

ANTIVIRAL IMMUNE RESPONSES TO INVERTEBRATE IRIDESCENT VIRUS 6
IN *DROSOPHILA*

A Dissertation Presented
By

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This work was undertaken in the Graduate School of Biomedical Sciences

Immunology and Virology Program

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January 2, 2018

“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world. Science is the highest personification of the nation because that nation will remain the first which carries the furthest the works of thought and intelligence.”

-Louis Pasteur

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ABSTRACT

The innate immune system is a critical first line of defense against invading pathogens. Innate immunity directly detects pathogens, sets up an appropriate adaptive response, and can directly kill pathogens.

Drosophila may lack an adaptive immune response, but have a robust innate immune system with a variety of defense effector mechanisms. While the responses to bacteria, fungi, and RNA viruses have been well characterized, not much is known about the response to DNA viruses. My studies have set out to characterize the *Drosophila* immune response to a DNA virus, utilizing the large dsDNA virus, Invertebrate Iridescent Virus 6 (IIV-6). IIV-6 infection causes shortened lifespan, and in later stages of infection, flies present with abdominal swelling and iridescent blue color. Our objectives were to identify pathways flies use to protect themselves from IIV-6 infection, determine how this protection is mediated, and to identify any immune inhibitors that IIV-6 uses to suppress innate immune signaling.

I have found that IIV-6 strongly up-regulates a class of stress proteins with unknown function, termed Turandots, after infection *in vivo* or *in vitro*. This induction is dependent upon viral replication, requires JAK-STAT activation, and activation of p38b MAPK. In addition, the *unpaireds*, which function as JAK-STAT ligands, are upregulated after IIV-6 infection in a p38b-dependent manner. Together, this data suggests that p38b activation leads to production of unpaired cytokines and activation of JAK-STAT signaling to induce *Turandots*.

I have also found that IIV-6 infected cells secrete protective factors. This response is induced within 12 hours of IIV-6 infection, exosome-mediated, and provides robust protection to naive cells challenged with an mCherry-expressing strain of IIV-6.

Additionally, IIV-6 inhibits two major immune responses in *Drosophila*, the IMD and Toll pathways. Stimulation of IIV-6 infected *Drosophila* S2* cells with either IMD or Toll stimulators results in very poor antimicrobial peptide responses. Yet, IMD and Relish are still cleaved upon stimulation in IIV-6 infected cells, indicating that the block is downstream. In support of this finding, IIV-6 infected flies respond very poorly to infection with the enterobacteria *Erwinia carotovora carotovora* compared to mock-injected flies.

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Figure 2.2 was compiled from FlyBase.org.

List of Abbreviations

AGO2	Argonaute 2
AIM2	absent in melanoma 2
ALR	AIM2-like receptor
AMP	antimicrobial peptide
ASC	apoptosis-associated speck-like protein containing CARD
BIR	baculovirus inhibitor of apoptosis repeat
BLAST	Basic Local Alignment Search Tool
CLR	C-type lectin receptor
CIV	Chilo Iridescent Virus (also known as IIV-6)
CM	conditioned media
CrPV	Cricket Paralysis Virus
cGAS	cyclic GMP-AMP synthase
DAP	diaminopimelic acid
DAMP	Damage-associated Molecular Pattern
DCV	<i>Drosophila</i> C Virus
DE	delayed-early
Dif	Dorsal-related immunity factor
DMelSV	Sigma virus of <i>Drosophila melanogaster</i>
DPI	diphenyleneiodonium chloride
DXV	<i>Drosophila</i> X Virus
EcD	20-Hydroxyecdysone
ERK	Extracellular signal-regulated kinase
FHV	Flock House Virus
IAP	inhibitor of apoptosis
IE	immediate-early
IFN	interferon
IIV-6	Invertebrate Iridescent Virus 6
IMD	immune deficiency
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
JAK	Janus Kinase
JNK	c-Jun Terminal Kinase
lys	lysine
MAK	Mitogen-activated protein kinase
mCh-IIV-6	ΔTS-MCP-mCherry-IIV6, IIV-6 strain expressing mCherry under MCP promoter.
MCP	major capsid protein
MDA5	Melanoma Differentiation-Associated protein 5
miRNA	micro-RNA
MyD88	Myeloid differentiation primary response gene 88

NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
PAMP	Pathogen-associated molecular pattern
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
piRNA	PIWI-interacting RNA
RVFV	Rift Valley Fever virus
RIG-I	retinoids acid-inducible gene I
RIP1	receptor interacting protein 1
RISC	RNA-induced Silencing Complex
RLR	RIG-I-like Receptor
RNAi	RNA interference
ROS	Reactive Oxygen Species
siRNA	small interfering RNA
SINV	Sindbis Virus
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
TLR	Toll-like Receptor
Tot	Turandot
TNFR	Tumor Necrosis Factor receptor
upd	unpaired
VACV	Vaccinia Virus
VSV	Vesicular Stomatitis Virus

List of Multimedia Files

November 2013 Nanostring: PBS-injected v IIV-6 infected whole male flies
harvested at 6, 12, 24 hours

November 2014 Nanostring: S2* cells infected with virus for 12 hours, stimulated
with Ecdysone and PGN, and harvested 12h post-PGN stimulation.

Preface to Chapter I

Figure 1.1 has been slightly modified from Anni Kleino and Neal Silverman.

CHAPTER I: INTRODUCTION

1.1 Significance of Innate Immunity

Everyday, organisms are challenged by pathogens seeking to exploit their resources. The first line of defense against these invading pathogens is the innate immune system. Sensing of pathogens by the innate immune system and responding to them is critical to the survival of an organism, and all organisms have developed detection systems of pathogen recognition. Innate receptors recognize molecular patterns from viruses, bacteria, or fungi to activate the appropriate downstream signaling pathways, ultimately inducing cytokine responses to protect neighboring cells, recruiting effector cells to the site of infection, and eliciting the appropriate immune responses to eliminate pathogens.

The concept of pattern recognition receptors was predicted by Charles Janeway (Janeway, 1989). The *Drosophila* Toll pathway, whose relevance in innate immunity was discovered in 1996 by Bruno Lemaitre and Jules Hoffman (Lemaitre et al., 1996), led to the discovery of Toll-like receptors (TLRs) in mammals by Charles Janeway and others (Medzhitov et al., 1997; Poltorak et al., 1998). The 13 mammalian TLRs sense a variety of pathogen-associated molecular patterns (PAMPs), including nucleic acids, proteins, and lipids. These landmark discoveries were awarded the 2011 Nobel Prize in Medicine or Physiology to Jules Hoffman and Bruce Beutler. Pathogens contain similar molecular patterns, foreign to the cell, that serve as signals. These patterns can be derived from bacterial cell walls, flagellin, lipids, or nucleic acid species. Additionally, the location of these signals, ie. cytosolic DNA, play an important

factor in whether it is considered a danger signal by the cell. Pathogens also elicit danger associated molecular patterns, or DAMPs, recognized by the cell.

Several classes of mammalian pattern recognition receptors have been discovered, TLRs, RIG-I like receptors (RLRs), nucleotide binding oligomerization domain receptors NOD-like receptors (NLRs), and the AIM2-like receptors (ALRs). The TLRs sense a large variety of PAMPs through an extracellular or endosomal LRR domain and signal via an intracellular TIR domain through which they associate with adapter proteins (Kawai and Akira, 2010). The RLRs, RIG-I, MDA-5, and LGP2, sense viral RNA in the cytosol and lead to type 1 IFN induction. The NLRs sense a wide variety of PAMPs and DAMPs, including bacterial ligands, K⁺ efflux, and viruses (Place and Kanneganti, 2017). ALRs sense dsDNA via a HIN200 DNA binding domain (Hornung et al., 2009). These receptors play a vital role in detecting and responding to pathogens, and their activation leads to production of inflammatory cytokines and recruitment of effector cells to limit pathogen replication.

Mammalian DNA Sensing

Mammals employ a variety of intracellular and cell surface receptors to detect DNA. Since DNA is also inherent, the immune recognition of DNA as a PAMP or DAMP lies mainly in its location, as DNA should mostly be contained within the nucleus. Notably, TLR9 detects unmethylated CpG DNA in the endosomal compartments to trigger type-1 interferon (IFN). In the cytosol, cyclic GMP-AMP

synthase (cGAS) senses DNA, leading to IFN production (Sun et al., 2013), and AIM2 senses AT-rich dsDNA in the cytosol leading to inflammasome activation (Holm et al., 2013). Both TLR and cGAS signaling culminate in the production of IFN, while AIM2 leads to the activation and cleavage of IL-1 β and IL-18, and in some instances, pyroptosis of the cell (Jorgensen et al., 2017).

Highlighting the inflammatory nature of DNA, mammalian systems encode mechanisms to limit inflammation by degrading endogenous DNA. Mammalian DNASE2 mutants are embryonic lethal because of rampant type-1 IFN production (Kawane et al., 2001). This lethality can be rescued by crossing DNASE2-deficient mice to type-1 IFN-receptor knock-out mice (Yoshida et al., 2005).

Intracellular detection of DNA occurs via cGAS (Sun et al., 2013). cGAS detection of cytosolic DNA, both endogenous and exogenous, results in the production of 2'3' cyclic (Wu et al., 2013). This cyclic dinucleotide is then bound by the ER-resident molecule stimulator of interferon genes (STING). This activates tank binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3), which then translocates to activate type-1 IFN genes (Cai et al., 2014). While many DNA sensors had been implicated prior to the discovery of cGAS and cGAMP, cGAS signaling has since shown to be the most prominent DNA sensor (Paludan and Bowie, 2013), and it is unclear how many of these may be cell-type specific (Gray et al., 2016).

AIM2 binds DNA via its HIN200 domain (Hornung et al., 2009). This binding causes a conformational change, allowing it to bind the adapter protein, apoptosis-associated speck-like protein containing CARD (ASC). This also leads to polymerization and the formation of the inflammasome. In addition, ASC contains a caspase activation and recruitment domain (CARD), which recruits caspase-1. Caspase-1 undergoes autoproteolysis, and is then able to cleave pro-IL-1 and pro-IL-18 into their mature forms, as well as Gasdermin D, leading to pyroptotic cell death (Broz and Dixit, 2016; Shi et al., 2015).

The model organism *Drosophila melanogaster* contains many homologous signaling pathways to mammalian innate immunity. Discoveries in *Drosophila* have often led to the discovery of homologs in mammals, such as the discovery of Toll. *Drosophila* are easily manipulated by genetic approaches, there are genome-wide screening libraries available, they have a short generation time, and are cost effective. In addition, studying invertebrate innate immune pathways is advantageous in order to gain a deeper understanding of the immune systems of vector insects, such as mosquitos. Understanding how insects clear or succumb to viral infections has many applications in vector control.

1.2 *Drosophila* Immunity

Like all organisms, *Drosophila* are constantly threatened by a wide variety of pathogens. Flies have a thick chitin exoskeleton that acts as a barrier defense.

Microorganisms able to breach this barrier immunity encounter an array of cell-mediated defenses, inducible humoral and transcriptional defenses, and a robust RNAi system. Flies lack an adaptive immune system in the sense that they do not have receptors created by V(D)J recombination, or express major histocompatibility complex (MHC) proteins for antigen presentation. Despite this, they are able to overcome a variety of immune challenges using their innate immune system.

Drosophila contain circulating hemolymph, within which several specialized differentiated cell types, crystal cells, plasmatocytes, and lamellocytes reside (Lemaitre and Hoffmann, 2007a). Crystal cells are round cells containing prophenoloxidase critical to wound healing. Cellular damage triggers a prophenoloxidase-activating cascade leading to melanization and clotting (Cerenius and Soderhall, 2004). The prophenoloxidase-activating cascade also produces a number of free radicals that serves to neutralize any pathogens that may have entered the wound. Plasmatocytes are the most numerous circulating cell, and phagocytose any pathogens that they encounter (Lanot et al., 2001). Additionally, larvae contain lamellocytes, which are large, flat cells responsible for walling off any organisms too large to be phagocytosed, such as the eggs of parasitic wasps.

In addition to the cell-mediated responses, a humoral response is also activated in response to infection by certain pathogens. Antimicrobial peptides (AMPs) are the hallmark of the *Drosophila* humoral response. Several families of AMPs exist and are robustly up-regulated in response to bacterial or fungal infections (Bulet et al., 1999). Some AMPs are directly lytic, while the mechanisms by which others kill their target microbes is unknown (Bulet et al., 1999). AMPs can be produced locally at the site of infection, such as the gut or barrier epithelia, or they can be produced systemically via the fat body and circulated through the hemolymph. AMP production is triggered via two NF- κ B-mediated pathways: Toll and IMD.

The IMD Pathway

The IMD pathway, homologous to the mammalian tumor necrosis factor receptor (TNFR pathway), is activated in response to diaminopimelic acid (DAP)-type peptidoglycan (PGN) found in the cell wall of Gram-negative and certain Gram-positive bacteria, such as *Micrococcus luteus* (Kaneko et al., 2004) (Figure 1.1). IMD signaling is triggered when the cell surface receptor peptidoglycan recognition protein (PGRP)-LC or the intracellular receptor PGRP-LE binds DAP-type PGN fragments (Kaneko et al., 2004). Receptor binding triggers amyloid fibril formation through the RIP Homotypic Interaction Motif (RHIM) domain of PGRP-LC (or PGRP-LE in the case of intracellular pathogens) and the recruitment and amyloid fibril formation of the RIP1 homologue IMD, reminiscent

of the functional amyloid formed in mammalian RIP1/RIP3-mediated programmed necrosis (Kaneko et al., 2004; Kleino et al., 2017; Li et al., 2012). In contrast to mammalian RIP1/RIP3 signaling, amyloid formation by IMD does not result in cell death (Kleino et al., 2017). IMD associates with dFADD via its death domain, and dFADD associates with the caspase 8-like DREDD via death effector domains (Hu and Yang, 2000). DREDD cleaves IMD thereby exposing an IAP binding motif and allowing it to become K63 ubiquitinated by the E3 ligase dIAP2 (Kleino et al., 2005), and E2 ligases Uev1a, bendless, and effete, and act as a scaffold for recruitment of dTAK1 and dTAB2 (Paquette et al., 2010). dTAK1 and TAB2 most likely phosphorylate the IKK complex, IKK β (or ird5) and IKK γ (or kenny) (Silverman et al., 2003). IKK β is phosphorylated, activated, and phosphorylates the NF- κ B protein, Relish (Ertürk-Hasdemir et al., 2009; Silverman et al., 2000b). This leads to the cleavage of Relish by DREDD, and the translocation of the Relish N-terminus into the nucleus, where it will activate transcription of a subset of AMP genes (Kim et al., 2014; Stöven et al., 2003).

TAB2 and dTAK1 also activate the c-Jun N-terminal kinase (JNK) arm of the IMD pathway through phosphorylation of Hemipterous, an MKK7 homolog (Boutros et al., 2002; Silverman et al., 2003). Hemipterous then phosphorylates basket (JNK), and in turn, activating the transcription factor JRA (AP1).

IMD signaling is negatively regulated by a number of proteins. Poor IMD response upon knock-in (Pirk) is a negative feedback regulator induced by

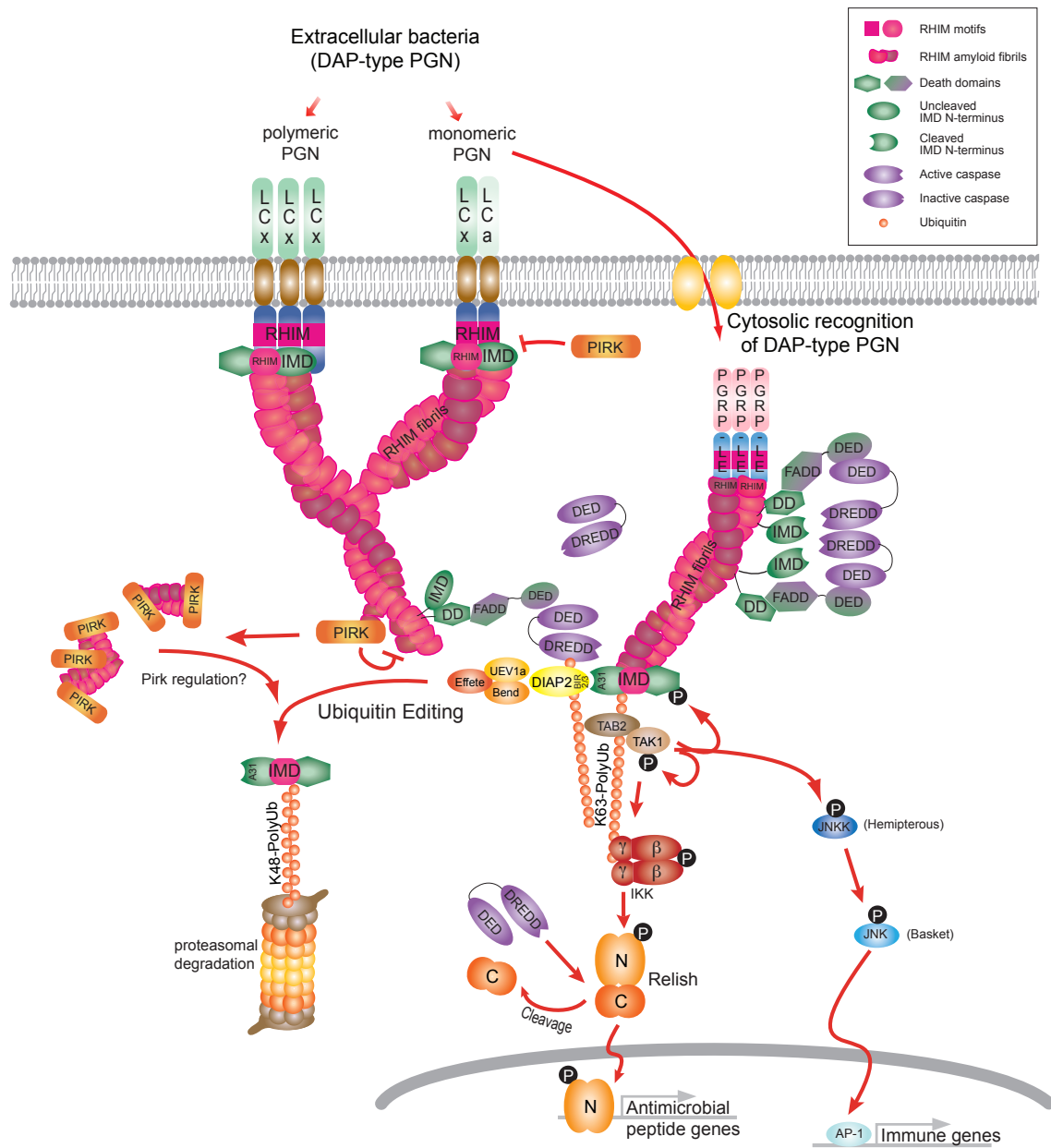


Figure 1.1 Overview of IMD Signaling

DAP-type PGN produced by Gram-negative bacteria is sensed by extracellular PGRP-LC or intracellular PGRP-LE. This recognition results in formation of functional amyloid through the RHIM domains of PGRP-LC and PGRP-LE, leading to association with IMD via its RHIM domain. IMD association with dFADD recruits the caspase 8 homolog DREDD, which cleaves IMD. This exposes an IAP binding site, leading to association with DIAP2, and K63-ubiquitination by UEV1a, Bendless, and Effete. These K63 ubiquitin chains act as a scaffold for the recruitment of TAB2 and TAK1, as well as the IKK complex. IKK phosphorylates Relish and is thought to mediate cleavage of Relish by DREDD. Upon cleavage, the N-terminus of Relish translocates into the nucleus to activate transcription of AMP genes. This pathway is negatively regulated through Pirk, which contains a RHIM domain and may interfere with amyloid fibril formation or act to cap it. IMD activation is also regulated by K48 ubiquitination of IMD. Additionally, TAK1 and TAB2 are able to activate JNK signaling through phosphorylation of the JNKK, Hemipterous.

Relish, and was recently shown to function via disruption of functional amyloid (Aggarwal et al., 2008; Kleino et al., 2008; Kleino et al., 2017).

The Toll Pathway

Before Toll was shown to play a key role in immune function, it was shown to be required during development in embryonic dorsal-ventral patterning by Christiane Nüsslein-Volhard's laboratory (Belvin and Anderson, 1996).

Drosophila encode eight toll genes, and although these transmembrane receptors all contain a leucine-rich repeat (LRR) domain and a Toll/IL-1R (TIR) domain, it remains unclear how many participate in the immune response (Lemaitre and Hoffmann, 2007a). Due to their higher expression profiles during embryonic stages, many of the *Drosophila* Tolls are thought to play a role in development rather than immune function (Tauszig et al., 2000). Toll2, also known as 18-Wheeler, functions in development but does not have a role in immune responses (Eldon et al., 1994; Ligoxygakis et al., 2002). Tehao, Toll5, has been shown to induce the antifungal AMPs *drosomycin* and *metchnikowin*, and to associate with Pelle upon transfection *in vitro* (Luo et al., 2001; Tauszig et al., 2000). Toll7 participates in antiviral autophagy (Nakamoto et al., 2012).

Unlike mammalian TLRs, which directly bind their ligands, *Drosophila* Toll is activated by binding the cleaved product of the protein spätzle. Spätzle is cleaved by a proteolytic cascade triggered by fungal or Gram-positive bacteria infections. Upstream, Gram-positive bacteria are detected by the receptors

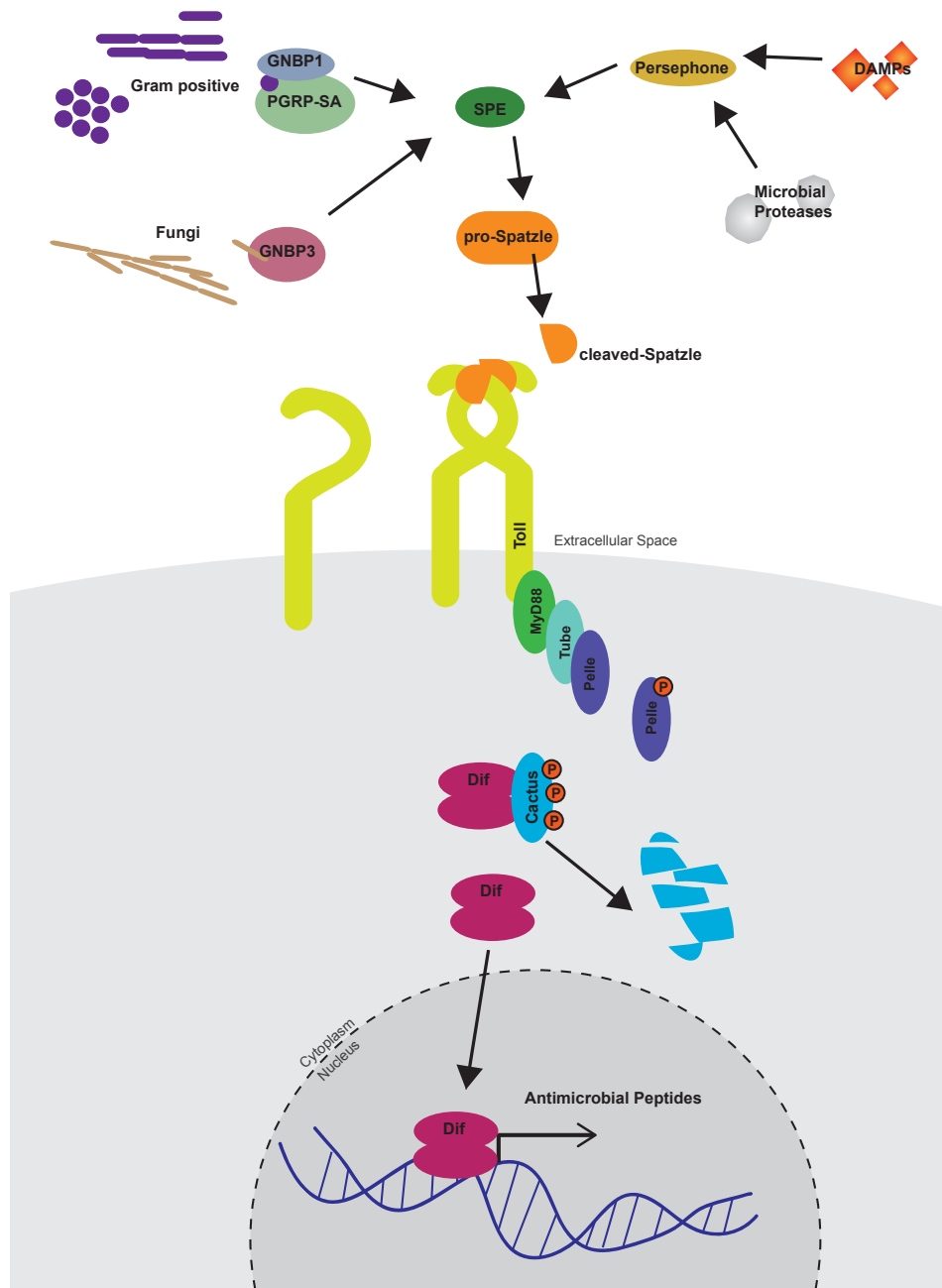


Figure 1.2 Overview of *Drosophila* Toll Signaling

Toll signaling is triggered in response to Gram-positive bacteria, fungi, detection of microbial proteases or endogenous DAMPs. These diverse PAMPs or DAMPs trigger a serine protease cascade through SPE to cleave the Toll ligand, spätzle. Once cleaved, spätzle can bind Toll, dimerizing the receptors, and causing MyD88 to associate via interaction with its TIR domain. MyD88 associates with Tube, which associates with Pelle. Pelle becomes activated, resulting in phosphorylation and K48 ubiquitination of Cactus. Cactus, the I κ B, is degraded via the proteasome, releasing the NF- κ B Dif or Dorsal, into the nucleus. Once in the nucleus, it can bind κ B sites, initiating transcription of target AMP genes.

PGRP-SA with Gram-negative binding protein 1 (GNBP-1), and fungi are detected by GNBP3 (Gobert et al., 2003). These receptors then activate serine proteases which cleave spätzle. Additionally, microbial proteases can activate the serine protease persephone to lead to spätzle cleavage (El Chamy et al., 2008). In development, spätzle is cleaved by the serine protease Easter (Chasan and Anderson, 1989), and upon immune challenge spätzle processing enzyme (SPE) cleaves spätzle (Jang et al., 2006).

Upon cleavage, spätzle binds Toll and induces receptor dimerization. This recruits dMyD88, Tube, and Pelle. This leads to the degradation of the *Drosophila* I κ B, Cactus. This releases the NF- κ B molecules Dif or Dorsal, freeing it to enter the nucleus and activate AMP transcription. Dorsal, named for its role in dorsal-ventral patterning (Santamaria and Nusslein-Volhard, 1983), functions in development and in some immune responses in larvae, while Dif primarily functions in the adult immune response (Lemaitre et al., 1995; Meng et al., 1999; Petersen et al., 1995).

Antimicrobial Peptides

The induction of antimicrobial peptides (AMPs) upon bacterial challenge was first discovered in the giant silkworm moth *Hyalophora cecropia* by Hans Boman and colleagues (Hultmark et al., 1980), who had previously demonstrated that flies first inoculated with an attenuated bacterial strain survived an otherwise lethal dose upon secondary challenge (Boman et al., 1972). AMPs are small,

cationic peptides that can be produced locally or secreted into the hemolymph. These small peptides are quite powerful; constitutive over-expression of any one AMP gene in a fly lacking both IMD and Toll pathways is capable of providing protection against its target class of microbe (Tzou et al., 2002). While some have been shown to directly depolarize bacterial membranes to lyse microbes, the function of others remains unclear (Bulet et al., 1999). The seven classes of AMPs can be stimulated by IMD or Toll signaling, or both (De Gregorio et al., 2002). Attacins, Cecropins, Defensin, and Diptericin are active against Gram-negative bacteria, while Drosocin is active against Gram-positive bacteria, and Drosomycin and Metchnikowin are antifungal (Lemaitre and Hoffmann, 2007a). Expression of some AMPs is also influenced by the steroid hormone 20-hydroxyecdysone (EcD). In addition to the requirement of EcD for expression of the IMD pathway receptor PGRP-LC, EcD is also required for induction of *Diptericin*, *Drosomycin*, and *Metchnikowin* (Rus et al., 2013).

1.3 Antiviral Responses in *Drosophila*

In addition to countering bacterial and fungal infections, *Drosophila* also face an array of viral pathogens (Table 1.4). While many pathways have been implicated in the *Drosophila* immune response to viral infections, the most robust antiviral pathway to date is the siRNA pathway. Additionally, the NF- κ B pathways, IMD and Toll, have been shown to be effective against Sindbis virus (SINV), Cricket Paralysis virus (CrPV), *Drosophila* X virus (DXV), and Sigma virus of

Drosophila melanogaster (DMelSV), although the mechanisms by which these pathways act is unclear. Autophagy restricts Vesicular Stomatitis virus (VSV) and Rift Valley Fever virus (RVFV). The JAK-STAT pathway is active against CrPV and *Drosophila C virus* (DCV). Erk signaling has been demonstrated to have antiviral effects in the gut. Other factors that can have antiviral roles include infections with the obligate intracellular bacterium *Wolbachia*, and transcriptional pausing of certain promoter regions to produce a rapid transcriptional response upon infection. The mechanisms of the siRNA pathway in response to virus infection is the best understood of these responses. How these pathways exert their antiviral effects, and what PAMPs most of them recognize remains unclear.

RNAi in Antiviral Defense

Insects have a robust RNAi system, comprised of three known pathways; the microRNA (miRNA), small interfering RNA (siRNA), and PIWI-interacting RNA (piRNA), and each serve a unique function.

The miRNA pathway functions to regulate gene expression. These small RNAs are derived from genome-encoded transcripts. Long primary miRNA transcripts containing dsRNA hairpins are bound by the endoribonuclease Drosha and dsRNA binding protein Pasha in the nucleus (Ghildiyal and Zamore, 2009). Drosha then cleaves these primary RNAs into smaller stem-loop RNAs, known as pre-miRNA (Lee et al., 2003). These pre-miRNAs are shuttled out of the nucleus, and in the cytosol, the loop is cleaved by Dicer-1 and Loquacious

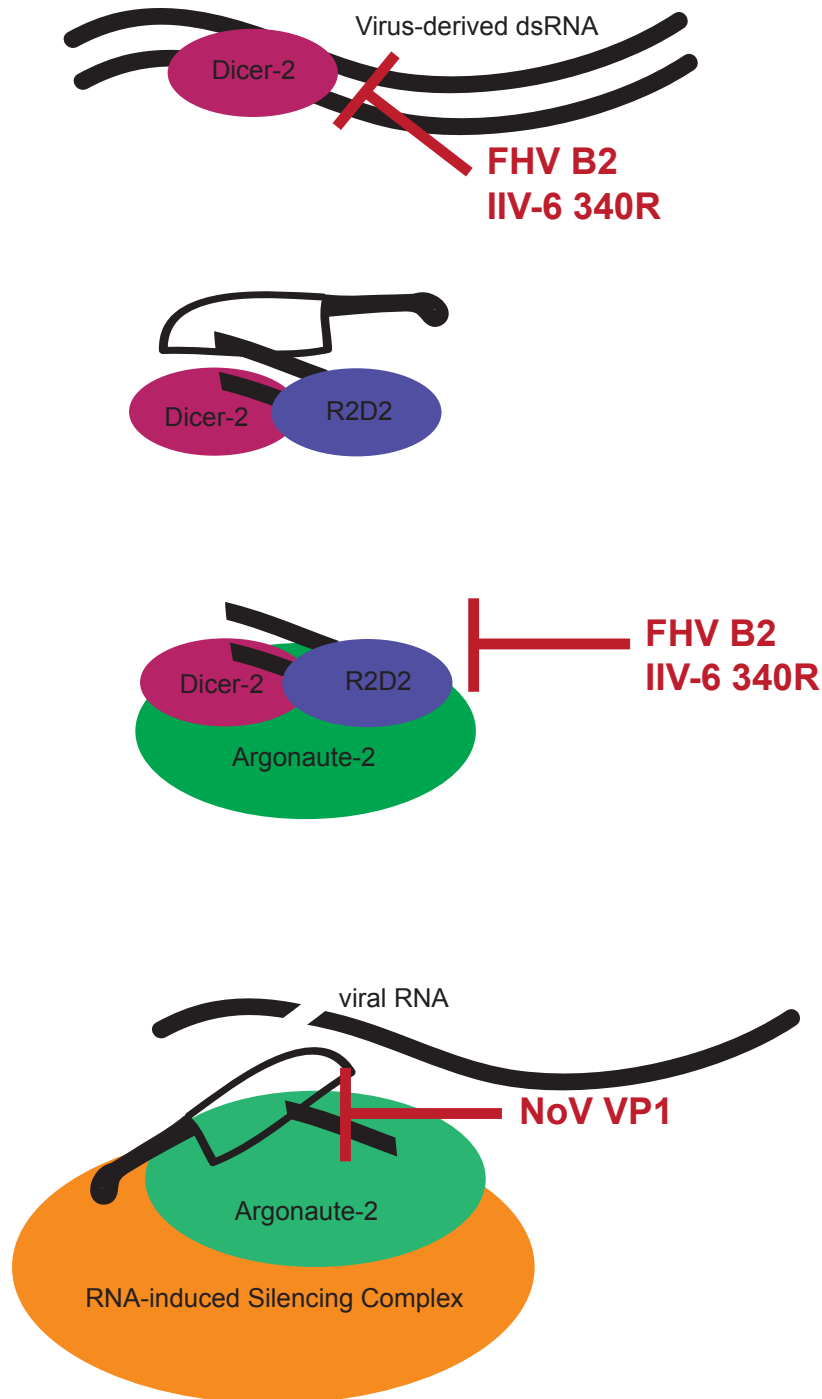


Figure 1.3 The *Drosophila* siRNA Pathway

Long, viral-derived dsRNA is sensed by Dicer-2. R2D2 promotes strand unwinding and Dicer-2 cleaves dsRNA into 21 basepair fragments. These short dsRNAs are loaded onto a AGO2/RISC. One of the two strands is selected, and the other degraded. The functional RISC may then slice any complimentary viral transcripts it encounters. Shown in red are virus-encoded inhibitors of RNAi. FHV B2 and IIV-6 340R bind dsRNAs preventing Dicer-2 cleavage and RISC loading. NoV VP1 prevents AGO2 slicing.

(Saito et al., 2005). These miRNAs are then loaded onto Argonaute-1, where strand selection occurs and a RISC is formed (Carthew and Sontheimer, 2009).

Interestingly, an antiviral role for Drosha has been described in response to RNA viruses. A number of RNA viruses, or even the RNA mimetic poly(I:C) was able to trigger translocation of Drosha from the nucleus to the cytosol, where it cleaved viral RNA (Shapiro et al., 2014).

The piRNA pathway functions within the germline, to protect the integrity of the genome from transposition of mobile genetic elements. Unlike the miRNA and siRNA pathways, the piRNA pathway does not use a dicer protein for cutting RNA, but only Piwi-clade Argonaute proteins (Khurana and Theurkauf, 2010). piRNAs are longer, and have a more variable length, typically 26-31 nucleotides. piRNAs are produced from regions of the genome known as piRNA clusters contain fragments of transposon sequences (Brennecke et al., 2007). Primary piRNAs transcribed from these clusters are bound by Piwi or Aubergine (Aub), and are antisense to active transposons. These transcripts have a 5' uridine. Binding and cleavage of a transposon by Aubergine creates a new 5' end, and piRNAs bound by Argonaute 3 (AGO3) are sense-strand and have an adenosine at position 10, suggesting a “ping-pong” amplification method (Brennecke et al., 2007).

The piRNA pathway has been proposed to function in antiviral defense in mosquitos (Vodovar et al., 2012), but does not appear to serve this function in *Drosophila* (Petit et al., 2016). Somatic mosquito cell lines infected with various

viruses have produced piRNAs, suggesting that piRNAs are not germline restricted in these species (Miesen et al., 2016). A mechanism for their production suggests that in addition to RNA feeding directly into ping-pong amplification, reverse transcriptases produce a DNA copy that is integrated into the genome or retained extrachromosomally, for production of future viral-derived piRNAs (Goic et al., 2016).

siRNA is the most robust and well-characterized antiviral response in *Drosophila*. Nucleic acid recognition is a vital part of innate immunity in both insects and mammals and in *Drosophila* dsRNA is recognized via the siRNA pathway. Mammals detect dsRNA using a variety of other proteins, however, RNAi has not been shown to function in innate immunity aside from a few very specific contexts where type 1 interferons seem to be absent (Li et al., 2013; MacKay et al., 2014; Maillard et al., 2013). Unlike mammals, which have only one Dicer protein for both siRNA and miRNA pathways, flies contain two Dicer proteins functioning in distinct pathways. In *Drosophila*, Dicer-1 functions in miRNA processing, and Dicer-2, functions in the siRNA pathway (Lee et al., 2004). The dsRNA binding protein Dicer-2 is a DEAD-box helicase, and shares similarities with the mammalian RNA sensor RIG-I (Deddouche et al., 2008). Dicer-2 activation may also lead to transcription of antiviral effectors, reminiscent of the mammalian DEAD-box helicases in IFN production, though its primary role is the generation of small RNAs to target complementary viral transcripts for destruction.

Table 1.4 Drosophila Antiviral Responses to Viral Infections

Virus	Family	Genome	RNAi	Toll	IMD	JAK-STAT	Autophagy
CrPV , Cricket Paralysis Virus ^{3,4}	<i>Dicistroviridae</i>	+ssRNA	X		X	X	
DCV , Drosophila C Virus ^{1,3,6}	<i>Dicistroviridae</i>	+ssRNA	X			X	
DMeISV Sigma Virus of <i>Drosophila</i> <i>melanogaster</i> ¹⁶	<i>Rhabdoviridae</i>	-ssRNA		X	X		
DXV , Drosophila X Virus ⁵	<i>Birnaviridae</i>	dsRNA	X	X			
FHV , Flock House Virus ^{2,6}	<i>Nodaviridae</i>	+ssRNA	X				
IIV-6 , Invertebrate Iridescent Virus-6 ^{12,13,14}	<i>Iridoviridae</i>	dsDNA	X				
NoV Nora Virus ⁸	picorna-like	+ssRNA	X				
RVFV Rift Valley Fever Virus ¹⁵							X
SINV , Sindbis Virus ^{6,7}	<i>Togaviridae</i>	+ssRNA	X		X		
VSV , Vesicular Stomatitis Virus ^{9,10,11}	<i>Rhabdoviridae</i>	-ssRNA	X				X
References	¹ Dostert <i>et al</i> Nat. Imm. 2005. ² Li <i>et al</i> Science 2002. ³ van Rij <i>et al</i> Genes Dev. 2006. ⁴ Costa <i>et al</i> PLoS One 2009. ⁵ Zambon <i>et al</i> PNAS 2005. ⁶ Galiana-Arnoux <i>et al</i> Nat. Imm. 2006. ⁷ Avadhanula <i>et al</i> PLoS Pathogens 2009. ⁸ van Mierlo <i>et al</i> PLoS Pathogens 2012. ⁹ Nakamoto <i>et al</i> Immunity 2012. ¹⁰ Shelly <i>et al</i> Immunity 2009. ¹¹ Mueller <i>et al</i> PNAS 2010. ¹² Bronkhorst <i>et al</i> Nucleic Acid Res 2014. ¹³ Bronkhorst <i>et al</i> PNAS 2012. ¹⁴ Kemp <i>et al</i> JI 2012. ¹⁵ Moy <i>et al</i> Immunity 2014. ¹⁶ Tsai <i>et al</i> Appl. Environ. Microbiol. 2008.						

The siRNA pathway protects flies against nearly every virus tested (Table 1.4), including DNA viruses (Bronkhorst et al., 2012; Kemp et al., 2013). The siRNA pathway is activated when long dsRNA, produced as intermediates of RNA virus replication in the case of ssRNA viruses; genomic RNA in the case of dsRNA viruses; or as a product of convergent transcription in the case of dsDNA viruses, is detected by the endoribonuclease Dicer-2. Dicer-2 will then cut the long dsRNA into 21-22 basepair fragments, and with the RNA binding protein r2d2, will load the fragment into an Argonaute 2 protein, where one strand is selected to be a guide RNA, and the other is degraded. This RNA-bound Ago2 complex is termed an RNA-induced Silencing Complex (RISC), and will slice any complementary RNA sequence that it binds (Figure 1.3).

Underscoring the importance of the RNAi pathway in antiviral immunity, many viruses encode dsRNA binding proteins to protect their dsRNA intermediates from being detected by Dicer2. The most notable is Flock House Virus (FHV) B2 (Li et al., 2002). FHV B2 binds dsRNA regardless of length, allowing it to inhibit both Dicer2 cleavage and Ago2 slicing (Chao et al., 2005). Additionally, Nora virus was found to be unaffected by the RNAi machinery (Habayeb et al., 2009). This virus encodes an RNAi suppressor, VP1, that inhibits AGO2 slicing (Van Mierlo et al., 2014).

IMD in Antiviral Defense

While the AMPs produced through activation of the Toll and IMD pathways are effective against bacteria and fungi, their role in viral infections is less clear. AttacinC and dptB are the only AMPs reported to play a role in viral infection (Huang et al., 2013). Knockdown of these AMPs resulted in a 2-3 fold increase in virus titres. In these studies, the authors utilized transgenic flies expressing SINV replicon to show a requirement for all of the core IMD pathway components with the exceptions of PGRP-LC and PGRP-LE, and drove this transgene using a ubiquitously expressed actin promoter (Avadhanula et al., 2009). SINV is naturally restricted to certain cell types (SINV does not infect hemocytes (Tassetto et al., 2017)), thus this replication system may inducing a response in cell types that would not naturally be infected.

Additional studies have also implicated a role for the IMD pathway in response to viral infections. Upon CrPV challenge, several IMD pathway mutants, including ird5 (IKKB), Relish, and PGRP-LC showed decreased survival rates and increased viral loads (Costa et al., 2009). However, IMD mutants, showed no phenotype. Whether a branch point downstream of IMD exists and is activated in response to viral infection remains to be determined. Additionally, the role PGRP-LC, which directly binds DAP-type PGN, in the context of viral recognition needs to be elucidated.

Another line of evidence pointing to the involvement of IMD signaling in antiviral responses is the discovery of viral homologues of a reported IMD

inhibitor, *diedel*. *Diedel* is induced in response to SINV and VSV infections, and homologues were found in several large DNA virus families, including Entomopoxviridae, Baculoviridae, and Ascoviridae. *Diedel* was first described as a JAK-STAT inhibitor (Boutros et al., 2002; Coste et al., 2012; Müller et al., 2005), then as an IMD inhibitor (Lamiable et al., 2016b). While *Diedel*'s role in the fly immune response remains unclear, the fact that many viruses have hijacked this protein to modulate the host immune response to their advantage suggests that *Diedel*'s function warrants further study.

While these reports all implicate roles for the IMD pathway in the host response to viral infections, the mechanism by which it plays a role is unclear. In response to the SINV replicon, it was reported that all IMD pathway components with the exceptions of PGRP-LC or PGRP-LE were required (Avadhanula et al., 2009). In another study with SINV, *Diedel* mutants were reported to show increased rate of death to SINV, but had no significant change in viral titres, suggesting IMD activation is not beneficial to infection (Lamiable et al., 2016b). In contrast, the response to CrPV required PGRP-LC, and all core IMD pathway components with the exceptions of IMD and dFADD (Costa et al., 2009). Whether these discrepancies are due to the systems used (the use of a *UAS-GAL4* SINV virus replicon versus the use of whole, replication-competent SINV), whether IMD activation is virus-specific, and which components of the pathway are required, is an area that will require further study.

Toll in Antiviral Defense

A study examining the roles of Toll and IMD in response to DXV found that although AMPs are upregulated upon DXV infection to the same levels as *E. coli* infection, only *Dif* mutants and *Toll^{10b}*, a line overexpressing a constitutively active form of the Toll receptor, showed any increased rate of death (Zambon et al., 2005). The authors hypothesized that since over expression of AMPs also did not increase resistance to DXV, that perhaps Toll signaling, which is activated in response to cleaved spätzle, not by direct binding to a PAMP, was being activated in response to damage generated by the virus. Supporting their hypothesis, later work has shown that the Toll pathway can be activated by DAMPs produced in apoptosis-deficient flies, through a mechanism activating the serine protease Persephone upstream of spätzle cleavage (Ming et al., 2014). This indicates that a virus capable of blocking apoptosis could potentially activate Toll signaling via endogenous danger signals.

Sigma virus (DMelSV), a rhabdovirus that is vertically transmitted through some fly lines, has been shown to upregulate a number of antimicrobial peptides regulated by both the IMD and Toll pathways (Tsai et al., 2008). Because DMelSV infection is vertically transmitted, this excludes the possibility that these AMPs were induced through the introduction of bacteria through the wound created by pricking or injection. While this study relied on transcriptional data, introducing DMelSV into *Relish* or *Dif* mutant flies and AMP mutant or over-expressing lines would be informative in determining whether induction of these

AMPs are truly beneficial to the fly in the context of viral infection, or whether they are being induced as the by-product of a non-canonical response mediated by Relish or Dif.

Autophagy in Antiviral Defense

Autophagy has also been shown to be restrictive to several viral infections. Autophagy breaks down cellular components as a means of recycling during periods of starvation or stress, and during many developmental processes. During autophagy, double-membraned vesicles, termed autophagosomes, are formed containing cellular components that then fuse with lysosomes to degrade their contents.

In mammals, autophagy plays dual roles in the context of viral infection, in a virus-dependent manner. Autophagy can either limit viral replication, or it can be exploited by viruses to benefit their replication cycles (Lennemann and Coyne, 2015). In *Drosophila*, autophagy is restrictive to VSV (Shelly et al., 2009) and Rift Valley Fever Virus (Moy et al., 2014b). This process is dependent upon Toll7 and requires MyD88 (Nakamoto et al., 2012). Other groups have not seen a role for Toll7, but have corroborated a role for autophagy in the response to VSV (Lamiable et al., 2016a). The reasoning for the discrepancies in these studies is unclear. In addition, this group has shown that autophagy can promote replication of FHV, showing that as in mammals, autophagy in *Drosophila* can act in either an antiviral or pro-viral manner, depending upon the virus.

ERK in Antiviral Defense

A common route of entry for many pathogens is the gastrointestinal tract. In the *Drosophila* gut, ERK signaling is activated upon feeding of insulin, and activated ERK resulted in protection from VSV, DCV, and SINV (Xu et al., 2013). This suggests that upon feeding, the fly gut prevents viral infection from its food source by precautionary activation of an antiviral pathway. It has also been shown that the microbiome plays a role in ERK activation by priming the immune system (Sansone et al., 2015). A gram-negative commensal, *Acetobacter pomorum* activates IMD signaling to induce the ligand Pvf2, which binds the receptor tyrosine kinase PVR and activates ERK. However, signals from the microbiome alone are not sufficient for *Pvf2* transcription, viral infection is also required. Acting as a second signal, viral infection releases the RNA pol II pausing machinery, allowing transcription of *Pvf2*.

The JAK-STAT Pathway

JAK-STAT signaling is critical for innate immune signaling in mammals, especially for type-1 interferon signaling (Villarino et al., 2015). While flies lack interferon homologues, they do have a conserved JAK-STAT pathway (Agaisse and Perrimon, 2004). Mammals have four JAK proteins and seven STAT proteins capable of being activated by a variety of cytokines and receptors. Flies have a vastly simplified pathway, comprised of a single gp130 receptor, *domeless*; a JAK

homologue, termed *hopscotch* (Binari and Perrimon, 1994; Perrimon and Mahowald, 1986); and a single STAT, *stat92E* (Hou et al., 1996; Yan et al., 1996). The closest mammalian JAK homologue of *hopscotch* is JAK2, while *stat92E* is most closely related to STAT5.

In *Drosophila*, this pathway is activated by three known ligands, distantly related to IL-6, known as the unpaireds (upd). These four helix bundle cytokines are secreted, though upd3 and upd1 seem to associate with the extracellular matrix (Oldefest et al., 2013). Upd2 is homologous to leptin, and plays a role in metabolism (Rajan and Perrimon, 2012). Upd1 was first discovered for its role in development, during segmentation of the embryo (Harrison et al., 1998). *Upd3* is induced upon bacterial challenge in the hemocyte (Agaisse et al., 2003b), and upd2 and upd3 are induced upon infection with DCV or CrPV (Kemp et al., 2013).

Conserved JAK-STAT signaling is initiated upon a pair of transmembrane receptors binding their cognate ligand. This binding event results in a conformational change in the cytosolic portion of the receptors, bringing their associated JAK proteins into proximity to phosphorylate each other. These activated JAK molecules then phosphorylate STAT proteins, which results in their dimerization and translocation into the nucleus. Here, they are able to initiate transcription of target genes (Myllymaki and Ramet, 2014)(Figure 1.5).

The main transcriptional targets of JAK-STAT activation are the *Turandots* (*Tot*) and *thioester-containing protein* (*Tep*) genes. The tep proteins show some

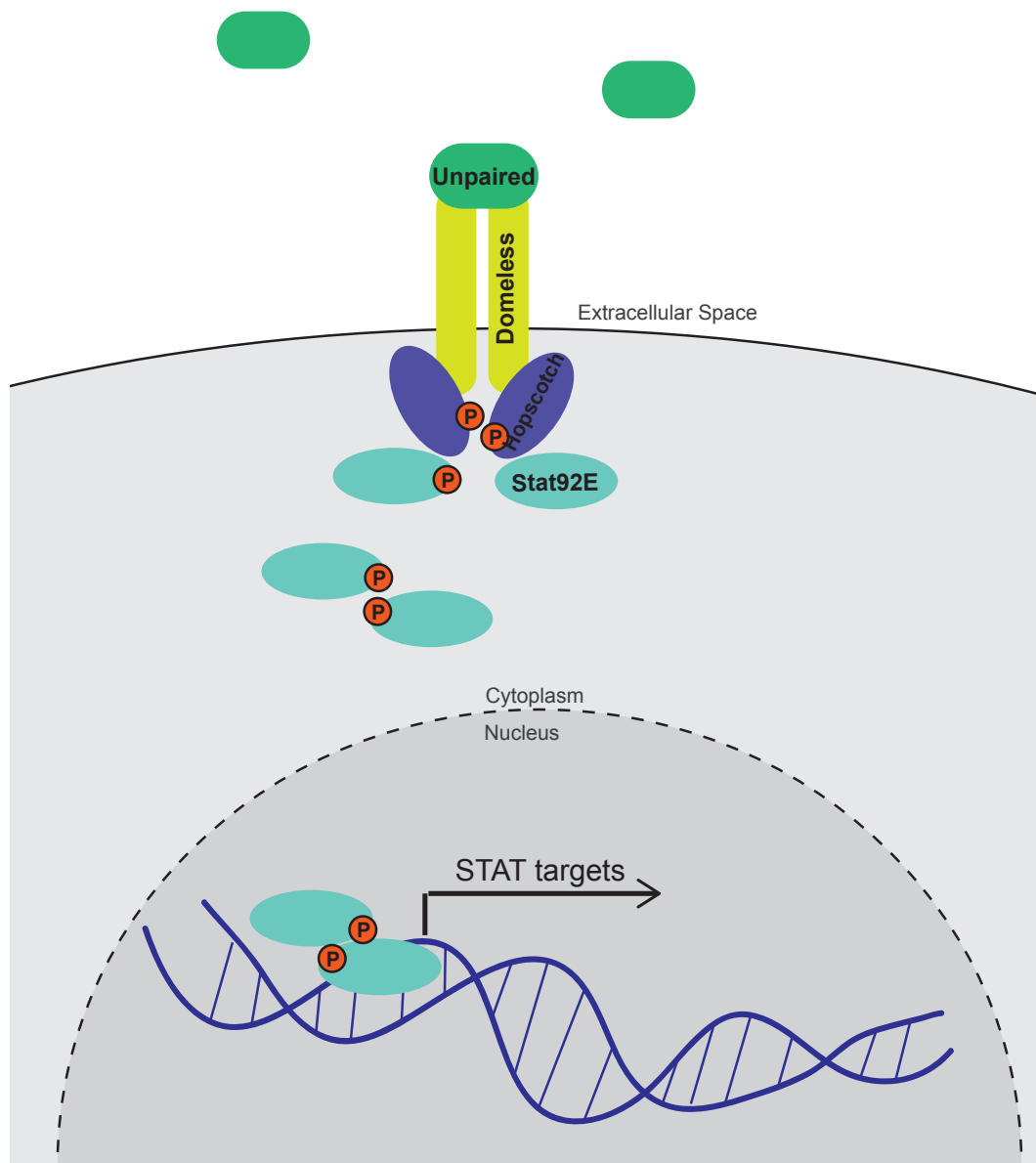


Figure 1.5 Overview of JAK-STAT Signaling

Drosophila encode a conserved and simplified JAK-STAT signaling pathway. Signaling is initiated upon receptor binding of one of three ligands, termed unpaired1, unpaired2, or unpaired3. The gp130 receptor, Domeless, triggers a conformational change in the JAK homolog, hopscotch. This leads to transphosphorylation of the receptor-associated JAK proteins, which are then able to phosphorylate the STAT, Stat92E, causing its dimerization. Dimerized STAT molecules are then able to enter the nucleus, bind and transcribe target genes.

homology to the α 2-macroglobulin/C3 complement family (Lagueux et al., 2000). It has long been speculated that the tep proteins function in opsonization and phagocytosis, after these roles were reported in *Anopheles gambiae* (Levashina et al., 2001). Recently, it was shown that tep proteins promote phagocytosis of pathogenic fungi and may play a role in activation of the Toll pathway (Dostalova et al., 2017).

The Tot proteins have no described function, but have been reported to be induced by a wide variety of factors, including heat shock, mechanical pressure, actin, septic injury, and bacterial and viral infections (Agaisse et al., 2003b; Brun et al., 2006b; Ekengren and Hultmark, 2001b; Ekengren et al., 2001; Merkling et al., 2015; Srinivasan et al., 2016). This family of eight, small, rapidly-evolved proteins is found only in *Drosophila* species (Obbard et al., 2009; Zhong et al., 2013). These genes are clustered together on chromosomes 2L and 3R, and will be discussed in greater detail in Chapter II (Gramates et al., 2017). The Tots contain highly charged residues and a signal sequence, indicating that they may be secreted. These genes are under positive selection and must be serving some important immune function. Figuring out what that function is will be a great task for a future graduate student.

Another transcriptional target of the JAK-STAT pathway is *diedel*. *Diedel* was first described as a negative regulator of JAK-STAT signaling (Boutros et al., 2002; Coste et al., 2012; Müller et al., 2005). Other studies have shown that *Diedel* may function in suppression of the IMD pathway (Lamiable et al., 2016b).

While its function in immune suppression needs further study to clarify these results, what is most fascinating about this gene is that it has been co-opted by many DNA viruses to suppress host immune signaling (Lamiable et al., 2016b).

Transcriptional Responses

JAK-STAT signaling is antiviral in response to several viruses, including the dicistroviruses DCV and CrPV (Dostert et al., 2005; Kemp et al., 2013). In response to DCV, a transcriptional response requiring JAK-STAT is induced. *vir-1*, a gene with unknown function, is robustly transcribed upon DCV infection and JAK-STAT activation is necessary but not sufficient for its induction (Dostert et al., 2005). *vir-1* is also upregulated upon infections with CrPV and FHV. While CrPV and DCV are both dicistorviruses, all three viruses are pathogenic in flies, suggesting that this response may be induced indirectly by the damage inflicted by these viruses (Kemp et al., 2013).

Another transcript induced in the fat body in response to DCV is *Vago* (Deddouche et al., 2008). *Vago* mutant flies had slightly increased DCV transcripts, suggesting it has antiviral activity. *Vago* induction was shown to require the DExD/H-box helicase domain of Dcr2 for its induction, indicating that RNA binding is essential and suggesting Dcr2 may have multiple roles in RNA recognition (Deddouche et al., 2008). This is interesting given that other DExD/H-box helicases such as RIG-1, and MDA-5 are involved in mammalian RNA sensing pathways. In both flies and mammals, the DExD/H-box helicase

DDX17, or Rm62, functions to restrict bunyaviruses through a mechanism that involves binding viral RNA (Moy et al., 2014a).

In mosquitoes, Vago homologues function as a ligand acting through an unknown receptor to activate JAK-STAT signaling, although this function has not been shown in *Drosophila* (Paradkar et al., 2012). Vago homologues are upregulated in response to flavivirus infections in several mosquito cell lines (Paradkar et al., 2014). Whether Vago functions in a similar manner in *Drosophila* will undoubtedly be the subject of future studies.

Transcriptional Pausing

Regulation of transcription during viral infection is another critical aspect of the immune response. In *Drosophila*, pausing of many immune genes is one way to rapidly employ a response to infection. Transcriptional pausing occurs when the transcriptional machinery has been recruited to a promoter, has initiated transcription, but then stops shortly after elongation. After stimulation, elongation resumes, allowing full transcription of the gene. This pre-recruitment of the transcriptional machinery allows for a rapid transcriptional response upon stimulation, and keeps the chromatin accessible. In *Drosophila*, transcriptional pausing regulates many antiviral genes across many antiviral pathways, including siRNA, JAK-STAT, and autophagy (Xu et al., 2012). About half of the genes induced by SINV and VSV were found to be dependent upon negative elongation factor (NELF) and positive elongation factor (P-TEFb), key

components of the pausing machinery. Additionally, antimicrobial peptide genes were not dependent upon NELF or P-TEFb, indicating that this response may be specific to viral infections.

Wolbachia

Another mechanism *Drosophila* utilizes to thwart viral infections is through the endosymbiont *Wolbachia*. *Wolbachia* is an obligate intracellular bacteria commonly present in a number of fly lines. Flies infected with *Wolbachia* vertically transmit the bacteria to their progeny. Flies infected with *Wolbachia* are more resistant to RNA virus infections (Teixeira et al., 2008). *Wolbachia* also naturally infects mosquitos, lending itself to be used as a method of vector control (Jiggins, 2017). *Wolbachia*-infected mosquitos are not only more resistant to arboviral diseases such as Dengue, but also lend reproductive advantages. Some *Wolbachia* strains induce cytoplasmic incompatibility, resulting in the death of embryos produced through the mating of a *Wolbachia*-negative female with a *Wolbachia*-positive male, while *Wolbachia*-positive females are able to mate with any males (Jiggins, 2017). This allows *Wolbachia*-positive females to outcompete females not infected with *Wolbachia*, and spread the benefits of disease resistance throughout the population.

1.4 Tolerance to Infections

Infections result in three outcomes on the organism they infect: either the organism clears the pathogen, the organism succumbs to the infection, or the organism finds a way to live with the infection, known as tolerance. In addition to mechanisms that actively clear viral infection, the host has evolved means of protecting itself to minimize the damage a pathogen is able to inflict. These tolerance mechanisms do not reduce pathogen load, but do protect tissues and thereby increase the survival rate of the host (Ayres and Schneider, 2012).

Mechanisms conferring tolerance seem to be pathogen-specific. A forward genetic screen looking for factors affecting *Listeria monocytogenes* survival and replication identified a number of genes that were important for fly survival but had no effect on bacterial replication (Ayres et al., 2008). These flies showed little overlap when challenged with *Salmonella typhimurium* or *Staphylococcus aureus* (Ayres et al., 2008). Anorexia was identified as a tolerance mechanism in response to *S. typhimurium*, but not *L. monocytogenes* (Ayres and Schneider, 2009). Mechanisms underlying tolerance are only beginning to be studied.

1.5 Invertebrate Iridescent Virus 6

Invertebrate Iridescent Virus 6 (IIV-6), also known as Chilo Iridescent Virus (CIV), is a large, dsDNA virus in the *Iridoviridae* family. Iridoviruses have a nucleocytoplasmic replication cycle, and are related to *Asfarviridae*, *Poxviridae*, and *Mimiviridae* (Asgari and Johnson, 2010). Their name derives from the

phenomenon that iridovirus virions pack into uniform paracrystalline arrays within their hosts, presenting an iridescent sheen at late stages of infection.

Host range

IIV-6 is able to replicate in a variety of invertebrate hosts, infecting over one hundred species, many of which are important to human health, the economy, and agriculture (Asgari and Johnson, 2010; Williams, 2008). One natural host is an agricultural pest, *Chilo suppressalis*, the stem borer moth, lending it the alternate name, Chilo iridescent virus. Studies investigating the cause of colony collapse disorder in honey bees claimed to have isolated IIV-6 peptides from hives (Bromenshenk et al., 2010), and it has been subsequently shown that these peptides may in fact be host peptides (Tokarz et al., 2011). Other iridoviruses infect honeybees (Bailey et al., 1976), but any deleterious effects caused by the virus have yet to be reported. In addition, IIV-6 is capable of infecting crickets, where it replicates in the fat body and can be transferred *per os* during mating rituals (Adamo et al., 2014).

Genome

IIV-6 is encoded by a linear dsDNA genome 212,484 base pairs in length, with 215 predicted ORFs (Ince et al., 2012; Jakob et al., 2001). It has circular permutations and is terminally redundant, consistent with its concatemeric DNA

and headful packaging (Asgari and Johnson, 2010). IIV-6 is AT-rich, with a GC content of only 29% (Asgari and Johnson, 2010).

Replication

Only limited work has been done on iridovirus replication, primarily with Frog Virus 3 (FV3), and many of the details remain unclear (Goorha, 2013).

IIV-6 is an infectious particle as either a naked virion, in cases where the virus has been released via cell lysis, or an enveloped particle, acquired from the plasma membrane from budding out of the cell. IIV-6 enters a cell by receptor-mediated endocytosis via an unidentified receptor in its enveloped form (Williams, 1996). Non-enveloped IIV-6 particles uncoat at the plasma membrane, likely via a different receptor than used by enveloped particles (Williams et al., 2005).

Once in the cytoplasm, the virus uncoats, and its genome enters the nucleus (Asgari and Johnson, 2010). Inside the nucleus, it transcribes its immediate-early (IE) and delayed-early (DE) genes via the host RNA polymerase II. Here, it also undergoes single-length replication of its genome via its virus-encoded DNA polymerase. These new genomes then migrate out of the nucleus and form viral replication factories adjacent to the nucleus in the cytoplasm. Here, the genomes undergo concatameric replication of several genome lengths, and transcription of late genes using the viral-encoded RNA polymerase occurs (Goorha, 2013).

The genomes appear to package via a headful-replication system, similar to some bacteriophages. Mature virions exit the cell via budding, or pack within the cytoplasm to form large paracrystalline arrays within the cell.

RNAi Inhibitors

Underscoring the importance of the RNAi pathway in the antiviral response, IIV-6 encodes an RNAi inhibitor, 340R. Transcription off of both DNA strands of IIV-6 results in areas of convergent transcription, producing dsRNAs that are sensed by the siRNA machinery (Bronkhorst et al., 2012; Kemp et al., 2013). 340R binds dsRNA and prevents both Dicer-2 processing into siRNAs and loading of siRNAs into RISC (Bronkhorst et al., 2014).

IAP proteins

IIV-6 encodes at least one functional inhibitor of apoptosis (IAP) protein. Three ORFs, 157L, 193R, and 332L, were discovered to have similarities to IAPs, determined by amino acid sequence similarities to a granulovirus IAP (Jakob et al., 2001). All three ORFs contain a C-terminal RING domain, however only 193R contains a baculovirus iap repeat (BIR) domain and has been shown to block apoptosis *in vitro* (Ince et al., 2008). Induction of apoptosis by IIV-6 has been reported to be JNK-dependent in other insect species, and to be mediated by a virion-associated kinase before being inhibited by an early gene product (Chitnis et al., 2008; Chitnis et al., 2011).

1.6 Thesis Objectives

The objective of this thesis is to better understand the mechanisms underlying antiviral immunity to DNA viruses in *Drosophila*. DNA virus infections in *Drosophila* have been understudied compared to RNA viruses on the basis that arbovirus infections affecting humans are mediated by RNA viruses. DNA viruses tend to be much larger and encode many more proteins than RNA viruses. Determining the host pathways that DNA viruses induce as well as the pathways they shut down can divulge a lot of information regarding host defense mechanisms. Some of the ways in which flies protect themselves against large DNA viruses may also apply to RNA viruses and provide insight into novel mechanisms of arbovirus antiviral defense. Additionally, the antiviral mechanisms deployed by flies to combat DNA viruses may have homologs in mammalian immunity. We sought to identify how flies protect themselves from infection by identifying pathways activated by the virus upon infection, by identifying how protection from viral infection is mediated, and by looking for virus-encoded immunomodulators to determine which host pathways the virus seeks to shut down.

PREFACE TO CHAPTER II

Portions of this chapter have been submitted for publication:

Cara West and Neal Silverman. *p38b and JAK-STAT Signaling Protect Against DNA Virus Infection in Drosophila*. (2017)

Cara West performed the experiments.

Cara West and Neal Silverman designed the experiments and wrote the manuscript.

**CHAPTER II:
TURANDOT INDUCTION BY IIV-6**

Abstract

The fruit fly *Drosophila melanogaster* is a powerful model system for the study of innate immunity in vector insects as well as mammals. For vector insects, it is particularly important to understand all aspects of their antiviral immune defenses, which could eventually be harnessed to control the transmission of human pathogenic viruses. The immune responses controlling RNA viruses in insects have been extensively studied, but the response to DNA virus infections is poorly characterized. Here, we report that infection of *Drosophila* with the DNA virus Invertebrate Iridescent Virus 6 (IIV-6) triggers JAK-STAT signaling and the robust expression of the *Turandots*, a gene family encoding small secreted proteins. To drive JAK-STAT signaling, IIV-6 infection more immediately induced expression of the *unpaireds*, a family of IL-6-related cytokine genes, via a pathway that required one of the three *Drosophila* p38 homologs, p38b. In fact, both *Stat92E* and *p38b* were required for the survival of IIV-6 infected flies. In addition, induction of the *unpaireds* required NADPH-oxidase generated reactive oxygen species (ROS). These results argue that ROS production, triggered by IIV-6 infection, leads to p38b activation and *unpaired* expression, and subsequent JAK-STAT signaling, which ultimately protects the fly from DNA virus infection.

Introduction

Like all multicellular organisms, insects face a constant threat of infection from a wide array of microorganisms, including viral, bacterial, and fungal pathogens. Insects combat these infections with both static and inducible defenses, including a chitinous exoskeleton, circulating phagocytes and the induction of host defense genes, such as antimicrobial peptides (Lemaitre and Hoffmann, 2007b). For example, *Drosophila melanogaster* respond to bacterial and fungal infections through two conserved NF- κ B signaling pathways, Toll and IMD, that drive the production of antimicrobial peptides and other inducible host defense molecules. Toll and IMD pathways are homologous to the TLR-MyD88 and TLR-TRIF signaling pathways in mammals, respectively. Unlike mammals, the *Drosophila* NF- κ B pathways seem to play a very limited role in response to invertebrate viral infections (Avadhanula et al., 2009; Costa et al., 2009; Sansone et al., 2015).

Insects, especially mosquitoes, are major vectors of arboviral diseases, and characterizing the pathways that they utilize to combat viral infections is necessary to gain a complete understanding of disease transmission. *Drosophila*, also a dipteran insect, has served as a productive model for studying insect antiviral immunity (Buchon et al., 2014). In *Drosophila*, the most intensely studied antiviral pathway is the siRNA response. The siRNA response is triggered when viral dsRNA intermediates, either derived directly from the viral genome or produced as an intermediate during replication or transcription processes, are

recognized by Dicer-2, which processes these dsRNAs into 21 base-pair fragments and loads them onto Argonaute-2. This complex, termed an RNA-Induced Silencing Complex (RISC), is then able to destroy its complementary target sequence. This mechanism is a potent antiviral defense against RNA viruses and, in some circumstances, against DNA viruses (Bronkhorst et al., 2012; Kemp et al., 2013). In addition, it has been suggested that Dicer-2, following the recognition of viral dsRNA, can trigger a signaling pathway that induces the transcription of the antiviral gene *Vago* (Deddouche et al., 2008). While antiviral effects of *Vago* have only been shown upon *Drosophila* C virus (DCV) infection in *Drosophila*, *Vago* has been shown to be induced upon flavivirus infections of several mosquito-derived cell lines and is suggested to be antiviral (Paradkar et al., 2014; Paradkar et al., 2012).

On the other hand, mammalian antiviral defenses are triggered after recognition of an array of pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs), including and extending beyond dsRNA. For example, various RNA species are recognized in the endosome by TLR3, 7 & 8, or in the cytosol by RIG-I or MDA-5. In addition, numerous viral proteins are recognized directly by various TLRs (Boehme and Compton, 2004), and viral-induced damage often leads to induction of inflammasome activation and pyroptosis or necroptosis. Inflammasome activation results in the production of pro-inflammatory cytokines to promote recruitment of effector cells to the site

of infection, while pyroptosis can act to restrict viral infection by killing the infected cell and thereby preventing viral replication (Jorgensen et al., 2017).

Compared to mammalian systems, the extent to which the invertebrate immune system recognizes PAMPs and DAMPs, beyond viral dsRNA, is less well-studied. Examples from *Drosophila* include Toll-7, which was demonstrated to sense vesicular stomatitis virus (VSV) and Rift Valley fever virus (RVFV), and activate autophagy as an antiviral defense (Moy et al., 2014b; Nakamoto et al., 2012). Additionally, JAK-STAT signaling has been shown to induce *vir-1* in response to DCV or cricket paralysis virus (CrPV), although the mechanism by which these viruses activate JAK-STAT signaling is unknown (Kemp et al., 2013). IMD signaling in the gut, triggered by commensals, is required for the activation of the ERK pathway, which protects against several enteric RNA viruses (Sansone et al., 2015). This commensal-mediated priming may explain the previously reported activity of IMD and Toll pathways in protecting against other viral infections (Avadhanula et al., 2009; Costa et al., 2009; Zambon et al., 2005). A study examining the origins of cGAS-STING signaling — an important cytosolic DNA sensing pathway in mammals, leading to Type I interferon production — concluded that cGAS homologs in insects lack regions required for DNA binding (Kranzusch et al., 2015). Thus, the role of the cGAS ortholog in insects remains unclear.

In fact, the pathways responding to DNA virus infections in *Drosophila* have not been characterized. Insect DNA viruses, such as baculoviruses, the

invertebrate iridescent viruses, and polydnaviruses are large, complex viruses ranging from 50 to more than 200 genes. These viral genes include many immunomodulators and other proteins that manipulate the cellular environment to facilitate replication (Alcami and Koszinowski, 2000). These large DNA viruses and their myriad of host targeting and manipulating factors suggest that many host immune response pathways, beyond RNAi, interfere with viral replication.

Here, we report that *Drosophila* infection with the DNA virus invertebrate iridescent virus 6 (IIV-6) induces a p38b-dependent pathway that activates JAK-STAT signaling and the robust transcriptional induction of a class of small secreted proteins called the Turandots. Activation of this pathway requires the production of reactive oxygen species, perhaps indicative of a response to damage. Moreover, two key components of this pathway, *p38b* and *Stat92E*, are essential for host defense against IIV-6 infection.

Results

Given the paucity of data on the insect response to DNA virus infection, we sought to identify the immune-related genes upregulated upon IIV-6 infection of adult flies. Male flies were injected with PBS (vehicle control) or IIV-6 at 1×10^4 TCID₅₀ for 12, 24, or 48 hours, RNA was isolated and analyzed by NanoString nCounter Analysis, with a codeset probing 139 immune-related genes (Figure 2.1A). Two members of the *Turandot* (*Tot*) family, *TotA* and *TotM* were strongly upregulated at all time points in the IIV-6 infected samples compared to PBS-

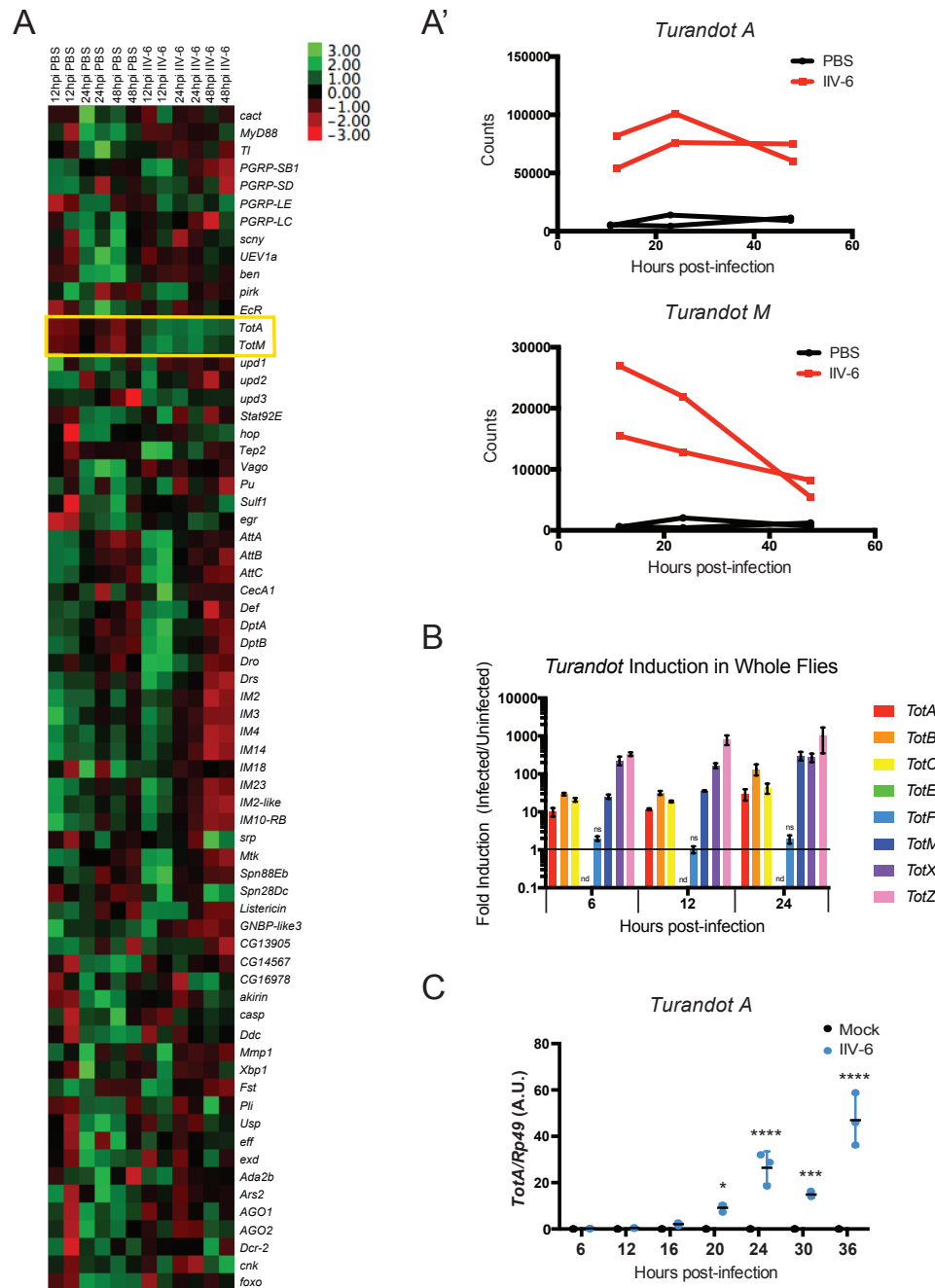


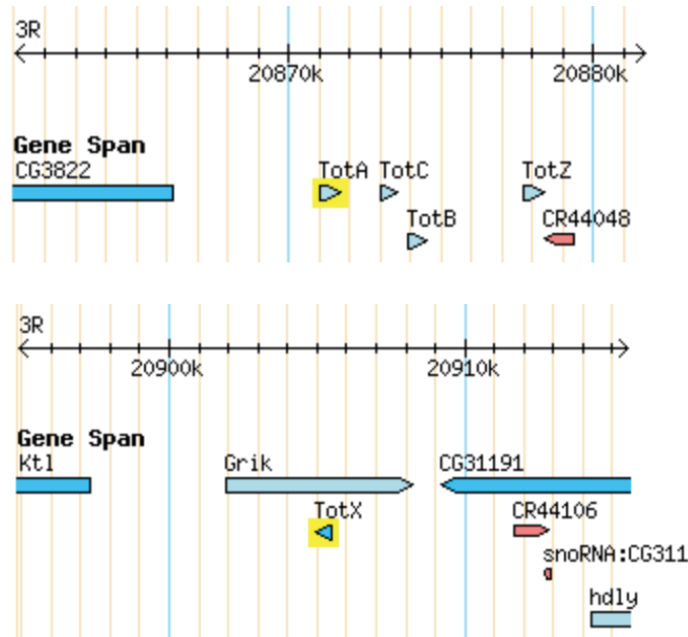
Figure 2.1 *Turandot* genes are Expressed Upon IIV-6 Infection

A) Heatmap of mRNA levels for selected immune-related genes following IIV-6 infection of adult w^{1118} flies for 12, 24 and 48 hours assayed by Nanostring nCounter. RNA was isolated from PBS-injected flies at the same time points as a control. Each data point is the mean of a biologically independent duplicate.

A') Detailed comparison of mRNA levels for of *Turandot A* or *Turandot M* from nCounter data. Biologically independent samples were analyzed in duplicate. **B)** Fold induction of all eight *Turandots* (from w^{1118} flies infected with IIV-6, relative to PBS injected controls at 12, 24, or 48 hours, quantified by qRT-PCR. *TotE* was undetectable (nd). N=3, SEM is indicated and statistical significance determined by Multiple t tests with correction for multiple comparisons using the Holm-Sidak method. By this analysis, all *Turandots* had p values between 0.05 and 0.0003 at all time-points with the exception of *TotF* (ns). **C)** S2* cells were infected with IIV-6 and *TotA* expression was assayed by qRT-PCR at the indicated time points.

Significance was determined by two-way ANOVA and Sidak's multiple comparisons test, comparing the infected sample to its uninfected control at that time point. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$ Error bars indicate standard deviation. Black bars indicate the mean. A.U., Arbitrary Units.

Chromosome 3R:
TotA, TotB, TotC, TotZ, TotX



Chromosome 2L:
TotE, TotF, TotM

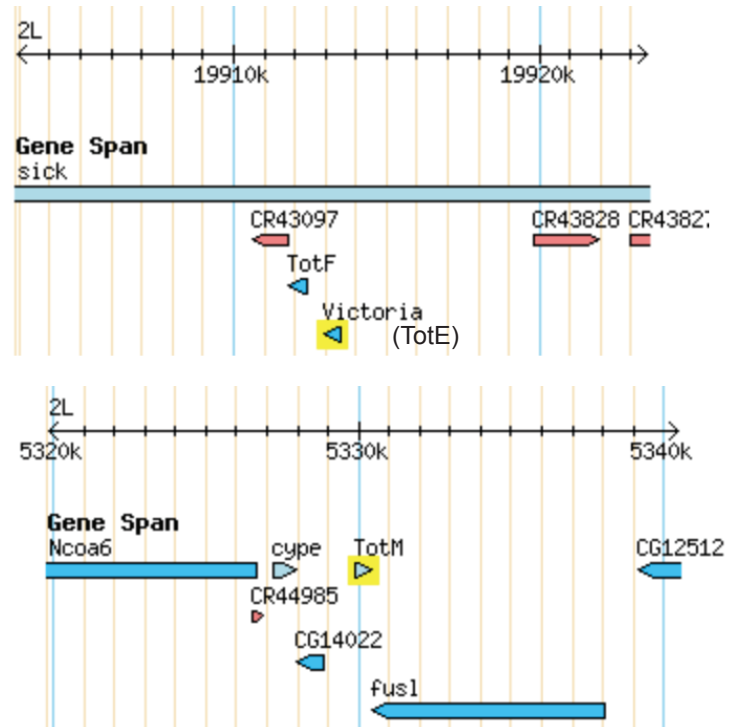


Figure 2.2 : *Turandot* loci on Chromosomes 3R and 2L

Compilation of images from FlyBase.org showing the chromosomal locations of the eight *Turandot* genes. *TotE* is also known as *Victoria*.

injected controls (Figure 2.1A, 2.1A'). These genes are part of a family of eight closely related, rapidly evolving genes that are induced by a variety of stressors including bacterial infection, heat shock, mechanical pressure, and UV-exposure (Ekengren and Hultmark, 2001a; Obbard et al., 2009). The *Tots* encode for small secreted proteins that have no known function (Ekengren and Hultmark, 2001a; Ekengren et al., 2001). As the NanoString codeset included only two *Tot* genes, we used qRT-PCR to examine the expression of all eight *Tot* genes following IIV-6 infection. Six *Tot* genes were induced 10-1000 fold, compared to the PBS injected controls, 6 to 24 hours following IIV-6 infection (Figure 2.1B). The two *Tot* genes not up-regulated, *TotE* and *TotF*, are clustered together on Chromosome 2, suggesting that these two closely related *Tots* may respond to a different set of stimuli (Figure 2.2). Note, *TotE* was undetectable, while *TotF* was detected but unchanged by IIV-6 infection. *Drosophila* S2* cells also induced *TotA*, peaking between 24-36 hours after IIV-6 infection, while in mock-treated controls *TotA* expression remained at baseline levels (Figure 2.1C).

To begin to dissect the mechanisms that lead from DNA virus infection to *Tot* gene induction, we tested the requirement for live virus infection and viral replication in this process. IIV-6 was heat- or UV-inactivated, which creates virus that can attach, enter cells, and possibly deliver damaged nucleic acids but is not replicative. These inactivated viruses were then used to stimulate S2* cells (Figure 2.3A). Both UV-inactivation and heat treatment significantly reduced *TotA* induction, to levels near baseline. Consistent with this observation,

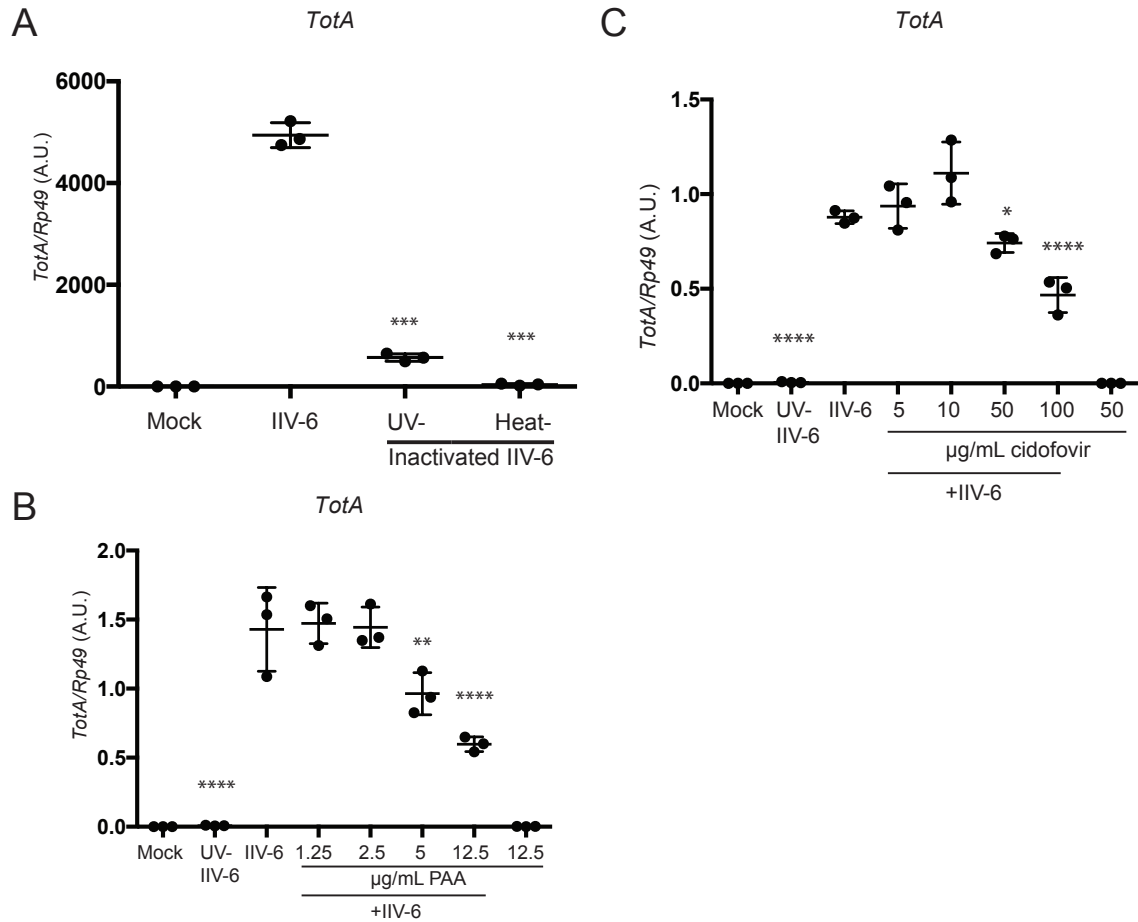


Figure 2.3 Viral Replication is Required for IIV-6 Induced *Turandot* Expression.

A) S2* cells were infected with heat- or UV-inactivated IIV-6 and *TotA* induction was assayed by qRT-PCR. **B,C)** S2* cells were treated with the viral polymerase inhibitors **B)** phosphonoacetic acid (PAA) or **C)** cidofovir at the indicated concentrations for one hour prior to IIV-6 infection. For all panels, cells were infected at an MOI of 2 and *TotA* induction was assayed at 24 hours post-infection by qRT-PCR. Significance, compared to IIV-6 infected samples without treatment or drugs, was determined by one-way ANOVA and Sidak's multiple comparisons test. Significance is indicated as * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$. Error bars indicate standard deviation. Black bars indicate the mean. A.U., Arbitrary Units.

pretreatment of S2* cells with viral DNA polymerase inhibitors, phosphonoacetic acid (PAA) or cidofovir, also resulted in significantly diminished IIV-6 triggered *TotA* induction (Figure 2.3B, C). These results indicate that the presence of viral DNA alone is not sufficient to trigger *Tot* induction, in contrast to mammalian systems where cytosolic DNA triggers a robust cytokine response and triggers inflammasome activation (Hornung et al., 2009; Wu and Chen, 2014). Studies with polymerase inhibitors suggest that virus entry and expression of early gene transcripts are also not sufficient for this response. Together, these data clearly demonstrate that active viral replication (or the processes downstream of viral replication) is required for IIV-6 induced *TotA* induction.

Previous studies demonstrated that the JAK-STAT signaling pathway is responsible for inducing *Tot* expression after Gram-negative bacterial infection (Agaïsse et al., 2003a). To test whether JAK-STAT signaling is also required for IIV-6-induced *Tot* expression, *domeless*, *hopscotch*, and *Stat92E*, the sole *Drosophila* homologs for the gp-130 receptor, JAK, and STAT, respectively, were targeted by RNAi in S2* cells. Knockdown of any of these genes led to a significant decrease in *TotA* induction (Figure 2.4A). We then asked whether the JAK-STAT pathway had any effect on survival following IIV-6 infection. *Stat92E* was ubiquitously knocked down using the *tubulin-GAL4* driver and these flies were challenged with 10⁴ TCID₅₀ of IIV-6. The *Stat92E* knockdown flies exhibited significantly increased lethality compared to the control strain (progeny of *w¹¹¹⁸* x *tubulin-GAL4*) after virus infection (Figure 2.4B). On the other hand, both control

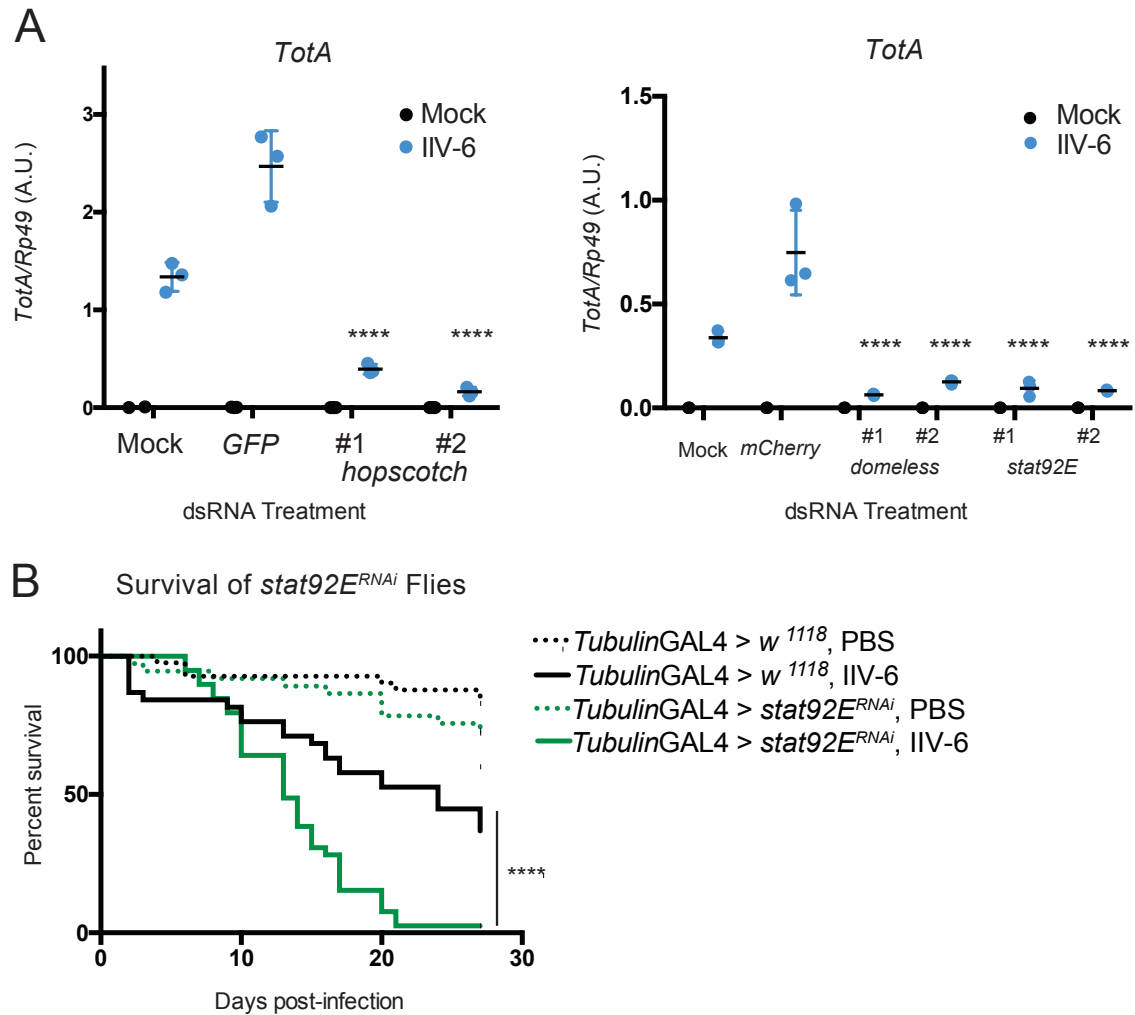


Figure 2.4 JAK-STAT Signaling is Required for IIV-6-Induced *Turandot* Expression.

A) S2* cells were treated with dsRNA targeting *domeless*, *hopscotch*, or *Stat92E*, and 48 hours later were infected with IIV-6 (MOI of 2) for 24 hours. RNA was then isolated and *TotA* induction was quantified by qRT-PCR. Data shown is three biologically independent assays, utilizing two distinct dsRNAs for each gene. Error bars indicate standard deviation, black bars indicate mean, with statistical analysis by two-way ANOVA, and corrections for multiple comparisons using Holm-Sidak testing, **** $p < 0.0001$. A.U., Arbitrary Units. **B)** Kaplan-Meier curve showing survival of *Stat92E^{RNAi}* flies expressing (UAS-*Stat92E^{RNAi}* x *tubulin-Gal4*) flies (green lines) or control (*w¹¹¹⁸* x *TubulinGAL4*) flies (black lines) following infection with IIV-6 (solid lines) or injection with PBS (dotted lines). Statistics were determined by Log-rank (Mantel-Cox) test. **** $p < 0.0001$

and *Stat92E* knockdown lines tolerated the control PBS injection to a similar degree. Previous studies examining the role of the JAK-STAT pathway in survival to IIV-6 have shown a non-significant decrease in survival (Kemp et al., 2013). We believe these different outcomes may be due to differences in injection method, dose, or the strains used in these experiments. The results shown here indicate that IIV-6 induced *Tot* expression is controlled by the JAK-STAT pathway, and this pathway is critical for survival following infection.

Next, we hypothesized that IIV-6 infection may induce the expression of one or more of the *unpaireds*, which encode the ligands for the gp130-like receptor Domeless. Unpaired 1, 2, and 3 are all distantly related to IL-6 (Oldefest et al., 2013). *Unpaired 1* is critical for embryonic development, and *upd1* null flies are embryonic lethal, while the *upd2*, *upd3* double deletion is viable. *Unpaired 3* is induced in hemocytes after septic injury (Agaisse et al., 2003a) and plays a role in gut regeneration following damage (Osman et al., 2013). In S2* cells, IIV-6 infection induced expression of all three *unpaireds*, ~10⁴-fold as measured by qRT-PCR (Figure 2.5A). Hypomorphic alleles of *upd1*, also known as *outstretched*, are viable and some of these alleles, such as *os^s*, also affect the expression of *upd3*, which lies nearby (Wang et al., 2014). In adult flies, IIV-6 induced *TotA* expression was significantly reduced in all *upd* alleles, with the largest decrease in the *os^s* allele (Figure 2.5B). *TotM* induction was similarly reduced by *os^s* to levels observed in the PBS injected control, with lesser but still significant reductions in the *upd3* mutant and *upd2^Δupd3^Δ* double mutant. Given

the phenotypes in the hypomorphic *os^s* allele and the *upd2^Δupd3^Δ* double mutant, these data indicate that the *unpaireds* function redundantly to drive JAK-STAT signaling in response to IIV-6 infection.

The IIV-6 triggered expression of *upds* suggests that virus infection may directly induce these cytokine genes, which in turn will drive JAK-STAT signaling and *Tot* expression. In other systems, such as gut renewal, it has been suggested that various MAPK pathways are responsible for driving *upd* expression (Brun et al., 2006a; Osman et al., 2013; Santabábara-Ruiz et al., 2015; Woodcock et al., 2015). Therefore S2* cells were treated with inhibitors targeting the three *Drosophila* MAPKs (JNK, p38, or ERK) and then infected with IIV-6. Treatment with p38 inhibitor significantly reduced *upd3* expression to near baseline levels, while ERK inhibitor had no effect and JNK inhibitor actually increased *upd3* expression (Figure 2.5C). On the other hand, the JAK-STAT inhibitor Tofacitinib blocked IIV-6 induced *TotA* expression but had no effect on the expression of *upd3* (Figure 2.5D). Together, these data indicate that while JAK-STAT is required for *Tot* induction, the virus-triggered expression of the *upds* (the JAK-STAT activating cytokines) involves the p38 MAPK pathway.

Drosophila encode three p38 isoforms, *p38a*, *p38b*, and *p38c*. *p38a* and *p38c* are close together on the third chromosome, and much of the early work on *p38* in *Drosophila* was originally performed in a *p38a/c* mutant. Therefore, many of the roles initially attributed to *p38a* may in fact be due to *p38c* or the nomination of both genes. *p38a* mutant flies are susceptible to oxidative stress and heat

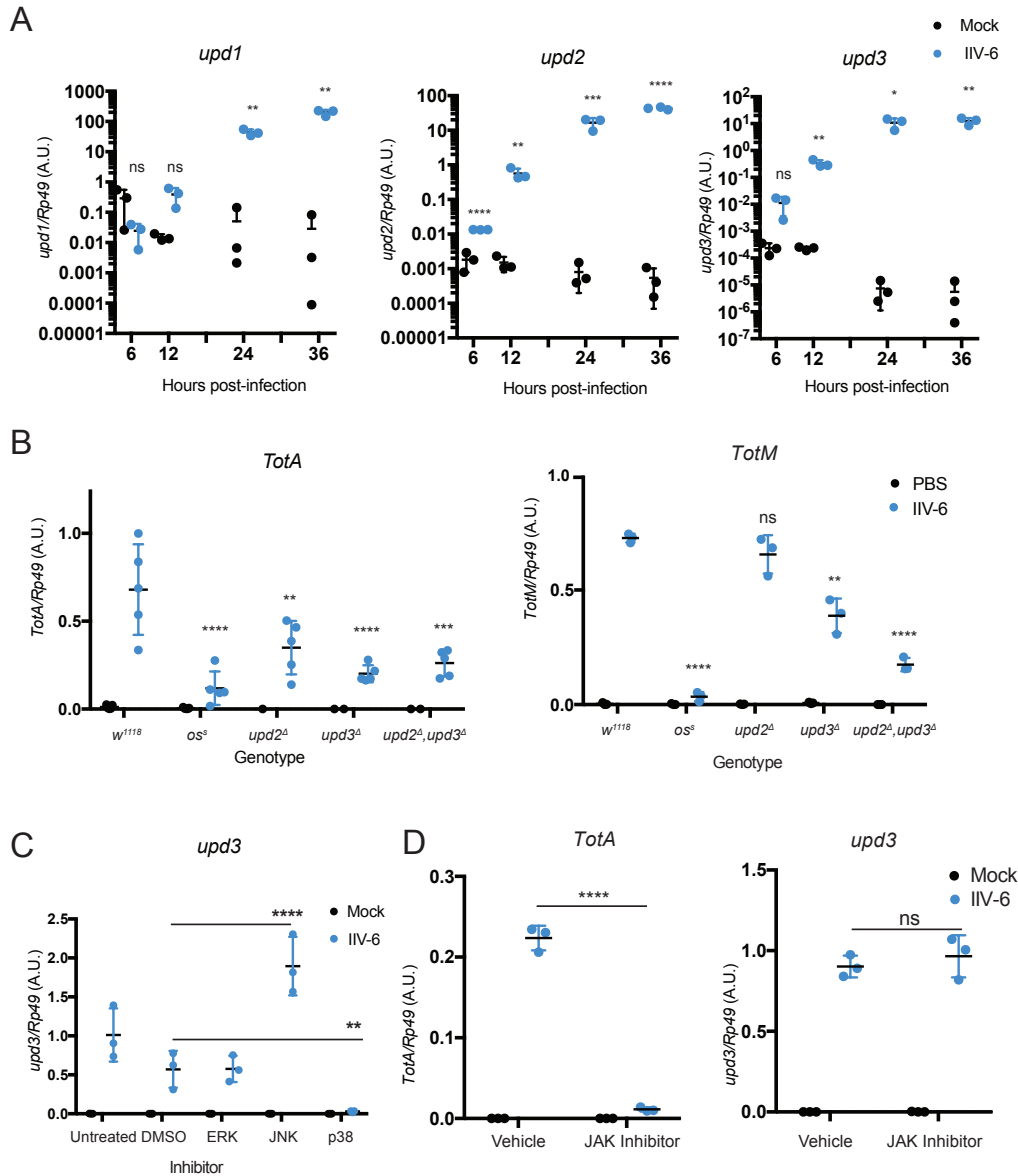


Figure 2.5 Unpaired ligands are Induced in vitro and are p38-dependent.

A) S2* cells were infected with IIV-6 at an MOI of 2 for the indicated times and induction of *upd1*, *upd2*, and *upd3* was monitored by qRT-PCR, and compared to PBS-injected controls. Three biologically independent replicates are shown and statistical analysis was performed by Multiple t tests and the Holm-Sidak correction for multiple comparisons. Error bars indicate standard deviation, black bars indicate mean. ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ **B)** *TotA* or *TotM* expression was measured by qRT-PCR from control *w¹¹¹⁸* flies as well as *oustretched^s* (*os^s*), *upd^{2Δ}*, *upd^{3Δ}*, or *upd^{2,3Δ}* mutant flies 24 hours after IIV-6 infection or injection with PBS. 3-5 biologically independent assays are displayed, error bars represent the standard deviation, black bars represent the mean, and statistics were determined by two-way ANOVA. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$ **C)** S2* cells were treated with inhibitors for the three MAPKs: JNK (SP600125, 25 μ M), ERK (U0125, 10 μ M), p38 (SB203580, 10 μ M), or treated with a vehicle control (DMSO), for one hour prior to IIV-6 infection or mock treatment. After 24 hours of infection, *upd3* expression was assayed by qRT-PCR. STATS determined by two-way ANOVA, **** $P < 0.0001$, ns, not significant. **D)** S2* cells were treated with the JAK inhibitor Tofacitinib (CP690,550), or treated with a vehicle control (DMSO), for one hour prior to IIV-6 infection or mock treatment. After 24 hours of infection, *TotA* and *upd3* expression was assayed by qRT-PCR. Statistics determined by two-way ANOVA, and Sidak's multiple comparisons test. A.U., Arbitrary Units.

shock, though not to osmotic shock (Craig et al., 2004). *p38b* has been shown to provide protection against pathogenic bacterial or fungal challenge, although the underlying mechanisms are unclear (Chen et al., 2010). *p38b* has also been linked to tolerance to infection with *Salmonella typhimurium* (Shinzawa et al., 2009). *p38c* has been implicated in gut homeostasis and reactive oxygen species (ROS) production in the gut upon infection with *P. entomophila* or *Erwinia carotovora carotovora* 15 (Chakrabarti et al., 2014). In addition, *p38c* is required for the induction of *DOPA decarboxylase*, which is required for the production of antimicrobial quinones produced in response to wounding (Davis et al., 2008). Mutant flies for all three p38 isoforms are viable to adulthood and were infected with IIV-6 to determine their ability to induce *Tots*. We found that *p38a* and *p38c* null flies displayed normal levels of *Tot* expression following IIV-6 infection, while *p38b* null flies had only basal levels of *Tot* expression (Figure 2.6A). Furthermore, *p38b* heterozygous flies also expressed wild-type levels of *Tots* upon IIV-6 infection. We also found that *p38b^{RNAi}* lines, expressed with a ubiquitous driver (*tubulin-GAL4*), had significantly reduced survival following IIV-6 infection compared to wild-type (driver alone) controls (Figure 2.6B). On the other hand, *p38a^{RNAi}* lines survived similarly to controls (Figure 2.6C). In addition, we a similar rate of death when *p38b* is knocked-down using a fat-body specific driver (*c564-GAL4*) (Figure 2.7A). These combined results show that *p38b* is required for *Tot* induction and survival following IIV-6 infection in adult flies. The S2* cell data further demonstrates that p38 is required for *unpaired* induction. Together,

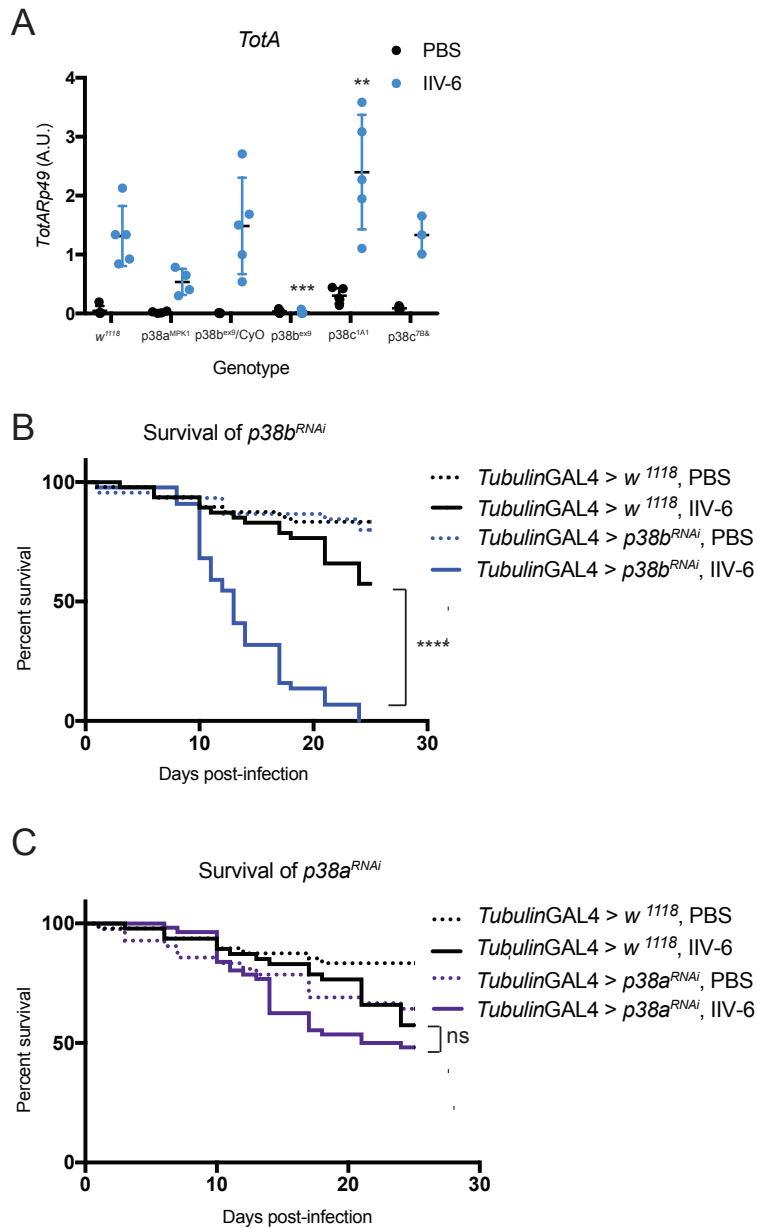


Figure 2.6 *p38b* is required for *Turandot* Induction and Survival

A) Mutant flies for the three p38 isoforms, *p38a¹*, *p38b^{ex9}*, and *p38c^{1A1}*, were infected with IIV-6 or injected with PBS, and *TotA* expression was measured by qRT-PCR 24 hours post-infection. 3-5 biologically independent replicates are shown, bar represents standard deviation, and statistical analysis by two-way ANOVA and corrected for multiple comparisons by Sidak's. *** $p = 0.0004$, ** $p = 0.0045$ **B)** Kaplan-Meier curves showing survival of *p38b^{RNAi}* expressing or **C)** *p38a^{RNAi}* expressing flies following IIV-6 infection (solid lines) or PBS-injected controls (dotted lines) compared to control flies. *UAS-p38a^{RNAi}* and *UAS-p38b^{RNAi}* flies were crossed to *tubulin-GAL4* for ubiquitous knock-down, while the control was generated by *w¹¹¹⁸* crossed to *tubulin-GAL4*. Statistics were determined by Log-rank (Mantel-Cox) test, **** $p < 0.0001$. ns, not significant. A.U., Arbitrary Units.

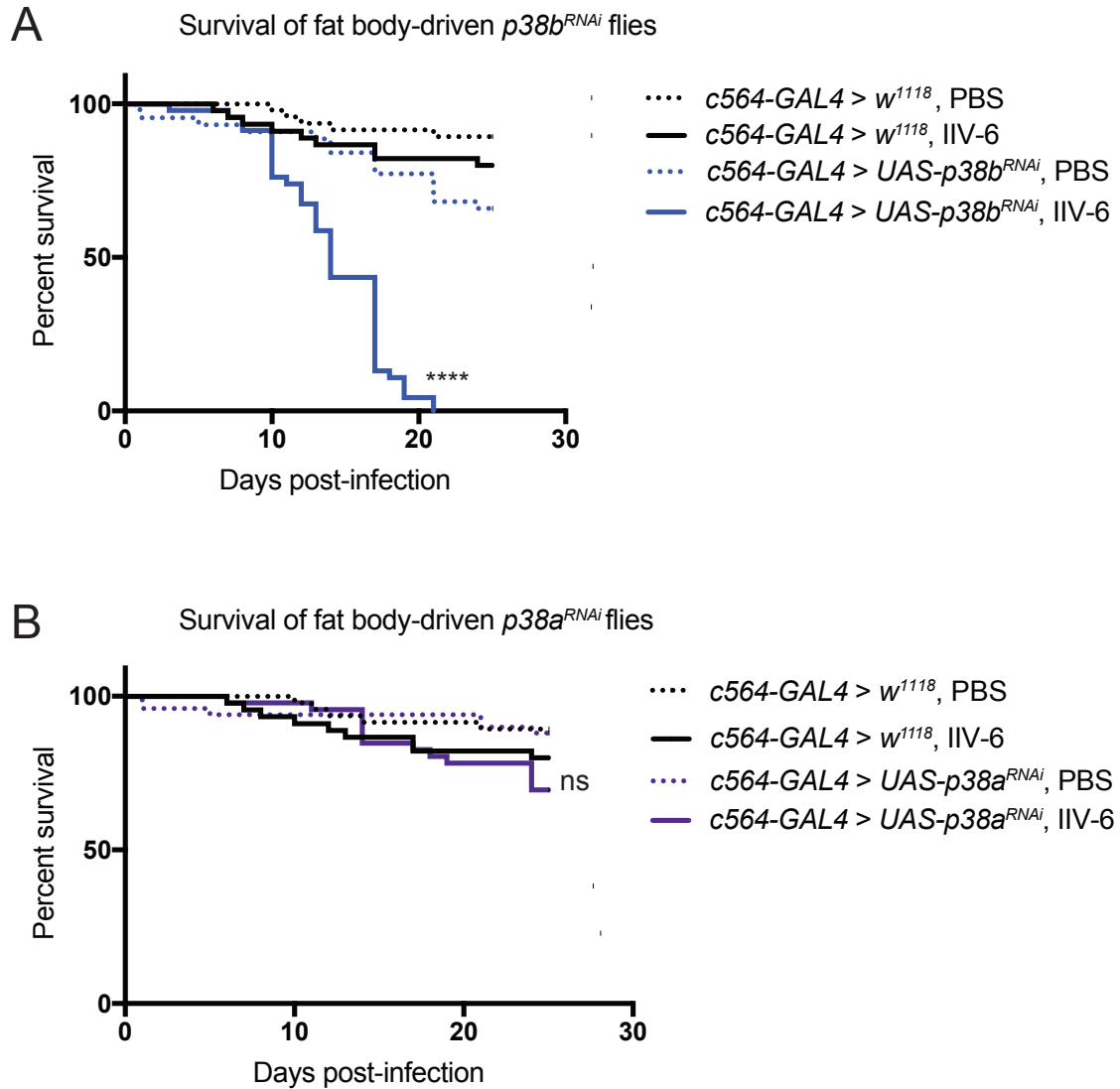


Figure 2.7 p38b is required in the fat body for IIV-6 protection.

A) Kaplan-Meier curves showing the survival of flies expressing *p38b*^{RNAi} or **B)** *p38a*^{RNAi} in the fat body following IIV-6 infection (solid lines) or PBS-injected (dotted lines) compared to control flies. *UAS-p38a*^{RNAi} and *UAS-p38b*^{RNAi} flies were crossed to *c564-GAL4* expressing flies for fat body-specific knock-down of progeny, while the control was generated by crossing *w¹¹¹⁸* to *c564-GAL4*. Statistics were determined by Log-rank (Mantel-Cox) test, **** $p < 0.0001$. ns, not significant.

these data suggest that some aspect of viral infection involving viral replication triggers p38b activation leading to Unpaired production, JAK-STAT activation, and *Tot* induction.

To determine if p38b was triggering an antiviral cascade, or whether the downstream effects of p38b were contributing to the tolerance of the organism to IIV-6 infection, viral loads were measured in *p38b* mutant flies. *p38b* mutants do not show any increase in viral titres (Figure 2.9), and had similar viral titers compared to their heterozygous siblings. *w¹¹¹⁸* flies, used as immunological controls, had ten-fold higher viral loads (Figure 2.9), whether this is due to background differences is unclear.

Viral infection damages cells, often inducing cell death (both apoptotic and necrotic) as well as the acute production of antivirals. These responses to viral infections can trigger the release of endogenous activators of inflammation, so called DAMPs. These DAMPs often lead to MAPK activation. In particular, it has been shown that ROS can trigger p38 MAPK signaling (Santabábara-Ruiz et al., 2015). Therefore, we sought to determine whether ROS generation is required for p38-induced *unpaired* expression upon IIV-6 infection. To this end, we treated S2* cells with diphenyleneiodonium chloride (DPI), an NADPH oxidase inhibitor, prior to infection with IIV-6. DPI treatment completely abrogated induction of both *upd3* and *TotA* 24 hours after IIV-6 infection (Figure 2.8). This suggests that IIV-6 infection triggers ROS, produced by a DPI-sensitive NADPH oxidase, that, in turn, stimulates p38b activation, Upd production and *Tot* induction (Figure 2.11).

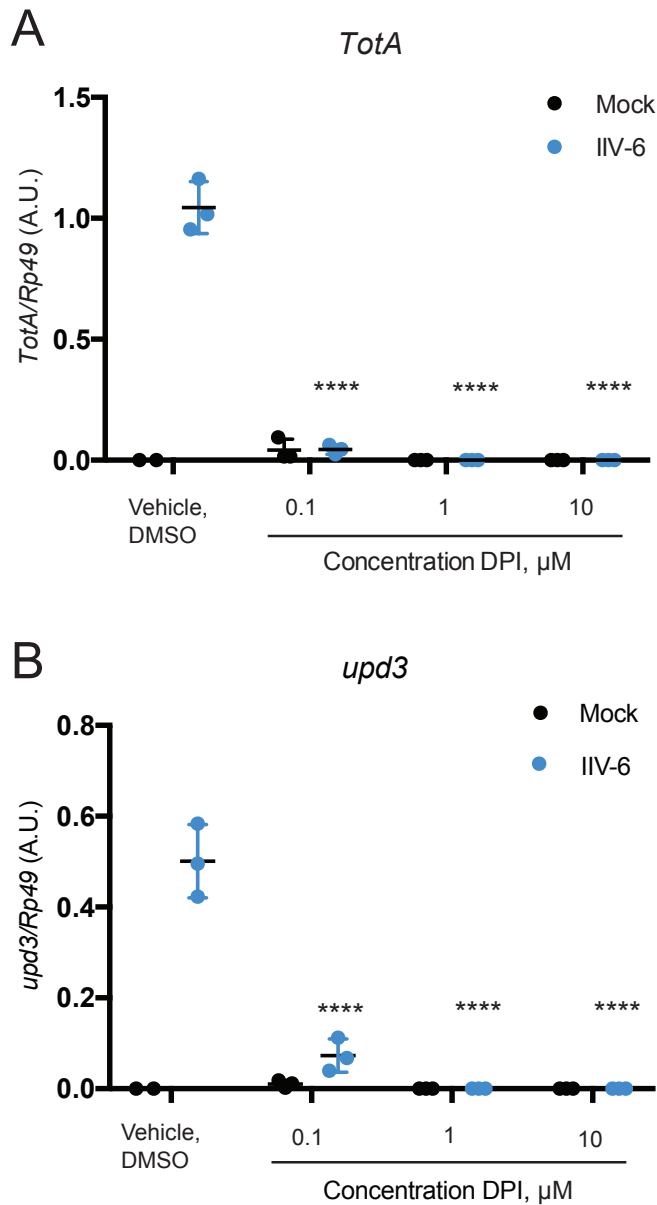


Figure 2.8 NADPH-oxidase inhibitor DPI prevents IIV-6 induced *Upd3* and *Turandot* Expression.

S2* cells were treated with the indicated concentrations of DPI, an inhibitor of NADPH oxidases, or vehicle control (DMSO) for one hour, and then infected with IIV-6 (MOI = 2) or mock treated with PBS. **A)** *TurandotA* expression, quantified by RT-PCR 24 hours post-infection, was significantly diminished after DPI treatment. **B)** *unpaired3* expression, quantified by RT-PCR 24 hours post-infection, was significantly diminished after DPI treatment. For both panels, significance was determined using two-way ANOVA. ****p<0.0001. A.U., Arbitrary Units.

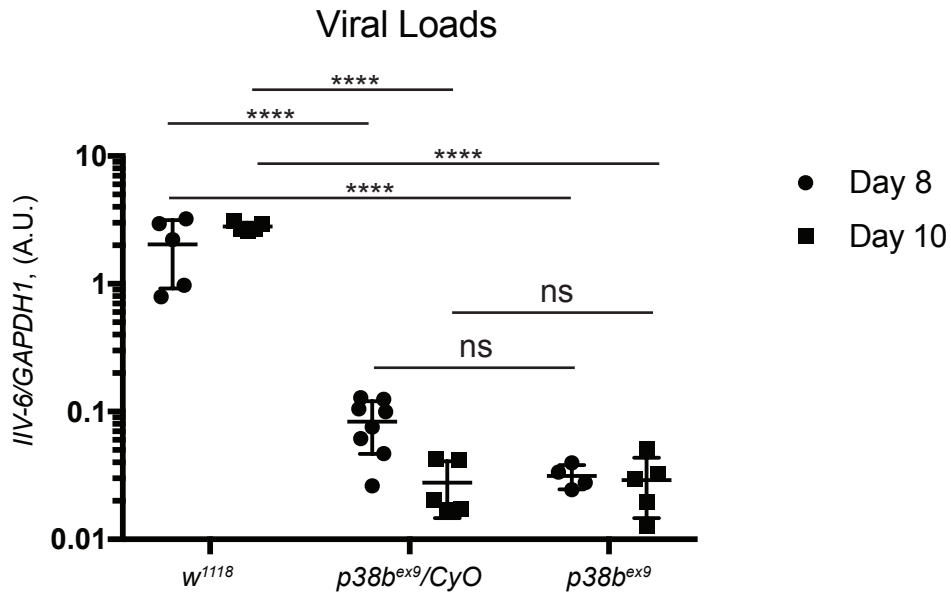


Figure 2.9 $p38b$ mutants show no increase in viral loads.

Viral loads as determined by QPCR for $p38b^{ex9}$ and $p38b^{ex9}/CyO$ heterozygous siblings and w^{1118} controls. Each data point represents 5 flies. Error bars indicate standard deviation, and black lines indicate the mean. A.U., arbitrary units. Statistics were determined using two-way ANOVA and Tukey's multiple comparison test. ****, $p < 0.0001$, ns, not significant.

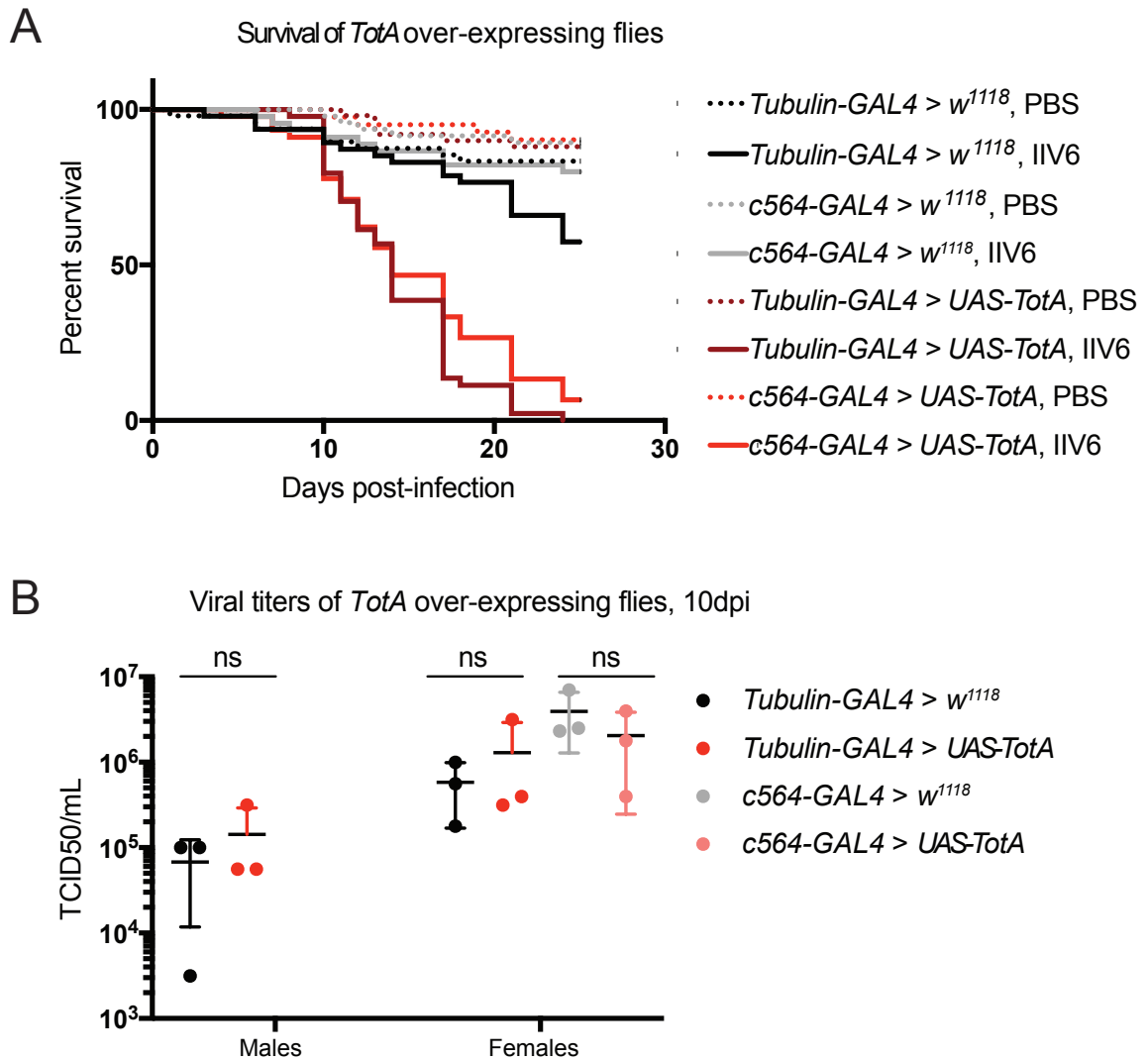


Figure 2.10. *TotA* over-expressing flies show no protection from IIV-6 infection

A) Kaplan-Meier curve showing survival of *TotA* over-expressing flies or control *w*¹¹¹⁸ flies under the control of a fat body (*c564-GAL4*) or ubiquitous (*Tubulin-GAL4*) driver, infected with IIV-6. *n*>50. B) Viral loads from *TotA* over-expressing flies infected with IIV-6 assayed by TCID50 at day 10 post-infection. Each data point

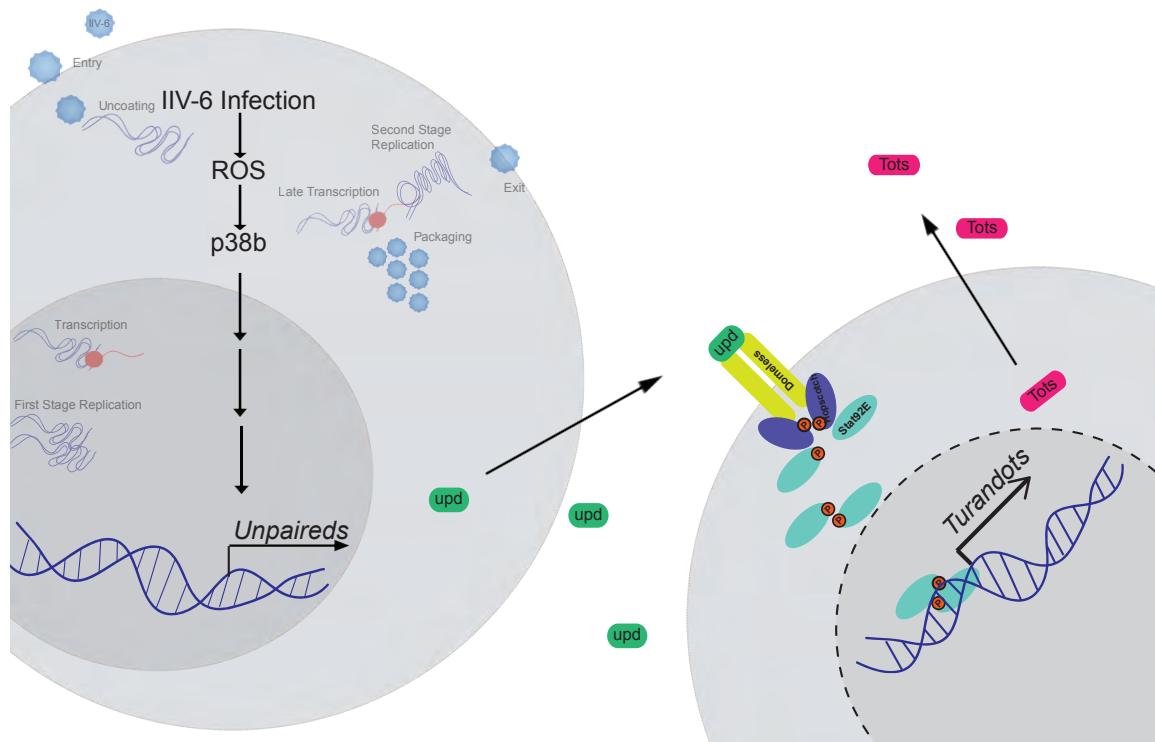


Figure 2.11 IIV-6 infection activates p38b and JAK-STAT signaling

IIV-6 infection induces ROS through an NADPH oxidase, leading to activation of p38b. p38b activation is required for induction of the *unpaireds*. The unpaired ligands activate JAK-STAT signaling, resulting in transcription of the *Turandot* genes.

In order to address whether the Turandots have any antiviral effects, we obtained an transgenic fly line over-expressing *TotA*. When expression was driven either ubiquitously (*Tubulin-GAL4*) or within the fat body (*c564-GAL4*), we saw no protection from IIV-6 infection compared to control flies (the progeny of *w¹¹¹⁸* crossed to either GAL4 driver line) (Figure 2.10A). We also assayed viral titers of these flies by TCID50 and saw no differences in viral load between control or *TotA* over-expressing flies (Figure 2.10B). While this result suggests that TotA on its own is not antiviral, we still cannot rule out a number of possibilities.

One is that the Turandots work in concert and over-expression of one is not sufficient for antiviral activity. Another is that TotA is not antiviral, or does not exert antiviral effects upon IIV-6 in particular. In order to fully explore the function of the Turandots in the context of viral infections, a number of experiments utilizing both over-expression and knock-downs, must be performed.

Discussion

Here, we show that infection of *Drosophila* with the DNA virus IIV-6 triggers a protective p38b-dependent response. While previous work has demonstrated that *Drosophila* p38b is critical for survival to bacterial or fungal infections and affects the tolerance to bacterial infections (Chen et al., 2010; Shinzawa et al., 2009), this is the first time p38b has been linked to antiviral defenses. Critical

targets for p38b for the protection against IIV-6 infection are the *unpaireds*, a family of three IL-6-like genes clustered together on Chromosome 1. The genetic data presented here argue that the three Unpaireds function together, in a partially redundant manner, to activate the JAK-STAT pathway following IIV-6 infection, thereby driving *Tot* gene expression. The JAK-STAT pathway also protects against IIV-6 infection, although the role of the Tots in antiviral defense requires more study. These results also imply that p38b is activated following IIV-6 infection. While the mechanisms leading from virus infection to p38 activation are unclear, they likely involve ROS-mediated signaling as the induction of *upd* expression is potentially blocked by an NADPH oxidase inhibitor. This is very similar to the activation of p38a following ROS generation from apoptotic cells in models of tissue regeneration (Santabárbara-Ruiz et al., 2015).

Interestingly, p38b has also been shown to provide tolerance to *Salmonella typhimurium* infections, promoting survival of the host without reducing bacterial burden (Shinzawa et al., 2009). This study suggested that p38b contributes to tolerance by enabling hemocyte enlargement, and hence, engulfment of larger quantities of bacteria. In the context of IIV-6 infection, p38b could be acting to promote engulfment of infected and damaged cells, thereby providing a repair mechanism to enable the animals to better tolerate and limit virus infection. Future studies will be necessary to probe all the roles of p38b in antiviral defense.

Although the data presented here demonstrate that the JAK-STAT pathway is protective against IIV-6 infection, the protective mechanisms require further study. In the case of the RNA virus DCV, the JAK-STAT pathway is also protective, possibly through the induction of *vir-1*. However, the JAK-STAT pathway is not broadly antiviral and *vir-1* was not induced by IIV-6 ((Kemp et al., 2013) and data not shown). The Tots are intriguing candidates for JAK-STAT induced antivirals. They are rapidly evolving with evidence of positive selection, typical for immune effectors (Obbard et al., 2009; Zhong et al., 2013). However, the Tots have not yet been demonstrated to provide direct antimicrobial activity. To date, we have been unable to demonstrate any antiviral activity for the Tots. In particular, over-expression of *TotA* resulted in reduced survival following IIV-6 infection (Figure 2.10), consistent with a general toxicity as reported previously (Ekengren et al., 2001). Further studies, examining all six of the IIV-6 induced *Tots*, with both loss- and gain-of-function approaches, will be necessary to more fully examine this possibility.

The sensitivity of STAT knockdowns to IIV-6 infection argues that JAK-STAT signaling is an important antiviral target of p38b. However, other p38b targets are also possible. For example, an established target of p38b is the heat shock response. In the context of bacterial and fungal infections, p38b is known to regulate Heat shock factor (Hsf) expression and the induction of heat shock proteins (Hsps) (Chen et al., 2010). In addition, another report has shown that Hsf protects flies against both RNA and DNA viral infections (Merkling et al.,

2015). Together, these results suggest that the antiviral effects of p38b could be mediated, at least in part, through Hsf and Hsps. Indeed, *Hsf* mutant flies display an increased rate of death after IIV-6 infection. It will be interesting to learn if the heat shock response is activated by p38b following IIV-6 infection, and how this response interacts with JAK-STAT dependent viral protection.

Successful host defenses detect multiple characteristics of an invading pathogen. For example, cellular damage is one common indicator pathogenic infection that can be sensed by the innate immune system. In mammals, several danger-associated molecular patterns (DAMPs) have been characterized, including HMGB1, F-actin, and histones (Ahrens et al., 2012; Chaput and Zychlinsky, 2009; Kono and Rock, 2008). Likewise, a recent report demonstrated that extracellular actin activates JAK-STAT signaling, using a model of sterile injury in *Drosophila* (Srinivasan et al., 2016). In this paradigm, detection of extracellular actin, via an unknown receptor, triggered Nox-dependent ROS generation, the activation of Src42A and Shark (Syk homolog), and induction of *unpaireds* and eventually *Tots*. This pathway is very similar to that reported above, although p38b was not examined, and suggests that IIV-6 infection may cause cellular damage and rupture and the release of actin, which in turn triggers ROS production, *unpaired* expression, JAK-STAT signaling and the induction of *Tots*. Formally testing this model will be facilitated by the identification of the extracellular actin receptor.

In summary, we have found a novel role for *Drosophila* p38b in protecting against DNA virus infection. Virus infection leads to a p38b dependent responses, including the induction of the JAK-STAT activating cytokines, the Unpaireds, and the induction of downstream target genes such as the *Tots* (Figure 2.11). While p38b does not appear to be directly antiviral (Figure 2.9), it contributes to the tolerance of the organism. The role of the Turandots, and whether they may have direct antiviral effects, will be the subject of future studies.

Materials and Methods

Reagents

p38 inhibitor SB203580 (CAT#13067), JNK inhibitor SP600125 (CAT#10010466), JAK inhibitor Tofacitinib CP690,550 (CAT#11598), and ERK inhibitor U-0126 (CAT#70970) were purchased from Cayman Chemical. Diphenyleneiodonium chloride DPI (CAS#4673-26-1) was purchased from Sigma-Aldrich. All inhibitors were dissolved in DMSO and used at the indicated concentrations.

Fly Stocks and Infections

p38a^{MPK1}, *p38b^{ex9}/CyO*, *p38c^{1A1}/TM6*, and *p38c^{7B&TM6}* flies were a kind gift of Bruno Lemaitre. *w¹¹¹⁸*; *P{UAS-STAT92E^{GD4492}RNAi}*v43866, and *w¹¹¹⁸*; *P{UAS-p38a^{GD17018}RNAi}*v52277 were obtained from the Vienna Drosophila

Resource Center (VDRC). *y¹ v¹*; *P{p38b^{TRiP.JF03341}RNAi}attP2*, *Ab(1)os^s*, *upd1^{os-s}* *upd3^{os-s}*(BIN#79), *Df(1)os^o*, *upd1^{os-o}* *upd3^{os-o}*(BIN#78), *w^{*} upd2^Δ* (BIN#55727), *w^{*} upd3^Δ* (BIN#55728), *w^{*} upd2^Δupd3^Δ*: (BIN#55729) were obtained from Bloomington Drosophila Stock Center (BDSC). *w¹¹¹⁸* flies were used as an immunologically wild-type control in all experiments, as these are the most similar background to the alleles listed above.

Three to five day old flies, maintained at 22°C, were used for all experiments. Flies were injected intrathoracically with 32.2 nL of virus (1x10⁴ TCID50) or vehicle (PBS) using a Nanoject II (Drummond). For survival assays, a minimum of fifty flies were used per treatment, per genotype and the dead were counted daily. Kaplan-Meier curves are shown and significance was determined by log-rank (Mantel-Cox) using GraphPad Prism. For qRT-PCR analysis, at least three independent replicates of 15-20 flies each were used for RNA extraction. In all cases, three or more independent replicates, as indicated in each figure legend, were performed in parallel on the same day. At least 2 additional trials, each with 3 or more biologically independent replicates, were performed at other times, with similar results.

nCounter Analysis

The expression levels of 139 *Drosophila* immune genes were assayed from 100 nanograms of RNA via a customized Nanostring nCounter codeset. Two biological replicates of 10-15 adult male flies 3-5 days of age were analyzed for each treatment and timepoint. The results were analyzed using nSolver 3.0

software according to the manufacturers instructions (NanoString Technologies, Seattle, WA, USA), and the heatmap was created using nSolver 3.0 software and JavaTree.

RNA Isolation and qRT-PCR

Total RNA from flies or S2* cells was extracted using TRIzol (Invitrogen). Samples were then DNase treated (RQ1, Promega) and RNA re-extracted by phenol-chloroform. cDNA was synthesized using iScript cDNA Synthesis kit (BioRad). Alternatively, the gDNAClear cDNA synthesis kit (BioRad) was used following TRIzol purification. qRT-PCR was analyzed normalizing to the housekeeping gene Rp49.

Cell Culture and RNAi

dsRNA was produced as previously described (Silverman et al., 2000b). S2* cells were cultured as previously described (Samakovlis et al., 1992; Silverman et al., 2000a) and were transfected with 3µg of dsRNA using Cellfectin II reagent (Invitrogen). Cells were split 24 hours after transfection to 5x10⁵ cells/mL and 24 hours later cells were infected with IIV-6 at an MOI of 2. As a control, cells were mock-treated with the same volume of PBS (virus diluent) as used in infections. Cells were harvested in TRIzol (Invitrogen) 24 hours post-infection. In experiments with small molecule inhibitors, cells were treated with the indicated inhibitor at the stated concentration or the appropriate vehicle control 1 hour prior to virus infection.

Virus Preparation

IIV-6 was provided by Luis Teixeira. IIV-6 was propagated and purified on DL-1 cells as previously described (9), with a final resuspension in PBS, and quantified on DL-1 cells by TCID₅₀. Cells were infected at an MOI of 2 unless otherwise noted, while flies were injected with 1×10^4 TCID₅₀, as detailed above.

Preface to Chapter III

Don Gammon and **Cara West** designed the recombinant, mCherry-expressing IIV-6 virus, Δ TS-MCP-mCherry IIV6.

Cara West, Don Gammon, and Ying Chen assisted with creating Δ TS-MCP-mCherry IIV6.

Jim Strassner and **Cara West** performed Nanosight Tracking analyses.

Don Gammon provided VSV-luciferase, VSV-GFP, SINV-luciferase, and SINV-GFP strains.

Cara West performed the experiments.

Cara West and Neal Silverman designed the experiments.

**CHAPTER III:
ANTIVIRAL EFFECTS OF IIV-6 CONDITIONED MEDIA**

Abstract:

Detection of virus infection and protecting naive cells from infection is critical for limiting viral spread and maintaining the health of the organism. In mammals, this is accomplished via the production of type-1 interferons, which set up a protective state in neighboring naive cells through activating transcription of interferon-stimulated genes (ISGs) (Schneider et al., 2014). *Drosophila* lack type-1 interferon homologues. It has been shown that viral replication is limited *in vivo* by RNAi spread at distal sites (Saleh et al., 2009). Recently, it was shown that RNAi spread is mediated by RNAi-packed exosomes secreted by hemocytes (Tassetto et al., 2017). Here, we show that infected *Drosophila* S2* cells secrete a factor into cell culture media that provides protection from virus infection to naive cells. This factor is induced within 12 hours of infection, is Proteinase-K resistant, large in size, and purifies with exosomes. While the contents and antiviral mechanisms of these exosomes have yet to be determined, they are not dependent upon endogenous reverse-transcriptases and we believe that they are generated in a distinct manner to those previously described (Tassetto et al., 2017). Our results show that a DNA virus is capable of generating antiviral exosomes to protect neighboring cells.

Introduction:

Mammals utilize a complex, protein-based antiviral innate defense system to protect cells from viral infections. The type-1 interferon system (IFN) is a potent antiviral defense that is able to be initiated from a number of upstream pattern recognition receptors in response to binding a variety of pathogen associated molecular patterns (PAMPs), including proteins, RNA or DNA species. These pathways all converge to induce type-1 interferons, which then signal through a JAK-STAT signaling pathway to induce hundreds of interferon-stimulated genes (ISGs). While some ISG products have potent antiviral activities, many remain uncharacterized (Schneider et al., 2014). Despite attempts to find a parallel system in *Drosophila*, no interferon homologues exist.

Detection of DNA viruses results in a potent inflammatory response, mediated by cyclic GMP-AMP synthase (cGAS) and AIM2. Activation of cGAS leads to type 1 interferon production, while AIM2 triggers inflammasome activation and IL-1 β release (Dempsey and Bowie, 2015). cGAS produces a second messenger, cyclic GMP-AMP (cGAMP). This cyclic dinucleotide binds and activates STING, activating TBK1 to phosphorylate IRF3, which can then dimerize, enter the nucleus and transcribe type-1 IFNs. While a number of ALR receptors have been implicated in cytosolic DNA sensing, it is unclear whether many of these are cell-type specific (Gray et al., 2016). In endosomes, DNA is detected by TLR9 leading to type-1 IFN production.

While *Drosophila* have a STING homologue, CG1667, it has not been shown to function in antiviral responses. In fact, *Drosophila* STING does not interact with 2',3' cGAMP, 3',3' cGAMP, cyclic di-GMP, or cyclic di-AMP (Kranzusch et al., 2015). This study also examined the *Drosophila* cGAS-like genes, CG7194 and CG12970. These genes are in the nucleotidyl transferase superfamily, however they lack the zinc-ribbon necessary for dsDNA recognition (Kranzusch et al., 2015).

Interestingly, although insects lack functional STING and cGAS homologs, more ancient species, such as the sea anemone (*Nematostella vectensis*), have retained them (Kranzusch et al., 2015). This suggests that insects may have lost cGAS/STING signaling over time, and perhaps that they developed a compensatory mechanism. Similarly, *Drosophila* lack NLR proteins.

In addition to a lack of IFN, *Drosophila* also lack an adaptive immune response. Flies have only a set of germline-encoded receptors, they do not undergo receptor rearrangement, such as V(D)J recombination. Additionally, flies lack MHC proteins and do not present antigen. However, several reports have described phenomena similar to that of trained immunity (Netea et al., 2016).

Recently, it was also shown that hemocyte engulfment of apoptotic corpses primes hemocytes to become more inflammatory (Weavers et al., 2016). This process appears similar to innate immune training. Hemocytes that have engulfed apoptotic cells become more rapidly recruited to wounds and bacterial infections.

Flies are able to respond more robustly upon a secondary immune challenge with the same bacteria. This phenomenon, known as priming, has been reported in response to certain bacterial infections (Pham et al., 2007). Flies challenged with heat-killed *Streptococcus pneumoniae* and then followed with lethal dose of *S. pneumoniae* survived, while flies not first challenged with heat-killed bacteria died. This effect is pathogen-specific, dependent upon phagocytosis, and unique to *S. pneumoniae*; flies primed with *S. pneumoniae* were not protected from challenge with other pathogens, nor were flies primed with other heat-killed bacterial strains protected from lethal doses with the same strain.

Extracellular vesicles are secreted or released by cells have important roles in intracellular communication. Several types of extracellular vesicles exist, categorized by size and origin (Jeppesen et al., 2014). Exosomes, which originate from multivesicular bodies, and are 50-120 nanometers. Microvesicles are larger, over 200 nanometers in diameter, and bud from the plasma membrane. Apoptotic bodies are released from dying cells. Within the immune response, extracellular vesicles have been reported to function in antigen presentation, activation or inhibition of cell subsets, and delivery of miRNAs (Silva et al., 2017; Valadi et al., 2007).

Recently, it was reported that exosome-like vesicles disseminate antiviral dsRNA to distal sites in the fly (Tassetto et al., 2017). While plants and nematodes encode an RNA-dependent RNA polymerase (RdRp) to amplify RNAi species, *Drosophila* lack RdRps and while it was known RNAi spread occurred in

the fly, the mechanism of RNAi spreading was never understood (Saleh et al., 2009). In this model, dsRNA derived from viral replication were engulfed by hemocytes, and transcribed into extrachromosomal DNA by endogenous reverse transcriptases (Goic et al., 2013). This enables the hemocyte to produce novel dsRNA from these longer-lived DNA copies. These novel dsRNAs are then packaged into exosome-like vesicles and secreted to provide protection to distal sites within the fly (Tassetto et al., 2017).

Here, we present an inducible, protective response to IIV-6 infection in *Drosophila* cells. Cells challenged with IIV-6 produce a factor that is secreted into the culture media and can provide protection to naive cells. This response is proteinase-insensitive and appears to be mediated by exosomes. Unlike the exosome-mediated methods of antiviral protection recently described in *Drosophila*, this response does not depend on the activity of endogenous reverse-transcriptases.

Results:

The ability to sense a viral infection and secrete protective factors in order to relay protection to nearby naive cells is a crucial aspect of the innate immune system. In mammals, this safeguard is accomplished by the secretion of type 1 interferons, leading to the transcription of antiviral effectors. While the interferon system has been intensely studied for more than sixty years in mammals, no homologous system exists in *Drosophila*. We sought to determine whether cells

might utilize a different system to secrete protective factors into their surroundings.

To test whether conditioned media from S2* cells was capable of providing protection to naive cells, we first harvested media from infected S2* cells 30 hours post-infection, and then used this media to treat naive S2* cells (Figure 3.1). These cells were then infected with a strain of IIV-6 we produced that expresses mCherry (mCh-IIV-6) under the Major Capsid Protein promoter, and examined for mCherry expression 24 hours post-infection (see Appendix for detailed strain information). Strikingly, cells treated with conditioned media produced by IIV-6 infected cells (IIV-6 CM) showed no mCherry expression, while cells treated with conditioned media from mock infected cells (Mock CM) exhibited robust mCherry expression. This indicates that a factor released into the media by infected cells is able to create an antiviral state, and block viral replication or viral entry in naive cells. Virus was removed via two subsequent centrifugations at 10,000g, followed by filtration through a 0.1 μ m filter to ensure that this effect was not due to the inability of the virus to super-infect cells (Figure 3.2A). To verify that this procedure removed all virus particles, a fluorescent tagged IIV-6 was used in place of WT-IIV-6. We treated naive cells with

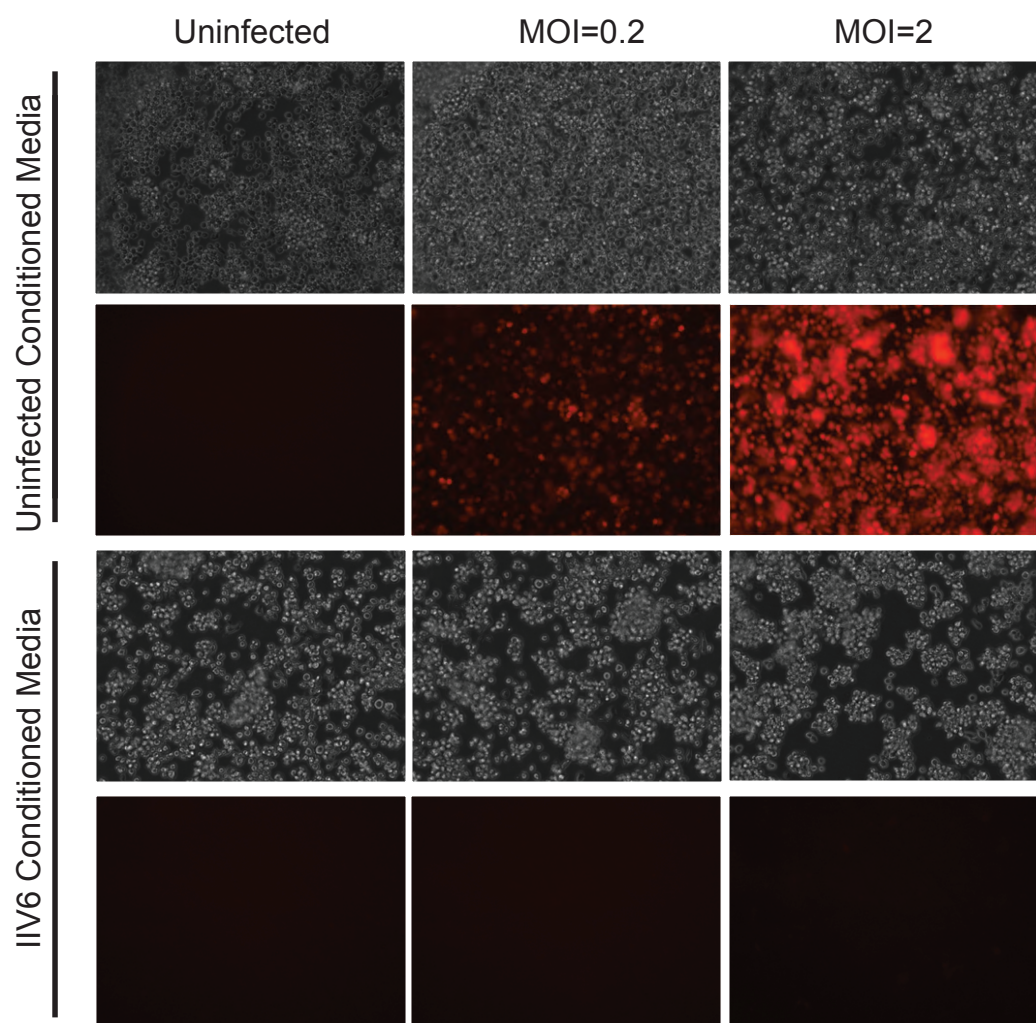


Figure 3.1 Naive cells were treated with conditioned media (CM) from Mock (Top rows) or IIV-6 infected cells (bottom rows), and infected with Δ TS_MCP-mCherry IIV-6 were indicated for 24 hours before imaging. Cells were either left uninfected (left panels) or infected with a low MOI of 0.2 (middle panels) or an MOI of 2 (right panels). Bright field image is shown above its corresponding fluorescence image.

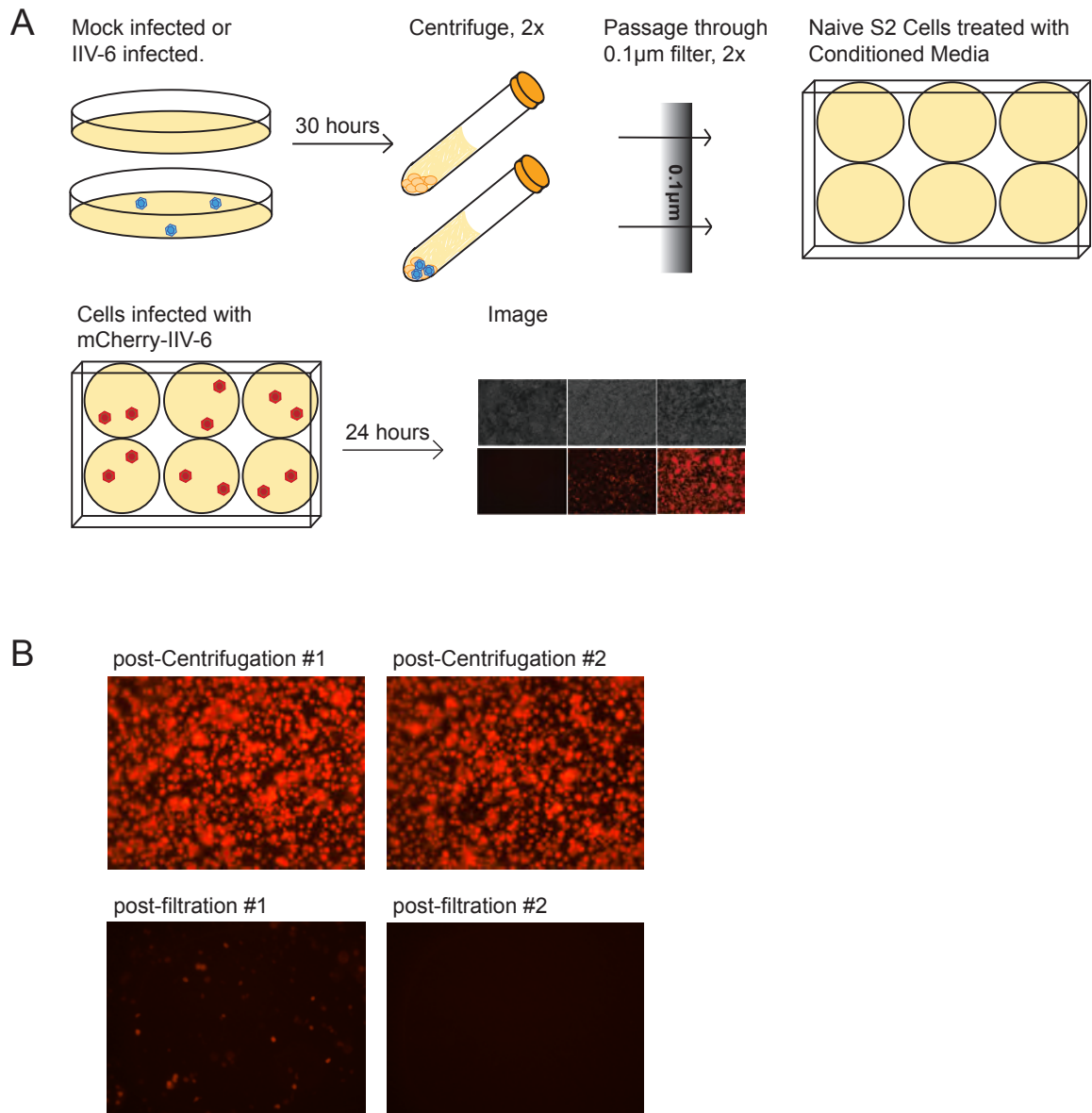


Figure 3.2 Conditioned media does not contain contaminating virus.

A) Conditioned media was prepared by infecting S2* cells with IIV-6 at an MOI of 2 or mock-infecting cells for 30 hours. Media was centrifuged to remove virus particles and cell debris, twice at 10,000g. Media was passed through 0.1m filters, twice, and then added to naive S2* cells. Cells were then infected with Δ TS_MCP-mCherry IIV-6 for 24 hours before imaging. **B)** Δ TS_MCP-mCherry IIV-6 was used to prepare conditioned media in order to assess whether contaminating viral particles were present in the media. Samples removed after each centrifugation and filtration step and were used to treat naive S2* cells. Cells were imaged after 24 hours.

conditioned media sampled after each step of processing to ensure that the final preparation did not contain any contaminating virus (Figure 3.2B).

This protective factor was further characterized by subjecting it to a range of temperatures. Conditioned media from IIV-6 infected cells was able to exert its protective effects after being heated at 37°C or 55°C for one hour, but lost its protective effects after being heated to 70°C for one hour or five minutes of 95°C treatment (Figure 3.3A). These results suggested that the protective factor may be proteinaceous.

To directly examine whether this factor is indeed a protein, conditioned media was treated with Proteinase-K bound to agarose beads. Much to our surprise, Proteinase-K treated IIV-6 CM retained its protective qualities (Figure 3.3B), protecting naive cells as well as control IIV-6 CM incubated at 37°C for one hour without Proteinase-K treatment. As expected, cells treated with Mock CM showed robust mCherry-IIV-6 infection regardless of Proteinase-K treatment. To verify that the agarose-bound Proteinase-K was active, we treated Mock or IIV-6 CM, or Schneiders media spiked with FBS with Proteinase-K, removed the Proteinase-K, and ran this material on an SDS-PAGE gel (Figure 3.3C). We visualized protein using silver stain, and found that Proteinase-K treated samples contained smaller molecular weight proteins than untreated samples, indicating that they had been processed into smaller molecular weight sizes (Figure 3.3C).

We looked at how quickly this protective factor is induced post-infection by isolating IIV-6 conditioned media at different times post-infection (Figure 3.4). By

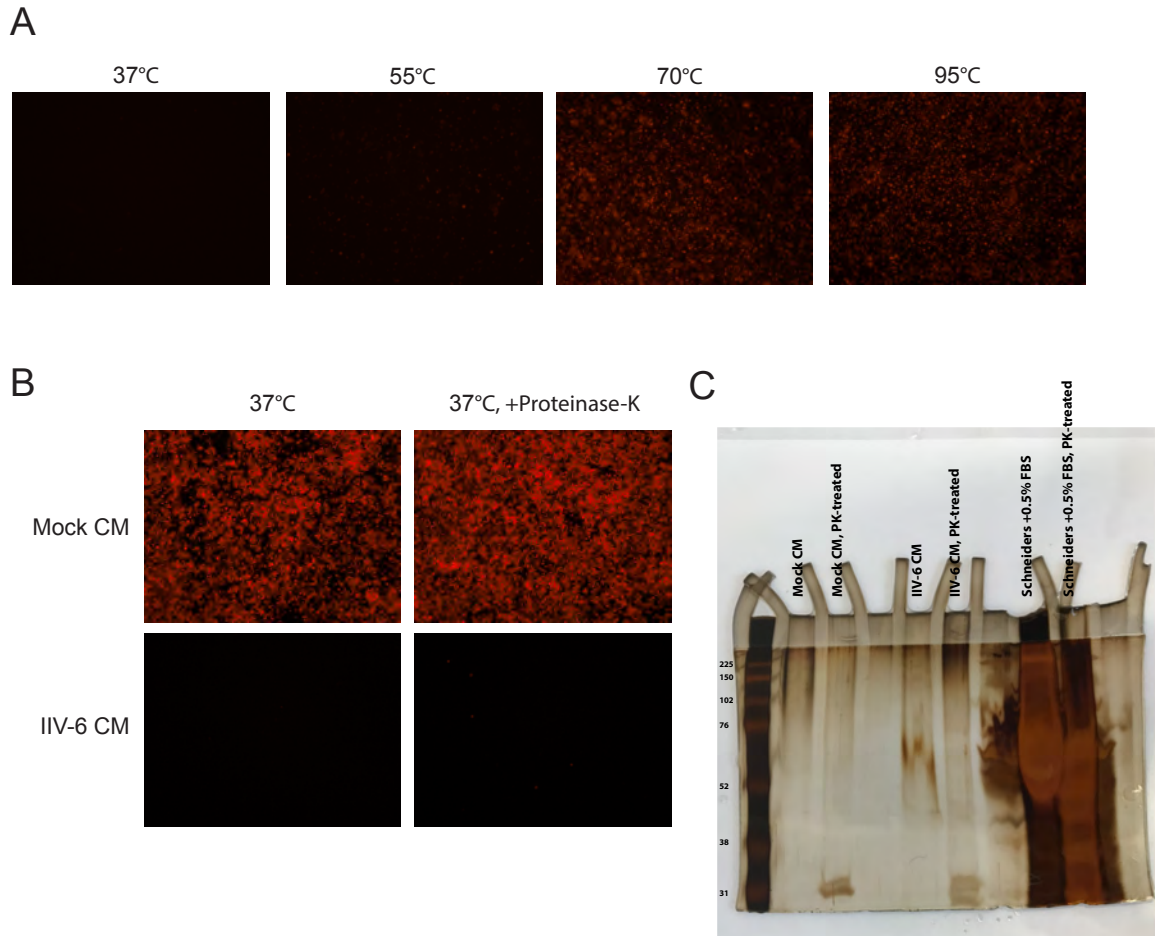


Figure 3.3 Antiviral factor is sensitive to heat but not to protease treatment.

A) IIV-6 CM was incubated at 37°C, 55°C, or 70°C for one hour, or 95°C for five minutes. Treated CM was then placed on naive cells, and infected with Δ TS_MCP-mCherry IIV-6 for 24 hours before imaging. **B)** Mock CM (top row) or IIV-6 CM (bottom row) were subjected to treatment with Proteinase K bound to agarose beads and incubated at 37°C (right panels). Control CM (left panels) were incubated at 37°C. Proteinase K was removed by centrifugation and the CM was placed on naive cells. Naive cells were infected with Δ TS_MCP-mCherry IIV-6 for 24 hours before imaging. **C)** Silver stained SDS-PAGE gel showing Mock or IIV-6 CM with and without Proteinase-K (PK) treatment. FBS-spiked Schneiders media was run as a control.

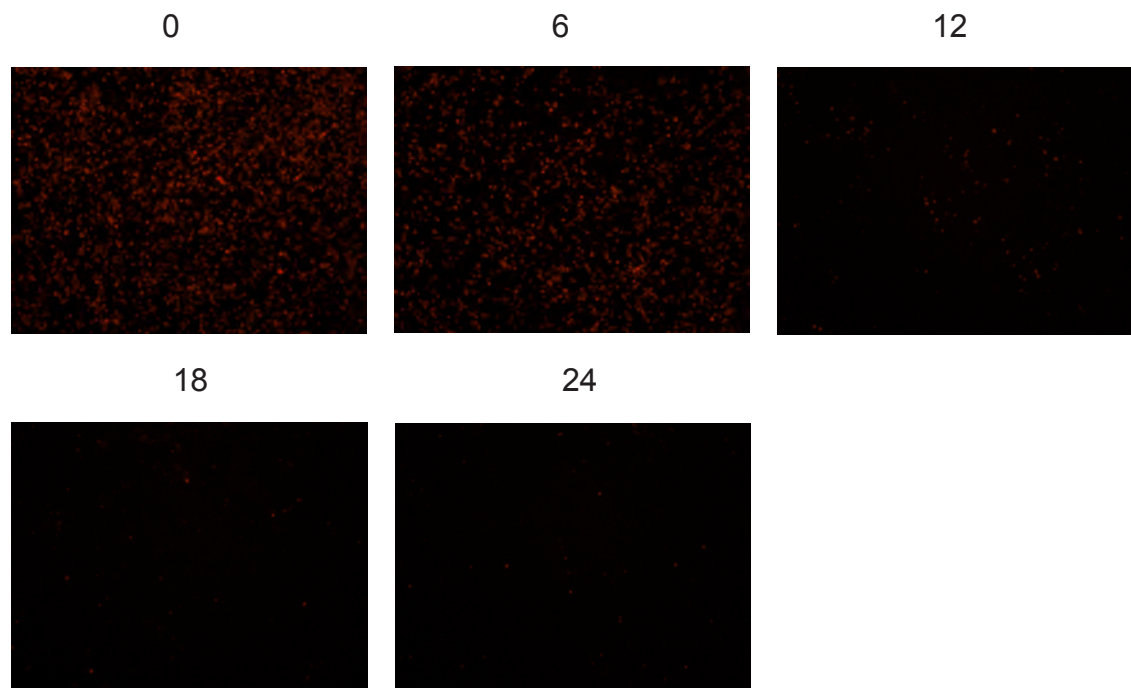


Figure 3.4 Protection is induced by 12 hours post-infection

Conditioned media harvested from WT-IIV-6 infected S2* cells at 0, 6, 12, 18, or 24 hours post-infection was used to treat naive S2* cells prior to mCh-IIV-6 infection. Cells were imaged 24 hours post mCh-IIV-6 infection.

12 hours post-infection, we saw increased protection of S2* cells upon mCh-IIV-6 challenge (Figure 3.4).

If the protective factor present in conditioned media is not a protein, this leaves a number of interesting possibilities. One attractive hypothesis is that the factor is a small molecule, such as cGAMP in mammals (Sun et al., 2013; Wu et al., 2013). The *Drosophila* genome contains a STING homolog, CG1667, and two cGAS-like genes, CG7194 and CG12970. While neither of these have been shown to function as homologues, it is also possible that another, unidentified, dinucleotide synthase exists. To probe whether a second messenger such as a cyclic dinucleotide was present, we subjected the conditioned media to a series of Amicon molecular weight cut-off centrifugal filters (Merck Millipore, Germany). The small size of a second messenger such as a cyclic dinucleotide would allow it to easily pass through these columns, and protective activity would be found in the flow-through fraction. Instead, we found that the activity in IIV-6 CM was retained even by the 100 kilodalton cutoff filters (Figure 3.5). These results suggest that a large, non-proteinaceous, secreted factor provides protective immunity to naive cells. A run of conditioned media over a Superose 6 gel filtration column resulted in a range of fractions that conferred antiviral activity to naive cells, further suggesting that this activity was not mediated by a protein (data not shown).

Having excluded the possibilities that the protective factor was a protein, as it retains its properties after proteinase treatment, or that it is a small molecule, as

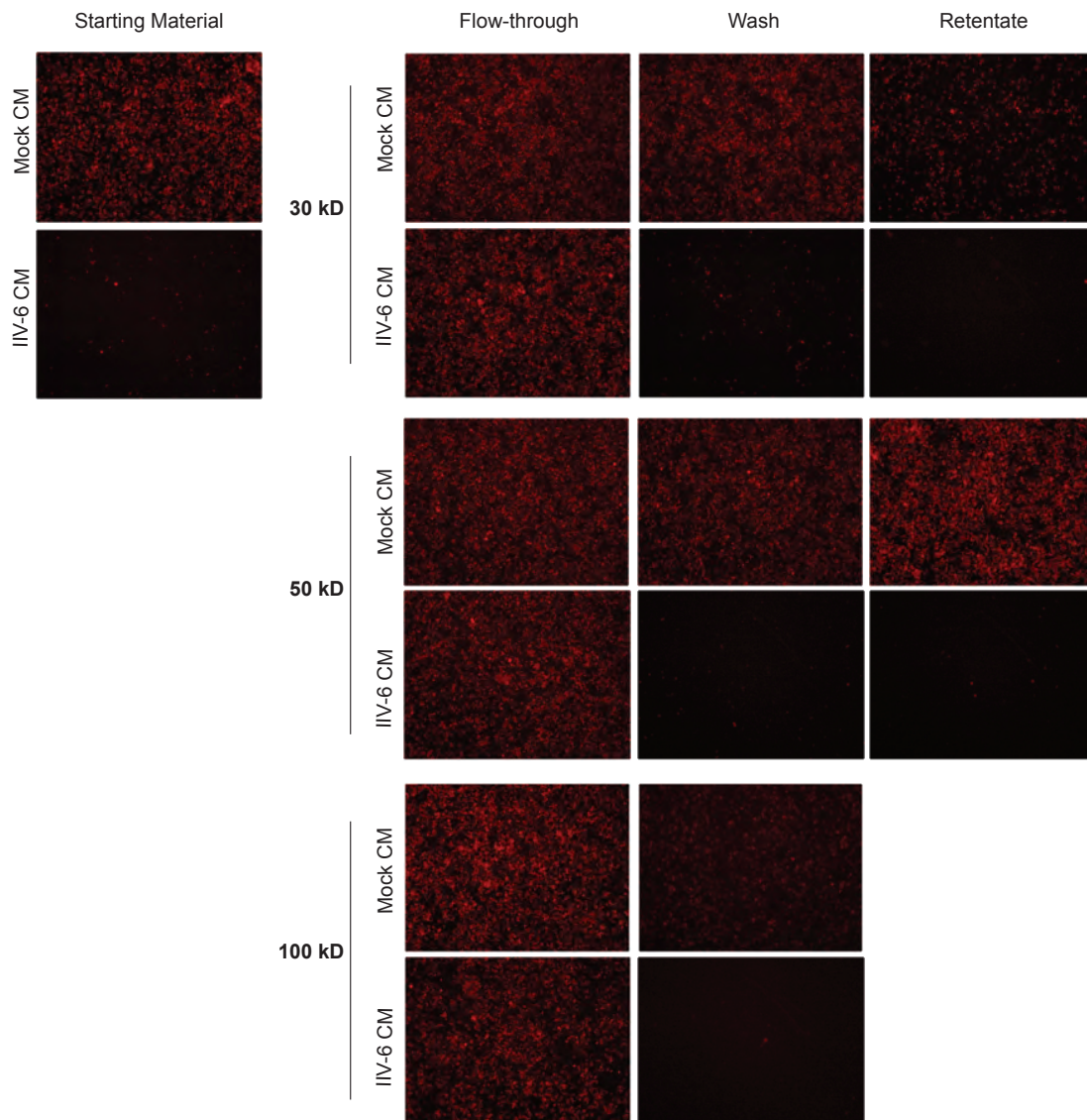


Figure 3.5 Antiviral activity is retained in 100 kD cutoff filters

Conditioned media from mock-treated or IIV-6 infected cells were centrifuged through 30, 50, or 100 kD molecular weight cut-off filters, and placed onto naive S2* cells. S2* cells were then infected with mCh-IIV-6 at an MOI of 2 for 24 hours and imaged. Flow through, wash from the retentate filter, or the retentate is shown. Starting material, unfiltered conditioned media, is shown as a control.

it is retained in the 100 kilodalton molecular weight cutoffs, we next sought whether to determine whether exosomes were responsible for this phenotype.

To determine whether exosomes were responsible for the protective effects observed in our conditioned media, we isolated exosomes from Mock or IIV-6 infected conditioned media via two methods: ultracentrifugation or an exosome isolation kit. Treatment of naive cells with the exosome-containing pellets isolated from ultracentrifugation of conditioned media from IIV-6 infected cells resulted in complete protection of cells from challenge with mCh-IIV-6 (Figure 3.6A). Treatment of naive cells with cleared supernatant from ultracentrifugal spun samples provided less protection than the starting material, while the pellet provided protection, indicating that the protective factor was pelleted with the exosome-containing fractions (Figure 3.6A). Similarly, treating naive cells with exosome-containing fractions obtained from exosome isolation kits also resulted in protection from mCh-IIV-6 (Figure 3.6B). This data shows that the protective factor is both the same density as an exosome and can also be isolated using a kit based on its hydrophobicity (Figure 3.6A,B).

To determine if this protection was being mediated by siRNAs in a manner as previously reported, we asked whether this response was dependent upon endogenous reverse transcriptases. We treated conditioned media producing cells with azidothymidine (AZT), a reverse transcriptase inhibitor, for one hour prior to infection with IIV-6, produced and processed CM as previously described. CM produced from AZT treated cells protected naive S2* cells as well as IIV-6

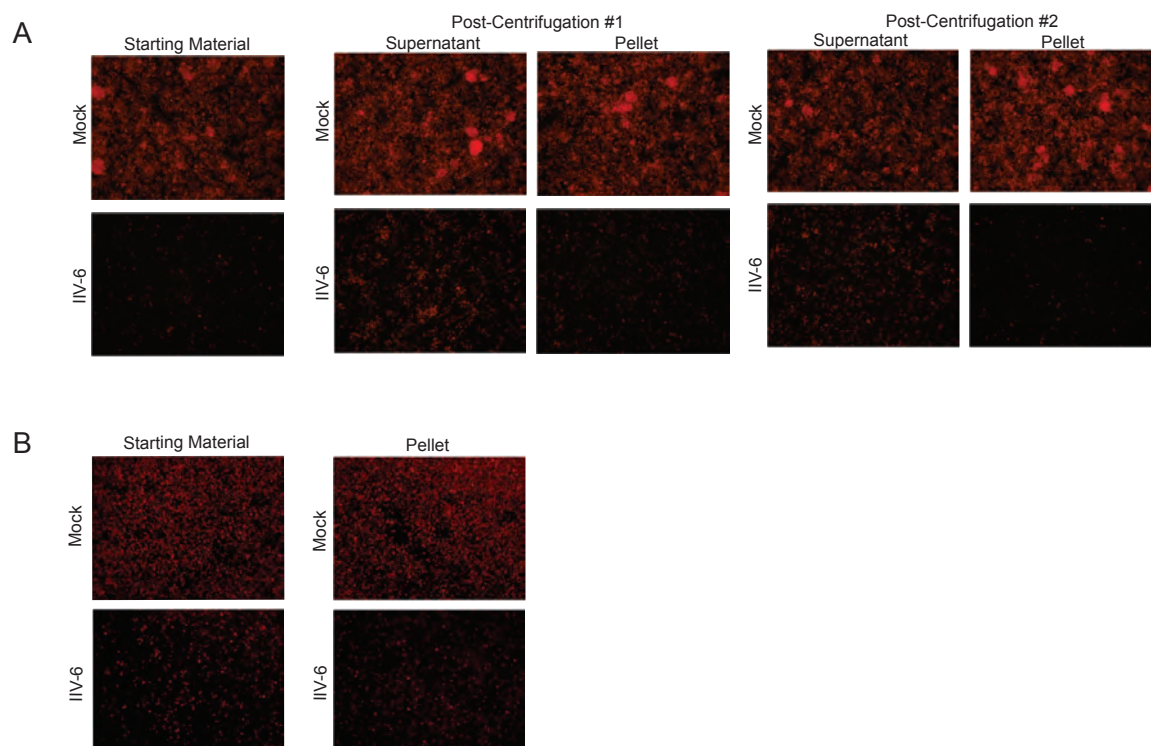


Figure 3.6 Exosome-containing fractions confer antiviral activity

A) Exosomes were isolated by ultracentrifugation from mock-treated or IIV-6 infected DL-1 cells. Starting material is non-centrifuged conditioned media, shown as a control. After the each centrifugation, supernatant was removed and the exosome-containing pellet was resuspended in a comparable volume of serum-free Schneider's media. Samples of each were used to treat naive DL-1 cells, and cells were subsequently infected with mCh-IIV-6 at an MOI of 2 for 24 hours and then imaged. **B)** Exosomes were isolated using "Total Exosome Isolation from Cell Culture Media" kit from Invitrogen, per the manufacturer's instructions, and the pellet was resuspended in a comparable volume of serum-free Schneider's media before being added to DL-1 cells. DL-1 cells were then infected with mCh-IIV-6 at an MOI of 2 for 24 hours before imaging. Starting material is untreated conditioned media.

CM (Figure 3.7). This suggests that endogenous reverse transcriptases are not required for the antiviral effects of IIV-6 CM.

Additionally, Nanoparticle tracking analysis was performed using Nanosight (Malvern Instruments) in order to further characterize the particles found in conditioned media harvested from both mock-treated and IIV-6 infected cells (Figure 3.8). This analysis revealed a population of particles with a mode size of 109 nanometers in diameter that were present in IIV-6 conditioned media, and absent in the conditioned media obtained from mock-treated cells. As a control, purified IIV-6 was also analyzed to rule out that these peaks were the result of contaminating virus. As expected, purified IIV-6 was slightly larger than the peak attributed to exosomes, with a mode appearing at 212 nanometers.

To further characterize these exosomes, conditioned media from IIV-6 infected or mock cells were subjected to 30, 50, or 100 kilodalton cutoff filters, and the retentates were boiled at 95°C for five minutes. These concentrated samples were run on an SDS-PAGE gel and silver stained. A single band was visible at 34kD (Figure 3.9A). This band was isolated and sent out for Mass Spec analysis. Surprisingly, the band sequence most closely aligned with the capsid protein of FHV (data not shown). While FHV is commonly present as a latent infection in many cell lines (Flynt et al., 2009; Wu et al., 2010), this band was absent from conditioned media derived from mock infected cells. Its possible that these cells are infected with FHV at a level below the limit of detection of this

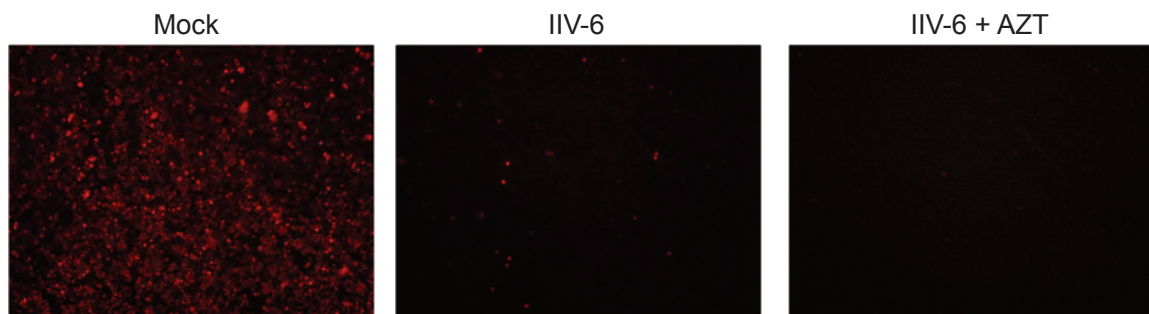


Figure 3.7 Antiviral effects are not dependent upon endogenous reverse transcriptase activity

Conditioned media was isolated from mock-treated cells, WT-IIV-6 infected cells, or cells treated with azidothymidine (AZT) for one hour prior to WT-IIV-6 infection. Naive S2* cells were treated with indicated CM and infected with mCh-IIV-6 for 24 hours prior to imaging.

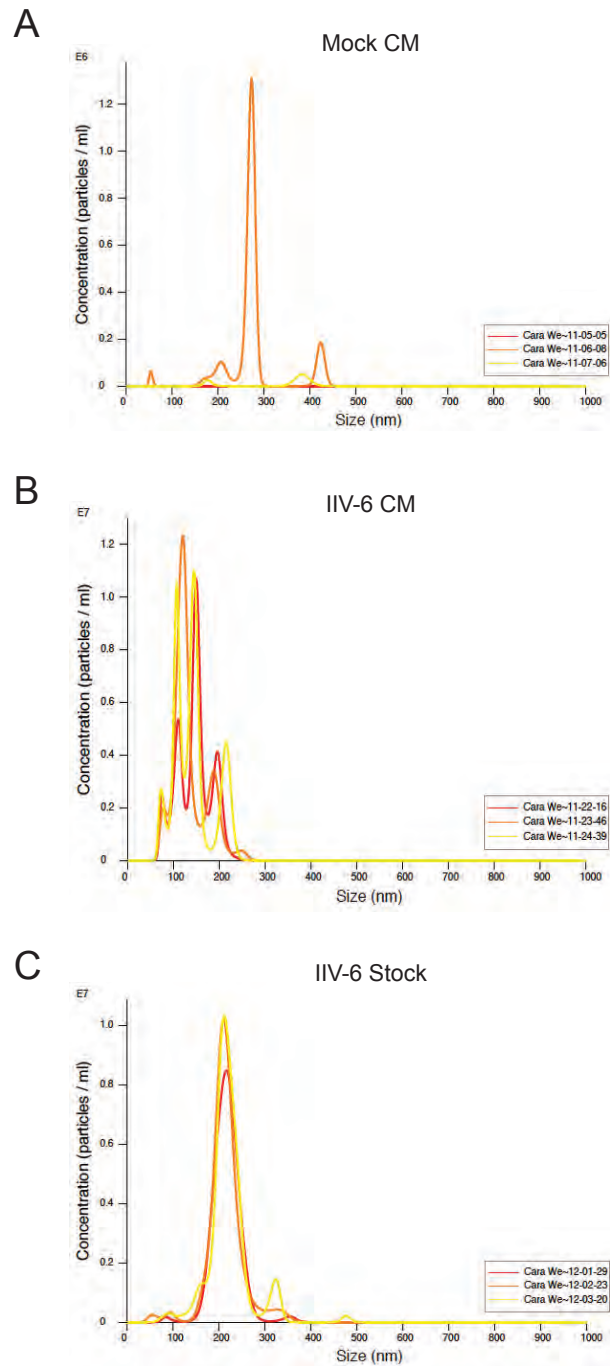


Figure 3.8 Exosome-like Particles in IIV-6 Conditioned Media

Nanosight analysis of conditioned media from **A)** Mock or **B)** IIV-6 infected DL-1 cells grown in serum-free Schneider's media. **C)** WT IIV-6 stock as a control for virus particle size. **A-C)** Each line represents the distribution of particle size in a given sample.

assay, and that IIV-6 infection is facilitating its replication to a detectable level by inhibiting the immune pathways preventing FHV from replicating.

Viruses are capable of modifying the host environment to best suit their needs, and the by-product of this can result in blocking infection of another virus, or in other instances, blocking restriction factors to facilitate its replication (Gammon et al., 2014). Since our method of purifying conditioned media does not exclude the possibility that contaminating FHV has purified with the exosomes, we asked whether FHV could block IIV-6 infection by infected cells with FHV for one hour and then infecting with IIV-6. Cells co-infected with FHV died at a high MOI of FHV infection, and therefore were not supporting mCh-IIV-6 infection. However, at lower MOIs, FHV did not inhibit the ability of mCh-IIV-6 to infect cells (Figure 3.9B). These cells do appear dimmer at MOI of 1 and 0.1, presumably because these cells are under considerably more duress than cells not co-infected with FHV.

Since RNAi relies on sequence specificity to exert protection, we asked whether IIV-6 CM protected S2* cells from other viruses, or whether these effects were specific to IIV-6. We treated S2* cells with mock or IIV-6 CM and then challenged with either VSV-luciferase (VSV-LUC), VSV-GFP, SINV-luciferase (SINV-LUC), or SINV-GFP. Infections in IIV-6 CM with either VSV-LUC or VSV-GFP resulted in increased infections compared to infections in Mock CM (Figure 3.10A, B). These experiments were performed in complete conditioned media, not with purified exosomes, and it may be that additional non-exosomal factors

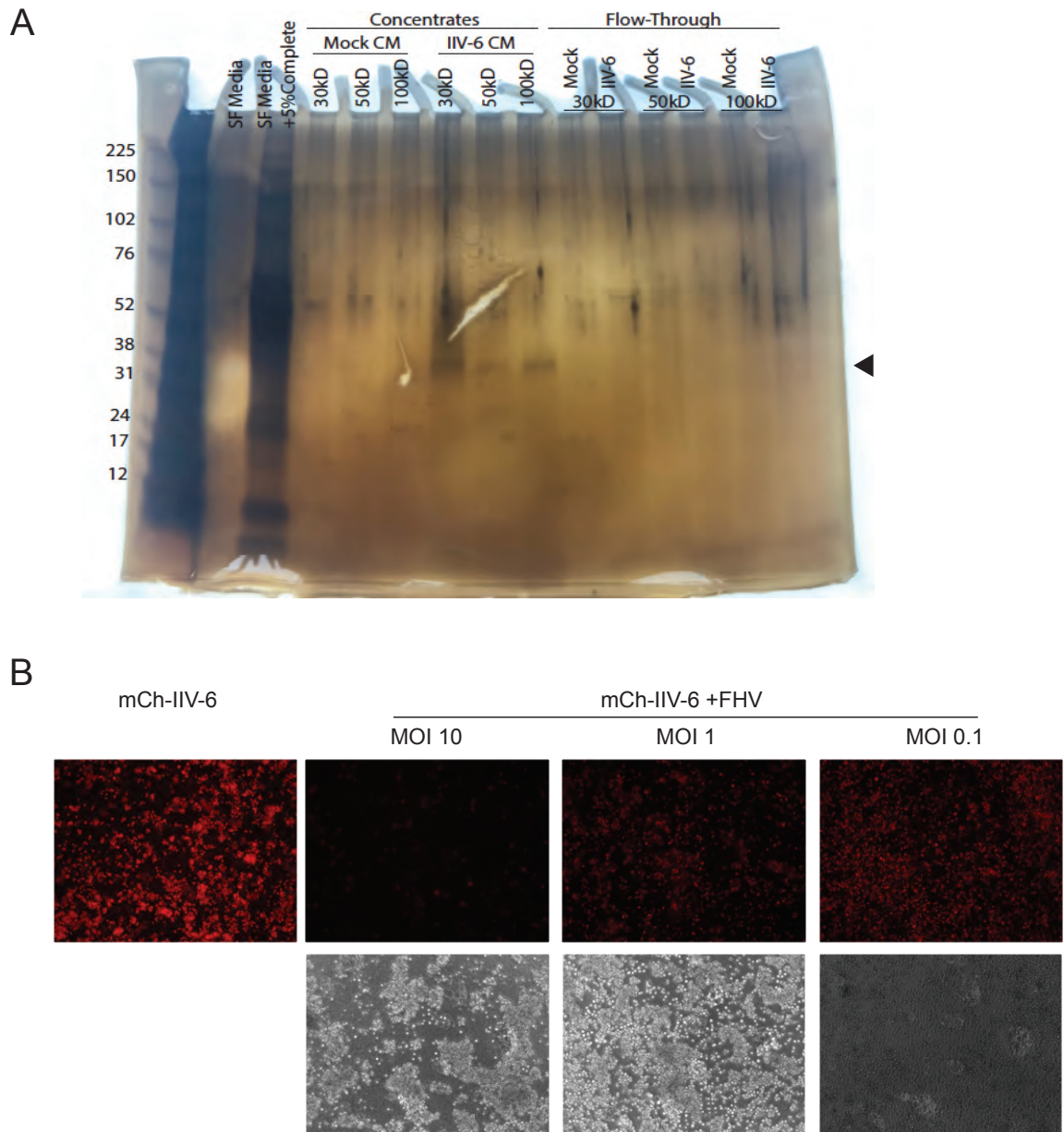


Figure 3.9 FHV does not inhibit mCh-IIV-6 infection
A) A silver-stained SDS-PAGE gel from concentrated CM preparations. Arrow indicates a single band present in lanes containing IIV-6 CM. This band was cut out and analysed by MS/MS, and was found to be a capsid protein of FHV. **B)** DL-1 cells were infected with FHV for one hour at various MOI before infecting with mCh-IIV-6. Infection with mCh-IIV-6 alone is shown as a control.

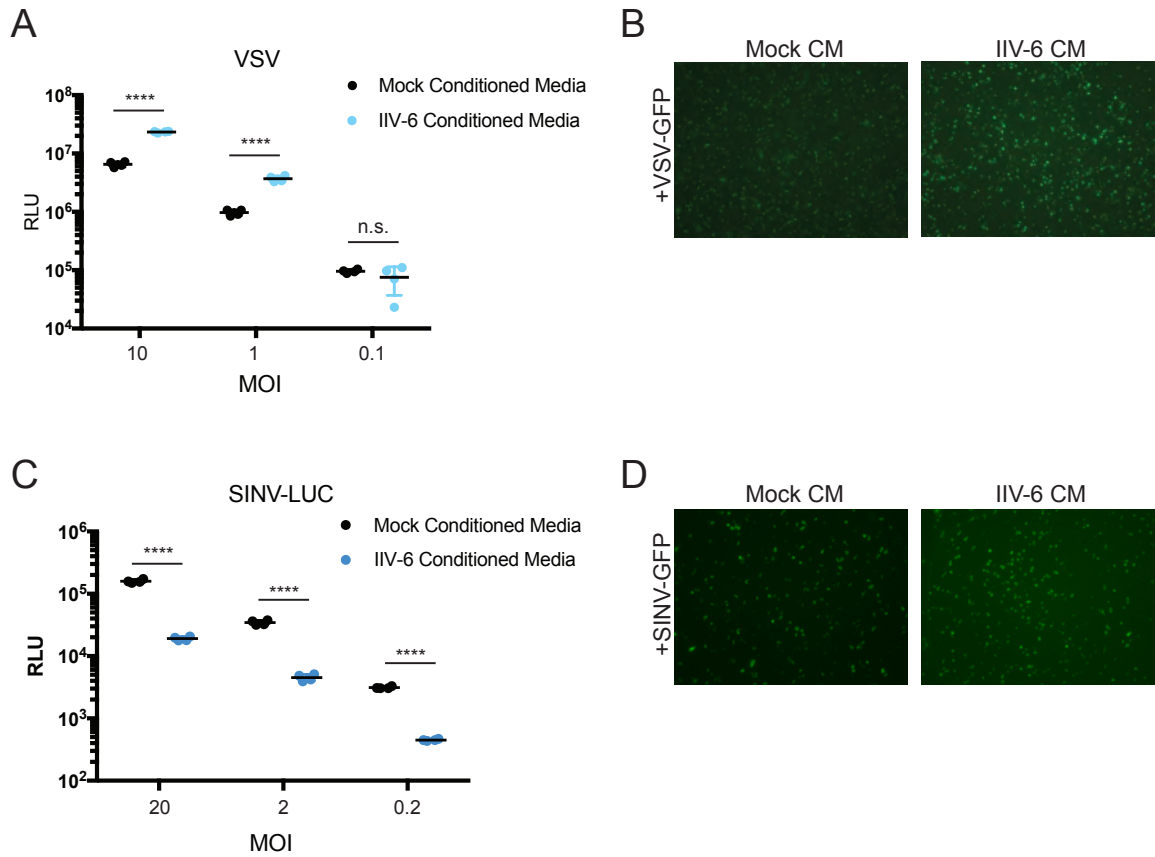


Figure 3.10 Varying Effects of IIV-6 Conditioned Media on RNA Virus Infections

A, B) Treatment of S2* cells with IIV-6 CM results in enhanced infection of VSV. **A)** Cells treated with IIV-6 CM and infected with VSV-luciferase. Cells were harvested and lysed after 24 hours of VSV-LUC infection. **B)** S2* cells treated with Mock or IIV-6 CM and infected with VSV-GFP. Cells were imaged after 24 hours of infection. **C, D)** Treatment of S2* cells with IIV-6 CM has varying effects on infection of SINV. **C)** Cells treated with IIV-6 CM and infected with SINV-luciferase. Cells were harvested and lysed after 24 hours of SINV-LUC infection. **D)** S2* cells treated with Mock or IIV-6 CM and infected with SINV-GFP. Cells were imaged after 24 hours of infection. **A, C)** Error bars indicate standard deviation, black bars indicate mean, with statistical analysis by two-way ANOVA, and corrections for multiple comparisons using Holm-Sidak testing. RLU, relative light units. ns, not significant; ****, $p < 0.0001$.

are responsible for this increased infectivity. For example, the cytokine Dieldel has been shown to enhance VSV infection through suppression of the IMD pathway. It is possible that Dieldel (or other IMD inhibitors, such as pirk) is present in conditioned media, and further studies using purified exosomes will be needed to determine whether exosomes or other factors in conditioned media are responsible for this effect.

However, infections with SINV showed varying results. Infections in IIV-6 CM with SINV-LUC resulted in a ten-fold reduction in SINV compared to SINV infections in Mock CM (Figure 3.10C). However, infections using SINV-GFP resulted in no difference in infections between Mock or IIV-6 CM treated cells (Figure 3.10D). It is possible that viral replication is restricted, but the point of restriction is after GFP expression, but before the luciferase reporter is turned on. The discrepancies between these two SINV strains will be explored in future studies by harvesting these cells and examining SINV replication via plaque assay.

Discussion

While some aspects of the *Drosophila* innate immune system bear striking homology to mammalian innate immune pathways, other critical aspects of mammalian innate immunity are conspicuously absent. *Drosophila* lack the type-1 interferon system, the hallmark of the mammalian antiviral response. While the RNAi system can be seen as an analogous pathway in response to

dsRNA, RNAi has not been shown to provide any lasting benefit whereas the type-1 interferon system not only elicits an array of various antiviral molecules, but also sets up the adaptive immune response to ensure long-lasting protection is created. The discovery of a long-lasting RNAi response in flies forces us to reconsider these ideas.

A striking difference between the report by Tassetto et al. and our work is the requirement of endogenous reverse transcriptase (RT). The protection provided by the exosome-like vesicles induced by SINV are RT-dependent, and activity is abolished in the presence of AZT. We find that the protection induced by IIV-6 CM is RT-independent, and find no loss of protection after AZT treatment, pointing to a distinct mechanism of protection. IIV-6 induces an RNAi response from areas of the genome with convergent transcription (Bronkhorst et al., 2012; Kemp et al., 2013), but presumably the dsRNA produced by these convergent transcripts is not significantly contributing to the antiviral activity of our exosomes. One possibility is that viral genomes are directly processed within hemocytes to produce novel extrachromosomal DNA for secondary dsRNA biogenesis. Future experiments inhibiting the siRNA pathway in secondary cells will have to be performed in order to address this question. Another possibility is that exosomes contain novel antiviral molecules that act independently of the siRNA pathway. Analysis of exosome components, including protein and lipid analyses, will be the basis of future experiments.

Our data shows that IIV-6 induces a protective response upon infection. This response is robust, induced within 12 hours, exosome-mediated, and independent of endogenous reverse transcriptase activity. The precise mechanisms of this activity, and whether they can be applied to other viruses, will be the directions of future studies.

Materials and Methods

Conditioned Media Preparations

S2* or DL-1 cells were infected with WT-IIV-6 at an MOI of 2 for 24 hours in a 27°C incubator (Figure 3.2A). Conditioned media was harvested from the cells by removing the supernatant and centrifuging the cells at 15,000g for 20 minutes at 4°C. The supernatant was removed and centrifuged again under the same conditions. The supernatant from this spin was then vacuum-filtered through a 0.1µm filter unit (Millipore). This filtration was repeated. As the virus capsid is 160-180 nanometers in diameter, this filtration removed all virus particles (Figure 3.2B), eliminating the possibility that the protection is caused by an inability of IIV-6 to super-infect cells.

For assays where protein analysis or exosome isolations were performed, cells were grown in serum-free media. Serum contains exosomes, as well as an abundance of protein, necessitating serum-free conditions for these assays. Cells were grown in complete media and infected for 6 hours in complete media. At six hours, cells were washed three times in equal or greater volumes of PBS

than the complete media used, and then incubated in serum-free media for 18 hours at 27°C. DL-1 cells are adherent, and PBS washings were performed in 10cm dishes the cells are grown in, while S2* cells were spun in a table top centrifuge at 2000g for 5 min after each PBS resuspension.

Exosome Isolations

Conditioned media was prepared from DL-1 cells as described, in serum-free media. Media was spun at 100,000g for 90 minutes in a 45Ti rotor (Beckman), using serum-free media to fill and balance the centrifuge tubes. Supernatant was carefully removed, and pellet was resuspended in ten milliliters PBS, and spun again at 100,00g in a 90Ti rotor (Beckman), using additional PBS to fill and balance the centrifuge tubes. Supernatant was carefully removed and pellets were resuspended in one milliliter of PBS. Exosome pellets were stored at 4°C for short-term storage and -80°C for long-term storage.

Where noted, exosomes were isolated using exosome isolation kits. Conditioned media was prepared from DL-1 cells in serum-free media, as described above. Total Exosome Isolation from cell culture media (Invitrogen, CAT#4478359), was used following the manufacturer's protocol.

Proteinase Treatment

Proteinase K bound to agarose beads (Sigma) was resuspended in milli-Q water, washed three times, and resuspended at a final concentration of 10mg/mL. Dilute Proteinase K was added to 500µL of conditioned media and incubated

at 37°C for one hour, per the manufacturers instructions. Control samples of conditioned media were incubated at 37°C without PK, for one hour.

Nanoparticle Tracking Analysis

Particles were tracked using a Nanosight (Malvern Technologies). For each sample, particles were tracked for 30 seconds before pushing an additional sample volume through the machine. This was performed 3 times, and 3, 30-second tracks were recorded per sample.

Preface to Chapter IV

Florentina Rus assisted with c-Rel Western blots and produced c-Rel antibody.

Cara West performed the remaining experiments.

Cara West and Neal Silverman designed the experiments.

**CHAPTER IV:
IMD INHIBITION BY IIV-6**

Abstract

The host immune response and virus-encoded immune evasion proteins pose constant, mutual selective pressure on each other. The immune evasion proteins that a virus encodes also indicates what host pathways are effective in protecting against that virus's replication. Here, we show that IIV-6 is capable of inhibiting the two *Drosophila* NF- κ B pathways, IMD and Toll. AMP induction downstream of either pathway is suppressed when cells infected with IIV-6 are stimulated with NF- κ B ligands. We find that cleavage of both IMD and Relish, two key points in IMD signaling, occurs as normal during IIV-6 infection, indicating that the mechanism of inhibition is farther downstream, at the level of Relish nuclear translocation or even promoter binding. Additionally, flies co-infected with IIV-6 and a Gram-negative bacterium, *Erwinia carotovora carotovora*, succumb to infection more rapidly than flies singly infected with either the virus or the bacterium. These demonstrate how pre-existing infections can have a dramatic and negative effect on secondary infections.

Introduction

The host immune system and the viruses that challenge it face constant, mutual, selective pressure for survival. This perpetual arms race has created a plethora of ways that the cell may thwart viral replication, and also a plethora of novel immune evasion tactics that the virus uses to evade the immune response, in some cases even stealing genes from their hosts to suit this purpose (Lamiabile et al., 2016b). In order to evade the innate immune response, viruses, especially large DNA viruses, encode a variety of immune evasion proteins to facilitate their replication. HIV-6 is a large DNA virus with an estimated 215 open reading frames (ORFs) (Ince et al., 2010). This suggests that it most likely encodes for many proteins to evade host detection, in addition to its known suppressor of RNAi (340L) (Bronkhorst et al., 2014), and Inhibitor of Apoptosis Protein (IAP) (193R) (Chitnis et al., 2008; Chitnis et al., 2011). For example, the model poxvirus *Vaccinia* encodes a variety of NF- κ B inhibitors (Smith et al., 2013).

The interplay between the virus and host, particularly what pathways the virus is devoting its resources to inhibit, provides information as to which pathways pose the most threat to viral replication. If a virus is devoting its resources to shutting down a host pathway, this is a good indication that the pathway has applied selective pressure against the virus.

Whether the Toll and IMD pathways play a role in antiviral defense has been a topic probed in several studies, often with mixed or conflicting results (Avadhanula et al., 2009; Costa et al., 2009). Here, we have examined whether

IIV-6 is capable of inhibiting these *Drosophila* NF- κ B pathways. We find that cells infected with IIV-6 have suppressed AMP production, while other genes (*Turandots*, JNK targets) remain induced, suggesting that this is not a global suppression of host transcription. Surprisingly, the cleavage of both IMD and Relish, key signaling events in the IMD pathway, remain intact. This indicates that the blockage must be at the level of Relish translocation into the nucleus, promoter binding, or transcription. In addition, flies infected with IIV-6 are more susceptible to the Gram-negative bacteria *Erwinia carotovora carotovora*.

Results

AMP Production is Suppressed in the Presence of IIV-6

One indicator of whether a pathway functions in suppressing viral infections is whether the virus encodes an inhibitor to that protein. Given the scarce and somewhat conflicting data on the antiviral effects of the NF- κ B pathways in response to viral infections (Costa et al., 2009; Huang et al., 2013; Lamiable et al., 2016b; Tsai et al., 2008; Zambon et al., 2005), we decided to test whether IIV-6 was capable of inhibiting these pathways. In order to test whether IIV-6 encodes an NF- κ B inhibitor, S2* cells were stimulated with 1 μ M 20-hydroxyecdysone, (EcD) for 18 hours followed by infection with IIV-6 for 6 hours. Treatment with EcD is required to induce the expression of the receptor PGRP-LC in S2* cells (Rus et al., 2013). After 6 hours of IIV-6 infection, cells were then stimulated with 2 μ g/mL of peptidoglycan (PGN). Cells were harvested for RNA

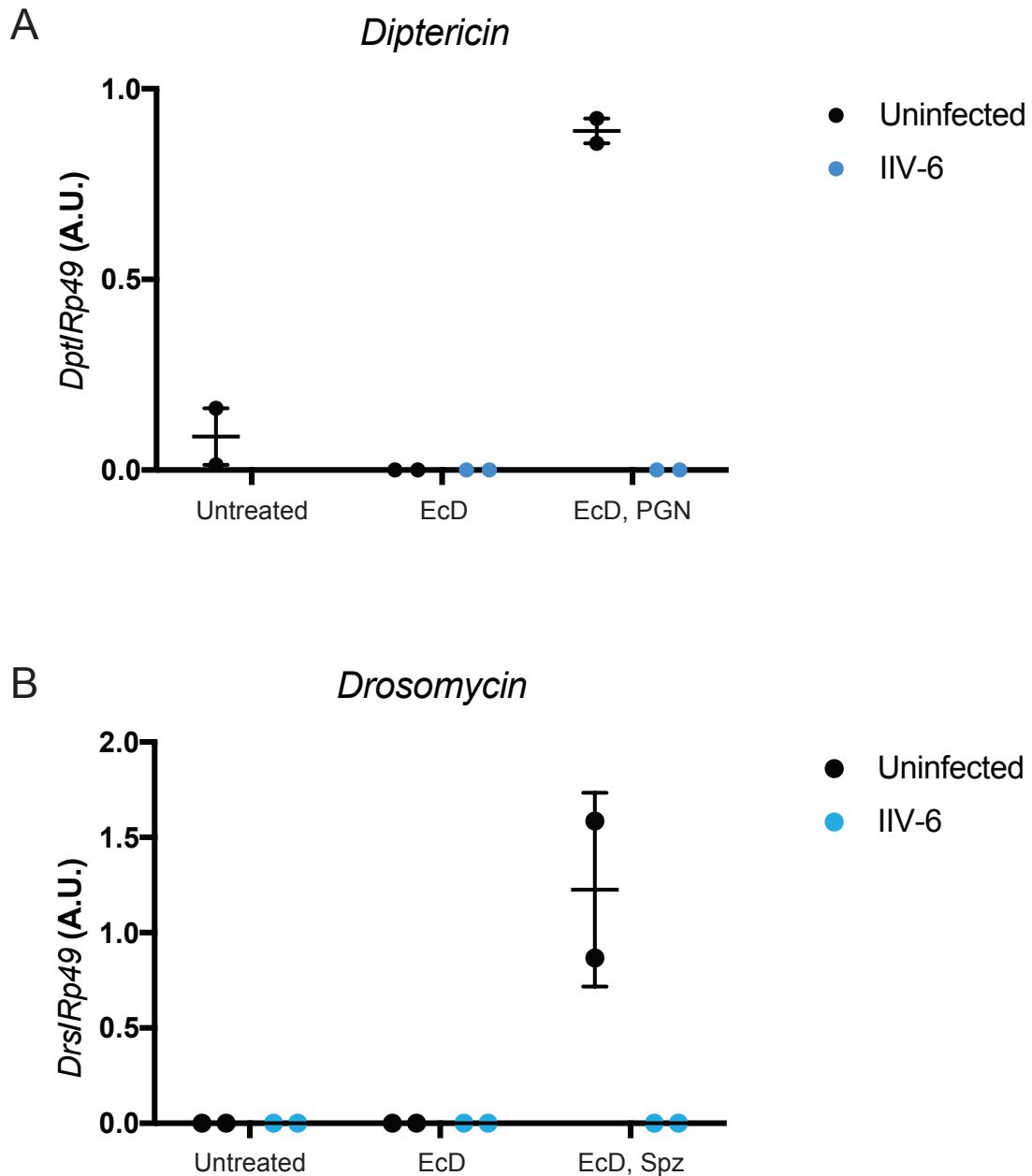


Figure 4.1 IIV-6 inhibits Imd and Toll Signaling

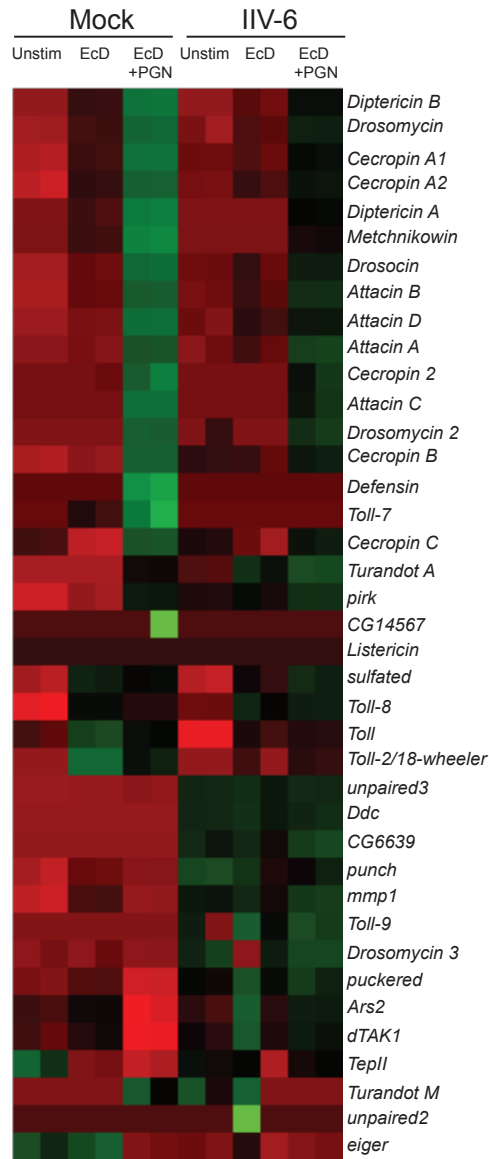
A) S2* cells were treated with 20-hydroxyecdysone (EcD) as indicated for 18 hours and then infected with IIV-6 (blue circles) or uninfected (black circles) for six hours. Cells were then stimulated with DAP-type PGN for six hours, where indicated. *Diptericin* levels were monitored by qRT-PCR. **B)** S2* cells were treated with 20-hydroxyecdysone (EcD) as indicated for 18 hours and then infected with IIV-6 (blue circles) or uninfected (black circles) for six hours. Cells were then stimulated with cleaved spätzle for 18 hours, where indicated. *Drosomycin* levels were monitored by qRT-PCR.

isolation after 6 hours of PGN treatment, and analyzed by QRT-PCR. Induction levels of the IMD-dependent AMP *Diptericin* were strongly reduced (Figure 4.1A).

To determine whether the IMD pathway is uniquely targeted by this inhibition or whether this is a general NF- κ B blockage, we cultured S2* cells in a similar manner, stimulating with EcD for 18 hours, infecting cells with IIV-6 for 6 hours, and then stimulating with the Toll ligand, cleaved spätzle, for 18 hours. We found that Toll signaling was similarly affected by IIV-6 infection (Figure 4.1B). To examine both of these pathways more broadly, we utilized NanoString nCounter Analysis, with a custom designed codeset probing 139 immune-related genes, including all of the known AMPs. Surprisingly, all of the antimicrobial peptides were down-regulated in IIV-6 infected samples stimulated with IMD (Figure 4.2A) or Toll ligands (Figure 4.2B) compared to uninfected controls stimulated with IMD or Toll ligands (Figure 4.2).

Notably, we also found groups of genes being induced by virus infection; the JNK targets *puckered* and *punch*, the p38b-dependent genes *upd3* and *Ddc*, and *Ars2*, which has been shown to function in antiviral defense (Figure 4.2A, 4B) (Sabin et al., 2009). Since JNK signaling can be initiated through TAK1, the fact that JNK targets are being transcribed suggests that IMD signaling is being initiated, and signaling successfully occurs through at least TAK1. This suggests that the virus is not simply shutting down all host transcription, but is specifically targeting the AMPs.

A



B

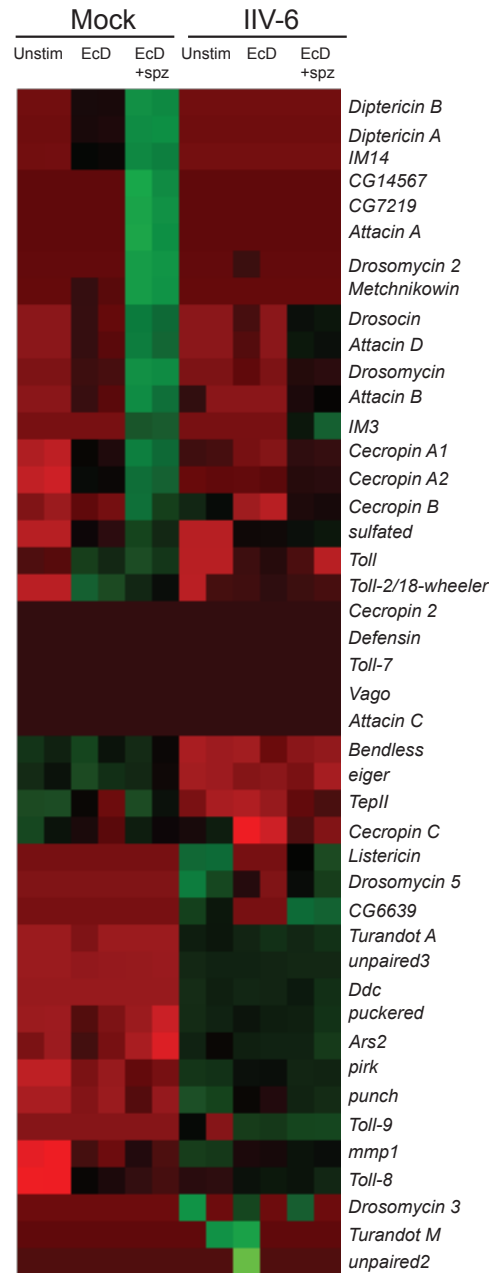


Figure 4.2 Both Imd and Toll -regulated AMPs are suppressed by IIV-6 infection
A) S2* cells were treated with 20-hydroxyecdysone (EcD) for 18 hours, and then infected with IIV-6 for 6 hours. Cells were then stimulated with peptidoglycan (PGN) for 6 hours prior to RNA isolation. **B)** S2* cells were treated with 20-hydroxyecdysone (EcD) for 18 hours, and then infected with IIV-6 for 6 hours. Cells were then stimulated with cleaved spaetzle (spz) for 18 hours prior to RNA isolation. **A, B)** Heatmap showing clustered mRNA levels of AMP genes in the presence or absence of virus and pathway stimulation by Nanostring nCounter analysis. Biologically independent duplicates are shown.

Since the virus is suppressing the NF- κ B pathways, this suggests that these pathways function to limit viral replication. We decided to look back at previous Nanostring data from whole flies to determine whether IIV-6 was inducing AMPs upon infection. IIV-6 appears to modestly induce AMP induction two-fold over PBS-injected controls 12 hours post-infection (Figure 4.3A'). However, by 24 hours, this induction has returned to or, in some instances, below baseline levels (Figure 4.3).

AMP Suppression is Downstream of IMD and Relish

Given the expertise of the Silverman laboratory, we focused on the IMD pathway in our attempt to tease apart the mechanism of inhibition by IIV-6. As IMD signaling requires cleavage of IMD by the caspase 8-like Dredd (Kim et al., 2014; Paquette et al., 2010), we probed for cleaved IMD in IIV-6 infected or uninfected cell lysates. We found robust cleavage of IMD upon PGN stimulation in both the presence and absence of IIV-6, indicating that the blockage of IMD signaling must occur downstream of IMD cleavage (Figure 4.4).

To probe further downstream, we then utilized a C-terminal Relish antibody to determine if Relish was being cleaved in the presence of IIV-6. As expected, samples mock treated or infected with IIV-6 alone show a prominent 110 kD band indicating full-length Relish, and control samples treated with EcD and PGN show complete processing of full-length Relish. To our surprise, Relish was fully

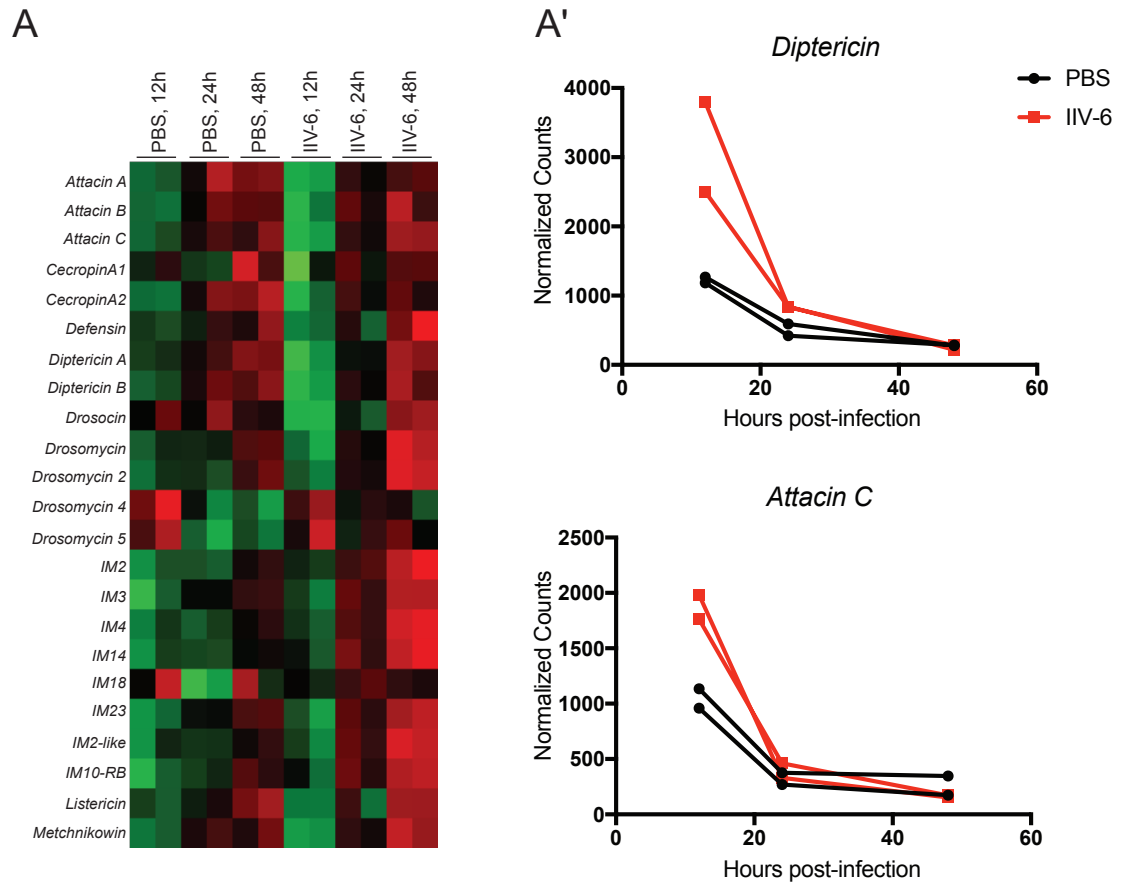


Figure 4.3 Some AMPs are elevated *in vivo* upon IIV-6 infection, before returning to baseline.
A) Heatmap of mRNA levels for AMP genes following IIV-6 infection of adult male w^{1118} flies for the indicated time points, assayed by Nanostring nCounter. RNA was isolated from PBS-injected flies at the same time points as a control. Biologically independent samples were analyzed in duplicate. **A')** Detailed comparison of mRNA levels for selected Imd-regulated AMP genes following IIV-6 infection of adult w^{1118} flies for 12, 24 and 48 hours assayed by Nanostring nCounter.

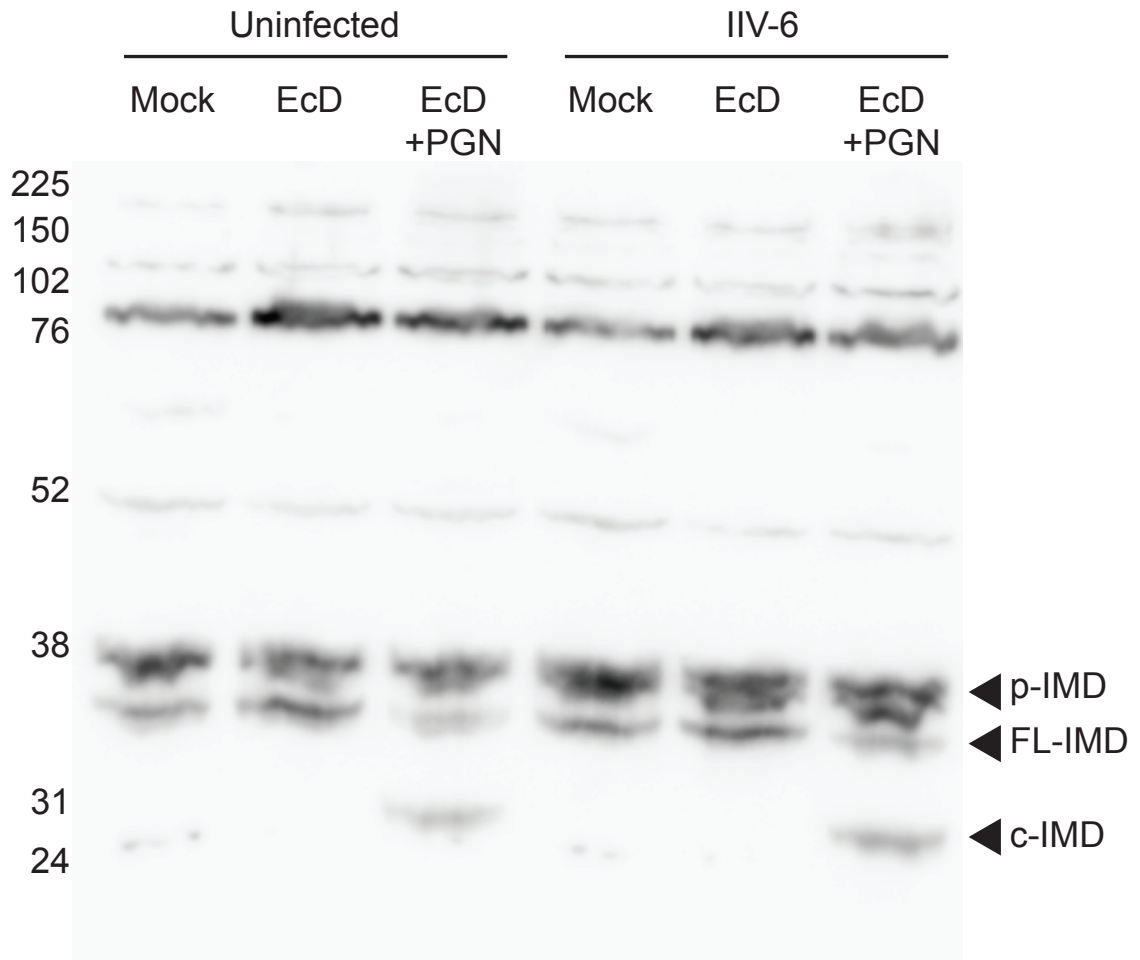


Figure 4.4 Imd is cleaved upon PGN stimulation in the presence of IIV-6

Cells were stimulated with 20-hydroxyecdysone (EcD), where indicated, for 18 hours. Cells were infected with IIV-6, where indicated, for six hours. Samples were then stimulated with PGN for 15 minutes, where indicated, and lysed in standard lysis buffer. Endogenous IMD was monitored by immunoblot. Arrows indicate the sizes of cleaved, full-length, or modified forms of IMD. c-Imd, cleaved IMD. FL-IMD, Full-length IMD. p-IMD, phospho-IMD.

cleaved in the presence of IIV-6, upon PGN treatment, even at a relatively high MOI (Figure 4.5, lanes 4-6).

Suppression of AMPs is mediated by an IE gene or is Virion-associated

Strategically, most—but not all—immune evasion proteins are immediate early genes. Shutting down the host defense as quickly as possible allows for the virus to achieve high levels of replication. Cells were treated with the viral polymerase inhibitor cidofovir, or infected with heat or UV-inactivated virus to determine whether inhibition of IMD signaling required DNA replication or transcription. *Diptericin* levels remained reduced compared to uninfected controls in all treatments, indicating that the inhibition was the result of immediate early genes, or associated directly with the virion.

Flies infected with IIV-6 are more susceptible to bacterial infection

Together, these results indicate that infection with IIV-6 results in the global suppression of NF- κ B signaling in flies— a major component of the immune response to bacteria and fungal infections. This suggests that flies infected with IIV-6 are highly susceptible to other infections. To test this hypothesis, we infected *Drosophila* adults with IIV-6 for seven days. On day eight post-IIV-6 infection, we pricked one group with a sterile needle, and the other group of flies with a needle dipped in *Erwinia carotovora carotovora*, a Gram-

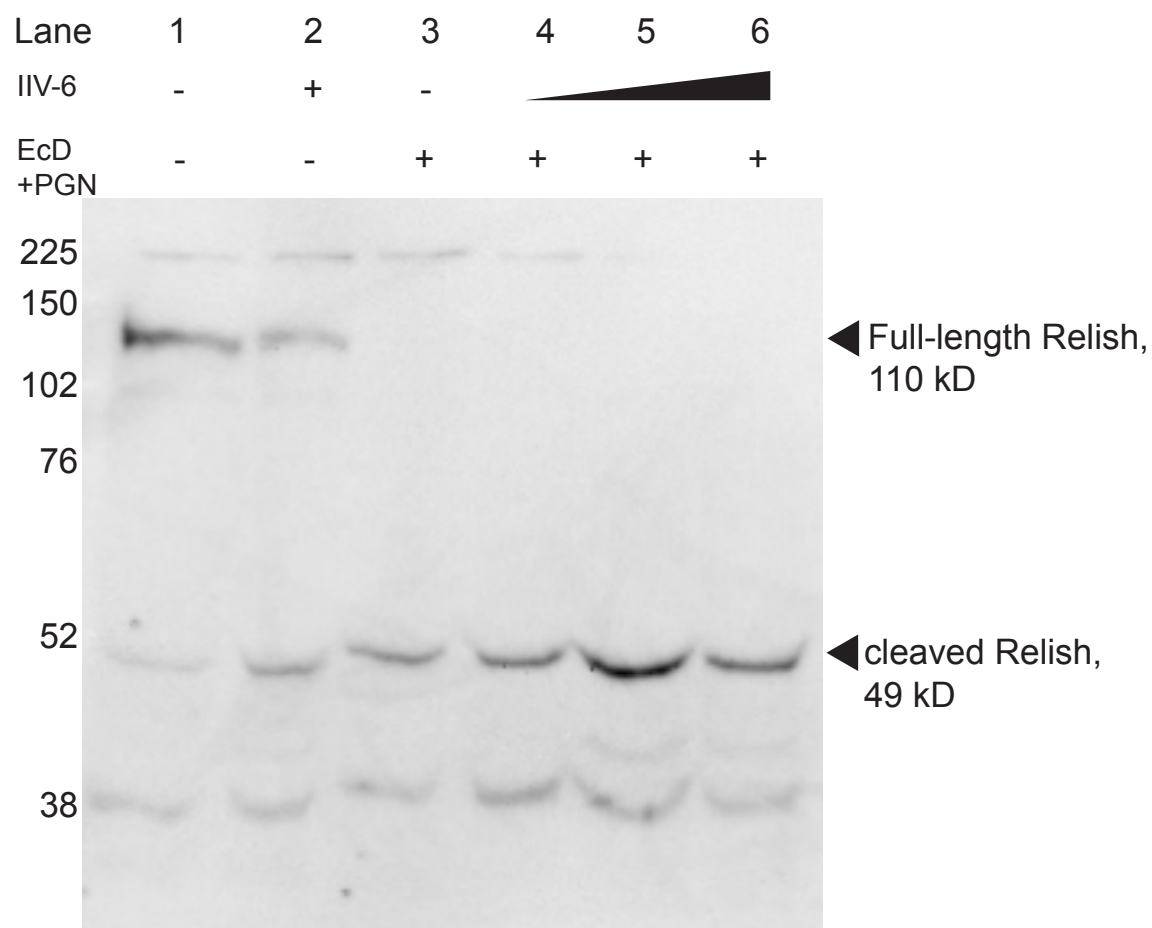


Figure 4.5 Relish is cleaved upon PGN stimulation in the presence of IIV-6

Cells were stimulated with 20-hydroxyecdysone (EcD) where indicated for 18 hours and infected with IIV-6, as indicated, for six hours. Samples stimulated with EcD were then stimulated with PGN for 15 minutes, and lysed in standard lysis buffer. Endogenous Relish was probed by immunoblot using a C-terminal Relish antibody. MOI used was as follows: Lane 2: MOI =2, Lane 4: MOI= 0.2, Lane 5: MOI = 2, Lane 6: MOI=5.

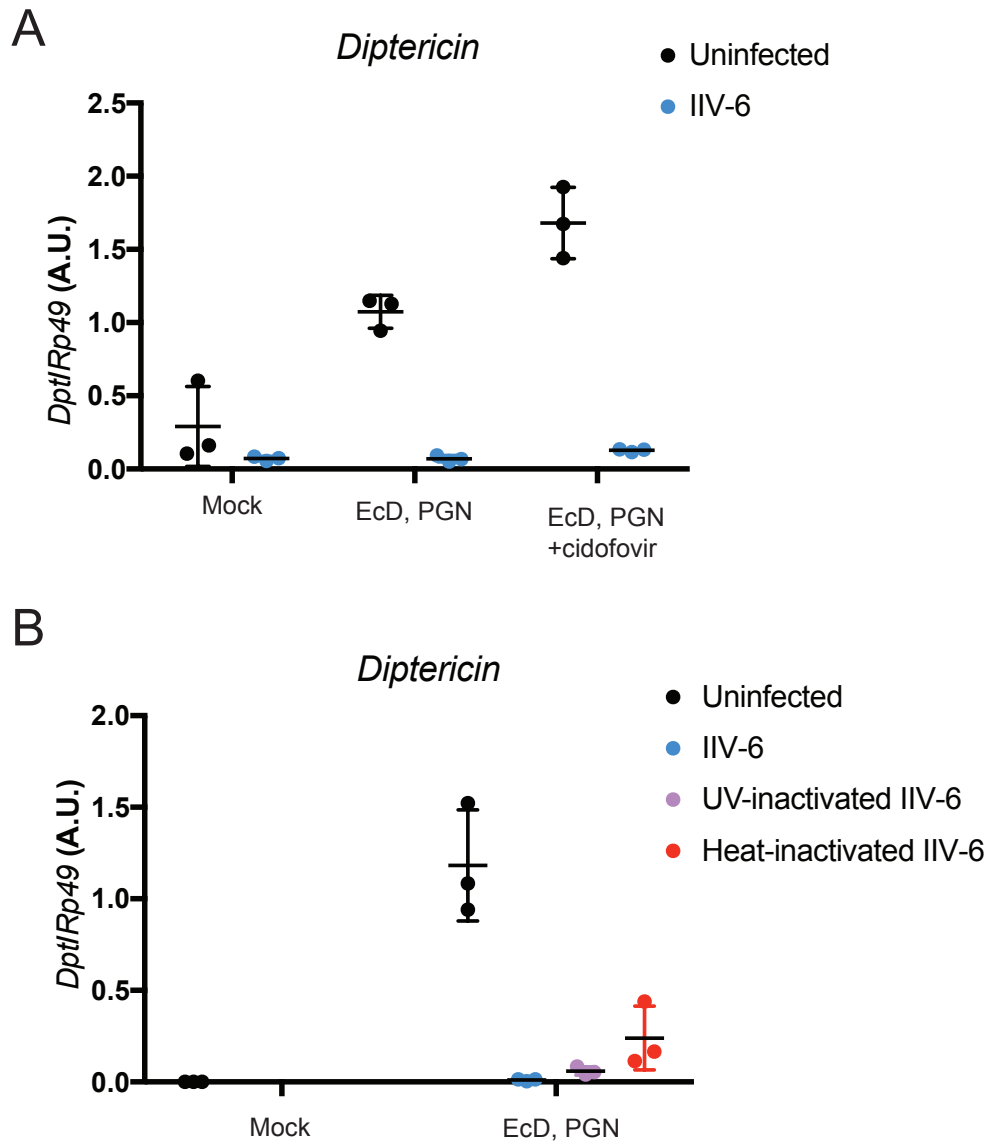


Figure 4.6 Viral replication is not needed for AMP suppression

A,B) S2* cells were treated with 20-hydroxyecdysone (EcD) as indicated for 18 hours and then infected with IIV-6 (blue circles) or uninfected (black circles) for six hours. Cells were then stimulated with DAP-type PGN for six hours, where indicated. Diptericin levels were monitored by qRT-PCR. **A)** Cells were treated with cidofovir, a viral polymerase inhibitor, where indicated. Mock cells untreated with EcD, PGN, or cidofovir, are shown as a control. **B)** S2* cells were infected with IIV-6 (blue circles), or treated with UV- (purple circles) or heat- (red circles) inactivated IIV-6. Uninfected controls are shown in black. Each data point is a biologically independent replicate, n= 3. Error bars represent standard deviation.

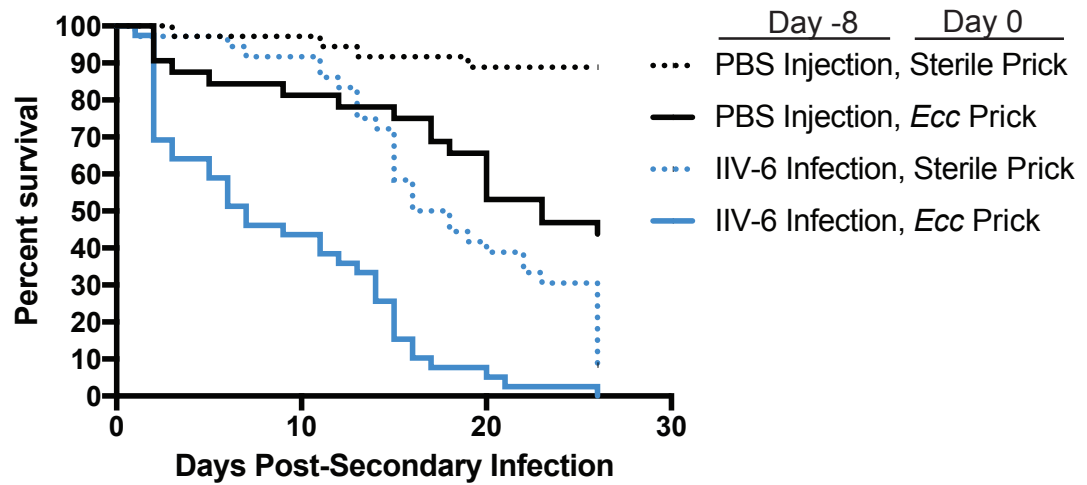


Figure 4.7 Flies co-infected with IIV-6 succumb more rapidly to *Ecc* infection
 Adult flies were infected with IIV-6 (blue lines) or PBS-injected (black lines) for 8 days prior to bacterial infection. On day 0, flies were pricked with *Ecc* (solid lines) or were sterile pricked (dashed lines) with a microsurgery needle. Flies were counted daily for survivors.

negative bacterium that is non-lethal in healthy, immunocompetent flies. By day seven post-secondary (bacterial) infection, 50% of the flies infected with both IIV-6 and *Ecc* had succumbed to infection (Figure 4.7). In contrast, flies that had been mock-injected with PBS prior to *Ecc* infection had a median survival time of twenty-three days post-secondary (bacterial) infection. This data shows that an underlying viral infection can have a dramatic effect on a secondary infection, causing flies to be far more susceptible to a bacterial infection that they would normally clear.

Discussion

Here, we show that IIV-6 infection interferes with the antimicrobial peptide response upon stimulation with bacterial or endogenous ligands through both NF- κ B pathways. In addition, we find that flies infected with IIV-6 succumb to an otherwise non-pathogenic bacterial infection *in vivo*. Together, our data shows that an underlying infection with a DNA virus can dampen the immune response and dramatically alter the outcome of a secondary bacterial infection, turning an otherwise innocuous infection into a lethal one.

While it appears that IIV-6 is suppressing the AMP response by actively inhibiting the NF- κ B pathways, the mechanisms of inhibition remain unclear. It is possible that IIV-6 encodes an NF- κ B inhibitor that acts downstream of Relish translocation. Several examples of inhibitory proteins acting within the nucleus exist. NF- κ B inhibitors functioning within the nucleus exist in VACV (Sumner et

al., 2014) as well as African swine fever virus A238L (Revilla et al., 1998). VACV encodes an inhibitor of Type 1 IFN, C6, that functions post-STAT1 and 2 translocation into the nucleus and binding of the ISRE by binding the STAT2 transactivation domain (Stuart et al., 2016). Another VACV protein, N2 inhibits IRF3 within the nucleus (Ferguson et al., 2013). Future studies examining nuclear translocation of the Relish N-terminal, either by nuclear fractionation or microscopy, in the context of IIV-6 infection and PGN stimulation will address where this NF- κ B inhibitor functions downstream of Relish cleavage. If Relish does translocate into the nucleus, chromatin immunoprecipitation (ChIP) experiments should also be performed to determine whether Relish is still able to bind κ B sites. A far less exciting prospect, is the possibility that the virus is simply blocking host transcription, and AMPs are not being transcribed as a result. However, this is in contrast to our data showing the up-regulation of other genes in response to IIV-6, such as the *Turandots*. Additionally, other studies have shown that apoptosis-deficient cells trigger DAMPs to activate Toll signaling and Toll-dependent AMP production suggests that we should see increased AMPs (Ming et al., 2014). IIV-6 encodes at least one functional IAP, 193R (Ince et al., 2008). Future studies should be performed over-expressing 193R to determine if Toll signaling is activated.

Future studies will also be required to determine which viral gene(s) are responsible for AMP inhibition. Since other large DNA viruses, such as VACV, encode multiple inhibitors targeting NF- κ B pathways at various points in

mammalian NF- κ B signaling pathways (Smith et al., 2013), knock-down of single viral genes may not reveal any phenotype due to redundancies. Our work shows that IMD signaling is inhibited at a step downstream of Relish cleavage, however, we have not investigated whether Toll signaling is inhibited at a similar step, or via a different mechanism. IIV-6 may encode more than one NF- κ B inhibitor, targeting the IMD and Toll pathways at different points of signaling. Determining which viral gene(s) is(are) responsible may require both over-expression and knock-down studies. One approach to begin screening through the estimated 215 ORFs encoded by IIV-6 by BLASTing for known NF- κ B inhibitors from related DNA viruses, such as ASFV and VACV.

Our data suggests that the increased susceptibility to bacterial infection of flies co-infected with IIV-6 is due to the suppression of AMPs. However, we have not yet formally shown *in vivo* that infection with IIV-6 and subsequent infection with bacterial strains result in suppressed AMP production during the relevant time frame. *In vivo* data with IIV-6 alone suggests that AMP are produced early upon IIV-6 infection and are then suppressed by 12 hours, but earlier time points are required to show that AMPs are truly induced by the virus, and later time points are required to show that they remain suppressed 8 days later, prior to, and more importantly, after, bacterial challenge.

IIV-6 has a broad host range, infecting a large variety of insects with agricultural and economic importance. The slow replication cycle of this virus results in infected insects surviving for weeks, allowing it to spread amongst a

population, and consequently, leaving that population with an increased susceptibility to a secondary bacterial—or perhaps fungal—infection it would have otherwise cleared. Given the current decline of honey bees and colony collapse disorder, a persistent viral infection could further damage this already precarious population by increasing susceptibility to a range of other pathogens. While previous studies have ruled out IIV-6 as the causative agent of colony collapse disorder (Tokarz et al., 2011), it should be noted that IIV-6 infection may poise other species to increased vulnerability to a secondary infection.

In summary, we have shown that IIV-6 infection results in an inability to mount an AMP response upon stimulation through both the IMD and Toll pathways. It appears that the virus may briefly induce AMP expression before shutting it down, however, this requires a more careful examination. Suppression of IMD signaling occurs downstream of both IMD and Relish cleavage, suggesting a viral inhibitor may act at the level of nuclear translocation or DNA binding/transcription. Nanostring data showing that IIV-6 induces transcription of JNK targets suggest that the IMD pathway is being successfully activated through at least the branch point of TAK1, bolstering our data showing that Relish cleavage remains intact, and that the block is downstream of Relish cleavage. Strikingly, flies infected with IIV-6 that were subjected to subsequent infection with *Ecc* died more rapidly than flies singly infected with virus or bacteria. Further studies will be aimed at determining whether AMPs are also suppressed *in vivo* during co-infection as well as the mechanisms of Toll and IMD inhibition.

Materials and Methods

RNA Isolation and qRT-PCR

Total RNA from flies or S2* cells was extracted using TRIzol (Invitrogen). Samples were then DNase treated (RQ1, Promega) and RNA re-extracted by phenol-chloroform. cDNA was synthesized using iScript cDNA Synthesis kit (BioRad). Alternatively, the gDNAClear cDNA synthesis kit (BioRad) was used following TRIzol purification. qRT-PCR was analyzed normalizing to the housekeeping gene Rp49.

nCounter Analysis

The expression levels of 139 *Drosophila* immune genes were assayed from 100 nanograms of RNA via a customized Nanostring nCounter codeset. Two biological replicates of S2* cells were analyzed for each treatment and timepoint. The results were analyzed using nSolver 4.0 software according to the manufacturers instructions (NanoString Technologies, Seattle, WA, USA), and the heatmap was created using nSolver 4.0 software and JavaTree.

Fly Stocks and *in vivo* studies

Three to five day old *w¹¹¹⁸* flies, maintained at 22°C, were used for all experiments. Flies were injected intrathoracically with 32.2 nL of virus (1×10^4 TCID₅₀) or vehicle (PBS) using a Nanoject II (Drummond). For survival assays, a

minimum of fifty flies were used per treatment, per genotype and the dead were counted daily. Kaplan-Meier curves are shown and significance was determined by log-rank (Mantel-Cox) using GraphPad Prism.

Erwinia carotovora carotovora 15, also known as *Pectobacterium carotovora*, was grown overnight in LB broth, spun, washed in PBS, and pelleted. Infections were performed by dipping a microsurgery needle into the concentrated bacterial pellet, and pricking in the thorax. The IIV-6 injection site was identified using melanization, and bacterial pricking was performed on the opposite side of the thorax.

Cell Culture

S2* cells were cultured as previously described (Samakovlis et al., 1992; Silverman et al., 2000a) and were stimulated with 1 μ M 20-hydroxyecdysone, (EcD) for 18 hours followed by infection with IIV-6 at an MOI of 2 for 6 hours. Cells were then stimulated with 2 μ g/mL PGN for 6 hours to stimulate the IMD pathway, or stimulated with cleaved spätzle for 18 hours for Toll pathway stimulations and were harvested in TRIzol (Invitrogen).

Immunoblots

S2* cells were cultured as previously described (Samakovlis et al., 1992; Silverman et al., 2000a) and were stimulated with 1 μ M 20-hydroxyecdysone, (EcD) for 18 hours followed by infection with IIV-6 at an MOI of 2 for 6 hours. Cells were then stimulated with 2 μ g/mL PGN for 15 minutes to stimulate the IMD

pathway. IMD (Paquette et al., 2010) or c-Rel (Ertürk-Hasdemir et al., 2009) antibodies were used as previously described.

Virus Preparation

IIV-6 was provided by Luis Teixeira. IIV-6 was propagated and purified on DL-1 cells as previously described (9), with a final resuspension in PBS, and quantified on DL-1 cells by TCID₅₀. Cells were infected at an MOI of 2 unless otherwise noted, while flies were injected with 1x10⁴ TCID₅₀, as detailed above. See appendix for additional information.

CHAPTER V: DISCUSSION

This work has focused on elucidating the immune response to DNA virus infections in *Drosophila*. Our goals were to identify any response induced by the virus, determine how protection from viral infection was mediated, and to determine whether the virus was inhibiting any host immune pathways. Using IIV-6 as a model for infection, we have shown that virus-infection triggers ROS production, activating the p38b MAK and JAK-STAT pathways, possibly through damage caused by the virus. This results in a robust induction of six of the eight *Turandots*, whose function remains unknown. p38b and Stat92E RNAi flies succumb more rapidly to infection, indicating that they are vital to the survival or tolerance of the organism. We have also shown that IIV-6 infected cells secrete exosomes that provide protection to naive cells. This response is independent of reverse transcriptase activity and therefore appears to protect via a distinct mechanism than reported for exosome-like vesicles induced by RNA virus infections (Tassetto et al., 2017). Lastly, we have shown that IIV-6 inhibits the NF- κ B-mediated AMP response. In the IMD pathway, signaling is blocked downstream of Relish cleavage. Additionally, IIV-6-infected flies present an increased susceptibility to bacterial infections.

One of the biggest questions our data leaves us with, is the role of the Turandot proteins. Highly induced by infection, and with a high dN/dS rate, indicating that they face strong adaptive pressure, the Tots remarkably have no ascribed function a decade and a half after their discovery (Obbard et al., 2009).

Although our data does not support that the Turandots are antiviral, it is also important to note that our current data does not rule out this possibility. Over-expression of TotA in flies resulted in increased mortality, as has been described by others (Ekengren et al., 2001). In addition, we obtained flies with a CRISPR deletion spanning the loci for TotA, TotB, TotC, and TotZ from Bruno Lemaitre (See Figure 2.2 for gene loci). Surprisingly, these flies are homozygous lethal. Experiments performed using heterozygous mutants showed no difference in survival rates or viral titres, but also showed relatively normal induction levels of *TotA* upon IIV-6 infection (data not shown). It is possible that the Tots function in a dominant manner. We also cannot rule out that antiviral effects are being mediated by TotX or TotM. TotE and TotF, located next to each other on Chromosome 3, are not induced or undetectable upon IIV-6 infection. It remains possible that the Turandots work in concert or that all or subset of them function redundantly. Future experiments in cell culture and in whole flies over-expressing individual or groups of Tots as well as knocking down the Tots singly and in combination would need to be performed to thoroughly address this question.

It is also possible that the Turandots function in a tolerance manner, and act to stabilize the cell during times of stress, such as heat shock proteins. Given that the Turandots are induced in response to a wide variety of viral, bacterial, and environmental stressors, this suggests their function may be general rather than specifically antiviral. It is also possible that the Turandots function in a completely novel mechanism. Our data suggests that exosome release is

ramped up by viral infection, and that these exosomes function in an antiviral manner. The mechanisms of how these exosomes are loaded with antiviral effectors (such as dsRNA), are upregulated, or function to inhibit IIV-6 replication will all be the subject of future studies. It is possible that the Turandots play a role in any of these events.

Whether activation of the p38b and JAK-STAT pathways result in direct antiviral activity is also a question that will be addressed in future experiments. Viral titers will be examined to determine whether one or both of these pathways has antiviral functions or promotes tolerance.

The discovery of antiviral exosomes are an exciting advance in *Drosophila* immunity. Our data suggests that these can be generated through more than one mechanism, and brings up dozens of questions regarding their generation, the content of their cargo, and whether they are possibly induced by the cell upon infection.

While others have shown a role for endogenous reverse-transcriptase in the formation of extrachromosomal DNA for the synthesis of secondary viral siRNAs, we find that exosomes derived from IIV-6 infected cells are protective independently of reverse transcriptases. This indicates that a novel mechanism may be providing protection; either via a non-dsRNA antiviral factor or via a novel mechanism of generating dsRNA for exosome secretion. Since IIV-6 is a DNA virus, and previous studies showing the requirement of reverse transcriptase in

the formation of extrachromosomal DNA were performed with SINV, an RNA virus, it is conceivable that the cell has an alternative mechanism for the processing and storing of viral DNA derived from DNA viruses. This is an interesting possibility to be explored in future studies, with many possible avenues.

Cellular DNases should be examined for any role in processing viral DNA. *Drosophila* contain a number of DNases, including dICAD, dDNase II, and stress-induced DNase (sid). Mutants of these fly lines could be tested for any survival defects upon IIV-6 infection. Additionally, silencing these genes in cell culture and looking for defects in the production and function of antiviral exosomes would provide insight to how or whether these DNases function in an antiviral context. Isolation of exosomes and sequencing of their DNA or RNA contents would be essential in order to continue these studies.

In addition, the mechanisms used in the formation of piRNA clusters, which contain fragments of transposon sequences, may provide insight and share similarities with the mechanisms at work in creating antiviral exosomes. Like viruses, transposons can be RNA or DNA, and piRNAs are generated to protect organisms against both. Recent studies examining antiviral piRNAs in mosquitos point to a similar mechanism for piRNA generation as for generation of formation of vDNA copies (Goic et al., 2016; Goic et al., 2013). While the piRNA pathway has not been shown to function in an antiviral context in *Drosophila*,

piRNA cluster formation may be a model for generation of virus-derived siRNAs in antiviral immunity.

RNA polymerase III has been shown to contribute to the mammalian response to DNA viruses, transcribing AT-rich dsDNA into dsRNA that is detected by RIG-I (Ablasser et al., 2009). Whether RNA polymerase III contributes to the *Drosophila* antiviral response has not been examined. An interesting possibility is that *Drosophila* RNA polymerase III functions in a similar manner to transcribe viral DNA into RNA transcripts to be detected by Dicer-2 (a DEAD-box helicase distantly related to RIG-I) or other, still unknown, RNA sensors. This would provide a mechanism of generating siRNAs without reverse transcriptase. To test this, RNA polymerase III could be inhibited using tagetitoxin, which inhibits polymerase III in *Drosophila*, but leaves polymerase II functional (Takada et al., 2000).

Our data indicates that exosome production may be increased by infection (Figure 3.7). Whether components of exosome processing are increased by infection, or have come up as hits in any previously published screens for antiviral factors should be examined. Examining whether any exosome processing factors are induced upon infection by PCR will be included in future studies.

Assessing the cargo of exosomes released by IIV-6 infected cells will direct many of the future experiments performed. While secondary viral dsRNA has been reported to be the cargo of antiviral exosomes in other systems, we

may find something entirely different is used in response to a DNA virus.

Signaling proteins have been reported on exosomes in *Drosophila*, and it is possible that these exosomes also act to activate immune pathways (Beckett et al., 2013). While running exosomes on a silver-stained SDS-PAGE gel did not produce many bands indicating potential protein cargo, its possible that not enough exosomes were loaded, or that they were not sufficiently lysed. Mass-spectrometry analysis of total exosomes will be performed in future studies to identify any proteins that may be in or on exosomes.

We have shown that IIV-6 infection suppresses the AMPs produced by IMD and Toll signaling, that IMD signaling is suppressed downstream of Relish cleavage, and that IIV-6 infected flies are more susceptible to bacterial infection. This work brings up a number of interesting questions to be addressed, such as whether viruses truly activate IMD signaling and how that is achieved, how IMD inhibition mediated, whether Toll is inhibited in a similar manner, what viral genes mediate these processes, and whether flies dying upon co-infection also have suppressed AMP levels.

As discussed in Chapters I and IV, the involvement of the *Drosophila* NF- κ B pathways in antiviral signaling is a topic that has not yet produced clear data. Depending upon the virus used, some but not all IMD pathway components seem to be restrictive. Identifying a viral-encoded IMD inhibitor would provide more solid evidence that this pathway functions in antiviral responses. However,

how IMD signaling is initiated and whether all components are necessary will need to be examined in greater detail. Since IMD has been shown to directly bind DAP-type PGN, it seems unlikely that PGRP-LC or PGRP-LE is involved in viral recognition. Antiviral studies implicating IMD signaling should be reevaluated in light of recent work showing that the microbiome plays an important role activating IMD signaling in the gut to prime an antiviral response via ERK. Whether commensals can act to influence antiviral responses in other organ systems may seem unlikely, however, the role of Malpighian tubules in antiviral immunity has not been explored. The Malpighian tubules are organs branching off from the *Drosophila* gut, which absorb waste from the hemolymph, but have also been shown to serve important roles in immune function, including induction and secretion of AMPs in response to infection (Verma and Tapadia, 2012). Whether the Malpighian tubules provide crosstalk between the gut and hemolymph during viral infections should be explored. Perhaps IMD is required only for the first signal in a two-step signaling process.

Our data suggests that IIV-6 inhibits both IMD and Toll signaling, indicating that activation of these pathways may indeed have antiviral effects. Identifying one or more NF- κ B inhibitors in IIV-6 would provide indication that these pathways do function to limit viral replication. Chapter IV discussed the use of ChIP and nuclear fractionation to determine if IMD inhibition occurred in the nucleus, and BLASTing known NF- κ B inhibitors from related viruses to look for homologs.

It should be noted that IIV-6 does not encode a homolog of *Diedel*, the putative IMD inhibitor, as other large DNA viruses do (Lamiabile et al., 2016b). We have looked for other viral inhibitors of IMD signaling in IIV-6, such as RHIM domain containing proteins (such as MCMV M45, which is capable of inhibiting NF- κ B in mammalian systems and forms functional amyloid), however we did not find any (Anni Kleino, personal communication).

Another approach to determine whether Toll and IMD have any antiviral effects would be to use constitutively active Toll (Toll^{10B}) and Relish (RelD) flies to determine if the virus is at all weakened by activation of these pathways, although any differences may be subtle due to the viral inhibitors (DiAngelo et al., 2009).

Whether Toll is also inhibited at the level of NF- κ B must also be explored. This could easily be determined by western blot, looking for degradation of the *Drosophila* I κ B, cactus. Challenging IIV-6 infected flies with *Beauveria bassiana*, a pathogenic fungi, and looking at survival and pathogen load *in vivo* would be informative.

As mentioned in Chapter 4, others have shown that apoptotic-deficient cells activate Toll signaling. Since IIV-6 contains an IAP, this suggests we should see induction of Toll-dependent AMPs upon IIV-6 infection, rather than their inhibition. Interestingly, the authors also noted that *TotA* was induced in

apoptosis-deficient cells (Ming et al., 2014), bolstering the notion that JAK-STAT signaling and *Turandots* are induced in response to damage.

An important question that arises from this work is where in the fly these events are happening. The fat body is an important organ for the systemic production of AMPs, and infection of this organ may be key to IIV-6 mediated suppression of NF- κ B pathways. Other routes of infection, such as feeding flies IIV-6 could be attempted to assay effects on AMP suppression, to avoid fat body infection or assay AMP production within the gut. IIV-6 infects the fat body, but whether it infects hemocytes is unknown. Hemocytes have been shown by other groups to produce not only the *Turandots* but also antiviral exosome-like vesicles (Agaisse et al., 2003b; Tassetto et al., 2017). Its possible that IIV-6 infection in the fat body activates *p38b* and the upds signal to the hemocytes to induce the *Turandots* and exosomes. This possibility should be explored using fat body (*c564-GAL4*) and hemocyte (*hemolactin-GAL4*) specific drivers to knock down *Stat92E*, *Tots* and genes required for exosome production and secretion in hemocytes, or *p38b* in the fat body. While the possible redundancies of the eight *Turandots* complicate the particular question of the role of the *Tots*, it should still be attempted.

This work also suggests that flies may not mount an antiviral response to DNA in the way that mammals do. None of our data suggests that *Drosophila* respond to DNA: *p38* activation appears to occur through damage—perhaps

through sensing of actin as others have shown for *Tot* induction—rather than via DNA, as replication incompetent IIV-6, which still exposes the cell to vDNA, does not trigger *Tot* induction. Additionally, we found that while DNase mutants in flies produced a strong, endogenous, AMP response, that this response correlated with bacterial load found in these animals, and was abrogated in axenic animals or by tetracycline treatment (Appendix II). This is interesting because DNA sensing of pathogens occurs not only in mammals and other higher-order organisms, but also in bacteria via CRISPR-Cas detection of bacteriophages, and other invertebrates, such as shrimp (Deveau et al., 2010; Wang et al., 2013). Studies examining the role of the *Drosophila* STING homologue in the context of DNA and RNA virus infections, and whether *Drosophila* STING functions in autophagy will provide more clues as to whether *Drosophila* may have lost a DNA sensing mechanism, or whether a non-homologous, compensatory system exists.

Perhaps the dispensability of DNA sensing lies within the lifespan of the organism and the cytopathic effects of the infecting DNA virus. If a DNA virus is producing relatively little damage and the organism is able to live relatively unaffected by the infection, having a robust DNA response may not be necessary, or may cause more harm than good through inflammation. *Drosophila* face a number of pathogenic RNA viruses, and its possible that evolution selected for flies with a robust RNAi response and a DNA response was left out. Additionally, flies are able to mount an RNAi response to DNA virus infections if

the infecting DNA virus has areas of convergent transcription. Its conceivable that this provides a manageable level of protection from the majority of infections that they face.

Overall, this work further strengthens the idea that JAK-STAT signaling in *Drosophila* seems to be induced in response to damage, verifies and builds upon reports that exosomes serve antiviral functions in flies, and demonstrates that NF- κ B pathways can be blocked by virus infection, indicating that in addition to protecting flies from fungal and bacterial responses, they may also play an important role in antiviral defense. This work raises many fascinating questions, illustrating how much is yet to discovered about antiviral immunity *Drosophila*.

APPENDIX I:
***DROSOPHILA* IIV-6 MODEL**

Virus Propagation

- DL1 cells were plated in T-150 flasks, and grown to ~75% confluency. IIV-6 was added at low (~0.1) MOI, and infected for 7 days.
- Virus was harvested using a cell scraper and transferred into 50mL conical tubes. Virus was freeze-thawed from cycling between ethanol baths in -80°C to water bath at RT, three times. Conical tubes were then placed in a sonicating ice bath for 30 seconds, then allowed to rest on ice for 1 minute, three times.
- Cell debris was pelleted out by centrifugation at 600g for 10 minutes.
- Cell lysates were transferred to a fresh conical tube, and re-centrifuged.
- Cell lysates were transferred to a fresh conical tube and centrifuged at 10,000g for ten minutes to pellet virus. Pellets were resuspended and added onto ____mL of autoclaved 30% sucrose (SIGMA CAT#____). Beckman centrifuge tubes (CAT#____) were submerged in ethanol for 15-30 minutes and subsequently UV-treated for 15-30 minutes in a biological safety cabinet. SW-28 swing buckets were also rinsed in ethanol and UV-treated for 15-30 minutes. **Balance tubes.** Tubes were balanced on a scale wiped with ethanol and placed in the hood thirty minutes prior to use with UV-treatment.

Virus was pelleted through 30% sucrose in ultracentrifuge at 30,000g for thirty minutes.
- Sucrose was removed and virus pellet was washed by resuspending in ddH₂O and spinning at 10,000g for ten minutes to pellet virus, twice.

- Virus pellet was resuspended in a final volume (depending upon size of virus pellet, approximately 5 milliliters for five flasks of initial cells) of PBS, aliquoted 50 micrometers per cryovial, snap-frozen, and stored at -80°C.

Virus Titering, TCID₅₀

DL1 cells were plated at 4×10^5 cells/mL, and aliquoted 500 μ L/well into 24-well plates. Cells were incubated overnight at 27°C .

Cell media carefully removed and ten-fold virus dilutions prepared in serum-free media were added at 250 μ L/well. After two hours of infection, 250 μ L of complete media was added to each well, and plates were assessed for infectivity 7 days later, comparing to uninfected wells to check density and size of cells in each well to determine infection. Reed and Muench infectivity calculator was used to determine titre.

IIV6 MCP PCR

Since IIV-6 is heavily AT-rich and has only 29% GC content, designing primers for QPCR proved difficult. To circumvent this, long primers were used. Therefore use of longer oligonucleotides necessitated a higher annealing temperature, and required only a two-step cycle.

F: GTATGGCAAAGCAGCAGTAGGAAGAGCAACTCCAGAATCGC

R: TCTCGCGATCGTAACTATTCTCTAATTTTTTTTACTTGCATGAAAAATAAG

1. 50°C, 2:00
2. 94°C, 2:00
3. 95°C, 0:15
4. 72°C, 1:00, + plate read
5. Go to 3, x39.

6. Melt curve, 58°C to 95°C, increment 0.5°C
7. 10°C, 0:30
8. End.

***In vivo* Infections**

Dec2015 IIV-6 stock was diluted 1:10, infecting flies with 10⁴ TCID₅₀. Flies were injected in the thorax using a pulled capillary needle fitted to a Nanoject II, and 33nL of fluid was dispensed.

In cases of co-infection with *Erwinia carotovora carotovora*, flies were first infected with IIV-6 for 8 days. The melanized injection site from IIV-6 was identified, and flies were pricked with a needle dipped in *Erwinia carotovora carotovora* on the opposite side of the thorax from the primary infection.

***In vitro* Infections**

Generation of Δ TS-MCP-mCherry IIV6

This strain was made with help from Don Gammon, at the time a post-doc in Craig Mello's laboratory. His student Ying Chen assisted with cloning and PCR to generate the constructs. IIV-6 genome AF303741.1 was used as the reference for nucleotide sequence and numbering.

To generate an mCherry-expressing strain of IIV-6, we first sought to determine which promoter regions would be best for driving mCherry expression. We utilized a published promoter analysis for IIV-6 as a guide, and we chose the MCP promoter to drive expression (Nalçacioğlu et al., 2003). We used published primer sequences to amplify the most effective promoter region (Nalçacioğlu et al., 2003).

Based on Don's previous experience making recombinant VACV strains expressing constructs from disruption of the non-essential thymidine kinase, we targeted a similar gene, the thymidylate synthase gene, 225R as a non-essential gene to disrupt with the mCherry construct (Jakob et al., 2001). Primers were designed to amplify left and right regions of homology, listed below.

Left homology F: atggatattaataatgaag
Left homology R: tacaacaattcaaaatgaac
Right homology F: ttggatagaagaatgata
Right homology R: aatggaaatggcgtataa

These regions of homology were cloned into a Topo vector, pCRZeroBlunt. All constructs were tested for their ability to drive mCherry prior to cloning and before being transfected for recombination.

Primers flanking

Heat- and UV- Inactivations (Nalçacioğlu et al., 2003)

Heat inactivations were made fresh before each use and performed by placing the appropriate amount of virus diluted in serum-free media in a microfuge tube. This was incubated in a dry heat block set to 55 for 90 minutes, vortexing every 30 minutes to ensure even heat distribution.

UV-inactivation was performed using a Stratalinker. Samples were made fresh before each use, and the appropriate amount of virus was diluted in serum-free media in a 15mL conical tube. Samples were treated for a total of 15 minutes, vortexing every 3 minutes.

APPENDIX II:
Drosophila DNases and DNA-dependent IMD Activation in *Drosophila*

Introduction

Whether *Drosophila* are capable of responding to DNA remains unknown, and the mechanisms that would be used to detect DNA do not appear to be completely conserved in *Drosophila*. None of the *Drosophila* Toll receptors have been shown to bind DNA, and there does not seem to be a clear, functioning, cGAMP homolog. Additionally, there are no HIN200 domains (the DNA-binding domain found in ALRs) in flies. While it is possible flies have evolved a novel compensatory mechanism for DNA sensing, none have been discovered to date.

It was reported that *dDNaseII* and *dICAD* mutants were capable of triggering IMD signaling due to their incapacity to break down chromosomal DNA, similar to mammalian DNaseII mutants (Mukae et al., 2002). We explored whether this phenotype was indeed in response to DNA, or whether an inability to phagocytose bacteria may have lead to increased bacterial burden, triggering IMD activation. We treated flies with the antibiotic tetracycline or produced axenic flies and tested their endogenous production of AMPs and bacterial loads.

Results

In order to determine whether *dICAD* and *dDNaseII* mutants were producing AMPs in response to the excessive levels of endogenous DNA in these animals or whether it was due to elevated levels of bacteria in the animals, we treated flies with the antibiotic tetracycline for three weeks, and then examined endogenous AMP levels in these animals by qRT-PCR. We found that

tetracycline reduced endogenous AMPs compared to flies treated with the vehicle control (Figure A2.1A).

Since tetracycline is only effective against certain classes of bacteria and the conditions flies are reared in are not sterile, we decided to produce axenic, "sterilized" flies and examine their AMP levels. Axenic flies also had reduced levels of AMPs compared to conventionally-reared flies (Figure A2.1B).

In order to determine whether axenic flies were truly free of bacteria, we plated homogenized flies on LB agar plates kept at RT, 22°C, the temperature at which fly gut microbes grow. Conventionally-reared fly homogenates produced a number of bacterial colonies, while homogenates plated from axenic-reared flies were nearly devoid of any bacterial growth (Figure A2.2). We used QPCR to further quantify microbial loads with primers to 16S rRNA. We found that flies reared under axenic conditions had 1000-fold less bacteria than conventionally reared animals and flies treated with tetracycline had 100-fold less bacteria (Figure A2.3). These results show that endogenous AMP production in *Drosophila* DNase mutants is influenced by the bacterial loads in these animals.

Discussion

While this work is preliminary, it suggests that the AMP response seen in *dICAD* and *dDNaseII* mutants is in response to bacterial loads rather than DNA specifically. Whether hemocytes in DNase mutants have increased bacterial burden due to an inability to phagocytose can be tested by directly inhibiting

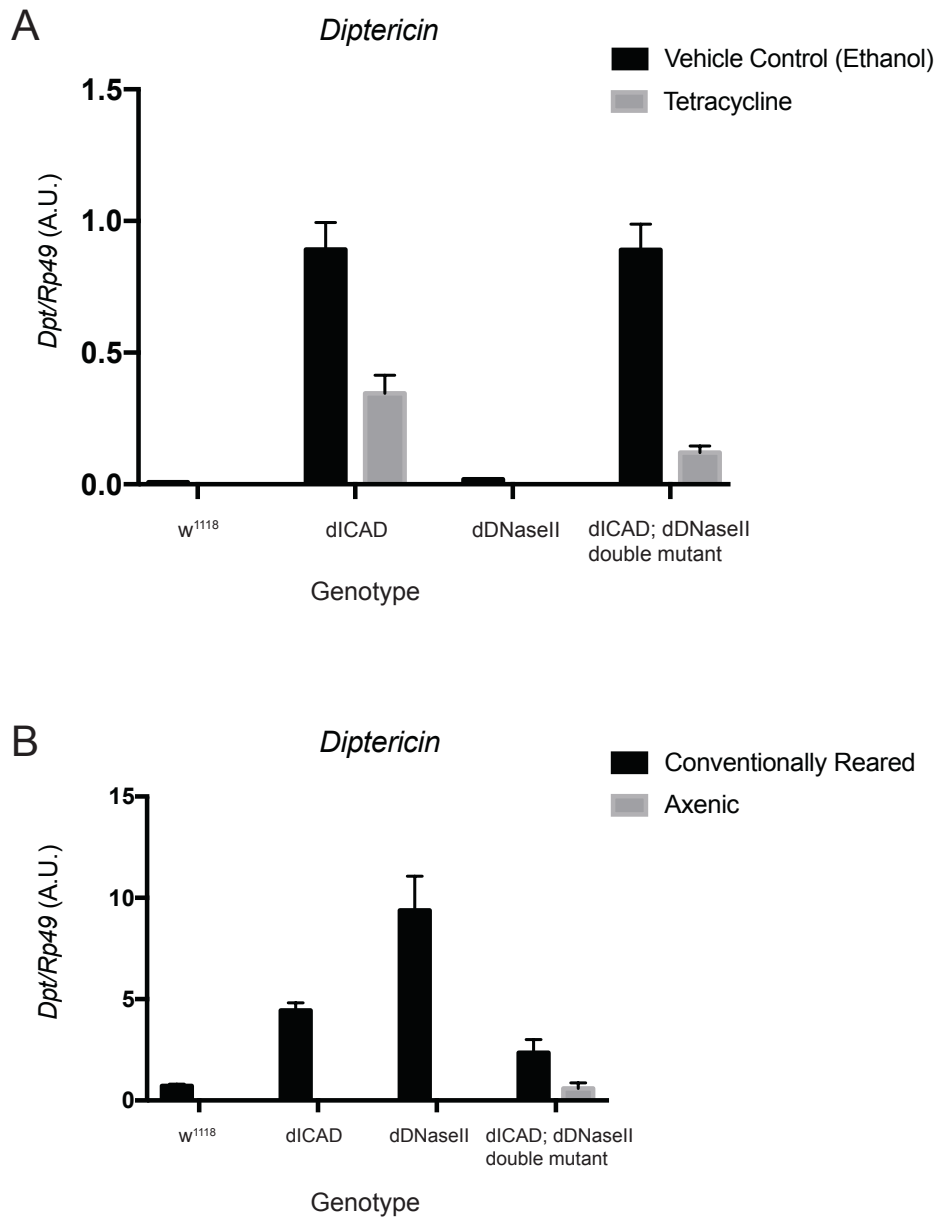


Figure A2.1 Endogenous AMP production in DNase mutant flies is bacterial-driven.

A) Flies were reared in vials treated with tetracycline (gray bars), or treated with vehicle (ethanol) for three weeks before RNA isolation from whole flies. Diptericin levels were measured by qRT-PCR, normalizing to the housekeeping gene Rp49. **B)** Flies were reared under conventional (black bars) or axenic (gray bars) conditions for one week before RNA isolation.

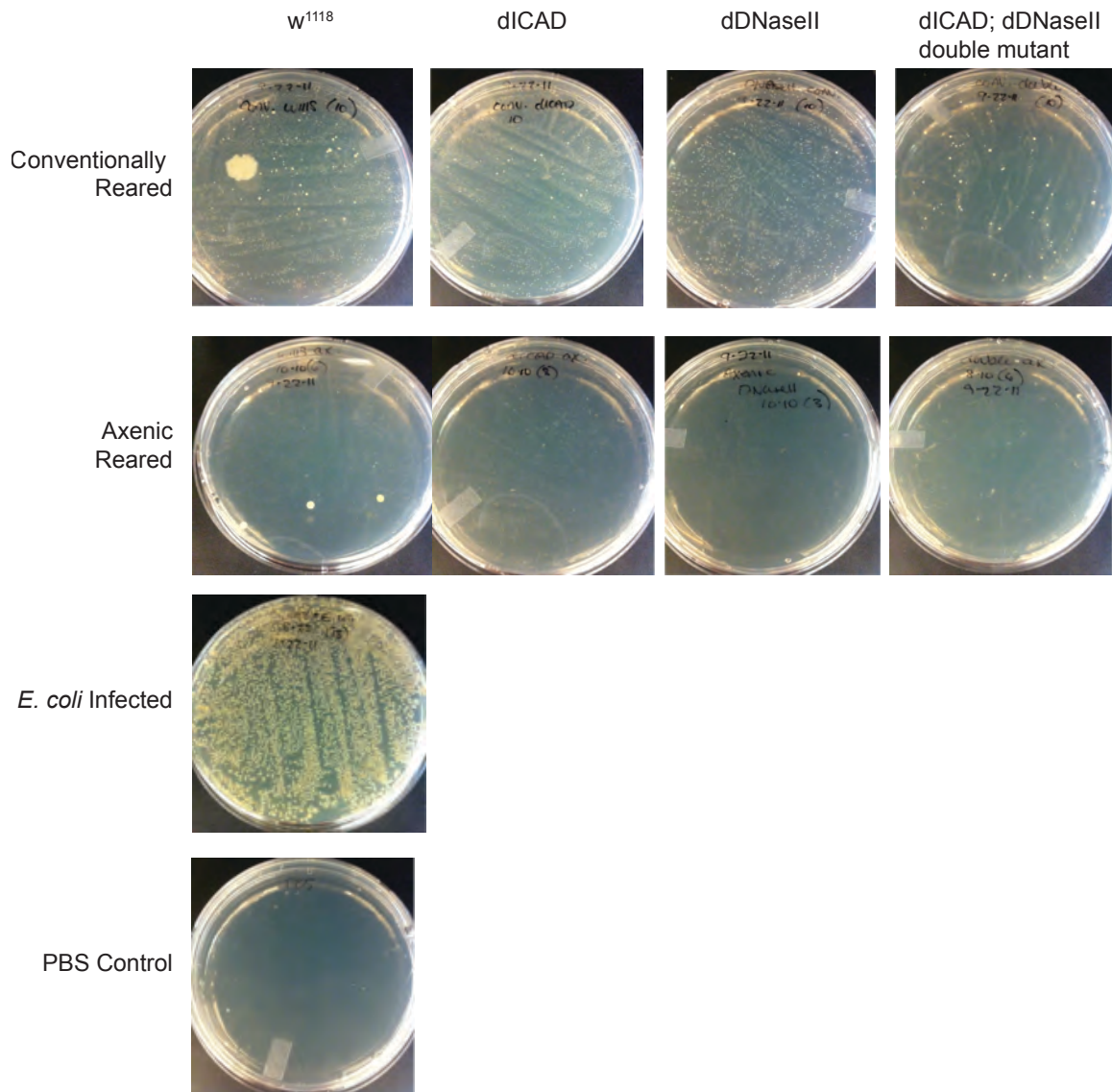


Figure A2.2. Axenic-reared flies have less bacterial growth.

Top row, LB agar plates plated with 5 conventionally-reared flies, ground with a mortar and pestle in 100 μ L PBS, and left at RT (22°C) for one week prior to imaging. Second row, flies reared under axenic conditions and processed in the same manner. Control plate (third row) was generated using *E. coli* infected w¹¹¹⁸ flies. Five flies were pricked with a surgical needle dipped in an *E. coli* pellet prior to processing as described above, and plate was grown overnight at 37°C before imaging. PBS was plated as a negative control (last row).

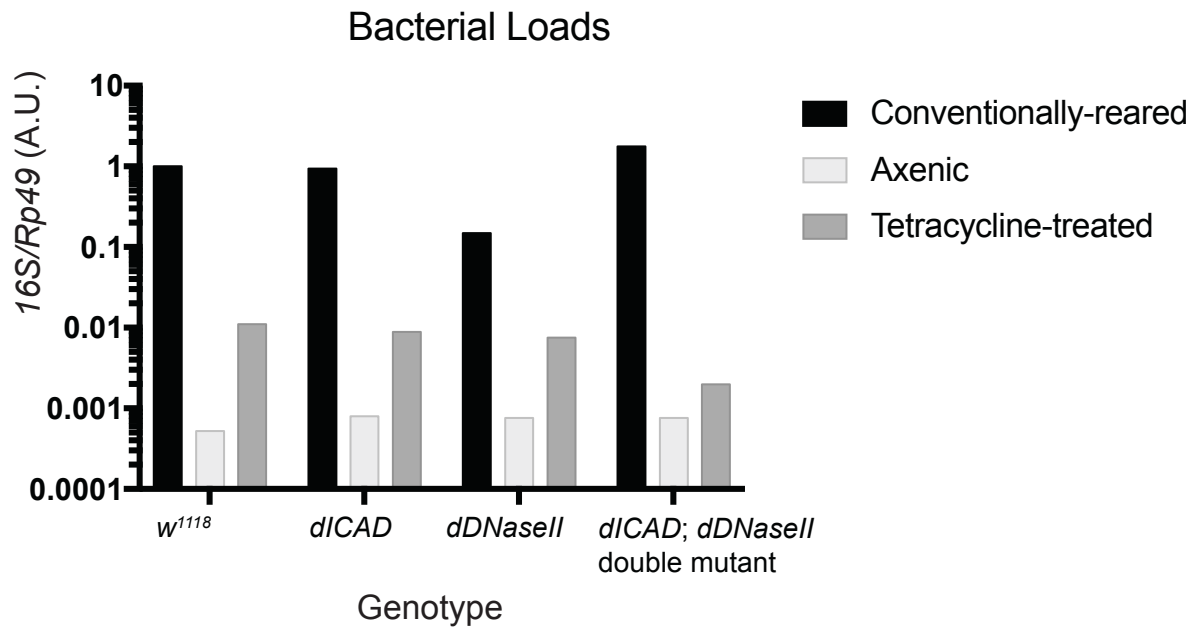


Figure A2.3 Axenic and tetracycline-treated flies have lower bacterial loads than conventionally-reared animals.

QPCR for 16S rRNA. Flies were grown under conventional, axenic, or tetracycline-treated conditions, and microbial DNA was isolated. A.U., arbitrary units.

phagocytosis with polystyrene beads, or using fluorescently labeled bacteria to monitor phagocytosis.

Genetic experiments, such as crossing *dICAD*; *dDNaseII* double mutants to flies with mutant IMD components such as PGRP-LC, PGRP-LE, or Relish, should also be performed to verify which components of IMD signaling are required for the elevated levels of endogenous AMPs seen in *dICAD* and *dDNaseII* mutants. Determining whether PGRP-LC or PGRP-LE, which directly bind DAP-type PGN, are required, would provide insight as to whether the AMP response is activated in response to PGN. If PGRP-LC and PGRP-LE are not required, but downstream IMD components are required, this implies that perhaps AMP production is being induced in response to other bacterial ligands, such as bacterial RNA or DNA.

These experiments also bolster our hypothesis that the *Drosophila* antiviral response does not respond to viral DNA but that the antiviral response to IIV-6 response is activated by other PAMPs produced by the virus (see Chapter V: Discussion).

Materials and Methods

Axenic Fly Rearing

Axenic flies were produced by collecting embryos overnight at 25°C on egg-production grape juice agar plates with a dab of yeast paste (dry yeast flakes mixed with sterile water). Embryos were collected in a biological safety cabinet

using sterile cell strainers (40um, BD CAT#352340), sterile PBS, autoclaved food vials (Wide Polypropylene Vials (28.5mm x 95mm), Genessee Scientific, CAT#32-114), and paint brushes cleaned in 75% ethanol and UV-treated for 15 minutes prior to use. Food vials were filled and then autoclaved on the liquid cycle in a plastic container covered with aluminum foil and opened in the hood. Cotton fly plugs were autoclaved before use in a covered container and opened only in the hood (more plugs than were required were autoclaved as they often become misshapen during autoclaving and many will not fit).

Embryos were collected from egg laying plates with a cleaned paintbrush and rinsed in sterile PBS in a cell strainer. The cell strainer was then moved to a sterile tissue culture plate containing 50% bleach for 2 minutes, to one containing 70% ethanol for 2 minutes, and then rinsed for 2 minutes in autoclaved Milli-Q water. Using a sterile brush, embryos were removed from the strainer and placed into autoclaved food vials and fitted with autoclaved plugs before being stored in the fly room at 25°C.

Conventional Fly Rearing

Flies were flipped into conventional low-yeast vials and stored at 25°C.

Microbial DNA Isolation

To isolate microbial DNA for 16S QPCR, Ultra Clean Microbial DNA Isolation Kit (MO-Bio, CAT# 12224-50) was used per the manufacturers instructions.

16S PCR

Primer sequences for 16S PCR (these primers give a 1.5kb product):

1492Ry: GGYTACCTTGTTACGACTT
8FE: AGAGTTTGATCATGGCTCAG
(Invitrogen, Y= C+T)

Reaction Mix,

0.5uL of 50pM primer stock
200uM dNTPs
1.5 mM MgCL₂
1 unit GoTaq-- Promega
50ng template
in 50 ul. +buffer, water as appropriate.

For QPCR, I use 5 ng template, 1uL of 2uM primer mix, 7.5ul SYBR per well.

Cycling Conditions,

1. 95°C, 5min
2. 95°C, 25s
3. 56.4°C, 25s
4. 72°C, 90s
5. Repeat #2-4 for 25 cycles
6. 72°C, 5 min

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