ABSTRACT

Title of Document: IDENTIFICATION OF GENES INVOLVED IN THE ANTIVIRAL RESPONSE THROUGH GENETIC SCREENS IN *DROSOPHILA*

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Innate immunity is essential for the host to defend against invading pathogens, such as viruses and bacteria. To identify novel genes or molecules that are involved in innate immunity, we carried out two genetic screens in *Drosophila*. From a forward screen of flies mutagenized with Ethyl methane sulfonate (EMS), four mutants with increased susceptibility to *Drosophia* X virus (DXV) were found. In this study, we focused on the *rogue* mutant and identified a novel antiviral gene *rogue.* The *rogue* mutant is highly susceptible to DXV infection and is unable to control viral replication during infection. The expression of *rogue* in either the hemocytes or the fat body is required for flies to control viral accumulation and to survive a viral infection. At an early stage of infection, *rogue* is induced and the amount of Rogue protein that locates to the nucleus increases. In addition, we confirm that the Rogue protein interacts with the polyA binding protein (PABP), and we propose that *rogue* restricts viral replication via translation regulation in *Drosophila*. The *rogue* mutant also has a phagosome maturation defect, which may

contribute to its susceptibility to *Staphylococcus aureus* infection. RNAi knockdown of *rogue* in the fat body or the hemocytes in wild type flies results in high bacterial susceptibility. Introducing the *rogue* transgene in the hemocytes of the *rogue* mutant can rescue the mutant survival to both DXV and *S. aureus*. Together, our results demonstrate that *rogue* plays a critical role in defending against DXV and *S. aureus* infections.

We performed another genetic screen on wild derived inbred flies from the Drosophila Genetic Reference Panel (DGRP). From a genome wide association study (GWAS) in these flies, we found four single nucleotide polymorphisms (SNPs) associated with susceptibility of flies to DXV. One allele contributed most to the susceptibility is located in the intron of Socs36E, a negative regulator of the JAK-STAT pathway, implicating that the JAK-STAT pathway plays a role in the immune responses against DXV. Our study also shows that natural genetic variation can be used as a tool for identifying novel genes or pathways involved in antiviral immunity.

IDENTIFICATION OF GENES INVOLVED IN THE ANTIVIRAL RESPONSE THROUGH GENETIC SCREENS IN DROSOPHILA

By

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Dedication

To my parents, my husband, and my daughters.

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Chapter 1: Introduction

Most hosts have powerful immune systems that provide protection against a large variety of pathogens, such as viruses and bacteria. The immune systems can operate through two types of responses: the innate immune response and the adaptive immune response. As the first defending line, the innate immune response is essential. Unlike adaptive immunity, innate immune responses do not rely on the clonal selection of antigen receptors, and instead make use of germline encoded receptors to recognize non-self-molecules known as: pathogen associated molecular patterns (PAMPs). This fast ancient innate response is indispensable for the control of pathogens, and for the establishment of balanced interactions with beneficial microorganisms in all metazoans. In the jawed vertebrates, innate immunity is required for the activation of adaptive immunity, while invertebrates seem to rely exclusively on the innate immune system for defense against infection (Janeway, 2005).

I. Innate Immunity in *Drosophila*

Drosophila has a robust innate immune system, which contains multiple lines of defense against pathogens: barrier epithelial immune responses, cellular responses and humoral responses. Epithelial barriers, such as the protective cuticle, the gut epithelial environment and the tracheal respiratory organs, are essential to limit the pathogen's entry (Kimbrell and Beutler, 2001). Cellular immunity is mainly conducted by hemocytes. In larvae, hemocytes freely circulate in the hemolymph, while in the adult fly they are primarily sessile cells (Elrod-Erickson et al., 2000; Lanot et al., 2001).

Hemocytes are functionally analogous to mammalian macrophages (Abrams et al., 1992). These cells are the very first cells to recognize and clear pathogens by phagocytosis. They also phagocytose apoptotic cells. Humoral immunity, on the other hand, produces effector molecules to defend against microbial infection. The humoral response is best characterized by the production of antimicrobial peptides (AMPs) by the fat body, a functional analog of the mammalian liver. The fat body is made up of adipose tissue, and distributed in the internal surface of the cuticle in adult flies (Arrese and Soulages, 2010). The inducible expression of AMPs in the fat body is controlled by two Nuclear Factor kappa B (NF- κB) signaling pathways: the Toll and Imd pathways.

A. Phagocytosis and the cellular immune responses

Phagocytosis is a form of endocytosis for uptake particles such as microbial organisms and apoptotic cells that are larger than 0.5μm (Mellman, 1996). The vesicles formed as a result of this uptake are known as phagosomes, and undergo sequential maturation events that culminate in fusion with lysosomes and degradation of the phagocytosed contents. The newly internalized phagosome fuses with the early/sorting endosome, subsequently matures to a late stage phagosome, and finally fuses with the lysosome. During these fusion events, distinct protein components specific to each stage are incorporated in the vesicle. Several of these proteins serve as markers for specific maturation stages. The small GTPase Rab5 is typically found on early phagosomes, while another small GTPase Rab7 replaces Rab5 on the late stage phagosomes (Rink et al., 2005). Lysosome-associated membrane protein (LAMP) serves as the most commonly used

phagolysosomal marker. Apart from a changing of proteins that regulate vesicle transport and functions, phagosome maturation involves significant acidification of the vesicle. The low pH in the lysosome activates the hydrolases that break down the microbial and cellular debris into recyclable small molecules (Kinchen and Ravichandran, 2008).

In *Drosophila*, there are three types of hemocytes: plasmatocytes, crystal cells and lamellocytes. Crystal cells mediate melanization, and lamellocytes encapsulate large objects. These two types of cells consist a small fraction of the hemocytes, and they can be induced upon infection in larval stages (Krzemien et al., 2007). The majority of hemocytes are plasmatocytes, which are the major phagocytic cells. Their phagocytic activity is essential for flies to survive some Gram-positive bacterial infections (Nehme et al., 2011). In other instances, phagocytosis alone is not sufficient to promote antibacterial protection. The Imd pathway induces humoral responses against Gram-negative bacteria. A mutant in this pathway, *imd,* shows increased susceptibility to *Escherichia coli* (*E. coli*) when the phagocytic capacity of hemocytes is blocked. However, inhibiting hemocytes function does not affect the susceptibility of wild type flies to *E. coli*. This indicates that the humoral response may be able to compensate for the lack of phagocytosis during infections with some Gram-negative bacteria (Elrod-Erickson et al., 2000).

Phagocytosis is mediated by the surface receptors on hemocytes (Table 1-1). The phagocytosis receptor involved in recognition and engulfment of different pathogens are Eater (Kocks et al., 2005), Croquemort (Stuart et al., 2005), SR-Cl (Ramet et al., 2001),

Drosophila melanogaster **phagocytosis receptors**	**Pathogens, apoptotic cells or dsRNA that are recognized**	**References**
TEP VI	*Candida albicans*	(Stroschein-Stevenson et al., 2006)
TEPIII	*Staphylococcus aureus*	(Stroschein-Stevenson et al., 2006)
TEP II	*Escherichia coli*	(Stroschein-Stevenson et al., 2006)
Integrin βv	Apoptotic cells and *S. aureus*	(Nonaka et al., 2013; Shiratsuchi et al., 2012)
Croquemort	Apoptotic cells and *S. aureus*	(Franc et al., 1999; Stuart et al., 2005)
SR-Cl	*S. aureus, E. coli* and dsRNA	(Ramet et al., 2001; Ulvila et al., 2006)
Eater	*S. aureus* and dsRNA	(Kocks et al., 2005; Ulvila et al., 2006)
Draper	Apoptosis cells and *S. aureus*	(Hashimoto et al., 2009; Manaka et al., 2004)
Nimrod C1	*S. aureus* and *E.coli*	(Kurucz et al., 2007)

Table 1-1: *Drosophila* cell-surface recognition receptors. *Drosophila* cell surface recognition receptors are listed in the table. TEP VI is an important phagocytosis receptor for *Candida albicans*. TEP II is required for phagocytosis of *E. coli* but not for *S. aureus*. All other receptors recognize *S. aureus* and mediate the phagocytosis of *S. aureus*. TEP, thioester-containing protein; SR, scavenger receptor.

TEPs (Stroschein-Stevenson et al., 2006), NimC1 (Kurucz et al., 2007), Integrin βv (Nonaka et al., 2013; Shiratsuchi et al., 2012) and Draper (Hashimoto et al., 2009; Shiratsuchi et al., 2012). Loss of any of these receptors in hemocytes can result in reduced phagocytosis of bacteria. Flies with RNAi knockdown of *Integrin β v*, *NimC1* or *Draper* are also defective in clearance of apoptotic cells (Manaka et al., 2004; Nagaosa et al., 2011; Shiratsuchi et al., 2012).

B. NF-κB signaling pathways and the humoral immune responses

Toll pathway

The Toll pathway is an evolutionarily conserved signaling pathway that was initially identified as essential for the establishment of the dorso-ventral axis of the *Drosophila* embryo (Morisato and Anderson, 1995). Later, Lemaitre et al. found that the *Toll* mutant had a defect in the expression of the antifungal peptide Drosomycin, implicating the Toll pathway in humoral immune responses (Lemaitre et al., 1996). The intensive study of the Toll pathway in *Drosophila* led to studies of mammalian Toll-like receptors (TLRs) (Medzhitov et al., 1997; Rock et al., 1998). In *Drosophila*, Gram-positive bacteria or fungi activate the Toll pathway. For Gram-positive bacteria, the lysine-type peptidoglycan of the bacteria is recognized by GNBP1 and PGRPs (PGRP-SA, PGRP-SD, PGRP-SC1a) (Garver et al., 2006; Michel et al., 2001; Wang et al., 2008). For fungi, the beta-glucans are recognized by GNBP3, and the virulence factor PR1 is recognized by Persephone (Lemaitre and Hoffmann, 2007). Any of these recognition events leads to the direct cleavage of the serine protease, Spätzle processing enzyme (SPE), which

proteolytically activates Spätzle. Once activated, Spätzle binds to the transmembrane receptor Toll, resulting in the dimerization and activation of the Toll receptor (Weber et al., 2003). Following this activation the intracellular adaptors MyD88, Tube (an IRAK-4 homolog) and Pelle (an IRAK-1 homolog) are recruited to the receptor, leading to the phosphorylation and proteasomal degradation of Cactus, an I-κB homolog (Horng and Medzhitov, 2001; Nicolas et al., 1998). Consequently, the Rel transcription factors Dif and Dorsal are released from Cactus and can translocate into the nucleus where they can initiate the transcription of AMP genes including *Drosomycin* (Lemaitre et al., 1996). Dif and Dorsal are NF-κB like trans-activators. They play redundant roles in larval antimicrobial peptide (AMP) responses, whereas only Dif is required for *Drosomycin* induction in adults (Lemaitre et al., 1995b; Meng et al., 1999). Only Dorsal is required for Toll pathway function in embryonic development (Steward, 1987).

Imd pathway

The Imd pathway is preferentially activated by direct recognition of Gram-negative bacteria diaminopimelic acid (DAP)-type peptidoglycan by the transmembrane receptor PGRP-LC or PGRP–LE (Choe et al., 2002; Ramet et al., 2002; Takehana et al., 2002). Once PGRP-LC or PGRP-LE is activated, the intracellular adaptor protein Imd is recruited. The signal from Imd goes through two branches, which ultimately converge to activate the NF-κB like transcription factor Relish. One branch leads to the recruitment and activation of *Drosophila* transforming growth factor-beta activated protein kinase 1 (dTak1), the TAK1-binding protein 2 (dTAB2), and the *Drosophila* inhibitor-of-apotosis protein 2 (DIAP2) (Gesellchen et al., 2005; Vidal et al., 2001). Activated dTak1 in turn

activates the I-κB kinase (IKK) complex (IRD5 and Kenny), which then phosphorylates Relish (Rutschmann et al., 2000). The other branch goes through the adaptor molecules *Drosophila* Fas-associated DD (dFADD) (Naitza et al., 2002) and death-related ced-3/NEDD2-like protein (Dredd), resulting in the cleavage of the I-κB like inhibitory domain containing ankyrin repeats in phosphorylated Relish. Consequently, the Rel domain of Relish translocates into the nucleus and initiates the transcription of AMP genes including *Diptericin* (Lemaitre and Hoffmann, 2007; Lemaitre et al., 1995a). The Imd pathway is highly homologous to the mammalian tumor necrosis factor receptor (TNF-R) pathway, an important pathway for inflammatory responses against viral infection (Georgel et al., 2001).

Antimicrobial peptides (AMPs)

Although the Toll and Imd pathways are activated independently by different microbes, the NF-κB transcription factors (*Dif*, *Dorsal* and *Relish*) are able to function synergistically. Compared to activation of only one of the pathways, activation of both the Toll and Imd pathways results in higher induction of AMP genes (Tanji et al., 2007). To date, 20 AMPs have been identified in *Drosophila* (Imler and Bulet, 2005). Most AMP genes have more than one κB motif. The κB motifs upstream of the AMP genes have different (either specific or non-specific) binding affinities for the NF-κB transcription factors; thus, induction of certain AMPs can be controlled by the two pathways (Busse et al., 2007; Tanji et al., 2007).

C. JAK-STAT Signaling Pathway

In *Drosophila*, the JAK-STAT (Janus kinase - signal transducers and activators of transcription) pathway was first identified for its role in embryonic segmentation (Binari and Perrimon, 1994). Later, it was found to have conserved roles in the control of cell proliferation, stem cell development and also in the immune system (Agaisse and Perrimon, 2004). In response to tissue damage and stress, the hemocytes release the cytokine Unpaired-3 (Upd-3) which binds Domeless receptor in the fat body and activates the pre-associated JAK (Hopscotch) kinase, resulting in the recruitment, phosphorylation and dimerization of STATs (Stat92E). The activated STAT transcription factors then translocate into the nucleus, inducing the expression of effector genes including: complement-like protein genes (Tep1 and Tep2), the *Turandot* family of genes (totA and totM) (Lagueux et al., 2000).

In adult flies, Tep2 and totA are induced by septic injury (Agaisse et al., 2003). However, overexpressing the Tot peptides in the NF-κB mutants cannot rescue the susceptibility of the mutants to bacterial infections. In addition, the JAK-STAT pathway mutants are not more susceptible to bacterial infection (Agaisse and Perrimon, 2004). Thus, the humoral factors induced by the JAK-STAT pathway may not have anti-microbial activity. Since totA expression also requires Relish (transcriptional activator of the Imd pathway), it is hypothesized that the Imd and JAK-STAT signaling pathways may be co-regulating downstream immune responses (Agaisse and Perrimon, 2004). A few recent studies are in agreement with this hypothesis**.** Listericin is a newly identified anti-microbial protein that is induced by the activation of PGRP-LE (a receptor of the Imd pathway) in response to

Listeria infection in flies. *Listericin* induction also requires STAT, suggesting the involvement of the JAK-STAT pathway in the regulation of *Listericin* induction (Goto et al., 2010). The JAK-STAT pathway is also suggested to play a role in controlling NF-κB signaling in *Drosophila*. STAT together with AP-1 form a repressosome complex, which competes with Relish for the κB binding sites and thereby restricts Relish transcriptional activation in flies (Kim et al., 2007).

Several negative regulators of the JAK-STAT pathway have been identified. *Socs36E*, the suppressor of cytokine signaling 36E (Callus and Mathey-Prevot, 2002), provides negative feedback to inhibit Hop. Another negative regulator is PIAS (Betz et al., 2001), protein inhibitor of activated STAT, which suppresses STAT activity. Recently, *eye transformer (ET)* was found as a negative regulator of the JAK-STAT pathway. It functions at the level of or upstream of Dome (Kallio et al., 2010).

II. Viruses

Studies of the immune responses to viruses in *Drosophila* have mostly focused on *Drosophila* C virus (DCV) (Deddouche et al., 2008; Dostert et al., 2005), Sigma virus (Tsai et al., 2008), and Nora virus (Cordes et al., 2013; Habayeb et al., 2006), which are viruses that naturally infect *Drosophila*. Also some non-*Drosophila* viruses can efficiently replicate in *Drosophila* after experimental introduction, like *Drosophila* X virus (DXV) (Zambon et al., 2005; Zambon et al., 2006), Flock House virus (FHV) (Li et al., 2002), Cricket paralysis virus (CrPV) (Costa et al., 2009; Wang et al., 2006),

Vesicular stomatitis virus (VSV) (Shelly et al., 2009; Xu et al., 2012), Sindbis virus (SINV) (Avadhanula et al., 2009), and West Nile virus (WNV) (Chotkowski et al., 2008). In the mosquito, Dengue virus (DENV) (Mukherjee and Hanley, 2010; Sanchez-Vargas et al., 2009; Xi et al., 2008), West Nile virus (WNV) (Vaidyanathan and Scott, 2006), Yellow Fever virus (YFV) (Colpitts et al., 2011) and O'nyong-nyong virus (ONNV) (Waldock et al., 2012) are the main viruses studied, because they can be transmitted to humans through mosquito vectors. DXV and DCV are the main viruses that were studied in this thesis.

A. *Drosophila* X virus (DXV)

Drosophila X virus (DXV) was first identified as a contaminant of Sigma virus by Teninges and co-workers (Teninges, 1979b). Although no isolates of DXV have been obtained from natural populations of *Drosophila*, it has been found in *Drosophila* cell lines (Teninges, 1979b). DXV belongs to the *Birnaviridae* family and is currently the only member of the *Entomobirnavirus* genus. The other two genera of *Birnaviridae* are: *Avibirnavirus* (type species: Infectious bursal disease virus, IBDV) and *Aquabirnavirus* (type species: Infectious pancreatic necrosis virus, IPNV) (Dobos et al., 1979). Not much is known about the replication cycle of DXV. Little is known for the prototypical virus of birnaviruses, IPNV. The replication cycle of IPNV consists of: entry, transcription and replication, assembly, and release. Entry occurs by receptor-mediated endocytosis. All the replication steps take place exclusively in the cytoplasm. Apoptosis and lysis of the

infected cells are the releasing strategies (Darragh and Macdonald, 1982; Dobos and Roberts, 1983).

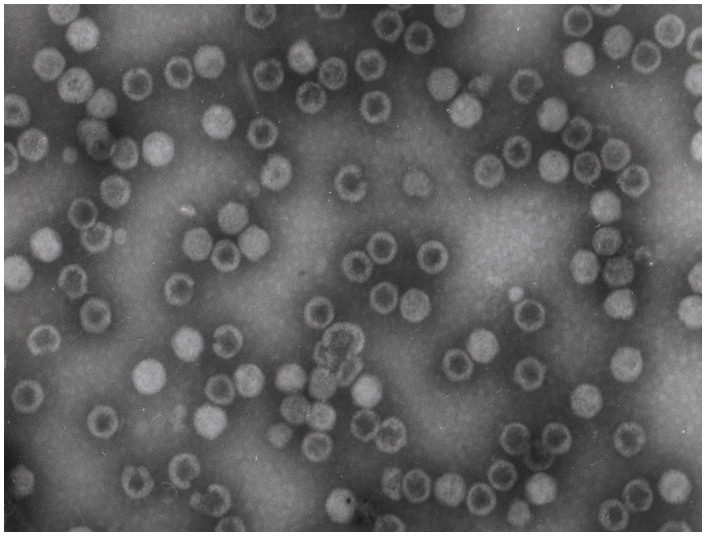
DXV has a non-enveloped, icosahedron shaped virion, which is about 59nm in diameter. The genome contains two segments of dsRNA, segment A and segment B. Segment A encodes two overlapping open reading frames (ORF) (Fig 1-1). One ORF encodes the major capsid protein VP2 (outer capsid) and VP3 (inner capsid), and the protease VP4. The other small ORF encodes a non-characterized NS protein. Segment B encodes the RNA dependent RNA polymerase (RdRp) (Teninges, 1979a).

DXV can cause the death of wildtype *Drosophila* 20-25 days after infection. Flies infected with DXV are also sensitive to anoxia, and exposure to anoxic conditions can induce an earlier death at approximately 6 to 7 days after infection (Zambon et al., 2005). Immune staining with an antibody against the virus showed that DXV spreads through the whole body of flies by Day 7 after infection, and evidence of cell death was observed at sites containing the virus.

B. *Drosophila* C virus (DCV)

Drosophila C virus (DCV) was first identified in a laboratory stock, which had unusually high lethality (Jousset et al., 1972). Later, DCV was found in wild populations of *Drosophila melanogaster* (Jousset, 1972, 1976). DCV belongs to the *Dicistroviridae* family, genus *Cripavirus* (Jousset and Plus, 1975). DCV particles are non-enveloped and

A



B

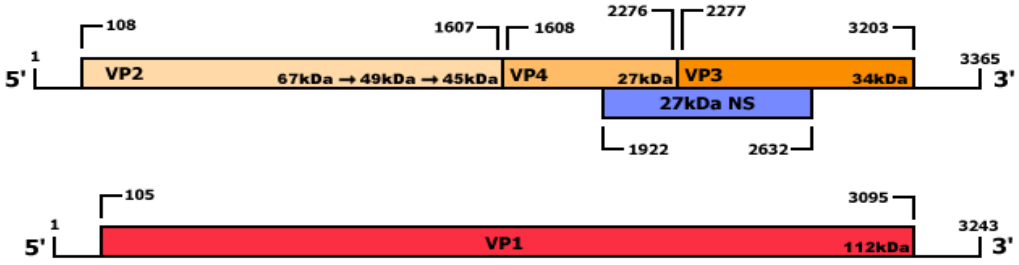


Figure 1-2: EM picture and the genome structure of DXV. The DXV virion has an icosahedron shape. The DXV genome has two segments of dsRNA molecules. Adapted from (Zambon, 2005).

icosahedral shaped. The genome of DCV contains a positive single stranded RNA (ss(+)RNA). DCV is one of the insect viruses whose viral replication cycle is well studied. Both *in vitro* and *in vivo* evidence showed that the viral particles are internalized by clathrin-mediated endocytosis (Cherry and Perrimon, 2004). Once inside the cell, DCV replicates in cellular vesicles derived from the remodeled Golgi apparatus. The virus can shut down CAP-dependent mRNA translation, causing the cell to preferentially translate viral mRNAs (Cherry et al., 2005).

DCV is strongly pathogenic to flies when injected, and causes the death of the flies 3-4 days after infection depending on the dose of virus used. Rapid spreading of the virus in multiple organs was seen in flies injected with the virus. However, when the virus was fed to flies, only a small portion of the flies could be infected, and in those flies viruses were limited to the epidermal cells (Lautie-Harivel, 1992). This indicates that the gut epithelial barrier is a vital host defense against DCV.

C. Other viruses

Most of the viruses used to examine the host antiviral innate responses in *Drosophila* are RNA viruses. The viral models are listed in Table 1-2.

Table 1-2: Viruses used in insect antiviral immunity studies.

Name	Family	Genome	Virion
DXV	*Birnaviridae*	ds RNA, bi-segmented	Non-enveloped
FHV	*Nodaviridae*	ss (+) RNA, bi-segmented	Non-enveloped
DCV	*Dicistroviridae*	ss (+) RNA, nonsegmented	Non-enveloped
CrPV	*Dicistroviridae*	ss (+) RNA, nonsegmented	Non-enveloped
SINV	*Togaviridae*	ss (+) RNA, nonsegmented	Enveloped
SFV	*Togaviridae*	ss (+) RNA, nonsegmented	Enveloped
WNV	*Flaviviridae*	ss (+) RNA, nonsegmented	Enveloped
DENV	*Flaviviridae*	ss (+) RNA, nonsegmented	Enveloped
YFV	*Flaviviridae*	ss (+) RNA, nonsegmented	Enveloped
Sigma virus	*Rhabdoviridae*	ss (-) RNA, nonsegmented	Enveloped
VSV	*Rhabdoviridae*	ss (-) RNA, nonsegmented	Enveloped
Invertebrate iridescent virus 6 (IIV6)	*Iridoviridae*	dsDNA, nonsegmented	Enveloped And Non-enveloped

Viruses that are commonly used to study the antiviral immunity in fly and mosquito are shown. DXV: *Drosophila* X virus; FHV: Flock House virus; DCV: *Drosophila* C virus; CrPV: Cricket paralysis virus; SINV: Sindbis virus; SFV: Semliki Forest virus; WNV: West Nile virus; DENV: Dengue virus; YFV: Yellow Fever virus; VSV: Vesicular stomatitis virus.

III. Antiviral Innate Immunity in *Drosophila*

A. Antiviral responses by the classical innate immune pathways

Toll pathway

The Toll pathway, which is required for anti-Gram-positive bacterial and fungal immunity, has been shown to be important against DXV infection in *Drosophila* (Zambon et al., 2005). AMP genes from both the Toll pathway and Imd pathway were induced by DXV, to a level similar to that induced by *Escherichia coli*. Furthermore, the mutant for *Dif*, the Toll pathway NF-κB transcription factor, had an increased susceptibility to DXV infection and an elevated viral RNA expression in the infected flies. In contrast, the mutant for *Relish*, the Imd pathway NF-κB transcription factor, had normal susceptibility to DXV infection. Induced expression of single AMPs could not rescue the susceptibility of the *Dif* mutant flies (Zambon et al., 2005). This indicates that the induction of AMPs may not be sufficient to defend against viral infection, or multiple AMPs are required for efficient antiviral immune responses.

The Toll pathway is also required for controlling the Dengue virus (DENV) infection in *Aedes aegypti* mosquitoes. Knockdown of the Toll adaptor protein MyD88 led to higher DENV accumulation in the midgut of the infected mosquito, and the Toll pathway related genes, *Toll*, *spatzle,* and *Defensin,* were induced with DENV infection *(Xi et al., 2008)*. In another study, a Toll-like receptor was induced in the midguts of mosquito by West Nile virus (WNV) infection (Smartt et al., 2009). These findings provide further support for the Toll pathway as an antiviral innate immune response in insects.

Imd pathway

In *Drosophila*, the Imd pathway plays a role in resistance to SINV and CrPV (Avadhanula et al., 2009; Costa et al., 2009). SINV replicon RNA has a higher replication rate in mutants from the Imd but not the Toll pathway, suggesting that the Imd pathway is involved in controlling SINV replicon replication. Similarly, the Imd pathway mutant *relish* had higher viral loads compared to wild type flies when injected with SINV. Injection of the flies with viruses also largely induced the expression of Diptericin (an Imd-dependent AMP) and Metchnikowin (a Toll and Imd-dependent AMP) (Avadhanula et al., 2009). Another study using CrPV infection in *Drosophila* demonstrated that even though there was no higher induction of AMPs with CrPV infection, the *Imd* mutant succumbed more rapidly and showed higher viral loads than wild type flies (Avadhanula et al., 2009; Costa et al., 2009). Thus, the Imd pathway is also involved in the immune responses against CrPV in *Drosophila*. This study also showed that the induction of AMPs was not required for the antiviral response against CrPV.

JAK-STAT pathway

A microarray study on DCV infected flies first implicated the JAK-STAT pathway as an antiviral pathway in *Drosophila* (Dostert et al., 2005). A number of genes were found to be up-regulated in response to virus infection but not to bacterial or fungal infection. Several of these induced genes, *vir-1*, *CG9080* (Listericin), *CG12780* (GNBP-like receptor), had active STAT binding regions in their promoters and were dependent on JAK for induction. The induction of these genes might require viral replication, since

UV-inactivated viral particles were not able to induce them (Hedges and Johnson, 2008). Dostert and colleagues showed that the highest induction of *vir-1* did not occur in the fat body, where the virus replicates. This suggests that an indirect signal might induce *vir-1* expression in uninfected cells. In addition, the loss-of-function *hopscotch* (JAK) mutant had a higher mortality and increased viral RNAs than the controls when infected with DCV. Although these mutant flies showed low induction of *vir-1*, the fact that overexpressing *vir-1* did not affect the susceptibility of flies in the infection suggests that *vir-1* might not be a direct effector for antiviral responses (Dostert et al., 2005). Therefore, the JAK-STAT pathway helps mount the antiviral immunity in flies, but the induction of *vir-1* by the JAK-STAT pathway may not be sufficient to defend flies from viral infection.

Another viral induced gene *vago* was identified through the study of the transcriptome of DCV-infected flies (Deddouche et al., 2008). This gene was induced in the fat body, and thus it might be directly induced by the virus. Higher viral RNA was detected in *vago* mutant flies, and more viral protein was found in the mutant fat body, indicating that *vago* was required for controlling DCV replication. It has been suggested that Vago may act as a ligand for the JAK-STAT pathway in mosquitoes, since viral induced secretion of Vago activated the expression of *vir-1* through the JAK-STAT pathway (Paradkar et al., 2012). Whether Vago is involved in activation of the JAK-STAT pathway by viral infection in *Drosophila* remains unclear.

Virus specific roles of the classical innate immune pathways

Although, the classical innate immune pathways (the Toll, Imd and JAK-STAT pathways) have been implicated in antiviral responses, they seem to contribute to the defense against certain viruses rather than all viruses. Moreover, it is not clear if the canonical effectors of these pathways such as: AMPs or STAT regulated molecules, are the effectors against viruses in *Drosophila*. Several studies have failed to detect the induction of these effector molecules with infection from certain viruses (Costa et al., 2009; Kemp et al., 2013). Studies that have observed induction of AMPs or STAT regulated genes have not provided direct evidence for an antiviral role of these molecules (Avadhanula et al., 2009; Dostert et al., 2005; Zambon et al., 2005). Ectopic expression of Defensin-A and Cecropin-A in the fat body of mosquito was able to restrict DENV replication, indicating an antiviral activity for these AMPs in the mosquitoes (Luplertlop et al., 2011; Pan et al., 2012). Whether these AMPs play an antiviral role in *Drosophila* remains to be studied.

The antiviral role of the JAK-STAT pathway is also virus specific. It is required for immune responses against DCV, SINV, and CrPV (Deddouche et al., 2008; Dostert et al., 2005), but not FHV, VSV and IIV-6 (Kemp et al., 2013). Also, although the JAK-STAT pathway is required for the restriction of some viral replication, the downstream genes activated by the JAK-STAT pathway are viral specific. Recently, a transcriptome study on flies infected with different viruses (DCV, FHV and SINV) showed that specific genes were activated via the JAK-STAT pathway in response to different viruses (Kemp et al., 2013).

B. Antiviral response by RNAi pathways

RNA interference is an important strategy in *Drosophila* for defense against viruses. It is a process in which different forms of double-stranded RNA (dsRNA) are cleaved by an RNase III-like enzyme into small interfering RNAs that guide the Argonaute complexes (the RNA-induced silencing complexes, RISC) to the complementary sequences to silence the target transcripts. Four RNA interference (RNAi) pathways have been identified in *Drosophila*: the micro RNA (miRNA) pathway, the exogenous small interfering RNA (exo-siRNA) pathway, the endogenous small interfering RNA (endo-siRNA) pathway and the Piwi-associated RNA (piRNA) pathway (Fig 3, Fig 4). These pathways are mainly classified by the different Argonaute proteins associated with the pathways and the distinct functions of each pathway.

RNAi pathways in *Drosophila*

miRNA pathway:

The miRNA pathway mainly uses the Argonaute protein AGO1, the RNase III-like enzymes Drosha (in the nucleus) and Dicer1 (in the cytoplasm) (Okamura et al., 2004; Okamura and Lai, 2008). It is involved in the regulation of endogenous gene expression, especially during development. The miRNA pathway has not been implicated in antiviral immunity in *Drosophila*.

siRNA pathway:

Exogenous dsRNAs, including viral RNAs, are recognized and processed by the RNase III-like enzyme Dicer2 with the help of a double-stranded RNA binding protein R2D2. siRNAs that are 21nt long are produced and incorporated into AGO2-containing complexes (Okamura et al., 2004; Okamura and Lai, 2008). This exo-siRNA pathway is important for cleavage of viral dsRNA and artificial dsRNA.

endo siRNA pathway:

Another group of 21nt long siRNAs is produced by the endo-siRNA pathway. *Drosophila* cells naturally generate endogenous dsRNAs from transposable elements (TEs), complementary annealed transcripts and long inverted repeats called hairpin RNAs (hpRNAs). These dsRNA sources are diced by Dicer2 and then are incorporated into AGO1-containing complexes (Okamura et al., 2004; Okamura and Lai, 2008). The endo-siRNA pathway helps maintain transposon silencing and endogenous mRNA regulation. If the endo-siRNA pathway plays a role in antiviral immune responses is not clear yet.

piRNA pathway:

The main RNAi pathway that regulates the transposon expression in germline stem cells is the piRNA pathway. Piwi-class Argonaute proteins-Piwi, AGO3, Aubergine (AUB)-are involved in this pathway. The 24-32 long piRNAs can be distinguished from the shorter miRNAs (~22nt) and siRNAs (21nt) (Okamura et al., 2004; Okamura and Lai, 2008). The piRNA pathway palys a role in protecting flies from DXV infection.

RNA interference is a general antiviral immune response in *Drosophila*

RNAi (RNA interference) is utilized by plants as a vital antiviral response. In other animal species like *Aedes albopictus, Caenorhabditis elegans* and *Litopenaeus vannamei* (Gaines et al., 1996; Lu et al., 2005; Robalino et al., 2005), RNAi is activated by viral infection, indicating that it is a likely antiviral strategy in these species. In *Drosophila*, the RNAi pathways have been explored for their antiviral role as well.

The siRNA pathway mutants, *dcr2, AGO2* and *r2d2*, have been shown to be more susceptible to a variety of viruses (Table 1-3) (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). These mutants also show increased viral loads or viral RNAs after infection with different viruses (Chotkowski et al., 2008; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). In addition to the genetic evidence, siRNAs derived from various viruses were detected by deep sequencing of the small RNAs produced in infected *Drosophila* cells and flies (Aliyari et al., 2008; Ding and Lu, 2011). These suggest an important role for the siRNA pathway in mediating broad antiviral defense in *Drosophila*. The endonuclease Dicer2 is important for immune defense against DCV, FHV, CrPV and SINV (Galiana-Arnoux et al., 2006; Wang et al., 2006); however, it is not required for flies to survive a DXV infection or to control the WNV replication (Chotkowski et al., 2008; Zambon et al., 2006). Thus, Dicer2 may play a role in antiviral immunity that is independent of the RNAi pathway.

Table 1-3: Mutants in the RNAi pathway have increased susceptibility to viruses.

RNAi mutants that are susceptible to viruses	**Viruses**
Dcr-2	DCV, FHV, SINV, CrPV
AGO2	DXV, DCV, CrPV
r2d2	DXV, DCV, FHV, SINV, CrPV
piwi	DXV
Increased lethality correlates with increased viral titers in infected flies. Higher viral titers of WNV were found in the *piwi* mutants.	

Mutants in the RNAi pathway show higher mortality after viral infection compared to control flies. DXV: *Drosophila* X virus; DCV: *Drosophila* C virus; FHV: Flock house virus; SINV: Sindbis virus; CrPV: Cricket paralysis virus; WNV: West Nile virus.

Deddouche and colleagues found that *Dcr-2* but not *AGO2* or *r2d2* was required for the induction of *vago,* a gene that its expression was required for restricting DCV replication (Deddouche et al., 2008). This indicates that the DCV susceptibility of the Dcr-*2* mutant may be partially caused by the lack of *vago* expression.

Another RNAi pathway, the piRNA pathway, is also involved in antiviral immunity in *Drosophila*. Previous studies in our lab demonstrated that mutants in this pathway, such as *piwi* and *aubergine*, are more susceptible to DXV compared to controls (Zambon et al., 2006). Later, Chotkowski and colleagues found that the *piwi* mutant has a defect in controlling WNV RNA accumulation in flies as well (Chotkowski et al., 2008). These studies suggest that the piRNA pathway is an important immune response against DXV and WNV in *Drosophila*. Similarly, in mosquito cells, viral derived piRNA are present after WNV infection (Chotkowski et al., 2008), and knockdown of piRNA pathway genes enhances the replication of SFV (Schnettler et al., 2014), suggesting that the antiviral role of the piRNA pathway may be shared by flies and mosquitoes.

In *Drosophila*, RNA interference not only plays a role in actively fighting against viral infection in virus-containing cells, it is also involved in the immune response in the neighboring uninfected cells to prevent the infection by the same pathogen. In the infected cells, viral dsRNA genome fragments or viral replication dsRNA intermediates are produced (Aliyari et al., 2008). These dsRNAs are thought to be able to elicit immune responses in the uninfected cells. Robalino and colleagues first showed, in an invertebrate model shrimp *Litopenaeus vannamei*, viral derived dsRNA that introduced into the

animal triggered potent antiviral responses (Robalino et al., 2005). Later, Saleh found that injecting viral sequence specific dsRNA was also able to induce immune responses against the corresponding virus in uninfected flies (Saleh et al., 2009). The fact that mutants in the RNAi pathways are not able to mount an antiviral response by exogenous dsRNA suggests that RNA interference in the uninfected cells mediates the responses. It has been shown that the endocytic pathway plays a role in the uptake of dsRNA in *Drosophila* (Saleh et al., 2006; Ulvila et al., 2006). Mutants that have defects in the dsRNA uptake pathway are hypersensitive to DCV and SINV (Saleh et al., 2009). Together, these indicate that dsRNA uptake is important for the systematic immune responses of RNA interference in *Drosophila*.

C. Other responses

Apoptosis

Apoptosis, a programmed cell death, can be triggered by either death ligands or by the cellular stress signals, which subsequently activate the death receptor or promote the cytochrome c release from mitochondria respectively, ultimately leading to cell death. These apoptotic cells are then cleared by phagocytosis (Manaka et al., 2004). Since infection is one of the sources of stress to a cell, apoptosis has long been speculated to play a role in intracellular pathogen clearance. With the finding of virus derived apoptosis inhibitors, it is believed that delaying apoptosis at early stage of infection is beneficial for virus to establish infection in the organism (Vaidyanathan and Scott, 2006). In *Drosophila*, it has been found that the pro-apoptotic genes, *hid* and *reaper,* are rapidly

induced following viral infections. The induction of these pro-apoptotic genes at an early infection stage is critical for limiting viral replication (Liu et al., 2013), suggesting that apoptosis plays a role in antiviral immunity in flies. Liu and colleagues also demonstrated that the rapid inductions of *hid* and *reaper* were completely blocked in the null mutant of transcriptional factor P53, which indicated a link between p53-mediated stress responses and the antiviral immunity via regulation of apoptosis.

Autophagy

Autophagy is a highly conserved process in response to cellular stress in eukayotic organisms, like yeast, fly and human. In the absence of nutrients, proteins and damaged organelles are captured by autophagy machinery. Amino acids are recycled so that cells can survive in nutrient stress situations. Autophagy also has a protective role against oxidative stresses. In cells that have lysosomal and autophagic degradative dysfunction, the oxidatively damaged proteins and injured organelles can accumulate and contribute to cell injury (Moore, 2008). A few studies implicate autophagy in cellular clearance of intracellular pathogens as well. Orvedahl and colleagues showed that the herpes simplex virus type 1 (HSV-1) inhibited autophagy function, which result in increased disease pathogenesis (Orvedahl et al., 2007). Recent studies also demonstrate that autophagy in macrophages is an effective mechanism to facilitate the intracellular killing of *Mycobacterium tuberculosis* (Songane et al., 2012). However, for some viruses such as coxsackievirus B3 (CVB3), the autophagy machinery provides a niche for viral replication (Kemball et al., 2010). In *Drosophila*, autophagy has been found to be

induced by VSV (Shelly et al., 2009). However, no solid evidence exists to support the direct interaction of autophagy machinery with this virus (Shelly et al., 2009).

Microbial symbionts: *Wolbachia*

Wolbachia are intracellular bacteria found in a great number of species of arthropods, including *Drosophila*. They can invade the germline cells and are mainly maternally transmitted. Depending on the strain of *Wolbachia* and the insect species that is infected, different phenotypes, such as cytoplasmic incompatibility (CI), increase or decrease of fertility, select killing of male offspring, and even converting genetic males into phenotypic females, have been observed in insects (Iturbe-Ormaetxe and O'Neill, 2007). In *Drosophila melanogaster*, *Wolbachia* causes less dramatic phenotypic impacts, with moderate effects on host development, fertility, viability and lifespan (Boyle et al., 1993). *Wolbachia* infection plays a role in resistance to viral infection in the mosquito and fly. In the mosquito, introducing *Wolbachia* effectively restricts replication of several viruses, such as DENV, YFV and Chikungunya virus (Sinkins, 2013). The DNA methyltransferase (AaDnmt2) was found to mediate the antiviral effect of *Wolbachia* infection in mosquito (Zhang et al., 2013). In *Drosophila*, flies infected with *Wolbachia* w^{Mel} stains were resistant to DCV, FHV and CrPV infection (Hedges et al., 2008; Teixeira et al., 2008). Since *Wolbachia*-infected *Dcr-2* and *AGO2* mutants also show delayed mortality to FHV or DCV, the main antiviral pathway (RNA interference) may not be involved in *Wolbachia*-induced protection against these viruses (Hedges et al., 2012). The mechanism by which this endosymbiont promotes antiviral protection in *Drosophila* remains largely unknown.

D. Host translation and immunity

Translation is another cellular program that has been found to have a role in immunity in several host species (Berlanga et al., 2006; Chakrabarti et al., 2012; Dunbar et al., 2012). The PolyA binding protein (PABP) interacts with the cap-dependent translation initiation complex eIF4F (Hinnebusch and Lorsch, 2012), and is particularly important for gene expression regulation at the step of translation initiation. PABP is an RNA binding protein that binds to the polyA tails of eukaryotic mRNAs. During translation initiation, eukaryotic initiation factor 4G (eIF4G) interacts with both PABP and the cap binding protein eIF4E, resulting in the formation of a "closed-loop" topology that links the 5' and 3' mRNA ends. After formation of this loop, the 40S subunit of the ribosome, the translation initiation factor eIF2 and the initiator tRNA (Met-tRNA) are all recruited to the site and translation is initiated (Hinnebusch and Lorsch, 2012).

In the mammalian systems, this process is regulated by the host serine/threonine kinase mTOR complex 1, which controls the phosphorylation of 4E-BPs (eIF4E binding protein1, 2, 3) (Pause et al., 1994; Poulin et al., 1998). Unphosphorylated 4E-BPs binds to eIF4E and prevents eIF4E from assembling into the eIF4F complex. In the presence of the mTOR complex 1, 4E-BPs are phosphorylated and activated, and subsequently release eIF4E, which promotes cap-dependent translation (Cully et al., 2010). Some viruses can interfere with this process and inhibit host translation. Encephalomyocarditis virus (EMCV) and poliovirus can dephosphorylate eIF4E to slow down host cap-dependent protein synthesis (Kleijn et al., 1996); VSV dephosphorylates 4E-BP1 and down-regulates host translation (Connor and Lyles, 2002). Since the translation of these

viruses do not require high concentrations of the active cap-binding eIF4F complex, reducing eIF4E and 4E-BP phosphorylation levels only inhibits the translation of the host but not the viruses. This helps these viruses establish an infection in the cell and also inhibits the production of host antiviral proteins.

An alternative translation regulation mechanism relies on the phosphorylation of the α subunit of eIF2 (eIF2α) (de Haro et al., 1996). Cellular stresses like oxidative stress (Lu et al., 2001) and ER stress (Ron and Walter, 2007) lead to elevated levels of the eIF2α kinase in the cells, which increases the phosphorylation of eIF2α, and decreases the guanine nucleotide exchange factor activity of eIF2, thereby inhibiting translation. Viral infection is another stress that can induce eIF2α kinase in the cell (Hovanessian, 1989; Walsh et al., 2013). Since viruses demand a high level of translation, inhibition of the eIF2-dependent translation can limit viral replication. However, many viruses can fight back by inhibiting the induction of eIF2α phosphorylation of the host (Mulvey et al., 2004). Some viruses such as DCV and CrPV can directly recruit the ribosome to viral RNA and do not require eIF2-mediated Met-tRNA$_i$ loading for their translation (Cherry et al., 2005; Pfingsten et al., 2010).

In conclusion, several signaling pathways and cellular processes are involved in antiviral immune responses in *Drosophila*. RNA interference is the only general mechanism that has been found so far in flies for defending against a variety of viruses (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). Other responses, such as the Toll pathway, the Imd pathway, the JAK-STAT pathway,

apoptosis and autophagy, are responding to and protecting flies from certain viral infections (Avadhanula et al., 2009; Costa et al., 2009; Dostert et al., 2005; Liu et al., 2013; Shelly et al., 2009; Zambon et al., 2005). As an important cellular program for controlling gene expression, host translation may also contribute to the integrity of the immune systems. Evidence in other species suggests an immune related role for the regulation of the host translation (Chakrabarti et al., 2012; Connor and Lyles, 2002; Dunbar et al., 2012; McEwan et al., 2012); however, whether translation is involved in the immune responses in *Drosophila* remains unknown. Given the complexity of antiviral immunity, new genes or pathways are likely involved in *Drosophila*. Thus, I use two types of genetic screens to discover novel genes or pathways that may play roles in antiviral immune responses in *Drosophila*.

Chapter 2: Identification and Characterization a Novel Antiviral Gene, *rogue*, Which Restricts *Drosophila* X Virus (DXV) Replication in *Drosophila*

Abstract

Drosophila has a robust and efficient antiviral innate immune system. RNA interference (RNAi) is a general immune response for defending against various viruses in the fly. The evolutionarily conserved signaling pathways, Toll, Imd and JAK-STAT have been shown to play roles in antiviral innate immunity against certain viruses in the fly. In addition, cellular processes, such as apoptosis and autophagy are involved in antiviral immune responses. With the goal of discovering novel antiviral genes or pathway, a pilot screen for mutants with increased susceptibility to a dsRNA virus, *Drosophila* X Virus (DXV), was done, and one of the mutants mapped to a novel antiviral gene, *rogue.* The *rogue* mutant is highly susceptible to DXV infection and is unable to control viral replication during infection. The expression of the *rogue* gene in either the hemocytes or the fat body is needed for flies to control viral accumulation and to survive viral infection. The *rogue* gene was induced in wild type flies, but not in *relish* mutants, indicating that it might be regulated by the Relish transcription factor. Increased numbers of hemocytes had nuclear localization of the Rogue protein at early stages of viral infection, suggesting that its localization responds to viral infection. In addition, our results showed that the Rogue protein interacts with the polyA binding protein (PABP) in adult flies. We propose that *rogue* is a novel antiviral gene that is involved with PABP in regulation of translation in *Drosophila*.

Introduction

Drosophila does not have an adaptive immune system, but they do have a robust innate immune system to defend against a variety of pathogens, including viruses (Lemaitre and Hoffmann, 2007). The immune pathways found in *Drosophila* share extensive similarity with other vertebrate or invertebrate organisms. The studies on the immune function of the Toll pathway in *Drosophila* led to the discovery of Toll-like receptors (TLR) in mammals (Lemaitre et al., 1996; Medzhitov et al., 1997; Poltorak et al., 1998b). Thus, studies on the antiviral immunity in *Drosophila* may contribute to the understanding of the principles of antiviral innate immunity in other organisms, including human and other insects. As a genetically tractable model organism, *Drosophila* has a short life cycle and produces many progeny at each generation; thus, it is amenable for large-scale forward and reverse genetics with low cost. Hence, it provides a powerful tool for discovering novel molecules or pathways that are involved in antiviral immune responses. In addition, *Drosophila* can serve as a good model for insect antiviral immunity studies. Findings in *Drosophila* may promote the development of transmission interventions of insect vectors.

In *Drosophila*, several pathways and cellular processes have been found to play antiviral roles. First, RNA interference is a general antiviral immune response in *Drosophila* (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). Upon infection, viral dsRNA intermediates are recognized and diced by an RNase III-like enzyme Dicer 2 (Bernstein et al., 2001) and its cofactor R2D2 (Liu et al., 2003) to produce virus-derived small interfering RNA (vsiRNA). The vsiRNA are then loaded onto the Argonaute 2 (AGO2) (Okamura et al., 2004) within the RISC complex. The

message strand of the vsiRNA duplex is then degraded, and the guide strand leads the RISC to the viral RNA by sequence complementarity. After the binding of RISC, the targeted viral RNA is degraded (Haley and Zamore, 2004). Thus, this process can restrict viral replication and provide efficient antiviral defense against different viruses. There are three sub-pathways of the RNAi pathway (Okamura and Lai, 2008): 1) The miRNA pathway, which regulates endogenous gene expression. AGO1, Dicer1 and Loq are involved in the miRNA pathway. 2) The siRNA pathway, which cleaves exogenously induced dsRNA, including viral RNA. AGO2, Dicer2 and R2D2 are used in the siRNA pathway. 3) The piRNA pathway, which controls transposon expression. Argonaute proteins Piwi, Aubergine and AGO3 are involved in piRNA pathway. So far, only the siRNA and the piRNA pathways have been implicated in antiviral responses.

In addition to the RNAi pathways, the classical immune pathways (Toll, Imd and JAK-STAT) play roles in defense against viral infections. However, the antiviral responses of these pathways appear to be virus specific. The Toll pathway, which is required for the fly to resist Gram-positive bacterial and fungal infection, has been shown to be important against *Drosophila* X virus (DXV) (Zambon et al., 2005). The Imd pathway, which is indispensable for defending against Gram-negative bacteria in the fly, is vital for defense against Cricket Paralysis virus (CrPV) and Sindbis virus (SINV) but not DXV (Avadhanula et al., 2009; Costa et al., 2009; Zambon et al., 2006). The JAK-STAT pathway, an important antiviral pathway in mammalian systems, also mounts antiviral responses against specific viruses, such as *Drosophila* C virus (DCV) and CrPV

(Deddouche et al., 2008; Dostert et al., 2005). Although, these pathways clearly respond to certain viruses, the mechanisms involved are largely unknown.

Besides the above conventional immune pathways, evidence for the involvement of other cellular processes in antiviral immunity has been emerging. Important cellular processes, such as apoptosis and autophagy, have been implicated in the defense against certain viruses in *Drosophila* (Liu et al., 2013; Shelly et al., 2009). Mutants of apoptosis or autophagy showed increased susceptibility to FHV and VSV respectively. Another important cellular process, translation, may also play a role in the immune responses against virus. Translation is essential for protein synthesis and for spatial and temporal gene expression in response to stresses (Lu et al., 2001), such as viral infection. On the other hand, viral protein synthesis relies on the host translational machinery. Viruses commonly block the translation of host mRNA, so that they can have privileged access to the host translational machinery (Connor and Lyles, 2002; Kleijn et al., 1996). Such virus-induced translation inhibition also dampens the expression of immune genes. Because of this tight host-virus interaction, it would not be surprising if translation serves as a form of host defense as well. It has been found that down regulation of inhibitors of translation by viral infection increases the basal cytokine expression in mouse fibroblast cells (Colina et al., 2008). However, if or how translation plays a role in antiviral responses in *Drosophila* remains to be studied.

Although these pathways have been found to play roles in antiviral immune responses, the mechanisms of most of them are not completely clear yet. Discovery of additional

genes of antiviral immunity may give insights into the mechanisms of these immune responses or help identify new antiviral pathways. To discover novel genes or pathways that may play a role in protecting the fly from virus infection, a screen for mutants that were more susceptible to *Drosophila X virus* (DXV) was conducted.

DXV is a non-enveloped dsRNA virus. It has not been found in wild populations of flies. DXV encodes its own RNA dependent RNA polymerase (RdRp) for replication, but depends solely on the host for its protein synthesis (Dobos et al., 1979; Zambon et al., 2005). Previous studies have found that exposure of flies to CO_2 can accelerate the death of the infected flies (Teninges, 1979b). In wild type flies, limited DXV can be detected at early infection stages, while the virions spread throughout the whole fly at later stages of infection. The siRNA pathway, the piRNA pathway and the Toll pathway are found to be important antiviral pathways against DXV (Zambon et al., 2005; Zambon et al., 2006).

Here we characterized one of the mutants from the screen, *rogue*, and investigated the role of *rogue* in antiviral immunity in *Drosophila*. Our results show that the *rogue* mutant is highly susceptible to DXV infection and has a defect in controlling viral replication during infection. RNAi knockdown of *rogue* in wild type flies also rendered flies more susceptible to viral infection. The expression of the *rogue* gene in either the hemocytes or the fat body was required for flies to control the viral accumulation in the adult fat body. The expression of the *rogue* gene is increased at early stages of infection, and the nuclear localization of the Rogue protein is induced by viral infection. We also find that Rogue is

a PABP interacting protein. Our findings suggest that Rogue may be a novel antiviral gene involved in PABP dependent functions.

Results

A) Identification of the X-men mutants

To identify novel genes or pathways involved in the antiviral responses against DXV, a genetic screen to identify mutants with increased susceptibility to DXV infection was conducted by Anne Macgregor, a former undergraduate student in the lab. A total of 161 adult viable mutant lines with mutagenized 2nd chromosomes (Koundakjian et al., 2004) were used in our study to identify mutants with increased susceptibility to DXV. As detailed below, homozygous flies (30-50 flies of each line) were injected with DXV, and the number of flies surviving one day after injection was used as the initial count. To accelerate the death of flies after DXV infection, anoxia treatments were applied on day 7 and day 10, and the number of flies that survived at day 8 and day 11 was used for survival analysis. The lines that had at least one standard deviation below the survival of the parental line (*iso*) for both days were selected for retesting. Four lines were found to have significantly higher mortality to DXV compared to *iso*. The four mutants were named after the comic book X-men characters: ***rogue*** (840), ***storm*** (906), ***pyro*** (1966) and ***mimic*** (4096). The original zuker line numbers of each mutant are showing in the parentheses.

To confirm the mutants found from the screen, I examined the survival of these mutants following DXV infection, with and without anoxia treatments. The four X-men mutants

and *iso* were injected with DXV, and the surviving flies were counted daily after infection. The anoxia treatments were conducted at day 7 and 10. I confirmed that *rogue, storm, pyro,* and *mimic* were more susceptible to DXV compared to *iso* (Fig 2-1A). The p-values were <0.001, 0.0229, <0.001, 0.003 respectively. The X-men mutants injected with water showed survival curves comparable to the *iso* flies (P-values > 0.05) (Fig 2-1B), indicating that the morbidity of flies was due to the viral infection, and not the injury caused by injection.

The death of pathogen-infected animals can be caused by either lack of resistance or tolerance to the pathogen. Upon infection, some animals succumb because of higher amounts of pathogen accumulation in the body; these animals have a lower ability to resist this pathogen. Other animals die faster even though the pathogen replication can be controlled by the host immunity; these animals have defects in tolerance to this pathogen. To investigate whether the increased susceptibility of the X-men mutants was caused by uncontrolled viral replication, viral RNA levels were measured following DXV infection by quantitative RT PCR (qPCR). *rogue*, *storm* and *pyro* had higher viral RNA levels compared to *iso* flies starting at day 5 (Figure 2-2 A and B), indicating that these mutants had defects in controlling viral replication. *mimic* showed similar viral RNA levels compared to *iso* flies, suggesting that *mimic* might have a lower ability to tolerate DXV infection (Figure 2-2 C).

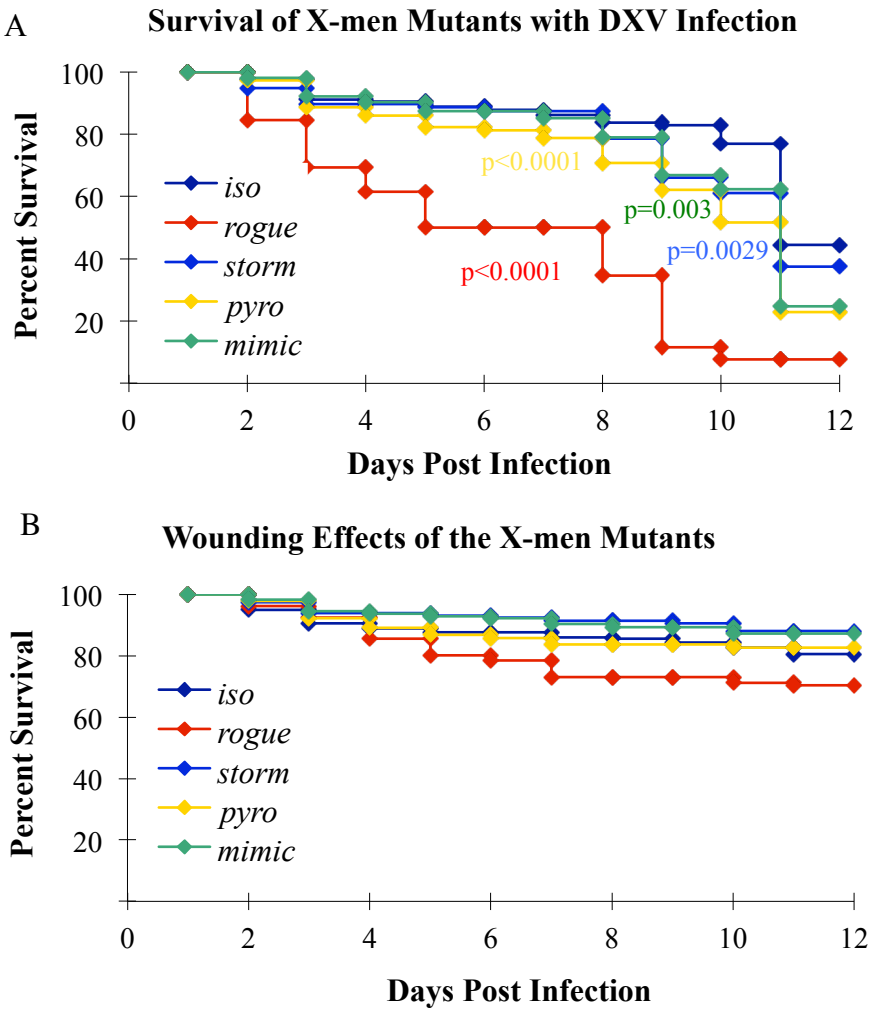
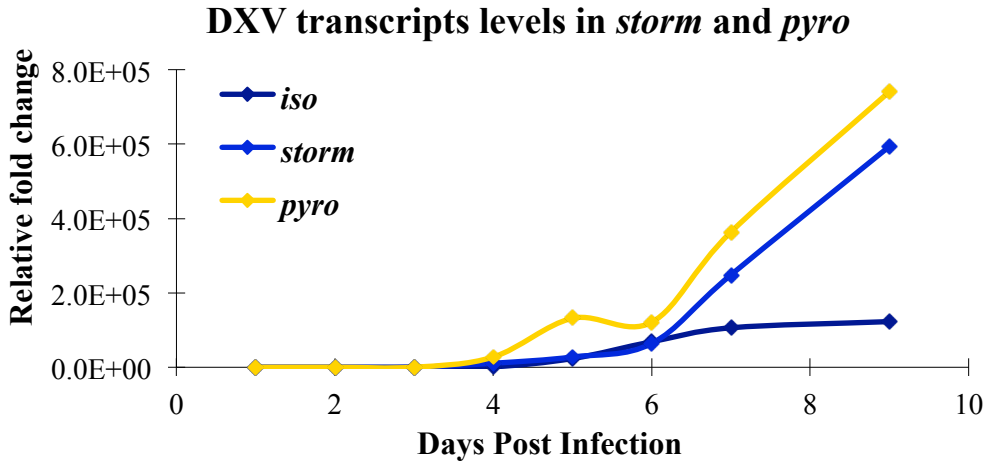
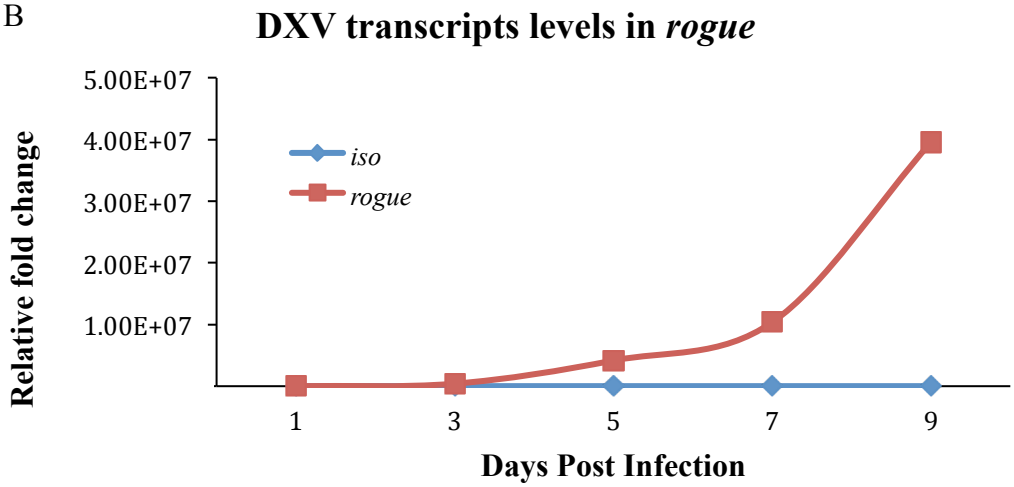


Figure 2-1: Survival curves of the X-men mutants following DXV infection or water injection. 50 flies (25 females and 25 males, 5-7 days old) of each line were injected with DXV or water. The morbidities were recorded daily after injection. The number of flies surviving at day 1 was used as the initial count. Anoxia treatments were performed at days 7 and 10 post injection. The survival of *rogue*, *storm*, *pyro*, *mimic* with viral infection were significantly different from *iso* flies, while the survivals of these lines after water injection were not significantly different from *iso* flies. The data shown represents the mean of triplicates. Log-rank tests were used to determine the p-values.

A


DXV transcripts levels in storm and pyro
Relative fold change
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
iso
storm
pyro
0
2
4
6
8
10
Days Post Infection

B


DXV transcripts levels in rogue
Relative fold change
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
iso
rogue
1
3
5
7
9
Days Post Infection

C

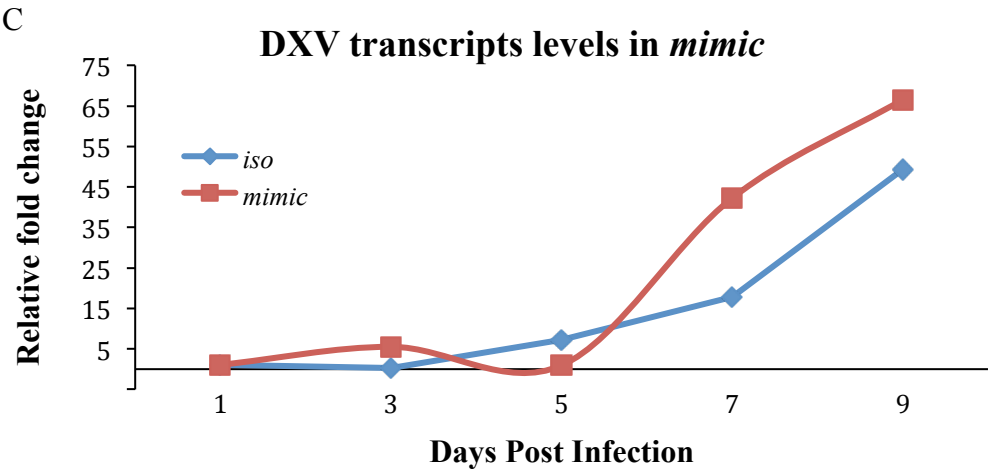

DXV transcripts levels in mimic
Relative fold change
75
65
55
45
35
25
15
5
iso
mimic
1
3
5
7
9
Days Post Infection

Figure 2-2: Viral RNA levels in the X-men mutants following DXV infection. The total RNAs of 10 flies (5 females and 5 males, 5-7 days old) were collected at each time point. The relative fold changes of the viral RNA levels were compared to those of *iso* at day 1 after infection. A, B) *storm*, *pyro* and *rogue* had much higher viral RNA levels over time compared to *iso* flies. C) *mimic* showed a similar viral RNA level as *iso*. Data shown are representative graph of at least 4 experiments.

EMS generates random point mutations on the chromosome. Different EMS mutations can affect the same gene. To determine if the X-men mutants are affecting the same gene, complementation tests among the X-men mutants were performed. None of the four mutants failed to complement each other, indicating that each mutant was affecting a different gene (Fig 2-3). Because several known genes involved in anti-DXV pathways, such as, *Dif, r2d2, vig(vas), piwi* or *aubergene*, are located on the 2nd chromosome, it is possible that the X-men mutants affect these genes. To explore this, complementation tests between X-men mutants and these genes were conducted (Fig 2-3). Our data showed that the mutants tested complemented the X-men mutants, except that *piwi* mutant failed to complement *mimic* in susceptibility to DXV. This suggests that *rogue, storm* and *pyro* are not mutants of *Dif, r2d2, vig(vas), piwi* or *aubergine*. However, *mimic* may have a mutation in the *piwi* gene, or that the mutated genes in *mimic* have genetic interactions with *piwi.* We also found that the *piwi* mutant and *r2d2* mutant appeared to fail to complement each other, indicating that these two genes might have a genetic interaction as the susceptibility was dose dependent on the *r2d2* and *piwi* alleles.

Since the original screen was done using anoxia treatment to trigger susceptibility, some of the mutants' susceptibility might be anoxia-dependent. To examine whether the susceptibility to DXV of the X-men mutants could be seen in the absence of anoxia, the mutants were subjected to survival analysis without anoxia.

Figure 2-3: Complementation tests between the X-men mutants and the Toll or RNAi pathway mutants. 50 flies (25 females and 25 males, 5-7 days old) of the progeny of each cross were injected with DXV. Anoxia treatments were performed at day 7 and 10 post injection. The morbidities were recorded at day 8 and 11 after injection. Log-rank tests were used to determine susceptibility.

The increased susceptibility to DXV compared to *iso* was lost in *storm* and *mimic* (Fig 2-4 A and B), suggesting that these two mutants have anoxia-dependent susceptibility to DXV. However, significantly higher mortality of *rogue* and *pyro* was still detected without the anoxia treatments, indicating there was anoxia-independent susceptibility to DXV in these two mutants (Fig 2-4 C and D). Because of the strong anoxia-independent susceptibility of *rogue* to DXV infection, it was chosen for the follow up studies. From this point on, all experiments were conducted without anoxia treatments.

To investigate if the increased mortality of *rogue* following DXV infection was caused by uncontrolled viral replication, viral RNA levels were measured following DXV infection by qPCR. We found that, even without anoxia treatment, *rogue* had higher viral RNA levels compared to *iso* flies over time (Fig 2-5). Thus the *rogue* mutant has a defect in controlling viral replication, and this defect is not due to sensitivity of the flies to anoxia treatments.

It is possible that the *rogue* mutant may be important for antiviral immunity against different viruses. To examine if *rogue* is sensitive to different viruses or specifically to DXV, the survival response to another virus, DCV, was examined. DXV is a birnavirus. The Toll, siRNA and piRNA pathway have previously been found to be important immune responses against DXV. DCV is a dicistrovirus and it is a natural pathogen of flies. DCV was found to activate the JAK-STAT pathway (Dostert et al., 2005). In addition, survival of DCV-infected flies also relied on the siRNA pathway (van Rij et al., 2006). Although DCV derived piRNA have been found by deep sequencing,

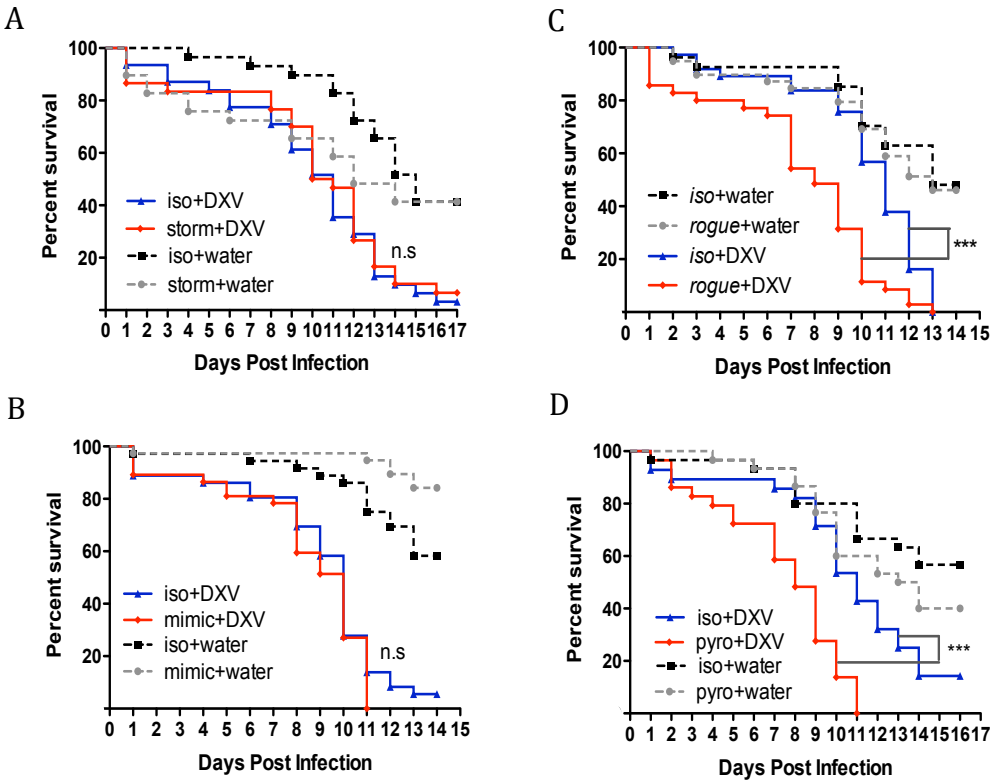


Figure 2-4: Survival of the X-men mutants following DXV infection without anoxia treatment. 30 flies (15 females and 15 males, 5-7 days old) of each line were injected with DXV or water. The morbidities were recorded daily after injection. The number of flies surviving at day 1 was used as the initial count. Among the four mutants, only *rogue* and *pyro* had significant higher morbidities compared to *iso* flies. The data shown are representative graphs of at least two experiments. The log-rank test was used to determine the p-values. *** $p<0.0001$

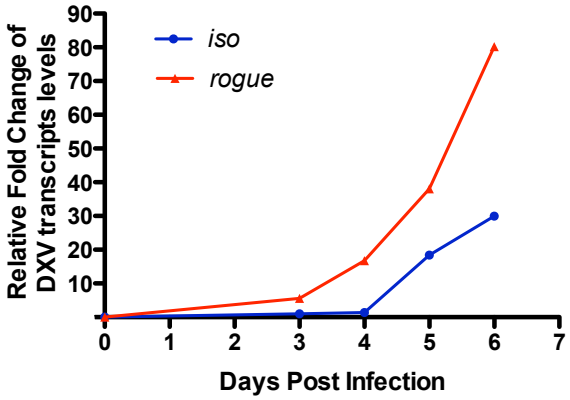


Figure 2-5: Viral RNA levels in the *rogue* mutant following DXV infection. The total RNAs of 10 flies (5 females and 5 males, 5-7 days old) were collected at each time point. The relative fold changes of the viral RNA levels were compared to those of *iso* at day 3 after infection. The data shown is a representative graph from three independent experiments.

it is not clear if the piRNA pathway plays a role in the immune responses against DCV. *rogue*, *iso* and the *dcr2* mutant were injected with DCV, and survivals were recorded daily. The homozygous *dcr2* mutant was significantly more susceptible to DCV; however, *rogue* was not susceptible to DCV (Fig 2-6). Our results suggest that the *rogue* mutation is not affecting the antiviral immunity against DCV. This result also indicates that the *rogue* mutant is not generally weak to pathogens.

B) Genetic mapping of the *rogue* mutant

Standard mapping crosses was carried out by crossing *rogue* and *mimic* to a *Drosophila* line with several visible recessive markers on the 2nd chromosome. Recombinants were collected and balanced. Because *rogue* is female sterile, and *mimic* is male sterile, the viral susceptibility and/or sterility were used to examine whether the recombinants inherited the mutations from *rogue* or *mimic*. Both *rogue* and *mimic* appeared to map to the interval between *dumpy* and *black*, which is 25A1-2 and 34D4-6 on the second chromosome (Fig 2-7). Complementation tests between deficiency lines in this region and the *rogue* mutant revealed that the sterility and the susceptibility of *rogue* were caused by different mutations. Deficiency line *Df(2L)BSC5* failed to complement *rogue* in sterility, suggesting that the mutation associated with the sterility of *rogue* was located between 26B1-2 and 26D1-2. Another two overlapping deficiency lines failed to complement *rogue* in survival to virus, indicating that the mutation associated with the susceptibility of *rogue* was located between 34A1 and 34A3. The two deficiency lines were *Df(2L)ED775* and *Df (2L)BSC277* (Fig 2-7).

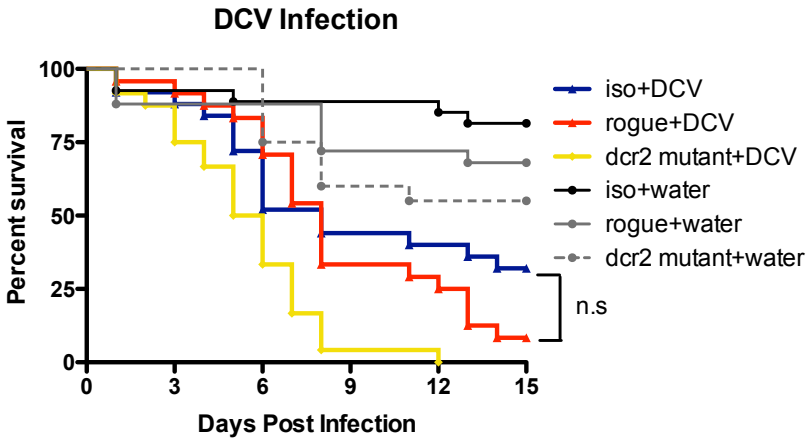


Figure 2-6: Survival of the *rogue* mutant following DCV infection. 50 flies (equal numbers of females and males) of each line were injected with DCV. The survival curves were shown. *rogue* and *iso* had similar susceptibility to DCV, while the homozygous *dcr2* mutant served as a positive control. Log-rank tests were used to determine the susceptibility. Data shown is a representative graph from two experiments.

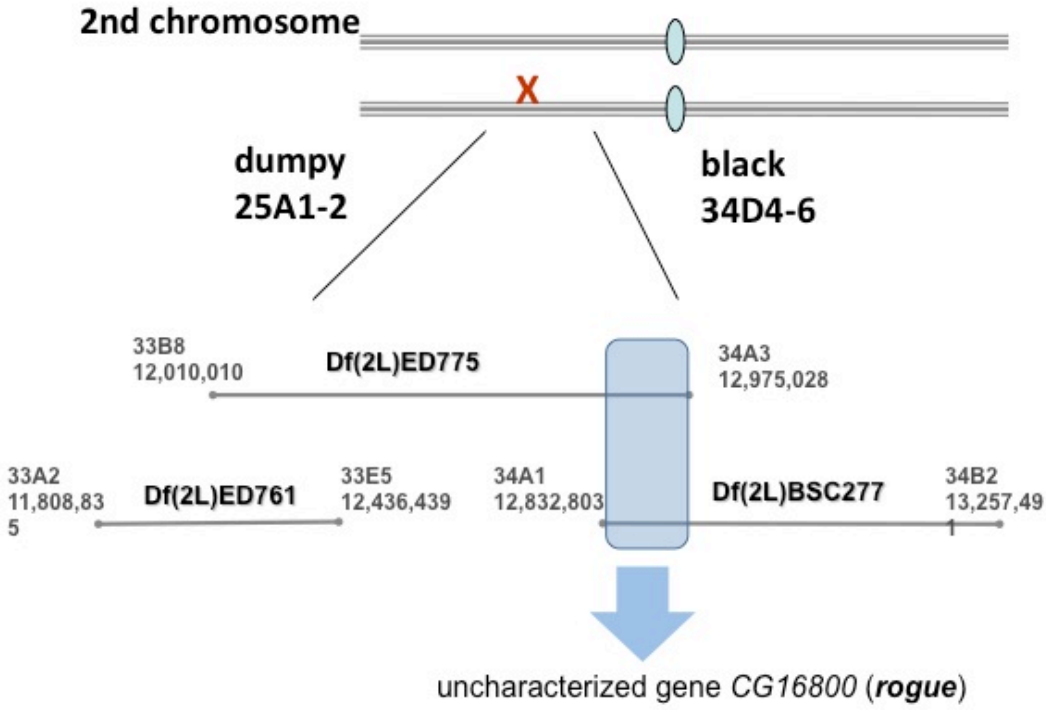


Figure 2-7: Mapping of the *rogue* mutant. The mutation was mapped between the mutations of *dumpy* and *black* by meiotic recombination mapping. Deficiency mapping in this region further located the mutation to a smaller region between 34A1 and 34A3.

Three genes and a small non-coding RNA were located in this region: *CR44183* (a small non-coding RNA); Adenylcyclase E (*ACXE*); V-type ATPase subunit 2 (*vha68-2*), and *CG16800* (an uncharacterized gene) (Fig 2-7).

To further narrow down the candidate genes, complementation tests between the *rogue* mutant and *ACXE* or *vha68-2* mutants were examined. Due to the lack of a *CG16800* or *CR44183* mutant, complementation tests between *rogue* and *CG16800* or *CR44183* were not conducted. As shown in Figure 2-8, the *ACXE* mutant is able to complement the *rogue* mutant in survival to DXV, indicating *rogue* is not an *ACXE* mutant. Meanwhile, *vha68-2* mutant fails to complement *rogue* (Fig 2-8), suggesting the possibility that *rogue* is a *vha68-2* mutant. However, our sequencing results found only a silent mutation in the coding region of the *vha68-2* gene in the *rogue* mutant. Together with the fact that the expression level of *vha68-2* gene in *rogue* is not decreased (Fig 2-9), the failed complementation of the two mutants is most likely due to a genetic interaction between *rogue* and *vha68-2*.

Since *rogue* was mapped to neither *ACXE* nor *vha68-2*, we shifted our focus to *CR44183* and *CG16800.* We first examined the transcript levels of *CR44183* and *CG16800* in the *rogue* mutant by qPCR (Fig 2-9). Surprisingly, both of them were significantly down regulated in the *rogue* mutant. However, in the *rogue* mutant, no mutation was found in *CR44183,* and only a silent mutation was found in the coding region of *CG16800* compared to the *iso* control. Noticeably, there is a big non-coding region (>10kb)

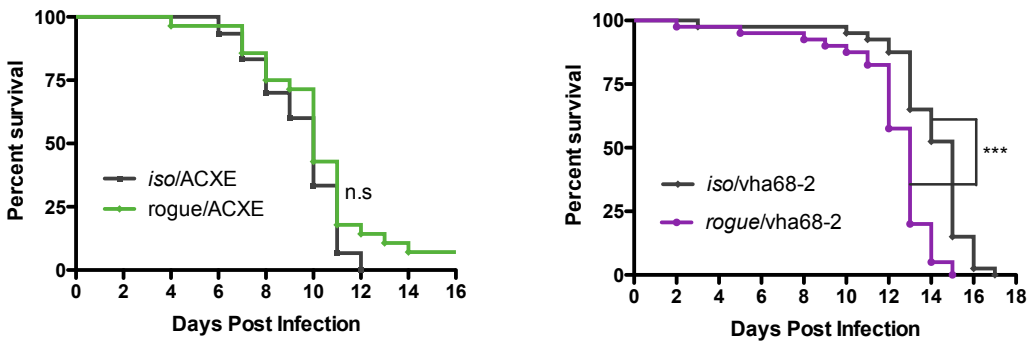


Figure 2-8: Complementation tests between the *rogue* mutant and the *ACXE* or *vha68-2* mutants. 50 flies (25 females and 25 males, 5-7 days old) of the progeny of each cross were injected with DXV. The morbidities were recorded daily after injection. The number of flies surviving at day 1 was used as the initial count. The *rogue* mutant was complemented by the *ACXE* mutant but not by the *vha68-2* mutant. The data shown are representative graphs of triplicates. Log-rank tests were used to determine susceptibility. ***p<0.0001

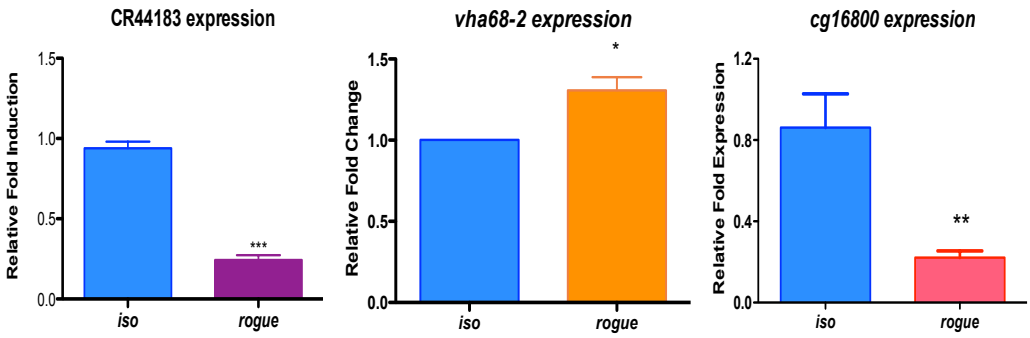


Figure 2-9: Expression levels of *CR44183*, *vha68-2*, and *CG16800* in the *rogue* mutant. Total RNAs were isolated and the expression levels of each gene were measured by quantitative PCR and were compared to *iso*. The data shown represents the mean of at least triplicates. The error bars show standard error. *** $p<0.0001$, **$p<0.01$, *$p<0.05$

between these two genes, and a mutation in this interval might be responsible for the effect on *CR44183* and *CG16800* expression. Thus, it is possible that the *rogue* mutant has mutations in the regulatory elements in this region and affects both *CR44183* and *CG16800* expression.

No mutant or RNAi line of *CR44183* was available; thus, we focused our study on the effect of *CG16800* on the antiviral response in the fly. Flies with a transgenic dsRNA hairpin against *CG16800*, were crossed to flies with the Actin5C GAL4 driver, a ubiquitous driver, and the progeny was subjected to DXV infection (Fig 2-10 A). Compared to the driver only flies, the *CG16800* knockdown flies showed a strong susceptibility to DXV. These flies also exhibited weakness as the flies were dying following PBS injection. Thus, other ubiquitous drivers Arm GAl4 and C564 GAL4 were used to express the dsRNA against *CG16800* in flies. These RNAi knockdown flies were not sensitive to PBS injection but were still significantly more susceptible compared to the driver only controls (Fig 2-10 B and C). These results confirmed that *CG16800* was required for flies to survive DXV infection.

The constructs of the dsRNA hairpin from these *CG16800* RNAi lines are predicted to produce two 19-mers that can potentially target another gene *CG16743*. Hence, the flies that express a dsRNA hairpin specifically against *CG16743* but not *CG16800* were also examined for susceptibility to DXV. In contrast, RNAi knockdown of *CG16743* did not affect the susceptibility of the flies to DXV (Fig 2-10D), suggesting that the survival

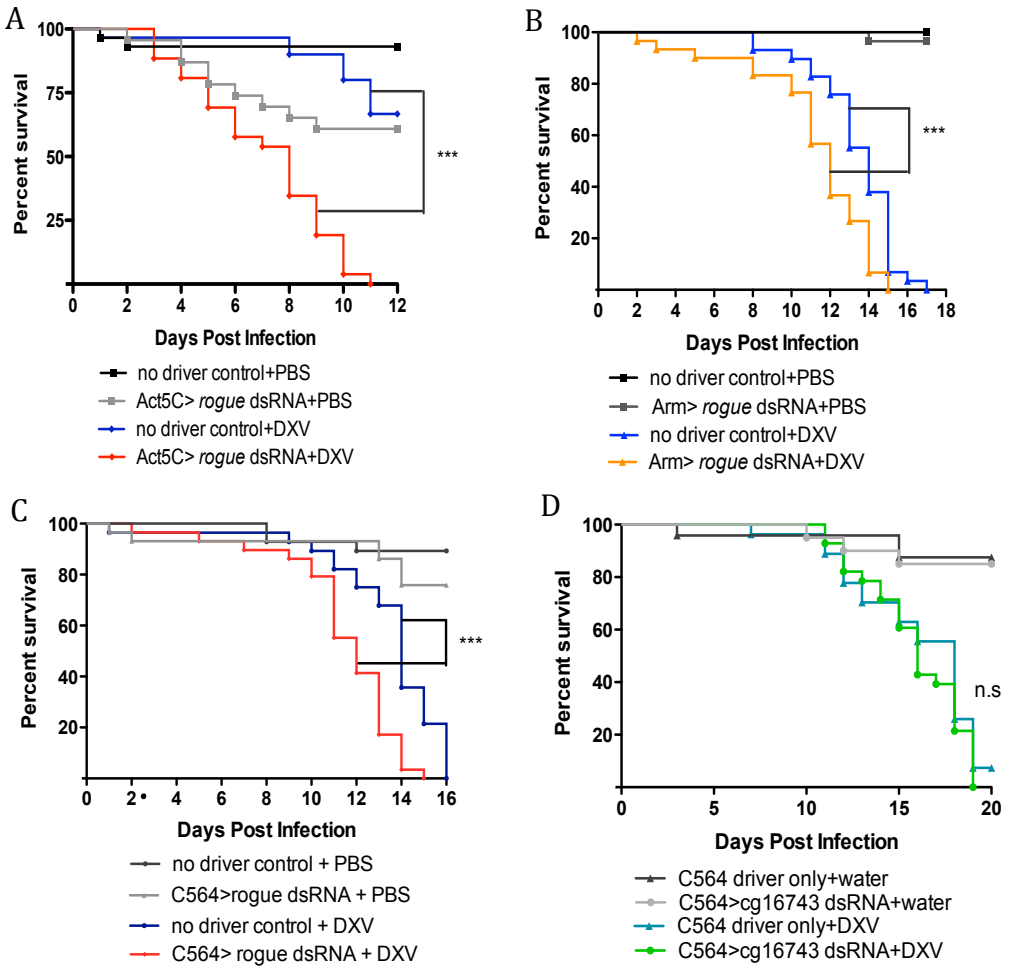


Figure 2-10: *CG16800* RNAi knockdown flies were more susceptible to DXV, while *CG16743* RNAi knockdown flies had similar susceptibility to virus as the control flies. Flies of each line were injected with DXV or PBS. The morbidities were recorded daily after injection. The number of flies surviving at day 1 was used as the initial count. Log-rank tests were used to determine susceptibility. The data shown are representative graph of triplicates. ***: $p<0.0001$

phenotype we saw from the *CG16800* RNAi knockdown flies was due to the decreased expression of *CG16800* but not *CG16743*.

To investigate if the susceptibility of the *rogue* mutant to viral infection was due to decreased *CG16800* expression, we expressed the *CG16800* transgene in the *rogue* mutant fly. To our surprise, overexpression of *CG16800* in the whole fly or fat body affected the overall viability in flies. Majorities of the progeny were not able to make it to adulthood, and the few progeny that did eclose were too weak to be used for the survival analysis. This suggests that the expression levels of *CG16800* are critical for the fitness of the flies. Or the timing and/or spatial regulation of CG16800 expression affects the health of the flies. We then attempted to make transgenic flies with the full length *CG16800* gene as well as the flanking regions (10kb upstream and 5kb downstream of the gene) so that it would contain the endogenous regulation elements. However, no transformants were obtained due to technical difficulties. Since expression of *CG16800* in the hemocytes produces relatively healthy flies, we used these flies for survival analysis. We found that the expression of *CG16800* in the *rogue* mutant resulted in flies that were less susceptible to DXV, which indicated that the low expression of *CG16800* contributed to the *rogue* mutant susceptibility to viral infection (Fig 2-11).

Although no clear mutations were found in *CG16800* in the *rogue* mutant, the flies express dramatically low levels of *CG16800* (Fig 2-9). Knockdown of the expression levels of the *CG16800* gene in wild type flies causes the flies to be more susceptible to DXV (Fig 2-10). Additionally, overexpression of *CG16800* in the *rogue* mutant rescues

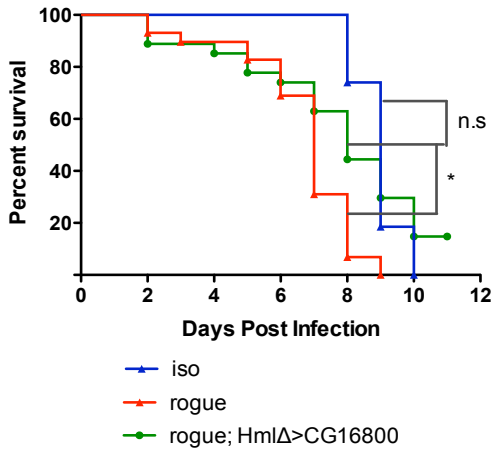


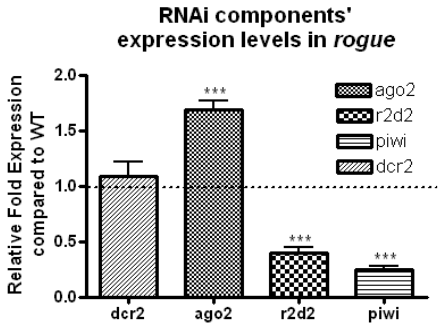
Figure 2-11: Expression of *CG16800* in hemocytes partially rescues the *rogue* susceptibility to DXV. 30 flies (equal number of females and males) of each line were injected with DXV. The morbidities were recorded daily after injection. The number of flies surviving at day 1 was used as the initial count. Log-rank tests were used to determine significance. The data shown is a representative graph. *: $p<0.05$

the susceptibility of the mutants to viral infection (Fig 2-11). Taken together, our results suggest that the susceptibility to DXV of the *rogue* mutant is most likely caused by the decreased expression of *CG16800*. Therefore, we named the *CG16800* gene ***rogue***.

C) Characterization of the *rogue* mutant

The RNAi pathway is a general antiviral immune response in the fly, and it has been found to be important for the fly to defend against DXV infection. To examine if *rogue* has any defects in the RNAi pathway, several genes involved in the RNAi pathway were examined in the *rogue* mutant by qPCR (Fig 2-12). Dicer2, an RNase III like enzyme, is the main generator of the siRNA. Its transcript level is not affected in the *rogue* mutant compared to *iso*. However, the mRNA level of *r2d2*, the co-factor of *Dcr-2*, is significantly lower in the *rogue* mutant, while the mRNA level of the Argonaute protein *AGO2*, the main component of RISC, is increased. The piRNA pathway gene *piwi* is also significantly down regulated in the *rogue* mutant. From our complementation tests between the *r2d2* and *piwi* mutant, we had noticed the possible interaction of these two genes. Thus, the expression of *AGO2* and *piwi* were measured in the *r2d2* mutant. Not surprisingly, the *r2d2* mutant showed expression patterns of AGO*2* and *piwi* similar to that of the *rogue* mutant (Appendix B Fig S1), which suggested that decreased expression of *r2d2* in the *rogue* mutant might cause the differential expression of the other RNAi pathway genes. Given that the *r2d2* mutant was susceptible to DXV, the low expression of *r2d2* in the *rogue* mutant might be associated with the increased susceptibility to DXV

A



B

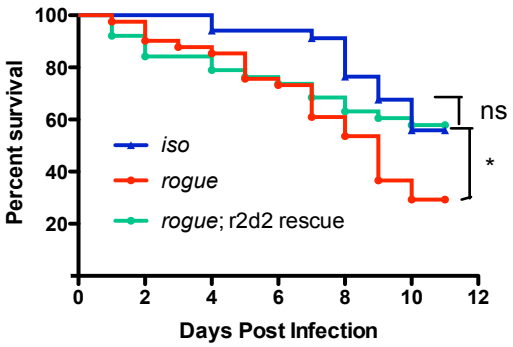


Figure 2-12: *r2d2* may contribute to the susceptibility of *rogue* to DXV. A) *rogue* had altered expression levels of *AGO2* (increased) , *r2d2* (decreased) and *piwi* (decreased). The expression levels of each gene were measured by qPCR and were normalized to that of *iso*. The data shown represents the mean of triplicates. The error bars show standard error. *** $p<0.0001$ B) Transgenic expression of *r2d2* in the *rogue* mutant background partially rescues its susceptibility to DXV. The data shown is representative experiments of at least three replicates. Log-rank statistics were used to calculate the p-value. * $p<0.05$

infection. To test this hypothesis, expression of *r2d2* was introduced into the *rogue* mutant. We found that expression of *r2d2* in the *rogue* mutant partially rescued the mutant survival to DXV infection, suggesting that the decreased expression of *r2d2* did contribute to the DXV susceptibility in the *rogue* mutant.

The Toll pathway is another known pathway that responds to DXV infection. However, we did not find any defect of the *rogue* mutant in the Toll pathway. The details are presented in the next chapter.

D) Characterization of the *rogue* gene

rogue (CG16800) encodes a protein with the predicted protein size of 32.2kD. Based on the amino acid composition, the Rogue protein is highly positively charged (pI=9.3). Secondary structure prediction showed that a large part of the Rogue protein contains helical structure. According to DNABinder, Rogue is unlikely to be a DNA binding protein. Limited studies have been done on this gene. According to the FlyAtlas Anatomical Expression Database, the highest expression of *rogue* was found in the adult fat body (Fig 2-13). It is also expressed at relatively higher levels in the late pupal central nervous system (CNS). The expression of *rogue* in larval or adult hemocytes was unknown. To examine if *rogue* is expressed in the hemocytes, the expression levels of *rogue* in larval hemocytes were performed using quantitative RT-PCR. The *rogue* mRNA is expressed higher in the hemocytes compared to the carcass (Fig 2-13), indicating that *rogue* is expressed in the larval hemocytes. To examine the expression patterns of *rogue*

FlyAtlas Anatomical Expression Data
(FlyAtlas-RNA.adult)
(FlyAtlas-RNA.larva)

[download data (TSV)]

Styles
- Linear
- Logarithmic
- Heatmap
- Back-to-back

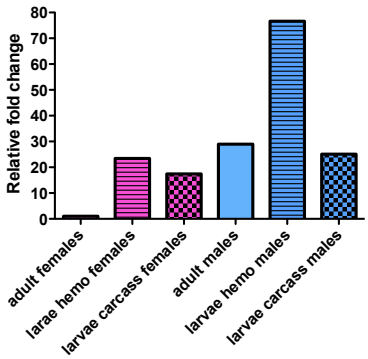
Scales
- gene max expression
- Moderate expression bin max
- High level expression bin max
- Very high expression bin max

Guide to FlyAtlas expression level colors
- No expression (0 - 9.999)
- Low expression (10 - 99.999)
- Moderate expression (100 - 499.999)
- High level expression (500 - 999.999)
- Very high expression (>999.999)

FlyAtlas Organ/Tissue Expression, larval vs. adult

Larval Expression Level	Tissue	Adult Expression Level
NA	Head	5.6
NA	Eye	6.125
NA	Brain	6.9
7.325	Central Nervous System	NA
NA	Thoracic-Abdominal Ganglion	9.1
NA	Crop	4.1
8.7	Midgut	6.5
8.2	Hindgut	4.6
3.9	Malpighian Tubules	5.4
5.3	Fat Body	15.7
6.3	Salivary Gland	*no informative data*
NA	Heart	5.2
8.275	Trachea	NA
NA	VirginFemale Spermatheca	*no informative data*
NA	InseminatedFemale Spermatheca	7.8
NA	Ovary	2.7
NA	Testis	6.4
NA	Male Accessory Gland	7.7
8.3	Carcass	3.4

cg16800 expression in hemocytes (mRNA level) (w1118)



cg16800 expression in different parts of adult fly

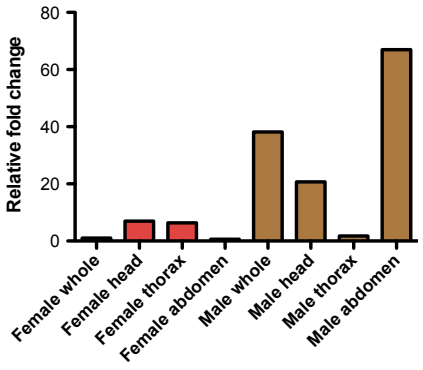


Figure 2-13: Expression of *rogue* in different tissues of adult fly. A) Expression data of *rogue* from the FlyAtlas Anatomical Expression Database. *rogue* gene expression in different tissues of larvae and adult flies is shown. The highest expression is found in the adult fat body. B) Expression of *rogue* in different parts of larval and adult flies. Total RNAs were extracted from different parts of the adult fly. 10 flies were used in each sample. The expression levels were measured by qPCR and were normalized to *rp49*. The data shown is from one experiment.

in the adult, tissues of female and male flies were examined for *rogue* expression (Fig 2-13). Higher expression of *rogue* was found in male flies compared to female flies. The expression of *rogue* was also higher in the abdomen of male flies than female flies. This suggests that *rogue* may be expressed in the male reproducing organ. In female flies, *rogue* was mostly found in the head and thorax.

The sequence of *rogue* was conserved in many insect hosts: different species of *Drosophila*, two species of mosquito and several species of bee (Table 2-1). The sequence alignments between Rogue and the few Rogue homologs in these species are shown in Figure 2-14. A few *C.elegans* homologs are predicted, but no *rogue* ortholog has been found in *H.sapiens* (human) or *M.musculus* (Mouse) (Table 2-2). The detailed information about these five orthologs is shown in appendix A.

Table 2-1: Sequences producing significant alignments

Protein ID	Gene	Species	Total score	E Value
NP_609594.2	CG16800	*Drosophila melanogaster*	335	2E-111
XP_002079269.1	GD23859	*Drosophila simulans*	330	1E-109
XP_002088520.1	GE18609	*Drosophila yakuba*	329	3E-109
XP_002042093.1	GM10082	*Drosophila sechellia*	327	1E-108
XP_001969697.1	GG23803	*Drosophila erecta*	300	3E-98
XP_001962866.1	GF14213	*Drosophila ananassae*	156	3E-42
XP_002003771.1	GI21259	*Drosophila mojavensis*	139	9E-36
XP_001355899.2	GA14158	*Drosophila pseudoobscura*	137	7E-35
XP_002051666.1	GJ11100	*Drosophila virilis*	137	7E-35
XP_001988845.1	GH11384	*Drosophila grimshawi*	131	6E-33
XP_003703702.1	LOC100880530	*Megachile rotundata*	162	3E-28
XP_002065168.1	GK15306	*Drosophila willistoni*	114	1E-26
XP_003402918.1	LOC100643328	*Bombus terrestris*	149	2E-23
XP_003491551.1	LOC10072061	*Bombus impatiens*	104	2E-21
XP_971797.1	CG16800	*Tribolium castaneum*	97.4	8E-21
XP_001604445.1	LOC100120847	*Nasonia vitripennis*	97.4	3E-19
XP_001946319.1	LOC100164442	*Acyrthosiphon pisum*	86.7	1E-16
XP_004533403.1	Cylicin-2-like	*Ceratitis capitata*	86.7	1E-16
XP_003491551.1	LOC100740058	*Bombus impatiens*	84.7	3E-16
XP_003402918.1	LOC100647166	*Bombus terrestris*	84.7	3E-16
XP_004925582.1	Histidine-rich Glycoprotein-like	*Bombyx mori*	84.0	6E-16

XP_006615292.1	spore wall protein 2-like	*Apis dorsata*	84.7	1E-15
XP_001120792.1	Histidine-rich Glycoprotein-like	*Apis mellifera*	80.1	1e-14
XP_006620015.1	Histidine-rich Glycoprotein-like	*Apis dorsata*	79.0	2E-14
XP_006562672.1	Protein starmaker-like	*Apis mellifera*	79.7	3E-14
XP_003243748.1	LOC100568636	*Acyrthosiphon pisum*	79.7	7E-14
XP_002366193.1	trichohyalin	*Toxoplasma gondii ME49*	256	1E-13
XP_791225.3	SCO-spondin	*Strongylocentrotus purpuratus*	247	2e-13
EJY57491.1	AAEL017367-PA	*Aedes aegypti*	76.3	2e-13
XP_004925585.1	Histidine-rich Glycoprotein-like	*Bombyx mori*	76.3	3e-13
XP_001844992.1	Conserved hypothetical protein	*Culex quinquefasciatus*	73.6	2e-12

Protein sequence of Rogue was used for the alignment. The non-redundant protein sequences were the database that used to search from. Domain enhanced lookup time accelerated BLAST program from NCBI was used. Predicted proteins that have p-values lower than 3e-12 are shown. The alignment was done in January, 2014.

Figure 2-14: Alignments of homolog sequences of Rogue. Clustal Omega program version 1.2.0 was used to generate the alignments of the homolog proteins of Rogue (EMBL-EBI). Colors indicate the residue groups as follows: small and hydrophobic residues are showing in RED; Acidic residues are showing in BLUE; Basic residues are showing in MAGENTA; hydroxyl/sulfhydryl/amine residues are showing in GREEN. AAEL017367-PA (*Aedes aegypti*); LOC100647166 (*Bombus terrestris*); LOC100740058 (*Bombus impatiens*); Rogue (*Drosophila melanogaster*); GD23859 (*Drosophila simulans*).

Table2-2: *rogue* Orthologs in *C.elegans*. (DRSC)

FlyBaseID	Fly Symbol	Worm Gene ID	Worm Symbol	Identity	Similarity	Program
FBgn 0032462	CG16800	180264	CELE_Y 39B6A.1	24%	32%	orthoMCL , Phylome
FBgn 0032462	CG16800	182736	C17F3.3	30%	44%	OMA
FBgn 0032462	CG16800	183174	C33G8.2	28%	41%	Isobase
FBgn 0032462	CG16800	189735	CELE_Y 39B6A.9	26%	43%	Phylome
FBgn 0032462	CG16800	181476	R01E6.5	24%	38%	Phylome

Five homologs were predicted from *C. elegans*. The details of these genes are shown in the appendix A.

E) *rogue* restricts viral replication in the hemocytes and the fat body of flies

In adult flies, the hemocytes and the fat body are important immune related tissues; the expression of *rogue* in these tissues might be important for mounting full immune responses against the virus. Thus, dsRNA against the *rogue* gene were expressed specifically in the hemocytes or the fat body using the Pxn GAL4 or Yolk GAL4 drivers respectively. These flies were then subjected to viral infection, and the survivals were compared to the driver only controls. Significantly higher susceptibility to viral infection was observed in these flies compared to the control flies (Fig 2-15). The susceptibility correlated with higher viral RNA levels in flies following infection (Fig 2-15). Thus, *rogue* expression in either hemocytes or fat body is essential for flies to resist viral infection and control viral accumulation in the whole fly. In wild type flies, viral levels in the fat body are usually not observable until the late infection stages (Zambon et al., 2005); however, RNAi knockdown of *rogue* in the hemocytes or fat body may promote earlier viral replication in the fat body. To examine if *rogue* expression in the hemocytes and fat body was required for the fat body to control viral replication, dsRNA against *rogue* was expressed in both the hemocytes and fat body using the C564 GAL4 driver. At three days after infection, viral accumulation was undetectable in the control flies. In contrast, virus was observed in the adult fat body in the *rogue* knockdown flies. This indicated that *rogue* expression in the hemocytes or fat body was necessary for the fat body to control viral accumulation (Fig 2-16).

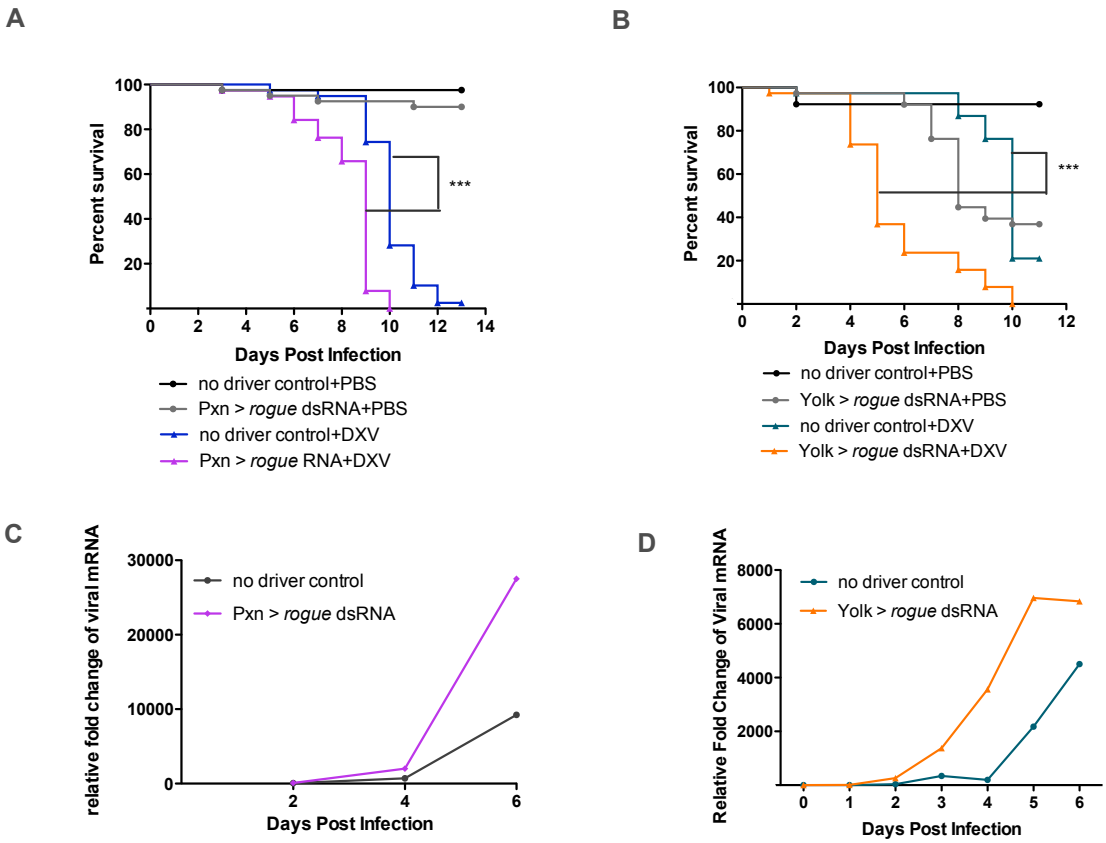


Figure 2-15: *rogue* expression in the hemocytes and fat body was required for the fly antiviral immunity. A, B) *rogue* expression in the hemocytes and fat body were required for fly survival with DXV infection. RNAi lines against *rogue* driven by hemocytes only (Pxn GAL4)(A) and fat body only (Yolk GAL4)(B) drivers were injected with DXV. The morbidities were recorded daily after injection. Log-rank statistics were used to calculate the p-value of survival. ***p<0.0001 C, D) Higher viral mRNA levels were seen in flies with RNAi knockdown of *rogue* in hemocytes (C) or fat body (D). Viral mRNA levels were measured by qRT-PCR at each time point. Driver only flies were served as controls. All data shown are representative experiments of at least three independent experiments.

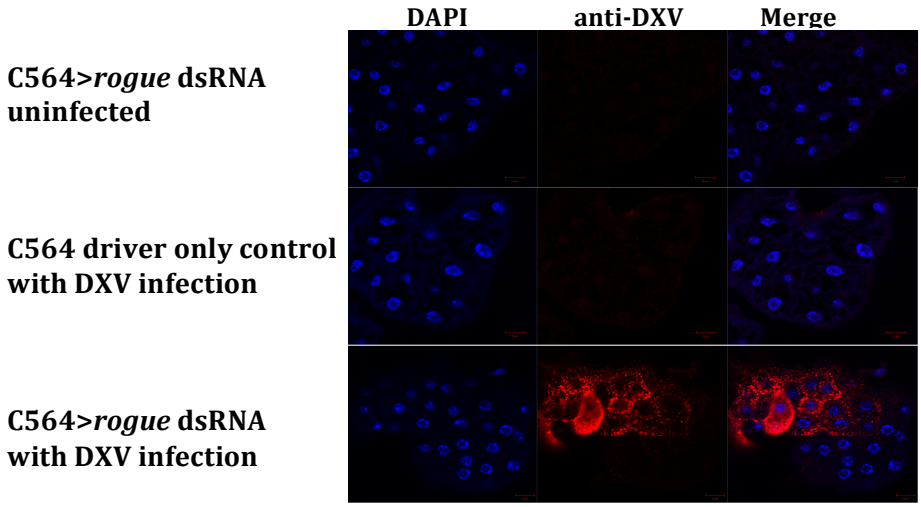


Figure 2-16: *rogue* expression in the hemocytes and fat body was required for the fly to control viral accumulation. Viral replication in the adult fat body of *rogue* RNAi knockdown flies. Driver only control and C564>rogue dsRNA flies were injected with DXV, and dissected at 3 days after infection. Viral accumulation in the fat body was detected by an anti-DXV antibody. Images were taken under the confocal microscope. Blue: DAPI; Red: DXV.

F) Rapid induction of the *rogue* gene following viral infection in fly

To examine if the *rogu*e gene can be induced by infection, DXV was injected into adult wild type flies. Quantitative RT-PCR was used to measure the expression level of the *rogue* gene at 6 hrs and 24 hrs. Following DXV infection, *rogue* was induced at 6 hrs post injection, but no induction was detected at 24 hrs (Fig2-17). Since *rogue* is induced before viral accumulation in the fly, this induction may be mediated by host cellular signals. To explore this, we examined the induction of *rogue* in response to viral infection in a variety of mutants or RNAi lines. Relish is a transcription factor that is activated by the IMD pathway (one of the NF-κB signaling pathways in the fly). In the control fly rel^{E23}, *rogue* was induced at 24 hrs but not at 6 hrs. This indicates that in different genetic backgrounds, the induction time of *rogue* by viral infection may vary. In the *relish* mutant rel^{E20}, the induction of the *rogue* gene was undetectable at both 6hrs and 24 hrs (Fig2-17). The abolished induction of *rogue* in the *relish* mutant suggested that this rapid upregulation of *rogue* expression might be mediated by the Relish transcription factor. However, we can not rule out two possibilities: first, the induction of *rogue* in the *relish* mutant happened at a later time point that was not examined; second, there might be less fat body tissues in the *relish* mutant, which resulted in the undetectable *rogue* induction.

G) Nuclear localization of Rogue in the hemocytes following viral infection

In the Rogue protein, two putative nuclear localization signals were identified (R.Nair. PredictNLS. Predict Protein. Appendix A), indicating that the Rogue protein may localize

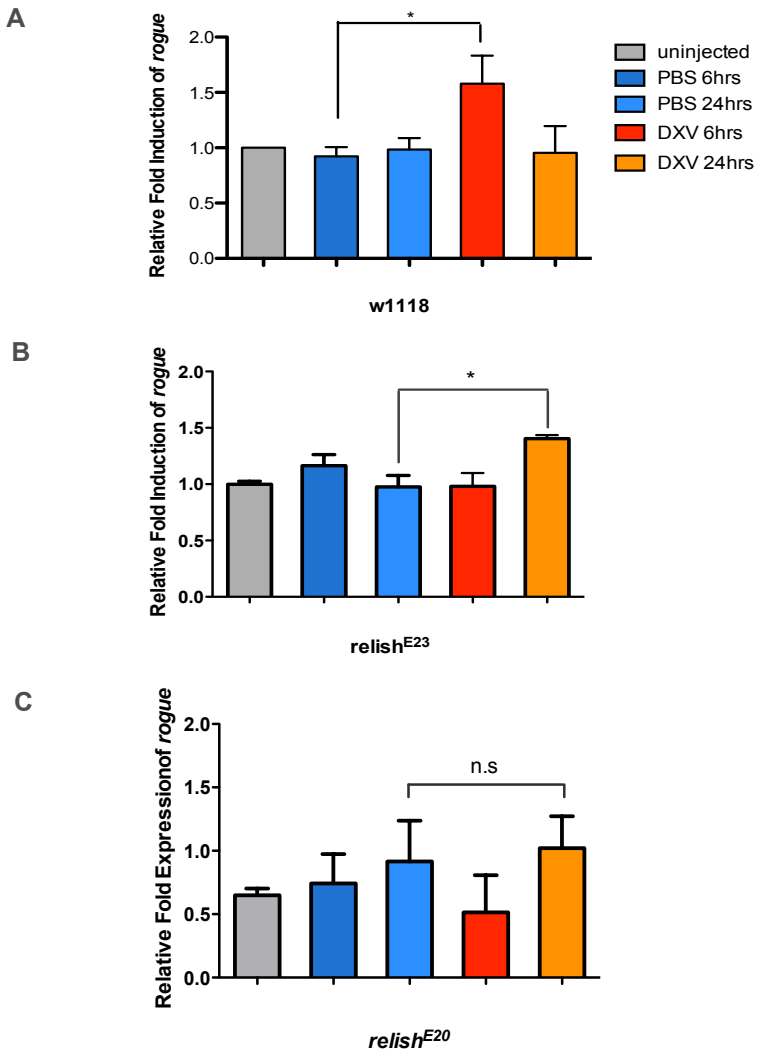


Figure 2-17: *rogue* induction in response to viral infection. *rogue* induction at 6 hrs or 24 hrs after PBS or DXV injection in *w1118* (A), $relish^{E23}$ control (B), and $relish^{E20}$ (C) flies. Quantitative PCR was used to measure the expression of *rogue*. The data shown represents the mean of triplicates. The error bars show standard error. Student t-tests were used to calculate the p-values. * $p<0.05$

to the nucleus. To determine the localization of Rogue, transgenic flies expressing a FLAG- tagged *rogue* gene in hemocytes were generated and the localization of the Rogue protein was examined. Hemocytes were bled out from 3rd instar wild type larvae and Rogue localization was determined by immunostaining for FLAG. Rogue expression was mainly found in the cytoplasm of larval hemocytes, and occasionally in the nucleus (Fig 2-18). To examine if this localization pattern changes after viral infection, the larvae were challenged with DXV, and hemocytes were collected at 1 hr and 5 hrs post-injection. Interestingly, at 1 hr after infection, 30% of the hemocytes from viral infected larvae showed nuclear localization of Rogue, while at same time point, only 5% of the hemocytes from PBS-injected larvae had nuclear localization of Rogue. At 5 hrs after infection, Rogue was mostly localized in the cytoplasm in both infected and uninfected cells (Fig 2-19). This suggests that viral infection triggers nuclear localization of Rogue in the hemocytes of larvae.

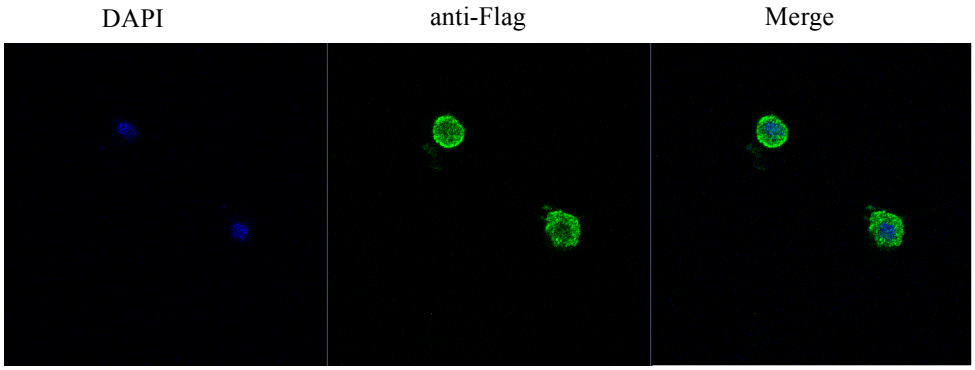
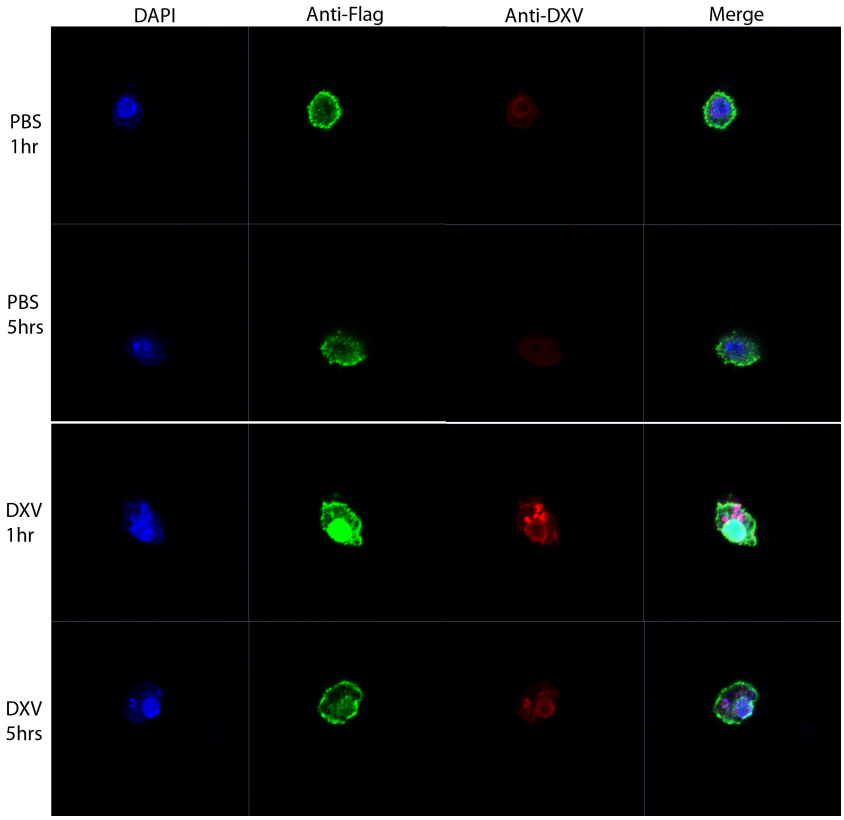


Figure 2-18: Rogue localization in the hemocytes of wild type larvae. Hemocytes were bled out from Pxn>GFP::FLAG-Rogue larvae, and imaged. Localization of Rogue was detected using an anti-FLAG antibody. Images were taken under the confocal microscope. Blue: DAPI; Green: Anti-Flag.


DAPI
Anti-Flag
Anti-DXV
Merge
PBS
1hr
PBS
5hrs
DXV
1hr
DXV
5hrs

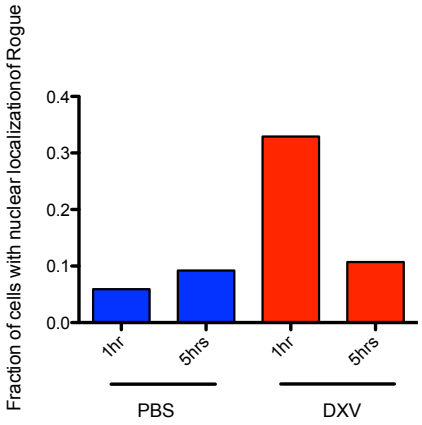

Fraction of cells with nuclear localizationof Rogue
0.4
0.3
0.2
0.1
0.0
1hr
5hrs
1hr
5hrs
PBS
DXV

Figure 2-19: Rogue localization in response to viral infection in the larval hemocytes. Pxn>GFP::FLAG-Rogue larvae were injected with PBS or DXV. Localization of Rogue was detected using an anti-FLAG antibody, and the virus was detected using an anti-DXV antibody at 1 hr or 5 hrs after injection. Images were taken under the confocal microscope. Blue: DAPI; Green: Anti-Flag; Red: DXV.

H) Rogue is a PABP interacting protein

Rogue has been reported to interact with the polyA binding protein (PABP) from the protein mapping project (Giot et al., 2003). We confirmed this interaction by co-immunoprecipitating FLAG-tagged Rogue with PABP in whole flies (Fig 2-20). Because the PABP antibody was generated against human PABP, a human cell lysis was used as a control. In the native environment, PABP is likely to associate with RNA. It is possible that both Rogue and PABP are binding RNA and that RNA provides a bridge for an indirect interaction of Rogue and PABP. Thus, we wanted to examine if the interaction was RNA-dependent. The fly lysis was treated with RNAase A before adding the antibody. PABP was still detectable in the Rogue complex but with a slightly lower amount of protein. This indicates that some of the Rogue-PABP interaction was independent of RNA. If the physical interaction between Rogue and PABP is important for the antiviral immunity, depletion of PABP may give a similar phenotype as the *rogue* knockdown flies. Indeed, a lab colleague Javier Robalino found that knockdown of PABP in the hemocytes resulted in increased viral protein accumulation in the flies, which indicates that PABP is required in the hemocytes to restrict viral replication in the flies. Together, our results suggest that both Rogue and PABP play important roles in anti-DXV immune responses.

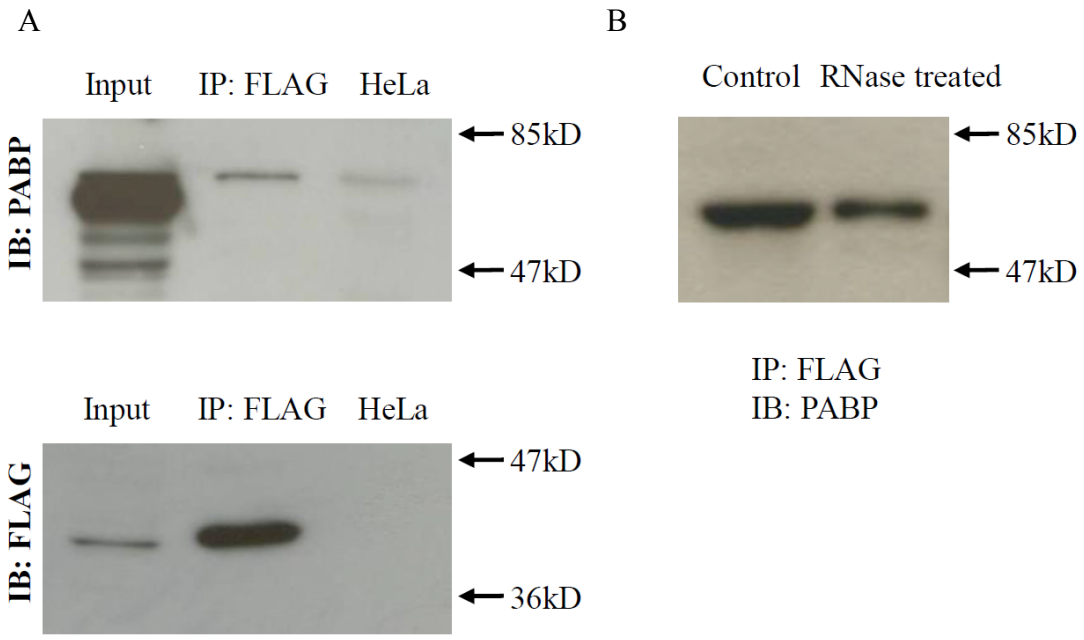


Figure 2-20: Rogue interaction with PolyA Binding Protein (PABP).

A) Co-immunoprecipitation assay and western blots analysis of PABP protein (upper panel) and FLAG-*rogue* protein (lower panel) levels in indicated samples. C564>FLAG-*rogue* flies were collected in the lysis buffer. Mouse anti-FLAG monoclonal antibody was used to precipitate the Rogue complex. Rabbit anti-PABP polyclonal antibody was used to detect PABP in the Rogue complex. Human HeLa cell lysis was used as a control. B) Co-immunoprecipitation assay and western blot analysis of FLAG-*rogue* protein in before and after RNase treated cell lysis. C564>FLAG-*rogue* flies were collected in the lysis buffer, and treated with RNase A for 30 min at 37C.

I) Overexpression of Rogue restricts the tissue size

PABP functions in translation and protein synthesis. If Rogue is involved in PABP dependent functions, it may facilitate or inhibit the translation and affect the growth of cells. To examine the possible effect of Rogue on translation, we ectopically expressed Rogue in fly wing discs, a tissue, which normally does not express *rogue*. We observed that the tissue on the side that *rogue* was expressed was much smaller (Figure 2-21), indicating that the Rogue protein may restrict tissue growth, possibly via translation inhibition.

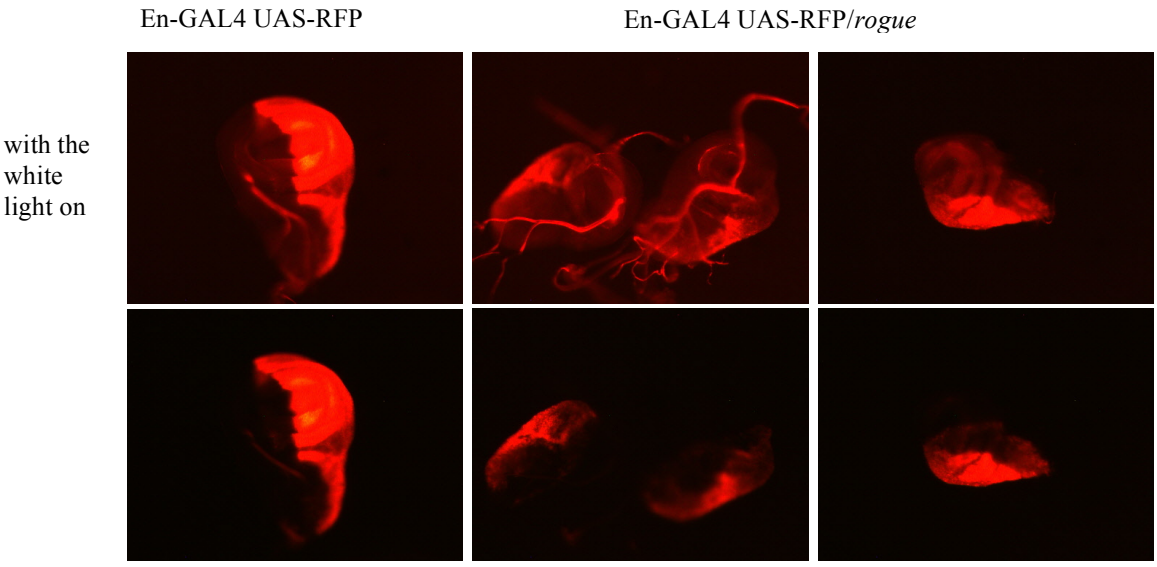


Figure 2-21: Rogue expression in the wing discs restricts the tissue size. RFP and Rogue were expressed in the posterior half of the wing discs using the En-GAL4 driver. In the En-GAL4 UAS-RFP sample, the RFP positive part is roughly half of the wing disc. The RFP positive tissue that had Rogue expression was smaller than the control part. The wing discs were dissected from the 3rd instar larvae. Images were taken using a Discovery V8 SteREO microscope (Zeiss). Images taken with the white light on show the whole discs. The data shown are representative images. Eight larvae of each genotype were examined.

Discussion

Previous antiviral innate immunity studies in *Drosophila* have mostly focused on the classical immune pathways and the RNA interference (Costa et al., 2009; Dostert et al., 2005; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2005; Zambon et al., 2006). Evidence for the involvement of other cellular processes in the immune system is just emerging (Liu et al., 2013; Nakamoto et al., 2012; Xu et al., 2012). As an important cellular process that both the host and the virus rely on, regulation of translation is an important antiviral response in many organisms (Berlanga et al., 2006; McEwan et al., 2012; Stopak et al., 2003). However, whether it played a role in antiviral immunity in *Drosophila* was not well studied. In this study, we identified a PABP-interacting protein, Rogue, as a novel antiviral protein. The expression of *rogue* was required for controlling the accumulation of DXV in the fly. The *rogue* gene responds to viral infection in two ways: first, the transcript level of *rogue* is induced, and this is possibly regulated by the Relish transcription factor; second, increased numbers of hemocytes have Rogue nuclear localization. Both of these responses are detected only at early infection stages, within hours after infection, and are undetectable at 5-6 hours after the infection. This indicates that Rogue may be involved in upstream events of the immune response, and that activation of *rogue* may be required for the subsequent antiviral reactions, such as transcription or translation regulation of immune related genes or proteins. The fact that the Rogue protein interacts with PABP, and PABP is important for translation, suggests the possibility that Rogue/PABP antiviral activity is mediated through translational regulation.

Translation is an important cellular program that the host relies on for its protein synthesis. PABP is an RNA binding protein that binds to the polyA tails of eukaryotic mRNAs. It directly interacts with eIF4G, a translation initiation factor that binds the cap binding protein eIF4E. These interactions lead to the recruitment of the 40S subunit of the ribosome, the translation initiation factor eIF2 and the initiator tRNA (Met-tRNA) on the starting site of the mRNA, so that translation can be initiated. In mammalian systems, this process is regulated by the host serine/threonine kinase mTOR complex 1, which phosphorylates and activates 4E-BPs (eIF4E binding protein 1, 2, 3) (Pause et al., 1994; Poulin et al., 1998). The phosphorylated 4E-BPs then release eIF4E and promote cap-dependent translation (Cully et al., 2010). Nutritional and environmental stress can result in the inactivation of mTOR and translation inhibition. An alternative translation regulation mechanism relies on the phosphorylation of the α subunit of eIF2 (eIF2α) (de Haro et al., 1996). Cellular stresses like oxidative stress (Lu et al., 2001), ER stress (Ron and Walter, 2007), and viral infection (Hovanessian, 1989) lead to the phosphorylation of eIF2α, and the reduction of the guanine nucleotide exchange factor activity of eIF2, thereby inhibiting translation.

Although ectopic expression of *rogue* may not represent its function in normal tissues, the fact that overexpression of *rogue* restricts the tissue size of wing discs hints at the possibility of *rogue* as an inhibitor of translation. Since the expression levels of the *rogue* gene and the localization of Rogue protein rapidly respond to viral infection, it is likely that Rogue is involved in translation inhibition caused by viral-induced stress. In many cases, viruses can selectively target and down-regulate host translation, and then utilize

the host translation machinery for the rapid production of viral protein (Cherry et al., 2005; Connor and Lyles, 2002; Kleijn et al., 1996). Since viral RNA translation is much faster than the host mRNA translation, global translation inhibition would have a bigger effect on viral replication than on host protein synthesis, and this may effectively dampen the accumulation of the virus. For example, the translation of DCV does not rely on cap-dependent translation machinery; instead its genome has several internal ribosome entry sites (IRES) to directly recruit ribosomes. It has been found that attenuated ribosome function protects flies from DCV infection (Cherry et al., 2005). The fact that the *rogue* mutant is not susceptible to DCV infection suggests that Rogue is not an inhibitor of global translation.

Thus, Rogue may be involved in translation inhibition of a specific group of proteins at early infection stages. In the *rogue* mutant and *rogue* RNAi knockdown flies, some RNAi pathway components are down regulated, such as *r2d2* (Fig 2-12 and Appendix B Fig S2). It is possible that a negative regulator of *r2d2* is induced, thereby restricting the expression of *r2d2*. So far, it is not clear which signal is the negative regulator of *r2d2*. In mouse, a study found that the phosphorylation of eIF4E was required for translational up-regulation of certain proteins, including Inhibitor of NF-κB (I-κB). When eIF4E loses the ability to be phosphorylated, the lower abundance of I- κB results in enhanced activity of NF-κB (Herdy et al., 2012). We also found that the *rogue* mutant and *rogue* knockdown flies had higher basal AMP gene expression (Fig 3-2 and Fig 3-4), which supports a model of increased NF-κB activity as a result of decreased *rogue* expression. It is possible that this high NF-κB activity somehow inhibits the expression of *r2d2*. Thus,

it would be interesting to examine if excessive activation of NF-κB activity restricts *r2d2* expression.

Despite the direct effect on viral replication, translational inhibition may also serve as an antiviral signal for the immune response. In plants, effector-triggered immunity (ETI) had long been known as an important immune defense mechanism (Jones and Dangl, 2006). Upon invasion by a pathogen, the host can sense the infection by recognizing the pathogen itself or by the effects of the pathogen. Translational inhibition initiated by the pathogen may be sensed by the host and trigger an immune responses. In *C.elegans*, the translational inhibition caused by the bacteria *Pseudomonas* triggered the activation of multiple immune signaling pathways (Dunbar et al., 2012; McEwan et al., 2012). In the mouse model, macrophages infected with the intracellular bacterial pathogen *Legionella pneumophila* show sustained activation of NF-κB. This host transcriptional response is due to the activity of secreted bacterial proteins that inhibit host translation (Fontana et al., 2011). In *Drosophila*, a recent study showed that host translation could be affected by *Pseudomonas* through inhibition of the TOR pathway. Flies in which host translation levels were decreased through TOR inhibition had higher immune responses against the bacteria *Erwinia carotovora* 15, indicating that translation inhibition induces immune responses in the *Drosophila* gut (Chakrabarti et al., 2012*)*. If the same surveillance mechanism seen in gut immunity is applicable to systemic infection, this mechanism may be affecting antiviral immunity. The defects of *rogue* in the immune responses against DXV may be caused by the inability of *rogue* in translation inhibition at early stages of an infection.

Another possible effect of loss of translational inhibition at early stages of infection is the failure to induce apoptosis. Apoptosis is an important for the systemic antiviral immune response (Liu et al., 2013; Vaidyanathan and Scott, 2006). A block of apoptosis at the early stage of infection in flies impairs the antiviral defense system (Liu et al., 2013). It has been shown that translation inhibition due to the absence of cellular PABP activates p53 and activates the apoptotic pathway (Thangima Zannat et al., 2011). If the binding of Rogue-PABP is important for translation inhibition and the induction of apoptosis, loss of Rogue expression will result in the inability of cells to initiate apoptosis in response to viral infection. If apoptosis limits DXV replication, like it does FHV (Liu et al., 2013), the loss of induction of apoptosis will significantly decrease the ability of the flies to control viral accumulation.

Finally, the translation inhibition may affect the miRNA pathway, which may regulate host antiviral gene expression and/or directly block viral genome synthesis. In *Drosophila*, it has been suggested that a physical interaction beween PABP and miRISC is important to stimulate gene repression by miRNAs (Moretti et al., 2012). If Rogue is involved in this process, the *rogue* mutant may have defects in miRNA function. So far, no evidence has been found for miRNA as an antiviral pathway. However, the antiviral role of miRNA might be overlooked because most mutations affecting the miRNA pathway are lethal to the fly. In our previous study, we had to use a heterozygous *dcr1* mutant as a miRNA pathway mutant (Zambon et al., 2006). We did not find this mutant to be sensitive to DXV infection, but one copy of the *dcr1* gene might be enough to produce adequate functional Dicer 1. When the AGO1 RNAi line became available, we

used RNAi to knockdown AGO1 in fly hemocytes and found that these flies were more sensitive to DXV infection (Appendix B Fig S3). This suggests that the function of AGO1 is important for host defense against DXV. In addition, the miRNA machinery suppresses the translation of target RNA when the sequences of the miRNAs are not completely complementary to the target RNA (Petersen et al., 2006; Thermann and Hentze, 2007). Thus, even though host miRNAs are not made for targeting the viral genome, they may be able to bind viral RNA and suppress viral protein synthesis. By comparing the DXV genome with the *Drosophila* miRNA database, we found several predicted host miRNAs that could target the viral genome. It would be interesting to screen these miRNAs for their ability to block viral replication. If any miRNAs were able to inhibit DXV replication, this would indicate that miRNAs in *Drosophila* might also be involved in antiviral immune responses.

Materials and Methods

Fly stocks

The EMS mutants were provided by Dr. Charles Zuker (Columbia University). The isogenic parental flies that were mutagenized to generate the Zuker mutants were used as a background control for the X-men mutants. The mapping kit and the deficiency lines were obtained from Bloomington Drosophila Stock Center. The $dcr2^{L811fsx}$ mutant was provided by Dr. Richard Carthew (Northwestern University). The $r2d2^{1}$ mutant and the *r2d2* transgenic flies were provided by Dr. Dean Smith (UT Southwestern). All other mutants, including Dif^{1}, $aub^{KG05389}$, $piwi^{EP1024}$, *vig* $vas^{EY07816}$, and $AGO2^{EY04479}$ were

obtained from Bloomington *Drosophila* Stock Center. The driver line Arm GAL4 was provided by Dr. Leslie Pick. The fly line En GAL4-UAS RFP was provided by Dr. Jian Wang. The ubiquitous driver flies, Actin5C GAL4, C564 GAL4; the fat body driver flies, yolkGAL4; the hemocyte-specific driver flies, hemlΔGAL4 and pxnGAL4 were provided by Bloomington *Drosophila* Stock Center as well. The *rogue* RNAi lines: w1118; P{GD9208}v32734; w1118; P{GD9208}v32733, P{KK105838}VIE-260B,GD line control v60000 and KK line control v60100 were provided by the Vienna *Drosophila* RNAi Center. Most *rogue* RNAi experiments were done three times using w1118; P{GD9208}v32734 and confirmed with one of the other RNAi lines. The Vienna *Drosophila* RNAi Center also provided the CG16734 RNAi line: P{KK112364}VIE-260B.

To make the *rogue* rescue construct, the *rogue* gene was amplified from the genomic DNA extracted from *iso* flies. Primers for *rogue* cloning are: forward primer containing a KpnI site: TTGGTACC ATGTGGCCGGCTTGGCAAGT; reverse primer containing an XbaI site: GCTCTAGA TTAATGATGTCCATGATCGTG. The FLAG tag, MDYKDDDDK, was added to the N-terminus of *rogue* by PCR using the following primers: forward primer containing KpnI site and the flag sequence: AAGGTACCATGGATTACAAAGACGATGACGATAAAATGTGGCCGGCTTGGCA AGTGATAA; and the reverse primer containing an XbaI site: GCTCTAGATTAATGATGTCCATGATCGTGTTC. The PCR products were cloned into the pCR 2.1 cloning vector (Invitrogen). Plasmids were transformed into TOP10 Chemically Competent *E.coli* Cells following manufacturer's instructions (Invitrogen).

Plasmids were amplified in the bacteria and then isolated using the Miniprep Kit (Qiagen), according to manufacturer's instructions. The constructs were sequenced, and cloned into a pUAST vector and transformed as above. The Plasmid Midi Prep Kit (Qiagen) was used according to manufacturer's instructions to isolate and purify the final constructs, which were submitted to BestGene for P-element mediated transformation in *Drosophila* embryos.

Virus preparation

The DXV used is a 10^{-5} dilution of the stock, and the DCV used is a $5x10^{-5}$ dilution of the stock. The TCID 50 of the DXV stock is $4.37x10^{10}$/ml. The DCV stock is the supernatant collected from the infected S2 cells. The doses are chosen as those causing around 50% death of the wild type flies at the day 11 for DXV and day 15 for DCV. Approximately 25nl of virus preparation (with 6% green food coloring dye) are injected into each fly using the manual injector in the screen and experiments in Fig 2-1, 2-2 and 2-3. For all other experiments 32nl of virus preparation are injected into each fly using a nano-injector (Drummond).

Survival analysis

All adult flies used are 5-7 days old and raised at 25°C on standard yeast/agar media. For all experiments, equal numbers of females and males are used unless otherwise stated. In each repeat, 30-50 flies of each line are used for survival tests. The survivals were recorded daily after injection. The number of flies surviving at day 1 was used as the initial count for calculation of the morbidities. Log-rank tests were used to determine the

susceptibility. Anoxia treatment is performed during the screen and mapping at 7 and 10 days post infection (d.p.i). Flies were exposed to CO_2 for 15min each time in a sealed chamber.

Reverse transcription and quantitative real-time PCR

RNA is isolated from adult flies using the STAT-60 kit (Isotex Diagnostics). Reverse transcription is performed using a reverse transcription kit (Invitrogen). SYBR Green RT-PCR is used for viral transcript levels measurement and gene induction assay. *Ribosomal protein 49* SYBR primers are used as the internal control in all experiments. To collect RNA from larval blood cells and carcasses, approximately 20 larvae were carefully lacerated with tweezers on their anterior end in 50µL nuclease-free water. Carcasses were allowed to rest in the water for an additional minute before being homogenized in 500µL STAT-60 (Tel-Test, Inc.). The liquid was then collected and homogenized in 500µL STAT-60. To collect RNA from adult flies, 6-10 animals (equal numbers of females and males) were anesthetized with CO_2 and homogenized in 800µL STAT-60. Once samples were in STAT-60, total RNA was extracted from all homogenized samples following manufacturer's instructions. The concentration of RNA was measured using the Nanodrop 1000 (Thermoscientific). Between 1ug of the total RNA was then used to make cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions.

To measure the expression of *rogue* after viral infection, flies were infected with DXV and samples were collected at 6hrs, 24hrs and 48hrs after infection. The uninfected flies

were also sampled as control. In each sample, 3 female and 3 male flies were homogenized. cDNA was synthesized as above. To ensure no genomic DNA contamination in the cDNA sample, a non-RT control was performed for each sample as quality control. To measure the viral RNA accumulation, flies were infected with DXV and samples were collected at day2, day4 and day6 after infection. In each sample, 3 female and 3 male flies were homogenized. cDNA was synthesized as above.

Quantitative PCR was conducted using the 7300 Real Time PCR System (Applied Biosystems) and programed as follows: 50°C for 2 minutes, 95°C for 10 minutes, and then cycled between 95°C for 15 seconds and 60°C for 1 minute 40 times. Each sample was measured in technical triplicate on the PCR plates. For each experiment, at least three biological repeats were included. The data were analyzed by one-tailed paired t-tests.

Immunostaining

To examine the viral induced *rogue* localization change in hemocytes, three instar pxn>FLAG-*rogue* larvae were injected with DXV or PBS. Larvae were put back in a fresh food vial after infection, and then incubated at 25°C for 5hrs. At 1hr and 5hrs after infection, approximately 8 larvae of each sample were bled into cold PBS on a poly-lysine coated coverslip. The carcass was removed after one minute to allow the blood cells to attach to the coverslip. Excess liquid was removed and cells were fixed in 4% formaldehyde, washed, blocked, and incubated with a 1:500 dilution of a mouse anti-Flag antibody (Sigma) at 4°C for overnight. Cells were then incubated with a 1:200 dilution of

the Alexa 488 conjugated α-mouse secondary antibody (Invitrogen) for 2 hours at room temperature, washed, and mounted in Prolong (Invitrogen). Slides were sealed and incubated at room temperature for at least 24 hours. The LSM 710 confocal microscope (Zeiss) was used for visualizing the cells.

To examine the viral accumulation in the fat body, *C564>rogue* dsRNA and the driver only flies were injected with DXV. At day3 after infection, approximately 7-9 DXV infected flies per genotype were dissected in the PBS, uninfected flies were also collected and dissected as control. The back wall of these flies were dissected out and fixed with 4% formaldehyde with 0.1% Triton X-100, then washed, blocked, and incubated with 1:500 dilution of the anti-DXV antibody at 4°C for overnight. Samples were then incubated with a 1:250 dilution of the Alexa 594 conjugated α-rabbit secondary antibody (Invitrogen) for 2 hours at room temperature, washed, and mounted in Prolong (Invitrogen) with the inside wall up on the slides. Slides were sealed and incubated at room temperature for at least 24 hours. The LSM 710 confocal microscope (Zeiss) was used for visualizing the cells.

Co-Immunoprecipitation

The C564>*rogue*-FLAG flies (6 females and 6 males in each sample) were collected in 400ul cold protein lysis buffer containing 50mM Tris-HCl at pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100 and proteinase inhibitor (BD). The fly extracts were centrifuged for 10 min at 13,500 rpm at 4°C. The supernatant was incubated with 4ul of mouse anti-FLAG antibody (Sigma) at 4°C overnight. For the RNase treatment, 200ul supernatant

was treated with RNase A at 37°C for 30min before the addition of antibody. 20ul of pre-cleaned protein A-agarose (Invitrogen) was then added into the protein complex for an additional 3hrs at 4°C. The protein complexes were precipitated by centrifugation for 10 min at 13,500 rpm and washed three times in 500ul wash buffer containing: 25mM Tris-HCl at pH 7.5, 250mM NaCl, 2mM EDTA, 0.1% Triton X-100 and proteinase inhibitor (BD). The precipitated protein complexes were suspended in SDS loading buffer and analyzed by Western Blotting using a rabbit PABP antibody and a mouse anti-FLAG antibody (Sigma).

Clearance of *Wolbachia* from infected flies

All stocks of flies were examined for the presence of *Wolbachia* by doing PCR for the *Wolbachia* specific surface protein gene *wsp* from genomic DNA extracted from the flies. Primers are: forward 5'-TGGTCCAATAAGTGATGAAGAAC; reverse 5'-AAAAATTAAACGCTACTCCA. For the Zuker mutants, no *Wolbachia* infection was found. For other lines, since the effect of *Wolbachia* infection on flies' response to DXV was limited (appendix B Fig S4), no further clearance of *Wolbachia* was performed. Because *Wolbachia* can be maternally inherited, in each experiments, the same genotype of virgin females were used for each cross, so that the progeny of control flies and the tested flies would have the same status of *Wolbachia* infection.

Chapter 3: Rogue is required for resistance to *Staphylococcus aureus* in *Drosophila*

Abstract

The innate immune system mounts an immediate response to invading microbes. Here we identify a novel gene, *rogue*, which is involved in protecting the host from *Staphlococcus aureus* infection. The *rogue* mutant is susceptible to *S. aureus* and shows higher bacterial loads compared to control flies, suggesting that the susceptibility is due to uncontrolled bacterial growth. The *rogue* mutant has a functional Toll pathway and is able to induce the Toll pathway antimicrobial peptide (AMP) *Drosomycin* following bacterial infection. However, we found that the *rogue* mutant had a phagosome maturation defect, which might contribute to the susceptibility of the mutant to *S. aureus* infection. Introducing a *rogue* transgene in to the hemocytes of the *rogue* mutant rescues in survival to *S. aureus* infection, suggesting that the lack of *rogue* expression results in the susceptibility of the *rogue* mutant. RNAi knockdown of *rogue* in the hemocytes or the fat body of wild type flies renders flies that are more susceptible to *S. aureus* infection. Flies with RNAi knockdown of *rogue* in the hemocytes also show defects in phagosome maturation. Altogether, our results indicate that *rogue* plays a critical role in defending against bacteria through the regulation of phagosome maturation.

Introduction

Innate immunity is important for host organisms to fight against microbial pathogens. It relies on germline encoded pathogen associated molecular patterens (PAMPs) to recognize the pathogen and initiate rapid responses. *Drosophila,* as a genetically tractable model, provides a useful tool to probe the mechanisms of innate immunity. The innate immune response in *Drosophila* mainly consists of barrier epithelial immune responses, cellular responses and humoral responses. Epithelial barriers include the cuticle protection, the gut epithelial environment and the tracheal respiratory organs (Kimbrell and Beutler, 2001). Cellular immune responses consist of phagocytosis, encapsulation and melanization, all of which involve hemocytes. Hemocytes are functionally analogous to the mammalian macrophage (Abrams et al., 1992). Humoral immune responses mainly induced by signaling pathways, such as the Toll, Imd and JAK-STAT pathways. The activation of these pathways produces effector molecules to defend against microbial infection. The humoral response is best characterized by the production of antimicrobial peptides (AMPs) by the Toll and Imd pathways in the fat body, a functional analog of the mammalian liver.

The Toll pathway is evolutionarily conserved with mammalian Toll like receptor (TLR) signaling pathways (Medzhitov et al., 1997; Poltorak et al., 1998a; Rock et al., 1998). In *Drosophila*, Gram-positive bacteria and fungi activate the Toll pathway, resulting in the nuclear translocation of the transcription factors Dif and Dorsal, which induce the transcription of AMP genes, including *Drosomycin* (Meng et al., 1999; Steward, 1987). The Imd pathway, on the other hand, is an essential pathway required for flies to defend

against Gram-negative bacteria (Elrod-Erickson et al., 2000). After recognition of the bacteria, the caspase signaling lead to the nuclear translocation of the Rel domain of Relish, which initiates the transcription of the AMP genes, including *Dipericin* (Hoffmann and Reichhart, 2002). The Imd pathway shares similarities with the mammalian tumor necrosis factor receptor (TNF-R) pathway (Georgel et al., 2001).

Compared to our knowledge of the humoral immunity, cellular immunity in *Drosophila* remains less well characterized. Upon infection, bacteria are internalized into cellular vesicles, termed phagosomes. These phagosomes then undergo maturation by fusing with the early/sorting endosome, and then the late endosome, and finally the lysosome. During this phagosome maturation process, the vesicles become progressively acidic inside (Rink et al., 2005), and eventually, the low pH in the phagolysosome causes the breakdown of the microbial and cellular debris inside of the vesicles. In *Drosophila*, hemocytes are the major phagocytic cells and their phagocytic activity is important for the immune response against certain Gram-positive bacteria (Nehme et al., 2011). However, blocking the phagocytic capacity of hemocytes does not decrease the flies' resistance to *E. coli*, indicating that phagocytosis may not be required for immune responses against certain types of Gram-negative bacteria (Elrod-Erickson et al., 2000).

Staphylococcus aureus (S. aureus) is a Gram-positive bacterium that is used to study the immune responses in *Drosophila* (Atilano et al., 2011; Defaye et al., 2009; Tabuchi et al., 2010). The cellular response, phagocytosis, has been shown to be vital for the host to survive an *S. aureus* infection (Nehme et al., 2011), and mutants affecting host

phagocytic uptake are more susceptible to *S. aureus* (Defaye et al., 2009; Shiratsuchi et al., 2012). Several cell surface receptors, such as Eater (Kocks et al., 2005), NimC1 (Kurucz et al., 2007), Integrinβ ν (Nonaka et al., 2013; Shiratsuchi et al., 2012) and Draper (Hashimoto et al., 2009; Shiratsuchi et al., 2012), are involved in the recognition and engulfment of *S. aureus*. Loss of any of these receptors in hemocytes can result in decreased phagocytosis of *S.aureus*. Phagosome maturation in hemocytes is an essential process controlling bacterial clearance. The phagosome maturation mutant *Rab14* showed striking sensitivity to *S. aureus* (Garg and Wu, 2013). In contrast, the importance of the humoral response to *S. aureus* was less studied. Tabuchi and colleagues had shown that the cell wall components of *S. aureus* have an inhibitory effect on host humoral responses. Mutant *S. aureus* that lack the D-alanylated wall teichoic acid triggered stronger Toll pathway activity than wild type bacteria. Flies infected with these mutant bacteria have lower bacterial loads and delayed mortality (Tabuchi et al., 2010), suggesting that the humoral responses play a role in immunity against *S. aureus.*

In this study, we find a mutant, *rogue*, is susceptible to *S. aureus* infection. The susceptibility was due to the uncontrolled bacterial growth, suggesting that the mutant is affecting the resistance of flies to *S. aureus* infection. The mutant can still induce the Toll pathway AMP *Drosomycin* following infection, but it has a phagosome maturation defect. This supports the model that the cellular response is important in protecting flies against *S. aureus* infection. Introducing the *rogue* transgene in the hemocytes rescues the *rogue* mutant's susceptibility to *S.aureus*, suggesting that the lack of *rogue* expression contributes to the susceptibility of the *rogue* mutant. Wild type flies with an RNAi

knockdown of *rogue* show a similar phenotype as the *rogue* mutant, indicating that *rogue* might be a novel factor affecting the phagosome maturation process.

Results

A) The *rogue* mutant is highly sensitive to *S. aureus* infection

We have identified a mutant, *rogue,* that has increased susceptibility to DXV infection as described in Chapter 2. Interestingly, the *rogue* mutant was consistently more susceptible to the Gram-positive bacteria *S. aureus* compared to the *iso* control (Fig 3-1A). The lethality associated with bacterial infection could be due to either the inability to control the accumulation of bacteria or the inability to tolerate the damage caused by the bacteria. To determine if the *rogue* mutant has a defect in resistance or tolerance with *S. aureus* infection, bacterial loads were examined. Significantly higher bacterial loads were detected in the mutant at 24 hrs after infection (Fig 3-1B), indicating that the susceptibility of the *rogue* mutant may be due to the uncontrolled bacterial growth.

B) The Toll pathway was not impaired in the *rogue* mutant

The Toll pathway is essential for flies to survive Gram-positive bacterial or fungal infection. It is also a known pathway that responds to DXV infection. Thus, we first examined if the *rogue* mutant has a defect in the Toll pathway. Mutants in the Toll pathway usually have lower hemocyte numbers; thus, the hemocyte counts in the fly can

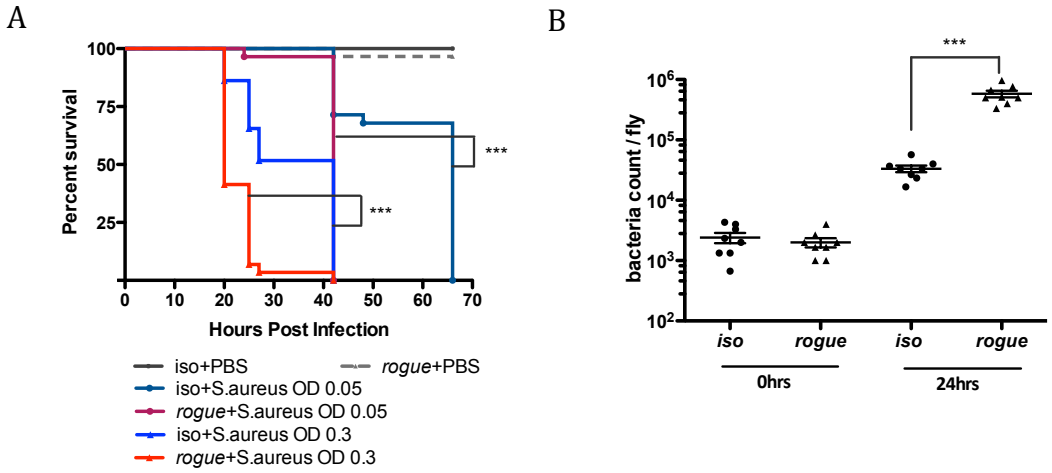


Figure 3-1: The *rogue* mutants die faster in response to *S. aureus* compared to the parental control *iso*. (A) The susceptibility correlates with higher bacterial loads in the fly (B). (A) 30 flies of each line were injected with log-phase *S. aureus* (diluted to OD=0.05 and 0.3). The morbidities were recorded hourly after injection. (B) Flies were injected with log-phase *S. aureus* (diluted to OD=0.05). Single flies at 0 hrs and 24 hrs of each line were homogenized, diluted and plated on agar plates and the bacterial number in each fly was determined. Data shown are representative experiments of three independent experiments. *** $p<0.001$

serve as an indicator of a Toll defect (Qiu et al., 1998). Because *rogue* is female sterile, the mutation is kept balanced by the CyO balancer chromosome. To get homozygous *rogue* larvae, the *rogue* mutant stock was crossed to flies with the 2nd and 3rd balancer chromosomes linked together. This allows the 2nd chromosome mutation to be balanced by the 3rd chromosome larval visible marker Tubby, so that the homozygous *rogue* larvae can be selected. The hemocyte numbers in the *rogue* mutant were not significantly different from that of the iso control (Fig 3-2A). This indicates that the conventional Toll pathway might not be affected in the *rogue* mutant.

Although the Toll function in hemocyte development was not affected, the *rogue* mutant might have defects in pathogen-induced Toll activity. Pathogen infection activates the Toll pathway and induces expression of the AMP gene, *Drosomycin*; hence, the expression levels of *Drosomycin* are a common output used to assess activation of the Toll pathway (Gobert et al., 2003). To examine if the *rogue* mutant had defects in the Toll pathway, the induction of *Drosomycin* was examined. The *rogue* mutant was able to induce *Drosomycin,* indicating that it is able to activate the Toll pathway (Fig 3-2B). Our results suggested that the high susceptibility of the *rogue* mutant was not caused by the inability to activate the Toll pathway. Rather, the *rogue* mutant might have defects in other aspects of their immune response.

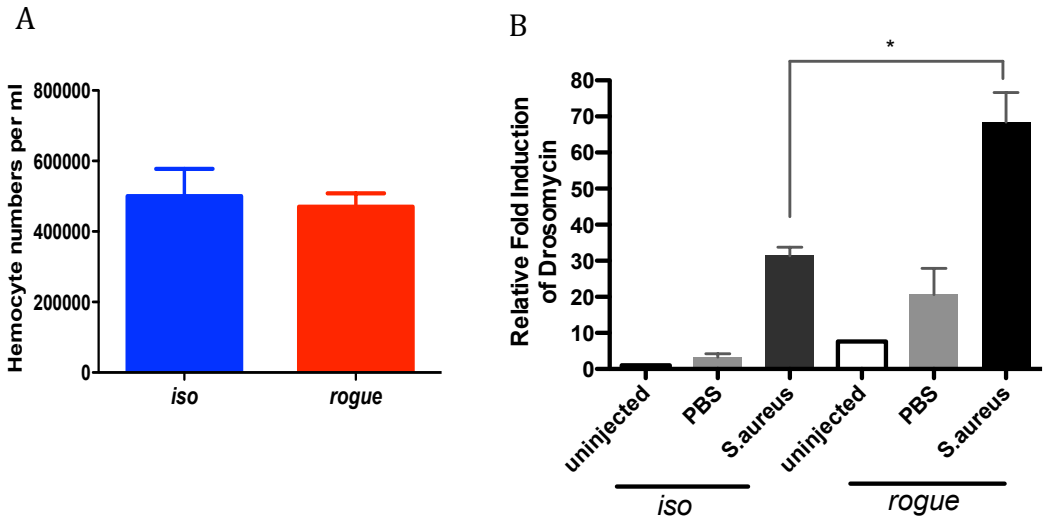


Figure 3-2: The Toll pathway was not impaired in the *rogue* mutant. A) The *rogue* mutant flies have normal amount of hemocytes as compared to wild type flies. Hemocytes from single larvae were bled into PBS and counted under the microscope. The numbers of hemocytes/µl were calculated. n = 6 B) The *rogue* mutant was able to induce *Drosomycin* after *S. aureus* infection. Adult flies were injected with *S. aureus* or PBS. 24 hrs after injection, *Drosomycin* expression were measured by qRT-PCR. The relative fold changes were compared to the induction of *Drosomycin* in *iso* uninjected flies. The data shown represents the mean of triplicates. Error bars show the standard errors. Student t-test was used to determine the p-value. * : $p<0.05$

C) The *rogue* mutant has a phagosome maturation defect

Previous studies have indicated the comparable roles of humoral and cellular immunity in defending against *S. aureus* infection in the fly (Garg and Wu, 2013; Gonzalez et al., 2013; Nehme et al., 2011). To test if phagocytosis, the major cellular immune response, was affected in the *rogue* mutant, phagocytosis and phagosome maturation assays were conducted. Hemocytes are the major phagocytic cells in fly. When they engulf fluorescently-labeled particles, these hemocytes can be visualized through the cuticle at the dorsal vessel. Hence, to examine if the *rogue* mutant had a defect in phagocytosing bacteria, fluorescently labeled *S. aureus* was injected into the adult fly, and 30 min after injection, the fluorescence intensity was measured at the dorsal vessel of the *rogue* mutant or the *iso* control. We found no significant difference regarding the uptake of fluorescently labeled *S. aureus* in the mutant or control fly, suggesting that the *rogue* mutant has normal phagocytic uptake (Fig 3-3A).

Phagosome maturation in the *rogue* mutant was examined using pHrodo-labeled *S. aureus* particles. The pHrodo dye is sensitive to pH: its fluorescence intensity increases when the surrounding pH decreases. This property of the pHrodo dye enables the tracking of the maturation of the bacteria-containing phagosome. Higher fluorescence from the pHrodo labeled *S. aureus* indicates that the phagosome has matured into a later stage. The *rogue* mutant and the *iso* control flies were injected with pHrodo-labeled *S. aureus*. To quench the extracellular fluorescent signals, Trypan Blue was injected into the same fly before the image was taken from the flies. Following pHrodo-labeled *S. aureus* injection, *iso* flies showed significantly increased fluorescence intensity over time (Fig 3-3B).

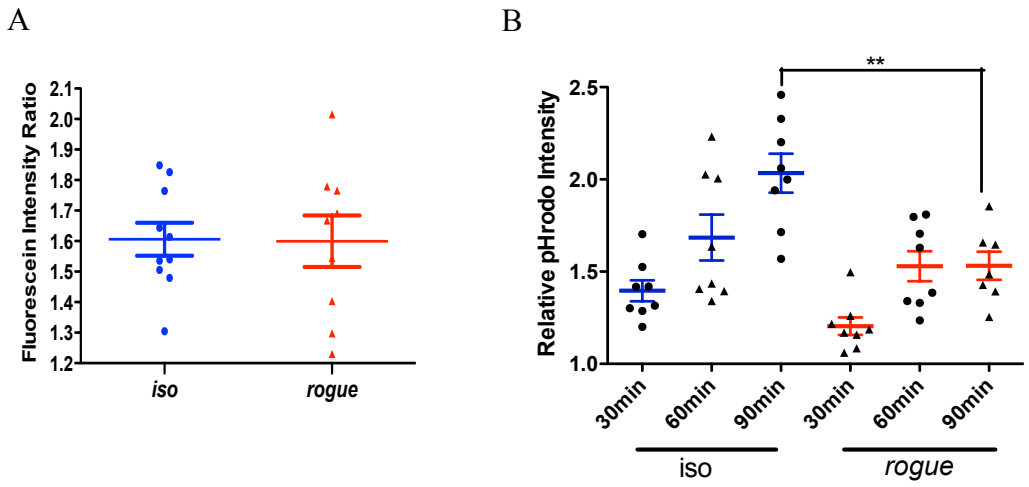


Figure 3-3: The *rogue* mutant has no defect in phagocytic uptake but has a defect in phagosome maturation compared to its parental control *iso*. A) Phagocytosis assay. Flies were injected with fluorescently-labeled *S. aureus*. Trypan Blue was injected into the same fly 30 min later to quench the extracellular fluorescence. The *S. aureus* associated fluorescein intensity was measured immediately following Trypan Blue injection. B) Phagosome maturation assay. Flies were injected with pHrodo-conjugated *S. aureus* and images were taken at 30, 60 or 90 min post-injection. Quantification of fluorescence intensity at the dorsal vessel was carried out using Axiovision 4.7. The dots shown represent each fly's dorsal vessel fluorescein intensity normalized to the fluorescein intensity of the adjacent area. The data shown are representative experiments of triplicates.

However, the fluorescence intensity failed to increase in the *rogue* mutant at 90 min after the initial injection, suggesting that the *rogue* mutant has a defect in a late stage of phagosome maturation. This defect may contribute to the susceptibility of the *rogue* mutant to *S. aureus* infection.

D) *rogue* was required in both the fat body and hemocytes for defense against *S. aureus*

To investigate if the high susceptibility of the *rogue* mutant to *S. aureus* was due to the low expression of the *rogue* gene, we examined the response of the *rogue* knockdown flies following *S. aureus* infection. With RNAi knockdown of *rogue* in the fat body, flies were more susceptible to *S. aureus* infection compared to the control flies (Fig 3-4A), indicating the importance of *rogue* expression in the fat body for the anti-*S. aureus* response. Next we examined if *rogue* expression in the hemocytes was required for flies to survive *S. aureus* infection. *rogue* hemocyte knockdown flies were subjected to *S. aureus* infection, and significantly higher susceptibility was also observed in these flies (Fig 3-4B). This suggests that the expression of *rogue* in both fat body and hemocytes is indispensable for the flies to defend against *S. aureus*.

To further explore if humoral or cellular immunity against *S. aureus* was affected by the low expression of *rogue*, AMP induction and phagosome maturation were examined in the flies with RNAi knockdown of *rogue* in the fat body and in the hemocytes respectively. Similar to the *rogue* mutant, flies with *rogue* knockdown in the fat body

were able to induce *Drosomycin* after bacterial infection (Fig 3-4C). A phagosome maturation defect was seen in the *rogue* hemocyte knockdown flies at 60 min after *S. aureus* infection (Fig 3-4E and F). This indicated that, similar to the *rogue* mutant, the *rogue* RNAi knockdown flies had cellular but not humoral immune defects. Finally, to examine if the low expression of the *rogue* gene in the mutant was indeed causing the mutant's susceptibility to bacteria, the transgenic *rogue* gene was expressed in the mutant. With the expression of *rogue* in the hemocytes, flies showed wild type survival to *S. aureus* infection (Fig 3-4D), suggesting that the low expression of the *rogue* gene was causing the susceptibility of the mutant. Hence, the expression of *rogue* is required for the immune response against *S. aureus* in the fly.

Discussion

The *rogue* mutant was initially identified to have increased susceptibility to DXV infection. During the characterization of the mutant, we found it to be extremely susceptible to the bacteria *S. aureus*. In this chapter, we explored the role of *rogue* in anti-bacterial immune responses. With *S. aureus* infection, higher bacterial loads are observed in the *rogue* mutant at 24 hrs post infection, indicating that the *rogue* mutant is unable to control bacterial growth. The *rogue* mutant has a functional Toll pathway because it has normal numbers of the blood cells and is able to induce the Toll pathway AMP *Drosomycin* after *S. aureus* infection. However, we found that the *rogue* mutant had a defect in phagosome maturation. Since previous studies demonstrate that a mutant

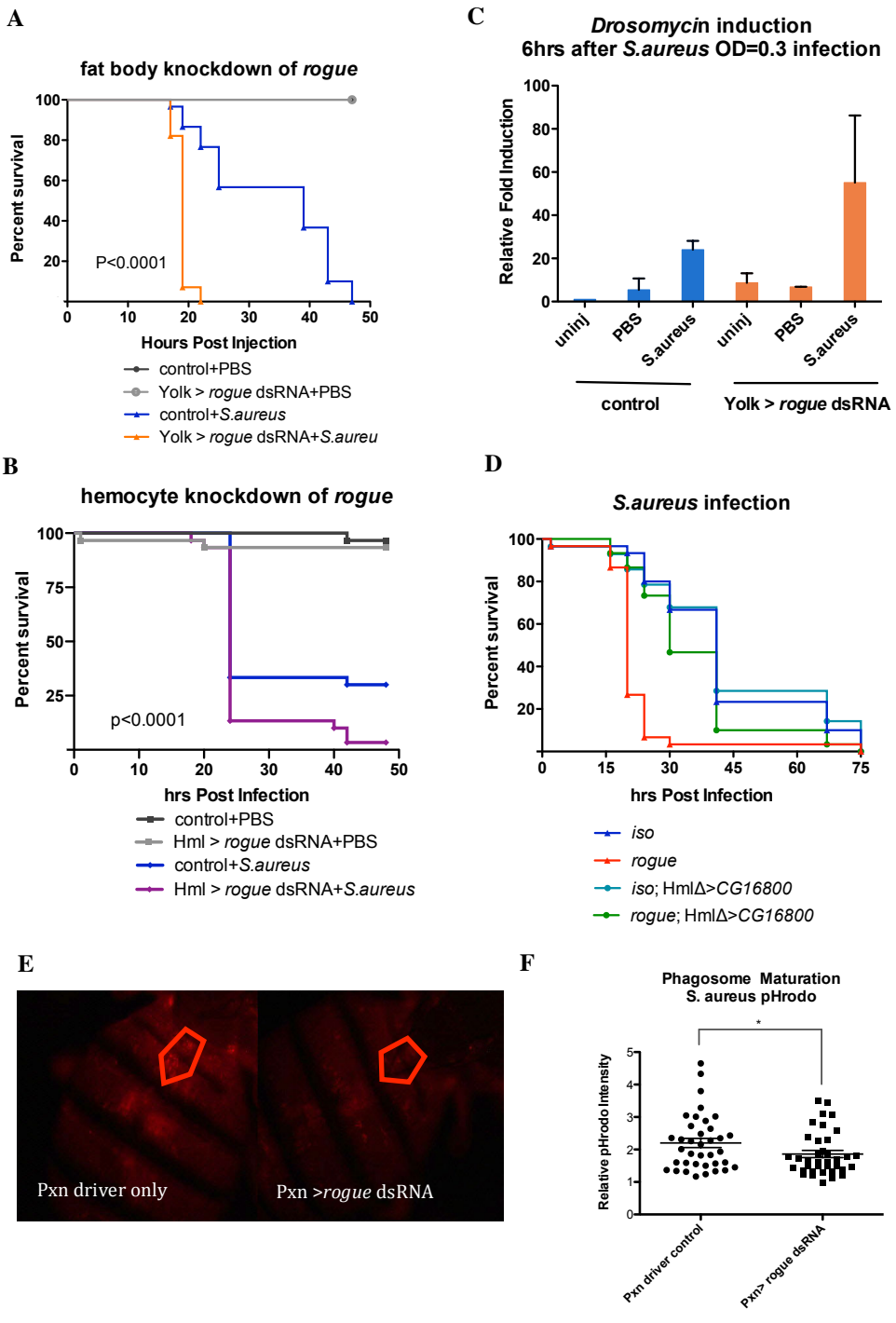

A
fat body knockdown of rogue
Percent survival
P<0.0001
Hours Post Injection
control+PBS
Yolk > rogue dsRNA+PBS
control+S.aureus
Yolk > rogue dsRNA+S.aureu
C
Drosomycin induction
6hrs after S.aureus OD=0.3 infection
Relative Fold Induction
uninj
PBS
S.aureus
uninj
PBS
S.aureus
control
Yolk > rogue dsRNA
B
hemocyte knockdown of rogue
Percent survival
p<0.0001
hrs Post Infection
control+PBS
Hml > rogue dsRNA+PBS
control+S.aureus
Hml > rogue dsRNA+S.aureus
D
S.aureus infection
Percent survival
hrs Post Infection
iso
rogue
iso; HmlΔ>CG16800
rogue; HmlΔ>CG16800
E
Pxn driver only
Pxn >rogue dsRNA
F
Phagosome Maturation
S. aureus pHrodo
Relative pHrodo Intensity
Pxn driver control
Pxn> rogue dsRNA

Figure 3-4: *rogue* RNAi flies recapitulate most of the *rogue* mutant phenotypes in response to *S. aureus* infection. A, B) Survival curves of flies with RNAi knockdown of *rogue* in the fat body or hemocytes in response to *S. aureus* infection. RNAi lines against *rogue* driven in fat body only (Yolk GAL4)(A), or hemocytes only (Pxn GAL4)(B) were injected with *S. aureus*. The morbidities were recorded after injection. Log-rank statistics were used to calculate the p-values of survival. ***$p<0.0001$ C) *Drosomycin* induction in *rogue* fat body knockdown flies. Adult flies were injected with *S. aureus* or PBS. Twenty four hrs after injection, *Drosomycin* expression was measured by qRT-PCR. The data shown represents the mean of triplicates. The error bars show standard error. D) Expression of *rogue* in the hemocytes completely rescues the *rogue* mutant's susceptibility to *S. aureus*. The data shown is a representative graph. E,F) Phagosome maturation in *rogue* hemocyte knockdown flies. Flies were injected with pHrodo-conjugated *S. aureus* and images were taken at 60 min post-injection. Representative pictures are shown (D). Quantification of fluorescence intensity at the dorsal vessel was carried out using Axiovision 4.7 (E), data shows the mean of four experiments. Student t-tests were used for the p-value calculation. * $p<0.05$

in phagosome maturation exhibits high susceptibility to *S. aureus* (Garg and Wu, 2013), the susceptibility of the *rogue* mutant to *S. aureus* is likely caused by the phagosome maturation defect. The mechanism by which Rogue affects phagosome maturation is not clear yet. As shown in the Chapter 2, Rogue may be involved in translation regulation. The expression of *rogue* may be required for the production of a group of proteins, which may include proteins necessary for normal phagosome maturation.

The hemocytes are the main phagocytic cells in *Drosophila*. Knockdown of the *rogue* gene in hemocytes result in the cells to have a defect in phagosome maturation, indicating the expression of *rogue* was required for the normal phagocytic function of the hemocytes. However, the phagosome maturation defects in the *rogue* mutant flies and the *rogue* RNAi knockdown flies were slightly different in terms of the strength and the time of onset of the defects. The *rogue* mutant had a stronger and prolonged effect on phagosome maturation compared to the *rogue* knockdown flies. This may be caused by two reasons: 1) The efficiency of the driver may affect the intensity of the knockdown in different flies, and create high variability among flies. 2) The *rogue* mutant may have multiple mutations that contribute to its phagosome maturation defect. For example, the gene that encodes the subunit 2 of the vacuolar ATPase 68 (*vha68-2*) is upregulated in the *rogue* mutant but not in the *rogue* RNAi knockdown flies (Appendix B Fig S5). This increased expression of *vha68-2* in the *rogue* mutant may contribute to its phagosome maturation defect. During maturation, the phagosomes become progressively acidified through a vacuolar ATPase pump (Lukacs et al., 1990). This pump is comprised of multisubunits: the transmembrane V_0 complex and the cytoplasmic V_1 complex. The V_0

complex, which is made of six copies of proteolipids (subunit H) (Umemoto et al., 1990), is a protonophore that transports protons from the cytoplasm into the phagosome. The V_1 complex consists of three copies each of subunit A and subunit B. Subunit A is the main catalytic subunit to hydrolyze ATP to ADP, while subunit B is considered to be the regulatory subunit (Davies et al., 1996; Liu et al., 1996). In *Drosophila*, *vha SFD* encodes subunit H, *vha55* encodes subunit B, and *vha68-1/2/3* encodes subunit A. Among the three genes that encode the subunit A, *vha68-2* is the most widely expressed. In the mosaic eye disc, the *vha68-2* mutant cells show very low levels of Lysotracker staining, which indicates the essential role of *vha68-2* in acidification of endocytic organelles (Vaccari et al., 2010). Compared to the *iso* flies, the *rogue* mutants showed higher lysotracker staining in the larval fat body (Appendix B Fig S6), indicating that the *rogue* mutant has higher acidification levels. Since the gradual decrease of pH inside the endocytic vesicles ensures the normal maturation of these vesicles (Beyenbach and Wieczorek, 2006; Vaccari et al., 2010), the high basal acidification levels in the *rogue* mutant may result in premature phagosomes. These phagosomes may not be equipped with the proteins necessary for the phagosome-lysosome fusion and thus, result in defects in maturation.

Both the *rogue* mutant flies and the *rogue* RNAi knockdown flies are able to activate the Toll pathway and induce *Drosomycin* expression. Despite this, they are still extremely susceptible to *S. aureus*. This is consistent with several other observations. The phagocytosis mutant, *Integrin βv*, is susceptible to *S. aureus* infection. The hemocytes of the mutant have impaired phagocytic activities, while the mutant has no defect in

Drosomycin induction after *S. aureus* infection (Shiratsuchi et al., 2012). Nehme and colleagues showed that injecting flies with latex beads blocked the phagocytic ability of hemocytes, and this resulted in flies that were highly susceptible to *S. aureus* infection. Additionally, ubiquitous expression of the AMP *Defensin*, or constitutive activation of the Toll pathway could not compensate for the lack of a cellular response in those flies (Nehme et al., 2011). Together, this indicates the predominant role of the cellular immune responses in defending against *S. aureus* infection in flies.

Materials and Methods

Bacteria preparation and survival analysis

S. aureus was cultured overnight at 37°C, and then sub-cultured at 1:100 dilution and grown to log phase (OD = 0.8-1.0). The culture was spun at 10,000 rpm for 3 minutes and cells were suspended at the appropriate OD in sterile PBS. The suspension used for the survival curves of *iso* and the *rogue* mutant were OD = 0.05, 0.2 and 0.3. For other experiments, OD = 0.3 was used. Five to seven day old adult flies were injected with 32nL of the appropriate bacterial suspension using the nano-injector (Drummond). Since the Yolk driver is only expressed in female flies, only females were used in the experiments that used the Yolk driver. In all other experiments, equal numbers of females and males were used. Flies were kept at 25°C, and death was monitored at different time points. None of the fly lines used for survival showed significant death after injection with sterile PBS. All survival curves were done, at minimum, in triplicate.

Bacterial Load

S. aureus was cultured overnight at 37°C, and then sub-cultured to an OD of 0.8-1.0.The bacteria was centrifuged at 10,000 rpm for 3 minutes and cells were suspended in sterile PBS to an OD of 0.3. Approximately 20 flies per genotype per experiment were injected with 32nL of the bacterial suspension. Eight flies from each genotype were then immediately homogenized in individual tubes with 200µL LB + 1% Triton X-100, serially diluted 1:10 twice in sterile PBS, and plated in triplicate on LB plates. The rest of the flies were incubated at 25°C. Twenty four hours after injection, 8 additional flies from each genotype were assayed as above, with the exception that each sample was serially diluted 1:10 five times. The plates were incubated for 24 hours at 37°C before the bacterial colonies were counted.

Hematopoiesis test

Individual larvae were bled in a 20µl PBS on a glass slide. 5 µl of it was placed on a hemocytometer and a coverslip was put on top of it. All hemocytes within a 0.16mm^2 square were counted under the microscope and the numbers of hemocytes/µl were calculated.

Reverse transcription and quantitative real-time PCR

RNA is isolated and reverse transcribed as described in chapter 2. To measure AMPs, flies were injected with 32nl of log-phase *S. aureus* at the dilution of OD = 0.3. Flies were collected at 24 hours post infection, and the expression of *Drosomycin* was measured using LUX-based qPCRs. The LUX-based primers were used, where one

primer of each pair was tagged with the fluorescent reporter FAM (Invitrogen). The ROX qPCR Mastermix (2X) (Fermentas) was used. Using the 7300 Real Time PCR System (Applied Biosystems) samples were placed at 50°C for 2 minutes, 95°C for 10 minutes, and then cycled between 95°C for 15 seconds and 60°C for 1 minute 40 times. Each sample was measured in technical triplicate on the PCR plates. For each experiment, at least three biological repeats were included.

Phagocytosis assay

To assay *S. aureus* phagocytosis, approximately 8 flies (5-7 days old with equal number of females and males) per genotype per experiment were injected with 50nl of 5 mg/mL fluorescein-labeled *S. aureus* bioparticles (Invitrogen) using the nano-injector (Drummond). After 30 minutes at room temperature in the dark, the flies were injected with Trypan Blue, and mounted onto black tape with the ventral side facing the tape. Fluorescently labeled particles were visualized immediately after the mounting using the Discovery.V8 SteREO Microscope (Zeiss). The quantification of fluorescence intensity was carried out by normalizing the fluorescence intensity at the dorsal vessel area to a neighboring background region. AxioVisionLE software was used to quantify the results.

Phagosome maturation assay

To assay phagosome maturation, approximately 8 flies (5-7 days old, equal numbers of females and males) per genotype per experiment were injected with 50nl of 5 mg/mL pHrodo-labeled *S. aureus* bioparticles (Invitrogen) using the nano-injector (Drummond). After incubating for 30, 60 or 90 minutes at room temperature in the dark, the flies were

mounted onto black tape. Images were taken as described above. For experiments with *iso* and *rogue* mutant flies, Trypan Blue was injected into the flies 5 min before the images were taken.

Chapter 4: A Genome Wide Association Study of the susceptibility to DXV in *Drosophila*

Abstract

Variation in susceptibility to infectious disease often has a genetic basis. To explore the genetic architecture underlying the susceptibility to *Drosophila* X virus (DXV) in *Drosophila* and to identify novel genes or pathways that are involved in antiviral immunity; we conducted a genome wide association study (GWAS) in a subset of wild derived inbred lines from Drosophila Genetic Reference Panel (DGRP). We have identified four single nucleotide polymorphisms (SNPs) that are associated with the phenotype of susceptibility to virus. From a second screen from a selected set of lines, we confirm that the presence of all four minor alleles of these SNPs in the same line of flies results in a significantly shorter mean time to death when injected with DXV; thus, the presence of the major alleles of all four SNPs is essential to protect flies from DXV infection. One of these SNPs was found to be highly associated with the susceptibility. This SNP is located in the intron of Socs36E, a negative regulator of the JAK-STAT pathway, implicating a role for the JAK-STAT pathway in immune responses against DXV. From this second screen, we identified several additional SNPs that are associated with the susceptibility of flies to DXV infection. These SNPs provide more candidate genes that may be involved in antiviral innate immunity in *Drosophila*. Our study shows that \ natural genetic variation can be used as a tool for identifying novel genes or pathways that are involved in antiviral immunity.

Introduction

The phenotypic variation of a population, especially for complex traits, often has a genetic base (Ayroles et al., 2009; Lewontin, 1974). However, these genetic variations that contribute to the phenotypes are often segregated and each have a small effect on the phenotypic traits (Consortium., 2007; Easton et al., 2007). In *Drosophila*, large numbers of loci affecting quantitative traits have been found in several studies, and high-resolution maps of segregating alleles of these quantitative trait loci (QTLs) have been generated (Harbison et al., 2013; Mackay and Anholt, 2006). Genome-wide association studies (GWAS) that focused on human immune responses have indicated that the natural variation is a mix of mutations with large and small effects on the selected quantitative traits (Chapman and Hill, 2012; Limou et al., 2010). Many loci contribute to the phenotypic variation with a different effect size on the phenotype. While a majority of these loci have small effect, some may have relatively higher impact. This rule may also apply to the natural variation underlying the susceptibility to pathogens in *Drosophila*. Recently one study found three single nucleotide polymorphisms (SNPs) that had major effects on the resistance to two fly natural viruses and these SNPs can explain up to 47% of the heritability in the susceptible individuals (Magwire et al., 2012). Thus, studying the genetic variation underlying the susceptibility of flies to pathogens may provide insights as to the major alleles that contribute to phenotypic variation.

Recently, the Mackay lab derived hundreds of highly inbred lines from the natural population of *Drosophila melanogaster* in North Carolina (Mackay et al., 2012). These lines contain homozygous polymorphisms that are different between individual lines.

Each of these lines has been fully sequenced, hence, the common single nucleotide polymorphisms (SNPs) segregating in these lines are known (Ayroles et al., 2009). Thus, these lines are great resources for identifying candidate genes for complex traits. Because these lines have known segregation of the SNPs, they can also be used for selection experiments that focus on studying specific loci (Harbison, McCoy et al. 2013). Since the lines and the sequencing information are open resources to the community, these flies can be used for studies of different quantitative traits (Chow et al., 2013; Harbison et al., 2013; Jumbo-Lucioni et al., 2012; Magwire et al., 2012; Turner et al., 2013). Thus, SNPs associated with multiple traits may be identified.

Susceptibility to infectious pathogens is one of the complex traits that can be deciphered through GWAS studies (Magwire et al., 2012). Viruses are among the most abundant pathogens in nature. Insects like *Drosophila* are constantly in contact with a variety of viruses in the wild. Under natural selection, a higher frequency of mutants with resistant alleles to natural viruses is expected. Magwire and Anholt studied the natural variation associated with resistance of flies to four different viruses, and found higher heritability of resistance against the two natural viral pathogens (Magwire et al., 2012). In addition, natural genetic variation in wild flies is also useful to identify polymorphisms that are associated with fly susceptibility to laboratory viruses. Because of natural selection, alleles that result in general antiviral defects would likely be selected out. However, alleles that affect the immune responses to laboratory viruses may still be present. Therefore, GWAS studies on viral susceptibility of the wild flies can be useful to identify antiviral genes and antiviral mechanisms.

Previous studies have shown that several signaling pathways and cellular processes are involved in antiviral immunity in *Drosophila* (Huszar and Imler, 2008). For example the Toll (Zambon, Nandakumar et al. 2005), Imd (Avadhanula, Weasner et al. 2009; Costa, Jan et al. 2009), JAK-STAT pathways (Dostert, Jouanguy et al. 2005), RNA interference (Sabin et al., 2009; van Rij et al., 2006; Zambon et al., 2006), autophagy (Shelly et al., 2009) and apoptosis (Liu et al., 2013) are important against different viruses. Except for RNAi, which is a general mechanism for antiviral immunity in the fly, all other pathways and cellular processes are responding to certain types of viruses. In our previous studies, we have identified that the RNAi pathway and the Toll pathway play roles in the immune response against *Drosophila* X virus (DXV) (Zambon et al., 2005; Zambon et al., 2006). These two pathways are activated by DXV infection. Also, mutants in the RNAi or Toll pathway have increased susceptibility to DXV. Besides DXV, these pathways were also found to respond to Dengue virus infection in *Aedes aegypti* mosquitoes too (Sanchez-Vargas et al., 2009; Xi et al., 2008). Whether other antiviral pathways play roles in the immune response against DXV is unknown.

Here, we explore the genetic variation underlying the viral susceptibility to DXV in the flies. We hope to identify novel genes or pathways involved in antiviral immunity. By looking at the specific responses of flies to virus, four single nucleotide polymorphisms (SNPs) were found and confirmed to be highly associated with the susceptible-to-virus phenotype. The minor allele of one of the SNPs contributes more to the susceptibility than that of the other SNPs. This allele is located at *Socs36E*, which is a negative

regulator of the JAK-STAT pathway, suggesting that the JAK-STAT pathway plays an important role in antiviral defense against DXV. Subsequently, in our second screen, twenty-four more SNPs were found to be associated with the fly susceptibility to viral infection. This provides more useful candidates for the studies on antiviral immunity.

Results

To identify SNPs associated with DXV susceptibility, we screened 35 different inbred lines from the Drosophila Genetic Reference Panel (DGRP) by injecting virus into the adult flies and monitoring their survival. These lines had their genomes sequenced first. From previous studies, we find that when flies are infected with DXV, the mortality of the flies usually starts at 10-11 days, with most of the flies dying within 4-5 days after. Because of the specific kinetics of fly survival to DXV infection, we used percent death on day 12 after infection as the indicator of susceptibility for each line. Four SNPs were found to have high effect ($p<10^{-6}$) on the variation to susceptibility to DXV (Table 4-1); all of them were associated with high susceptibility to the virus. For an easier way to refer to the SNPs, we assigned each of them a letter (Table 4-1).

Table 4-1: SNPs associated with susceptibility to DXV (initial screen).

Chrs	Position	Letter	Features	Effect size	Gene	p-value
2L	18148677	B	intronic	-0.185	Socs36E	2.29E-06
2R	14762291	C	intergenic	-0.184	Downstream CG43109, Upstream sano	4.98E-06
2R	19133176	D	intergenic	-0.208	Downstream yip3, Upstream RpL22-like	4.65E-06
3R	26303629	A	intronic	-0.181	PH4alphaEFB	8.69E-06
3R	26303629	A	exon;CDS	-0.181	spdo	8.69E-06

An ANOVA with the model - phenotype = mean + M, where M is the Marker (SNP) was used; 10^{-6} was used as the cutoff for significance;

Effect sizes were determined by [(Major allele mean) - (Minor allele mean)]/2;

SNPs were assigned a letter A-D;

SNP A is located within two overlapping genes: in the intron of *prolyl-4-hydroxylase-alpha EFB (PH4alphaEFB)* and in the coding region of *sanpodo (spdo*). The minor allele of SNP A creates a silent mutation in *spdo*. SNP B lies in the intron of *Socs36E* gene. The other two SNPs (C and D) are intergenic.

To investigate if these SNPs have additive effects, we selected lines with some or all four minor alleles of the SNPs and lines the major alleles of these SNPs to form our second screen. A different indicator, the mean time to death of each line, was used to assess susceptibility, so that a general survival effect could be included. Each line was repeated three times. The average of the three repeats was obtained and used to determine the mean time to death in each groups (Table 4-2). The mean time to death in Group N, in which the lines contain the reference alleles (major alleles) of these four SNPs, was used as the control. Our results showed that the flies in Group ABCD, in which the lines contained all four minor alleles of the SNPs, had significantly (3 days) shorter mean time to death compared to control flies (Fig 4-1). Although flies in Group CD have no defect in survival, flies in Group ABCD are much more susceptible than flies in Group AB, which indicates that SNPs C and D together may have an additive effect over SNP A and B together. Flies containing the minor allele of SNP B but not the minor alleles of the other three SNPs are also significantly more susceptible to virus compared to control flies. In contrast, flies with the minor allele of SNP A but not the minor alleles of the other three SNPs are slightly resistant to DXV, which explains the lower viral susceptibility of flies in Group AB compared to Group B.

Table 4-2: Lines used for the screening.

SNP Group	Line number	Average mean time to death	SNP Group	Line number	Average mean time to death
ABCD			ACD		
	324	7.3		**859**	10.7
	712	7.2	BCD		
	786	9.0		**217**	12.0
AB			CD		
	21	7.3		**158**	11.3
	41	12.7		**357**	10.0
	195	12.3		**373**	12.3
	761	8.3		**884**	10.3
	787	8.7	C		
A				**774**	11.8
	399	11.7	D		
	437	12.3		**440**	11.2
B			N		
	26	9.0		**307**	12.2
	69	9.0		**315**	9.7
	208	8.8		**375**	10.7
ABC				**732**	10.8
	149	11.3	ABCD progeny*		
	228	9.5		**712/786**	6.5
	707	10.7		**712/324**	6.17
	810	9.3		**324/714**	7
ABD				**149/195**	7.5
	304	9.0			
	317	10.7			
	374	10.5			
	491	10.7			
	705	9.3			

Flies were injected with DXV. The average mean time to death shown was generated from at least three repeats of the same line. *Parental lines were shown for the progeny that were examined.

SNPs:

A: 26303629 (spdo / PH4αEFB)

B: 18148677 (Socs36E)

C: 14762291

D: 19133176

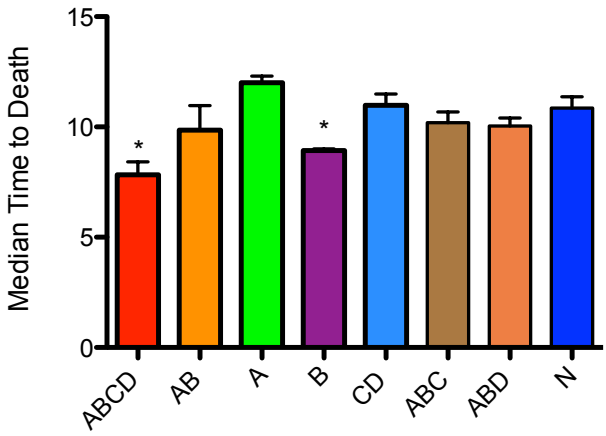


Figure 4-1: Susceptibility of flies in the different SNP groups. 34 lines were divided into different SNP groups. Group ABCD include lines that have all four SNPs, group AB include lines that have SNP A and B, but not SNP C and D, group N include lines that do not have any of these four SNPs. Each SNP group contains the mean from at least three lines (except group A), and each line has been repeated at least three times to get the mean. The bars show the standard error from the means of each SNP group. For line numbers see Table 4-2.

Flies in Group ABCD contain all four minor alleles of the target SNPs; however, each line may also contain other alleles that contribute to the susceptibility of flies to virus. To confirm that the susceptibility of flies in Group ABCD is due to these four alleles, but not homozygosity at other SNP sites, lines in this group were randomly selected and mated with each other. The progeny from these crosses would contain homozygous minor alleles at SNP A, B, C and D, but heterozygous alleles at other SNPs. These flies were subjected to DXV infection. Highly significant susceptibility to virus was found in these progeny compared to the control flies (Fig 4-2), indicating that the presence of minor alleles of these four SNPs in the same flies was sufficient to cause the flies to be highly sensitive to viral infection.

Flies in Group B were slightly less susceptible to DXV compared to Group ABCD, but showed significantly higher susceptibility to the virus than flies in Group N, which suggested that the presence of the minor allele of SNP B contributes the most to the susceptibility of flies in Group ABCD. To examine if the presence of the minor allele of SNP B by itself is sufficient to promote the susceptibility of flies to virus, the flies in the second screen are rearranged into two groups: one with flies that contain the major allele of SNP B, the other with flies that present the minor allele of SNP B. The susceptibility of flies to viral infection in these two groups was compared. Flies with the presence of the minor allele of SNP B showed significantly shorter mean time to death than flies with the major allele of SNP B (Fig 4-2), suggesting that the presence of the minor allele of SNP B decreased the resistance of flies to viral infection.

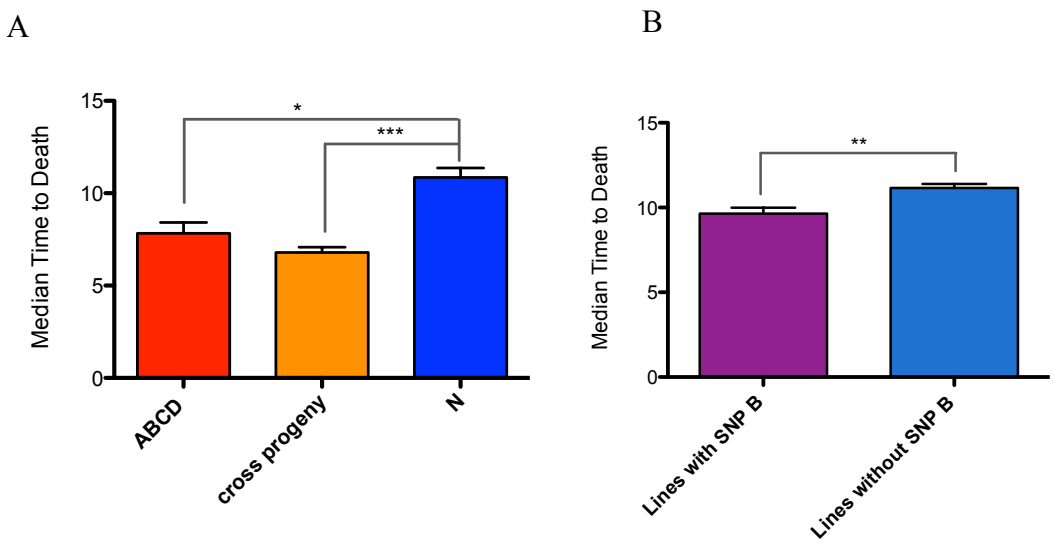


Figure 4-2: The presence of the minor alleles of SNP A, B, C and D is sufficient to cause the susceptibility of flies to DXV infection; the allele at SNP B contributed the most to the phenotype. A) Flies that had all four SNPs were crossed to each other (Lines that were used are shown in Table 4-2). Four progeny lines were infected with DXV and the average survival of them was compared to the control flies. B) Mean time to death of lines with SNP B and lines without SNP B were grouped and compared.

SNP B is located in the intron of gene *Socs36E*, which indicates that the presence of the minor allele of SNP B may affect *Socs36E* expression. Thus, we examined the expression levels of *Socs36E* in randomly selected lines by qPCR. Compared to the lines that contained major allele of SNP B, the lines with the presence of the minor allele of SNP B had higher expression of the gene; however, the difference was not significant (Fig 4-3). This suggested that alleles in SNP B might not significantly affect the transcriptional levels of the *Socs36E* gene.

To identify additional polymorphisms associated with resistance or susceptibility to DXV, we performed a genome-wide association studies on the second screen using the DGRP online analysis tool. We found a number of SNPs associated with the susceptibility of flies after viral infection using a $p<10^{-5}$ cut off threshold (Table 4-3). Many SNPs were located in intergenic regions (27.3%) and in introns (59.1%), while fewer SNPs were found in coding regions (13.6%). One of the SNPs was in the coding region of the gene *CG42382*. The minor allele of this SNP causes a Phenylalanine to Leucine change in the amino acid sequence; thus, it may affect the protein function of this gene. Out of 24 SNPs found from the screen, 13 SNPs were associated with the phenotype of increased susceptibility to viral infection, while 11 were associated with the phenotype of resistance. Strikingly, the top three SNPs that had the most significant p-values were found to affect the same gene: *sp2637* (Table 4-3), and they were all strongly associated with the increased susceptibility to viral infection. This result indicated that *sp2637* might be an important gene involved in the immune responses against the viruses.

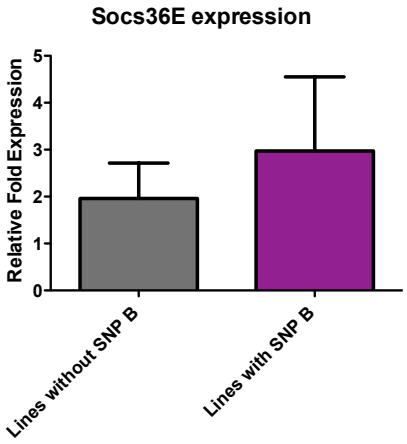


Figure 4-3: Expression levels of *Socs36E* in random lines. Total RNAs from 11 randomly chosen lines were collected. The expression levels of Socs36E of each line were measured by quantitative RT-PCR (qPCR). The data were grouped into two categories: lines without SNP B (5 lines) and lines with SNP B (6 lines). The bars shown are the standard error from the means of each SNP group.

Table 4-3: Genes with SNPs associated with susceptibility to DXV (second screen).

Gene	chrs	position	site	P-value
S*	2L	1053028	synonymous	2.42E-06
CG43321	2L	7011296	intergenic	7.82E-06
uif	2L	7011296	intergenic	7.82E-06
KdelR*	2L	10426784	intergenic	4.38E-06
SmB*	2L	10426784	intergenic	4.38E-06
CG6144*	2L	10489606	synonymous	8.99E-06
bun*	2L	12489439	intronic	5.62E-07
CG42382	2R	5054763	coding	8.60E-06
CG43729*	2R	11300639	intronic	2.35E-06
SP2637*	2R	14534994	intronic	3.12E-07
SP2637*	2R	14535001	intronic	4.60E-07
SP2637*	2R	14535003	intronic	4.60E-07
CG5549	2R	19710620	intronic	9.21E-06
CG5549	2R	19710621	intronic	5.15E-06
CG32365	3L	7875165	intronic	2.79E-06
CG32365	3L	7875188	intronic	6.20E-06
A2bp1*	3L	10470734	intergenic	6.74E-06
A2bp1*	3L	10470834	intergenic	9.70E-06
CG34050*	3L	10934597	intergenic	3.06E-06
Or69a*	3L	12959458	intronic	8.20E-06

Cad87A	3R	7759971	intronic	7.64E-06
Cad87A	3R	7759980	intronic	7.64E-06
Cad87A	3R	7759994	intronic	7.64E-06
CG12688	X	4273048	intergenic	3.13E-06

The mean times to death of each line from the second screen were uploaded to the DGRP website for analysis. The SNPs that were significantly associated with the survival phenotype are shown above. $p<10^{-5}$ was used as the cut off. *: SNPs that were associated with phenotype of increased susceptibility.

Following these three most significant SNPs, the SNP with the fourth lowest p-value is in the intron of the gene, *bun*, which encodes the fly homolog of the mammalian tumor suppressor TSC-22. On the other hand, most of the SNPs associated with the increased resistance to viral infection were at the relatively high allele frequency, with minor allele frequencies between 0.34-0.43 (Fig 4-4). In addition, we observed a few instances of local linkage disequilibrium (LD) regions associated with the susceptibility of flies to viral infection (Fig 4-4). For increased susceptibility, one LD region including the three SNPs of *sp2637* was found on 2R. For decreased susceptibility, two adjacent SNPs on 2R within *CG5549* were highly linked. A 24bp LD region was found within *Cad87A* on 3R.

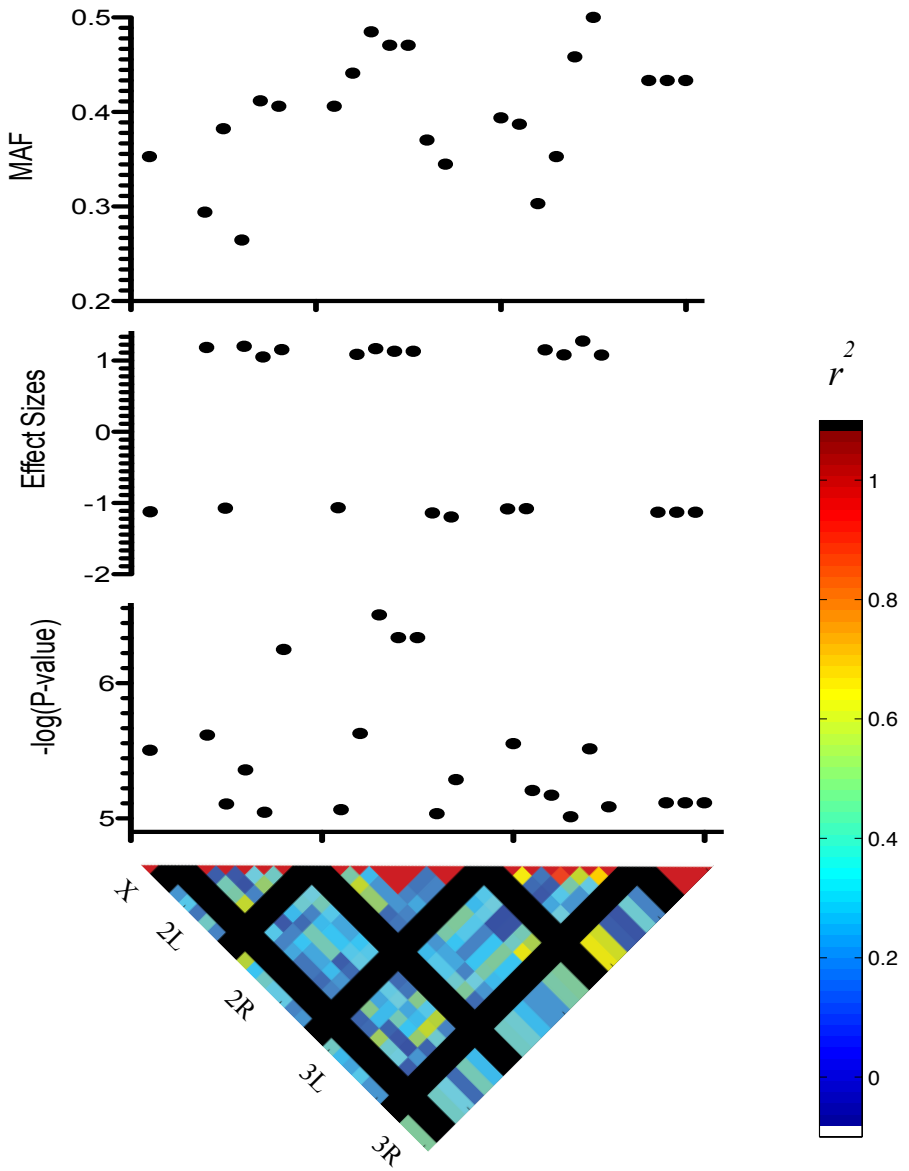


Figure 4-4: Genome wide association results for the survival trait. Significant SNPs ($p<10^{-5}$) are plotted. The top panel shows the minor allele frequency (MAF) for each significant SNP. The middle panel shows the effect sizes of each significant SNP. p-values are plotted as $-\log_{10}$(p-value) in the bottom panel. The lower triangle shows the distribution of linkage disequilibrium among the SNPs as r^2. Solid black lines separate the five major chromosome arms.

Discussion

In *Drosophila*, genome wide association studies have been conducted to look for genetic variations affecting quantitative traits and alleles that associate with various phenotypes have been found (Harbison et al., 2013; Mackay and Anholt, 2006). Because the animals are constantly interacting with various pathogens in nature, the pressure of natural selection helps concentrate the variances that benefit the survival of animals. Thus, major effect alleles that contribute to the resistance of infectious disease can be seen in the natural population (Hill, 2012; Limou et al., 2010). However, it was proposed that significant association of polymorphisms with the resistance to non-natural pathogens would be difficult to detect (Magwire et al., 2012).

In our study, we asked if the natural variation could be associated with susceptibility to DXV, a virus that has not been found in the natural fly population. By looking at the specific survival responses of flies to virus, we were able to identify four SNPs that were highly associated with a susceptible-to-virus phenotype. Mutant alleles that can lead to susceptibility to natural viruses will occur at a decreased frequency under the process of natural selection, because animals with these alleles often die faster when infected. However, alleles that are associated with susceptibility to non-natural viruses should maintain its own frequency in the natural population. This is because the animals are not interacting with these viruses; therefore there is no selection pressure against these alleles. Although there is no selection pressure against these alleles, there is also no selection favoring them either. The frequency of the SNPs of interest is still moderate. An allele that was associated with rapid progression to AIDS in humans was identified in

disease association studies (Chapman and Hill, 2012; Limou et al., 2010). Previous studies implicated that incorporating biological information into the analytical approach could increase the power of detection (Quintana et al., 2012). Thus, to gain power, we used a virus-specific survival phenotype in our initial screen to identify SNPs associated with the susceptibility of flies to DXV. Following the initial screen, we conducted a second screen in a set of flies with selected combinations of SNPs, to experimentally confirm the effects of the SNPs from the initial screen. We used a different indicator, mean time to death, for susceptibility in the second screen to minimize the bias.

Flies that contain homozygous minor alleles of SNPs A, B, C and D but are heterozygous at other SNPs were still more susceptible; this confirmed that these four SNPs are the major alleles that affect the susceptibility of flies to virus. Also, it indicated that the presencne of other alleles in the flies of Group ABCD might be compensating the effects that came from the minor alleles of these four SNPs. In terms of how the minor alleles of these four SNPs contribute to the phenotype, both additive effects and synergistic effects are seen. For example, although the presence of the minor allele of SNP B contributes most to the susceptibility of flies to viral infection, the presence of the minor alleles of the other three SNPs results in flies that are even more susceptible. Thus, SNPs A, C and D together may have an additive effect to SNP B. On the other hand, the presence of the minor allele of SNP A compensates the effect of the minor allele of SNP B as long as the flies do not contain both the SNP C and D minor alleles. Therefore, SNP A probably has a synergistic effect on the susceptibility when not all of the SNP B, C and D minor alleles are present.

Our screen found an allele that contributed most to the susceptibility, the minor allele of SNP B. This allele affects the gene *Socs36E*, which is a negative regulator of the JAK-STAT pathway, suggesting that the pathway may play an important role in antiviral defense against DXV. In the mammalian system, the JAK-STAT pathway is the signaling cascade downstream of interferon, and it is required for the animal to survive viral infections (Darnell et al., 1994; Meraz et al., 1996). In *Drosophila*, the JAK-STAT pathway is found to be important to protect flies against DCV and SINV. But it is not activated with FHV infection (Deddouche et al., 2008; Dostert et al., 2005). Here we have implicated an important role of the JAK-STAT pathway in immune responses against the DXV virus. It would be interesting to further explore if and how the minor allele of SNP B affects the function of the JAK-STAT pathway.

From the second screen, additional SNPs were found to be associated with the viral susceptibility of the flies, which provided more candidate genes for follow up studies. The top three SNPs with the most significant p-values affect the same gene: *sp2637* (Table 4-3), indicating that *sp2637* might be an important gene involved in the immune responses against virus. *sp2637* has not been well studied in *Drosophila*. But its mouse and human homologs, NTAN1 and hNTAN1, are N-terminal asparagine amidases and are involved in the N-end rule pathway protein degradation. They function in the deamidation of the N-terminal L-Asn of the target protein into L-Asp, a process that promotes ubiquitin-dependent protein degradation (Cantor et al., 2011; Grigoryev et al., 1996). If Sp2637 has a similar function as its homologs NTAN1 and hNTAN1, our

results may indicate that ubiquitin-dependent protein degradation plays a role in antiviral immune responses in *Drosophila*.

After *sp2637*, the fourth lowest p-value SNP was in the intron of the gene *bun*. *bun* encodes the protein Bunched, a fly homolog of the mammalian tumor suppressor TSC-22 (Transforming Growth Factor-beta1 stimulated clone-22) (Nakashiro et al., 1998). It is involved in regulating cell growth and apoptotic processes (Gluderer et al., 2008; Wu et al., 2008). It is also associated with neuronal cell proliferation (Kim et al., 2009). Interestingly, it is also implicated as a negative regulator of the Notch signaling pathway (Dobens et al., 2005). Since Notch signaling can activate the JAK-STAT pathway (Kamakura et al., 2004), this SNP might affect the JAK-STAT pathway as well, further supporting a model for the JAK-STAT pathway in antiviral signaling.

Finally, from GWAS screening for susceptible phenotypes with a non-natural virus, our study has found multiple potential antiviral genes. We anticipate that these genes will serve as candidates for further in-depth analyses. Understanding the precise function of these candidates may provide insights to the antiviral defense mechanisms in *Drosophila*.

Materials and Methods

Fly stocks

A total of 49 lines (35 in the initial screen, 34 in the second screen, 20 lines overlapping between the two screens) of wild derived flies from the Drosophila Genetic Reference

Panel (DGRP) were used in this study. These flies are homozygous inbred lines that were created by 20 generations of full sibling mating of progeny of wild females from Raleigh, North Carolina (Mackay et al., 2012).

Virus preparation

The DXV used is a 10^{-5} dilution of the stock, and the DCV used is a $5x10^{-5}$ dilution of the stock. The TCID50 of the DXV stock is $4.37x10^{10}$/ml. DCV stock is the supernatant collected from infected S2 cells. The doses chosen are those causing around 50% death of wild type flies at day 11 for DXV and day 15 for DCV. 32nl of virus preparation (with 6% green food coloring dye) are injected into each fly using the Nanoinjector II (Drummond).

Survival analyses

All adult flies used are 5-7 days old and raised at 25 °C on standard yeast/agar media. For all experiments, equal numbers of females and males are used. In each repeat, 30-50 flies of each line were subjected to viral infection. The survivals were recorded at day 1 and day 12 after injection for the initial screen. For the second screen, the survivals were recorded daily. The number of flies surviving at day 1 was used as the initial count for calculation of the morbidities.

Genotype-phenotype associations

For the initial screen the percent death of each line was associated with all segregating sites in the first 40 lines that had been sequenced. For the second screen the line mean of

mean time to death of each line was associated with all segregating sites in the DGRP for which the minor alleles are present in four or more lines (Mackay et al., 2012). Associations were tested using ANOVAs of model $Y= \mu + M + \varepsilon$, where M is the genotype effect. These tests were implemented using the tools provided from dgrp.gnets.ncsu.edu (Mackay et al., 2012).

Chapter 5: Summary and future directions

My work mainly focuses on the discovery of novel genes or molecules that are involved in anti-viral immunity in *Drosophila*. Two genetic screens for flies with increased susceptibility to *Drosophila* X virus (DXV) were conducted, one was a forward screen using flies that were mutagenized by ethyl methane sulfonate (EMS) (Koundakjian et al., 2004), and the other was a genome wide association screen (GWAS) using the wild-derived inbred flies from the Drosophila Genetic Reference Panel (Mackay et al., 2012). From the forward screen, four mutants were identified to have increased susceptibility to DXV when anoxia treated. Two of these mutants (*rogue* and *pyro)* were sensitive to virus without the anoxia treatments. We were able to map the *rogue* mutant to a novel antiviral gene *rogue*. From the genome wide association studies we identified four single nucleotide polymorphisms (SNPs) that were associated to the phenotype of increased susceptibility to DXV. Absence of the major alleles of all four SNPs in the same flies significantly decreased the mean time to death of these flies. The presence of the minor allele of one of these SNPs was found to contribute most to the susceptibility. This allele is located in the intron of *Socs36E*, a negative regulator of the JAK-STAT pathway (Callus and Mathey-Prevot, 2002), implicating a role for the JAK-STAT pathway in immune responses against DXV. We also identified additional SNPs that associated with susceptibility of flies to DXV infection; the genes or pathways that these SNPs affected could serve as candidates for further investigation. Together, our study had identified one novel antiviral gene, *rogue*, and several possible candidate genes and pathways that might be involved in antiviral responses in *Drosophila*.

Future directions

Does the virus directly interact with Rogue?

Since increased numbers of hemocytes show the nuclear localization of the Rogue protein at the early infection stages, it is possible that direct interaction with the viruses induce the nuclear localization of Rogue. Thus, it would be interesting to examine if Rogue and the virus directly interact. In larval hemocytes of infected animals, viral protein (VP2 and VP3) is mainly found in the cell cytoplasm (Fig 2-19). Occationally, a small fraction of PABP and the FLAG-tagged Rogue can be seen in the same location, but in the majority of cells they show distinct localization (data not shown). This indicates that the viral protein might not be in close contact with Rogue or activated Rogue. We also have tried to determine if viral protein can co-immunoprecipitate with the Rogue-PABP complex, but have failed to detect any viral proteins in the complex at 1 hour after infection (data not shown). This indicates that the viral protein might not directly interact with Rogue. However, the dsRNA genome of DXV might be interacting with Rogue or activated Rogue. Thus, it would be interesting to examine if the viral genome can be found in the Rogue complex.

Does the Rogue-PABP interaction affect translation?

Although we have showed that the Rogue protein is physically part of the same protein complex as PABP, we have not yet provided direct evidence that it affects translation. As was mentioned in Chapter 2, we overexpressed the Rogue protein in fly wing discs and found the tissue size was decreased, indicating that the Rogue protein might have an inhibitory role on translation. In humans, two PABP interacting proteins, Paip1 and

Paip2, can regulate PABP-dependent translation. Paip1 stimulates translation, while Paip2 inhibits translation and can compete with Paip1 for binding to PABP (Craig et al., 1998; Khaleghpour et al., 2001). The *Drosophila* homolog of human Paip2, dPaid2, was found to interact with *Drosophila* PABP and inhibit translation and cell growth (Roy et al., 2004). Overexpression of dPaip2 in fly wings or wing discs resulted in smaller wings or wing discs (Roy et al., 2004). Since overexpression of Rogue showed a similar phenotype as overexpression of dPaip2, Rogue is more likely to be involved in translation inhibition. Because fly wing discs normally do not express the *rogue* gene, this ectopic expression might not represent the normal function of the Rogue protein, thus, it would be useful to examine the effect of loss or gain of expression of the Rogue protein in a tissue that normally expresses the *rogue* gene – the adult fat body. Because the cell size of the fat body in different flies can be different, it may be hard to compare cell size between different flies. Thus, fat body mosaic clones of the *rogue* mutant may be necessary. In addition, dPaip2 inhibited up to 80% of translation activity *in vitro*, but it only showed a moderate (10%-20%) decrease of tissue size *in vivo*. This could be because *in vivo* translation is under the control of many regulators. The loss of one regulator might be compensated by others. Thus, an *in vitro* translation assay may be useful to determine if *rogue* is involved in repressing translation.

As discussed in Chapter 2, Rogue may affect the translation of a set of proteins rather than general protein synthesis. Thus, it would be useful to look for proteins or RNAs that are associated with the Rogue protein. If certain proteins or RNAs were pulled down with

the Rogue protein, it would indicate that Rogue might be regulating the translation of these proteins or RNAs.

How does loss of *rogue* cause flies to be susceptible to both virus and bacteria?

Interestingly, the *rogue* mutant and the *rogue* RNAi knockdown flies were not only susceptible to DXV but also to *S. aureus* infection. Our phagocytosis assay indicated that these flies had phagosome maturation defects, which could contribute to the susceptibility of these flies to the bacterial infection. But it was not clear if the phagosome maturation defect could contribute to the susceptibility of these flies to virus. Due to the small virion size, DXV is unlikely to be internalized by a phagosome; hence, phagosome maturation may not be responsible for viral clearance in the cell as it is for the bacteria. However, there are two possibilities as to how a phagosome maturation defect may be related to the antiviral responses.

First, phagosome maturation is required for apoptotic cell clearance and nutrition recycling, both of which may be important for flies to survive a viral infection. Impairment of apoptotic cell clearance can result in cellular lipid accumulation and autoimmune responses to self-antigen in the mouse (Mukundan et al., 2009). In viral infected flies, the failure to digest apoptotic cells may also lead to the accumulation of cellular debris and elevated stress levels inside of cells, which would affect normal immune responses. This may explain the high basal and bacterial induced Drosomycin levels observed in the *rogue* mutant (Fig 3-2) and the *rogue* knockdown flies (Fig 3-4). In

addition, some of the apoptotic cells may contain viral particles, engulfment but not clearance of these cells may spread the virus to the uninfected cells.

Secondly, the *rogue* mutant may have a general defect in endocytic pathways. Since endocytic pathways are important for dsRNA uptake and systematic antiviral RNAi, a defect in the endocytic pathway may render the flies to be unable to control viral spreading inside of the animal. In the adult fat body of *rogue* knockdown flies, viral particles were often found in cells located at the interior part of the tissue, indicating that the viral infection was able to spread into the tissues in the knockdown flies (Fig 2-15). Thus, Rogue may play a role in antiviral responses mediated by the dsRNA endocytic uptake.

The *rogue* mutant has a phagosome maturation defect in a late stage. It is possible that the defect is affecting the lysosomes, and the lysosomes were not able to fuse with phagosomes or endosomes (Mellman, 1996). This lysosomal fusion defect may affect the flies' viral susceptibility, since it is important for some intracellular pathogens to escape from the endosome to replicate in the cytoplasm (Maier et al., 2012; Xiao and Samulski, 2012). If DXV escapes from the endosome before or at the lysosomal fusion step, the failure of the endosome-lysosome to fuse in the *rogue* mutant will favor the release of virions and promote the replication of viruses. Thus, it would be interesting to trace the cellular location of virus, and examine when the virus escapes into the cytosol.

Alternatively, it is also possible that the phagosome maturation defect does not play a major role in the antiviral responses. Rogue may be involved in both phagosome maturation and the regulation of translation, which individually affect the immune responses against bacteria and virus respectively.

Appendix A: Predicted features of the *rogue* gene and the Rogue protein

1) Gene sequence:

The sequence is obtained from flybase.com.

Legend: *gene span*:xxxx *RNA*:XXXX *CDS*:XXXX

>2L:12966590,12967810
GTGGCAGGGCGAGAAGGGTTTGGATAAAAAGCTTCGGCGGCTCGAGGCGAG
TCCAGTAGACTGCGATAAGCGGGCGTGGCAGCCACACCAGAAGCAGGGGAA
AAACCAGCGGGCAACAGCAGCCAGCCAGGATAACACGGAGCAGCAGTGGCA
TCAGCAATGTGGCCGGCTTGGCAAGTGATAACGCTGCTGGGGCTTTTGGCCA
GGGCTTTGGCCCTCCACTCAACGCCAGATGGGGCAATGGCCATAAGCGCGGC
GCTGCTTGGCCAGGACTTTGAGGATTTTCAGCCGTACTTCGCACATAAACAGG
AGCAGgtaaggaattctaaatttatatggattttatagggatttagctgtcgttacaaaagaataattgaattttgctataattac
ttaaaattcactttgaaggttattataatgttgagctctgaaagttatattttgtatattaagtatacgccatgttgcagtcacattttaattt
aaacatcatattttagGAAGAAGATCAGCTAGTTGCTGCCACAAAGCACGAGGAGCATT
CCGAGGGTGGCGAGGAGGAATCTGGGGAGGAGCACCACAGTGAGCACTTCC
ACAAGAAGGGGGGAAAGAGCAAGAAGGGTCACAAGCACGGCGAGCACTCC
GAGAAGGGCGAGAAAGGTCACCACGACAAGGAGGGCAAGAAGGGGGAACA
CGGCGAGGAGGAGGGTCACGAGAAGAAGCACAAGCACTCGGAGTCTCATCA
CAAGAAGAAGAAGAAAGGCTCCAAGGGCGAGAAGGGCAGCGAGTTCGAGG
ATCACGGCTCCTATAAGAAAGGACACTCCATCAAGGGCAAGCACAACATCCA
CAAACTAGACGAGAACAAGAAAGAGAAAAAGTTCTACGATGAGGATCACAA
TGAGGGCGGCGAGGAAAAGCACGGCGGATTCGAGGAGTCCAAGAAGCACAA
AAAGGGCAGCAGCTTCAAGAAGGGTCACCACAAAAAGGGCGGCCACGAGGA
GAACTACGGCAAGAAGGGTCACAGCAAGAAGGGTCACAAAAAGAAGGGCCA
CAAGGGGCACAAGAAGAAGCACGAGGAGTCCAAGAAGTGGGGCCACAAAA
AGGAGCACGGCAAAAAGGGCGGCGAGGAGCACAAGAAGAAGTGGCACAAA
TCGCACAAACAGAGCAGCGAACACGATCATGGACATCATTAAATCTCAAGCC
GACCACCCTGCG

2) Protein sequence:

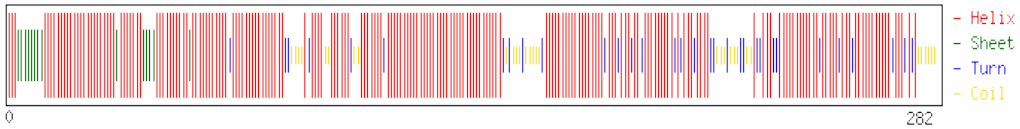
The sequence is obtained from flybase.com.

Rogue consists of 282 amino acids:

MWPAWQVITL LGLLARALAL HSTPDGAMAI SAALLGQDFE DFQPYFAHKQ
EQEEDQLVAATKHEEHSEGG EEESGEEHHS EHFHKKGGKS KKGHKHGEHS
EKGEKGHHDK EGKKGEHGEEEGHEKKHKHS ESHHKKKKKG SKGEKGSEFE
DHGSYKKGHS IKGKHNIHKL DENKKEKKFYDEDHNEGGEE KHGGFEESKK
HKKGSSFKKG HHKKGGHEEN YGKKGHSKKG HKKKGHKGHKKKHEESKKWG
HKKEHGKKGG EEHKKKWHKS HKQSSEHDHG HH

3) Second structure prediction:

(CFSSP Server- Chou & Fasman Second Structure Prediction)



4) Nuclear localization signal prediction:

Program used: PredictNLS

MWPAWQVITLLGLLARALALHSTPDGAMAISAALLGQDFEDFQPYFAHKQEQE
EDQLVAATKHEEHSEGGEEESGEEHHSEHFHKKGGKSKKGHKHGEHSEKGEKG
HHDKEGKKGEHGEEEGHEKKHKHSESHHK**KKKKGSKGEKGSEFEDHGSYKK**
GHSIKGKHNIHKLDENKKEKKFYDEDHNEGGEEKHGGFEES**KKHKKGSSFKK**
GHHKKGGHEENYGKKGHSKKGHKKKGHKGHKKKHEESKKWGHKKEHGK
KGGEEHKKKWHKSHKQSSEHDHGHH

Nuclear localization signals are highlighted in red.

5) Predicted homologs of *rogue* in *C. elegans*:

Y39B6A.1: A protein-coding gene.
Phenotypes:
1) RNAi knockdown of this gene in worms causes maternal sterility (Simmer et al., 2003).
2) RNAi knockdown of this gene increases the Pgpdh-1:: GFP expression in worms (Lamitina et al., 2006).

C17F3.3: A protein-coding gene.
Expression level of it is regulated by TGFβ signaling (sma-2)(Luo et al., 2010).

C33G8.2: A protein-coding gene.
Phenotypes:
1) RNAi knockdown of this gene in worms causes embryonic lethality (Skop et al., 2004).

CELE_Y39B6A.9: uncharacterized

R01E6.5: uncharacterized

Appendix B: Supplemental figures

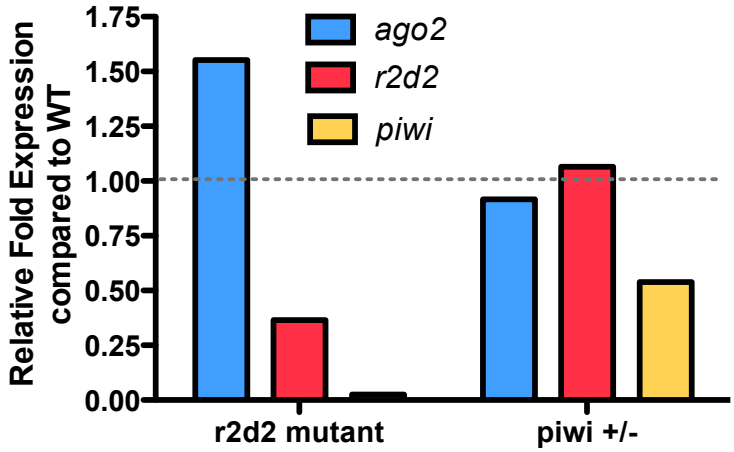


Figure S1: The *r2d2* mutant has a similar expression pattern of *AGO2*, *r2d2* and *piwi* as the *rogue* mutant.

r2d2 has increased expression levels of *ago2*, decreased expression levels of both *r2d2* and *piwi*. The expression levels of each gene were measured by qPCR and were normalized to that of *iso*.

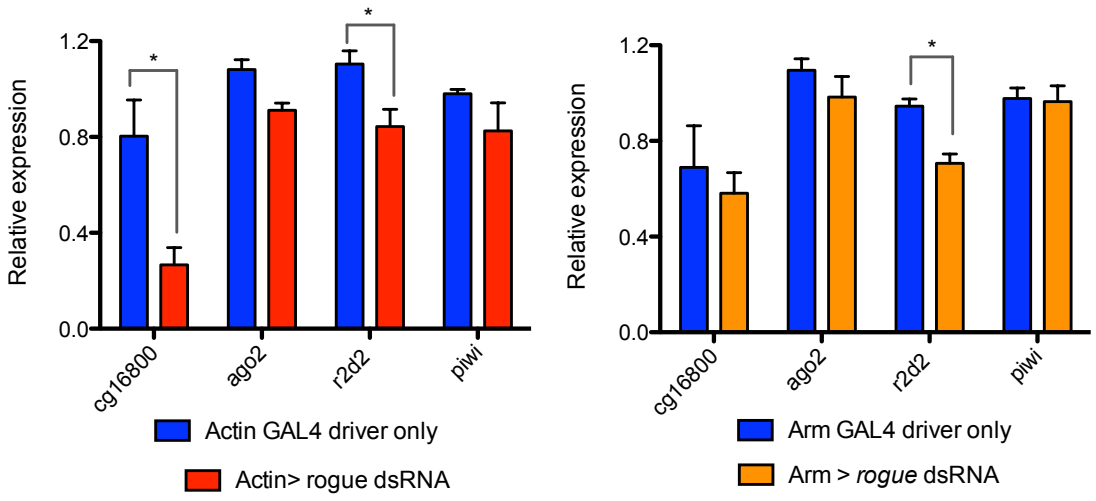


Figure S2: *r2d2* expression in rogue RNAi knockdown flies.

rogue knockdown with (A) Actin or (B) Arm drivers significantly decreases the expression level of *r2d2*. The expression levels of each gene were measured by qPCR. The data shown represents the mean of triplicates. The error bars show standard error. * $p<0.05$

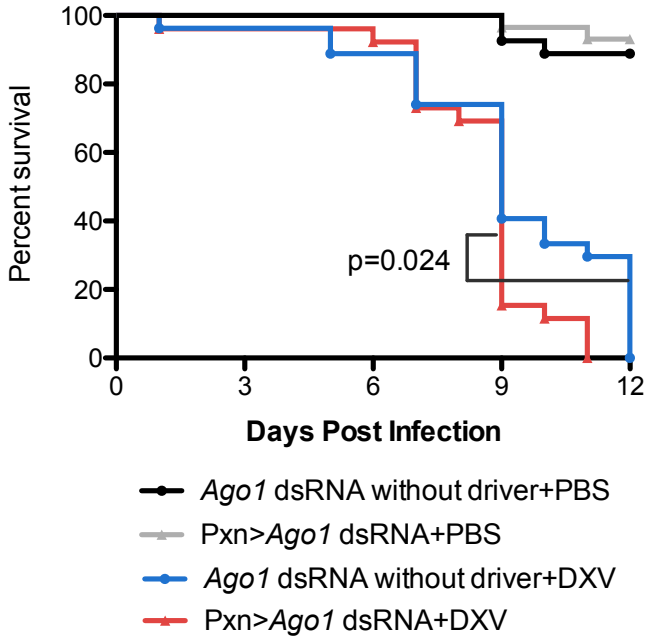


Figure S3: Flies with *AGO1* knockdown in the hemocytes are susceptible to DXV compared to control flies.

Flies of each line (15 females and 15 males) were injected with DXV. The morbidities were recorded daily after injection. The number of flies surviving at day one was used as the initial count. Log-rank tests were used to determine susceptibility. The data shown are representative graph of two experiments.

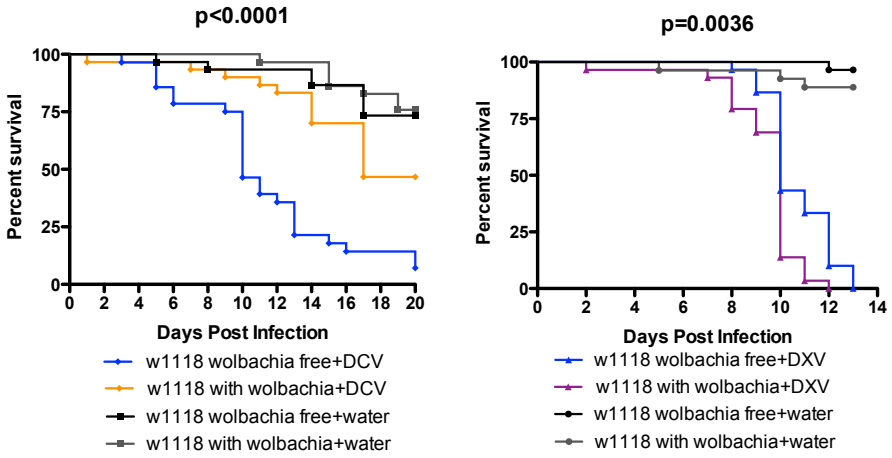


Figure S4: *Wolbachia* infected flies are more resistant to DCV but more susceptible to DXV as compared to *Wolbachia* free flies.

Flies of each line (15 females and 15 males) were injected with DCV at the dose of 10-3, DXV at the dose of 10-4 or water. The morbidities were recorded daily after injection. The number of flies surviving at day one was used as the initial count. Log-rank tests were used to determine susceptibility. The data shown are representative graph of three independent experiments.

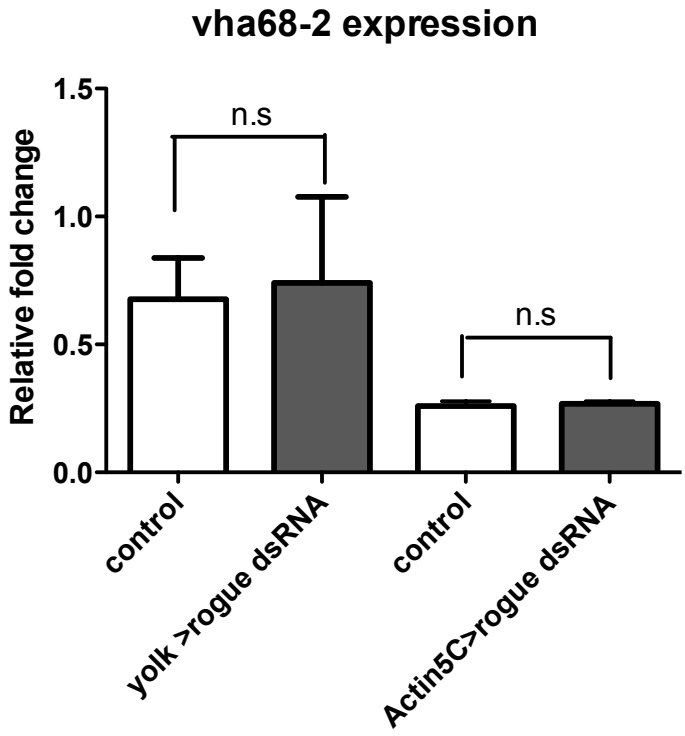


Figure S5: *vha68-2* expression in *rogue* RNAi knockdown flies.

rogue knockdown with Yolk or Actin drivers had similar expression levels of *vha68-2* compared to control flies. The expression levels of each gene were measured by qPCR. The data shown represents the mean of triplicates. The error bars show standard error.

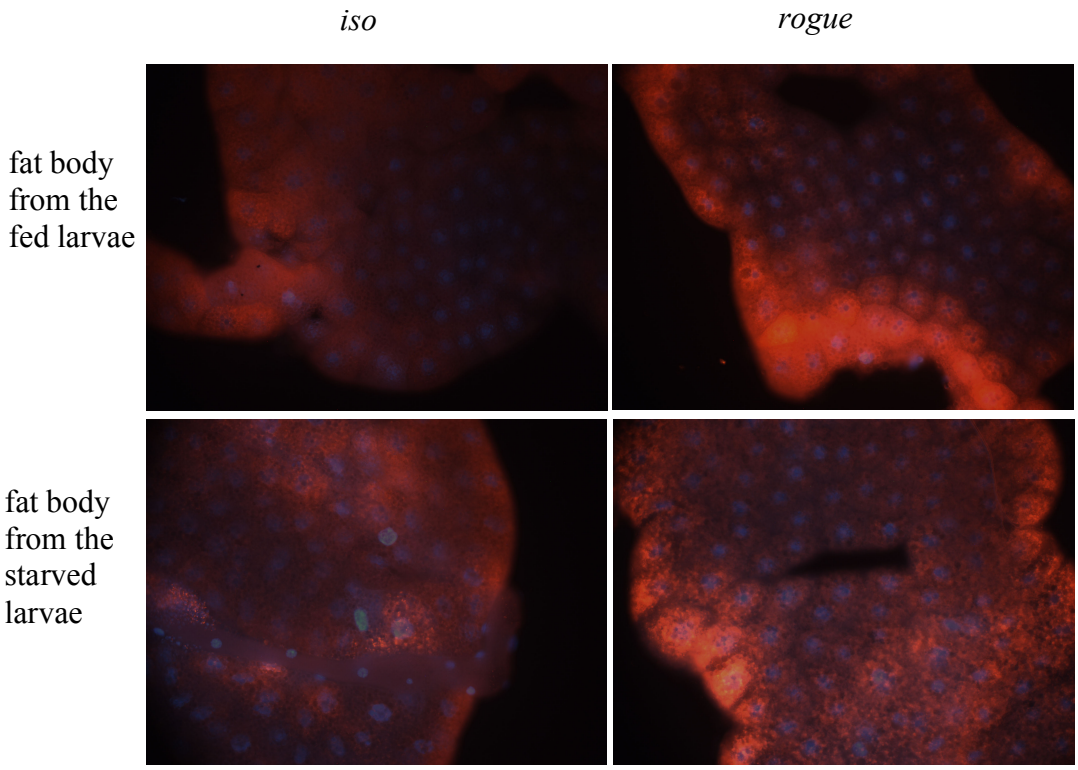


Figure S6: Lysotracker staining of larval fat body.

Third instar larvae of *iso* or *rogue* were fed on fresh food overnight and then starved for four hours. The fat body was dissected from the fed and starved larvae of each genotype. The fat body was stained with Lysotracker at room temperature for 1 min and then washed by PBS twice. Images were taken using the Discovery V8 SteREO microscope (Zeiss). The data shown are representative images. Four larvae from each group were examined.

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