavy metals, and xenoestrogens often leads to disrupted gap junction signalling [190]. Gap junctions have even been proposed to be targets of endocrine signalling disruptors, such as Bisphenol-A, possibly through the inhibitory binding of compounds to Cx43 residues, thus disrupting gap junction-mediated signalling [45]. By gaining a better understanding of gap junction-mediated signalling in the testis, the causes of infertility upon misregulation of juxtacrine signalling can be addressed and eventually treated.

Gap junctions are essential for soma-germline signalling during gametogenesis. *Drosophila* is an excellent system to study gap junction-mediated signalling during spermatogenesis. In particular, the fly testis is a well-characterized system, with a suite of available genetic tools, and is relatively simple compared to mammals while sharing remarkable conservation [29-31]. Thus, lessons from gap junction-mediated signalling in the fly testis can yield crucial insight into the basic mechanisms of soma-germline interactions and the study of infertility.

1.5. Aim and Scope of Thesis

The aim of this thesis is to provide insight into how gap junction-mediated juxtacrine signalling regulates spermatogenesis. Early events in *Drosophila* spermatogenesis, such as the regulation of stem cell self-renewal and the initiation of differentiation programmes in the soma and germline, necessitate coordinated signalling within the testis. Previous studies have provided insight into the importance of many of the signalling mechanisms utilized throughout spermatogenesis; to date, however, little is known about the juxtacrine signalling mechanisms utilized in the testis to regulate important cellular decisions. Many *in vitro* mammalian studies have contributed to our understanding of how neighbouring cells take advantage of gap junctions to signal with one another; how this contributes to cell behaviour within tissues, though, is not well-understood. While advances have been made in understanding the genetic bases of stem cell behaviour and infertility in humans, it is often difficult to gain mechanistic insight into stem cell regulation and gametogenesis. Thus, the goal of this work was to use the genetic model organism *Drosophila melanogaster* to provide insight into how gap junction signalling contributes to the regulation of somatic and germline stem cells, the stem cell niche, and the differentiation of gametes in an *in vivo* context. Firstly, I characterize the function of Zpg in the germline. Secondly, I identify the somatic gap junction partner of Zpg in the soma. Thirdly, I explore how Zpg and Inx2 function together to contribute to stem cell regulation and daughter cell differentiation of both the soma and germline. Finally, I begin to use a structure-function approach to gain insight into the molecular mechanisms by which Zpg may regulate gap junctional signalling. Overall, my study strives to provide important insight into the role of gap junctional signalling in regulating stem cell behaviour and gametogenesis in order to contribute to understanding the

importance of juxtacrine signalling in complex processes such as gametogenesis and the causes of infertility.

1.6. Chapter 1 Figures

Figure 1.1 Schematic of spermatogenesis in the Drosophila *testis.*
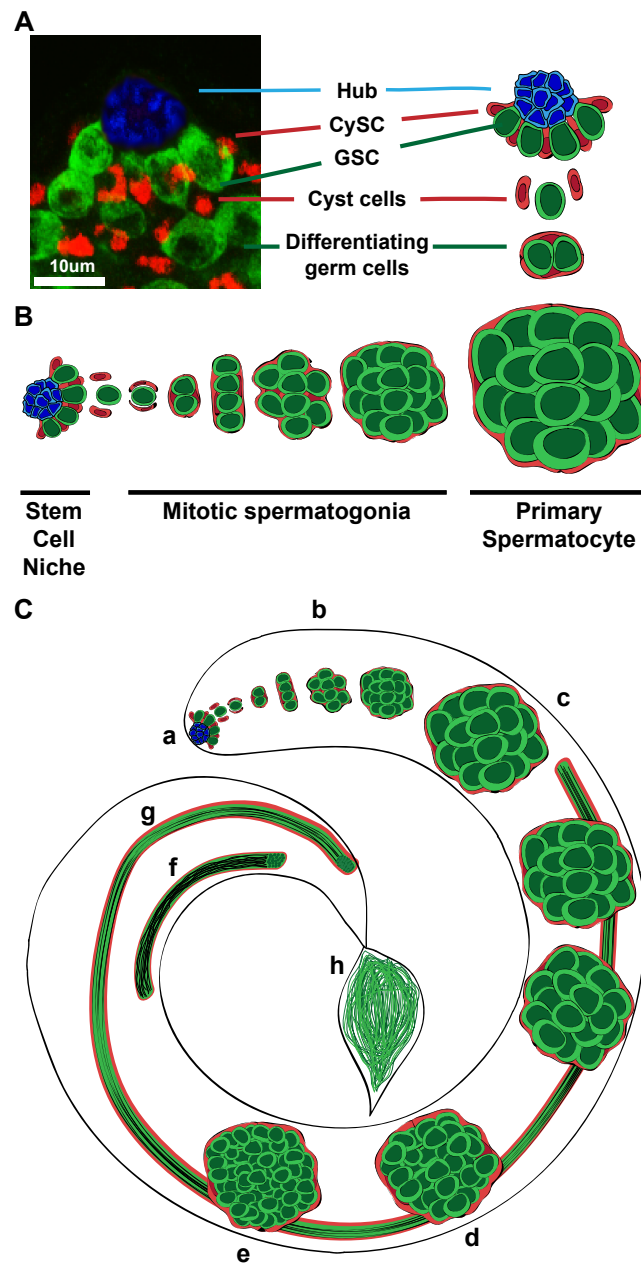
(A) Depiction of the testis stem cell niche in *Drosophila melanogaster*. The hub cells (false-coloured blue) form a signalling centre that physically anchors GSCs (green) and CySCs (red). Testis stained with Traffic Jam to label the soma and Vasa to label the germline. (B) The early mitotic stages of spermatogenesis. A single-celled gonialblast/spermatogonia divides four times, forming an interconnected germline cyst. After the mitotic divisions, the spermatocyte grows in size, in preparation for meiosis. (C) Schematic of germline development in the fly testis: (a) The stem cell niche; (b) transit-amplifying mitotic divisions; (c) primary spermatocytes; (d) secondary spermatocytes, following meiosis I; (e) post-meiotic round spermatids; (f) elongating spermatid bundles; (g) individualizing spermatids undergoing terminal differentiation, with the head cyst cell adhering to the terminal epithelium at the basal end of the testis; (h) fully developed sperm, extruded into the seminal vesicle.

Figure 1.2. Schematic of signalling within the testis stem cell niche.
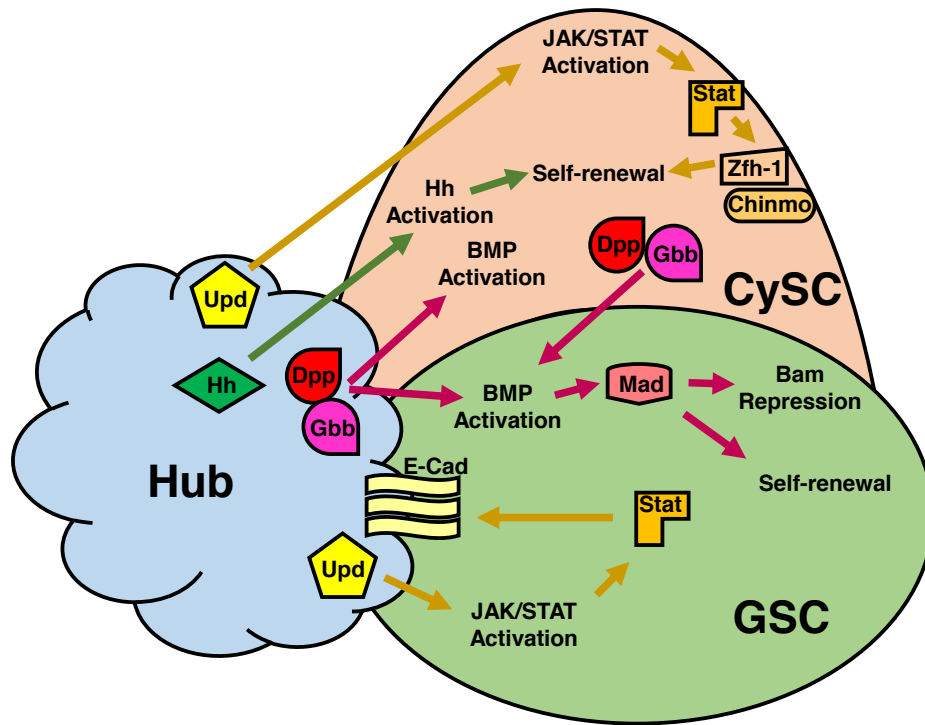
Summary of signalling pathways active in the hub, cyst stem cells (CySC), and germline stem cells (GSC).

Figure 1.3. Gap junction protein families in vertebrates and invertebrates.

A

Connexin　　　　Innexin　　　　Pannexin

Extracellular

Cytosolic

B Vertebrates	Invertebrates	Vertebrates
Can dock with hemichannels to form gap junctions	Can dock with hemichannels to form gap junctions	Cannot form gap junctions due to extracellular glycosylations
Can function as a hemichannel	No described role as hemichannel	Only functions as hemichannel
Can permit small molecule signalling between cells	Can permit small molecule signalling between cells	Can signal by secreting small molecules into extracellular space
No sequence homology to innexins	Some sequence homology to pannexins	Some sequence homology to innexins

C

Extracellular

Cytosolic

Connexon　　　　Innexon　　　　Pannexon

(A-C) Gap junctions are 4-pass transmembrane proteins, with two extracellular-facing loops, a single cytoplasmic loop, and a cytoplasmic N-terminus and C-terminus. Connexins and Innexins form gap junctions in vertebrates and invertebrates. Pannexins, a recently discovered family of proteins with sequence homology to invertebrate gap junction proteins, do not form gap junctions due to post-translational modifications on the extracellular

domains of the protein. Gap junction proteins hexamerize to form hemichannels. Hemichannels formed by Connexins, termed Connexons, and Innexins, termed Innexons, can dock with other hemichannels on apposing cell surfaces. The docking of two hemichannels forms a functional gap junction, which can permit rapid passage of small molecules or ions between two or more cells.

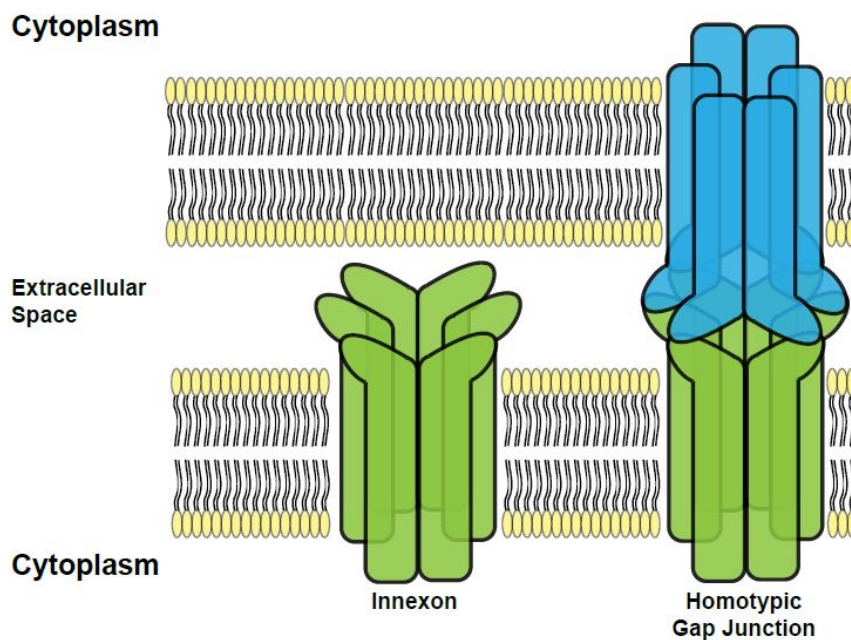*Figure 1.4. The assembly of a gap junction channel*

Diagram depicting the docking of two hemichannels between apposing cell surfaces. Once two hemichannels/innexons dock with one another, small molecules and ions such as cAMP, IP$_3$, and Ca^{2+} can be transmitted between neighbouring cells.

Chapter Two: Analysis of gap junction-mediated signalling during *Drosophila* spermatogenesis

2.1. Introduction

To date, little is known about gap junction-mediated signalling in the fly testis. Genetic screens attempting to identity genes required for fertility in males identified the gene *zero population growth* (*zpg*) [135]. The *zpg* locus was predicted to encode a four-pass transmembrane protein, similar to other identified innexins in the fly. Preliminary analysis of *zpg* mutant males described the phenotype as a reduction in germ cells, which may be unable to differentiate [135]. The role of *zpg* was also investigated in ovaries in the same study, which showed that Zpg localized to germline boundaries and may form gap junctions between the soma and germline or between germ cells [135]. Rudimentary analysis suggested that *zpg* may also be required for GSC maintenance in the ovary. Subsequent analysis in females showed that *zpg* is required for differentiation of the germline. By expressing a differentiation factor under the control of an inducible promoter, Gilboa *et al*. demonstrated that germ cells in a *zpg* ovary may die if forced to differentiate [179]. Taken together, these studies suggested that Zpg might mediate both the maintenance and differentiation of GSCs in the ovary. Since the initial study that described a phenotype in the testis, the role of Zpg has not been further studied.

Studies in the mammalian testis have revealed the importance of gap junction-mediated signalling in regulating somatic proliferation and germline development [184, 186]. To date, only Zpg has been shown to be expressed strongly in the germline, making it a likely candidate to mediate gap junctional signalling in the testis. Additionally, it is unknown which innexins function in the soma during spermatogenesis, and at what stage gap junction

signalling becomes important for soma-germline signalling. To address this, I sought to thoroughly characterize the *zpg* phenotype in both the germline and soma, identify which innexins are required during spermatogenesis, and identify the gap junction partner or partners of Zpg.

2.2. Results

2.2.1. *zpg* is required specifically in the germline for GSC maintenance and differentiation

To understand the role of *zpg* in regulating soma-germline communication, we expanded on previous analysis of the germline phenotypes of *zpg* mutant testes [135]. As described, testes in *zpg* null flies are rudimentary and contain fewer germ cells compared with wild-type (**Fig. 2.1. A, B**) [135]. To determine if *zpg* flies developed fewer PGCs, we looked in *zpg* embryos. Zpg protein can be detected in PGCs by at least Stage 15 of embryogenesis, and at the end of embryonic development, there were no obvious differences in PGC numbers, compared to wild-type (**A.1. B-D**). Previous analysis of the germline did not distinguish between differentiating germ cells and GSCs [135], defined as Vasa$^+$ germ cells that contact the hub [105, 191]. We found that *zpg*-deficient testes had far fewer GSCs than did wild-type sibling controls (1.2±0.3, *n*=49 versus 10.8±0.3, *n*=37; **Fig. 2.1. C, D, G**), and in 62.1% of *zpg* mutant testes, no GSCs were present at 1 day post-eclosion (DPE) (*n*=100), although 95% of *zpg* mutant testes contained germ cells (**A.2. A**) (*n*=101). The reduction in GSCs in *zpg* mutant testes might be due to an inability of GSCs to respond to stem cell maintenance signals secreted from the hub, such as Upd [23, 24]. To test this possibility, wild-type and *zpg* mutant testes were stained for Stat92E, a downstream effector protein of JAK/STAT signalling, which accumulates in GSCs and promotes their maintenance [93]. In both wild-type and *zpg* mutant testes, Stat92E protein was detected in

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GSCs (**Fig. 2.1. E, F**). This suggested that although GSCs in *zpg* mutant testes can respond to Upd, as observed by the accumulation of Stat92E, they are not maintained. This was tested directly by generating negatively-labelled control and *zpg* clones and counting the number of GSC clones present. For the wild-type control, GSC clones were detected at 4 and 5 days post-clone induction (dpci) in 78% (*n*=32) and 73% (*n*=41) of testes, respectively (**Fig. 2.1. H, L**). However, *zpg* mutant GSC clones were only detected in 3.2% (*n*=32) testes at 4 dpci and never detected at 5 dpci (*n*=59; **Fig. 2.1. I**). *zpg* mutant germline clones were always observed as single cells several cell lengths away from the hub. By comparison, CySC maintenance was not affected by loss of *zpg*. When *zpg* clones were induced in CySCs (defined as Traffic jam (Tj)$^+$ cells less than one cell diameter from the hub; [32]), they were found in 53% of the testes scored at 5 dpci in (*n*=15; **Fig. 2.1. K, L**). This was similar to control wild-type CySC clones, which could be detected at 5 dpci in 69% of testes (*n*=13; **Fig. 2.1. J, L**). Overall, these findings argue that *zpg* is specifically required for GSC maintenance in the testis. These results are consistent with analysis of the *zpg* phenotype in the fly ovary, which showed that GSCs are lost over time in *zpg* mutants [135].

2.2.2. *zpg* is required for germ line differentiation and association with the soma

As work in the *Drosophila* ovary showed that germ cells in *zpg* mutants begin to differentiate but do not survive [135, 179], we examined the differentiation of the germline in *zpg* mutant testes. The maturation of the spectrosome, a round, cytoskeletal-rich organelle in the GSC, to become a branched fusome within a spermatogonial cyst is a well-established indicator of germline differentiation [30]. In wild-type testes, the spectrosome could be detected in GSCs and gonialblasts, forming a large, branched fusome as the spermatogonia underwent further mitotic divisions (**Fig. 2.2 A**). As described previously, GSCs and

gonialblasts in *zpg* mutant testes were observed to contain spectrosomes (**Fig. 2.2. B**) [135]. In some instances, dumbbell-shaped fusomes were detected in *zpg* mutant testes between adjacent germ cells, indicating the presence of 2-cell-stage spermatogonia. However, unlike in wild-type testes, in the 2-cell-stage spermatogonia seen in *zpg* mutant testes, ectopic spectrosomes were also observed alongside early fusomes, consistent with a differentiation defect (**Fig. 2.2. B, inset**). As a second indicator of differentiation, we also examined expression of Bam. In wild-type testes, Bam is expressed in gonialblasts following displacement from the hub in 2- to 4-cell-stage spermatogonia, and is detected until the 16-cell stage [102]. Analysis of Bam expression in *zpg* mutant testes revealed it to be expressed in germ cells which resided outside of the niche, similar to wild-type (compare **Fig. 2.2. C** with **D**). However, Bam$^+$ germ cells in *zpg* mutant testes often appeared to be single-celled spermatogonia, rather than 2- to 16-cell-stage spermatogonia. Taken together, these results are consistent with earlier analyses [135, 179], suggesting that germ cells initiate differentiation in *zpg* mutants but cannot complete the process.

Previous analysis of *zpg* mutant testes indicated defective association between the soma and the germline [135]. We confirmed this result by labelling for the septate junction component Coracle (**Fig. 2.2. E**), which is a useful marker for encapsulation expressed in the soma [32]. This analysis showed that in *zpg* mutant testes, germ cell clusters lacked a detectable belt of Coracle, indicating encapsulation defects (**Fig. 2.2. F**). Together, these data suggest that germ cells require Zpg either to send a signal to the cyst cell or be in a certain state for encapsulation to occur.

2.2.3. *zpg* is required non-autonomously for differentiation of the soma

Markers that label specific stages of soma development were identified following the initial characterization of the *zpg* mutant phenotype [135]. Three such stage-specific somatic cell markers were chosen (**Fig. 2.3. A**): Zinc Finger Homeodomain 1 (Zfh-1) to label CySCs and their immediate daughters [93], Tj to label early-stage somatic cells [76], and Eyes Absent (Eya) to label late-stage somatic cells [86]. Analysis with these markers revealed that the size of somatic cell populations in *zpg* mutant testes was misregulated compared with wild-type. Wild-type testes contained an average of 37.1±1.5 Zfh-1$^+$ cells per testis (*n*=17; **Fig. 2.3. B**), whereas testes from *zpg* flies contained an average of 160.0±9.7 Zfh-1$^+$ cells per testis (*n*=27; **Fig. 2.3. C**), a 331% increase. Wild-type testes were observed to have an average of 98.4±1.8 Tj$^+$ cells per testis (*n*=23; **Fig. 2.3. D**), compared with an average of 160.6±8.2 in *zpg* mutant testes (*n*=55; **Fig. 2.3. E**), a 63% increase. Finally, an average of 185.1±6.1 Eya$^+$ cells were detected in wild-type testes (*n*=19; **Fig. 2.3. F**), compared with 79.3±4.3 for *zpg* mutant testes (*n*=26; Fig. **2.3. G**), a 67% reduction. These results suggest a large increase in the number of CySCs and early somatic cells in *zpg* mutant testes, but a significant decrease in the late-stage somatic cells, relative to wild type (**Fig. 2.3. H**). Furthermore, the stochastic variability in somatic cell numbers in *zpg* mutant testes is consistent with the idea that loss of gap junction-mediated regulatory cues have substantial effects on the soma. Particularly, the number of Tj$^+$ cells per testis was not correlated with the number of germ cells present in each testis (**A.2. B**). Taken together, these results suggest that somatic differentiation is disrupted, possibly delayed or partially blocked in *zpg* mutant testes, and that *zpg* is required non-autonomously to regulate the proliferation of the early soma.

2.2.4. *zpg* may be required non-autonomously in the soma for repressing the ectopic expression of hub cell markers

We next set out to investigate if the hub cell population exhibited any clear defects. The JAK/STAT ligand Upd is expressed specifically in the hub and secreted into the stem cell niche for GSC and CySC maintenance [23, 24, 93, 94]. We used *upd-GAL4* to drive expression of *UAS-nls-RFP* specifically in hub cells and then counted the number of hub cells in wild-type and *zpg* testes. Wild-type testes were observed to have an average of 11.3±0.4 Upd>nls-RFP$^+$ cells per testis (n=25; **Fig. 2.4. A, D**), while *zpg* testes contained 29.2±4.9 Upd>nls-RFP$^+$ cells on average, with significant variability between testes (n=24; **Fig. 2.4. B-D**), representing a 258% increase. The increase in RFP$^+$ cell number in *zpg* testes, however, was not due to an increase in the size of the hub itself, but from non-hub cells expressing a hub cell marker (**Fig. 2.4. C**). N-Cad antibody reliably labels the hub in both wild-type and *zpg* testes. More than 40% of observed Upd>RFP$^+$ cells were not cells that resided in the hub, based on N-Cad staining (**Fig. 2.4. E**). These results suggest that *zpg* is required non-autonomously either to repress the ectopic expression of hub cell markers or to repress the proliferation of hub cells. This supports our finding that *zpg* functions non-autonomously in the soma to regulate somatic cell differentiation.

2.2.5. *zpg* is required non-autonomously to regulate the proliferation of the early soma

The differentiation defect of somatic cells in *zpg* mutant testes likely results from failures in gap junction-mediated soma-germline communication. However, the germline in *zpg* mutants is disrupted in two ways: first, there are fewer germ cells present; second, the residual germline is blocked in differentiation. We sought to determine which of these two germline defects might lead to the somatic defects we observed. Although the somatic

defects could be due to both fewer germ cells and disrupted germline differentiation, we tested these separately. To test if fewer germ cells are responsible for the somatic defects, soma differentiation was analysed in *tudor* mutant flies, which lack a germline [62, 64, 192]. In *tudor* mutant testes, the number of Tj^+ early somatic cells was not significantly higher relative to a wild-type control, with an average of 112.0±10.9 (*n*=14) cells per testes versus 103.3±2.9 (*n*=14), respectively (**Fig. 2.5. A, B, E**). This contrasts with the substantial and significant increase in the number of Tj-expressing cells observed in *zpg* mutant testes (**Fig. 2.5. D, E**). Our results differ to previous work from that of Gónczy & DiNardo, who suggested that agametic *oskar* mutant testes exhibited a large increase in early somatic cell number [48]. This variance between *tudor* and *oskar*, which would be expected to have similar effects, might simply be because we performed this analysis in adult flies at <1 day post-eclosion versus 1-5 days post-eclosion for the previous study. Thus, the somatic phenotypes of *zpg* and *tudor* are at least partially distinct, arguing that the reduction in germ cells in *zpg* mutant testes does not by itself account for the somatic defects.

To test whether the somatic defects observed in *zpg* mutant testes were due to germline differentiation defects, constitutively active transgenes for the Type-I BMP receptors Thickveins (Tkv) and Saxophone (Sax) were expressed in early germ cells [193]. It has been previously shown that over-activation of the BMP pathway disrupts germline differentiation [84, 85, 194]. Quantification of early somatic cells revealed an increase of Tj^+ cells upon activation of the BMP pathway, compared with controls, an average of 130.7±4.4 (*n*=27) compared with 101.8±2.0 (*n*=26) cells per testis, respectively; this equates to a 28% increase (**Fig. 2.5. C-E**). This increase was similar to that observed in *zpg* mutant

testes and it is therefore possible that the somatic defects in *zpg* mutant flies were related to disrupted germline differentiation.

To explore further the possibility that the *zpg* mutant phenotype resulted from the inability of the germline to differentiate, additional analysis was performed. We hypothesized that the increase in early somatic cells could possibly result from delays in somatic differentiation or from abnormal proliferation. Therefore, CySC and early daughter cell proliferation was assayed by labelling cells actively synthesizing new DNA with an 5-ethynyl-2′-deoxyuridine (EdU) pulse and co-staining for Zfh-1 [93]. Interestingly, in agametic *tudor* testes or when BMP signalling is constitutively activated, the number of proliferating somatic cells was similar to controls at ~17 cells per testis (**Fig. 2.5. H**). This implies that constitutively activating germline BMP signalling leads to an increase in Tj$^+$ cells by delaying their differentiation rather than through changes in proliferation. By comparison, in *zpg* mutant testes the number of proliferating somatic cells increased by 113% (**Fig. 2.5. H**; 19.0±1.3 in the control to 40.6±4.6; *n*=18 and 14, respectively). Furthermore, whereas proliferating somatic cells were only detected proximal to the hub in wild-type testes (**Fig. 2.5. F**, arrowheads), Zfh-1$^+$/EdU$^+$ cells could be detected many cell lengths away from the hub in *zpg* mutant testes (**Fig. 2.5. G**, arrowheads). These results show that the increase in the number of Tj$^+$ cells in *zpg* mutants is at least partially due to a specific defect in proliferative regulation of the early soma. Although we cannot rule out the possibility that disrupted germline differentiation and a reduction in germ cells both contributed to the effects we observed, the phenotype of *zpg* mutants is distinct from that obtained by either removing the germline or blocking germline differentiation (**Fig. 2.5. I**). This suggests that

the somatic misregulation observed in *zpg* mutants likely represents a specific defect in gap junction-mediated soma-germline communication.

2.2.6. Analysis of innexins in the testis

Our results showing specific somatic defects in *zpg* mutant testes point to a possible role for innexin-mediated signalling between the soma and germline. Because Zpg is expressed only in the germline [135], it must interact with other innexin proteins on the somatic side of soma-germline contact sites to form gap junctions. To identify which of the eight fly innexins might be implicated in Zpg-dependent, gap junction-mediated communication during spermatogenesis, we carried out a small RNAi-based fertility screen using *tj-GAL4* to drive RNAi expression specifically in the soma (**Fig. 2.6. A**; **Fig. 2.7. A-E**). Somatic knockdown of only one innexin, Inx2, resulted in a phenotype. Somatic knockdown of Inx2 resulted in sterility and subsequent histological analysis revealed small, rudimentary testes, which did not contain any sperm in the seminal vesicles (**Fig. 2.6. A**; **Fig. 2.7. A-E**; **A. 3**).

To determine if any other innexins may be important in the germline, we performed two additional RNAi-based fertility screens to drive RNAi expression in the early and late germline. Using *nos-GAL4*, which is expressed in GSCs and their daughters, only knockdown of Zpg resulted in a clear fertility defect (**A.4. A**). This coincides with where we see Zpg expression. Knockdown of innexins during the transit-amplifying stages with *bam-GAL4*, however, did not produce a consistent result. Only the knockdown of Innexin5 (Inx5) with one RNAi line, but not another, resulted in a fertility defect, with sperm absent from the seminal vesicle (**A.4. B**; **A.5**). Further experiments with Inx5 will be necessary to clarify what role if any Inx5 plays during spermatogenesis. *bam-GAL4* knockdown of Zpg would be

expected to result in a phenotype, as Zpg is strongly expressed during the transit-amplifying stages, but it is possible that Zpg expression is too high at this stage to be efficiently knocked down.

Previous work in the fly ovary demonstrated that both Innexin2 (Inx2) and Innexin3 (Inx3) are present in the somatic follicle cells in developing egg chambers. Furthermore, Inx2 was found to co-localize with Zpg at soma-germline boundaries [180]. Phenotypic analysis of a hypomorphic *inx2* mutation in the ovary suggested a role for Inx2 in the soma [181]. Therefore, expression of Inx2 was analysed in the testis (**Fig. 2.6. B**). Previously published Inx2 antibodies did not yield good results in immunohistochemical analyses, so a new Inx2 antibody was generated (see Materials and Methods). Staining of wild-type testes using this new antibody revealed that Inx2 localized to the soma-germline boundary (**Fig. 2.6. B**; **Fig. 2.7. G**; **Fig. 2.10**). Our Zpg data suggested that Inx2 should be expressed at the earliest stages of spermatogenesis. An *inx2* enhancer trap line expressing GFP revealed that Inx2 expression could be detected weakly in the hub and CySCs, and, more strongly, in differentiating somatic cells (**Fig. 2.7. F, F'**, insets) [195]. As previously published, Zpg also localized to the soma-germline boundary, visualized with a Zpg-specific antibody (**Fig. 2.6. C-D**) and a GFP-tagged Zpg rescue construct (**Fig. 2.6. E**; see Materials and Methods). Labelling Inx2 in testes expressing the Zpg::GFP transgene showed that Zpg co-localized with Inx2 (**Fig. 2.6. B**). Intriguingly, although weak expression of Zpg was detected in GSCs at the hub interface, Inx2 was not detected at this stage by antibody staining (**Fig. 2.6. C**). As in the ovary, Inx3 also co-localized with Zpg at soma-germline boundaries, although its knockdown did not give rise to detectable defects (**Fig. 2.6. A, F**). Overall, these results argue that Inx2 is

required in the soma for spermatogenesis and its expression largely overlaps with that of Zpg.

2.2.7. Ultrastructural analysis of gap junctions in the testis

Previous work has demonstrated the presence of gap junctions between GSCs and niche cells in the fly ovary [135]. To determine when gap junctions form in the testis, we performed ultrastructural studies using electron microscopy. At the apical tip of the testis (Fig. 2.5.8. A), gap junctions were observed between GSCs and adjacent CySCs (**Fig. 2.8. B, B'**). Gap junctions could also be observed between 1-cell-stage gonialblasts and cyst cells immediately outside of the niche (**Fig. 2.8. C, C'**). In differentiating spermatogonia, gap junctions were visible between germline cysts and cyst cells (**Fig. 2.8. D, D'**). Interestingly, germline-germline gap junctions were observed in spermatogonia (**Fig. 2.8. E, E'**), although these were infrequent and small in comparison to soma-germline gap junctions (compare **Fig. 2.8. E'** with **B'-D'**). Together, these results indicate that gap junctions are formed early during spermatogenesis, that these junctions persist during early stages of germline differentiation and that gap junctions occur within a germline cyst.

2.2.8. Inx2 is required in the soma for the subcellular localization of Zpg

The expected mode of innexin function would predict coupling between innexins in the soma and the germline. If Inx2 was indeed the main somatic innexin and it coupled to Zpg on the surface of germ cells, then Inx2 knockdown could affect distribution of Zpg. This prediction was verified directly by knocking down Inx2 in the soma and staining for Zpg. Quantification of the relative enrichment of Zpg at soma-germline and germline-germline interfaces demonstrated that in wild-type testes (**Fig. 2.9. A**) there is 3.5 ± 0.2-fold enrichment of Zpg at soma-germline interfaces and a 1.5 ± 0.1-fold enrichment of Zpg at germline-

germline interfaces, compared with background staining (**Fig. 2.9. C**). Upon knockdown of Inx2 in the soma, Zpg redistributed from the soma-germline to the germline-germline interface (**Fig. 2.9. B**), with an approximately 1.2±0.1-fold enrichment of Zpg at soma-germline interfaces and a 3.7±0.1-fold enrichment of Zpg at germline-germline interfaces, compared with background cytoplasmic staining (**Fig. 2.9. C**). By comparison, a disruption in the subcellular localization of Zpg was not observed upon knockdown of Inx3 (**Fig. 2.9. D-F**). This shows that Inx2 is required in the soma to maintain the distribution of Zpg at the soma-germline interface, consistent with a coupling of somatic Inx2 with germline Zpg. Moreover, as Zpg is the only fly innexin known to be expressed in the germ line [169, 180], these results suggest a possible homotypic coupling of Zpg at germline-germline boundaries that competes with the heterotypic coupling of Inx2-Zpg at soma-germline boundaries.

2.2.9. Inx2 is required in the soma for GSC maintenance and germline differentiation

Our data thus far suggested that germline signals travel through Zpg-Inx2 gap junctions to regulate somatic differentiation and proliferation and vice versa. If this were the case, somatic knockdown of Inx2 should resemble the *zpg* mutant phenotype; indeed the small, rudimentary testes we observed following somatic Inx2 knockdown closely mirrored the *zpg* phenotype (**Fig. 2.11. A, B**). Because the *inx2* locus is located on the X chromosome, clonal analysis of *inx2* mutants in the testis proved to be exceptionally difficult. To draw further comparisons between Zpg and somatic Inx2 knockdown, we extended our Inx2 analysis using the RNAi line that gave the strongest and most penetrant phenotypes. RNAi-mediated Inx2 knockdown in the soma using this line strongly reduced Inx2 protein levels in both the testis and the ovary (**Fig. 2.7. G, H**; **Fig. 2.10 A, B**).

Previous work has suggested that innexins may affect cadherin-mediated cell-cell adhesion [175, 176, 196]. To determine if disruption of gap junctions altered levels of E-Cad in the testes, we stained *zpg* and *inx2RNAi* testes for E-Cad. We did not observe differences between *zpg*, *inx2RNAi* and control testes (**Fig. 2.12. A-D**). Furthermore, we used clonal over-expression of a wild-type *zpg* transgene in the ovary follicular epithelium to investigate whether Zpg could modify cell-cell adhesion, using Armadillo (β-catenin) expression as a marker. Again, we did not detect changes in Armadillo levels in *zpg* over-expression clones, nor did cell-cell adhesion appear to be altered (**Fig. 2.12. E, F**).

In addition to having similar morphology, Inx2 knockdown testes resembled *zpg* mutants in several other regards. First, Inx2 knockdown reduced GSC numbers, consistent with the GSC maintenance defects observed in *zpg* mutants (an average of 5.9±0.3 per knockdown testis, *n*=62 versus 9.6±0.3 per control testis, *n*=16; **Fig. 2.11. C-E**). Second, somatic knockdown of Inx2 partially blocked germline differentiation (**Fig. 2.11. F-N**). Although most germline cysts developed a fusome (**Fig. 2.11. F, G**), almost half of testes analyzed did not contain germline cysts that reached meiotic stages and only 10% had late-spermatid stage cysts (identified by Boule protein staining; [197]; **Fig. 2.11. L-N**). However, Bam expression was similar to that observed in wild type (**Fig. 2.11. H, I**). Furthermore, encapsulation, as judged by staining for the somatic marker Cora, was largely normal (**Fig. 2.11. J, K**). This is consistent with the Inx2 knockdown phenotype being less severe than the *zpg* phenotype in some aspects (compare **Fig. 2.11 J, K** with **Fig. 2.2. E, F**). Nonetheless, testes at the severe end of the phenotypic spectrum greatly resembled *zpg* mutant testes (**Fig. 2.11. M**). The weaker phenotype may reflect the limitations of RNAi or a partial redundancy with Inx3, which is also expressed in the soma. To test for this latter

option, a double RNAi-knockdown experiment targeting both Inx2 and Inx3 was performed (**Fig. 2.10 C-H**). Knockdown of Inx3 alone in the soma did not disrupt spermatogenesis, despite reducing Inx3 protein levels, determined by antibody staining (**Fig. 2.10. C-D**). Knockdown of Inx2 disrupted localization of Inx3, reduced protein expression levels of Inx2 and disrupted spermatogenesis (**Fig. 2.10 E**). Simultaneous knockdown of Inx2 and Inx3 phenocopied the Inx2 knockdown phenotype (**Fig. 2.10. F-I**). These results suggested that Inx3 is dispensable in the early soma for spermatogenesis, that Inx3 may require Inx2 for its localization and that Inx2 is required to regulate the proliferation and differentiation of the early soma.

2.2.10. Inx2 is required to regulate the proliferation and differentiation of the early soma

Because the *zpg* mutant germline phenotypes resembled those observed upon Inx2 somatic knockdown, we analysed somatic differentiation and proliferation in Inx2 knockdown testes. Similar to *zpg*, somatic Inx2 knockdown gave rise to an increase in early somatic cells and a decrease in late somatic cells (**Fig. 2.13 I**). The number of Zfh-1^{+} cells compared with controls was 48% higher (67.5±2.9 per testes, *n*=30 versus 45.5±1.4, *n*=23, respectively; **Fig. 2.13. A, B, I**). The number of Tj^{+} cells compared with control was slightly lower, although not statistically significant (99.8±3.0 per testes, *n*=12 versus 92.6±2.7, *n*=43; **Fig. 2.13. C, D, I**). Finally, the number of late somatic Eya^{+} cells was decreased in Inx2 knockdown testes compared with controls by 19% (149.8±6.2 per testes, *n*=13 versus 184.8±7.5, *n*=9, respectively; **Fig. 2.13. E, F, I**). Thus, similar to *zpg*, somatic knockdown of Inx2 shows an increase, albeit smaller, in early somatic cells and a significant decrease in late somatic cells compared with wild type. These results show that Inx2 acts to promote differentiation of the soma. To determine whether somatic Inx2

knockdown resulted in aberrant proliferation of the early soma, as observed in *zpg* mutants, the number of proliferating early somatic cells was quantified by co-labelling for EdU and Zfh-1. In control testes, an average of 20.7±0.8 Zfh-1$^+$/EdU$^+$ cells were detected (*n*=23; **Fig. 2.13. G, J**). In Inx2 knockdown testes, an average of 18.5±1.3 Zfh-1$^+$/EdU$^+$ cells were observed (*n*=30; **Fig. 2.13. H, J**). Taken by itself, this result appeared to suggest that early somatic proliferation was not misregulated in Inx2 somatic knockdown testes. However, because the Zfh-1 population is in general larger in Inx2 knockdown testes compared with controls, the actual proportion of proliferating early somatic cells following Inx2 knockdown was in fact much lower than in controls. In addition, it was suspected that non-CySCs might be proliferating upon somatic knockdown of Inx2. To correct for this and ensure that only CySC proliferation was being assayed, the total number of Zfh-1$^+$ cells per testis was quantified in addition to Zfh-1$^+$/EdU$^+$ cells that were less than one cell-length from the hub. We have previously used this method to gain insight into patterns of CySC proliferation [32]. This analysis revealed that in control testes, 25-27% of CySCs were labelled for EdU. In Inx2 somatic knockdown and *zpg* mutant testes, only 13% and 7% of CySCs were labelled, respectively (**Fig. 2.13. K**). This result suggested that CySC proliferation is disrupted upon somatic Inx2 knockdown, similar to observations in *zpg* mutant testes.

It was further noted that in Inx2 knockdown testes, cells proliferated further away from the hub, similar to observations in *zpg* mutant testes (**Fig. 2.5. G**; **Fig.2.13. L**). The average distance from the hub of Zfh-1$^+$/EdU$^+$ cells was determined. Zfh-1$^+$/EdU$^+$ cells were detected within 4.9 μm±0.2 (*n*=310) of the hub in control testes, whereas upon knockdown of Inx2, the average distance of Zfh-1$^+$/EdU$^+$ cells from the hub grew by 43% to 7.0 μm±0.3

(n=338). This was comparable to *zpg* mutant testes, where proliferation events occurred on average 97% further from the hub, or 11.0 μm±0.4 (n=595), relative to wild-type controls.

Taken together, these results show substantial overlap between Inx2 somatic knockdown and *zpg* mutant phenotypes. These results suggest that gap junction signalling between the germ line and soma is required to regulate the soma by controlling CySC proliferation rates, by limiting proliferation to near the hub cell niche, and by promoting differentiation of the soma.

2.2.11. Zpg may mediate calcium signalling in the soma

Gap junctions facilitate signalling by permitting the rapid transport of small molecules between neighbouring cells. These small molecules may be ions or small metabolites. To attempt to gain insight into how gap junction signalling may be disrupted in *zpg* testes, we used a genetically-encoded calcium sensor to monitor relative Ca^{2+} *in vivo*. Changes in Ca^{2+} concentrations can be detected in live-imaging experiments, using a transgenic calcium sensor, GCaMP, that combines a circularly permutated enhanced GFP, the calcium-binding protein calmodulin, and the M13 fragment of myosin light chain kinase [198]. When Ca^{2+} binds to the calmodulin domain, a conformational change is caused in the GFP, leading to an increase in fluorescence intensity [198]. By expressing the GCaMP sensor under the control of the UAS-GAL4 system, tissue-specific changes in calcium signalling could be assayed *in vivo*. This would permit the study of calcium signalling alterations in the soma and germline of wild-type and *zpg* testes.

A transgene encoding the GCaMP sensor was expressed in the testes of wild-type and *zpg* mutant flies. To optimize the visibility of the GCaMP sensor in live testes, larvae were dissected, as their gonads are much smaller than in adult flies. Following the germline-

specific expression of UAS-GCaMP, GFP could be detected in GSCs and their immediate progeny of both wild-type and *zpg* testes (**A.6. A, B**). No changes in GFP intensity were observed in the germline over a 10-minute time course in wild-type or *zpg* testes. This could be due to an inability of the sensor to detect subtle changes in calcium levels or calcium signalling may not be important at this stage of spermatogenesis. In contrast, *tj-GAL4*-driven expression of GCaMP in the soma revealed momentary increases in GFP intensity in the soma of *zpg* mutant testes (**A.6. D, arrowhead**). These bursts in GCaMP activity were observed primarily in CySCs and their immediate daughters. This may suggest that Zpg is required non-autonomously in the soma for the regulation of calcium signalling. However, as this experiment was technically difficult to do, subsequent attempts to obtain a greater sample size for image analysis and quantification could not be completed.

2.2.12. Preliminary structure-function analysis of Zpg

To gain insight into the molecular mechanisms by which Zpg contributes to soma-germline signalling, a structure-function approach was developed to identify domains and residues of Zpg that are important for its function. Little is known about how fly innexins are regulated. It would be expected that there are similarities between invertebrate innexins and the structurally-analogous vertebrate connexins, and how general domains contribute to their function. Because only a small number of studies have been done on fly innexins, insights from vertebrate and invertebrate gap junction studies were used to inform the design of transgenic Zpg constructs carrying mutations which were predicted to disrupt different functions of Zpg (**Fig. 2.14.**) [136, 142, 155, 156, 171, 199].

First, as a proof-of-principle experiment, a wild-type *zpg* transgene was generated and introduced into a *zpg* mutant fly [200]. The ability of the transgene to rescue the *zpg*

mutant phenotype was then assessed. The wild-type *zpg* transgene was able to rescue *zpg* mutant flies, restoring wild-type morphology and fertility levels (**Fig. 2.15. A**, compared to **Fig. 2.1**. **A, B**; **Fig. 2.15. B**). Furthermore, Tj$^+$ cell numbers were also restored to wild-type levels (**Fig. 2.15. C-F**). Thus, the wild-type transgene can successfully rescue the *zpg* mutant phenotype.

A fluorescent tag was then added to the wild-type transgene to determine if a Zpg::GFP protein would still localize to the plasma membrane and rescue *zpg* mutant flies. Indeed, Zpg::GFP mimicked the localization of endogenous Zpg in PGCs and adult testes (**A.1. A**; **Fig. 2.6. E**). The GFP-tagged transgene also rescued morphological defects in *zpg* null testes (**Fig. 2.16. A-C**) and restored fertility to wild-type levels (**Fig. 2.16. D**), indicating that the GFP tag likely did not interfere with the function of Zpg.

Important regulatory sites for gap junction proteins are often found in the cytoplasmic C-terminal region of the protein [155]. An *inx2* transgene with a deletion of the C-terminus has been shown to be unable to rescue *inx2* mutant flies [178]. To determine if the C-terminus of Zpg is required for its function, we generated a *zpg* transgene with a 78 amino acid C-terminal deletion. Because the Zpg antibody we developed recognizes epitopes on the C-terminus, a GFP tag was added to the *zpgΔCT* transgene in order to detect it in a *zpg* null fly. Unlike the previous transgenic rescue experiments, the *zpgΔCT::GFP* transgene was unable to rescue zpg mutant flies. In both wild-type and *zpg* mutant testes, the transgenic protein failed to reach the plasma membrane, and instead exhibited a perinuclear localization (**Fig. 2.17. A-C**). This result suggested either that the C-terminus of Zpg is required for its export from the endoplasmic reticulum or for stability of Zpg at the plasma membrane. As expected, the *zpgΔCT::GFP* transgene also failed to rescue the *zpg* somatic cell

differentiation phenotype (**Fig. 2.17. D-G**). Thus, the C-terminus of Zpg is essential for its function.

2.3. Discussion

2.3.1. Gap junctions link the soma and germ line

The work presented here demonstrates that gap junctions between the soma and germline are essential for fly spermatogenesis. Previous work revealed an essential role for Zpg in the fly gonad and raised the possibility that signals either from the soma or other germ cells travel through gap junctions to regulate germline survival and differentiation [135]. Subsequent work in fly ovaries showed that Zpg was also required for GSC maintenance [179]. Our analysis supports and extends these conclusions by finding a cell-autonomous requirement for Zpg in GSC maintenance and differentiation in the fly testis. We also demonstrate a similar role for Inx2 in the soma. Furthermore, we find that gap junction-mediated signals from the germline also play unique and essential roles in the soma during spermatogenesis, independent of general germline defects. In particular, gap junctions are required to control the proliferation of CySCs and promote the differentiation of their daughters. Our work illustrates that the main type of gap junction between the soma and the germline in the fly testis is a heterotypic channel coupling Inx2 in the soma and Zpg in the germline. Preliminary analysis using an *in vivo* calcium sensor points to a possible role for gap junction-mediated calcium signalling within the stem cell niche. Importantly, disrupting gap junctions in the soma by knocking down Inx2 phenocopies the *zpg* mutant phenotype in both the germline and soma. Therefore, gap junction-mediated soma-germline regulation in the testis is bi-directional.

2.3.2. Gap junctions contribute to stem cell regulation in the testis

Recent work has highlighted the importance of gap junctions in stem cell regulation in a number of systems [165, 171, 201, 202]. In line with results from other stem cell models, our data illustrates a specific role for gap junctions in both GSCs and CySCs. The role of gap junctions in stem cell regulation in the testes was illustrated by the requirement for Zpg in the germline and Inx2 in the soma for GSC maintenance. Loss of Zpg or somatic knockdown of Inx2 also affected CySC proliferation. Furthermore, ultrastructural analysis revealed the presence of gap junctions between GSCs and CySCs. These results, as a whole, suggest a requirement for gap junction-mediated soma-germline communication in both stem cell populations and at the earliest stages of sperm differentiation.

2.3.3. Gap junctions facilitate signalling between the soma and germline

Following the stem cell stage, strong expression and co-localization of Zpg and Inx2 was consistently detected starting at the 4-cell cyst stage. Expression of Zpg and Inx2 began to diminish after the early spermatocyte stages and was not detected past meiotic stages. The timing at which Inx2 and Zpg expression were most prominent corresponds to a period during which niche signals such as BMP are lost [84, 85]. Loss of these signals causes the germ line to undergo rapid differentiation and specialization [30, 31]. Recently, work from our lab has shown that as somatic cells move away from the niche and begin differentiating, the soma forms a permeability barrier around the germline, isolating the germline from the outside environment [32]. This transition corresponds with a switch whereby soma-germline communication shifts from predominantly paracrine to juxtacrine signalling. Thus, as the germline becomes increasingly isolated, it becomes more dependent on differentiation signals that arrive via gap junctions from the soma. Once the germline becomes isolated, gap

junctions may also play an important nutritive role and permit the movement of essential small metabolites between the germline and soma. Similarly, the soma requires gap junction-mediated signals to allow it to accommodate the increasingly expanded, differentiated and specialized germline.

Our observations that gap junctions regulate germline differentiation and soma proliferation are in line with studies from both vertebrate and invertebrate models. In *C. elegans*, it was recently shown that gap junction-mediated signals are required to maintain GSCs in the niche and for germline differentiation [165]. Similarly, work in vertebrates has shown that loss of gap junction-mediated signalling in the soma increased proliferation in post-mitotic Sertoli cells [184, 203, 204]. It is therefore likely that an early role for gap junctions in coordinating soma-germline differentiation is an evolutionarily conserved mechanism. One recurring feature of soma-germline gap junctions is the expression of different gap junction proteins, resulting in heterotypic gap junctions, exemplified by the Inx2-Zpg gap junctions we observe in the fly testis [165, 185]. A key problem in understanding the role of gap junctions in mediating soma-germline communication is identifying the transported signalling cargos. Some possible signals are cAMP, Ca^{2+} and cGMP [136, 205], which have been implicated in regulating meiosis in the germline [206, 207]. Our initial attempts to study Ca^{2+} in the testis have been encouraging but the experiments have been difficult to consistently replicate. However, recent work in *Drosophila* ovaries has suggested that somatic gap junctions may play roles in regulating pH, membrane potential and ion transport [182]. Overall, multiple signals are probably exchanged between the soma and germline through gap junctions and elucidating their respective functions is a complex task that should be further studied.

Based on the results presented here and on previous studies, we propose the following model (**Fig. 2.19.**): GSCs receive multiple cues that control their behaviour, with gap junctions mostly providing a supporting role, allowing the passage of cues from the soma that facilitate long-term GSC maintenance. After stem cell division, the germline undergoes rapid differentiation, becoming increasingly isolated from the outside environment, and a permeability barrier is formed by the soma. As outside signals from the niche are lost, the germ line relies more heavily on gap junctions to allow the passage of small molecules and metabolites from the soma to promote differentiation and provide nourishment. To ensure coordinated growth and differentiation of the soma and germline, signals pass from the germline through the gap junctions into the soma. The work presented here defines gap junction-mediated juxtacrine signalling as an additional signalling mechanism in the fly testis. Furthermore, our study provides a clear illustration of the bi-directional regulatory action of soma-germline gap junctions. As we demonstrate, disrupting innexins in the soma or germline leads to a specific regulatory effect in the other tissue. Therefore, bi-directional gap junction-mediated signalling plays a vital role in ensuring proper coordination of the soma and germline during spermatogenesis.

2.4. Materials and Methods

2.4.1. Fly stocks

The following lines were used: the somatic drivers *tj-GAL4* and *c587-GAL4*; the germline driver *nos-GAL4-VP16; zpg^{z-2533},st/TM3,Sb; zpg^{z-5352}/TM6B; hs-flp/FM7;FRT2a/TM3,Ser; His2a::RFP/TM3,Ser; UAS-mCD8::GFP; w ^{1118}; tud^1 /CyO, tud^{B42}/CyO, and tud^{B45}/CyO,* [64]; *UAS-tkv^{Act},UAS-sax^{Act}/CyO* [193]; *hs-flp; +; Dr/TM3,Sb; w - ; +; act5c-GAL4-FRT-stop-FRT-RFP/TM3, Sb; UAS-inx1RNAi JF02595; UAS-inx2RNAi*

JF02446; UAS-inx2RNAi HM05126; UAS-inx2RNAi KK111067; UAS-inx2RNAi 4590R-2; UAS-inx3RNAi HM05245; UAS-zpgRNAi GL00447; UAS-zpgRNAi JF02753; UAS-inx5RNAi JF02877; UAS-inx6RNAi JF02168; UAS-inx7RNAi JF02066; UAS-inx7RNAi KK112684; UAS-shakBRNAi JF02603; UAS-shakBRNAi JF02604; UAS-shakBRNAi GD12666; and UAS-shakBRNAi GD7794. The innexin2 enhancer trap line, *inx2>nlsGFP P01999*, was obtained from the Cooley lab [195].

2.4.2. Genetics

Crosses were set up at room temperature, flipped each day, and raised at 25°C on standard media. To generate *zpg* flies, *zpg^{z-2533}/TM3,Sb* flies were crossed to *zpg^{z-5352}/TM6B* flies; heterozygous siblings were used as a control. *tudor* flies were generated using *tud^{B42}/CyO* or *tud^{B45}/CyO* crossed to *tud^{1}/CyO*, which were then crossed to *w^{1118}* males to generate germ cell-less flies. CA-BMP flies were generated by crossing *nos-GAL4::VP16* flies to *UAS-tkv^{Act},UAS-sax^{Act}/CyO* flies. RNAi knockdowns, were performed using *UAS-Dcr2; tj-GAL4/CyO* crossed to the corresponding UAS-innexin RNAi line. For inx2RNAi phenotypic analysis, UAS-JF02446 was used as it provided the strongest knockdown based on testis morphology and sterility assays. Controls were *UAS-Dcr2; tj-GAL4/CyO* males. Clones were *hs-flp, c587-GAL4>UAS-mCD8::GFP; +; His2a::RFP, FRT2a/TM3,Ser* crossed to *w^{-}; +; FRT2a* for control clones and to *w^{-}; +; zpg^{z-5352},FRT2a/TM6B* for mutant clones. Progeny were raised at 25°C, clones were induced in males less than 1 day post-eclosion (DPE), using two 60-minute heat shocks at 37°, 90-minutes apart, and aged for 4 and 5 days at room temperature. Clones in the ovary follicular epithelium were *hs-flp; +; act5c-GAL4-FRT-stop-FRT-GAL4-RFP/TM3, Sb* crossed to UAS-JF02446 for the Inx2

knockdown experiment and w^- ; UASt-zpg^{cDNA} for the Zpg overexpression experiment. Clones were induced in females <1DPE as above and aged for three days prior to dissection.

2.4.3. Fertility assays

Fertility assays were performed using single males, 5DPE, crossed to three w^{1118} virgin females, aged 15 days, and scored as sterile if no larvae/pupae were present, and semi-sterile if <50 larvae/pupae were present.

2.4.4. Immunostaining

Polyclonal antibodies were raised in rabbits (GenScript) to peptides corresponding to the C-terminal amino acids (345-367, RKLLEELYEAQSLIKIPPGADKI) of Zpg. A peptide corresponding to amino acids 348-367 of Inx2 (DLSREMSGDEHSAHKRPFDA) was injected into guinea pigs (GenScript). Polyclonal antisera were affinity-purified for Zpg and Inx2, and determined to be epitope-specific by ELISA.

Flies <1DPE were dissected in phosphate buffered saline (PBS) pH 7.4, fixed in 4% paraformaldehyde in PBS, rinsed in 0.2% Tween20, and washed and incubated in 0.3% Triton X-100 in PBS with 0.5% BSA. Primary antibodies used were rabbit anti-Vasa (R. Lehmann, P. Lasko, 1:5000), guinea pig-anti-Traffic jam (D. Godt, 1:3500), rabbit anti-Zfh-1 (R. Lehmann, 1:1000), guinea pig-anti-Zfh-1 (J. Skeath, 1:500), rabbit anti-Stat92E (E. Bach, 1:500), rabbit anti-Inx3 (J. Davies, 1:1000), rabbit anti-Boule (S. Wasserman 1:1000), mouse anti-Cheerio/Filamin (L. Cooley, 1:1000), rabbit anti-Zpg (1:20000), guinea pig anti-Inx2 (1:1000), rat anti-DN-Cadherin (Developmental Studies Hybridoma Bank (DSHB), 1:50), rat anti-DE-Cadherin (DSHB, 1:50), mouse anti-spectrin (DSHB, 3A9, 1:5), mouse anti-Bam (DSHB and D. McKearin, Bam, 1:50), mouse anti-Coracle (DSHB, C566.9 and C615.16 1:500), mouse anti-Armadillo (DSHB, N2 7A1, 1:1000), mouse anti-Eya (DSHB, eya10H6,

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1:500), mouse anti-GFP (Invitrogen, A11120, 1:1000), and rat anti-dsRed (Chromotek, 5f8, 1:1000). Bam stainings were supplemented with additional Bam every 24 hours for a total of 72 hours. This was followed by a one-day wash, before addition of secondary antibodies to detect Bam. For Stat stainings, samples were incubated with primary antibodies overnight at room temperature (M. Amoyel and E. Bach, personal communication). Alexa Fluor 488-, Cy3- and Cy5-conjugated secondary antibodies were used at 1:500 (Molecular Probes).

2.4.5. Imaging and analysis

All analyses were performed on males <1DPE, unless otherwise stated. GSCs were defined as Vasa$^+$ or Tj$^-$ cells contacting the hub. CySCs were defined as Zfh-1$^+$ or Tj$^+$ cells <10 μm from the hub. Somatic cells in S phase were labelled by vivisecting testes in Testis Buffer (TB) [208] and culturing for 30 min with EdU in TB prior to fixation and staining using a Click-iT Kit (Life Technologies). Distance of proliferation events from the hub was measured as the linear distance from the hub edge to the nearest Zfh-1$^+$/EdU$^+$ nuclei edge. Images were acquired on an Olympus FV1000 inverted confocal microscope using an UplanSApo 20x0.75, an UplanFL N 40×, 1.30NA oil objective, and an UplanSApo 60×, 1.35 NA oil objective. Image analysis was performed in Olympus Fluoview (Ver.1.7c) unless otherwise stated. Images were processed using Adobe Photoshop CS4 (version 11.0.2).

2.4.6. Zpg subcellular localization measurements

Fluorescence-intensity measurements were performed in ImageJ (NIH) on confocal images of germline cysts, using the line tool. Soma-germline boundaries were defined as borders where Zpg co-localized with Armadillo. Background intensity was measured in the cytoplasm of germline cysts. In *tj>inx2RNAi* testes, the soma-germline boundary was defined as a cell boundary without an adjacent germ cell (based on Zpg staining). A minimum of

seven measurements were made from soma-germline and germline-germline boundaries, and cytoplasm from multiple cysts within each testis and averaged.

2.4.7. Electron microscopy

Testes were dissected in PBS and fixed for 2 h in fixative [1.5% paraformaldehyde (Sigma-Aldrich), 0.1 M sodium cacodylate (Electron Microscopy Sciences), 1.5% glutaraldehyde (Electron Microscopy Sciences), pH 7] at room temperature. Samples were washed three times for 10 min with 0.1 M sodium cacodylate (pH 7.3) and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M Na cacodylate on ice. Samples were washed three times for 10 min with ddH$_2$O, stained for 1 h '*en bloc*' with 1% aqueous uranyl acetate, washed three times for 10 min with ddH$_2$O, then dehydrated through an ascending concentration ethanol series, ending with three changes of 100% ethanol for 10 min each. Dehydration was followed by two changes of 100% propylene oxide for 15 min each. Samples were placed in a 1:1 mixture of propylene oxide:EMBED 812 Resin (Electron Microscopy Sciences) overnight. Testes were embedded in 100% EMBED 812 Resin and polymerized for 48 h at 60°C. Thin sections were cut on a Leica EM UC7 ultramicrotome (Leica Microsystems), placed on 200 mesh copper grids (Electron Microscopy Sciences) and stained with uranyl acetate and lead citrate. Sections were imaged on a Tecnai G2 Spirit electron microscope (FEI North America NanoPort) operated at 120 kV.

2.4.8. Live Imaging

For live-imaging of GCaMP, crosses were set up in a cage with yeast paste on apple juice agar plates at room temperature. Plates were changed approximately every 24 hours for fresh plates and yeast. Embryos were shifted to 29°C for 4-5 days. 3rd instar larvae were collected and dissected in Schneider's *Drosophila* media (ThermoFisher Scientific) to obtain

testes. Testes were transferred to a dissection dish with a coverslip. A 0.8% agarose-PBS pad was used to restrict movement of the testes during imaging. Three-dimensional scans of testes were performed every 10-30 seconds, for up to 10 minutes. Stills from the resulting movies were analyzed for easily detectable changes in GCaMP fluorescence intensity.

2.4.9. Molecular biology

To generate *zpg* transgenes for use in structure-function analysis, a 6.15kb rescue fragment, encompassing the *zpg* genomic locus and an additional 1.5kb upstream and downstream of the locus [135] was cloned into a pattB (addgene) vector using BamHI. For the *zpg::GFP* transgene, a short linker sequence (LAAA) was inserted after the last amino acid of Zpg [209] followed by a GFP transgene. Similarly, for the *zpgΔCT::GFP* transgene, the sequence encoding amino acids 295-367 was removed, replaced with a short linker sequence (LAAA), and followed by a GFP coding sequence. The construct sequences were verified prior to injection (BestGene) into *Drosophila w*⁻ flies carrying *attP40* integration sites on the second chromosome. For *UASt-zpgcDNA*, a vector containing the cDNA of *zpg* was obtained from the Berkley Drosophila Genome Project, care of the *Drosophila* Genomics Resource Centre (cDNA clone RE18536). The zpg cDNA was removed from the vector and cloned into a pTWR vector after removing the C-terminal RFP sequence (*Drosophila* Genomics Resource Centre). The construct sequence was verified prior to injection (BestGene) into Drosophila *w*⁻ flies. Transformants were generated using attP40 integration sites on the second chromosome

2.4.10. Statistics

The mean and standard error of the mean (s.e.m.) are shown. Prism (GraphPad) was used to test significance using unpaired t-tests with Welch's correction. P-values indicated are **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

2.5. Chapter 2 Figures

Figure 2.1. zpg *is required specifically in the germline for GSC maintenance and*
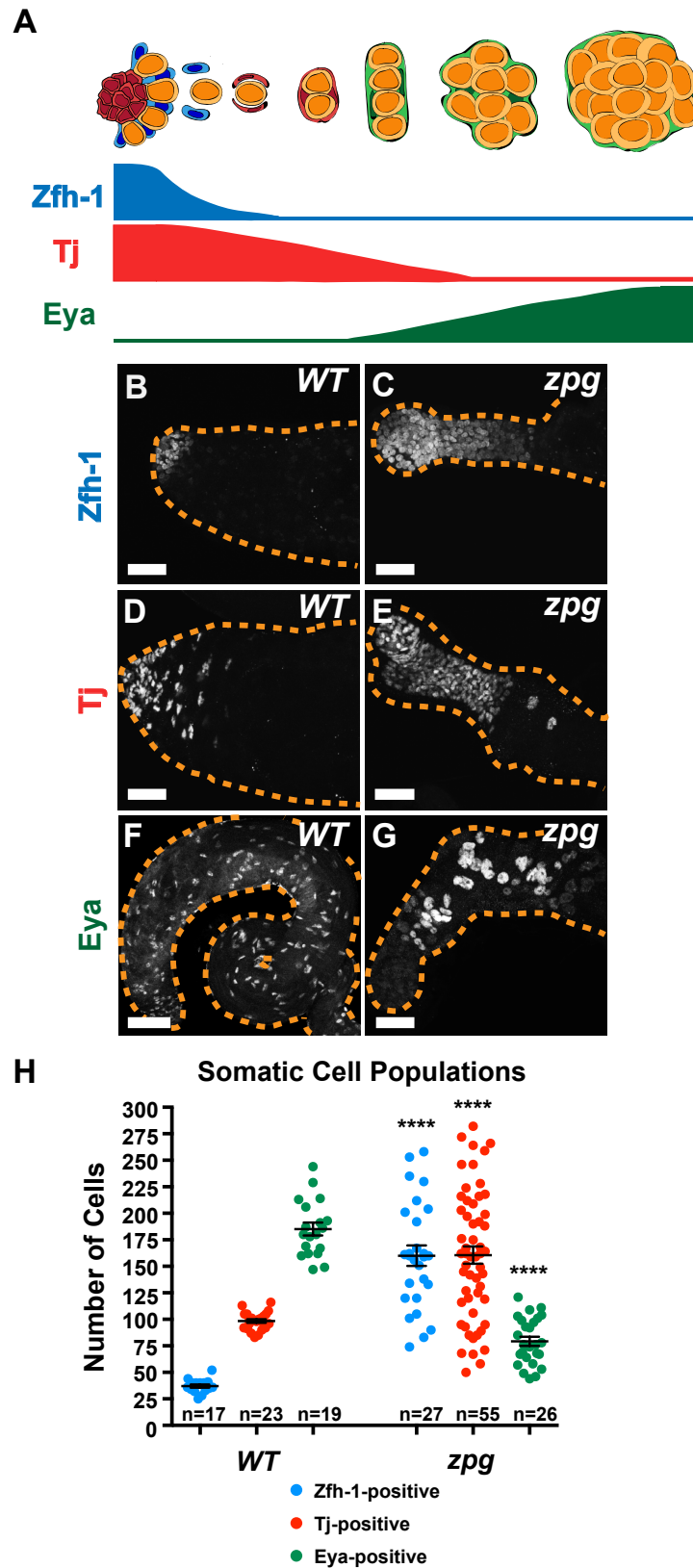
differentiation.
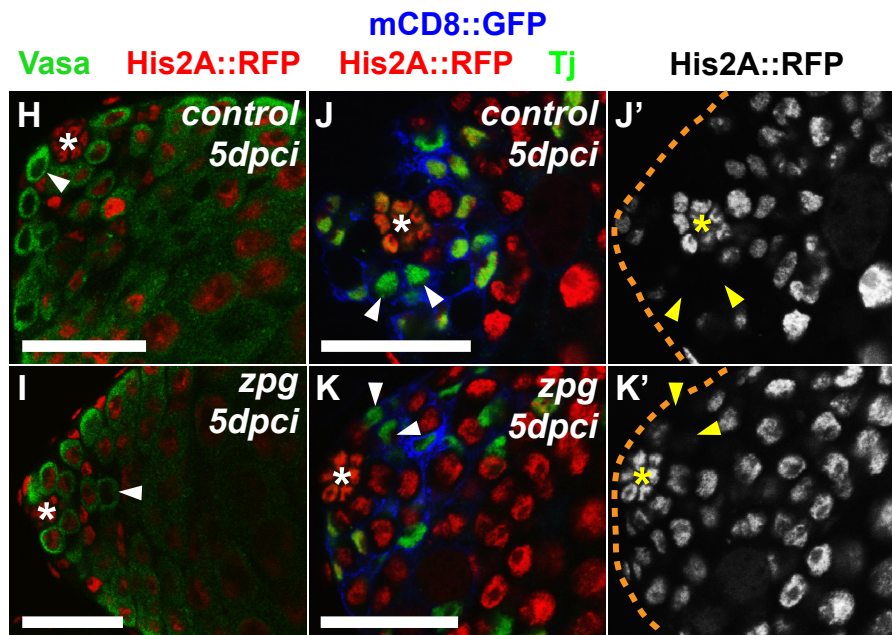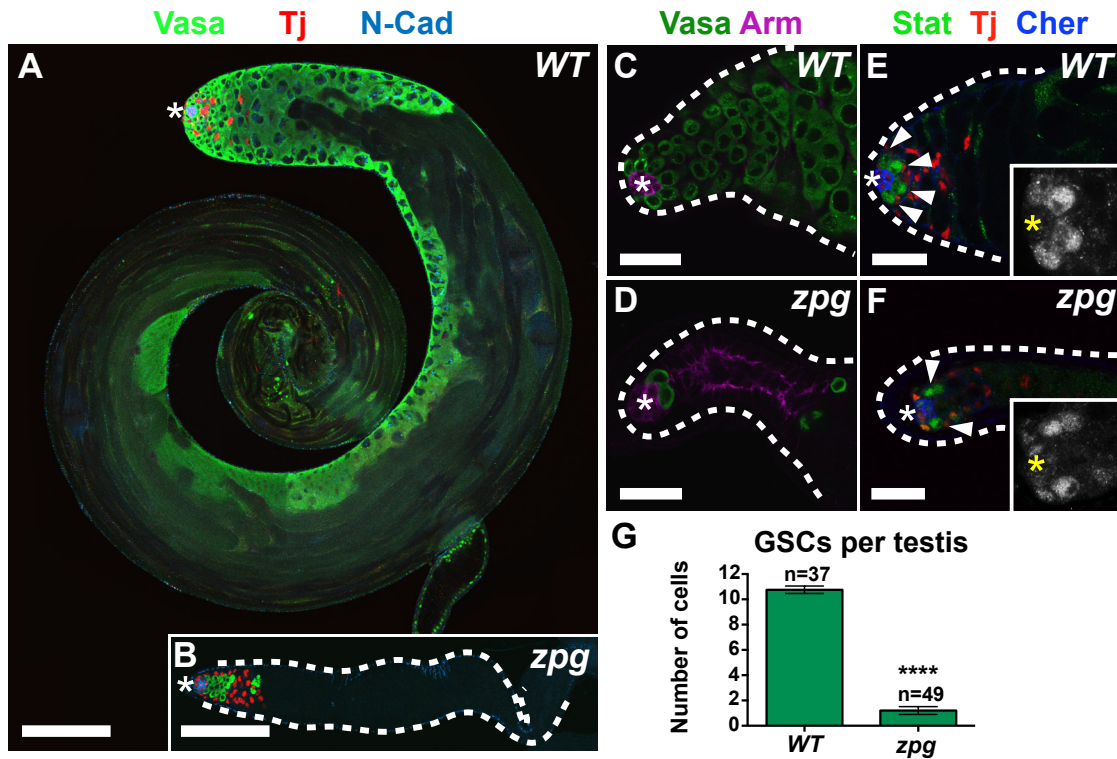
(A, B) Compared with wild-type testes, (A) *zpg* mutant testes (B) are rudimentary and contain few germ cells. (C) Wild-type testes contain GSCs arrayed around the hub. (D) *zpg* mutant testes contain few GSCs, if any. (E, F) In response to JAK/STAT pathway signals from the hub, Stat92E accumulates in GSCs (arrowheads) in both wild-type (E, inset) and *zpg* mutant testes (F, inset). (G) GSC quantification in wild-type and *zpg* mutant testes at 1 day post-eclosion. Error bars show mean±s.e.m., ****$P<0.0001$; *n*, number of testes counted. (H, I) Negatively-labelled germline clones (loss of His2A::RFP) were either wild-type controls or homozygous for a *zpg* null allele, *zpg*$^{z-5352}$. (H) Control clones (arrowhead) were maintained as GSCs at 5 days dpci. (I) *zpg*$^{z-5352}$ clones were never successfully maintained as GSCs at 5 dpci, and rarely at 4 dpci. Where *zpg*$^{z-5352}$ clones were detected, they existed as single-cell clones, displaced from the niche. (J-K′) Negatively-labelled somatic clones were either wild-type controls or homozygous for *zpg*$^{z-5352}$. Both control (J) and mutant clones (K) were detected in the CySC position at 5 dpci (arrowheads). Germline labelled for Vasa (green; A-D,H,I) CySCs and early soma with Tj (red in A,B,E,F; green in J,K), hub with N-Cad (CadN − FlyBase; blue; A,B), Arm (magenta; C,D), Cheerio (blue; E,F), Stat92E detected by antibody (green; E,F), and clones labelled by loss of His2A::RFP (red), mCD8::GFP labels soma (blue; J,K). Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 100 µm in A, B; 30 µm in C-F, H-K.

*Figure 2.2.* zpg *is required for germline differentiation and association with the soma.*
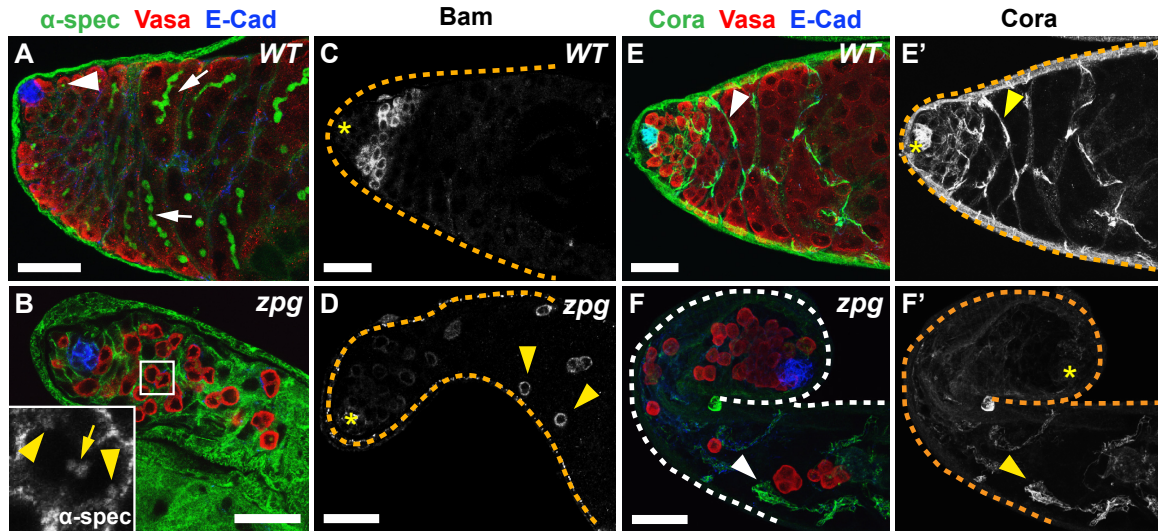
(A) In wild-type testes, small dot spectrosomes (arrowhead) enlarge and branch, forming fusomes (arrows). (B) In *zpg* mutant testes, germ cells contained spectrosomes and occasionally small dumbbell-shaped fusomes (inset, arrow). However, ectopic or fragmented spectrosomes were also detected, indicative of abnormal differentiation (inset, arrowheads). (C) Bag of Marbles (Bam) expression is repressed in GSCs by niche-derived BMP signalling. Bam expression is detected in germ cells beginning at the 2- to 4-cell stage in wild-type testes until the 16-cell stage. (D) In *zpg* mutant testes, Bam is detected in single-celled germ cells that are far from the hub (arrowheads). (E, E′) In wild-type testes, germ cells are wrapped by somatic cells shortly after exiting the niche and remain encysted throughout spermatogenesis, detected by Cora staining (arrowhead). (F, F′) In *zpg* mutant testes, germ cell clusters far from the niche are not encysted by the soma (arrowhead). Germline labelled for Vasa (red; A, B, E, F); spectrosomes/fusomes with α-spectrin (green; A, B); the hub with E-Cad (blue; A, B, E, F); and encystment by Cora (green; E, F). Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 30 μm.

Figure 2.3. zpg *is required non-autonomously for differentiation of the soma.*

(A) Schematic summarizing stage-specific expression of somatic cell differentiation markers Zfh-1, Tj and Eya in the testis. (B) In wild-type testes, Zfh-1 is highly expressed in CySCs and weakly in their immediate daughters. (C) In *zpg* mutant testes, the apical tip is filled by Zfh-1^{+} cells. (D) In wild-type testes, Tj is expressed in the hub, CySCs and early somatic cells. (E) In *zpg* mutant testes, Tj^{+} cells fill the tip of the testis, similar to expression of Zfh-1 in *zpg* mutant testes. (F) In wild-type testes, Eya is detected in the soma beginning near the 4-cell cyst stage until the end of spermatogenesis. (G) In *zpg* mutant testes, fewer Eya^{+} cells were observed than in the wild-type. Dashed lines outline the testis. (H) Quantification of Zfh-1^{+}, Tj[+] and Eya^{+} somatic cells in wild-type and *zpg* mutant testes. *n*, number of testes counted. Error bars show mean±s.e.m.; ****P<0.0001. Scale bars: 30 μm in B-E, G; 100 μm in F.

Figure 2.4. zpg *may be required non-autonomously in the soma for repressing the ectopic expression of hub cell markers.*
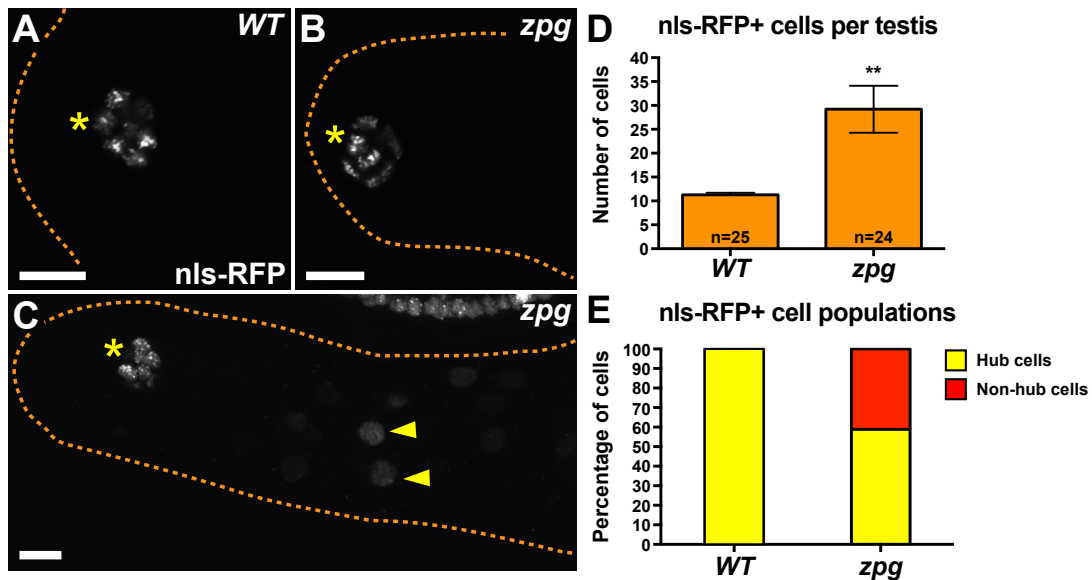
(A-C) Expression of a *UAS-nls-RFP* under the control of the hub-specific *upd-GAL4* promoter. (A) In wild-type testes, nls-RFP expression is restricted to the hub. (B) Expression of nls-RFP in *zpg* mutant testes is often restricted to the hub; however, ectopic expression of nls-RFP was also detected far from the hub (C, arrowheads). (D) Quantification of *upd>nls-RFP$^+$* cells in wild-type and *zpg* mutant testes. (E) Breakdown of *upd>nls-RFP$^+$* populations shown in D. nls-RFP cells are only detected in the hub in wild-type testes; in *zpg* mutant testes, a large proportion of total nls-RFP cells are not hub cells. Testes were stained with antibodies to detect RFP. Asterisks indicate the hub. Dashed lines outline the testis. Scale bars: 10µm. n, number of testes. Error bars show mean+/-s.e.m.; *P<0.01*.

Figure 2.5. zpg *is required non-autonomously to regulate the proliferation of the early soma.*
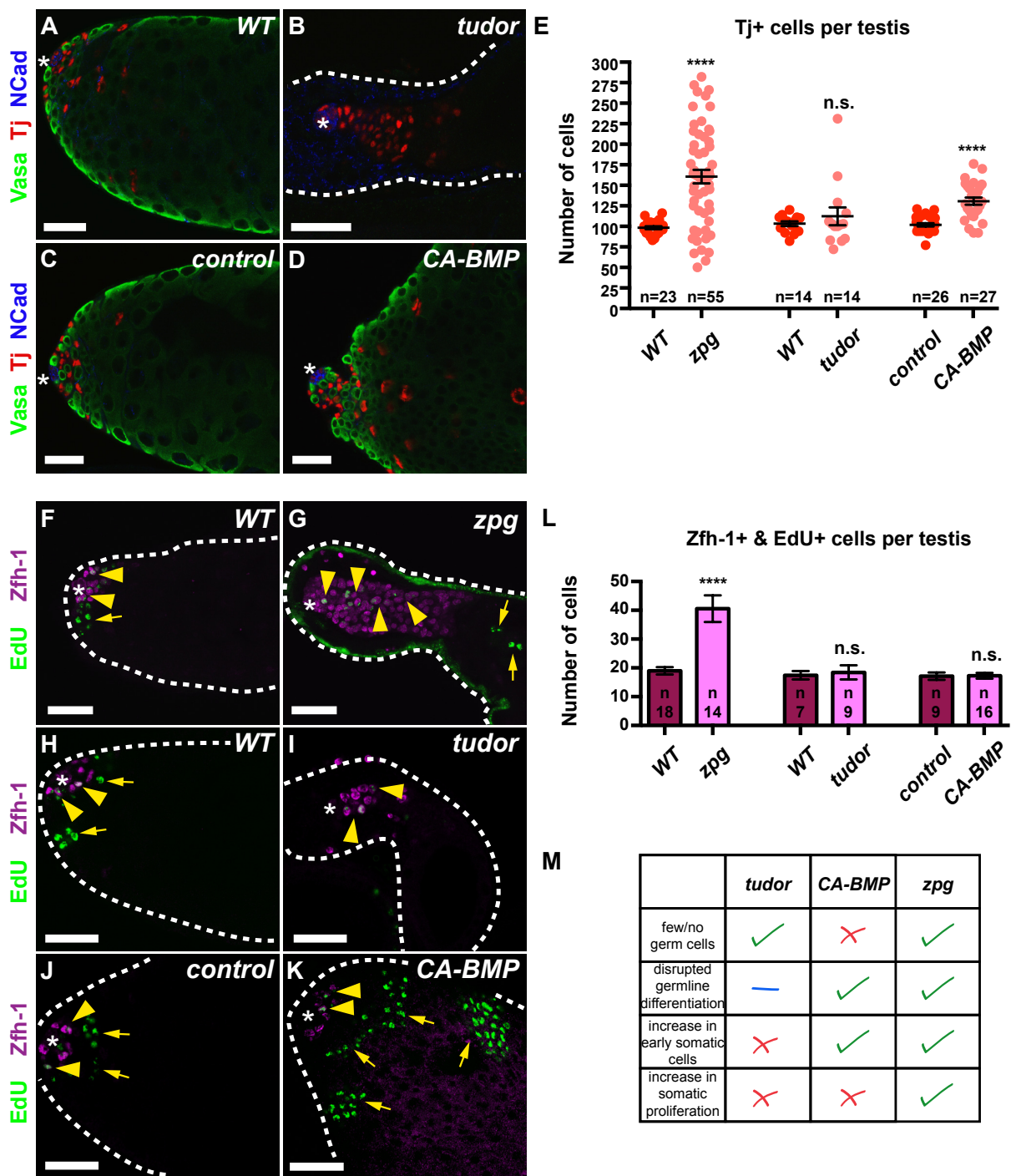
(A-E) Analysis of early somatic cell numbers in agametic testes and testes expressing constitutively active BMP receptors in the early germ line using *nos-GAL4*. Germline labelled by Vasa (green), early soma by Tj (red) and the hub by N-Cad (blue). (A, B)

Tj$^+$ cells in w^{1118} (WT) controls (A) and in *tudor* mutant testes (B), which do not form a germ line. (C, D) Tj$^+$ cells in control (w^{1118}; *UAS-tkv^{Act}, sax^{Act}*/+) testes (C) and in testes expressing CA-BMP receptors in the early germ line (*nos-GAL4 >UAS-tkv^{Act}, sax^{Act}*/+; D). (E) Quantification of Tj$^+$ cells in *zpg*, *tudor* and *nos*>CA-BMP testes versus their respective controls (in order: *zpg*/+, w^{1118}, w^{1118}; *UAS-tkv^{Act}, sax^{Act}*/+). Loss of Zpg or constitutive-activation of BMP signalling led to a significant increase in Tj$^+$ cells. (F-K) Quantification of S-phase somatic cells labelled with Zfh-1 and EdU in wild-type, *zpg*, *tudor*, control and CA-BMP testes. (F) In wild-type testes, Zfh-1$^+$/EdU$^+$ cells were detected adjacent to the hub (arrowheads). (G) In *zpg* mutant testes, Zfh-1$^+$/EdU$^+$ cells were detected throughout the apical tip (arrowheads). Germ cells in S phase were observed in both backgrounds (arrows). (H, I) Zfh-1$^+$/EdU$^+$ cells (arrowheads) were detected adjacent to the hub in wild-type and *tudor* testes, as were germ cells in wild-type testes (arrows). (J, K) In control and CA-BMP testes, Zfh-1$^+$/EdU$^+$ cells were detected near the hub (arrowheads), in addition to germ cells in S phase (arrows). (L) Quantification of Zfh-1$^+$/EdU$^+$ cells in *zpg*, *tudor* and *nos*>CA-BMP testes versus their respective controls (in order: *zpg*/+, w^{1118}, w^{1118}; *UAS-tkv^{Act}, sax^{Act}*/+). A significant increase in proliferating somatic cells was only seen in *zpg* mutant testes. (M) Table summarizing germline and somatic phenotypes in the different genotypes studied. *n*, number of testes. Error bars show mean±s.e.m.; ****$P<0.0001$. Asterisks indicate the hub, detected by N-Cad or Cheerio staining. Dashed lines outline the testis. Scale bars: 30 μm.

Figure 2.6. Analysis of innexins in the testis.

Inx2 expression in the testis

Zpg expression in the testis

Inx3 expression in the testis
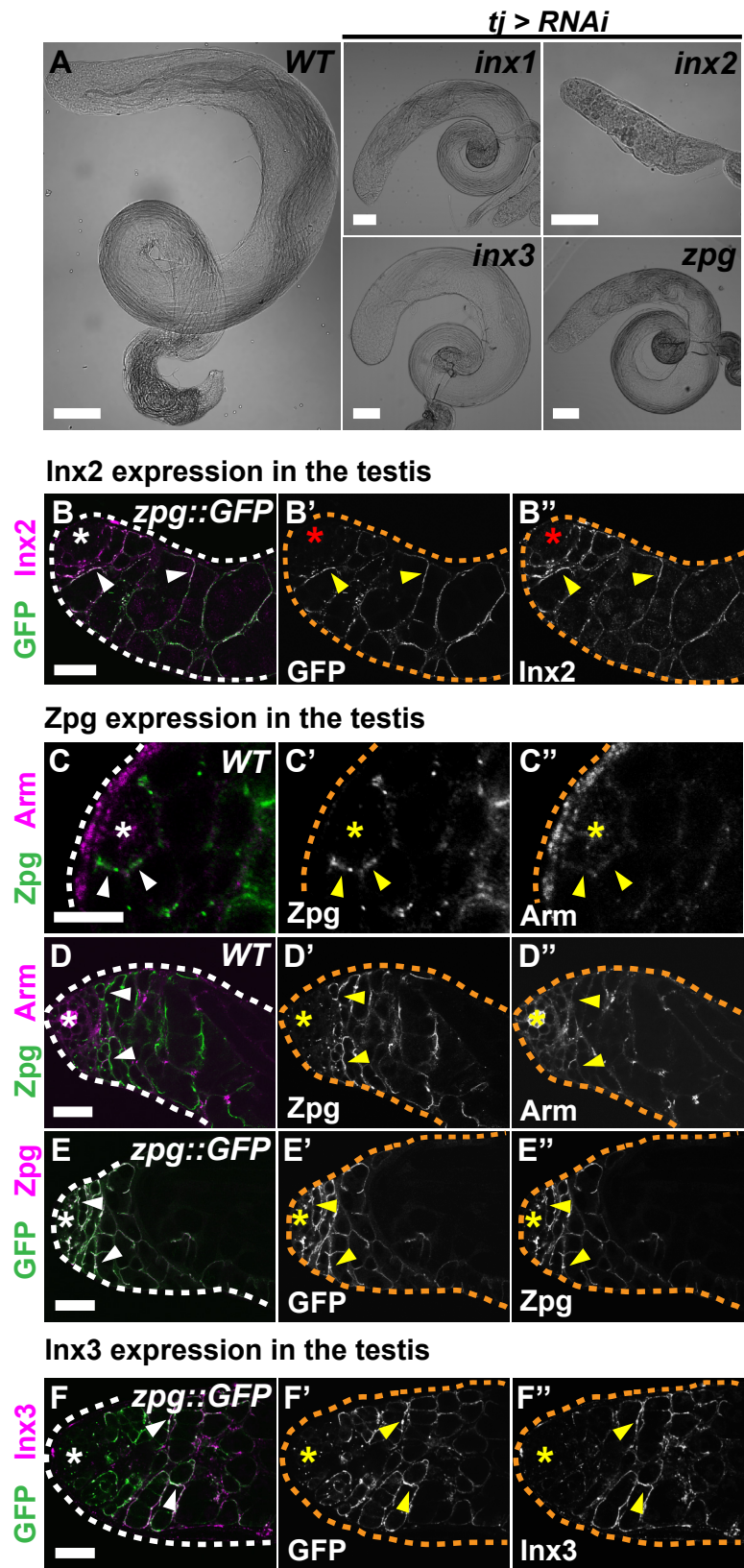
(A) Representative differential interference contrast images of wild-type testis and testes in which Innexins 1-4 were somatically knocked down using RNAi driven with *tj-GAL4*. Only Innexin 2 (Inx2) knockdown produced a mutant phenotype. (B) Inx2 (magenta) prominently co-localized (arrowheads) with Zpg (green; visualized using a GFP-tagged rescue construct) beginning at the 4-cell cyst stage. (C-C″) Weak Zpg (green) expression was detected at the hub-GSC interface (arrowheads, hub marked with Arm in magenta). (D-D″) Strong Zpg expression (arrowheads; green) was observed in 4- to 16-cell-stage cysts (hub marked with Arm in magenta). (E-E″) The GFP-tagged Zpg rescue construct expression (arrowheads) mimicked endogenous Zpg protein expression, detected with Zpg-specific antibody (magenta). (F-F″) Inx3 (magenta) also co-localized (arrowheads) with Zpg (green; GFP-tagged rescue construct) during early spermatogenesis. Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 100 μm in A; 10 μm in C; 30 μm in all other panels.

Figure 2.7. Inx2 alone is required in the soma for fertility and Inx2 is expressed early in spermatogenesis.
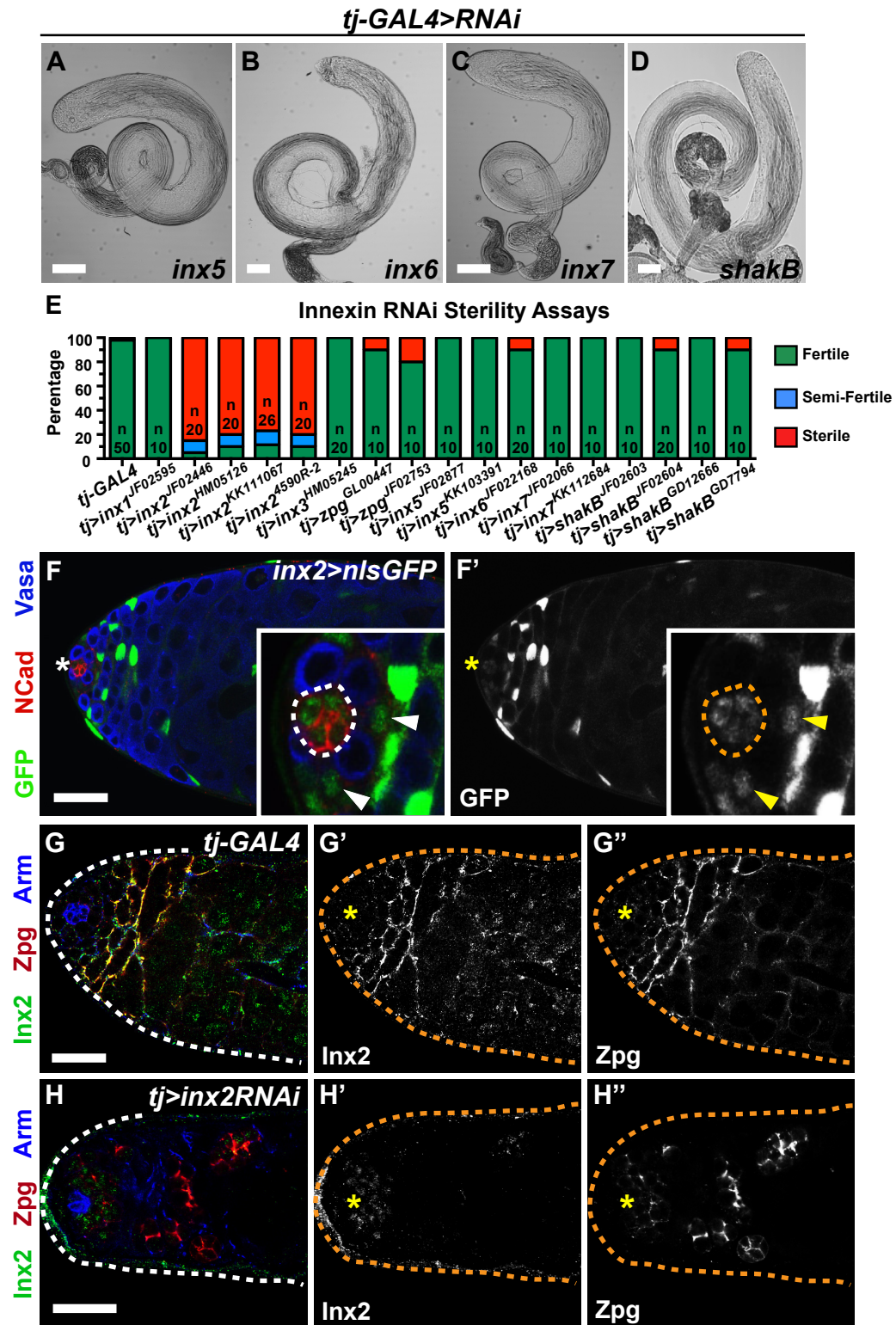
(A-D) Representative DIC images of testes in which Innexin 5, 6 ,7 and 8 (shaking-B) were knocked down in the soma using RNAi lines (see materials and methods) driven with *tj-GAL4*. No visible phenotypes were observed with these knockdowns. (E) Summary of fertility assays carried out with various RNAi lines targeting every fly innexin driven by *tj-GAL4* in the soma (*n*=number of independent fertility assays). Inx2 was the only innexin that was consistently required for fertility. (F-F') Expression of nls-GFP under the control of the *inx2* promoter. Reporter expression can be detected weakly in hub cells and CySCs, before increasing in early somatic cells. Arrowheads indicate CySCs. (G) control and (H) *tj>inx2RNAi* testes that were stained with antibodies to detect Inx2 (green) and Zpg (red) proteins show that RNAi-mediated knockdown substantially reduced Inx2 expression (Armadillo in blue was used to highlight the soma and the hub). Asterisk indicates the hub. Dashed lines outline the hub in F'F', and the testis in G-H. Scale bars A-D are 100μm and 30μm in all other panels.

Figure 2.8. Ultrastructural analysis of gap junctions in the testis.
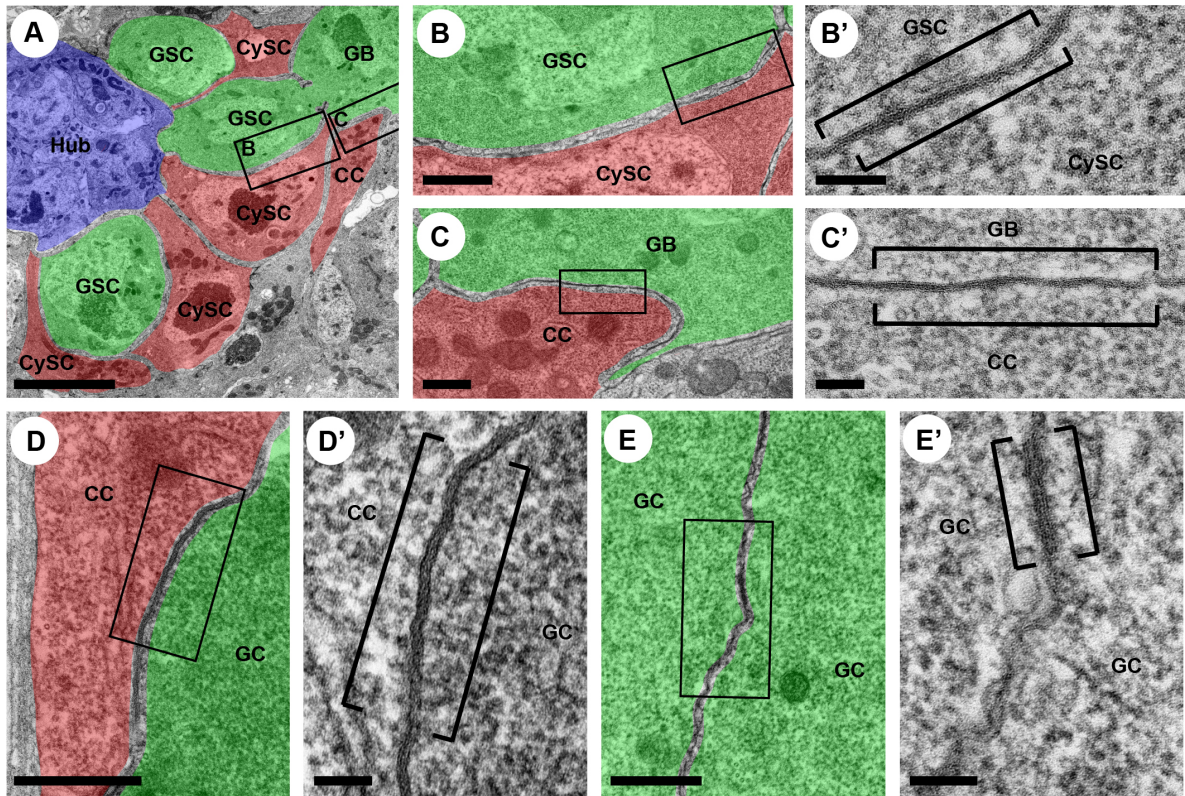
(A-E) Electron micrographs of wild-type testes. (A) Overview of the hub cell niche. (B, C) Gap junctions were detected early during spermatogenesis between both GSCs and CySCs (B, B′) and between cyst cells (CCs) and 1-cell-stage gonialblasts (GBs; C, C′). (D, D′) Outside the niche, germ cells (GCs) exhibited large gap junctions with associated CCs. (E,E′) Gap junctions were also observed between neighbouring germ cells (E′, brackets), although these were less common and smaller than those between germ cells and cyst cells (compare E′ with B′-D′). The hub is highlighted in blue, germ line in green and soma in red. Square brackets indicate gap junctions. Scale bars: 5 μm in A; 1 μm in B, C; 500 nm in D, E; 100 nm in B′-E′.

Figure 2.9. Inx2 is required in the soma for the subcellular localization of Zpg.
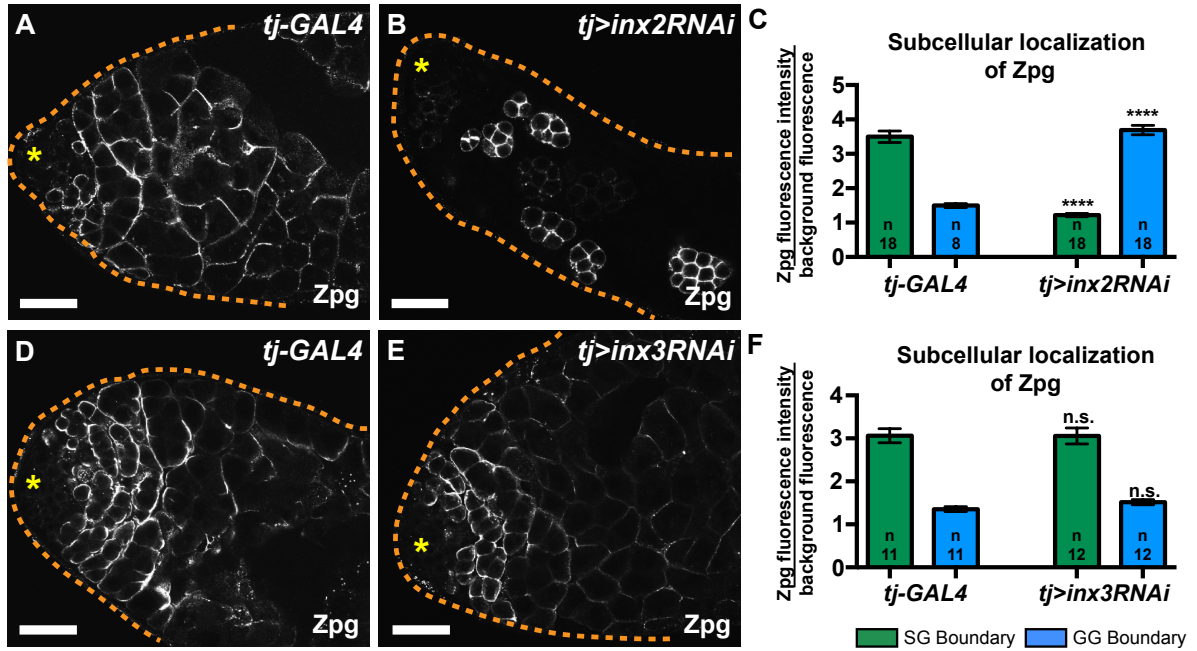
(A, B) Control (*tj-GAL4*) and *tj>inx2RNAi* testes labelled with anti-Zpg antibody. (A) Zpg localized predominantly to soma-germline boundaries. (B) Following Inx2 somatic knockdown, Zpg was enriched at germline-germline boundaries. (C) Quantification of Zpg enrichment based on normalized fluorescence intensity. Zpg was recruited to soma-germline boundaries at approximately three times greater levels than to germline-germline boundaries in control testes; this recruitment was disrupted following Inx2 knockdown and Zpg became enriched at germline-germline boundaries compared with controls. (D-F) Analysis of Zpg recruitment upon somatic Inx3 knockdown. Inx3 knockdown did not affect the subcellular localization of Zpg compared with controls. *n*, number of testes quantified. Error bars show mean±s.e.m.; ****P<0.0001. Significance indicates differences between either soma-germline boundaries between samples, or germline-germline boundaries between samples, respectively. Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 30 μm.

Figure 2.10. Inx3 is not required in the soma for spermatogenesis, but Inx2 is required in the soma for the localization of Inx3.

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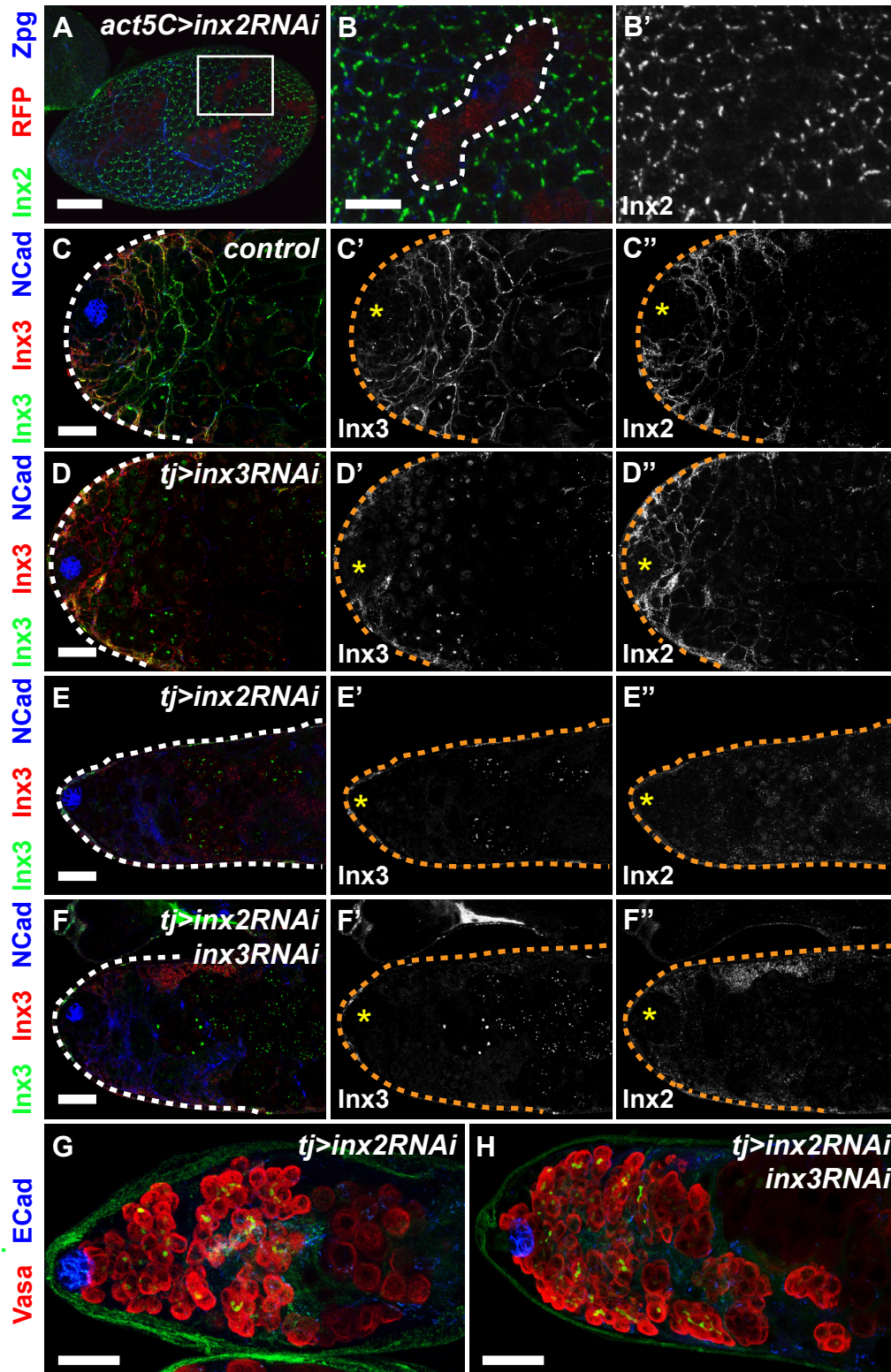
(A-B) Clonal overexpression of *inx2RNAi* in developing egg chambers. At 2 days post-clone induction, Inx2 protein levels were greatly reduced in clones, compared to neighbouring cells based on Inx2 antibody staining. (C-F) Control, *inx3RNAi*, *inx2RNAi*, and *inx2RNAi+inx3RNAi* testes stained for Inx3 (green), Inx2 (red), and N-Cad (blue). (C) Inx3 and Inx2 co-localized in control testes. (D) Upon knockdown of Inx3, Inx3 staining was reduced, but Inx2 staining remained similar to controls. (E) Somatic knockdown of Inx2 disrupted Inx3 localization, in addition to reducing Inx2 protein levels. (F) Knockdown of both Inx2 and Inx3 in the soma greatly decreased levels of both Inx2 and Inx3. (H) *tj>inx2RNAi* and *tj>inx2RNAi, inx3RNAi* testes show that double knockdown of Inx2 and Inx3 in the soma results in a phenotype similar to that obtained from knockdown of Inx2 alone (Vasa in red; E-Cad in blue; α-spectrin in green). Asterisk indicates the hub. Dashed lines outline the clone in B, the testis in C-F. Scale bars are 30µm in all panels.

*Figure 2.11. Inx2 is required in the soma for GSC maintenance and germline differentiation.*
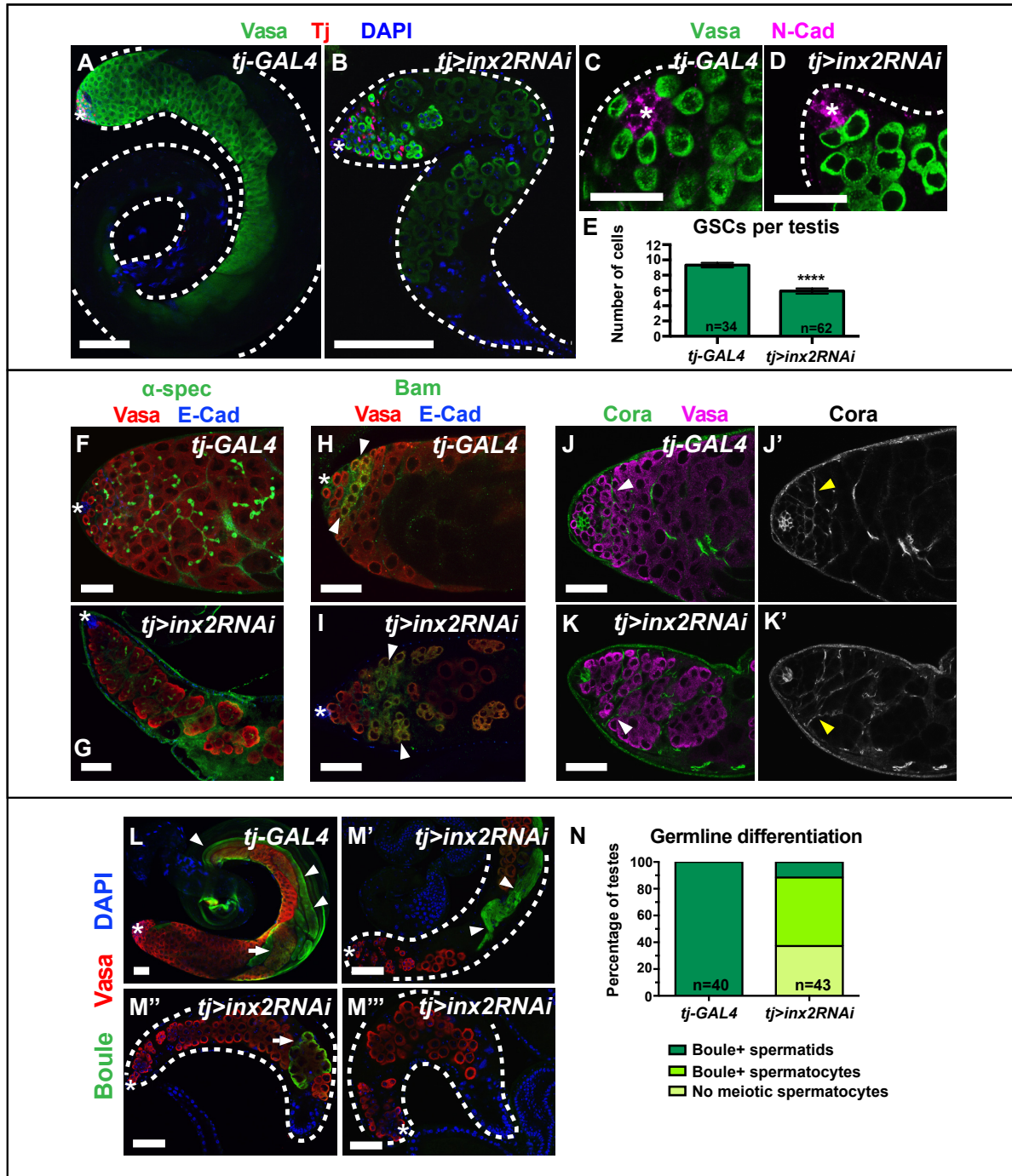
(A, B) Control (*tj-GAL4*) and *tj>inx2RNAi* testes labelled for Vasa (green), Tj (red) and DAPI (blue). Somatic Inx2 knockdown resulted in small rudimentary testes. (C, D) Close-up of the apical tip of control and *tj>inx2RNAi* testes (hub highlighted with N-Cad in magenta,

Vasa in green). (E) GSC number was significantly reduced upon Inx2 knockdown in the soma compared with controls. (F, G) Labelling with the spectrosome/fusome marker α-spectrin (green) revealed mostly normal fusomes in *tj>inx2RNAi* and control testes (Vasa in red; E-Cad in blue). (H, I) Bam expression (green) was similar in control and *tj>inx2RNAi* testes (arrowheads; Vasa in red; E-Cad in blue). (J-K′) Encystment, marked by labelling for Cora (green; Vasa in magenta) appeared largely normal in *tj>inx2RNAi* testes. Arrowheads indicated encystment, detected by Cora staining. (L) In wild-type testes, meiotic spermatid stages were marked by expression of Boule protein (green; Vasa in red; DAPI in blue). (M-M″) Examples of the phenotypic range observed in *tj>inx2RNAi* testes arranged from the least (M) to most (M″) severe. (L-M″) Arrowheads indicate Boule[+] spermatids and arrows indicate Boule[+] spermatocytes. (N) Quantification of the effect of Inx2 knockdown on germline differentiation, showing the percentage of testes observed in each phenotypic class in control and *tj>inx2RNAi* testes. Nearly one-third of *tj>inx2RNAi* testes exhibited no Boule expression. *n*, number of testes. Error bars show mean±s.e.m.; ****P<0.0001. Asterisks indicate hub. Dashed lines outline the testis. Scale bars: 100 μm in A-B, L-M; 30 μm in all other panels.

Figure 2.12. Cadherin expression is not misregulated upon changes in zpg *or* inx2 *expression.*
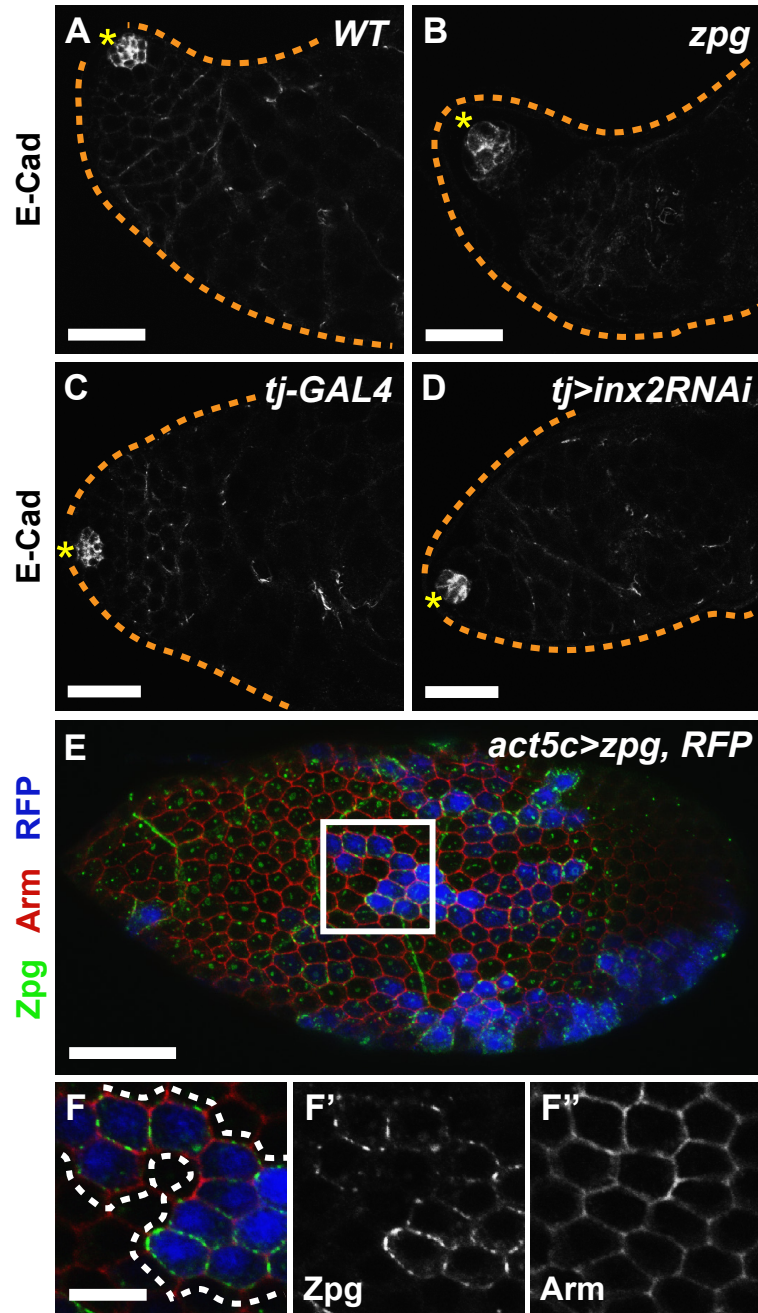
(A-B) Wild-type and *zpg* testes stained for E-Cad. In both wild-type (A) and *zpg* testes (B), E-Cad was enriched in the hub and could be detected in differentiating germline cysts. (C-D)

In control testes and upon somatic knockdown of Inx2, E-Cad staining showed a similar staining pattern, with enrichment in the hub, and lower levels of expression outside of the niche. (E) Overview of a stage 9 developing egg chamber. A heat-shock-inducible *act5c-GAL4* drives expression of RFP (blue) and a *UASt-zpg^{cDNA}* transgene (green). Armadillo (red) labels cell-cell junctions. (F) Inset of clone from E. Expression of Zpg (F') does not visibly alter levels of Armadillo within clones (F''). Asterisk indicates the hub. Dashed lines outline the testis in A-D, the clone in F. Scale bars are 30μm in A-E, 10μm in F.

Figure 2.13. Inx2 is required to regulate the proliferation and differentiation of the early soma.
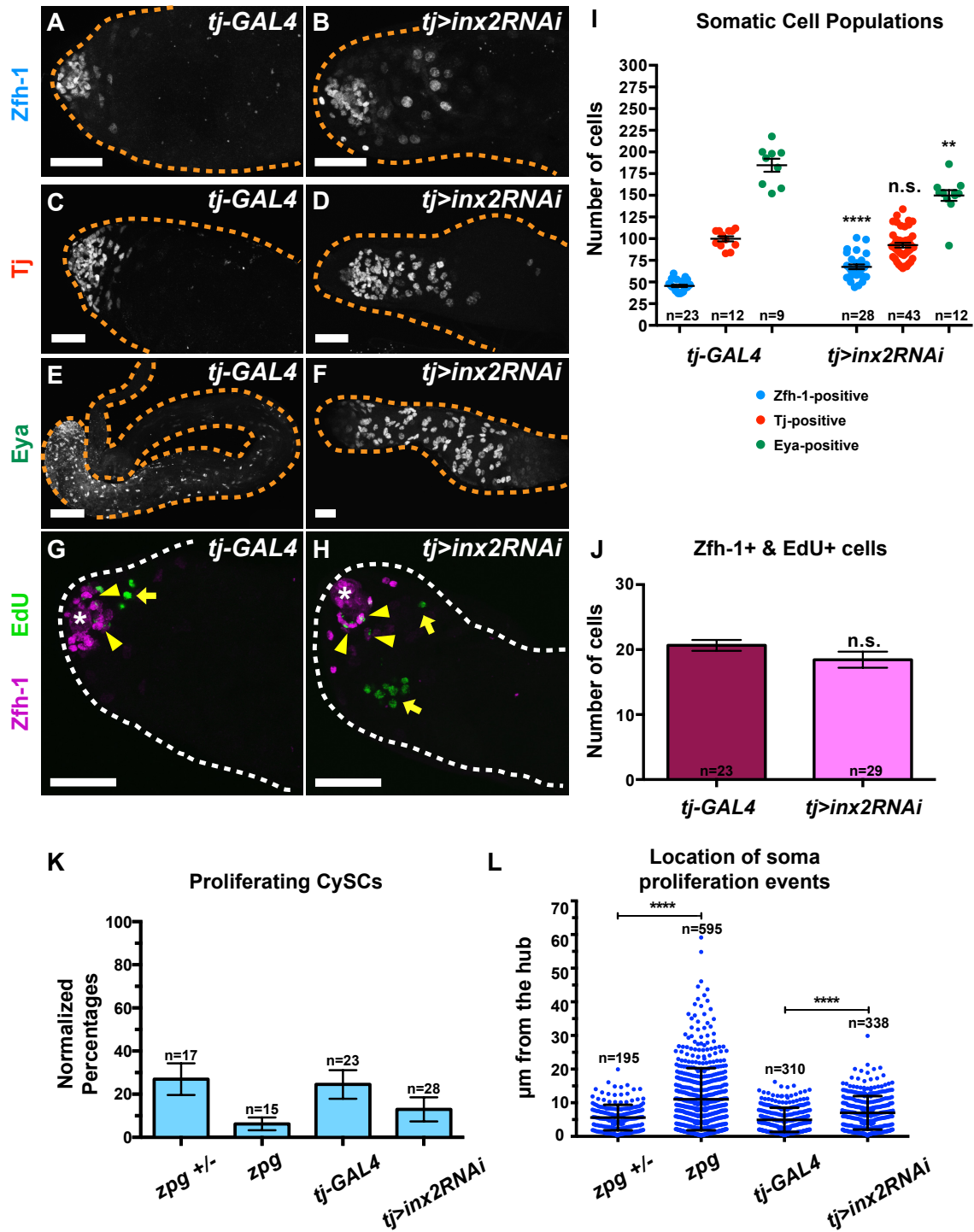
(A-F) Expression of the stage-specific somatic cell markers Zfh-1 (A, B), Tj (C, D) and Eya (E, F) in control (*tj-GAL4*) and *tj>inx2RNAi* testes. (G, H) S-phase somatic cells are labelled for Zfh-1 (magenta) and EdU (green) in control and *tj>inx2RNAi* testes. Arrowheads indicate proliferating CySCs, arrows indicate germ line. (I) Quantification of Zfh-1$^+$, Tj$^+$ and Eya$^+$ cells in control and *tj>inx2RNAi* testes. (J) Quantification of Zfh-1$^+$/EdU$^+$ cells. (K) Percentage of proliferative CySCs in wild-type (*zpg*$^{+/-}$), *zpg*, control and *tj>inx2RNAi* testes normalized to total number of Zfh-1$^+$ cells per testis. (L) Quantification of distance of somatic proliferation events from the hub in wild-type, *zpg*, control and *tj>inx2RNAi* testes. *n*, number of testes quantified in I-K; single Zfh-1$^+$/EdU$^+$ cells in L. Error bars show mean±s.e.m.; **$P<0.01$; ****$P<0.0001$. Asterisks indicate the hub. Dashed lines outline the testis. Scale bars: 30 μm in A-D, F-H; 100 μm in E.

Figure 2.14. Transgenes for structure-function analysis of Zpg.

Transgene	Diagram	Question it will address
genomic rescue *UAS-rescue*		**Can our construct rescue *zpg*?** **Zpg only required in germline?**
zpg::GFP		**Can Zpg be fluorescently-tagged without disrupting protein localization?**
zpg^{Cys-less}		**Does Zpg function as a hemi-channel?**
GFP::zpg		**Does Zpg need to form a channel to function?** **Can it mediate cell adhesion?**
zpg^{ΔC-terminus}		**Is the C-terminus required for function?**
zpg-C-terminus		**Is the C-terminus sufficient to mediate signalling?**
zpg^{phospho-dead}		**Is phosphorylation of the C-terminus important?**

Transgenes to be used to investigate functional domains/residues of Zpg and the questions each transgene may be used to answer.

Figure 2.15. A wild-type zpg *transgene rescues fertility in* zpg *mutant testes.*
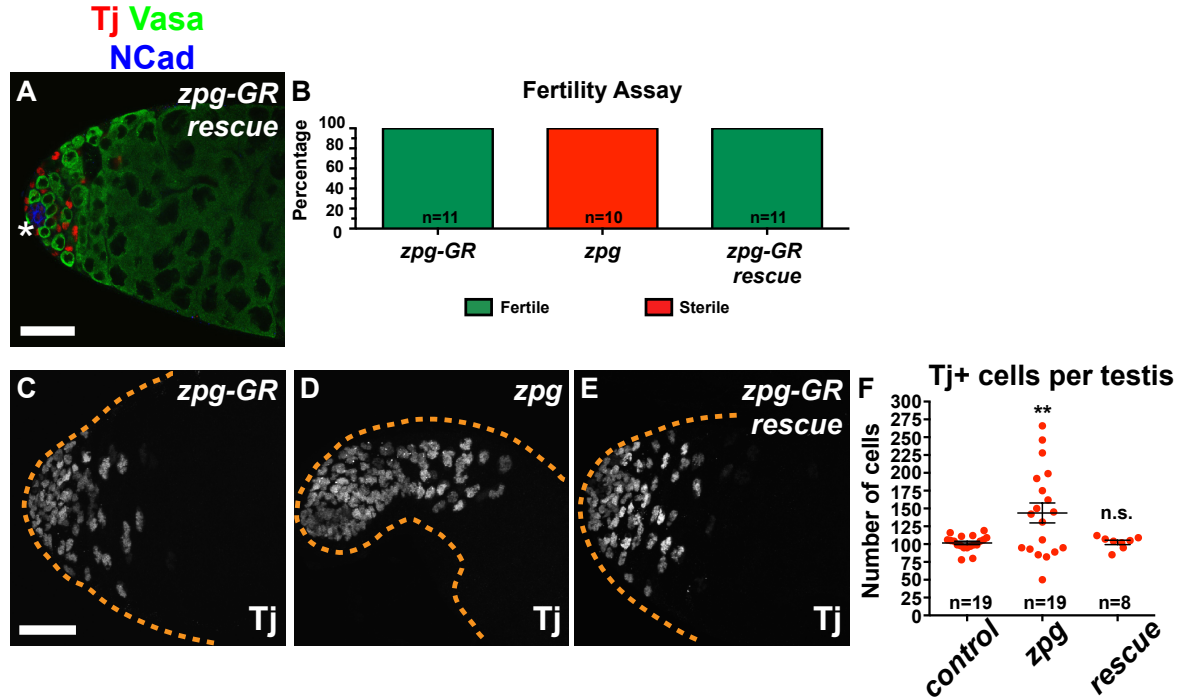
(A) A wild-type *zpg* transgene, encoding the *zpg* locus and 1.5kb upstream and downstream of the locus, was able to rescue the *zpg* mutant phenotype, observed by immunohistochemistry. (B) Fertility assays demonstrate that the wild-type *zpg* transgene restored fertility in *zpg* mutant males to wild-type levels. (C-F) Analysis of Tj+ cell counts shows that the wild-type *zpg* transgene can restores Tj+ cell numbers to wild-type levels. (compare C and E to D, quantified in F). Testes were stained with antibodies to detect Zpg (green), the early soma by Tj (red), and N-Cad labels the hub (blue) in A; Tj labels the early soma (white) in C-E. Asterisk indicates the hub. Dashed lines outline the testis. Scale bars: 30µm. n, number of testes. Error bars shown mean +/-s.e.m.; ** *P<0.01*, n.s., not significant.

Figure 2.16. A GFP-tagged, wild-type zpg transgene rescues fertility in zpg mutant testes.
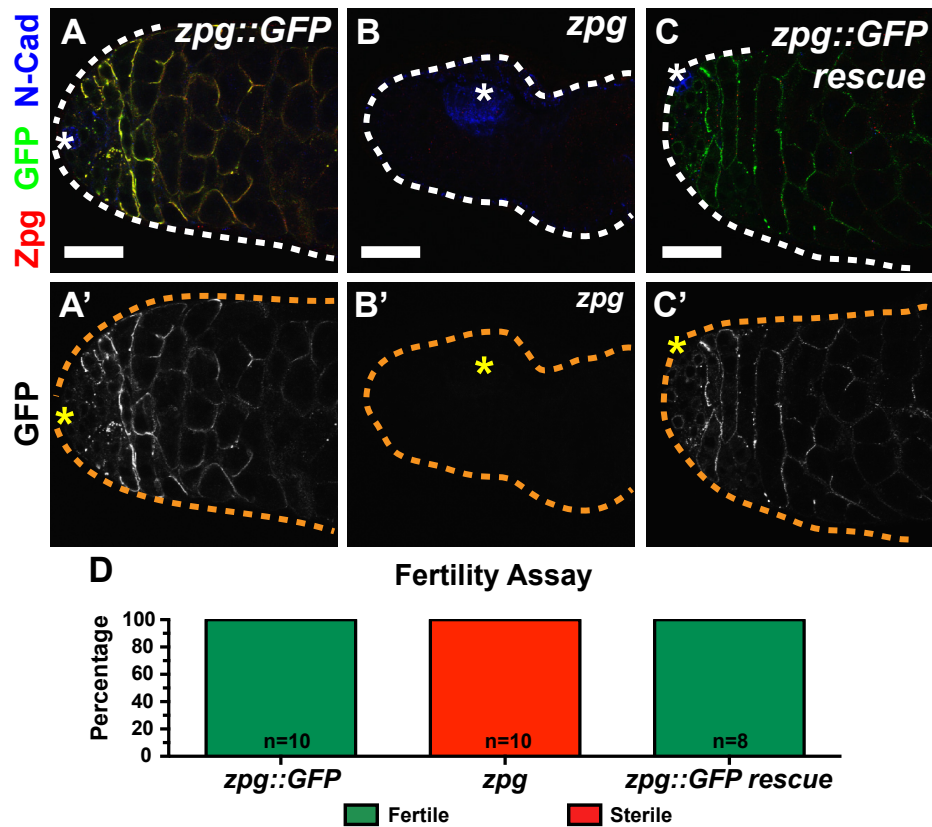
(A-C) Expression of a GFP-tagged *zpg* transgene under the putative endogenous promoter of *zpg* co-localized with endogenous Zpg (A). The *zpg::GFP* transgene (C) was able to rescue the *zpg* mutant (B) phenotype, observed by immunohistochemistry. (D) Fertility assays demonstrate that the *zpg::GFP* transgene restored fertility in *zpg* mutant males to wild-type levels. Testes were stained with antibodies to detect Zpg (red), GFP (green), and N-Cad (blue). Asterisk indicates the hub. Dashed lines outline the testis. Scale bars are 30μm in all panels.

Figure 2.17. The Zpg C-terminus is essential for the function of Zpg.
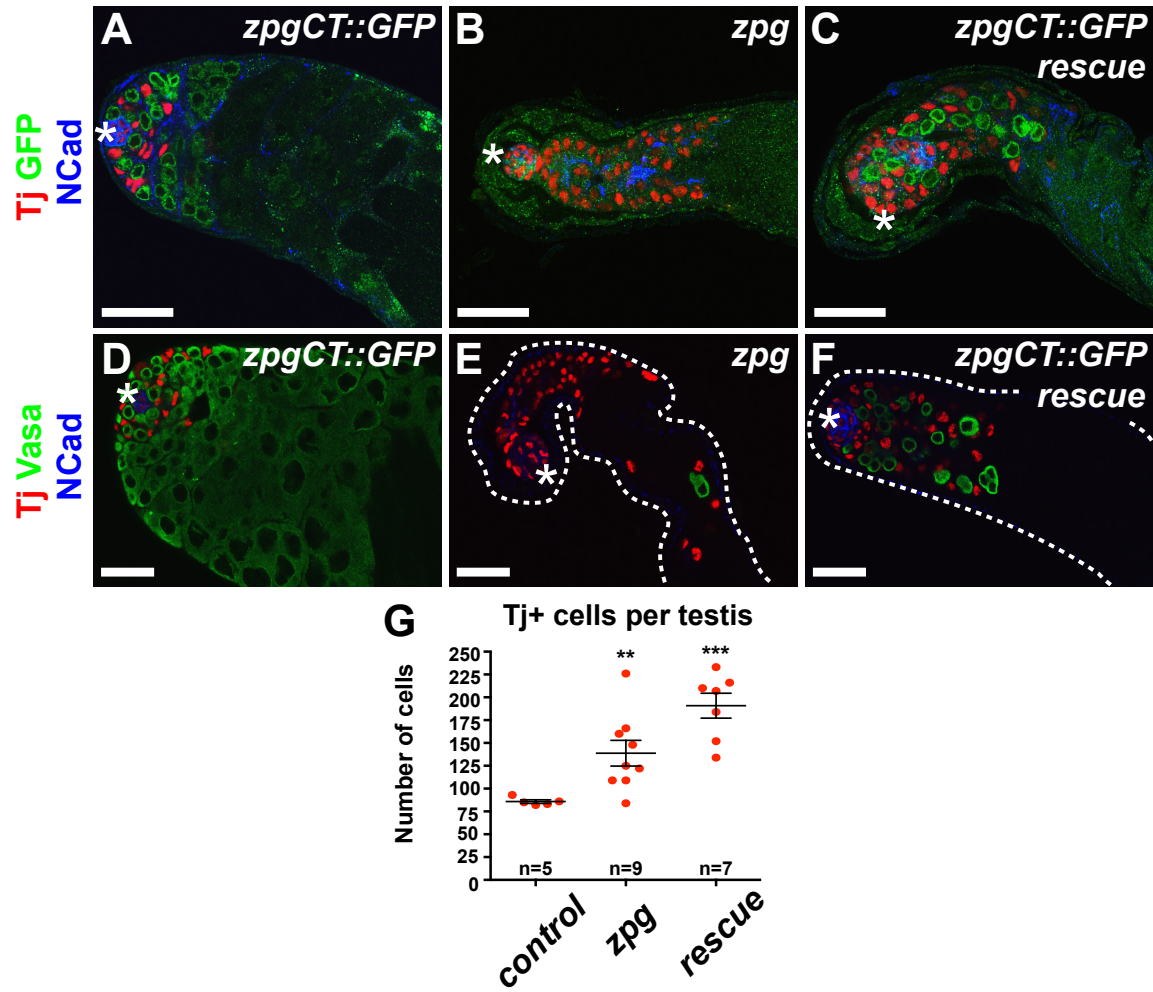
(A-F) Expression of a GFP-tagged *zpg* transgene with a deletion of the cytoplasmic C-terminus. (A) ZpgΔCT::GFP is not trafficked to the plasma membrane in wild-type testes and appears to be trapped in the endoplasmic reticulum. (B) *zpg* mutant testis without the transgene. (C) ZpgΔCT::GFP does not localize to the plasma membrane, even in zpg mutant testes. (D-G) Tj+ cell counts demonstrate that *zpgΔCT::GFP* does not rescue the zpg mutant phenotype. Testes were stained with antibodies to detect ZpgΔCT::GFP in germ cells (green) in A-C, Vasa (green) labels germ cells in D-F, Tj (red) to detect early somatic cells in A-F, and N-Cad (blue) labels the hub in A-F. Asterisk indicates the hub. Dashed lines outline the

testis. Scale bars: 30um. n, number of testes. Error bars show mean=-s.e.m.; *****, *****, *P<0.01,
P<0.001.*

Figure 2.18. Model of gap junction signalling in the testis.
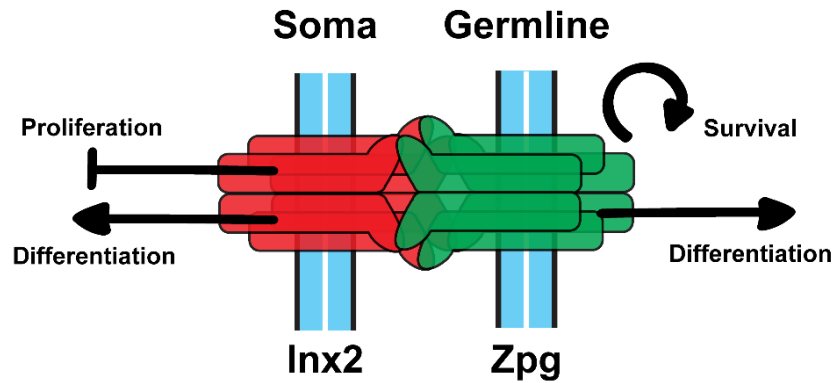
Zpg in the germline forms gap junctions with Inx2 in the soma. Zpg is essential for germ cell survival via an unknown mechanism. Signals from the soma to the germline, mediated by Zpg/Inx2 gap junctions, are required for germline differentiation. Similarly, gap junction-derived signals from the germline to the soma promote somatic differentiation and repress ectopic somatic proliferation. The signal transmitted across the gap junction is not known.

Chapter 3: General Discussion and Conclusions

3.1. Overview of findings

The goal of this thesis was to provide insight into how juxtacrine signalling, mediated by gap junctional signalling, regulates spermatogenesis. I focused on the invertebrate gap junction protein Zero population growth (Zpg) and identified important roles for Zpg in stem cell maintenance, self-renewal, and differentiation of both the germline and soma in the *Drosophila* testis. Importantly I was able to demonstrate that disruptions to gap junction signalling between the soma and germ line lead to specific regulatory defects in both tissues.

The major conclusions of this research are as follows: 1) gap junctions are formed at the earliest stages of spermatogenesis and mediate soma-germline signalling; 2) Zpg-mediated signals are essential for stem cell maintenance and proliferative control in the soma and germline; 3) gap junction signalling is essential for somatic and germline differentiation.

While many studies have identified specific roles for Cx43 in mammalian Sertoli cells, less is known about the role of gap junction proteins in the germline. In *Drosophila*, the importance of gap junction signalling during gametogenesis is poorly understood. Using a variety of genetic tools, cell markers, microscopy, my work has defined specific roles for gap junction signalling in early spermatogenesis, identified which innexins are essential for this signalling, and laid the foundation for experiments that can provide mechanistic insight into the molecular underpinnings of gap junction-mediated signalling in the testis. In addition, my work illuminates the importance of one mechanism of juxtacrine signalling in regulating many different events during gametogenesis.

3.2. Emergent themes and implications of this thesis

3.2.1. Coordinated bi-directional signalling is a mechanism for the regulation stem cell proliferation and differentiation

The unifying theme of this work is that gap junction-mediated juxtacrine signalling is essential for regulating basic cell behaviours. Gap junction signalling is required for cell fate maintenance versus differentiation decisions in the testis, as well as in exercising proliferative control in a tissue. The observations that loss of Zpg or Inx2 in either the soma or the germline led to specific defects in both cell types supports the idea that gap junctions mediate bi-directional, juxtacrine signalling in the testis. The disruption of this signalling capacity has severe consequences for the tissue as a whole. Specifically, the requirement for Zpg and Inx2 for GSC maintenance, differentiation of the germline, proliferative control of the somatic cyst cells, and differentiation of CySC daughters underscores the multitude of ways in which disruption of a single signalling mechanism can result in the dysfunction of a whole tissue.

In Chapter 2, I characterized the autonomous requirement of Zpg and non-autonomous requirement of Inx2 for GSC maintenance. In *zpg* mutant testes, few GSCs are present, if any, and clonal analysis of demonstrates that *zpg* is essential for GSC maintenance; *zpg* mutant GSCs are rapidly lost from the stem cell niche, less than two days after mutant clones are induced, and are undetectable by three days. Similarly, in testes where *inx2* has been somatically knocked down, GSCs are depleted from the niche, relative to wild-type controls. These experiments reveal that juxtacrine signalling plays a very important role during spermatogenesis, beginning in the stem cell niche. This is further

supported by the ultrastructural analysis that shows the presence of gap junctions between GSCs and CySCs.

Similar concepts from the mammalian testis indicate that gap junction-mediated signalling is an evolutionarily conserved mechanism of soma-germline regulation. Cx43 is required in the somatic Sertoli cells to prevent proliferation in the post-mitotic Sertoli cells [184, 186, 204]. Similarly in flies, CySC daughters that exit the niche cease to divide and are post-mitotic, but this repression of proliferation is lost upon disruption of either Zpg in the germline or Inx2 in the soma. Given this commonality, it is likely that a germline connexin functions with Cx43 in Sertoli cells to prevent proliferation in the somatic tissue; failure to do so could give rise to Sertoli cell tumours [204].

Gap junction signalling may play other important roles in regulating soma-germline communication. While I could not identify gap junction-like structures in the hub itself, the expression of the Inx2 reporter reveals that Inx2 is indeed expressed in the hub (Fig. 2.5.7. F, F', inset). The driver line used to knockdown Inx2 throughout my experiments, *tj-GAL4*, is expressed both in the early somatic cells, as well as the hub itself. Subsequent experiments that disrupt innexins solely in the hub cells would be helpful for identifying which innexins are required for hub integrity, quiescence, or promoting the maintenance of GSCs and CySCs. Inx2 may play other roles in regulating the hub cell population, or transmitting signals from the hub to CySCs and GSCs and vice versa. Gap junction signalling in this context, may be required for stem cell maintenance or self-renewal.

The discovery of small gap junctions between germ cells within a germline cyst in this work is unusual (Fig. 2.5.8. E'). Freeze-fracture analysis in the mink testis identified gap junction structures between germ cells, but this appears to be rare, at least with common

microscopy techniques [189]. Because germ cells develop in a syncytium and share their cytoplasm, gap junction signalling between germ cells in a cyst seems unnecessary. After each round of cytokinesis, an intercellular bridge remains between a germ cell and its daughters. However, as the germ cells increase in size over the course of spermatogenesis, the single open window in a massive space that intercellular bridges represent may be insufficient for rapid communicating across a cyst. Coordination across a large area is especially important for ensuring synchronous cell divisions and executing the spermatogenic differentiation programme across 64 spermatids (or more, depending on the species). Gap junctions would thus enable rapid communication across that tissue; after all, it is the defining feature of gap junction signalling [7].

In the mammalian testis, it is thought that gap junctions may be important for nourishing germ cells after they are isolated by the blood-testis-barrier [204]. A similar permeability barrier is formed around the germline in the *Drosophila* testis, shortly after encapsulation, before the onset of meiosis [32]. This coincides with the highest expression levels of both Zpg and Inx2, suggesting that gap junctions may play a role in soma-germline signalling once the germline is isolated. As the somatic cells stretch to remain around the growing spermatocyte and elongating spermatids after meiosis, gap junctions may provide the quickest means of signalling across the expanse of the soma and germline. Studies in the ovary of mice, worms, and flies have demonstrated that gap junction signalling is critical for the regulation of meiotic maturation [206]. Given this, it is possible that gap junctions play a similar role in the testis. This could be investigate by using a simple RNAi screen to knockdown metabolic pathway components and observe which knockdowns result in a phenotype that resembles *inx2RNAi* or *zpg* mutants.

3.3. Limitations and proposed future directions

3.3.1. Differences between vertebrate and invertebrate gap junctions

One of the major limitations of this thesis is the challenge of translating insight from gap junction-mediated regulation of stem cells from flies to mammals and humans. While the basic mechanisms of gap junction regulation appear to be conserved between invertebrates and vertebrates, applying specific lessons from innexins to connexins will be difficult as there is no sequence similarity between the two protein families [136]. Structure-function analysis will be useful for understanding the essence of how gap junction control the cargoes they transmit, how stability at the plasma membrane is achieved, or how certain types of cytoskeletal proteins may interact with gap junctions. But translation of that knowledge into designing medical treatments or drugs without common targets will be challenging. Therefore, while in-depth analysis of innexin regulation may not provide direct medical insight that is relative to patients, understanding the general mechanisms of biology are central to make medical advances possible.

The suite of mutations generated for the structure-function analysis of Zpg hold great promise. The existing mutations can quickly be tested for their ability to rescue the *zpg* null phenotype completely, or any one of the specific phenotypes I have described. Live-imaging of fluorescently tagged versions of the *zpg* transgenes can be used to study how specific mutations affect the trafficking and stability of Zpg at the membrane, and what consequences this may have for Inx2 in the soma. Furthermore, by genetically manipulating the *Drosophila* testis, such as generating germ cell tumours, ablating the germline, or preventing differentiation, the role of gap junction signalling in different disease states could be studied. Such experiments are both financially forgiving and involve a minimal investment of time,

compared to studies in rodents. The general lessons from these experiments could inform subsequent experiments in the mammalian testis, and improve our understanding of gap junction signalling in disease models.

Furthermore, as more and more genetic tools, such as genetically encoded biosensors become available, the questions that can finally be addressed will grow. Rather than trying to determine what moves through gap junctions *in vitro*, gap junction signalling can be studied *in vivo*. Indeed, a study from Speder & Brand has already used live imaging of GCaMP to visualize coordinated organism-wide pulses of calcium signalling in a fly larva [171]. These experiments revealed that calcium signalling propagated by gap junctions from glial cells is essential for neuroblasts to re-enter the cell cycle. The calcium pulses may be initiated by changes in insulin-mediated endocrine signalling. This serves as a superb example of how an animal can integrate endocrine and juxtacrine signals to affect cell fate. And the lessons from such analyses may very well be conserved.

3.3.2. What molecules are transported by gap junctions during spermatogenesis?

The second major limitation of this work, perhaps surpassing the translational nature of studying invertebrate gap junctions, is the matter of cargo: what is being transmitted through gap junction channels during spermatogenesis? Is the cargo moving in a uni-directional manner, depending on the developmental timing? Are multiple cargoes, such as cyclic nucleotides and metal ions moving across a gap junction at the same time? Are the signalling inputs from gap junctions additive; that is, does a cell need to integrate gap junction-mediated signals with other signalling pathways to perform a specific behaviour?

Within the testis stem cell niche, there are many signalling pathways that act simultaneously to govern cell fate decisions. For instance, Hh signalling regulates CySC self-

renewal [31, 111, 112]. Recent work has demonstrated that cAMP signalling can modulate Hh signalling, through a G-protein receptor kinase [210, 211]. As gap junctions can transmit cAMP signals, it is possible that Zpg/Inx2 may mediate cAMP signalling from the germline to the soma, thus regulating the output of Hh signalling in CySCs. While this would be difficult to study, it may be possible to disrupt Hh signalling in a manner that can be rescued by a corresponding change in cAMP signalling. Given the multitude of signalling that occurs in the stem cell niche in the testis, an additional level of regulatory control could make the system more robust. Thus small challenges that could compromise the integrity of the tissue, such as loss of proliferative control, could be tempered to prevent a disastrous outcome.

Gap junctions facilitate juxtacrine signalling via the movement of small molecules, ions, and metabolites between neighbouring cells [7]. While there is evidence that gap junctions in the fly ovary may mediate changes in ion concentration and pH in somatic follicle cells, and meiotic maturation of worm oocytes is regulated by gap junction-mediated cAMP signalling, it is unclear what signals pass through gap junctions in the testis [164, 182]. While it is difficult to investigate the minutiae of gap junction signalling in a live organism, particularly during the complex process of spermatogenesis, several tools and approaches could be utilized to begin to address this in the fly testis.

Genetic approaches can be useful to investigate possible cargos that may be transported across gap junctions. Performing tissue-specific RNAi knockdowns of components required for calcium, cAMP, or IP_3 signalling may result in phenotypes that resemble gap junction mutant phenotypes. This would be useful to determine which signals are required in the soma versus the germline.

Changes in small molecule concentrations in a live testis can be detected through the use of fluorescent biosensors. For instance, in Chapter 2, GCaMP was used to detect changes in calcium concentration near the stem cell niche over short time periods. By employing a variety of biosensors, alterations in the relative levels of small molecules in the soma and germline could be determined through different stages of spermatogenesis. This type of experiment may provide insight into how changes in the concentration of small molecules may be correlated with stem cell behaviour and differentiation events. For instance, cAMP signalling from GSCs to CySCs may be important for regulating Hh signalling in the soma; calcium signalling may be required for the encystment of the germline by the soma; and IP_3 and insulin signalling from the soma may be transduced to the germline via gap junctions during transit—amplifying stages and before meiosis. As gap junction-mediated cAMP signalling has been demonstrated to be important for meiotic maturation in several systems for meiotic maturation of the oocyte, it is possible that cAMP may play a similar role in the testis. Upon identifying cell- and stage-specific changes in biosensor activity, the biosensors could then be introduced into innexin-mutant backgrounds to investigate how their activities differ when gap junction-mediated signalling is disrupted.

In addition, post-translational modifications to gap junction proteins or assembly of heteromeric hemichannels can alter the permeability of the intercellular channels to different cargos [136, 155]. Indeed, there is evidence from both *C. elegans* and *Drosophila* that the ability of gap junctions to perform tissue-specific functions is dependent on which innexins form a hemichannel. For instance, in the worm gonad, INX-14 is required in the germline for the formation of functional INX-14/INX-21/INX-22 hemichannels, whereas loss of either INX-21 or INX-22 does not disrupt hemichannel formation [165]. Loss of either INX-8 or

INX-9 prevents the formation of somatic hemichannels that can dock with INX-14/INX-21/INX-22 hemichannels in the germline [165]. During embryogenesis in the fly, amnioserosa cells express Ogre/Inx1, Inx2, and Inx3; embryos that lack Inx3 are still able to form Ogre/Inx2 gap junctions, but do not complete unless Inx3 expression is restored [176]. In terms of post-translational modifications, little headway has been made in identifying relevant modifications for innexin function in flies. The C-terminus of Inx2 has been shown to be essential for its function in developing foregut; loss of the C-terminus phenocopies *inx2* mutant and *inx2RNAi* embryos [178]. The N-terminus of Inx2 plays a role in regulating the permeability state of a hemichannel; disrupting the N-terminus through attachment of a RFP molecule causes a dominant-negative phenotype [171]. A similar has been described for Connexin26 [212]. Preliminary experiments presented in this work demonstrate that the C-terminus of Zpg is required for export from the endoplasmic reticulum, although the required residues have not been identified. Subsequent experiments using our current array of rescue transgenes could provide insight into post-translational regulation of Zpg.

3.3.3. The utility in investigating basic biological questions

By better understanding the role of gap junctions in such complex processes as stem cell regulation or developmental differentiation, new mechanisms of cell control may be elucidated. Previously unknown levels of control in a system can provide opportunities for new medical treatments or diagnostic tools. Investigating altered patterns of gap junction-mediated signalling could become a new diagnostic for identifying damaged tissues or instances where un-regulated proliferation has the potential to contribute to cancer development. While understanding the basic questions that govern cell behaviour is

complicated, addressing such questions is even more fraught in more complex organisms, unless we have a firm grasp of the biological underpinnings of such behaviour.

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Appendix:

A.1. Zpg protein expression is detected in PGCs and zpg is not required for gonadogenesis.
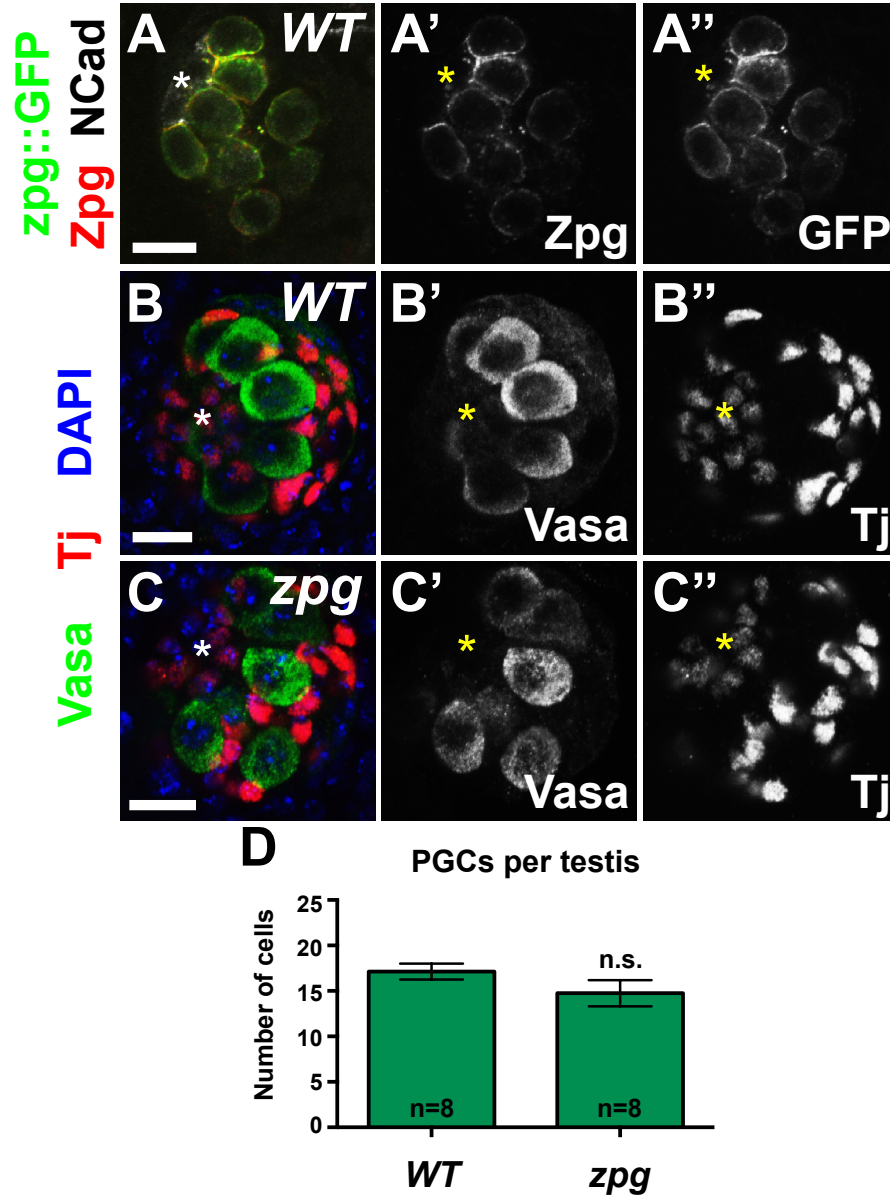
(A) Expression of endogenous Zpg and a Zpg::GFP transgene in Stage 15 embryonic gonads. Zpg (red) labels endogenous Zpg expression in PGCs, GFP (green) labels transgene expression in PGCs, N-Cad (white) labels the hub. (B-D) Stage 17 embryonic gonads. In wild-type and *zpg* mutant embryos, PGCs populate the gonad and orient themselves adjacent

to the hub. Vasa (green) labels PGCs, Tj (red) labels SGPs and hub cells, DAPI (blue) labels heterochromatin. *n*, number of testes. Error bars show mean±s.e.m.. Asterisk indicates the hub. Scale bars 10μm throughout.

*A.2. Germ cell number is varied in zpg testes and does not correlate with Tj+ cell numbers.*
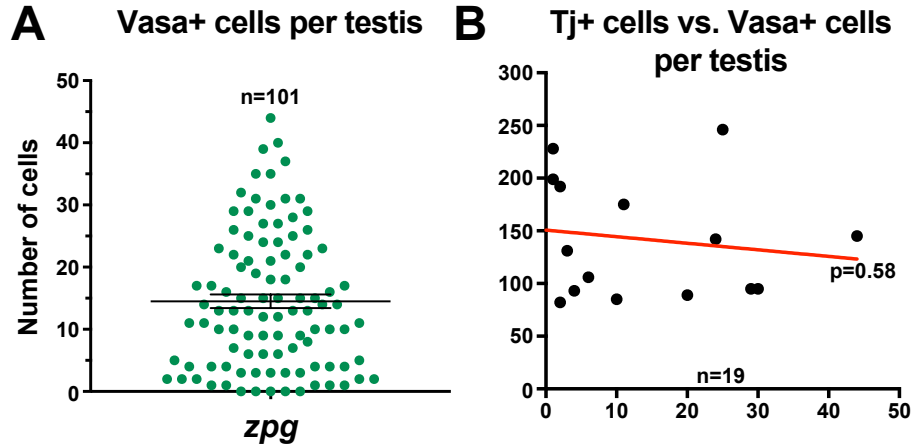
(A) *zpg* testes contain few germ cells. Number of germ cells per testis shown, detected by Vasa staining. (B) Tj$^+$ cells per testis (y-axis) relative to Vasa$^+$ cells per testis (x-axis). *p*-value is not significant. *n*, number of testes. Error bars show mean±s.e.m.

A.3. Inx2 is required in the soma for spermiogenesis.
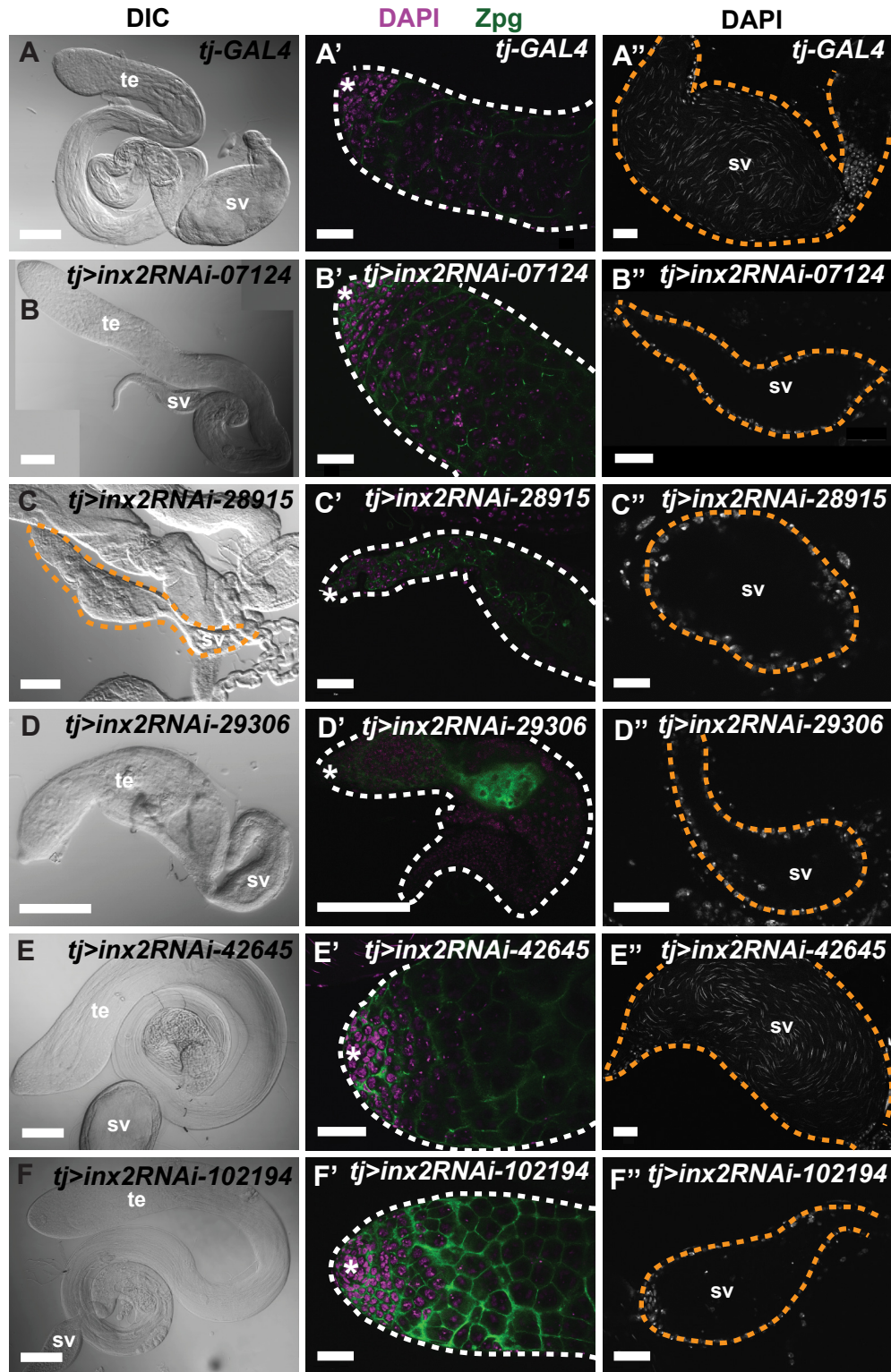
(A-F) DIC images showing knockdown of Inx2 using five independent RNAi lines. (A) Control testes are large and filled with sperm. (A') Zpg staining (green) is not disrupted and seminal vesicle (A'') contains sperm, detected by needle-like DAPI-staining. (B, F) Representative testes following weak Inx2 knockdowns. (B', F') Zpg staining is not disrupted, but the seminal vesicles (B'', F'') lack sperm. (C, D) Strong knockdown of Inx2 results in small, rudimentary testes. (C', D') Zpg staining appears abnormal and sperm is absent from the seminal vesicles (C'', D''). (E) Ineffective knockdown of Inx2 appears wild-type, with wild-type like Zpg staining (E'), and sperm in the seminal vesicle (E''). Dashed lines outline the testis. *te* = testis; *sv* = seminal vesicle. Asterisk indicates the hub. DAPI staining highlights nuclei. Scale bars: 100μm in A, B, E, F; 30μm in C, D, A'-F', A''-F''.

A.4. Knockdown of innexins in the early and late germline.
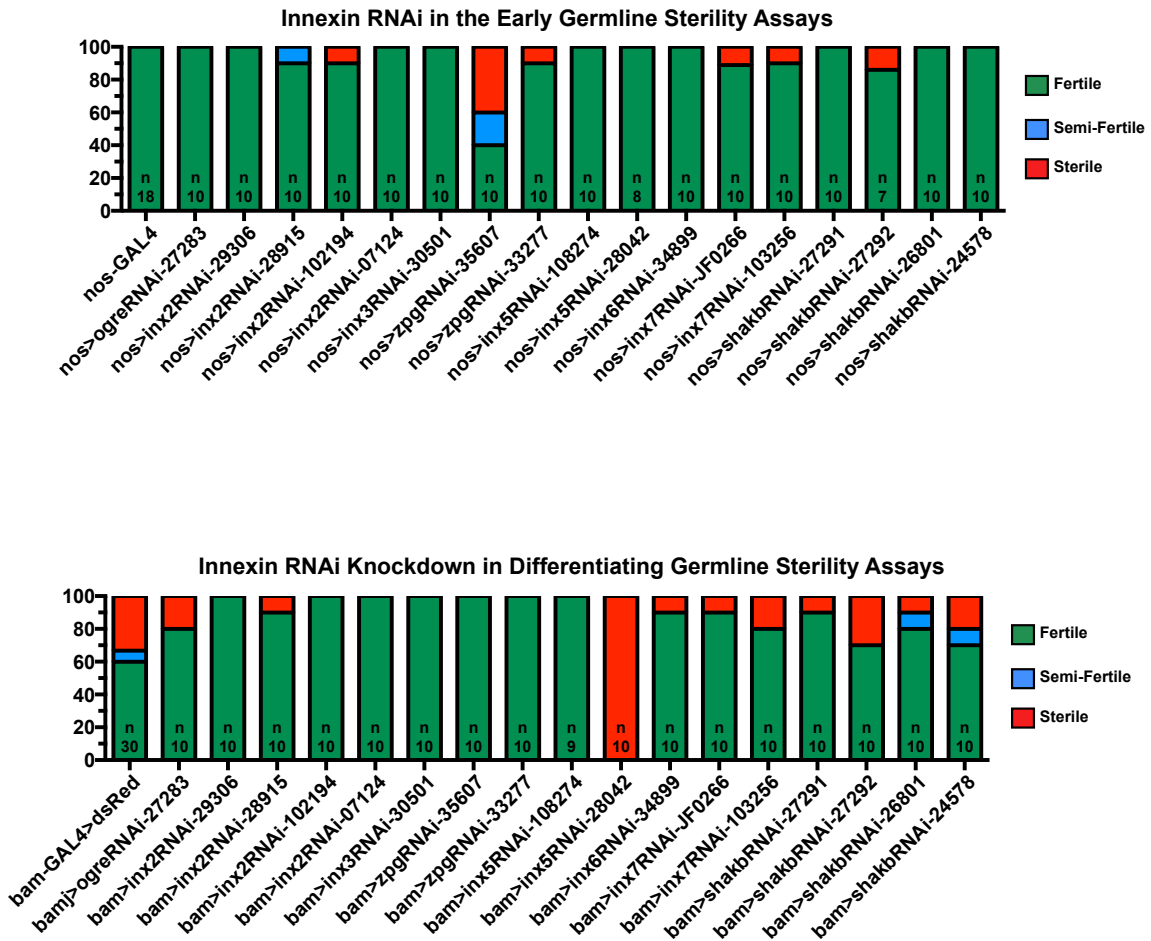
Innexin RNAi in the Early Germline Sterility Assays

Innexin RNAi Knockdown in Differentiating Germline Sterility Assays

Innexin RNAi in the Early Germline. Sterility assays show the phenotype following knockdown of each innexin with *nos-GAL4*. Only *zpgRNAi* produces a clear defect in fertility.

Innexin RNAi Knockdown in Differentiating Germline. Sterility assays show the phenotype following knockdown of each innexin with *bam-GAL4*. *inx5RNAi-28042* results in completed sterility, but *inx5RNAi-108274* is fertile. *n* = number of independent fertility assays.

A.5. Inx5 may be required in differentiating germ cells for spermiogenesis
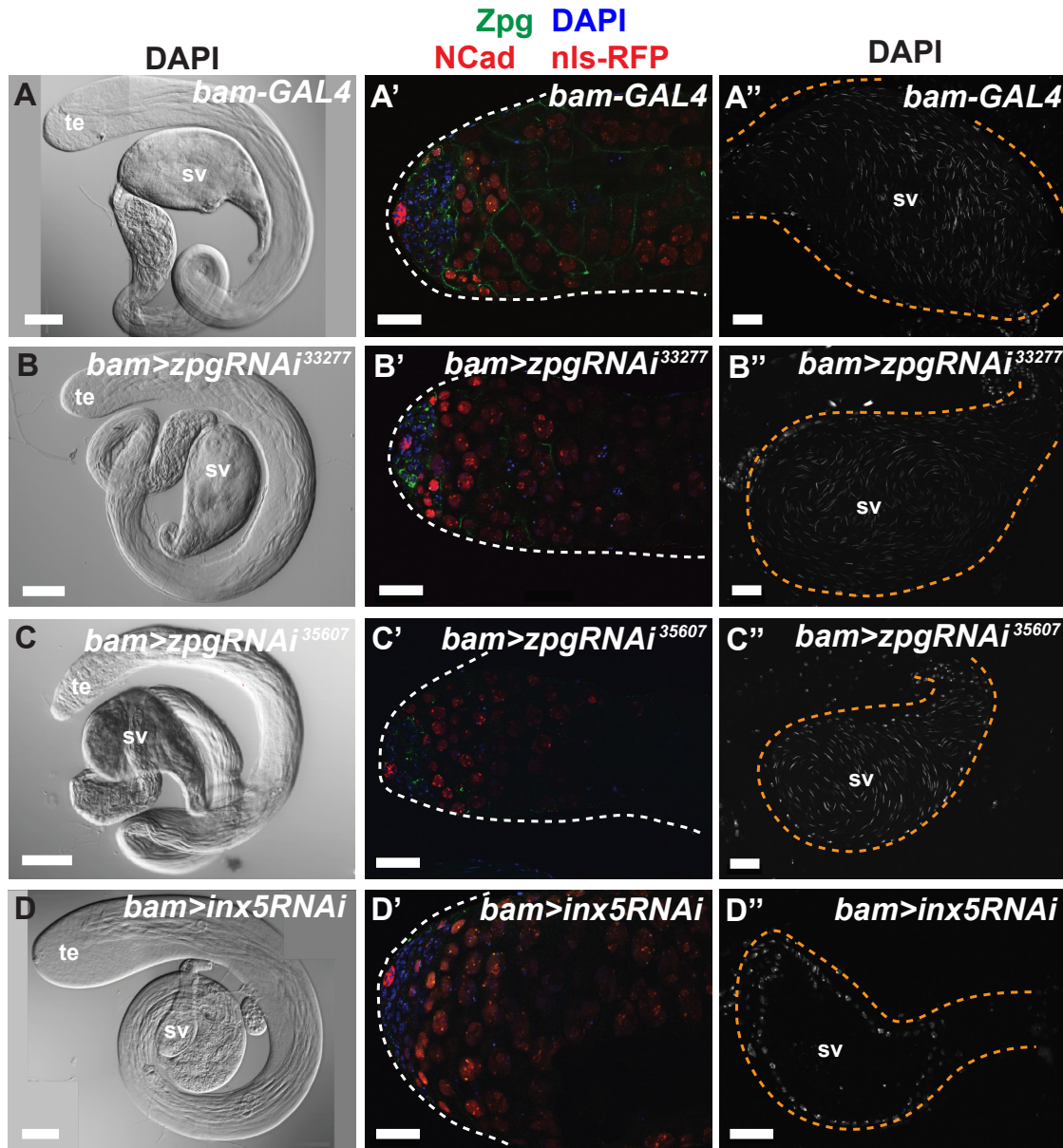
(A-D) Knockdown of Zpg and Inx5 with *bam-GAL4*-driven RNAi. All testes appear wild-type, but *inx5RNAi-28042* testes do not contain sperm in the seminal vesicle. *Te* = testis; *sv* = seminal vesicle. DAPI labels round nuclei in (A'-D') and needle-like sperm nuclei in A''-C''. Note the absence of sperm nuclei in D''. Scale bars 30µm throughout.
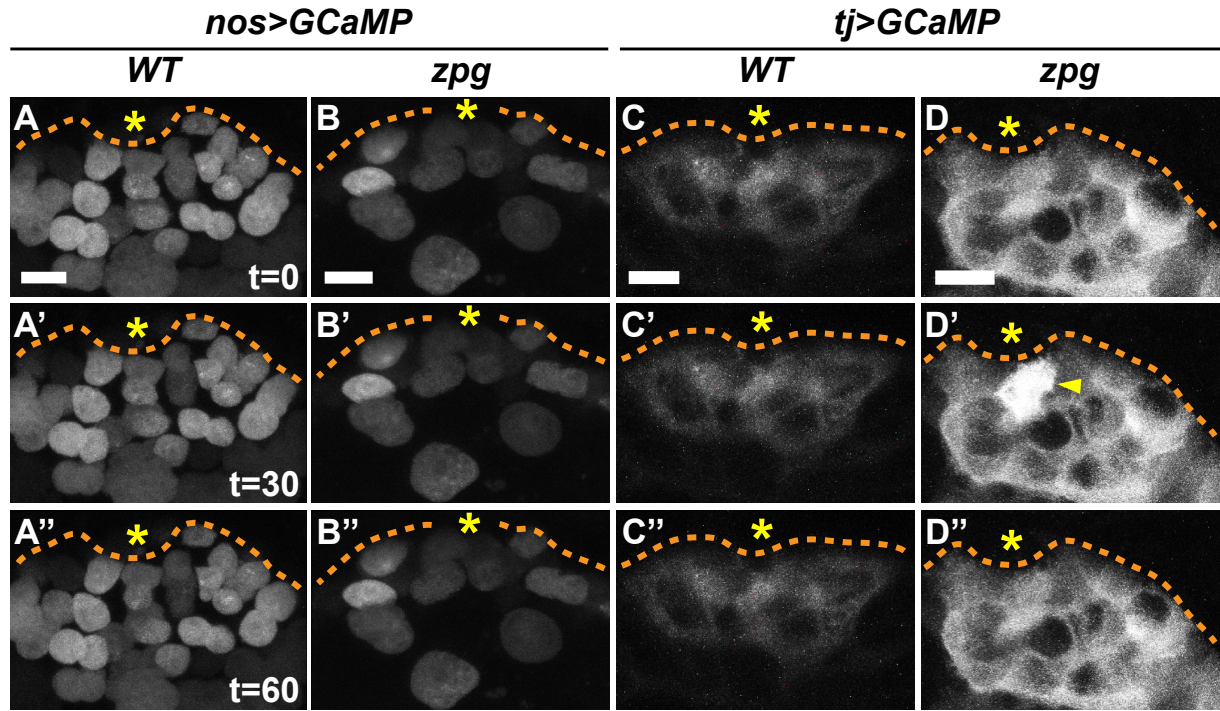
(A-B) Expression of a GCaMP calcium sensor in the early germline of wild-type (A) and *zpg* mutant third-instar larval testes, driven by *nos-GAL4* (B). GFP signal can be detected in germ cells adjacent to the niche and up to several cell lengths away from the hub. No changes in signal could be detected over the course of 360-600 second movies. (C-D) Expression of GCaMP in the early soma of wild-type (C) and *zpg* mutant (D) larval testes, driven by *tj-GAL4*. GFP signal can be detected in the somatic cells closest to the hub, likely CySCs, in wild-type (A). Increased GFP intensity is detected in *zpg* testes (D), as well as a large, momentary increase in GFP signal (D', arrowhead), indicating increased Ca^{2+} levels. *t =* seconds. Representative movie stills shown for each. Asterisk indicates the hub. Dashed lines outline the testis.