FUNCTIONAL CHARACTERIZATION OF UPD3 IN DROSOPHILA DEVELOPMENT

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ABSTRACT OF DISSERTATION

Liqun Wang

The Graduate School

University of Kentucky

2008
FUNCTIONAL CHARACTERIZATION OF UPD3 IN DROSOPHILA DEVELOPMENT

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
Liqun Wang
Lexington, Kentucky

Director: Dr. Douglas Harrison, Associate Professor of Biology
Lexington, Kentucky

2008

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ABSTRACT OF DISSERTATION

FUNCTIONAL CHARACTERIZATION OF UPD3 IN DROSOPHILA DEVELOPMENT

The JAK/STAT pathway is a non-receptor tyrosine kinase signaling pathway that is well conserved and highly re-utilized in many mammalian and Drosophila developmental processes. Compared to dozens of ligands and receptors in mammalian JAK/STAT, Drosophila JAK/STAT pathway is simpler with one receptor and three ligands, Upd, Upd2 and Upd3, which have similar amino acid sequences. Previous literature shows that upd and upd2 exhibit the same dynamic striped expression pattern in embryos and have semi-redundant functions during embryogenesis. Do Upd and Upd3 also have redundant functions? To answer this question, the functions of Upd3 in Drosophila development were investigated in this dissertation. In addition, the coordinate expression mechanism of upd and upd3 in eye discs was also analyzed.

To study the functions of Upd3 in development, the expression pattern of upd3 was examined and detected in larval eye discs, wing discs, haltere discs, lymph glands and adult ovaries with in situ hybridization to upd3 mRNA and an upd3 reporter line. Consistent with the expression pattern, the loss of function mutants of upd3 exhibit small eyes, outstretched wings, downward extended halteres and reduced circulating blood cell concentration, demonstrating the roles of Upd3 in these tissues’ development. However, functions of Upd3 in other aspects of immune response were not detected.

To investigate the mechanism of the coordinate expression of upd and upd3, the genetic and molecular relationship of upd, upd3 and os was dissected. The os alleles, os\(^o\), os\(^s\) and os\(^l\), are a group of classical alleles which display outstretched wings, small eyes, or both, respectively. The genetic complementation tests of upd, upd3 and os showed that both upd and upd3 failed to complement os while upd complemented upd3, suggesting functions of both upd and upd3 are affected in os alleles. Consistent with the genetic tests, the expression of upd and upd3 in eye discs is lost in os allele. Molecularly,
putative enhancer regions are deleted at the 5’ end of upd3 in os alleles. Hence, a transcriptional co-regulation model of upd and upd3 is proposed in which upd and upd3 share a common cis-regulatory region, lesions of which cause the os phenotype.

KEYWORDS:  JAK/STAT pathway, upd, upd3, os, Drosophila immune response
FUNCTIONAL CHARACTERIZATION OF UPD3 IN DROSOPHILA DEVELOPMENT

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July 30, 2008
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This work is dedicated to my husband Hongyang, my parents and my brother.
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Chapter One
Background

The JAK/STAT signaling pathway

The JANus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is a non-receptor tyrosine kinase signaling pathway first identified in mammals through the study of interferon-α (IFN-α) and interferon γ (IFN-γ) induced transcriptional activation (Darnell et al., 1994). The induced genes that are transcriptionally responsive to IFN-α and IFN-γ share conserved DNA response elements specific to each (Cohen et al., 1988; Kessler et al., 1988; Levy et al., 1986; Levy et al., 1988; Lew et al., 1991; Reich et al., 1987; Rutherford et al., 1988; Shirayoshi et al., 1988). Later, the class of proteins specifically binding to the conserved response elements were purified and found to have tyrosine phosphorylation upon activation by IFN-α and IFN-γ (Decker et al., 1991; Fu et al., 1992; Schindler et al., 1992; Shuai et al., 1992; Shuai et al., 1993). These proteins are STAT (signal transducer and activator of transcription) proteins. Furthermore, a kinase family, JAK (Janus) kinase, was identified to be required for the phosphorylation of STAT proteins in IFN induced transcriptional activation (Muller et al., 1993; Pellegrini et al., 1989; Velazquez et al., 1992; Watling et al., 1993). The discovery of STATs and JAKs defines the JAK/STAT pathway.

Besides IFN-α and IFN-γ, many other extracellular signaling proteins, such as interleukins (IL) and growth factors (GF), also can activate the JAK/STAT pathway in mammals. Consistent with various ligands, the receptors of the mammalian JAK/STAT pathway are also heterogeneous, including IL family receptors, IFN family receptors, and more. Despite the huge diversity of ligands and receptors, four Janus kinases, JAK1-3
and Tyk2, were identified in mammals and they have distinctive domains of a JAK: a tyrosine kinase domain at the C-terminus and an adjacent pseudo-kinase domain with no catalytic function next to the functional kinase domain (Firnbach-Kraft et al., 1990; Schindler and Darnell, 1995; Wilks et al., 1991; Wilks et al., 1989). A total of seven STAT proteins, STAT 1-4, 5a, 5b and 6 have been found in mammals with a SH2 domain and a single conserved tyrosine residue at the C-terminus which will be phosphorylated by JAKs upon activation (Schindler and Darnell, 1995). In addition to the principle components, positive regulators and negative regulators are also identified. STAM (signaling transducing adaptor molecules), StIP (STAT interacting proteins) and SH2B/Lnk/APS family proteins are thought to facilitate the JAK signaling as adaptor proteins (Lohi and Lehto, 2001; Rawlings et al., 2004b) while SOCS (suppressors of cytokine signaling), PIAS (protein inhibitors of activated STATs) and PTPs (protein tyrosine phosphatase) are three major classes of negative regulators (Starr and Hilton, 1999). The SOCS family, the largest negative regulator family for JAK signaling, is composed of eight members: CIS and SOCS1-7, which all share a SH2 domain and a SOCS box at the C-terminus. The SOCS proteins inhibit JAK signaling either by binding to JAKs and activated cytokine receptors or by inducing the proteosomal degradation of JAKs (Cooney, 2002).

Despite numerous components, the signal transduction mechanism of JAK/STAT pathway is simple. The binding of ligands to transmembrane receptors brings receptor associated JAK kinases into close proximity, which facilitates the trans-phosphorylation of JAKs (Figure 1.1). The activated JAKs can further phosphorylate the latent cytoplasmic signaling molecules, STATs, on a conserved tyrosine residue at the C-terminus. Activated STATs can form dimers which serve as transcription factors after translocating into the nucleus. In the nucleus, phosphorylated STAT dimers bind to
conserved DNA response elements and induce the transcription of downstream target genes.

As an evolutionarily conserved pathway, the complete cascade and components are found in all vertebrates and *Drosophila*. *unpaired* encodes a ligand of the *Drosophila* JAK/STAT pathway, a glycosylated, secreted and extracellular matrix binding protein (Harrison et al., 1998). The *domeless* gene encodes a transmembrane signal transducing receptor for the *Drosophila* JAK/STAT pathway with most similarity to mammalian IL-6 receptor family (Brown et al., 2001; Chen et al., 2002). The *hopscotch* gene encodes a *Drosophila* JAK homologue and is maternally required for the establishment of normal embryonic segmentation (Binari and Perrimon, 1994; Perrimon and Mahowald, 1986). STAT92E is the signal transducer and transcription activator of *Drosophila* JAK/STAT pathway which is phosphorylated on Tyr-704 by *Drosophila* Hopscotch upon activation (Chou and Perrimon, 1996; Hou et al., 1996; Yan et al., 1996a). In more divergent organisms such as *C.elegans* and *Dictyostelium*, only STAT homologs are found (Wang and Levy, 2006a; Wang and Levy, 2006b; Williams, 2000). The completeness and simplicity of the JAK/STAT signaling pathway in *Drosophila* make it a good model to study the components, functions and regulation of the pathway.

**Developmental functions of Drosophila JAK/STAT signaling pathway**

The *Drosophila* JAK/STAT pathway is a pleiotropic signaling cascade which has critical functions in many developmental processes. In *Drosophila* eye development, JAK signaling functions in cell proliferation, photoreceptor differentiation and the establishment of equator and ommatidia polarity (Luo et al., 1999). Ligand Unpaired (Upd) is expressed in the posterior region of eye discs where the dorsal/ventral boundary
intersects with the posterior margin (Dominguez and Casares, 2005; Tsai and Sun, 2004). Upd functions as a long range signaling molecule which promotes cell proliferation ahead of the morphogenetic furrow (MF) and up-regulates cyclin $D$ ($cycD$) transcription at the anterior edge of the MF (Tsai and Sun, 2004). Reduced Upd results in the small eye phenotype and increased Upd level by misexpression results in eye enlargement (Bach et al., 2003; Tsai and Sun, 2004). This function of Upd in cell proliferation is regulated by Notch signaling, which localizes in the dorsal/ventral boundary and regulates the global growth of Drosophila eye by acting on eye selector gene eye gone ($eyg$) and unpaired ($upd$) (Chao et al., 2004; Kenyon et al., 2003). In addition to its role in cell proliferation, Upd also regulates the polarity of ommatidia through an unknown second signal and affects the position of the equator by inhibiting mirror ($mirr$) (Zeidler et al., 1999). Ectopic expression of Upd at the dorsal/ventral poles of eye discs causes the inversion of ommatidia polarity and loss of a regular equator between dorsal and ventral parts (Treisman and Heberlein, 1998; Zeidler et al., 1999). In addition, JAK signaling also functions in photoreceptor differentiation. In trans-heterozygous hop alleles, which have reduced JAK activity, loss of photoreceptor cells is observed (Luo et al., 1999).

In addition to its role in eye development, JAK signaling establishes patterns in the wing. Ectopic wing vein near the posterior crossvein is observed in hypomorphic alleles $Stat92E^{HJ}$ and $hop^{msv/m38}$, which have reduced JAK signaling (Rawlings et al., 2004a; Yan et al., 1996a). The ectopic wing vein phenotype caused by reduced JAK signaling can be partially rescued by $hop^{Tum-1}$, a dominant gain of function mutation of hopscotch (Yan et al., 1996a). Consistent with this, the overexpression of $Socs36E$ and $Socs44A$ causes abnormal wing vein development and/or outstretched wing phenotype, suggesting the role of JAK signaling in wing development (Callus and Mathey-Prevot, 2002; Rawlings et al., 2004a).
The JAK/STAT pathway is also well known to function in *Drosophila* immune response. In blood cell development, overexpression of JAK signaling stimulates both blood cell proliferation and differentiation. In *hop*<sup>Tum-l</sup>, leukemia-like abnormalities with significantly increased number of total circulating blood cells and lamelllocytes are observed (Luo et al., 1995). Lamelllocytes are specially differentiated blood cell. In addition, melanotic tumors and hypertrophy of lymph glands, the hematopoiesis organ, are observed in *hop*<sup>Tum-l</sup> and misexpression of *hop* alleles (Harrison et al., 1995). In humoral response, a few antimicrobial peptides are known to be JAK/STAT dependent. The Tep (thiolester-containing proteins) protein family, including Tep1, Tep2, Tep3 and Tep4, is constitutively activated in *Hop*<sup>Tum-l</sup> flies (Agaisse and Perrimon, 2004; Lagueux et al., 2000). The synthesis of the Tot (Turandot) protein family, TotA, TotC and TotM, will not be induced by septic injury in hypomorphic *hop*<sup>msv/m38</sup> background (Agaisse and Perrimon, 2004; Agaisse et al., 2003). The synthesis of a small Cys-rich antimicrobial peptide, CG11501, is also not induced by septic injury in hypomorphic *hop*<sup>msv/m38</sup> flies (Boutros et al., 2002).

The role of the JAK/STAT pathway in *Drosophila* oogenesis has also been well established. JAK signaling is required for the differentiation of the interfollicular stalk cells and polar cells (McGregor et al., 2002). Reduced JAK activity results in the fusion of developing egg chambers due to the expansion of the polar cell population and the loss of stalk cells (McGregor et al., 2002). In addition, the anterior-posterior patterning of the follicular epithelium cells is determined by a gradient of JAK activity, stimulated by the ligand Upd, which is expressed in the polar cells of ovaries (McGregor et al., 2002; Silver and Montell, 2001; Xi et al., 2003). Reduced JAK activity results in the reduced number of border cells and defects of their migration while increased JAK activity results in more border cells (Silver and Montell, 2001; Xi et al., 2003). Furthermore, the *Drosophila* JAK/STAT pathway also has functions in embryonic segmentation, sex determination.
and germline stem cell maintenance in male flies (Harrison and Harrison, 2006; Kiger et al., 2001; Sefton et al., 2000; Tulina and Matunis, 2001; Zeidler et al., 2000).

**The evolution of the **upd** gene family**

In evolution, duplication of individual genes, chromosome segments and even the entire genome is an important source for new gene functions and expression patterns (Lynch and Conery, 2000). With an average of 1% duplication per gene per million years, duplicated genes arise fast and face the fate of either being preserved in the genome or being rapidly lost in evolution. To be selected and preserved by natural selection, the equality or complete redundancy between duplicated genes must be disrupted one of two ways, neofunctionalization or subfunctionalization (Lynch, 2002; Lynch and Conery, 2000). In neofunctionalization, one copy of the duplicated genes gains novel and beneficial function while the other copy keeps the original function (Lynch and Conery, 2000). In subfunctionalization, both duplicated copies are compromised with mutations to the point that the total capacity of both copies equals to that of one original ancestral gene (Lynch, 2002; Lynch and Conery, 2000)). In addition to these two fates, duplicated genes can also accumulate degenerative mutations in one copy resulting in silence and finally being selected against, which is called nonfunctionalization (Lynch and Conery, 2000). For the **upd** gene family, three duplicated genes, **upd**, **upd2** and **upd3**, exist in *Drosophila melanogaster* genome and they cluster within a 70 kb desert region on the X chromosome. **upd3** is in the middle and has opposite transcriptional direction to **upd** and **upd2** (Figure 1.2). Upd2 and Upd3 were identified by similar amino acid sequences with the founding member of the family, Upd (Figure 1.3) (Hombria and Brown, 2002). Three Upd proteins share a few conserved amino acid blocks (underlined in Figure 1.3) and the overall similarity of the three proteins is about 36%. By searching orthologs of **upd**, **upd2** and **upd3** in other species, it appears that **upd** and **upd2** genes are under relaxed selection, which is common for duplicated genes.**
upd3 in other Drosophila species, the upd gene family is identified in all twelve Drosophilidae species whose genome sequences have been revealed (Clark et al., 2007; Stark et al., 2007; http://flybase.bio.indiana.edu/). In addition, the upd gene family has the same cluster organization in the other eleven Drosophilidae species as it is in Drosophila melanogaster. This suggests that the duplication of upd gene family occurred at least 40 million years ago when the speciation of Drosophilidae began. Homologs of upd2 are found in Anopheles gambiae (African malaria mosquito) and Nasonia vitripennis (jewel wasp) while homologs of upd3 are found in Tribolium castaneum (red flour beetle) with the E-value (a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size) ranging from 0.001-0.007. No homolog of upd is identified in any of the genome sequenced insect species, suggesting that upd is probably more recently duplicated compared to upd2 and upd3. Are the upd genes preserved in the Drosophila genome by neofunctionalization or subfunctionalization? In this dissertation, functions of Upd3 in Drosophila development were investigated and its functional relationship with Upd was analyzed, which help to elucidate the evolutionary mechanism of the upd gene family.

In addition to the mechanism of evolutionary conservation of upd genes, the cluster organization of upd genes on the X chromosome is also interesting. Literature suggests that genomically neighboring genes are usually transcriptionally coupled across tissues (Purmann et al., 2007; Vogel et al., 2005). upd2 displays the same dynamic striped pattern with upd in embryos. Do upd and upd3 also have coordinate expression pattern? If so, what is the mechanism? To answer these questions, the expression pattern of upd and upd3 in Drosophila was examined and a mechanism for coordinate expression of upd and upd3 is proposed based on the molecular characterization of the upd/upd3 region.
Figure 1.1. Overview of the JAK/STAT signaling pathway. This figure shows the general activation and negative regulation mechanisms of the JAK/STAT signaling pathway (see text for details). Specific proteins in the parenthesis are the JAK/STAT components in Drosophila.
Figure 1.2. Genomic arrangement of *upd* and *upd-like* genes on the X chromosome.

The *upd*, *upd2* and *upd3* genes are in a 70 kb region of X chromosome. The transcriptional directions of *upd*, *upd2* and *upd3* are indicated by the directions of arrows. *CG15057* and *CG15059* are two predicted genes between *upd2* and *upd3*. However, no EST clone has been found for either.
Figure 1.3. Amino acid sequence alignment of Upd, Upd2 and Upd3. Alignment analysis of predicted amino acid sequences of Upd, Upd2 and Upd3 was performed with ClustalW2 software. The stars (*) indicate the same amino acids in all three proteins. While one or two dots (· or :) indicate the same amino acids in two proteins or similar amino acids in three proteins. The blocks of conserved amino acid sequence are highlighted with red lines and numbered.

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Chapter Two
Roles of Upd3 in *Drosophila* Development

Introduction

**Upd, Upd2 and Upd3**

While the mammalian JAK/STAT pathway has dozens of various ligands including cytokines and growth factors, the *Drosophila* JAK/STAT pathway only has three ligands with protein sequence homology, Upd, Upd2 and Upd3. As the first characterized primary ligand of the JAK/STAT pathway, Upd displays multiple functions in *Drosophila* development through the activation of JAK signaling. For one, Upd regulates cell proliferation in eye discs; loss of Upd results in small eye phenotype with fewer ommatidia (Bach et al., 2003; Tsai and Sun, 2004). In addition, Upd sets up a JAK signaling gradient in egg chambers to instruct follicular epithelium cell patterning (Xi et al., 2003). Furthermore, Upd also has functions in embryonic segmentation, sex determination and germline stem cell maintenance in male flies (Harrison et al., 1998; Kiger et al., 2001; Sefton et al., 2000; Tulina and Matunis, 2001). For Upd2, it is shown to be expressed in the same dynamic striped pattern as *upd* in embryos (Hombria et al., 2005). However, amorphic alleles of *upd2* are viable and fertile without visible phenotype while *upd* null alleles are homozygous lethal and display embryonic structural defects, indicating the possible compensation of Upd to loss of Upd2 in embryos (Hombria et al., 2005). Despite this, loss of Upd2 can slightly enhance the embryonic structural defects caused by *upd* alleles, suggesting the subtle function of Upd2 in embryogenesis (Hombria et al., 2005). For Upd3, very little work has been done to characterize its function in *Drosophila* development. The only work on Upd3 so far is to
show the hemocyte specific expression of upd3 upon septic injury which is required for the induction of the synthesis TotA and the expression of upd3 in Drosophila lymph gland with a GFP reporter line (Agaisse et al., 2003; Jung et al., 2005). Does upd3 have the same expression pattern as upd? Does Upd3 have other functions in development? What is the functional relationship between Upd and Upd3? To answer these questions, the expression pattern and functions of Upd3 in Drosophila development were investigated in this chapter,
Results

**upd3 is expressed in several tissues of Drosophila.**

To investigate the roles of Upd3 in development, the expression pattern of *upd3* in *Drosophila* was examined, assuming that *upd3* would be expressed in the tissues where it has functions. Since *upd* and *upd2* have the same expression pattern in embryos, it is assumed that *upd3* would also have the same expression pattern with *upd* in a subset of tissues where *upd* is expressed. *upd* is known to be expressed in the posterior region of eye discs, wing discs and polar cells of ovaries (McGregor et al., 2002; Mukherjee et al., 2005; Tsai and Sun, 2004). Thus, the expression of *upd3* in these tissues was examined with *in situ* hybridization to *upd3* mRNA. Consistent with the hypothesis, the expression of *upd3* was detected in the posterior region of the second and early third larval eye discs and two polar cells of ovaries, the same as the expression pattern of *upd* (Figure 2.1, A-D). The coordinate expression of *upd* and *upd3* suggests that they may have related functions in eye and ovary development. However, in contrast to the *upd* expression in wing discs and haltere discs, no expression of *upd3* was found in these two tissues, which suggests either no expression of *upd3* in wings and halteres or the expression level of *upd3* was too low to be detected by *in situ* hybridization (Figure 2.1, I and J). In the wing and haltere discs, *upd* staining was also seen at the margins of discs and this is likely to be non-specific due to the inconsistency in all the stainings (indicated by asterisk in Figure 2.1, I and J). The staining of *upd* at the margin of wing discs is also different from what is reported in the presumed ventral hinge region (Mukherjee et al., 2005). With an *upd3* reporter fly line, *upd3-GAL4 UAS-GFP* which has a 4 kb *upd3* promoter region constructed in front of *GAL4* gene, the expression of *upd3* in wing discs and haltere discs was revealed by the GFP expression. The GFP expression in wing discs is strong and restricted to four regions of presumed dorsal hinge, dorsal wing surface, ventral wing
surface and ventral hinge areas. The GFP expression in haltere discs is a more uniform ring (Figure 2.1, K and L). The detection of *upd3* in wings and halteres through the GFP expression in *upd3-GAL4 UAS-GFP* suggests that the failure to detect *upd3* in these two tissues by *in situ* hybridization may be because of the low transcription level of *upd3*, which makes it difficult to be detected by *in situ*. However, unlike the overlapping expression pattern of *upd* and *upd3* in eye discs and ovaries, the GFP expression of *upd3-GAL4 UAS-GFP* in wing and haltere discs is different from the expression of *upd* in the dorsal hinge region of these two tissues. This may suggest the expression pattern of *upd* and *upd3* are different in wing and haltere discs or that the promoter included in the *upd3* reporter fly does not contain the complete regulatory region.

In addition to the expression of *upd3* in larval imaginal discs and adult ovaries, literature also suggests the expression of *upd3* in lymph glands (Jung et al., 2005). Lymph glands are the hematopoiesis organ of *Drosophila*, which are composed of two or three pairs of lobes arranged bilaterally along the dorsal vessel. The biggest pair of lobes is called the primary lobe (1°), which contains two morphologically distinct regions: the cortical zone (a peripheral region with loosely arranged cells) and the medullary zone (a region with compactly arranged cells) (Jung et al., 2005). In the *upd3-GAL4 UAS-GFP* reporter, *upd3* was reported to be expressed in the medullary zone and the posterior signaling center of the lymph gland, which is defined by the expression of the Notch ligand Serrate and transcription factor Collier (Jung et al., 2005). To validate the expression of *upd3* in lymph gland, *in situ* hybridization was used to detect both * upd* and * upd3* mRNA with antisense and sense probes. However, the detected expression of * upd* and * upd3* in the cortical zone of lymph glands is non-specific, demonstrated by the same staining with both antisense and sense probes (Figure E-H). By examining the native GFP expression in the lymph gland of * upd3-GAL4 UAS-GFP*, GFP expression was observed uniformly throughout the lymph gland with slightly stronger expression at the posterior
signaling center (Figure 2.1, M). This observation is inconsistent with the expression of *upd3* in the medullary zone of the lymph gland, reported by the Banerjee group (Jung et al., 2005). This can be due to the methodology difference. In Banerjee group’s report, antibody staining was performed to visualize the GFP expression while native fluorescence was observed in this dissertation. The antibody staining treatment may disturb the GFP distribution pattern and result in the difference of GFP expression pattern with this dissertation. Overall, considering the inconsistency of the GFP expression and the *upd* expression in wing and haltere discs, it remains uncertain that whether this *upd3* reporter line truly represents the expression pattern of *upd3 in vivo*.

**The *upd3* mutants display structural defects.**

The expression of *upd3* in tissues of *Drosophila* larvae and adults suggests its potential functions in *Drosophila* development. To further investigate the functional role of Upd3 in development, mutants of *upd3* were generated by P element mobilization mutagenesis with *P{XP}upd3*<sup>d00871</sup>, which has a P element inserted in the last intron of *upd3* (Bellen et al., 2004; Thibault et al., 2004). *P{XP}upd3*<sup>d00871</sup> has yellow eyes, due to the presence of a mini *white* gene, an eye color gene, on the P element. The P element in *upd3* was removed from the original site by a transposase, which sometimes resulted in excision mutants or local hop mutants (Figure 2.2). Excision mutants were generated when the excision of the P element takes away flanking genomic DNA region with it and they were identified by white eyes due to the loss of mini *white* gene carrying P element. Local hop mutants were generated when the P element duplicated itself and the duplicated P element re-inserted in the genome. The local hop mutants were identified by dark eyes due to the existence of two mini *white* genes carrying P elements. Some flies, which had the same eye color of the parental *P{XP}upd3*<sup>d00871</sup> line, were also recovered. They may be generated by the partial excision of the P element and flanking DNA. In this
screen, mutants with outstretched wings, small eyes and/or downward extended halteres were recovered. Both the small eye phenotype and outstretched wing phenotype exhibit variability. \textit{upd3}^{x21c} has mild small eyes while \textit{upd3}^{d2332a}, \textit{upd3}^{x25c} and \textit{upd3}^{x21b} have strong small eye phenotype compared to wild type eyes (Figure 2.3, A, B and E-H). For the outstretched wing phenotype, \textit{upd3}^{d2332a} displays the strongest phenotype with wings about 90 degrees away from the body and \textit{upd3}^{x21b} only holds the wings slightly away from the body (Figure 2.3, I, J and L). In addition, the small eye phenotype of \textit{upd3} mutants exhibits 100\% penetrance while the outstretched wings phenotype has variable penetrance. The outstretched wing penetrance of \textit{upd3}^{d2332a} is 100\% while that of \textit{upd3}^{x21b} and \textit{upd3}^{x21c} is only about 20\%. In addition, the downward extended haltere phenotype is only observed in the \textit{upd3} mutants with outstretched wing phenotype (Figure 2.3, M and N, Figure 2.4). In evolution, \textit{Drosophila} originates from four-winged ancestors. Presumably, the posterior flight appendage slightly reduced in size and finally became morphologically distinct halteres, which serve to maintain balance during flight and motion (Roch and Akam, 2000; Weatherbee et al., 1998). This phenotypic linkage between outstretched wings and downward extended halteres is consistent with the homologous origin of these two tissues. The distinct outstretched wing, small eye and downward extended haltere phenotype of \textit{upd3} mutants is similar to the phenotype of \textit{os} alleles, which include \textit{os}^{l}, \textit{os}^{o} and \textit{os}^{s}. \textit{os}^{l} has mild small eyes, outstretched wings and downward extended halteres (Figure 2.3, D and K; Figure 2.4) (Verderosa and Muller, 1954). \textit{os}^{o} has outstretched wing and downward extended haltere phenotype with wild type eyes (Figure 2.4) while \textit{os}^{s} only has mild small eye phenotype (Figure 2.3, C; Figure 2.4) (Verderosa and Muller, 1954; Morgan, Bridges and Sturtevant, 1925). The similar phenotype of \textit{upd3} and \textit{os} alleles suggests that they may be in the same genetic pathway.

To find out whether the phenotypes are due to lesions of \textit{upd3} and the molecular nature of these mutants, PCR was used to either locate the position of local hop P element
or map the deletions in the excision mutants. Out of 63 recovered viable and lethal upd3 mutants, a few lines were chosen as representatives of various phenotypes and for molecular mapping. The summary for the P element mobilization mutagenesis is shown in figure 2.5 and the molecular mapping is shown in figure 2.6. Three local hop mutants, upd3^{x21b}, upd3^{x25b} and upd3^{74b}, have a second P element inserted at 134 bp, 76 bp and 134 bp upstream of the original P element respectively. The original P element also remains in the genome. The viable excision mutant upd3^{d232a} has a 1.8 kb deletion including the complete last exon of upd3 while upd3^{x21c} has a 1.0 kb deletion including part of the last exon of upd3. Three lethal mutants of upd3 contain big deletions. upd3^{d49a} has a 4.1 kb deletion including the last exon of upd3. Compared with viable allele upd3^{d232a}, upd3^{d49a} has 2.3 kb more deletion than upd3^{d232a} at 3’ end of upd. The lethality of upd3^{d49a} suggests that there may be some essential elements in this 2.3 kb region. upd3^{d76a} has a deletion of at least 44.3 kb removing both upd2 and upd3. upd3^{d127a} has a deletion of 43.7 kb including upd3 and most of the intergenic region between upd, upd2 and upd3. In conclusion, consistent with the expression upd3 in eye discs, wing discs and haltere discs, loss of function alleles of upd3 display defects in eye size, wing extension and haltere extension, suggesting the roles of Upd3 in the development of these tissues.

*upd3 genetically interacts with JAK/STAT signaling pathway.*

The os alleles are classical alleles closely linked to the JAK/STAT pathway. The outstretched wing and/or small eye phenotype of os was thought to be due to the loss of JAK signaling (Eberl et al., 1992). The phenotypic similarities of os and upd3 mutants suggest that the phenotype of upd3 mutants is also due to the loss of JAK signaling and that Upd3 is an activating ligand of the *Drosophila* JAK/STAT pathway. To test this hypothesis, potential genetic interaction of upd3 with other components of the JAK/STAT pathway was examined. According to the hypothesis that the structural
defects of \textit{upd3} are due to the loss of JAK activity, further reduction of the JAK signaling should enhance or exaggerate the \textit{upd3} mutant phenotype. Namely, the outstretched wings of \textit{upd3} alleles are expected to extend further away from the body and the small eyes of \textit{upd3} alleles are expected to become even smaller with fewer ommatidia. On the other hand, increased JAK signaling is expected to suppress or compensate the \textit{upd3} mutant phenotype. Thus, the outstretched wings of \textit{upd3} alleles are expected to be closer to the body and the small eyes of \textit{upd3} alleles are expected to become bigger with more ommatidia.

To test whether reduced JAK signaling can enhance the mutant phenotype of \textit{upd3}, two homozygous lethal alleles of \textit{Stat92E}, \textit{Stat92E}^{J6C8} and \textit{Stat92E}^{06346} were used (Hou et al., 1996; Yan et al., 1996b). In heterozygous \textit{Stat92E}^{J6C8} or \textit{Stat92E}^{06346} flies, the JAK/STAT activity is reduced due to the loss of one functional copy of \textit{Stat92E}. Thus, the outstretched wing and small eye phenotype of \textit{os}^{1}, \textit{upd3}^{d232a} and \textit{upd3}^{v21b} in the heterozygous \textit{Stat92E}^{J6C8} \textit{FRT82B} and \textit{Stat92E}^{06346} \textit{FRT82B} background is expected to be enhanced compared to that in a wild type \textit{FRT82B} background. To compare the outstretched wing phenotype, a numerical system was used. In the non-anesthetized animals, when the wings were extended 90° away the body (completely outstretched), it was recorded as 1. When the wings were extended 45° away the body (partially outstretched), it was recorded as 0.5. When the wings were extended 0° away the body (wild type), it was recorded as 0. Thus, by scoring the wing extension of all the animals in one genotype, the average wing extension number for each genotype was obtained. The bigger the extension number, the stronger the outstretched wing phenotype. As expected, the average wing extension of \textit{os}^{1}, \textit{upd3}^{d232a} and \textit{upd3}^{v21b} in the heterozygous \textit{Stat92E} mutants was much stronger than that in wild type \textit{FRT82B} background, suggesting that reduced JAK signaling enhanced the outstretched wing phenotype of \textit{os}^{1} and \textit{upd3} alleles (Figure 2.7, A). In addition, the enhancement of \textit{os}^{1} outstretched wing
phenotype in heterozygous \(\text{Stat92E}^{16C8}\) background was significantly greater than that in heterozygous \(\text{Stat92E}^{06346}\) background. Similarly, the enhancement of the \(\text{upd3}^{x21b}\) outstretched wing phenotype in heterozygous \(\text{Stat92E}^{16C8}\) was greater than that observed in a heterozygous \(\text{Stat92E}^{06346}\) background although the difference was not statistically significant. This suggests \(\text{Stat92E}^{16C8}\) may be a stronger allele than \(\text{Stat92E}^{06346}\). For the eye phenotype, the size of the ommatidial area of each fly was measured and the average was calculated for each genotype. The bigger the average number, the weaker the small eye phenotype. However, the average eye size of \(\text{os}^{1}\), \(\text{upd3}^{d232a}\) and \(\text{upd3}^{x21b}\) in the heterozygous \(\text{Stat92E}\) mutant backgrounds did not show significant reduction compared to that in wild type \(\text{FRT82B}\) background (Figure 2.7, B). This can be due to two reasons. On one hand, in \(\text{os}^{1}\) and \(\text{upd3}\) alleles, the ligand level has already been reduced. Reduced ligand level requires less \(\text{STAT92E}\) for signal transducer and transcription activation function than wild type. Thus, the loss of one functional copy of \(\text{Stat92E}\) may only slightly reduce the JAK activity in \(\text{os}^{1}\) and \(\text{upd3}\) alleles. On the other hand, the JAK activity required in the wings may be less than that in the eyes. Thus, slight reduction of the JAK activity in heterozygous \(\text{Stat92E}\) mutant background may result in the dramatic reduction of the overall signaling in the wing while it only caused a small percentage of the signaling reduction in the eye. Therefore, the wing extension phenotype was very sensitive to this slight reduction of JAK signaling while the eye phenotype was not.

To test whether increased JAK signaling suppresses the mutant phenotype of \(\text{upd3}\), \(\text{Socs36E}\) and \(\text{Socs44A}\) alleles were used. In \textit{Drosophila}, three \textit{Socs} genes have been defined based on their sequence similarity with mammalian \textit{Socs} and named after their cytological locations, \(\text{Socs16D}\), \(\text{Socs36E}\) and \(\text{Socs44A}\). \(\text{Socs36E}\) participates in the JAK/STAT pathway in a negative feedback loop. Namely, the expression of \(\text{Socs36E}\) is responsive to the JAK activity and in turn \(\text{SOCS36E}\) protein can inhibit the JAK activity. However, \(\text{Socs44A}\) behaves in a different way. The expression of \(\text{Socs44A}\) is not
responsive to the JAK activity and it regulates the JAK activity in a tissue specific manner. *Socs44A* can suppress the JAK signaling in wings but it has no effect on the JAK activity in oogenesis (Rawlings et al., 2004a). In addition, *Socs44A* enhances the EGFR/MAPK signaling in wings, in contrast to the suppression of EGFR/MAPK by *Socs36E* (Rawlings et al., 2004a).

Alleles of *Socs36E* and *Socs44A* were made in the lab through P element mobilization mutagenesis (Thesis of Qian Guo, 2007). *Socs36E*<sup>189a</sup> is an incomplete excision mutant with 38 base pairs of the P element left in *Socs36E*. It displays ectopic wing vein phenotype. *Socs44A*<sup>291a</sup> is an excision line removing the whole *Socs44A* gene without displaying visible phenotype. Fly line *Socs36E*<sup>189a</sup>*Socs44A*<sup>291a</sup> is a double mutant of these two alleles. *Socs36E*<sup>rev330b</sup> is a revertant mutant of *Socs36E*<sup>189a</sup>, which serves as a wild type genetic background control for *Socs36E*<sup>189a</sup> and *Socs36E*<sup>189a</sup>*Socs44A*<sup>291a</sup>. In *Socs36E*<sup>189a</sup> and *Socs36E*<sup>189a</sup>*Socs44A*<sup>291a</sup>, the JAK activity is assumed to be increased due to the loss of a functional copy of negative regulators. Thus, the small eye phenotype of *os*<sup>1</sup> and *upd3* is expected to be partially suppressed in heterozygous *Socs36E*<sup>189a</sup> and *Socs36E*<sup>189a</sup>*Socs44A*<sup>291a</sup> background and the suppression of *Socs36E*<sup>189a</sup>*Socs44A*<sup>291a</sup> is expected to be stronger than that of *Socs36E*<sup>189a</sup> because of the loss of functions of two *Socs* genes. By measuring the size of the ommatidial area of each fly, the average eye size for each genotype was calculated. As expected, the eye size of *os*<sup>1</sup> and *upd3* alleles in heterozygous *Socs36E*<sup>189a</sup> and *Socs36E*<sup>189a</sup>*Socs44A*<sup>291a</sup> was bigger than that in wild type *Socs36E*<sup>rev330b</sup> background, showing increased JAK activity can suppress the mutant phenotype of *os* and * upd3* (Figure 2.8). However, the suppression by *Socs36E*<sup>189a</sup>*Socs44A*<sup>291a</sup> was not consistently stronger than the suppression of *Socs36E*<sup>189a</sup>. The eye size of *upd3*<sup>d232a</sup> in heterozygous *Socs36E*<sup>189a</sup>*Socs44A*<sup>291a</sup> was significantly smaller than that in heterozygous *Socs36E*<sup>189a</sup>. This could be due to the complex genetic relationship between *Socs44A* and JAK signaling. *Socs44A* only negatively regulates the
JAK signaling in certain developmental process such as wing vein patterning and it functions oppositely to Socs36E in regulating the EGFR/MAPK signaling (Rawlings et al., 2004a). Thus, it is difficult to compare the strength of JAK activity between Socs36E189a and Socs36E189aSocs44A291a. In conclusion, the genetic interactions of upd3 with Stat92E and Socs alleles support the hypothesis that Upd3 is an activating ligand of the JAK pathway and the mutant phenotype of upd3 is due to the loss of JAK signaling.

Upd3 displays mild function in Drosophila immunity.

Drosophila is a great model to study innate immune response, an ancient and essential system for insects to combat microbial infection. Lacking an adaptive immune system, Drosophila has a very elegant and sophisticated innate immune system consisting of two complementary aspects: humoral response of antimicrobial peptides synthesis and cellular response of blood cell proliferation and differentiation. Usually, these two processes are thought to interact and cooperate to defend flies from infection (Elrod-Erickson et al., 2000). The synthesis of antimicrobial peptides occurs in Drosophila fat body, a counterpart of mammalian liver. Four signaling pathways: Imd, Toll, JAK/STAT and JNK, have been implicated in this process (Figure 2.9). Imd and Toll pathways are homologous to mammalian tumor necrosis factor receptor pathway and Toll-like receptor signaling pathway, respectively. As two complementary signaling pathways, Imd and Toll have their own specificities. Infection of gram-negative bacteria triggers Imd pathway while fungi and gram-positive bacterial infection triggers Toll pathway. When Imd signaling pathway is activated, Relish, a NF-κB homologue, will translocate into nucleus to activate gram-negative bacteria specific antimicrobial peptides (Kaneko and Silverman, 2005). Similarly, when Toll pathway is activated, transcription factors Dif and/or Dorsal, are released from cytoplasm and translocate into nucleus to activate the expression of gram-positive bacteria or fungi specific antimicrobial peptides.
Besides the well studied Imd and Toll signaling pathways, JAK/STAT and JNK signaling pathways have also been reported to induce certain antimicrobial peptides upon septic injury challenge (Boutros et al., 2002).

Cellular response is an immune mechanism that depends on blood cells. In *Drosophila*, there are three major mature blood cells: plasmacytes, lamellocytes and crystal cells (Figure 2.10). Plasmacytes are the largest group of blood cells that constitute about ~90% of total blood cells. They are small and round cells which have phagocytic function, similar to mammalian monocyte/macrophage cells. The plasmacytes are responsible for removing foreign microorganisms and apoptotic corpses by tethering, engulfing and then destroying objects in phagosomes with lysosome enzyme, reactive oxygen species and nitric oxide (Meister and Lagueux, 2003). Crystal cells contain the precursor for prephenoloxidase enzyme and function in quick melanization of wound sealing before more elaborate and complete epithelial wound healing. Lamellocytes are big flat blood cells accounting for less than 5% of total blood cells in wild type *Drosophila*. The presence of wasp eggs in *Drosophila* haemocoel sends a cue to prohemocytes and triggers massive differentiation of lamellocytes which then surround and melanize wasp eggs by forming capsules. The parasites will finally be killed by cytotoxic molecules such as reactive intermediates of oxygen and nitrogen (Nappi et al., 1995; Nappi et al., 2000).

The involvement of the JAK/STAT pathway in *Drosophila* immune response is shown in both humoral response and cellular response. The synthesis of a few antimicrobial peptides, the Tep protein family, the Tot protein family and CG11501, is JAK/STAT dependent (Agaisse et al., 2003; Boutros et al., 2002; Lagueux et al., 2000). In cellular response, significantly increased number of total circulating blood cells and lamellocytes is observed in *hop*<sup>Tum-l</sup> (Luo et al., 1995). Despite the important functions of
JAK signaling in *Drosophila* immunity, it is not clear which ligand or ligands are performing this function. However, literature shows that hemocyte specific expression of *upd3* upon septic injury is required for the JAK/STAT dependent expression of TotA (Agaisse et al., 2003). In addition, *upd3* is also expressed in the lymph gland of *upd3-GAL4 UAS-GFP* reporter fly line (Figure 2.1) (Jung et al., 2005). Thus, it is hypothesized that Upd3 is the JAK ligand that functions in *Drosophila* immune response. To test this hypothesis, several immunity assays were performed and stated below.

*Adult survival assay after septic injury*

It has been shown that immune compromised flies have reduced survival rate after septic injury with bacteria (Elrod-Erickson et al., 2000; Lemaitre et al., 1995). The homozygous *imd/imd* mutants, which have lost the ability to induce gram-negative bacteria specific antimicrobial peptides, display reduced survival rate when challenged with bacteria (Lemaitre et al., 1995). Homozygous mutants of *Rsh/Rsh* also display significant susceptibility to *E.coli* infection and exhibit even higher and faster lethality than *imd/imd* with bacteria challenge (Elrod-Erickson et al., 2000). To test whether loss of Upd3 also results in immune compromise, septic injury was applied to *upd3*<sup>d232a</sup>, a partial deletion mutant of *upd3*, by pricking the dorsal thorax of flies with a glass needle that was previously dipped into GFP expressing *E.coli* culture. The same septic injury treatment was also applied to wild type *CG6023*<sup>d04993</sup> and immune compromised *imd/imd* and *Rsh/Rsh* flies. *CG6023*<sup>d04993</sup> is from the same screen of *upd3*<sup>d00871</sup> with a P element inserted in the intron of an *upd3* unrelated gene *CG6023* (Bellen et al., 2004; Thibault et al., 2004). No phenotype is observed in *CG6023*<sup>d04993</sup> and it was used as wild type control for the same genetic background with *upd3* alleles. At the same time, the non-challenged control group for each genotype was set up without being performed with septic injury. Both the survival rates of adult flies and the growth of GFP expressing bacteria were monitored over a 10 day period. Immune compromised flies are expected to have
bacterial growth and significantly reduced survival rate in challenged flies compared to the same genotype non-challenged flies. Over the 10 day period, both wild type and $upd3^{d232a}$ flies did not have bacteria growth based on GFP expression while about 50% $imd/imd$ and 100% $Rsh/Rsh$ flies had GFP expressing bacteria growth (Figure 2.11). The survival rates of both challenged and non-challenged wild type flies were greater than 95% after 10 days with no significant difference between them. For $imd/imd$ and $Rsh/Rsh$ flies, the flies challenged with $E.coli$ bacteria displayed significant low survival rate compared to the non-challenged flies. Challenged $imd/imd$ had only about 30% survival rate while non-challenged $imd/imd$ still showed almost 100% survival rate on day 10. Challenged $Rsh/Rsh$ had 0% survival rate on day 4 while non-challenged $Rsh/Rsh$ had ~95% survival rate on the same day. For $upd3^{d232a}$, the survival rates of challenged and non-challenged groups were the same although both of them had only ~70% survival rate on day 10. The lethality of $upd3^{d232a}$ is not due to bacterial infection but rather the outstretched wings which easily stick in the food, leading to death. In conclusion, this adult septic injury assay shows that loss of Upd3 in $upd3^{d232a}$ does not result in reduced adult survival rate upon $E.coli$ infection.

**Larval survival assay with septic injury**

Although no reduced survival rate was observed in adult $upd3^{d232a}$ upon bacteria infection, it was not clear whether the larvae of $upd3^{d232a}$ would have reduced immune response. To investigate the function of Upd3 in larval immunity, septic injury was applied to the early third instar larvae of $upd3^{d232a}$ with a fine glass needle which was previously dipped into GFP expressing $E.coli$ bacteria culture. The same septic injury performance was applied to the early third instar of wild type $CG6023^{d04993}$, immune compromised $imd/imd$ and $Rsh/Rsh$. Non-challenged control group for each genotype was set up at the same time without septic injury. Over a two day period, the survival rates of control group and experiment group for each genotype were monitored and compared.
The significant reduction of the survival rate in experiment group compared to that in the same genotype control group would suggest the immune deficiency of that genotype. However, after two days, the survival rates of non-challenged $\text{upd}^{d232a}$ control group and septic injury challenged $\text{upd}^{d232a}$ experiment group were 95% ± 3.54% and 92.5% ± 2.50% respectively with no significant difference. Similarly, the survival rate of non-challenged wild type group (95% ± 2.89%) was not significantly different from the survival rate of septic injury challenged wild type group (93.75% ± 4.73%). In addition, adult immune compromised $\text{imd/imd}$ and $\text{Rsh/Rsh}$ also did not display significant reduction of survival rates in septic injury challenged experiment groups (86.38% ± 3.45% and 92.5% ± 1.44%, respectively) compared to those in non-challenged control groups of $\text{imd/imd}$ and $\text{Rsh/Rsh}$ (91.67% ± 3.82% and 96.25% ± 1.25%, respectively), which was unexpected. This may be because the amount of bacteria used was too little that it did not efficiently challenge larvae immunity. Alternatively, it may be also due to the different defense strategies used by larvae and adults. The adult flies may rely very much on the synthesis of antimicrobial peptides, loss of which will lead to dramatic lethality upon bacteria challenge. However, larvae may rely more on cellular response. Thus, loss of a humoral response signaling pathway does not affect the immunity significantly. Overall, no defect of $\text{upd}^{d232a}$ was observed in both adult and larval septic injury assay.

Antimicrobial peptide synthesis in septic injury challenged $\text{upd}^{d232a}$

$\text{upd}^{d232a}$ did not show immune deficiency in either adult or larval survival assays upon septic injury, which could be due to the compensation by other immune signaling pathways such as Imd, Toll and JNK. To more specifically dissect the immune response of $\text{upd}^{d232a}$, the synthesis of JAK/STAT dependent antimicrobial peptides, TotA, TotM and CG11501, was examined in septic injury challenged $\text{upd}^{d232a}$ by RT-PCR. If Upd3 is the functional JAK/STAT ligand in immune response, the synthesis of TotA, TotM and
CG11501 would not be induced in septic injury challenged \textit{upd3}^{\text{d232a}}. For comparison, two JAK/STAT independent antimicrobial peptides, CecA1 (Cecropin A1) and Drs (Drosomycin) were used as control. Therefore, the mRNA level of TotA, TotM, CG11501, CecA1 and Drs was quantified by RT-PCR in four different groups: septic injury challenged and non-challenged wild type and \textit{upd3}^{\text{d232a}} flies. It was expected that the mRNA level of all five antimicrobial peptides would be very low in non-challenged wild type and \textit{upd3}^{\text{d232a}}. After septic injury challenge, the mRNA level of CecA1 and Drs was expected to increase significantly and equally in challenged wild type and \textit{upd3}^{\text{d232a}} flies. It was expected that the mRNA level of all five antimicrobial peptides would be very low in non-challenged wild type and \textit{upd3}^{\text{d232a}}. After septic injury challenge, the mRNA level of TotA, TotM and CG11501 was expected to increase significantly in challenged wild type flies but not in challenged \textit{upd3}^{\text{d232a}} flies. Just as expected, there was very low expression of all five antimicrobial peptides in unchallenged wild type and \textit{upd3}^{\text{d232a}} (Figure 2.14). The mRNA level of CecA1 and Drs increased significantly in challenged wild type and \textit{upd3}^{\text{d232a}} flies compared to non-challenged wild type and \textit{upd3}^{\text{d232a}} flies, but there was no difference of CecA1 and Drs mRNA in challenged wild type and \textit{upd3}^{\text{d232a}} flies (Figure 2.14). However, for three JAK/STAT dependent antimicrobial peptides, only CG11501 had reduced mRNA level in challenged \textit{upd3}^{\text{d232a}} flies compared to that in challenged wild type flies and the reduction was not statistically significant. The synthesis of TotA and TotM was not affected in septic injury challenged \textit{upd3}^{\text{d232a}} compared to septic injury challenged wild type flies. This was different from previous reports that the induction of TotA, TotM and CG11501 was significantly reduced in hypomorphic alleles of \textit{hopscotch} upon septic injury (Agaisse et al., 2003; Boutros et al., 2002). One possible explanation for this inconsistency is that the JAK signaling may be not reduced in \textit{upd3}^{\text{d232a}} due to the redundant functions of other ligands such as Upd while the JAK signaling is reduced in the hypomorphic \textit{hopscotch} background.
Circulating blood cell concentration assay

In *Drosophila* larvae, total circulating blood cell concentration is a good indicator of cellular immune response. The significant increase of total circulating blood cells is observed in *hop<sup>Tum-l</sup>* larvae, suggesting that increased JAK signaling results in blood cell proliferation (Luo et al., 1995). Thus, *upd3<sup>d232a</sup>* is expected to have reduced total circulating blood cell concentration due to reduced JAK activity. To test this hypothesis, total circulating blood cell concentrations from late third instar larvae of wild type, *upd3<sup>d232a</sup>*, *Rsh/Rsh* and *hop<sup>Tum-l</sup>* were counted on a hemacytometer (Figure 2.15). As expected, the *hop<sup>Tum-l</sup>* larvae had highest blood cell concentration of 11171 ± 1080 cells/ul (n=7). The blood cell concentration of *upd3<sup>d232a</sup>* was 4238 ± 300 cells/ul (n=26), which was significant less than 6418 ± 318 cells/ul (n=50) of wild type. But this reduction of circulating blood cell concentration in *upd3<sup>d232a</sup>* was not as much as that in *Rsh/Rsh* larvae, which has only 1671 ± 142 cells/ul (n=51).

Despite the significant reduction of circulating blood cell concentration in *upd3<sup>d232a</sup>* , its biological consequence is not clear. Will this affect blood cell proliferation in *upd3<sup>d232a</sup>* upon immune challenge? To answer this question, total circulating blood cell concentration of septic injury challenged *upd3<sup>d232a</sup>* was examined. The early third instar larvae of wild type and *upd3<sup>d232a</sup>* were subjected to septic injury with *E.coli* and their circulating blood cell concentrations were measured 24 hours after challenge. The same age non-challenged larvae of wild type and *upd3<sup>d232a</sup>* were used as control. For wild type larvae, significant increase of total circulating blood cell concentration from non-challenged control group (813 ± 89 cells/ul, n=40) to septic injury challenged group (1234 ± 127 cells/ul, n=41) was observed (Figure 2.16). However, no significant difference of total circulating blood cell concentration was observed in non-challenged *upd3<sup>d232a</sup>* larvae (1369 ± 171 cells/ul, n=26) and septic injury challenged *upd3<sup>d232a</sup>* larvae.
(1369 ± 227 cells/ul, n=26) (Figure 2.16). But it is noticeable that non-challenged $\text{upd}^3_{d232a}$ early third instar larvae had more total circulating blood cells (1369 ± 171 cells/ul) than non-challenged wild type early third instar larvae (813 ± 89 cells/ul), which suggests that either non-challenged $\text{upd}^3_{d232a}$ larvae were already immune challenged somehow without septic injury or the basal level of the circulating blood cell concentration of $\text{upd}^3_{d232a}$ early third instar is higher than that of the same age wild type larvae. But from the previous experiment, the total circulating blood cell concentration of $\text{upd}^3_{d232a}$ late third instar larvae was less than that of the same age of wild type. This inconsistency of the blood cell concentration comparison suggests that blood cell concentration is highly variable at different developmental stages and between different genotypes. It is probably more valuable to compare the blood cell concentrations of the same genotype non-challenged and challenged larvae than comparing the blood cell concentrations of different genotypes.

**Wasp encapsulation assay**

To evaluate whether reduced circulating blood cell concentration in late third instar of $\text{upd}^3_{d232a}$ results in lamellocyte differentiation defects, the wasp encapsulation assay was adopted. When an avirulent wasp, *L. boulardi G486*, lays eggs in *Drosophila* larvae, lamellocytes will be triggered to differentiate and then surround wasp eggs by forming capsules in which wasp eggs will be melanized and killed (Sorrentino et al., 2004). Although the complete encapsulation process is not well understood, the encapsulation capacity of flies, which is measured by the percentage of larvae which form capsules around wasp eggs, is thought to be a good indicator of lamellocyte differentiation. To perform this assay, second instar larvae of wild type and $\text{upd}^3_{d232a}$ were exposed to female wasps for parasitization and the encapsulation events were examined in late third instar by the presence of visible black capsules in *Drosophila* larvae (Figure 2.17, A). To control variability, the same age and number of *Drosophila* larvae and female wasps were
used in the experiment. With similar infection rates (99.55% ± 0.45% in wild type and 99.76% ± 0.24% in $upd3^{d232a}$), which were the percentage of larvae parasitized with wasp eggs, the encapsulation capacity of wild type larvae (21.09% ± 2.4%) was lower than that of $upd3^{d232a}$ (46.45% ± 3.55%) (Figure 2.17, B). The difference of encapsulation capacity between wild type and $upd3^{d232a}$ was significant. This suggests that $upd3^{d232a}$ is not only able to induce lamellocyte differentiation, but it has stronger encapsulation capacity compared to wild type.

In addition to the wasp encapsulation capacity, another way to assay lamellocyte differentiation is to examine lamellocytes in lymph glands directly with antibody staining. In the primary lobes of lymph glands, both undifferentiated prohemocytes and differentiated mature blood cells exist. In unchallenged healthy larvae, mature blood cells usually only include plasmocytes and crystal cells which will be released into hemolymph for circulation from primary lobes. Lamellocytes will be triggered to differentiate upon wasp infection. To investigate the effect of loss of Upd3 on lamellocyte differentiation in the lymph gland, beta-PS antibody, a marker for lamellocytes in lymph glands and dorsal vessel, was applied to both wasp challenged and non-challenged wild type and $upd3^{d232a}$ larvae. In unparasitized wild type and $upd3^{d232a}$ larvae, no beta-PS staining was visible in the primary lobes of lymph glands, indicating there was no lamellocyte differentiation (Figure 2.18, A1-A3 and C1-C3). However, upon wasp infection, beta-PS staining was visible in lymph glands of both wild type and $upd3^{d232a}$, suggesting that $upd3^{d232a}$ is able to induce lamellocyte differentiation in lymph glands upon wasp infection (Figure 2.18, B1-B3 and D1-D3), consistent with the wasp encapsulation capacity of $upd3^{d232a}$.
Misexpression of *upd3* in the lymph gland

Misexpression of *hop* in lymph glands leads to lymph gland hypertrophy and tumor formation in gastric cecae of third instar larvae due to the overactivation of JAK signaling \cite{Harrison95}. According to the hypothesis that Upd3 is the activating ligand of JAK/STAT, it is predicted that misexpression of *upd3* in lymph glands will also result in similar lymph gland hypertrophy and tumor formation in third instar larvae. To test this hypothesis, either genomic *upd3* region (UAS-upd3g) or *upd3* cDNA (UAS-upd3ss1) were misexpressed with lymph gland GAL4 drivers 76B-GAL4, e33C-Gal4 and c355-Gal4. The UAS-upd3ss1 is a chimeric construct of the signal sequence from *upd* and the *upd3* cDNA lacking 5’ end signal sequence. In addition, UAS-hop was used as positive control and UAS-lacZ was used as negative control. In late third instar larvae, pigmentation and posterior structure defects were observed in larvae with misexpression of *hop* (76B>hop and e33C>hop), *upd3g* (76B>upd3g) and *upd3ss1* (e33C>upd3ss1 and c273>upd3ss1) (Figure 2.19, A). The misexpression phenotype of *hop* or *upd3* was at low frequency and no tumor formation was observed in gastric cecae even with misexpression of *hop*. The failure to reproduce the misexpression phenotype of *hop* could be due to the experimental condition. Previous misexpression of *hop* was driven by hs-GAL4 at 29 °C while this experiment was performed at room temperature. It is known that the GAL4 activity is temperature sensitive and a wide range of expression levels can be obtained by altering temperature \cite{Duffy02}. Thus, the temperature difference may be the reason for the failure to repeat the misexpression phenotype of *hop*.

To examine more specific effects of *upd3* misexpression on lymph glands, the morphology of the lymph gland and lamellocyte differentiation were examined. The lymph glands of 76B>upd3g were dissected in PBS and compared with that of *hop_Tum-1* and wild type. Lymph glandS of 76B>upd3g, both at 29 °C and room temperature, were bigger than wild type lymph glands but smaller than *hop_Tum-1* lymph glands (Figure 2.19,
B), suggesting Upd3 may have the similar function of Hop in lymph glands on blood cell proliferation and differentiation. However, different from $hop^{Tum-1}$, no lamellocyte differentiation was observed in lymph glands of $76B>upd3g$ with beta-PS antibody staining (Figure 2.19, C), suggesting that the role of Upd3 in the lymph gland was weaker than that of Hop, consistent with the weaker lymph gland hypertrophy phenotype of $upd3$ than that of $hop^{Tum-1}$. 
Discussion

Functional relationship of Upd and Upd3 in *Drosophila* development

*Drosophila* JAK/STAT pathway is a simple pathway compared to its mammalian counterpart with unique receptor, JAK kinase and STAT protein. However, three potential ligands, Upd, Upd2 and Upd3, complicate this neat pathway. Do they all have functions in *Drosophila* development? Do they function independently or redundantly, synergistically or oppositely? Work on Upd2 shows that although Upd2 is not required for embryogenesis, it may support the function of Upd in embryo development (Gilbert et al., 2005; Hombria et al., 2005). In this work, functional relationship of Upd and Upd3 in multiple developmental events was analyzed and the tissue specific functional relationship of Upd and Upd3 is revealed.

Consistent with the overlapping expression of *upd* and *upd3* in the posterior region of eye discs, both Upd and Upd3 have functions in eye development. Loss of Upd and Upd3 causes similar small eye phenotype which contains less ommatidia than wild type (Tsai and Sun, 2004). In addition, loss of either Upd or Upd3 also leads to the wing and haltere defects of outstretched wings and downward extended halteres, suggesting that Upd and Upd3 have similar functions in the eye, wing and haltere development. Since the phenotype is visible in either loss of Upd or loss of Upd3, the functions of Upd and Upd3 are not redundant and both of them are required for the development of these tissues, probably in an additive manner by activating the JAK signaling.

Although structural defects are observed in Upd3 loss of function mutants, the defects in *Drosophila* immunity and oogenesis are not obvious in loss of function mutants of *upd3*. The synthesis of antimicrobial peptides of TotA, TotM and CG11501 is
induced in $upd3^{d232a}$ flies equally to that in wild type upon septic injury. However, previous literature suggests that in the hypomorphic $hopscotch$ background, the synthesis for these three peptides is significantly reduced or blocked (Agaisse et al., 2003; Boutros et al., 2002). Similar results are also observed in oogenesis. In $Drosophila$ oogenesis, the JAK/STAT pathway is known to function in follicular epithelium patterning. Reduced number of border cells is observed in reduced $hopscotch$ background (Xi et al., 2003). But in loss of function mutant of $upd3$, the border cell number is not changed compared to that in wild type (Travis Sexton, unpublished data). A few possibilities can explain the failure to detect the functions of Upd3 in $Drosophila$ immunity and oogenesis. One is that Upd3 does not have functions in these two processes. However, considering the lymph gland hypertrophy phenotype caused by $upd3$ overexpression in lymph glands and the expression of $upd3$ in lymph glands and ovaries, this explanation seems unlikely. The second possibility is that the assays used are not proper. This probably holds true for the functions of Upd3 in oogenesis since only the border cell number was examined. But given the thorough and complete investigation of Upd3 in $Drosophila$ immunity, this is not a good explanation. The third possibility is that the functions of Upd3 in immune response and oogenesis are masked somehow. Given the protein homology and same expression pattern of Upd and Upd3 in a few tissues, it is very likely that Upd and Upd3 have redundant functions in $Drosophila$ immune response and oogenesis. The defects caused by loss of Upd3 could be compensated and masked by the endogenous redundant function of Upd. Overall, through the functional analysis of Upd3 in $Drosophila$ development, a tissue specific functional relationship of Upd and Upd3 is revealed: both Upd and Upd3 are required for the proper development of $Drosophila$ eyes, wings and halteres. Loss of either one causes visible structural defects. But in $Drosophila$ immune response and oogenesis, a possible redundant function of Upd and Upd3 is suggested. Loss of Upd3 function does not results in defects in the synthesis of antimicrobial
peptides, blood cell proliferation and border cell number, may be due to the redundant function of Upd.

With the finding of the Upd and Upd3 functions in multiple developmental processes, it remains unclear how they can fulfill their complex developmental roles through the same JAK/STAT signaling pathway. A few models can be proposed. One is that Upd and Upd3 may act independently through different receptors. Although Domeless is the only receptor described so far, a predicted gene CG14225 encodes a protein structurally similar to Domeless and the vertebrate JAK receptor gp130 (Hombria and Brown, 2002). Thus, it is possible that Upd and Upd3 can activate JAK signaling through either Dome or CG14225, respectively. In addition, Upd and Upd3 may share the same receptor Domeless and they compete to activate the JAK signaling based on their affinity with Domeless. The stronger the affinity, the stronger the signaling. Furthermore, Upd and Upd3 may also physically interact and form homo- or hetero-dimers to activate the JAK signaling synergistically. This model of the ligands interaction has been proposed for the BMP signaling pathway (O'Conner et al., 2006). Two ligands of the BMP pathway, Dpp and Scw, form homo-dimers at dorsolateral region of early embryos and output mild signaling while they form hetero-dimers at dorsal midline of early embryos and output synergistically strong signaling (O'Conner et al., 2006). Upd and Upd3 may also be able to form different homo- or hetero-dimers at different developmental stages and tissues to fulfill specific functions. In conclusion, although all these three models are just speculations, it is important to know the functional mechanisms of three JAK ligands for further understanding the regulation of the *Drosophila* JAK/STAT pathway.
Figure 2.1. Overlapping expression pattern of \textit{upd} and \textit{upd3}. Both \textit{upd} and \textit{upd3} mRNAs were detected in the posterior region of the second instar and early third instar eye discs (arrows in A and B) and polar cells of ovaries (arrows in C and D). Non-specific staining of \textit{upd} and \textit{upd3} was detected in the lymph gland with both antisense (as) and sense (s) probes (arrows in E-H). \textit{upd} was also detected in the hinge region of wing discs and in haltere discs (arrows in I and J) while the expression of \textit{upd3} in wing discs, haltere discs and lymph glands are revealed by the GFP expression in \textit{upd3-GAL4 UAS-GFP} flies (arrows in K, L and M). The asterisk (*) in I and J shows the non-specific staining at the margin region of discs. The asterisks (*) in K-L indicates the GFP expression in trachea.
Figure 2.2. P element mobilization mutagenesis scheme for upd3 mutants. The parental line, $P\{XP\}^{upd3\text{d00871}}$, has a P element inserted in the last intron of $upd3$. $P\{XP\}^{upd3\text{d00871}}$ has yellow eye color. By crossing $P\{XP\}^{upd3\text{d00871}}$ with a fly line carrying P element transposase ($\Delta 2\cdot3$), excision mutants and local hop mutants of $upd3$ were generated. Excisions of $upd3$ were recognized by white eye color due to the loss of P element and the local hop mutants of $upd3$ were recognized by red eye color due to the presence of two white gene containing P elements in the genome.

Excision mutant: w- (white eye)
Local hop mutant: w++ (dark red eye)
Figure 2.3. Structural defects of upd3 mutants. Compared to the eyes of wild type (A) and parental line upd3^{d00871} (B), upd3^{x21c}, upd3^{d232a}, upd3^{x21b} and upd3^{x25c} show the small eye phenotype (E-H), which is similar to os^s and os^l flies (C and D). The upd3^{d232a} and upd3^{x21b} also exhibit outstretched wings (J and L), similar to that of os^l (K). The halteres of upd3^{d232a} extend downward toward the ventral part of flies (arrow in N), which is opposite of the upward extended halteres in wild type (arrow in M).
Table 2.4

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Figure 2.4. Haltere extension phenotype of upd3 and os alleles. Wild type flies have their halteres extended upward toward the dorsal part of flies while some upd3 mutants and os flies have their halteres extended downward toward the ventral part of flies. upd3<sup>d232a</sup> flies have the highest percentage of downward extended halteres, followed by os<sup>o</sup>, os<sup>l</sup> and upd3<sup>x21b</sup>. The os<sup>s</sup> flies have upward extended halteres same as wild type.
Figure 2.5. **Summary of the P element mobilization mutagenesis of upd3.** Out of 63 upd3 mutants recovered, six viable lines and three lethal lines were selected as representatives for molecular characterization while the rest was not investigated.
**Figure 2.6. Molecular characterizations of *upd3* mutants.** In this schematic representation of 70 kb *upd* and *upd-like* region, *upd3* is blown up above. Black boxes indicate the predicted exons and gray boxes indicate the UTRs. P element *d00871* locates in the last intron of *upd3* indicated by an open triangle. The deletions of *upd3* excision mutants are indicated by gray bars below the genomic schemes and the secondary P element insertion sites of * upd3* local hop mutants are indicated by filled triangles (each line still has the original *d00871* P element). The breakpoint of *upd3* is not precisely mapped and indicated by “?”.
Figure 2.7. Genetic interaction of *upd3* and *Stat92E*. In figure A and B, the outstretched wing phenotype and the small eye phenotype of *os¹*, *upd3²d²₃₂a* and *upd3ₓ²₁₁₅b* in heterozygous *Stat92E¹₆C₈ FRT82B* and *Stat92E⁶₃₄₆ FRT82B* background were compared with that in wild type *FRT82B* background, respectively. The numbers in each column are sample sizes. ***: P<0.001 (student t-test comparing *Stat92E¹₆C₈ FRT82B* and *Stat92E⁶₃₄₆ FRT82B* with wild type *FRT82B*). Δ: P<0.05 (student t-test comparing *Stat92E¹₆C₈ FRT82B* and *Stat92E⁶₃₄₆ FRT82B*).
Figure 2.8. Genetic interaction of upd3 and Socs alleles. The small eye phenotype of os\textsuperscript{f}, upd3\textsuperscript{d232a} and upd3\textsuperscript{x21b} in the heterozygous Socs36E\textsuperscript{189a} and Socs36E\textsuperscript{189a}Socs44A\textsuperscript{291a} background was compared with that in wild type Socs36E\textsuperscript{rev330b} background. 

- ***: P<0.001 (student t-test comparing Socs36E\textsuperscript{189a} and Socs36E\textsuperscript{189a}Socs44A\textsuperscript{291a} with Socs36E\textsuperscript{rev330b}).
- ∆∆: P<0.01 (student t-test comparing Socs36E\textsuperscript{189a} and Socs36E\textsuperscript{189a}Socs44A\textsuperscript{291a}).
- ∆: P<0.05 (student t-test comparing Socs36E\textsuperscript{189a} and Socs36E\textsuperscript{189a}Socs44A\textsuperscript{291a}).
Figure 2.9. *Drosophila* humoral immune response. Microbe infection triggers the humoral immune response of *Drosophila* by activating different signaling pathways such as Imd, Toll, JAK/STAT and JNK to synthesize antimicrobial peptides in the fat body.
Figure 2.10. *Drosophila* blood cells. Three mature blood cells, plasmocytes, lamellocytes and crystal cells, exist in *Drosophila*. All of them derive from undifferentiated prohemocytes in the lymph gland, the *Drosophila* hematopoiesis organ (see A). Under normal condition (no immune challenge), most of the blood cells are plasmocytes (>90%). Lamellocytes can be induced to differentiate with wasp infection. The plasmocytes are small and round cells while lamellocytes are large and flat cells (see B). The crystal cells function in melanization of wound sealing and turn black when heated at 70°C for 10 minutes because they contain precursors for enzyme phenoloxidase (see C).
Figure 2.11. Bacteria growth in septic injury challenged adults (29°C). Upon septic injury challenge at the dorsal thorax of flies with GFP expressing *E.coli*, bacterial growth was observed in immune compromised flies of *imd/imd* (C) and *Rsh/Rsh* (D). However, both wild type (A) and *upd3<sup>d232a</sup>* (B) did not have bacteria growth. The yellow arrow in B indicates the melanized injury wound.
Figure 2.12. Adult survival rates after septic injury (29°C). One week old adult flies from four different genotypes, wild type, upd3^{d232a}, imd/imd and Rsh/Rsh, were subjected to septic injury on thorax with *E.coli* bacteria. The survival rates of challenged flies over a 10 day period are indicated with continuous lines. For the same genotype non-challenged control group, the survival rate is indicated with the same color dashed line. Each genotype had 5 replicates with 15 adults in each replicate.
Figure 2.13. Larval survival rates after septic injury (29°C). Early third instar larvae of wild type, $\textit{upd3}$d$^{232a}$, $\textit{imd}/\textit{imd}$ and $\textit{Rsh}/\textit{Rsh}$ were subjected to septic injury with \textit{E.coli}. For each of the following two days, the survival rates of the non-challenged control group (gray column) and the septic injury challenged experiment group (black column) were recorded and graphed above. Each genotype had 4 replicates with 20 larvae in each replicate.
Figure 2.14. The induction of the antimicrobial peptide mRNA synthesis upon septic injury. The mRNA level of TotA, TotM, CG11501, CecA1 and Drs was examined by RT-PCR in the bacterial challenged experiment group and the non-challenged control group of wild type and upd3<sup>d232a</sup> flies. The mRNA level of all five antimicrobial peptides is low in the control wild type and upd3<sup>d232a</sup> flies and then significantly increased in experimental groups after septic injury. However, no significant difference of the mRNA level of TotA, TotM and CG11501 is observed in bacteria challenged wild type experiment group and upd3<sup>d232a</sup> experiment group, just like JAK/STAT independent antimicrobial peptides CecA1 and Drs. Each sample had two independent replicates with 30 flies in each replicate.
Figure 2.15. Circulating hemocyte concentration in unchallenged larvae. Total circulating hemocyte concentrations from late third instar larvae (120 hours after egglaying) of wild type, \textit{upd3}^{d232a}, \textit{Rsh/Rsh} and \textit{hop}^{Tum-1} were measured using a hemocytometer. *: P<0.05 (student t-test).
Figure 2.16. Circulating hemocyte concentration in challenged larvae. Total circulating blood cell concentrations of septic injury challenged (balck column) and non-challenged (gray column) wild type and $upd3^{d232a}$ larvae were counted on a hemocytometer 24 hours after challenge. **: P<0.01 (student t-test)
Figure 2.17. Wasp encapsulation assay for $upd3^{d232a}$ mutant. The second instar larvae of wild type and $upd3^{d232a}$ were subjected to female wasp L. boulardi G486 infection for 24 hours and the encapsulation event was visible in late third instar larvae with the presence of black capsules (arrows in A). The wasp infection rate of wild type ($99.55\% \pm 0.45\%, n=10$) and $upd3^{d232a}$ ($99.76\% \pm 0.24\%, n=10$) was similar, but the encapsulation capacity of $upd3^{d232a}$ ($46.45\% \pm 3.55\%, n=10$) was significantly higher than that of wild type ($21.09\% \pm 2.40\%, n=10$) (B). ***: $P<0.001$ (student t-test).
Figure 2.18. Lamellocyte differentiation in lymph glands. Beta-PS antibody is a marker for lamellocytes in the primary lobes (1°) of lymph gland and dorsal vessel (DV). In unchallenged wild type and $upd3^{d232a}$ larvae, no lamellocyte was visible in the lymph glands (A1-A3 and C1-C3). However, upon wasp infection, both wild type and $upd3^{d232a}$ had clear beta-PS staining in the primary lobes (1°) of lymph glands, indicating the presence of lamellocytes (red arrows in B3 and D3).
Figure 2.19. Phenotype of upd3 misexpression in the lymph gland. A. Larvae with misexpression of hop, upd3 genomic region (upd3g) and upd3 cDNA (upd3ss1) showed pigmentation and posterior structural defects (indicated by arrows). B. The primary lobes (1°) of 76B>upd3g lymph gland displayed hypertrophy phenotype at 29 °C and room temperature compared to that of wild type, a phenotype similar to but weaker than that of hop\textsuperscript{Tum-I}. C. No beta-PS staining was visible in the primary lobes (1°) of 76B>upd3g lymph gland. DV: dorsal vessel
Chapter Three
Molecular and Genetic Characterization of *upd, upd3* and *os*

**Introduction**

**Complex molecular and genetic relationship of *upd, upd3* and *os***

Upd and Upd3, two ligands of the *Drosophila* JAK/STAT signaling pathway, have very close genetic and molecular relationship. In chapter two, the expression pattern of *upd* and *upd3* was examined and they show coordinate expression in the posterior region of the second and early third instar eye discs and two polar cells of ovaries (Figure 2.1). But the mechanism of this coordinate expression is unknown. In addition, both *upd* and *upd3* genetically relate to a third allele class: *os*. The *os^o^* and *os^l^* alleles were generated from X-ray mutagenesis while *os^s^* is a spontaneous mutation (Verderosa and Muller, 1954; Morgan, Bridges and Sturtevant, 1925). No molecular information is available for any of the three alleles. The *os^o^*, *os^s^* and *os^l^* alleles display outstretched wings, small eyes or both. Because of the similarity with the phenotype of *upd3* mutants, possible genetic relationship of *upd3* and *os* is suggested. Beyond this, *os^o^*, *os^s^* and *os^l^* have long been recognized as alleles of *upd* due to the failure of zygotic lethal *upd* alleles, *upd^YM55^* and *upd^YC43^* to complement *os* alleles. Further, *os* is thought to be lesions in a regulatory region of *upd* because some *os*/*upd^+^* alleles have lesions at least 13 kb away from the *upd* transcript (Eberl et al., 1992; Harrison et al., 1998). With the misty and intriguing genetic and molecular relationship of *upd, upd3* and *os*, a few questions are raised: What is *os* exactly? How does it relate to both *upd* and *upd3* molecularly and genetically? How is the coordinate expression of *upd* and *upd3* regulated? Partial resolutions to these questions will be provided in this chapter.
Results

**upd and os have complex genetic relationship.**

To test whether *upd* and *os* are truly allelic to each other, a set of complementation tests were done between flies with putative deletions around the *upd/os* locus and *os* and *upd* (Table 3.1). These deletion mutants are all homozygous lethal alleles generated by different methods in different labs. They are thought to be deletions around the *upd/os* locus based on complementation tests and no molecular information is available except *os* and *os* (Eberl et al., 1992). *os* deletes all three *upd*, *upd2* and *upd3* genes while *os* deletes *upd* and the last exon of *upd3* (Hombria et al., 2005). Out of nine tested mutant lines, three groups of alleles are defined. The first group of *D2* is an *os* allele which complemented both the outstretched wing and small eye phenotype of *os* and the lethality of *upd*. The second group of alleles, including *os* and *os*, are *os* alleles which failed to complement both the outstretched wing and small eye phenotype of *os* and the lethality of *upd*. The third group of alleles, including *os* and *os*, are *os* alleles which complemented the lethality of *upd* but not the outstretched wing and small eye phenotype of *os*. This third group of alleles, *os* and *os*, separates the lethality phenotype of *upd* and the outstretched wing and small eye phenotype of *os*, indicating that *upd* and *os* are not completely allelic to each other. They have their own independent functions which can be separated by other alleles.

In addition to the complementation tests, the allelic relationship of *upd* and *os* was further challenged by a rescue test for *os* alleles. *P[sisc*, *w*]*10 is a genomic construct of *upd* which contains the complete transcription unit of *upd* and 6.5 kb upstream sequence (Sefton et al., 2000). It is able to rescue the sex specific defects of *upd* alleles (Sefton et
al., 2000). However, this upd construct rescued neither the small eye phenotype nor the outstretched wing phenotype of three os alleles (Table 3.2), indicating that either this transgenic construct is not complete enough to contain all the regulatory region of upd for the os phenotype or the os phenotype is not caused by lesions in upd, but other genes. Since upd2 and upd3 are next to upd on the X chromosome and share overlapping expression pattern with upd, it is possible that lesions in these two genes may result in the os phenotype.

Based on the complementation tests and the rescue test of os, upd and os exhibit more complex genetic relationship than simple allelism. Thus, to find out what os is molecularly and its relationship with upd, upd2 and upd3, PCR was used to analyze upd, upd2 and upd3 genes in three os alleles. Due to the big size of the complete 70 kb upd and upd-like region and high abundance of non-coding sequences, a simplified strategy was adopted to just examine the coding sequences of upd, upd2 and upd3. The purpose was to see whether the lesions of os could be identified in any of the upd genes. With two overlapping pairs of primers, each gene was amplified into two fragments and then sequenced with multiple gene specific primers. With this method, two amino acid changes were found in os1, one in upd (N365K) and the other in upd3 (Q289P) (Figure 3.1, A). However, no mutation was found in upd2 of os1 and no mutation was found in any upd gene in either os8 or os9. Due to the loss of the parental lines for os alleles, it is not clear whether these two changes in os1 are just polymorphisms or functional mutations. But from the protein alignment of Upd and Upd3 in twelve Drosophila species, the amino acid change of Q289P in Upd3 is likely to be a polymorphism because the presence of both Q and P at the same conserved site in different species (Figure 3.1, B). However, whether the amino acid change of N365K in Upd is polymorphism is less clear because there is no amino acid K at the same site in other Drosophila species (Figure 3.1, C).
Lesions of \textit{os} are found at the 5’ end of \textit{upd3}.

The mysterious genetic relationship between \textit{upd} and \textit{os} and the unknown molecular nature of \textit{os} may be elucidated with the generation of \textit{upd3} mutants. The similar phenotype of \textit{os} and \textit{upd3} alleles suggests that they may have genetic relationship. To clearly understand the genetic relationship of \textit{upd}, \textit{upd3} and \textit{os}, complementation tests were performed. The \textit{upd3} alleles, \textit{upd3}^{d232a} and \textit{upd3}^{s21b} showed wild type phenotype with \textit{upd} allele \textit{upd}^{YM53}, suggesting \textit{upd} and \textit{upd3} complemented each other and were two independent complementation groups (Table 3.3). However, the heterozygous allele of \textit{upd3}^{d232a} or \textit{upd3}^{s21b} with \textit{os} alleles, \textit{os}^{1}, \textit{os}^{o} and \textit{os}^{8} still showed the outstretched wing and small eye phenotype, indicating \textit{upd3} and \textit{os} alleles failed to complement each other (Table 3.3). Considering that \textit{upd} fails to complement \textit{os} alleles, three complementation groups exist in the \textit{upd/upd3} region, \textit{upd}, \textit{upd3} and \textit{os}. Somehow, the functions of both \textit{upd} and \textit{upd3} are affected in \textit{os}. Given the fact that \textit{upd} and \textit{upd3} are neighboring genes on the X chromosome and have overlapping expression patterns in some tissues, a model for \textit{os} is proposed: \textit{upd} and \textit{upd3} may have a common \textit{cis}-regulatory region, lesions of which cause the \textit{os} phenotype.

To test the hypothesis that \textit{os} alleles are mutations in the common \textit{cis}-regulatory region of \textit{upd} and \textit{upd3}, PCR was used to find out \textit{os} lesions in the complete 43 kb \textit{upd/upd3} region. Both the coding and non-coding sequences were examined with about 50 overlapping primer pairs representing about 1 kb amplicons. A 7.2 kb deletion and a 3.1 kb deletion were found in \textit{os}^{1} and \textit{os}^{o} alleles, respectively (Figure 3.2, A). Both lesions of \textit{os}^{1} and \textit{os}^{o} locate in the intergenic region between \textit{upd2} and \textit{upd3}, which are 3.1 kb and 6.3 kb away from the 5’ end of \textit{upd3}. The small deletion of \textit{os}^{o} falls within the big deletion of \textit{os}^{1}. Since \textit{os}^{1} displays both outstretched wing and small eye phenotype while \textit{os}^{o} only displays outstretched wing phenotype, it is assumed that the deletion in \textit{os}^{1}
includes both wing and eye enhancer elements while the deletion in \( \text{oos}^o \) only removes wing enhancer element. Therefore, the region deleted in \( \text{oos}^1 \) but not in \( \text{oos}^o \) may contain the eye enhancer element. No lesion was detected in \( \text{oos}^s \) allele, a spontaneous mutation. This may be because the lesion of \( \text{oos}^s \) is small deletion or other lesion that is not detectable by PCR or the lesion of \( \text{oos}^s \) is not in this examined 43 kb \( \text{upd}/\text{upd}3 \) region. In addition to the \( \text{oos}^1 \) and \( \text{oos}^o \) lesions, lesion of \( \text{oos}^{c18} \), a homozygous lethal \( \text{oos}^-\text{udp}^+ \) allele, was also found at the 5’ end of \( \text{upd}3 \) (Figure 3.2). However, the breakpoint of \( \text{oos}^{c18} \) lesion could not be precisely mapped because of the failure to amplify the fragment across the lesion. This may be due to a potential DNA rearrangement such as inversion, which could explain the lethality of \( \text{oos}^{c18} \). The lesion of \( \text{oos}^{c18} \) does not fall in the range of the lesion of \( \text{oos}^1 \); rather it is located between the \( \text{oos}^1 \) lesion and \( \text{upd}3 \). Since the \( \text{oos}^{c18} \) lesion is not in the presumed eye and wing enhancer region of \( \text{oos}^1 \), it may have other regulatory functions such as facilitating the enhancer effect on the promoters. In conclusion, the molecular mapping of the \( \text{oos} \) lesions defines a non-coding region at the 5’ end of \( \text{upd}3 \) as potential common \textit{cis}-regulatory region for both \( \text{upd} \) and \( \text{upd}3 \).

The expression of \( \text{upd} \) and \( \text{upd}3 \) is altered in \( \text{oos} \) alleles.

The model of \( \text{oos} \) as a common \textit{cis}-regulatory region for both \( \text{upd} \) and \( \text{upd}3 \) predicts that the expression of \( \text{upd} \) and \( \text{upd}3 \) will be altered or lost in \( \text{oos} \) alleles. To test this, the expression pattern of \( \text{upd} \) and \( \text{upd}3 \) was examined in \( \text{oos} \) alleles and compared with that in wild type by doing \textit{in situ} hybridization to \( \text{udp} \) and \( \text{udp}3 \) mRNA. Since both \( \text{udp} \) and \( \text{udp}3 \) are expressed consistently and strongly in the posterior region of wild type eye discs, the eye disc was chosen as the tissue to check the expression of \( \text{udp} \) and \( \text{udp}3 \) in \( \text{oos} \) alleles which have small eye phenotype, \( \text{oos}^1 \) and \( \text{oos}^s \). As predicted, the expression of \( \text{udp} \) and \( \text{udp}3 \) in \( \text{oos}^1 \) eye discs was completely lost, suggesting that the lesion in \( \text{oos}^1 \) affects the
transcription of both * upd* and * upd3* (Figure 3.3, C and D). However, the situation was complicated in * os* allele. Although the expression of * upd* was lost in * os* allele, the expression of * upd3* still remained (Figure 3.3, E and F). But it is not clear whether there is a quantitative difference of the * upd3* expression in * os* eye discs and wild type eye discs. Nevertheless, the altered expression of * upd* and * upd3* in * os* and * os* alleles supports the hypothesis that * os* alleles are caused by mutations in a common * cis*-regulatory region of * upd* and * upd3*.

**Enhancer reporter assays reveal the enhancer property of os lesions.**

The model of * os* as a regulatory region indicates that the regions deleted in * os* should have enhancer properties. To test this, enhancer reporter assays, which make use of reporter genes such as * GFP* and * lacZ*, to analyze the expression pattern of enhancers and promoters, were performed (Barolo et al., 2000). The hypothetical enhancer regions for both wing and eye (* WE*, 7.8 kb fragment covering the deletion in * os*), just wing (* W*, 4.4 kb fragment covering the deletion in * os*), and just eye (* E*, 3.4 kb fragment including the region deleted in * os* but not in * os*) were amplified by PCR and constructed in a * GAL4* vector * pPelican-GAL4* (Figure 3.2). Transformant flies carrying * WE-GAL4*, * W-GAL4*, and * E-GAL4* constructs were generated respectively. According to the hypothesis, * WE-GAL4*, * W-GAL4*, and * E-GAL4* should have the * GAL4* expression driven by both wing and eye enhancers, just wing enhancer and just eye enhancer. By crossing three * GAL4* fly lines with * UAS-GFP*, the expression patterns of three enhancer regions were assayed through the expression of GFP in imaginal discs with antibody staining.

In * WE>GFP* (* WE-GAL4 UAS-GFP*), GFP expression is expected in both eye and wing imaginal discs. However, only the eye discs showed the expression of GFP at the
posterior region of late third instar eye disc. This expression is similar to the upd and upd3 mRNA expression in the posterior region of eye discs detected with in situ hybridization. However, the GFP expression pattern was broader than the in situ staining and the GFP expression was detected only in the late third instar while the in situ staining of upd and upd3 shows up at the second and early third instar. To specify the cells expressing GFP, ELAV antibody staining was performed. ELAV is a molecular marker for differentiated photoreceptor cells. With the merge of GFP antibody staining and ELAV antibody staining, all the GFP positive cells were also ELAV positive while not all ELAV positive cells were GFP positive, suggesting that the enhancer is active in a subset of differentiated photoreceptor cells (Figure 3.4, top panel).

Consistent with no GFP expression detected in wing discs of WE>GAL4, GFP expression was also not detected in wing discs of W>GFP (W-GAL4 UAS-GFP), which is supposed to have the wing enhancer (data not shown). In addition, no GFP expression was detected in other imaginal discs of W>GFP. For E>GFP (E-GAL4 UAS-GFP), GFP expression is expected in eye discs, the same as WE>GFP. But rather, the GFP expression was detected in the tarsus region of the late third instar leg discs (Figure 3.4, bottom panel). This is different from the expression of upd in the presumed tibia, femur and coax regions detected in an upd enhancer reporter line (Ayala-Camargo et al., 2007).

Overall, the results of enhancer reporter assays suggest that the os lesions do have enhancer properties by driving the expression of GFP in eye discs and leg discs. However, the temporal and spatial expression pattern of GFP is not as neat as expected. In WE>Gal4 and W>GAL4, which are supposed to contain the same wing enhancer, no GFP expression is detected in wing discs, suggesting that the deletion in os° allele is required but not sufficient for the wing enhancer function. This could be due to the
incompleteness of the wing enhancer constructed in $WE>GAL4$ and $W>GAL4$. In addition, although both of $E>GFP$ and $WE>GFP$ are supposed to have the eye enhancer, the expression of GFP is only detected in the eye discs of $WE>GFP$ but not $E>GFP$. This suggests that the presumed eye enhancer in $WE>GFP$ is sufficient for the eye enhancer function while the presumed eye enhancer in $E>GFP$ is not. Given the fact that the only difference between $E>GFP$ and $WE>GFP$ is the lack of $os^o$ lesion region in $E>GFP$, it suggests that the $os^o$ lesion includes part of the eye enhancer region. Additionally, the expression of GFP in the leg discs of $E>GFP$ but not $WE>GFP$ suggests that there may be silencers in the $os^o$ deletion region which prevents the expression of GFP in leg discs of $WE>GFP$. Furthermore, although GFP expression is detected in eye discs of $WE>GFP$, its expression pattern is broader and its expression time is later than those of the $upd$ and $upd3$ mRNA expression in the posterior region of eye discs. This mismatch of the expression pattern and time could be due to the properties of GFP reporter and GAL4 protein, which may have delayed and augmented the expression. But it may also indicate the missing of other necessary cis-regulatory elements required for the proper tuning of GFP expression. One candidate for such cis-regulatory elements could be the $os^{c18}$ lesion region, which locates between the $os^I$ deletion and $upd3$. The $os^{c18}$ lesion may be able to regulate the expression of $upd$ and $upd3$ in the second and early third instar and restrict the GFP expression pattern through the binding with certain transcription factors. Taken together, the enhancer reporter assays suggest that the $os$ enhancer region is bigger and more complicated than just the deletion recovered in $os^I$. Besides the eye and wing enhancers, there may be other cis-regulatory elements, including both positive and negative regulators. They function cooperatively with the eye and wing enhancers to ensure the correct temporal and spatial pattern of $upd$ and $upd3$. 
Discussion

A model for *os*

In this chapter, the genetic complementation tests between *upd, upd3* and *os* show that both *upd* and *upd3* fail to complement *os* while *upd* and *upd3* complement each other, demonstrating three complementation groups, *upd, upd3* and *os* in the *upd/upd3* region. Somehow, functions of both *upd* and *upd3* are affected in *os* alleles. Consistent with the complementation tests, the coordinate expression of *upd* and *upd3* is lost in *os* alleles. Therefore, a model for *os* is proposed that *os* alleles are mutations in a common *cis*-regulatory region of *upd* and *upd3*, lesions of which cause the outstretched wing and/or small eye phenotype. Later, the molecular mapping of *os* alleles defines lesions of *os* at the 5’ end of *upd3*. The deletion of *os<sup>o</sup>* is within the deletion of *os<sup>l</sup>* while the deletion of *os<sup>c18</sup>* is between *os<sup>l</sup>* lesion and *upd3*. Furthermore, the enhancer property of the *os* lesion regions is supported by enhancer reporter assays with GFP as a reporter. However, the inconsistency of the GFP expression pattern with the predictions suggests the complexities of the *os* regulatory region. Thus, the model for *os* is modified and shown in figure 3.5: the expression of *upd* and *upd3*, especially in eyes and wings, is co-regulated by a common *cis*-regulatory region at the 5’ end of *upd3*. This regulatory region contains both eye and wing enhancers for *upd* and *upd3*. Both eye and wing enhancers are affected in *os<sup>l</sup>* deletion while only the wing enhancer is affected in *os<sup>o</sup>* deletion. According to the enhancer reporter assays, more *cis*-regulatory elements may be involved in this transcriptional network. A potential silencer in the *os<sup>o</sup>* lesion prevents the expression of *upd* and *upd3* in leg discs. The lesion of *os<sup>c18</sup>* may be also part of the enhancer regulatory region. In addition to the *cis*-regulatory elements, *trans*-elements or chromatin modification such as conformational change may be needed for the
transcriptional regulation of *upd*, which is one gene away from the *os* enhancer region. However, the exact mechanism is not clear.

**Rescue constructs for os alleles (hypothetical)**

To fully understand the molecular nature of *os* and the transcriptional co-regulation mechanism of *upd* and *upd3*, the complete *os* regulatory region must be recovered. However, the molecular mapping of classical *os* alleles only reveals part of this region. Future research is needed to define the complete *os* regulatory region and two strategies can be adopted. One is to make a rescue construct for *os* alleles. So far, no construct, including an *upd3* genomic construct and an *upd* genomic construct P{**sisc**+, **w**+}10, has been able to rescue the outstretched wing and/or small eye phenotype of *os* alleles (Figure 3.6, black lines). According to the *os* model, the complete rescue construct for *os* must include *upd*, *upd3* and a big portion of the intergenic region between *upd2* and *upd3* (Figure 3.6, blue line). If this construct can rescue the outstretched wing and/or small eye phenotype of both *os* and *upd3* alleles, it suggests that the intergenic region included in this construct contains the complete *os* regulatory elements. Then, deletion mapping can be used to precisely locate the eye enhancer, wing enhancer, or other individual regulatory elements respectively by generating different deletion constructs in the *os* region. To distinguish from this complete rescue construct, two partial constructs can also be made: One partial construct would only contain *upd3* and the same intergenic region between *upd2* and *upd3* as the complete rescue construct (Figure 3.6, yellow lines). Same as the previous construct, if the intergenic region contains the complete *os* regulatory element, this partial construct should rescue the *os* phenotype of *upd3* alleles but not *os* alleles due to the missing of *upd*. The other partial construct would only contain *upd* and *upd3* but not the intergenic region between *upd2* and *upd3* (Figure 3.6, pink line). This
construct should not rescue the os phenotype of either upd3 or os alleles due to the missing of the regulatory region. With these three constructs, the components of the os regulatory region and the transcriptional relationship of os, upd and upd3 will be more evident and explicit.

In addition to making a rescue construct for os, generating new os alleles is another way to map the complete os regulatory region. By performing P element mobilization mutagenesis with $P\{XP\} upd3^{04951}$, which has a P element inserted at the 5’ end of upd3, new os alleles are expected to be recovered and they should fail to complement both upd and upd3 (Bellen et al., 2004; Thibault et al., 2004). Molecular mapping of the new os alleles, especially excision mutants which have genomic regions deleted, will provide detail information for the locations and sizes of the cis-elements in the os regulatory region.
Table 3.1. Complementation tests between different mutations around $upd/os$ locus and $os^I$ and $upd^{YM55}$ alleles.

<table>
<thead>
<tr>
<th></th>
<th>$os^I$</th>
<th>$upd^{YM55}$</th>
<th>$os^I A$</th>
<th>Class</th>
</tr>
</thead>
<tbody>
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<td>$D2$</td>
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<td>WT</td>
<td>L</td>
<td>os$^+upd^+$</td>
</tr>
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<td>L</td>
<td>L</td>
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<td>L</td>
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<td>L</td>
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<td>L</td>
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<tr>
<td>$os^{1A}$</td>
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<td>L</td>
<td>os$^+upd^-$</td>
</tr>
<tr>
<td>$os^{109}$</td>
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<td>S; O</td>
<td>L</td>
<td>os$^+upd^-$</td>
</tr>
<tr>
<td>$os^{c18}$</td>
<td>S; O</td>
<td>S; O</td>
<td>L</td>
<td>os$^+upd^-$</td>
</tr>
</tbody>
</table>

WT: wild type; S: small eye; O: outstretched wing; L: lethal

This table represents complementation results of alleles listed in the first column with alleles on the first row. Tested alleles in the first column are homozygous lethal and presumed deletion mutations around $upd/os$ region. They all failed to complement $os^{1A}$.

Based on complementation results with $os^I$ and $upd^{YM55}$, they were classified into three groups: os$^+upd^+$, os$^+upd^-$ and os$^+upd^+$. The os$^+upd^+$ allele group complemented both $os^I$ and $upd^{YM55}$ and showed wild type phenotype with both alleles. The os$^+upd^-$ allele group failed to complement both $os^I$ and $upd^{YM55}$ allele by showing os phenotype with $os^I$ and lethal phenotype with $upd^{YM55}$. The os$^+upd^+$ allele group failed to complement $os^I$ but did complement $upd^{YM55}$ by showing os phenotype with both $os^I$ and $upd^{YM55}$. 


Table 3.2. Rescue test of os alleles by an upd genomic construct.

<table>
<thead>
<tr>
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<th>wing</th>
</tr>
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<tbody>
<tr>
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<td>O</td>
</tr>
<tr>
<td>os/o</td>
<td>WT</td>
<td>O</td>
</tr>
<tr>
<td>os/s</td>
<td>S</td>
<td>WT</td>
</tr>
</tbody>
</table>

WT: wild type; S: small eye; O: outstretched wing; L: lethal;

Transgenic construct $P\{\text{sisc}^+, \text{w}^+\}10$ is a genomic construct of upd which contains the complete transcription unit of upd and 6.5 kb upstream sequences (Sefton et al., 2000). It failed to rescue the outstretched wing and/or small eye phenotype of os/l, os/o and os/s.
Table 3.3. Complementation tests between *upd*, *upd3* and *os*.

<table>
<thead>
<tr>
<th></th>
<th><em>upd</em>&lt;sup&gt;YM55&lt;/sup&gt;</th>
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<th>*os&lt;sup&gt;o&lt;/sup&gt;</th>
<th>*os&lt;sup&gt;s&lt;/sup&gt;</th>
<th>*upd3&lt;sup&gt;d232a&lt;/sup&gt;</th>
<th>*upd3&lt;sup&gt;x21b&lt;/sup&gt;</th>
<th>*os&lt;sup&gt;UE69&lt;/sup&gt;</th>
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<td>WT</td>
<td>L</td>
<td>L</td>
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<tr>
<td>*os&lt;sup&gt;l&lt;/sup&gt;</td>
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<td>S; O</td>
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<td>S; O</td>
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<td>*os&lt;sup&gt;o&lt;/sup&gt;</td>
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<td>O</td>
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<td>S</td>
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<td>S; O</td>
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<td>S; O</td>
<td>O</td>
<td>S</td>
<td>S; O</td>
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<td>S; O</td>
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<td>*os&lt;sup&gt;c18&lt;/sup&gt;</td>
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<td>*os&lt;sup&gt;UE69&lt;/sup&gt;</td>
<td>L</td>
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<td>*os&lt;sup&gt;1A&lt;/sup&gt;</td>
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WT: wild type; S: small eye; O: outstretched wing; L: lethal; NA: non-available

In this table, all tested *upd*, *os* and *upd3* alleles failed to complement both *os<sup>UE69</sup>* and *os<sup>1A</sup>*. *upd<sup>YM55</sup>* complemented both *upd3<sup>d232a</sup>* and *upd3<sup>x21b</sup>*. However, both *upd<sup>YM55</sup>* and *upd3* alleles of *upd3<sup>d232a</sup>* and *upd3<sup>x21b</sup>* failed to complement the *os* alleles of *os<sup>l</sup>* , *os<sup>o</sup>* and *os<sup>s</sup>*. Alleles *os<sup>109</sup>* and *os<sup>c18</sup>* failed to complement all *upd*, *os* and *upd3* alleles while N19 can complement the small eye phenotype but not the outstretched wing phenotype of *os<sup>l</sup>* , *os<sup>s</sup>* and *upd3<sup>d232a</sup>*.
Figure 3.1. Amino acid changes found in *upd* and *upd3* genes of *osI*. (A). With PCR, the *upd*, *upd2* and *upd3* genes were amplified with two pairs of primers for each gene in *osI*, *osO* and *osS* alleles. Two amino acid changes were found in *upd* (N365K) and *upd3* (Q289P) of *osI*. But no change was found in *upd2* of *osI* allele and no change was found in any *upd* genes of *osO* and *osS*. (B, C) Proteins of Upd and Upd3 from twelve and eleven *Drosophila* species were aligned with ClustalW2 software and the amino acid changes found in Upd and Upd3 of *osI* allele were indicated at their mutation sites in red and blue respectively. NC: no change.
Figure 3.2. Mapping of os allele lesions. (A) With PCR, lesions of os\textsuperscript{1}, os\textsuperscript{0} and os\textsuperscript{c18} were found at the 5' end of upd3. The breakpoint of os\textsuperscript{c18} lesion could not be precisely mapped and indicated by “?”. No lesion was found in os\textsuperscript{s}. (B) Presumed wing and eye enhancers (WE), just wing enhancer (W) and just eye enhancer (E) were amplified with PCR and constructed in \textit{pPelican-GAL4} vector for enhancer reporter assays.
Figure 3.3. Comparison of the \textit{upd} and \textit{upd3} expression in wild type and \textit{os} alleles.

With \textit{in situ} hybridization, both \textit{upd} and \textit{upd3} were detected in the posterior region of wild type eye discs (arrows in A and B). However, this expression of \textit{upd} and \textit{upd3} was completely lost in \textit{os}^1 eye discs (C and D). In \textit{os}^s eye discs, the expression of \textit{upd} was lost (E) but expression of \textit{upd3} remained (arrow in F).
Figure 3.4. Enhancer reporter assays. In *WE-GAL4 UAS-GFP (WE>GFP)* (top panel), GFP expression was detected in the posterior region of late third instar eye discs. With ELAV staining, which marks the differentiated photoreceptor cells, all the GFP expressing cells were shown to be ELAV positive cells (big arrow) while not all the ELAV positive cells expressed GFP (small arrow). In *E-GAL4 UAS-GFP (E>GFP)* (bottom panel), GFP expression was detected in the tarsus region of the late third instar leg discs (indicated by arrow).
Figure 3.5. Transcriptional co-regulation model for *upd* and *upd3*. This is a schematic model of the transcriptional co-regulation of *upd* and *upd3* by a common *cis*-regulatory region at the 5' end of *upd3*. This common *cis*-regulatory region includes both wing enhancer (W) and eye enhancer (E) for *upd* and *upd3*. In addition, a potential silencer for the expression of *upd* and *upd3* in leg discs (S(L)) which helps to finely tune the spatial and temporal expression pattern of *upd* and *upd3* is also shown in the figure.
Figure 3.6. Potential rescue constructs for os alleles. Both the upd3 genomic construct and upd genomic construct could not rescue os alleles (black lines). A complete rescue construct for os will include os regulatory region, upd and upd3 genes (blue line) which can completely rescue the outstretched wings and small eyes of os and upd3. Two partial constructs will also be made for comparison. One partial construct containing os regulatory region and upd3 gene will only rescue the os phenotype of upd3 alleles but not os alleles (yellow line). The other partial construct containing upd and upd3 genes without os regulatory region will not rescue the os phenotype of either os or upd3 alleles (pink line). The “+” in the parenthesis indicates the predicted rescue of the os phenotype while the “−” in the parenthesis indicates the predicted failure to rescue the os phenotype.
Chapter Four
Conclusions and Discussion

In this dissertation, the functional roles of Upd3 in Drosophila development and its coordinate expression with udp were investigated. As a ligand of the Drosophila JAK/STAT pathway, Upd3 has similar functions with Upd in eye size regulation, wing development and haltere development while Upd3 has potential redundant function with Upd or Upd2 in immune response. The expression of upd3 is coordinately regulated with upd, possibly by a common cis-regulatory element, lesions of which lead to the outstretched wing and/or small eye phenotype.

Subfunctionalization of duplicated genes in upd gene family

The upd gene family contains three duplicated genes that have similar amino acid sequences. Based on the identification of all three upd homologs in other species of Drosophilidae family, the duplication of the upd gene family is assumed to be before Drosophilidae speciation, about 40 million years ago. This hypothesis is consistent with the functional expression of all three upd genes in Drosophila. A genomic analysis for gene expression in C.elegans suggests that most newly duplicated genes are not expressed (Mounsey et al., 2002). Therefore, functional expression patterns of upd genes suggest their duplication occurred at long time ago. But how did the upd genes avoid being selected against after duplication: neofunctionalization or subfunctionalization? The functional analysis of Upd, Upd2 and Upd3 provides some clues.

In chapter two, the functional relationship of Upd and Upd3 is revealed as tissue specific. In Drosophila eye development, Upd and Upd3 have similar functions in eye
size regulation. Loss of either leads to small eye phenotype, suggesting both Upd and Upd3 are required for eye development. However, in *Drosophila* immune response and oogenesis, a redundant functional relationship of Upd or Upd2 with Upd3 is suggested. In loss of function mutants of *upd3*, no defect was observed in antimicrobial peptide synthesis, blood cell differentiation, and migrating border cell number, presumably due to the compensation by the endogenous redundant Upd or Upd2. In addition, the functional relationship of Upd and Upd2 during embryogenesis has been reported by other research groups. In embryogenesis, null alleles of *upd* are lethal and display structural defects in embryonic segmentation, posterior spiracle and head skeleton while *upd2* null alleles are viable and fertile, indicating the loss of Upd2 may be compensated by endogenous Upd or Upd3 (Hombria et al., 2005). However, loss of *upd2* can slightly enhance the embryonic defects caused by *upd* alleles, suggesting that although Upd2 is not required for embryo development, it may have subtle function in supporting the role of Upd (Gilbert et al., 2005; Hombria et al., 2005).

Collectively, the studies of Upd, Upd2 and Upd3 suggest subfunctionalization phenomenon for *upd* duplicated genes: The redundant functions of *upd* gene are probably the results of their original duplicated gene fate. However, the similar but not redundant functions of Upd and Upd3 in eye size regulation and others and that of Upd and Upd2 in embryonic segmentation imply that they are dividing or have divided the functional capacity of the original ancestral gene. Each copy only represents part of the original ancestral gene function. Furthermore, the subfunctionalization of *upd* genes is asymmetric in dividing functions of the ancestral genes, which means the functional capacities of Upd, Upd2 and Upd3 are not equal. The loss-of-function phenotype of *upd2* and *upd3* is weaker than that of *upd*. Loss of Upd3 in ovaries does not result in visible defects and reduced number of border cells while reduced Upd results in reduced border cell number and migration defect (Silver and Montell, 2001; Xi et al., 2003). The
embryonic segmentation defects caused by loss of Upd2 are much weaker than that caused by loss of Upd (Hombria et al., 2005). Therefore, it seems the functional capacity of Upd2 and Upd3 is less than that of Upd in most developmental processes studied. In addition, the redundant functions of Upd with Upd2 and Upd3 may provide flies with a tissue specific buffering system for mutations in one of the genes.

**New insight into transcriptional co-regulation for clustered duplicated genes**

Transcriptional regulation, one of the most important eukaryotic gene expression regulation mechanisms, is achieved by the interplay between cis-acting DNA elements and trans-acting protein elements such as transcription activators and repressors. The cis-acting DNA elements include core promoter, enhancer, and silencer (Blackwood and Kadonaga, 1998; Lee and Young, 2000). The core promoter is sufficient for the initiation of the basal transcription by binding to the basal (or general) transcription factors. Transcription activators bind to enhancers where they recruit chromatin-modifying complex and transcription apparatus to induce spatial and temporal specific gene expression (Lee and Young, 2000). The silencers can inhibit gene transcription by binding to sequence specific repressors. The inhibition mechanism is various including preventing the binding of activators, preventing the recruitment of the transcription apparatus by activators and chromatin modification (Hanna-Rose and Hansen, 1996). Chromatin modification includes non-covalent modification (conformational remodeling) and covalent modification of histone acetylation, phosphorylation, methylation, and ubiquitination (Cairns, 1998; Davie and Murphy, 1990; Davie and Murphy, 1994; Davie and Spencer, 1999; De Cesare et al., 1998; Grunstein, 1997; Hendzel and Davie, 1989; Hendzel and Davie, 1991; Imbalzano, 1998; Spencer and Davie, 1999). The influence of chromatin modification on gene transcription is dependent on the requirement of promoters and the higher order chromatin structure. Usually, the packaging of DNA into
nucleosomes and then into higher order chromatin structure is thought to repress gene transcription. For example, histone acetylation is thought to induce transcription by disrupting the high order chromatin structure and histone deacetylation is thought to repress transcription by forming high order chromatin structure (Davie and Spencer, 1999; Garcia-Ramirez et al., 1995; Grunstein, 1997; Spencer and Davie, 1999).

In eukaryotic genomes, genes were previously assumed to be randomly distributed and one enhancer only acts on one gene whose promoter is the closest. However, with the completion of genome sequencing in many organisms such as yeast, plants, fruit flies, mice and human, more evidence suggests that genes with similar functions and/or expression patterns are likely to cluster together in the genome (Hurst et al., 2004; Vogel et al., 2005). Furthermore, clustered genes may share the same set of cis-acting regulatory elements for coordinate expression. Specific examples are Drosophila yp1-yp2 genes, Drosophila AS-C complex and human β-globin gene family. The Drosophila yp1 and yp2 are two neighboring genes encoding yolk proteins and only expressed in female ovarian follicle cells and fat bodies (Barnett et al., 1980; Brennan et al., 1982; Garabedian et al., 1985; Garabedian et al., 1986). The expression of yp1 and yp2 is controlled by two cis-regulatory elements between them: the ovarian enhancer and the fat body enhancer which specify the expression of yp1 and yp2 in ovaries and fat bodies respectively (Logan et al., 1989). In addition, a cis-acting element in the second exon of yp2 influences the expression level of yp1 in ovaries (Logan et al., 1989). This example of yp1-yp2 provides a very simple transcriptional co-regulation model for two divergently transcribed neighboring genes with shared enhancers between them. However, the coordinate transcription regulation can be more complex with the shared enhancers being far away from the regulated genes such as the Drosophila AS-C complex.
In *Drosophila*, four *ac-sc* complex (*AS-C*) genes arise from three independent gene duplications in a 90 kb genomic region (Skaer et al., 2002). The expression of the unidirectionally transcribed genes *achaete* and *scute* is restricted to a few proneural clusters of imaginal discs and they have the same pattern with identical position, size, shape, time of emergence and time of disappearance (Cubas et al., 1991; Skeath and Carroll, 1991). This amazing co-expression pattern of *ac* and *sc* results from the regulation of a single set of *cis*-regulatory elements scattered along the 90 kb *AS-C* genomic region, with each element having unique temporal and spatial properties (Gomez-Skarmeta et al., 1995). The enhancer elements are located upstream of, downstream of, or between *ac* and *sc*. It is assumed that the enhancers act on the promoters of *ac* and *sc* with equal efficiency due to similar expression level of *ac* and *sc* (Gomez-Skarmeta et al., 1995). Additionally, other elements in *AS-C* region are found to regulate the transcription of *ac* and *sc*. The removal of a group of E-boxes, which are binding sites for basic-helix-loop-helix (bHLH) proteins and present upstream of the promoters of both *ac* and *sc*, promotes the expression of *ac* and *sc* in sites where they are not normally expressed, suggesting the E-boxes may be silencers (Gomez-Skarmeta et al., 1995; Martinez et al., 1993). In conclusion, the transcriptional regulation of *AS-C* complex suggests that shared enhancers can regulate the transcription of genes independent of orientation and distances. Enhancers can even act on genes that are one gene away. In addition, the *AS-C* complex also suggests a model of transcriptional regulation controlled by multiple *cis*-acting elements including both enhancers and silencers.

In addition to *Drosophila*, transcriptional co-regulation has also been seen in other organisms such as yeast, *C.elegans*, humans and more, suggesting this is a common transcription mechanism for clustered genes in eukaryotes. For example, the human β-globin genes cluster in a 70 kb region on chromosome 11 with five expressed genes
arranged in order of 5' - ε - Gγ - Aγ - δ – β – 3', which are all exclusively expressed in erythroid cells at specific developmental stages consistent with their gene order: ε is expressed during early embryogenesis, Gγ and Aγ are expressed in fetal period, δ and β are expressed in adult (Martin et al., 1996). The β locus control region (β LCR), 6-26 kb non-coding region upstream of ε gene, is a cis-regulatory element for the tissue specific and high level expression of five β-globin genes. In the deletion of human β-globin LCR, the complete loss of transcription is observed (Reik et al., 1998; Schubeler et al., 2000). However, the LCR region alone does not explain regulatory switching at different developmental stages. Although the mechanism of gene switching is still not very clear currently, it is thought be controlled by many factors including both cis and trans-acting factors in the chromatin context (Shen et al., 2001): At embryonic stages, β LCR recruits chromatin remodeling complexes such as SWI/SNF and HAT (histone acetyltransferase) to open the local chromatin upstream of ε (Shen et al., 2001). Meanwhile, SSP (stage specific proteins) interacts with SSE (stage-selector element) in the ε promoter to activate RNA Pol II and induce the expression of ε. Later, ε globin gene is silenced autonomously and γ gene starts transcription. γ is silenced by some transcription factors during fetal/adult stage which may results in the opening of the local β gene chromatin. Then the cooperation of the looped LCR and adult β gene transcription factor EKLF induces the transcription of β gene. Compared to the Drosophila yp1-yp2 and AS-C complex model, transcriptional co-regulation of human β-globin gene requires multiple levels’ regulation of many cis-acting elements, trans-acting elements and chromotin modifications, demonstrating the complexity of coordinate transcriptional regulation.

Herein, a neat and well regulated co-expression model is proposed for upd and upd3. The upd gene family, upd, upd2 and upd3, clusters in a 70 kb region on the X chromosome. upd and upd3 are convergently transcribed and have overlapping expression pattern in the second and early third instar eye discs and female adult ovaries.
The genetic and molecular analysis of the coordinate expression of \textit{upd} and \textit{upd3} suggests that there may be a common \textit{cis}-regulatory region for \textit{upd} and \textit{upd3} at the 5’ end of \textit{upd3}. Deletions of this region result in the loss of \textit{upd} and \textit{upd3} expression. In addition, enhancer reporter assays with the genomic DNA in this presumed common \textit{cis}-regulatory region suggest that multiple DNA elements may exist in this region, including enhancers, silencer and some other \textit{cis}-acting elements facilitating the fine tuning of the spatial and temporal expression \textit{upd} and \textit{upd3}. Furthermore, chromatin conformational change may be also needed for the regulation of \textit{upd} which is one gene away from the \textit{cis}-regulatory region. Overall, the proposed \textit{upd} and \textit{upd3} co-regulation model potentially contains varieties of regulatory elements for transcriptional regulation. The complete dissection of each individual element will be necessary for understanding the regulation of \textit{upd} and \textit{upd3}. Considering the conservation of gene clusters and transcriptional co-regulation mechanism, the study of the coordinate expression of \textit{upd} and \textit{upd3}, along with the examples of \textit{yp1-yp2} genes, \textit{AS-C} complex and human \(\beta\)-globin genes, will provide detailed information for the co-regulated transcription of clustered genes not only in \textit{Drosophila} but also in other eukaryotic organisms.

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Fly strains, bacteria strain and wasp strain

All the flies were raised at 25°C unless otherwise noted. $P\{XP\}^{upd3d00871}$ and $CG6023^{d04993}$ were from Bloomington Drosophila stock center. $Socs36E^{rev330B}$, $Socs36E^{i89a}$, $Socs44A^{291a}$ and $Socs36E^{i89a}Socs44A^{291a}$ were generated in the lab (Thesis of Qian Guo, 2007). $Rsh$ and $Imd$ were obtained from Dr. Schneider’s lab (Elrod-Erickson et al., 2000). Other flies, $os^t$, $os^o$, $os^s$, $upd^{TM55}$, $upd^{YC43}$, $Stat92E^{16C8}$, $FRT82B/TM3$, $Stat92E^{06346}$ $FRT82B/TM3$ and $ry^{506}$ $FRT82B$ were all from Dr. Perrimon. Bacterial $E. coli (pHC60)$ is a gift from Dr. Schneider’s lab. It contains plasmid $pHC60$ which constitutively expresses GFP and is tetracycline resistant (Elrod-Erickson et al., 2000). Wasp $L. boulardi G486$ is a gift from Dr. Govind’s lab (Sorrentino et al., 2004).

In situ hybridization

Sense and antisense probes of $upd$ and $upd3$ were generated by linearizing plasmids $PBS-GR51$ ($upd$ plasmid) and $PBS-1FK/2RX$ ($upd3$ plasmid) with Rsal enzyme. Then digoxigenin labeled DNA probes were made by asymmetric amplification with appropriate primers. Primers used for making $upd$ antisense probe were $upd-488R$, $upd-1049R$ and $upd-1498R$. Primers used for making $upd$ sense probe were $upd-381$, $upd-987$ and $upd-679$. Primers used for making $upd3$ antisense probe were $upd3-7732R$, $5963-2R-Xba$, $5963-4367R$ and $5963-GSP1$. Primers used for making $upd3$ sense probe were $5963-281F$ and $5963-GSP-2$. The PCR program was 30 cycles of 95°C for 45 seconds, 55°C for 30 seconds and 72°C for 1 minute. Obtained probes were stored in 300
ul hybridization buffer (50% formamide, 5X SSC, 0.2 mg/ml Salmon Sperm DNA, 0.1 mg/ml yeast tRNA, 0.5 mg/ml heparin, 0.1% Tween-20). For in situ hybridization, 1:5 or 1:3 diluted probes were used and each probe could be re-used for at least three times.

Primer sequences:
Upd-488R: CGAAGTTGCGATAGTCGATCC
Upd-1049R: AGCGCAGCTTCAAACGCTTGTCA
Upd-1498R: GAGTCCTGAGGTAAGGGGAAATGG
Upd-381: CGGCTTCAGCTCAGCATCCC
Upd-679: GTCCCTCCACACGCACAACCTAC
Upd-987: GTTGGCGGCACCAC
5963-281F: CCCAGTTGCCCTCTCCGGC
5963-2R-Xba: CGTCTAGAGTTTCTTCTGGATCGCC
5963-4367R: ATCAGCTTGCGCGGCAGTATTTGTA
5963-GSP1: CGTCGGAGAGCACGCTCTTCGC
5963-GSP-2: GAGAACACCTGCAATCTGAAGCCCACGG
Upd3-7732R: CTGCGTAGGTGGTCAGCGCGAAAGTGGCC

Ovary in situ hybridization

The solution volume was 1 ml and washing time was 5 minutes on a rotator unless otherwise noted. Ovaries were dissected in PBS (7 mM Na2HPO4, 3 mM NaH2PO4, 130 mM NaCl ) and fixed in 3.7% formaldehyde for 20 minutes on a rotator at room temperature, followed by one time washing with 1 ml methanol and rehydrating in methanol:PBT (PBS, 0.1% Tween-20) (3:1, 1:1, and 1:3). Then, ovaries were washed 3 times with PBT before incubated in 50 ug/ml protease K/PBT for 1 hour at room temperature without rotation. The protease K digestion was stopped by quick rinsing ovaries in 2 mg/ml glycine/PBT once and then incubating ovaries in 2 mg/ml
glycine/PBT for 2 minutes with rotation. Ovaries were re-fixed in 3.7% formaldehyde for 20 minutes with rotation after two times’ washing with PBT. After fixation, ovaries were washed 5 times with PBT and then incubated with 100 ul hybridization buffer:PBT (1:1) and 100 ul hybridization buffer without rotation for 10 minutes. Ovaries were prehybridized in 100 ul hybridization buffer at 45°C for 1 hour and then hybridized with appropriate DNA probes (1:5 dilution) at 45°C for overnight. Probes were boiled at 100°C for 3 minutes before use. After hybridization, ovaries were subjected to washing with 100 ul hybridization buffer and 100 ul PBT:hybridization buffer (1:1) at 45°C for 10 minutes each, followed by five times PBT washing. Then mouse anti-dig antibody (1:2000) was applied to ovaries for 4 hours at room temperature. After washed with PBT for 3 times and pH 9.0 solution (0.1 M Tris9.2, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20) for 3 times, ovaries were developed in 1ml pH 9.0 solution with 4.5 ul 175 mg/ml NBT and 8.75 ul 120 mg/ml BCIP in dark for 30 - 45 minutes. The development was stopped by washing ovaries in 10 mM EDTA/PBT for several times.

*Imaginal discs and lymph glands in situ hybridization*

The solution volume was 1 ml and washing time was 5 minutes unless otherwise noted. No rotation was allowed in the whole process. Imaginal discs or lymph glands were dissected in PBS by tearing larvae apart. Dissected discs or lymph glands were fixed in 4% paraformaldehyde for 20 minutes at room temperature. Following fixation, tissues were washed three times in PBT and then protease K digestion (12.5 µg/ml) was applied for 10 minutes, stopped by washing tissues with 2 mg/ml glycine/PBT for two times. Tissues were re-fixed in 4% paraformaldehyde/PBT for 20 minutes, followed by 4 times PBT washing. Before prehybridization, discs or lymph glands were incubated in 100 ul PBT:hybridization buffer (1:1) and 100 ul hybridization buffer for 10 minutes. Discs or lymph glands were prehybridized in 100 ul hybridization buffer at 45°C for 1 hour and then hybridized with appropriate probes (1:5 dilution) at 45°C for overnight.
Probes were boiled at 100°C for 3 minutes before use. After hybridization, discs or lymph glands were washed in 100 ul of hybridization buffer: PBT (100:0, 80:20, 60:40, 40:60, 20:80) solutions for 20 minutes each at 45°C, followed by two times washing in PBT for 20 minutes each. The following antibody staining with mouse anti-dig antibody was the same as the staining in ovary in situ hybridization.

**Immunological staining in imaginal discs and lymph glands**

The solution volume was 1 ml and washing time was 5 minutes unless otherwise noted. No rotation was allowed in the whole process. Imaginal discs or lymph glands were dissected in PBS and then fixed in 3.7% formaldehyde for 10 minutes, followed by three times washing in PBT. Discs or lymph glands were blocked in 100 ul 5% BSA/PBT for 1 hour and then incubated in primary Ab/5% BSA at 4°C for overnight. Secondary antibody was applied to discs or lymph glands for 4 hours at room temperature after several times washing with PBT. After secondary antibody staining, discs or lymph glands were washed in PBT for a few times and mounted in 70% glycerol with anti-fading agent (DABCO). DAPI (1:1000) staining was applied to discs or lymph glands for 10 minutes during the first or second time PBT washing after secondary antibody incubation.

Primary antibodies used were rabbit anti-GFP (1:500), mouse anti-GFP (1:500), mouse beta-PS (1:50) and rat anti-ELAV (1:25). Secondary antibodies used were Alexa 488 anti-mouse (1:500), Alexa 488 anti-rabbit (1:500) and Texas-red anti-rat (1:100).

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**Septic injury assay**

One week old adult flies were subjected to *E.coli* infection at thorax with a glass needle. The glass needle was dipped into 10x overnight *E.coli (pHC60)* bacterial culture every time. Septic injury challenged and control flies were placed in a 29°C incubator for better bacteria growth. The survival rate of adult flies and bacterial growth were checked everyday and alive flies were transferred to a new vial every day. The length of the experiment was 10 days.

**Egglaying and wasp encapsulation assay**

Egglaying took place in a collection cup on molasses plates. Newly hatched first instar larvae in 8 hour window were placed onto standard corn meal food (vial) with 80 larvae in each vial. 6-8 female wasps (10-15 days old) were applied to the second instar larvae for 24 hours infection. Late third instar larvae were observed for the appearance of black capsules. The total number of larvae with black capsules was recorded as X. The larvae without black capsules were torn apart to check the presence of wasp larvae. The number of larvae with wasp larvae but without black capsule was recorded as Y and the number of larvae without wasp larvae was recorded as Z. The encapsulation capacity was calculated as X/(X+Y). The infection rate was calculated as (X+Y)/(X+Y+Z).

**RT-PCR**

Newly hatched adults flies of *CG6023*<sup>d04993</sup>, *upd3*<sup>d232a</sup> and *hop<sup>msv/m38</sup>* were kept at 25°C on corn meal food. One day before septic injury, they were flipped into new vials and kept at room temperature. One week old flies were subjected to septic injury with
10x overnight *E.coli (pHC60)* bacterial culture. After septic injury, challenged flies and non-challenged control flies were kept at 25°C for 6 hours. After 6 hours, total RNA of 30 flies from each genotype was isolated with Invitrogen TRIzol® solution. Two batches of RNA from each genotype were isolated as replicates. Obtained total RNAs were kept in RNaseqTM suspension solution (Ambion Inc) and treated with DNase for removing genomic DNA (TURBO DNA-free™, Ambion Inc). RNA concentration was measured with the NanoDrop® ND-1000 Spectrophotometer machine in Dr. Bruce O’Hara’s lab. One microgram of total RNA was used for cDNA synthesis with iScript™ cDNA synthesis kit (BIO-RAD). Total 20 ul of cDNA was obtained for each sample and then diluted in 20 ul DEPC H2O to make a 40 ul cDNA pool. Use 1 ul or 2 ul of each of diluted cDNA for PCR. PCR was set up as followed: Cycle 1 (1x): 94°C for 01:30; Cycle 2 (40x): 94°C for 00:15, 60°C for 01:00, 72°C for 01:00; Cycle 3 (1x): 95°C for 1:00; Cycle 4 (80x): 60°C for 00:10, increase set point temperature by 0.5°C after cycle 2, melt curve data collection and analysis enabled; Cycle 5 (1x): 4°C hold. The iQ™ SYBR Green Supermix was from BIO-RAD Company. The data was analyzed according to the 2−ΔΔCT method (Livak and Schmittgen, 2001; Pfaffl, 2001).

Primers used in RT-PCR:

- **TotA**: tgctcttatgtgttcttctctctgtgctttgactg, gagcaagctttgaacccaattc
- **TotM**: aagccaagcctgcactatgaat, ttgactccctcagaggcaattt
- **CG11501**: aatcatggcatccccagtagtc, tgtgatgcaaggggttaaaatg
- **CecA1**: gctcagacctcactgcaatatca, ttgttttatttacagggagcaacag
- **Drs**: tcatttaccaagctccgtgaga, agctaaacgcgcttttcagaac
- **Rp49**: cagcatacaggccccagatcg, cttactcgtctcttttgagaacgcag
**Blood cell counting**

Larvae of *CG6023<sup>d04993</sup>, upd3<sup>d232a</sup>, Rsh/Rsh and Imd/Imd* at 120 hours after egglaying were washed in PBS and slightly dry on kimwipe. Larvae were torn apart at the posterior region with forceps and the circulating blood cells were collected in 9 ul 1% trypsin/PBS. After about 10 minutes in 1% trypsin/PBS, total 10 ul diluted blood cell sample was applied to a FISHER SCIENTIFIC hemacytometer (CAG #0267110) for blood cell concentration counting. The middle large square (1 mm<sup>2</sup>, including 25 small squares) was chosen as the counting area. The number of blood cells in the 1 mm<sup>2</sup> region was recorded as X. Considering the 0.1 mm depth of the chamber and the 1 to 10 dilution of the original blood cell (Assume 1 ul of original blood cell sample diluted in 9 ul 1% trypsin/PBS), the original blood cell concentration was calculated as 100X cells/ul.

**Enhancer reporter construction**

Genomic DNA sequences for the deletions in *os<sup>l</sup>* (WE), *os<sup>o</sup>* (W) and the deletion in *os<sup>l</sup>* but not in *os<sup>o</sup>* (E) were obtained by PCR with the following primers:

- U2/U3-23887F-BglII: CCAGATCTCCTCCGTGTTGCTCAATGTGTT
- U2/U3-28287R-EcoRI: CCGAATTCGGCCCAGGGTATAATTAACG
- U2/U3-28288F-EcoRI: CCGAATTCTGAACTGAACCGAACTGAGCCG
- U2/U3-31675R-XbaI: CGGAATGCCCTACACCGATGG

The obtained fragments were constructed into *pPelican-GAL4* vector (from Dr. O’Connor’s lab). The *pPelican-GAL4* was cut with BglII and XbaI and ligated with the deletion in *os<sup>l</sup>* (WE) (7.8kb). The *pPelican-GAL4* was cut with BglII and EcoRI and ligated with the deletion in *os<sup>o</sup>* (W) (4.4 kb). The *pPelican-GAL4* was cut with EcoRI...
and XbaI and ligated with the deletion in os¹ but not in os² (E) (3.4 kb). Three constructs were sent to Rainbow Transgenic Flies, Inc. for making transformants. Four independent lines for \( pPelican-WE-GAL4 \) were recovered. Two of them have the construct inserted on the second chromosome and two of them have the construct inserted on the third chromosome. Fourteen independent lines for \( pPelican-W-GAL4 \) were recovered. Two of them have the construct inserted on the first chromosome. Three of them have the construct inserted on the second chromosome. Nine of them have the construct inserted on the third chromosome. Five independent lines for \( pPelican-E-GAL4 \) were recovered. One of them has the construct inserted on the first chromosome. Four of them have the construct inserted on the second chromosome.

**Image capturing and processing**

The fly eye, wing and haltere images were taken on a Nikon SMZ1500 scope with a SPOT camera. The fly eye size was measured by choosing the entire ommatidia area and the area size was given out by Scion Image software (Scion Corporation). *In situ* hybridization images were taken on a Nikon E800 microscope with a SPOT camera and converted to grayscale modes in Adobe Photoshop. The Confocal images of the eye discs in the enhancer report assay were taken on a Leica TCS-SP laser scanning Confocal and merged in Adobe Photoshop. Other fluorescent images were taken on a Nikon E800 microscope with a SPOT camera and merged in Adobe Photoshop.

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References


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PUBLICATIONS
1. Functional characterization of Upd3 in Drosophila development (in preparation)
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2. Reduced and misexpression of 5-HT2 receptor alters development, behavior and CNS activity in Drosophila melanogaster (submitted)
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