2009

THE DISTRIBUTION OF UNPAIRED DURING DROSOPHILA OOGENESIS

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ABSTRACT OF DISSERTATION

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The Graduate School

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the

Requirements for the degree of Doctor of Philosophy in the

College of Arts and Sciences at the University of Kentucky

By

Travis Sexton

Lexington, Kentucky

Director: Dr. Douglas Harrison, Associate Professor of Biology

Lexington, Kentucky

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Janus Kinase (JAK) activity specifies the cell fates of the follicular epithelium during *Drosophila* oogenesis by establishing a gradient of JAK activity with highest levels at the A/P poles. Unpaired (Upd), a ligand for the pathway, is expressed and secreted exclusively from the polar cells potentially establishing the JAK activity gradient. This project proposed that Upd acts as a morphogen to directly establish the JAK activity gradient, specifying the fates of the follicular epithelium. The aims of this work were to investigate the extracellular distribution of Upd and, in addition, factors that may be involved. Furthermore, *upd3*, a gene encoding a protein with sequence similarity to Upd, is also co-expressed with *upd* in the polar cells. An additional aim of this project was to determine what role, if any, Upd3 plays in follicular development.

Immunostaining was used to reveal Upd distribution during oogenesis. The data revealed an Upd gradient on the apical membrane of the follicular epithelium. By virtue of the extracellular gradient, Upd fulfills the requirements necessary to be classified as a morphogen.

Some morphogens are dependent on heparan sulphate proteoglycans (HSPGs) for distribution. Using mitotic recombination to make mosaics, this work reveals that Dally, a glypican, is essential for the distribution of Upd and establishment of the JAK gradient during oogenesis. The data suggests Dally is involved with stability of extracellular Upd. Mosaic analysis of an additional HSPGs revealed that they are not essential for the Upd gradient or JAK activity during oogenesis.

upd3 mutant flies have small eyes and outstretched wings, a phenotype consistent reduced JAK activity. In *upd3* mutant ovaries it is shown that there is a higher frequency of deteriorating egg chambers, a higher frequency of egg chamber fusions, and a decrease in border cells per egg chamber compared to wildtype controls; all of which support a reduction of JAK activity. Furthermore, ovarian phenotypes of *upd3* get worse as the fly ages suggesting that *upd3* is required over time. The data presented suggests that Upd3 does act to maintain JAK activity in the ovary as the fly ages.

KEYWORDS: JAK/STAT signaling, *upd*, *upd3*, morphogen, HSPG

Travis R. Sexton

September 16, 2009

THE DISTRIBUTION OF UNPAIRED DURING *DROSOPHILA* OOGENESIS

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This work is dedicated to my fiancé Samantha, my parents Don and Karen Sexton, my grandparents Adrian and Olive Rose Hall and Andrew and Zina Sexton, my sister Megan, my brother-in-law Tony, my niece Madeline, and my nephew Nicholas (Niko).

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Chapter 1

Background

The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway has been shown to be involved in several developmental processes in metazoa. This work investigates the role that the JAK/STAT pathway plays in Drosophila oogenesis. Previously, a gradient of JAK activity was revealed in the anterior/posterior (A/P) axis of the follicular epithelium of developing egg chambers, with the highest levels being at the anterior and posterior poles (Xi et al., 2003). Furthermore, the level of JAK signaling specifies the cell fates of the developing follicular epithelium, which subsequently, will play a major role in the establishment of the A/P axis of the oocyte. The primary aim of this work was to investigate how the JAK gradient is established. The focus is on the activating ligand, Unpaired (Upd), which is exclusively expressed at the anterior and posterior poles of the developing egg chambers. It is hypothesized that Upd acts a morphogen by establishing an extracellular gradient, which, in turn, establishes the JAK activity gradient that specifies follicular cell fates. As described below, Upd is associated with the extracellular matrix (ECM) where it acts as a paracrine signal and can activate JAK several cell diameters away. Therefore, an additional aim of this work was to determine what factors may be involved in the movement of Upd. Heparan Sulfate Proteoglycans (HSPGs), a family of ECM proteins has been shown to be involved in the distribution of other extracellular ligands. Therefore, the genes encoding HSPG core protein and enzymes involved in their modifications were the primary focus of my investigations of Upd distribution factors. In addition to Upd, a related protein, Upd3, was investigated for its role in establishing the JAK gradient during oogenesis.

The Janus Kinase Signaling Pathway

The JAK/STAT cell signaling pathway plays an important role in the development of organisms ranging from mammals to *Drosophila*. The JAK/STAT pathway, originally identified in vertebrates in response to cytokine signaling (Darnell et al., 1994), was later found to be conserved in *Drosophila* (Binari and Perrimon, 1994; Hou et al., 1996). This pathway is a streamlined signaling cascade receiving an extracellular signal leading to the activation of transcription without the need of a second messenger. The components of this pathway include an extracellular ligand, a single-pass transmembrane receptor, a Janus tyrosine Kinase (JAK) that is constitutively bound to the cytoplasmic domain of the receptor, and a signal transducer and activator of transcription (STAT) protein. Upon binding of the ligand to the receptor dimer, the receptors undergo a conformational change which brings the attached JAKs into close proximity to one another (Figure 1.1). The JAKs will then transphosphorylate each other as well as phosphorylate tyrosine residues on the receptor. Phosphorylation of the tyrosine residues allows the cytoplasmic STATs to bind to the receptor via an SH2 domain, where they will be activated by the JAKs. Activated STATs will dimerize and translocate to the nucleus where they bind to DNA and act as transcription factors (Figure 1.1). The activation of the JAK/STAT pathway triggers a cellular events such as cell proliferation, differentiation, apoptosis, and cell migration. In vertebrate development, the pathway has been shown to be involved in events such as hematopoiesis, immune development, adipogenesis, and sexually dimorphic growth (Rawlings et al., 2004). In *Drosophila*, this pathway is essential during embryonic segmentation, eye development, sex determination, oogenesis, spermatogenesis, stem cell maintenance, hematopoiesis, and tracheal development (Arbouzova and Zeidler, 2006; Denef and Schupbach, 2003; Hombria and Brown, 2002).

Although the JAK/STAT pathway is streamlined, it can become more complex by having multiple homologues of each pathway member. The mammalian genome contains 4 JAKs, 7 STATs, and around 25 receptors most of which form homodimers,

heterodimers, or other types of multimers (Kisseleva et al., 2002). The *Drosophila* JAK/STAT pathway, however, is simplified in that it contains only one known receptor, a single JAK, and a single STAT, and three ligands: Upd, Upd2, and Upd3 (figure 1.1). The receptor for the Upd protein is encoded by the gene *domeless* (*dome) (Brown et al., 2001)*. The JAK is encoded by *hopscotch (hop)(Binari and Perrimon, 1994)* and the STAT gene is *stat92E (Hou et al., 1996; Yan et al., 1996)*. The *Drosophila* JAK-STAT pathway is activated by the Upd family of ligands. The family consists of *unpaired* (*upd*), *upd2*, and *upd3*. *unpaired* (*upd*) was the first confirmed ligand for the *Drosophila* JAK pathway (Harrison et al., 1998). Upd is a secreted glycosylated ligand that associates with the extracellular matrix (ECM) in cell culture. Upd2 has been shown to activate the pathway during embryogenesis, however, its function is redundant with that of Upd (Hombria et al., 2005). *upd3* encodes a potential ligand for the pathway having domains similar to that of Upd and Upd2, however, the ability for it to activate JAK signaling is unclear. Upd3 has been shown to be upregulated in response to septic injury (Agaisse et al., 2003) and RNAi experiments have suggested a function in immunity (Malagoli et al., 2008). Furthermore, *upd3* mutant flies have small eyes and outstretched wings, a phenotype consistent with mutants with reduced JAK activity (Wang, 2008). Although the sequence similarity between the Upd family of proteins is limited (~10% identity, ~45% similarity, figure 1.2), their coexpression during development (Wang, 2008) and ability to form heterodimers in *Drosophila* cell culture suggest a functional relationship between them (Pei, 2007).

The JAK/STAT pathway is essential to many developmental processes. Among them, and the focus of this work, is oogenesis. As discussed below, the JAK signaling pathway is involved in several aspects of oogenesis and plays an essential role in the proper production of the mature egg.

Drosophila oogenesis

Drosophila oogenesis produces a mature egg in which patterns and polarities have already been established. The establishment of such an egg is accomplished by a coordinated interaction among and between the germline cells and overlying somatic follicular epithelium. Proper coordination between these two cell types ensures that each egg is properly setup to undergo fertilization and embryogenesis. Each ovary consists of approximately 16 structures called ovarioles. An ovariole is a chain of sequentially developing egg chambers which begin development at the anterior end in a structure called the germarium and ends with a mature egg at the posterior end (Figure 1.3). The germarium contains germline stem cells as well as two populations of somatic stem cells that will give rise to the follicular epithelium. Each egg chamber begins as a 16 cell germline cyst that will be enveloped by a single layer of primordial epithelial cells as it moves towards the posterior of the germarium. As the cyst is moving towards the posterior of the germarium, one germline cell will become the oocyte leaving the rest of the germline cells of the cyst to become nurse cells. As the cyst exits the germarium, two distinct populations of somatic cells arise; two polar cells at the anterior and posterior of each cyst and approximately 7 stalk cells that form a bridge between adjacent egg chambers. After leaving the germarium, the germline cyst grows while the undifferentiated follicle cells proliferate. During this time, the anterior-posterior polarity of the egg chamber is determined. Differentiation of the follicle cells occurs upon the onset of Notch signaling, beginning at stage 7. As differentiation occurs, the follicle cells will begin to undergo morphological and molecular changes. The result is 5 distinct follicle cell populations: border cells (violet in figure 1.3), stretched cells (green in figure 1.3), centripetal cells (yellow in figure 1.3), posterior cells (blue in figure 1.3), and main body cells (white in figure 1.3). At stage 10, border cells will undergo an epithelial to mesechymal (EMT) transition and migrate, along with the anterior polar cells, between the nurse cells to the anterior of and oocyte. This border cell cluster will later form the micropyle of the mature egg. The stretched cells will become flat and

form a thin epithelium overlying the nurse cells. The centripetal cells will invaginate between the nurse cells and the oocyte, providing a physical separation between the nurse cells and oocyte. The posterior cells will undergo very subtle morphological changes and will provide signals to the oocyte that cause it to polarize and rearrange its cytoskeleton. The main body cells are necessary for the deposition of eggshell components. To produce a mature egg with correct structures and polarity, it is essential that these specialized follicle cells be at their appropriate positions along the epithelium.

The Functions of JAK Activity in Oogenesis

JAK activity has been shown to be essential in many aspects of oogenesis. In the germarium, JAK is active in the germline stem cells (GSCs) and is required for stem cell maintenance during asymmetric division (Decotto and Spradling, 2005). As the germline cyst leaves the germarium, JAK activity is essential for the formation of the polar and stalk cells (Baksa et al., 2002; McGregor et al., 2002). After differentiation of the follicular epithelium has taken place at stage 7 constant JAK activity is necessary within the migrating border cells. Alteration of JAK in the border cells will slow down or stop this migration (Silver et al., 2005). A fourth role of JAK activity is in the anterior posterior patterning of the follicular epithelium (Xi et al., 2003). JAK activity forms an activity gradient throughout the follicular epithelium with the highest activity occurring at the anterior and posterior poles. In the anterior egg chamber, the level of JAK activity specifies the anterior cell fates. JAK activity, along with EGFR activity, is essential for the specification of the posterior cell fate at the posterior pole. As mentioned in the opening of this chapter, the central aim of this work was to investigate how the JAK gradient in the follicular epithelium is established. Upd, the ligand for the JAK pathway, is expressed only in the posterior and anterior polar cells. The overall hypothesis of how the JAK gradient is established is that locally expressed Upd is distributed in a gradient

which then establishes the known JAK activity gradient, and in turn, specifies the follicular cell fates. The ability of Upd to establish a gradient of JAK activity during oogenesis is consistent with its activity as a morphogen. If Upd is acting as a morphogen during oogenesis, and the results indicate that it is, this would be a novel role for the JAK pathway as it has never been shown to have morphogenic activities in any system. Furthermore, because Upd is associated with the ECM, it was particularly intriguing to determine if ECM components are involved in its distribution in a gradient.

Morphogens

Morphogens are molecules with the ability to specify cell fates, over a distance, in a concentration dependent manner (Wolpert, 1989). Morphogens are molecules that are distributed from a localized source and distributed in a gradient over an epithelium, thus establishing polarity in structures such as limb buds in vertebrates and imaginal discs in *Drosophila*. The key feature to a morphogen is that the gradient that they form will specify cell fates in a concentration dependent manner. The roles of morphogens are quite conserved between *Drosophila* and vertebrates. In *Drosophila*, Wingless (Wg), Hedgehog (Hh), and Decapentaplegic (Dpp), a TGF-β homologue, have been studied for their roles as morphogens during many aspects of *Drosophila* development. Like Upd, all three of these proteins have posttranslational modifications; glycosylations on Wg and Dpp and a cholesterol moiety on Hh, all of which has been shown to be important in their extracellular distribution.

How morphogens move from source to target cells across epithelial tissue has been an intensely studied and debated issue for the past several years. Initially, it was believed that morphogens were distributed by simply diffusion, however, work over the last decade has revealed that their distribution is likely to be more complex. Current models of transport include planar transcytosis (figure 1.4, A), transport via argosomes (lipid vesicles) (figure 1.4, B), and transport through interaction of ECM proteins such as

HSPGs (figure 1.4, C). Planar transcytosis involves receptor-mediated endocytosis of the ligand, trafficking through the endocytic pathway, and release of the ligand to an adjacent cell via exocytosis (figure 1.4, A) (Zhu and Scott, 2004). Another model involves the use of argosomes, lipid vesicles capable of being distributed from cell to cell. In this model, argosomes are loaded with ligand in the source cell and distributed and fractioned from cell to cell across the epithelium for ligand distribution (figure 1.4, B). Perhaps the most promising model, and the one that was the focus of this work, is the association of the morphogen with Heparan sulfate proteoglycans (HSPGs) (figure 1.4, C). HSPGs are cell surface and ECM glycoproteins that have been reported to be essential in morphogen distribution (Lin and Perrimon, 2002). Importantly, the HSPG model is not mutually exclusive with the other models as it is possible that the HSPGs could be influencing both transcytosis and argosome-mediated movement. As mentioned above, Upd is associated with the ECM in cell culture. Furthermore, Upd is released from the ECM upon the addition of heparin to the culture medium suggesting that this interaction is through heperan sulfate interactions. There are multiple ways that HSPGs could play a role in the distribution of morphogens. They could function as facilitators of diffusion, co-receptors, or stabilizers or retainers of the ligand in the ECM.

Upd and the formation of the JAK gradient during oogenesis

The aim of this work was to investigate how the JAK gradient is established during oogenesis. This study reveals that the Upd ligand is distributed in an extracellular gradient overlapping that of the JAK gradient during *Drosophila* oogenesis and therefore acting as a morphogen. This is a novel role for the JAK/STAT signaling pathway as no JAK activating ligand has ever been shown to act as a morphogen in any system. In addition, Upd is shown to depend on the HSPG, Dally, for proper distribution. It is shown that loss of Dally results in the destabilization of Upd in the ECM. The role of *upd3*, which is coexpressed with *upd* in the polar cells, was examined for the potential role it may play

during oogenesis. *upd3* mutant animals are shown to have ovaries that degenerate at a higher rate than those of wildtype control animals. Furthermore, the number of border cells per egg chamber in *upd3* mutant animals is statistically lower than that in wildtype animals. Despite these ovarian phenotypes, misexpression of *upd3* cDNA was unable to activate JAK activity during oogenesis.

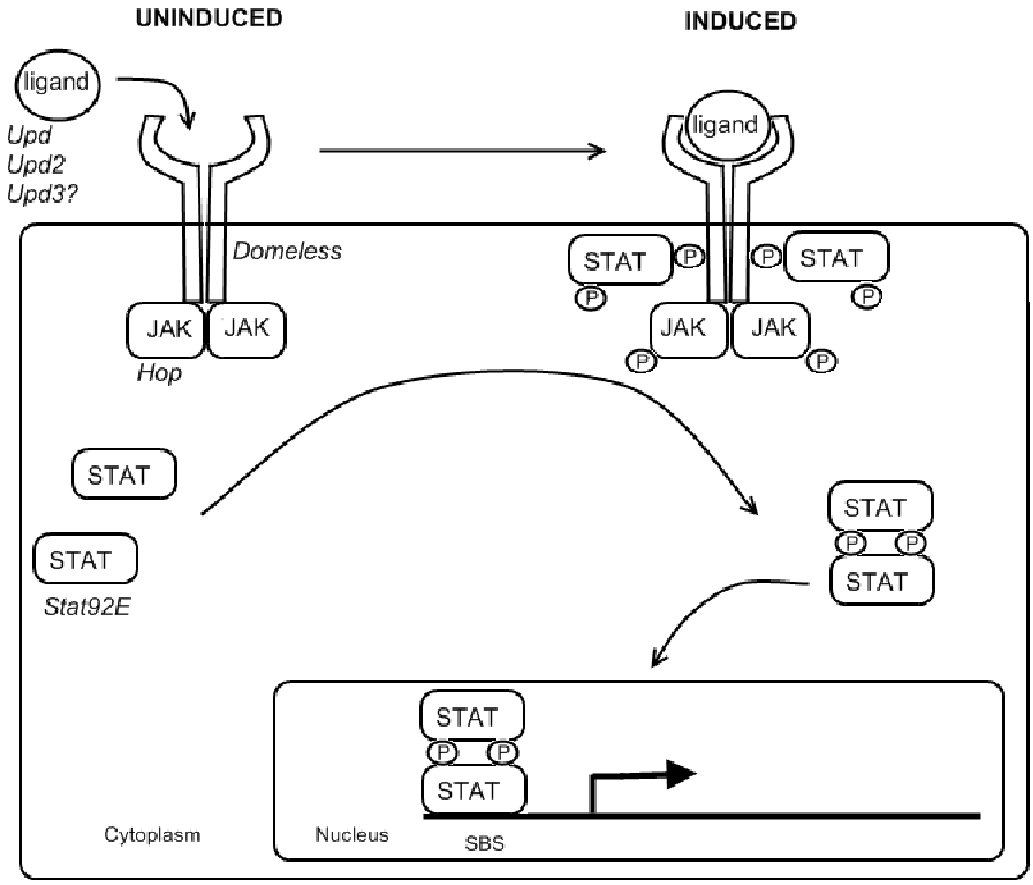


Figure 1.1. Activation of the JAK-STAT Pathway. Activation of the pathway initiates with the binding of the ligand to the receptors. Binding of the ligand causes a conformational shift of the cytoplasmic side of the receptor, bringing the attached Janus Kinases (JAK) in proximity to one another. Transphosphorylation occurs between the two JAKs which allows each to phosphorylate tyrosine residues on the receptor. Cytoplasmic STATs are able to bind to the phosphorylated receptor which allows the JAKs to phosphorylate the STAT proteins. Phosphorylated STATs form homodimers which permit translocation into the nucleus. The STAT homodimers bind to the STAT binding sites (SBS) which recruit the transcriptional machinery to allow transcription to begin. Drosophila homologues of JAK pathway members are shown in italics on un-induced half of the figure.

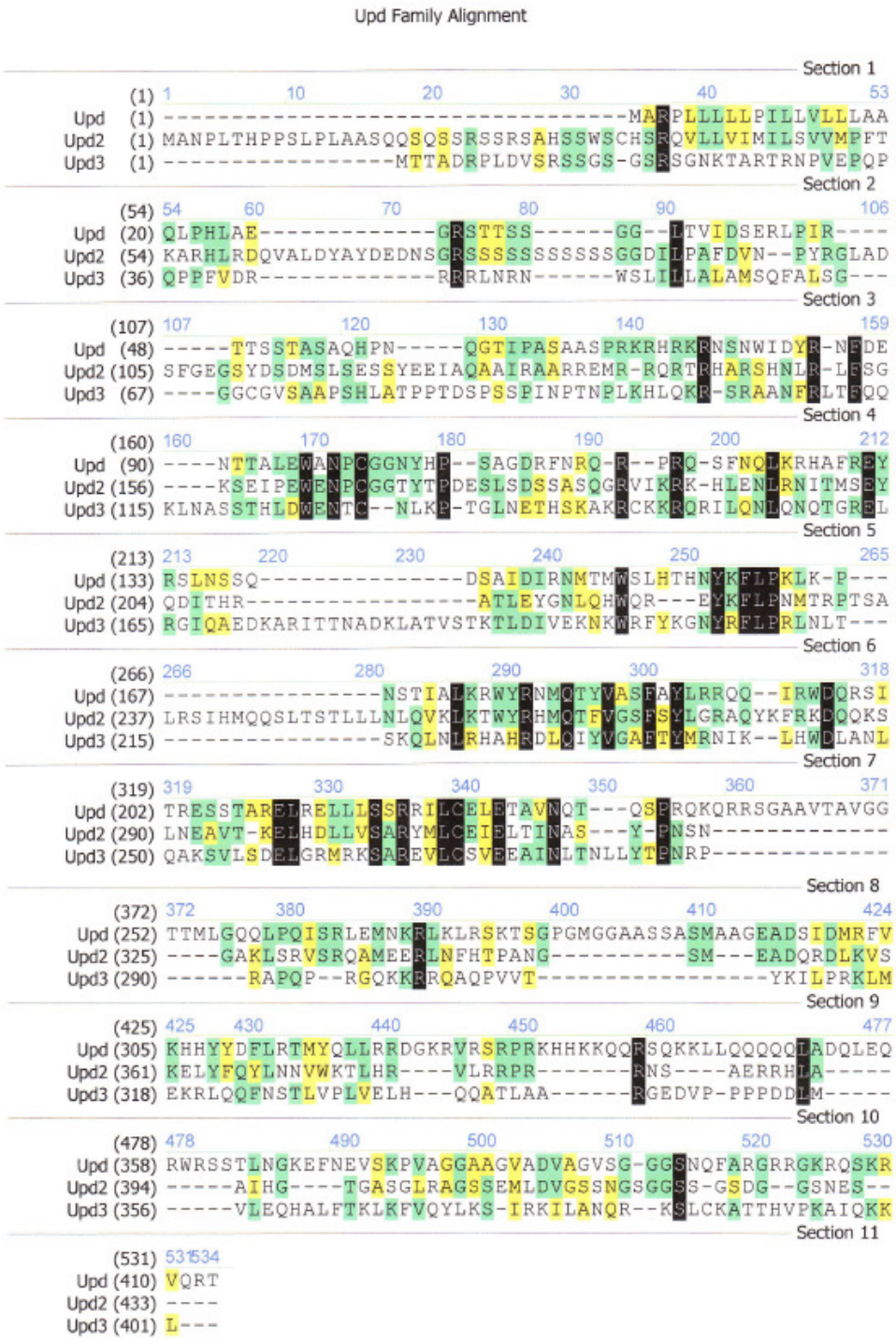


Figure 1.2. Alignment of the Upd family of proteins. The overall primary structure of the Upd family of proteins is ~45% similar (green and yellow) and ~10% identical (black). Similar residues conserved between two proteins are in green and yellow and those conserved between three are in yellow.

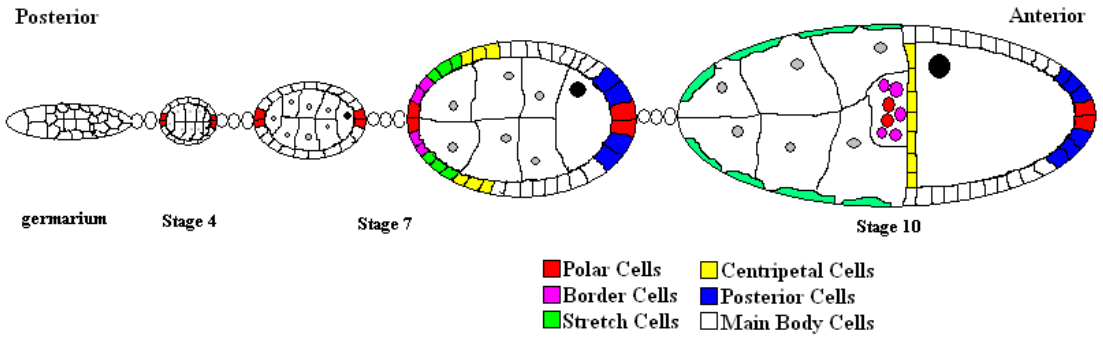


Figure 1.3. *Drosophila* oogenesis. *Drosophila* oogenesis is a process that depends on both germline cells as well as somatic cells for the formation of a mature egg. Oogenesis begins in the anterior region in the germarium. Germline stem cells release cystoblasts, which then undergo 4 mitotic divisions with incomplete cytokinesis to give a germline cyst. Each germline cyst is then surrounded by a monolayer of somatic cells derived from the SSCs in the middle of the germarium. Two distinct populations of cells appear in the follicular epithelium; polar cells (red) and stalk cells (white cells connecting adjacent egg chambers). The remaining follicle cells will continue to divide until stage 7 where they will differentiate into Border Cells (violet), Stretched Cells (green), Centripetal cells (yellow), Posterior cells (blue), or main body cells (white).

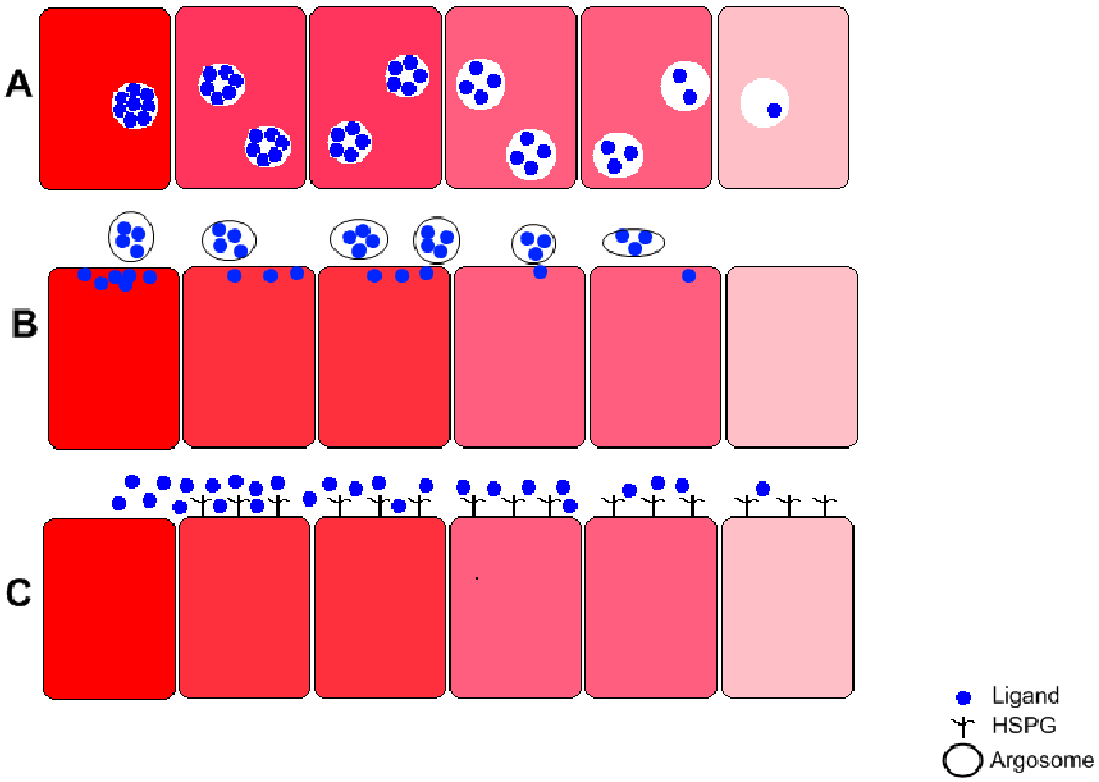


Figure 1.4. Models of morphogen distribution. There are three commonly used models for extracellular distribution of ligands. A) In Planar Transcytosis, packets of ligand are distributed throughout an epithelium through a series of receptor-mediated events exo-endocytosis along the basalateral membrane. B) In the argosome model, ligands are packed into exocytic vesicles and released into the extracellular environment to be taken up into nearby cells. C) In the HSPG mediated distribution, HSPG molecules on the cell surface interact with the ligand to regulate its distribution

Chapter 2

Upd Distribution During oogenesis

Introduction

Drosophila oogenesis produces a mature egg in which patterns and polarities have already been established. These features are important because they will be transmitted to the embryo upon fertilization. The development of each egg is accomplished by interaction between and among germline cells and the overlying somatic follicle cells. The JAK/STAT pathway has several roles during oogenesis. In the germarium, JAK activity is necessary to maintain stem cell fate in dividing germline stem cells and somatic stem cells (Decotto and Spradling, 2005; Lopez-Onieva et al., 2008). When early cysts are leaving the germarium, JAK activity regulates the formation of stalk cells (Baksa et al., 2002; McGregor et al., 2002). Later in oogenesis, when the border cell cluster is migrating towards the posterior of the egg chamber, a constant JAK activity is necessary for proper migration (Beccari et al., 2002; Silver et al., 2005; Silver and Montell, 2001). The fourth role of JAK activity is to specify follicle cell fates prior to differentiation by establishing a gradient of JAK activity with the highest activity at the anterior and posterior poles (Xi et al., 2003). This anterior-posterior gradient of the JAK activity was detected by STAT nuclear localization as well as an in vivo reporter of JAK activity. In both cases, the highest activities were observed at the anterior and posterior poles with a steady decline towards the middle region of each egg chamber (Xi et al., 2003). Previously it was found that a viable combination of hypomorphic JAK alleles led to aberrantly migrating border cells expressing a reporter ordinarily expressed only in stretched cells (Xi et al., 2003). In this case, the border cells expressed a marker exclusive to cells that, in wildtype chambers, have less JAK activity. This observation led to the hypothesis that the JAK activity gradient is responsible for specifying follicular cell fates. Consistent with this hypothesis, in gain of function experiments, misexpression

of *upd* in clones of main body cells was able to induce each cell. *In situ* hybridization experiments revealed that *upd* is expressed exclusively in the polar cells at both anterior and posterior poles, precisely where JAK activity is highest. These data suggest the activator of the pathway, Upd, may act as a morphogen during *Drosophila* oogenesis.

A morphogen is defined as a molecule that is released from a localized source that can specify cell fates in a concentration dependent manner (Wolpert, 1989). By definition, a morphogen is required to have 4 characteristics; 1) To be released from a localized source, 2) form a concentration gradient from source to targets, 3) initiate at least two different responses in neighboring cells in addition to the default response of no activity, and 4) be able to shift cell fates when either over or underexpressed. Previous work has shown that Upd has each of these characteristics except an ability to form a concentration gradient from source to target. If Unpaired is acting as a morphogen, it would be expected to form a concentration gradient reflecting that of the JAK gradient. Unpaired is an extracelluar glycosylated protein that is exclusively expressed in and secreted from the polar cells at the anterior and posterior ends of each developing egg chamber. Interestingly, Upd interacts with the extracellular matrix in cell culture which suggests that components of ECM may play a role in establishing the Upd gradient. Our hypothesis is that Upd acts as a morphogen and forms an extracellular gradient that activates JAK in a concentration dependent manner, thus establishing the gradient of JAK activity, and, in turn, specifies cell fates (figure 2.1).

Results

Upd is distributed in a gradient during oogenesis

Previous work has led to the hypothesis that Upd protein may form an extracellular gradient during oogenesis and thus establish the gradient of JAK activity that has been reported (Xi et al., 2003). Prior to this work, the only localization studies of Upd were done through misexpression of Upd-GFP in the eye disc using GMR-Gal4, a very strong Gal4 driver that expresses throughout the posterior eye disc (Tsai and Sun, 2004). When misexpressed, Upd-GFP was indeed to be found in a gradient. However, the GMR-Gal4 driven expression of Upd-GFP was presumably well beyond the endogeneous levels of *upd* expression. In order to examine the distribution of Upd during oogenesis two immunohistological approaches were taken. One approach examined and the other examined C-terminally tagged Upd driven from a polar cell. One immunohistological staining protocol used tissue that was fixed directly after dissection. This protocol detects extracellular as well as intracellular Upd that has been taken up into endocytic vesicles via receptor mediated endocytosis. The other protocol is specific for extracellular molecules and has been utilized to detect the Wg gradient in wing discs through incubation of the tissue with the primary antibody before fixation (Strigini and Cohen, 2000). Since cellular membranes are not permeable prior to the antibody incubation in this protocol, only extracellular protein will be detected.

The initial effort to detect the Upd protein during oogenesis utilized a C-terminally GFP-tagged version of Upd controlled by a UAS promoter (UAS-Upd-GFP). Using an Upd-Gal4 driver, UAS-Upd-GFP was expressed exclusively in the polar cells of developing egg chambers (For description of the GAL4/UAS expression system, see figure 6.1). Conventional staining protocols detected the Upd-GFP within the polar cells themselves as well as in a gradient on the apical side of the follicular epithelium (figure 2.2A). Using the extracellular specific protocol, Upd-GFP was detected on the basal surface of the follicular epithelium, however, there was no apparent gradient (figure

2.2B). In younger egg chamber in figure 2.2B, the Upd-GFP is forms a ring-like pattern around the area of the polar and stalk cells in the anterior side of the chamber (arrow). Furthermore, Upd-GFP was detected at high levels where the posterior and anterior of adjacent egg chambers overlay. In both cases, Upd-GFP was detected at relatively high levels on the basal surface near the polar cells (figure 2.2B, arrowheads), however, the signal is undetectable a very short distance towards to the middle of the egg chamber. This could be due to interference of the overlying sheath surrounding each ovariole with antibody penetration. For example, in the middle of the egg chamber, the sheath would be tightly stretched around the egg making it more difficult for antibodies to diffuse into these regions, whereas the sheath would be more relaxed in the polar regions, thus, making it easier for antibodies to diffuse into the area.

The Upd gradient seen in the conventional staining was consistent with the central hypothesis that Upd acts a morphogen, however, this is not a detection of the native Upd protein. Antibodies against Upd were previously produced (Harrison et al., 1998) and were reported to work well in western blotting, but gave very poor results in immunohistological staining of the tissues. Nevertheless, we also used these antibodies to detect the endogenous Upd protein in Canton S flies. Both extracellular and conventional staining protocols detected a gradient of Upd on the apical side of the follicular epithelium (figure 2.3A). Antibodies against Fas3, an integral membrane protein present at high levels in polar cells in late stage egg chambers and in undifferentiated follicle cells in early stage chambers, as well as Orb, a protein found exclusively in the oocyte, were used along with rabbit anti-Upd. In extracellular staining, Fas3 was observed at high levels in the polar cells (figure 2.3, asterisk, red) while there was no detection of Orb in the oocyte. This suggests that, as expected for this protocol, the antibodies do not penetrate the cell, thus showing that the detected Upd is exclusively extracellular. Conventional staining of Upd was also done using anti-Upd, anti-Orb, and anti-Fas3 (figure 2.3B-B''). Upd was detected in a gradient resembling that which was seen in the extracellular protocol with the exception that Upd overlaps with

Orb within the oocyte, suggesting that the observed Upd is not exclusively extracellular. This could be an artifact as a result of the fixation procedure, however it is possible that Upd is being taken up into the oocyte and what is detected is Upd that has been taken up into endocytic vesicles. Nevertheless, because Upd is distributed in a gradient in both the conventional and extracellular staining protocols it fulfills the criteria of a morphogen.

The Upd gradient is conserved among species of *Drosophila*

Because of their importance in development, both in structure and function, morphogens are conserved across animal phyla. The identification of Upd as a morphogen has led to the prediction that the Upd protein should be well conserved among the dipteran order. It is also predicted that the role as a morphogen during oogenesis will be conserved among different species within *Drosophila*. Utilizing data from the 12 species of *Drosophila* that have been sequenced, the protein similarity and identity among Dpp, Wg, and Upd were determined using AlignX (Clustal X algorithm) with a Blosum62mt2 scoring matrix (figure 2.5). Upd homologues among the 12 species were determined to be 55.9% similar and 15.5% identical. Sequences taken from other morphogens revealed that the Wg protein had 86.6% similarity and 39.6% identity while Dpp had 76.3% similarity and 46.4% identity. By comparison Upd is not as conserved as Dpp and Wg across the 12 sequenced species. Despite the modest conservation of the Upd proteins across the 12 species, it was still predicted that Upd would form gradients based on the morphogenic function of the JAK/STAT pathway during oogenesis. Antibody staining using rabbit anti-Upd^{melanogaster} was used on ovaries taken from *D. melanogaster*, *D. simulans*, *D. ananassae*, *and D. virilis*. In species more closely related to *D. melanogaster*, the Upd gradient is clear (figure 2.4). These data are consistent with the conservative nature of morphogens. *Drosophila virilis* however failed to reveal

staining. This could be due to the divergence between the two species resulting in a failure of the antibody to recognize *virilis* protein.

Discussion

Upd is secreted from the anterior and posterior polar cells during oogenesis. Previously, it was shown that there is a gradient of JAK activity in the follicle cells around the polar cells with the highest levels closest to the polar cells. This was one of the observations that led to the hypothesis that Upd was acting as a morphogen during oogenesis. In this work, a gradient of Upd is revealed in the egg chambers that is consistent with the idea that it is responsible for establishing the gradient of JAK activity, thus making it a morphogen.

Both endogenous and misexpressed Upd are distributed in a gradient

Both endogenous and misexpressed Upd were utilized in order to determine if Upd forms a gradient in the follicular epithelium. Previously, it had been reported that Upd-GFP forms a concentration gradient in the eye discs when misexpressed using GMR-Gal4, an extremely strong Gal4 driver that expresses throughout the posterior of the eye disc (Tsai and Sun, 2004). GMR-Gal4 expresses well outside the cellular range of upd, and presumably, at a much higher amplitude. Because of the dramatic increase in the concentration of Upd, it becomes problematic when assuming that the endogenous Upd would behave in a similar manner. Recall that Upd associates with the ECM. It is hypothesized that this interaction with the ECM is affecting the distribution of Upd. Therefore, it is likely that when excessive Upd protein is present, the association with the ECM could become overridden, thus leaving the unassociated Upd to diffuse away from the source or simply degrade. In order to determine what the distribution of Upd was in the ovary, the Upd-Gal4 driver was used. The advantage of this gal4 driver over the GMR-Gal4 driver is that it restricts the expression of Upd-GFP to the cells where Upd is endogenously expressed. Furthermore, it is a fairly weak Gal4 driver. Because expression is restricted to the polar cells, it is likely that Upd-GFP is processed and secreted properly. However, it is important to note that the Upd-GFP will presumably

be present at higher levels than the endogenous Upd protein, but still generally low. Conventional staining using an anti-GFP molecule revealed that Upd-GFP is present in a gradient on the apical side of the follicular epithelium. In addition to the staining outside the polar cells, there appeared to be quite an accumulation of the Upd-GFP signal remaining in the polar cells themselves (figure 2.2A) indicating that Upd-GFP may have perturbed secretion (discussed later). When Upd-GFP was observed using the extracellular staining protocol, Upd-GFP was localized to the basal side of the follicular epithelium. Additionally, there was not an obvious gradient, however, the signal was strongest in proximity to the border cells. At the time, it was assumed that there was no apical signal because the antibody could not penetrate the follicular epithelium.

The gradient of Upd-GFP using the conventional staining protocol was consistent with our hypothesis that Upd was acting as a morphogen during oogenesis, however, it still did not reveal the distribution of the endogenous Upd protein. Antibodies against Upd have been available since its discovery (Harrison et al., 1998), however, they have only been demonstrated to work in western blots. Nevertheless, when the Upd antibodies were used in the ovaries, they appeared to work surprisingly well. Both conventional and extracellular staining protocols revealed a gradient of Upd on the apical side of the follicular epithelium, thus strongly supporting our hypothesis that Upd is acting as a morphogen during oogenesis. The conventional protocol using the anti-Upd antibody revealed a similar concentration gradient outside of the polar cells, however, there was noticeably less Upd within the polar cells as compared to the Upd-GFP. Also, in contrast to the Upd-GFP staining, the extracellular staining protocol using anti-Upd revealed that a gradient of Upd exists on the apical side of the follicular epithelium indicating that the antibodies can penetrate the follicular epithelium and access the apical side without actually going into the follicle cells themselves. It was later found that Upd-GFP could be detected on the apical membrane with a longer incubation period (data not shown), however, it remained prevalent on the basal side. Nevertheless, it has been shown that the Domeless receptor in the follicular epithelium

is located exclusively on the apical membrane (Devergne et al., 2007; Ghiglione et al., 2002). Therefore, it would seem likely that the apical Upd is more important in the formation of the JAK activity gradient.

There was clearly more Upd protein detected in the conventional staining protocol was used, as to the extracellular staining protocol when either endogenous or misexpressed Upd-GFP were examined. This observation could be explained by the fact that the conventional method not only detects extracellular Upd, but also that which is bound to the Domeless receptor and has been taken into endocyctic vesicles. Alternatively, it could simply be an artifact of the fixation. Antibodies can penetrate the follicular epithelium more readily in fixed tissue rather than unfixed tissue. Furthermore, conventional staining revealed that there is an abundance of Upd protein overlapping with Orb, suggesting that Upd is within the oocyte. It could be that this is simply an artifact of the fixation, or that Upd is normally endocytosed into the oocyte, however, it could be that the oocyte is redistributing Upd back to the follicular epithelium via transcytosis. This would influence the overall distribution of Upd along the follicular epithelium. Consistent with this idea, it is consistently observed that the Upd protein distribution is much broader in the posterior of the egg chamber than in the anterior even though, presumably, both anterior and posterior polar cells have the same level of *upd* expression. In order to test this, one could disrupt exocytosis within the germline by expressing a dominant negative form of the *Drosophila* dynamin, Shibire, driven by the germline specific nanos-Gal4 driver. If transcytosis through the oocyte was necessary for the distribution of Upd, one would expect shrinkage in the Upd distribution at the posterior as well as a failure of Upd protein to overlay Orb. If transcytosis through the germline is shown to be involved in Upd distribution it would be a new model of morphogen distribution. Most of the work done on morphogen distribution has been done in imaginal discs, which are surrounded by luminal space, thus isolating them and preventing interaction with other tissues. This would make this transcytosis model impossible to study in the imaginal disc. Thus, the possibility that

Upd is being transcytosed through the germline to expand the gradient along the follicular epithelium makes this model particularly interesting in that it provides a system that most resembles developmental processes in other metazoans where tissues are adjacent to one another.

The secretion of Upd from polar cells is likely to be regulated

An interesting observation made in the conventional staining protocol was that both the endogenous and misexpressed Upd were detected at similar concentrations and ranges on the apical side of the follicular epithelium. However, there was noticeably more Upd-GFP detected in the polar cells as compared to the endogenous staining. This led to the consideration that the secretion of Upd may be regulated, or in other words, the secretion of Upd is a rate limiting step in the activation of JAK in the follicular epithelium. Of course, one could argue that because Upd-GFP is a recombinant protein, the processing of it could be slowed down by other factors like chaperone proteins. However, a separate observation seems to suggest otherwise. When misexpressed in follicle cells other than the polar cells (main body cells), misexpressed Upd leads to an increase in the number of border cells, presumably because of the increased JAK activity due to the excess of extracellular Upd being secreted from both polar cells and a subpopulation of main body cells (Xi et al., 2003). However, when observing border cells in flies expressing Upd-GFP only in the polar cells, there was no obvious increase in the number of border cells. Because ectopic border cells only appeared when there were more Upd expressing cells (polar cells + subpopulation of main body cells) rather than elevated expression within polar cells (Upd-Gal4; UAS-Upd-GFP) alone, it is speculated that the secretion of Upd is regulated in the follicular epithelium.

Upd is a morphogen during Drosophila oogenesis

With the demonstration of the extracellular gradient of Upd, we now have evidence to support all 4 characteristics of a morphogen. This is the first time, in any model organism, that a ligand that activates the JAK signaling pathway has been shown to act as a morphogen. In identifying Upd as a morphogen, it is added to a list of very well known *Drosophila* morphogens such as Dpp, Hh, and Wg. Morphogens, by virtue of their importance to multiple processes in development, are well conserved. The completion and the current annotations to the 12 genomes of *Drosophila* species allows for the comparison of these molecules quite readily. Comparisons of the protein sequences among the 12 genomes revealed that Upd is less conserved than are Dpp and Wg. The 12 species that have been sequenced have diverged for approximately 40 million years. However, regardless of the limited sequence similarity between Upd across the 12 species, antibody staining using the anti-Upd specific for the *melanogaster* antigen revealed gradients in *Drosophila* species that are closely related to *D. melanogaster* suggesting that the observed Upd gradient as well as its morphogenic activities are conserved within *Drosophila*. Noticeably, there was no gradient present in *D. virilis* when using anti-Upd^{melanogaster}, most likely due to the divergence of the two species.

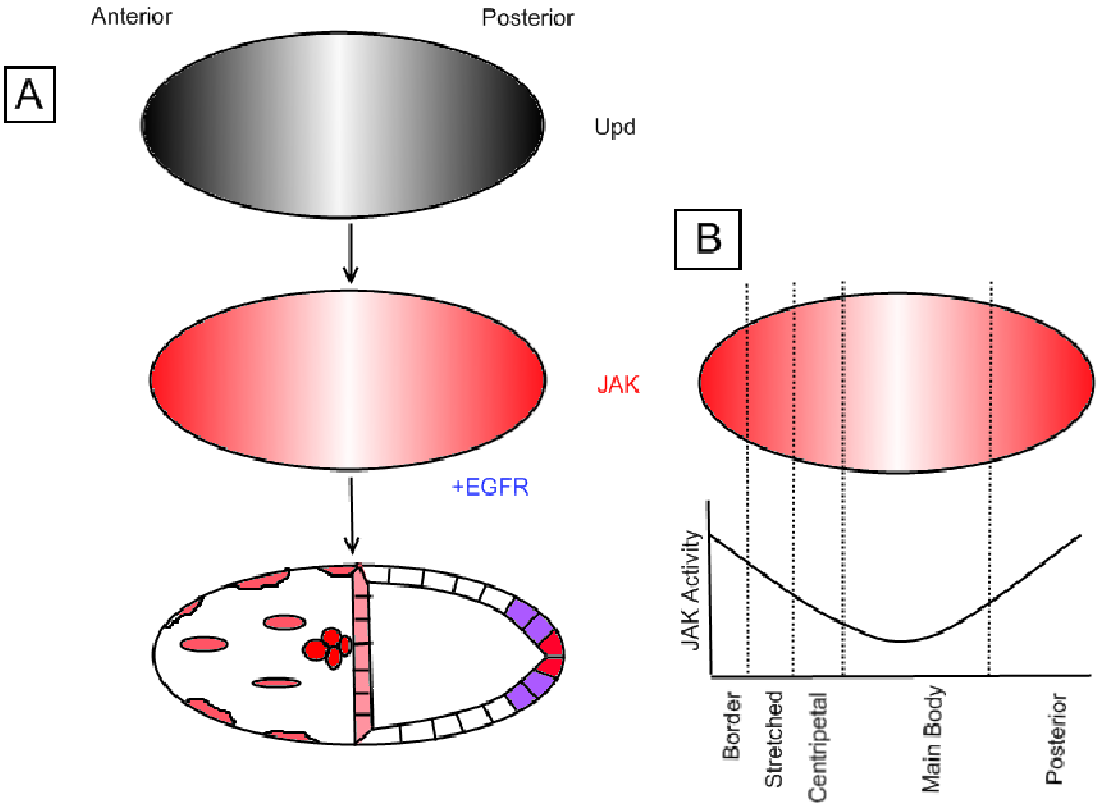


Figure 2.1. Upd Morphogen Model. JAK signaling is known to form a gradient at which certain levels of JAK activity will specify cell fates of the undifferentiated follicle cells. (A) Upd, the activator of the JAK pathway, is expressed in the polar cells at the anterior and posterior of each egg chamber. In the Upd morphogen hypothesis, Upd is secreted from the polar cells and is distributed along the follicular epithelium in a graded fashion. The graded distribution of Upd will establish the gradient of JAK activity. (B) Specification of cell fates are established by the amount of JAK activity. Anterior cells are specified by which threshold of JAK activity they lie within (dotted lines). Posterior cells are specified by the presence of Gurken (activating EGFR) from the oocyte and Upd from the polar cells.

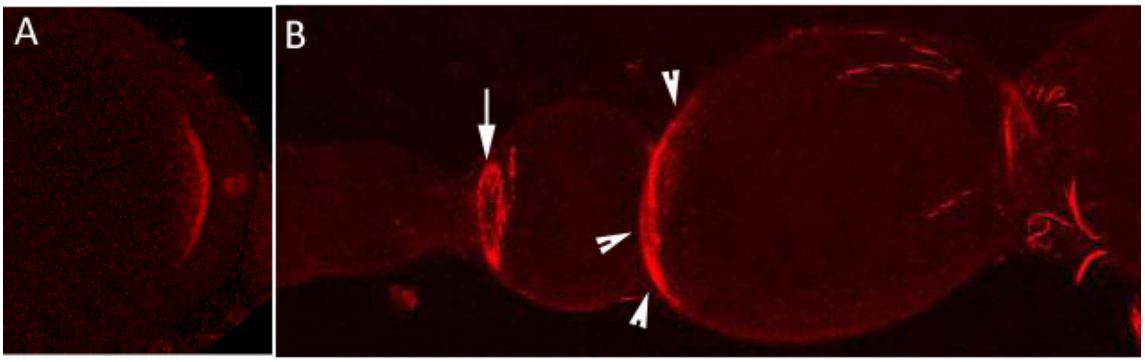


Figure 2.2. Upd-GFP is detected on both the basal and apical sides of the follicular epithelium depending on the staining protocol. The initial examination of extracellular Upd was done utilized a UAS-Upd-GFP construct expressed exclusively in the polar cells using the Upd-Gal4 driver. (A) Conventional staining protocols revealed both intracellular Upd-GFP as well as Upd-GFP on the apical side of the follicular epithelium (rabbit α-Upd). (B) Using an extracellular staining protocol, Upd-GFP was detected at the poles of each egg chamber, but on the basal surface (mouse α-GFP).

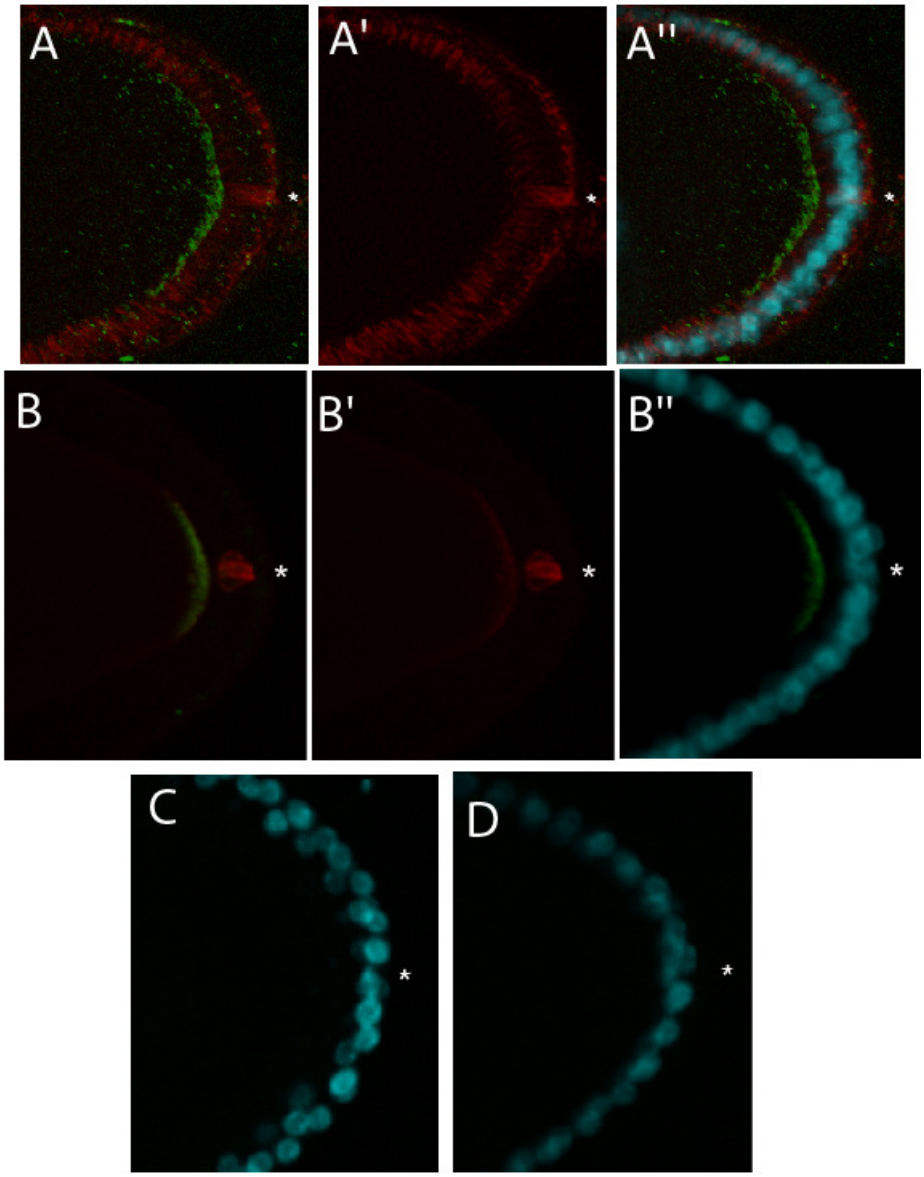


Figure 2.3. Upd is distributed in a gradient in both extracellular and conventional immunohistological staining. Upd (A-D, green) is detected in a gradient from its source, the polar cells (A-D, red at asterisk)(rabbit α-Upd, mouse α-Fas3). (A-A'') Extracellular staining protocol detects Upd and Fascicilin 3 (Fas3)(red in follicle cells) but excludes detection of Orb (red in oocyte). (B-B'') A conventional staining protocol detects Upd, Fas3, and Orb. Upd and Orb are colocalized with one another in the posterior of the oocyte. Controls using serum taken from preimmunized rabbits were done using extracellular (C) and conventional (D) staining protocols

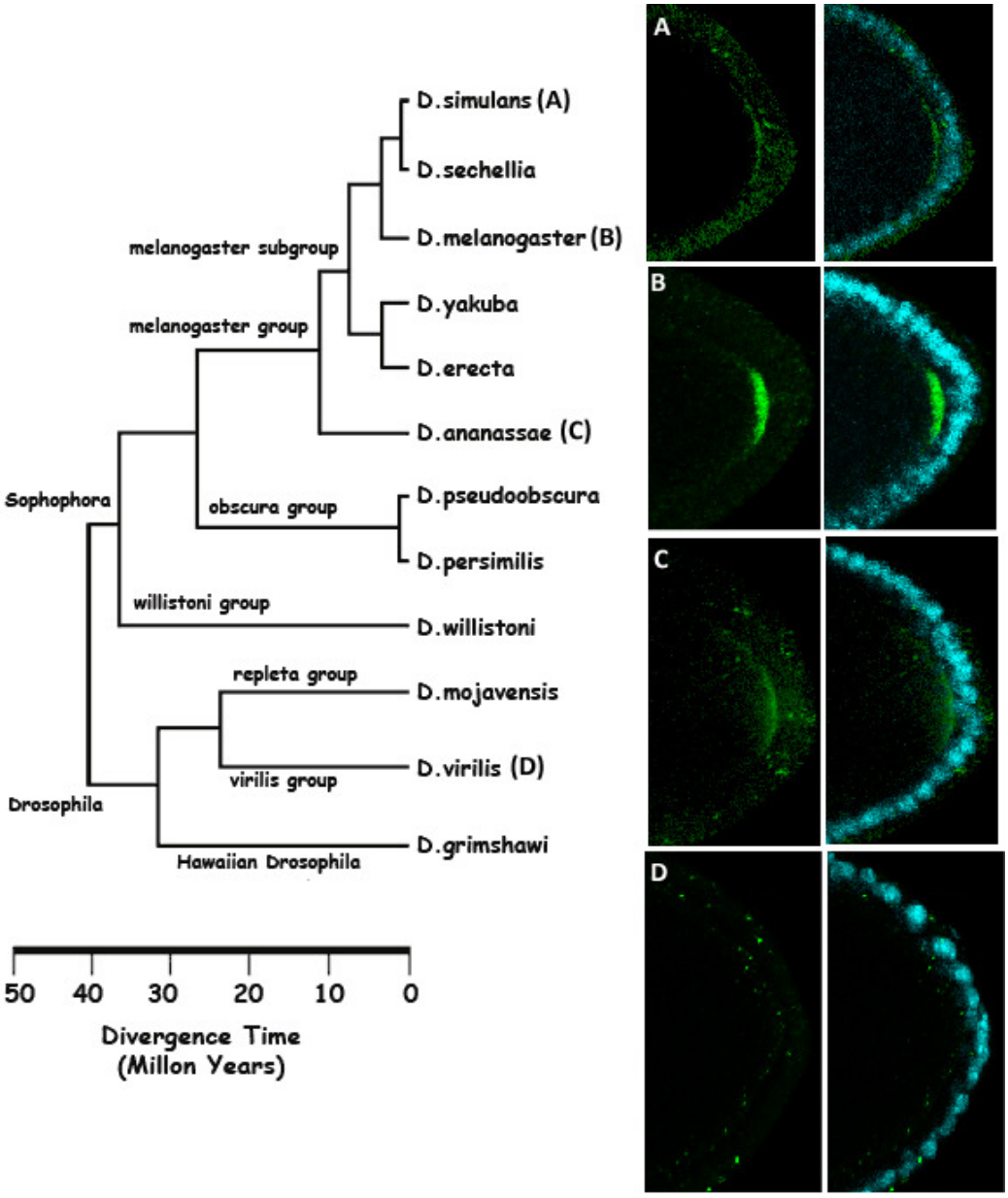
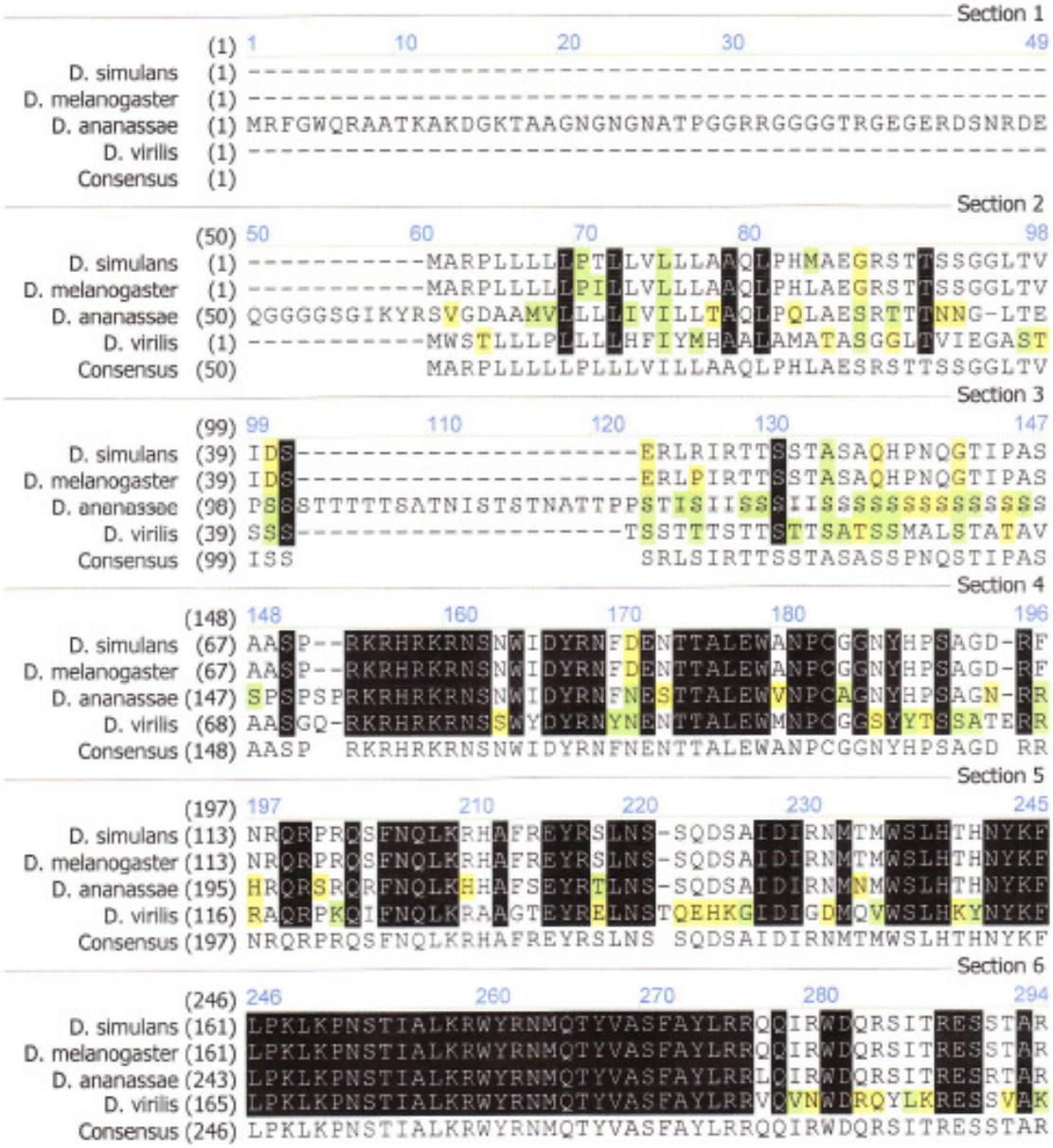


Figure 2.4. The Gradient of Upd is Conserved in Closely Related *Drosophila* species. Conventional staining using rabbit α-Upd (Upd from melanogaster) was carried out on ovaries from other species of *Drosophila*. The letters next to the species in the phylogenic chart correspond to the lettered antibody staining. Upd (A-D, green) is detected in a gradient in *Drosophila simulans* (A), *Drosophila melanogaster* (B), and *Drosophila anannassae* (C). There was no signal detected in *Drosophila virilis* (D).

4 Species Upd Alignment



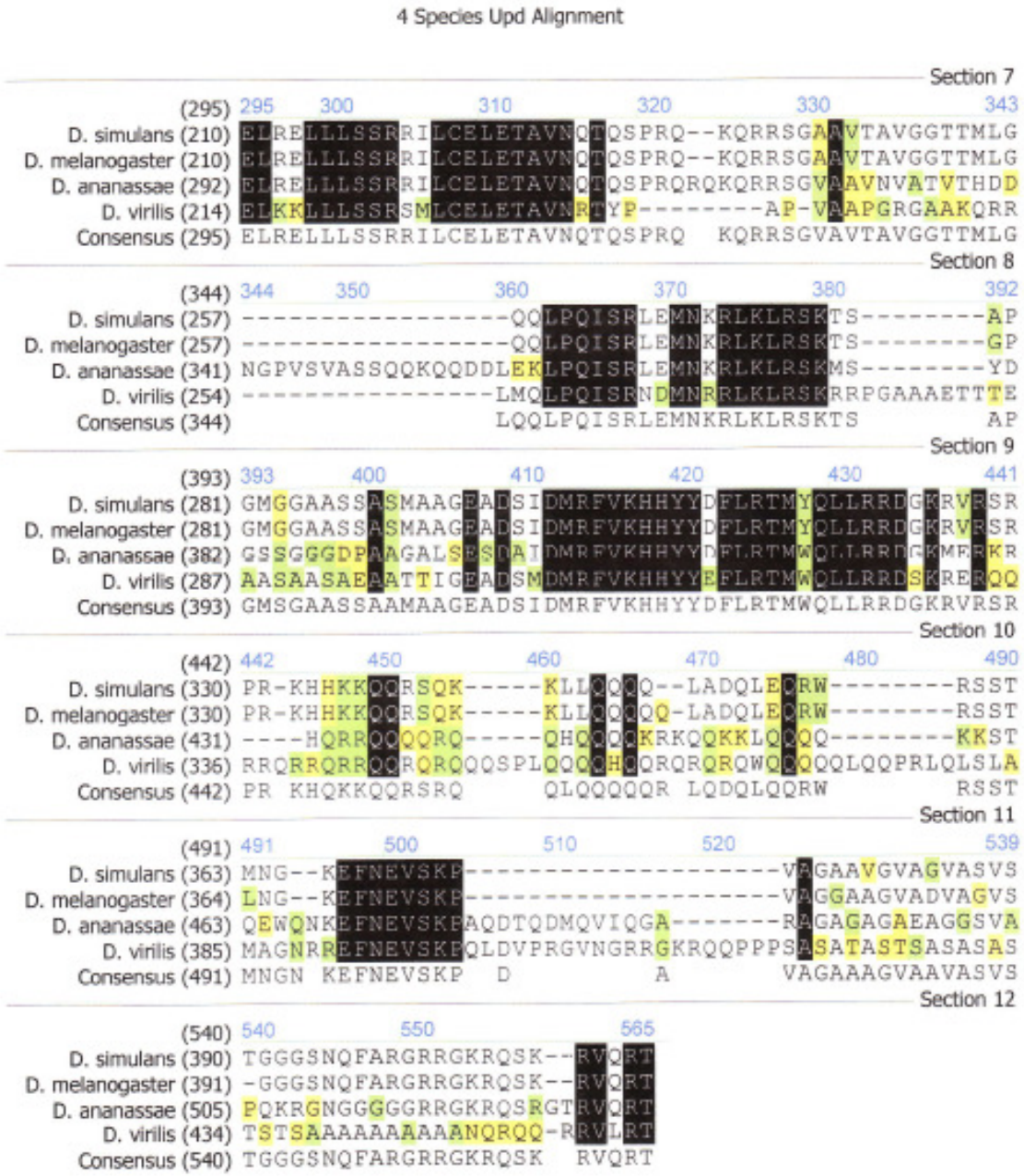


Figure 2.5. There are several highly conserved domains within Upd between distantly related species of Drosophila. The Upd protein has ~75% similarity and ~31% identity between *D. virilis, D. simulans*, *D. melanogaster*, and *D. ananassae* . Identical sequences are shown in black boxes, with similar sequences being shown in yellow and green boxes. Notice that there are around 7 domains within the Upd primary structure that are highly conserved.

Chapter 3

The Role of HSPGs in the Distribution of Upd

Introduction

The data in chapter 2 suggest that Upd acts as a morphogen during oogenesis. How morphogens move from source cells to target cells across epithelia has been intensely studied over the past several years with the focus being on the Wnt, Hh, and TGF-β families of morphogens. A key feature of all of these morphogens is that they are post-translationally modified, having multiple glycoslations on the Wnt and TGF-β molecules and a cholesterol moiety added to the Hh family members. These modifications allow interaction with the ECM or the cell membrane that play a key role in regulating or facilitating the movement of the ligand (Han et al., 2004a). In *Drosophila*, Decapentapalegic (Dpp, a TGF-β homologue) and Wingless (Wg, a Wnt homologue) proteins are both glycosylated and interact with members of a family of ECM proteins known as Heparan sulfate proteoglycans (HSPGs) (Baeg et al., 2004; Belenkaya et al., 2004; Kreuger et al., 2004).

In chapter 2 of this work it was shown that Upd forms an extracellular gradient within the follicular epithelium with the highest level at its source, the polar cells. However, as mentioned previously, Upd associates with the ECM in *Drosophila* cell culture and can be released upon the addition of heparin into the culture media (Harrison et al., 1998). Together, these results beg the question: how does the extracellular Upd protein, which is associated with the ECM, move from the source cells to the target cells? Could it be that Upd, like Dpp and Wg, depends on HSPGs for proper distribution? The facts that Upd is a glycosylated protein, forms a concentration gradient, interacts with the ECM, and can be released from the ECM with the addition of heparin support this hypothesis.

HSPGs are a family of extracellular matrix and cell surface molecules. Each HSPG consists of a core protein to which glycosaminoglycan (GAG) chains are attached (Esko and Selleck, 2002). The HSPG family is divided into three subgroups: glypicans, syndecans, and perlecans. Each subgroup is distinguished by its core protein structure and how it interacts with the cell membrane or extracellular matrix. Glypicans are a family of HSPGs that are distinguished by their connection to the cell membrane via GPI-linkage. *Drosophila melanogaster* has two known genes encoding glypicans: *division abnormally delayed* (*dally*) and *dally-like protein* (*dlp*). Syndecan is a class of HSPG that features a transmembrane domain, of which there is one known in *Drosophila*: *syndecan* (*sdc). terribly reduced optic lobes* (*trol*), encodes the sole *Drosophila* Perlecan, which is a class of HSPGs that are secreted from the cell into the ECM.

The Glypican family of HSPGs has been shown to be involved in BMP, Wnt, and Hh signaling. The *Drosophila* glypicans, *dally* and *dlp*, are required for both Hh and Wg in the patterning of the embryonic epidermis (Baeg et al., 2001; Han et al., 2004b). In the wing disc, mutants in *dally* and/or *dlp* disturb the distribution of Dpp and Wg (Belenkaya et al., 2004; Lin and Perrimon, 1999). Furthermore, glypicans have roles in vertebrates consistent with the roles in *Drosophila*. Members of the glypican families have been shown to regulate Wnt dependent cell movements during gastrulation in both *Danio rerio* (zebrafish) and *Xenopus* (Ohkawara et al., 2003; Topczewski et al., 2001). The *Drosophila* Syndecan, *sdc*, plays several distinct roles within the organism. Sdc is critical in the development of the *Drosophila* CNS by regulating the distribution of Slit, an axonal repellent protein (Johnson et al., 2004). In cell cultured hemocytes (Kc167 cells), Sdc is essential for the cell adhesion to the basal lamina (Yamashita et al., 2004). Thus far in *Drosophila*, *sdc* has not been linked to any morphogenic signaling, however, in mammals, *sdc1* disrupts Wnt1 signalling (Alexander et al., 2000; Haerry et al., 1997). The sole *Drosophila* perlecan, *trol*, regulates cell division of stem cells in the larval brain (Datta and Kankel, 1992), promotes progression through mitosis in arrested

neuroblasts by regulating FGF and Hh signaling (Park et al., 2003), as well as maintaining cell polarity in the follicular epithelium during oogenesis (Schneider et al., 2006).

The common feature of all three familes of HSPGs is the HS glycosaminoglycan (GAG) chains that are attached to the core protein. GAGs are assembled in the golgi and contain repeating disaccharide units of uronic acid linked to glucosamine (Esko and Selleck, 2002). HS GAG biosynthesis consists of 3 general stages; chain initiation, chain polymerization, and chain modification. Several enzymes are required for proper synthesis of GAGs and thus, proper formation of the HSPG complex. These enzymes and their functions are well conserved from vertebrates to invertebrates. It is generally hypothesized that the ligands that associate with HSPGs do so by interactions with the HS GAG chains, although recent evidence suggests that, at least in the case of Dpp, the ligand has some affinity to the core protein (Kirkpatrick et al., 2006). Because of their generalized role in the formation of all HSPGs, genes that encode enzymes in GAG biosynthesis were the first of the HSPG associated genes found to be involved in cell signaling. In the case of Hh signaling, *tout-velu* (*ttv*), a gene encoding a heparan sulfate co-polymerase (chain polymerization enzyme), was shown to be essential for the traverse of Hh across the wing disc epithelium (Bellaiche et al., 1998). *Sugarless* (*sgl*), which encodes a protein with homology to UDP-glucose dehydrogenase (chain initiation enzyme), was shown to be essential for FGF signaling during embryogenesis (Lin et al., 1999). In cuticle formation during embryogenesis, *sugarless* and *sulfateless* (*sfl*) are essential for the Wg and Hh signaling required for segment polarity (Binari et al., 1997; Hacker et al., 1997; Lin and Perrimon, 1999). *sfl* encodes a protein similar to vertebrate N-deacetylase/N-sulfotransferases (Chain Modification).

There are three non exclusive models that are commonly used to describe how HSPGs influence the distribution of extracellular ligands. These are commonly referred to as the HSPG mediated transport model, the HSPG facilitator model (coreceptor model), and the Stability-Retention model (figure 3.1). In the HSPG Mediated model, the ligand will interact with the HSPGs on the cell surface as a template for

transportation, much like a car uses a street. In this simple model, loss of HSPGs in cells distant from the source of the ligand will result in an accumulation of ligand in the HSPG containing wildtype cells. In the HSPG Mediated model, the ligand binds to its receptor in an HSPG dependent process. In this model the HSPG could be required for 1) increasing the affinity of the ligand-receptor interaction, 2) stabilizing the receptor, or 3) the binding to the receptor to allow activation. In the Stability-Retention Model, HSPGs on the cell surface retain and/or stabilize the ligand at the cell surface. Loss of the HSPG would cause the ligand to either decay or to be lost from the ECM rather than limiting its movement to the 2-dimensional surface of the epithelium. It is also important to realize that these models are not mutually exclusive. It is theoretically possible for any combination of these models to be supported for the distribution of a given ligand. Furthermore, because of what has already been seen in Hh and Wg signaling, the supported models may be different between ligands and even tissues.

Results

Glypicans promote JAK signaling in the follicular epithelium

Both Dally and Dlp have been shown to be involved in the distribution of known morphogens, although, their roles differ depending on the type of tissue and ligand being studied (Belenkaya et al., 2004; Han et al., 2004b; Kreuger et al., 2004). Because glypicans are essential for multiple signaling pathways, loss of function of either *dally* or *dallylike* is lethal. Therefore, homozygous mutant clones were generated using FLP-mediated recombination (Chou and Perrimon, 1992)(For description of the FLP-mediated recombination, see figure 6.2). Mitotic recombination was induced in females of the appropriate genotypes and egg chambers were observed 3 to 5 days post induction. Upd distribution or JAK activity was subsequently observed using antibodies against Upd or a molecular marker for JAK activity, respectively. Mosaic analysis revealed that the concentration of extracellular Upd protein is abruptly decreased on those cells mutant for *dally* (figure 3.2 A, arrow) but appears to be normal in cells mutant for *dallylike* (figure 3.2 B, arrows). Although the Upd signal declines sharply upon reaching a *dally* mutant cell, it is possible that Upd remains present along *dally* mutant cells at an undetectable level, yet a level high enough to activate JAK signaling. Therefore, similar mitotic recombination experiments were carried out utilizing a JAK activity marker rather than observing extracellular Upd. *In situ* hybridization has revealed that *domeless*, the gene encoding the *Drosophila* JAK-STAT receptor is in a positive feedback loop with JAK activation (Hombria et al., 2005; Xi et al., 2003). Therefore, the expression of *domeless* can be utilized as a JAK pathway reporter *in vivo*. An allele of *domeless*, $dome^{G0367}$, is caused by the insertion of the p{lacW} transposable P-element within the locus causing it to be expressed by the *domeless* regulatory elements, or in other words, a *domeless* enhancer trap which results in the expression of β-galactosidase rather than the Domeless protein. Immunohistology of *domelacZ* flies using a β-galactosidase antibody detected a clear gradient in egg chambers which is reminiscent of the JAK gradient displayed by STAT localization (Xi et al., 2003) as well as

the Upd distribution shown in chapter 2 of this work. Consequently, for the remainder of this work, *dome*^{G0367} will be referred to simply as *domelacZ*.

Clonal analysis of follicle cells homozygous for a null mutation of *dally* reveals a sharp decline of the *domelacZ* signal as compared to their wildtype counterparts (figure 3.3 B, arrows). Importantly the JAK activity, as observed with *domelacZ* signal, is present in the first row of *dally* mutant follicle cells, however, the signal is drastically reduced in cells further into the clone. This result is reminiscent of results seen for Dally mediated transport of Dpp in wing discs (Belenkaya et al., 2004). The observation of JAK signaling in *dally* mutant cells adjacent to wildtype cells could suggest that Dally is involved in transporting Upd from cell to cell, promotes stability and/or retention of the Upd on the ECM, or acts as a co-receptor in presenting Upd to domeless receptors on the mutant cell. In egg chambers containing very small clones homozygous for *dally*, there is clearly an absence of the *domelacZ* signal distal to the *dally* mutant which appears as a "shadow" of *domelacZ* expression (figure 3.3 C, arrows). Importantly, there was a response to JAK activity in the *dally* clones, just as was seen previously. The absence of JAK activity in cells distal to the *dally* mutant clone suggests that Upd cannot traverse the cells lacking Dally. If Upd did not rely on Dally for transport, it would have reached the distal wildtype cells and would have activated JAK signaling. Furthermore, If Dally was acting as a coreceptor, there would not be strong JAK in the *dally* clone. The observations that *dally* mutant cells respond with JAK activity, and that Upd cannot be distributed across a small *dally* clone suggest that Dally is being used as a facilitator for the distribution of Upd along the epithelium or that it is stabilizing/retaining Upd to the apical membrane. If Dally was needed as a facilitator, it would be expected that there would be an accumulation of Upd at the mutant-wildtype border. However, this is not seen, suggesting that Dally is functioning by stabilizing or retaining Upd.

The three models of HSPG involvement in ligand transport (figure 3.1) derive from studies done mostly within imaginal discs, which are isolated from other tissues and surrounded by luminal space. Because of the proximity of the follicular epithelium

to the oocyte, it becomes possible to differentiate between stability and retention. Therefore, if Dally was acting to retain Upd to the membrane, one would expect to still observe the Upd protein, however, it would be more diffuse than Upd on wildtype cells. This was not the case, therefore, supporting a role for Dally in the stability of Upd.

The Drosophila Perlecan, Trol, is not required for proper JAK activation or Upd distribution.

Perlecans have been shown to act as coreceptors in FGF signaling in *Drosophila* larval brain (Park et al., 2003). Furthermore, *trol* is involved in the maintenance of cell polarity in the follicular epithelium (Schneider et al., 2006). Knowing that Trol has been shown to be involved in extracellular signaling, as well as the role that it plays during oogenesis, makes studying its possible effect on Upd distribution/JAK activation during this process particularly interesting. Because both *trol* and *domelacZ* are on the X chromosome, it is not possible to make clones of *trol* using *domelacZ* to mark JAK activation. Therefore, the posterior cell fate marker *pnt-lacZ* was utilized to assay JAK activity. Posterior cell fate is established by the combination of JAK signaling activated by the Upd deriving from the polar cells (Xi et al., 2003) and EGFR signaling activated by the TGF-α molecule Gurken released from the oocyte (Gonzalez-Reyes and St Johnston, 1998). Like *domelac*Z, *pnt-lacZ* is also expressed in a JAK activity determined gradient (Xi et al., 2003). In small clones of cells lacking JAK pathway components, cells in the posterior fail to differentiate into posterior cell fate and instead adopt the main body cell fate (Xi et al., 2003). Therefore, in large *trol* mutant clones, if Upd distribution is effected, it would be manifested in lack of *pnt-lacZ* expression. Large *trol* clones were generated in posterior egg chambers; however, regardless of size or localization of the clone, there remains a consistent, unaltered pnt-lacZ expression (figure 3.4 A, B). Upd distribution was also unaltered in egg chambers containing large mutant clones of *trol* (figure 3.4, C). A caveat to these results is that, since Trol is secreted, there can be a non

autonomous compensation of mutant cells by wildtype neighbors. Although Trol is known to be secreted, the extracellular range of the protein in not known. However, in a previous study, Trol was shown to be localized exclusively to the basal side of the follicular epithelium (Schneider et al., 2006). Furthermore, Domeless-containing endosomes, and therefore JAK activation (Devergne et al., 2007), as well as the Upd gradient presented in this work form on the apical side of the follicular epithelium. These two observations indicate that Trol plays no direct role in regulating JAK activity through affecting the distribution of the Upd protein during oogenesis.

The *Drosophila syndecan* is not essential for Upd distribution.

The *Drosophila* Syndecan (sdc) is required for proper *slit/robo* signaling during the formation of the *Drosophila* CNS (Johnson et al., 2004). Although Sdc has never been observed to influence known morphogens, by virtue of the *sdc* role in *slit/robo* signaling, it remains possible that it could play a role in the establishment of the Upd gradient during oogenesis. Examination of the role of *sdc* in the establishment of JAK activity during *Drosophila* oogenesis was carried out by mosaic analysis. In egg chambers containing clones of *sdc* mutants there is no disruption of the Upd ligand (Figure 3.5, A). Thus, Sdc is not acting as a mediator for Upd transport or for the stability and/or retention of Upd to the ECM. It does remain possible that Sdc could be acting as a cofactor in the activation of JAK. The *sdc* mutant used in these studies is caused by an insertion of a P-element into the *sdc* locus. The P-element present contains a *lacZ* reporter making it impossible to assay JAK activity using the *domelacZ* reporter. As a means for examining the role of Sdc on the activity gradient the morphology of the anterior cell fates within *sdc* clones were examined in stage 10b egg chambers. Previously it was shown that *hop* clones in the anterior do not undergo the morphological transitions that are typically seen in stretched and centripetal cells, rather, they remain as main body cells (Xi et al., 2003). In *sdc* clones in the anterior egg

chambers, it can clearly be seen that the mutant cells have undergone the morphological changes consistent with stretched and centripetal cells, thus, providing indirect evidence that JAK signaling is occurring within them (figure 3.5, B).

GAG modifying enzymes have varying effects on Upd distribution.

HS GAG biosynthesis consists of three general stages; chain initiation, chain polymerization, and chain modification. In order to test the hypothesis that the interaction of Upd with the ECM is dependent on the GAG chains associated with the HSPG core proteins, mutants from different stages of GAG biosynthesis were obtained. The mutants; *sgl*, *ttv*, and *sfl* which encode enzymes responsible for chain initiation, chain polymerization, and GAG modification respectively were analyzed for their ability to alter JAK activity or Upd distribution.

The *sgl* gene encodes an enzyme for UDP-glucose dehydrogenase activity. Specifically it catalyzes the production of UDP-glucuronate (UDP-GlcA) which is an essential building block for all GAGs. Therefore, mutant clones of *sgl* should carry only non-glycosylated HSPG core proteins on the ECM. Based on the general hypothesis that extracellular ligands interact with HSPGs in a GAG dependent manner, it was predicted that Upd will not be able to be transported across mutant clones of *sgl*. Surprisingly, the induction of large *sgl* clones did not lead to Upd disruption in mosaic egg chambers. In *sgl* clones, Upd can clearly be seen at two cell diameters away from the polar cells on both wildtype and mutant sides at equal concentrations (figure 3.6, A, arrows). Based on this observation alone, one may conclude that GAGs are not required for Upd distribution. However, it was previously reported that embryos that are *sgl*/*sgl*, which receive the maternal contribution from their *sgl*/+ mothers, survive until late larval to early pupal stages (Toyoda et al., 2000). Furthermore, it has been proposed that UDP-GlcA stores are shared among the follicle cells and germ cells on the developing egg chambers by being passed through the gap junctions that exist between the cells

(Goldberg et al., 2004; Zhu et al., 2007). Therefore, it is possible that the lack of UDP-GlcA production in *sgl* clones is non-autonoumously compensated for by the wildtype cells within the epithelium.

In *ttv* mutant clones, *domelacZ* expression is reduced in mutant cells when compared to their wildtype counterparts (figure 3.6, B arrows). *domelacZ* expression is visible in the first two cells of the clone closest to the polar cells, but is not present in the neighboring mutant cells. Furthermore, it was unclear as to how Upd distribution is affected in *ttv* clones. In the egg chambers that were observed, there were no *ttv* clones close enough to the polar cells to assess their effect on Upd distribution (data not shown). This was most likely due to a combination of small *ttv* clone size and the limited range at which Upd can be detected. Nevertheless, the reduction of JAK activity within *ttv* mutant clones suggest that GAGs play a role in the activation of JAK signaling, presumably by promoting Upd distribution. Nevertheless, the data are not definitive enough to rule out the other mechanisms discussed.

Mutant analysis of *sfl* was complicated by small size of the mutant clones generated in the egg chambers. In most cases, *sfl* mutant clone consisted of 1 to 2 mutant cells while the homozygous wildtype *sfl* sister clones were quite large. This was an unexpected result as large *sfl* clones have been generated previously within the follicular epithelium (Zhu et al., 2005; Zhu et al., 2007). However, the allele that was previously used in mosaic studies (sfl^{03844}) was not utilized in this study. Like the sfl^{03844} allele, the sfl^{9B4} allele used in this study is a homozygous lethal mutation caused by the insertion of a P-element into the locus, or another lesion cause by P-element mobilization. The lethality occurs during embryogenesis, presumably because of the role of Sfl in Wg and Hh signaling during embryonic segmentation. Interestingly, sfl^{9B4}/sfl^{03844} flies overcome the embryonic lethality and survive until at least the pupal stage (Baeg et al., 2001). This may be an indication that neither one of these alleles is a true null allele, however, it is also possible that something else was affected on the sfl^{03844} chromosome during the mutagenesis, such as insertion of the P-element

somewhere else on the chromosome. Nevertheless, in the *sfl*^{9B4} clones that were localized near the polar cells, there was no change in the JAK signaling as reported by the *domelacZ* reporter (figure 3.6, C). Attempts to observe changes in the Upd distribution in *sfl* clones was similar to the result obtained in the *ttv* clones. However, like in the *ttv* experiments, *sfl* clones were too small to observe effects on Upd distribution. Although the unaltered JAK activity in *sfl* clones indicate that *sfl* is not required for JAK signaling, a major caveat to this interpretation is that there could be non-autonomous compensation of HSPG to the small *sfl* mutant clones by the surrounding wildtype cells.

Discussion

Dally is required for long-range JAK signaling by stabilizing Upd at the apical surface.

In Chapter 2 of this thesis, Upd was shown to form a gradient on the apical membrane of the follicular epithelium. In mosaic analysis of *dally* effects on Upd distribution it was observed that there was a very rapid decline in Upd protein on *dally* mutant cells. When assaying for JAK activity in *dally* mutant clones, it was observed that mutant cells closest to the source of Upd were positive for JAK activity, however, JAK was noticeably reduced or absent in mutant cells further from the Upd source as compared to wildtype cells. In both assays, Dally was shown to clearly have a role in the overall formation of the JAK gradient in oogenesis. As mentioned above, there are 3 non-exclusive models in which HSPGs are influencing the distribution of extracellular ligands (figure 3.1). The data presented here support a stability-retention type model for Dally in the distribution of Upd. If Dally was required only as a template for Upd to travel, we would have expected to see an accumulation of Upd at the wildtype-*dally* mutant border. This however, was not the case. Furthermore, if Dally was strictly playing a coreceptor role, we would have expected to see JAK signaling distal to small *dally* clones. This, too, was not the case. Furthermore, JAK signaling could still be detected (slightly) in *dally* cells that were not adjacent to wildtype cells. These data together do not completely rule out a coreceptor model, however, they do rule out an exclusive coreceptor model. The loss of Upd and the diminished JAK activity in *dally* clones is the expected result if distribution occurs through a stability-retention model. The previous support fro the stability-retention model in morphogen distribution comes from data gathered on morphogen distribution within imaginal discs. The imaginal discs consist of a rather isolated epithelium surrounded by luminal space. In oogenesis, because of the juxtaposition of the oocyte and apical membrane of the follicle cells, it becomes possible to distinguish between stability and retention. If the HSPG was simply required for retention of the ligand, one might expect to see a more diffuse extracellular signal with loss of HSPG. If the HSPG was required for stability, the ligand might be lost

altogether as a result of protein degradation. In the case of Upd in *dally* mutant clones, the Upd signal was drastically reduced and virtually no Upd could be detected within one cell diameter of the mutant clone. Together, these observations support a role of Dally in stabilizing Upd in the establishment and maintenance of the Upd gradient during oogenesis. In *dlp*, *trol,* or *sdc* mutant clones, neither Upd distribution nor JAK activity was altered leading to the conclusion that Dally of the glypican family is the only *Drosophila* HSPG that influences JAK signaling in the ovary.

The Role of GAGs in the Distribution of the Upd protein

Genes responsible for the 3 general steps in the production of GAGs were tested for their influence on Upd distribution or JAK activity. *sgl*, a gene encoding the enzyme responsible for producing the GAG building blocks, was shown to form large mutant clones in the *Drosophila* ovary. Furthermore, Upd distribution was not altered in these mutant clones. Oddly, genes encoding enzymes acting downstream of Sgl gave only small mutant clones when compared to their homozygous wildtype sister clones. Because of the pleitropic role played by GAGs in cell biology, it is not surprising to see an effect in cell division, however, it was surprising to see *sgl* mutants form such large mutant clones. This could be due to another gene encoding a UDP-glucose dehydrogenase, however, there is no evidence that another exists. It has recently been proposed that UDP-GlcA, the product of the Sgl reaction, can be shared amongst the germ line and follicle cells by use of the gap junctions that exist between them. It is estimated that molecules up to 1 KDa in size can be shared through the gap junction, making the sharing of 0.577kDa UDP-glucose a reasonable prediction. Nevertheless, the data gathered here on the requirement for the GAG chain in Upd distribution and the JAK activity gradient are inconclusive.

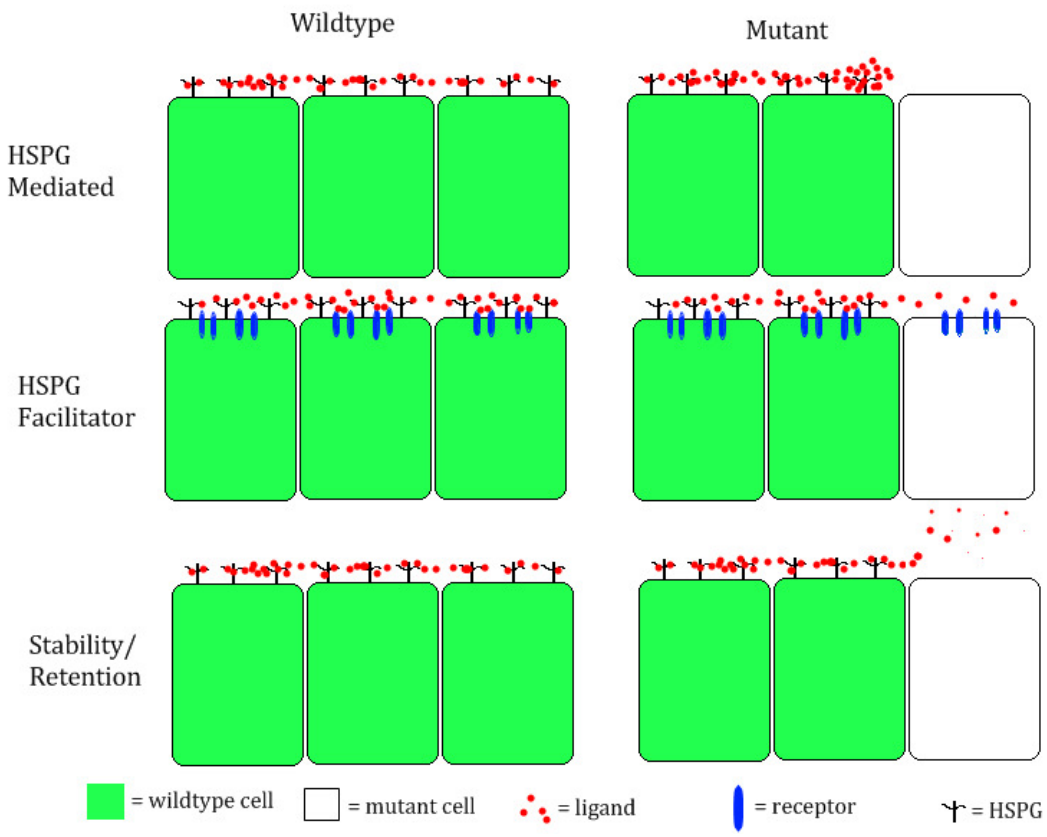


Figure 3.1. Models of HSPG dependent distribution of extracellular ligands. The HSPG mediated model proposes that the HSPG on the cell surface acts as a substrate for ligand transport. The HSPG facilitator model propose that HSPG increases the efficiency that which the ligand binds to the receptor or how receptor responds to the ligand. In the stability/retention model, HSPGs present on the cell surface stabilize the ligand or retain it on the cell surface.

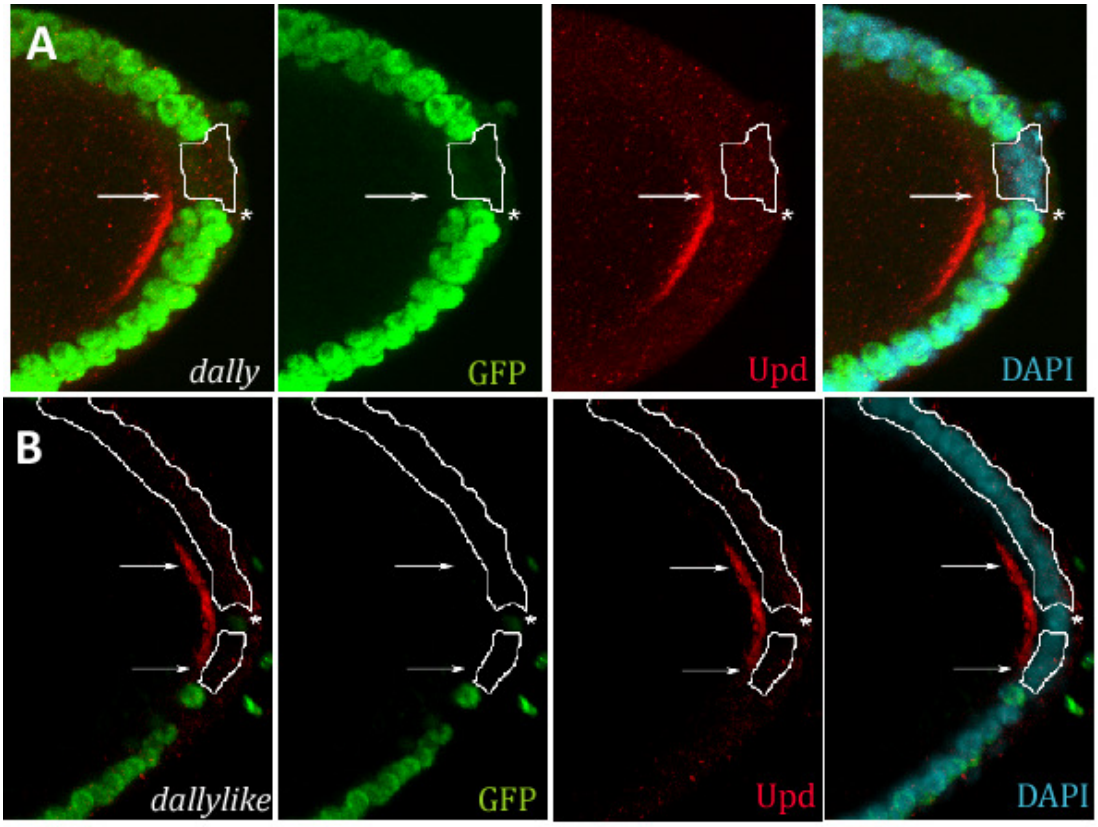


Figure 3.2. Upd protein is abruptly reduced along *dally* mutant cells but not *dallylike* mutant cells. Upd ([A and B], red) is distributed in a gradient from the source of its expression, the polar cells (A and B, asterisk). In *dally* mutant cells (A, loss of GFP) Upd is abruptly reduced (arrow) when compared with wildtype sister cells (A, GFP). In egg chambers containing *dallylike* mutant cells (B, loss of GFP), Upd distribution is normal and can be detected at least two cell diameters away from the polar cells (B, arrows).

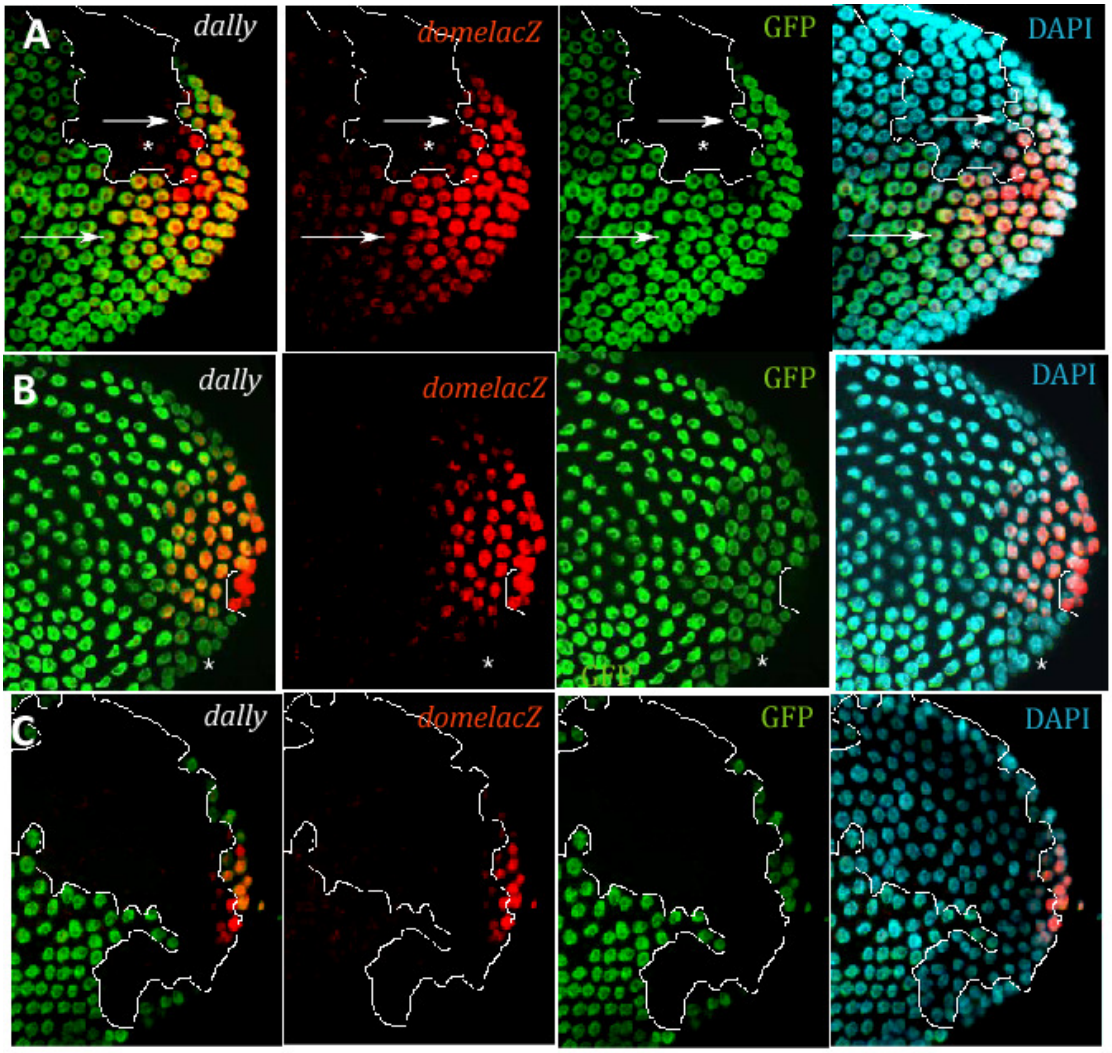


Figure 3.3. JAK activity is dramatically decreased in *dally* mutant cells. JAK activity, marked by *domelacZ* (red) occurs in a graded manner with the highest level at the A/P poles. *domelacZ* is noticeable reduced in cells mutant for dally (A-C, lack of GFP). In dally mutant cells closest to the poles *domelacZ* is detected (A, asterisk) and there is clearly a loss of signal in mutant clones as compared to wildtype cells (A, arrows). In small *dally* mutant clones (B, lack of GFP), *domelacZ* is detected, however there is an obvious lack of *domelacZ* in neighboring cells distal to the polar cells (B, asterisk) creating a "shadowing" effect. In large *dally* mutant clones (C, loss of GFP), there is a drastically reduced *domelacZ* signal. B and C represent the two sides of a single egg chamber.

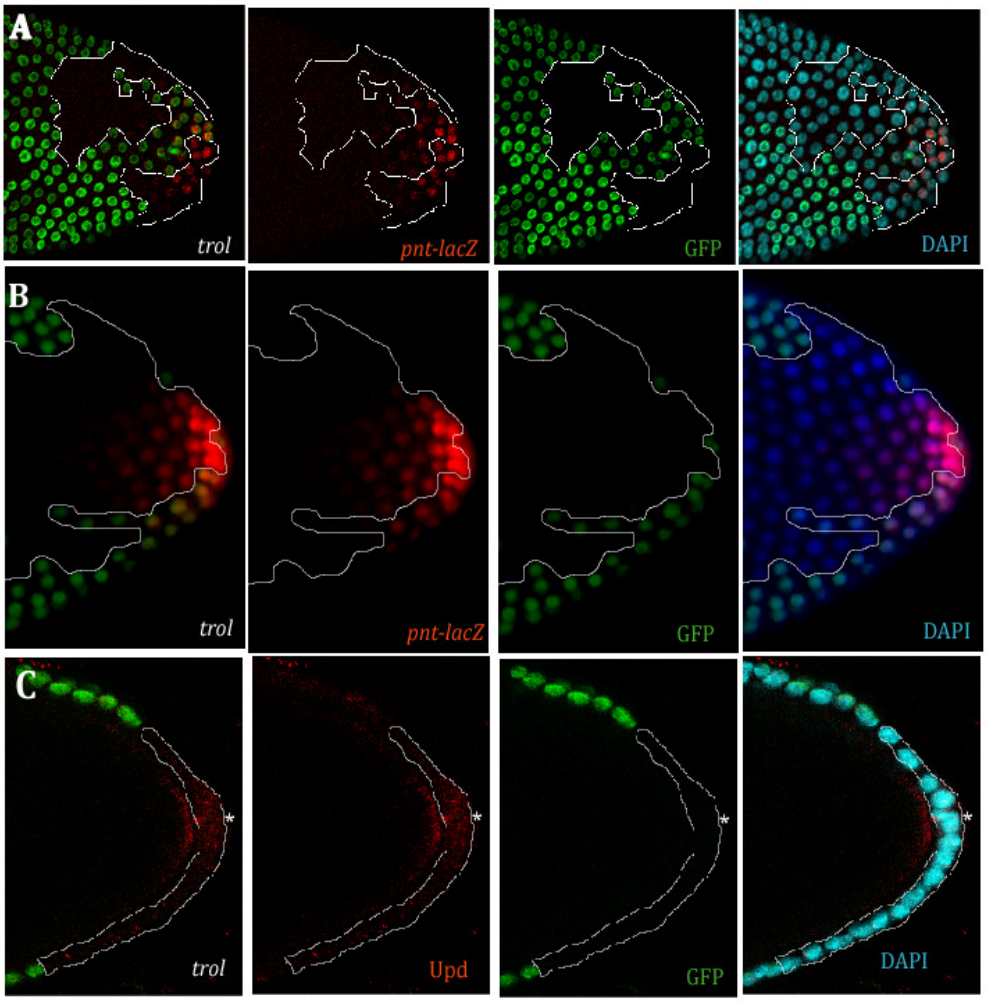


Figure 3.4. *trol* and *sdc* do not influence the distribution of Upd during oogenesis. *domelacZ* (A and B, red) is expressed in a gradient with the highest levels closest at the polar cells (asterisk). In large *trol* clones (A and B, loss of GFP outlined in white), *domelacZ* expression maintains a gradient when compared to wildtype cells (A and B, GFP). Upd (C, red) is distributed in a gradient is from the polar cells (asterisk). The gradient is remains constant over a very large *trol* clone (C, loss of GFP outlined in white). DAPI marks all nuclei in the follicle cells (A-C, blue).

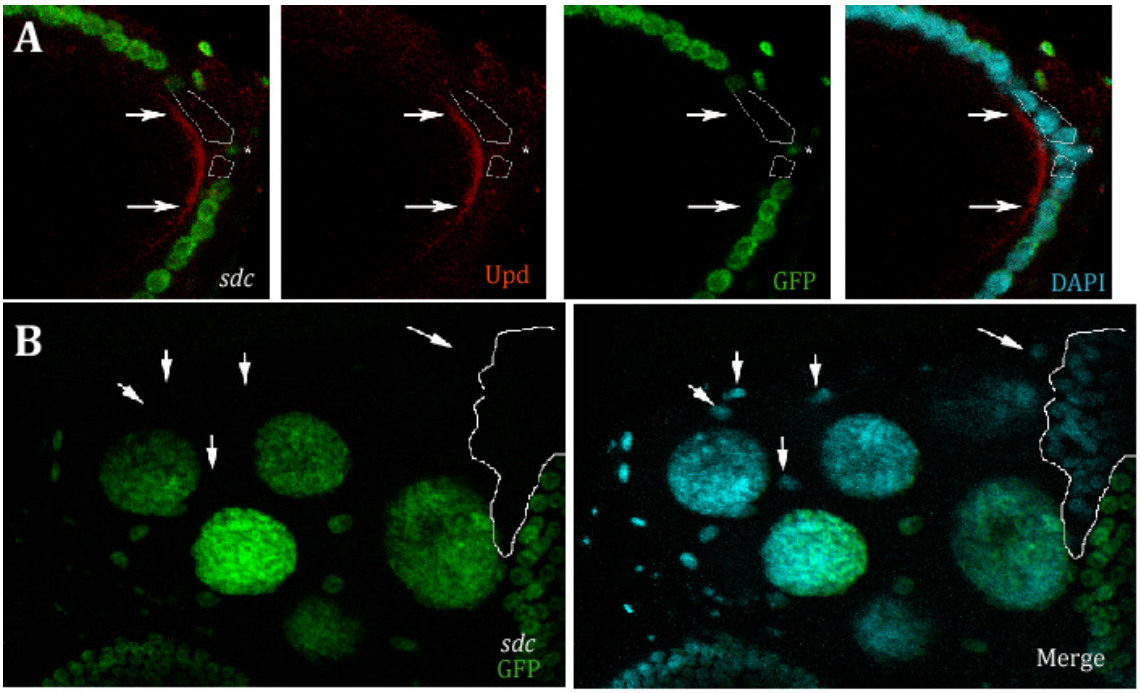


Figure 3.5. *sdc* mutants have normal Upd distribution and have normal follicle cell morphology. Upd (A, red) is distributed in a gradient along wildtype cells (A, GFP) and sdc mutant cells (A, loss of GFP outlined in white). Upd can be seen at least two cell diameters (arrows) away from the polar cells (asterisk). Anterior follicle cells mutant for sdc (B, loss of GFP, arrows) undergo normal morphological transitions and cannot be differentiated from the wildtype anterior follicle cells (B, GFP). Nuclei are positive for DAPI (A-B, blue).

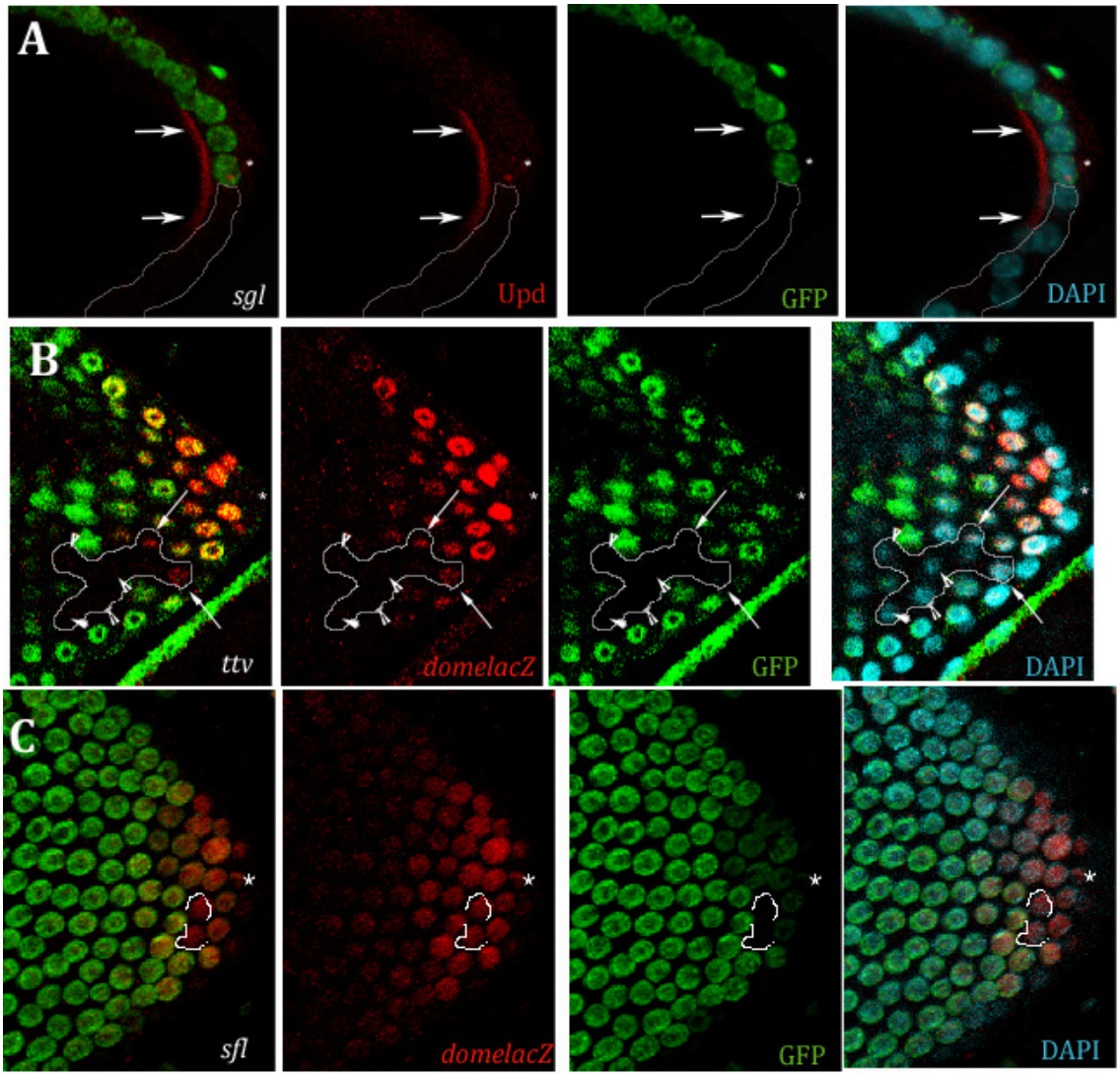


Figure 3.6. Loss of GAG modifying enzymes has various effects on Upd distributions and JAK activity. Upd Distribution (A, red) can be seen in a gradient at least two cell diameters away from the polar cells (asterisk) on the wildtype (A, GFP) and *sgl* mutant (A, loss of GFP outlined in white) follicle cells. In clones of *ttv* (B, loss of GFP outlined in white) domelacZ is reduced on the side closest to the polar cells (arrows) and absent in this further from the polar cells (arrowheads). Mitotic recombination with *sfl* always resulted in very small clones (C, loss of GFP outlined in white). *domelacZ* expression was indistinquishable from those of wildtype cells.

Chapter 4

The role of Upd3 in oogenesis

Introduction

Upon completion of sequencing of the *Drosophila melanogaster* genome, it was found that two other putative genes encode proteins with amino acid similarity to that of Upd. Although the overall similarity between these three genes is very low (27.4% similarity between Upd and Upd3), there are individual domains within each peptide that have a very high degree of similarity (Figure 1.3). These genes have been designated *upd2* and *upd3*. Upd2 has been shown to activate JAK signaling during embryogenesis and does so in a manner that is redundant to Upd (Zeidler, 2005). *upd3* has also been studied, although the ability for it to regulate JAK activity is unclear. In *Drosophila* fat bodies, *upd3* expression is induced upon septic injury and is necessary, as shown by RNAi experiments, for the expression of TotA (Agaisse et al., 2003). Exposure of human Interleukin to *Drosophila* SL2-Macrophage like cells resulted in an increased level of macrophage cells expressing *upd3*, suggesting a role for *upd3* in *Drosophila* immunity (Malagoli et al., 2008). Consistent with a role in immunity, *upd3* along with the known JAK receptor Domeless, were both strongly upregulated in the intestine following ingestion of bacteria (Buchon et al., 2009). Recent work in our lab has shown that mutants of *upd3* result in a small eye, outstretched phenotype that is typical of mutations within the JAK/STAT pathway (Wang, 2008). Furthermore, in *situ hybridization* has revealed that *upd3* is co-expressed with *upd* in the polar cells. This could indicate that *upd3* could contribute to the JAK activity during oogenesis. Because *upd3* expression overlaps with *upd* during oogenesis and null alleles of *upd3* show phenotypes consistent with that of JAK pathway mutants, it is hypothesized that Upd3 contributes to the formation of the JAK activity gradient during oogenesis.

Results

upd3 mutant ovarioles degenerate at a higher frequency than wildtype ovaries

Newly emerged *upd3* mutant adults are fertile with ovaries that appear morphologically normal. However, as the mutants age, their ovaries exhibit a higher frequency of defects than ovaries of wildtype controls of the same age (figure 4.1A). As *upd3* females age, their ovaries tend to have a higher frequency of degenerating egg chambers. These egg chambers usually appear in mid-oogenesis and appear to be undergoing apoptosis (figure 4.1). These presumably apoptotic egg chamber have multiple DAPI-positive vesicles of varying sizes (arrows, figure 4.1D), which is consistent with the apoptotic signature of DNA fragmentation that has been seen in egg chambers undergoing apoptosis (McCall, 2004).

In order to quantify the degeneration, ovarioles from a null mutant of *upd3*, $upd3^{d232a}$, and a wildtype control, $upd3^{37E}$, were examined at 12 and 18 days post-eclosion (figure 4.1B). At both times, there was a significant difference between the two genotypes. At 12 day post-eclosion, control flies had a 3.6% occurrence of degenerating egg chambers compared to a 51.4% in *upd3* mutant ovaries. In 18 day old flies, both the control and *upd*3 mutant experienced an increase in the frequency of degenerating egg chambers, 16.9% in control and 65.0% in *upd3* mutants. When compared to their 12 day old counterparts, this was nearly a 5-fold increase in the wildtype flies by day 18, with only a 13.6% increase in *upd3* mutants. A healthy egg chamber contains 15 nurse cells having relatively similar chromatin size, shape, and densities (figure 4.1A). There are many external factors that can contribute to the degeneration of an egg chamber, such as exposure to cytotoxic chemicals and nutritional deprivation, as well as developmental defects resulting in abnormal numbers of nurse cells, multiple oocytes, or too few follicle cells (McCall, 2004; Peifer et al., 1993; Zhang and Kalderon, 2000). Thus, the degeneration of the egg chambers in *upd3* flies may be an indirect effect of the mutation. Recall in Chapter 1 that in early oogenesis JAK is necessary for somatic

stem cell (SSC) maintenance as the egg chambers leave the germarium. It is conceivable that loss of *upd3* could result in diminished amounts of SSCs, which may lead to improper ratios of germline and somatic cells. Therefore, a possible explanation is that loss of *upd3* signaling leads to reduced numbers of stem cells, eventually resulting in improper ratios of germline and somatic cells.

Another role that JAK has in early oogenesis is the establishment of stalk cells (McGregor et al., 2002). Reduction of JAK signaling leads to a reduction in the number of stalk cells and an expansion of the polar cell population. The reduction of stalk cells often leads to egg chamber fusions. Egg chamber fusion events are easily distinguished as egg chambers containing multiples of 15 nurse cells as detected by DAPI staining (figure 4.2, B) as compared to 15 nurse cells in wildtype egg chambers (figure 4.2, A). Along with the degenerating phenotype described above, *upd3* ovaries often contain fused egg chambers. In order to quantify the fusion phenotype, fusion events counted in wildtype flies and *upd3* mutant flies at both 12 days old and 18 days old. Control flies that were scored had 0% ovarioles with egg chamber fusions at 12 days post eclosion (n=74) whereas 18 day old flies had 2.15% of the ovarioles containing egg chamber fusions (n=93) (figure 4.2). 12 day old *upd3* mutants had 12.5% ovarioles containing egg chamber fusions (n=40), whereas ovaries taken from 18 day old *upd3* mutants had 20.5% ovarioles containing egg chamber fusions (n=39). The presence of egg chamber fusions in *upd3* mutants strongly suggests that it influences the JAK/STAT pathway. There are only a few signaling pathways that have been associated with egg chamber fusions. These include the JAK/STAT pathway, the Notch/Delta signaling pathway, and Hedgehog signaling pathway. Hedgehog is expressed and secreted from the terminal filament cells and is required mainly for the proliferation the SSCs (Zhang and Kalderon, 2000, 2001). Reduction of Hh results in some egg chamber fusions through a reduction in the number of follicle cells. Reduction of Notch signaling results in loss of both polar cells and stalk cells, which causes egg chamber fusions (Keller Larkin et al., 1999; Lopez-Schier and St Johnston, 2001). Reduction of JAK, on the other hand, causes egg

chamber fusions by generating too many polar cells at the expense of the stalk cells (McGregor et al., 2002).

There are fewer border cells in the developing egg chambers of *upd3* mutants.

While it is clear that *upd3* mutant ovaries have an increased frequency of egg chamber degenerations, it is unclear that this effect is due to a decrease in JAK activity. In addition to stem cell maintenance, JAK activity also specifies follicular cell fates during oogenesis. The anterior cells that are specified by the highest levels of JAK activity are the border cells. There are 6-8 border cells in wildtype ovaries. Previously it has been shown that alteration of JAK activity also has a direct impact on the number of border cells. In viable combinations of hypomorphic alleles for components of the JAK pathway, the presumptive border cells exhibit aberrant migrations and expressed a marker specific for a cell fate that receives less JAK activity (Xi et al., 2003). Therefore, in order to assess the contribution to JAK activity of Upd3, the number of border cells was assayed in *upd3* mutants and compared to wildtype. An advantage to this assay over the egg chamber degeneration assay is that it is more sensitive to small changes in JAK activity and also is a more specific functional assay. In the degeneration assay, egg chambers were either degenerating or they were not. In this assay, all egg chambers will have border cells, however, slight changes in JAK activity should result in changes in border cell number. It is predicted that *upd3* egg chambers will have fewer border cells than their wildtype counterparts. Furthermore, because of the age dependent degeneration, it is predicted that the number of border cells will decrease over time.

Using 5A7, a β-galactose reporter gene specific for border cells, it becomes possible to mark the border cells and make them easily countable. The border cells were counted in stages 10a-10b, where the border cells are migrating or have just reached their position along the nurse cell-oocyte border. Consistent with the previous assay, a major portion of *upd3* ovaries were degenerated, however, in those that were

not, border cells were counted. At 13 days post-eclosion, *upd3* mutant egg chambers had an average 4.50 border cells compared to the control having 5.05 border cells per egg chamber (figure 4.3). At 23 days post-eclosion, *upd3* mutants had 4.00 border cells per egg chamber as compared to the wildtype control having 4.92 border cells per egg chamber (figure 4.3). At both time points, *upd3* mutants had significantly fewer border cells per egg chamber as affirmed by t-test. Interestingly, the difference between the number of border cells in mutant and wildtype ovaries increased as the flies aged. This is consistent with the observation that the degeneration of egg chambers in upd3 mutants worsen over time. Together, these results suggest that *upd3* does play a role, albeit a small one, in the regulation of JAK activity throughout oogenesis, a role that becomes more important as time goes by.

Wing specific misexpression of *upd3* leads to aberrant wing development

In the previous two loss-of-function assays, *upd3* mutants displayed results consistent, though not conclusive, with a role for it in the regulation of JAK during oogenesis. An alternative method to examine the role of Upd3 is by gain-of-function experiments. By utilizing a subset of the many available Gal4 drivers, it becomes possible to misexpress *upd3* within a tissue, during a specific time within development, or at a different transcriptional level than that of the endogenous *upd3* (Brand and Perrimon, 1993). These gain-of-function experiments can provide insight that a loss-of-function or hypomorphic mutations cannot reveal. Three separate *upd3* cDNAs were expressed using a variety of wing specific Gal4 drivers. The Gal4 drivers were used to drive expression of cDNA construct with an incomplete signal sequence whose protein product accumulates in the nucleus ($upd3^{nuc}$), a signal sequence from Upd on the 5' end ($upd3^{ss1}$), or the endogenous *upd3* signal sequence ($upd3^{wt}$)(Figure 4.4A). $upd3^{nuc}$ is the first *upd3* cDNA that was recovered in our lab. It contained what was thought at the time to be the start methionine, however, recent work led to the discovery of an

alternate upstream start methionine. The Upd3^{NUC} protein localizes to the nucleus in cell culture as well as when misexpressed in vivo (figure 4.4B). Because the protein product of *upd3*^{NUC} localized to the nucleus, it was presumed that it was not being properly processed through secretory pathway due to lack of a complete signal sequence. Consistent with this, work in Martin Zeidler's lab observed that when the signal sequence is removed from Upd, Upd localizes to the nucleus (personal communication). *Upd3*^{SS1}, which is *Upd3*^{NUC} with the signal sequence from Upd spliced to the 5' end, was created to force Upd3 into the secretion pathway. u*pd3*^{WT} is an *upd3* cDNA that was isolated by BDGP and contains what is most likely the actual start methionine, based on its ability to rescue the *upd3* small eye phenotype (figure 4.6, discussed below). Using the *patched-Gal4* driver, which drives expression at the anterior/posterior border of the developing wing (figure 4.5A) the three *upd3* constructs were misexpressed. In *upd3*^{nuc} flies, as expected, the wing appeared wildtype (figure 4.5B). In the *upd3*^{ss1} there was noticeable aberration at the anterior cross vein and the L4 vein was slightly pitched towards L3 at the site of the anterior cross vein (figure 4.5C). *patched-Gal4* misexpression of *upd3*^{wt} resulted in complete loss of the anterior cross vein (figure 4.5D). Interestingly, the *upd3*^{wt} and *upd3*^{ss1} gave significantly different effects even though the only virtual difference between the two is the origin of the signal sequence. *engrailed-Gal4* drives expression throughout the posterior of the developing wing (figure 4.5A'). *engrailed-Gal4* misexpression of *upd3*^{nuc} resulted in a very slight reduction of the L5 vein (figure 4.5B'), which is most likely due to the *engrailed*-Gal4 transgene, as these animals show a slight reduction of the L5 vein in the absence of an *upd3* transgene. *engrailed-Gal4* misexpression of *upd3*^{ss1} resulted in an extremely shortened posterior cross vein (figure 4.5C'). *engrailed*-G*al4* expression of *upd3*^{wt} resulted in the reduction of the L5 vein. Interestingly, *upd3*^{wt} misexpression did not exhibit the anterior cross vein phenotype seen in *upd3*^{ss1} expression (figure 4.5D'). The region at which the anterior cross vein is present is on the fringe of where the *engrailed-Gal4* driver expresses. Together, these data indicate that Upd3 can have a

biological function. Furthermore, the activity and range of effect of Upd3 seems to be influenced by the signal sequence.

Upd3-Gal4 driven Upd3 cDNA rescues *upd3* mutant eye phenotype

upd3 mutant flies show phenotypes consistent with that of flies with reduced JAK activity. Specifically, *upd3* mutants show a small eye and an outstretched wing phenotype (Wang, 2008). A transgenic line of *upd3-Gal4* had been previously developed to analyze *upd3* response in flies subjected to septic injury (Agaisse et al., 2003). The construct used in this *upd3-Gal4* construct is a 7.5kb fragment of the *upd3* promoter region fused the Gal4 coding sequence. Importantly, this transgenic construct does not necessarily contain relevant regulatory elements of the endogenous *upd3* gene. To determine if the *upd3* cDNA could rescue the *upd3* phenotype, *upd3* mutant animals were crossed to those containing the *UAS-upd3-gfp* and the *upd3-gal4* constructs. *upd3-Gal4* expression of $upd3^{WT}$ led to the rescue of both *upd3* alleles tested as well as suppression of the *os* small eye phenotype. In $upd3^{d232a}$ flies, the small eye phenotype was completely suppressed and the eye size was comparable to the wildtype control (figure 4.6, 101.9% area as compared with the control). Also, in $upd3^{x21c}$ flies, the small eye phenotype was rescued to the wildtype eye size (figure 4.4, 109.6% area as compared with the control). Furthermore, $upd3^{WT}$ also suppressed the small eye phenotype associated with outstretched (os^{1}) (figure 4.6). *os* is a locus previously considered to be *upd*, but recent evidence produced in our lab suggests that it is more likely to be a regulatory region that is common to both *upd* and *upd3* (Wang, 2008). Interestingly, expression of *UAS-Upd-GFP* using this *Upd3-Gal4* driver was lethal, suggesting that Upd and Upd3 are not equal in their ability to activate JAK signaling. However, because Upd3 was able to rescue the small eye of *os*, it suggests that Upd3 can compensate for the reduction of Upd as well as Upd3 in the os^{1} mutant. This suggests additive functions for Upd and Upd3 in the eye. Interestingly, *upd3* cDNA was

unable to rescue the wing phenotype in the upd3 mutants (data not shown). This is most likely due improper spacial expression from the *upd3-gal4* construct which may not include all required elements from the endogenous promoter.

Upd3 misexpression does not activate JAK in mid-stage follicle cells.

In order to assess the ability for *upd3* to activate JAK signaling in developing egg chambers, misexpression was carried out using a flp-mediated flip out cassette with detection of JAK activity accomplished with *domelacZ* (for description of the flp-mediated flip out cassette, see figure 6.3). A flip out cassette is a construct of DNA having an actin promoter upstream of two coding sequences. In this case, the coding sequence (CDS) for *yellow* is directly downstream of the *act5C* promoter followed by the CDS for *Gal4*. The central CDS, *yellow*, is flanked by two FRT sites. When FLP-recombinase is present it can act on the two FRT sites resulting in the excision of the *yellow* cds leaving *gal4* directly downstream of the Act5C promoter. Only cells that have had the *yellow* CDS removed will express and produce Gal4 protein, which can then activate transcription at any UAS site. Misexpression of *upd* in follicle cells (figure 4.7A, green) resulted in the non-autonomous activation of JAK (figure 4.7A, red) indicating that the flip-out system is working correctly. Misexpression of $upd3^{NUC}$ (figure 4.7B, green), as expected, did not activate JAK signaling (figure 4.7B, red). In egg chambers containing clones of cells misexpressing the $upd3^{WT}$ cDNA using an actin promoter (figure 4.7D, green), there was no apparent response in JAK activity in the expressing clone or the wiltype cells (figure 4.7D, red). Interestingly, in egg chambers containing clones of cells misexpressing $upd3^{SS1}$, there appeared to be an increase in JAK activity on the polar edge of the clone (figure 4.7C, anterior pole). This could be due to the additive effects of the endogenous Upd and Upd3 being secreted from the polar cells, as well as the ectopically expressed $upd3^{SS1}$. Recall that the difference between the $upd3^{wt}$ and $upd3^{SS1}$ constructs are the signal sequences, whereas the $upd3^{wt}$ carries the endogenous

signal sequence and the *upd3*^{SS1} carries the signal sequence from *upd*. Together, these results indicate that Upd3 does seem to be able to activate the JAK pathway in follicle cells in mid-oogenesis.

Discussion

upd3 ovaries display phenotypes consistent with loss of JAK activity

upd3 flies have outstretched wings and small eyes, phenotypes consistent with the reduction of JAK activity. Furthermore *upd3* is expressed in an overlapping pattern with *upd* in ovaries as revealed by *in situ* hybridization (Wang, 2008). Because of this, it was hypothesized that Upd3 contributes to JAK activity during oogenesis. Work here has shown that *upd3* mutant flies have an increased frequency of degenerating egg chambers that worsen with age. Because there are multiple factors that can cause degeneration of the egg chamber, this is probably an indirect result of loss of *upd3*. One of the factors that cause degeneration is an improper ratio of germline and somatic cells. Recall that one of the roles of JAK activity is the maintenance of the stem cell populations within the germarium. It seems reasonable to hypothesize that loss of JAK activity caused by lack of *upd3* could reduce the number of stem cells. In another assay the number of border cells were counted between *upd3* and wildtype control. In this assay there was a small, yet significant reduction of border cells per egg chamber in *upd3* flies. Another role of JAK activity is the specification of follicular cell fates, which include border cells. Thus, the observed reduction of border cells is consistent with reduced JAK activity in oogenesis.

Although the results of these two loss-of-function assays are not conclusive that *upd3* mutants have reduced JAK activity, the phenotypes observed are certainly consistent with loss of JAK activity. In further support of a role of Upd3 in the regulation of JAK activity during oogenesis it was observed that there is a higher frequency of egg chamber fusions in the *upd3* mutants. Recall that in addition to a role in stem cell maintenance and follicular cell specification, JAK is involved in the specification of stalk cells as the young egg chambers exits the germarium. The loss of stalk cells caused by reduction of JAK activity results in egg chamber fusions. Thus, *upd3* mutants show

phenotypes consistent with reduced JAK activity in three of the processes in which it is known to be involved during oogenesis.

Upd3 is Required with Age

In the loss-of-function assays, it was observed that the aberration worsened over time in the *upd3* mutants. As the flies aged, the frequency of degenerating egg chambers increased while the number of border cells per egg chamber decreased. Furthermore, a separate assay conducted in the lab showed that there was an increase in the frequency of egg chamber fusions as the flies aged. These results strongly suggest that Upd3 becomes increasingly necessary as the fly ages. But why would this be? One possible explanation would be that Upd3 becomes necessary to maintain JAK levels as negative regulators of JAK accumulate. Alternatively, the expression and concentration of positive regulators of *upd* could become reduced with age in the germarium, resulting in the requirement of Upd3 to maintain JAK activity levels.

Upd and Upd3 do not activate JAK signaling equally

In situ hybridization has revealed that *upd2* or *upd3* are often expressed along with *upd*. *upd2* is expressed in an overlapping pattern with upd during embryogenesis, however, null alleles of *upd2* do not have any obvious phenotype. Furthermore, Upd2 seems to be functionally redundant with Upd during this process. *upd3* flies have outstretched wings and small eyes, a phenotype reminiscent of reduction of JAK activity. An interesting observation in these studies is that when using the *upd-Gal4* driver to express *upd* or *upd3*, both genotypes are viable and fertile. However, in the *upd3* rescue experiments in this study, *upd3-gal4* misexpression of Upd3^{WT}-GFP resulted in a suppression of the small eye phenotype, while *upd3-gal4* misexpression of Upd-GFP was lethal. This indicates that Upd and Upd3 do not activate JAK signaling equally and that

Upd may be more potent than Upd3. Furthermore, while Upd3^{WT} and Upd3^{SS1} gave varying results when misexpressed in the wing using *engrailed-gal4* and *patched-gal4*, misexpression using Upd-GFP was lethal. Possible explanations for these differences include the affinity each ligand has for the receptor, the location of the P-element insertion (although all transgenics used had similar eye color), ligands differ in their stability, or that each ligand is processed/secreted at different rates.

Upd3 may be processed differently than Upd

In a previous study, it was observed that the signal sequences between *upd* and *upd2* played an important role in the ligand's ability to activate JAK signaling in cell culture (Zeidler, 2005). Furthermore, it was predicted that Upd2 contained an anchor sequence which kept it membrane bound. In support of this, immunohistology suggested that the majority of Upd2 was intracellular, presumably in the ER and golgi networks. A signal sequence swap between the Upd and Upd2 molecules resulted in the secretion of Upd2 and an increased ability of it to activate JAK signaling in cell culture. In this work, there was clearly a difference in the response from misexpression of *upd3*^{WT} and *upd3*^{SS1}. Misexpression in the wing disc using *engrailed*-Gal4, which expresses throughout the posterior of the disc results in defects in the anterior cross-vein with *upd3*^{SS1} and no effect with *upd3*^{WT}. Furthermore, *upd3*^{SS1} was able to cause a robust, non-autonomous activation of JAK in mid stage follicle cells whereas there was no detectable response from egg chambers misexpressing *upd3*^{WT}. Assuming that there is not a significant difference in the accessibility of the P-element for Gal4 transcription factors, this demonstrates that there is a difference in either the production and/or the stability of the ligand that is determined by its signal sequence.

Upd3 may not contain a signal sequence

In considering that *upd3^{WT}* and *upd3^{SS1}* had different ranges of influence in wing discs it seemed possible that the only difference between the two, the signal sequence, was playing a key role in the activity of Upd3. In order for a protein to be secreted, it must first be transferred to the ER. This is accomplished by a signal sequence that is usually near the N-terminal end of the protein. In addition to the signal sequence there is also a proteolytic cleavage site that separates the signal sequence from the core protein after being transferred to the ER. Previously, in studies of Upd2, it was shown that the differences between signal sequences of Upd and Upd2 directly influence their ability to activate JAK signaling in cell culture. In the current study, it was shown that the misexpression of *upd3^{wt}* and *upd3^{SS1}* in the wing result in considerably different wing vein aberrations (figure 4.3). Furthermore, when misexpressed in follicle cells, only Upd3^{SS1} was shown to affect JAK signaling. Comparison between the N-terminal ends of Upd, Upd3^{SS1}, and Upd3^{wt} reveal hydrophobic regions consistent with signal sequences. However, using the SignalP 3.0 server, a hidden Markov model (Bendtsen et al., 2004; Nielsen et al., 1997) predicts that Upd3^{wt} has a 0% chance of having a signal sequence, whereas Upd and Upd3^{SS1} both have a 100% chance of having a signal sequence. Interestingly, it does predict (100%) that Upd3^{wt} carries a signal anchor, which would indicate the possibility of being membrane bound. Both Upd and Upd3^{SS1} had a 0% chance of carrying a signal anchor. The signal sequence present on Upd3^{SS1} and Upd are consistent with the observations of non-autonomous activation of domelacZ when misexpressed main body cells, while the prediction of an anchor sequence of Upd3^{WT} could explain why it could not activate JAK signaling when misexpressed in main body cells (figure 4.7). Furthermore, the broader effect of Upd3^{SS1} in the wing when expressed with *engrailed-Gal4* suggests that the release of the two ligands is not equal. Possible mechanisms for the processing and secretion of Upd3 are discussed in Chapter 5.

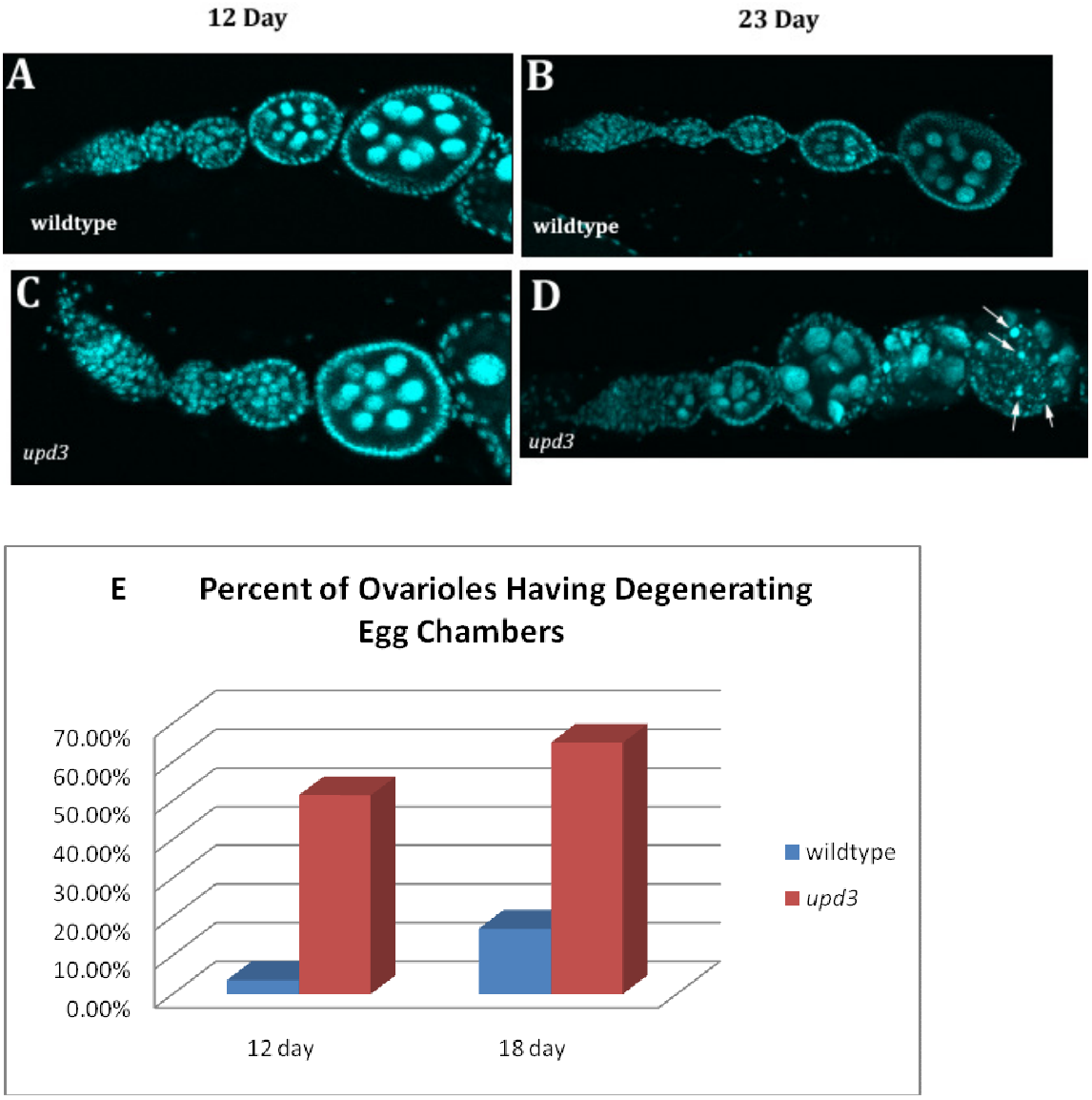
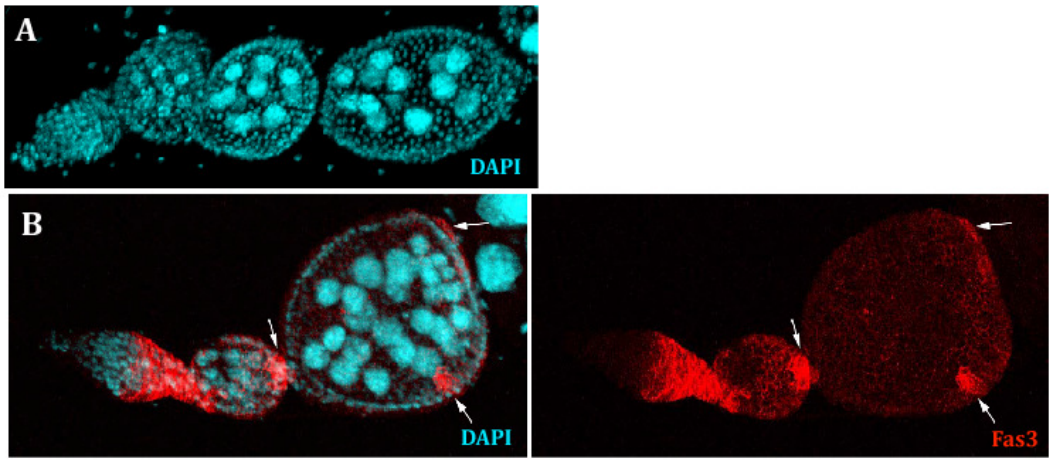


Figure 4.1. *upd3* egg chambers degenerate at a higher rate than wildtype. (A-D) Dapi staining of ovaries showing typical results on young (A, C) and older (B, D) flies. The degeneration of an egg chamber is detected by DAPI positive vesicles of varying sizes (D, arrows). At 12 days post-eclosion, ovaries from wildtype are normal with 3.64% of ovarioles containing degenerating egg chambers while *upd3* mutant ovaries contain 51.4%. In ovaries taken from flies 18 post-eclosion, 16.9% of wildtype ovarioles contained degenerating egg chambers while *upd3* mutant ovarioles jumped to 65.0%.



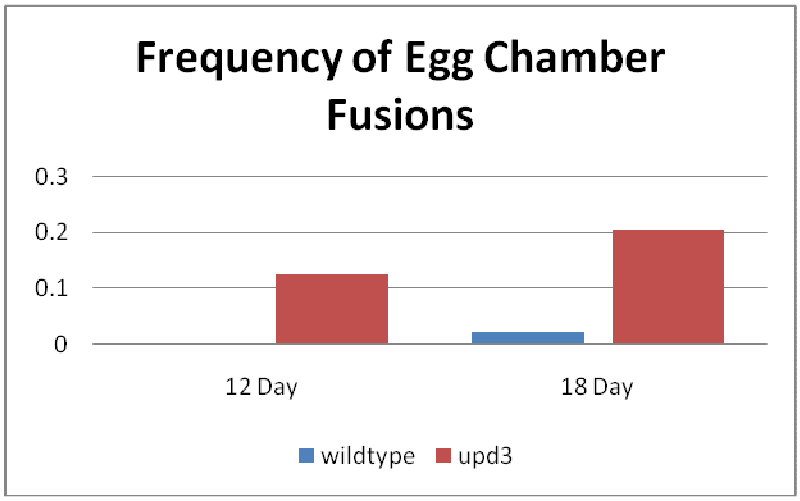
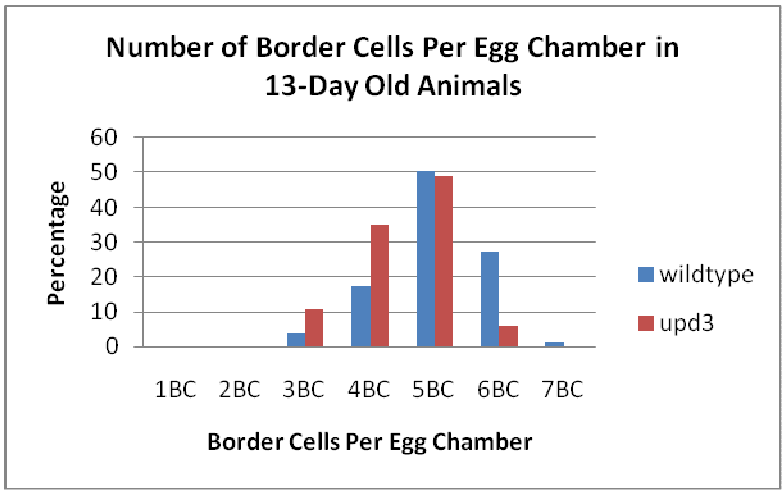
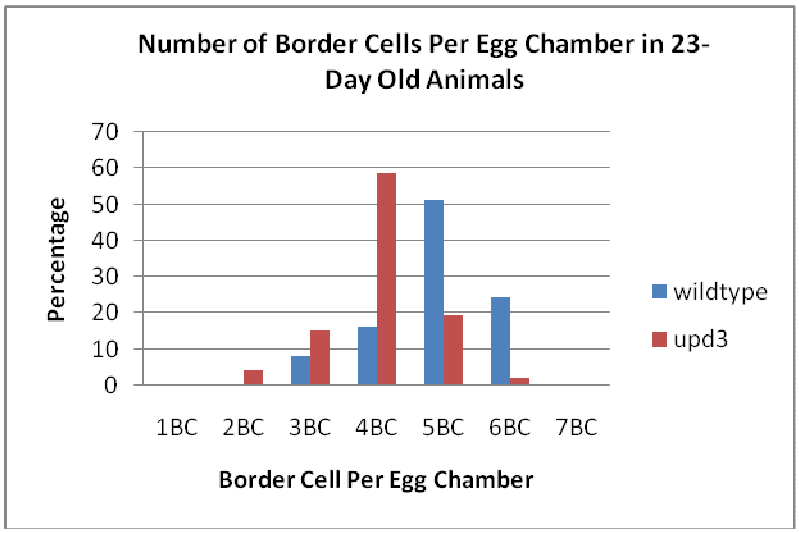


Figure 4.2. *upd3* mutants display an increased frequency of egg chamber fusions than wildtype. In wildtype egg chambers there are 15 nurse cells and 1 oocyte (A). *upd3* mutants display a higher frequency of egg chamber fusions as indicated by >15 nurse cells per egg chamber (B). In *upd3* fused egg chambers, there is an expansion of the polar cell populations (B, arrows). Egg chamber fusions remain very low in both younger and older wildtype flies, however, the frequency of fusions increases with age in *upd3* flies (C).





	BC average per egg chamber at 13 days	BC average per egg chamber at 23 days	Δ of BC average between 13 and 23 days
wildtype	5.05	4.92	-0.13
upd3	4.50	4.00	-0.5

Figure 4.3 Border cells are reduced in *upd3* mutant egg chambers. In ovaries taken from 13 day post eclosion flies, *upd3* egg chambers (stages 10 and 10b) contained an average of 4.50 border cells per egg chambers (n=86) compared to an average of 5.05 in wildtype ovaries (n=81)($p= 1.2x10^{-5}$, t-test). At 23 days post-eclosion *upd3* females contained 4.00 border cells per egg chamber (n=46) as compared to 4.92 border cells per egg chamber in wildtype (n=37)($p=3.6x10^{-6}$, t-test). The difference between *upd3* with wildtype extended from -0.13 border cells at 13 days to -0.50 at 23 days.

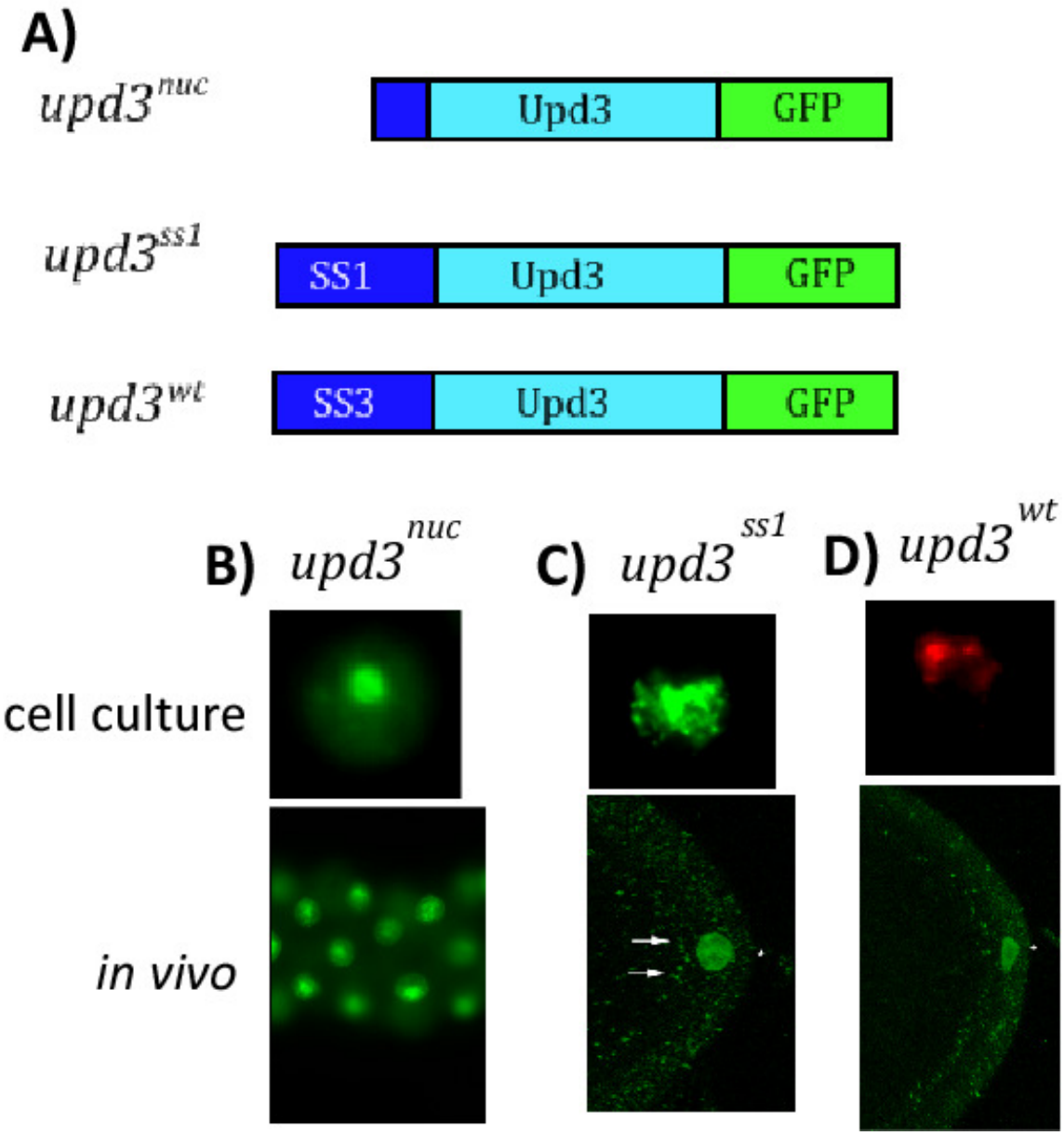


Figure 4.4. Three *upd3* cDNA constructs display different localization patterns. The three constructs used in *upd3* misexpression contain the same core protein (A, cyan), a GFP tag (A, Green), and differ only by the signal sequence (A, blue). $upd3^{NUC}$, an *upd3* cDNA missing a complete signal sequence localizes to the nucleus in cell culture (B) and when expressed in salivary glands (C). $upd3^{SS1}$ is an *upd3* cDNA that has had the signal sequence from *upd* fused onto the 5' end of the CDS. The protein product is localized in a pattern consistent of the golgi, ER, or endosomes in cell culture (D) and polar cells (E). The $upd3^{WT}$ cDNA contains the endogenous signal sequence for Upd3 and is localized in a in a similar pattern as $Upd3^{SS1}$ in cell culture (F) and polar cells (G).

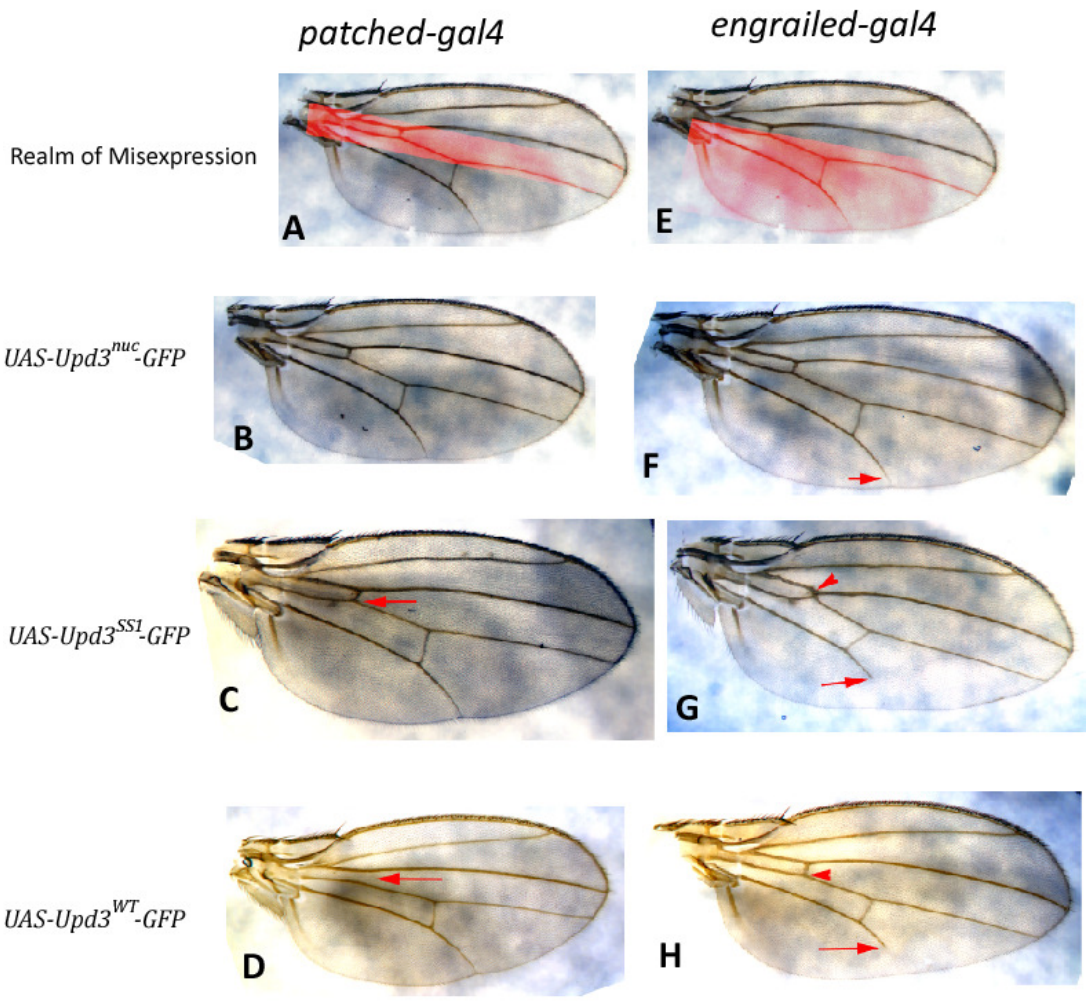
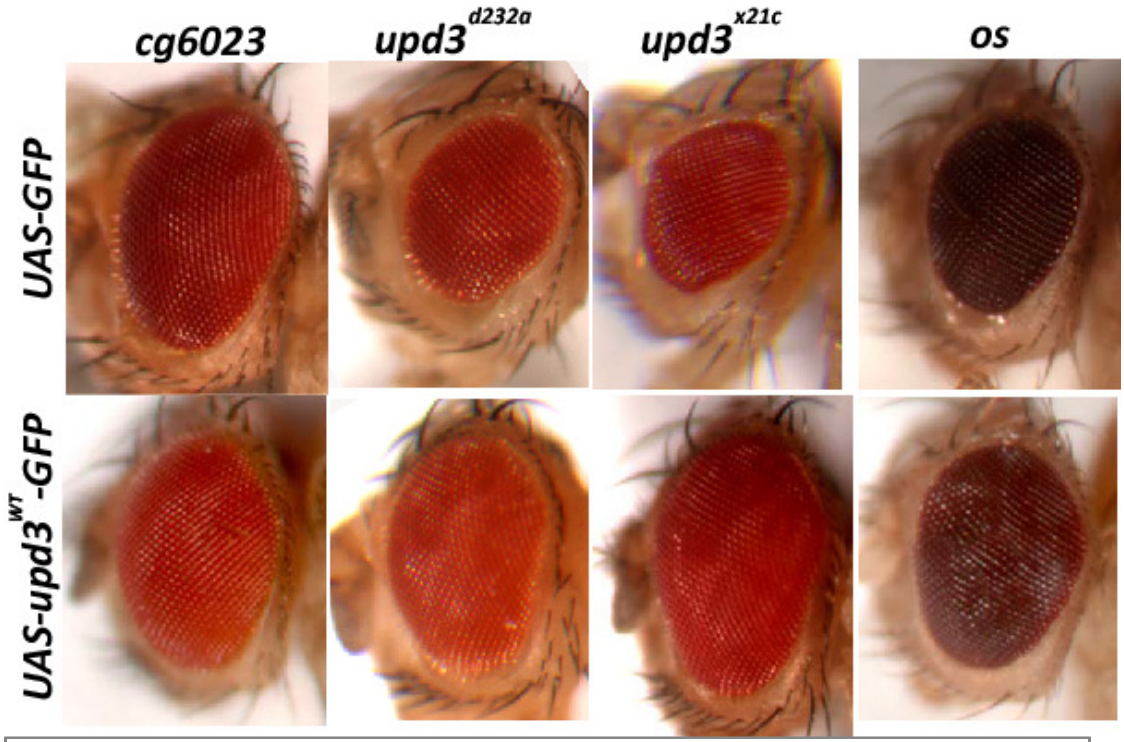


Figure 4.5. Misexpression of *upd3* in the wing leads to vein defects. *Patched-gal4* misexpression is specific for the anterior-posterior midline of wing development (cooresponding to the red shaded area in A). Misexpression of $upd3^{NUC}$ (negative control) results in wildtype wing development (B). Misexpression of $upd3^{SS1}$ resulted in slight aberration of vein formation in the area of the anterior cross vein (C, arrow). Expression of upd^{WT} resulted in a complete loss of the anterior cross vein (D, arrow). *Engrailed-gal4* expressed throughout the posterior of the wing disc (cooresponds to the red shaded area in E). Misexpression of $upd3^{NUC}$ results in wildtype wing development (F, arrow). Misexpression of $upd3^{SS1}$ resulted in a reducting in the L5 vein (G, arrow) as well as shortening of the anterior cross vein, pinching L3 and L4 closer together (G, arrowhead). Misexpression of $upd3^{WT}$ resulted in reduction in the L5 vein (H, arrow), however the anterior cross vein appeared normal (H, arrowhead).



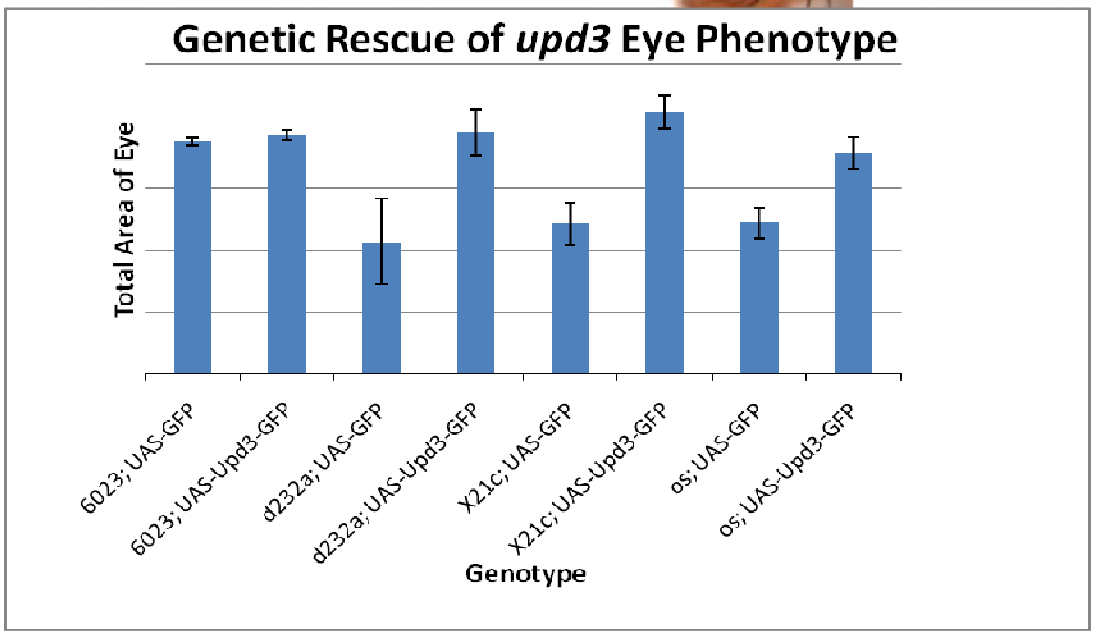


Figure 4.6. *upd3* cDNA rescues the eye size phenotype of *upd3* mutants. Eye sizes taken at equal magnification of *upd3-gal4* flies carrying either *UAS-GFP* or *UAS-UpdWT-GFP* in 6023, $upd3^{d232a}$, $upd3^{x21c}$, or *os* background. 6023 (*cg6023*) is a wildtype control for the two null alleles $upd3^{d232a}$ and $upd3^{x21c}$. The small eye size phenotyoe of both *upd3* null alleles and *os* allele were suppressed when expressing $upd3^{WT}$ with *upd3-Gal4*.

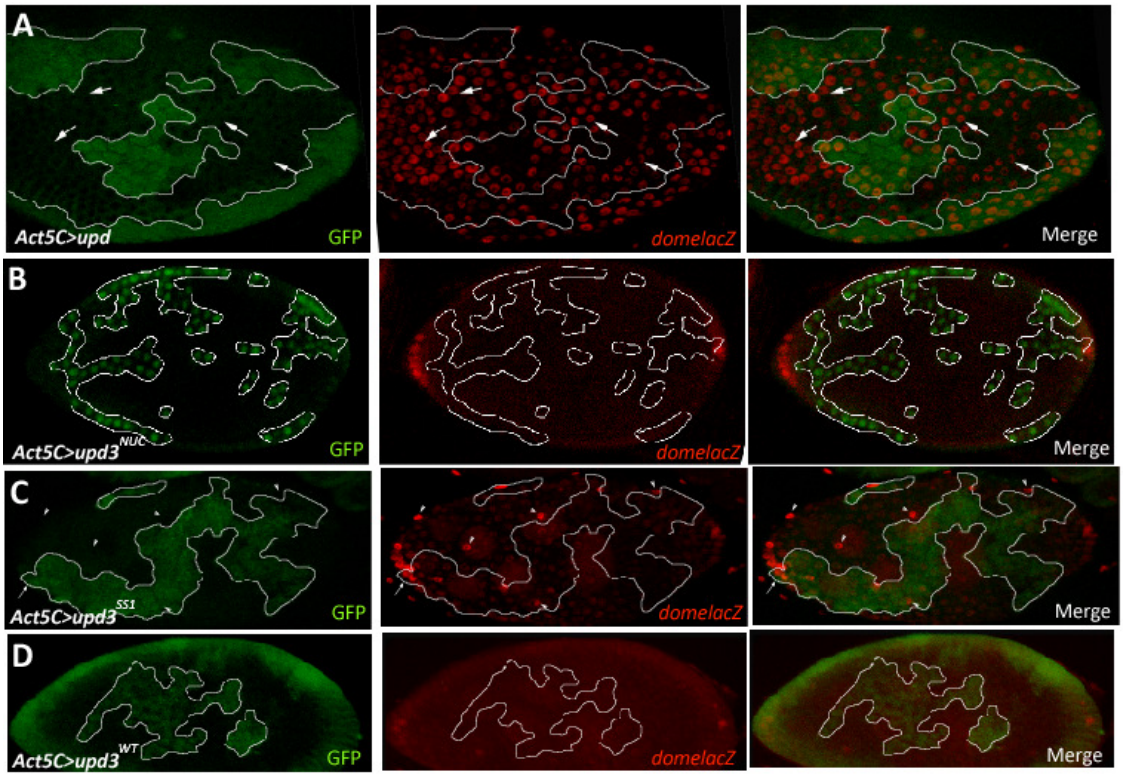


Figure 4.7. *upd3* expressed in main body cells does not activate JAK signaling. Misexpression of *upd* and *upd3* were carried out using a flip-out cassette. Follicle cells misexpressing upd (A, GFP outlined in white) activated JAK activity non-autonomously (A, red, arrows). Follicle cells misexpressing $upd3^{NUC}$ (B, GFP outlined in white) did not result in ectopic activation of JAK (B, red). Misexpression of $upd3^{SS1}$ in follicle cells (C, GFP outlined in white) did not appear to activate JAK signaling, although JAK levels were high in the anterior part of the clone (C, red, arrow). Cells overlying the egg chamber were also expressing *domelacZ* (arrowheads in C) and should not be considered follicle cells. Misexpression of $upd3^{WT}$ (D, GFP outlined in white) did not activate JAK signaling (D, red).

Chapter 5

Conclusions and Discussion

Previously, it was shown that there is a JAK activity gradient within the follicular epithelium of developing egg chambers with the highest levels at the poles and lower levels towards to middle of the egg chambers (Xi et al., 2003). Furthermore, genetic studies revealed that the level of JAK activity specifies cell fates within the follicular epithelium, suggesting that JAK signaling is functioning as a morphogenic pathway. The central aim of this project was to investigate how the gradient is established. The hypothesis was that the ligand Upd, which is expressed and secreted from the anterior and posterior polar cells, is distributed in an extracellular gradient, which in turn, establishes the JAK gradient. This work reveals that Upd is indeed distributed in an extracellular gradient, thus supporting our hypothesis and indicating that Upd functions as a morphogen setting up the JAK gradient.

An additional aim of this project was to identify factors involved in the distribution of Upd. When it was identified, Upd was also shown to be associated with the ECM. Therefore, it seemed likely that the factors that allow Upd to associate with the ECM may also affect its distribution and the formation of the JAK activity gradient. In this work the HSPG Dally was shown to be essential for both the proper distribution of Upd and the proper formation of the JAK activity gradient. Currently, there are three nonexclusive models that are used to explain how HSPGs interact with extracellular ligands: the HSPG mediator model, the facilitator model (coreceptor), and the stability/retention model (figure 3.1). Interestingly, JAK activity was observed in *dally* mutant clones, however, it only occurred in the cells closest to the Upd source, the polar cells (figure 3.3, A). JAK activation decreased dramatically in the cells located more distal from the source within the clone. Also, in even small mutant clones of *dally*, there was no JAK activity in the wildtype cells on the distal side of the clone (figure 3.3, B).

Taken together, these observations suggest that Dally does not simply play a role as a coreceptor for JAK signaling. Furthermore, it was observed that there was no accumulation of Upd protein at the mutant/wildtype border suggesting that Dally is not required to act as a mediator (figure 3.2, A). It was seen, however, that the Upd protein is lost at within the Dally clones, suggesting a role for it in the stability of Upd on the ECM.

An additional aim of this project was to investigate the role of Upd3, a protein having some similarity to Upd, during *Drosophila* oogenesis. *upd3* is expressed along with *upd* in the polar cells of developing egg chambers and *upd3* mutant flies display outstretched wings and small eyes, phenotypes consistent with reduction of JAK activity. Interestingly, it was found that the ovaries of *upd3* mutants displayed a high frequency of chamber degeneration and a reduction in the number of border cells per egg chambers, with each phenotype worsening as the fly aged. In both cases, these loss-of-function phenotypes were consistent with a reduction in JAK activity. As for gain-of-function phenotypes, misexpression of *upd3* in the wing disc resulted in ectopic venation similar to that seen with misexpression of other JAK pathway components. Furthermore, the *upd3* cDNA was able to rescue the small eye phenotype of the *upd3* mutants. However, it did not activate JAK activity when misexpressed in follicle cells. Together, the results suggest that Upd3 is involved in regulating JAK activity during oogenesis, but most likely does so in a cooperative manner with Upd. How this happens exactly remains to be seen, however, there are several possibilities which include forming active heterodimers with Upd, being processed and secreted differently than Upd, and/or compensating for Upd as its transcription decreases with age.

Upd: A novel morphogen that activates JAK signaling

Prior to the discovery of the gradient of JAK activity, it was hypothesized that the polar cells acted as an organizer for the development of the follicular epithelium

(Grammont and Irvine, 2002). The discovery that *upd* is expressed in the polar cells and that the JAK gradient specifies cell fate led to the hypothesis that Upd was acting as a morphogen (Xi et al., 2003). In this work, *upd* is shown to be distributed in a gradient, thus confirming it as a morphogen by providing evidence for the final necessary characteristic of a molecule to be designated a morphogen. This is the first time a ligand for the JAK-STAT pathway has been classified as a morphogen. Most known morphogens in animal development are ligands of the Wnt, TGF-β, or Hedgehog signaling pathways. Although they may differ in the roles that they play during particular aspects of development, their morphogenic properties have been evolutionarily conserved across phyla from *Drosophila* to vertebrates. The discovery that Upd is acting as a morphogen during oogenesis is significant because it adds the JAK/STAT signaling pathway to the very short list of morphogenic cascades.

Morphogens are not only conserved across animal development, they are utilized in multiple developmental processes and various tissues within a given species. Considering the developmental importance of morphogens, it is unlikely that the morphogenic activity of Upd and the JAK/STAT pathway is restricted to the development of the follicular epithelium. Indeed, there have been other gradients of JAK activity reported in the eye discs (Zeidler et al., 1999) and within the tubular epithelium (Johansen et al., 2003). In the eye disc, *in situ* hybridization has shown that *upd* (and *upd3*) is expressed within a small group of cells in the posterior disc (Wang, 2008) and the reported gradient of JAK activity is highest towards the posterior tapering down towards the anterior of the disc (Zeidler et al., 1999). These two observations are consistent with the idea that Upd is forming an extracellular gradient in the developing eye disc as well. During the development of the tubular epithelium, cells are stimulated by multiple signaling pathways to undergo rearrangements to create an elongated, narrow hindgut. *In situ* hybridization reveals that *upd* is expressed specifically in the anterior hindgut, whereas a gradient of STAT nuclear localization is observed in an anterior to posterior direction from the Upd source (Johansen et al., 2003). Reduction

of JAK activity using hypomorphic alleles of JAK pathway components during the development of the tubular epithelium results in shorter, wider hindguts when compared to those of wildtype animals suggesting that subsets of cells failed to undergo rearrangements required for tubular elongation. In oogenesis, all three anterior cells whose fates are specified by JAK signaling undergo rearrangements of their own upon differentiation. Although Upd may not be acting as the morphogen in each of these contexts, the events that are induced by the JAK activity gradient in the eye (proper initiation of morphogenic furrow) and tubular epithelium (causing cell rearrangements) are clearly important for proper development.

How morphogens move from their source to their target cells has been an extremely active field of research. Originally, it was assumed that these molecules simply diffused away from their sources. However, evidence over the last decade suggests that the mechanism of distribution is more complex than diffusion alone. In *Drosophila*, the bulk of the work has been done in the imaginal discs looking at Dpp, Wg, and Hh. The collective data resulted in the three models for morphogen movement that were presented in Chapter 1 of this work (figure 1.4). Now that Upd has been identified as a morphogen in oogenesis, it becomes an additional molecule of focus in investigations of the mechanism of morphogen movement. This work has identified one factor in the movement of Upd across the follicular epithelium, the HSPG Dally. However, there are multiple other factors that could also contribute to the distribution of Upd, such as homodimer/heterodimer dynamic with Upd3, the concentration of the Domeless receptor, the regulation of Upd secretion, and genes that are upregulated by JAK activity.

Work in our lab has shown that Upd and Upd3 are able to form heterodimers in *Drosophila* S2 cells (Pei, 2007). Furthermore, the co-expression of the *upd* and *upd3* in the polar cells during oogenesis make it possible that they also form heterodimers in the polar cells. As perhaps a precedent for this idea, it has been reported that Dpp homodimers and Dpp-Scw heterodimers are responsible for establishing the two types

of dorsal tissues in the embryonic blastoderm. Dpp homodimers are shown to have a long range effect, whereas the Dpp-Scw heterodimers are much more restricted in their range (Shimmi et al., 2005). It is certainly possible that an Upd-Upd3 dimer could contribute greatly to the overall distribution of the JAK ligands and the establishment of the JAK gradient. More on the role of Upd3 will be discussed later in this chapter.

Another factor to consider in generating the JAK activity gradient during oogenesis is that the concentration of the Domeless receptoris likely to be altered over time of activation. The expression of *domeless* is in a positive feedback loop with the JAK pathway, therefore cells that are closest to the source of Upd will upregulate *domeless* at a higher rate than those farther away. It is likely that this would result in the sequestration of higher concentrations of Upd among cells with highest JAK activity, thus maintaining an extracellular Upd gradient.

The process secreting Upd could also contribute to the formation of the extracellular gradient. Both loss-of-function and gain-of-function experiments have suggested that the JAK gradient in oogenesis is sensitive to small changes of Upd concentration (Xi et al., 2003). Therefore it must also be important to regulate the production and secretion of Upd in the polar cells. Perhaps consistent with this idea, Upd-Gal4 driven misexpression of Upd-GFP in the polar cells did not drastically alter the specification of the anterior somatic cells (as observed in ovaries from figure 2.2, A). Furthermore, a higher concentration of Upd is seen in the polar cells of Upd-Gal4::UAS-Upd-GFP flies when compared to Canton S flies (compare anti-Upd staining from figure 2.2 A, with figure 2.4 B). This could possibly be due to the Upd-GFP chimera protein being slowed in processing or experiencing difficulty in folding, however, this is most likely not the case because Upd-GFP rescues *upd* null alleles and Upd-GFP can be detected outside of the polar cells in the ovaries (figure 2.2, B) or eye discs misexpressing Upd-GFP (Tsai and Sun, 2004). Given the cumulative data, Upd is most likely sequestered in the golgi or in other secretory vesicles and secretion is controlled.

Oogenesis as a new model system for the study of morphogen distribution

Morphogens have been shown to play roles in many developmental processes, As already mentioned, most studies of morphogen distribution in *Drosophila* have taken place in the wing or eye imaginal discs. While certainly informative, it is important to consider that imaginal discs are surrounded by luminal space and therefore are relatively isolated when compared to other types of epithelial tissues. The follicular epithelium of the egg chamber, on the other hand, is not an isolated tissue. Instead, like many other developing tissues among metazoans, it shares borders with other cell types, which in this case, are the nurse cells and oocyte of the germline. Consequently, it was possible, in this work, to show that Dally is involved in the stabilization of Upd on the ECM whereas if the experiments were done in the imaginal discs, it would have been quite difficult to distinguish between stability and retention. Furthermore, as seen in figure 2.3 B, Upd seems to also be distributed in the posterior of the oocyte making it possible that the oocyte may be involved in the redistribution of Upd to ECM thus affecting the gradient. Such cooperation between two tissue types would not be possible in imaginal discs. Additionally, from a technical standpoint, the ovary is more easily accessible, is much larger, and has larger cells than any of the imaginal discs from any larval stage, which makes manipulation and microscopy more efficient.

The Role of Glypicans in JAK activation and Upd Distribution

In this work the glypican Dally was shown to be essential for the stabilization of Upd in the ECM during oogenesis, whereas the other glypican Dallylike, was not required at all in Upd distribution or JAK activation in the follicular epithelium. Because Dallylike has been shown to be involved in morphogen distribution in the imaginal discs, it was particularly interesting that mutations did not have an effect. As part of an ongoing collaboration with his lab, work from Dr. Hiroshi Nakato (University of Minnesota, Department of Genetics) has revealed through *in situ* hybridization, that

both *dally* and *dallylike* are expressed in the germarium, while only *dally* is expressed in the follicle cells in later stages of oogenesis (personal communication). Consistent with his data, in anti-Dlp immunostaining of oocytes by our lab, no Dlp was detected in the follicular epithelium (data not shown). This raises an interesting question regarding JAK activity during early oogenesis. Recall that prior to the establishment of the JAK gradient in the follicular epithelium, JAK activity is involved in stem cell maintenance and stalk cell specification. Could Dlp be involved in the distribution of Upd (or Upd3) and/or the activation of the JAK pathway during these early processes? JAK is required in both germline stem cells (GSCs) and somatic stem cells (SSCs) in the *Drosophila* testes, however, is only required for the maintenance of SSCs in the ovary. *upd* is expressed in the cap cells and terminal filament of the ovary, which is quite far from the source of Upd. In wing imaginal discs Dlp is involved in the long range signaling of Wg, whereas Dally is essential for a shorter range (Han et al., 2005). This long range signaling effect could be occur through cleavage of Dlp by Notum, an extracellular peptidase, to release it from its GPI linkage. Association of Upd with cleaved Dlp might similarly enable it to have long range effects on the SSCs.

The role of Upd3 in the regulation of JAK activity during oogenesis

It is shown in this work that *upd3* mutant flies have a higher frequency of egg chamber degenerations, a higher frequency of egg chamber fusions, and a decreased number of border cells per egg chamber. Because similar phenotypes are observed in *hop* mutants, these results are consistent with the hypothesis that Upd3 regulates JAK signaling in oogenesis. Interestingly, in every assay conducted, defects associated with *upd3* mutants worsened as the fly aged. Therefore, if the hypothesis that Upd3 is regulating JAK activity during oogenesis is accurate, then Upd3 must be required to maintain JAK activity over time. But how is this accomplished? Why would Upd3 be required over time? What happens to JAK activity over time?

One possibility of how Upd3 activates JAK signaling over time would be to form heterodimers with Upd. In this work, it is shown that misexpression of *upd3* alone within the follicular epithelium is unable to activate JAK signaling (figure 4.5). Because of the similarity between the phenotypes associated with reductions of JAK activity and *upd3* mutations, this result was somewhat unexpected. However, work done in our lab has shown that Upd3 and Upd form dimers in *Drosophila* S2 cells as well as when misexpressed in salivary glands of 3rd instar larvae (Pei, 2007). Therefore, it is entirely possible that Upd3 can activate JAK signaling in an Upd dependent way; by forming active heterodimers with Upd.

The possibility of a functional heterodimer of Upd and Upd3 inspires a number of questions based on what has been observed in this work. First, is there a difference in the amount of JAK activity that is activated by Upd/Upd3 heterodimers versus Upd or Upd3 homodimers? A difference between the activities of these 3 dimers would certainly influence the observed gradient. Furthermore, the distance of distribution, the stability of, and the potential HSPG interactions between the homodimer and heterodimer could also contribute greatly to gradient formation. Also, could Upd3 be involved in the possible regulated secretion of Upd? It was suggested in this work that the secretion of Upd from the polar cells is regulated, that is to say, much of the Upd is sequestered in what may be the golgi. In *upd2* studies, it was shown that most of Upd2 is retained within the golgi and very little was found to be extracellular (Zeidler, 2005). Furthermore, the *upd2* study went on to show that this retention was dependent on the signal sequence of the protein as a signal sequence switch between Upd and Upd2 gave the very opposite results; Upd^{SS2} was retained in the golgi while Upd2^{SS1} was mostly secreted (Zeidler, 2005). Similarly, in this work, Upd3^{SS1} was shown to be able to non-autonomously activate JAK signaling when misexpressed in follicle cells, however, the Upd3^{WT} was not. Is it possible that like Upd2, Upd3 is also retained in the golgi? If so, the formation of heterodimers with Upd could slow the rate at which Upd is secreted. In regards to the role of *upd3* with age, loss of *upd3* could result in the secretion of

higher levels of Upd in young flies, which would lead to an abnormal accumulation of negative regulators of JAK signaling. Over time, this could cause desensitization of the follicle cells to JAK and therefore produce results consistent with reduction of JAK activity.

Another possibility in considering the accumulation of negative regulators of JAK activity is that Upd3 is required to boost the levels of ligand over time. Some negative regulators of JAK activity in *Drosophila*, such as SOCS36E, are in a negative feedback loop with JAK activity. Unfortunately, nothing is known about the turnover of these negative regulators, however, it is likely that they will accumulate within cells receiving a constant JAK stimulus. This would most likely occur in the SSCs in the germarium. Given the lifespan of a SSC is potentially much longer than any follicle cell deriving from them, it seems possible, and likely, that the accumulation of negative regulators will occur within them. Slowly, these SSCs would produce follicle cells that have a higher resistance to JAK activity and would therefore require more ligand to maintain appropriate levels of JAK. In this scenario, Upd3, by formation of hetero or homodimers could compensate and boost the levels of ligand, thus maintaining the developmentally important levels of JAK activity.

Insights into the subfunctionalization of the Upd family

The three members of the *upd* family are the result of a duplication event at least 40 million years ago as all 12 sequenced *Drosophila* species have all three members. Other insects that have been sequenced do not appear to have all three members, however, there is a homologue of *upd3* in *Tribolium casteneum* (Red Flour Beetle) and a homologue of *upd2* in both *Nasonia vitripennis* (jewel wasp) and *Anopheles gambiae* (African malaria mosquito). When a duplication event occurs, it has been proposed that natural selection must disrupt the equality and redundancy of the duplication in one of two ways: Neofunctionalization or Subfunctionalization (Lynch and

Conery, 2000). Previous work in our lab has led to the proposal that the Upd family has undergone subfunctionalization, splitting the roles of activating JAK signaling between the 3 family members (Wang, 2008). The collective data suggests that Upd is the principal ligand in activating JAK during embyogenesis and oogenesis, however *upd2* and *upd3* also appear to have a role in JAK activation during embryogenesis and oogenesis respectively. Also consistent with subfunctionalization, it has been reported that *upd3* has a role in immunity, whereas *upd* and *upd2* do not. In this work, when using Upd3-Gal4 misexpression of *upd3* cDNA, Upd3 was shown to suppress the small eye phenotype associated with *upd3* and *os* mutants, while *upd3-Gal4* directed expression of *upd* was lethal. These data suggest that Upd and Upd3 have additive effects on eye development as Upd3 can rescue the small eye phenotype. Further support of the subfunctionalization of the Upd family comes from work done with Upd2. It was shown that *upd2* is expressed in an overlapping pattern with *upd* during embryogenesis, however, there were not obvious defects and flies were viable and fertile with loss of *upd2* (Zeidler, 2005). However, loss of both *upd* and *upd2* resulted in an enhanced embryonic segmentation defect compared to loss of *upd* alone. Furthermore, mutations in *stat92E* were shown to enchance the small eye phenotype of *upd3* (Wang, 2008). In both cases, it appears that Upd2 and Upd3 have less functional capacity to activate JAK than Upd, however, it is clear that both genes genetically interact with JAK pathway members.

A model for the establishment of the JAK gradient during oogenesis

This work has led to our current model for how the gradient of JAK is established during oogenesis (figure 5.1). In our model, Upd and Upd3 are secreted from the apical surface of the polar cells (figure 5.1, red and green, respectively). The secretion of Upd and Upd3, as discussed throughout this work, is likely to be differently regulated and not equal to one another. The Upd ligands then migrate along the ECM forming a

concentration gradient with the highest levels present at the anterior and posterior poles. The HSPG Dally stabilizes at least Upd in the extracellular environment and likely stabilizes Upd3 as well. Additionally, Upd3 could potentially interact with other HSPGs that are present. This hypothesis will, however, require further testing. Ligands bind to the Domeless receptors on the apical surface of the receiving follicle cells and activate JAK signaling, thus transmitting their extracellular gradient to the JAK gradient observed in the follicular epithelium.

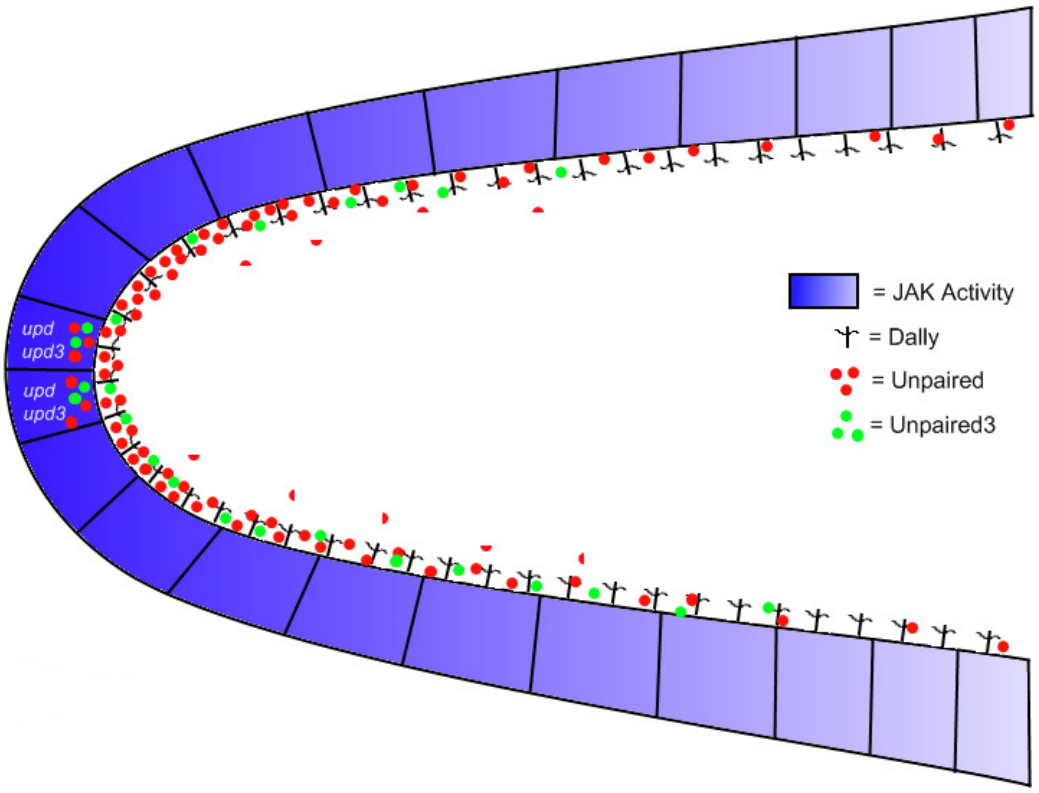


Figure 5.1. The Upd Morphogen Model of JAK Gradient Formation. Upd (red) and Upd3 (green) are secreted from the polar cells (most left) onto the apical surface of the follicular epithelium. The ligands then migrate, depending on the HSPG Dally for stability along the apical surface to target cells. Upd that is lost from the ECM is destabilized in the absence of Dally (red, half circles). The ligands form a concentration gradient with highest levels at the anterior and posterior poles. Binding of the ligand to the receptors activate JAK signaling thus transposing the extracellular ligand gradient to a JAK gradient.

Chapter 6

Materials and Methods

Fly strains and markers

Flies were raised at 25°C unless otherwise stated. *dally*^{80} and *dly*^{A187} are both loss-of-function (LOF) alleles caused by deletions (Han et al., 2004b). *sdc*^{10608} and *sgl*^{08310} are each null alleles caused by insertions of a P-elements (Hacker et al., 1997; Rawson et al., 2005). *trol* is a strong hypomorph caused by a P-element insertion (Datta and Kankel, 1992). s*fl*^{9B4} is a LOF allele caused by an insertion of a P-element (Lin and Perrimon, 1999). *ttv*^{63} is a C to T transition resulting in a nonsense allele deleting most of the protein (Han et al., 2004a). 5A7 and H20 (*pnt-lacZ*) are both enhancer trap lines specific for border cells and posterior cells respectively (Roth et al., 1995). *Dome*^{367} (*domelacZ*) is an enchancer trap in the *domeless* locus that responds to JAK activity (Brown et al., 2001). *Upd3*^{d232a} results from an imprecise excision of *upd3*^{d00871} removing the last exon. *Upd3*^{x21c} results from a local hop of a P-element into the CDS of *upd3*. *upd3*^{X37E} is a precise excision of *upd3*^{d00871}. Gal-E132 (Upd-Gal4) is an enhancer trap in the *upd* locus (Tsai and Sun, 2004). The *engrailed*-Gal4 and *patched*-Gal4 lines are Gal4 drivers described in Flybase.

Generation of LOF clones

Mosaic egg chambers having mutant clones for *dally*, *dlp*, *sdc*, *trol*, *sfl*, *sgl*, or *ttv* were generated by Flp-mediated recombination (Chou and Perrimon, 1992)(figure 6.1B). Expression of Flp recombinase was induced by incubating animals carrying a hsFLP construct for 2 hours at 37°C (McGregor et al., 2002; Xi et al., 2003).

The genotypes of animals in which clones were induced by heat shock were:

y w^{1118} hsFLP1/*dome*^{367}; *dally*^{80} FRT2A/{histone-GFP}^{62A} FRT2A

y w^{1118} hsFLP1/+; *dally*^{80} FRT2A/{histone-GFP}^{62A} FRT2A

y w^{1118} hsFLP1/+; *dlp*^{A187} FRT2A/{histone-GFP}^{62A} FRT2A H20 (aka *pnt-lacZ*)

y w^{1118} hsFLP/+; *dlp*^{A187} FRT2A/{histone-GFP}^{62A} FRT2A

trol^{SD} FRT101/*y w* [histone-GFP]JD1 FRT101; FLP38/+; H20 (aka *pnt-lacZ*)

trol^{SD} FRT101/y w [histone-GFP]JD1 FRT101; FLP38/+

w^{1118} hsFLP1/+; *sdc*^{10608} FRTG13/{histone-GFP} FRTG13 *bw*

w^{1118} hsFLP1/+; FRTG13 *ttv*^{63}/FRTG13 Ubn-GFP; H20 (aka *pnt-lacZ*)/+

y w^{1118} hsFLP1/*dome*^{367}; *sfl*^{9B4} FRT2A/{histone-GFP}^{62A} FRT2A

y w^{1118} hsFLP/+; *sgl*^{A31} FRT2A/{histone-GFP}^{62A} FRT2A

Generation of misexpression clones

Tissue directed misexpression was accomplished by using the UAS-Gal4 system (Binari and Perrimon, 1994)(figure 6.1). For expression of *upd* in the polar cells, *upd-Gal4* was utilized. For wing misexpression of *upd3*, flies carrying pUAS-*upd3*^{nuc}-GFP, pUAS-u*pd3*^{SS1}-GFP, or pUAS-*upd3*^{wt}-GFP transgenes were crossed to the *patched*-GAL4 or *engrailed*-GAL4 lines. Flies carrying both *upd3* and Gal4 contructs were selected. Wings were dissected and mounted in Hoyer's solution.

Misexpression clones of *upd3* in follicle cells were made by utilizing a flip-out cassette (Struhl and Basler, 1993) (figure 6.3). Clones were induced by a 30 minute incubation at 37^{o}C. Ovaries from induced flies were taken 2-4 days later.

The genotype of the misexpressing clone are:

w^{1118} hsFLP/ $dome^{367}$; [Act>y>Gal4][UAS-nGFP]/pUAS-$upd3^{WT}$-GFP(TS5)

w^{1118} hsFLP/ $dome^{367}$; [Act>y>Gal4][UAS-nGFP]/pUAS-$upd3^{SS1}$-GFP

w^{1118} hsFLP/ $dome^{367}$; [Act>y>Gal4][UAS-nGFP]/pUAS-$upd3^{NUC}$-GFP

w^{1118} hsFLP/ $dome^{367}$; [Act>y>Gal4][UAS-nGFP]; pUAS-*upd*-GFP

Immunological Stainings

Conventional Stainings. Ovaries were dissected in PBT (1XPBS, 0.1%Tween 20) and fixed for 10-15 minutes in 3.7% formaldehyde in PBT. Ovaries were washed in PBT. Ovaries were blocked in 5% BSA in PBT for 1 hour at RT^{o}, followed by an overnight incubation with primary antibodies at $4^{o}C$. Secondary antibodies were incubated 4 hours at RT^{o} or overnight at $4^{o}C$. Ovaries were washed 5X in PBT after fixation, primary antibody incubation, and secondary antibody incubation. DAPI was administered in the 3^{rd} wash after secondary antibody treatment at 1ug/mL. Ovaries were mounted in 70% Glycerol/2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO) in 1XPBS. When using anti-βGal antibody, 3.7% formaldehyde fixation was replaced by a 1:1 MeOH:PBT fixation for 1 hour at RT^{o} with rotation.

Extracellular Stainings. Extracellular staining was adapted to ovaries from the established protocol for imaginal discs (Strigini and Cohen, 2000). Ovaries were dissected in ice cold Complete Schneider's media (2.5% fly extract and 5% FBS). Ovaries were incubated overnight on ice in primary antibody diluted in Complete Schneider's media. After primary incubation, ovaries were washed 2X in Complete Schneider's media followed by 3 washes with PBS. Ovaries were fixed in ice cold 3.7% formaldehyde in PBS for 20 minutes, followed by 2 washes with PBS, then 2 washes with PBT. Ovaries were then blocked using 5% BSA in PBT. Secondary antibodies were incubated 4 hours at RT^{o} or overnight at $4^{o}C$. Ovaries were washed 5X in PBT after fixation, primary

antibody incubation, and secondary antibody incubation. DAPI was administered in the 3rd wash after secondary antibody treatment at a 1:1000 dilution from a mg/mL stock. Ovaries were mounted in 70% glycerol/2.5% Dapco in 1XPBS.

Primary antibodies and dilutions used were: rabbit α-GFP at 1:500, rabbit α-Unpaired (final bleed, animal #1111) at 1:800 (Harrison et al., 1998), mouse α-GFP at 1:500, rabbit α-βGal at 1:500, mouse α-Fas3 at 1:30 (7G10, DSHB), and mouse α-Orb at 1:30 (4H8, DSHB)

Upd3^{ss1}, *upd3^{nuc}*, and *upd3^{wt}* construction

upd3^{nuc} is a cDNA construct obtained by Rapid Amplification of cDNA Ends (RACE, Clonetech) and cloned into pBlueScript (p1FK/2RX). u*pd3^{SS1}* was created by cloning the first 159 bp of *upd*, which contains both signal sequence and cleavage site, onto the 5' end of p1FK/2RX. *upd3^{wt}* was amplified by using Upd3-0F-att5' and 5963-att3' from the IP04620 clone (DGBC) and cloned into pDONR-201 via Gateway Technology (Invitrogen). Sequences of all constructs were verified at the core sequencing facility at CCHMC (Cincinnati Children's Hospital and Medical Center).

Generation of transgenic lines

upd3^{SS1}, *upd3^{nuc}*, and *upd3^{wt}* were each amplified with the primers listed below and cloned into a pDONR-201 vectors via the BP reaction from Gateway Technology (Invitrogen) to create the entry vectors: pENTR-upd3$^{1FK/2RX}$, pENTR-upd3^{SS1}, and pENTR-upd3^{IP04620}. Entry vectors were then cloned into pUAST-Dest-EGFP via LR reaction from Gateway Technology (Invitrogen) to create the expression vectors: pUAS-*upd3^{NUC}-gfp*, pUAS-*upd3^{SS1}-gfp*, and pUAS-*upd3^{WT}-gfp*. Purified vectors were microinjected into

transposase containing flies to make transgenics as previously described (Rubin and Spradling, 1982; Spradling and Rubin, 1982).

Primers

5963-att5' (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCCATGTCCCAGTTTGCCCTC-3') for *upd3*^{NUC}. Upd3-0F-att5' (5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTCAAAATGACGA CAGCTGACCGCC-3') for *upd3*^{IP04620}. upd-att-5' (5'-GGGGACAAGTTTGTACAAAAAAGCAG GCTCGGCGATGGCTCG TCCGCTGC-3') for *upd3*^{SS1}. 5963-att3' (5'- GGGGACCACTTTGTAC AAGAAAGCTGGGTCG AGTTTCT TCTGGATCGCCTT-3') for *upd3*$^{1FK/2RX}$, *upd3*^{IP04629}, and *upd3*^{SS1}.

Image capture and processing

Images of fly eyes and wings were taken on a Nikon SMZ 1500 scope with a SPOT camera as previously described (Harrison et al., 2005). The fly eye area was measured using Scion Image software (Scion Corporation) in which the entire ommatidia of the eye was selected. Epifluorescent images were taken using a SPOT camera on a Nikon E800 microscope. Confocal images were collected on a Leica TCS-SP laser scanning confocal microscope. Images were exported in TIFF format and processed in Adobe Photoshop 7.0.

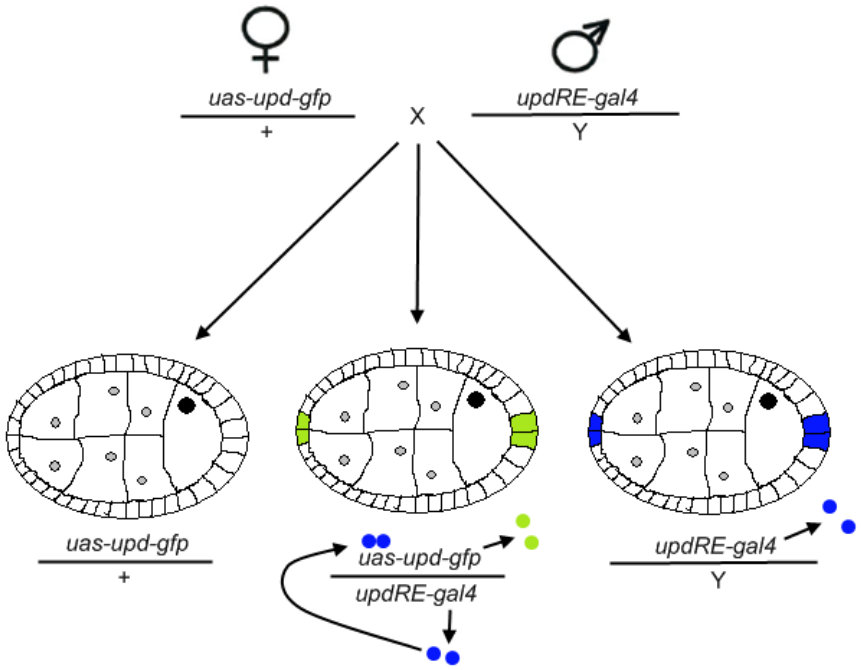


Figure 6.1. Gal4 driven expression of *upd-gfp* in the polar cells of the ovary. The UAS-Gal4 is a bipartite system utilizing a Gal4 Transcription factor to specifically enable the transcription at the Upstream Activating Sequence (UAS). In chapter 2, *upd-gfp* was expressed in the polar cells of developing egg chambers. Crossing a female containing a P-element with *uas-upd-gfp* to a male having a P-element containing a basal promoter-*gal4* coding sequence downsteam of the upd regulatory element (*updRE-gal4*) gives rise to 3 progeny with distinct phenotypes. Upd-GFP (green) is only present in progeny having both the *uas-upd-gfp* sequence and *updRE-gal4* sequence. In these flies, *upd-gfp* expression is restricted to those cells specified by the *updRE* sequence.

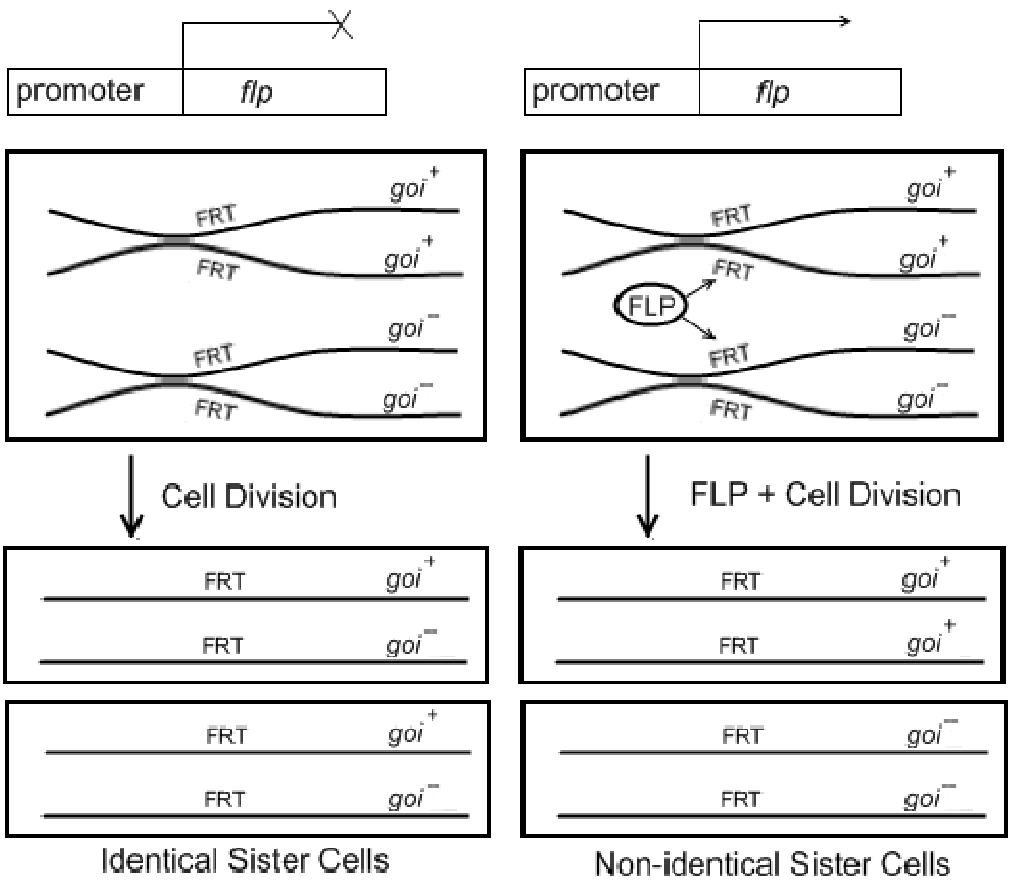


Figure 6.2. FLP-FRT mediated mitotic recombination. The goal of mitotic recombination is to alter the genotype of daughter cells following mitosis. In the exemplified case, the goal is to produce cells homozygous for our gene of interest (*goi*). FLP-FRT mediated mitotic recombination in *Drosophila* relies on a FLP-recombinase and FLP-Recombinase Recognition Tag (FRT) sequences, which in this case are located near the centromere. Briefly, FLP-Recombinase exchanges partial sequences complimentary sequences from two identical FRT sites resulting in the rearrangement of chromosomal DNA attached to given FRT sites. In the absence of FLP-Recombinase (left), cells resulting from mitosis are identical, as is normal during mitosis. When FLP-recombinase is present there is a chance of exchange between non sister chromatids. If exchange occurs between non-sister chromatids, the result will be non identical sister cells, in which each will be homozygous for goi^{+} or goi^{-}. These recombined cells will then give rise to identical cells via mitosis producing populations of both goi^{+} and goi^{-} homozygote cells.

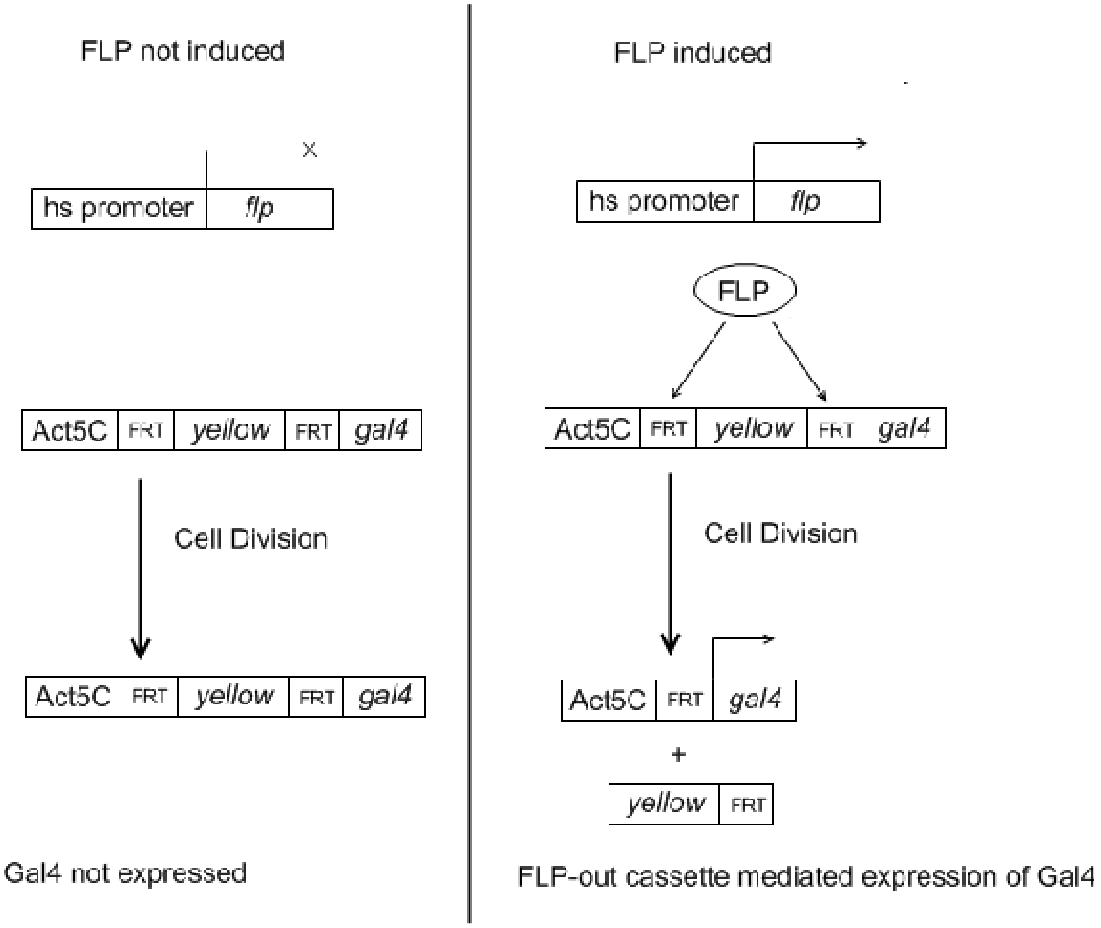


Figure 6.3. FLP-FRT mediated Flp-out cassette. The goal of the "flip-out" cassette in Drosophila is to misexpress a gene of interest in a subset of cells. This technique relies on both the UAS-Gal4 system (figure 6.1) and the FLP-mediated recombinase (figure 6.2). The "flip-out" cassette is located within a transposable element integrated into the genome and consists of a strong promoter, Act5C (actin promoter), the *yellow* cds, and the *gal4* cds with FRT sites flanking the *yellow* cds. When FLP-recombinase is not present, the cassette is unchanged and only the *yellow* transcript is produced (left). If FLP-recombinase is present it becomes possible to recombine the two flanking FRT sequences resulting in the clipping out of the *yellow* cds resulting in the transcription of *gal4*. Subsequent translation will produce the Gal4 transcription factor to induce transcription at whatever UAS sites are available throughout the genome. Note that, as opposed to mitotic recombination (figure 6.2), this process does not depend on mitosis because the FRT sites are in trans.

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