THE JAK/STAT PATHWAY IS REUTILIZED IN DROSOPHILA SPERMATOGENESIS

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THE JAK/STAT PATHWAY
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DISSERTATION

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

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In the Drosophila testis, sperm are derived from germline stem cells (GSCs) which undergo a stereotyped pattern of divisions and differentiation. The somatic cells at the tip of the testis form the hub, which is the niche for both the somatic cyst stem cells (CySCs) and GSCs. The hub expresses Upd, a ligand for the JAK/STAT pathway that has roles in the maintenance of CySCs and GSCs. Male mutants of upd3, another ligand of the JAK/STAT pathway, become sterile much earlier than the wild-type, leading to the hypothesis that similar to upd, upd3 also promotes the self-renewal of stem cells in testis. It was found here that upd3 is also expressed in the hub, and that mutants of upd3 have fewer CySCs and GSCs. Using a GFP reporter of the JAK/STAT pathway, it was found that the JAK/STAT pathway is not only activated in the stem cells, consistent with its known function in the maintenance of stem cells, but is also activated in the elongated cyst cells that encapsulate late stage differentiating spermatids. The reduction of JAK/STAT activity in the somatic cyst cells led to impaired spermatid individualization, a late stage of spermatogenesis during which the syncytial spermatids are separated. The impairment of individualization was shown by the loss of three characteristic structures: individualization complexes (ICs), cystic bulges (CBs), and waste bags (WBs). The failure of IC formation implies STAT activity is required for the initiation of individualization, and the loss of CBs and WBs suggests STAT activity is required for the progression of individualization. Activation of caspases in elongated spermatids is known to be required for individualization. The reduction of JAK/STAT activity in cyst cells almost completely eliminated the activation of two effector caspases: drICE and DCP-1. It was concluded that JAK/STAT activity in somatic cyst cells promotes individualization by stimulating caspase activity in spermatids. The JAK/STAT pathway is not only required for the maintenance of stem cells at the tip, but also required for individualization away from the tip during late differentiation, thus is reutilized in Drosophila spermatogenesis.
KEYWORDS: JAK/STAT pathway, *Drosophila*, Spermatogenesis, Individualization, Caspase
THE JAK/STAT PATHWAY IS REUTILIZED IN DROSOPHILA SPERMATOGENESIS

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Aging of the society

Due to the improvement of life-expectancy and decline of fertility rate, defined as the average number of children a women produces over her lifetime, our world is facing an unprecedented situation: the aging of the society. A society is considered to be old when people aged 65 or older account for more than 8%-10% of the population (see review Muramatsu and Akiyama, 2011). This proportion was about 5% in 1960 when the proportion of children under 5 years old was 14%. In 2016, it was estimated that the proportion of people aged 65 or older will start to outnumber children under 5 for the first time, after both of them account for about 8% of the population. The trend of rapid aging will not stop. In 2050, our society will be super-aged, when about 18% of people will be aged 65 or more, and 7% of people will be less than 5. The number of people aged 65 or more was estimated to increase rapidly from 524 million in 2010 to 1.5 billion in 2050, almost tripled (World Health Organization, 2011).

Although the proportion of people aged 65 or more is high at present, it is still increasing not only in the developing countries, but also in the developed countries. In China and India, the two countries that have the largest population in the world, older people (aged 65 or older) will increase rapidly in the near future. In China, the number will increase from 110 million in 2011 to 330 million in 2050, and it will grow from 60 million in 2010 to 270 million in 2050 in India (World Health Organization, 2011). While Japan is enjoying the longest life expectancy, which is 86 for women and 80 for men in 2009 (World Health Organization, 2011), Japan also has the highest proportion of people aged 65 or older: 23% in 2009. And there is no trend for aging to stop, which will further increase the percentage to 40% in 2050 (World Health Organization, 2011). In the US, the number of people aged 65 or more has increased from 4.1% to 12.9% over the last century, and will continue to increase to 20% by 2030 (Anderson et al., 2012).
Aging is associated with the decline of function in almost every organ, and the prevalence of many diseases such as cardiovascular diseases, neural degenerative diseases and diabetes increases. In China, the incidence rate of hypertension increases from about 8% at age 18-49, to about 19% at age 50-59, and to 33% at age 70-79, and 41% at age 81 or older (World Health Organization, 2011). Dementia prevalence also increases rapidly with age. The prevalence of Alzheimer's disease, the most common form of dementia, almost doubles every five years after 65. In Economic Cooperation and Development (OECD) countries, which are composed of 34 developed countries, the prevalence of dementia is less than 3% at 65-69, but increases dramatically to nearly 30% at 85-89 (World Health Organization, 2011). The prevalence of diabetes also increases with age. In China, the prevalence of diabetes in men is 2.6% at 20-30, 15.5 at 50-60, and 21.8% at 70 or older; in women, it is 1.5% at 20-30, increases to 13.1% at 50-60, and reaches 22.0% at 70 or older (Yang et al., 2010).

Aging also has a negative effect on economics. It was estimated that economic loss from heart disease, stroke and diabetes in twenty three low and middle income countries was $83 billion between 2006 and 2015 (World Health Organization, 2011). It was estimated that from 2010 to 2030, direct medical costs for cardiovascular disease will increase from $273 billion to $818 billion, and indirect costs will increase from $172 billion to $276 billion (Heidenreich et al., 2011). According to Alzheimer's Diseases International, the world spent more than $600 billion in 2010 on the treatment of dementia (World Health Organization, 2011). Aging even leads to public policy concerns. It was speculated that the elderly may prefer to support medical expenditure, which benefits them more, than environmental expenditure, although no direct data support this speculation (Balestra and Dottori, 2012).

As mentioned above, the fertility rate keeps decreasing in recent decades and is one of the two main factors that lead to the aging of the society (World Health Organization, 2011). Decreased fertility rate is caused by late marriage, increased cost for child raising, increased numbers of woman working outside the home, and
increased infertility, defined as the failure to conceive and give birth to a child in full term after at least one year of unprotected intercourse etc. (World Health Organization, 2011). About 15% of couples suffer from infertility worldwide (Pang et al., 2005).

While fecundity decreases with age (see reviews Baird et al., 2005; Kidd et al., 2001), the age at first marriage has also increased in recent decades. In the US, the age of first marriage increased from 20 in 1950-1960 to 26 in 2010 for women, and from 23 in 1950-1960 to 28 in 2010 for men (Lehrer and Chen, 2013). In Japan, the mean age for the first marriage increased from 24.5 to 27.7 for women, and 27.6 to 30.7 for men between 1975 and 1995 (Retherford et al., 2001). Because fecundity decreases with age, late marriage is associated with lower fertility rates. When the age at marriage is 20-24, the possibility of childlessness is 5.7%. The risk of childlessness increases rapidly with age: 15.5 if the age at marriage is 30-34, and 63.5 if the age of marriage is 40-44 (see review Baird et al., 2005). These studies suggest that the function of gonads decline with age.

Besides the gonads, aging is also associated with the function decline of other tissues, organs or systems. For example, during aging, there is a loss of muscle mass caused by declined number of muscle fiber and fiber atrophy (see review Sadoun and Reed, 2003). Aged muscle has reduced amount of aerobic enzymes and generate less force (Kirkendall and Garrett, 1998). Aging of the skin is characterized by wrinkles, senile plaque, decreased sebaceous gland activity and decreased hair growth (see review Bolognia, 1995). Aged heart is associated with loss of myocytes, decline in maximum achievable heart rate, increased arterial stiffness, cardiac fibrosis and ventricular hypertrophy (see review Pugh and Wei, 2001). To better understand aging of the organisms, we need to understand aging of specific tissue, organs and systems well.

Lifespan and reproductive lifespan are closely related in both male and female. In a study of 43,227 men, semen quality, including sperm concentration and motility, are
positively associated with life expectancy, regardless of whether they have a child or not (Jensen et al., 2009). In women, as the age at menopause increases, life expectancy also goes up, suggesting female fertility is also positively associated with lifespan (Ossewaarde et al., 2005). Thus, investigating the aging of gonads may help in understanding aging of the whole organism.

In addition, as mentioned above, age at marriage keeps increasing but, fecundity decreases with age. However, the mechanism underlying this age associated fecundity decline is not well investigated. Investigating this mechanism may help to find solutions to slow down the age related decline of fertility, hence counteract the unwanted low birth rate associated late marriage, and increase fertility rate.

*Drosophila* testis is not only a great model to investigate tissue aging, but also to investigate spermatogenesis. *Drosophila* testes are easy to dissect, and the morphological changes during each stages of spermatogenesis can be visualized by DIC or phase-contrast microscopy easily (see review Fuller, 1993). In addition, there are established indicators for the aging of *Drosophila* testis, for example, the number of stem cells (Boyle et al., 2007), and male fertility (Wang et al., 2014). Compared to mammalian testis, *Drosophila* testis has several advantages. First, *Drosophila* has a short lifespan, which is only about 50 days (Peng et al., 2012). But mouse has a lifespan of about 2-3 years (see review Ladiges et al., 2009). In addition, *Drosophila* spermatogenesis is very fast. Spermatogenesis takes about 72 days in human, 38 days in mouse (see review Franca et al., 2005), but only 10 days in *Drosophila* (see review Fuller, 1993). Male *Drosophila* lose their fertility at about 38 days (Wang et al., 2014), so it will not take long to see the result of testis aging. Due to these reasons, we chose *Drosophila* testis to investigate spermatogenesis and aging of the testis.

*Drosophila* spermatogenesis

*Drosophila* spermatogenesis occurs in the testis which is a coiled tube. Spermatogenesis starts at the tip where stem cells and the hub are located, and
progresses to the base of the testis. Finally mature sperm are produced and released to the seminal vesicle that is attached to the base of testis (see reviews Fuller, 1993; Fuller, 1998; Loveland and Hime, 2005). Spermatogenesis can be divided into seven stages: First, dividing populations of both germline and somatic stem cells must be maintained. Second, daughters of germline stem cells, termed gonialblasts, undergo four cycles of mitotic transit amplification. After mitosis, a cyst containing sixteen primary spermatocytes is produced. Third, the sixteen primary spermatocytes undergo meiosis. After meiosis II, sixty four haploid spermatids are produced. Fourth, the round haploid spermatids elongate and become spermatid bundles. Fifth, the interconnected syncytial spermatids are separated by individualization. After individualization, free sperm are produced. Sixth, the sperm coil into the base of testis. Seventh, coiled sperm are released into the seminal vesicle. Stages four through seven are termed spermiogenesis, which is the maturation of spermatids into mature sperm (see reviews Fuller, 1993; Fuller, 1998).

The first step of spermatogenesis is the maintenance of stem cells. Stem cells have two key characteristics: the ability to differentiate into specialized cells and the ability to self-renew (see review Weissman, 2000). The microenvironment that is required for the maintenance (self-renewal) of stem cells is termed the niche. Although there might be some disputes, it is widely believed that stem cells will die or undergo differentiation without the niche (see reviews de Cuevas and Matunis, 2011; Li and Xie, 2005; Zhang and Li, 2008). The niche-stem cell interaction is controlled by signaling pathways in most cases (see review Li and Xie, 2005). In many tissues such as the skin, digestive tract and circulating system, continuous production of terminal cells from adult stem cells is important to replenish lost cells and repair damage (Amcheslavsky et al., 2009; Li and Clevers, 2010). Drosophila has a short lifespan, so the continuous replenishment of terminal cells may be unnecessary in most tissues. As a result, only limited types of adult stem cells are found in Drosophila. In testes and ovaries, continuous production of sperm or oocytes requires active stem cells that can provide the progenitors of sperm or oocytes throughout the reproductive lifespan. As
a result, GSC is one the most intensively investigated stem cells in *Drosophila* (see reviews de Cuevas and Matunis, 2011; Li and Xie, 2005).

The *Drosophila* testis serves as a great model for investigating stem cells. Besides the sheath, the testis is only composed of two lineages of cells: germline cells and somatic cyst cells (including CySCs, cyst cells and hub cells). At the tip of testis is the hub, which is composed of 9-12 non-dividing cells and serves as the niche. Both GSCs and CySCs directly attach to the hub, and each GSC is enclosed by two CySCs (see reviews de Cuevas and Matunis, 2011; Fuller, 1993; Tulina and Matunis, 2001).

After division of the stem cells, one daughter cell of the GSC that still contacts the hub retains GSC identity, and the other daughter cell becomes a gonialblast, the founder germ cell of all spermatids in one cyst. The two daughter cells of the two CySCs that still contact the hub retains CySC identity, and the other two daughter cells become the two cyst cells enclosing the gonialblast. The two cyst cells and their enclosed germline cell(s) are called a cyst. After that, the gonialblast undergoes four cycles of mitosis and becomes a cyst containing 16 spermatogonial cells. The mitosis is incomplete; there are cytoplasmic bridges called fusomes interconnecting daughter cells which are used for cytoplasm exchange and cell cycle synchronization. Cyst cells do not divide, but their size will increase as the size of the germline cells increases. After mitosis, the spermatogonial cells will enter meiosis.

After meiosis, each cyst will contain 64 spermatids. Then the cyst will elongate. Flagellar axonemes will be assembled and elongate to about 1.8mm, the nuclei of spermatids will condense and elongate, then finally become needle shaped (see review Fuller, 1993). After elongation, individualization will occur to get rid of cytoplasmic bridges and most of the cytoplasm and to separate the spermatids within a cyst (Figure 1.2). Individualization initiates at the head of the spermatid bundles where the nuclei are located (Arama et al., 2003). At the head of each spermatid (nuclei end, toward the base of testis), F-actin based structures called investment...
cones form. 64 investment cones in one cyst form an individualization complex (IC). Then the IC moves toward the tail (to the tip of the testis), forming a cystic bulge (CBs) which contains the unneeded cytoplasm for mature sperm. As individualization goes on, the CBs becomes larger and larger, and finally reaches the tail of the spermatid bundle and becomes a waste bag (WBs), containing the materials that are not needed in mature sperm (Figure 1.2). The WBs then is released from the tail of spermatid bundles and degrades to fragments. After individualization, individual sperm are made. These sperm then coil into the base of the testes and finally are released to the seminal vesicle (see review Fuller, 1993).

The JAK/STAT signaling pathway

Cells use cell signaling pathways to cooperate with each other by detecting and responding to the signals sent by other cells. As a result, cell signaling pathways are vital for the development and normal physiology in multicellular organisms. The abnormality of cell signaling pathways may lead to diseases such as tumors, malformation and metabolic diseases. Although there are numerous physiological processes, there are only a limited number of important cell signaling pathways. Among them, the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is an important signaling pathway that is conserved from invertebrates to mammals. It was first discovered in mammals through the investigation of interferon alpha in the mammalian immune system (Fu et al., 1990; Fu et al., 1992; Schindler et al., 1992). After that, it was discovered that not only interferon alpha, but also many cytokines and growth factors, can also activate the JAK/STAT pathway in mice (see review Stark and Darnell, 2012). The JAK/STAT pathway has many components in mammals: dozens of ligands (including more than 50 cytokines, including hormones such as prolactin, growth hormone, erythropoietin, and growth factors), dozens of receptors, four JAKs and seven STATs (see reviews Harrison, 2012; Rawlings et al., 2004; Schindler and Plumlee, 2008). In mammals, it is involved in cell proliferation and differentiation, apoptosis, stem cell maintenance and cell movement at the cellular level (see reviews Ghoreschi et al., 2009; Kamran et al., 2013; Katoh and
Katoh, 2007). At the organismal level, the JAK/STAT pathway plays a role in many processes, including haematogenesis, immunity, adipogenesis, brain development, hematopoiesis, mammary gland development and lactation (see reviews Ghoreschi et al., 2009; Rawlings et al., 2004).

The JAK/STAT pathway is also conserved in Drosophila. Compared to that of mammals, the pathway is much simpler in Drosophila: there are only three ligands (Upd, Upd2 and Upd3), one receptor (Domeless), one JAK (Hopscotch), one STAT (Stat92E). Several regulators, including the negative regulators Eye transformer (Et, also named Latran/Lat), Suppressors of cytokine signaling (Socs36E, Socs44A), and more have also been found in the Drosophila JAK/STAT pathway (see review Chen et al., 2014). Without the ligand, the Dome receptors form homodimers with each other and are constitutively associated with Hopscotch (Hop), the Drosophila homologue of JAK. Upon binding of Upd family ligands to a pair of receptors, the two Hops activate each other by trans-phosphorylation. The phosphorylated Hop also activates the receptor by phosphorylating specific tyrosine residues on Dome. Phosphorylated receptor recruits the cytoplasmic STAT92E to the phosphorylated residue on the receptor through the SH domain on STAT. STAT92E then is phosphorylated by Hop. Activated Stat92E dimerizes and translocates to the nucleus to activate gene transcription, including genes for the negative regulator Socs36E (see review Chen et al., 2014). In Drosophila, JAK signaling is involved in cell proliferation and differentiation, apoptosis, stem cell maintenance and cell movement at the cellular level, which is essentially the same as in mammals, and in sex determination, embryonic segmentation, development of wings and eyes, immunity, gametogenesis, insulin production, and response to bacterial infection at the organismal level (see reviews Bina and Zeidler, 2009; Chen et al., 2014).

One of the most intensively investigated functions of the JAK/STAT pathway in Drosophila is its ability to promote the self-renewal of stem cells, especially stem cells in gametogenesis. In the testis, upd is expressed and released from the hub and
activates JAK/STAT in GSCs and CySCs. Loss of function (LOF) of Stat92E leads to the loss of both GSCs and CySCs in testes, and ectopic activation of Stat92E leads to ectopic GSC like cells (Kiger et al., 2001; Tulina and Matunis, 2001). Activation of JAK/STAT in CySCs is required for their self-renewal. CySCs then release Dpp and activate the BMP pathway in GSCs which is required for their self-renewal. Activation of JAK/STAT in GSCs is not required for their self-renewal, but is required for the attachment of GSCs to the hub through the expression of cadherin (Leatherman and Dinardo, 2010). Similar to the JAK/STAT pathway, the Hedgehog pathway is also required for the maintenance of stem cells in Drosophila testis. Hedgehog (Hh), the ligand of the Hh pathway in Drosophila, is expressed and released from the hub; Patched (ptc) and Smoothened, the two receptors of the Hh pathway are expressed in stem cells (Amoyel et al., 2012; Michel et al., 2012). Loss of function (LOF) of the Hh pathway leads to loss of CySCs by premature differentiation, and gain of function (GOF) of the Hh pathway leads to more CySCs and stimulates their proliferation; Although both the JAK/STAT pathway and the Hh pathway support the maintenance of stem cells, they do not interact with each other (Amoyel et al., 2012; Michel et al., 2012).

Compared to Upd and Upd2, the functions of Upd3 are less well characterized. Upd and Upd2 have different functions. Upd plays a role in eye and wing development, immunity, gametogenesis, the maintenance of stem cells in testis and ovaries, the proliferation of intestinal stem cells, pattern formation of follicle cells in egg chambers, development of tracheal, and embryonic segmentation (see reviews Bina and Zeidler, 2009; Jiang and Edgar, 2011; Lucchetta and Ohlstein, 2012). upd2, the Drosophila functional homologue of leptin, is co-expressed in the embryo with upd and contributes to the embryonic segmentation (Mukherjee et al., 2005). In addition, upd2 is also expressed in the fat body upon feeding. After secretion, it travels a long distance to reach the insulin production cells in the brain and stimulate the production of Drosophila insulin-like peptides there (Rajan and Perrimon, 2012). In contrast to Upd, there are only limited reports on the expression profile and function of upd3. Upon bacterial infection, Upd3 is released from the hemocytes and activates JAK/STAT
signaling in the fat body, which expresses genes in response to septic injury (Agaisse et al., 2003). Under stress, such as during bacterial infection, aging or apoptosis, upd3, like upd and upd2, is expressed in the enterocytes, activating cell division of intestinal stem cells to replenish the loss of enterocytes (Buchon et al., 2009a; Buchon et al., 2009b; Jiang et al., 2009; Osman et al., 2012; Zhou et al., 2013). upd3 is also expressed in the larval lymph gland and embryonic gonads, but the function of this expression remains unknown (Hombria et al., 2005; Jung et al., 2005).

To investigate the function of Upd3, several mutants were made using P element mutagenesis. upd3<sup>d232a</sup> is a deletion mutant, with the largest exon deleted. upd3<sup>x21b</sup> is an insertion mutant, with two P elements inserted into the third intron. upd3<sup>x21b</sup> may be a hypomorphic mutant, while upd3<sup>d232a</sup> may be a null mutant. Flies with precise excision of the p element was used as the wild-type (upd3<sup>+</sup>). It was found that upd3 mutants have a reduced lifespan (personal communication with Lakshmi Pilla), male upd3 mutants become sterile earlier than wild-type, and upd and upd3 are co-expressed in the eye discs and the polar cells of egg chambers (Wang et al., 2014). The reduced male reproductive lifespan may suggest Upd3 has a role in spermatogenesis, which is consistent with the known function of Upd in testis. In addition, the co-expression of upd and upd3 in eye discs and polar cells raises the possibility that they may also co-express in other tissues, such as the hub of testis. Thus, the original goal of this study was to find out why upd3<sup>d232a</sup> male become sterile earlier, and to test whether Upd3 plays a similar function as Upd in the maintenance of stem cells in the testis. This hypothesis was supported, however, it was found unexpectedly that JAK/STAT is also activated in cyst cells after elongation, and subsequent investigations found that the STAT activation in cyst cells is required for individualization.
Figure 1.1. Illustration of *Drosophila* early spermatogenesis. At the tip of testis is the hub (Green. Blue indicates nuclei) which consists of 9-12 non-dividing somatic cells and serves as the niche for CySCs (red) and GSCs (yellow). Both GSCs and CySCs contact the hub, while one GSC is enclosed by two CySCs. After an asymmetric division, one daughter cell of the GSC that does not contact the hub becomes a gonialblast (light yellow), and the two daughter cells enclosing the gonialblast become two early cyst cells (light red). The gonialblast then undergoes four cycles of mitosis, making a cyst containing 16 interconnected primary spermatocytes. During the mitosis of germline cells, the cyst cells do not divide, but their size increases as the size of the cyst increases. After the completion of mitosis, the cyst enters meiosis (Modified from Tran et al., 2000).
Figure 1.2. Illustration of individualization. (A) An elongated cyst is composed of two elongated cyst cells (yellow) and 64 interconnected elongated spermatids (green) with axoneme (purple) in the center of each spermatid bundle. The head is where the nuclei are located. (B) Individualization initiates at the head of the spermatid bundles. 64 F-actin based investment cones (red) form at the nuclei, and 64 investment cones form an individualization complex (IC). (C) IC moves toward the tail of spermatid bundles, squeezes the cytoplasmic bridges and cytoplasm to the cyst bulge. (D) Cystic bulge moves toward the tail and the size increases as individualization progresses. Finally it reaches the tail and becomes a waste bag which contains IC and the materials that are not needed by mature sperm. The waste bag will detach from the spermatid bundles and degrade, and individualized sperms (shown by solid lines) have been produced.
Figure 1.3. The JAK/STAT signaling pathway. Upon the binding of the ligand, receptor-associated JAK activates each other by trans-phosphorylation. Activated JAK then phosphorylates the receptor, providing the docking site for STATs. STATs are also phosphorylated by JAK and form homodimers which translocate to the nucleus, binding to the STAT binding site and driving the expression of target genes.
Chapter 2: Upd3 plays a role in the molecular niche of stem cells in *Drosophila* testis

**Introduction**

Compared to the dozens of ligands of the JAK/STAT pathway in mammals, the only known ligands in *Drosophila* are Upd, Upd2 and Upd3 (see reviews Rawlings et al., 2004; Stark and Darnell, 2012). The *upd* family is a set of duplicated genes located in a 70kb region on X chromosome. *upd3* is located between * upd* and * upd2*, and its transcriptional direction is opposite to that of * upd* and * upd2* (see review Hombria and Brown, 2002). All of them are able to activate the JAK/STAT pathway, although the abilities differ: Upd is the most potent ligand, while Upd2 is the least potent, at least as shown by in vitro experiments (Wright et al., 2011).

*upd* and * upd3* are co-expressed and play similar functions in some tissues. During eye development, both * upd* and * upd3* are expressed in the posterior region of the eye discs; and both promote cell proliferation during eye development (Chao et al., 2004; Tsai and Sun, 2004; Wang et al., 2014). * upd* and * upd3* are also co-expressed in the wing discs and haltere discs, and loss of function of either * upd* or * upd3* leads to outstretched wings or downward extended halteres (Wang et al., 2014). In addition, they are also co-expressed in the polar cells of egg chambers, and loss of function of * upd* or * upd3* leads to fused egg chamber phenotypes (Wang et al., 2014). When the midgut is damaged, * upd* and * upd3* are both expressed in the enterocytes to promote the proliferation and differentiation of intestinal stem cells and replenish the intestinal epithelium in the midgut (Jiang et al., 2009).

Upd serves as the molecular niche of stem cells in *Drosophila* testis. Both in situ hybridization and * upd*-lacZ, a lacZ reporter of * upd*, shows * upd* is expressed in the hub (Boyle et al., 2007; Kiger et al., 2001; Tulina and Matunis, 2001). In addition, ectopic expression of * upd* leads to ectopic GSC-like and CySC-like cells (Boyle et al., 2007; Kiger...
et al., 2001; Tulina and Matunis, 2001). Since upd and upd3 are co-expressed and play similar roles in several tissues as mentioned above, it was hypothesized that upd3 is co-expressed with upd and both promote the self-renewal of stem cells in the testis.

To investigate the functions of Upd3, several mutants of upd3 were made by others in our lab, including a presumably null allele (upd3d232a) and a presumably hypomorphic allele (upd3x21b) (Wang et al., 2014). The mutants show some phenotypes, including reduced lifespan (personal communication with Lakshmi Pillai), smaller eyes, outstretched wings, downward halteres and premature male sterility (Wang et al., 2014). Wild-type and upd3 mutant males become sterile at 38 days and 21 days, respectively, supporting the hypothesis that upd3 is expressed in the testis and plays a role in spermatogenesis (Wang et al., 2014). But why does the testis need two ligands of the JAK/STAT pathway for spermatogenesis?

The activity of the Dpp pathway decreases with age in the germaria of ovaries, where stem cells are located, leading to the age related decline in the number and division rate of GSCs (Zhao et al., 2008). BMP signaling in GSCs is positively regulated by JAK/STAT activity in cap cells to maintain GSCs in the germaria (Lopez-Onieva et al., 2008). Thus, decreased BMP signaling in old germaria suggests the activity of JAK signaling may also decrease with age, consistent with the reported decline of Upd in the hub of testes (Boyle et al., 2007). Ovaries are composed of germaria and developing egg chambers. Although it has not been reported that the upd level in egg chambers also diminishes with age, upd level and the activity of the JAK/STAT pathway in the whole ovaries, including upd from the germaria and egg chambers decreases with age (personal communication with Claire Venard). In wild-type, the percentage of unhatched eggs increases as the mother ages. In upd3d232a, this percentage is higher than that of the wild-type at the same age (Wang et al., 2014), suggesting Upd3 is additive to Upd and may have an anti-aging effect which counteracts the age related decline of Upd in the ovaries. Does Upd3 also have an anti-aging effect in testis?
In testis, the expression level of *upd* and number of GSCs both decrease with age. When *upd* expression in the hub was increased by experimental manipulation, the loss of GSCs in old testes was slowed or prevented, suggesting that the decline of *upd* expression level is the cause of GSCs loss in old testis (Boyle *et al.*, 2007). IGF-II messenger RNA binding protein (Imp) binds to the 3'UTR of *upd* mRNA and protects it from degradation. As a fly get old, the expression level of Imp in the hub cells drops due to its targeting by the heterochronic microRNA let-7, whose expression increases with age. Thus, the let-7-Imp axis leads to the age related diminish of Upd in the hub (Toledano *et al.*, 2012). As the fly ages, male fertility also declines (Snoke and Promislow, 2003). Reducing the number of GSCs caused by decreased Upd level is correlated with decreased male fertility, suggesting that there is a positive association between *upd* level and male fertility. Upd3 mutant males become sterile earlier than the wild-type, suggesting that Upd3 may be additive to Upd and counteract the age related diminishment of Upd, hence Upd3 may have an anti-aging effect in spermatogenesis. To test this possibility, the number of stem cells in different ages of testes from wild-type and mutants of *upd3* was quantified and compared.
Results

Upd plays a role in the molecular niche of stem cells in testis by activating the JAK/STAT pathway in both CySCs and GSCs (Kiger et al., 2001; Leatherman and Dinardo, 2010; Tulina and Matunis, 2001). Since upd is expressed in the hub of the testis (Kiger et al., 2001; Leatherman and Dinardo, 2010; Tulina and Matunis, 2001), we tested whether upd3 is also expressed there by in situ hybridization (ISH). upd was used as a positive control, the sense probes were used as negative controls. The results show that similar to upd, upd3 is expressed in the hub of testis (Figure 2.1).

Upd3 released from the hub may be able to activate JAK/STAT in GSCs and CySCs, hence promote the self-renewal of both type of stem cells. To test this possibility, the number of CySCs and GSCs in the testis of wild-type and upd3 mutants upd3\textsuperscript{d232a} and upd3\textsuperscript{d21b} were quantified by immunostaining. Compared to the wild-type males, which lose their fertility at about 38 days, upd3\textsuperscript{d232a} males lose their fertility at about 21 days (Wang et al., 2014). In order to test the change of stem cell number as the flies age, the number of GSCs and CySCs in testis of one, two and three week old wild-type and upd3 mutant testes were counted. Fas III marks hub cells (Wang et al., 2006), vasa marks germline cells. Small vasa\textsuperscript{+} cells contacting the hub were counted as GSCs (Tulina and Matunis, 2001). Traffic Jam (Tj) is a transcription factor that is expressed in the nuclei of CySCs and early cyst cells (Li et al., 2003). Since the cytoplasmic extensions of CySCs contact the hub directly and the nuclei of CySCs are located near the GSCs, Tj\textsuperscript{+} cells within one cell distance from the hub were counted as CySCs.

In testis, the null mutant upd3\textsuperscript{d232a} have fewer GSCs than upd3\textsuperscript{+} and the hypomorph mutant upd3\textsuperscript{x21b} at all time-points, and have fewer CySCs than upd3\textsuperscript{+} at one and three weeks. At two weeks, although the difference between upd3\textsuperscript{+} and upd3\textsuperscript{d232a} for CySCs/testis is not statistically significant, the P value for student T test is 0.058, very close to 0.05. The differences didn’t change significantly as the fly aged (Figure 2.2, B-E). There is no difference for stem cell numbers between upd3\textsuperscript{+} and upd3\textsuperscript{x21b} (Figure 2.2, C, D), probably because upd3\textsuperscript{x21b} is a weaker allele than upd3\textsuperscript{d232a}.
The number of stem cells in the same genotype was also compared at one, two and three weeks with each other (Figure 2.2, C, and D). As the age increases for upd3+, the number of CySCs increases from one week to two weeks, and then from two to three weeks, although the increase is statistically significant only between one and three week old upd3+. The P value for student T test between one and two weeks upd3+ is bigger than 0.05 (0.083). In the upd3d232a mutant, the number of CySCs increases from one week to two weeks, although the P value for this difference is bigger than 0.05 (0.057). A slight decrease was observed from two weeks to three weeks testes of upd3d232a, but the decrease is not statistically significant (P=0.56). It is possible that CySCs will decrease in very old testis of upd3+ such as four or five weeks. If that is the case, the fact that CySC decreases at three weeks upd3d232a may suggest aging of stem cells is accelerated in the testis of upd3d232a. All three genotypes have more GSCs from one week to two weeks, and then had a decrease from two weeks to three weeks, although only the increase from one to two weeks in upd3x21b and upd3d232a mutants were statistically significant. In the ovaries, JAK/STAT activity peaks at 10-13 days, then decreases by 17-20 days (personal communication with Claire Venard). If JAK/STAT activity also increase from one week to two weeks, then decreases from two weeks to three weeks in the testis, that could explain the observed change of GSCs in testis.

Complete loss of Upd3 (upd3d232a) led to loss of stem cells. Whether gain of Upd3 leads to gain of stem cells was also tested. Ectopic expression of upd led to ectopic GSCs as shown by ectopic bright DAPI staining with small and spherical fusomes (Tulina and Matunis, 2001). Tj-Gal4, a Gal4 driver which is active in CySCs and early cyst cells, was used to misexpress upd3 (Tj-Gal4 UAS-upd3, or Tj>upd3 in short), while misexpression of upd (Tj>upd) was used as a positive control and Tj-Gal4 was used as a negative control. Testes from all the genotypes (Tj-Gal4, Tj>upd and Tj>upd3) were stained with antibodies to vasa, hts and Zfh1 to visualize stem cells, and DAPI to probe the density or size of cells. Misexpression of both ligands led to ectopic bright DAPI
staining which is an indicator of small cells including stem cells and hub cells (Figure 2.3, A.), ectopic Zfh1 staining which is a marker for CySCs (Broihier et al., 1998) (Figure 2.3, B), and ectopic vasa+ cells with small and spherical hts+ fusomes which resemble GSCs (Tulina and Matunis, 2001) (Figure 2.3, D.). These results indicate that misexpression of upd3 generated ectopic CySCs and GSCs, and support the hypothesis that similar to Upd, Upd3 also plays a role in the molecular niche of stem cells in Drosophila testis. Misexpression of upd or upd3 led to enlarged testes, probably caused by over-proliferation, as shown by increased number of germline cells and brighter DAPI staining (Figure 2.3, A and C). However, Tj>upd testes have larger ball-like tips compared to Tj>upd3 testes (Figure 2.3, A). In addition, Tj>upd3 testes has more CySCs and GSCs like cells compared to Tj>upd3 (Figure 2.3, B, C). In addition, all Tj>upd testes (27/27) formed ectopic stem cells, while 80% Tj>upd3 testes (20/25) showed this phenotype. These results may be caused by different expression level of upd or upd3, or because Upd3 is less potent than Upd in activating the JAK/STAT pathway in testis.
Conclusions and Discussion

We found that similar to *upd*, *upd3* is also expressed in the hub. The deletion mutant of *upd3* (*upd3^{d232a}* ) caused fewer CySCs and GSCs compared to the wild-type at all ages. Misexpression of *upd3* led to ectopic CySCs and GSCs, similar to the ectopic stem cells phenotype when *upd* was misexpressed. All these results are consistent with the fact that *upd* and *upd3* have redundant functions in the eye discs, wing discs, haltere discs and polar cells in egg chambers. These results are also consistent with our predictions for the hypothesis that *upd3* has a similar function to *upd* in testis. Taken together, we conclude that similar to Upd, Upd3 also participates in the molecular niche of stem cells in Drosophila testis. What is the relationship between them? Why does the testis need two ligands for the JAK/STAT pathway in testis?

Although the Drosophila JAK/STAT pathway has three ligands, only one receptor, one JAK (Hop) and one STAT (Stat92E) have been reported. To date, the only known function of the binding of Upd, Upd2 or Upd3 to the receptor is the phosphorylation or activation of Stat92E. Although Upd, Upd2 and Upd3 have different potencies in activating the JAK/STAT, these differences can simply be realized by adjusting the expression levels of just one ligand. Why does Drosophila need three ligands for just one STAT? At least for Upd and Upd2, their functions are not completely the same. The phenotypes of null mutant of *upd2* can be completely rescued by *upd*, however, the loss of Upd phenotypes cannot be completely rescued by Upd2. This is possibly because Upd is closely associated with the extracellular matrix, while Upd2 is more freely diffusible (see review Bina and Zeidler, 2009). In fact, Upd2 is secreted from the fat body cell and travels a long distance to reach the brain where it stimulates the insulin like peptide production (Wright et al., 2011). Although Upd and Upd2 may have different functions, *upd* and *upd3* are usually co-expressed in tissues and seem to play similar functions as mentioned above. Why does Drosophila need both Upd and Upd3 for the JAK/STAT pathway?

A possible explanation is that *upd* and *upd3* may have different enhancers and
untranslated regions which increases the diversity of expression pattern of ligands, both spatially and temporally. In fact, even if they are co-expressed, their expression levels may be different. For example, upon apoptosis and JNK-mediated stress in the *Drosophila* midgut, the JAK/STAT pathway is activated in the ISCs and promote their proliferation. Activation of the JAK/STAT pathway is caused by up-regulation of *upd* ligands. However, the fold change of *upd3* is at least 3 times that of *upd*, suggesting Upd3 is more responsive than Upd for replenishing the lost enterocytes in the middle gut (Jiang et al., 2009). The stronger up-regulation of Upd3 than Upd is possibly due to their different enhancers which usually determines the expression level of specific genes. In addition, as mentioned above, the expression level of *upd* in the hub diminishes with age due to decline in Imp activity in binding to the 3'UTR of *upd* mRNA and protecting it from degradation (Toledano et al., 2012). *upd3* has different 3'UTR, hence the degradation of its mRNA may not be regulated by Imp whose level declines with age, so it is possible that *upd3* expression won't decrease with age. What is more, *upd* may form heterodimers with *upd3*, which may affect the stability, distribution and JAK/STAT activating function of the ligands (Chen, 2014).

*upd* and *upd3* are co-expressed in several tissues, including the hub of the testis. When two ligands are co-expressed, there are several possible effects, including, but not limited to: additive, redundant, antagonistic, or qualitatively different effects. In the testis, loss of *upd3* led to loss of stem cells, so it is not antagonistic or redundant, but appears to be an additive effect. Which is more important for the maintenance of stem cells in testis? Since the effect of direct LOF of *upd* on stem cells in testes has not been reported or performed, it is difficult to directly compare their function. However, partial decline of *upd* in 30 days old testes is associated with about 30% reduction of the number of GSCs compared with 1 day old testis, while complete loss of Upd3 only led to 25-30% reduction of stem cells in one week *upd3*^{d232a} compared to 1 week *upd3*+, and about 15% reduction of GSCs for three weeks old *upd3*^{d232a} compared with one week *upd3*+, suggesting that Upd may be more important than Upd3 for the maintenance of stem cells in testis. Complete loss of *upd3* causes a 25-30% reduction
of the number of stem cells compared with the wildtype, suggesting that Upd is able to maintain the majority of stem cells. Why does the testis need additive effect from upd3? As mentioned above, Upd level diminishes with age, which may be partly responsible for the age related decline of male fertility. A minimum level of JAK activity may be required for normal male fertility. As the fly gets older, the activity of the JAK/STAT pathway may decrease due to the age related decline of Upd. When the JAK activity is lower than the threshold of JAK signaling, the fly becomes sterile. Upd3 may add some JAK activity to Upd, hence increase the male reproductive lifespan. This model is consistent with the result that upd3 mutants become sterile earlier than wild-type. Thus, upd3 may have an anti-aging effect for male fertility in testis.

It should be noted that at three weeks when upd3d232a males become sterile, they only has a 25% reduction of the number of CySCs and a 30% reduction of the number of GSCs compared to wild-type. In addition, three week old upd3d232a does not have fewer CySCs and GSCs than one week old upd3d232a. These results raise the possibility that the reduced number of stem cells may not be the major cause of loss of fertility. For example, the quality of stem cells may be poor, so they cannot differentiate into sperm. Or alternatively, upd3, as well as the JAK/STAT pathway may also participate in other stages of spermatogenesis. Therefore, upd3d232a not only have fewer stem cells, but also have defects at other stages of spermatogenesis.
Figure 2.1. *upd3* is expressed in the hub of testis. The tips of wild-type testes were shown after in situ hybridization. (A) *upd* mRNA can be detected in the hub (arrow) of testis by in situ hybridization (ISH). (B) The sense control of *upd* shows there is no staining in the hub. (C) *upd3* mRNA can be detected in the hub (arrow). (D) The sense control of *upd3* shows no signal in the hub.
Figure 2.2. *upd3<sup>d232a</sup> has fewer stem cells than *upd3<sup>+</sup> in the testis*. One, two and three week old testes from *upd3<sup>+</sup>, *upd3<sup>x21b</sup>* and *upd3<sup>d232a</sup>* were stained to visualize the stem cells. (A) The tip of one week old *upd3<sup>+</sup>*. Fas III (green and asterisks) stains the hub (asterisks). Vasa (yellow) stains all germline cells. Small germline cells attached to the hub were counted as GSCs (arrow head). Tj (red) stains the nuclei of CySCs and early cyst cells. Tj<sup>+</sup> nuclei within one cell distance were counted as CySCs (arrow). (B) GSCs (arrow) and CySCs (arrowhead) at the tip of testis from one week old *upd3<sup>d232a</sup>*. (C, D) Quantization of CySCs/testis (C) or GSCs/testis. Difference between different genotypes at the same age, or different age of testes for the same genotype was compared by student T-test. n=15-23. Error bar: SE. *: P<0.05; **: P<0.01; ***: P<0.001.
Figure 2.3. Ectopic expression of *upd3* leads to ectopic stem cells in testis. Column one, two and three are testes from Tj-Gal4 (control), Tj>*upd* (ectopic expression of *upd*) and Tj>*upd3* (ectopic expression of *upd3*), respectively. Row one shows the whole testes, while rows two to four only show the anterior ends of the testis. (A1-A3) DAPI staining and transmitted light images were merged. In Tj-Gal4 (control), bright DAPI staining can only be seen at the tip (arrow) where stem cells are located or at the base of testis (yellow arrow head) where the elongated nuclei of spermatids are located, and in the seminal vesicles (yellow arrow) where mature sperms are stored. Upon ectopic expression of *upd* (A2) and *upd3* (A3), ectopic bright DAPI (red arrows) can be
seen away from the tip (white arrow). (B1-B3) Zfh1 (yellow) marks CySCs and early cyst cells (similar to Tj in Figure 2.2), Fas III (Green) marks the hub. In Tj-Gal4 (B1), Zfh1+ cells can only be seen around the hub (arrow). In ectopic expression of upd (B2) and upd3 (B3), ectopic Zfh1+ cells (red arrows) can be seen away from the normal hub (white arrow in B3. In B2, the normal hub cannot be seen). (C1-C3) GSCs are small vasa+ cells with small and spherical fusomes, normally attach to the hub. In Tj-Gal4, small vasa+ cells can only be seen at the tip (white arrow). As differentiation proceeds, germline cells become larger (green arrow in C1). In ectopic expression of upd (C2) and upd3 (C3), ectopic small vasa+ cells can be seen (red arrows). (D1-D3) Hts marks fusomes (purple). In GSCs of Tj-Gal4 (D1), fusomes are small and spherical (yellow arrow). As differentiation goes on, fusomes become larger and branched (white arrow). In ectopic expression of upd (D2) and upd3 (D3), ectopic small and spherical fusomes (red arrow) can be seen everywhere.
Chapter 3: The JAK/STAT pathway plays a role in individualization

Introduction

As the organism ages, the function of testes and ovaries declines. In female Canton S, a widely used wild-type *Drosophila* strain, egg produced per day decreased from about 50 at 3 days old to less than 10 at 30 days old, and the proportion of eggs with morphological defects increased from 0% at 1 day old to more than 60% at 35 days old, while the proportion of eggs that hatch decreased from 90% at 1 day old to about 33% at 35 days old (Zhao et al., 2008). The decreased quantity and quality was associated with a decline in the number and division rate of GSCs, and with a reduction of BMP pathway activity and Hh expression level. When *dpp*, the ligand for the BMP pathway that promotes self-renewal of GSCs, was over-expressed in the niche, the number of Brdu+ GSCs as well as the number of eggs produced per day increased dramatically, while the apoptotic cells associated with aging decreased significantly (Zhao et al., 2008). The decline in GSCs with age can also be seen in *Drosophila* testis. The number of GSCs decreased from 8.3/testis at 1 days old to 5.1/testis at 50 days old (Boyle et al., 2007). Similar to ovaries, the decline in GSCs with age is associated with reduction of *upd* expression in the hub and STAT expression in the stem cells, and a decline of division rate of GSCs (Boyle et al., 2007). When *upd* is over-expressed in the hub, the decline of GSCs is partly delayed (Boyle et al., 2007). Although they didn't test the effect on male fertility after misexpression of *upd* in the hub, it is known that as male *Drosophila* get older, their fertility decreases (Snoke and Promislow, 2003). Taken together, these two studies raise the possibility that the decline of *upd* expression, which leads to a decline in stem cells division rate and numbers, may contribute to the decline in fertility of *Drosophila* males as they age.

Although stem cells are very important for gametogenesis, this is not the only step that is necessary for fertility. Oogenesis and spermatogenesis are composed of many steps. The defects for each step can impair or even block the successful
production of oocyte and sperm. For example, *bam* is expressed in 4-8 cell spermatogonia and is required for their differentiation. Mutants for *bam* fail to produce sperm, hence they are sterile (McKearin and Spradling, 1990). Caspase activation is required for individualization, while cytochrome C is required for caspase activation. *Drosophila* mutants for cytochrome C cannot go through individualization, and as a result, cannot produce sperm and are sterile (Arama et al., 2003).

*upd3* males become sterile at three weeks old (Wang et al., 2014), however, it was shown in the previous chapter that they only have a 25%-30% reduction in stem cells. More importantly, three week old sterile *upd3* males do not have fewer stem cells than one week old fertile *upd3* males (GSCs: 6.95 vs. 5.29 /testis, p=0.1006; and CySCs: 12.04 vs. 9.71 /testis, p=0.1871 for three week and one week). These results raise the possibility that three week old *upd3* males do not mate with the virgin females due to behavioral problems during the fertility test or alternatively, they also have problems at other stages of spermatogenesis. Is it possible that Upd3 and the JAK/STAT pathway play roles at another stage of spermatogenesis? This possibility is consistent with the observed pleiotropic function of the JAK/STAT pathway in *Drosophila* oogenesis.

Oogenesis is the production of the oocyte, the female haploid gametocyte that can develop into a new organism after fertilized by sperm, and is a process that is similar to spermatogenesis. Ovaries and testes share similar structures and utilize the same signaling pathways for similar process as detailed later. Thus the investigation of oogenesis, especially the role of the JAK/STAT pathway may shed light on the functions the JAK/STAT plays in spermatogenesis.

Each female fly has two ovaries, each ovary composed of about 18 ovarioles, egg production lines (Figure 3.1, A). At the anterior end of each ovariole is the germarium which contains two types of stem cells: germline stem cells (GSCs) and somatic follicle stem cells (SSCs), which develop to follicle cells which cover the outer layer of the egg.
chamber. At the very anterior end of the germarium are terminal filament cells, followed by several cap cells. Cap cells directly contact equal numbers of GSCs located posterior to the cap cells but at the anterior tip of the germarium. Two escort cells which also contact the cap cells wrap a single GSC with cytoplasmic extensions, similar to the two CySCs wrapping a single one GSC in Drosophila testes. After an asymmetric division, the one daughter cell of the GSC that does not contact the cap cells becomes a cystoblast, similar to the gonialblast in testes and is enclosed by two escort cells, which are similar to cyst cells in testes. The cystoblast then goes through four cycles of incomplete mitosis, forming a 16 cell cyst. 15 of them become nurse cells which are located at the anterior region of the cyst and provide nutrients and necessary materials for the oocyte located in the most posterior region of the cyst. After mitosis, the oocyte will enter meiosis but arrest in prophase I before exiting the germarium. After the division of germline cells, the cyst is composed of sixteen germline cells enclosed by a single layer of follicle cells derived from SSCs. The cyst then buds off from the germarium, and becomes the earliest stage egg chamber (Figure 3.1, B).

The egg chambers move toward the posterior end of the ovariole as it matures. It takes about 7 days for oogenesis to complete, and egg chamber development can be divided into 14 stages (see review Bastock and St Johnston, 2008). Each egg chamber is connected to the neighboring egg chambers by somatic stalk cells. In stage one egg chambers, the size of the oocyte is similar to that of nurse cells. As the egg chamber matures, the oocyte receives materials synthesized in the nurse cells, and its size increases dramatically. Meanwhile, the number of follicle cells also increases to a maximum of 1000 cells (see review Bastock and St Johnston, 2008).

Oogenesis and spermatogenesis use the same signaling pathways in similar biological processes. During embryonic development, Notch signaling controls the formation of the stem cell niche in both processes, which are caps cells in the ovaries (Song et al., 2007), and are hub cells in testis (Kitadate and Kobayashi, 2010). The Hedgehog, BMP and the JAK/STAT pathways contribute to the maintenance of stem
cells in both the ovaries and testis. In the ovaries, Hedgehog released from the cap cells promote the self-renewal of somatic follicle stem cells (Apionishev et al., 2005; Forbes et al., 1996; Margolis and Spradling, 1995). In the testis, Hedgehog released from the hub controls the self-renewal of somatic CySCs (Amoyel et al., 2012; Michel et al., 2012). JAK/STAT activation promotes the self-renewal of stem cells both in the ovaries and testis (see review Gregory et al., 2008)). In addition, the BMP pathway is activated in GSCs and promotes the self-renewal of GSCs in both the ovaries and testis (Lopez-Onieva et al., 2008). These reports suggest that the ovaries and testis use the same signaling pathways for similar processes.

As a highly reutilized signaling pathway, the JAK/STAT pathway not only participates in the maintenance of stem cells in ovaries, but also plays functions at later stages of oogenesis. In early oogenesis, Upd released from polar cells activate JAK signaling in the neighboring follicle cells, making them adopt the stalk cell fate (McGregor et al., 2002). After the specification of stalk cells, 3-6 polar cells are produced at each end. Upd released from polar cells activates STAT in the neighboring follicle cells, stalk cells and polar cells themselves. Activated STAT signaling in these cells then activates apoptosis in polar cells through an unknown relay signal, making sure that there are only 2 polar cells at each pole (Borensztejn et al., 2013). At later stages of oogenesis, Upd released from the polar cells form a gradient concentration, activating JAK signaling in surrounding follicle cells, and controls the fate of follicle cells according to the concentration of Upd that they receive (Xi et al., 2003) (Figure 3.1, C). Thus, in the differentiation of oogenesis, JAK signaling not only controls cell death, but also determines cell fate of follicle cells.

 upd3d232a loses its fertility at three weeks when it does not have fewer stem cells than it has at one week old. Because, ovaries and testis use the same signaling pathway for similar events, and the JAK/STAT signaling is known to play a role in the patterning of somatic follicle cells as a morphogen in the ovaries (Xi et al., 2003), it is interesting to ask whether the JAK/STAT signaling has functions in later stages of
spermatogenesis. If it plays a role at other stages of spermatogenesis, JAK and STAT should be expressed at those later stages. To test the potential function of \textit{upd3} and the JAK/STAT at other stages of spermatogenesis, the expression pattern of \textit{upd3} and the activation pattern of the JAK/STAT signaling pathway were examined by in situ hybridization and using reporters of the JAK/STAT pathway. Surprisingly, we found that the JAK/STAT pathway is also activated in elongated cyst cells, which has never been reported before. By knocking down components of the JAK/STAT pathway at stages at which it is expressed, it was found that JAK signaling in cyst cells is required for individualization.
Results

The JAK/STAT pathway is also activated in elongated cyst cells

To determine the cause of reduced fertility of upd3 mutant males, the possibility that old upd3\(^{d232a}\) males did not mate with virgin females was first tested. Each 48 day old upd3\(^{d232a}\) male was placed in a vial with three young virgin females, and the copulation was observed every 10 minutes in two hours. It was found that 80% (16/20) of the males mated with virgin females, hence ruling out a behavioral problems.

To determine if a later function of upd3 in spermatogenesis is responsible for the reduced fertility, the expression profile of upd3 at later stages of spermatogenesis, i.e., after stem cell maintenance, in testis was examined using ISH (data not shown). However, ISH in testis is notoriously difficult (personal communication with Erica Matunis), especially after meiosis. Even in the sense-strand negative control, high background in the spermatid bundles away from the tip was always seen. This is probably due to the somatic permeability barrier which becomes much less permeable by late spermatocyte stage (Fairchild et al., 2014). This technical problem made it very difficult to detect by ISH where upd3 is expressed in regions located distantly from the tip. Another way to detect the expression pattern of upd3 in testis is to use the upd3 reporter upd3-Gal4, a Gal4 driver that is under the control of the hypothetical enhancer which is a 4kb fragment located 1.7kb upstream to 2.3 downstream of the transcription start of upd3 (Agaisse et al., 2003). Upd3-Gal4 was crossed with UAS-GFP. However, upd3>Gal4 (> denotes Gal4::UAS) showed no GFP at all in the testis, including the hub. This is inconsistent with the ISH result that upd3 is expressed the hub (Figure 2.1). Since the upd3-Gal4 only contains a hypothetical enhancer region, which only contains a small proportion of the sequence upstream of upd3, it may not faithfully reflect the all aspects of the expression pattern of upd3.

Compared with detecting the expression pattern of upd3, it should be much easier to detect the activity of JAK signaling in testes, because several in vivo reporters
of the *Drosophila* JAK/STAT pathway such as 10X STAT-GFP, 3X GAS-lacZ, dome-lacZ are available (*Bach et al., 2007; Gilbert et al., 2005; Xi et al., 2003*). Among these reporters, 10x STAT-GFP is the most commonly used (*see review Chen et al., 2014*). The first intron of the inhibitor *Socs36E* contains a 441bp fragment which has at least two potential Stat92E binding sites. Five tandem repeats (10 Stat92E binding site in total) of this 441bp fragment were placed upstream of a minimal heat-shock promoter and a cDNA encoding enhanced GFP to drive the expression of enhanced GFP in vivo. GFP expression from this transgene therefore reflects activation of the JAK/STAT pathway (*Bach et al., 2007*). This reporter has been successfully used to detect JAK/STAT activity in several tissues, including midgut (*Singh et al., 2012; Zhou et al., 2013*), eye discs (*Woodfield et al., 2013*) and lymph glands (*Mondal et al., 2011*). 10x STAT-GFP was used to test where the JAK/STAT pathway is activate in testes. Anti-STAT staining showed that STAT is expressed in both GSCs and CySCs, but is absent from the hub (*Leatherman and DiNardo, 2008*). Consistent with this result, weak GFP can be seen in both GSCs and CySCs using confocal microscopy (data not shown). Stronger GFP than in the stem cells was also seen in the hub (Figure 3.2, A, arrowhead), which is inconsistent with the anti-STAT staining (*Leatherman and DiNardo, 2008*), raising the possibility of false positive result of this reporter.

Interestingly, GFP is much stronger in a region away from the tip than in the hub. The GFP+ cells seem to be in elongated layers (Figure 3.2, A, arrow), suggesting it is expressed in cysts after elongation. If STAT is activated at later stages of spermatogenesis, is it activated in somatic cyst cells, or germline spermatids? In which specific stage is it activated? To answer these questions, immunostaining combined with confocal microscopy, was performed to visualize the cell type and morphology of the cells that expresses GFP. The receptor of the Wnt pathway, Frizzled, is clearly expressed in all of the elongated germline spermatid bundles. The GFP positive cells were seem to enclose, but do not overlap all Fz+ cells (Figure 3.2, D-F), demonstrating that GFP is expressed in the elongated cyst cells surrounding germline cells. This result shows that the JAK/STAT pathway is also activated in the elongated cyst cells,
suggesting that it may play a role at later stages of spermiogenesis.

Since 10x STAT-GFP may have false positive result just as in the hub, we also tried other reporters to see whether the activity of the JAK/STAT away from the tip shown by 10x STAT GFP is real. Sbs-lacZ and Dome-lacZ were used to verify the result (Hombria et al., 2005; Xi, 2002; Xi et al., 2003). Unfortunately, Sbs-lacZ showed diffused staining all over the testis, while Dome-lacZ shows no signal in testis (data not shown). This is contradictory to the fact that the JAK/STAT pathway is activated in the stem cells in testis (see review Gregory et al., 2008), so Dome-lacZ may not be a responsive reporter in the testis. An alternative way to verify the result of 10x STAT-GFP in elongated cyst cells is to knockdown the components of the JAK/STAT pathway in cyst cells. If the loss of function of the JAK/STAT signaling leads to defects of spermatogenesis, it would be strong support that STAT is activated in elongated cyst cells.

What functions does JAK/STAT play in the elongated cyst cells, where it is strongly activated? Since the next step of spermatogenesis after elongation is individualization, we hypothesized that it may play a role in individualization. After meiosis, the cyst, composed of 2 cyst cells enclosing 64 spermatocytes, elongates. During elongation, the length of spermatids will increase from 10 um to 1850 um, but the volume remains the same. Elongation involves the development of an axonemal-based tail for motility. As the spermatid elongates, the nuclei also condense to a compact needle-shaped. During this process, the spermatids within one cyst are still interconnected by ring canals which are the conduits between syncytial germline cells as mentioned in the background. After elongation, long spermatids with condensed needle-shaped nuclei and a flagellar tail are formed. The flagellum is composed of axonemes and is associated with mitochondria, and is used for the motility of sperm (see reviews Fuller, 1993; Fuller, 1998).

After elongation, individualization occurs to eliminate the cytoplasmic bridges and most of the cytoplasm that is useless for the mature sperm. F-actin based
microfilament structures, called investment cones form near the nuclei of elongated spermatids. 64 investment cones within one cyst form an individualization complex (IC). Both dynein and Myosin V participate in the assembly of investment cones. Dynein light chain 1 regulates F-actin assembly by retaining dynamin to the investment cones (Ghosh-Roy et al., 2005). The movement can be inhibited by actin inhibitors, but not tubulin inhibitors, indicating the movement requires actin, but not microtubules (Noguchi and Miller, 2003). The membrane remodels at the place where ICs are located, forms a characteristic structure called the cystic bulge (CBs) which contains ICs. CBs also move toward the tail of spermatids as ICs move. Cytoplasm and organelles that are unnecessary for the mature sperm are squeezed out of the spermatids and contained in the CBs as the IC moves toward the tail, making the CBs bigger and bigger. Finally, the CBs reaches the tail and become a waste bag (WBs), containing all the materials that are no-longer needed for the sperm (Arama et al., 2003; Noguchi and Miller, 2003).

As mentioned above, individualization has three characteristic structures: IC, CBs and WBs. The IC is an F-actin based structure and can be stained by phalloidin (Wulf et al., 1979). CBs and WBs can be visualized by acridine orange (AO), which is a vital dye and stains apoptotic cells (Arama et al., 2003). The reason why AO stains apoptotic cells is because AO can enter the acidic compartments and be sequestered there (Millot et al., 1997), while apoptosis have very low PH (Lagadic-Gossmann et al., 2004). Although there is no cell death involved in individualization, caspase activity is required. Two effector caspases in Drosophila, DCP-1 and drICE, can be stained in the CBs and WBs (Arama et al., 2003). In wild-type testes, AO stains the CBs, WBs and degrading WBs fragments (Arama et al., 2003), probably because these structures are acidic. AO can also be used to differentially stain DNA and RNA. When AO intercalates into double-stranded nucleic acids, which is usually DNA, when excited in blue light, the maximum emission wavelength is 530 nm, When intercalates into single strand nucleic acids, which usually is RNA, the maximum emission wavelength is 640 nm (Darzynkiewicz, 1990). The concentration used for nucleic acid staining is about
10µg/ml, while for CBs, WBs and apoptosis detection is only 0.5µg/ml (Arama et al., 2003).

**upd3^d232a testis has individualization defects**

*upd3^d232a* males lose their fertility at three weeks when they only have about 25-30% reduction of stem cells in testis compared to wild-type. More importantly, three week *upd3^d232a* males do not have fewer stem cells in the testis than one week old *upd3^d232a* when they are still fertile. These results suggest that the decline of stem cells in *upd3^d232a* is not the only cause of sterility, and *upd3^d232a* must have other defects that affect fertility, perhaps in a later stage of spermatogenesis. Since the JAK/STAT appears to be activated in elongated cyst cells, *upd3* may also be expressed and contribute to the activity of JAK signaling during or after elongation. The next step of spermatogenesis is individualization, so it was tested whether *upd3^d232a* has individualization defects.

Compared to the wild-type control, the testes of *upd3^d232a* have fewer CBs and WBs based on AO staining both at one week and three weeks old (Figure 3.3, A1-B2, and C). At one week, *upd3^d232a* testes have fewer ICs than those from wild-type (Figure 3.4, A1-C). ICs are formed at the base of the testis first, then move toward the hub. The proportion of more progressed non-basal ICs, the ICs located away from the base of testis (the base of testis is defined as within about 400µm from the seminal vesicle), to all ICs may be an indicator of the progression of individualization. Does Upd3 not only play a role in the initiation, but also in the progression of individualization? To answer this question, the non-basal IC/all IC proportion was quantified. No difference was seen between the wild-type and control for this ratio (Figure 3.4, D), suggesting non-basal IC to all IC ratio may not be a sensitive indicator for individualization progression, or Upd3 does not play a major function in the progression of individualization.
The JAK/STAT pathway is required for individualization

An individualization defect phenotype in upd3[d232a] testes was not significant: at one week, there was only about 10%-20% reduction of CBs, WBs and ICs. This may be because there are three ligands for the JAK/STAT pathway in Drosophila, and Upd3 is not the major one to activate the JAK/STAT in elongated cyst cells. To determine the effect of strong loss of the JAK/STAT pathway activity in spermatogenesis, the only STAT, stat92E, was knocked down in cyst cells by crossing eya-Gal4, a driver that is specifically active in all the differentiated cyst cells (Leatherman and DiNardo, 2008), with a UAS-STAT RNAi line from the Transgenic RNAi Project (Ni et al., 2011). In the eya-Gal4 line alone which is the control, CBs and WBs could be seen, and there was no AO staining in spermatid bundles (Figure 3.5, A1 and A1'). When STAT was knocked down in cyst cells (genotype: eya>STAT RNAi), CBs and WBs were almost completely eliminated, and the testis had some AO staining throughout spermatid bundles (Figure 3.5, A2 and A2'). As mentioned above, low concentration of AO may stain the acidic cellular compartments. It seems in the control, the PH in CBs and WBs are low, while in the remaining area in the spermatids are normal. It is possible that when JAK signaling is reduced in cyst cells, some of the spermatid bundles have lower pH, or the low pH material cannot be sequestered to CBs and WBs, thus formed the AO+ spermatid bundles.

In the control, many ICs (10.65/testis, or 0.4088 for ICs/spermatid bundles) can be seen (Figure 3.4, B1 and B1'). When STAT is knocked down in cyst cells, the number of IC was greatly reduced (2.25/testis, or 0.0978 for ICs/spermatid bundles) (Figure 3.6, A2 and A2'). ICs are formed at the base of testis where the nuclei of elongated spermatids are located, then moves toward the tip of testis. In eya>STAT RNAi testes, the very few ICs are all located at the base of testis (Figure 3.6, D), suggesting STAT activity is not only required for the formation of IC, but also for the movement of ICs. The great reduction of CBs, WBs and IC in testes with reduced STAT activity indicates that individualization is blocked. Sperm can easily be visualized in the seminal vesicle by DAPI staining based on the bright needle shaped nuclei (Figure 3.6, B1). No sperm
can be seen in the seminal vesicle of testes in which STAT is knocked down in cyst cells, and the seminal vesicle is much smaller than the control (Figure 3.6, B2), suggesting individualization is completely blocked, hence no sperm can be produced. All these results support the hypothesis that STAT activity in cyst cells is required for individualization.

To confirm the phenotypes of STAT knockdown in cyst cells, the receptor Dome was also knocked down using two independent lines of UAS-Dome RNAi. RNAi may also knock down other genes non-specifically, which is termed off-target phenotypes (Jackson et al., 2003). Since we used several different RNAi lines to knockdown the components of the JAK/STAT pathway, the possibility that they will produce the same off-target phenotype is very low. To further confirm the result from RNAi, the inhibitor et (lat) was misexpressed in the cyst cells. If each treatment leads to similar phenotypes, then the phenotypes are reliable. To test whether upd is expressed in the cyst cells, upd was also knocked down by eya-Gal4. Knock down of Dome (with 2 independent RNAi lines), STAT (as mentioned above), upd, and misexpression of et in cyst cells were performed. The number of CBs, WBs and ICs were quantified. Knock down of STAT, Dome (2 lines), and misexpression of et in cyst cells led to significant reduction of CBs, WBs and ICs (Figure 3.11, A-D), supporting the idea that the JAK/STAT is activated in cyst cells and this activation is required for spermatid individualization.

When upd was knocked down in cyst cells, it did not significantly reduce the number of CBs, WBs or ICs, supporting the idea that Upd is not produced by the cyst cells (Figure 3.6, D-G). To test whether it is from the spermatids, upd was knocked down by bam-Gal4 which is active in 4-8 cell spermatogonial stage germline cells (Chen and McKearin, 2003). The knockdown of upd in germline led to about 78% reduction of CBs and WBs (Figure 3.7, B), 23.5% reduction of ICs (Figure 3.8, B), and increased AO+ spermatid bundles dramatically (Figure 3.7, C), supporting the idea that Upd is secreted from the spermatids to initiate individualization. When upd was knocked down in upd3 mutant background, no stronger reduction of CBs, WBs and IC compared
to the knock down of *upd* only was seen. This result implies that Upd3 plays a minor role in individualization compared to Upd.

The non-basal IC/all IC percentage was also quantified. While *upd* KD in the germline reduced CBs, WBs and ICs, there is no reduction of non-basal IC percentage. However, when STAT and Dome was knocked down in the cyst cells, all ICs were located at the base of testis. The vast majority (>95%) of the remaining IC in testes when *Stat92E, dome* was knocked down, or *et* was misexpressed in testes were located at the base (Figure 3.6, D), suggesting that the progression of individualization requires stronger JAK activity than the initiation of individualization.

The Gal4-UAS system and RNAi knockdown does not work well in the germline (Brand and Perrimon, 1993; Ni et al., 2008). To make the Gal4-UAS system work, Gal4 must be transcribed and translated, before it can drive the transcription of genes downstream of a UAS promoter. In the male germline, transcription and translation are strictly controlled. After meiosis, there is almost no transcription. The genes required for spermatogenesis after meiosis are transcribed before meiosis, saved and translated when needed after meiosis (see review White-Cooper, 2010). This makes it difficult for the Gal4-UAS system to work well in the germline after meiosis. Bam-Gal4 is active during the mitotic stages, so it still can drive gene expression. Although bam-Gal4 is not active after mitosis, Gal4 protein may persist for a long time, and the double strand RNA driven by Gal4 may last even longer. Thus bam-Gal4 may be used to knock down mRNA in spermatids even after elongation. In fact, bam-Gal4 has been successfully used to knockdown genes in the germline of testis (Yu et al., 2014). Vectors used to make the UAS lines also have an important effect on the efficiency of transcription in the germline. It has been known that the original vector pUAST does not lead to sufficient expression in the germline, while pUASP and pVALIUM20, pVALIUM22 or pMF3 work well (Dietzl et al., 2007a; Ni et al., 2011; Rorth, 1998; Wang and Elgin, 2011). To rule out potential false negative results, only germline compatible UAS-RNAi lines were used, except for UAS-et because only a pUAST version of it was
available. Thus if bam>et shows no individualization defects, it may be a false negative result, or the inhibition of JAK/STAT activity in the germline has no effect on individualization.

Although 10X STAT-GFP is strongly activated in cyst cells, we cannot rule out the possibility that it is also activated in spermatids at a lower level. To test whether there is STAT activity in the spermatids play a role in individualization, STAT and Dome were also knocked down, and et was misexpressed in the germline by bam-Gal4. Surprisingly, all of the RNAi genotypes showed reduced CBs and WBs, suggesting JAK signaling in the germline also participates in individualization. However, only bam>Dome RNAi-2 had significantly increased AO⁺ spermatid bundles (Figure 3.7, B and C). Individualization defects are characterized by reduced number of CBs and WBs, and increased number of AO⁺ spermatid bundles. Thus, the unchanged number of AO⁺ spermatid bundles in bam>STAT RNAi, bam>Dome RNAi1 is contradictory to their reduced number of CBs and WBs. Since all the RNAi genotypes showed reduced number of CBs and WBs, it is possible that the reduction was caused by a non-specific effect of RNAi. Thus, AO staining results of bam>STAT RNAi, bam>Dome RNAi1 and bam>Dome RNAi2 may not indicate function of the JAK pathway in the germline. To further test the possible role of JAK signaling in the germline, et was misexpressed by bam-Gal4. Bam>et did not reduce the number of CBs and WBs, and did not increase the number of AO⁺ spermatid bundles. These results are consistent with the hypothesis that JAK signaling is activated in cyst cells. However, since the UAS-et was made from the germline incompatible vector pUAST (Makki et al., 2010), it is unclear whether the misexpression of et was successful or not. Thus, we cannot come to any conclusion based on the phenotypes of bam>et expression in the testis. Since we cannot make a safe conclusion based on the AO staining results for bam>STAT RNAi, bam>Dome RNAi1, bam>Dome RNAi2 and bam>et, phalloidin staining was performed to test the role of JAK signaling in the germline.

The knockdown of Dome in the germline, i.e. Bam>Dome RNAi1 and Bam>Dome
RNAi2, did not reduce the number of ICs (Figure 3.8, B), suggesting that JAK signaling in the germline does not play a role in individualization. But surprisingly, STAT knockdown in the germline led to slightly more ICs (Figure 3.8, B), suggesting STAT activity in the germline did not facilitate individualization, but inhibited it. Alternatively, the impairment was incomplete and spermatids were able to begin individualization, but cannot complete the whole process, leading to an accumulation of ICs. Without ligand activation, unphosphorylated STAT can bind to chromosomes and maintain heterochromatin, a non-canonical role of JAK/STAT signaling (see review Mohr et al., 2012). Bam>Dome RNAi1 and bam>Dome RNAi2 did not change the number of ICs, while bam>STAT RNAi increased it. It is possible that increase in IC formation in bam>STAT RNAi was caused by the reduction of non-canonical STAT activity. Since the increase of ICs in bam>STAT RNAi is mild, it is also possible that this may be a false positive result. Testes of bam>STAT RNAi, bam>Dome RNAi1 and bam>et did not change the proportion of non-basal IC/all IC, but bam>Dome RNAi2 reduced this proportion significantly (Figure 3.8, C). Since the testes of bam>Dome RNAi1 and bam>Dome RNAi2 showed different phenotypes, it is difficult to reach a conclusion regarding the role of Dome in the germline for non-basal IC/all IC proportion. However, for IC/spermatid bundles proportion, both bam>Dome RNAi1 and bam>Dome RNAi2 did not show any change compared with the control, suggesting IC/spermatid bundles result may be a better indicator for individualization defects.

It is unknown why AO stains CBs and WBs, but we do know that ICs can be stained by phalloidin because ICs are F-actin based structure. In addition, AO staining for all the bam>RNAi testes showed reduced number of CBs and WBs, suggesting that RNAi in the germline led to non-specific reduction of AO+ CBs and WBs. Thus, it is possible that IC quantification by phalloidin is a more reliable assay of individualization than AO staining. UAS-Dome RNAi and UAS-Dome RNAi2 are made from germline compatible vector, and have no off targets, while UAS-et was made from germline incompatible vector, and it is unknown whether UAS-STAT RNAi has off targets or not. Thus, the result from Bam>Dome RNAi1, Bam>Dome RNAi2 are most reliable than Bam>STAT.
RNAi and Bam>et. They both had no reduction of ICs, suggesting that JAK signaling is not required for individualization in the germline, and RNAi in the germline does not leads to non-specific reduction of ICs. Thus, the reduction of ICs in bam>upd RNAi may be real, supporting the hypothesis that Upd is released from the spermatids.

To sum up, bam>et may not lead to misexpression of et, bam>STAT RNAi and bam>Dome RNAi caused inconsistent results. In addition, no GFP can be seen in 10X STAT-GFP in the elongated spermatids, indicating STAT is not activated there (Figure 3.2, C, D). Taken together, we concluded that there is no solid result to support the idea that canonical JAK signaling in the germline play a role in individualization.

Caspase in spermatids is downstream of the JAK/STAT activation in cyst cells in controlling individualization

The cytoplasmic bridges and most of the cytoplasm in the spermatids are squeezed out and degraded during individualization. Although there is no cell death, individualization is similar to apoptosis for that both require the activity of cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases (Caspase) (Arama et al., 2003). The caspase family is composed of initiator caspases and effector caspases (also termed executioner caspases). All of them exist in the cell as inactive pro-caspases containing a prodomain. Initiator caspases have a long prodomain, while effector caspases have a very short prodomain. Upon receiving of a cell death signal, such as activation of death receptors and apoptosome that is regulated by cytochrome c, initiator caspases (2, 8, 9 and 10 in humans) are clustered and then activated. The activated initiator caspase then activates the effector caspases (3, 6, 7 in humans), which lead to cleavage of other proteins to cause cell death. The protease activation cascades can be sub-divided into two apoptotic signaling pathways: intrinsic and extrinsic pathways. In the extrinsic pathway, pro-apoptotic ligands bind to the pro-apoptotic receptors Fas, TRAIL and tumor necrosis factor (TNF) receptor and activate initiator caspase 8 and 10, which then activate the effector caspases. In the intrinsic pathway, pro-apoptotic signals such as ionizing radiation, chemotherapeutic drugs and
mitochondrial damage lead to the release of cytochrome C from the mitochondria. Released cytochrome C forms the apoptosome, in which caspase 9 becomes activated. Activated caspase 9 then activates the effector caspases (see review Boatright and Salvesen, 2003).

In *Drosophila*, there are three initiator caspases: Dronc, Strica (Dream), and Dredd; four effector caspases: drICE, DCP-1, Decay, and Damm (Daydream) (Hay and Guo, 2006), and two closely linked cytochrome C genes, cyt-c-d and cyt-c-p. Cyt-c-p is expressed at much higher level than Cyt-c-d, and is the major form of cytochrome C in *Drosophila*. After elongation, the effector caspase drICE is activated at the head (nucleus) of spermatid bundles. When caspase inhibitor Z-VAD, which has been shown to block drICE activity, is added to cultured testes, or an inhibitor of caspase is misexpressed in spermatids, CBs and WBs became flattened, ICs cannot move toward the tail, and the 64 investment cones of a single IC become scattered, indicating caspase is required for the progression of individualization (Arama et al., 2003). Normally, when an effector caspase is activated, it stimulates nuclear fragmentation. In a mutant of dBruce, a potential inhibitor of caspases, the nuclei of elongated spermatids become more condensed and degenerate, indicating dBruce functions against drICE to protect the nuclei of spermatids from degeneration (Arama et al., 2003). When DCP-1, another effector caspase DCP-1 was knocked down in the germline by RNAi, although western blot showed the level of drICE was not reduced, individualization was prevented, suggesting DCP-1i is required for the individualization in *Drosophila* spermatogenesis (Huh et al., 2004). In a mutant driceless which lacks drICE activation completely, about 50% of the elongated cysts individualize normally, and the remaining cysts fail to individualize, suggesting drICE activation is partly required for individualization (Huh et al., 2004). In cyt-c-d mutants, although investment cones can be formed at the nuclei of elongated spermatids, they cannot move towards the tail, indicating cyt-c-d or caspases are required for the progression of individualization, but not the assembly of ICs (Arama et al., 2003).
JAK/STAT is activated in elongated cyst cells, while effector caspases are activated in some of the elongated spermatids. Both are required for individualization. Based on this association, it was hypothesized that the JAK/STAT signaling in cyst cells is required for caspase activity in spermatids. Immunostaining using antibodies against DCP-1 or active drICE and were performed to test this hypothesis. In the control eye-Gal4, DCP-1 or active drICE can be seen in some elongated spermatids, and are enriched in CBs and WBs (Figure 3.9, A and C). The reason why DCP-1 and drICE staining are not seen in some elongated spermatid bundles may be because those spermatid bundles have not yet initiated, or have already finished individualization. DCP-1 or active drICE was completely eliminated in testes when STAT or Dome was knocked down, or et was misexpressed in cyst cells (Figure 3.9, A-D).

The number of DCP-1+ CBs, WBs and spermatid bundles were quantified in all the RNAi and misexpression combinations. When upd was knocked down in the germline (genotype: Bam>upd RNAi), DCP-1+ CBs and WBs were reduced, but the number of DCP-1+ spermatid bundles did not change (Figure 3.9, E-F). The reduction in the number of DCP-1+ CBs and WBs is consistent with the hypothesis that upd is expressed in the spermatids, and the unchanged number of DCP-1+ spermatid bundles in Bam>upd RNAi testes suggested that DCP-1+ spermatid bundles is less sensitive than DCP-1+ CBs and WBs to the reduction of STAT activity. upd was also knocked down by bam-Gal4 in the upd3 mutant background (genotype: d232a; Bam>upd RNAi), and it did not lead to more severe reduction of DCP-1+ CBs and WBs than upd knockdown by bam-Gal4 in an upd3+ background (Figure 3.9, E-F), suggesting Upd3 plays a minor role, if any, in the activation of STAT and caspases. When STAT or Dome was knocked down in the germline (genotype: Bam>STAT RNAi or Bam>Dome RNAi), or et was misexpressed in the germline (genotype: Bam>et), the number of DCP-1+ CBs, WBs and spermatid bundles was not reduced, in fact there were even more DCP-1+ spermatid bundles in Bam>STAT RNAi testes (Figure 3.9, E-F). These results are consistent with the hypothesis that STAT is not required in the germline spermatids for individualization.
When STAT or Dome was knocked down (genotype: Eya>STAT RNAi or Eya>Dome RNAi), or et was misexpressed in the cyst cells (genotype: Eya>et), there was more than 90% reduction in the number of DCP-1+ CBs and WBs, and 50-90% reduction in the number of DCP-1+ spermatid bundles (Figure 3.9, E-F), supporting the hypothesis that germline expression of DCP-1 depends on JAK/STAT activity in the cyst cells. When upd was knocked down in the cyst cells (genotype eya>upd RNAi), there was no reduction in the number of DCP-1+ CBs and WBs and spermatid bundles (Figure 3.9, E-F), consistent with the hypothesis that upd is released from the spermatids, activating JAK signaling in the cyst cells. The drICE staining is almost the same as DCP-1 staining (Figure 3.9, C-D) based on results from a smaller sample size of testes, but it has higher background, so the quantifications of drICE+ CBs, WBs and spermatid bundles were not completed in all genotypes and not shown here.

It has been reported that caspase is not required for the assembly of ICs. In the testes of mutants of cytochrome c (bln1), ICs can still be seen at the base of testis, although they cannot move toward the tip (Noguchi and Miller, 2003). We found that STAT is required for the expression or activation of caspases. When STAT or Dome is knocked down in cyst cells, no caspase activation and no IC can be seen in testes, suggesting STAT activity is required for the assembly of ICs. Sheath cells of the testis contain smooth muscle which contain actin, and the sheath cells usually stain with phalloidin. When ICs are assembled at the base of testis, the signal from phalloidin staining is usually weak. In the presence of background staining from sheath cells, newly formed ICs may be hard to seen. To rule out the possibility of a false negative result of phalloidin staining in the testes with reduced STAT activity, the protocol of phalloidin staining was modified. Before mounting, the testes were squashed to break the sheath at the base and expose the cysts. To test whether STAT activity is required for the assembly of ICs, eya-Gal4 was used as the positive control, and eya>Dome RNAi2 which showed the strongest individualization defects was used to test the effect of JAK signaling on IC formation. bln1 was used to control the sensitivity of phalloidin
staining since it has been reported that $bln^I$ testes have IC assembly at the base (Noguchi and Miller, 2003).

In eya-Gal4 testis, ICs are formed at the base of testis, and ICs overlap the nuclear clusters of elongated spermatids as shown by DAPI staining (Figure 3.10, A1-A3). When Dome was knocked down in cyst cells, no ICs could be seen, suggesting that JAK signaling is required for the assembly of ICs. However, $bln^I$ testes also had no ICs, which is contradictory to the reported result that $bln^I$ testes are able to assemble ICs but fail to progress. (Noguchi and Miller, 2003). This may be because our protocols are different, and my protocol is less sensitive than theirs in visualizing ICs, or cyt-c is in fact required for the assembly of ICs.

### Requirement of STAT activity for individualization is mainly in elongated cyst cells

Eya-Gal4 is active in early cyst cells before elongation (Leatherman and DiNardo, 2008), while 10X STAT-GFP shows that STAT is strongly activated in cyst cells after elongation (Figure 3.2), so it is possible that the phenotype of eya>STAT RNAi is caused by STAT knockdown in cyst cells before elongation where STAT may be weakly activated. To test this possibility, the activity of the JAK/STAT pathway needs to be specifically reduced in cyst cells after elongation. Several Gal4 drivers that were reported to be active in testis were screened by crossing with UAS-GFP. Among them, A90-Gal4 showed strong GFP only in elongated cyst cells enclosing elongated spermatid bundles (Figure 3.11).

Because A90-Gal4 is activated exactly in elongated cyst cells where 10X STAT-GFP was seen, it was used to direct RNAi knock-down of Stat92E. However, no reduction of CBs, WBs and ICs was seen in testes of A90>STAT RNAi, A90>Dome RNAi based on AO and phalloidin staining (data not shown). RNAi can only knockdown a gene before translation and it also takes time to mobilize the RNAi machinery and effectively knock down gene products. However, in the elongated cyst cells, STAT may have already been at least partly translated and activated. In order to reduce the activity of the JAK/STAT
pathway after the translation of STAT, et, a negative regulator of the JAK/STAT pathway which inhibits JAK signaling through inhibition of Dome (Kallio et al., 2010; Makki et al., 2010), was misexpressed by A90-Gal4. The misexpression was performed at 25°C at first. Fewer CBs, WBs and ICs, more AO⁺ spermatid bundles, reduced non-basal IC/all IC percentage, and smaller seminal vesicle with fewer sperm were seen (Figure 3.12, A1-D2), indicating individualization was inhibited when et is misexpressed in elongated cyst cells. However, the phenotypes are not as strong as that of STAT or Dome KD in cyst cells. The activity of Gal4 increases as temperature increases (see review Duffy, 2002). To increase the activity of Gal4, misexpression was also performed at 28°C. At 28°C, the misexpression of et lead to more severe reduction of CBs, WBs and ICs, non-basal IC/all IC percentage, and stronger increase of AO⁺ spermatid bundles (Figure 3.12.F-1), and a complete block of sperm production in the seminal vesicle (data not shown). DCP-1 staining was also performed. At both 25°C and 28°C, DCP-1⁺ CBs, WBs and spermatid bundles were reduced, and at 28°C, the reduction is more severe (Figure 3.12, A-F). Although the possibility that the JAK/STAT is weakly activated in cyst cells before elongation has not been ruled out, these results support the idea that STAT activation is required for individualization mainly in cyst cells after elongation.
Conclusions and Discussion

JAK/STAT is required for individualization

Using the reporter of the JAK/STAT pathway 10x STAT-GFP, strong GFP can be seen in elongated cyst cells, but not spermatids. In addition, when STAT or Dome was knocked down, or et was misexpressed in the cyst cells, individualization was almost completely blocked. When STAT or Dome was knocked down in the germline, or et was misexpressed in the germline, no individualization defects were observed. These results support the idea that after elongation, STAT is mainly, if not exclusively, activated in cyst cells. The activation of STAT in cyst cells is not only required for the initiation of individualization, including the assembly of ICs, but also for the progression of individualization. JAK/STAT activation in cyst cells is required for individualization through caspase activity in elongated spermatids. It has been known that the JAK/STAT is required for the maintenance in stem cells at the tip of testis (Kiger et al., 2001; Tulina and Matunis, 2001). We found that it is also activated and required for later stage of spermatogenesis after meiosis, hence the JAK/STAT pathway is reutilized during Drosophila spermatogenesis.

Upd family ligands can be both paracrine and autocrine. In the Drosophila midgut, Upd is released from the intestinal stem cells (ISCs) and activates the JAK/STAT signaling in ISCs. Upon bacterial infection, Upd2 and Upd3 are released from the enterocytes, and activate JAK/STAT in ISC which promotes the division of ISCs. Thus in the midgut, Upd ligands serves both as an autocrine and a paracrine ligand (Osman et al., 2012). In the mushroom body of the Drosophila brain, upd also serves as a paracrine ligand (Copf et al., 2011). In the testis, Upd is released from the hub and activates the JAK/STAT pathway in the surrounding CySCs and GSCs, thus activating as a paracrine ligand (Kiger et al., 2001; Leatherman and DiNardo, 2008; Tulina and Matunis, 2001). In the germaria of the ovary, upd is expressed in the TFCs and ECs, while STAT is activated in the TFCs, CpCs and ECs, thus Upd is a paracrine and autocrine ligand in the germarium (Lopez-Onieva et al., 2008). In the developing egg chambers,
upd and upd3 are expressed in polar cells (McGregor et al., 2002; Wang et al., 2014), while STAT is activated in surrounding follicle cells, stalk cells and polar cells themselves, so upd and upd3 are autocrine and paracrine ligands in the egg chambers (Assa-Kunik et al., 2007; Borensztejn et al., 2013).

STAT is strongly activated in the cyst cells, but where are the ligands coming from? As described above, Upd has been found to act as an autocrine and a paracrine ligand, so it could be from the spermatids, or cyst cells, or even the sheath cells. When upd was knocked down in the cyst cells, no individualization defect was seen, indicating upd is not required in the cyst cells for this activity. When upd was knocked down in the germline, individualization was greatly impaired, indicating that upd expression in the germline is required for individualization. The only remaining cell type that has not been tested is the sheath cell. As an extracellular paracrine ligand, the diffusion of Upd requires the heparan sulfate proteoglycans Dally or Dally-like protein in the ovary. In the ovaries, dally and dally like are co-expressed in the posterior of the germarium and the follicle cells of egg chambers (Hayashi et al., 2012). dally is expressed in the hub of the testis but is not expressed in sheath cells (Guo and Wang, 2009). Sheath cells are located at the outer layer of the testis, as shown in Figure 3.1, 10x STAT-GFP positive cyst cells do not contact sheath cells, in some regions, they are located far from the sheath. If upd is expressed in the sheath cell, it may be difficult to reach the cyst cells located in the center of testis. Thus may lead to weaker GFP in cysts at the center of testis than in those near the sheath. However, as can be seen from Figure 2.1, the intensity of GFP in cysts near or far away from the sheath is not different. These results suggest that it is unlikely that upd is also expressed in sheath cells. To test whether upd3 expression from germline or cyst cells is also required for individualization, it was knocked down in both cell types. Knocking down in neither led to individualization defects (data not shown). However, upd3 is co-expressed with upd in many tissues, thus it may also be co-expressed with upd in the spermatids. In addition, as discussed above, Upd ligands usually act in paracrine fashion, also support the idea that Upd3 is expressed in the germline.
Although the \textit{upd3}^{d232a} mutant showed minor individualization defects, the phenotype is much less significant than knocking down of \textit{upd} in the germline. Even when \textit{upd} was knocked down in the \textit{upd3} mutant background, the phenotype was not more significant than when \textit{upd} was knocked down in a wild-type \textit{upd3} background. This is probably because \textit{upd3} is not the main ligand required for individualization. However, Upd3 can be a major, minor, or equally important ligand compared to Upd in the JAK/STAT pathway, depending on the context. JAK/STAT activation controls the number of polar cells in developing egg chambers through apoptosis. When \textit{upd} is knocked down in the polar cells, 30-50% egg chambers have more than 2 polar cells \cite{Borensztejn2013}, however, the number of polar cells is not changed in the egg chamber of \textit{upd3}^{d232a} (personal communication with Carlos Sandfoss). In addition, when \textit{upd} is knocked down in polar cells, STAT activity is greatly reduced based on 10x STAT-GFP expression \cite{Van2011}, however, in the egg chambers of \textit{upd3}^{d232a}, 10x STAT-GFP did not show detectable difference in comparison with the egg chambers of \textit{upd3}+. These two results suggest that Upd3 is a minor ligand compared to Upd in developing egg chambers. However, \textit{os}^{s}, which carries a mutation in a potential enhancer of \textit{upd}, has normal \textit{upd3} but no \textit{upd} expression in the eye discs. \textit{os}^{s} and \textit{upd3}^{d232a} have similar reduced eye phenotypes, suggesting that Upd3 is as important as Upd in eye development \cite{Wang2014}. During apoptosis and JNK-mediated stress in the \textit{Drosophila} midgut, the JAK/STAT pathway is activated in the ISC, promoting the proliferation of ISCs. Activation of the JAK/STAT pathway in this context is a result of up-regulation of \textit{upd} ligands, with \textit{upd3} expression at least 3 times higher than that of \textit{upd}, suggesting that Upd3 is more important than Upd in replenishing lost enterocytes in the middle gut \cite{Jiang2009}.

\textbf{How JAK/STAT controls individualization}

The first step of individualization is the assembly of investment cones. After the assembly of investment cones, 64 investment cones in one cyst form an IC at the head of the spermatid bundles, and the IC then moves toward the tail of the spermatid
bundles. It was reported that caspase activation is not required for assembly of ICs, as \textit{bln1} mutant testes show normal IC assembly at the head of elongated spermatids \cite{Arama2003}. However, our results showed that no ICs can be seen in the head of spermatids of \textit{bln1} testes. This difference in results is probably because our phalloidin staining protocol is different from theirs. In their protocol, the testes were transferred to siliconized slides after dissection, and opened by thin forceps, then sandwiched with a poly-L-lysine-coated slide. After that, the slides were frozen in liquid nitrogen. Then, the testes were stained on the slide \cite{Arama2003}. In my protocol, intact testes were not frozen, and were stained using the whole-mount protocol (see materials and methods for detail). When the testes were mounted on slides, the coverslip was squashed to break the testes and expose the spermatid bundles at the base of testes. It is possible that their protocol leads to better permeability, thus the ICs at the base of \textit{bln1} testes can be stained. But our results indicate that cytochrome C, or possibly caspase as well, is in fact required for the assembly of ICs. In addition, when Dome was knocked down in the cyst cells, no ICs were formed at the head of elongated spermatids, suggesting that STAT activity in cyst cells is required for the assembly of ICs. As mentioned above, dynein and Myosin V are required for the assembly of investment cones. It is possible that STAT activity in cyst cells affects the activity of Dynein and Myosin V in spermatids in the formation of IC. In addition, based on our results, caspase activity is required for the assembly of ICs. Therefore, STAT activity in cyst cells may also be required for caspase activity in IC formation in spermatids.

RNAi usually does not completely eliminate expression of the target gene. While eya>STAT RNAi and eya>Dome RNAi1 led to 75\% and 50\% reduction of IC formation respectively, almost all the remaining ICs remained at the base of the testis. After the initiation of ICs, ICs move towards the tip of the testis. Since all remained ICs are located at the base of testes, JAK/STAT activity may also be required for the progression of individualization. The movement of ICs requires actins dynamics, thus JAK signaling may promote the translocation of ICs through the regulation of actin dynamics. In fact,
JAK signaling is required for the migration of border cells in egg chamber development through the regulation of actin dynamics (Silver et al., 2005). During the progression of ICs, there is also membrane remodeling of spermatids and cyst cells to form CBs and WBs. When STAT activity was reduced in cyst cells, CBs and WBs were also greatly reduced, suggesting that STAT is also directly or indirectly required for the membrane remodeling of cyst cells and spermatids during individualization.

JAK/STAT is activated in cyst cells, while caspase is activated and individualization takes place in the elongated spermatids. STAT activation cannot lead to individualization directly because these two events occur in two type of cells. It is possible that after STAT activation, cyst cells signal to the spermatids through one or more cell signaling pathways. The BMP pathway (Wang et al., 2008) (Kawase et al., 2004; Wang et al., 2008), the Notch pathway (Kitadate and Kobayashi, 2010), the Wnt pathway (Leatherman and DiNardo, 2008) and the Hh pathway (Michel et al., 2012) have been known to play roles in spermatogenesis. Therefore, these pathways were screened to see whether they are downstream of JAK signaling in cyst cells to control individualization.
Figure 3.1. Schematic of oogenesis. (A) An ovariole is composed of a gerarium at the anterior end and egg chambers at increasing stages pinched off the germarium. (B) The germarium has several terminal filament cells (TFC) at the anterior end, followed by two cap cells (CpC). Two germline stem cells (GSC) contact the CpC, and each GSC is enclosed by two escort cells (EC) which also contact the CpC. After an asymmetric division, one daughter cell of the GSC that is away from the CpC becomes a cystoblast (CBs) enclosed by two ECs. The CBs undergoes four cycles of incomplete mitosis and becomes a 16 cell interconnected germline cyst. Follicle stem cells (FSC) gives rise to follicle cells which encloses the 16 germline cyst. The germline cyst pinches off the germarium and forms a stage one egg chamber. The egg chamber is connected to the germarium and the next stage egg chamber through stalk cells, and at each end of the egg chambers there are two polar cells (red) which release Upd ligands. (C) Upd serves as a morphogen and activates the JAK/STAT signaling in the follicle cells. The JAK signaling decreases as the distance between the follicle cells to the polar cells increases. At stage seven, the anterior somatic follicle cells adopt one of four cell fates: border cells, stretched cells, centripetal cells, or main body cells based on the level of the JAK signaling. In the wild-type, JAK signaling cooperates with EGFR signaling at the posterior end, so the follicle cells adopt the posterior terminal fate in the posterior end (Xi et al., 2003). Modified from (Bastock and St Johnston, 2008; Xi et al., 2003)
Figure 3.2. The JAK/STAT pathway is activated in elongated cyst cells. Testes of 10x STAT-GFP were observed directly or after immunostaining. (A) Direct observation of testis of 10x STAT-GFP shows that GFP can not only be seen at the tip of testis (arrow head) but also away from the tip (arrow). (B-D) Immunostaining of testis of 10x STAT-GFP shows GFP (green) can be seen at the hub (arrow head in B) and in cyst cells after elongation (arrow in B). Fas III (yellow) stains the hub (arrowhead in C), Frizzled (yellow) stains elongated spermatid bundles (arrow in C); GFP positive cyst cells enclose spermatid bundles as shown by Fz staining (D).
Figure 3.3. *upd3*<sup>d232a</sup> shows individualization defects based on acridine orange staining. Whole mount testes of one and three week old *upd3*<sup>+</sup> and *upd3*<sup>d232a</sup> were stained by acridine orange to visualize waste bags (WBs, white arrows) and cystic bulges (CBs, arrowheads). (A1) AO+ WBs (white arrow) and CBs (arrow head), and degrading WBs at the tip (yellow arrow) in one week old *upd3*<sup>+</sup> (A1), one week old *upd3*<sup>d232a</sup> (A2), three week old *upd3*<sup>+</sup> (B1) and three week *upd3*<sup>d232a</sup> (B2). (C) Quantifications of CBs and WBs normalized by the number of spermatid bundles in one and three week old testes of *upd3*<sup>+</sup> and *upd3*<sup>d232a</sup>. n=20, *: P<0.05; **: P<0.01; ***: P<0.001
Figure 3.4. **upd3<sup>d232a</sup>** shows individualization defects observed by phalloidin staining.

Whole mount testes of **upd3<sup>+</sup>** and **upd3<sup>d232a</sup>** were stained by phalloidin to visualize individualization complexes (ICs). (A1-A2) ICs (red) in testis of one week old **upd3<sup>+</sup>** (A1), one week old **upd3<sup>d232</sup>** (A2), three week old **upd3<sup>+</sup>** (B1) and three week old **upd3<sup>d232</sup>** (B2). Yellow arrow shows ICs are located at the base of testis, white arrow shows some ICs away from the base of testis (non-basal IC), and one non-basal IC is enlarged in the square. (C) Quantifications of ICs normalized to the number of spermatid bundles in each testis. (D) Quantifications of non-basal IC/all IC in each testis. n=20, *: P<0.05; **: P<0.01; ***: P<0.001
Figure 3.5. Reduction of JAK signaling in cyst cells leads to fewer CBs and WBs and more AO\(^+\) spermatid bundles. One week old testes from eya-Gal4 (the control) and eya>STAT RNAi (STAT knockdown in cyst cells) or other genotypes as shown in B and C are stained by acridine orange to visualized CBs, WBs and AO\(^+\) spermatid bundles. (A1 and A1\(^\prime\)) AO staining shows CBs (arrow head, magnified in A1\(^\prime\)) and WBs (arrow, magnified in A1\(^\prime\)) in the control. (A2 and A2\(^\prime\)) There is no CBs or WBs, but many AO\(^+\) spermatid bundles (red arrow) in eya>STAT RNAi. (B) Quantifications of AO\(^+\) CBs, WBs normalized to the number spermatid bundles in each testis. (C). Quantifications of AO\(^+\) spermatid bundles in each testis. n=17-20 for each bar. Error bar is SE. *: P<0.05; **: P<0.01; ***: P<0.001
Figure 3.6. Reduction of JAK signaling in cyst cells leads to fewer ICs and blocks sperm production. One week old testes from eya-Gal4 (the control) and eya>STAT RNAi (STAT knockdown in cyst cells) or other genotypes as shown in B and C are stained by phalloidin to visualize ICs, and by DAPI to visualize nuclei. (A1 and A1') IC in the control (arrow, magnified in B1'). (B2 and B2') No IC can be seen in eya>STAT RNAi. (C) The
seminal vesicle of the control (C1) is large, and contains sperm as shown by needle-shaped nuclei (arrow). (C2) The seminal vesicle of eya>STAT RNAi is much smaller and contains no sperm. (B) Quantifications of ICs normalized to the number spermatid bundles in each testis. (C). Quantifications of Non-basal IC/all IC in each testis. n=17-20. Error bar is SE. *: P<0.05; **: P<0.01; ***: P<0.001
**Figure 3.7. Upd knockdown in the germline impairs individualization based on AO staining.** One week old testes from Bam-Gal4 (the control) and Bam>upd RNAi (upd knockdown in the germline after 4 cell spermatogonial stage) or other genotypes as shown in B and C are stained by acridine orange to visualize CBs, WBs and AO+spermatid bundles. (A1) CBs (arrow head) and WBs (arrow) in testis of Bam-Gal4. (A2) No CBs and WBs, AO+spermatid bundles (white arrow) in testis of Bam>upd RNAi. (B) Quantification of CBs, WBs normalized to the number of spermatid bundles in the testis of each genotype. (C) Quantifications of AO+spermatid bundles in the testis of each genotype. n=17-20 for each bar. Error bar is SE. *: P<0.05; **: P<0.01; ***: P<0.001
Figure 3.8. Knockdown of Upd in the germline impairs individualization based on phalloidin staining. One week old testes from Bam-Gal4 (A1, the control) and Bam>upd RNAi (A2, upd knockdown in the germline after 4 cell spermatogonial stage) or other genotypes as shown in B and C are stained by phalloidin to visualize ICs. (A1) ICs both away from the base (green square) and at the base of testis (blue square) in testis of Bam-Gal4. (A2) ICs both away from the base (green square) and at the base of testis (blue square) in testis of Bam>upd RNAi. (A1’-A1'b) Inset to show the non-basal ICs (A1'a) and base ICs (A1'b) in testis of Bam-Gal4. (A2'-A2'b) Inset to show the non-basal ICs (A2'a) and base ICs (A2'b) in testis of Bam>upd RNAi. (B) Quantification of ICs normalized to the number of spermatid bundles in the testis of each genotype. (C) Quantifications of Non-basal IC/All IC percentage in the testis of each genotype. n=17-20 for each bar. Error bar is SE. *: P<0.05; **: P<0.01; ***: P<0.001
Figure 3.9. Caspase is downstream of JAK/STAT in individualization. One week old testes from eya-Gal4, eya>STAT RNAi, and other genotypes are stained by anti-DCP-1 or anti-active drICE to visualize the expression or activation of caspases in testis. (A) DCP-1 (A) or drICE (C) antibody stains CBs (arrow heads), WBs (white arrows) and some of the spermatid bundles (red arrow) in testis of eya-Gal4, but no DCP-1 staining (B) or drICE staining (D) can be seen in testis of eya>STAT RNAi. (E) Quantification of DCP-1+ CBs and WBs normalized to the number of spermatid bundles in testis with different genotypes. (F) Quantification of DCP-1+ spermatid bundles in testis with different genotypes. n=17-20. Error bar is SE. *: P<0.05; **: P<0.01; ***: P<0.001
Figure 3.10 JAK signaling is required for the assembly of IC. One week old testes from eya-Gal4 (A1-A3), eya>Dome RNAi2 (B1-B3) and bln1 (C1-C3) were stained by DAPI and phalloidin. The testes were then squashed to break the sheath and expose the nuclei of spermatid bundles located at the base of testis. Nuclei of spermatid bundles and ICs at the base of testes are shown here. DAPI staining overlaps DIC of nuclei clusters (arrow) of spermatid bundles (A1), Phalloidin stains the sheath (arrow head) and ICs (arrow) (A2) at the base of testis of Eya-Gal4. (A2) Phalloidin stains the sheath (arrow head) and ICs (arrow). (A3) Overlay of DAPI and phalloidin staining. (B1) DAPI staining of nuclei clusters (arrow) of spermatid bundles and sheath cells (arrow head) overlaps DIC at the base of Eya>Dome RNAi2 testis. (B2) Phalloidin stains the sheath (arrow head). (B3) Overlay of DAPI and phalloidin staining. (C1) DAPI staining of nuclei clusters (arrow) of spermatid bundles overlaps DIC at the base of bln1 testis. (C2) Phalloidin stains the broken sheath (arrow heads) at the base of bln1 testis. (C3) Overlay of DAPI and phalloidin staining shows the elongated sperm nuclei clusters (arrow) and sheath (arrow head).
Figure 3.11. A90-Gal4 is activated in cyst cells after elongation. Less than 7 days old testes of 10x STAT-GFP (Column A), eya>GFP (Column B) and A90>GFP (Column C) were stained by antibody against GFP, Fas III and Fz to visualize where A90-Gal4 is activated. The tips of testis are shown here. (A1) GFP in testis of 10x STAT-GFP (green) can be seen in the hub (arrow head) and in elongated cyst cells (arrow). (A2) Fas III staining (yellow) shows the hub (arrowhead) and Frizzled staining (yellow) shows elongated spermatid bundles (arrow) in testis of 10x STAT-GFP. (A3) Merge of A1 and A2 to show that away from the hub, GFP positive cyst cells (white arrow) exactly enclosed elongated spermatid bundles. (B1) GFP staining in testis of eya>GFP shows weak GFP (green) in cyst cells before elongation (red arrow) and strong GFP in cyst cells after elongation (white arrow). (B2) Fas III and Fz (yellow) staining shows the hub (arrowhead) and elongated spermatid bundles (arrow) in testis of eya>GFP. (B3) Merge of B1 and B2 shows besides the elongated cyst cells, eya is also weakly active in cyst cells before elongation (red arrow). (C1) GFP staining (green) in testis of A90>GFP shows GFP in elongated cyst cells (arrow). (C2) Fas III and Fz staining (yellow) show the hub (arrowhead) and elongated spermatid bundles (arrow) in testis of A90>GFP. (C3) Merge of C1 and C2 shows that GFP in testis of A90>GFP can only be seen in cyst cells (white arrow) enclosing elongated spermatid bundles.
Figure 3.12. The requirement of JAK/STAT signaling for individualization is mainly from cyst cells after elongation. Testes from A90-Gal4 and A90>et aged at 25°C or 28°C for eight days were stained by AO, phalloidin, anti-DCP-1 and DAPI to visualize AO+ CBs, WBs and spermatid bundles, ICs and DCP-1+ CBs, WBs, and spermatid bundles. Quantifications of AO+ CBs and WBs (A), AO+ spermatid bundles (B), ICs (C), DCP-1+ CBs and WBs (E), normalized to the number of spermatid bundles in each testis and the percentage of Non-basal IC/all IC in each testis (D) are summarized here. n=20, *: P<0.05; **: P<0.01; ***: P<0.001
Chapter 4: Pathways downstream of JAK/STAT signaling that control cyst cell-spermatid interaction

Introduction

At the tip of testis, two CySCs enclose one GSC and the CySCs serve as the niche of GSCs by inhibiting Bam expression in GSC (Kawase et al., 2004; Leatherman and Dinardo, 2010). BMP pathway is utilized to convey the signal of JAK/STAT activation in CySCs to GSCs. Upon the JAK/STAT activation, dpp, the ligand of the BMP pathway, is expressed in the CySCs (Wang et al., 2008). The released Dpp then activates the BMP pathway and inhibits Bam expression in GSCs (Kawase et al., 2004; Wang et al., 2008). After elongation, STAT is activated in cyst cells (Figure 2.2), while individualization occurs in spermatids (Figure 3.9). How is the signal of STAT activation in cyst cells conveyed to the enclosed spermatids to activate individualization there? Similar to the CySC-GSC interaction, there must be a signaling pathway that is responsible for the cyst cell-spermatid interaction. The downstream pathway that mediates the cyst cell-spermatid interaction in inducing individualization should meet these criteria: 1). its activity should be affected by the JAK/STAT signaling. 2). If JAK/STAT positively (or negatively) regulates this pathway, increased (or decreased) activity of this pathway in spermatids should cause similar phenotypes to that of loss of the JAK/STAT activation in cyst cells. 3). If JAK/STAT positively (or negatively) regulates this pathway, increased (or decreased) activity of the pathway in spermatids should be able to at least partly rescue the phenotypes of loss of the JAK/STAT activation in the cyst cells. To test which pathway mediates the cyst cell-spermatid interaction, various conserved signaling pathways, including Notch, BMP, Wnt, Hh pathways, were screened for such interaction. The pathways chosen to test were those that have been previously reported to interact with the JAK/STAT pathway.

In ovarioles, a series of egg chambers at increasing stages of development are connected by stalk cells (see review Bastock and St Johnston, 2008). At the end of each egg chamber there are two polar cells which secrete Upd and activate JAK/STAT in the
neighboring stalk cells and follicle cells, including main body cells (McGregor et al., 2002; Xi et al., 2003). Notch is highly activated in the polar cells, and increased levels of Notch activity in polar cells leads to more polar cells (Assa-Kunik et al., 2007). Notch is activated at a lower level in stalk cells, and increased level of Delta, the ligand of Notch pathway, leads to more stalk cells (Assa-Kunik et al., 2007). In the egg chamber, the JAK/STAT and Notch pathways have mutually antagonistic activities. In main body cells and polar cells, Notch activity inhibits JAK signaling. In stalk cells, JAK signaling reduces Notch activity (Assa-Kunik et al., 2007). The Notch pathway has an anti-apoptotic effect in human T cells (Sade et al., 2004). Thus, it is possible that when STAT is knocked down in cyst cells, the activity of Notch may increase and inhibit the activation of individualization in the spermatids.

As mentioned above, JAK/STAT activation in CySCs promotes the expression of dpp in CySC of ovaries, which then activates the BMP pathway in GSCs. The activation of the BMP pathway inhibits the expression of bam and promotes self-renewal of GSCs in testes and ovaries (Leatherman and Dinardo, 2010; Lopez-Onieva et al., 2008; Wang et al., 2008). In addition, in smooth muscle cells lining the human pulmonary artery, activation of caspase-8 and caspase-9 is dependent on the BMP pathway (Lagna et al., 2006). BMP also induces the Smad-dependent apoptotic cell death of mouse embryonic stem cell-derived neural precursors (Gambaro et al., 2006). Taken together, it is possible that STAT activation in cyst cells promotes dpp expression in cyst cells. Dpp released from cyst cells might then promote the activation of the BMP pathway in elongated spermatids, then the activated BMP signaling could lead to caspase activation which is required for individualization.

In developing eye discs, activation of the JAK/STAT pathway leads to retina formation, while activation of the Wnt pathway leads to formation of head cuticle. 10XSTAT-GFP is expressed in the posterior domain of first and second instar eye discs, while wg is expressed in the region abutting the region where STAT is activated. In mutants for hop (JAK) mutant which impairs JAK signaling, ectopic wg is expressed in
the cells where STAT is normally activated. In the region with ectopic STAT activation, 
wg is inhibited autonomously. STAT inhibits wg expression by binding to a 263 base 
pair enhancer region located 3’ of the wg gene (Ekas et al., 2006). Besides the eye discs, 
STAT also inhibits wg expression autonomously in the antennal disc, leg discs and wing 
discs (Ayala-Camargo et al., 2007; Tsai et al., 2007). In cultured rat fibroblast cells, Wnt 
pathway activation inhibits apoptosis (Chen et al., 2001). Taken together, it was 
hypothesized that STAT may inhibit wg expression in cyst cells, and that when STAT is 
knocked down in cyst cells, wg expression is increased, activating the Wnt pathway in 
spermatids and blocking individualization, perhaps by inhibiting caspase activation.

The JAK/STAT pathway and Hh pathways act cooperatively in some tissues such 
as in promoting self-renewal of stem cells in testis, but they are independent of each 
other (Kiger et al., 2001; Michel et al., 2012; Tulina and Matunis, 2001). Although there 
is no report of the JAK/STAT and Hh pathways interacting in Drosophila, interaction has 
been reported in other organisms such as mice and rats. In cerebellar granule neurons 
of STAT2 null mutant mice, the expression level of the ligand for the Hh pathway, shh, 
is significantly increased, indicating that STAT2 inhibits shh expression. However, in the 
cultured cerebellar granule neurons, IFN-γ induced STAT1-dependent expression of 
shh, indicating that STAT1 promotes shh expression (Wang et al., 2003). In cultured rat 
and mouse hepatic stellate cells, leptin activated shh expression, probably through 
STAT activation (Choi et al., 2010). In addition, Shh inhibits apoptosis in neuroepithelial 
in mouse (Thibert et al., 2003). It is possible that Shh inhibits apoptosis through the 
hindering of caspase activity which is required for individualization. Thus, the JAK/STAT 
pathway in cyst cells activates individualization in spermatids may through negative 
regulation of hh expression in cyst cells.
Results

BMP and Notch pathway

Immunostaining of the components of the BMP and the Notch pathway was performed to see whether they are expressed in elongated cyst cells or spermatids. The role of the Notch pathway in individualization was examined by the immunostaining of Notch. Notch can be seen at the tip of testis (Figure 4.1, A and B, yellow arrow). In ovaries, stem cells signal back to the niche through the Notch pathway. The Notch ligands Delta and Serrate are released from GSCs, activating the Notch pathway in neighboring somatic cells and contributing to the function of the niche (Ward et al., 2006). Our result is consistent with a model in which the Notch pathway may also be activated at the tip of testis and stem cells may also signal back to the hub through the Notch pathway. Weak Notch staining can be seen at the base of testis in the control (Figure 4.1, A, white arrow), and this staining was stronger with reduced STAT activity (Figure 4.1, B, white arrow). The base of the testis is where individualization initiates, however, it is also the place where non-specific staining often occurs. For example, Eya can be seen in the nuclei of cyst cells (Figure 4.1, E, red dots), but non-specific staining can also be seen at the base of testis (Figure 4.1, E, white arrow). Even in the absence of primary antibody, when the testes were stained with secondary antibody only, non-specific staining can be seen at the base of testis (Figure 4.1, F, white arrow). Eya-Gal4 was used to knockdown STAT in cyst cells, so if STAT knockdown leads to increased Notch expression, the increased Notch expression should be consistent with eya staining (Figure 4.1, E). Since eya is in the nucleus and Notch is the transmembrane receptor, Notch staining should be seen in the membrane of cyst cells. After elongation, the elongated cyst is about 1.8 mm long, which is almost 2/3 of the length of testis (see review Fuller, 1993). However, the bright Notch staining at the base of testis with reduced STAT activity is only 1/10 length of the testis. Taken together, the bright Notch staining is most likely background, and STAT knockdown in cyst cells did not change the expression level of Notch, and Notch pathway appears not to be the downstream pathway of JAK signaling that controls individualization.
A role for the BMP pathway was examined by staining of phosphorylated (activated) SMAD (pSMAD) transcription factor. pSMAD staining can be seen at the tip of the testis (Figure 4.1, C and D, yellow arrow). This is consistent with the fact that the BMP pathway is activated in the GSCs and promotes the self-renewal of them (Kawase et al., 2004). pSMAD staining can also be seen in the testis away from the tip, indicating BMP pathway may also have other functions in addition to maintaining GSCs in Drosophila spermatogenesis. When STAT activity is reduced in cyst cells, there is much stronger staining of pSMAD at the base of testis (Figure 4.1, D, white arrow) than in the control (Figure 4.1, C, white arrow). But compared to the dotted nuclear pSMAD staining in the nuclei at the tip or middle of testis, the staining at the base is not nuclear, and seems to be in the spermatid bundles. This implies that the staining of pSMAD at the base is background, and reduced STAT activity in cyst cells did not change the activity of BMP pathway in elongated cysts or spermatids. Thus, BMP signaling seems not to be the target of JAK signaling that controls individualization in spermatids.

**Wnt pathway**

As mentioned above, JAK signaling inhibits wg expression in several tissues. In addition, based on wg-lacZ, there is no wg expression in elongated cyst cells (Figure 4.2, A). Thus, it was hypothesized that the JAK/STAT pathway inhibits wg expression in cyst cells, and the phenotypes in testes with reduced JAK signaling were caused by increased wg expression. To test whether individualization defects in testis with reduced STAT activity were caused by increased wg expression, misexpression of wg by eya-Gal4 or A90-Gal4 was performed. However, the progeny (Genotype: eya>wg and A90>wg) did not survive to adulthood. It was also tested whether wg expression is affected by STAT knockdown in cyst cells using the Wg-lacZ reporter of wg expression (Figure 4.2). In contrast to the prediction that STAT knockdown in cyst cells increases wg expression, reduced STAT signaling in fact decreased wg expression at 30°C, but did not affect it at 22 °C. Based on these results, we concluded that the Wnt pathway is not responsible for relaying the signal of STAT activation in cyst cells to spermatids and
initiating individualization.

**Hedgehog pathway**

To test whether individualization defects were caused by increased *hh* expression in cyst cells, over-expression of *hh* was performed by eya-Gal4. Overexpression of *hh* in cyst cells led to loss of caspase staining in CBs, WBs and spermatid bundles, similar to the phenotype observed with reduction of JAK/STAT signaling in cyst cells (Figure 4.3, F and G). If the Hh pathway acts downstream of the JAK/STAT pathway, and reduction of JAK/STAT activity leads to increased Hh expression, then Hh knockdown in cyst cells should be able to rescue the phenotype of STAT knock-down in cyst cells. Since eya>STAT RNAi males are sterile, we used Gal80ts to inhibit Gal4 activity at 25 °C, but remove the inhibition at 30 °C. Flies with reduced STAT activity in cyst cells (genotype: tub-Gal80<sup>ts</sup>, eya-Gal4/+; UAS-STAT RNAi/TM3) and reduced STAT and Hh activity in cyst cells (The rescue, genotype: tub-Gal80<sup>ts</sup>, eya-Gal4/+; UAS-STAT RNAi/UAS- Hh-RNAi) were aged for seven days at 30°C. Gal80 inhibits the activity of Gal4, Gal80ts is a temperature sensitive Gal80 which inhibits Gal4 at permissive temperature (about 25 °C), but lose its activity and cannot inhibits Gal4 activity at restrictive temperature (30 °C), and then testes were immunostained by DCP-1. Preliminary results show that Hh knockdown partly rescued DCP-1 staining as shown by more DCP-1+spermatid bundles, CBs and WBs (Figure 4.3, H, I). These results support the hypothesis that STAT inhibits Hh expression which inactivates caspases. However, Hh-lacZ, the lacZ reporter of Hh expression, is not affected by STAT knockdown in cyst cells (data not shown). Since Hh-lacZ only represents the transcription of *hh*, not the translation, it is possible that the Hh protein level may be increased, or other changes that alter the function of Hh, may occur in testis with reduced STAT activity.
Conclusions and Discussion

If a single signaling pathway acts downstream of the JAK/STAT pathway to mediate the cyst cell-spermatid interaction in individualization, this pathway should be affected by reduced JAK signaling in cyst cells, and manipulation of this pathway should be able to mimic or rescue the STAT knockdown in cyst cell phenotypes. The Notch and BMP pathways were not affected by STAT knockdown in cyst cells. Also, the Wg-lacZ expression was not affected by STAT reduction in cyst cells at 22 °C when STAT knockdown in cyst cells showed significant individualization defects. These results indicate that the Notch, BMP and Wnt pathways do not function alone in conveying the signal of STAT activation in cyst cells to spermatids to initiate individualization.

hh is expressed in cyst cells, and when hh was misexpressed in cyst cells, it caused phenotypes similar to the LOF of STAT in cyst cells. More importantly, when we knocked down hh and STAT simultaneously, the elimination of DCP-1 staining phenotype was partly rescued. Although it seems that STAT reduction in cyst cells did not change the expression level of hh based on Hh-lacZ reporter, this reporter only reflects the expression of hh at the transcriptional level, not at the translational level. In the future, western blot of Hh should be performed to check whether the level of protein Hh is increased in testis with reduced JAK/STAT activity. If so, then the Hh pathway is very likely to be the downstream pathway of the JAK/STAT that mediates the cyst cell-spermatid interaction controlling individualization. If not, a microarray can be performed to compare the expression profile of thousands of genes, including the components of all pathways, in the testis with normal versus reduced JAK signaling. Then, the pathways whose expressions are changed by STAT reduction can be experimentally manipulated to see whether this manipulation can mimic or rescue the phenotypes of STAT knockdown in cyst cells.

Based on these preliminary results, a model was proposed. 10x STAT-GFP is seen in the elongated cyst cell, not in spermatids. In addition, STAT or Dome knockdown and misexpression of et, the inhibitor of the JAK/STAT pathway, in the cyst cells, but not in
spermatids, blocked individualization. These results indicate that STAT is activated in cyst cells (Figure 4.4). Because the knockdown of upd in the germline, but not in cyst cells, led to individualization defects, Upd must be released from the spermatids to activate JAK/STAT signaling in the somatic cyst cells (Figure 4.4). Although upd3 mutants showed minor individualization defects, when upd3 was knocked down in the germline or cyst cells no defect of individualization can be seen. Therefore it is unknown where upd3 comes from. However, since upd and upd3 are co-expressed in several tissues, including the polar cells of egg chambers and hub in testis, upd3 may also be co-expressed with upd in the spermatids (Figure 4.4). Although hh expression in cyst cells is not affected by reduction of JAK signaling based on hh-lacZ reporter, since hh knockdown can partly rescue the phenotype of STAT knock down in cyst cells, it is possible that STAT inhibits hh expression at the translational or posttranslational level in cyst cells (Figure 4.4). Since STAT is strongly activated in cyst cells based on the 10x STAT-GFP reporter, Hh in cyst cells may be low, and Hh may fail to, or weakly, activate Ci, the transcription factor of the Hh pathway, in the spermatids, hence cannot inhibits caspases (Figure 4.4). Without the inhibition from the Hh pathway, caspase will be activated which subsequently initiates and drives the progress of individualization. When STAT activity is reduced in cyst cells, Hh protein may be increased, activating Ci in the spermatids, inhibiting caspase activity and blocking individualization of spermatids.

Although BMP pathway may not act downstream of the JAK/STAT pathway in individualization, it is activated in the testis in regions away from the tip, suggesting that it plays other functions in Drosophila spermatogenesis other than promoting self-renewal of GSCs. Reducing the activity of the BMP pathway by RNAi or misexpression of inhibitors through eya-Gal4 or A90-Gal4 may lead to some interesting phenotypes and reveal the function of the BMP pathway at later stages of Drosophila spermatogenesis.
Figure 4.1. Possible downstream pathways: Notch and BMP pathway. One week old testis of eya-Gal4 (A and C, control) and eya>STAT RNAi (B and D, STAT knockdown in cyst cells) were stained by anti-Notch or anti-pSMAD (red), or wild-type testes were stained by anti-eya plus secondary antibody (E) or secondary antibody only (F). The whole testes are shown here. (A) In the control testis, Notch staining can only be seen at the tip of testis (yellow arrow). At the base of testis, faint staining can be seen (white arrows). (B) In testis of eya>STAT RNAi, normal Notch staining can be seen at the tip (yellow arrow), and also strong Notch staining can be seen at the base of testis (white arrow). (C) pSMAD staining in testis of eya-Gal4 can be seen at the tip (yellow arrow) or away from the tip (red arrow). The staining can also be seen at the base of testis.
(white arrows). (D) Ineya>STAT RNAi testis, pSMAD staining can be seen at the tip (yellow arrow), away from the tip (red arrow), and strong pSMAD staining can be seen at the base (white arrow). (E) Eya staining (yellow arrow) can be seen in wild-type (upd3+) testis, and there is also non-specific staining at the base of testis (white arrow). (F) Staining without primary in wild-type (upd3+) testis shows non-specific staining at the base of testis (white arrow).
Figure 4.2. The effect of STAT knockdown in cyst cells on Wg-lacZ expression. Testes with genotype eya-Gal4/Wg-lacZ (column one) or eya-Gal4/Wg-lacZ; UAS-STAT RNAi (column two) were stained by anti-beta galactosidase to show the Wg-lacZ
expression. (A, B) At 22°C, Wg-lacZ (green) can only be seen at the tip of testis both in eya-Gal4/Wg-lacZ and eya-Gal4/Wg-lacZ; UAS-STAT RNAi (Square in A, B and enlarged in A', B'). (C, D) After raising at 30°C for three days, eya-Gal4/Wg-lacZ shows ectopic Wg-lacZ (square in C and C'). However, eya-Gal4/Wg-lacZ; UAS-STAT RNAi did not show any ectopic Wg-lacZ in the testis (square in D and enlarged in D').
Figure 4.3. Hh pathway may be downstream of the JAK/STAT pathway in individualization. (A) X-Gal staining of testis of ptc-lacZ shows Patched is expressed in some elongated spermatids (arrow) and enriched in waste bags (arrow head). (B)
Similar to the X-Gal staining of ptc-lacZ, anti-DCP-1 staining (green) can also be seen in some of the spermatid bundles (arrow) and enriched in waste bags (arrow head) in testis with normal STAT activity (eya-Gal4 testis is shown here). (C-E) Antibody staining of testis of Hh-lacZ overlaps Eya, the marker of cyst cells. (F) DCP-1+ spermatid bundles can still be seen in testis of eya-Gal4 after 10 days at 30°C. (G) No DCP-1 staining can be seen in testis of eya>Gal4 after 10 days at 30°C. (H) No DCP-1 staining in STAT KD (eya-Gal4 tub-Gal80ts/+; UAS-STAT RNAi/+ after seven days at 30°C). (I) Partial rescue of DCP-1 staining can be seen when STAT and Hh are knocked down simultaneously (eya-Gal4 tub-Gal80ts/+; UAS-STAT RNAi/UAS-Hh RNAi after seven days at 30°C).
Figure 4.4. A proposed model to show how the JAK/STAT pathway controls individualization. 64 elongated spermatids (yellow) are enclosed by two cyst cells (red). STAT activation in cyst cells may signal to the spermatids and initiate individualization through the Hh pathway. See text for detail.
Chapter 5: Conclusions and Discussion

**Soma-germline interaction is important for spermatogenesis**

Spermatogenesis is a very complex cellular process. Within about 10 days, one small, spherical GSC develops into 64 free swimming and viable sperm with long (1.8mm) tails (see reviews Fuller, 1993; Witman, 2003). Not only has the size dramatically increased, but also the morphology has been completely changed (see review Fuller, 1993). This is one of the most dramatic morphology changes of any cell in adult animals. In such a complex cellular process, germline cannot develop to sperm by itself in *Drosophila*. During gametogenesis, the germline cells are always enclosed by somatic cells and their interactions are important during *Drosophila* spermatogenesis.

The soma-germline interactions have been extensively investigated at the niche and stem cell stage. In *Drosophila* testes, the somatic hub cells promote the self-renewal of GSCs indirectly, while the somatic CySCs promote the maintenance of GSCs directly. Upd released from the hub cells activates JAK/STAT signaling in the surrounding CySCs and promotes the self-renewal of these CySCs (Kiger et al., 2001; Tulina and Matunis, 2001). Two CySCs enclose one GSC. Activation of JAK/STAT in the CySCs positively regulates DPP or BMP expression in CySC, DPP or BMP then activate the BMP pathway in the enclosed GSC. The activation of the BMP pathway is required for the maintenance of GSCs (Leatherman and Dinardo, 2010). In addition, Zfh1 is also required in CySCs for the self-renewal of GSCs (Leatherman and DiNardo, 2008). Interestingly, although CySCs not only support their self-renewal, but also support the differentiation of GSCs. Loss of EGFR activity in the CySCs leads to more GSC-like cells in the testis, indicating that EGFR activity in CySCs is required for the differentiation of GSCs (Kiger et al., 2000). The activity of Raf, a serine/threonine kinase, a downstream effector of EGFR pathway, in CySCs also supports the differentiation of GSCs, suggesting a requirement for the EGFR pathway in CySCs for differentiation of GSCs through Raf (Tran et al., 2000). In addition, when CySCs and cyst cells are ablated by
forced expression of the apoptotic activator Grim by C587-Gal4, a Gal4 driver that is active in all somatic cells, 49% of testes had no GSCs, while 51% had GSC-like cells which are ectopic small vasa+ cells with small and spherical fusomes. The authors concluded that somatic cells are required for the differentiation, but not for the maintenance, of GSCs. It was explained that the reason why 49% of testes lost their GSCs was because of leaky expression of C587-Gal4 in the GSC, or compromised function of the hub, since hub cells are slowly replenished by CySCs (Lim and Fuller, 2012). In somatic cyst cells, the transcription factor Eya which normally plays a role in eye development, is required for spermatocyte development. When eya is mutated, the spermatocytes degenerate (Fabrizio et al., 2003). While somatic cells affect germline cells, germline cells also affect somatic cells, although compared to the effect of CySCs on GSCs, the effect of GSCs on CySCs are relatively limited. Normally, in a cyst the germline cells divide, but the two cyst cells do not divide. In the absence of germline cells, the cyst cells proliferate (Gonczy and DiNardo, 1996).

After mitosis, soma-germline interactions have been much less well investigated. In cultured elongated cysts, although it is unclear whether cyst cells are required for the initiation of individualization. Individualization continued normally when the cyst cells were removed, suggesting that cyst cells are not required for this process (Noguchi and Miller, 2003). This result seems to be controversial to my finding that STAT activity in cyst cells is vital for individualization. However, based on the model that I proposed at the end of chapter 4, their result is consistent with mine. According to the model, the reason why STAT activity is required for individualization is because hh expression in cyst cells needs to be inhibited by STAT. Without STAT activation, increased hh expression in cyst cells activate the Hh pathway in spermatids and blocks the expression or activation of caspases. However, if the cyst cells are completely removed, although STAT activation is lost, Hh from cyst cells also is lost, therefore caspase activation and individualization will also not be blocked. Even if the inhibitor is not hh, another signaling ligand that is negatively regulated by STAT in cyst cells may act on spermatids. However, if STAT in cyst cells positively regulates a ligand that is
required for individualization, then complete removal of cyst cells would impair individualization.

Our preliminary results show hh, the ligand of the Hh pathway, is expressed in the cyst cells, while ptc, the receptor of the Hh pathway is expressed in the spermatids; moreover, frizzled, the receptor of the Wnt pathway, is expressed in elongated spermatids; and pSMAD, the activated transcription factor of the BMP pathway is detected in elongated cyst cells. While further research will be required to determine if the ligand and receptor of a given pathway are expressed in reciprocal lineages of cells in later stages of spermatogenesis, these pathways usually work as paracrine signaling (see reviews Bejsovec, 2013; Huangfu and Anderson, 2006; Li and Xie, 2005). Thus, it is possible the detection of the ligand of the Wnt pathway and the transcription factor of the BMP pathway in cyst cells may suggest they play functions in cyst cell-spermatid intercalations at later stages of spermatogenesis. It should be interesting to learn the nature of these interactions, and what functions Wnt, Hh and BMP signaling play during spermatogenesis after elongation. The Hh and Wnt pathways may be activated in the spermatids, given that the ligand of the Hh pathway is expressed in cyst cells, and the receptor of the Wnt pathway is expressed in spermatids. However, for the BMP pathway, pSMAD staining can be seen in cyst cells. If the ligand is released from the spermatids, it may suggest that spermatids also have an effect on cyst cells. In fact, the finding that Upd is required in spermatids and STAT is activated in cyst cells already shows that the germline also affect cyst cells after meiosis. Thus, based on these preliminary results, it seems not only that cyst cells affect spermatids through the Hh and possibly also the Wnt pathway, but also spermatids affect cyst cells through the JAK/STAT and possibly also the BMP pathway.

Somatic cells not only affect germline cells through cellular signaling pathway, but may also protect the germline cells by providing a mechanical barrier. Spermatogenesis is a very important process that requires a safe and stable environment. In mammals, the blood-testis barrier which is composed of the somatic
Sertoli cells, protects the germline cells from toxic reagents and the attack from immune system during spermatogenesis. Although no blood-testes barrier exists in *Drosophila* testes, the cyst cells, which are the counterparts of Sertoli cells in mammals, plays a similar function through septate junction. When the permeability barrier is destroyed, germline differentiation is delayed or disrupted (*Fairchild et al.*, 2014).

Soma-germline interaction may also be affected by transcriptional and translational control of genes that affect soma-germline interaction. Transcription and translation are strictly regulated in the germline during spermatogenesis. Almost all transcription is finished before meiosis, and some mRNAs are stored and translated later (which can be as long as several days) when the protein is needed (see review *White-Cooper, 2010*), only a very limited number of genes are transcribed after meiosis (*Barreau et al.*, 2008). For example, Frizzled staining can be seen in elongated spermatids, it is possible that *frizzled* is expressed before meiosis, and then translated after elongation. If premature translation of *frizzled* occurs, it may lead to premature Wnt pathway activation and impair spermatogenesis. If the proposed model is right, the transcription and translation of Hh may also be controlled. According to 10X STAT-GFP, the JAK/STAT pathway is activated in all elongated cyst cells. However, only a proportion of spermatid bundles are DCP-1⁺ and drICE⁺ and are undergoing individualization. There must be a mechanism to control the timing of individualization. Is it possible the transcription and/or translation or posttranslational control of Hh is used to control the timing? For example, in the elongating or elongated spermatid bundles that are not ready for individualization, Hh will be translated and inhibits caspase activity in the spermatids. When spermatid bundles are ready for individualization, Hh expression will be reduced and individualization can occur.

**Link to mammalian spermatogenesis and clinical application**

The JAK/STAT pathway is evolutionarily conserved, so it may play similar functions in *Drosophila* and mammals. Some of the components of the JAK/STAT pathway are also expressed in the mammalian testis, but studies on its role in mammalian testes
are limited, the results are preliminary, the expression pattern are sometimes inconsistent between different studies, and thus its function in spermatogenesis mainly remains unknown. While there are only three ligands for the *Drosophila* JAK/STAT pathway, the ligands in mammals include growth factors and cytokines such as TNF, IL, and leptin. Leptin, the mammalian functional homologue of Upd2, is a protein hormone secreted by fat cells that is well known for its anti-obesity function (see review Ricci and Bevilacqua, 2012). Leptin also plays roles in inflammation, immunity, angiogenesis and hematopoiesis (see review Blüher and Mantzoros, 2007). The leptin receptor is expressed in the germline of the mouse testis. In cultured seminiferous tubules, STAT3 becomes phosphorylated with the addition of leptin to the cultivation medium (El-Hefnawy et al., 2000). Unlike in mice in which the leptin receptor is expressed in the germline of mice testis, in the human testis, the leptin receptor is mainly expressed in the somatic interstitium, primarily in Leydig cells, which are the counterparts of sheath cells in *Drosophila*, and leptin is expressed in the germ cells, primarily spermatocytes. The ratio of leptin positive cells to all cells of the testis is negatively correlated with testosterone concentration and sperm concentration; the ratio of leptin receptor positive cells to all Leydig cells is also negatively correlated with testosterone level, indicating the JAK/STAT pathway also plays a role in human spermatogenesis (Ishikawa et al., 2007). STAT4 is expressed in germ cells of the mouse testis after meiosis; STAT4 doesn't translocate to the nucleus, and was localized to the condensing perinuclear theca of spermatids, where selective biochemical extraction of thecal proteins occurs. The theca depolymerize in the cytoplasm of the zygote within several hours after fertilization, so it was speculated that STAT4 may affect transcription in the zygote, rather than in the sperm (Herrada and Wolgemuth, 1997). Leukemia Inhibitory Factor activates the phosphorylation of STAT3 and STAT1 in the cultured rat Sertoli cells, but not in the germ cells (Jenab and Morris, 1998). The activated STAT in Sertoli cells then drives the expression of C-fos (Jenab and Morris, 1998), which is well known as an oncogene and is also driven by action potential in neurons (see review Flavell and Greenberg, 2008). It has also been reported that STAT3 is expressed in the developing acrosomes of round spermatids in the adult testis of the
mouse (Murphy et al., 2005). Besides, protein inhibitor of activated signal transducer and activator of transcription (PIAS1), a negative inhibitor of the JAK/STAT pathway, is also expressed in Sertoli cells and Leydig cells of human testes (Tan et al., 2000).

In sum, although the functions of the JAK/STAT pathway in Drosophila spermatogenesis are well investigated, it is less well investigated in mice and human. This is probably due to the difficulty of knockdown or tissue specific knockout in mammals. The only study that investigated the function is the correlation between leptin/leptin receptor and testosterone concentration and sperm count (Ishikawa et al., 2007). However, this is not known to be a causal relationship. Since no knockdown or knockout of leptin or leptin receptor has been done in mammals, the exact function of the JAK/STAT pathway in the mammalian testes largely remains unknown. Our research, in addition to the research of others, in Drosophila may shed some light on the role of the JAK/STAT pathway in mammals. For example, the molecular mechanism that supports the self-renewal of spermatogonial stem cells (SSC) in mammals, the counterpart of GSCs in Drosophila, is poorly understood. Glial cell line–derived neurotrophic factor (GDNF) has been reported to support the spermatogonial stem cell self-renewal as an extrinsic factor (see review Oatley and Brinster, 2008). As JAK/STAT is required for the maintenance of GSCs in the Drosophila testis. Is JAK signaling also required for the maintenance of SSC in mammals? Cytokine colony–stimulating factor is able to activate STAT1, and preliminary results suggest that cytokine colony-stimulating factor-1 may influence SSC in mice (see review Shimoda et al., 1997), suggesting that the JAK/STAT pathway also plays a role in the maintenance of SSC in mammals.

In humans, common causes of male infertility include oligospermia (less sperm) and aspermia (no sperm). The expression levels of leptin and leptin receptor are negatively correlated with sperm concentration (Ishikawa et al., 2007), suggesting that JAK/STAT activity may affect spermatogenesis in humans. In Drosophila, we found that loss of function of JAK/STAT signaling leads to premature male infertility. Signaling
pathways play important roles in the development of cancer. As a result, targeting signaling pathways is a major strategy for the prevention and treatment of tumors (see reviews Bjornsti and Houghton, 2004; Cho, 2012; Qi and Zhu, 2008; Waldner and Neurath, 2012). In clinical practice, there are many ways to treat male infertility, but the manipulation of signaling pathways has never been reported. Several small molecule inhibitors of the JAK/STAT pathways have been reported (Deng et al., 2007; Ivanenkov et al., 2011; Liu et al., 2010; Meng et al., 2009; Pardanani et al., 2007). Since the JAK/STAT pathway may also play a role in male infertility, the manipulation of the JAK/STAT pathway, as well as other pathways which play a role in human spermatogenesis, may provide novel therapies for male infertility.

Implications for aging

As mentioned in chapter 3, the number and division rate of GSCs in Drosophila ovaries as well as the fertility of females decreases with age, and these decreases may be caused by the decline of Dpp and Hh level (Zhao et al., 2008). In the testis, Upd decreases as the fly ages, and the decline of Upd may lead to decreased numbers and division rates of GSCs (Boyle et al., 2007). As we discussed above, the JAK/STAT, BMP and Hh pathways all have been known to promote the self-renewal of GSCs directly or indirectly both in the ovaries and testis, and oogenesis shares many similarities with spermatogenesis. The level of Upd in the hub drops in old testes (Boyle et al., 2007), but does it also decline with age in the ovaries? Hh and BMP diminish with age in the ovaries (Zhao et al., 2008), so do they also decline with age in the testis? By combination, the decline of Hh, Dpp and Upd may contribute to the decreased number and division rate of CySCs and GSCs both in the ovaries and testis.

While upd3<sup>d2232a</sup> has minor individualization defects both at one week and three week old, three week old testis of upd3<sup>+</sup> also has 71% reduction of ICs in testis compared to one week old upd3<sup>+</sup>. What causes the individualization defects in the three week old wild-type testis? Having a look at oogenesis in old wild-type ovaries may help us to understand individualization defects in old wild-type testes. In the
ovaries, there was about a 50% decrease in the expression of *Socs36E, dome* and *stat92E* between 10-13 days and 24-27 days (personal communication with Claire Venard). While the JAK/STAT pathway is weakly activated in the gerarium where the stem cells and niche are located, the vast majority of STAT activity in the ovaries is in the developing egg chambers based on 10x STAT-GFP reporter expression (data not shown). Thus the decreased expression of *Socs36E, dome* and *stat92E* in old ovaries may mainly represent the expression level of these genes in egg chambers. *Socs36E, dome* and *stat92E* are all positively regulated by JAK/STAT activation, and the enhancers of these genes have been used to make reporters of JAK/STAT signaling (see review Chen et al., 2014). Thus, decreased expression level of *Socs36E, dome* and *stat92E* in ovaries with age may reflect the decreasing activity of JAK signaling in the ageing egg chambers. While STAT activity in egg chambers may decline with age, the proportion of eggs with morphological defects increases dramatically as the fly ages (Zhao et al., 2008). In addition, the proportion of unhatched eggs increases dramatically with age, and in eggs laid by *upd3d232a* which should have reduced JAK signaling, there are more unhatched eggs compared to the eggs laid by wild-type females. These results may suggest that decreased JAK/STAT activity in egg chambers with age may cause the morphological defects or decreased potential for hatching of eggs produced by old female. Since JAK signaling may decrease with age during differentiation in ovaries, it is possible that JAK signaling also decreases with age during differentiation in the testis after elongation. The impaired individualization of *upd3*+ testes may be caused by decreased Upd expression in the spermatids and STAT activation in the cyst cells. To test this, real-time PCR of Upd, Dome and STAT can be done using aged and young testes. To make sure the RNAs are from cysts after elongation, but not from the stem cells, hub or seminal vesicle, the tip and seminal vesicle should be removed before RNA extraction.

Wild-type males lose their fertility at about 38 days (Wang et al., 2014). The aging of the testis may include not only the aging of stem cells and impairment of individualization, but also the impairment of other stages of spermatogenesis such as...
mitosis, meiosis and elongation. As described in chapter 4, the preliminary results show that the BMP pathway, Hh pathway and Wnt pathway components are expressed in the testis away from the tip. If the activity of JAK/STAT in elongated cyst in testis decreases with age, it is possible that the activity of the BMP pathway, Hh pathway and Wnt pathway away from the tip also decrease or increase with age, and the change of these pathways contribute to the impairment of spermatogenesis at the corresponding stage. If the Hh pathway is inhibited by JAK/STAT signaling, and JAK/STAT activity decreases with age, then Hh expression, hence Hh activity may increase with age in the cyst cells. To test the activity of these pathways, real-time PCR can be performed to compare the wild-type young and old testis without tip and seminal vesicle. If these pathways do change with age, manipulation to the opposite direction can be performed to see whether aging can be slowed down or reversed.

The association of decreased activity of signaling pathways and aging is not limited to the ovaries and testis. Notch is expressed in the adult Drosophila brain (see review Ables et al., 2011), and it is required for the long term memory formation in Drosophila (Ge et al., 2004; Presente et al., 2004). The JAK/STAT pathway is activated in the mushroom body, the memory center in the Drosophila brain, and is required for the long term memory formation for Drosophila (Copf et al., 2011). As the animal ages, the functions of the brain declines. One important functional decline is memory impairment (see review Rosenzweig and Barnes, 2003). What causes memory to decline with age? Since the JAK/STAT and Notch pathway are required for long term memory formation in Drosophila, do their activities decline with age and cause, or partly cause the memory impairment with age? Notch protein does not decrease in the Drosophila brain between 0 day and 20 days old (Presente et al., 2001). However, Notch needs to be cleaved to be activated, so the total Notch protein does not reflect the activity of the Notch pathway. In mammals, Notch is cleaved by γ-secretase to form the activated Notch. Compared to young mice, the γ-secretase activity that cleaves Notch decreases dramatically with age, suggesting Notch activity in the mouse brain decreases with age (Placanica et al., 2009). Although it is unknown when the
expression level or activity of the JAK/STAT pathway in the *Drosophila* brain decreases in relationship to age, in the rat brain, STAT3 protein expression decreases dramatically with age. There is about 40% decline between 3 and 8 months, and about 90% decline by 26 months ([De-Fraja et al., 2000](#)). Taken together, these results may suggest that the memory impairment with age is partly caused by the reduced activity of the JAK/STAT and Notch pathways in the brain. Memory impairment is one indicator of brain aging, so the decline of JAK/STAT and Notch activity may contribute to the aging of the brain.

The lesson we learned from testes that reduced JAK/STAT activity contributes to aging may be a general rule and may also be extended to other tissues including the brain. The decline of regenerative ability is an important character of tissue aging. The ability to regenerate in muscle decreases with ages, and this decline is caused by reduced Notch activity in satellite cells, the skeletal muscle stem cell in mice ([Conboy et al., 2003](#)). The decline in Notch activity in satellite cells is probably due to an elevation in TGF-beta pathway activity which antagonizes Notch ([Carlson et al., 2009](#)). Sonic Hh signaling, which is required for angiogenesis, also decreases with age, and this decline leads to decreased angiogenesis in old animals ([see review Carlson et al., 2008](#)).

Interestingly, while the JAK/STAT and Notch pathways are required for the maintenance of stem cells for regeneration in specific tissues, the global loss of function of these pathways decreases the lifespan of the organism. *upd3* mutants have reduced lifespan (personal communication with Lakshmi Pillai). A global Notch mutant in *Drosophila* also shows reduced lifespan ([Presente et al., 2001](#)). These results validate the usefulness of investigating aging of a specific organ to understand the aging of the whole organism.

In conclusion, the activity of many signaling pathways change with age, and these changes may lead to aging of specific tissues individually, and by combination, lead to
the aging of the whole organism. If we can find a way to maintain the activity of these important pathways at a young level, aging may be slowed down, and lifespan, as well as life-quality, may be increased.
Chapter 6: Materials and methods

Fly strains

Flies were raised at 25°C under standard condition unless otherwise noted. $upd^3^{221b}$ and $upd^3^{d232a}$ were made from P-element mutagenesis. $upd^3^{221b}$ has a p-element inserted into the 2nd intron and $upd^3^{d232a}$ has the third and largest exon deleted. $upd^3^+$ is a precise excision of the inserted P-element (Wang, 2008). UAS-$upd$-GFP was made by (Chen, 2014). Tj-Gal4 (P(GawB)NP1624-5-1) (Hayashi et al., 2002) was obtained from Erika A. Bach (Hayashi et al., 2002). eya-Gal4 (Leatherman and DiNardo, 2008) was provided by Stephen Dinardo, A90-Gal4 (Manseau et al., 1997) was provided by Celeste A. Berg, bam-Gal4-VP16 (Chen and McKearin, 2003), c587-Gal4 (Voog et al., 2008) and Gal-E132 ($upd$-Gal4) (Tsai and Sun, 2004) were provided by Erika Matunis, Nanos-Gal4-VP16 (nos-Gal4) (Van Doren et al., 1998) was provided by Ruth Lehmann, tai-Gal4 (P[GT1]taiBG01746. BDSC, 12751) (Bunt et al., 2012), $upd$-lacZ (Sun et al., 1995) was provided by Henry Sun, 10X STAT-GFP (Bach et al., 2007) was provided by Erika A. Bach, UAS-latran (Makki et al., 2010) was provided by Michèle Crozatier. These lines are from BDSC: Cyt-c-d$b^{11}_{b1}$/CyO (P[PZ]Cyt-c-d$b^{11}_{b1}$ CG31808$b^{11}_{b1}$ cn$^1$/CyO. BDSC, 11763) (Arama et al., 2003), hs-hid (y1 w*/Dp(2;Y)G, P[hs-hid]Y. BDSC, 8846), tub-Gal80ts (BDSC, P[tubP-GAL80ts]x19, w/FM7c, #7016). These lines are from TriP (Ni et al., 2011): UAS-STAT RNAi (P[Trip.HMS00035]attP2. BDSC, 33637), UAS-GFP (P[UAS-GFP.S65T]Myo31DF2. BDSC, 1521), UAS-Wg RNAi (P[Trip.HMS00794]attP2. BDSC, 32994). These lines are from VDRC stock center (Dietzl et al., 2007b): UAS-Dome RNAi1 (VDRC36356), UAS-Dome RNAi2 (VDRC106071), UAS-$upd$ RNAi (VDRC, 3282). Hh-lacZ (Jiang and Struhl, 1995), ptc-lacZ (Ingham et al., 1991), UAS-Hh RNAi (VDRC #v1402) were provided by Jianhang Jia. Wg-lacZ (P[PZ]wg02657 cn$^1$/CyO; ry$^{506}$, BDSC, #11205), UAS-Wg RNAi (TriP.HMS00794 BDSC, 32994) were provided by Xinhua Lin.

RNAi, misexpression and rescue experiment

Misexpression of $upd$ and $upd^3$ was performed by crossing Tj-Gal4 with UAS-$upd$ or UAS-$upd^3$-GFP at 25 °C, F1 was aged at 25 °C for one week before staining. Crosses
to make eya>STAT RNAi (eya-Gal4 UAS-STAT RNAi. > refers to Gal4-UAS), eya>Dome RNAi1, eya>Dome RNAi2, eya>upd RNAi, eya>et were performed at 25 °C, F1 were shifted to 22.5°C for seven days, then analyzed by immunostaining. Crosses to make bam>STAT RNAi, bam>Dome RNAi1, bam>Dome RNAi2, bam>upd RNAi, bam>et were performed at 25 °C; F1 were aged at 25 °C for seven days, then analyzed by immunostaining. Misexpression of et by A90-Gal4 were crossed at 25 °C, F1 were aged at 25°C or 28 °C for eight days, then analyzed by immunostaining.

To test whether Hh pathway is downstream of the JAK/STAT in cyst cells, eya-Gal4 tub-Gal80ts/+; UAS-STAT RNAi/Hh-lacZ and eya-Gal4 tub-Gal4ts/+; +/-Hh-lacZ then were aged at 30 °C for seven days to see whether STAT KD affect Hh expression level; eya-Gal4 tub-Gal80ts/+; UAS-STAT RNAi/UAS-Hh RNAi and eya-Gal4 tub-Gal4ts/+; UAS-STAT RNAi/UAS-Hh-RNAi were aged F1 at 30 °C for seven days to test whether Hh KD rescues the STAT KD phenotypes. To test whether Wnt pathway is downstream of the JAK/STAT pathway in cyst cells, eya-Gal4/Wg-lacZ and eya-Gal4/Wg-lacZ; UAS-STAT RNAi/+ were aged at 25 °C and 30 °C for seven days before analyzing. Hs-hid was used to rescue the phenotype caused by STAT knockdown. Flies (genotype: hs-hid; eya-Gal4; UAS-STAT RNAi and eya-Gal4; UAS-STAT RNAi) were heat shocked in a 37°C incubator for seven consecutive days, 70 min per day then analyzed.

**Acridine orange staining**

The protocol was modified from (Arama et al., 2003). Testes were dissected in PBT (phosphate-buffered saline containing 0.1% Tween 20, pH 7.4), stained in PBS (phosphate-buffered saline, pH 7.4) with 0.5ug/ml of acridine orange (Sigma) for 5min, then rinsed 2 times in PBT and mounted on slides. The slides were observed no longer than 30 min after the staining.

**Immunostaining and microscopy**

Immunostaining was essentially the same as (Xi et al., 2003). The basic protocol is as follows: The testis were dissected in PBT (PBS with 0.1% Tween). Then the testes
were fixed in PBT or BBT (Buffer B with 0.1% Tween 20, recipe from (Fabrizio et al., 2003)) with 3.7% formaldehyde at room temperature (RT) for 20min. The testes then are rinsed 4 times in PBT, blocked in 100µL of 4%DS.2%PBX (4% of normal donkey serum diluted in PBS containing 2% Triton X-100). Then primary antibodies diluted in 4%DS.2%PBX was added. The testes were incubated at 4°C overnight. Then the primary antibody was removed, and washed 5x5min in PBT. Then 2nd antibody diluted in 4%DS.2%PBX was added, and incubate at RT for 3 hours. Then the 2nd antibody solution was removed, and washed in PBT with 0.1%DAPI for 5min, followed by 4x5min in PBT. After that, 100 µL of mounting medium (70% glycerol with 2% 1,4-diazabicyclo[2.2.2]octane (DABCO) was added. When all the testes settle to the bottom of the Eppendorf tube, they were mounted on slides and observed using epifluorescence microscopy or confocal microscopy. Different antibodies require different conditions. The solutions, fixation conditions and incubation conditions are optimized for each antibody and are reported in Table 1.

The primary antibodies used were: rabbit anti-vasa (1:100, Santa Cruz, Catalog #: sc-30210), rabbit anti-galactosidase (1:250, Cappel, Catalog #: AB1211-5MG), rabbit anti-GFP (1:500, Abcam, Catalog #: ab6556), rabbit anti-DCP-1 (1:1000, a gift from Bruce Hay(Huh et al., 2004)), rabbit anti-drICE (Arama et al., 2003) (1:500, a gift from Bruce Hay), rabbit anti-Zfh1 (Lai et al., 1991) (1:2000, a gift from James Skeath), mouse anti-GFP (1:250, Millipore, Catalog #: MA83580), mouse anti-eya (1:50, DSHB), mouse anti-Frizzled (1:10, DSHB), mouse anti-Fas III (1:50, DSHB), mouse anti-hts (1:250, DSHB) and Guinea Pig anti-Traffic Jam (Li et al., 2003) (Tj, 1:10000, a gift from Dorothea Godt). The secondary antibodies used were: DyLight488-conjugated donkey anti-rabbit (1:500, Jackson ImmunoResearch, Catalog #:711-485-152), Alexa Fluor 594-conjugated anti-mouse (1:1000, Jackson ImmunoResearch, Catalog #: 715-585-150), DyLight 649-conjugated anti-mouse (1:250, Jackson ImmunoResearch, Catalog #: 705-495-003) and Rhodamine Red-XRRX-conjugated donkey anti-guinea pig (1:500, Jackson ImmunoResearch, Catalog #: 706-295-148). Nuclei were counterstained in 4'-6-diamidino-2-phenylindole (DAPI, 1 µg /ml) at RT for 5 min during the first wash after
the incubation with secondary antibody. The samples were imaged by a Spot Camera (Diagnostic Instruments) on a Nikon E800 microscope, or taken from Leica TCS-SP laser scanning confocal. Images were processed by Adobe Photoshop.

**Phalloidin staining**

Phalloidin staining is modified from (Arama et al., 2003). It is generally the same as immunostaining. Alexa 594 conjugated phalloidin (Molecular Probes) was used at 1:1000. It was added with the 2nd antibody during the DCP-1 staining, and then testes were incubated at 37 °C on a rotator for 2 hours. The remaining steps are the same as DCP-1 staining.

**Table 1. Detailed conditions for each primary antibody and phalloidin**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>PBT or BBT</th>
<th>Fixation condition*</th>
<th>Block testes by</th>
<th>Diluted antibody in</th>
<th>2nd Ab incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-vasa</td>
<td>1:100</td>
<td>PBT</td>
<td>4%PFM.PBT 2min</td>
<td>4%DS.2%PBX</td>
<td>4%DS.2%PBX</td>
<td>RT 3 hrs.</td>
</tr>
<tr>
<td>Mouse anti-hts</td>
<td>1:500</td>
<td>PBT</td>
<td>4%PFM.PBT 2min</td>
<td>4%DS.2%PBX</td>
<td>4%DS.2%PBX</td>
<td>RT 3 hrs.</td>
</tr>
<tr>
<td>Rabbit anti-DCP-1</td>
<td>1:1000</td>
<td>PBT</td>
<td>4%PFM.PBT 20min</td>
<td>4%DS.2%PBX</td>
<td>4%DS.2%PBX</td>
<td>37°C on rotator for 2 hrs.</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>1:1000</td>
<td>PBT</td>
<td>4%PFM.PBT 20min</td>
<td>4%DS.2%PBX</td>
<td>4%DS.2%PBX</td>
<td>37°C on rotator for 2 hrs.</td>
</tr>
<tr>
<td>Mouse anti-Fas III</td>
<td>1:50</td>
<td>BBT</td>
<td>4%PFM.BBT 2min</td>
<td>4%DS.2%BBX</td>
<td>4%DS.2%BBX</td>
<td>RT 3 hrs.</td>
</tr>
<tr>
<td>Rabbit anti-Zfh1</td>
<td>1:2000</td>
<td>BBT</td>
<td>4%PFM.2min</td>
<td>4%DS.2%BBX</td>
<td>4%DS.2%BBX</td>
<td>RT 8-10 hrs.</td>
</tr>
<tr>
<td>Guinea Pig anti-Tj</td>
<td>1:1000</td>
<td>BBT</td>
<td>4%PFM.BBT 20min</td>
<td>4%DS.2%BBX</td>
<td>4%DS.2%BBX</td>
<td>RT 8-10 hrs.</td>
</tr>
</tbody>
</table>
In situ hybridization

ISH was performed according to (Morris et al., 2009) except that after Proteinase K digestion, 1ml of 0.1M triethanolamine (pH 8.0) with 25µL of acetic anhydride were added, incubated at RT for 10min to reduce the background by inhibiting the endogenous alkaline phosphatase. The plasmid used for upd ISH is pBS-GR51 (Harrison et al., 1998), which was digested by Bgl II. The digested plasmid then was used for in vitro transcription by T7 RNA polymerase to make the sense probe, and by T3 RNA polymerase to make the anti-sense probe. The plasmids used for upd3 ISH is pBS-upd3 (Wang et al., 2014). To make sense probe, the plasmid was digested by XbaI, then transcribed in vitro to make the sense probe by T3 RNA polymerase. For anti-sense probe, the plasmid was digested by Kpn1, then transcribed in vitro by T7 RNA polymerase.
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