ECDYSONE SIGNALING ANTAGONIZES EGF SIGNALING IN THE CYST CELL LINEAGE OF THE ADULT MALE GONAD OF *DROSOPHILA*

by

RICHARD WALTER ZOLLER

(Under the Direction of Cordula Schulz)

ABSTRACT

This thesis describes the role that the steroid hormone ecdysone plays in influencing interactions between germline cells and somatic cells in the adult *Drosophila melanogaster* testes. In epidermal growth factor (EGF) mutants, germline cells do not associate with their somatic microenvironment cells, the cyst cells. As a consequence, testes are small and germline cells fail to differentiate and form early stage germ cell tumors. The first chapter introduces a brief history of stem cells and the use of *Drosophila* testes as a stem cell model. Chapter 2 reviews of the role of cyst cells in testes. Chapter 3 shows that reduction of ecdysone signaling in cyst cells promotes the differentiation of germline cells in EGF mutant testes. I have confirmed that reducing ecdysone is responsible for the differentiation of germline cells in EGF mutants and I have also found ecdysone signaling components that are expressed in cyst cells.

INDEX WORDS: *Drosophila melanogaster*, EGF, EcR, spermatogenesis, ecdysone, 20E, male, cyst cell

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DEDICATION

This thesis is dedicated to my family, friends and my beautiful wife-to-be, Erin, without whose support would have made this venture exponentially more arduous.

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CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Throughout the life of a multicellular organism, cells are constantly lost through various biological processes. To combat this loss and maintain tissue function, this must be rectified through the regeneration of specific cell types. Long-lived, adult stem cells are charged with the task of re-stocking organs and tissues with differentiated cells as an organism ages. Populations of stem cells can be found in numerous tissues, including the intestine, epidermis, hematopoetic and nervous tissue (Verfailie, 2006; reviewed in Wagers and Weissman, 2004). In all of these tissues, a defining characteristic that stem cells share is the ability to asymmetrically self-renew, that is, produce one daughter that retains its stem cell identity and another that will undergo a series of amplifying divisions and eventually undergo development into a terminally differentiated cell (Weissman, 2000). How do stem cells retain their stem identity? How do daughters become specialized and how is this development into mature, differentiated cells regulated? These questions are central to the field of stem cell biology and are being answered through different *in vitro* and *in vivo* approaches.

Brief History of Stem Cell Biology

In 1963, James Till and Ernest McCulloch showed that mouse donor hematopoietic cells transplanted into a recipient animal amplified and differentiated from a single precursor, showing for the first time incontrovertible evidence for the existence

of stem cells (Becker *et al*, 1963). A year later, it was discovered that mouse embryo-derived carcinoma cells could regenerate themselves and also produce differentiated progeny *in vitro* (Kleinsmith and Pierce, 1964). Although stem cells were known to exist, further characterization and their ability to be used in *in vivo* and *in vitro* studies were limited until 1981 when Sir Martin John Evans and Matthew Kaufman discovered that pluripotent embryonic stem cells (ESCs) derived from the inner cell mass of the blastocyst stage of mouse embryos could be isolated and passaged indefinitely in an undifferentiated state but also retain their ability to differentiate *in vitro* (Evans and Kaufman, 1981). Nearly two decades later, James Thomson and his colleagues were able to replicate Evans and Kaufman's results from a human blastocyst, isolating human embryonic stem cells (hES cells), stimulating excitement for the potential of stem cells to be used as molecular tools and clinical therapeutic possibilities to treat injuries and combat degenerative diseases such as Parkinson's, Alzheimer's and diabetes (Thomson *et al*, 1998; Zeng *et al*, 2004). hES cells are characterized by their ability to differentiate into all of the different cell types of the three primary germ layers: ectoderm, endoderm, and mesoderm (Zeng *et al*, 2004). Although there are many potentials for the usage of hES cells, obtaining these cells requires the destruction of the human embryo at its earliest stages of development, which brings about a controversy of ethics and has polarized the field of human stem cell biology (Steinbock, 2007). In 2006, Shinya Yamanaka and his team generated pluripotent stem cells from adult mouse fibroblast cells by culturing them in ESC conditions and introducing four key factors, and termed the cells resulting from this treatment induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Soon after this achievement, Yamanaka and colleagues as well as

Thomson and his team were able to create the first human iPSCs using similar approaches (Takahashi *et al*., 2007; Yu *et al*, 2007).

These advancements in the field opened doors to a multitude of useful applications. Entire organs can potentially be created from a patient's own cells *in vitro*, thereby eliminating the complications of tissue rejection. Muscle tissue could be cultured *in vitro,* potentially increasing the global availability of meat products and is also potentially more environmentally-friendly than raising livestock. Although the potential utilities of stem cells are great, the exact mechanisms of how daughter cells from asymmetric divisions undergo amplification and differentiation to contribute to the function of different tissue systems remain to be fully explained. To investigate these mechanisms, we look to the process of gametogenesis for mechanisms that influence the differentiation of stem cells to mature eggs and sperm.

Germ Cells in Vertebrates & Invertebrates

In the gonad of most metazoan animals, the germline cells normally differentiate in intimate contact with somatic cells. In the mammalian gonad, the differentiating male germline cells are enclosed in large compartments between Sertoli cells (Desjardins and Ewing, 1993; Nel-Themaat *et al*., 2010; Nel-Themaat *et al*., 2011) and the oocytes are enclosed in a layer of somatic granulosa cells (Erickson, 1986; Hsuesh and Schomberg, 1993). Interactions between the germline cells and somatic cells guide gametogenesis in both genders (Bitgood *et al*., 1996; Marziali *et al*., 1993; Matzug, 2000; Ojeda *et al*., 2000; Pesce *et al*., 1997). In the gonad of *Caenorhabditis elegans,* early germline cells contact the somatic distal tip cell, and signals from the distal tip cells govern germline

proliferation and differentiation (Berry *et al*., 1997; reviewed in Kimble and Crittenden, 2007). At later stages, the germline cells in *C. elegans* are associated with somatic sheath and spermathecal cells (Church *et al*., 1995; Hall *et al*., 1999; McCarter *et al*., 1997). Finally, in insects, germline cells are enclosed by somatic microenvironment cells which play key regulatory roles in germ cell fate (Bünning, 1994; Hardy *et al*., 1979; reviewed by Kiger and Fuller, 2001; King, 1970; Morris and Spradling, 2012; Sarkar *et al*., 2007; Schulz *et al*., 2002; Xie and Spradling, 2000; reviewed in Zoller and Schulz, 2012).

Male Drosophila melanogaster Gonad as a Stem Cell Model

The male gonad of *Drosophila melanogaster* is an excellent model for studying the dynamic nature and differentiation of stem cell populations. The testes are a long, coiled organ that house germline and somatic support cells, the arrangement of which follows a spatio-temporal organization (Fig 2.1). Analysis of the development of germline and cyst cells in the testes of *Drosophila* has shown that differentiation of the germline daughters depends on signals from their microenvironment cyst cells (Schulz *et al*., 2002). For example, the exit of the germ line cells from mitotic amplification divisions before they enter terminal differentiation into sperm is regulated by TGFβ signaling in cyst cells that engulf the germline (Matunis *et al*., 1997). The similarity in the association between germline and soma in *Drosophila* and mammalian testes suggests that mechanisms and molecules maintaining the organization and the communication pathways between germline and soma may be conserved. This study aims to characterize the role the invertebrate steroid hormone 20-hydroxyecdysone (20E) plays in influencing germline-soma interactions in adult male *Drosophila*. Investigation into the expression of

20E signaling components and the role that these play in interacting with another known signaling pathway that affects germline-soma association, Epidermal Growth Factor Receptor (EGFR) signaling, will shed light on how these two cell types communicate to ensure proper co-differentiation during spermatogenesis.

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CHAPTER 2

THE *DROSOPHILA* CYST STEM CELL LINEAGE – PARTNERS BEHIND THE SCENES?[1]

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Abstract

In all animals, germline cells differentiate in intimate contact with somatic cells and interactions between germline and soma are particularly important for germline development and function. In the male gonad of *Drosophila melanogaster*, the developing germline cells are enclosed by somatic cyst cells. The cyst cells are derived from cyst stem cells (CySCs) of somatic origin and codifferentiate with the germline cells. The fast generation cycle and the genetic tractability of *Drosophila* has made the *Drosophila* testis an excellent model for studying both the roles of somatic cells in guiding germline development and the interdependence of two separate stem cell lineages. This chapter will focus on our current understanding of CySC specification, CySC self-renewing divisions, cyst cell differentiation, and soma-germline interactions. Many of the mechanisms guiding these processes in *Drosophila* testes are similarly essential for the development and function of tissues in other organisms, most importantly for gametogenesis in mammals. In chapter 3, we report findings that implicate the steroid hormone ecdysone in regulating cyst cell differentiation and genetic interaction with EGF signaling. In the fourth and concluding chapter, we discuss possible mechanisms and interactions of EcR signaling in testes and future experiments that can reveal details of how ecdysone influences somatic niche cells in male *Drosophila melanogaster*.

Background & Introduction

Adult stem cells have been identified from many tissues where they constantly produce highly specialized cells, such as germline, blood, and skin cells (Huckins, 1971a; Huckins, 1971b; Hardy *et al*., 1979; Blanpain and Fuchs, 2006; Metcalf, 2007).

A *Drosophila* testis contains two types of stem cells, germline stem cells (GSCs) and cyst stem cells (CySCs), which give rise to the germline cells (blue in Fig. 2.1A), and cyst cells (yellow in Fig. 2.1A), respectively (Hardy *et al*., 1979; Gönczy and DiNardo, 1996). Electronmicroscopy studies revealed that GSCs and CySCs are arranged in rosettes around a single group of terminally differentiated somatic cells, called the hub, at the apical tip of a wildtype testis (Hardy *et al*., 1979). Each GSC is flanked by a pair of CySCs which extend cytoplasmic protrusions around the GSC and into the hub (illustrated in Fig. 2.1A). GSCs and CySCs divide asymmetrically to produce daughter cells (gonialblasts and cyst cells, respectively) that form developmental units, termed cysts. During cyst formation, two cyst cells grow cytoplasmic extensions around a single gonialblast to completely enclose it, thereby isolating it from direct contact with any other cell type. Once a cyst is formed, the two cyst cells and the enclosed gonialblast codifferentiate. The gonialblast undergoes four rounds of transit amplification divisions to produce 16 spermatogonia, which then become spermatocytes, and grow in size. After the growth phase, the spermatocytes undergo meiosis and differentiate into elongated spermatids. During all stages of germline differentiation, the two cyst cells continue to enclose the germline cells, grow tremendously in size, and codifferentiate with the germline. During terminal differentiation, morphological differences between the two cyst cells become apparent, as one cyst cell, the tail cyst cell, becomes much larger than

the other cyst cell, the head cyst cell (TCC, HCC; Fig. 2.1A). The developing cysts become progressively displaced toward more basal regions of the testis. This results in a spatio-temporal arrangement of cysts along the apical to basal axis of the wildtype testis (Hardy *et al*., 1979; Tates, 1972; Fuller, 1993; Schulz *et al*., 2002).

The cyst cell lineage can be visualized easily by immunofluorescence microscopy. Several cell surface markers, such as Semaphorin (green in Fig. 2.1B), are highly expressed on the membranes of cyst cells (Zoller and Schulz, unpublished data) and allow for visualization of the cytoplasmic extensions enclosing the germline cells (red in Fig. 2.1B). Antibodies raised against the transcription factors Zinc finger homeodomain-1 (Zfh-1), Traffic jam (Tj), and Eyes absent (Eya) can be used to assess the developmental stage of the cyst cells. Zfh-1 (red in Fig. 2.1C) is expressed at high levels in CySCs, fading rapidly in early stage cyst cells (cyst cells associated with proliferating germline cells (Leatherman and DiNardo, 2008). Tj (green in Fig. 2.1C and 2.1D) is expressed at equal levels in CySCs and in early stage cyst cells (Li *et al*., 2003). Eya (red in Fig. 2.1D) shows a very low level of expression in early stage cyst cells and a high level of expression in late stage cyst cells (cyst cells associated with postmitotic germline cells) (Fabrizio *et al*., 2003). In addition to these antibodies, marker genes expressed from transposon insertion lines or driven by tissue-specific Gal4-transactivators provide useful tools for identifying and staging cyst cells throughout the *Drosophila* testis (Gönczy *et al*., 1992; Metaxakis *et al*., 2005; White-Cooper, 2012).

The arrangement of the germline and the cyst cells in Drosophila testes is similar to the arrangement of germline and somatic cells in mammalian testes. Just as in a Drosophila testis, the germline cells in a mammalian testis are arranged in a

spatiotemporal order along the axis, with the youngest stage germline cells located next to the basal membrane and the more differentiated germline stages located toward the lumen of the seminiferous tubules (Griswold, 1998; Gilbert, 2006). Mammalian germline cells are enclosed in large somatic Sertoli cells, which act as a physical barrier as well as a source for nutrients and regulatory molecules. Physical isolation of the differentiating germline cell clusters from each other is achieved via localization of specialized cell junctions between the Sertoli cells and the germline cells (Griswold, 1998; de Kretser *et al*., 1998; Lui and Cheng, 2007; Nel-Themaat *et al*., 2010; Boyle and DiNardo, 1995). It appears that the enclosure of the developing germline cells, therefore, is dependent upon different mechanisms in *Drosophila* and mammals. While each cluster of germline cells in a *Drosophila* testis has a pair of microenvironment cyst cells generated by CySC divisions, each cluster of germline cells in a mouse testis sits in a compartment within the microenvironment Sertoli cell marked by the localization of junction proteins. The similarity in the tight association between germline and soma in *Drosophila* and mammalian testes, though, suggests that mechanisms and molecules setting up and maintaining the organization, and the communication pathways between germline and soma may be conserved.

The Orgin & Identity of CySCs

In most organisms, the germline and somatic cells of the gonad are specified independently and coalesce during development to form the gonad (Gilbert, 2006). The *Drosophila* somatic cyst cell lineage originates from somatic gonadal precursor cells (SGPs) specified in parasegments 10–12 during embryogenesis. As the primordial

germline cells migrate from the posterior end of the embryo toward the position of the future gonad in parasegment 10, they pass through parasegments 12 and 11 where they associate with the SGPs. Together, the germline and the SGPs from parasegments 12 and 11 migrate anteriorly and join the SGPs of parasegment 10. There, the germline cells and the SGPs coalesce to form the gonad and the SGPs become specified into the different somatic cell types of the testis (Boyle and DiNardo, 1995; Riechmann *et al*., 1998; Moore *et al*., 1998).

A common origin of cyst cells and hub cells from SGPs was shown by lineage tracing experiments. When gonadal precursor cells were labeled in parasegment 11, label-positive progeny were subsequently found among both the hub cells and the cyst cells of adult testes (DiNardo *et al*., 2011). Consistent with the common origin of the two somatic cell types, the cyst cells and the hub cells both express Zfh-1, Tj, and marker genes from enhancer trap lines (Leatherman and DiNardo, 2008; Li *et al*., 2003; Gönczy *et al*., 1992).

The specification of the SGPs as CySCs vs. hub cells was suggested to depend on signaling via the Notch (N), the Epidermal Growth Factor (EGF) and the Sevenless (Sev) signaling pathways. N signaling between the SGPs was sufficient to specify hub fate in SGPs throughout the embryonic gonad. Throughout the gonad, except for the most anterior region, N is opposed by EGF and Sev signaling from the germline to the SGPs. This results in specification of SGPs as hub cells at the anterior tip of the embryonic gonad, and in the specification of SGPs as CySCs in the remainder of the embryonic gonad (Kitadate *et al*., 2007; Kitadate and Kobayashi, 2010; Okegbe, 2011).

Both specification and maintenance of CySC vs. hub cell fate in *Drosophila* testes requires the antagonistic roles of *lines* (*lin*) and *brother of odd with entrails limited (bowl)*(DiNardo *et al*., 2011). The *bowl* gene encodes a zinc finger transcription factor and *lin* encodes a cytoplasmic protein with catalytic activity. During *Drosophila* epidermal cell differentiation, Lin binds directly to Bowl to reduce Bowl abundance, possibly by targeting Bowl for degradation. Another protein, Drumstick (Drm), competes with Lin for binding to Bowl and, thus, promotes Bowl accumulation (Hatini *et al*., 2005). Transcription of Bowl was upregulated in testes with excess stem cells based on microarray analysis (Terry *et al*., 2006). This observation led to the investigation of a requirement for Bowl and it's antagonist Lin in testes. While *bowl* mutant embryonic gonads had fewer hub cells, *lines* mutant embryonic gonads had an increased number of hub cells in comparison to wildtype gonads. Likewise, when CySCs in adult testes were depleted of *lines*, their progeny accumulated in aggregates that expressed a variety of hub-specific marker genes, including Cactus and *hedgehog*-reporter. Similar to hub cells, these hub-like aggregates appeared to recruit CySCs. The hub-like aggregates failed to recruit functional GSCs based on the expression of several GSC specific markers genes, implying that they are not identical to hub cells (DiNardo *et al*., 2011). How exactly Bowl specifies CySC vs. hub cell fate has not been revealed and no target genes of Bowl have been identified in testes.

Substantiating the common origin of cyst cells and hub cells, CySCs are able to convert into hub cells: they can associate with the hub cells and express hub cell markers. In testes devoid of germline cells, CySCs obtain hub cell identity at a high frequency, suggesting that germline cells play a central role in maintaining the CySC population

(Gönczy and DiNardo, 1996). The ability of CySCs to convert into hub cells was also observed in wildtype testes, yet reports on the frequency of this conversion are conflicting (DiNardo *et al*., 2011; Voog *et al*., 2008). Voog *et al*., 2008 reported a high frequency of conversion (one converted CySC in 46% of 74 testes examined), while others reported that CySCs very rarely generated daughter cells that adopted hub cell fate. Dinardo *et al*., 2011 did not find any converted CySCs out of 40 testes, even though they used the same tools for lineage tracing as exploited by Voog (Voog *et al*., 2008). It remains to be resolved whether different genetic backgrounds or growth conditions may be the reason for the wide range in conversion frequency.

Lineage tracing experiments confirmed beyond any doubt that CySCs are indeed functional stem cells, as they both self-renew and produce differentiating daughter cells. In a key experiment, a Flippase recombinase was transiently expressed to induce a recombination event that randomly and permanently marked single cells and their progeny by reconstitution of a β-galactosidase (*lacZ*) marker gene. LacZ-positive cells persisting next to the hub had to be either GSCs or CySCs. GSCs produce many clusters of LacZ-positive, proliferating germline cells. CySCs, in contrast, produce daughter cyst cells that do not divide further, but together with a second cyst cell, enclose the developing germline. As expected, LacZ-positive CySCs were observed next to the hub and these stem cells produced a series of single, LacZ-positive cells that associated with developing germline cells (Gönczy and DiNardo, 1996). With these experiments, it was established that the CySCs in *Drosophila* testes are indeed stem cells, as they self-renew and produce differentiating daughter cells. Subsequent research shifted toward studying the specification of both stem cell populations, the GSCs and the CySCs.

CySC Self-Renewal Depends on Signals from the Hub

A series of elegant genetic experiments demonstrated how the cell fate decisions of CySCs to become either new CySCs or cyst cells are regulated (illustrated in Fig. 2.2A). CySC fate is dependent on signaling from the hub cells via the Janus kinase and Signal Transducer and Activator of Transcription (JAK/STAT) pathway (Kiger *et al*, 2001; Tulina and Matunis, 2001). The highly conserved JAK/STAT signaling pathway is essential in a number of developmental processes in *Drosophila* and in mammals, and has been associated with a variety of cancers (Ward *et al*., 2000; Luo and Dearolf, 2001; Rawlings *et al*., 2004b; Darnell, 2005; Mohri *et al*., 2012; Levine, 2009). The JAK/STAT signaling pathway was originally discovered as a cytokine-induced signaling pathway required by the myelid and lymphoid cell lineages and is now known to regulate many stem cell populations, including stem cells in the *Drosophila* ovary and intestine, and murine embryonic stem cells (Ihle, 1995; Matsuda *et al*., 1999; Decotto and Spradling, 2005; Gregory *et al*., 2008). The core JAK/STAT signaling pathway is relatively simple. Binding of the cytokine to its receptor induces conformational changes that lead to activation of associated JAKs. Activated JAKs phosphorylate the cytokine receptors and the tyrosine-phosphorylated motifs in the cytokine receptors serve as docking sites for the SH2 domains of STATs. Once bound to the receptor, the STATs become activated by tyrosine phosphorylation. Phosphorylated STATs dissociate from the receptors, dimerize, and translocate into the nucleus to regulate transcription of target genes (Schindler and Darnell, 1995).

Drosophila males carrying a temperature sensitive allele of the JAK, *hopscotch* (*hop*), or *stat* were raised at a permissive temperature and then shifted to a restrictive

temperature to induce a phenotypic change in the gonad. In testes from shifted animals, CySCs and GSCs were progressively lost. This suggested that GSCs and CySCs failed to self-renew and instead differentiated into cyst cells. Conversely, hyperactivation of JAK/STAT signaling had the opposite effect. Overexpression of the ligand Unpaired (Upd), that is normally only expressed in the hub, throughout the germline resulted in the excessive accumulation of CySCs and GSCs, as evident by their expression of stem cell specific marker genes (Kiger *et al*., 2001; Tulina and Matunis, 2001). These studies demonstrated that JAK/STAT signaling induces CySC and GSC fate in *Drosophila* testes.

Additional studies revealed that two proteins act downstream of JAK/STAT to regulate CySC fate in *Drosophila* testes. These are Zfh-1 and chronologically inappropriate morphogenesis (*chinmo*), a protein that may act as a transcriptional regulator or play a role in protein degradation (Leatherman and DiNardo, 2008; Perez-Torrado *et al*., 2006; Flaherty *et al*., 2010). Analogous to *hop* and *stat* mutants, *zfh-1* or *chinmo* deficient CySCs failed to self-renew. Analogous to Upd overexpression in the germline, overexpression of Zfh-1 or Chinmo in the cyst cells resulted in an accumulation of CySCs and GSCs (Leatherman and DiNardo, 2008; Flaherty *et al*., 2010). These findings strongly suggest that the activities of Zfh-1 and Chinmo depend on JAK/STAT signaling and link the JAK/STAT signaling event to transcriptional regulation of target genes. However, Zfh-1 and Chinmo appear to act in an independent manner, based on their expression patterns and genetic interaction. While Zfh-1 is predominantly expressed in CySCs, Chinmo appears to be expressed at similar levels in CySCs and in early stage cyst cells. Zfh-1 and Chinmo expression was unaffected in testes mutant for the other

gene and overexpression of *zfh-1* did not restore CySCs in *chinmo* mutants (Flaherty *et al*., 2010).

In addition to the core signaling event from the hub cells to the CySCs, the levels of JAK/STAT signaling is further regulated cell autonomously within the CySCs (Leatherman and DiNardo, 2010; Singh *et al*., 2010). Suppressors of Cytokine Signaling (SOCS) are highly conserved transcriptional targets of JAK/STAT signaling and antagonize the JAK/STAT pathway via several distinct mechanisms (Callus and Mathey-Prevot, 2002; Rawlings *et al*., 2004a; Arbouzova and Zeidler, 2006). In *Drosophila* testes, Socs36E is expressed in the hub cells and the CySCs. Yet surprisingly, testes from animals carrying a viable allele of *socs36E* showed a defect in the germline. The testes from these *socs36E* mutant animals progressively lost their GSCs from the position next to the hub. Notably, the CySCs in *socs36E* mutant testes had abnormally broad contact regions with the hub and expressed increased levels of βPS-Integrin at the hub-CySC interface. This indicated that JAK/STAT signaling acts via *socs36E* to regulate the expression, stability, or localization of cell adhesion molecules (Issigonis *et al*., 2009). The authors further hypothesized that increased cell adhesion between CySCs and hub cells in testes from *socs36E* mutant animals displaces the GSCs away from their position next to the hub. Consistent with these ideas, overexpression of βPS-Integrin in CySCs phenocopied the loss of *socs36E*, and reduction of βPS-Integrin from *socs36E* mutant animals rescued the GSC loss in the testes from *socs36E* mutant animals (Issigonis *et al*., 2009). While *socs36E* downregulates JAK/STAT signaling, the Nucleosome Remodeling Factor (NURF) appears to positively regulate JAK/STAT signaling within the CySCs (Issigonis *et al*., 2009; Cherry and Matunis, 2010). Mosaic analysis experiments revealed

that CySCs were not maintained when the cells were mutant for subunits of the NURF complex, specifically *nurf301, nurf38,* and *iswi*, suggesting a role for the NURF complex in CySC self-renewal. Restoring STAT expression in *nurf301* mutant CySCs restored the CySC loss, showing that STAT acts downstream of the NURF complex, and suggesting that *stat* may be a transcriptional target of the NURF complex (Cherry and Matunis, 2010). Following up on the importance of JAK/STAT signaling for stem cells, microarray studies were performed that identified transcripts up- or downregulated in testes with excess stem cells that are now being investigated for their roles in gametogenesis (DiNardo *et al*., 2011; Terry *et al*., 2006).

CySCs Specify GSC Fate

JAK/STAT signaling is required for the self-renewal of GSCs and CySCs yet specification of GSCs also requires crosstalk between the two stem cell populations (Kiger *et al*., 2001; Tulina and Matunis, 2001; Leatherman and DiNardo, 2010). When animals were created that expressed *stat* in the CySCs but lacked *stat* specifically in GSCs, surprisingly, the GSCs were maintained. These GSCs had lost contact with the hub yet remained associated with the CySCs and appeared functional. These observations suggested that *stat* activation in the CySCs is sufficient to mediate self-renewal of the GSCs, and that a second pathway from the CySCs to the GSCs has to induce, or reiterate GSC fate. This other pathway is the Transforming Growth Factor-β (TGFβ) signaling pathway (Leatherman and DiNardo, 2010).

TGFβ is homologous to the Bone Morphogenetic Protein (BMP) and the signaling pathways activated by TGFβ and BMP play important roles in development and stem cell

function across species (Blanpain and Fuchs, 2006; Hogan, 1996; Raftery and Sutherland, 1999; Varga and Wrana, 2005). BMP signaling has also been implicated in regulating mammalian spermatogenesis, yet studying the role of the BMP pathway in mammals is more difficult due to the high number of BMP family members that may act redundantly (Zhao *et al*., 1998; Meng *et al*., 2000; Puglisi *et al*., 2004; Itman and Loveland, 2008). The core BMP signaling pathway consists of the active receptor and the SMAD transcription factors. The active TGFβ family receptor is a transient complex of two receptors with serine-threonine activity and a ligand. The active receptor phosphorylates receptor-regulated Smad type proteins which complex with nonreceptor- regulated Smad proteins, enter the nucleus, and regulate transcription of target genes (Raftery and Sutherland, 1999; Varga and Wrana, 2005).

The *Drosophila* TGFβ ligands Decapentaplegic (Dpp) and Glass bottom boat (Gbb) are expressed in CySCs and hub cells of wildtype testes, and pathway activation has been observed in GSCs of wildtype testes (Shivdasani and Ingham, 2003; Schulz *et al*., 2004; Bunt and Hime, 2004; Kawase *et al*., 2004). Leatherman reported that the TGFβ pathway was activated in GSCs from testes with germline- depleted *stat* (Leatherman and DiNardo, 2010). The GSCs in these testes had high levels of phosphorylated SMAD in their nuclei, and expression of TGFβ antagonists in the *stat*-depleted testes led to a loss of GSCs. This strongly suggested that CySCs signal to neighboring germline cells via the TGFβ pathway to induce GSC fate (Leatherman and DiNardo, 2010). It remains to be investigated whether the Dpp and Gbb signals from the hub are also sufficient to induce GSC fate and whether Dpp and Gbb activity in CySCs is directly dependent on the JAK/STAT pathway.

Regulation of CySC Divisions

Stem cells have to adjust to the demand for differentiated cells in order to keep a tissue fully functional. For example, skin stem cells have to produce more skin cells during childhood when an individual grows than they have to produce during adulthood. Likewise, GSCs and CySCs have to adjust to the overall metabolism of the fly and to the demand for sperm. This novel aspect of stem cell biology is only recently being addressed and the *Drosophila* gonad has emerged once again as one of the pioneer systems to approach this unknown territory. A few studies have already addressed the mechanisms by which stem cells regulate their division frequency to produce more or less differentiating germline and cyst cells (illustrated in Fig. 2.2B). One factor regulating the division frequency of CySCs and GSCs is nutrient availability. When animals were starved, CySCs and GSCs divided at lower frequencies compared with CySCs and GSCs in testes from fed animals (McLeod *et al*., 2010; Wang *et al*., 2011). This suggested that insulin signaling regulates the division frequencies of stem cells in the testis in a manner similar as has been proposed for insulin signaling to the GSCs of the ovary (Drummond-Barbosa and Spradling, 2001; Hsu *et al*., 2008; LaFever *et al*., 2010). Complicating matters, CySCs, in turn, influence the division frequency of the enclosed GSCs in an only partially understood feedback loop. In testes from animals mutant for EGF signaling, GSCs divided at frequencies two to three times higher than GSCs of control testes (Parrott *et al*., 2012).

The EGF signaling pathway is highly conserved and plays multiple roles in embryonic development, stem cell biology, and gametogenesis of several species (Wiley *et al*., 1995; Shilo, 2003; Moghal and Sternberg, 2003; Normanno *et al*., 2006). In

Drosophila, the major ligand for the pathway, Spitz (Spi), and its receptor, the Epidermal Growth Factor Receptor (EGFR), are assumed to be ubiquitously expressed in many tissues and pathway activation depends on the cell type-specific activity of ligand processing proteases (Shilo, 2003). In testes, the germline cells process Spi into the active, secreted form via activity of the protease Stet, while EGFR is stimulated on the CySCs and cyst cells (Schulz *et al*., 2002; Parrott *et al*., 2012; Kiger *et al*., 2000). Once activated, the tyrosine kinase EGFR phosphorylates cascades of downstream signal transducers (Lemmon and Schlessinger, 2010).

Removal of either *spi* or *stet* from the germline cells, or removal of the EGFR from the soma resulted in increased division frequencies of GSC but did not affect the division frequencies of CySCs (Parrott *et al*., 2012). This strongly suggested that EGF signaling normally downregulates GSC divisions. However, this effect is indirect as EGF is produced by the germline and received by the CySCs which presumably respond with an unknown return signal to the GSCs. Thus, the division frequency of stem cells depends on both systemic factors and interaction between the CySCs and GSCs.

Germline stem cells show a characteristic orientation of their mitotic spindles during cell division in both mouse and *Drosophila* testes. In *Drosophila* testes, the spindles of the GSCs are oriented perpendicular to the hub (Yamashita *et al*., 2003). In rat testes, mitotic spindles of spermatogonia were mainly oriented with angles ranging from 60 to 90° perpendicular in relation to the basement membrane of the seminiferous tubules (Lagos-Cabré and Moreno, 2008). CySCs in *Drosophila* testes also undergo strict asymmetric divisions yet they employ a different cellular mechanism for orienting their plane of division than GSCs do (Yamashita *et al*., 2003; Cheng *et al*., 2011). In GSCs, the

mitotic spindle is oriented perpendicular to the hub throughout mitosis (Yamashita *et al*., 2003). CySC spindles do not show a consistent orientation. Instead, one of the spindle poles is repositioned to the hub-CySC interface specifically during anaphase (Cheng *et al*., 2011). Spindle positioning in both CySC and GSC divisions requires Centrosomin, as loss of Centrosomin disrupts the spindle orientation in both GSCs and CySCs. While GSCs also require activity of Adenomatous Polyposis Coli (APC), this factor is dispensable for CySC spindles (Yamashita *et al*., 2003; Cheng *et al*., 2011). Instead, CySCs require Moesin, a linker between the membrane and the cytoskeleton (Cheng *et al*., 2011; Bretscher *et al*., 1997). Moesin knockdown via RNA Interference (RNAi) in CySCs resulted in reduced spindle repositioning during anaphase. In contrast, Moesin knockdown in GSCs had no effect on spindle positioning. In the course of their experiments, the authors noticed that both overexpression and loss of Moesin increased the number of GSCs, CySCs, and cyst cells. Though the mechanism for the dose effect of Moesin is not understood, these results suggested that the correct expression level of Moesin is required for maintaining the correct number of stem cells in wildtype testes (Cheng *et al*., 2011).

The above discussed studies showed that a combination of multiple mechanisms, including Upd signaling from the hub, the cell autonomous activity of *lines* and *bowl*, and attachment of the CySCs to the hub via cell adhesion molecules assure the maintenance and self-renewal of CySCs, as well as proper interactions between CySCs and GSCs in *Drosophila* testes. In the following section, we will focus on discussing cyst cell differentiation and the roles the cyst cells play in guiding germline proliferation and germline differentiation.

Cyst Formation is Regulated by EGF Signaling

The cysts in *Drosophila* testes are one of the few examples in animal development where one cell type completely encloses another. Other examples are the enclosure of nerve cells by the myelin sheath and the formation of biofilm during the mating of yeast (Quarles, 2002; Sahni *et al*., 2010; Alby and Bennett, 2011). Research in *Drosophila* testes has revealed insights as to how a specific arrangement between two different cell types is established. Genetic experiments showed that signaling from the germline cells via EGF to the cyst cells is required to induce and organize the growth of the cyst cells around the germline cells (illustrated in Fig. 2.2C). A conditional allele of *spi* displayed germline enclosure defects of increasing severity with increasing temperature. Removing one copy of the small monomeric GTPase, *rac1*, from *spi* mutant animals raised at an intermediate temperature drastically enhanced the germline enclosure defects resembling testes from *spi* mutant animals raised at higher temperatures. Likewise, reducing the expression of the docking protein and guanidyl exchange factor, Vav, or Rac1 specifically from the cyst cells of *spi* mutant resulted in a similarly strong enhancement of the germline enclosure defects. Together with binding studies of EGFR and Vav, the above genetic data suggested that Vav and Rac1 act in a signaling branch downstream of the EGFR. Conversely, reducing the expression of the small monomeric GTPase Rho1 from the cyst cells had the opposite effect on testes from *spi* mutant animals, restoring germline enclosure even when the animals were raised at high temperatures. This indicated that Rho1 acts in a pathway opposing EGFR for germline enclosure (Sarkar *et al*., 2007). In cultured mammalian cells, Rac and Rho play antagonistic roles in regulating cell shape changes and growth via different effects on the

actin cytoskeleton (Sander *et al*., 1999; Jaffe and Hall, 2003; Kurokawa *et al*., 2004; Hall, 2005). Based on electron microscopy, expression of dominant negative Rac1- and Rho1-constructs in the cyst cells of otherwise wildtype testes specifically affected the structure of the cyst cell membranes. In wildtype testes, the cyst cell membranes were wavy. Loss of Rac1 from the cyst cells resulted in smoother membranes than those seen in cyst cells of wildtype testes, while loss of Rho1 resulted in the appearance of filopodia in the cyst cell membranes. These findings supported the idea that EGF signaling from the germline cells produces a differential of Rac- and Rho-activities across the cyst cells that, perhaps by organizing the actin cytoskeleton, leads to the directional growth of the cyst cells around the germline cells (Sarkar *et al*., 2007). In mammalian testes, the displacement of the differentiating germline cells toward the lumen of the seminiferous tubules requires reorganization of the germline-Sertoli cell contacts (Kopeta *et al*., 2010). The Sertoli cells in mouse testes contain a most elaborate cytoskeleton that is essential for their structure and function and likely to play an active role in the constant reorganization of the germline-Sertoli cell contacts (Lui and Cheng, 2007; Amlani and Vogl, 1988). Interestingly, the mammalian EGFR homolog was reported to localize to Sertoli cells opening the possibility that some of the roles for EGF signaling in somatic cell shape or function are conserved between *Drosophila* and mammals (Amlani and Vogl, 1988; Vogl, 1988; Suárez-Quian and Niklinski, 1990).

Further studies in *Drosophila* led to the identification of several other conserved proteins that are required for the physical interactions between germline and cyst cells. For example, the large musculo-aponeurotic fibrosarcoma (Maf) factor homolog Tj acts cell autonomously in cyst cells to mediate cyst cell association with the germline by

activating the transcription of cell adhesion molecules (Li *et al*., 2003). Similarly, the conserved motor protein Dynein Light Chain 1 (DDLC1) is required for the presence of several cell adhesion molecules on the cyst cell membranes and the presence of Eya in the cyst cell nuclei (Joti *et al*., 2011). However, it remains to be addressed whether this is due to a role of DDLC1 in regulating gene expression, protein localization, or protein stability. Likewise, it would be interesting to learn if loss of *ddlc1* has an effect on EGF signaling and whether it plays a parallel role in gametogenesis of other organisms.

Cyst Cells Regulate Germ Cell Proliferation

In most tissues maintained by stem cells, the stem cell daughters undergo several rounds of transit amplifying divisions to increase the numbers of precursors for terminally differentiated cells (Huckins, 1971a; Blanpain and Fuchs, 2006; Metcalf, 2007; Hanahan and Weinberg, 2000). The proliferation of cells needs to be tightly regulated to avoid loss or tumorous growth of a tissue and the mechanisms restricting cell proliferation are of wide interest in development, stem cell biology, and cancer biology. The *Drosophila* testis is an excellent model tissue in which to study tumorigenesis (Loveland and Hime, 2005; Hime *et al*., 2007). In *Drosophila* testes, proliferation of the stem cell daughters relies on both germline intrinsic and extrinsic factors (Gönczy and DiNardo, 1996; Schulz *et al*., 2002; Gönczy *et al*., 1997). The requirement for germline-soma interactions to restrict cell proliferation is most evident in testes that lack either germline cells or germline enclosure by cyst cells. In testes without a germline (agametic testes), the cyst cells lost the ability to differentiate into late stage cyst cells and proliferated as early stage cyst cells instead. The somatic cells appeared to be properly

specified and initially behaved normally. However, after five days, the number of early stage cyst cells increased and the number of late stage cyst cells decreased. In addition, the cyst cells underwent mitotic divisions, a trait normally restricted to CySCs. This suggests that germline cells normally restrict the ability of cyst cells to reenter the CySC proliferation program (Gönczy and DiNardo, 1996). Interestingly, cyst cells associated with gonialblasts normally fail to elongate their centrioles and to recruit pericentriolar material, possibly due to downregulation of SAS-6, a main regulator of centriole architecture (Riparbelli *et al*., 2009). It remains to be investigated whether downregulation of SAS-6 is the cause for the withdrawal of cyst cells from the cell cycle and whether cyst cells in agametic testes continue to express SAS-6.

Conversely, germline proliferation is restricted by surrounding cyst cells. In *spi* or *stet* mutant animals, germline cells and cyst cells were present but the cyst cells did not enclose the germline. In these testes, the germline cells proliferated at early stages and failed to differentiate (Schulz *et al*., 2002; Sarkar *et al*., 2007). Though previous experiments had suggested that EGF signaling produces a feedback loop from the cyst cells to the enclosed germline cells to restrict germline proliferation, these experiments did not address germline enclosure (Kiger *et al*., 2000; Tran *et al*., 2000). Therefore, it remained unclear whether the germline proliferation defects in the *egfr* mutants were due to defects in enclosure or whether EGF also restricts germline proliferation past the enclosure event. Recent studies have shed more light on the specific roles of EGF in restricting germline proliferation and cyst maturation (Hudson and Schulz, unpublished data). By using many alleles and conditions for *spi, stet, raf, and egfr*, as well as overexpression studies, we and others demonstrated that EGF signaling has a range of

effects on cyst cells and germline cells. EGF signaling from the germline to the soma regulates the division frequency of GSCs, the enclosure of the gonialblast by cyst cells, the progression of the spermatogonia through amplification divisions, and EGF signaling also promotes the entry of the germline cells into the spermatocyte stage (illustrated in Fig. 2.2B–E) (Schulz *et al*., 2002; Parrott *et al*., 2012; Kiger *et al*., 2000; Sarkar *et al*., 2007; Tran *et al*., 2000; Hudson and Schulz, unpublished data). This suggests that the cyst cells send different sets of return signals to the enclosed germline cells at different stages of spermatogenesis. The EGFR also appears to be required cyst cell-intrinsically for their viability, as some EGFR alelles are cell lethal (Kiger *et al*., 2000).

In order for the spermatogonia to enter the spermatocyte stage, they first have to exit from the mitotic amplification divisions. The exit of the spermatogonia from mitosis specifically at the 16-cell stage depends on TGFβ signaling (illustrated in Fig. 2.2E). Loss of the TGFβ receptors Punt (Pnt) or Saxophone (Sax), the signal transducer Smad on X (Smox), or the downstream transcription factor Schnurri (Shn) from cyst cells resulted in the accumulation of large clusters of spermatogonia of multiples of 16. For example, these cysts contained 32, 64, or 128- spermatogonia (Matunis *et al*., 1997; Li *et al*., 2007b). In addition to JAK/STAT, EGF and TGFβ signaling, a number of candidate genes have been identified that affected germline proliferation but their mode of action and whether their homologs may play a role in tissue homeostasis in other organisms or cancer is yet to be determined (Schulz *et al*., 2004).

Germline proliferation was suggested to depend on motor-based processes within the cyst cells, based on the phenotypes of mutations in a cytoplasmic dynein, motor proteins and a GTPase (Joti *et al*., 2011). Animals carrying a hypomorphic allele of *ddlc1*

displayed a testes phenotype in which the germline cells over-proliferated and accumulated as single cells and 2-cell spermatogonia. In an effort to investigate the mechanism of DDLC1 action, the potential binding partners of DDLC1 were knocked down via RNA Interference and the effect on germline and cyst cells investigated (Joti *et al*., 2011; Makokha *et al*., 2022). The binding partners for DDLC1 are Dhc64C, which encodes a microtubule motor protein, and Myosin V (*didum*), which encodes an actin binding protein (FlyBase Consortium, 2003). The mammalian homolog of Myosin V, Myosin 5b, displayed motor protein function during membrane recycling, suggesting a similar role as a motor protein in *Drosophila* (Joti *et al*., 2011; Wang *et al*., 2008). Knockdown of either *didum* or *dhc64C* in the cyst cells resulted in a similar germline over-proliferation phenotype as knockdown of *ddlc1* and removing one copy of *didum* or *dhc64C* from animals carrying a hypomorphic allele of *ddlc1* enhanced the germline proliferation phenotype. This suggested that the three proteins act in a pathway to restrict germline proliferation. However, the effect on cell adhesion molecules observed in the *ddlc1* mutants was not recapitulated upon reduction of *didum* or *dhc64C*, suggesting that *ddlc1* acts in two distinct pathways, one involved in cell adhesion, and the other in restricting germline proliferation. Finally, cyst cell-reduction of the GTPase, Rab11, which was shown to play a role in Myosin V-dependent secretion in developing photoreceptors, also resulted in germline over-proliferation (Joti *et al*., 2011; Li *et al*., 2007a). The authors hypothesized that Rab11 acts in the same pathway as DDLC1, Myosin V, and Dhc64C, and that they play a role in the exocytosis of a yet-to-be-identified cyst cell derived signal regulating germline proliferation (Joti *et al*., 2011). Interestingly, another Dynein, Dhc1, has been associated with EGF secretion in the

developing eye and cytoplasmic dynein has been identified in rat Sertoli cells suggesting conserved roles for Dyneins in regulating intracellular processes during development and germline differentiation (Nelly *et al*., 1990; Iyadurai *et al*., 2008).

Cyst Cells Regulate Germline Survival

In addition to providing regulatory cues for the development of the germline cells, cyst cells are also required for germline survival, yet for most of the genes mentioned below, their exact mechanisms of action and how they relate to each other are unknown. Germline survival specifically at the spermatocyte stage depends on the transcription factors Eya and Sine oculis (So), suggesting a checkpoint after mitotic amplification divisions. Eya and So are both expressed in cyst cells associated with spermatocytes and clonal analysis revealed that they act in the cyst cells to prevent the death of spermatocytes prior to their maturation. Mutations in *eya* and *so* show synergistic genetic interactions, suggesting that they function in different pathways (Fabrizio *et al*., 2003).

Several other factors are required for germline survival prior to the spermatocyte stage. The *zero population growth* (*zpg*) gene encodes a gap junction protein which is expressed in the germline. Mutations in *zpg* were associated with under-proliferating spermatogonia that eventually died, which suggested that germline cells and surrounding cyst cells exchange small molecules for promoting their viability (Tazuke *et al*., 2002). Likewise, mutations in the tumor suppressor gene *discs large (dlg),* which encodes a septate junction protein, resulted in the death of spermatogonia as well as their enclosing cyst cells, suggesting that germline viability depends on direct connections between the germline and the cyst cells (Papagiannouli and Mechler, 2009). *slow motion (slowmo),* a

mitochondrial gene of unknown function, is expressed in cyst cells and affected survival of germline cells at all stages of development (Reeve *et al*., 2007).

When the role of cyst cells in GSC self-renewal and GSC daughter differentiation was first explored, two outstanding studies also suggested a role for EGF signaling in CySC viability or maintenance (Kiger *et al*., 2000; Tran *et al*., 2000). This was not recapitulated in testes from animals carrying a temperature sensitive allele of *spi*, animals mutant for the protease *stet*, or animals with CySC-depleted EGFR (Schulz *et al*., 2004; Parrott *et al*., 2012; Sarkar *et al*., 2007). Kiger reported a failure to detect *egfr* mutant CySCs or cyst cells in clonal analysis experiments. This failure to detect EGFR mutant somatic cells is not surprising as the mutant alleles used in the clonal analysis experiments had been reported to be cell lethal (Kiger *et al*., 2000; FlyBase Consortium, 2003). Maybe, the EGFR produced from the cell-lethal alleles folds in a manner interfering with basic cellular functions and, therefore, induces cell death. As the alleles have no effect on the germline, it suggests that the folding defects would depend on ligand binding. We tested the temperature sensitive allele of the EGFR used by Kiger (Kiger *et al*., 2000). We observed that, after the shift to the restrictive temperature, the animals themselves died within a few days supporting the idea that this allele is cell-lethal (Hudson and Schulz, unpublished data).

The other study reported that the somatic cells next to the hub in adult animals depleted of the Mitogen activated protein (Map)-Kinase, Raf, expressed the late cyst cell marker Eya, suggesting that the CySCs may have died or differentiated into late stages (Tran *et al*., 2000). In these animals, Raf activity was systemically reduced, leaving open in which cell type Raf acts to maintain CySCs. As Raf acts downstream of several

signaling pathways it is plausible that the observed defects do not reflect loss of EGF signaling from the germline (Wassarman *et al*., 1995; Schnorr *et al*., 2001). Consistent with this, we never detected somatic cells next to the hub with high levels of Eya expression in testes maternally and zygotically depleted for EGF signaling (Hudson and Schulz, unpublished data). The strongest argument to refute the idea that EGF signaling from the germline to the cyst cells directly prevents CySC death or differentiation is the observation that somatic cells next to the hub in agametic testes did not undergo cell death or express Eya (Gönczy and DiNardo, 1996).

Cyst Cells Regulate Late Stages of Germline Differentiation

Somatic and germline cells remain intimately associated throughout spermatogenesis in many species, suggesting that interactions between the two cell types also regulate late stages of spermatogenesis (Huckins, 1971a; Griswold, 1998; Kimble and Crittenden, 2007). An excellent example for this was observed in advanced stages of *Drosophila* spermatogenesis. As spermatids elongate, the cyst cells undergo a series of morphological changes whereby they become structurally distinct from one another. Tail cyst cells grow dramatically in size to accommodate the elongating sperm tails, while the head cyst cells grow to a much lesser extent. Following elongation of the cysts, the head cyst cells associate with the terminal epithelium of the testes, upon which the entire cysts coil to form compact structures (Fuller, 1993). As the spermatids mature, the head cyst cells grow a cap around the sperm heads (illustrated in Fig. 2.2F). This cap is rich in F-actin and cell adhesion molecules, and organized by the Arp2/3 complex. A requirement for the cap was shown by phenotypic analysis of testes from animals carrying mutant

alleles and expressing RNAi constructs for genes encoding cap proteins specifically in the cyst cells. These studies included knockdown of Wsp, the Arp2/3 complex proteins Sop2 and Arp3, Wasp-interacting protein (WIP), the cell adhesion protein Sticks and stones (SNS), and the small monomeric GTPase Cdc42. Any of these mutants resulted in abnormal caps and were associated with an arrest in development of the germline cells prior to cyst coiling. Furthermore, it was shown that release of mature sperm from the cap depends on the activity of the dynamin Shibire. Interestingly, spermatogenesis was also arrested when the mammalian WASp homologue was disrupted in mouse Sertoli cells, and the nature of the molecular mechanisms and the degree of parallels have become a future target of this line of research (Desai *et al*., 2009; Rotkopf *et al*., 2011).

Though the cyst cells are clearly required for proper germline proliferation and differentiation *in tissue*, it appears that germline cells have some cell-intrinsic properties for differentiation. An interesting cell culture study of *Drosophila* male germline cells suggested that the entry into terminal differentiation may be independent from surrounding cyst cells. When the authors dissociated 16-cell spermatogonia from the encapsulating cyst cells, the dissociated spermatogonia transitioned into spermatid-like elongated cells. Unfortunately, it was not addressed whether these germline cells had undergone meiosis, whether they had normal DNA-content or chromatin structure, or whether the mitochondria assembled properly to produce the long flagellum of the sperm tail. The authors nevertheless concluded that cyst cells are dispensable for spermatocytes to undergo spermiogenesis (Kawamoto *et al*., 2008). Similarly, in some mutant situations, germline cells can enter the spermatocyte stage independent from surrounding cyst cells. For example, germline cells from animals double-mutant for *stet* and the *nucleoporin98*–

96 locus were not enclosed by cyst cells but developed into spermatocyte-like cells (Parrott *et al*., 2011).

Although the intrinsic properties of germline cells may give them some independence from the soma, the constant and coordinated production of mobile sperm from GSCs remains under cyst cell control. All of the findings discussed in this review (see Table 2.1 for overview of all discussed molecules) clearly prove this point and have changed our view of the cyst cell lineage in *Drosophila* testes. While cyst cells were originally thought of as nurturing cells in *Drosophila* testes, the research discussed in this review shows that the CySC lineage in *Drosophila* testes plays an active role in gametogenesis and put them into the spotlight. In *Drosophila* testes, germline and cyst cells undergo coordinated interactions that regulate their co-differentiation and assure the production of functional sperm. A similar interdependence of stem cell lineages was discovered in mammals and similar mechanisms of interactions are being discussed (Devine and Hoffman, 2000; Nishimura, 2011). For example, melanocyte stem cells are closely associated with epithelial stem cells in the hair follicle of the skin, and here as well signaling between the two lineages is an important key in coordinating the differentiation of the two stem cell lineages to make pigmented hair (Rabbani *et al*., 2011).

Outlook

The interdependence of germline and cyst cells has been demonstrated for both the early and the final stages of spermatogenesis in *Drosophila*. Cyst cells regulate spermatogonial divisions, germline cell survival, and sperm maturation via several

conserved molecules and pathways. Though research on *Drosophila* cyst cells has dramatically advanced our knowledge in this respect, many questions remain to be addressed. For example, we are only beginning to understand how the divisions of CySCs and GSCs are regulated to assure that two cyst cells are generated for each gonialblast. To start to address these questions, the molecules and pathways regulating stem cell division frequencies need to be identified and studied in detail, followed by the identification of the means by which the two stem cell populations coordinate their activities.

What are the molecular natures of the signals presented by the cyst cells to the enclosed germline cells? Despite the importance of these signals for germline differentiation, not a single one has been identified. A possible explanation is that redundant pathways regulate the behavior of the enclosed germline. Consistent with this idea, attenuation of signaling pathways in sensitized backgrounds can have a strong effect on germline development while not causing an effect in an otherwise wildtype background.

Molecules and pathways regulating early and late stages of germline and cyst cell development have been described. However, no reports are available that describe interactions between germline and cyst cells during germline meiosis or the initiation of terminal differentiation. Several factors, including Protein Phosphatase Y, the NOA fatty acid elongase, and Ku80, a protein involved in DNA damage repair, are expressed in late stage cyst cells and the study of these genes may shed light on these questions (Armstrong *et al*., 1995; Boutanaev *et al*., 2007; Jung *et al*., 2007). However, we still need to create a pool of late stage cyst cell-specific markers and transcriptional profiles of

differentiating cyst cells in order to study germline and cyst cell differentiation. These tools will ultimately aid us in identifying potential interactions and show whether cyst cells, like germline cells, pass critical cell-intrinsic checkpoints during their differentiation program.

Future research will shed light on these and other key questions. As discussed, many of the principles unraveled by studying gametogenesis in *Drosophila* testes may apply to gametogenesis in other species.

Figure 2.1: *The organization of somatic cells and germline cells in wildtype testes* (A) Graphic depicting the organization and development of the cysts along the apical (left) to basal (right) axes of the testis. CySC, cyst stem cell; GSC, germline stem cell; GB, gonialblast; SG, spermatogonia; SC, spermatocytes; CC, cyst cells; HCC, head cyst cells; TCC, tail cyst cells. (B–D) Immunofluorescent labeling of wildtype testes as indicated. (B) Visualization of cyst cells enclosing the germline cells in a whole testis. (C) The apical tip of a testis showing co-localization of Zfh-1 and Tj. (D) The apical region and the testis coil. Tj is expressed in early stage cyst cells near the tip, and Eya is expressed strongly in late stage cyst cells. *hub cells; scale bars, 50 μm.

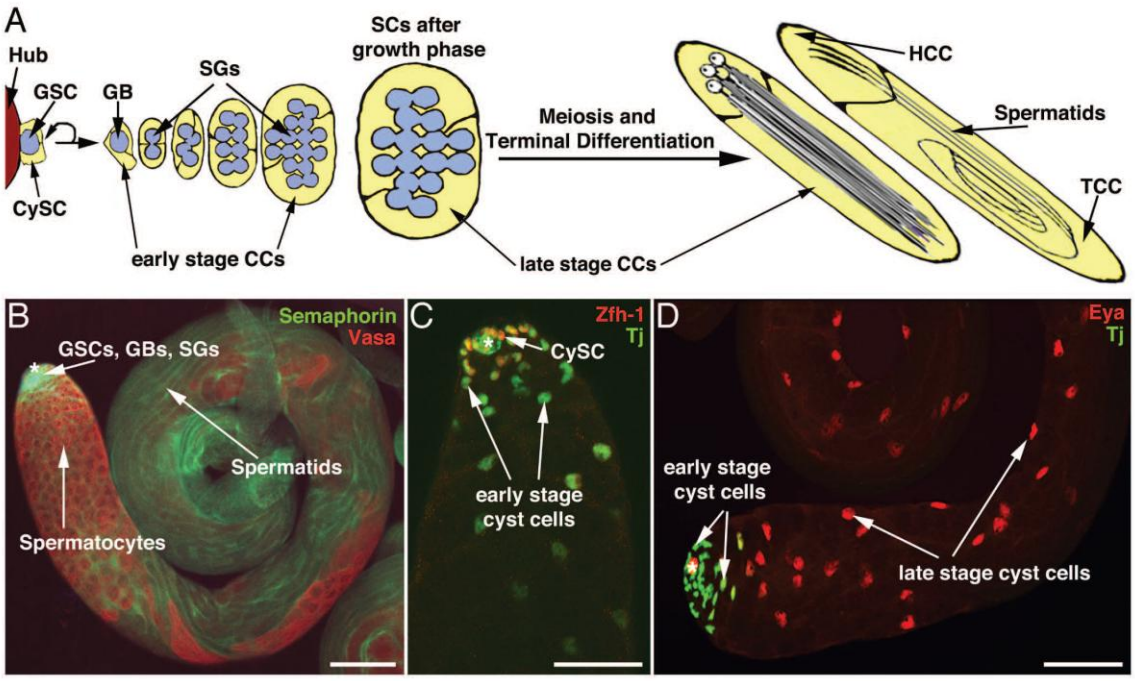
A
Hub
GSC
GB
SGs
CySC
early stage CCs
SCs after
growth phase
Meiosis and
Terminal Differentiation
HCC
Spermatids
TCC
late stage CCs
B
Semaphorin
Vasa
GSCs, GBs, SGs
Spermatids
Spermatocytes
C
Zfh-1
Tj
CySC
early stage
cyst cells
D
Eya
Tj
early stage
cyst cells
late stage cyst cells

Figure 2.2: *Regulation of cyst development* **(**A) Signaling events at the tip of the testes regulating CySC and GSC fate. (B) Signaling events regulating CySC and GSC division frequency (black round arrows). (C) The regulation of germline enclosure by EGF signaling. (D) The regulation of germline proliferation by EGF signaling. (E) Signaling events regulating the transition of the spermatogonia into the spermatocyte stage. (E) Sperm maturation depends on the cap proteins in the head cyst cells.

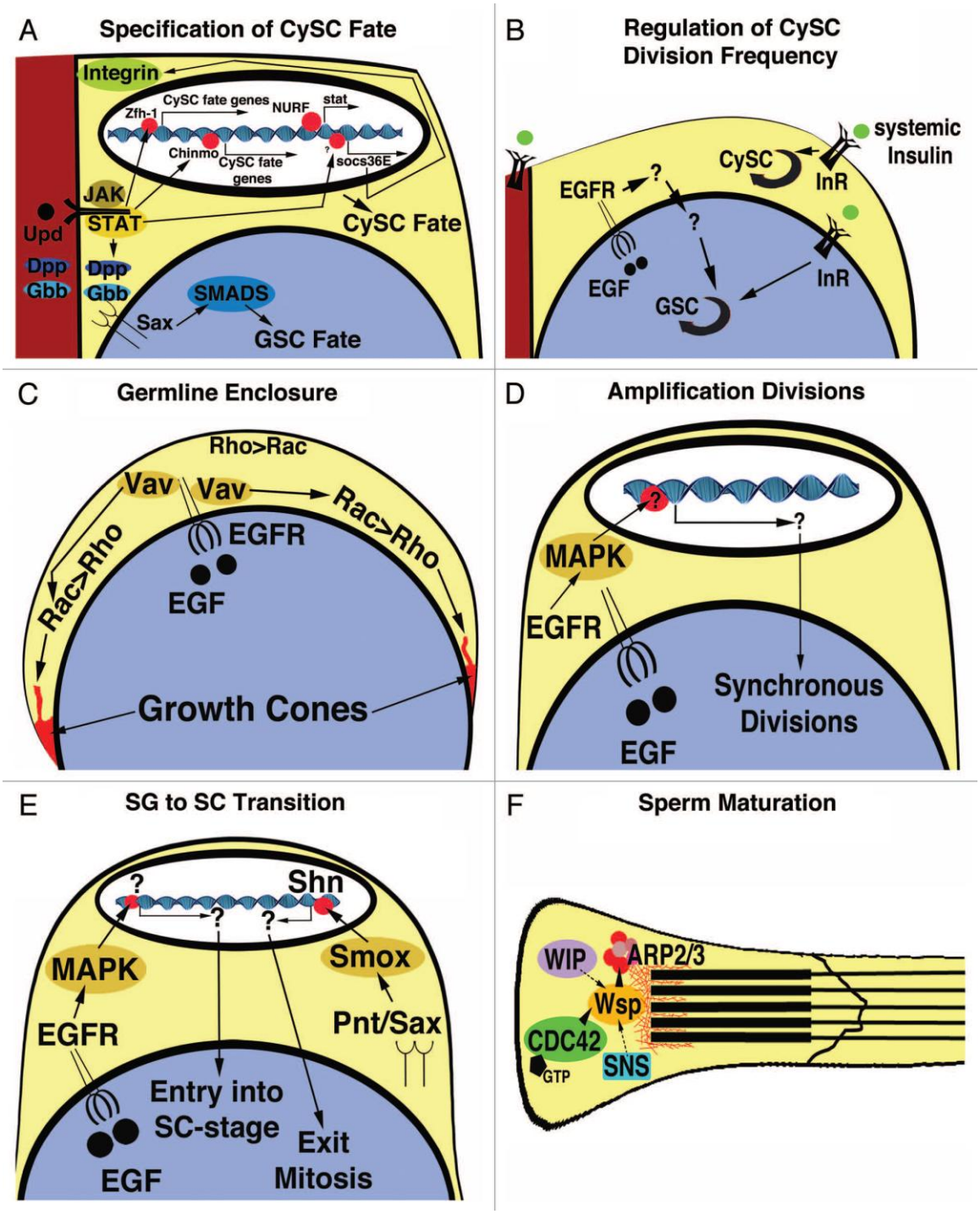
A
Specification of CySC Fate
Integrin
CySC fate genes
NURF
stat
Zfh-1
Chinmo
CySC fate genes
socs36E
JAK
STAT
Upd
Dpp
Gbb
Dpp
Gbb
SMADS
Sax
CySC Fate
GSC Fate
B
Regulation of CySC Division Frequency
systemic Insulin
CySC
InR
EGFR
?
?
EGF
GSC
InR
C
Germline Enclosure
Rho>Rac
Vav
Vav
Rac>Rho
Rac>Rho
EGFR
EGF
Growth Cones
D
Amplification Divisions
?
?
MAPK
EGFR
Synchronous Divisions
EGF
E
SG to SC Transition
?
Shn
?
?
MAPK
Smox
EGFR
Pnt/Sax
Entry into SC-stage
Exit Mitosis
EGF
F
Sperm Maturation
WIP
ARP2/3
Wsp
CDC42
GTP
SNS

Table 2.1: *Alphabetical listing of the genes discussed that play roles in or for the CySC lineage.* CySC, cyst stem cell; GSC, germline stem cell; GL, germline; SGP, somatic gonadal precursor.

Gene name/Abbreviation	Molecular nature	Discussed function
Arp2/3 complex (Sop2, Arp3)	Cytoskeletal regulator	Sperm maturation
Brother of odd with entrails limited (bowl)	Transcription factor	CySC/hub cell specification
CdC42	GTPase	Sperm maturation
Centrosomin (cnn)	Protein binding	Stem cell fate
Chronologically inappropriate morphogenesis (chinmo)	Transcription factor or protein degradation	CySC fate
Decapentaplegic (dpp)	Protein/ligand binding	GSC fate
Discs large (dlg)	Protein binding	Cyst survival
dhc64C	Microtubule motor activity	GL proliferation
Dynein Light Chain 1 (ddlc1)	Cytoplasmic Dynein	GL-soma adhesion; GL proliferation
Epidermal growth factor receptor (egfr)	Receptor	CySC specification; CySC division frequency; GL proliferation; Cell viability
Eyes absent (Eya)	Transcription factor	Spermatocyte survival
F-actin	Cytoskeletal component	Sperm maturation
Glass bottom boat (Gbb)	Protein/ligand binding	GSC fate
Hopscotch (hop) = JAK	Protein tyrosine kinase	CySC fate
Insulin	Protein/ligand binding	GSC & CySC division frequency
Insulin Receptor (InR)	Receptor	GSC & CySC division frequency
Ku80	Unknown	Unknown
Lines (lin)	Catalytic activity	CySC specification
Moesin	Protein binding	Stem cell fate
Mothers against Dpp (mad)	Signal transducer	GSC fate
Myosin V (didum)	Actin binding	GL proliferation
NOA	Fatty acid elongase	Unknown
Notch (N)	Receptor	Hub specification from SGPs
NURF complex (nurf301, nurf38, iswi)	Chromatin remodeling	CySC fate
Protein Phosphatase (ppy)	Phosphatase	Unknown
Punt (pnt)	Receptor	GL proliferation
Rab11	Endosome transport	GL proliferation
Rac1	GTPase	GL enclosure
Rho1	GTPase	GL enclosure
Saxophone (sax)	Receptor	GL proliferation
SAS-6	N/A	Cyst cell centriole architecture
Schnurri (shn)	Transcription factor	GL proliferation
Sevenless (sev)	Receptor	CySC specification from SGPs
Shibire (shi)	Actin binding	Sperm release
Sine oculis (so)	Transcription factor	GL survival

Slow motion (slomo)	N/A	Cyst survival
Smad on X	Signal transducer	GL mitosis
Signal transducer and activator of transcription (stat)	Signal transducer	CySC fate
Suppressor of cytokine signaling 36E (socs36E)	N/A	CySC fate
Spitz (spi)	Protein/ligand binding	CySC division frequency; germline enclosure; GL proliferation
Stet	Protease	CySC division frequency; Germline enclosure; GL proliferation
Sticks and Stones (sns)	Protein binding	Sperm maturation
Traffic jam (tj)	Transcription factor	GL-soma adhesion
Unpaired (upd)	Protein/ligand binding	CySC fate
Zero population growth (zpg)	Gap junction protein	Cyst survival
Vav	Guanidyl exchange factor	GL enclosure
Wsp	Protein binding	Sperm maturation
Wasp-interacting protein (wip)	Actin filament binding	Sperm maturation
Zinc finger homeodomain-1 (zfh-1)	Transcription factor	CySC fate

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CHAPTER 3

ECDYSONE SIGNALING ANTAGONIZES EGF SIGNALING IN THE MALE GONAD OF ADULT *DROSOPHILA*[2]

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Abstract

The mechanism of tissue replenishment in multicellular organisms depends upon the proper, coordinated differentiation of precursor cells that are constantly produced from long-lived stem cells. In the gonad of male *Drosophila melanogaster*, signaling via the Epidermal Growth Factor (EGF) pathway is necessary to induce and organize the growth of cyst cells around the germline cells and for the cyst cells to guide the germline cells through the early steps of spermatogenesis. Here we show through RNAi-mediated knockdown of the ecdysone receptor (EcR) and other ecdysone signaling components that ecdysone signaling in cyst cells antagonizes EGF signaling. We confirm that ecdysone is responsible for this interaction with EGF, as ecdysone biosynthesis genes had the same effect when attenuated in one copy from *spi*-mutant animals. Furthermore, we show that EcR signaling components are expressed in cyst cells, based on RT-PCR and immunofluorescence experiments with specific antibodies.

Background & Introduction

During development, the different organ and tissue systems in multicellular organisms arise from precursor cells. For example, appendages develop from imaginal discs in D*rosophila* while muscles and bones develop from somites in vertebrates (Cohen, 1993; Hofmann, 2003). Throughout postembryonic life in vertebrates, specialized, long-lived cells, the adult stem cells, are responsible for the maintenance of adult tissues. Adult stem cells are found in many vertebrate tissues, such as the blood, skin, nervous system, various internal organs, and the gonad (reviewed in Wagers and Weissman, 2004; Oatley and Brinster, 2012). Adult stem cells are also found in

invertebrate tissue including nervous, gonadal, and intestinal tissue where they both self-renew and reproduce short-lived, differentiated cells (reviewed in Spradling *et al*., 2001; reviewed in Brand and Livesey, 2011; reviewed in Morris and Spradling, 2008). The programming of stem cell fate is influenced by multiple cell-intrinsic factors and complex interactions with other cells that constitute a microenvironment, or niche for these resident stem cells (reviewed in Watt and Hogan, 2000; Morrison and Spradling, 2008; Sugiyama and Nagasawa, 2012).

The male gonad of *Drosophila* has emerged as an excellent model for studying stem cell dynamics, the interdependence of cell types, and the differentiation of stem cell progeny because of the genetic tractability of the animal, number of tools and resources available, and the simplicity of the model tissue (reviewed in Zoller and Schulz, 2012). Research on the male *Drosophila* gonad has shown that differentiation of the stem cell daughters also depends on signals from their external microenvironment (Schulz *et al*., 2002). For example, the exit of the germ line cells from mitotic amplification divisions prior to terminal differentiation into sperm is regulated by TGFβ signaling in the surrounding cyst cells (Matunis *et al*., 1997).

Spermatogenesis in Drosophila melanogaster

Germline stem cells (GSCs) are located at the apical tip of the testes around a single group of post-mitotic, apical hub cells. Each GSC is flanked by two somatic cyst stem cells (CySCs) that extend cytoplasmic extensions around the GSCs and into the hub (Fig 2.1A) (Hardy *et al*., 1979; Fuller, 1993). Both GSCs and CySCs divide asymmetrically to produce new stem cells and daughter cells that initiate differentiation,

the gonialblasts (GBs) and the cyst cells (CCs), respectively (Cheng *et al*. 2011; Yamashita *et al*. 2003;). The cell fate decision of the stem cell daughters to either self-renew or to initiate development depends on signals from the hub. The hub cells signal via the Janus kinase and Signal Transducer and Activator of Transcription (JAK/STAT) and the Hedgehog signaling pathways to induce and maintain stem cell fate in the CySCs (Amoyel *et al*. 2013; Kiger *et al*. 2001; Leatherman and DiNardo 2010; Michel *et al*. 2012; Tulina and Matunis 2001). CySCs then signal for stem cell identity and maintenance to the enclosed GSCs via the Transforming Growth Factor β (TGFβ) signaling pathway (Kawase *et al*. 2004; Leatherman and DiNardo 2010; Shivdasani and Ingham 2003).

A gonialblast proliferates through exactly four rounds of synchronous mitotic amplifying divisions with incomplete cytokinesis so that its progeny, the spermatogonia (SG), remain interconnected by cytoplasmic bridges as they develop from 2-cell spermatogonia into 16-cell spermatogonia. After mitosis, the 16 interconnected spermatogonia enter terminal differentiation where they are then referred to as spermatocytes. Spermatocytes grow in size, divide twice by meiosis, and differentiate into elongated spermatids (Fuller 1993). While CySCs continue to divide by mitosis throughout the reproductive life of the fly, their daughter cyst cells exit the cell cycle and undergo differentiation. When a gonialblast is produced, two of the cyst cells grow cytoplasmic extensions around it to form the germline microenvironment. Next, the cyst cells grow in size as they co-develop with the germline cells and express different sets of stage-specific molecular markers (Gönczy and DiNardo 1996; Hardy *et al*. 1979; Riparbelli *et al*. 2009; Schulz *et al*. 2002). Co-development of germline cells and cyst

cells is regulated via cellular interactions of the two cell types, including signaling via the TGFβ and EGF signaling pathways (Matunis *et al*., 1997; Schulz *et al*., 2002)

The EGF signaling pathway is necessary for proper spermatogenesis

EGF signaling is a conserved pathway that is involved in embryonic development, biology of stem cells and gametogenesis in numerous species (Moghal and Sternberg, 2003; Normanno *et al*., 2006; Shilo, 2003; Wiley *et al*., 1995). In *Drosophila*, the major ligand for the pathway, Spitz (Spi), and its receptor, the Epidermal Growth Factor Receptor (EGFR), are expressed in multiple tissue types and activation of the pathway is dependent on cell type-specific activity of ligand-processing proteases (Shilo, 2003). In the testes, the germline cells process Spi into the active, secreted form via the protease Stet. The secreted form of Spi binds to EGFR found on the CySCs and cyst cells (Fig. 2.2B-E) (Kiger *et al*., 2000; Parrot *et al*., 2012; Schulz *et al*., 2002). Once activated, autophosphorylation of tyrosine residues on the intracellular domains of the receptor elicits activation and signaling cascades of downstream signal transducers (Lemmon and Schlessinger, 2010). Electron microscopy studies revealed that expression of dominant negative Rac1- and Rho1 constructs in the cyst cells of wildtype testes affected the cell membrane structure of the cyst cells. In wildtype testes, cyst cell membranes are wavy and undulate. Loss of Rac1 from the cyst cells results in smoother membranes than those seen in cyst cells of wildtype testes, while loss of Rho1 produced filopodia in the cyst cell membranes. These findings suggest that EGF signaling from the germline cells produces a differential of Rac- and Rho-activities across the cyst cells and, by organizing the actin cytoskeleton, leads to the directional growth of the cyst cells around the germline cells

and is required for the normal progression of the germline during spermatogenesis (Fig. 2.2C) (Sarkar *et al*., 2007; Schulz *et al*., 2002).

Testis from animals homozygous for a temperature-sensitive allele of *spi*, $spi^{77\text{-}20}$ (*spi/spi*, *spi*-mutants or *spi*-testes) displayed defects in cyst cells enclosing the germline at a restrictive temperature of 26.5°C. Removal of one copy of the small monomeric GTPase, *rac1*, from *spi*-mutant animals raised at an intermediate temperature exacerbated these germline enclosure defects resembling testes from *spi*-mutant animals raised at higher temperatures. Similarly, reducing the expression of the docking protein and guanidyl exchange factor, Vav, or Rac1 specifically from the cyst cells of *spi* mutants resulted in a strong enhancement of the germline enclosure defects. Along with binding studies of EGFR and Vav, the previous data suggested that Vav and Rac1 act in a signaling branch downstream of the EGFR pathway. Conversely, reducing the expression of the small monomeric GTPase, Rho1, from the cyst cells had the opposite effect on testes from *spi* mutant animals, rescuing germline enclosure even when the animals were raised at higher temperatures. This indicated that Rho1 acts in a pathway opposing EGFR for germline enclosure (Fig. 2.2C) (Sarkar *et al*., 2007). Along with Rho1 acting in cyst cells as an antagonist of EGF signaling, we have identified an additional antagonist to EGF signaling in cyst cells, the Ecdysone Receptor (EcR).

Ecdysone & the Ecdysone Receptor (EcR)

Developmental events that occur during the molting and metamorphosis of larva to adult insects are controlled by the rise and fall of ecdysteroids, the major molting hormones. Ecdysteroids are polyhydroxylated compounds that are synthesized from

dietary cholesterol in a multi-step biosynthetic process to produce 20-hydroxyecdysone (hereafter referred to as 20E). The Halloween genes (*phantom, disembodied, shadow* and *shade*) encode for cytochrome P450 enzymes that catalyze the final four hydroxylations of steroid precursors (Fig. 3.1) (reviewed in Rewitz *et al*., 2006). During larval development, pulses of ecdysone and other ecdysteroids are released from the prothoracic gland portion of the ring gland of insects and converted to 20E in peripheral tissues, where it is the most active form of the hormone (Gilbert *et al*., 1996). These pulses initiate the molting process as well the formation of the puparium and the metamorphosis of larval to pupal to adult tissue (Gilbert *et al*., 1997; Gilbert *et al*., 2002; Riddiford, 1993; Warren *et al*., 1988). The differing metamorphic responses of various target tissues are dramatically apparent in the transitioning from larva to adult where many larval tissues, such as the larval salivary glands, gut, and prothoracic glands undergo programmed cell death and degrade while cells in the imaginal discs proliferate to form the future appendages of the adult (Fristrom and Fristrom, 1993; Jiang *et al*., 1997; Riddiford *et al*., 2000). The prothoracic gland degenerates early in the pupal period and the source of adult ecdysteroids is not clear. However, 20E is still detected in both the adult male and female (Dai and Gilbert, 1991). Studies have shown that 20E signaling is required in the adult male for proper sleep-wake cycle, long-term memory, courtship, stress response and lifespan. Additionally, 20E signaling is known to be required for oogenesis, and wake-sleep cycle in the female (Garen *et al*., 1977; Morris and Spradling, 2012; Riddiford, 1993; reviewed in Schwedes and Carney, 2012).

In *Drosophila*, 20E facilitates these diverse tissue-specific responses through binding to EcR. EcR is a member of the nuclear steroid receptor superfamily that

contains DNA- and hormone-binding domains, indicating that it is a ligand-regulated transcription factor. EcR is capable of binding ecdysone weakly, but exhibits the highest degree of transcriptional activation when bound to 20E, and binds to ecdysone response element (EcRE) sequences of DNA (Koelle *et al*., 1991). It was later discovered that high-affinity binding of 20E to EcR and of EcR to EcREs was dependent on heterodimerization of EcR with a homologue of the human retinoid X receptor, Ultraspiracle (USP) (Yao *et al*., 1993). In order to initiate transcription of target genes, 20E must bind to the EcR/USP complex and dissociate from co-repressors, such as Alien and SMRTER. The complex then recruits co-activators, such as Hsp70/90, Taiman (Tai) and Trithorax-related (Trr), to form the active transcriptional complex (Arbeitman *et al*., 2000; Arbeitman and Hogness, 2000; Bai *et al*., 2000; Dressel *et al*., 1999; Sedkov *et al*., 2003; Tsai *et al*., 1999). Once bound to the active receptor complex, 20E directly induces expression of a small group of early regulatory genes, e.g. E74, E75 and at pupariation, Broad-Complex (Br-C). The protein products of these "early genes" repress their own transcription and also activate or induce the transcription of a larger set of downstream "early-late" and "late-late" genes, producing a genetic hierarchy of transcription (Fig. 3.2) (Ashburner & Richards, 1976). Here we investigate a role for EcR signaling in testes. We discovered that 20E signaling components are expressed in cyst cells of wildtype testes and that reduction in 20E signaling antagonizes germline differentiation defects found in *spi*-mutants. We propose that EGF and 20E signaling act antagonistically in guiding the progression of germline cells through early developmental stages.

Results

Components of ecdysone signaling are present in adult testes

Based on an expression study, components of the EcR signaling cascade are expressed in adult wildtype testes. To detect components of EcR signaling at the level of RNA transcription, we amplified transcripts from wildtype adult testes by RT-PCR. When primers specific for *USP, E74, Br-C* and the B1 isoform of EcR (*EcR-B1*) were used in PCR reactions with testes cDNA as a template, we obtained products of the expected sizes (Fig 3.3A). Sequencing of these products confirmed the identity of the amplified products to be *USP, E74, BR-C,* and *EcR-B1*.

In order to determine if components of EcR signaling are expressed in somatic cells of the testes, we used available antibodies specific for EcR, USP, Br-C, and the coactivator Taiman in immunofluorescence staining of adult testes in conjunction with antibodies against somatic cyst cells at different stages of development. The cyst cell marker Tj is specific for CySCs and early-stage cyst cells while the cyst cell marker Eya is expressed weakly in early-stage cyst cells and strongly in late-stage cyst cells (Fig. 2.1D). We discovered that EcR signaling components are indeed expressed in cyst cells. The co-activator Tai co-localized with both Tj-positive (Fig. 3.3B", arrowhead) and Eya-positive (Fig. 3.3C", arrowhead) cyst cells of wildtype testes. Antibodies against EcR (Fig. 3.3D', D") also detected signals in early-stage cyst cells (Fig. 3.3D", note co-localization with Tj, arrowhead) and expression in late stage cyst cells (Fig. 3.3D',D", arrows). Likewise, USP was expressed in the early-stage cyst cells (Fig. 3.3E", arrows) and strongly co-localized with Eya-positive cyst cells (Fig. 3.3 E", arrowhead).

Interestingly, we detected Br-C only in cyst cells at the apical tip, where it co-stained with Tj-positive cyst cells (Fig. 3.3F', F", arrowhead), but not in late-stage cyst cells.

In addition to detecting EcR signaling components via RT-PCR, we also detected transcripts of Halloween genes amplified from adult testes cDNA. Using gene-specific primers, we obtained and sequenced products that corresponded to *disembodied* (*dib*), *phantom* (*phm*), *shadow* (*sad*), *shade* (*shd*), and *spook* (*spo*) (Fig. 3.4). Based upon immunofluorescence results, it is apparent that cyst cells express the proteins necessary to respond to 20E. Not only are cyst cells capable of responding, it is apparent that early-stage, Tj-positive cyst cells are responding to 20E signaling based on their expression of Br-C. Additionally, RT-PCR results suggest that testes may also be a potential source of 20E in adult male *Drosophila*.

EcR signaling antagonizes EGF signaling in cyst cells

The germline cells are arranged in a spatio-temporal order along the apical to basal axis. When stained with the DNA dye 4', 6-diamidino-2-phenylindole (DAPI), early-stage germline cells (germ line stem cells, gonialblasts and spermatogonia) have small, bright DAPI-stained nuclei (Fig. 3.5A', arrowhead) and are found in the apical region of the testes (Fig. 3.5A). The spermatocytes have larger, less brightly stained nuclei (Fig. 3.5A' small arrows) and are located in the apical region basal to spermatogonia (Fig. 3.5A). The testis coil and base are filled with meiotic and post-meiotic germline cells. Bundles of spermatids are found at the basal end (Fig. 3.5A) and have sickle-shaped DAPI-stained nuclei (Fig. 3.5A" large arrows).

Animals homozygous for a conditional allele of the EGF ligand Spitz have testes that are short and filled with early-stage germline cells (Sarkar *et al*., 2007; Schulz *et al*., 2002). *spi*-mutant testes display phenotypes of differing severity dependent on the temperature at which animals are raised and maintained. When raised at 26.5°C, the majority of the testes (80-90%, n=100) from *spi*-mutant animals are tiny and filled with small, bright DAPI-stained nuclei (Fig. 3.5B, arrowheads).

Expression of RNAi constructs against EcR signaling components in cyst cells promotes the differentiation of the germline cells in *spi*-mutants. When raised at 26.5°C, *spi*-testes that have reduced EcR signaling are longer than *spi*-testes. For example, expression of RNAi constructs against conserved regions of EcR (*UAS-EcRi*) in the cyst cells of *spi*-testes via the UAS/Gal4-expression system partially restored spermatogenesis. The majority of *spi/spi; cyst cell-EcRi*-testes were longer and contained germline cells at all stages of spermatogenesis compared to *spi*-testes. We detected small, bright DAPI-stained nuclei at the apical tip and throughout the *spi/spi*; *cyst cell-EcRi*-testes (Fig. 3.5C, arrowheads) as well as larger, less bright DAPI-stained nuclei (Fig. 3.5C, small arrows) and sickle-shaped DAPI-stained nuclei (Fig. 3.5C, large arrow).

To quantify the effects of reduced EcR signaling on the germline cells in s*pi*-testes, control testes and *spi/spi; cyst cell-EcRi* -testes were classified into three phenotypic groups. Group I testes contain only small, bright DAPI-stained nuclei, indicative of early stage germline cells. Group II testes contain small, bright DAPI-stained nuclei and large, less brightly stained nuclei, indicative of early stage germline cells and spermatocytes. Group III testes contain small, brightly stained nuclei, large, less brightly stained nuclei, and sickle-shaped stained nuclei, indicating that all stages of

spermatogenesis are present in these testes (Fig 3.5C). After quantifying and categorizing testes from control and experimental animals, it was apparent that expression of RNAi constructs against *EcR, USP, E74* and *Br-C* in cyst cells of *spi*-testes was able to suppress the mutant phenotype and promote the differentiation of germline cells as evidenced by the presence of all three types of nuclear morphology revealed by DAPI staining (Fig. 3.6). A transgene of the dicer-2 enzyme (*UAS-Dicer*), which is involved in the RNAi silencing mechanism, was coexpressed along with RNAi constructs in order to enhance the silencing effects of RNAi (Dietzl *et al*., 2007).

Reduction of ecdysone promotes germline differentiation in spi-mutants

In an alternative approach to reduce 20E signaling, we generated *spi*-mutant flies that carried strong mutant alleles of genes involved in 20E biosynthesis in one copy. Embryos heterozygous for mutations in *shadow* displayed decreased ecdysteroid levels, suggesting that mutations in genes involved in ecdysteroid biosynthesis in one copy are able to appreciably reduce 20E levels (Warren *et al*, 2002). In these *spi*-animals with mutant alleles for Halloween genes, 20E titers are expected to be reduced in the entire animal and, thus, also in the testes. At 26.5°C, the majority of control *spi*-testes were small and contained only small DAPI-stained nuclei while the majority of *spi*-testes that were heterozygous for either sad^{1} (*shadow*), spo^{1} (*spook*), dib^{2}, or dib^{B17} (*disembodied*) alleles were longer and contained germ cells at all stages of development (Fig. 3.7) when raised at the same temperature. After quantifying testes according to their phenotype severity and comparing control and experimental testes, it was apparent that reduction in 20E signaling promoted the differentiation of germline cells and suppressed the *spi*

mutant phenotype. This indicates that the EcR signaling complex or downstream components of this pathway antagonize signaling via EGFR.

Discussion

Here we present expression studies of EcR signaling components and suppression data of *spi*-mutant testes, suggesting that signaling via EcR plays an instructive role in the somatic cyst lineage in *Drosophila* testes. Though we are currently gathering more data to develop a model as to the specific role of EcR signaling and how the role of EcR opposes EGF, I will discuss our present point of view in the following discussion.

Ecdysone signaling is present in adult testes

RT-PCR using adult testes cDNA and primers specific for components of 20E signaling revealed that RNA of EcRB1 and USP, as well as RNA of the early-response genes *E74*, *E75* and *Br-C* are expressed in testes. Though these data suggest that EcR signaling is present in testes, it does not provide information as to which cells are competent to or express the proteins involved in 20E signaling. In immunofluorescence experiments using available antibodies against EcR, USP, Taiman and Br-C, we showed that all of these proteins are present in cyst cells, suggesting that cyst cells are competent and 20E-responsive. While EcR, USP and Taiman colocalized to early and late cyst cell markers, Br-C was found to only localize to Tj-positive cyst cells. This indicates that either late Br-C expression is not within the detectable limits of the antibody, or more likely, that Br-C functions only in early-stage cyst cells. Similar to its role in timing of development, EcR could play a role in the temporal regulation of cyst cell development,

such that early-response genes are activated in early-stage cyst cells, which in turn activate late-response genes in later-stage cyst cells.

We also detected transcripts of the Halloween genes *phantom*, *disembodied*, *shadow*, and *shade* by RT-PCR, as well as *spook*, an additional enzyme that is believed to play a role in the 20E biosynthesis pathway, although its function is not yet clear (Fig 3.2) (Ono *et al*., 2006; reviewed in Rewitz *et al*., 2006). The Halloween enzyme *shade* is responsible for catalyzing the final hydroxylation in the 20E biosynthesis pathway and is expressed in peripheral tissues that are known to be active in response to 20E in numerous insects, suggesting that cell type(s) within the male gonad are 20E-responsive (Petryk *et al*., 2003). Although we detected *shade* RNA in testes, Petryk *et al*. (2003) were unable to do so using semi-quantitative RT-PCR using different primers.

Due to the presence of these 20E biosynthesis transcripts, we investigated the possibility that testes are ecdysteroidogenic organs. In preliminary experiments to detect whether *Drosophila* testis is an ecdysteroidogenic tissue, extracts of testes and incubated testes media were subjected to radioimmunoassay (RIA) to detect levels of ecdysone and 20E according to procedures in Sieglaff *et al*. (2005). These preliminary RIA experiments were unable to detect appreciable levels of ecdysone or 20E in either incubated media or extract samples (Brown, M.B. and Zoller, R.W., unpublished observations). Given the small number of potentially 20E producing cells in testes, it was not surprising that the amount of 20E in RIA samples were not at a detectable level.

Expression of RNAi constructs against EcR signaling components in cyst cells promotes the differentiation of the germline in spi-mutants

Signaling from the germline cells via the EGF ligand, Spi, to the cyst cells is required to induce and organize the growth of the cyst cells around the germline cells and is required for normal progression of the germline during spermatogenesis (Fig 2.2C) (Sarkar *et al*., 2007; Schulz *et al*., 2002). In *spi*-mutants raised at a restrictive temperature of 26.5 °C, the majority of testes show a very severe phenotype. These testes are small and filled with early stage germ cells (Group I). EcR, USP, Br-C and E74 depleted in cyst cells of *spi*-mutants statistically significantly increased the prevalence of testes that had a less severe (Group II or Group III) *spi*-mutant phenotype (Fig. 3.6). These results implicate 20E signaling functioning as an antagonist to EGF signals in cyst cells, possibly by repressing genes necessary for encasement and association with the developing germline, or by modifying the fate or developmental timing of the germline and cyst cells.

We noted that when compared to *spi/spi* animals, the control *spi*-mutant animals carrying only the Gal-4- or only the UAS- constructs (*spi, eya-gal4/spi; UAS-Dicer/+* or *spi, UAS-EcRi/spi; UAS-EcRi/+*, respectively) were also significantly different in their prevalence of Group II and Group III testes (Fig 3.6, grey asterisks). This opens up the possibility that the effects seen in experimental spi mutants (*spi, eya-gal4/spi; UAS-RNAi/UAS-Dicer*) are additive and not due to a decrease in 20E signaling in cyst cells. Although we cannot exclude this possibility, together with additional substantiating evidence provided in this thesis, we propose that 20E is indeed interacting with EGF signaling in testes. In a genetic modifier screen, Ward *et al*. (2003) discovered that 20E

signaling, acting through Br-C, interacts with the small GTPase Rho1 in an unknown fashion to influence imaginal leg disc development during morphogenesis. Since Sarkar *et al.* (2007) has shown that Rho1 antagonizes EGF signaling in testes, it is possible that Br-C interacts with EGF signaling in testes, through Rho1 or other cytoskeletal factors, to influence cell morphology of cyst cells and affects their ability to associate with germline cells in the adult. Alternatively, EcR signaling could regulate germline differentiation. Hudson (personal communication) recently proposed a model in which increased EGF signaling between germline cells and cyst cells provides a temporal signature regulating early steps of male gametogenesis. Loss of EcR could, for example, oppose EGF signaling by promoting germline differentiation. Thus, timing provided by EGF and the timing provided by EcR-signaling may have to be in balance for proper gametogenesis. Strong reduction of EGF may result in hyperactivity of EcR, explaining why reduction of EcR can suppress the phenotype of *spi*-testes. However, whether and how EGF and EcR cross-talk remains to be addressed.

Reduction of ecdysone promotes germline differentiation in spi-mutants

Using a different approach to reduce EcR signaling, we established *spi*-mutant stocks that carried strong alleles of genes involved in 20E biosynthesis. *spi*- mutants that were heterozygous for alleles of *disembodied, spook* and *shadow* were crossed to *spi*-mutants and the F_1 progeny raised and maintained for approximately ten days as adults at 26.5 °C. These animals are expected to have reduced ecdysteroid titers and, thus, reduced levels of EcR signaling in testes and throughout the entire animal. We compared testes from *spi*-mutants with *spi*-mutants heterozygous for *disembodied, spook* or *shadow* and

found that the prevalence of Group II and Group III testes were significantly lower in the simple *spi*-mutants versus *spi*-mutants carrying alleles for ecdysone biosynthesis genes (Fig. 3.7). This result coupled with our RNAi suppression data for genes involved in the ecdysone signaling pathway indicates that 20E signaling influences germline and cyst cell development and interacts with EGF signaling in the male gonad of *Drosophila melanogaster*.

We propose that EGF signaling most likely interacts with EcR via two different fashions (Fig. 3.8). In the first and most direct form, transcriptional products of Map kinase signaling could repress activation of EcR target genes or Map signaling components themselves could inhibit the transcription of EcR target genes. In *Drosophila,* both EcR and USP have multiple different protein kinase consensus serine/threonine and tyrosine phosphorylation sites, and the phosphorylation of which affects their function (Raush *et al.*, 2008; Song and Gilbert, 1998; Song *et al.*, 2003; Sridhara, 2012). Protein kinase C (PkC) phosphorylates USP and is is required for 20E-induced gene expression in larval salivary glands in *Drosophila* (Sun and Song, 2006). It is possible that the Map kinase protein, Raf, which phosphorylates downstream Map2 kinase, also phosphorylates an intermediate protein that is responsible for the inactivation of PkC. This de-activation of PkC could lead to de-activation of USP as well, an in turn, result in repression of EcR target genes.

In conclusion, we have shown that components of 20E biosynthesis and signaling are present in adult testes and that a reduction of 20E signaling both in cyst cells and also in the entire animal is able to promote the differentiation of the germline in *spi*-animals. Together, these results indicate that 20E signaling influences and antagonize EGF

signaling in the adult male gonad of *Drosophila melanogaster,* although exactly where and how this interaction occurs is yet to be determined. One possible explanation for this opposing nature of these two pathways is that EcR signaling may be up-regulated in EGF mutants, whereby over-activation of this pathway contributes to the enclosure and differentiation defects seen in *spi*-animals, and a reduction of 20E signaling in *spi*-animals relieves this over-active response. Future experiments will address this and other questions in regard to how and exactly where these two pathways crosstalk.

Experimental Procedures

Genetics & UAS-Gal4 expression studies

All fly stocks in this study were raised and maintained on standard cornmeal molasses agar at room temperature. Flies used in *spi*-mutant experiments were raised and maintained at 26.5 °C. Fly stocks used in this study include $spi^{77\text{-}20}$ (Sarkar *et al*., 2007) and the cyst cell driver *eyaA3*-Gal4 (Leatherman and DiNardo, 2008). The following flies carrying RNAi constructs, Halloween alleles and overexpression constructs were obtained from the Bloomington Stock Center*: UAS-EcR-*$RNAi^{97}$ [BL#9326]; *UAS-EcR-*$RNAi^{104}$ [BL#9327]; *UAS-USP-*$RNAi^{TRiP.HMS01620}$ [BL#36729]; *UAS-Eip74EF-*$RNAi^{TRiP.JF02515}$ [BL#29353]; *UAS-BrC-*$RNAi^{TRiP.JF02585}$ [BL#27272]; spo^{1}[BL#3276]; dib^{2}[BL#2776]; dib^{B17}[BL#5159]; sad^{1}[BL#2087]; *UAS-Dicer-2* [BL#24651]. p-values were calculated using Fisher's and chi-squared exact test.

Molecular techniques

Total RNA from testes was isolated and purified using TRIzol® Reagent with the PureLink® RNA Mini Kit as described by the manufacturer. First strand cDNA was generated using Oligo-dT and random hexamer primers provided in Invitrogen® SuperScript® III First-Strand Synthesis System for RT-PCR kit. Gene-specific PCR primers were designed using GeneRunner® oligo analysis. PCR reactions were run on 1% agarose gel to purify products, which were then excised and purified using the QIAquick® Gel Extraction Kit and used as a template for secondary PCR to further amplify products and then sequenced by an Applied Biosystems™ 3730xl 96-capillary DNA Analyzer for Sanger sequencing at the Georgia Genomics Facility. Forward and reverse primers for RT-PCR were designed from neighboring exons, ensuring that products obtained were from RNA and not genomic contamination. Additionally, when products obtained from RT-PCR were sequenced, we noted no intronic sequences present. The following primers were used in this study:

E74 *F*: 5'-CGCACACAGAAACTACACGAC-3'

E74 *R*: 5'-CGGATGAGAGTGCGGATGTGAG-3'

E75 *F*: 5'-GATGGCCAGCAGAACAAGTCGC-3'

E75 *R*: 5'-CTTGTAGGCCTCGTCCATCAGC-3'

Br-C *F*: 5'-GCAGAGGACACACACAGCCATC-3'

Br-C *R*: 5'-GTCGTTGGCATTGGCATTGTTG-3'

EcRA *F*: 5'-GCTCAGTTGCTAGGAAATGATG-3'

EcRB1 *F*: 5'-CGCGCAGACAACCATCATTCC-3'

EcRcom *R*: 5’-CTTCGCATCGCAGCTTTCGTTC-3’

USP *F*: 5’-CAGCAGTATCCGCCTAACCATC-3’

USP *R*: 5’-CGACTGTGGAATAGGGACCAA-3’

Phm *F*: 5’-GAGCACTTCCTAGCCGTTCGAG-3’

Phm *R*: 5’-CCTGAGGATGCGACCCGTAAAG-3’

Dib *F*: 5’-CTTACCTTTGGAGCTCGTCTGC-3’

Dib *R*: 5’-GATAGCTCGTCCTTGGCACTCG-3’

Sad *F*: 5’-GGAGAGTGGTGAAATACGAAGC-3’

Sad *R*: 5’-CTGCTGCAATGACCAAGTCTAC-3’

Shd *F*: 5’-GATGGGTCTGGAAGCTGTGTGC-3’

Shd *R*: 5’-GCACCGGGATCTCCAGTAACAG-3’

Spo *F*: 5’-CTACCAGCGACACCTGAACAAG-3’

Spo *R*: 5’-GGCTTTGTTCCTTTGACGGTTC-3’

Immunofluorescence & fluorescence microscopy

Testes were dissected in tissue isolation buffer (10 mM Tris-HCl, ph 6.8, 180 mM KCl) and fixed with 4% formaldehyde in 1x PBT (1x PBS with 0.1 % Triton X-100, pH 7.5; 1x PBS: 3 mM NaH_2PO_4, 7 mM Na_2HPO_4, 130 mM NaCl, pH 7.4) for 30 minutes at room temperature. Tissue was then washed at least 3x20 minutes in 1xPBT and incubated with primary antibodies overnight at 4 °C in PBT. After incubation, tissue was washed at least 3x20 minutes in 1x PBT, incubated with fluorophore-conjugated secondary antibodies in 1x PBT for 2 hours at room temperature, and washed again at least 3x20 minutes in 1xPBT. Tissue was then embedded in Vectashield (Vector Laboratories)

mounting medium with DAPI. The following hybridoma/monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242: mouse α-EcR (Aq10.2) [1:10], mouse α-Eya (10H6) [1:10], mouse α-BrC (25E9.D7) [1:10], mouse α-spectrin (3A9)[1:10]. Goat α-Vasa [1:500] and rabbit α-Armadillo [1:500] was obtained from Santa Cruz Biotechnology. Rabbit α -phosphorylated Histone-H3 [1:500] was obtained from Millipore. Mouse α-USP [1:200] – a gift from Dr. Rosa Barrio (used in Morris & Spradling, 2012); guinea pig α –TJ [1:500] – a gift from Dr. Dorothea Godt (used in Li *et al*., 2003); rabbit α-Taiman (JPAS)[1:2000] – a gift from Dr. Denise Montell (used in Bai *et al*., 2000). Alexa-488-, Cy3-, and Cy5-conjugated secondary antibodies were used at 1:500 (Invitrogen). Immunofluorescence was performed following standard procedures (Ashburner, 1989). Tissues were observed using a Zeiss Axiophot microscope in fluorescent microscopy. Images were taken with a CCD camera using an Apotome and Axiovision Rel Software.

Figure 3.1: *Synthesis of 20-hydroxyecdysone from dietary cholesterol.* The biosynthesis of 20-hydroxyecdysone from dietary cholesterol is carried out by multiple enzymes. The last four sequential hydroxylations are performed by the Halloween enzymes: *phantom*, *disembodied*, *shadow* and *shade*. The positions at which enzymes catalyze hydroxylations are indicated by color-coded arrows.

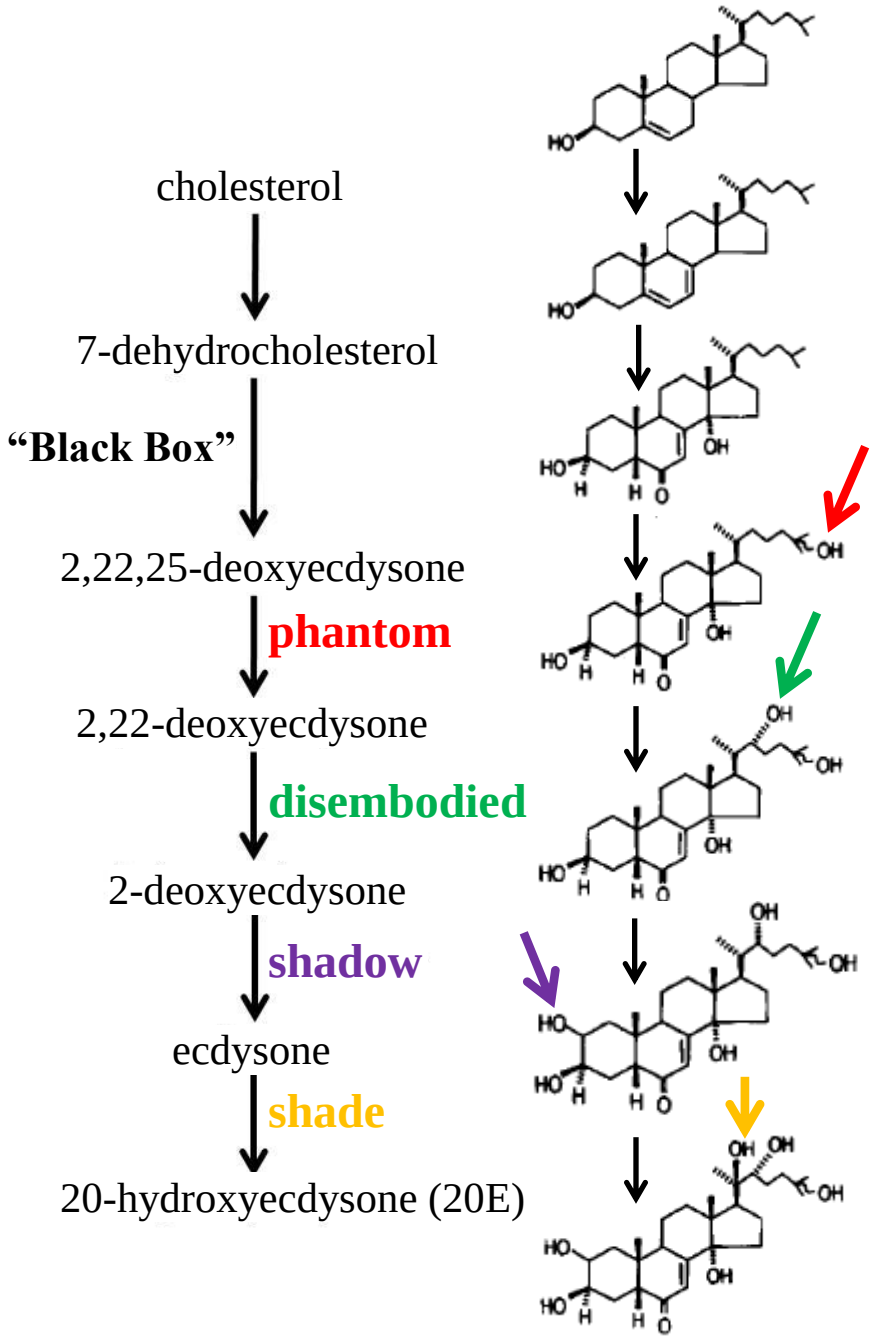

cholesterol
7-dehydrocholesterol
"Black Box"
2,22,25-deoxyecdysone
phantom
2,22-deoxyecdysone
disembodied
2-deoxyecdysone
shadow
ecdysone
shade
20-hydroxyecdysone (20E)

Figure 3.2: *The active EcR complex creates a transcriptional cascade.* 20E binds to the EcR/USP heterodimer, displacing corepressors and recruiting coactivators (CoA) to initiate early gene transcription. Activated EcR/USP complex also initiates transcription of early-late genes as well as inhibition of late gene transcription. The products of the early genes repress their own transcription and activate the early-late and late genes.

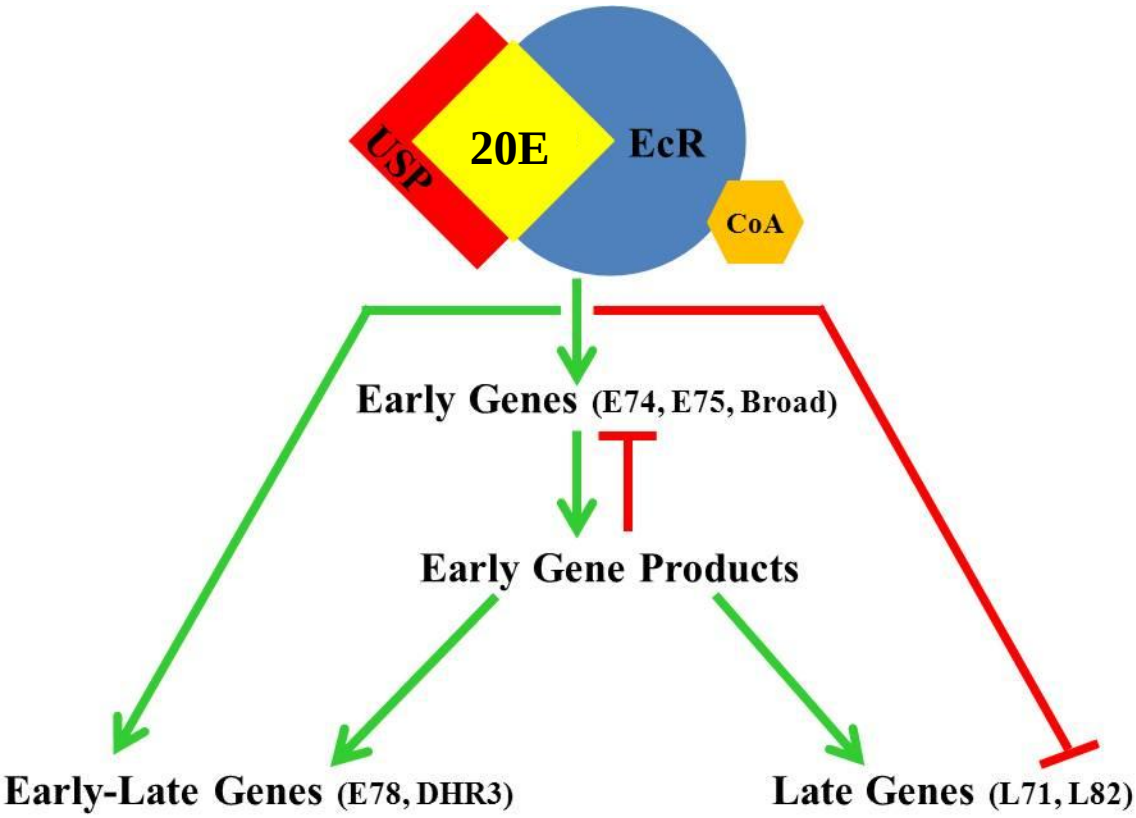

USP
20E
EcR
CoA
Early Genes (E74, E75, Broad)
Early Gene Products
Early-Late Genes (E78, DHR3)
Late Genes (L71, L82)

Figure 3.3: *Components of ecdysone signaling are expressed in cyst cells of wildtype testes.* (A) *EcR, USP* and downstream *EcR* signaling products are present in adult testes as revealed by RT-PCR. Amplicons were purified and sequenced to determine specificity of primes and product. Asterisk denotes band of anticipated size for *USP* that was sequenced. (B-F") Apical tips of wildtype testes labeled with antibodies as indicated. Tj is expressed in CySCs and early-stage cyst cells while Eya is expressed weakly in early stage cyst cells and strongly in late-stage cyst cells. EcR, USP, and Br-C proteins are present in cyst cells of adult testes. Asterisks denote the apical tips, arrowheads indicate early-stage cyst cells, and arrows indicate late-stage cyst cells. Scale bars: 30 μm.

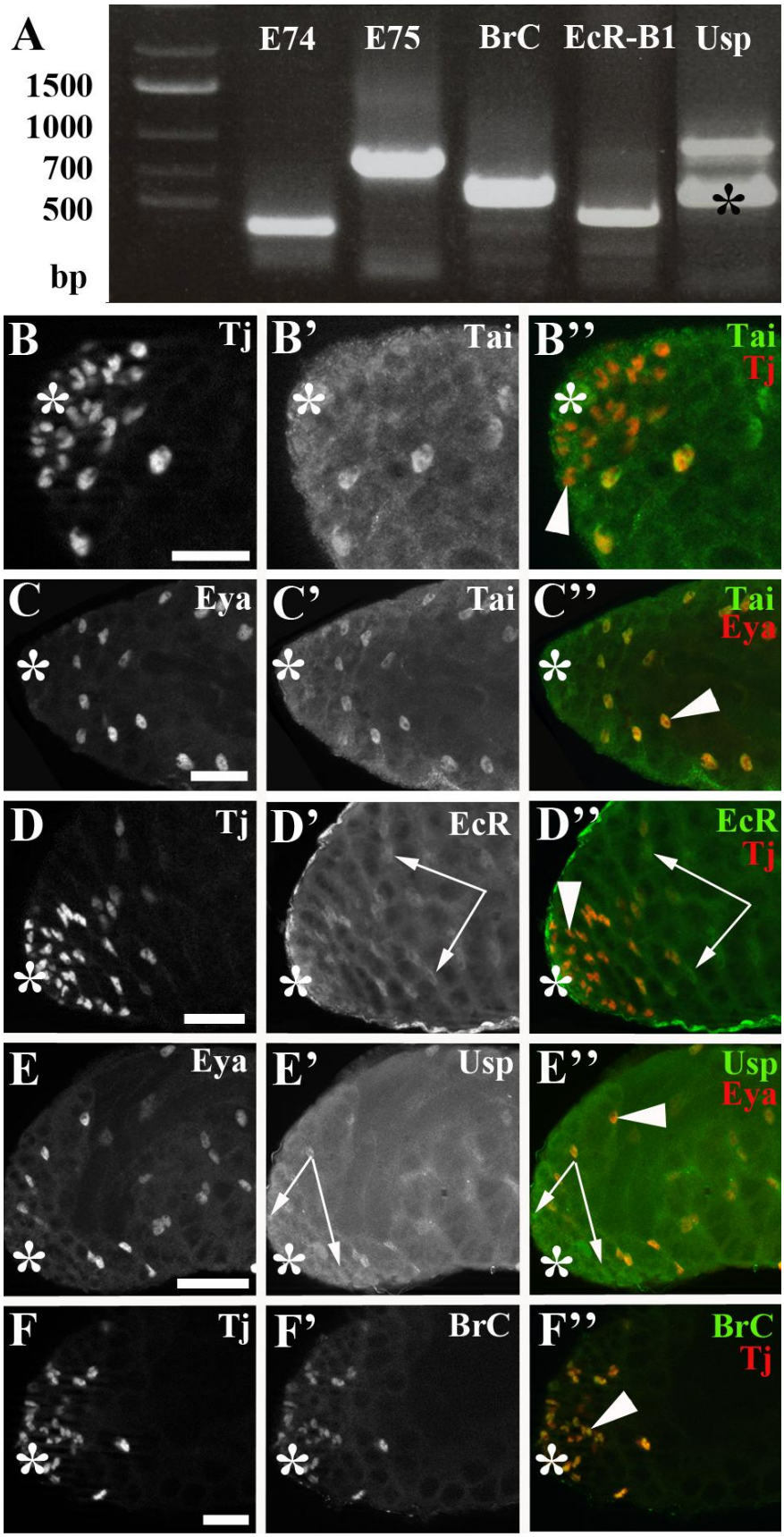

A
E74
E75
BrC
EcR-B1
Usp
1500
1000
700
500
bp
B
Tj
B'
Tai
B''
Tai
Tj
C
Eya
C'
Tai
C''
Tai
Eya
D
Tj
D'
EcR
D''
EcR
Tj
E
Eya
E'
Usp
E''
Usp
Eya
F
Tj
F'
BrC
F''
BrC
Tj

Figure 3.4: *Halloween gene transcripts are detected in adult testes.* cDNA generated from wildtype adult testes was used as a template with gene-specific primers for RT-PCR. Arrows indicate which band was excised, purified and sequenced to determine product identity. + control – EcR isoform A, USP - *Ultraspiracle*, dib – *disembodied*, phm – *phantom*, sad – *shadow*, shd – *shade*, spo – *spook*

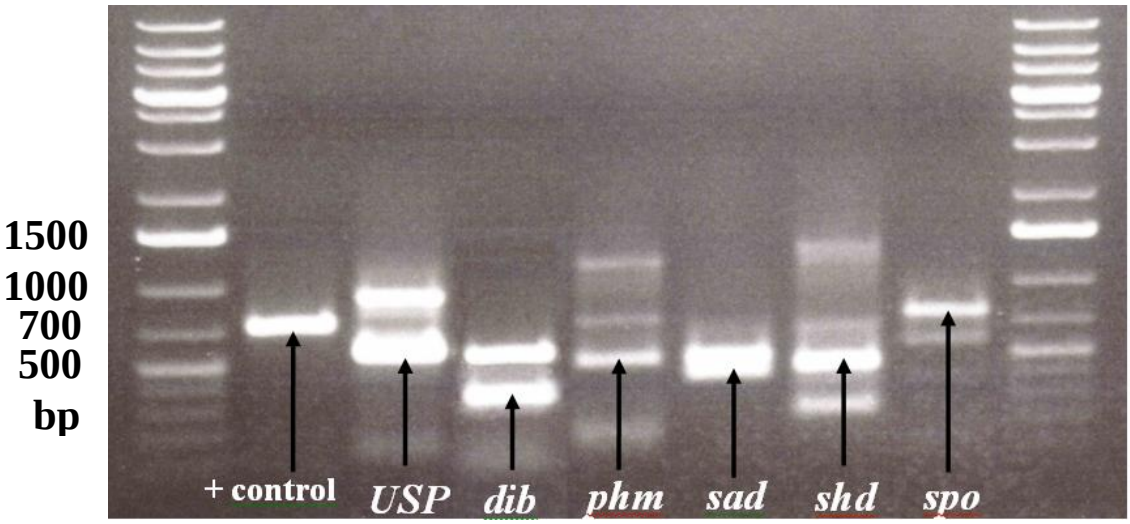

1500
1000
700
500
bp
+ control
USP
dib
phm
sad
shd
spo

Figure 3.5: *Wildtype & spi-testes are distinct in their nuclear germ cell stainings*. (A-C) DAPI-stained testes. (A) A testis from a wildtype animal contains all stage of germline cells, (A') shows a high magnification of the wildtype testis tip with the early-stage germline cells at the tip, followed by the larger spermatocytes basally, based on the size and intensity of the DAPI-stain. (A'') high magnification of the sperm bundle heads from the testis in (A). (B) Most *spi*-testes are small and contain only early-stage spermatogonia, but no later-stage germline cells when animals are raised at 26.5°C. (C) A *spi/spi*; *cyst cell-EcR-RNAi*-testes at 26.5°C attains the length of a wildtype testes but with clusters of early-stage germline cells in positions away from the apical tip. Asterisks denote the apical tips, arrowheads point to small, bright stained nuclei of early-stage germline cells (spermatogonia), small arrows point to large, less bright stained nuclei of later-stage germline cells (spermatocytes), and large arrows point to sickle-shaped nuclei (spermatids). Scale bars: 30 µm.

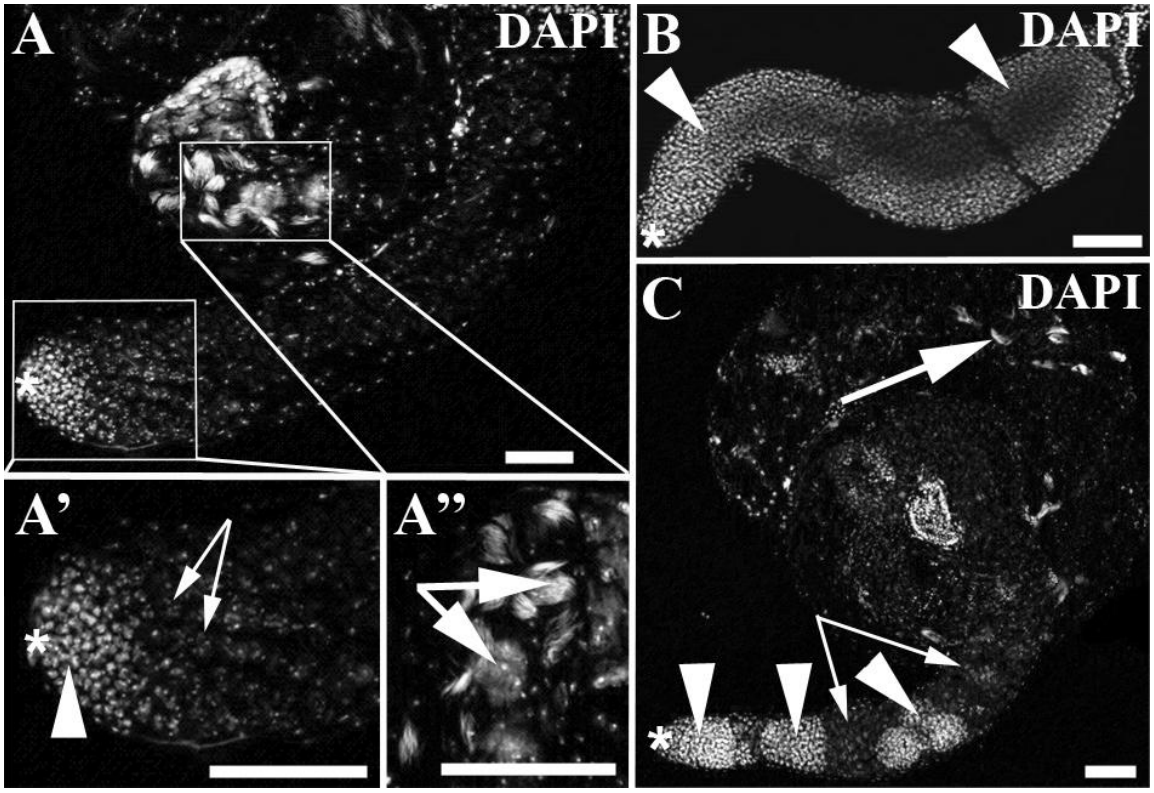

A
DAPI
B
DAPI
C
DAPI
A'
A''

Figure 3.6: *Reduction of EcR signaling via expression of RNAi constructs in cyst cells of spi-testes rescues the spi-mutant phenotype.* Light grey bars represent testes that are small and filled with only germ cells in the spermatogonia stage (Group I). Dark grey bars represent testes that contain germ cells in the spermatogonia and spermatocyte stage (Group II). Black bars represent testes that are larger and filled with germ cells in all three developmental stages: spermatogonia, spermatocytes and spermatids (Group III). Black (experimental) and grey (control) triple asterisks represent $p < 0.0001$. All p-values were calculated by comparing the number of Group II + Group III testes in *spi/spi* animals to the number of Group II + Group III testes in each other data set using two-tailed Fisher's & chi-squared exact test. BL#24651 was used to create *spi*-mutants that carry the UAS-Dicer construct.

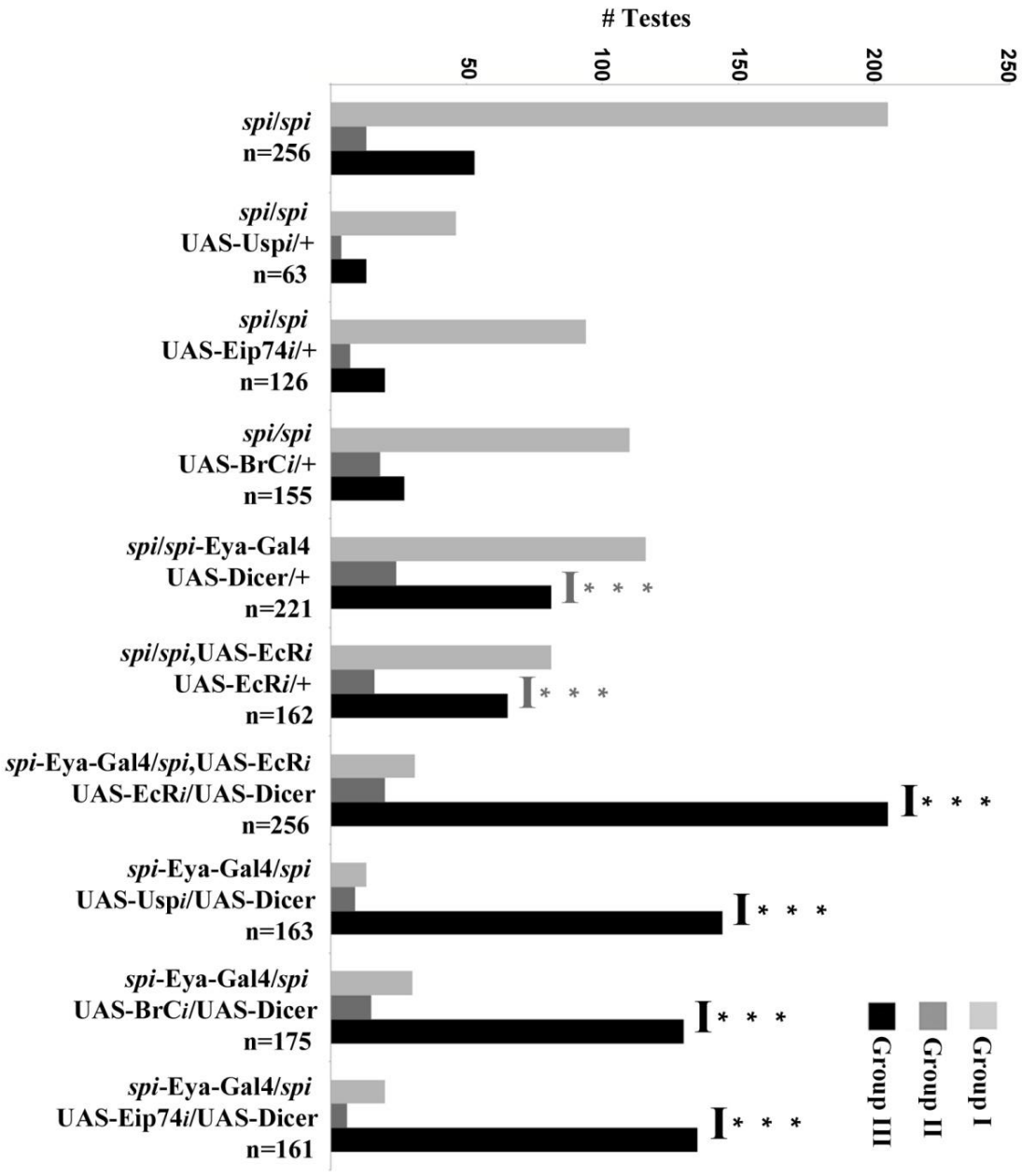

Testes
50
100
150
200
250
spi/spi
n=256
spi/spi
UAS-Uspi/+
n=63
spi/spi
UAS-Eip74i/+
n=126
spi/spi
UAS-BrCi/+
n=155
spi/spi-Eya-Gal4
UAS-Dicer/+
n=221
spi/spi,UAS-EcRi
UAS-EcRi/+
n=162
spi-Eya-Gal4/spi,UAS-EcRi
UAS-EcRi/UAS-Dicer
n=256
spi-Eya-Gal4/spi
UAS-Uspi/UAS-Dicer
n=163
spi-Eya-Gal4/spi
UAS-BrCi/UAS-Dicer
n=175
spi-Eya-Gal4/spi
UAS-Eip74i/UAS-Dicer
n=161
* * *
Group III
Group II
Group I

Figure 3.7: *Reduction in ecdysone in the entire animal rescues the spi phenotype. spi/spi* mutant animals heterozygous for strong alleles of genes involved in ecdysone biosynthesis have more testes that contain germline cells at all stages of development compared to control *spi*-testes when raised at 26.5°C. Black triple asterisks represent $p < 0.0001$. All p-values were calculated by comparing the number of Group II + Group III testes in *spi/spi* animals to the number of Group II + Group III testes in each other data set using two-tailed Fisher's & chi-squared exact test.

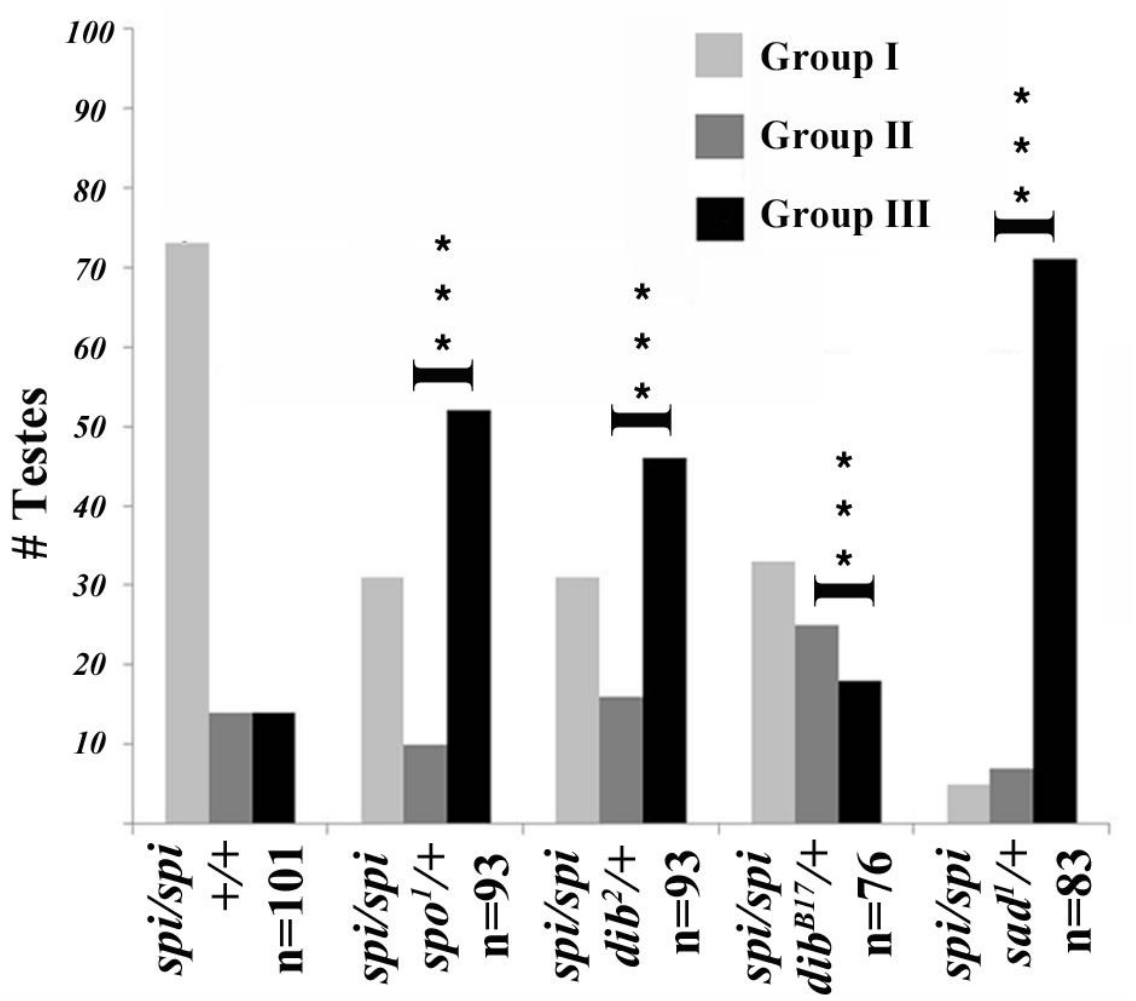
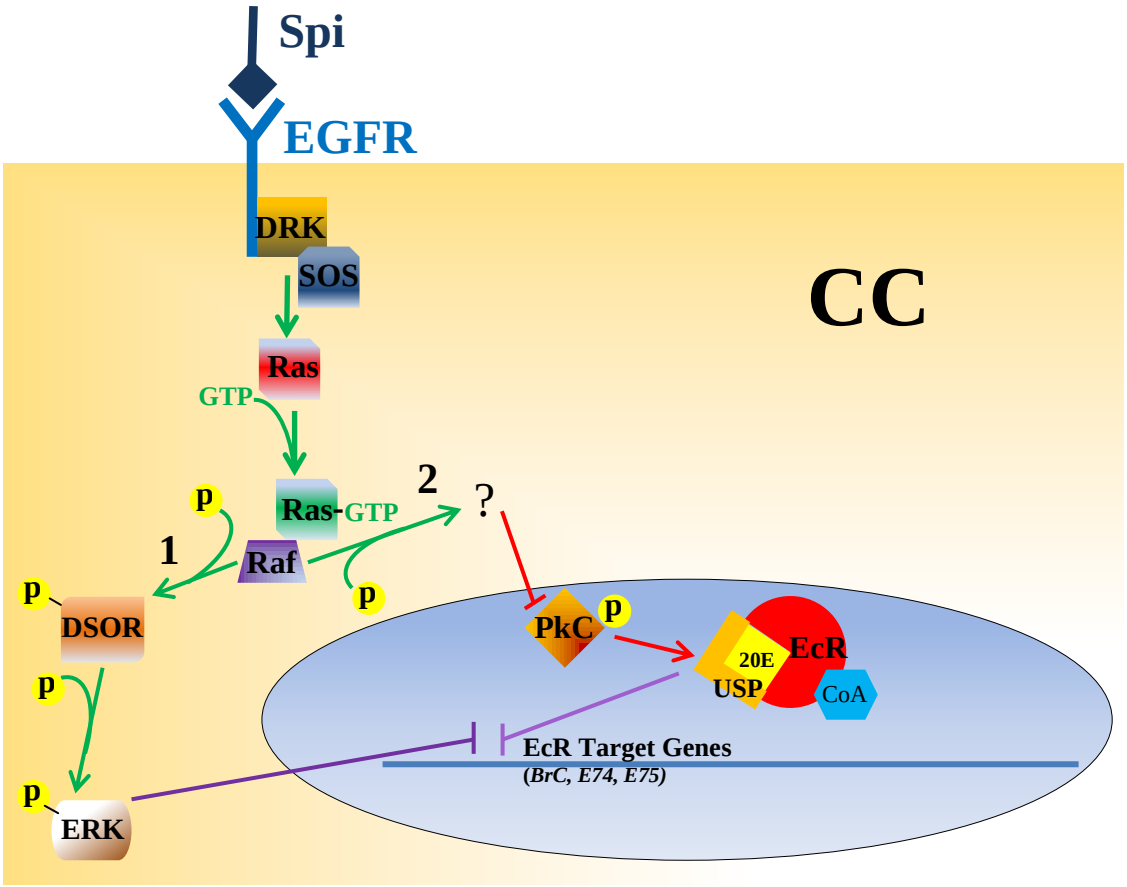

Group I
Group II
Group III
Testes
100
90
80
70
60
50
40
30
20
10
spi/spi +/+ n=101
spi/spi spo1/+ n=93
spi/spi dib2/+ n=93
spi/spi dibB17/+ n=76
spi/spi sadt/+ n=83
*
*
*

Figure 3.8: *Cartoon depicting DRK branch of EGF signaling pathway, regulation of transcription via EcR/USP heterodimer, and how the two may crosstalk.* Inhibition of EcR signaling may be accomplished by 1) transcriptional inhibition of EcR target genes or intermediates by Map kinase [indicated by purple lines] or 2) deactivation of protein kinase C, which phosphorylates USP to activate gene transcription, thereby inhibiting the transcription of EcR target genes [indicated by red lines]. CC- cyst cell, DRK – Downstream Receptor Kinase (docking protein), SOS – Son of Sevenless (Guanidyl exchange factor), Ras (GTPase), Raf (polehole, MapKKK), DSOR (Downstream of Raf, MapKK), RL (Rolled, MapK), PkC (Protein kinase C), CoA – Co-activators.


Spi
EGFR
DRK
SOS
CC
Ras
GTP
Ras-GTP
2
?
p
1
Raf
p
p
DSOR
p
p
ERK
PkC
p
20E
EcR
USP
CoA
EcR Target Genes
(BrC, E74, E75)

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CHAPTER 4

CONCLUSION

In this thesis, I have discussed the role of cyst cells and their requirement for the proper development of the germline during spermatogenesis in *Drosophila melanogaster*. In Chapter 2, I presented an up-to-date review of the cyst stem cell lineage in *Drosophila* on how these cells are specified, how their division is regulated, the process by which they enclose germline cells and co-differentiate, and the mechanisms by which they regulate the different developmental aspects of the encased germline. In Chapter 3, I presented data that implicate the 20E signaling pathway in antagonizing the EGF pathway in the enclosure and differentiation of the germline by cyst cells. From these data, it appears that 20E signaling opposes EGF signaling in regulating the development of cysts into late stages. In the model proposed, EcR signaling may be over-active in EGF mutants, whereby excess stimulation of this pathway contributes to the enclosure and differentiation defects seen in *spi*-animals, and a reduction of EcR signaling in *spi*-animals relieves this hyper-active response. I discuss the possibilities that these two pathways interact either at the post-translational or transcriptional level.

Future experiments will address this and other questions in regard to how and exactly where these two pathways crosstalk. In addition to showing that cyst cells are responding to 20E signaling and that there is cross-talk between this pathway and EGF signaling, we have also provided evidence for testes being a potential source of ecdysone or 20E in the adult male animal. Current experiments on the role of EcR in wildtype

testes should reveal its precise mode of action and the role that 20E has in influencing gametogenesis in the male gonad of *Drosophila.*