JAK/STAT SIGNALING REGULATES GAMETOGENESIS AND AGE-RELATED REPRODUCTIVE MAINTENANCE

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JAK/STAT SIGNALING REGULATES GAMETOGENESIS AND AGE-RELATED REPRODUCTIVE MAINTENANCE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
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Lexington, KY

Director: Dr. Douglas Harrison, Professor of Biology
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2018

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ABSTRACT OF DISSERTATION

JAK/STAT SIGNALING REGULATES GAMETOGENESIS AND AGE-RELATED REPRODUCTIVE MAINTENANCE

Cell signaling is central to integration of internal and external cues that regulate development and homeostasis. Most development is thought of as pre-adult, but limited developmental processes occur in adults. Gametogenesis incorporates elements of both these facets, with a distinct developmental plan for gamete synthesis which is regulated by integration of homeostatic inputs such as nutrient status, and environmental cues. Signaling pathways integrate and transduce information from these cues to evoke a response. A decline in homeostasis and subsequent cues occurs over time, in the case of reproductive tissues leading to a progressive loss of fertility. The Janus Kinase and Signal Transducer and Activator of Transcription or Jak/Stat signaling pathway is conserved between vertebrates and invertebrates and is necessary for numerous functions needed to maintain organism and reproductive homeostasis, as well as contributing to various developmental events. The pathway in the fruit fly *Drosophila melanogaster*, is composed of a single receptor, Domeless, one Janus kinase, Hopscotch, one known effector, Stat92E, and the Unpaired family of ligands consisting of Upd, Upd2, and Upd3. Jak/Stat signaling is highly pleiotropic in both sexes with involvement in homeostasis and reproduction, making it an ideal model for studying the role of signaling in reproductive aging. Reduction of pathway activity in females results in a higher proportion of unfertilized eggs, which increases with age, and in males leads to a premature onset of infertility. Central to both is integration through cell signaling to evoke an appropriate response. This dissertation explores two of the requirements for Jak/Stat signaling: the pleiotropic requirement for Jak/Stat activity during oogenesis and male reproductive maintenance.

Jak/Stat functions from the beginning of oogenesis, in the stem cell niche. From there it participates in multiple functions including specification of a subset of somatic cells called the border cells through the polar cells, a pair of cells at either pole of the egg. Pathway stimulation in the border cells drives their migration with the polar cells to the oocyte boundary, where the polar cells each form an extension in a coordinated manner into the micropyle, the means for sperm entrance during fertilization. Loss of Jak/Stat activity in the border cells prevents border cell migration. While border cell
migration has been well studied, polar cell involvement after completion of border cell migration is less well known. To investigate the requirements for polar cell activity and Jak/Stat activity after the completion of border cell migration, we reduced Jak/Stat signaling in the polar cells which, while having no effect on border cell migration, results in blocked micropyles due to loss of coordination of extensions during their outgrowth. Reduced function in the polar cells did not significantly affect expression of adhesion molecules. But, the loss of Stat92E is phenocopied by loss of DE-cadherin. Hence, these results indicate a previously unknown autocrine requirement for Jak/Stat activity in the polar cells.

The testes also have a continuous requirement for Jak/Stat activity for stem cell maintenance and differentiation of the germline into mature sperm. Reproductive maintenance not only requires sustained production of gametes, but reproductive tissues are also subject to deterioration of homeostatic functions that contribute to organismal aging. Males from thirty-nine lines of the Drosophila Genetic Reference Panel (DGRP), a panel of inbred, fully sequenced lines, were screened for age at infertility. Data were used to perform a genome-wide association study (GWAS) to identify the genetic architecture of reproductive aging. Candidate variants associated with cell signaling regulators, genes with functions in maintaining cell homeostasis, and organism behavior were uncovered. Notably, several SNPs fell in and near Ptp61F, a negative regulator of Jak/Stat activity. While variants in the primary components of the Jak/Stat pathway were not identified, the general classes of candidate loci functions reflect the requirements for homeostasis, metabolism, and development that have been shown by other studies examining the genetics of aging and fecundity. Thus, we show that Jak/Stat has an amazing amount of pleiotropy that encompasses both the real-time functions of fertility and the time related process of aging.

KEYWORDS: Jak/Stat pathway, Drosophila, micropyle, polar cells, senescence, fertility

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Chapter One: Background

The fecundity of an organism is reflected in its ability to produce offspring. Numerous genetic and environmental factors define the degree of fecundity of an individual. If environmental limitations are removed, there are two major determinants: the baseline production of gametes capable of successful fertilization and development, and the ability to maintain fertility for a significant period over the lifetime of an organism. Gametogenesis consists of a set of defined and predictable events to create a mature gamete. This process is repeated on a continuous basis within an animal that is aging. Hence, the direct impacts of the aging gonad and the indirect effects of the aging organism influence the production of gametes.

Gamete quality and rate of production decline with age, contributing to a loss of fertility. Maintenance of fertility requires cellular and developmental homeostasis in reproductive tissues, but is subject to age-related decline. Loss of germline stem cell homeostasis due to accumulation of misfolded proteins, increased DNA damage due to defective DNA repair mechanisms, and oxidative stress are cellular mechanisms that are factors in the inability to maintain sufficient production of quality gametes and organism viability (Höhn et al., 2017; Tilly and Sinclair, 2013; Wang and Jones, 2011; Wang et al., 2014a). Signaling pathways regulate the response of these processes to nutrient and environmental inputs to maintain homeostasis and gametogenesis (Hsin and Kenyon, 1999; Kenyon, 2011; Pletcher, 2009). Signaling is, in turn, subject to regulation. Given the variety of processes that are required to maintain fertility, the genetic architecture of reproductive senescence is quite complex.

In this dissertation, I describe efforts to address the genetic regulation of aspects of fecundity. In the first project, I examine the genetic program required during oogenesis to construct a functional micropyle, the egg structure through which the sperm enters. In
the second project, I identify genes that contribute to the maintenance of fertility in males. Although these projects are at very different levels of investigation, they both emanate from preliminary observations that each is strongly influenced by activity of the Janus kinase/Signal transducer and activator of transcription (Jak/Stat) pathway. This evolutionarily conserved signaling cascade has functions in development, immunity, cellular homeostasis, and gametogenesis.

Gamete Structure

Reproduction in metazoans requires the production and fusion of viable gametes. Sperm and egg each contribute a haploid copy of parental genetic information, while the egg also supports embryonic development by providing nutrients and protection from the environment. This requires structural specializations that permit sperm and egg to complete each step of fertilization: gamete location, binding of sperm to egg, fusion of sperm and egg membranes, and delivery of sperm genetic material to the egg (Gilbert, 2010). Sperm structure is specialized to allow it to travel and interact with the egg.

While sperm vary in size among species, structure is generally conserved with mature sperm consisting of three regions: head, midpiece, and tail (Figure 1.1). Covering the anterior of the nucleus is the acrosomal vesicle which contains enzymes required to penetrate the outer layers of the egg. Next is the mid-piece consisting of tubulin, dynein and mitochondria to form the axoneme which serves as a “motor” to drive the flagellum forming the tail of the sperm (Gilbert, 2010).

Spermatogenesis in mammals and flies begins with mitotic divisions of a germline stem cell. The daughter cells then undergo a series of meiotic divisions, with the daughter cells remaining connected by cytoplasmic bridges. During spermatogenesis, the differentiating germline cells are in close association with somatic cells. In humans these are the Sertoli cells, somatic cells that secrete factors that
support the differentiating spermatids. *Drosophila* spermatogenesis has a similar cell type, the cyst cells, somatic support cells that surround the developing spermatids (de Cuevas and Matunis, 2011; Fuller, 1993; Gilbert, 2010; Zoller and Schulz, 2012). In both flies and mammals, each haploid germline cell undergoes a complex series of morphological changes, including nuclear condensation to form a compressed head, elongation to form the tail, and individualization in which the connections between each sperm are broken down to form individual spermatids (Figure 1.1) (Fabian and Brill, 2012; Gilbert, 2010).

While sperm morphology is largely conserved between organisms, egg morphology is much more variable, reflecting species life history. All eggs, apart from supplying half the genetic material to the future embryo, must also supply nutrients in the form of yolk, proteins, and mRNA to the embryo until transcriptional machinery can take over while protecting the embryo usually through the production of a hardened layer or eggshell. The presence of an eggshell is problematic for fertilization as it forms an impenetrable barrier that prevents sperm directly binding to and fusing with the egg. A solution to this problem utilized by many insects and some fish is the creation of a sperm entry portal, or micropyle, to permit fertilization of the egg. Formation of the micropyle and its functions during fertilization will be discussed here and in the following chapter.

**Egg/Sperm Interactions**

The first step of fertilization requires that the sperm locate the egg. Animals in which fertilization occurs outside the female must contend with the problem of location of the egg by the sperm in a species-specific manner. In external fertilizers, such as the sea urchin, the outer most layer of the egg, a carbohydrate “jelly” layer contains chemotactic agents such as speract or resact that act in a species-specific manner to attract sperm (Gilbert, 2010; Oulhen et al., 2013; Wessel and Wong, 2009). Less is known about
location mechanisms in internal fertilizers such as mammals, birds, and insects. It is known, however, that these species do employ chemotaxis, rheotaxis, and thermotaxis during sperm-egg location. (Bahat et al., 2012; Cohen-Dayag et al., 1995; Pérez-Cerezales et al., 2015).

Once the sperm and egg have located each other, the sperm must attach to the egg and penetrate the outermost layers for fertilization to proceed. Much of what is known about events that occur during fertilization have been discovered in sea urchins due to fertilization occurring externally. In sea urchins, upon recognizing the egg, sperm bind to the jelly layer and digest their way through it to the vitelline layer, a non-cellular layer resembling the extra-cellular matrix. Sperm bind to the vitelline layer via a bindin receptor on the sperm surface (Gilbert, 2010; Wessel and Wong, 2009). Once a sperm has successfully bound to the egg, the sperm and egg membranes fuse and sperm contents are released into the egg.

Unlike the sea urchin, mammalian fertilization is internal, making the study of fertilization more difficult. The zona pellucida of mammalian eggs is analogous to the vitelline layer in sea urchins and is non-cellular in nature. In humans, it is composed of four glycoproteins termed ZP1, ZP2, ZP3, and ZP4. During mammalian fertilization sperm recognize and bind to ZP-2. (Gilbert, 2011). Mammalian sperm are believed to interact with the egg via expression of the protein Izumo that is located on the sperm surface after the acrosome reaction occurs and interacts with Juno, a protein found on the egg surface (Bianchi et al., 2014).

In *Drosophila*, fertilization is internal and occurs as the egg moves toward the uterus. However, an eggshell is formed during oogenesis and creates a physical barrier that prevents sperm from directly binding to and fusing with the egg. A specialized structure termed the micropyle is part of the eggshell and provides an entry point for fertilization. Located on the egg surface around and inside the micropyle lumen are
fucose, mannose, and β-N-acetylglucosamine residues. Sperm possess the complementary enzymes on their surface that recognize and interact with these residues, cleaving them as they bind and move toward the egg (Intra, 2006; Intra et al., 2015). The importance of these residues in fertilization is demonstrated by the male sterile casanova (csn) mutant. csn codes for an β-N-acetylglucosaminidase. In mutants, the sperm fails to bind to the residue, resulting in reduced fertility (Perotti et al., 2001).

In contrast to other organisms, Drosophila sperm puncture the oocyte membrane during fertilization rather than fuse with the egg, and the entire sperm including the tail enters the oocyte. Because there is no membrane fusion of the sperm head and oocyte membranes, once inside the oocyte the sperm membrane must disintegrate to release the male pronucleus. An active participant in this process is coded by sneaky (snky) a transmembrane protein that is localized to the sperm acrosome. GFP tagged forms of the protein show that upon fertilization, the acrosome is retained within the egg cytoplasm and does not disintegrate (Wilson et al., 2006).

**Chorion Formation**

Insect eggs occur in a wide array of forms that reflect insect phylogeny and life history, but they all act as a protective structure to support embryonic development. Eggs can be deposited onto a surface, or dispersed by a variety of mechanisms including attachment to hair or fur. These tasks are accomplished by the formation of specialized secreted structures during chorion development. Presence, morphology, and number of structures such as micropyles, dorsal appendages, or attachment mechanisms on the eggshell surface are a useful tool for insect classification (Hinton, 1981; Kristensen, 1981; Niepielko et al., 2014). The process of chorion development and secretion of specialized chorion structures, with variations, is shared among insect
species.

The insect eggshell is a multi-layer, proteinaceous structure deposited in spatial and temporal manner by subsets of specialized follicle cells during choriogenesis (Figure 1.2). Typically, the vitelline membrane is the first and innermost layer to form during Stage 8 (Figure 1.3) (Fakhouri et al., 2006; Manogaran and Waring, 2004; Mauzy-Melitz and Waring, 2003; Spradling, 1993). Over this are a lipid layer, the inner chorion layer, a protein-rich endochorion, and the waxy exochorion, all of which are successively secreted by the follicular epithelium as oogenesis progresses (Pascucci et al., 1996; Spradling, 1993; Waring and Mahowald, 1979). The endochorion consists of many layers with chorion bridges linking the upper and lower layers, thereby providing stability (Figure 1.2) (Pascucci et al., 1996; Zarani and Margaritis, 1986). Upon completion of eggshell deposition, there is a hardening step.

The egg shell serves a protective role by acting as a physical barrier to the environment, however it also interferes with the passage of oxygen and sperm. Therefore, there are a variety of structural specializations that account for this including the operculum, an exit point for the hatching larva, respiratory appendages, such as the dorsal appendages in *Drosophila*, attachment structures that permit dispersal, and micropyles that permit sperm entry for fertilization of the oocyte. The types and morphologies of these structures vary widely between groups. Chorionic structures on the surface of the egg have long been used as criteria for classification (Hinton, 1981; Kristensen, 1981).

Found as part of both insect and, interestingly, teleost fish eggs, micropyle morphology varies widely and can take the form of a protuberance with one to multiple pores, or can be simply multiple pores on the surface of the egg (Hinton, 1981; King, 1970). Variation in micropyle location on the egg varies as well, with micropyles forming at either the anterior or posterior of the egg. Some insects such as the termite
Reticulitermes speratus, regulation of micropyle development is employed as a switch between sexual and parthenogenic reproduction. The micropyle is located at the egg posterior, and takes the form of a variable number of pores. In older females, the micropyles do not form, resulting in parthenogenesis and production of new queens (Yashiro and Matsuura, 2014).

While number and morphology of these structures can vary among insects, underlying mechanisms of their formation appear to be conserved (Wenzel et al., 1990). Specifically, there is a rearrangement or migration of specialized follicular epithelial cells, while a subset of these cells forms cellular extensions toward the oocyte. Regardless of whether the micropyle is a pore or protrusion, the formation of at least one cellular extension by a subset of specialized follicle cells to form the micropyle lumen is required. Additionally, some micropyle developmental events require that a subset of follicle cells migrate to the oocyte (Arukwe and Goksøyr, 2003; Marlow and Mullins, 2008; Montell et al., 1992; Zarani and Margaritis, 1986; Zarani and Margaritis, 1991a).

Micropyle Formation in Drosophila

Drosophila is an excellent tool for dissecting the genetic pathways required for eggshell formation. Surrounding the developing follicle are a layer of somatic epithelial cells. Distinct subsets of these are required for the secretion and sculpting of specialized eggshell structures. Central to micropyle development are the polar cells, a specialized pair of somatic cells. The anterior polar cells play an important role in oogenesis at Stage 6-7 (Figure 1.3), acting as an organizer to specify follicle cell fates through secretion of the morphogen Upd and activating Jak/Stat signaling in the surrounding follicular epithelial cells (McGregor et al., 2002; Xi et al., 2003). The highest signaling activity is in a group of 4-6 cells closest to the polar cells, which adopt a border cell fate at Stage 8 of oogenesis (Starz-Gaiano et al., 2009).
At Stage 9 of oogenesis the border cells undergo an epithelial to mesenchymal transition, delaminate from the follicular epithelium and migrate posteriorly toward the oocyte. They take the non-motile polar cells with them, marking a loss of symmetry in the egg chamber and the beginning of micropyle formation. The polar cells, which are non-migratory and sit at the center of the cell cluster, initiate and maintain border cell migration through secretion of Upd, activating the Jak/Stat pathway in the border cells (Beccari et al., 2002; Montell et al., 1992; Silver et al., 2005). Migration ends when the border cell cluster reaches the nurse cell/oocyte boundary by the end of Stage 10. There the border cells, along with a subset of follicular epithelial cells, secrete and shape chorion to form the micropyle structure (Zarani and Margaritis, 1986). The timing of migration is critical. Delayed border cell migration leads to an increased incidence of defective micropyles (Montell et al., 1992; Silver et al., 2005). Likewise, if the border cells are ablated, the micropyle forms but is blocked (Montell et al., 1992).

As with many insects, follicle cell projections are associated with micropyle development and creating the lumen in Drosophila oogenesis (Edwards et al., 1997; Hinton, 1981; King, 1970; Zarani and Margaritis, 1986). The polar cells were later shown to form extensions associated with the micropyle, but the specific function of the extensions was not investigated (Edwards et al., 1997). Given what is known about micropyle formation this observation suggests the polar cells are involved in creating the micropyle lumen. Little is known about the signaling pathways and molecular mechanisms underlying the relocation and development of the follicle cell extensions forming the pores in most insects. Given the available genetic tools, and the large amount known about oogenesis, the best model for understanding the signaling and cytoskeletal rearrangements driving formation of the micropyle is Drosophila melanogaster. My work to characterize polar cell extension dynamics and the cell signaling networks that regulate it are described in Chapter 2.
Reproductive Senescence

While gamete production is essential to fertility, the length of time an individual can remain fecund is also a vital contribution for iteroparous species. However, over time individuals are subject to a progressive decline in fitness, and the rate at which individuals senesce varies within a population. This is driven in part by a loss of cellular homeostasis due to the cumulative effects of degeneration of such processes as DNA repair mechanisms, elimination of defective proteins, oxidative damage, and alterations in cell signaling. Subsequently, as cell processes degenerate there is a decline in cell proliferation and differentiation, and tissue and organ function. Changes at the cellular level are affected, in part, by the environment, behaviors, and neurological factors that contribute to the ability to sense nutrients or a potential mate. Underlying all of this is genetic variability that regulates how efficiently the cellular, behavioral, sensory, and environmental contributions interact.

Trade-Off Between Reproduction and Lifespan

There is much work showing that reproduction and lifespan are linked, and the nature of this relationship is beginning to be understood. Reproductive tissues exhibit homeostatic populations of stem cells, developmental processes in the form of gametogenesis, and sensitivity to other factors, such as behavioral, environmental, and sensory inputs, that affect aging on a whole organism level. Therefore, these tissues may serve as a model for understanding senescence overall. Thus, the question regarding what drives maintenance of fertility over the reproductive lifetime of an organism emerges. Female reproductive lifespans, particularly human female fertility, are well-studied. Human females exhibit a post-reproductive phase, where fertility has significantly decreased, yet the organism is viable. Male fertility also decreases with
age, however less is known about what is driving this decline (Thompson et al., 2007; Tilly and Sinclair, 2013; Wiener-Megnazi et al., 2012).

Reproduction requires a significant allocation of resources that must be invested into generation of gametes, courtship, and competition for a mate. For example, male members of the genus *Drosophila* produce some of the largest documented sperm of any animal. *Drosophila bifurca* males produce massive sperm that approach 6cm in length and require testes that occupy 11% of their body mass to produce and store them (Bjork et al., 2007). However, producing such large gametes comes at a cost. There is a tendency that the larger the sperm, the fewer sperm are produced (Pitnick, 1996) and sexual maturity occurs at a later age (Pitnick et al., 1995). Because of the enormous cost of reproduction, allocation of resources often favors maintenance of reproductive processes over that of somatic tissues.

The disposable soma theory of aging considers the trade-off between the soma and germline. This theory holds that resources are insufficient to adequately sustain both the soma and germline, so investment in somatic repair is sacrificed to provide for maintenance of the germline (Drenos and Kirkwood, 2005; Kirkwood, 1977). It is well-documented in many species that there is a relationship between organism longevity and reproduction. Age of reproductive onset has an impact on lifespan, while early fertility leads to shortened lifespans, and conversely, late life fertility and fecundity are correlated with a longer lifespan (Partridge and Fowler, 1992; Prowse and Partridge, 1997). Sexual reproduction has a negative influence on organism lifespan. Both mating male and female flies show declines in lifespan, an effect that can be eliminated by inhibiting perception of the opposite sex. Expression of loss of function mutations in odorant receptors interfere with pheromone perception and essentially reduce mating (Gendron et al., 2014). Similar connections between reproduction and lifespan have been documented in *C. elegans* (Kenyon, 2010; McCormick et al., 2012). Hydra
provides an interesting example. When reproducing asexually, the animal appears to be essentially immortal (Bellantuono et al., 2015; Martinez, 1998). Yet, when Hydra undergoes a sexual form of reproduction, signs indicative of senescence appear (Schaible et al., 2015; Yoshida et al., 2006).

Using flies and worms, the signals underlying reproduction and lifespan have been extensively studied. Involvement of insulin signaling have been shown in the worm C. elegans (Kenyon, 2010, 2011) and fly (Flatt et al., 2008; Tatar, 2010). In C. elegans, daf-2 and daf-16 appear to be key in the link between reproduction and longevity. daf-2 codes for an insulin/IGF-1 receptor implicated in the interaction of somatic gonad and germline regulation of lifespan (Hsin and Kenyon, 1999; Yamawaki et al., 2008). Evidence shows that the germline regulates lifespan through activity of daf-2. Ablation of the germline, or reduction of daf-2 in the soma extends lifespan through the accumulation of daf-16 (the transcription factor FOXO) in nuclei of intestinal cells (Hsin and Kenyon, 1999; Kenyon et al., 1993; Yamawaki et al., 2008).

Further evidence for the link between germline and longevity is observed in Drosophila. Similar lifespan extension effects are seen in Drosophila as well when germline cells are ablated. In the absence of insulin signaling, dFOXO accumulates in somatic cell nuclei. Elimination of GSCs caused a nearly 28% increase in male lifespan and upregulation and nuclear accumulation of dFOXO in somatic tissues. Additionally, the insulin binding protein IMP-L2 and Drosophila insulin-binding peptides (DILPs) were upregulated in flies lacking germline cells. IMP-L2 binds to insulin, inhibiting insulin signaling, thus interference with the insulin signaling pathway in the germline has a positive effect on lifespan (Flatt et al., 2008).

In turn, activation of these pathways and their effects on lifespan and reproduction are influenced by sensory inputs. Indeed, experimental evidence has demonstrated a link between sensory input and aging in flies. Loss of chemosensory
bristles extends lifespan, and interestingly exposure to yeast odorants without access itself is sufficient to negate effects of dietary restriction (Pletcher, 2009). Additionally, chemosensory perception of potential mates also impacts lifespan. Expression patterns of cuticular hydrocarbons, essentially fly pheromones, change with age and result in a decrease in mating behavior by reducing sexual attractiveness and thus fertility (Kuo et al., 2012).

Mechanisms of Cellular Homeostasis

Maintenance of cellular and tissue homeostasis is a key aspect of longevity. Degeneration of processes regulating cell function ultimately contribute to decline of tissue function. Proper DNA repair, elimination of defective proteins, and control of reactive oxygen species all support normal cellular functions. Over time, the efficiency of these processes is reduced. Furthermore, each individual process requires each other process to function properly, as they all form a complex network of interactions to maintain cellular equilibrium. Thus, a dysfunction in one process can lead to a gradual cascade of effects as dependent functions degenerate.

An important part of maintaining cellular homeostasis is protein turnover. The proteasome is a molecular complex that degrades proteins that are designated for destruction through ubiquitination. Functions include housekeeping through elimination of misfolded or otherwise damaged proteins, such as proteins that have undergone oxidative damage, and specialized functions in immunity for processing and delivery of antigens (Rodriguez et al., 2010). Protein degradation helps maintain cell homeostasis by maintaining the cellular pool of amino acids and ensuring that functional proteins are produced to maintain processes required for normal cellular function. Proteasomes also function during developmental processes such as Drosophila spermatid differentiation and individualization by control of caspases leading to apoptosis (Bader et al., 2011).
Thus, malfunctioning proteasomes not only can adversely affect cell homeostasis by allowing a build-up of defective proteins, thereby depleting the cell amino acid pool, but on a developmental level can have a negative effect on fertility by disrupting gametogenesis.

Another method used in cells to cope with not only protein aggregates, but also damaged organelles is autophagy. The autophagic response is another cellular stress coping mechanism that has been shown to regulate lifespan (Gelino and Hansen, 2012). Studies in *Drosophila* have shown that mutation of a core autophagy gene, *Atg7*, which activates two other autophagy proteins, *Atg8* and *Atg12*, exhibit reduced lifespan and poor response to stress (Juhász et al., 2007). Components of the autophagy pathway decrease with age, and likely contribute to neurodegeneration (Gelino and Hansen, 2012; Simonsen et al., 2008). Regulation of organism lifespan is also observed if autophagy is upregulated in the nervous system. Loss of *Atg8a* in the fly nervous system leads to degeneration and reduction in longevity. Neurodegeneration is due in part to accumulated protein aggregates. These diminish and lifespan is extended if *Atg8a* is misexpressed (Simonsen et al., 2008).

DNA, both nuclear and mitochondrial, accumulates damage during the lifetime of an organism contributing to aging (Shaposhnikov et al., 2015). A variety of mechanisms are in place to repair DNA damage, including Base Excision Repair, Homologous End Joining, and Mis-Match Repair (Gredilla et al., 2010). Errors in DNA repair can introduce potentially harmful mutations. The importance of DNA repair in regulation of lifespan was shown in *Drosophila*. Overexpression of DNA repair genes caused an extension of lifespan of up to 40% (Shaposhnikov et al., 2015).

Taken together, reproductive senescence is under the control of a complex network of molecular, behavioral, and environmental factors. Within a population, individuals senesce at varying rates. Thus, individuals must have differences in the
functionality of the processes described above that help determine the rate of decline. However, the genes involved in senescence can be difficult to identify given standard techniques. Most studies have focused on genetic characterization of female lifespan and fecundity. In this dissertation, I used the DGRP to examine the genetic architecture of male reproductive aging. Using publicly available sequence data, a genome-wide association study (GWAS) was used to identify potential regulators and targets of reproductive maintenance. This provided an unbiased screen for identification of individual genes with roles in reproductive aging. Candidate loci can then be validated for their involvement in the phenotype.

One factor that has been identified as a factor to regulate the length of fertility in *Drosophila* is the activity of the Jak/Stat pathway. Previous work has shown that males with reduced Jak/Stat activity have a decreased reproductive lifespan (Wang et al., 2014b). Loss of the pathway ligand *upd3* in males, though they are viable and fertile, experience an earlier onset of infertility with age. Two ligands are expressed in the testis hub of *Drosophila* males, *upd* and *upd3*. Expression of *upd* by the male germline stem cell niche is required for maintenance of the germline and somatic stem cells which will eventually give rise to sperm (Bausek, 2013; Boyle et al., 2007; Sinden et al., 2012). A molecular method involving miRNA regulation of *upd* expression has been described. In the hub, the IGF-II mRNA binding protein (Imp) binds to and protects the *upd* mRNA from degradation triggered by *let-7* miRNA. In aging males, protection of the *upd* transcript by Imp decreases, resulting in a reduction in number of germline stem cells (Toledano et al., 2012). While Jak/Stat signaling is important to maintaining fertility by sustaining gametogenesis over the lifespan of a male, there are also many other factors that contribute to reproductive maintenance. But what are these, and what role do they play in maintaining male fertility?
The Jak/Stat pathway has multiple functions in gametogenesis and is a molecular target of age-related decline (Bausek, 2013; Beccari et al., 2002; Borensztejn et al., 2013; Boyle et al., 2007; Sinden et al., 2012; Toledano et al., 2012). The pathway is conserved between invertebrates and vertebrate, but flies are an ideal model for studying the Jak/Stat pathway due to its relative simplicity. In *Drosophila*, the pathway consists of a single receptor (*domeless*), one Janus kinase or Jak (*hopscotch*), the Unpaired family of ligands consisting of *upd*, *upd2*, and *upd3*, and one known effector, Stat92E (Figure 1.4). In contrast the human pathway is much more complex, with four Jaks, and seven Stats, and a high number of ligands, making *Drosophila* an ideal model for exploring pathway function. Binding of the ligand to the receptor causes dimerization of the receptor and activation of the Jaks leading to phosphorylation of the Jaks. The active Jaks phosphorylate Stat92E which then dimerize. Stat92E can then enter the nucleus and promote transcription. The pathway is also subject to the action of different regulators including Eye Transformer/Latran, the SOCs family of proteins, Ptp61F, and Ken and Barbie which either positively or negatively affect signaling activity.

Jak activity has numerous functions in both male and female gametogenesis. In females, the pathway is required for events at several different stages of oogenesis. Specifically, the anterior polar cells have a major role in directing egg development through the activation of Jak activity in surrounding cells (Xi et al., 2003). Jak activity in the testis is a target of senescence. Evidence from the male testis demonstrates age-related regulation of *upd* expression in the testis stem cell niche, leading to a reduction of germline stem cells (Boyle et al., 2007; Toledano et al., 2012).

The projects discussed in this dissertation build upon previous work characterizing the functions of the Jak/Stat pathway ligand Upd3. It was found that loss of *upd3*, while not affecting female viability, caused a higher frequency of unfertilized
eggs with blocked micropyles, which increased with age. This suggests a role for Jak activity in ovarian senescence as well. As the polar cells are required for Jak/Stat activity and have an important role in egg development, they were the focus for investigation of the cause of blocked micropyles. However, males mutant for upd3 are also viable and fertile but they become infertile earlier than wild type males (Wang et al., 2014b). The Jak pathway is highly pleiotropic, making it a useful model for understanding how this and other such factors contribute to gametogenesis. It furthermore provides a framework for understanding how senescence affects the function of this and other pleiotropic factors required for process utilized in the maintenance of fertility.
Figure 1.1: *Drosophila* spermatogenesis and general sperm morphology. (A)

Spermatogenesis begins with asymmetrical division of germline and somatic stem cells. The germline cell undergoes a series of cell divisions to form a cyst. Each germline cell then undergoes a process of elongation and individualization to form a mature sperm.

(B) Fully mature sperm typically consist of three regions: head, mid-piece, and tail. The head contains a highly-condensed nucleus. The acrosomal vesicle is located anterior to the nucleus and contains enzymes necessary for sperm entry into the
egg. The mid-piece contains mitochondria which produce the necessary energy to power the tail.
Adapted from (Margaritis et al., 1980; Zarani and Margaritis, 1985, 1986)

**Figure 1.2: Chorion structure of the *Drosophila* main body eggshell and micropyle.**

VM=Vitelline Membrane, ICL= Inner Chorion Layer, WL= Wax Layer, EC= Endochorion, EX= Exochorion, ECP=Endochorion pore, PC=Paracrystalline material. A mature egg showing the layers of the eggshell. (A) Eggshell covering the main body of the egg is composed of five layers: vitelline membrane, inner chorion layer, a very thin wax layer, endochorion, and exochorion. The endochorion is spongy in structure, with many pores throughout. (B) The inset has been rotated 90 degrees clockwise. Eggshell structure around the micropyle consists of similar layers as the main body, with the addition of a “paracrystalline layer” believed to be secreted by the projection forming cells, in this case the polar cells (in green).
Figure 1.3: A *Drosophila ovariole*. The *Drosophila* ovary is composed of ovarioles, or chains of developing egg chambers. The ovariole is essentially an egg production line with the least mature eggs at the anterior of the ovariole. There is a total of 14 stages of egg development, with Stage 14 as the most mature. This dissertation focuses on Jak activity in the polar cells from Stage 10 (end of border cell migration) through the end of Stage 13.
Figure 1.4: The *Drosophila* Jak/Stat pathway. The pathway consists of a single receptor (*domeless*), a single Janus Kinase (*hopscotch*), the Upd family of ligands (*unpaired, unpaired2, unpaired3*), and a single effector (*Stat92E*). Binding of the ligand causes dimerization of the receptor and a series of phosphorylation events, culminating in the phosphorylation of Stat92E (red balls). In its phosphorylated form, Stat92E can dimerize and enter the nucleus where it activates transcription of target genes.
Chapter 2: The Tails of Two Cells: Autocrine Jak/Stat Signaling in the Polar Cells is Required During Micropyle Development

Abstract

The micropyle is a small tube located at the anterior of the Drosophila egg and serves as the point of entry for sperm during fertilization. However, micropyle formation does not follow canonical methods of tubulogenesis, rather it is secreted and shaped by specialized follicle cells including the border cells and polar cells. The functions of polar cells in follicle cell specification and border cell migration are well studied, however, their role in late stage oogenesis is less well known. We show that the polar cells extend processes that undergo a series of predictable, coordinated morphological changes directed into the micropyle to the oocyte. Formation of extensions relies upon the activities of polarity proteins, including atypical Protein Kinase C (aPKC), Par6, and Bazooka/Par3. Polar cells mutant for upd3, a gene that encodes one of the Jak/Stat ligands, form extensions but these frequently fail to be located in the micropyle at the time of chorion deposition, resulting in micropyle defects. Reduction of Jak/Stat activity specifically in the polar cells perturbs extension morphology and coordination, suggesting an autocrine requirement for pathway activity to maintain polar cell cohesion and extension coordination, leading to an increase in blocked micropyles and unfertilized eggs. Thus, the polar cells have a necessary role in oogenesis after border cell migration is completed, and require continued Jak/Stat activity to carry out their functions.

Introduction

The insect eggshell is a multi-layer secreted structure, formed by specialized somatic cells of the follicular epithelium. In Drosophila deposition of the vitelline membrane, the first and innermost layer, begins at Stage 8 of oogenesis, with chorion...
deposition occurring during Stage 11 through Stage 14 (Pascucci et al., 1996; Waring and Mahowald, 1979; Zarani and Margaritis, 1986), forming an impermeable barrier that protects the developing embryo from a potentially harsh environment. Yet the eggshell must permit sperm entry and oxygen uptake during embryogenesis, requiring structural adaptations to accommodate these requirements.

During chorion deposition, many insects secrete specialized structures that are an integral part of the eggshell. Oxygen uptake, occurring through the dorsal appendages in Drosophila (Berg, 2008), attachment structures for egg dispersal as in the Phthiraptera (Zawadzka et al., 1997), and micropyles that permit sperm entry and fertilization as direct penetration of the thick chorion is not possible (Mazzini, 1976; Mouzaki et al., 1991; Zarani and Margaritis, 1986; Zarani and Margaritis, 1991a; Zarani and Margaritis, 1991b; Zarani and Margaritis, 1994; Zawadzka et al., 1997), are all examples of chorionic specialization. Micropylar morphology varies among insect orders, ranging from simple pores in the surface of the egg, to a protrusion at the anterior with anywhere from one to as many as 20 pores (Hinton, 1981; Kubrakiewicz et al., 2005; Zarani and Margaritis, 1986; Zarani and Margaritis, 1994).

Different populations of follicular epithelial cells cooperate to form the micropyle (Mouzaki et al., 1991; Zarani and Margaritis, 1986; Zarani and Margaritis, 1991a, 1994). Generally, for insects in which the micropyle takes the form of a protrusion, multiple subsets of follicle cells secrete, shape the structure, and form one or more pores (Edwards et al., 1997; Hinton, 1981; Zarani and Margaritis, 1986). In Drosophila, electron microscopy of sectioned eggs has shown that two follicle cells form microtubule-rich extensions that protrude into the micropyle to form the single central lumen (Zarani and Margaritis, 1986). The extension forming cells were later identified as the anterior polar cells (Edwards et al., 1997). Beginning at Stage 10, the somatic follicle cells that comprise the follicular epithelium secrete the chorion (Waring and Mahowald, 1979),
while other subsets of follicle cells will participate in formation of eggshell specializations such as the dorsal appendages (Boyle et al., 2010) and micropyle. During Stage 6-7, the polar cells, a pair of somatic cells located at either end of the egg chamber, specify the identity of the follicular epithelial cells. Anterior follicle cell fates are determined by secretion of the morphogen Upd by the anterior polar cells, which act as an organizer (Xi et al., 2003). The highest amount of signaling activity is in a group of 4-6 cells closest to the polar cells, which adopt a border cell fate at Stage 8 of oogenesis (Xi et al., 2003).

At Stage 9 of oogenesis the border cells undergo an epithelial to mesenchymal transition, delaminate from the follicular epithelium and migrate posteriorly toward the oocyte, taking the polar cells with them. The polar cells sit at the center of the cell cluster, and initiate and maintain border cell migration through secretion of Upd, which activates the Jak/Stat pathway in the border cells (Beccari et al., 2002; Montell et al., 1992; Silver et al., 2005). Migration ends when the border cell cluster reaches the nurse cell/oocyte boundary by the end of Stage 10, prior to appearance of the micropyle. Border cells and other follicle cells have long been attributed with secreting and shaping the chorion to form the micropyle (Zarani and Margaritis, 1986). However, a micropyle forms even if the border cells are ablated, although it is blocked and suggests that other follicle cells also contribute to micropyle (Montell et al., 1992; Silver et al., 2005).

As with many insects, during Drosophila oogenesis follicle cell projections are associated with creation of pores during micropyle development (Edwards et al., 1997; Hinton, 1981; King, 1970; Zarani and Margaritis, 1986). Expression of fluorescently tagged moesin in the polar cells showed that they indeed form extensions associated with the micropyle, but the specific function of the extensions was not investigated (Edwards et al., 1997). Given that the production of follicle cell extensions in other species is associated with pore development during micropyle formation, this observation suggests the polar cells are involved in creating the micropyle lumen.
Here we show that the polar cells, through production of extensions into the micropyle, form the central pore. We further show that autocrine Jak/Stat signaling in the polar cells is required for micropyle formation.

Materials and Methods

Fly Lines and Crosses

Lines used in this study were as follows: UAS-Stat92E RNAi (BDSC 33637), UAS-lat (Crozatier), UAS-dome RNAi (BDSC 34618), UAS-aPKC<sup>DN</sup> (BDSC 51673), UAS-par6 RNAi (BDSC 35000), UAS-baz RNAi (BDSC 65869), UAS-Cdc42<sup>DN</sup> (BDSC 6288), UAS-Rac1<sup>DN</sup> (BDSC 6292), UAS-Rho1<sup>DN</sup> (BDSC 7327), UAS-lifeact.GFP (BDSC 57326), UAS-mCD8::GFP (BDSC 5137), UAS-myr RFP (BDSC 7118), slbo-lifeact.GFP (BDSC 58364), upd3<sup>d232a</sup>, X37E, UAS-shg RNAi (BDSC 32428), UAS-mys RNAi (BDSC 33642), UAS-EB1::GFP (BDSC N35512), Upd Gal4, UAS-mCD8::GFP (BDSC 5137), slbo-Gal4 (a gift from Denise Montell, (Montell et al., 1992))

Antibody Staining

Ovaries were stained as described previously (McGregor et al., 2002). The following primary antibodies were used: polyclonal goat anti GFP (Rockland Immunochemicals catalog No. 600-102-215, 1:500), mouse monoclonal anti-Fas3 (DSHB, 1:20), rat monoclonal anti-E-Cadherin (DSHB, 1:10), mouse monoclonal anti beta PS (DSHB, 1:10), mouse monoclonal anti-singed (DSHB, 1:5). The following secondary antibodies were used: Alexa-Fluor 488 anti-goat (Jackson Immunolabs, 1:1000), Alexa-Fluor 594 anti-mouse (Jackson Immunolabs, 1:1000), Alexa Flour 488 anti-rat (Jackson Immunolabs, 1:1500).

Imaging of Live and Fixed Samples

Both confocal and lightsheet microscopy was used for live imaging. Samples
were dissected in Schneider’s medium (US Biological Cat no. S0100) prepared per manufacturer’s instructions, and supplemented with 200µg/mL insulin, 0.6X pen/strep, and 10% fetal bovine serum (Prasad et al., 2007; Prasad and Montell, 2007). To prepare samples for lightsheet imaging, Stage 10 through Stage 12 egg chambers were selected and mounted in 1% low gel agarose containing Schneider’s medium, 15% FBS, 200µg/mL insulin, and 0.6X pen/strep in a glass capillary tube. During imaging, the mounted sample was immersed in Schneider’s medium containing 0.6X pen/strep. Live imaging was performed using a Zeiss Z.1 lightsheet microscope with a 20X objective. Images were processed using Adobe Photoshop, ImageJ, and Arivis Vision 4D software. Additional lightsheet imaging was performed using a Leica SP8 Digital Lightsheet microscope. Samples were dissected in Schneider’s medium, and appropriately staged eggs selected as described. A stock of 2% low gel temperature agarose was diluted to 0.5% with Schneider’s medium supplemented with insulin, FBS, fly extract, and pen/strep. Egg chambers were selected and mixed with approximately 20µl of agarose and transferred to a 25mm glass bottomed petri dish containing a small open capillary. Using a dissection needle, egg chambers were positioned near the surface of the agarose. The ends of the capillary were plugged with a drop of 2% agarose to keep the sample in place. The petri dish was filled with 1% Schneider’s medium during live imaging. Egg chambers were imaged using a 25X objective with a 5mm twinflect mirror. Images were collected using a Hamamatsu Orca-Flash 4.0 digital camera and were processed using Leica LASX.

For confocal live imaging, a Leica SP8 confocal microscope was used. Samples were dissected in Schneider’s medium as described above and mounted on an aluminum slide as described by Dorman et. al. (Dorman et al., 2004). Eggs were mounted in a small amount of 0.5% agarose supplemented with insulin, FBS, and fly extract to prevent the follicles from drifting out of the field of view. A small amount of
Schneider’s medium supplemented with 15% FBS and 200ug/mL insulin was also added to the preparation to prevent drying during imaging. Imaging was performed for varying lengths of time using a 40X objective.

Fixed samples were imaged using a Leica SP8 confocal at 63X, and 100X magnification. Images were processed using LasX, and ImageJ. Deconvolution of selected images was performed using SVI Huygens Essential software.

**Egg Hatching Assays**

Females were collected and placed with wild type males at a ratio of 1:1 in fly cages with grape juice plates and wet yeast paste. Flies were allowed two days to equilibrate with daily plate changes before egg collection. For hatching assays, at least 100 eggs were transferred to a fresh juice plate and incubated for 48 hours. Unhatched eggs were quantified and placed in 50% glycerol for examination.

**Quantification of Extension Dimensions**

Stage 10B egg chambers from females expressing mCD8::GFP in the polar cells were photographed using a Nikon E800 microscope. Measurements were performed using ImageJ. Extension length was determined by measuring from the base of the polar cell bodies to the end of the extensions. Width was measured at the midpoint of the extensions using ImageJ (Schindelin et al., 2015).
Photon Counting

Age matched females were reared on yeast paste at 29°C for 36 hours prior to dissection to enhance Gal4 expression (Duffy, 2002). Upd-Gal4>mCD8::GFP or Upd-Gal4>mCD8::GFP;UAS-Stat92E RNAi females were dissected and ovaries processed in parallel for either Fas3 or DE-Cadherin simultaneously as described previously. DE-Cadherin and Fas3 immunofluorescence were captured using a hybrid detector on a Leica SP8 confocal microscope in counting mode. A step size of 0.33µm was used during stack collection. Using the 3D quantification function in LASX, the GFP expression throughout the stack was used to define the region of interest, and expression of either Fas3 or DE-Cadherin immunofluorescence was quantified for the entire polar cell volume.

Results

Characterization of extension formation

Involvement of follicle cell projections in micropyle lumen formation has been demonstrated using electron microscopy for a range of insects (Garbiec et al., 2016; Mazzini, 1976; Zarani and Margaritis, 1986; Zawadzka et al., 1997). The number of projection forming cells varies among species and appears to be dependent upon the number of pores that form. Furthermore, specialization and migration of subsets of somatic follicle cells to the site of micropyle development is also observed in some groups. Drosophila micropyle formation follows this pattern of follicle cell involvement, requiring migration of the border cell cluster and subsequent formation of a protrusion by the polar cells to shape the micropyle and form the lumen. While the general timing of micropyle development in Drosophila has been documented using electron microscopy of sectioned eggs (Zarani and Margaritis, 1986), we were interested in understanding the dynamics of the morphological changes of polar cells in intact eggs. Upd-Gal4 was used to specifically label the polar cells using UAS-mCD8::GFP, a membrane-bound
GFP, allowing morphological changes in the cells to be tracked. Polar cells undergo a consistent and predictable series of changes that create protrusions directed into the developing micropyle. During border cell migration, the polar cells have no visible protrusions, retaining their round shape until migration is complete at Stage 10B. Each polar cell then forms a short lamellipodial extension at the interface with the oocyte (Figure 2.1 A), with numerous short filaments that project down to the oocyte (Figure 2.1A”). At Stage 11-12, as the oocyte increases in size, the polar cells remain at the anterior tip and each extension narrows and elongates, while filaments form. These filaments are less numerous, finer, and longer than those seen at Stage 10B, and give the polar cells an octopus-like appearance (Figure 2.1 B). As oogenesis progresses to Stage 13, each polar cell forms a primary extension that elongates into the micropyle (Figure 2.1 C), from which multiple filaments form and elongate, contacting the oocyte (Figure 2.1 E, Movie 1). At the end of oogenesis, the extensions do not retract, but rather the polar cells remain in place at the top of the micropyle and undergo apoptosis, while the extensions fragment inside the micropyle channel (Figure 2.1 D).

*Cell polarity regulation and cytoskeletal activity during extension outgrowth*

Alterations in cell polarity and adhesion are required in some cell types during development, such as those that undergo an epithelial to mesenchymal transition (EMT). The neural crest cells in vertebrate development undergo such a transition. Similarly, metastatic cancers also exhibit these traits (Gallik et al., 2017; Jie et al., 2017). With EMT, there is a loss of apical-basal polarity and cell contact with the epithelium, and establishment of new contacts. During oogenesis in *Drosophila*, cellular processes are formed by the border cells at the beginning of, and during their migration to the oocyte, responding to guidance cues (Fulga and Rørth, 2002; Poukkula et al., 2011). Similarly, outgrowth of the polar cell extensions occurs in a directed manner from the apical cell
surface toward the oocyte, though it is not known if this is in response to a signal or guidance cue (Bianco et al., 2007; Fulga and Rørth, 2002). We hypothesized that regulation of cell polarity is required during formation and growth of the polar cell extensions. In *Drosophila*, cell polarity is determined through the partitioning of specific protein complexes at either the apical or basal surface (Figure 2.3). Apical identity is determined through the localization of the Par/Bazooka complex to that surface, while the basal compartment is determined by Dlg/Scb (Pinheiro and Montell, 2004). Action of atypical protein kinase C (aPKC) either targets or excludes these complexes from the appropriate region. It was predicted that interference with components of these complexes would either prevent extension formation or lead to defects in extension orientation.

*Par6* and *bazooka* were knocked down in polar cells marked with mCD8::GFP to follow extension development. At Stage 10B, knockdown of *baz* and *par6* did not prevent extension outgrowth, but did affect lamellipodium formation at Stage 10B (Figure 2.2 A-D). Knockdown of *Par6* in the polar cells caused morphological defects in approximately one-third of Stage 10B egg chambers including elongated extensions, and approximately 8% of these exhibited reduced cohesion (Figure 2.2 D). RNAi of *baz* had similar effects on polar cell morphology; lamellipodia were formed at Stage 10B, but polar cells either formed elongated extensions or had reduced cohesion. In an extreme instance, one polar cell appeared to form two extensions, one oriented correctly and the other directed away from the oocyte (Figure 2.2 C). Expression of dominant negative aPKC prevented lamellipodium formation in 80% of Stage 10B egg chambers (Figure 2.4 A). At Stage 13, polar cells still formed extensions directed toward the micropyle, but they appeared shorter and thinner (Figure 2.2 H-K). Eggs collected from aPKC mutant females were not fertilized, and upon examination, showed a high proportion of micropyles that lacked channels (Figure 2.5). It was possible that the loss of aPKC
influenced overall development. Rearing flies at 19°C can lessen the effect of detrimental alleles during development. To test if this was the case, flies were housed at 19°C and 25°C to determine if oogenesis at a lower temperature would reduce the penetrance of the defect. While the proportion of blocked micropyles from females grown at 19°C decreased from 95% to 70%, it was still greater than females with only upd-Gal4. Notably, wild type females reared at 19°C had an increase in the proportion of eggs with defective micropyles compared to those grown at 25°C. This is attributed to the nature of the upd-Gal4 construct used, which is an insertion located in a regulatory region of upd. In our hands, it causes defects at a lower temperature when heterozygous. In instances where eggs laid by aPKC mutant females had channels, they appeared to be much narrower than in wild type eggs. While there was an increase in polar cell extension defects when cell polarity regulators were targeted, aPKC^DN had the greatest effect. This can most likely be explained by the dominant negative form of aPKC was more efficient at impacting the phenotype than RNAi constructs of Par6 and Baz.

Early studies using electron microscopy showed the extensions in Drosophila were microtubule-rich (Margaritis, 1984). Work on follicle cell projections formed during micropyle development in other insects has demonstrated actin involvement in mallophagan insects (Zawadzka et al., 1997) and the silkworm moth Bombyx mori (Yamauchi and Yoshitake, 1984). To examine changes in cytoskeletal distribution during extension initiation and outgrowth, fluorescently labeled cytoskeletal proteins were expressed specifically in the polar cells. F-actin dynamics in the polar cells was followed using Upd Gal4 to drive expression of Lifeact-GFP, a GFP tagged form of actin (Riedl et al., 2008; Spracklen et al., 2014). Tubulin was visualized using either a GFP tagged form of the microtubule end capping protein EB1 or GFP tagged alpha tubulin. During border cell migration at mid Stage 9, the polar cells have no visible protrusions, with an even distribution of actin and tubulin over the surface of each cell (Figure 2.6 A, D). Upon
completion of border cell migration at Stage 10B, an apical concentration of actin and tubulin, visible as bright GFP expression in a narrow region where the extensions contact the oocyte (Figure 2.5 A-D, E-H). This localization of cytoskeletal components to the apical region of the polar cells continues as extension outgrowth progresses.

Reorganization of the actin and microtubule cytoskeleton are required for changes in cell morphology and growth, for example during axonal development (Dent et al., 2011). Promotion or inhibition of cytoskeletal dynamics requires interaction of the Rho family of GTPases (Ng and Luo, 2004). Both actin and tubulin interact at regions of cell outgrowth with the small GTPases to initiate and form lamellipodia, and filopodia (Montell et al., 2012). The small GTPases are also required for reorganization of the actin cytoskeleton during border cell migration. Border cells expressing dominant negative Rac failed to migrate, failing to form processes (Murphy and Montell, 1996). We attempted to prevent or reduce extension outgrowth using dominant negative forms of the small GTPases Cdc42, Rac1, and Rho1. At Stage 10B, polar cells expressing dominant negative forms of these small GTPases formed lamellipodia. However, polar cells expressing Rho1DN and Cdc42DN exhibited reduced cohesion and cell bodies were slightly distorted (Figure 2 B). While effects were not as extreme as those observed when cell polarity determinants were altered, extension outgrowth was reduced or prevented at Stage 13 in cells expressing Rho1DN (Figure 2.2M).

Loss of Upd3 affects polar cell extensions but not border cell migration

The Jak/Stat ligands Upd and Upd3 are both expressed by the polar cells and loss of Upd3 results in an increased frequency of unfertilized eggs and eggs with blocked micropyles (Figure 2.7 E). Wild type polar cells produced extensions directed
into the micropyle (Figure 2.7 A) In contrast, extensions from approximately 20% of upd3 mutant polar cells either missed the micropyle completely (Figure 2.7 B, E), or became separated leading to uncoordinated outgrowth and only one extension entering the micropyle (Figure 2.7 B, D). Mis-targeted projections are correlated with an increase in blocked micropyles (Figure 2.7 E).

We then wished to resolve the phenotypes of upd3 mutants with the known functions of Jak/Stat activity in micropyle formation. Timing of border cell migration is important to ensure development of the micropyle proceeds normally. Activation of Jak/Stat signaling in the border cells is required for initiation, organization, and maintenance of migration of the border cells (Silver et al., 2005; Silver and Montell, 2001). Expression of hypomorphic alleles of Stat92E specifically in the border cells leads to significantly delayed migration of the border cell cluster (Silver et al., 2005). Mutation of Stat92E targets such as the transcription factor slow border cells (slbo) also lead to migration impairment (Montell et al., 1992). Previous work has shown that if migration of the border cell cluster is delayed or is not completed, blocked micropyles are formed (Beccari et al., 2002; Montell et al., 1992; Silver et al., 2005). However, loss of the Jak/Stat ligand Upd3 also causes blocked micropyles, albeit at a frequency of only approximately 10% (Figure 2.6E). Both upd and upd3 are expressed in the polar cells (Wang et al., 2014b), and perhaps have an additive effect in stimulating Jak activity in the border cells during their migration. So, perhaps loss of upd3 leads to reduced Jak activity in the border cells, slowing migration of the cluster.

Egg chambers from upd3 mutant females were examined for delayed border cell migration. Migration distance at Stage 10B was assessed semi-quantitatively using an assay adapted from Ghiglione et al. (Ghiglione et al., 2002) (Figure 2.7). A migration index for the polar cell pair was determined by estimating the distance the migrating border cell cluster had traveled by Stage 10B, with a migration index of 1 being
essentially no migration, and full migration of the cluster assigned a migration index of 4. Proximity of the polar cells to the oocyte was determined by labeling the polar cells with anti-Fas3 or mCD8::GFP. The mutant polar cells were located appropriately at the oocyte boundary at Stage 10B and still produced extensions. As a control Stat92E RNAi was expressed in the border cells using slbo-Gal4. Unsurprisingly, reduction of Stat92E in the border cells had a large effect on border cell migration (Figure 2.7), with all Stage 10B egg chambers demonstrating defective border cell migration (Silver et al., 2005). We conclude that Jak/Stat activity in the polar cells was not required for border cell migration.

**Autocrine Jak/Stat activity is required by the polar cells**

While perturbation of Jak/Stat activity in the polar cells did not disrupt border cell migration, defective micropyles were still observed in 25% of eggs when Stat92E was knocked down. This made us reconsider the mechanism and we surmised that there was a process independent of failure to fully activate the pathway in the border cells. An alternate explanation was that autocrine activity is required by the polar cells. To test this possibility, both Stat92E and the receptor domeless were knocked down specifically in the polar cells using an upd-Gal4 driver to increase the effect of the loss of Jak/Stat signaling. Neither knockdown of Stat92E, or domeless alone in the polar cells significantly affected border cell migration, with nearly all border cell clusters migrating normally (Figure 2.8). In contrast, misexpression of eye transformer/latran, a negative regulator of the Jak/Stat pathway (Kallio et al., 2010; Makki et al., 2010), in the polar cells did affect migration and cell morphology (morphology data not shown). et/lat codes for a short receptor with a truncated internal portion and lacks ligand binding ability (Fisher et al., 2016; Makki et al., 2010). Negative regulation of Jak/Stat activity by Et/Lat occurs when Et/Lat dimerizes with Dome, preventing it from forming a functional receptor. The ability of Et/Lat to form a heterodimer with Dome suggests that Et/Lat may
act in a non-autonomous manner, and would explain our observation that expression of Et/Lat by the polar cells impairs border cell migration.

As noted above, upd3 polar cell extensions appeared to be less coordinated in upd3 mutants. Wild type polar cells each produce an extension that maintains a close association to the extension of its partner, entering the micropyle as a single unit. However, upd3 mutant polar cell extensions become separated, losing coordination, with one extension entering the micropyle and one missing it (Figure 2.7 B), or missing the micropyle altogether (Figure 2.7 C). Extensions exhibited uncoordinated behavior in approximately 20% of upd3 mutant egg chambers, and at slightly greater frequency of egg chambers in which Stat92E was knocked down in the polar cells (Figure 2.7 D). Reduction of Stat92E also increased the proportion of polar cell extensions that missed the micropyle completely (Figure 2.7 D). Jak activity is known to be required for regulation of both Fas3 and E-cadherin which are both expressed by the polar cells (Silver and Montell, 2001; Wells et al., 2013). Reduction of pathway function in the polar cells by the loss of Upd3, may be sufficient to maintain cluster integrity during migration but insufficient to ensure proper adhesion of the polar cells to their target and/or each other.

While polar cells with reduced Jak/Stat activity still produced extensions that were oriented toward the oocyte, extension morphology strongly differed from wild type at Stage 10B. Knockdown of Stat92E in the polar cells resulted in development of longer extensions at Stage 10B (Figure 2.9 B, C). Furthermore, polar cell bodies and extensions showed reduced cohesion (Figure 2.9 B). In instances of lost polar cell body cohesion, each polar cell appeared to form an extension but extensions did not contact each other, despite projecting toward the oocyte boundary (Figure 2.9 A, B, Figure 2.10). We concluded that Jak/Stat activity is not required for extension formation or directing them toward the oocyte, however may be required to regulate extension morphology. To
determine if there was a difference in extension morphology between wild type and reduced Stat92E polar cells, extensions were measured from the base of the cell bodies to the area where the extension contacted the oocyte boundary. Width was measured as the width of the area of contact with the oocyte. Quantification of extension dimensions showed extensions were significantly longer in polar cells in which Stat92E was knocked down (Figure 2.9 C). Egg hatching assays were performed on both females carrying upd-Gal4 as a control and those expressing Stat92E RNAi in the polar cells (Figure 2.9 D). Quantification of unhatched eggs showed that 25% of eggs laid by females with reduced Stat92E activity failed to hatch and were unfertilized. Of these, 30% had blocked micropyles (Figure 2.9 D-F). While Jak/Stat activity in the polar cells is not required to maintain timely border cell migration, it appears that there is an autocrine requirement for Jak activity in the polar cells to maintain extension morphology, coordination, and adhesion.

Quantification of adhesion molecules

One possible explanation for the observed increase in altered extension morphology and uncoordinated polar cell extension behavior is reduced adhesion of the extensions to each other and to the surrounding cells. Because their expression has been demonstrated to be regulated by Jak/Stat activity in some tissues (Niewiadomska et al., 1999; Wells et al., 2013), Fas3 and DE-cadherin expression by the polar cells was quantified. Photon counting confocal microscopy was performed on cells immunostained for Fas3 or DE-cadherin to determine if decreased Stat92E activity affected adhesion through decreased expression of these genes. Egg chambers from age matched wild type or Stat92E RNAi females were processed and stained for either DE-Cadherin or Fas3 under the same conditions. Intensity of staining was determined by measuring photon counts over the entire volume of the polar cells. As distribution patterns varied
somewhat, this approach accounted for differences in cell size and distribution patterns. Measurements revealed that there was no significant measurable difference in fluorescence intensity for cadherin or Fas3 between wild type and Stat92E RNAi polar cells (Figure 2.11).

**Distribution of adhesion molecules**

Although the levels of total E-cadherin and Fas3 proteins in Stat92E knockdown polar cells did not differ significantly from wild-type in polar cells in which Stat92E was knocked down, it was possible that changes in distribution of these proteins could contribute to alterations in polar cell morphology. Distribution patterns of Fas3 and E-cadherin were observed for immunostained wild type and Stat92E knockdown polar cells in Stage 10B and Stage 13 egg chambers. In 3D volume projections of Stage 10B follicles, Fas3 is located around the periphery of the polar cells and between the polar cell extensions of both wild type and Stat92E RNAi cells (Figure 2.12 A, B, Movies 2, 3). Consistent with observations by others (Cai et al., 2014; Niewiadomska et al., 1999) DE-cadherin is expressed strongly by the border cells during border cell migration, surrounding the polar cells. Interestingly, at the site of polar cell contact with the oocyte, cadherin expression forms a star shape around a central pore where the polar cell extensions eventually protrude (Figure 2.12 E, F, Movies 4, 5).

At oogenesis Stage 13, Fas3 is still observed between each polar cell. However, there is a region of very strong Fas3 staining where the polar cells contact the top of the micropyle. Fas3 surrounds the base of the polar cell bodies, and extends between and around the polar cell extensions (Figure 2.12 E, F, Movies 6, 7). Distribution of E-cadherin does not show a dramatic difference from that observed at Stage 10B. Still visible is the star shaped pattern around the base of the polar cells. The central pore in
E-cadherin is still visible and is located at the top of the developing micropyle. The polar cell extensions pass through this pore into the growing micropyle to form the channel (Figure 2.12 G, H, Movies 8, 9). Overall, the distribution of both Fas3 and E-cadherin in both wild type and Stat92E egg chambers is similar and is consistent with the photon counting results.

*Loss of DE-Cadherin phenocopies knockdown of Stat92E in the polar cells*

Expression of a dominant negative DE-Cadherin, and knockdown of DE-Cadherin in the polar cells phenocopied reduction of Stat92E. An upd-Gal4 driver was used to express either RNAi of the *Drosophila* E-Cadherin, *shotgun (shg)*, or dominant negative DE-Cadherin in the polar cells. At Stage 10B, evidence of reduced cohesion was seen in both shg RNAi and dominant negative DE-cadherin polar cells. As with Stat92E RNAi, while still maintaining some contact, polar cells showed various degrees of separation, and in extreme cases, one cell is not in contact with the oocyte. Misexpression of DE-cadherin does not affect polar cell extension development (Figure 2.13 A-D). At Stage 13, loss of extension coordination is observed when DE-cadherin is knocked down or eliminated (Figure 2.13 E-H). One extension is either directed into the micropyle while one misses (Figure 2.13 F), or extensions are directed properly, but exhibit a loose association (Figure 2.13 G). Polar cells in which wild type DE-cadherin is mis-expressed do not exhibit extension coordination defects at Stage 13. Quantification of extension morphology at Stage 10B found no significant difference between genotypes (Figure 2.13 I). However, a significant increase of uncoordinated extensions in which one extension enters the micropyle and one misses it, and extensions that miss the micropyle completely was observed in polar cell that had a complete loss of DE-cadherin (Figure 2.13 J).
Discussion

The polar cell extensions are correlated with development of the micropyle lumen

Chorion formation in insects occurs through secretion of proteinaceous material to form a cross-linked series of layers that comprise the eggshell (Pascucci et al., 1996; Waring and Mahowald, 1979). Not limited to Diptera, micropyle formation occurs during egg development in a wide variety of insect orders and can assume different forms. Micropyles can either take the form of a simple pore or occur as a small tube. The number of pores can vary as well; ranging from one to 20 simple pores in the chorion as in the termite Reticulitermes speratus (Yashiro and Matsuura, 2014), a single lumen in a protrusion as in Drosophila, or as in the Neuropteran insects that have multiple pores on the periphery of a protrusion (Garbiec et al., 2016; Zarani and Margaritis, 1986; Zawadzka et al., 1997). Eggs of teleost fish also possess a micropyle, and in zebrafish this takes the form of a funnel-shaped pore located at the animal pole of the egg (Hart et al., 1992). Interestingly, micropyle formation in both insects and fish requires the involvement of one or more specialized follicle cells that form a projection to create the pore.

The requirement for the polar cells in follicle cell specification and border cell migration is well documented, but functions of the polar cells after border cell migration are not well studied. Indeed, the polar cells have an active role in oogenesis after Stage 10B, and are in fact needed for a functional micropyle. Evidence from the work described here and from others (Edwards et al., 1997) shows that the polar cell extensions are correlated with formation of the micropyle lumen. Furthermore, we have shown that coordinated extension outgrowth is necessary for proper formation of the micropyle channel. Micropyle formation follows a “Bundt-cake” model of formation in which the border cells act in the role of the pan to help shape the micropyle, while the polar cells extend processes into the growing micropyle, forming a central pore upon
polar cell apoptosis.

**Autocrine requirement for Jak activity in the anterior polar cells**

Paracrine Jak/Stat activity is required to pattern the follicular epithelium and drive migration of the border cells. Perturbation of the pathway in the epithelium leads to alteration in the number of border cells and other follicle cell fates (Beccari et al., 2002; Silver and Montell, 2001; Xi et al., 2003), and slowing or prevention of border cell migration (Montell et al., 1992; Silver and Montell, 2001). We show here that there is also an autocrine requirement for Jak/Stat activity in the polar cells to coordinate extension formation during micropyle development. Knockdown of Stat92E or domeless in the polar cells causes altered extension morphology and reduced polar cell cohesion without significantly altering rate of migration of the border cell/polar cell cluster.

Autocrine Jak/Stat activity occurs in other tissues, contributing to tissue homeostasis. In the *Drosophila* midgut, autocrine signaling through Upd secretion is required to maintain the intestinal stem cell population, while the other two ligands function in a paracrine fashion to regulate cell proliferation and differentiation of progenitor cells. Thus a combination of paracrine and autocrine pathway activity functions to regulate cellular proliferation, differentiation, and maintenance of gut tissue (Osman et al., 2012).

Other autocrine pathway requirements are seen earlier in oogenesis and in the developing wing. During oogenesis, specification of the polar cells occurs in the germarium (Margolis and Spradling, 1995; Ruohola-Baker et al., 1993) and produces three to five polar cells at each pole that must be reduced to a pair of cells through the cell death pathway. Reduction of polar cell numbers early in follicle development integrates secretion of Upd by both the polar cells and the stalk cells to activate the apoptotic pathway in the supernumerary polar cells (Khammari et al., 2011). Failure of
apoptotic elimination of excess polar cells is a vital necessity during oogenesis. Excess polar cells interfere with border cell migration and specification of follicle cell fates, critical functions in egg development (Besse and Pret, 2003). During wing development, there is a requirement for spatially-restricted Jak/Stat signaling to form the wing hinge. Another developmental requirement for autocrine Jak activity is seen in the developing hinge cells. Jak/Stat activity is restricted to the wing hinge through inhibition of Stat92E and Upd by factors specific to other regions of the developing wing (Ayala-Camargo et al., 2013).

Uncontrolled autocrine activity can be detrimental to organism viability. Autocrine Jak/Stat function is necessary for performing necessary functions during development. But dysregulation of the pathway contributes to a number of cancer types in both flies and humans from excessive cellular proliferation (Amoyel et al., 2014). In humans, elevated Stat3 is associated with a range of different cancer types including breast, prostate, and nasopharyngeal cancer (Bowman et al., 2000). In breast cancer, Tamoxifen resistance is driven by autocrine Stat3 activity. This comes about by an autocrine interaction between the chemokine CCL5 and Stat3 which initiate anti-apoptotic processes which promote drug resistance (Yi et al., 2013). Thus, the polar cells may be a suitable candidate for the study of the role of autocrine signaling in tumor progression and identification of potential anti-cancer therapies. Thus, autocrine Jak/Stat activity is important for development, when there is a need for tight control of expression.

Adhesion molecules are required for coordination of invasive behaviors

Border cell migration is initiated when the border cells undergo an epithelial to mesenchymal transition (EMT) in response to stimulation by Upd secretion from the polar cells (Montell et al., 1992). EMT involves dissolution of cellular contacts with the epithelium, formation of new contacts, and alterations in cell polarity (Montell et al.,
Migration of the cluster involves regulators of cellular polarization and cytoskeletal dynamics to form the protrusions needed for direction sensing (Fulga and Rørth, 2002; Murphy and Montell, 1996; Pinheiro and Montell, 2004). DE-cadherin expression is important for proper migration of the border cell cluster, as it provides a mechanism for gripping or traction during their passage through the nurse cells (Cai et al., 2014; Montell, 2001; Niewiadomska et al., 1999). Upregulation of DE-cadherin in the border cells is dependent upon Jak/Stat activity through activation of the Stat92E target slbo (Montell, 2001). If DE-cadherin adhesion between within the border cells in the cluster, or between the border cells and nurse cells is disrupted, migration is negatively affected. Adhesion within the cluster also depends upon the proper localization of the cell polarity components Baz and Par6. Proper localization of these molecules in the border cells requires expression of the transcription factor slbo. Mutation of slbo leads to a failure of Baz and Par6 to properly relocate away from the junction between border and polar cells. Localization defects are also observed in Jak/Stat mutant border cells. Loss of Baz and Par6 in the border cells decreases cohesiveness of the cluster during migration. It also increases the number of protrusions formed by the border cells, in addition to altering protrusion morphology (Pinheiro and Montell, 2004). Furthermore, both loss and mis-expression of Par6 and Baz altered cluster migration. Therefore, maintenance of proper adhesive contacts between cells, cytoskeletal regulation, and polarity is critical for overall cluster stability and guidance.

While the border cells express high levels of DE-cadherin during cluster migration to the oocyte, the polar cells produce elevated levels of the homophilic adhesion molecule Fas3 (Wu et al., 2008). This molecule functions in tissue shaping during several developmental processes. In the nervous system, it aids in neuronal guidance and targeting (Chiba et al., 1995; Snow et al., 1989), contributes to the curvature of the developing Drosophila hindgut, and maintenance of wing folds in the
larval imaginal wing disk (Wells et al., 2013). Shaping of the hindgut to create a curve is accomplished through spatial expression of Fas3. Like the use of DE-cadherin in the border cells for migration, localized and asymmetric distribution of Fas3 creates tension to shape the hindgut (Wells et al., 2013). Evidence of a Jak/Stat activity requirement during these events is seen as a relaxing of the tight curve normally observed when upd is lost during hindgut development (Wells et al., 2013).

We quantified Fas3 and DE-cadherin expression by the polar cells. The difference in adhesion molecule expression was not significant between wild-type and Stat92E knockdown polar cells, and distribution of DE-cadherin and Fas3 was similar between the two genotypes. We could phenocopy reduction of Stat92E in the polar cells through cadherin knockdown. However, while distribution patterns may be similar between wild type and Jak/Stat mutant polar cells, concentration of adhesion molecules in different regions of the polar cells could differ. This in turn could cause changes in the tension that the polar cells are subjected to during border cell migration and extension formation. The observed disparities in extension morphology between mutant and wild type cells thus may be due to biologically significant differences in adhesion protein distribution.

The polar cells are required for a novel mechanism of tube formation

Tube formation from a flat epithelial sheet is required in mammalian development for creation of the neural tube, formation of the heart, lung, and kidneys. Tubulogenesis is employed during Drosophila development of the salivary glands, wing veins, and tracheal tubes. This diversity of structures is formed by the actions of subpopulations of cells in an epithelial sheet that undergo changes in shape and adhesive properties. Typically the mechanisms employed follow one of five models of tubulogenesis (Lubarsky and Krasnow, 2003), including wrapping, budding, cord hollowing, cavitation,
and cell hollowing, to produce a structure composed of cells. In flies, a combination of wrapping and budding form the salivary glands, while wing veins are formed by a variation of cord hollowing. In all cases, the final product is cellular in nature and in stark contrast to the micropyle, which is acellular in form.

Fertilization and embryogenesis occur after eggshell deposition and hardening, necessitating a means to permit these functions to occur. The solution takes the form of tubular specializations present on the surface of the fly eggshell. But the acellular nature of the eggshell requires an alternate method of tube formation. Epithelial involvement occurs, but rather than directly forming the structure, cells secrete and sculpt the tube and are shed after development is complete. A well-studied example of this method are the dorsal appendages, paddle shaped tubes that oxygenate the embryo. These develop through the cooperation of two populations of follicle cells, the floor cells and roof cells. The cells undergo a series of shape and adhesive changes to form the structure in a manner resembling wrapping, but the epithelial cells secrete chorion into the tube lumen to form the final product (Berg, 2005, 2008; Boyle et al., 2010; Dorman et al., 2004). These cells are later shed at the end of oogenesis. Thus, the epithelial cells act as a mold to shape a long, acellular tube utilizing a conventional method of tubulogenesis. Similarly, the *Drosophila* micropyle is essentially a short, secreted tube, the formation of which is the result of cooperative action of specialized subsets of somatic cells in the follicular epithelium.

**Future Directions**

The function of the anterior polar cells after completion of border cell migration is not well studied. This work has shown that the polar cells are indeed required throughout the later stages of oogenesis and their activity is dependent in part upon autocrine Jak signaling. However, there are many more questions that remain to be answered to attain
an understanding about the polar cell functions during micropyle development. While we have demonstrated a correlation between the polar cell extensions and creation of the micropyle lumen, the timing for requirement of the extension has not been determined. Spatial and temporal ablation of the polar cells at various stages of egg development, beginning at completion of border cell migration at Stage10B can be performed to address this. One approach we have attempted is mis-expression of cell death activators, the pro-apoptotic genes grim, reaper, and hid, and the inhibitor of apoptosis Diap1, specifically in the polar cells, with little success. Progression from Stage 10B to 14 in Drosophila takes approximately 14 hours, but the duration of apoptosis ranges from 12-24 hours (Saraste, 1999). This method of cell death induction therefore lacks the speed for the specific targeting of developmental stages we wished to achieve. An optogenetic approach was then tested. This technique uses a form of channel rhodopsin, a light activated cation channel, that has been engineered for greater sensitivity to blue light. Through expression of this channel in the polar cells it was hoped that activation would overwhelm the cell with calcium and sodium ions, stressing the cell sufficiently to promote cell death. Females carrying the construct in the polar cells under the control of an upd-Gal4 driver were exposed to blue light for 24 hours. With this method, polar cells appeared to be damaged, showing defects in cell shape, but this method also lacked the temporal control that was desired. Ideally, laser ablation would be the best option for ablating the polar cells at a specific stage, but access to a laser with sufficient power is lacking.

One other approach that is possible is exploitation of the ability of dominant negative aPKC to prevent extension outgrowth. Controlled expression of dominant negative aPKC could have a similar effect to polar cell ablation by preventing extension development. Flies expressing aPKC\textsuperscript{DN} in the polar cells were reared at 19°C, to reduce Gal4 activity. However, this still resulted in nearly three-quarters of eggs possessing
blocked micropyles. Use of temperature sensitive Gal80, an inhibitor of Gal4 at 19°C, with upd-Gal4 could provide the temporal specificity desired by inhibiting Gal4 at low temperatures. Shifting to higher temperatures would then activate aPKC through alleviation of Gal80 repression. Testing would have to be performed to determine the amount of time needed to relieve repression and promote aPKC expression.

Most of what is known about polar cell extension development has been gleaned from static images. Although the polar cells are actively altering their morphology during the later stages of oogenesis, most of the results described here are based on snapshots. For a more thorough understanding of the nature of the relationship of the polar cell extensions to the micropyle and the relationship of Jak/Stat signaling to its development, live imaging is required. Limited live imaging has been performed on wild type eggs, providing a tantalizing look at later stage polar cell activity. But, the present movies created from live imaging sessions do not paint a complete picture of extension development, as they begin around Stage 11-12 after the polar cells have already begun to develop protrusions and the micropyle is beginning to develop. Imaging beginning earlier in oogenesis and continuing through to Stage14 is required to achieve a better understanding of the process. Image collection beginning at Stage 9 would permit visualization of the entire course of micropyle development and polar cell extension outgrowth. This also needs to be performed on pathway mutants, assisting in determining the timing of the requirement of the polar cell extension in formation of the micropyle channel. It would also be valuable for determining whether the cause of blocked micropyles in mutants is due to loss of adhesion of the polar cell to the surrounding cells and becoming dislodged from the micropyle. Procedures for maintaining Drosophila follicles in culture for imaging purposes have been published and used by this lab for live imaging. The duration of proposed imaging is longer than most of the live imaging experiments performed by others, thus keeping the tissue alive.
pose a potential problem. We have successfully performed live imaging for 12 to 16 hours, but ideally 24 hours of imaging should span events from Stage 9 onward. Minor modifications to previous protocols and optimization of mounting for light sheet microscopy should permit us to complete these experiments in both wild type and mutant egg chambers.
Figure 2.1: Polar cells form extensions that grow in a coordinated fashion into the micropyle. Formation of extensions was visualized in ovaries from otherwise wild type females in which Upd Gal4 was used to drive expression of a membrane-bound GFP in the polar cells. Ovaries were dissected, fixed, and immunostained with anti-GFP (green), and anti-Fas3 (red). (A, A’) At stage 10B prior to appearance of the micropyle, each polar cell forms a foot-like protrusion that contacts the oocyte. Multiple short filaments (A”, arrowheads) are produced at the periphery of each protrusion. (B, B’) Stages 11-12 show a constriction of the extensions, they begin to narrow and elongate prior to appearance of the micropyle. Each extension begins to form multiple filamentous processes (B”, arrowheads). (C, C’, E) During Stage 13 the micropyle appears and the extensions elongate, extending into the micropyle. (E) Deconvolved image that shows the degree of branching that occurs within the micropyle. Broad green auto fluorescence from the chorion marks the micropyle. Primary extensions from each polar cell form multiple filaments (arrowheads) that spread throughout the interior of the micropyle. (D, D’) At Stage 14 micropyle development is complete and the extensions disintegrate.
within the micropyle lumen (arrowheads). Bar is 10µm. Anterior is to the left.
Figure 2.2: Disruption of cell polarity determinants and regulators of skeletal dynamics disrupts formation of the polar cell extensions. Anterior to left. Bar=10µm.

Upd-Gal4 was used to inhibit regulators of either cell polarity or cytoskeletal dynamics specifically in the polar cells. Cell morphology was tracked using mCD8::GFP. (A-G) Dotted lines indicate oocyte boundary. Disruption of cell polarity proteins affected
extension outgrowth at Stage 10B, either inhibiting it completely (B) or causing a 
misoriented extension to form (C, D). Knock down of small GTPases in polar cells did 
not have a dramatic effect on extension development at Stage 10B, but did affect polar 
cell cohesion (G). (H-N) Asterisks indicate location of the micropyle. Expression of 
dominant negative $aPKC$ in polar cells prevented formation of the polar cell extension. 
(J, K) Knock down of $baz$ and $par6$ reduced extension length and width, but extensions 
still formed. (L-N) Mis-expression of dominant negative Rho GTPases disrupted 
extension coordination and outgrowth. Most extreme phenotype of dominant negative 
Rho shown Anterior to the left. Bar is 10µm
Figure 2.3: Model of polarity/small GTPase in establishment of cell polarity and cytoskeletal rearrangements during formation of the polar cell extensions. Prior to Stage 10B, (A) aPKC phosphorylates Bazooka. (B, C) Phosphorylated Bazooka is excluded from the Par6/aPKC/Cdc42 complex (C) and localizes with DE-Cadherin to form the Adherens Junction (B), determining apico-lateral polarity. Interaction of Cdc42 with Par6 is required to establish apical polarity, possibly through activation of aPKC. (D) Rac1 is initially localized at the AJ, but is eventually excluded from the AJ and relocates apically. (E) Rho1 interacts with both Par6/aPKC/Cdc42 and with phospho-Baz. It is believed to be required for stabilization of the AJ through regulation of endocytosis of
DE-Cadherin by Par6/aPKC/Cdc42 and through linking of the AJ to the actin cytoskeleton. (bottom two cells) During extension outgrowth beginning at Stage 10B, Cdc42 and Rac1 are responsible for apical expansion of the cell surface and actin polymerization to regulate development of lamellipodia and filopodia (brown bars). Rho1 regulates actomyosin assembly and contractility (purple bars).
Figure 2.4: Loss of cell polarity components or regulators of cytoskeletal dynamics causes polar cell defects at Stage 10B. Regulators of cell polarity were knocked down in the polar cells, and dominant negative forms of cytoskeletal dynamics were expressed in the polar cells to interfere with extension outgrowth. (A) Polar cells with reduced expression of Par6 or baz had a variety of morphological defects, but most polar cells formed lamellipodia at Stage 10B. In contrast, expression of dominant negative aPKC eliminated lamellipodium development in 80% of the polar cell pairs. (B) Dominant negative Rho1 and Cdc42 reduced polar cell cohesion.
Figure 2.5: Expression of dominant negative aPKC increases the proportion of micropyle defects. Unhatched eggs collected from females reared at 19C or 25C had a higher proportion of blocked micropyles than wild type females.
Figure 2.6: Actin and tubulin are both found in the polar cell extensions. Fixed egg chambers were immunostained for GFP labeled actin or EB1 to detect actin and microtubule contribution to the polar cell extensions. (A-D) Polar cells expressing Lifeact.GFP. During border cell migration actin is distributed as puncta over the surface of the cell. At Stage 10B (panel B, arrowhead) actin is found at a high concentration at the interface of the polar cells and oocyte. Actin is present in the elongating extensions. Interestingly, the presence of rings of actin can be seen on the polar cell bodies (panel D, arrowhead). (E-H) Tubulin was visualized using a GFP tagged form of EB1, a microtubule end capping protein. EB1 is present in the elongating extensions. Bar is 10µm. Anterior to the left.
Figure 2.7: Polar cell extensions from *upd3* mutants show loss of coordination and targeting defects. Polar cells from wild type and *upd3* mutants were labeled with mCD8::GFP. (A-A”) Wild type polar cell extensions elongate in a coordinated manner to form the micropyle lumen (arrowhead). (B-B”) The polar cells in *upd3* mutants form projections, but there is a loss of coordination during formation of the micropyle lumen. One extension enters the micropyle, but the other, rather than entering the micropyle, exits off to one side (B, arrowhead). A micropyle channel is formed, (B’, arrowhead), but it exits out of the side of the micropyle, away from the oocyte (B”). (C-C’) In a small fraction of egg chambers, the extension completely misses the micropyle (arrowheads point to extension and micropyle). (D) The proportion of eggs in which extensions exhibit uncoordinated or complete loss of targeting to the micropyle is greater when Jak/Stat activity is reduced in the polar cells. (E) Quantification of micropyle defects shows a higher proportion of blocked micropyles from eggs laid by *upd3* mutant females.

Anterior is to the left. Bar=10µm.
Figure 2.8: Reduction of Stat92E activity in the polar cells does not significantly affect border cell migration. Polar cells/border cell migration was assessed by quantifying polar cell proximity to the oocyte at Stage 10B using a previously described migration index assay (Ghiglione et al., 2002). Reduction of Stat92E or domeless in the polar cells did not significantly affect migration of the border cell cluster. Misexpression of latran, the negative regulator of Jak activity, in the polar cells did partially slow migration.
Figure 2.9: Polar cells with reduced Stat92E activity form longer extensions stage 10B than wild type. Differences in extension morphology were noted between WT and Stat92E knockdown polar cells. Image intensities are oversaturated in cell bodies to show extension detail. Anterior is to the left. (B) Polar cells in which Stat92E is knocked down have visibly longer extensions at Stage 10B than wild type cells (A), and extension outgrowth appeared uncoordinated. (C) Measurements were performed in ImageJ. Extension length was determined by measuring from the base of the cell body to the interface with the oocyte in ImageJ. Width was measured along the base of the extension at the polar cell/oocyte interface. Polar cells in which Stat92E was knocked
down had significantly longer extensions than wild type cells. (D) Knockdown of Stat92E in the polar cells resulted in a significantly higher proportion of unhatched eggs and eggs with blocked micropyles. (E) Egg from a wild type female showing a normal micropyle channel (arrowhead). (F) Egg from a Stat92E RNAi female showing a blocked micropyle (arrowhead).
Figure 2.10: Reduction of Jak/Stat activity in the polar cells reduces polar cell cohesion at Stage 10B. Polar cells from wild type and Stat92E knockdown flies were examined at Stage 10B for polar cell cohesion. Polar cells were either scored as normal (maintained full contact with each other) or exhibited reduced cohesion (began to separate from each other, but still retained contact). In polar cells expressing Stat92E RNAi, while still adhering to each other, exhibit reduced cohesion causing them to incompletely separate. This was not observed in wild type cells.
Figure 2.11: Reduction of Stat92E in the polar cells does not affect Fas3 or DE-Cadherin total protein levels. Upd Gal4>mCD8::GFP and Upd Gal4>mCD8::GFP; Stat92E RNAi flies were stained for Fas3 or DE-Cadherin. Volumetric measurements were made of Fas3 and DE-Cadherin immunofluorescence in the polar cells. Knockdown of Stat92E in the polar cells did not significantly change the levels of immunofluorescence measured.
Figure 2.12: Distribution patterns of Fas3 and DE-cadherin in wild type and 

Stat92E knockdown egg chambers. Upd Gal4 was used to drive expression of lifeact-GFP in the polar cells. Fas3 is expressed by both wild type polar cells and cells in which Stat92E is reduced (C-C’), with a region of extremely high expression detected by Fas3 staining at the site of extension outgrowth for each polar cell at Stage 10B (A-A’). At Stage 10B, cadherin expression is seen surrounding the polar cells of both wild type (B-B’) and Stat92E knockdown (D-D’) flies. There is a region underneath the extension in both resembling a star. At Stage 13, Fas3 surrounds is still seen between the polar cells, but is also found around the outside of the growing extension of both wild type (E-E”) and Stat92E knockdown (G-G”) polar cells. Furthermore, both genotypes show bright Fas3 staining at the region where the polar cells contact the micropyle. In contrast, at Stage 13, cadherin distribution does not appear to significantly change from that observed at Stage 10B.
Figure 2.13: Reduction of DE-Cadherin causes altered extension morphology and loss of coordinated outgrowth. (A-C) Dotted line indicates nurse cell oocyte boundary. (A) Wild type polar cells at Stage 10B remain in close contact and form a short foot that contacts the oocyte. (B, C) Knockdown of E-cadherin or mis-expression of dominant negative DE-Cadherin specifically in the polar cells causes polar cells to exhibit reduced cohesion, and alterations in extension morphology at Stage 10B. (D) Mis-expression of wild type cadherin has no effect on lamellipodium formation at Stage 10B. (E-H) Asterisk indicates location of the micropyle. (E) Wild type polar cells at Stage 13. (F, G) Stage 13 polar cells with reduced E-cadherin expression through either knockdown of E-Cadherin or expression of dominant negative cadherin exhibit a loss of coordinated extensions. (H) Mis-expression of E-cadherin has no effect on extension coordination or outgrowth. (I) Anterior polar cell pairs were quantified for either a long or uncoordinated extension at Stage 10B. While differences are observed, they are not significant. (J) Location of the polar cell extensions relative to the micropyle was quantified. Uncoordinated extensions
were scored as one extension in the micropyle and one missing the micropyle opening. Reduction of Stat92E activity or DE-Cad\textsuperscript{ON} significantly increased the frequency of uncoordinated extensions or extensions that missed the micropyle. Anterior is to the left. Bar=10\textmu m.

Supplemental movies

**Movie 1: Movie\_1\_wt\_lightsheet.mp4.** Live imaging using lightsheet microscopy of a Stage 12 egg chamber. The polar cells are marked with mCD8::GFP to permit visualization of extension development.

**Movie 2: Movie\_2\_wt\_s10b\_gfp\_fas3.mp4.** 3-D volume projection of polar cells at stage 10B showing the relative distribution of Fas3. Polar cells are labeled with GFP (green), Fas3 (red).

**Movie 3: Movie\_3\_statrnai\_s10b\_gfp\_fas3.mp4.** Upd-Gal4>Stat92E RNAi. 3-D volume projection of polar cells at stage 10B showing the relative distribution of Fas3. Polar cells are labeled with GFP (green), Fas3 (purple).

**Movie 4: Movie\_4\_wt\_s13\_gfp\_fas3.mp4.** 3-D volume projection of polar cells at stage 10B showing the relative distribution of Fas3. Polar cells are labeled with GFP (green), Fas3 (purple).

**Movie 5: Movie\_5\_statrnai\_s13\_gfp\_fas3.mp4.** Upd-Gal4>Stat92E RNAi. 3-D volume projection of polar cells at stage 10B showing the relative distribution of Fas3. Polar cells are labeled with GFP (green), Fas3 (purple).
**Movie 6: Movie_6_wt_s10b_gfp_cad.mp4.** upd-Gal4>Lifeact.GFP. 3-D volume projection of polar cells at stage 10B showing the relative distribution of Fas3. Polar cells are labeled with GFP (green), Fas3 (purple).

**Movie 7: Movie_7_wt_s13_gfp_cad.mp4.** upd-Gal4>Lifeact.GFP. 3-D volume projection of polar cells at stage 10B showing the relative distribution of Fas3. Polar cells are labeled with GFP (green), Fas3 (red).

**Movie 8: Movie_8_statrnai_s10b_gfp_cad.mp4.** Upd-Gal4>Stat92E RNAi. 3-D volume projection of polar cells at stage 10B showing the relative distribution of Fas3. Polar cells are labeled with GFP (green), Fas3 (purple).

**Movie 9: Movie_9_statrnai_13_gfp_cad.mp4.** Upd-Gal4>Stat92E RNAi. 3-D volume projection of polar cells at stage 10B showing the relative distribution of Fas3. Polar cells are labeled with GFP (green), Fas3 (purple).
Chapter Three: Genetic Architecture of Male Reproductive Lifespan

ABSTRACT

Aging is a progressive degeneration of cellular function leading to a decline in organism viability. Reproductive tissues also age, but at a rate that may be separable from the whole organism, and do so without affecting organism viability. Senescence is subject to a range of processes that act individually or interact with each other or the environment to determine the rate at which it occurs. Previously it was shown by our lab that reduction of Jak/Stat activity in male *Drosophila* shortened reproductive lifespan. However, Jak/Stat signaling is subject to a variety of regulatory mechanisms and is required for many of the processes that sustain fertility. The *Drosophila melanogaster* Genetic Reference Panel (DGRP), a panel of over 200 wild-derived, inbred, fully sequenced lines, was used to perform an unbiased screen for natural variants that regulate reproductive aging. Longitudinal analysis of thirty-nine lines for age at infertility uncovered candidate loci in functions relating to regulation of cellular homeostasis, Jak/Stat signaling regulation, and stem cell maintenance and proliferation. A cross sectional assay of males at 28 days of age presented candidate loci with functions in regulation of Jak/Stat signaling, and neurological development. Furthermore, both assays identified candidates with Stat92E binding sites and functions in cellular differentiation and sensory input. Thus, Jak/Stat signaling and its regulation either directly or through action of its targets is a natural component of reproductive maintenance.

Introduction

In contrast to semelparous organisms, iteroparous species reproduce multiple times during their lifespan. Underlying this ability to maintain fertility for an extended length of time are multiple genetic and environmental inputs. Integration of these stimuli
in the reproductive tissues helps promote the continuous production of viable gametes, one necessary component of reproductive maintenance. Gametogenesis depends on the maintenance and proliferation of a pool of germline stem cells, and proper differentiation of germline cells to form egg or sperm. But, there are other factors that contribute to fertility. Behaviors, such as courtship, nutrient sensing, maintenance of cellular homeostasis, and environment all interact with gametogenesis to determine fecundity. Environmental factors are difficult to identify and measure, but behaviors, signaling, gametogenesis, metabolism, etc. are at least partially under the control of genetic components that can be identified and their relative contribution to a phenotype measured.

Maintenance of cellular homeostasis is regulated by inputs from these factors. With time, the ability of a cell to integrate signals and cope with damage decreases, contributing to senescence. The cumulative effect of deterioration at the cellular level leads to a decline in tissue and organism viability. Several tissues in adults have a pool of stem cells that can undergo a low level of proliferation (Liu et al., 2010; Osman et al., 2012; Zhou et al., 2013). In contrast, reproductive tissues have a population of continuously proliferating stem cells that contribute to gametogenesis. While testes and ovaries senesce, they degenerate without affecting organism viability, which distinguishes them from other tissues such as intestine and skin. Furthermore, many of the same factors that influence organismal longevity promote reproductive maintenance (Hsin and Kenyon, 1999; Partridge et al., 2011). In addition to environmental effects, these include decreased protein homeostasis, DNA repair defects, changes in behaviors, and decrease in the stem cell population. There are likely additional unidentified elements that also contribute to senescence. Therefore, reproductive senescence is a model that can be used to understand the individual elements that influence aging as a whole. Previous work showed that, although fertile and viable, a
reduction of Jak/Stat activity through loss of upd3 results in premature loss of fertility in males (Wang et al., 2014b). The pathway is required for maintenance of male germline stem cells, and decreased activity in the testis leads to a decrease in the stem cell population (Boyle et al., 2007; Tang, 2014; Toledano et al., 2012). However, there are likely other pathway regulators and targets of Jak signaling required for maintaining fertility that are currently not known. It is therefore desirable to use a method that uses the natural variation in a population to identify candidate genes in an unbiased way. A genome-wide association study (GWAS) is an ideal way to pinpoint loci contributing to a trait of interest through association of sequenced genetic markers with a given phenotype.

Jak/Stat signaling is required in the testis for maintenance of the germline stem cell pool (Boyle et al., 2007), and the pathway ligands Upd and Upd3 have overlapping expression in the testis at the hub (Tang, 2014). We previously showed males with reduced Jak/Stat activity due to loss of upd3 had shortened reproductive lifespans compared to wild type males (Wang et al., 2014b). The Jak pathway also has functions beyond stem cell maintenance. Given the complex nature of reproductive senescence, and the pleiotropy of Jak/Stat signaling, it is likely that there are other functions of Jak/Stat required for maintaining fertility over the lifetime of a male. For example, there is evidence that Jak/Stat signaling has a critical function in the process of spermatid individualization (Tang, 2014), a step required to produce sperm. However, the identity of regulators and targets of signaling in this process is not known. Jak/Stat activity has functions beyond gametogenesis required for cellular homeostasis at tissue and organ level, thus the number of potential processes contributing to reproductive lifespan is quite large.

Degeneration in a wide variety of functions beyond gametogenesis contribute to the rate at which reproductive senescence occurs. Each function is itself subject to
genetic control and interaction with the environment, making identification of pathways leading to senescence a daunting task. An unbiased approach to screening the genome for candidate loci with associations to reproductive maintenance is required. One suitable method is a genome-wide association study or GWAS, which associates genetic variants with a trait of interest to identify candidate loci. This approach was used to determine the genetics underlying male reproductive lifespan.

_Drosophila melanogaster_ is an excellent tool for unearthing the genetics underlying reproductive senescence. With a large variety of available genetic tools, short development period, and reproductive tissues that are easy to manipulate, the fruit fly is a useful model for addressing this problem. One such tool is the _Drosophila melanogaster_ Genetic Reference Panel (DGRP). The DGRP was developed to explore the architecture of quantitative traits. Consisting of over 200 lines derived from wild-caught flies, the panel is fully sequenced and has over 4 million genetic markers identified, encompassing natural variation present in the population. Combined with publicly available sequence and phenotypic data, and an online analysis pipeline, the DGRP is a useful tool for performing a GWAS (Huang et al., 2014; Mackay et al., 2012).

A GWAS of a subset of DGRP lines was used to identify potential targets of Jak/Stat signaling responsible for regulation of reproductive lifespan. A benefit of this method is that it permits a screen for all variants in the genome that contribute to reproductive senescence, regardless of whether their function relates to Jak/Stat activity or not. Among the results were genes with roles in chemosensory bristle development, and courtship behaviors, as well as stem cell maintenance and differentiation. Many genes with functions in neurogenesis and processing of sensory input were also identified, consistent with findings from other groups that found sensory perception and processing are an important factor in determining lifespan.
Materials and Methods

Fly Strains and Husbandry

Testing was performed using males from the *Drosophila melanogaster* Genetic Reference Panel (DGRP). The DGRP is a set of over 200 publicly available lines created from wild-caught flies, thus capturing the natural genetic variation present within the population. Each line was created through 20 generations of inbreeding of siblings produced by gravid females. The panel is fully sequenced, with over 4 million variants in the form of SNPs, microsatellites, insertions, and deletions characterized (Huang et al., 2014; Mackay et al., 2012). A subset of thirty-nine DGRP lines was obtained for testing from the Bloomington Drosophila Stock Center. Unless otherwise stated, flies were reared at 25°C.

Production of Virgins for Mating Assays

Transgenic “virginator” flies, w[1118]/Dp(2;Y)G, P[w+[mC]=hs-hid]Y, were used to generate virgin females for the mating assays. The stock is w1118 with a hs-hid transgene on the Y chromosome (Van Doren, 2005). Presence of hs-hid on the Y chromosome, permits selective elimination of males. Virgin females were obtained by heat shocking bottles containing embryos for 4-5 hours at 37°C for four to five days. Females were collected upon eclosion and maintained in vials at room temperature. Females older than one week old were not used for crosses.

Longitudinal Reproductive Lifespan Assay

Reproductive lifespans for individual males were tracked using a longitudinal assay. From each line, 36 males were collected within one day of eclosion were collected and placed in 5mL polystyrene vials (Globe Scientific catalog no. 110410F) containing 1mL Nutri-Fly BF fly food (Genessee Scientific Catalog no.66-112) prepared
per manufacturer’s instructions and crossed with two virgin females no older than a week old. The males were crossed to new virgin females every Monday, Wednesday, and Friday, using CO₂ to anesthetize animals during each transfer and crosses were incubated at 25°C. Mated females were retained in the original vial and vials were scored for progeny within one week. (Figure 3.1). Any males that died or escaped prior to becoming infertile were excluded from all calculations, thus the actual number of males tested per line could be less than the original 36 males. A list of total sample size is shown in Table 3.1. Male fertility was determined by the presence or absence of progeny after four days. If larvae were present, the male was scored as fertile, and infertile if no larvae were observed in the vial. The age at infertility was specified as the age of the male on the date of the last transfer to produce offspring after two consecutive crosses without larvae observed. This is a conservative estimate of the true age at infertility because males were not crossed daily with new virgin females. Thus, the actual age of infertility is not known, and may be slightly longer than that recorded. Scoring was performed the same way for all lines, therefore relative reproductive lifespans between lines should be accurately reflected.

Cross-Sectional Reproductive Lifespan Assay

Instead of tracking individual male reproductive lifespan, an alternate approach was used to determine the proportion of males fertile at a given age. Males aged 0-1 days were collected from each line and aged in groups of no more than 10 with an equal number of females in fly vials containing cornmeal fly medium and a small piece of tissue on the surface to prevent flies from becoming stuck in the food. Males were aged at 25°C and transferred to new vials every few days. At 28 days old, males were crossed to females as described above, but were left with females for a week. Vials were scored for progeny and the proportion of fertile males was calculated (Figure 3.2).
Once phenotypic data were obtained, a GWAS was performed using the DGRP analysis pipeline on the DGRP website (Huang et al., 2014; Mackay et al., 2012). The pipeline uses the programs PLiNK, and R, to perform a statistical association between phenotypic data and sequenced genetic variants for each line. Annotation of results is performed utilizing SNPEff. Typically, a genome-wide significance threshold is set at $p=10^{-8}$. Variants with an association meeting or exceeding this threshold are considered to have a significant association with the trait of interest. The significance threshold is conservative, as it employs a Bonferroni correction to reduce the number of false associations.

One requirement for performing a GWAS is variation between genetically distinct individuals in a population. The DGRP consists of inbred lines that exhibit approximately 97% homozygosity (Mackay et al., 2012). Hence, every individual in a line, in theory, is genetically identical to every other individual at most loci. Therefore, each line can be treated as a single individual and the total number of lines is designated as the test population. Males from thirty-nine lines were tested using a longitudinal assay. The calculated mean age at infertility ranged from 7 days to 19 days and showed that the DGRP is an appropriate tool for studying this phenotype, as variation is observed between lines. (Figure 3.3 A). However, variation within lines is also high. Additionally, when lines were re-tested for age at infertility using the longitudinal assay, there was a high amount of variability observed between trials (Figure 3.3 B).

For the longitudinal study, several approaches to data analysis were used. First a GWAS of mean reproductive lifespan was performed. But given the high level of inter- and intra-line variation, it was decided to also perform a GWAS of median reproductive lifespan to reduce the effect of outliers. Data were formatted and uploaded to the DGRP analysis pipeline (Huang et al., 2014; Mackay et al., 2012). Determination of significant
SNPs at individual timepoints was evaluated by calculating the proportion of fertile males at six, twelve, and eighteen days and analyzed as described above. Proportion of males fertile at 28 days was used to perform the GWAS of cross-sectional assay data.

**Determination of Sources of Variation**

To determine whether reproductive lifespan variation was attributable to differences in experimenter manipulation three experimenters each tested 36 males from each of three DGRP lines using the longitudinal assay. Each experimenter consistently used their preferred transfer method, either forceps or paintbrush, and testing was performed at the same workstation over the duration of the assay. Contribution of workstation and transfer method to variation was tested, controlling for experimenter. Again, three experimenters performed the testing. Each was assigned a different DGRP line to test using the longitudinal assay. A total of seventy-two males were tested per line, by testing half at a workstation at the main bench, and the other half at a station at an auxiliary bench. Transfer method was tested simultaneously by transferring 18 males using forceps and 18 males using a paintbrush at each of the two workstations. Results from each analysis were compared using ANOVA (Sigma Plot).

**Quantification of Chemosensory Bristles**

Lines carrying either the major or minor allele for *Poxn* were selected. Front left legs of males were removed and mounted in glycerol, and chemosensory bristles were counted using a Nikon E800 microscope with a 40X DIC objective. The foreleg of male *Drosophila* is composed of five tarsal segments and the tibia, which is the largest segment closest to the body. The number of chemosensory bristles on each segment was quantified based on bristle morphology. Chemosensory bristles were identified based on two criteria, their long, fine shape, and the presence of bracts at the base of
the bristle. In contrast, mechanosensory bristles are shorter and thicker in shape, and are not associated with bracts.
GWAS of mean male reproductive lifespan using a longitudinal assay

The DGRP reports and annotates variants that exceed a significance level of $p=10^{-5}$ as nominally significant. Results are corrected for the effect of Wolbachia infection. For mean age at infertility, a total of 59 variants reached a nominally significant $p$-value for reporting. Variants reported included SNPs, insertions, and deletions (Figure 3.4, Table 3.2, Figure 3.7). An LD heatmap showed a large block of linkage disequilibrium on Chromosome 2R (Figure 3.6). This may be reflective of the influence of outliers on the results of the mean reproductive lifespan and indicate a high degree of linkage between SNPs in this region. Therefore, the resolution of the GWAS in this region cannot reach the level of single gene. Thus, when interpreting results, genes in the surrounding genomic region should also be investigated.

Eight variants fell in intergenic regions. Of the top annotated variants that met the level for nominal significance, several fell within or near genes with documented functions in metabolism, immunity, and neurogenesis as determined by observation of mutant phenotypes (Table 3.2). The top variant was located downstream of Cyp4ad1, a gene implicated in oxidation/reduction activity. Regulation of oxidative stress is of importance at the cellular level to prevent DNA and protein damage (Höhn et al., 2017). A gene ontology analysis was conducted using the Panther Gene Ontology website. The list of genes associated with annotated variants was submitted to the website to generate a list of biological functional classes. Genes active in metabolism and cellular processes constituted the largest proportion of functional classes identified (Figure 3.5).
GWAS of median reproductive lifespan

Male reproductive lifespans as determined by the longitudinal assay demonstrated high inter- and intra-line variability (Figure 3.3 B). There were many outliers that were initially sterile, or were fertile for only one transfer. Likewise, some males were fertile much longer than the rest of the cohort. The reproductive lifespan data generated for the previous analysis was re-analyzed by determining the median age at infertility for each line and these data were used to perform a second GWAS. Use of the median age should lessen the effect of outliers on the results. Median age at infertility ranged from 1 day to 20 days old.

Confidence levels for associations from the GWAS of median age at infertility failed to reach a genome-wide significance level of $10^{-8}$. A total of thirteen variants were nominally significant (Figure 3.8, Table 3.3, Figure 3.10). Analysis of linkage disequilibrium for median reproductive lifespan gave a moderate sized block with high linkage on Chromosome 3R, and smaller blocks scattered along the arms of the other chromosomes (Figure 3.9). Comparison of LD for median reproductive lifespan with that of mean reproductive lifespan shows a decrease in linkage with the median analysis. This suggests that outliers in the data in the mean analysis had a large effect on the association analysis.

Four variants were located within intergenic regions, with the remaining variants falling in or near annotated genes. As with the GWAS of mean results, many of the variants identified fell in or near genes with roles in reproduction, neurogenesis, and cellular homeostasis, functions all required for fecundity and longevity (Table 3.3). The most significant variant located near or in a named gene was a SNP located approximately 1800 bases downstream of the gene Pox-neuro (Poxn) in a transcription factor binding site. This gene encodes the highly pleiotropic transcription factor Poxn, which has multiple roles in neurogenesis, specification of chemosensory neurons,
development of the ventral nerve chord, and functions in the neurons of the antennal lobe during brain development (Boll and Noll, 2002; Minocha et al., 2017). It also is required for proper development of male genitalia, and male courtship behaviors (Boll and Noll, 2002).

Cellular homeostasis is a determinant of longevity. A candidate locus with functions in homeostasis were associated with median reproductive lifespan. The first SNP is located in an intron of CG30377, a gene that codes for a protein of unknown function, but mutants have defects in copper homeostasis (Norgate et al., 2007). A primary mechanism to prevent damage from reactive oxygen species is through action of catalase and cooper zinc superoxide dismutase (sod) an enzyme that defends against oxidative stress by disposing of oxygen free radicals (Seto et al., 1990). Sequestration of copper and zinc is critical to cell survival, and if not controlled properly metal ions can themselves contribute to oxidative damage (Kozlowski et al., 2009).

Candidate SNPs were also associated with genes functioning in reproduction. One SNP fell within an intron of the gene gone early (goe/CG9634), a metalloendopeptidase. A role for this gene in regulation of the size of the germline stem cell pool in the ovary have been demonstrated (Matsuoka et al., 2014), and identification of this gene as a candidate locus could indicate a requirement in males as well. A second candidate locus was associated with a SNP located in an intron of the gene vibrator (vib). This gene has no known molecular function, but participates in male meiosis during spermatogenesis (Giansanti et al., 2004). The location of variants in introns of these genes could conceivably changes in protein function through altered splicing.

Investigation of Poxn as a candidate locus for reproductive aging

The variant in an annotated gene with the highest significance was located
downstream of the gene *pox neuro* (*Poxn*). *Poxn* lies within a much larger gene, *tungus* (*tun*) that has functions in olfactory learning and memory (Figure 3.11). *Poxn* codes for a transcription factor with numerous functions during development including formation of the male genitalia, and determination of chemosensory bristle fate from mechanosensory bristle fate (Awasaki and Kimura, 2001; Boll and Noll, 2002). *Poxn* mutant males have normal testes, but are behaviorally sterile, failing to exhibit courtship behavior resulting in failure to mate (Boll and Noll, 2002). This is due, in part, to the chemosensory bristles adopting a mechanosensory fate. When reproductive lifespans were compared for males, shorter reproductive lifespans were associated with lines carrying the minor allele (Figure 3.12 A).

*Poxn* could also affect reproduction through its involvement in development of genitalia. However, this is a less likely scenario because a genitalia defect should not change with age, having no effect on reproductive lifespan. It was thus hypothesized that the SNP affected male mating activity by affecting the number of chemosensory bristles. Easily identified by their morphology, chemosensory bristles on the forelegs of males are used for pheromone sensing during mating. Complete loss of function of *Poxn* results in a loss of chemosensory bristles in favor of a mechanosensory fate. Thus, a reduction in the number of chemosensory bristles should impair the ability of a male to sense and mate with a female. Four lines each homozygous for the major or minor allele for *Poxn* were selected and chemosensory bristles were quantified for each segment of the front left leg (Figure 3.12 B). While difference in bristle number was not significantly significant, males carrying the minor allele had fewer bristles per segment and overall. This might be biologically significant, but given the function of *Poxn* during development the ventral nerve cord, there may be alterations in behavior or neuronal functioning contributing to the phenotype.
Evaluation of fertility at specific ages

Previous work has shown an age-dependent requirement for given SNPs in female fecundity and aging (Durham et al., 2014). In the study, candidate genes were examined for their impact on fecundity and lifespan at various ages. To determine how factors in male reproductive maintenance vary with age, the proportion of fertile males was determined for each line and the data for each age were analyzed using the DGRP analysis pipeline.

GWAS of data at six days old resulted in 16 annotated variants with a nominal p<10^{-6}. Two variants fell within an intron of a negative regulator of the Jak/Stat pathway, \textit{Ptp61F} (Figure 3.13 A, A’, Figure 3.14 A). Other variants lay in or near genes with annotated roles in cell proliferation, neurogenesis, and GPCR signaling. At ages four and five days, \textit{trol} and \textit{ttv} were identified as candidate loci (data not shown). \textit{trol} is a heparan sulfate proteoglycan (HSPG), while \textit{ttv} modifies HSPGs. Heparan sulfate proteoglycans are a component of the extra-cellular matrix and as such, contribute to neuronal guidance, and signaling through regulation of ligand diffusion, stability, and presentation to the receptor. One variant \textit{CG10738}, is a gene of unknown function, predicted to code for a guanylate cyclase. Through experimental evidence, it was shown to be a positive regulator of cell proliferation (Zacharogianni et al., 2011). The variant associated with this gene lies in a coding region, and is predicted be a modifier of gene activity. As with the GWAS of median reproductive lifespan, \textit{Poxn} was identified as a candidate locus. However, this SNP fell upstream of the gene rather than downstream. It is possible that these loci reflect issues with development rather than reproduction that could contribute to lower fitness.

At twelve days, candidate variants were identified that fell in genes that have functions in aging and reproduction (Figure 3.13 B, B’, Figure 3.14 B). Defects in DNA repair mechanisms, protein ubiquitination, and oxidative stress are all known to
contribute to the loss of cellular homeostasis observed during senescence. One candidate locus lies in the coding region of *stall* (*stl*), an extra-cellular metalloendopeptidase. Gene activity is needed in females during stalk cell development (Willard et al., 2004), thus its presence in a GWAS of male reproductive senescence is surprising. It is possible that it has an undiscovered function in males as well. A second, developmental requirement for this gene during migration of the v’ch1 sensory neuron during embryogenesis (Lhamo and Ismat, 2015). Male courtship behavior is a large component of male fertility. A variant located with an intron was associated with *Pde1c*. The protein coded for by this gene is a phosphodiesterase, and is required for male fertility. Mutation of the gene in males causes changes in courtship behavior and copulation latency. However, mutants have reduced fertility or are sterile. Males that do eventually mate either appear to transfer fewer sperm to the female than wild type males, or the females either degrade or expel the sperm from the reproductive tract (Morton et al., 2010).

At eighteen days, loci with documented links to lifespan and fecundity were identified. The most significant variants identified had roles in neurological functions, lying in genes that have been identified as having roles in chemosensory functions, and learning and memory (3.13 C, C’, Figure 3.14 C). *dpr12* does not have an identified molecular function but work has shown it is required for synaptic targeting (Kurusu et al., 2008) and based on sequence similarity to *dpr1*, is believed to have a role in chemosensory perception (Nakamura et al., 2002). One candidate SNP fell upstream of *Drip*, an aquaporin that regulates water balance, a gene associated with female fecundity. Reduction of *Drip* expression has a negative effect on fecundity, appearing to do so through modulating expression of two neuropeptides: *corazonin* and *pale* (Bergland et al., 2012). *Drip* has also been associated with lifespan, though the exact mechanism is not known (Paik et al., 2012).
Durham et. al., performed a GWAS examining age-specific effects of SNPs on female *Drosophila* lifespan and lifetime fecundity (Durham et al., 2014). When the results of the GWAS results at specific timepoints, GWAS of mean reproductive lifespan, and GWAS of median reproductive lifespan are compared with the results of Durham et al., similarities in candidate loci are seen. A significant gene identified in the group’s screen also identified in the median GWAS is A2bp1 (aka Rbfox1). This gene binds to RNA and regulates mRNA alternative splicing, with roles in development of germline cysts in females. The role of this gene in regulation of spermatogenesis has not been documented.

*Differences between experimenters are the largest source of variation in the longitudinal assay*

Fitness traits are subject to the influence and interaction of both genetic and environmental factors. However, the effects environmental contributions are more difficult to pinpoint, quantify, and control. As discussed earlier, repeated tests of the same line for reproductive lifespan gave varying results. We therefore wanted to identify potential sources that contributed to the observed variation for controlling their effects on the assay. The large quantity of crosses that had to be conducted to generate results required the assistance of many undergraduate experimenters. Prolonged exposure to CO₂, which was used to anesthetize the flies during transfer, can lead to alterations in fertility and behaviors such as courtship. Each experimenter varied in their fly handling ability and their speed at performing the crosses, creating a source of variability. Additionally, given the number of students required to perform crosses, it was necessary to use auxiliary workstations at another bench. These used a separate CO₂ tank and regulator, potentially adding to the variability in CO₂ exposure during transfers. Students were given the option of using either paintbrush or forceps to transfer males.
Paintbrushes are a gentler method of manipulating flies, and students were encouraged to use them. However, some students preferred to use forceps to move flies, but the likelihood of physically damaging the animals is greater. Experimenter, workstation, and method of manipulation are all easy to test and correct for, contributing to their selection.

Three DGRP lines were selected for testing student effect on variability. Three experimenters each tested all three lines using their preferred transfer method and workstations at the main bench. A total of 36 males from each line were tested using the longitudinal assay and the mean reproductive lifespan for each line was determined (Figure 3.20 A). For all three lines, one experimenter produced significantly higher results, with a p-value <0.001 between experimenter 3 and the other two students performing the testing.

Contribution of manipulation method and workstation to variability was also tested. Experimenter effects were controlled for by assigning each experimenter a different line for testing. A total of 72 males were tested, with 36 males transferred at a workstation at the main bench and the other 36 crossed at the auxiliary bench. Of the males tested at each workstation, half were manipulated using a paintbrush and the others using forceps. Reproductive lifespans for each trial was determined and plotted (Figure 3.20 B). There was no significant difference between transfer methods or workstations used to perform the assay, as determined using a Mann-Whitney test of significance. Thus, the experimenter performing the testing is a contributor to variability within lines.

Cross-sectional analysis of reproductive lifespan

The mean and median reproductive lifespans using the longitudinal assay were unexpectedly short, with variation in assay results attributed to the experimenter performing the analysis. It is likely that repeated handling resulted in physical damage to
males over the duration of the assay, causing a premature cessation of fertility. Therefore, an attempt was made to find an alternate approach to assess reproductive lifespan by reducing the effect of experimenter contribution to variability. A cross-sectional assay was developed by aging males with females, then performing a reproductive lifespan assay at a single timepoint, in this case 28 days. However, this assay is selective toward longer-lived individuals, and thus could bias the results of the GWAS (Figure 3.15).

A total of 77 annotated SNPs met or exceeded a nominal significance level of $10^{-5}$, although no SNPs exceeded a genome-wide significance level of $10^{-8}$ (Figure 3.16, Table 3.4, Figure 3.19). Linkage disequilibrium was higher on Chromosome 3R than in the longitudinal assays, and there were smaller blocks distributed throughout other genomic regions (Figure 3.18). This may reflect the nature of the assay method. The GWAS was performed on larger samples and tested the proportion of fertile males rather than an average or median age. This may have eliminated some of the observed variability. High LD is still present so identification down to a single gene is less likely, but they are smaller, which narrows down regions that contain candidate loci.

GO analysis of candidate loci found many more functional classes than the previous analyses. Like the longitudinal assay results, metabolism is a large component of gene functional classes. Genes responsible for maintaining cellular processes and homeostasis constitute and even greater portion of gene ontology results. Of these, 15 SNPs fell in regions without an annotated gene nearby (Figure 3.17). One significantly associated SNP lay in an intron in the gene *serrano* (*sano*). This gene has no known molecular function, but is an apically expressed regulator of planar cell polarity and tracheal tube length (Chung et al., 2009). Furthermore, it is differentially expressed between males and females in the genital disk (Takahara and Takahashi, 2015) and thus may have a role in development of genitalia.
Regulation of signaling can occur through direct molecular control, turnover of surface receptors, and modification of ECM components, for example. The importance of signaling modulation was also detected through identification of candidate loci associated with regulation of signaling pathways. As with the longitudinal assays, a SNP fell within an intron for *Ptp61F*, a negative regulator of Jak/Stat signaling (Muller et al., 2005). It also has been demonstrated to negatively regulate the MAPK pathway and insulin signaling (Tchankouo-Nguetcheu et al., 2014). Another potential signaling regulator identified in this analysis was *abnormal wing discs (awd)*, a nucleotide diphosphate kinase. This is a homolog of a NM23, a suppressor of tumor metastasis in humans. *awd* is a participant in cell motility, organization of cell junctions, and tracheal cell migration. It could potentially act as a regulator of Jak/Stat signaling, as it functions during migration of tracheal cells through modulating the turnover of FGF receptors at the cell surface via endocytosis (Dammai et al., 2003).
Discussion

The first aim of this study was to identify potential Jak/Stat pathway regulators and targets required for male reproductive maintenance. The benefit of using a GWAS is that it is unbiased, also providing insight into other mechanisms that are required during the aging process. Use of the DGRP permitted screening the natural variation present in the panel for variants associated with reproductive senescence. The beauty of the GWAS as a gene discovery method is that using a high number of variants permits identification of potential causal variants down to the level of a single gene.

Mechanisms for maintaining homeostasis are common between mean and median reproductive lifespan

Loss of tissue and organism viability results from many factors including loss of cellular homeostasis, changes in stem cell proliferation and differentiation, defects in DNA repair, efficiency of processing sensory inputs, and changes in behavior. Similarly, fertility is dependent upon these functions with an additional developmental aspect regarding formation of gametes. Both lifespan and reproduction are linked, with age at reproduction influencing organism lifespan (Partridge and Fowler, 1992; Prowse and Partridge, 1997). Most of what is known about aging and reproduction has been learned by examining females, while less work has been done to understand male reproductive aging. In this dissertation, we set out to understand the genetics underlying male reproductive senescence.

Functional classes of candidate loci are shared between analyses

A GWAS was performed for mean and median reproductive lifespan. These analyses uncovered genes required for cellular homeostasis. Homeostasis is regulated
by protein and organelle turnover, DNA repair, and response to oxidative stress, among other things. Oxidative stress is a known contributor to aging and is implicated in DNA damage, protein damage, and neuronal degeneration (Korovila et al., 2017). Previously, another group has assessed genetics underlying oxidative stress in *Drosophila*. Using the DGRP to perform a GWAS they found a sexually dimorphic response to oxidative stress and survival varied with the type of stress inducer each sex was grown on (Weber et al., 2012). While genes identified in their analysis were not found in this study, candidate loci shown to be involved with redox activity were identified, implicating this process in reproductive maintenance.

Related to control of oxidative stress is a requirement for metal ion homeostasis. Metal ion homeostasis is a critical requirement for control of oxidative stress. Ions such as Cu and Fe possess high redox activity, thus if allowed to exist unchecked in a biological system can be highly deleterious through generation of oxidative damage (Norgate et al., 2007). Therefore, cells must have systems in place in the form of chaperone proteins that control transport of metal ions thereby limiting their ability to create oxidative damage (Kozlowski et al., 2009). Furthermore, many proteins require metal ions for proper function, with requirements in proteins active in electron transfer, and regulation of oxygen radicals and degradation of ROS such as the enzyme copper-zinc superoxide dismutase (Sod) (Holm et al., 1996; Korovila et al., 2017; Kozlowski et al., 2009).

The analyses performed in this dissertation found variants associated with copper-ion homeostasis. The candidate locus associated with these variants has an unknown molecular function. As described above, oxidative stress prevention utilizes sequestration and enzymatic function to control damage from oxygen free radicals. Thus, the locus uncovered in this analysis may be required for metal ion transport, or interact with enzymes such as Sod. These results differ from a study performed by
others for sex-differences in oxidative stress resistance (Weber et al., 2012) in which males and females were subjected to paraquat or menadione sodium bisulfite (MSB). The candidates identified by Weber et al., had not been previously associated with resistance to oxidative stress.

A critical process needed for fertility is the production of gametes. This requires maintenance of a stem cell population and differentiation into either egg or sperm. Variants were located in or near candidate loci required for neuronal stem cell maintenance. It is possible that this gene also participates in germline stem cell maintenance. Other candidates fell in genes regulating the cell division, including genes that code for cell cycle proteins or gene products required during meiosis in males. This is not surprising given that reproductive lifespan was being tested. Gametogenesis is an ongoing process in males, and begins with mitosis of a germline stem cell to produce a daughter cell which then undergoes meiosis and differentiates. An inability to produce sufficient daughter germline cells that can go on to differentiate would impact fertility.

The largest gene ontology category in the GWAS analyses was for genes with metabolic processes. Diet is a known regulator of lifespan and reproduction, with insulin signaling a significant regulator of longevity in flies, worms, and mammals (Dantzer and Swanson, 2012; Kenyon, 2010; Partridge et al., 2011; Rajan and Perrimon, 2012; Tatar, 2010). We found genes associated with carbohydrate and glycogen metabolism. Furthermore, a candidate locus in a gene with a role in regulation of insulin receptors at the cell surface was identified. Furthermore, the mean GWAS results also gave SNPs associated with genes required for immunity, and development. These differences between the two approaches to analysis may possibly be attributed to the presence of data from outliers in the mean GWAS, driving the results in the direction of loci regulating organism viability in contrast to loci with functions in viability and reproduction.
There is a high level of inter- and intra-line variability in the longitudinal assay

The longitudinal assay allows tracking of individual male reproductive lifespan. Reproductive maintenance is subject to both genetic and environmental inputs. The genetic contribution to this trait is relatively easy to determine, but the effects of environmental factors are more difficult to assess. One possible explanation for the high inter and intra-line variability is a high contribution of an environmental influence on male fertility. Things such as food composition, dehydration, and exposure to CO2 and handling could all factor into reproductive lifespan. A genetic component could conceivably contribute to variability, either alone or more likely through interaction with environment. The DGRP males are highly inbred and they may possess alleles that, while not lethal, do impair viability. They may directly influence overall health of the organism, or their interaction with the environment may have affect reproductive status. The lack of reproducibility makes verification through replication of the GWAS difficult.

Reproducibility of the longitudinal assay was repeated for several lines, with some trials providing significantly different results. Analysis of possible sources of variability investigated included experimenter, workstation, and transfer method. The primary source of variability was the experimenter performing the test, with workstation and transfer method having little effect. To attempt to achieve statistical power, many student experimenters were used to perform fly transfers. The use of CO2 is a common method of anesthetizing flies, but exposure for as little as 5 minutes can have adverse effects on Drosophila behavior, motor activity, and immunity (Bartholomew et al., 2015; Helenius et al., 2009). It is likely that transfer using anesthetization was affecting behavior and fertility in both males and females.

The effect of handling also can explain the difference in reproductive lifespan observed between the longitudinal and cross-sectional assays. The longitudinal assay tested 36 males per line, although for some lines this was not possible. Males that died
or escaped prior to becoming infertile were not included in the analysis, so data from fewer than 36 males was typically analyzed. This, taken with the fact that only 39 lines were tested, the study lacks power to identify rare variants associated with reproductive senescence. While the cross-sectional assay reduced the effects of handling, a minimum of 20 males were tested for each line. Again, this sample size could not be met due to fitness issues. Other investigators who have tested more lines have eliminated lines with low fitness from the analysis which was not feasible here.

**Sensory perception and behaviors are required for reproductive maintenance**

Perception of environmental cues is a primary requirement to organism survival and reproduction. The ability to sense potential dangers, nutrient sources, or mates, and to respond appropriately requires a means of sensing and processing environmental cues. Extensive work has shown a relationship between sensory inputs and lifespan and reproduction (Alcedo and Kenyon, 2004; Gendron et al., 2015; Pletcher, 2009). Insulin signaling is central to the connection between environmental inputs and lifespan. In flies and worms, gustatory input is separate from caloric intake, and the nature of the inputs can have a positive or negative effect on lifespan (Alcedo et al., 2013; Ostojic et al., 2014). Action of the pathway occurs through gustatory neurons in worm by modulation of the insulin pathway. In contrast, odorant receptors have only a modest effect on lifespan, but also appear to influence reproduction in worm through the insulin pathway (Alcedo and Kenyon, 2004). Furthermore, select neurons can positively effect lifespan while others have the opposite effect (Alcedo and Kenyon, 2004; Ostojic et al., 2014).

**Poxn may affect reproductive lifespan through modulated chemosensing or courtship behavior**

In *Drosophila*, *Poxn* which was associated with a variant with the highest
significance in an annotated gene in the GWAS of median reproductive lifespan, has been demonstrated to be required for courtship and chemosensory bristle development in males (Boll and Noll, 2002). *Poxn* is also required for development of taste bristles. Mutation of *Poxn* in flies results in a loss of chemosensory innervation. Mutant flies lacking subsets of taste bristles, such as labellar or leg bristles, lived longer than control flies with all gustatory bristles present. Furthermore, flies lacking taste bristles consumed more food than control flies, yet still had a longer lifespan and similar reproductive output (Ostojic et al., 2014).

*Poxn* is highly pleiotropic, with multiple functions during development including development of chemosensory neurons and chemosensory bristles that subsequently have effects on male courtship behaviors (Boll and Noll, 2002). *Drosophila* male courtship consists of a complex series of behaviors requiring many different sensory cues (Markow, 1987). How these behaviors are performed can potentially effect whether mating occurs. Genetic architecture of courtship and mating behaviors is quantitative, the composition of which was determined by another group using the DGRP. A GWAS of mating progression led to the identification of two candidate loci: *Serrate* (*Ser*), and *Furin1* (Mackay et al., 2015). *Serrate* is a component of the Notch signaling pathway and is an active participant during neurogenesis. *Furin1* codes for an endopeptidase that has functions in receptor clustering and organization and targeting of synapses. *Poxn* is also active in neurogenesis. While the Mackay lab study did not identify *Poxn* in their screen, they also analyzed many more lines and focused solely on courtship progression. However, the mating progression analysis and the work in this dissertation indicate neurological development is an important part in regulation of behaviors such as courtship. The significance of neurogenesis to lifespan and fertility is also seen in the results from the cross-sectional assay, in which three of the top ten variants were located in or near genes with neuronal functions. Thus, while the candidate loci were not
identical to those identified previously, loci such as *Poxn* carry out functions required for neurogenesis and courtship, thereby promoting fertility.

Variants are associated with various mechanisms of cell signaling regulation

Central to the processes that promote aging are signaling pathways. Signaling integrates environmental inputs, relaying them to downstream effectors that carry out a suitable response. Alterations in pathway functions occur with age, reducing the efficiency of the ability to respond to signal, signal transduction, and cross-talk with other pathways (Carlson et al., 2008). Deterioration in pathway function can influence stem cell maintenance, cell proliferation, and lead to tissue degeneration. For example, in aged mammalian muscle tissue, regeneration of damaged muscle is inhibited due to a decrease in Delta, the ligand for the Notch signaling pathway in satellite cells (Conboy et al., 2003).

Jak activity is required during gametogenesis in both male and female flies, and regulation of signaling activity is accomplished through several mechanisms. Modulation of signaling activity is a critical determinant of the ability to form gametes, and a decline in Jak signaling is associated with a reduction of germline stem cell numbers in the male testis through decreased protection by Imp leading to degradation of *upd* transcript by the miRNA let-7 (Boyle et al., 2007; Toledano et al., 2012). In *Drosophila*, there are three miRNAs, let-7, miR-100, and miR125, that compose the let-7 complex (Sokol, 2012). Micro RNAs have dual functions in organisms, as they promote development through regulation of timing of events such as larval progression in *C. elegans*, neurological development in Drosophila, and pigmentation in butterflies (Pasquinelli and Ruvkun, 2002; Wu et al., 2012). They can also promote aging through regulation of transcript expression (Toledano et al., 2012), or repression of transcription of transcription factors such as *chinmo* (Chawla et al., 2016), which is itself a target of
Jak/Stat activity. miRNAs function through the recognition of a seed sequence in the 3’ UTR of transcripts, preventing translation. Presumably, variants in the 3’UTR of Jak/Stat pathway components and targets could affect activity by enhancing or reducing expression of ligands and effectors. While many variants identified in this study were located in introns, increasing the size of the study population may locate causal SNPs in potential miRNA seed sequences of Jak pathway targets which could alter expression of pathway components.

A second approach to signaling modulation is through physical control of ligand diffusion. This can be accomplished by delivering ligand directly to a target cell, such as has been documented in the *Drosophila* testis stem cell niche (Inaba et al., 2015), or through interaction of the ligand with components of the extra-cellular matrix (ECM). Of particular importance in control of signaling are the heparan sulfate proteoglycans (HSPGs). Interaction of ligand with the HSPGs is a proposed mechanism of modulating pathways such as FGF and Jak/Stat during development and gametogenesis (Hayashi et al., 2012; Yan and Lin, 2007; Yan et al., 2009). Our analysis of reproductive lifespan found SNPs associated with ECM components or modifiers. These loci either are components of the ECM or act to modify proteoglycans. Thus, alterations in the activity of these genes due to the influence of causal variants could potentially impact signaling affecting developmental processes required during gametogenesis.

There are number of proteins that modulate Jak/Stat activity, including the SOCs proteins, Ptp61F, and BRWD3, Ken and Barbie, and Eye Transformer (Arbouzova and Zeidler, 2006; Makki et al., 2010). The results from this study identified variants in *Ptp61F* in several of the analyses. However, variants in other components such as ligands, receptor, and effector molecules did not appear in the results. A final way signaling could be affected is through variants carried in target genes that affect the ability of the protein to carry out its function or in how they interact with Stat92E or other
transcription factors activated by the pathway. Potential target genes that contain Stat92E binding sites were identified in the GWAS that could impact reproductive aging fell into gametogenesis, sensory input and behavior including courtship behaviors, genes with roles in immunity. In summary, there are multiple levels of signaling regulation and modulation, all potentially influenced by the presence of a minor change at the nucleotide level.

Conclusions and future directions

Reproductive senescence is a highly complex process, integrating developmental, sensory/behavioral, signaling, and homeostatic functions to establish the length of male fertility. GWAS results for highly significant variants associated with components of the Jak/Stat pathway identified Ptp61F, a known negative regulator of activity. Indirect regulators were identified in the form of components or modifiers of the ECM. Furthermore, targets of Jak/Stat activity were identified that have functions in processes required in fertility and longevity. The general functional classes of candidate loci were consistent between type of analysis, and these classes of loci were identified in other studies as well.

The next step is validation of candidate loci to confirm their role in regulating reproductive lifespan. RNAi and mis-expression studies in wild-type flies can be utilized to determine if the gene affects reproductive lifespan. Another possible approach is to replace the minor allele in a line carrying the major allele and vice versa using Cas/CRISSPR, looking for an alteration in phenotype. Finally, a validation set approach could be employed. An equal number untested DGRP lines that carry either the major or minor allele for each candidate locus can be selected using the information on the DGRP2 website. These lines would act as a validation set, and the assay would be repeated using these lines to determine if the allele appears in the GWAS. Given the
complexity of the phenotype, this work has only begun to uncover the underpinnings of reproductive senescence. Further work is required to validate, and determine how these findings regulate male reproductive aging.
Table 3.1: Sample size for mean and median reproductive lifespan assay. The number of individuals that completed the entire assay were analyzed.

<table>
<thead>
<tr>
<th>Line</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAL26</td>
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</tr>
<tr>
<td>RAL28</td>
<td>42</td>
</tr>
<tr>
<td>RAL42</td>
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<tr>
<td>RAL45</td>
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<td>RAL59</td>
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<td>RAL88</td>
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<td>RAL93</td>
<td>41</td>
</tr>
<tr>
<td>RAL101</td>
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<td>RAL109</td>
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<td>RAL129</td>
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<td>RAL138</td>
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<td>RAL208</td>
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<td>RAL301</td>
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<td>RAL324</td>
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<td>RAL358</td>
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<td>RAL362</td>
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<td>RAL852</td>
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</table>
Table 3.2: Annotated genes with the lowest significance value for mean reproductive lifespan. Listed above are genes correlated with SNPs with the lowest p-value. Variants that fell in intergenic regions were excluded. Results were generated by the DGRP analysis pipeline, which uses SNPEff to annotate results. Among the information produced by the program are gene name and location of the variant (intron, exon, etc.). Flybase (flybase.org), was used to identify the molecular and biological functions of the genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>p-value</th>
<th>Molecular Function</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp4ad1</td>
<td>downstream</td>
<td>3.65x10^-8</td>
<td>oxidoreductase activity</td>
<td>redox activity</td>
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<td>CG30377</td>
<td>intron</td>
<td>2.12x10^-7</td>
<td>unknown</td>
<td>copper ion homeostasis</td>
</tr>
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<td>Gbs-70E</td>
<td>intron</td>
<td>4.71x10^-7</td>
<td>glycogen binding</td>
<td>glycogen metabolism</td>
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<tr>
<td>pirk</td>
<td>downstream</td>
<td>1.08x10^-6</td>
<td>receptor binding</td>
<td>neg. regulation of immune system</td>
</tr>
<tr>
<td>Mmp2</td>
<td>intron</td>
<td>1.51x10^-6</td>
<td>metalloendopeptidase</td>
<td>ECM modification</td>
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<td>CG30499</td>
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<td>ribulose-phosphate 3-epimerase</td>
<td>carbohydrate metabolism</td>
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<td>upstream</td>
<td>2.39x10^-6</td>
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<td>sbb</td>
<td>intron</td>
<td>2.71x10^-5</td>
<td>transcription corepressor</td>
<td>neg. regulation of transcription</td>
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<td>6.26x10^-5</td>
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<tr>
<td>byn</td>
<td>intron</td>
<td>6.26x10^-5</td>
<td>DNA binding</td>
<td>hindgut morphogenesis</td>
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<tr>
<td>tow</td>
<td>intron</td>
<td>9.08x10^-6</td>
<td>unknown</td>
<td>planar cell polarity</td>
</tr>
</tbody>
</table>
Table 3.3: GWAS results for median male reproductive lifespan tested using the longitudinal assay. The most strongly associated variant with a named gene was pox neuro (Poxn). This and other candidates were identified in genes with documented functions in processes of importance in reproduction and fertility, including behavior and cellular reproduction and differentiation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>p-value</th>
<th>Molecular Function</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>poxn</td>
<td>downstream</td>
<td>1.05x10^-5</td>
<td>transcription factor</td>
<td>chemosensory bristle development</td>
</tr>
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<td>Rbfox1</td>
<td>intron</td>
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<td>transcription regulation</td>
<td>oogenesis</td>
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<td>CG30377</td>
<td>intron</td>
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<td>vib</td>
<td>intron</td>
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<td>phospholipid transporter</td>
<td>male meiosis</td>
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<td>CG12885</td>
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<td>2.77x10^-5</td>
<td>metalloendopeptidase</td>
<td>neg. regulation of stem cell differentiation</td>
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</table>
Table 3.4: Cross-sectional assay GWAS results for 28 day old males. Top SNPs lying in or near an annotated gene are listed. The GWAS identified SNPs associated with candidate loci with annotated functions related to cell signaling, development, immunity, and homeostasis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>p-value</th>
<th>Molecular Function</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>plum</em></td>
<td>intron</td>
<td>1.07x10⁻⁶</td>
<td>unknown</td>
<td>neg. reg. of synaptic growth</td>
</tr>
<tr>
<td><em>Ef1alpha100E</em></td>
<td>upstream, intron</td>
<td>7.92x10⁻⁷</td>
<td>translation elongation</td>
<td>elongation factor</td>
</tr>
<tr>
<td><em>sano</em></td>
<td>intron</td>
<td>1.72x10⁻⁶</td>
<td>unknown</td>
<td>planar polarity</td>
</tr>
<tr>
<td><em>mspo</em></td>
<td>intron</td>
<td>2.92x10⁻⁶</td>
<td>unknown</td>
<td>myoblast fusion</td>
</tr>
<tr>
<td><em>Ptp61F</em></td>
<td>intron</td>
<td>1.36x10⁻⁶</td>
<td>tyrosine phosphatase</td>
<td>neg. reg. of Jak/Stat</td>
</tr>
<tr>
<td><em>awd</em></td>
<td>upstream</td>
<td>3.28x10⁻⁶</td>
<td>kinase</td>
<td>cell polarity cell cycle</td>
</tr>
<tr>
<td>CG31431</td>
<td>non-synonymous coding</td>
<td>4.69x10⁻⁶</td>
<td>unknown</td>
<td>unknown</td>
</tr>
<tr>
<td><em>Flo-2</em></td>
<td>intron</td>
<td>1.01x10⁻⁵</td>
<td>structural molecule</td>
<td>melanotic encapsulation</td>
</tr>
<tr>
<td><em>tinc</em></td>
<td>intron</td>
<td>1.56x10⁻⁵</td>
<td>unknown</td>
<td>photoreceptor development</td>
</tr>
<tr>
<td><em>spg</em></td>
<td>intron</td>
<td>1.15x10⁻⁵</td>
<td>Rho-GEF</td>
<td>nervous system development</td>
</tr>
</tbody>
</table>
Figure 3.1: Longitudinal Assay of DGRP Reproductive Lifespan. DGRP males were collected at 0-1 days old and crossed with virgin females. Crosses were performed Monday, Wednesday, and Friday by removing the male and crossing him to new virgin females. Mated females were retained for one week in the old vials. Males were deemed fertile if larvae were present. After two consecutive crosses without larvae present, males were scored as infertile and no more crosses were performed. Age at infertility was specified as the age on the last day larvae were present.
Figure 3.2: Cross-sectional assay to determine DGRP male reproductive lifespan.

Males were aged in vials in groups of 10 males and 10 females. Flies were transferred to new vials every few days until males were 28 days old. Males were crossed to two virgin females and scored after seven days for the presence of larvae. The presence of any larvae was scored as fertile.
Figure 3.3: Reproductive lifespans of males using the longitudinal assay have a high amount of inter- and intra-line variation. Error bars = ±1SD. The same data set was used to generate the results for mean and median reproductive lifespan. Sample sizes for the mean and median analysis range from 8 to 67. (A) Depicted is the mean male reproductive lifespan in days for 39 lines, showing the large amount of variability among lines. Median reproductive lifespan in days for the same dataset depicted in
Figure 3.3A. There is still variability between lines for age at infertility. (B) Repeated trials for selected DGRP lines tested using the longitudinal assay. While the variability within lines is not significant between trials, others do show failure to reproduce results.
Figure 3.4: Manhattan plot of mean reproductive lifespan GWAS results. The red line indicates a genome-wide significance level, while the blue line is the nominal significance level. Each point indicates a variant in the DGRP and the statistical significance of its association with reproductive lifespan. SNPs are plotted sequentially along the X-axis, relative to their chromosomal location. Variants that met a nominal significance level of $p<10^{-5}$ were annotated.
Figure 3.5: GO analysis of candidate loci identified with a GWAS of mean reproductive lifespan. The Panther Gene Ontology website was used to determine the fraction of biological processes associated with candidate loci identified in the analysis.
Figure 3.6: There is a large region of linkage disequilibrium on Chromosome 2R for mean reproductive lifespan results. A linkage disequilibrium heatmap generated for the analysis of mean reproductive lifespan. Degree of linkage ($R^2$) between SNPs is indicated by color. Shown is the degree of linkage disequilibrium for the lines tested, with a large block of linkage disequilibrium on chromosome 2R.
Figure 3.7: Quantile-Quantile (QQ) plots for GWAS of mean reproductive lifespan.

The QQ plots were generated by the DGRP analysis pipeline for the GWAS of mean reproductive lifespan. The quantile distribution of observed p-values is plotted on the Y-axis and the quantile distribution of expected p-values is plotted on the X-axis. No causal variants would result in all the dots lying on the red line. Deviation from the 45-degree line indicates a significant difference between the two distributions and that there are causal SNPs associated with the trait.
Figure 3.8: Manhattan plot of median reproductive lifespan GWAS results. The red line indicates a genome-wide significance level, while the blue line is the nominal significance level. Each point indicates a variant in the DGRP and the statistical significance of its association with reproductive lifespan. SNPs are plotted sequentially along the X-axis, relative to their chromosomal location. Variants that met a nominal significance level of $p<10^{-5}$ were annotated.
Figure 3.9: LD heatmap of median male reproductive lifespan GWAS results shows a region of high linkage on Chromosome 3R. A linkage disequilibrium heatmap generated for the analysis of mean reproductive lifespan. Degree of linkage ($R^2$) between SNPs is indicated by color. Shown is the degree of linkage disequilibrium between SNPs detected for mean reproductive lifespan. There is a moderately large block of high linkage disequilibrium on chromosome 3R.
Figure 3.10: qq plots of male median reproductive lifespan. The QQ plots were generated by the DGRP analysis pipeline for the GWAS of median reproductive lifespan. The quantile distribution of observed p-values is plotted on the Y-axis and the quantile distribution of expected p-values is plotted on the X-axis. No causal variants would result in all the dots lying on the red line. Deviation from the 45-degree line indicates a significant difference between the two distributions and that there are causal SNPs.
associated with the trait.
Figure 3.11: The SNP associated with *pox neuro* is located downstream. A portion of chromosome 2R in which the *Poxn* gene lies is depicted. The SNP (red dot) associated with reproductive lifespan lies within a transcription factor binding site (blue bar) downstream of *Poxn*. The SNP and *Poxn* lie within the much larger gene *tungus*. 
Figure 3.12: Males with carrying the minor allele of *Poxn* had a shorter median reproductive lifespan and slightly fewer chemosensory bristles. (A) This is an alternate depiction of the data from Figure 3.3B showing median reproductive lifespan for DGRP lines color-coded for whether they possess the major or minor
allele of Poxn. (B) Chemosensory bristles were counted for selected lines carrying either the major or minor Poxn allele. Bristles were counted for each tarsal segment and the tibia.
Figure 3.13: GWAS of reproductive lifespan at specific timepoints. The data set used for mean and median reproductive lifespan was analyzed for the possibility that certain SNPs may be more important at a specific time during reproductive senescence.
(A-C) Plots of the proportion of fertile males at six, twelve, and eighteen days. (A'-C')

GWAS performed on data at six, twelve, and eighteen days identified SNPs in or near
candidate loci with functions in behavior, homeostasis, regulation of signaling pathways,
and lifespan determination.
Figure 3.14: Gene ontology results showing prevalence of biological functions for three selected ages. A gene ontology analysis was performed to determine the classes of biological functions for the candidate loci associated with variants identified in the analysis for SNPs at specific timepoints. The Panther Gene Ontology website was used to determine functional classes at six, twelve, and eighteen days. (A) At six days, metabolism was the largest functional class, constituting nearly 50% of the candidate
loci. (B) At twelve days of age, metabolic function was still the greatest process associated with reproductive aging, but the number of biological processes identified increased from six days. (C) At eighteen days of age, the number of classes of processes increased over that seen at six and twelve days. Metabolism, while still a large component of gene ontology, is reduced in comparison to the other two timepoints.
Figure 3.15: Proportion of males fertile at 28 days using the cross-sectional assay.

The number of males tested ranged from 5-52 per line, with most of the lines having n-values between 25-40. The proportion of fertile males was calculated, and ranged from 5-95%. There is still inter-line variability observed between DGRP lines tested using the cross-sectional assay.
Figure 3.16: Manhattan plot of cross-sectional assay GWAS results. The red line indicates a genome-wide significance level, while the blue line is the nominal significance level. Each point indicates a variant in the DGRP and the statistical significance of its association with reproductive lifespan. SNPs are plotted sequentially along the X-axis, relative to their chromosomal location. Variants that met a nominal significance level of $p<10^{-5}$ were annotated.
Figure 3.17: Biological process gene ontology results for cross sectional assay

GWAS results. Panther Gene Ontology website was used to determine the distribution of biological processes for the top annotated variants generated by the cross sectional assay.
Figure 3.18: LD heatmap of male reproductive lifespan using the cross-sectional assay shows small blocks of linkage scattered throughout the genome. A linkage disequilibrium heatmap generated for proportion of males fertile at 28 days. Degree of linkage ($R^2$) between SNPs is indicated by color. Shown is the degree of linkage disequilibrium for the lines tested. Blocks of LD are scattered throughout the genome, ranging from small to somewhat moderate in size.
Figure 3.19: QQ plots for cross-sectional assay GWAS results. The QQ plots were generated by the DGRP analysis pipeline for the GWAS of the proportion of males fertile at 28 days. The quantile distribution of observed p-values is plotted on the Y-axis and the quantile distribution of expected p-values is plotted on the X-axis. No causal variants
would result in all the dots lying on the red line. Deviation from the 45-degree line indicates a significant difference between the two distributions and that there are causal SNPs associated with the trait.
Figure 3.20: Analysis of factors contributing to variation in assay results. Mean age at infertility ± 1 S.D. shown. (A) Three experimenters tested the same three lines using the longitudinal assay. Results between experimenters was statistically significant, although experimenters were consistent between lines. (B) Transfer method and workstation had no influence on assay results.
Chapter 4: Conclusions

The projects discussed in this dissertation initially began with results from work delving into the pleiotropic functions of Jak/Stat activity, and the function of the ligand Upd3 (Wang et al., 2014b). There it was found that reduction of pathway activity by loss of one ligand lead to eggs that could not be fertilized. Specifically, loss of Jak activity in the polar cells caused blocked micropyles in approximately ten percent of eggs. While the spatial and temporal activity of Jak signaling in the developing egg prior to Stage 10B is well known, relatively little is known about Jak requirements in oogenesis during development of the micropyle. Furthermore, the same work found males mutant for upd3 were fertile and viable, but became infertile earlier than wild type males. Gametogenesis is a continuous process, and there is a requirement for Jak/Stat signaling throughout.

Gametogenesis in both males and females requires Jak activity. Stem cell maintenance in females occurs through secretion of Upd by the terminal filament and cap cells in the gerarium. In males, the pathway is required in the hub of the testis, a group of somatic cells at the apical end that form a supportive niche for the germline and somatic stem cells. Jak/Stat signaling has multiple additional roles during differentiation into mature egg and sperm. Functions of pathway activity extend beyond gametogenesis, being required for maintaining cellular homeostasis. Other things contribute to maintenance of homeostasis as well, including nutritional status, sensory inputs, and environmental cues and are integrated through cell signaling networks. Cellular homeostasis declines with age, reducing tissue function. These observations led me to ask how the pleiotropic Jak/Stat pathway operates in fertility, through regulation of micropyle development in females. I furthermore wished to uncover the roles this pathway and other factors have in regulating the length of time an individual remains fertile during aging.
There is a continuous and spatial requirement for Jak/Stat activity during gametogenesis

Jak activity is required in a continuous and spatially restricted fashion in the testis hub to maintain the number of germline and somatic stem cells. Secretion of the Jak/Stat ligand Upd by the hub cells is required to maintain the stem cell fate. Spatial control of Jak signaling is regulated by short cytoneme-like processes formed by the germline stem cells contacting the hub cells. Signaling appears to occur through these nanotubes, controlling and limiting which cells receive the signal to retain a stem cell fate by limiting the ligand to a single cell (Inaba et al., 2015).

In the testis stem cell niche upd is regulated at the level of transcript through the interaction of Imp and the miRNA Let7. Imp protects mRNA from degradation by the miRNA Let7 in young males. However, this protection is decreased in older males, leading to a reduction in the number of germline stem cells (Toledano et al., 2012). The pathway has additional functions in spermatogenesis beyond stem cell maintenance. The germline daughter cells undergo a series of morphological changes to become mature spermatids. A late step in sperm production, during which Jak/Stat activity is required, is individualization, in which connections between the spermatids are broken down and excess cytoplasm is eliminated. (Tang, 2014).

In females, as in males, there are similar requirements for Jak in multiple distinct steps throughout oogenesis, influencing the specification and function of most of the somatic follicle cells to produce a functional egg. In the gerarium, the stem cell niche of the ovariole, the stem cell population is maintained by pathway activity (Bausek, 2013). Upon formation of a germline cyst, the polar cells become the source of Jak/Stat ligand during follicle development. Figure 4.1 illustrates processes requiring polar cell-derived ligand during oogenesis. Early in oogenesis, a combination of Jak/Stat and Notch signaling acts to specify the polar cells and the stalk cells, a group of cells that separate each follicle, from a pool of pre-polar cells (Figure 4.1). While Notch signaling from the
germline specifies the polar cells, paracrine Jak activation by the polar cells is required to promote the formation of the stalk cells (McGregor et al., 2002). There is typically an excess of polar cells early in oogenesis. Elimination of supernumerary polar cells is initiated by autocrine Jak/Stat signaling, and by Stage 5 of oogenesis, polar cell number is reduced to two at each pole (Besse and Pret, 2003). The anterior polar cells act as a developmental organizer through the secretion of the Jak/Stat pathway ligands Upd and Upd3, to activate the pathway in the surrounding cells to pattern the follicular epithelium (Xi et al., 2003).

After reduction of polar cell number, secretion of the Jak/Stat ligand Upd by the anterior polar cells specifies anterior follicle cell fates. Among these are the border cells, 4-6 cells that surround the polar cells. As described more extensively in Chapter 2, during Stage 9, the polar cells promote an epithelial to mesenchymal transition in the border cells through activation of Stat92E in the border cells (Silver et al., 2005; Silver and Montell, 2001). As the border cells delaminate, they take the polar cells with them. The polar cells continue to promote Jak activation in the border cells, driving migration of the cell cluster (Montell et al., 1992).

In this dissertation, I have shown yet another function for Jak/Stat signaling in oogenesis. I demonstrated that persistence of signaling is required well beyond border cell migration completion at Stage 10B. A need for signaling is required at subsequent stages to promote coordination of extension outgrowth and guidance. Furthermore, I showed that requirement for Jak pathway activation resides in the polar cells themselves, indicating autocrine function of the pathway. I have also provided evidence for additional polar cell functions during oogenesis. The importance of the anterior polar cells prior to Stage 10B is well established. But they continue to be required throughout all of oogenesis. There is a continual requirement for both paracrine and autocrine Jak/Stat activity from the polar cells, contributing to the amazing level of pleiotropy of
the pathway during oogenesis.

Future Directions-Polar cell functions in micropyle development and oogenesis

While it has been shown that the polar cells are required for formation of the micropyle channel, it is possible that they have other functions beyond this. At the tip and inside the micropyle channel are lectin residues (Intra et al., 2015) that act as recognition and binding sites for sperm during fertilization (Intra et al., 2015; Perotti and Riva, 1988; White et al., 1984). The source of the residues is not documented. The polar cells act as a placeholder during micropyle growth, forming extensions to maintain a passage for sperm during development. Given their temporal and spatial requirement during micropyle development, the polar cells are a good candidate source for depositing these at the top of and inside the micropyle channel. Lectins can be stained using Coomassie Blue (Yanagimachi et al., 2013), or fluorescently tagged Wheat Germ Agglutinin (Perotti and Riva, 1988; White et al., 1984; Yanagimachi et al., 2013). Eggs from either wild-type or aPKC<sup>DN</sup> mutant females can be stained and examined for fluorescence around the top and in the micropyle channel. If the polar cells indeed secrete the lectin used for sperm recognition, fluorescence should be absent.

Extensions formed by the polar cells are directed toward the oocyte, most likely due to their function in formation of a passage that must allow sperm access to the oocyte for fertilization. Directed extension outgrowth could be guided by cues produced by the oocyte. The polar cells rely on the border cells for localization at the nurse cell-oocyte boundary. During migration, the border cells are guided by the growth factors Pvf and Egf secreted by the oocyte (Bianco et al., 2007; Jekely et al., 2005), forming long extensions that appear to sense guidance cues produced by the oocyte (Fulga and Rørth, 2002). The nature of the signal guiding the polar cell extensions is unknown, but likely candidates are the same signals that guide the border cells during their migration.
Preliminary investigation into whether Pvf and Egf act as guidance cues for the polar cell extensions have been conducted. Single knockdown of receptors to these growth factors in the polar cells has not shown any effect on extension guidance. Both factors are required for migration of the border cell cluster (Bianco et al., 2007). The failure of knockdown of either component alone to impair extension guidance may indicate redundant functions of the cues, or that a completely different signal is required.

As with males, gametogenesis in females occurs continuously throughout the life of the animal. As discussed in Chapter 3, male reproduction is subject to effects of aging. The lab has previously shown that while upd3 mutant females have an initial higher proportion of unfertilized eggs compared to wild type flies, this continues with age (Wang et al., 2014b). We have shown that the incidence of blocked micropyles is correlated with unfertilized eggs. The current study could be extended to determine how aging affects the frequency of micropyle defects. It is possible that with age there are changes in the process of micropyle development, and egg chambers could be observed for changes in the rate of border cell migration, and alterations in polar cell extension dynamics and targeting. These events are under the regulation of a variety of signaling pathways which are subject to changes in expression over time, as is seen in the regulation of upd expression in the testis stem cell niche (Toledano et al., 2012). It is predicted that there would be defects in border cell migration, such as delayed migration, and failure of the polar cell extensions to properly target the micropyle. Degeneration of these processes would thus lead to an age-related increase in micropyle defects.

Requirement for Jak activity for cellular homeostasis and aging

Alterations in homeostatic processes (Biteau et al., 2010; Wang et al., 2014a), sensory and nutrient sensing (Grandison et al., 2009; Ostojic et al., 2014; Partridge et
al., 2011), and environmental cues (Gendron et al., 2014; Pletcher, 2009; Waterson et al., 2014) all contribute to the rate of aging at the organism and tissue level. In reproductive tissues, homeostatic and environmental triggers are integrated by signaling pathways, directing formation of gametes, and maintenance of reproductive output over the life of the animal (Figure 4.2). However, the response of signaling pathways to these cues diminishes with age (Carlson et al., 2008). Cell signaling can be extremely complex, with cross-talk between pathways often occurring to promote cellular responses to a given stimulus. The impacts of aging on cell signaling are not well understood. However, there is evidence that failure to upregulate the ligand Delta in the Notch signaling pathway contributes to senescence-related loss of regenerative potential in mouse muscle tissue. Injured tissue of older mice compared to that of younger mice had fewer activated satellite cells and decreased expression of the ligand Delta, contributing to tissue degeneration in older animals. Reduced regenerative ability can be reversed experimentally by introduction of a Notch activator (Conboy et al., 2003).

Another study comparing the effects of exercise on MAPK signaling in muscle tissue from both young and old males. They found that older men had higher levels of phosphorylated showed that resting muscle tissue from older males had higher levels of phosphorylated components of MAPK signaling compared to younger males. A bout of resistance work caused the levels to decrease in older males while increasing in younger males, indicating differential activation of the signaling pathway with age. The authors concluded that this was reflective of muscle tissue from older individuals operating under stress like conditions at rest (Williamson et al., 2003).

The Jak/Stat pathway is highly conserved between vertebrates and invertebrates and is highly pleiotropic in function. As described earlier in this dissertation, the pathway functions in both flies and mammals, to regulate stem cell maintenance and proliferation in both the gonad and intestine. I have also discussed the its multiple roles in
differentiation and development of *Drosophila* gametes and functions in maintenance of stem cell populations in the intestine (Osman et al., 2012). Furthermore, as discussed in Chapter 3, the pathway also is required for maintaining cellular homeostasis.

Nutrient perception and metabolism are required for organism growth and cellular homeostasis. Central to this is insulin signaling which regulates energy generation and has been shown experimentally to regulate reproduction and lifespan (Flatt et al., 2008; Yamamoto and Tatar, 2011). Jak activity is required in *Drosophila* to maintain proper functioning of the insulin pathway and maintain cellular and organism homeostasis. It does so through the secretion of Upd2 by the fat body. Presence or absence of Upd2 acts on GABAergic neurons to signal the nutritional state of the animal, thereby regulating growth and metabolism (Rajan and Perrimon, 2012). Further evidence of tissue regulating systemic homeostasis through Jak/Stat interaction with insulin signaling comes from examination of the role of muscle tissue regulation of lipid homeostasis. Activity of the transcription factor FOXO in muscle tissue regulates *upd2* expression in muscle. This in turn stimulates the release of AKH from neuroendocrine cells to maintain lipid homeostasis (Zhao and Karpac, 2017).

As described above, Jak/Stat has important functions in sustaining cellular homeostasis and formation of both egg and sperm. Disruption of the pathway can lead to stem cell loss (Boyle et al., 2007; Kiger et al., 2001; Toledano et al., 2012) and defective eggs and sperm. Maintenance of stem cell pools and other functions required for longevity are controlled by a multitude of genes. Genetic variation between individuals contributes to how well these processes operate and the rate of aging and fertility loss for each member of a population. Thus, I attempted to determine if there was a detectable genetic contribution to reproductive aging, and if so, if natural variation in components of the Jak/Stat pathway could contribute to the rate of reproductive aging. The genetic architecture of longevity and fertility in flies (Durham et al., 2014; Leips
and Mackay, 2002; Mackay, 1999, 2002; Magwire et al., 2010; Nuzhdin et al., 1997), yeast (Stumpferl et al., 2012), mouse (Gotoh et al., 2012; Leduc et al., 2010; Nelson et al., 2012; Rikke et al., 2010), and humans (Broer et al., 2015; Kosova et al., 2012; Sebastiani et al., 2012; Zeng et al., 2016) has been explored separately using either QTL analysis or GWAS, to exploit natural individual variation to identify candidate loci. In this study, I attempted to understand if the genetics underlying the relationship between these two traits could be detected using an unbiased assay.

Reproductive senescence and aging in general are influenced through the contributions of many factors that are shared among species. Cellular homeostatic processes, development, behaviors, and signaling pathways interact with each other and the environment to regulate the rate of aging. Signaling acts as a central hub, taking in environmental stimuli and directing the responses of the other components (Figure 2). While environmental factors are difficult to identify and quantify, the other components of reproductive senescence are easier to determine, given their genetic basis. An important factor in reproductive aging is the development of gametes that are capable of undergoing fertilization, and to maintain this ability for a considerable part of an individual’s lifespan.

Cellular homeostasis requires the involvement and balance of many functions and the rate of homeostatic decline is a contributor to senescence. These include protein turnover, metabolic processes, DNA repair, and the prevention of oxidative damage and stress due to reactive oxygen species (ROS). A failure in one or more of these processes results in a degeneration of overall cellular and tissue function. GWAS results for the DGRP identified candidate loci with documented functions in these processes. The importance of protein homeostasis as a link between reproduction and lifespan has recently been demonstrated in C. elegans. Proteomic analysis showed that proteins produced by the soma that are typically cleared from the uterus in young animals,
accumulate with age and have a negative effect on lifespan. The authors found that increasing the length of reproduction reduced the amount of accumulated protein. It was concluded that egg laying cleared the protein from the uterus, thereby regulating the rate of aging (Zimmerman et al., 2015).

GO analysis of the genes found in this study revealed a significant contribution of genes required for metabolism. This is not surprising. Nutrient sensing and metabolism and its role in lifespan and fecundity is well-studied in organisms ranging from yeast to humans. It has been shown that for many organisms, caloric or dietary restriction (DR) extends organism lifespan (Mair et al., 2003; Partridge et al., 2011; Rikke et al., 2010), and is mediated through the action of insulin/mTOR signaling. The relationship of these pathways to lifespan and reproduction has been well studied in worms. In worm, it was shown that mutation of daf-2, the C. elegans insulin receptor extends lifespan (Hsin and Kenyon, 1999; Kenyon, 2011). Ablation of the germline also extended lifespan if daf-16, the FOXO transcription factor, was also present. Thus, the group concluded that FOXO is required to block insulin signaling to extend lifespan (Hsin and Kenyon, 1999; Yamawaki et al., 2008). A requirement for insulin/mTOR in regulation of longevity and fertility has also been demonstrated in mice (Nelson et al., 2012), and flies (Flatt et al., 2008; Yamamoto and Tatar, 2011).

A role for insulin/mTOR in regulation of lifespan in humans has been shown in humans as well. A GWAS for human longevity in a Han Chinese population identified four SNPs associated with FOXO3, as well as SNPs associated with carbohydrate metabolism (Zeng et al., 2016). In contrast, a GWAS for female longevity using the DGRP found an enrichment for genes in the mTOR pathway, which is also implicated in lifespan extension. An array of inputs activates mTOR including amino acid and oxygen levels, hormones, and growth factors. Inhibition of mTOR extends lifespan in flies, worms, and humans (Bjedov et al., 2010; Moskalev et al., 2016; Wu et al., 2013) The
GWAS for male reproductive lifespan performed as part of this dissertation did not uncover SNPs with a direct association to either the *Drosophila* insulin pathway or mTOR signaling. However, SNPs associated with carbohydrate metabolism were found. The lack of SNPs associated with known longevity pathways is somewhat surprising. However, the population screened was much smaller than that of previous GWASs performed on longevity. However, the focus of this study was reproductive maintenance, and other processes may have a greater effect on reproductive lifespan than the longevity pathways.

Another study of human longevity performed a GWAS meta-analysis on data generated from studies performed by the Cohorts for Heart and Aging Research in Genomic Epidemiology consortium. The population for this study consisted entirely of Caucasians. Data were analyzed either in its entirety to identify disease-associated SNPs or time to event to find SNPs associated with healthy aging. In contrast to the Han Chinese lifespan GWAS, the major associations were associated with developmental and neuronal activities including axonal guidance, and Wnt signaling (Walter et al., 2011). This is similar to results from the analyses performed for reproductive lifespan for the DGRP in which identified variants associated with axonal guidance and targeting. These results are consistent with work by others using mutant flies demonstrating the importance of sensory perception to regulation of lifespan and reproduction (Gendron et al., 2014; Ostojic et al., 2014; Waterson et al., 2014).

GWAS of male reproductive lifespan did identify a candidate locus shared with a study of genetic determinants of fitness traits. The GWAS of median male reproductive lifespan found a variant in the gene *Rbfox1* (also known as *A2bp1*), which codes for a protein involved in alternative splicing. This was also found in a screen conducted by Durham et al. They examined lifespan and lifetime fecundity of females. Knockdown of
*Rbfox1* at weekly ages showed that at three weeks of age, reduced activity had asignificant impact on fecundity (Durham et al., 2014). There are no documented effects in males, but the results from this dissertation may suggest that it is required in males as well to maintain fertility. Furthermore, the results from the work by Durham et al. also found variants associated with ECM components and regulators of neural function, although these differed from those found in this work.

Overall, elements of both longevity and fecundity were found in the GWAS performed as part of this dissertation. This is to be expected, as there is a relationship between lifespan and age of onset of reproduction (Kirkwood and Rose, 1991; Tabatabaie et al., 2011). Furthermore, cellular processes promoting organism senescence should also be functioning at the level of reproductive tissues. However, most candidate loci identified in this screen were not identified in other analyses. Replication of GWAS results can be problematic, and attributable to factors including sample size and the population tested (Kraft et al., 2009). The number of lines tested in this assay were less than those used in other studies, and it is possible that increasing the size of the test population would replicate results from other studies. The study described in this dissertation addressed the idea of the genetic architecture or reproductive senescence, which differs from other studies in that rather than investigating one process, we attempted to dissect the genetics of two processes that are loosely intertwined.

**Future Directions**

It is known that *upd* expression is regulated through the action of the miRNA Let-7 (Toledano et al., 2012) in the testis. It is unknown if this is a factor in age-related reduction of *upd* expression in the ovary or if this mechanism of regulation even occurs in the female gonad. Similar regulation in the ovary has not been performed. Both *upd*
and upd3 are expressed in the ovary by the terminal filaments and cap cells of the germarium which is essential for stem cell maintenance, and the polar cells. miRNA regulation of upd expression is known to function in testes. But, it is unknown if upd3 expression is subject to the same type of regulation. It is already known that the upd mRNA transcript carries a seed sequence for Let-7. The 3’UTR of upd3 lacks a seed sequence for Let-7, but it may be possible that upd3 expression could be subject to regulation of by a different miRNA, possibly one of the other two that compose the Let-7 complex. Aligning the 3’UTR of the upd3 transcript with the recognition sequences of other candidate miRNAs could identify possible candidates. Knockdown or mis-expression of the miRNA could then be performed and the effect on oogenesis evaluated. Additionally, the role of Imp as a protector of transcript could also be examined through knockdown and mis-expression studies.

Other studies have focused on identifying genetic determinants of either lifespan or fertility/fecundity, and test subjects have been primarily female. This work differed by attempting to discover variants driving reproductive maintenance in males. However, there are differences in male and female life histories that would suggest that there would be some differences in loci underlying reproductive lifespan. It would be expected that the processes underlying reproductive maintenance in both sexes would be the same, but enrichment for a certain process could possibly differ. Many of the GWAS conducted on the DGRP have shown sex related differences even when there is no obvious sexual dimorphism (Arya et al., 2015; Jordan et al., 2012; Mackay et al., 2012; Weber et al., 2012). The GWAS for male reproductive lifespan produced several candidate loci with functions in behavior such as courtship and aggression. It is likely that these would be absent from a similar study of females as male courtship comes at a cost to longevity in male Drosophila (Cordts and Partridge, 1996). Rather, genes controlling post-mating responses such as egg laying behaviors could be important.
Additionally, the seminal fluid of males contains Acps (accessory gland proteins) that female behaviors and sperm storage (Wolfner, 2002). Furthermore, male sex peptides have been demonstrated to dampen the female immune response to microbial infection (Short et al., 2012). Hence, it is possible that variants located in or near genes regulating immunity would be found. Some loci identified in the current study were stated to have functions in oogenesis. It is possible that they have previously unknown functions in spermatogenesis. The assay to assess female age at infertility would have to be adapted to suit female reproductive physiology. Females will store sperm after mating, a cohort style mating assay may be appropriate, where a group of males and females (ten males and ten females, for example) are housed together. As with the longitudinal assay, males would be exchanged for fresh males periodically. Flies could be transferred to fresh vials every other day, and the old vials retained to screen for larvae. This would continue until either only two females survive, or no offspring are present. Thus, a lifespan assay could be conducted simultaneously, and assessment of female post-reproductive lifespan could also be determined.

Summary

Jak/Stat activity is crucial for maintaining cellular and organism homeostasis and appropriate developmental processes. Deterioration or mutation of pathway components can have serious effects on cellular and organism functions. Examination of pathway functions in the polar cells during oogenesis uncovered a previously undescribed autocrine requirement for Jak/Stat activity for proper polar cell function during micropyle development. Oogenesis in other insects utilizes similar cellular processes in micropyle formation. Knowledge from this work could provide insight into the evolution of insect gametogenesis.

The fidelity of gametogenic processes are subject to age-related changes to the organism in which they are happening. Jak/Stat signaling is highly pleiotropic and is
utilized in a variety of processes that regulate both gametogenesis and homeostasis at a cellular/tissue and organism level. Signaling pathways integrate numerous types of inputs to regulate fertility. In this dissertation, I used an unbiased approach to dissect the genetics of reproductive aging. A GWAS of male reproductive maintenance found variants associated with a number of processes associated with lifespan and fertility. Furthermore, categories of processes for candidate loci are consistent with those found in other studies, and included metabolism, and cellular processes required for homeostasis. Results from this work demonstrate how not only a single signaling pathway alone can affect a sole process such as fertility, but, with contributions from other genes and pathways, can also regulate a multitude downstream functions necessary to maintaining reproductive viability.
**Figure 4.1: Jak/Stat activity is required for a range of activities during oogenesis.**

Jak activity is required during several stages of oogenesis. (A) At Stage 2, the polar cells (darker green) specify the stalk cells (light green) through paracrine Jak/Stat activation. Follicular epithelium cells are yellow. (B) At Stage 3-5 the excess polar cells (green) undergo apoptosis through autocrine Jak/Stat activity to reduce the number of anterior and posterior polar cells to two. (C) Paracrine Jak/Stat activity during Stages 6-7 by the polar cells (green) specifies the fate of the border cells (red) and other cells of the follicular epithelium (yellow). (D) During Stage 9, the polar cells (green) signal in a paracrine manner to activate Jak/Stat activity in the border cells (red), driving an epithelial to mesenchymal transition and migration to the oocyte. (E-E") Border cells in red. Autocrine Jak activity is required by the polar cells (green) to coordinate extension outgrowth beginning at Stage 10B and continuing through Stage 13. See text for details.
Figure 4.2: Reproductive lifespan is determined by the interaction of many factors.

Cell signaling is central to relaying environmental cues that drive downstream responses. Each on their own contributes to reproductive lifespan, but they are interconnected and a change in one can lead to alterations in efficiency of others.
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