TARGETING INSULIN-MEDIATED ACTIVATION OF THE PI3K/AKT AXIS TO REDUCE FLAVIVIRUS INFECTION IN HUMANS AND MOSQUITOES

By

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Abstract

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Arthropod-borne viruses (arboviruses) pose a significant health threat that continues to grow due to mosquito habitat expansion and activity during the year. To address this growing concern, more effective vector control protocols and prophylactic treatments are needed. Understanding host immunity to viral infection, whether it be at the mosquito vector or human clinical level, would provide insight and a means to base future protocols or treatments on. This work targets insulin-mediated activation of antiviral immune signaling associated with the PI3K/AKT axis to address mosquito-borne viruses at two approaches: 1) to identify and characterize evolutionarily conserved insulin-mediated antiviral immunity against flaviviruses between the *Drosophila melanogaster* insect model and humans, and 2) to demonstrate the therapeutic potential that targeting specific proteins in the PI3K/AKT axis in *Aedes aegypti* mosquitoes has in reducing viral infection and transmission. In the first approach, the work demonstrates that insulin treatment, previously shown to reduce West Nile virus, Kunjin strain (WNV-Kun) replication in *Drosophila* and *Culex quinquefasciatus* mosquitoes through induction of canonical antiviral signaling, activates other signaling pathways that are both canonical immune responses and

previously unidentified antiviral mechanisms. Specifically, endothelin signaling appears to be involved in the antiviral immune response against WNV-Kun that is conserved between *Drosophila* and humans and is also protective against the more virulent WNV, New York-99 (WNV-NY99) strain. The work presented in the second approach demonstrates that through small molecule targeting that selectively alters the activation state of the insulin-like receptor (InR) and AKT in *Aedes aegypti* cells and mosquitoes, canonical immune pathways RNA interference (RNAi) and JAK/STAT can be simultaneously induced and provide more potent protection against Zika virus (ZIKV). Notably, small molecule treatment significantly reduces a mosquito's ability to become infected and transmit ZIKV which may be a potential avenue in reducing transmission in the field. Taken together, this work compares insulin-mediated immunity among *Drosophila*, *Aedes*, and humans against different flaviviruses to evaluate their potential as broad and more efficient vector control protocols and clinical markers for disease and intervention.

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LIST OF ABBREVIATIONS

ADE – antibody-dependent enhancement

AGO2 – Argonaute 2

cGAS – cyclic GMP-AMP synthase

CHIKV – chikungunya virus

DCs – dendritic cells

DENV – dengue virus

DMAQ-B1 – demethylasterriquinone B1

d.p.i. – days-post infection

EDN1 – endothelin 1

FC – fold change

FOXO – forkhead box O

GO – gene ontology

GSEA – gene set enrichment analysis

HBV – hepatitis B virus

HCV – hepatitis C virus

IFN – interferon

IGF-1 – insulin-like growth factor-1

IIS – insulin/insulin-like growth factor signaling

ILPs – insulin-like peptides

InR – insulin receptor

JAK – Janus kinase

JEV – Japanese encephalitis virus

MDA5 – melanoma differentiation-associated protein 5

MOI – multiplicity of infection

NK – natural killer cell

NPV – nucleopolyhedrovirus

PRR – pathogen recognition receptor

qRT-PCR – Quantitative Reverse Transcriptase PCR

RISC – RNA-induced silencing complex

RLR – RIG-I-like receptors

RNAseq – RNA sequencing

RVFV – Rift Valley fever virus

SINV – Sindbis virus

siRNA – small interfering RNA

SLEV – St. Louis encephalitis virus

STAT – Signal Transducer and Activator of Transcription

TLRs – Toll-like receptors

upd – unpaired

vir-1 – virus-induced RNA-1

WNV – West Nile virus

WNV-Kun – West Nile virus-Kunjin strain

WNV-NY99 – West Nile virus-New York 99 strain

YFV – Yellow Fever virus

ZIKV – Zika virus

Dedication

To Y.B. Rodger,

You had the audacity to believe in me. Take care. Too-da-loo.

CHAPTER ONE: GENERAL INTRODUCTION

Vector-borne diseases, which utilize a blood-feeding arthropod as a transmitter of infectious pathogens to humans, make up a significant portion of all infectious diseases with an increasing incidence each year. Approximately 17% of all human infectious disease are vector-borne (Chala and Hamde, 2021). Climate change and human activity have contributed to the growth of disease incidence. Specifically, these factors have influenced vector habitation and feeding activity that have resulted in increased exposure and likelihood of transmission. Mosquitoes are significant contributors to vector-borne disease. Of particular concern with the most rapid growth are mosquito-borne viruses such as West Nile virus (WNV), Zika virus (ZIKV), dengue virus (DENV), and Yellow Fever virus (YFV). Mosquito-borne viruses are a prevalent global health concern that results in numerous cases and deaths each year and while mosquito range and activity has expanded, vector control and prophylactic therapeutic development has lagged (Chala and Hamde, 2021; Baker et al., 2022; Swei et al., 2020).

WNV was introduced into the Americas in 1999 in New York state and has since become endemic within the continental United States (Briese et al., 1999; Centers for Disease Control and Prevention, 1999; Lanciotti et al., 1999; Nash et al., 2001; Tsai et al., 1998). ZIKV was introduced into the Americas in 2013 and following the 2015-16 Brazil epidemic has become endemic within Central and South America (Faria et al., 2016; Hennessey, Fischer, and Staples, 2016). Both WNV and ZIKV are members of the *Flaviviridae* virus family which include a variety of other human-disease causing pathogens. Viruses within the *Flaviviridae* family are characterized as possessing a positive-sense, single-stranded RNA (+ssRNA) linear genome that is translated as a single polyprotein. WNV and ZIKV are vector-borne diseases as they are

predominately spread by *Culex* or *Aedes* mosquitoes, respectively. While most individuals are asymptomatic when infected with either WNV or ZIKV, both viruses possess similar clinical manifestations for those that develop more severe symptoms. This can range from manageable symptoms such as a rash, joint pain, or flu-like symptoms that can resolve on their own with little to no hospital intervention required. For more severe cases, the resulting viremia and disease pathology can advance to neurological damage and disorders like encephalitis for WNV (Alli et al., 2021; Sejvar, 2014) or microcephaly or Guillan-Barré syndrome for ZIKV patients (Calvet, Santos, and Sequeira, 2016; Pan American Health Organization, 2015). Currently there are no vaccines or specific prophylactic therapeutics available in preventing or treating viral infection. Because of the growing spread and number of cases caused by mosquito-borne viruses, research that results in a more direct means of addressing infection, whether at the vector or clinical level, is necessary.

The host immune system is a key determinant that recognizes and responds to infection. Higher-order organisms like humans possess an adaptive immune system that provides more targeted and long-term responses in the form of cellular and humoral antibody-mediated responses using B- and T-cells. The innate immune system is an evolutionarily conserved form of protection that is responsible for pathogen recognition and immediate response during early stages of infection. Because of its necessary role in pathogen control and presence across species, research regarding the innate immune system has expanded to identify their potential as an intervention target. In the case of mosquito-borne illnesses, research has been focused on characterizing and targeting host innate immunity as a means of approaching disease from both the mosquito and human level (Ahlers et al., 2019; Aliota et al., 2016; Dutra et al., 2016; Hedges et al., 2008).

Understanding how the mosquito responds to infection and identifying intervention targets would reduce a mosquito's ability to become infected and in turn reduce vector competency for virus transmission. Likewise, understanding human antiviral immunity during early stages of infection would reduce the likelihood of progression into severe disease and possibly provide some level of protection against subsequent or related viral infections. Because of the evolutionarily conserved nature that is the innate immune system, there is potential overlap in responses shared between mosquitoes and humans that can be targeted in both species as a means of addressing viral infection (Trammell and Goodman, 2021).

While clinical research and application of effective antivirals or vaccines has been limited, there have been advancements regarding vector control protocols implemented in the field to reduce transmission. Early efforts specifically focused on limiting exposure to potentially infectious mosquitoes through community netting and education followed by use of potent insecticides. While this mitigation has been helpful in addressing disease incidence and education within affected communities, mass insecticide use has also permitted the emergence of insecticide-resistant mosquito populations (Kupferschmidt, 2016). To circumvent this, the development of vector-control protocols has pivoted to focus more on targeting mosquito physiological processes to reduce vector-competence and population growth. Examples of this include the introduction of mosquito populations that possess either genetic modification (Evans et al., 2019; Waltz, 2021; Williams et al., 2020) or symbiotic parasites (Aliota et al., 2016; Dutra et al., 2016) that renders them less likely to become infected with virus while also outcompeting with native mosquito populations. While these methods have proven effective in a lab setting and in limited field trials, there remains some debate regarding their effectiveness across a range of co-circulating viruses

(Dodson et al., 2014) as well as their long-term efficacy (Resnik, 2017). As such, it is necessary to develop a multitude of approaches to address vector competency and transmission that can be readily implemented in a field setting in response to active outbreaks.

The insulin signaling pathway, a highly organized and conserved process present across species including insects, arthropods, and vertebrates, is an important regulator in host metabolic and nutritional processes (Barbieri et al., 2003). Insulin or insulin-like peptides (ILPs) binds to the insulin receptor (InR) which induces a phosphorylation cascade to activate a multitude of intracellular pathways including the PI3K/AKT and MAPK/ERK axes (Barbieri et al., 2003; Vinayagam et al., 2016). This cascade in turn regulates transcription factors or secondary signaling pathways that impact a wide variety of processes including cellular growth (Nielsen et al., 2008), lifespan (Altintas, Park, and Lee, 2016; Drexler et al., 2014; Hwangbo et al., 2004; Sharrock et al., 2019), and reproduction (Wittes and Schüpbach, 2019). Additionally, further investigation has demonstrated that the insulin signaling pathway is also involved in host immune signaling against a range of pathogens including bacteria (McCormack et al., 2016; Suzawa et al., 2019), parasites (Cator et al., 2015; Pietri et al., 2015), and viruses (Ahlers et al., 2019; Haqshenas et al., 2019; Xu et al. 2013). Various viral infections including influenza (Ohno et al., 2020), SARS-CoV-2 (Yu et al., 2021), and hepatitis C virus (HCV) (Banerjee et al., 2008; Campo et al., 2012; Gao et al., 2015; Hsieh et al., 2012) have been implicated to impact host metabolic processes like insulin signaling that can result in long-term metabolic disorders even after viral clearance. It is only recently that various mosquito-borne pathogens like *Plasmodium spp.*, the causative agent of malaria (Cator et al., 2015; Pietri et al., 2015; Luckhart et al., 2013), and viruses like WNV (Ahlers et al., 2019; Kumar et al., 2012, 2014), ZIKV (Harsh

et al., 2018; Nielsen and Bygbjerg, 2016; Trammell et al., 2022), and DENV (Lee at al., 2020; Sansone et al., 2015) have been shown to also impair insulin signaling in mosquitoes and humans. While there have been numerous studies that connect how infection can impair insulin signaling in both mosquitoes and humans, there remains a limited understanding regarding the potential functional role that the pathway may have as a mediator of antiviral immunity and its therapeutic potential.

The work presented here investigates the role that insulin signaling, specifically that mediated by the PI3K/AKT axis, has on host immunity to flaviviral infection and its potential as a therapeutic target. Chapter 2 will discuss host factors present in mosquitoes and humans that are important for antiviral immunity against flaviviruses, analyze their impact on host-pathogen interactions, and evaluate similarities and differences present between both species. Chapter 3 characterizes the transcript profile of *Drosophila melanogaster* during West Nile virus, Kunjin strain (WNV-Kun) in the presence of insulin. This work identifies novel gene sets associated with host survival and antiviral immunity that we demonstrate are relevant to their orthologous genes in humans. Additionally, this work demonstrates that insulin signaling and its induction of downstream endothelin signaling are conserved across species and, more importantly, is effective against more virulent and clinically relevant flaviviruses. Chapter 4 will discuss the role and connections that insulin signaling has on various canonical antiviral responses present in the *Drosophila* system. This review expands upon how insulin signaling may impact and influence host antiviral responses and their potential role in disease dynamics and vector control. Chapter 5 investigates the potential that targeting insulin-mediated antiviral immunity possesses as a viable vector-control measure in *Aedes aegypti* mosquitoes against ZIKV infection. The canonical

PI3K/AKT axis can be targeted by coupled small molecule treatment that renders downstream antiviral signaling active and reduces viral replication and dissemination in the mosquito vector. This work expands the current model in which insulin connects to antiviral RNA interference (RNAi) and JAK/STAT and provides a novel means of addressing ZIKV infection at the transmission level which can possibly be translated into vector control protocols. Chapter 6 will discuss the presented findings by providing insight regarding the impact of this work, future directions, and remaining concerns left to be addressed that were beyond the scope of this study.

CHAPTER TWO: HOST FACTORS THAT CONTROL MOSQUITO-BORNE VIRAL INFECTIONS IN HUMANS AND THEIR VECTOR

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ABSTRACT

Mosquito-borne viral infections are responsible for a significant degree of morbidity and mortality across the globe due to the severe diseases these infections cause, and they continue to increase each year. These viruses are dependent on the mosquito vector as the primary means of transmission to new vertebrate hosts including avian, livestock, and human populations. Due to the dynamic host environments that mosquito-borne viruses pass through as they are transmitted between vector and vertebrate hosts, there are various host factors that control the response to infection over the course of the pathogen's life cycle. In this review, we discuss these host factors that are present in either vector or vertebrate models during infection, how they vary or are conserved between hosts, and their implications in future research pertaining to disease prevention and treatment.

INTRODUCTION

Vector-borne diseases pose a significant global health burden. Global climate changes have enabled various arthropod vectors to expand into previously uninhabitable regions which increases potential exposure to at-risk population (Morin and Comrie, 2010; Kamal et al., 2018). In particular, mosquito populations have expanded, and this has resulted in increasing occurrence of mosquito-borne disease (Kraemer et al., 2015; Leta et al., 2018), notably viruses such as West Nile virus (WNV), dengue virus (DENV), Zika virus (ZIKV), and chikungunya virus (CHIKV) (Rosenberg et al., 2018). While mosquitoes are also responsible for transmission of hazardous parasitic diseases such as plasmodia and filariasis parasites, their role as transmitters of viral pathogens is of particular interest to epidemiologists and infectious disease monitoring groups. Recent reports by the World Health Organization (WHO) have indicated a steady or downward

trend of global malaria cases (Guglielmi, 2019), while viral cases have appeared to increase in both frequency and severity (Rosenberg et al., 2018; Giovanetti et al., 2019; Gutiérrez, 2015). This has resulted in the WHO to designate DENV and related mosquito-borne viruses as significant concerns that need to be addressed at the global level in 2020 (World Health Organization, 2019).

Mosquito-borne viruses utilize the mosquito as a natural reservoir for replication and vector for transmission into vertebrate populations. Depending on the virus, transmission can occur in both sylvatic and urban settings during the bloodmeal between mosquito and vertebrate hosts such as primates, birds, and humans (Ahlers et al., 2018; Gutiérrez-Bugallo et al., 2019). Once infected, some vertebrate hosts are unable to clear acute infection and can develop viremia which results in infectious virions circulating in the blood and lymphatic fluid. This would, in turn, result in potential virus transmission from vertebrate to mosquito in subsequent bloodmeals (Ahlers et al., 2018). The transmission cycle between mosquito and vertebrate populations requires constant monitoring to identify new infections in each host type as this would indicate active viral transmission within a community or region.

Viruses that undergo this transmission cycle encounter various environmental and host factors as virions move between mosquito and vertebrate hosts. Immune responses to mosquito-borne viruses vary between vector and vertebrate hosts as the sophistication and complexity in the cellular environment vary between species. This does not, however, mean that there are no evolutionarily conserved responses that are shared between organisms. In fact, at the level of innate immunity, there are many shared antiviral mechanisms that are conserved between

species. In the following review, we provide a summary of conserved host factors responsible for initiating antiviral responses against mosquito-borne viruses in mosquito and human systems, how these factors vary between organisms, and how these responses provide a foundational understanding for future vector control and therapeutic research.

MOSQUITO ANTIVIRAL IMMUNITY

Mosquito-borne viruses utilize multiple mosquito species as the primary means of transmission into higher-level vertebrate species such as birds and mammals. While these viruses can be transmitted within a vertebrate population by routes such as blood transfusion (Harrington et al., 2003; Tambyah et al., 2008), sexual transmission (D'Ortenzio et al., 2016), or in utero transmission (Nguyen et al., 2017), the mosquito is the predominate means of transmission and is responsible for a majority of disease cases within a community. Mosquitoes acquire a viral infection during the bloodmeal exchange from an infected vertebrate, but there is evidence suggesting vertical transmission of certain viruses such as ZIKV from female mosquitoes to eggs (Armstrong et al., 2020; Comeau et al., 2020). Once the virus-containing bloodmeal is ingested and digested in the midgut, the virus escapes the midgut and systemically infects distal tissues including the ovaries, fat body, and salivary glands (Merwaiss et al., 2021). As viremia is reached in the mosquito, the saliva becomes infected with high levels of infectious virions that induces activation of chemosensory-related genes that affects feeding behavior (Sim et al., 2012). Once the salivary glands become infected, the mosquito becomes a competent vector for future virus transmission during subsequent bloodmeals. Mosquitoes can be co-infected with different viruses simultaneously without compromising vector competence or survival, such as

Aedes aegypti infected with DENV, ZIKV, and CHIKV; the mosquito can then transmit these viruses simultaneously (Göertz et al., 2017; Rückert et al., 2017).

From the initial digestion of the virus-containing bloodmeal to transmission, the mosquito initiates different cellular responses to control the virus without affecting host survival. Physical tissue barriers are present along the mosquito digestive tract, and the virus must pass through this barrier in order to become systemic. Then, the virus reaches the salivary glands and ovaries for horizontal and vertical transmission, respectively. Broad and specific antiviral immune signaling responses are also crucial to reduce a virus's ability to establish itself within the mosquito. The antiviral barriers involved in protecting mosquitoes from mortality due to viral infection are generally well conserved across genera such as *Culex* and *Aedes*.

Innate Immune Responses in the Mosquito. The mosquito, like other invertebrate species, lacks the canonical adaptive immune system found in more complex vertebrate species such as humans (Ahlers et al., 2018). Instead, mosquitoes utilize response pathways that are heavily conserved across metazoa and are critical in the insect immune system and innate immunity. As opposed to the development of immunological memory via the adaptive immune system, the innate immune system involves recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) that activate immune signaling pathways which ultimately: (1) induces transcription of downstream antiviral effector proteins, or (2) recruits immune cells that can initiate cellular and humoral signaling to localized infection. The primary focus of innate immune signaling is to initiate an immediate response to infection that aims to clear and keep the virus localized. The innate immune system has been heavily dissected and

studied in the context of mammalian systems, the *Drosophila melanogaster* model organisms, and mosquito species to varying degrees. Taken together, the role and significance of innate immune signaling against mosquito-borne viruses are a well-established and continuously growing field of study.

One of the more significant defense responses against a multitude of viral infections in the mosquito is the RNA interference (RNAi) pathway (Figure 1A). RNAi signaling is a heavily conserved pathway across invertebrate and vertebrate organisms and is involved in regulating gene expression (Schuster, Miesen, and van Rij, 2019). In invertebrate organisms, the RNAi pathway also functions as an antiviral response pathway that is activated by nucleic acids that are the by-products of the replicative process during viral infection (Saleh et al., 2009; Mukherjee and Hanley, 2010; Campbell et al., 2008). Specifically, viral nucleic acids such as double-stranded RNA (dsRNA) are detected by the endoribonuclease Dicer-2 which binds to and cleaves the larger dsRNA into smaller RNA duplexes (Lee et al., 2004; Galiana-Arnoux et al., 2006). R2D2 then binds to the small RNA duplexes, and the protein–viral nucleic acid complex is loaded into the RNA-induced silencing complex (RISC) (Liu et al., 2003). Once associated with RISC, the effector proteins AGO2 and p400 bind to specific sequences within the viral mRNA target to cleave bound viral nucleic acids (Okamura et al., 2004; van Rij et al., 2006; McFarlane et al., 2020). The purpose of this degradation of viral nucleic acids is to clear the intracellular virus before viral replication proteins can hijack the host translational machinery and generate more infectious virion copies. The importance of this pathway has been demonstrated against flaviviruses such as DENV2 and WNV in *Aedes* and *Culex* mosquitoes, respectively (Scott et al., 2010; Paradkar et al., 2014; Brackney, Beane, and Ebel, 2009), and

ZIKV and WNV in *D. melanogaster* (Harsh et al., 2018; Chotkowski et al., 2008). RNAi is also important in controlling viral replication and mosquito survival against alphaviruses such as Sindbis virus (SINV) (Cirimotich et al., 2009) and Semliki Forest virus (SFV) (Siu et al., 2011). While RNAi is present in higher-level organisms such as humans, the extent and importance of its role as an antiviral immune regulator remain unclear and it is not yet defined as an antiviral mechanism in mosquito-borne viral infections (Cullen, 2014; Schnettler et al., 2012; Li, Y. et al., 2013).

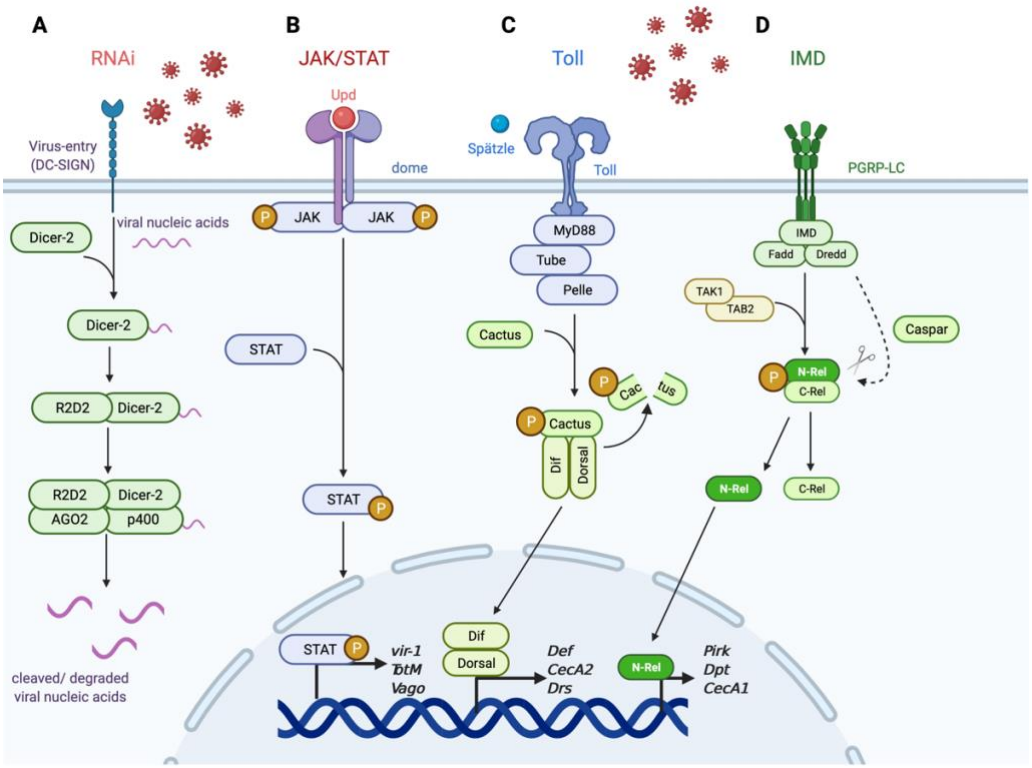

Figure 1. Innate immune signaling in insect and mosquito systems. The innate immune system is heavily conserved across species and involves various signaling pathways induced in response to viral nucleic acids (RNAi) or detection of upstream antiviral effectors (JAK/STAT, Toll, IMD). (**A**) The RNA interference (RNAi) pathway functions to detect cytosolic dsRNA or DNA that is indicative of viral infection. (**B**) The JAK/STAT pathway, which is conserved between mosquito and human species, induces transcription of downstream antiviral effector

genes. (**C**) The Toll pathway functions to respond to Gram-positive bacterial and fungal infection and is present in the form of TLR in mammals. (**D**) The IMD pathway is activated during Gram-negative bacterial infection and is similar to the TNF/NF-kB pathway. Adapted from "Blank Pathway (Linear)", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates (Biorender, 2021).

Another important signaling response involved in mosquito antiviral immunity is the JAK/STAT pathway (Figure 1B). Similar to RNAi, JAK/STAT is linked to various host processes beyond immunity including cellular division, maintenance, and regeneration (Osman et al., 2012; Wright et al., 2011), along with regulating oogenesis in insect species (Wittes and Schüpbach, 2019; Ghiglione et al., 2002). Unlike the RNAi pathway, however, the functional role of JAK/STAT as a cellular and antiviral regulator is well conserved between vertebrate and invertebrate hosts (Herrera and Bach, 2019; Levy, 1999) and is associated with responding to various viral infections including WNV (Ahlers et al., 2019), ZIKV (Harsh et al., 2020; Angleró-Rodríguez et al., 2017) , and DENV (Terradas, Jourbert, and McGraw, 2017; Jupatanakul et al., 2017; Souza-Neto, Sim, and Dimopoulos, 2009). JAK/STAT is an effector response pathway that activates an intracellular signaling cascade and induces downstream antiviral genes in response to infection (Dostert et al., 2005). Upon infection, the extracellular ligand unpaired (Upd) is secreted (Agaisse et al., 2003; Harrison et al., 1998) and binds to the receptor domeless (dome) expressed on neighboring hemocytes or related immune cells (Brown, Hu, and Hombría, 2001). Binding results in the activation of the intracellular Janus kinase hopscotch (hop) (Binari and Perrimon, 1994) and phosphorylation of transcription factor STAT (Yan et al, 1996; Hou, Melnick, and Perrimon, 1996). Phosphorylation then induces the dimerization and nuclear import of STAT to

promote transcription of downstream antiviral effectors such as *vir-1* (Dostert et al., 2005) and *TotM* (Agaisse et al, 2003). Vago is a secreted cytokine that activates JAK/STAT signaling in *Cx. quinqufasciatus*, and *Vago* is induced in a Dicer-2-dependent manner (Paradkar et al., 2012). JAK/STAT signaling is conserved between invertebrate and vertebrate species as well as its functional role in an innate immune response to viral infections. In humans, JAK/STAT signaling is partially involved in the generation of type-I interferon (IFN)-stimulated responses and has been shown to be active in the presence of ZIKV (Wu et al., 2017), WNV (Keller et al., 2006), and Japanese encephalitis virus (JEV) (Lin et al., 2004). Since Vago restricts WNV in *Cx. quinquefasciatus*, its role as an antiviral secreted cytokine is similar to that of mammalian IFN (Paradkar et al., 2012).

The Toll and IMD pathways, while primarily associated with antibacterial and antifungal immunity, have also been implicated in antiviral protection within the mosquito and insect models (Figure 1C,D) (Valanne, Wang, and Rämet, 2011; Myllymäki, Valanne, and Rämet, 2014). The Toll pathway is important for defense against Gram-positive bacterial and fungal infections (Michel et al., 2001; Lemaitre et al., 1996), whereas IMD is important against Gram-negative bacteria (Leulier et al., 2003; Kaneko 2004). The mechanistic events involved in Toll and IMD signaling have been heavily dissected using the *D. melanogaster* model, and the pathways are evolutionarily conserved in *Aedes* and *Culex* mosquitoes. Toll signaling is initiated when the ligand Spätzle binds to the Toll receptor to activate adaptor proteins MyD88, Tube, and kinase Pelle to induce a phosphorylation cascade that activates degradation of the regulatory factor Cactus following its phosphorylation (Horng and Medzhitov, 2001; Tauszig-Delamasure et al., 2002; Sun et al., 2002). Once Cactus is degraded, the transcription factors Dif and Dorsal

are able to translocate into the nucleus to induce transcription of downstream antimicrobial peptides (AMPs) and response genes (Wu and Anderson, 1998). The IMD pathway is activated by DAP-type peptidoglycans recognized by peptidoglycan recognition proteins (PGRP-LC) which act as transmembrane receptors that induce a series of phosphorylation and cleavage events in the cytosol. These intracellular events ultimately result in induction of downstream AMPs and response genes (Choe, Lee, and Anderson, 2005). Binding to PGRP-LC induces the formation of a signaling complex composed of the proteins IMD, Fadd, and Dredd (Leulier et al., 2003; Georgel et al., 2001; Stöven et al., 2003). Dredd cleaves IMD which recruits the Tab2/Tak1 protein complex that induces the phosphorylation and cleavage of the transcription factor Relish (Rel) (Silverman et al., 2000; Paquette et al., 2010; Dushay, Asling, and Hultmark, 1996). Caspar acts as a negative regulator of IMD by targeting Dredd-mediated cleavage (Kim et al., 2006). Cleavage of Rel results in the nuclear translocation of the N-terminus of Rel to induce transcription of effector genes responsible for regulating AMPs and other immune response elements (Stöven et al., 2003; Stöven et al., 2000). The Toll and IMD pathways have been heavily dissected in the context of antibacterial and antifungal immunity but have also been linked to humoral and cellular antiviral responses in the insect system. For example, *Ae. aegypti* knocked down for *Cactus* and *Caspar* by RNAi exhibited increased Toll and IMD immune signaling during DENV infection and reduced viral replication (Barletta et al., 2016; Xi, Ramirez, and Dimopoulos, 2008). Toll and JAK/STAT signaling has also been shown to be induced during ZIKV infection in *Ae. aegypti* (Angleró-Rodríguez et al., 2017). In addition, WNV infection in *Culex pipiens* induces Toll signaling in addition to the canonical RNAi and JAK/STAT pathways (Zink et al., 2015). It is also important to note that each pathway is involved in hemocytes' functional role as circulating immune cells against insect-specific

(Dostert et al., 2005; Zambon et al., 2005; Wang et al., 2015; Costa et al., 2009) and vector-borne viruses such as DENV and ZIKV (Angleró-Rodríguez et al., 2017; Terradas, Joubert, and McGraw, 2017; Barletta et al., 2016; Zink et al., 2015; Nanfack-Minkeu et al., 2019). Finally, Toll and IMD signaling is conserved in the human immune system as the Toll-like receptor (TLR) signaling pathway (Medzhitov, Preston-Hurlburt, and Janeway, 1997) and NF-kB/TNF signaling pathways (Engström et al., 1993), respectively. Both TLRs and NF-kB/TNF signaling pathways have been linked as critical defense mechanisms against various RNA viruses including WNV (Kumar, Belcaid, and Nerurkar, 2016; Daffis et al., 2009; Szretter et al., 2010; Thackray et al., 2014).

Physical Barriers in the Mosquito. Mosquitoes primarily become infected through ingestion of a bloodmeal containing a virus. Due to this infection route, virions undergo various environmental pressures and conditions as they move from the midgut to distal tissues such as the salivary glands and ovaries which are involved in horizontal and vertical transmission, respectively. The physical barriers that the viruses overcome in order to reach viremia conditions are a significant component in the mosquito's response to infection. Comparatively, the physical barriers involved in the mosquitoes' and humans' response in infection vary significantly as the different organ systems and cellular pressures encountered would utilize different host factors to respond to viral infection.

Perhaps the most significant tissue functioning as a physical barrier in preventing viremia in the mosquito is the midgut, the organ responsible for the digestion of an ingested bloodmeal and absorbance of essential nutrients (Okuda et al., 2002). The virus must first overcome the midgut

infection barrier, which is when the virus moves from the gut lumen to the midgut epithelial cells. Upon infecting and replicating in the midgut epithelial cells, the virus then passes the midgut escape barrier and basal lamina to disseminate into the hemocoel (Okuda et al., 2002; Romoser et al., 2004). Once the virus breaches the midgut, virions enter the hemocoel and induce activation of humoral immune responses such as melanization (Rodriguez-Andres et al., 2012) to limit dissemination (Kumar et al., 2018). Failure to keep infection localized results in viremia that systemically infects distal tissues such as the fat body, hemocytes, and salivary glands (Sim, Ramirez, and Dimopoulos, 2012; Parikh, Oliver, and Bartholomay, 2009; Girard, Klinger, and Higgs, 2004). It is at this point when the virus reaches, modulates signaling events, and establishes itself in the salivary glands that the mosquito becomes a competent vector for virus transmission in future bloodmeals. The dissemination rate into midgut epithelial cells varies between virus and mosquito species as JEV disseminates faster in *Culex* mosquitoes when compared to DENV2 dissemination in *Aedes* (Mourya and Mishra, 2000; Zhang, Zhenge, et al., 2010). Dissemination rates are also enhanced based on the frequency of subsequent bloodmeals, regardless of whether they are infected or not, due to the digestive impact on the midgut integrity and permeability (Armstrong et al., 2020; Girard et al., 2005; Weaver et al., 1988).

For a virus to reach the blood–lymphatic system and become systemic, virions must overcome the physical and chemical barriers of skin tissue, in addition to avoiding immune cells. These differences in how viremia is achieved in the vector and mammalian hosts pose as a potential target for vector control intervention. The different cellular environments that the virus is exposed to during dissemination in the mosquito and human hosts implies that targeted therapeutics would have varying degrees of success. For example, recent studies have

demonstrated that targeting signaling events involved in digestive and nutritional acquisition may prime mosquitoes for viral infection and reduce viral replication and the likelihood of transmission to subsequent hosts (Ahlers et al., 2019; Xu et al., 2013; DiAngelo et al., 2009; Drexler et al., 2014). This is evident in the implementation of using *Wolbachia*, an endosymbiont present in various insect and arthropod species, as a means of vector control due to its established effect on reducing vector competence and viral replication in mosquito populations (Terradas, Joubert, and McGraw, 2017; Haqshenas et al., 2019; Hedges et al., 2008). Additionally, stimulating the insulin/IGF-1 signaling pathway has been shown to reduce infection in mosquito vectors (Ahlers et al., 2019; Xu et al., 2013; DiAngelo et al., 2009). While targeting nutritional and digestive events may be effective in limiting viral activity in the mosquito, targeting similar processes in humans or other vertebrates may not be as effective as the virus would not undergo the same cellular pressures. In the case of insulin/ IGF-1 signaling, in which insulin has a broad effect on transcriptional activity beyond immune signaling, it may be possible to implement similar insulin-dependent strategies for both by targeting different insulin targeted-downstream host factors (Drexler et al., 2014; Luckhart et al., 2013; Altindis et al., 2018; Liang et al., 2016).

Variability between Mosquito Species and Viruses. Specific virus transmissions are generally linked to certain mosquito genera or species. For example, *Aedes* mosquitoes are primarily associated with hemorrhagic- or arthritic-inducing viruses such as DENV, CHIKV, and yellow fever virus (YFV) transmission, whereas *Culex* mosquitoes are associated with encephalitic viruses such as WNV, JEV, and St. Louis encephalitis virus (SLEV). Each genus's geographic range does overlap to a certain degree with significant overlap within the Northern and Southern

tropics, but each genus does possess a certain unique range as habitation becomes more polar within Africa and Southeast Asia (Kraemer et al., 2015; Leta et al., 2018; Samy, Elaagip, et al., 2016; Alaniz et al., 2018). There is also evidence of specific genus activity within the Northern and Southern tropics based on present environmental pressures such as elevation, population density, and available nutritional sources (Kamal et al., 2018; Muttis et al., 2018). This correlates to disease incidence within these areas as well as expansion of mosquito populations within the regions (Giovanetti et al., 2019; Miller and Loaiza, 2015).

Current studies have primarily focused on how specific mosquito species respond to viral infection without comparison to how other species may respond to the same pathogen. ZIKV and Rift Valley fever virus (RVFV), for example, are able to infect both *Aedes* and *Culex* mosquitos but to differing levels of success depending on the species and virus strain (Weger-Lucarelli et al., 2016; Elizondo-Quiroga et al., 2018; Jupp et al., 2002). Research thus far as indicated that certain immune signaling and physical barriers play an important role in antiviral responses, but there is limited understanding as to how multiple canonical or novel signaling pathways may interact with one another to achieve the most effective immune response. While there is still much to discover regarding how different mosquito populations respond to and regulate the multitude of arboviruses that pose a threat to human populations, new studies are beginning to compare the related and unique host factors within mosquito populations and how they may lead to either broad or mosquito-specific intervention targets.

HUMAN ANTIVIRAL IMMUNITY

Mosquito-borne viruses pose a global health threat as they can be transmitted to humans with limited therapeutics or preventatives available. Unlike the mosquito vector, vertebrate hosts have evolved to possess two forms of immunity: the innate immune response, which is heavily conserved across species as previously discussed, and the adaptive immune response. The latter form of immunity is responsible for specific and long-lasting immunological memory associated with humoral and cellular immune responses. In the context of immune responses during an active viral infection, both branches of host immunity are involved and impact disease morbidity, mortality, and long-term immunity. Many of the host factors and signaling pathways present in the mosquito are also conserved in humans (Table 1). Specifically, the innate immune response pathways previously discussed are present to some orthologous or functional degree. Variability between mosquito and human host factors active during viral infections primarily exists in the form of physical defense barriers and adaptive immune responses.

Table 1. Host factors involved in responses to mosquito-borne viruses.

	RNA Interference	JAK/STAT	Toll	IMD/TNF	Physical Barriers	Adaptive Immunity
Mosquitoes	Dicer-2 AGO2 Drosha R2D2 p400 piwi4 *Ppo8*	Hop STAT1 *Vago* *vir-1*	Toll dMyD88 Dorsal DIF Cactus *Spätzle*	Relish Caspar Fadd Dredd	Hemocytes *Midgut epithelium*	N/A
Humans	Dicer AGO Drosha TARBP2 Piwi	JAK STAT1/2 *RIG-I* *MDA5* *IFN*-α/β	TLRs MyD88 NF-kB *IFN*-α/β *IRF7*	NF-kB FAF1 Caspase8/10	Blood–brain barrier *Epidermis* *Dermis*	*B cells* *T cells*

Italicized factors are unique to the specified host or no ortholog exists in the other host.

Transmission and Physical Barriers in Human Hosts. The human host initiates an immediate immune response following a mosquito bloodmeal in which the virus-infected saliva is ejected from the mosquito hypopharynx into the skin epidermis. The saliva contains various host-derived salivary factors that can enhance viral transmission and reduce pro-inflammatory responses initiated by the human host (Moser et al., 2016; Sun et al., 2020; Styer et al., 2011). This includes various mosquito-derived factors including anticoagulants (Stark and James, 1996) and deregulators that disrupt recruitment of immune cells such as macrophages and neutrophils (Schneider et al., 2010; Bai et al., 2010; Ben-Nathan et al., 1996; Vogt et al., 2018). Ultimately, these salivary factors result in enhanced cell infection and viral replication that assists in viral dissemination (Pingen et al., 2016). Virions infect local resident cell populations including keratinocytes (Lim et al., 2011), dermal dendritic cells (DCs), and Langerhans cells (Figure 2A) (Johnston et al., 2000). DCs are responsible for movement of a virus into the local lymph node where replication and viremia are induced (Johnston et al., 2000). Permissive tissues typically vary between virus type but include the spleen (DENV, RVFV), liver (YFV, WNV), and neuronal tissue (ZIKV, JEV), amongst others. This tissue tropism coincides with the disease manifestation caused by each virus.

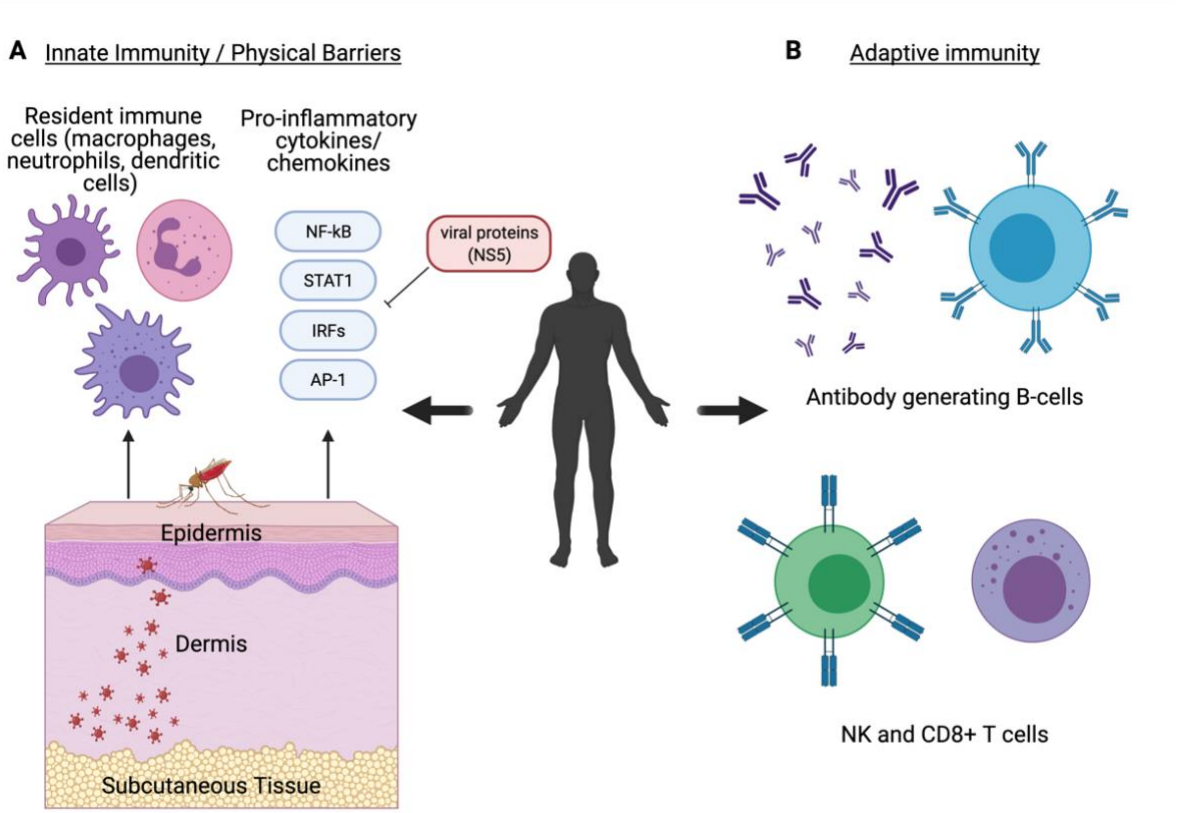

Figure 2. Innate and adaptive immune signaling in the human system. Humans defend against viral infections using physical barriers, innate immune signaling, and adaptive immune signaling responses. (A) Innate immune responses occur at physical barriers and involve the secretion of pro-inflammatory cytokines and chemokines by resident immune cells such as macrophages, neutrophils, and dendritic cells (Schneider et al., 2010; Bai et al., 2010; Ben-Nathan et al., 1996; Vogt et al., 2018; Lim et al., 2011; Johnston et al., 2000; Samuel and Diamond, 2005). Components of the innate immune system are well conserved in both mosquitoes and humans. (B) The adaptive immune response primarily involves the generation of specialized and specific antibody-producing B cells, NK cells, and CD8+ T cells (Hershkovitz et al., 2009; Zhang, Daniel, et al., 2010; Xin et al., 2010; Purtha et al., 2008; Pinto et al., 2011; Hong et al., 2019; Lazear et al., 2013). The adaptive immune response is a more evolved form of

immunity that is unique to vertebrate organisms. Created with BioRen-der.com (BioRender, 2021).

Innate and Adaptive Immune Responses in the Human Host. Innate immune responses to viral infection in the human host involve both conserved responses present in the mosquito as well as more effective, specific responses evolved in vertebrate organisms. Specifically, the innate immune signaling pathways previously discussed within the mosquito are functionally present in humans in the forms of more evolutionarily advanced responses that can act both independently and cross-talk with other pathways (Table 1). Local immune cells induce expression of pro-inflammatory cytokines and type I interferon (IFN) responses including IFN-α and IFN-β (Samuel and Diamond, 2005). Induction of IFN and downstream antiviral response genes are regulated by PRRs such as the RIG-I-like receptors (RLR), TLRs, and cGAS/STING that regulate transcription factors such as NF-kB and IRF3, which then stimulates JAK/STAT signaling to induce IFN-stimulated genes (ISG) (Akira, Uematsu, Takeuchi, 2006). This cytokine-mediated signaling indicates the presence of active infection and results in the recruitment of immune cells (Figure 2A) (Keller et al., 2006, Lin et al., 2004). Proper function of these response elements is critical for host survival (Lazear et al., 2016; Shresta et al., 2004). The NS5 protein expressed by WNV (Lubick et al., 2015), ZIKV (Grant et al., 2016), DENV (Morrison et al., 2013), JEV (Lin et al., 2004), and YFV (Laurent-Rolle et al., 2014) has shown to mediate host immune evasion as an antagonist to IFN signaling which results in enhanced viral replication (Figure 2A). CHIKV NS2 also functions as an antagonist of IFN signaling by inhibiting activation of JAK/STAT (Fros et al., 2010).

In addition to the innate immune signaling conserved across species, vertebrate organisms have an evolved adaptive immune system to more effectively respond to infection. Components of the adaptive immune system make up the foundation of what is referred to as immune memory by developing established B and T cell populations that can more effectively and specifically recognize, neutralize, and degrade infectious virions during and in subsequent infections. DCs and macrophages act as antigen-presenting cells and link innate and adaptive immune responses to induce expression of pro-inflammatory cytokines and chemokines and recruitment of cell populations involved in the cell-mediated response including NK cells (Hershkovitz et al., 2009; Zhang, Daniel, et al., 2010) and neutrophils (Bai et al., 2010; Xin et al., 2010). As infection progresses, IgG-secretory B cells and CD8+ T cell populations develop to effectively neutralize and inhibit further viral replication (Figure 2B) (Purtha et al., 2008; Pinto et al., 2011; Hong et al., 2019; Lazear et al., 2013). While the host factors involved in innate immune signaling are conserved between mosquitoes and humans, the adaptive immune response has evolved its own unique subset of host factors to enhance host immunity against viral pathogens (Table 1).

Impacts on Morbidity and Mortality. Following a successful immune response, humans are typically able to clear viral infection and generate some protective immunity for potential future infections. There is evidence, however, indicating that even upon clearing an active infection, certain host factors can greatly influence the outcome of future infections against the same or related mosquito-borne viruses. Perhaps the most significant example is the phenomenon known as antibody-dependent enhancement (ADE). ADE is a result of a prior infection generating antibodies that, upon subsequent infections with a similar or related virus, enhances viral entry, replication, and the likelihood of severe disease (Tirado and Yoon, 2003). This permits partially

bound virion–antibody complexes to bind to Fc receptors present on immune cells to mediate increased entry, mass virion replication, and premature release of virions (Gollins and Porterfield, 1984; Gollins and Porterfield, 1985; Dejnirattisai et al., 2010). DENV is the best example of mosquito-borne virus ADE as subsequent infection with a different serotype (Dejnirattisai et al., 2010) or ZIKV (Charles and Christofferson, 2016) may result in this increased viral uptake in immune cells and an increased likelihood of presenting symptoms associated with disease such as hemorrhagic fever or neurological damage. This, in turn, is followed by greater disease severity and risk of mortality (Kliks et al., 1988; Kliks et al., 1989; Guzmán et al., 2002). ADE is a significant concern in the production and implementation of DENV vaccines and as such has caused delays in their effective development and implementation to the general public (de Silva and Harris, 2018).

Dysfunctional insulin signaling in humans is linked to impaired immunity to mosquito-borne viruses. Diabetic individuals are more prone to developing severe disease symptoms and mortality against ZIKV (Nielsen and Bygbjerg, 2016), WNV (Kumar et al., 2012, 2014), and DENV (Lee et al., 2020). Previously medical professionals thought this was due to an overall reduction in host signaling and regulatory processes, but now studies have begun to identify that such viruses impact and target components of insulin signaling to cause disease pathology. The NS4A and NS4B proteins expressed on ZIKV reduce AKT-mTOR signaling, which is targeted in insulin treatment and causes destruction of neuronal tissues that is a hallmark of disease (Liang et al., 2016). While the effect that insulin has on human immunity during arboviral infection is still a relatively unexplored field, it may be an ideal candidate for future disease intervention that could be applied at the vector level as well.

Co-infection with multiple arboviruses poses different effects between human and mosquito hosts. While humans may present with more severe clinical symptoms or competing viral replication (Vogels et al., 2019), mosquitoes co-infected with different viruses experience little obvious hazardous phenotypes (Göertz et al., 2017; Rückert et al., 2017) and may even enhance viral replication and the likelihood of transmission (Chaves et al., 2018). Whether this is due to variability in host factors or viral replication mechanisms is still under investigation, but this phenomenon does present another example in which mosquito and human immune responses to arboviruses vary at the molecular level due to available host factors.

OUTLOOK

As evident in the gradual increase in the number and severity of clinical cases as well as the expansion of mosquito activity across the globe, the looming threat that mosquito-borne viruses pose is of significant concern and must be addressed at both the vector and clinical levels. Understanding the host immune responses and how they are varied between organisms is an important step in identifying more effective targets for vector control and therapeutics. More importantly, understanding how the responses are similar between mosquito-borne viruses is of great value as it permits research in broad or virus-specific targeting. As summarized in Table 1, the host factors involved during viral infection in mosquitoes and humans are well conserved with some variability regarding host physical barriers and adaptive immunity.

Further investigation is required into identifying and evaluating the importance of certain antiviral immune responses in both humans and mosquitoes. For example, vector-control

mechanisms such as the endosymbiont *Wolbachia*, while reducing viral load in mosquitoes infected with ZIKV and DENV (Haqshenas et al., 2019; Hedges et al., 2008), may be pro-viral for other related viruses such as WNV (Dodson et al., 2014). This indicates that there may be virus-specific variations among related pathogens that result in potential, broad antiviral preventatives being less effective. This is also the case in humans as responses that are important against one virus may be insignificant or detrimental for another (de Silva and Harris, 2018). One example of a potential cross-species antiviral target, as previously discussed, is how insulin/IGF-1 signaling regulates mosquito and human immunity. Since this pathway possesses a broad effect on homeostatic activity in both organisms, insulin-mediated immunity may be achieved by targeting different downstream host factors or pathways. While studies into insulin-mediated arboviral immunity are not well established in humans, there is an established effect of dysfunctional insulin signaling on patient survival and virus activity for ZIKV and WNV (Liang et al., 2016; Kumar et al., 2012). Research in and implementation of more effective antivirals in both mosquitoes and humans are necessary and require a greater understanding regarding the conserved and differing host factors that respond to these zoonotic infections.

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AUTHOR CONTRIBUTIONS

C.E.T. wrote the manuscript in consultation with A.G.G. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER THREE: INSULIN-MEDIATED ENDOTHELIN SIGNALING IS ANTIVIRAL DURING WEST NILE VIRUS INFECTION

Trammell, C.E., Rowe, E.H., Jones, B.J., Char, A.B., Fawcett, S., Ahlers, L.R.H., and Goodman, A.G. Insulin-mediated endothelin signaling is antiviral during West Nile virus infection. In preparation.

Attributions:

C.E. Trammell was responsible for experimental design and execution. They completed experiments with assistance of E.H. Rowe, B.J. Jones, A.B. Char, and S. Fawcett. They completed data collection, analysis, and wrote the text of this manuscript in consultation of A.G. Goodman.

E.H. Rowe was responsible for fly survival, titer, and western blot studies presented in Figure 2 in consultation with C.E. Trammell.

B.J. Jones was responsible for optimization of siRNA knocked-down qRT-PCR and titer studies presented in Figure 3 in consultation with C.E. Trammell

A.B Char and S. Fawcett were responsible for interferon titer analysis presented in Figure S3 in consultation with C.E. Trammell.

L.R.H. Ahlers was responsible for generating mRNA samples used for RNAseq analysis present in Figure 1 in consultation with C.E. Trammell and A.G. Goodman.

A.G. Goodman was responsible for funding and revisions in consultation with C.E. Trammell.

ABSTRACT

West Nile virus (WNV) is the most prevalent mosquito-borne virus in the United States with approximately 2,000 cases each year. There are currently no approved human vaccines and a severe lack in prophylactic treatments. Understanding host responses to infection may reveal potential intervention targets to reduce virus replication and disease progression. The use of *Drosophila melanogaster* as a model organism to understand innate immunity and host antiviral responses is well established. Previous studies revealed that insulin-mediated signaling regulates WNV infection in invertebrates by regulating canonical antiviral pathways. Because insulin signaling is well-conserved across insect and mammalian species, we sought to determine if results using *D. melanogaster* can be extrapolated for the analysis of orthologous pathways in humans. Here, we identify insulin-mediated endothelin signaling using the *D. melanogaster* model and evaluate an orthologous pathway in human cells during WNV infection. We demonstrate that endothelin signaling reduces WNV replication through the activation of canonical antiviral signaling. Taken together, our findings show that endothelin-mediated antiviral immunity is broadly conserved across species and reduces replication of viruses that can cause severe human disease.

INTRODUCTION

West Nile virus (WNV) is a member of the *Flaviviridae* family and is transmitted predominately between *Culex quinquefasciatus* and birds with humans as incidental "dead-end" hosts (Ahlers and Goodman, 2018). WNV was introduced to the Western Hemisphere through New York in 1999 and has since become endemic in the United States (Centers for Disease Control and Prevention, 1999; Nash et al., 2001; Lanciotti et al., 1999). Like other arthropod-borne viruses,

WNV poses a significant health threat due to the expansion of mosquito range and activity (Gorris et al., 2021; Harrigan et al., 2014; Ludwig et al., 2019) without effective means to address these concerns at a transmission or clinical level. While our ability to address arboviruses at the vector-transmission level has progressed significantly in the past decade through genetic (Evans et al., 2019), microbial (Hedges et al., 2008), or small molecule (Trammell et al., 2022) targeting of mosquito responses, addressing WNV clinical cases has lagged. There are currently no vaccines or specific treatments available for treating WNV with the best approaches being disease management and pain relief (Alli et al., 2021). Understanding host responses to viral infection and identifying potential therapeutic targets would allow us to better address WNV infection and disease at a clinical level.

Drosophila melanogaster is an established model organism that has been used for studying host responses. This is due to its readily accessible and annotated genome that permits broad or targeted study of specific signaling pathways or interactions. *D. melanogaster* has been successfully used to study innate immune responses to infection including WNV (Yasunaga et al., 2014; Ahlers et al., 2019) and Zika virus (ZIKV) (Liu et al., 2018). Previous investigation identified insulin-mediated induction of JAK/STAT as a critical component of host survival and immunity to WNV in *D. melanogaster* that was conserved in *Culex quinquefasciatus* (Ahlers et al., 2019). Because of the broad conservation that the insulin signaling pathway is across species, especially from *D. melanogaster* to human systems (Puig et al., 2003; Barbieri et al., 2003), we rationalize that insulin-mediated antiviral immunity may exist in the human innate immune system as well. Previous studies have shown that viral infection may target components of insulin signaling that can result in insulin resistance and dysfunction (Šestan et al., 2018; Campo

et al., 2012; Yu et al., 2021; Liang et al., 2016; Chan et al., 2018), but there is limited investigation about how this host-virus interaction can be a potential intervention target. Because of the substantial number of downstream signaling pathways insulin signaling impacts, we sought to identify previously unidentified signaling pathways that canonical insulin signaling regulates and may have important roles in the host response to viral infection. In addition, because of the significant conservation that insulin signaling possesses across species and the genetic power of the *D. melanogaster* model, we propose that we can extrapolate identified pathways from *D. melanogaster* and their orthologous pathways in the human system.

In this study, we performed RNA sequencing (RNAseq) in *D. melanogaster* during WNV infection to identify novel antiviral response elements that are activated in the presence of insulin. We find that insulin induces a large number of both canonical antiviral response elements as well as genes that are were previously unidentified components of host immunity. Specifically, we identified the endothelin signaling pathway and evaluated its importance for host survival and reducing WNV infection. We then used this information to evaluate endothelin signaling in human cells. We similarly found that endothelin signaling was important for regulating viral replication and regulating insulin-mediated responses to infection against both attenuated and virulent WNV strains. These results suggest that insulin regulates endothelin signaling such that the loss of endothelin results in deficient host antiviral immunity. These pathways are conserved across species and may be a potential avenue for future therapeutic research.

RESULTS

Transcriptomic profiling of *D. melanogaster* S2 cells identifies antiviral pathways linked to insulin-signaling. We first sought to generate a complete transcript profile of *D. melanogaster* S2 cells following 24 h treatment with 1.7 μM bovine insulin and 8 h infection with WNV-Kun (MOI 1 PFU/cell). Gene expression in treated and/or infected cells was measured relative to that in controls receiving neither bovine insulin or virus (Fig. 1A). These experimental conditions were selected based on previous showing that bovine insulin treatment induces sufficient insulin and JAK/STAT signaling in S2 cells (Ahlers et al., 2019). The average number of sequence reads mapped to the *D. melanogaster* genome is approximately 93.22% (Table S1, Sheet 1).

Gene set enrichment analysis (GSEA) was performed to identify and compare enriched gene sets in 0 μM insulin + WNV-Kun, 1.7 μM insulin + mock infection, and 1.7 μM insulin + WNV-Kun (Fig. 1B). Analysis was completed to identify previously unidentified gene sets for further analysis as well to compare to previous, targeted qRT-PCR analysis showing enrichment of insulin and JAK/STAT signaling (Ahlers et al., 2019). Gene sets were filtered for $p < 0.05$ in at least one experimental condition and were selected based on their association with immunity and WNV disease. We identified eight gene sets that are significantly enriched in the presence of insulin including immune response elements (response to oxidative stress, regulation of JAK-STAT cascade), canonical insulin signaling (phosphoinositide 3-kinase activity, insulin-like growth factor receptor signaling pathway, positive regulation of TOR signaling pathway, Ras protein signal transduction), and physiological development (establishment of glial blood-brain barrier, heart development). (Fig. 1B).

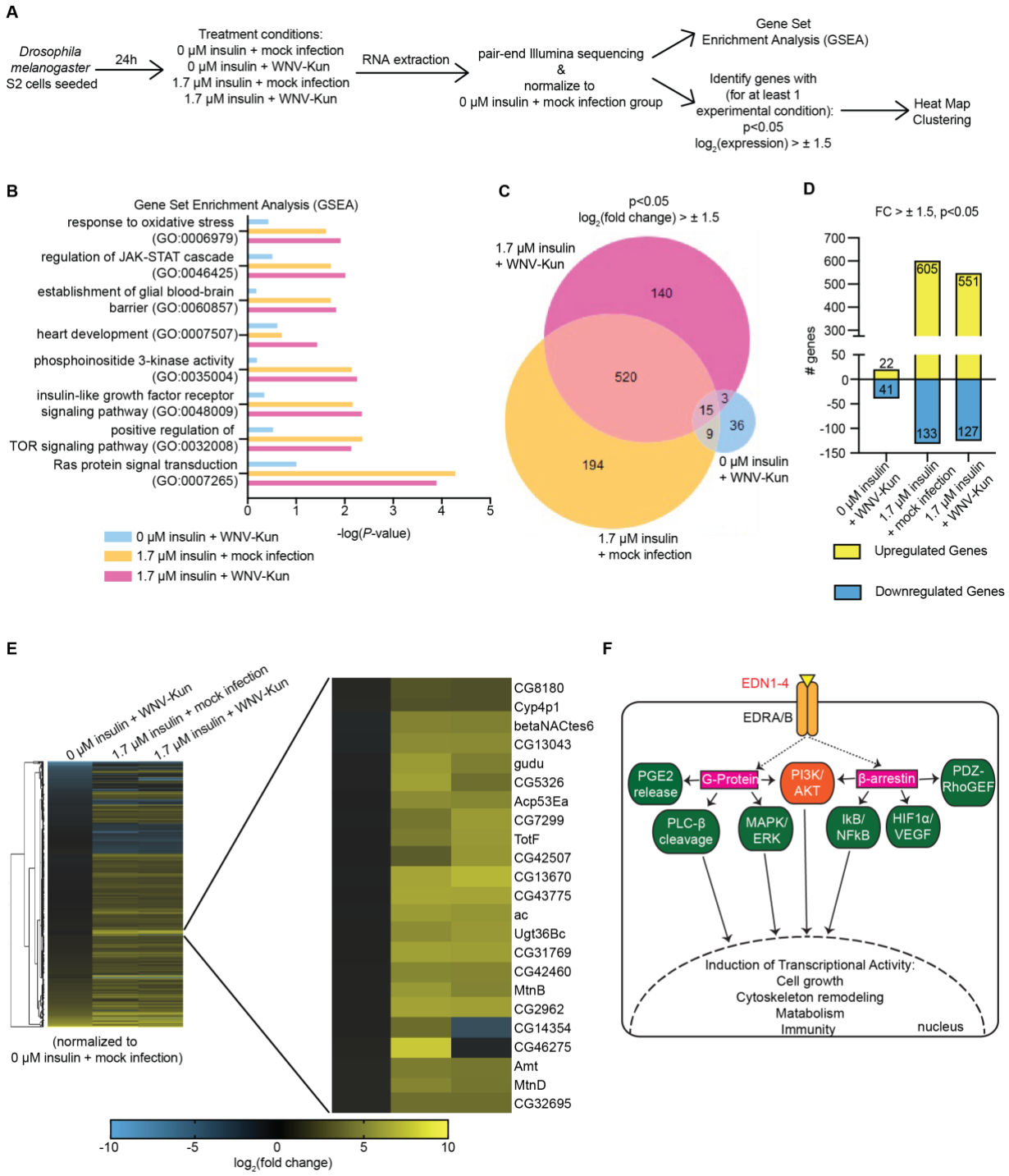

Fig. 1: Insulin treatment during WNV-Kun infection in *D. melanogaster* S2 cells induces canonical and previously unidentified signaling pathways. (A) Schematic illustrating experimental design for RNA extraction and RNA sequencing (RNAseq) analysis of *D. melanogaster* S2 cells with or without insulin treatment or WNV-Kun infection. (B) Gene Set

Enrichment Analysis (GSEA) using transcript levels for each experimental condition normalized to 0 μM insulin + mock infection from the RNAseq analysis. Gene ontology (GO) categories were selected based on GSEA p value ($p < 0.05$) for at least one experimental condition. (C) Venn Diagram of all transcripts enriched or suppressed for each experimental condition normalized to 0 μM insulin + mock infection. Transcripts were selected based on their $\log_2$(fold change) (FC) > ± 1.5 and $p < 0.05$ for at least one experimental condition. (D) The number of genes transcriptionally enriched (yellow) or suppressed (blue) for each experimental condition normalized to 0 μM insulin + mock infection. (E) Hierarchal clustering and heat map expression of genes transcriptionally enriched or suppressed as identified in (C-D). Genes shown in enlarged cluster identify a subset of genes that showed the most up-regulation compared to no insulin treatment. GO analysis identifies this get set associated with endothelin signaling. (F) Schematic of canonical endothelin signaling in mammals and its intracellular and transcriptional activity.

Further analysis into the specific genes that were transcriptionally induced or suppressed was carried out to better understand the impact that infection or insulin treatment have on the *D. melanogaster* transcriptome. Genes were filtered for $p < 0.05$ and a $\log_2$(fold change) > ± 1.5 for at least one experimental condition. There was a ~10-fold increase in the number of differentially expressed genes in cells that received insulin treatment and those that received no insulin (Fig. 1 C-D). 535 genes were commonly regulated in the presence of insulin regardless of WNV-Kun infection status (Fig. 1C). Together, this suggests that insulin treatment enriches or suppresses transcriptional activity with a high overlap in genes affected between mock infection and WNV-Kun infection. Cells that were not treated with insulin but were infected with WNV-Kun only exhibited 22 upregulated genes and 41 downregulated genes. Cells that received only insulin

treatment reported 605 upregulated genes and 133 downregulated genes. Cells that received insulin treatment and WNV-Kun infection exhibited 551 upregulated genes and 127 downregulated genes (Fig. 1D). These results suggest that insulin-treatment regulates a large set of genes during early stages of infection that can potentially impact later virus-specific responses.

Genes that were transcriptionally altered in Fig. 1C-D were used to generate a hierarchical clustering heatmap (Fig. 1E). As the goal of this study was to investigate effectors involved in insulin-mediated antiviral immunity, we were specifically interested in identifying and evaluating gene clusters that were enriched in the presence of insulin treatment (Fig. 1E, expanded node). Genes identified within the selected cluster were then imported into PANTHER Classification System to identify gene ontology (GO) categories that were overrepresented (Mi et al., 2019; Thomas et al., 2022) (Table S1, Sheet 2). Using this gene set, only the endothelin signaling pathway was identified. Endothelin signaling is primarily associated with cardiovascular function and smooth muscle constriction (Dagamajalu et al., 2021; Davenport et al., 2016). Through this functional role, endothelin signaling also interacts and impacts associated components linked to insulin signaling including the PI3K/AKT/FOXO axis (Chahdi and Sorokin, 2008; Cifarelli et al., 2012; Jiang et al., 1999; Li, Q. et al., 2013; Lu et al., 2014; Nihei et al., 2021; Renga et al., 2015) and MAPK/ERK axis (Chen, Edvinsson, and Xu, 2009; Foschi et al., 1997) (Fig. 1F). The endothelin signaling pathway is not a canonical immune pathway; however, it has been linked to *Mycobacterium tuberculosis* (Correa et al., 2014) and Hepatitis B/C virus (HBV) (HCV) infection (Notas et al., 2001; Ersoy et al., 2006) which leads us to consider that endothelin may also be involved during WNV infection and should be further

analyzed. Further analysis of *D. melanogaster* genes associated with endothelin signaling outside the heatmap shows that insulin treatment + mock infection or insulin treatment + WNV-Kun infection cells had significant transcriptional activity compared to only WNV-Kun infected cells (Fig. S1). These endothelin-related genes were selected based on their PANTHER GO classification and designation (Mi et al., 2019; Thomas et al., 2022). Because of the lack of knowledge pertaining to endothelin signaling in the insect or in the context of WNV, we further investigated this pathway to determine if it may be a mediator of insulin-mediated antiviral immunity against WNV.

***D. melanogaster CG43775* contributes to insulin-mediated antiviral immunity.** To validate and expand upon our RNAseq results, we more closely examined the magnitudes of fold changes presented in Fig. 1E. *CG43775* was one of the most up-regulated genes in the insulin treatment conditions (Table S1, Sheet 3), so we examined *CG43775* induction under the same conditions using qRT-PCR. We observed significant induction of *CG43775* in S2 cells with 1.7 μM insulin + WNV-Kun relative to other experimental conditions (Fig. 2A). We next experimented with adult flies that contained a transposable element insertion in *CG43775* to disrupt its expression ($CG43775^{MB08418}$) compared to genetic control flies (w^{1118}) (Bellen et al., 2011; Metaxakis et al., 2005). Survival of female flies that received either 5,000 PFU/fly or a mock infection was measured over 30 days. A hazard ratio was generated as a metric of host mortality which compares the mortality rate of the virus-infected mutant flies to that of the virus-infected control flies (Fig. 2B). We observed significant mortality in *CG43775* deficient flies with a mortality rate approximately 7-times greater compared to the control flies. To expand the role that *CG43775* has on host survival to viral infection, we measured viral titer in mutant and parental

flies at 1-, 5-, and 10-days post infection (d p.i) by standard plaque assay (Fig. 2C). We observed significantly higher virus replication in mutant flies by 10 d. These data suggest that *CG43775* is important for host survival to WNV-Kun infection due to its ability to reduce virus replication.

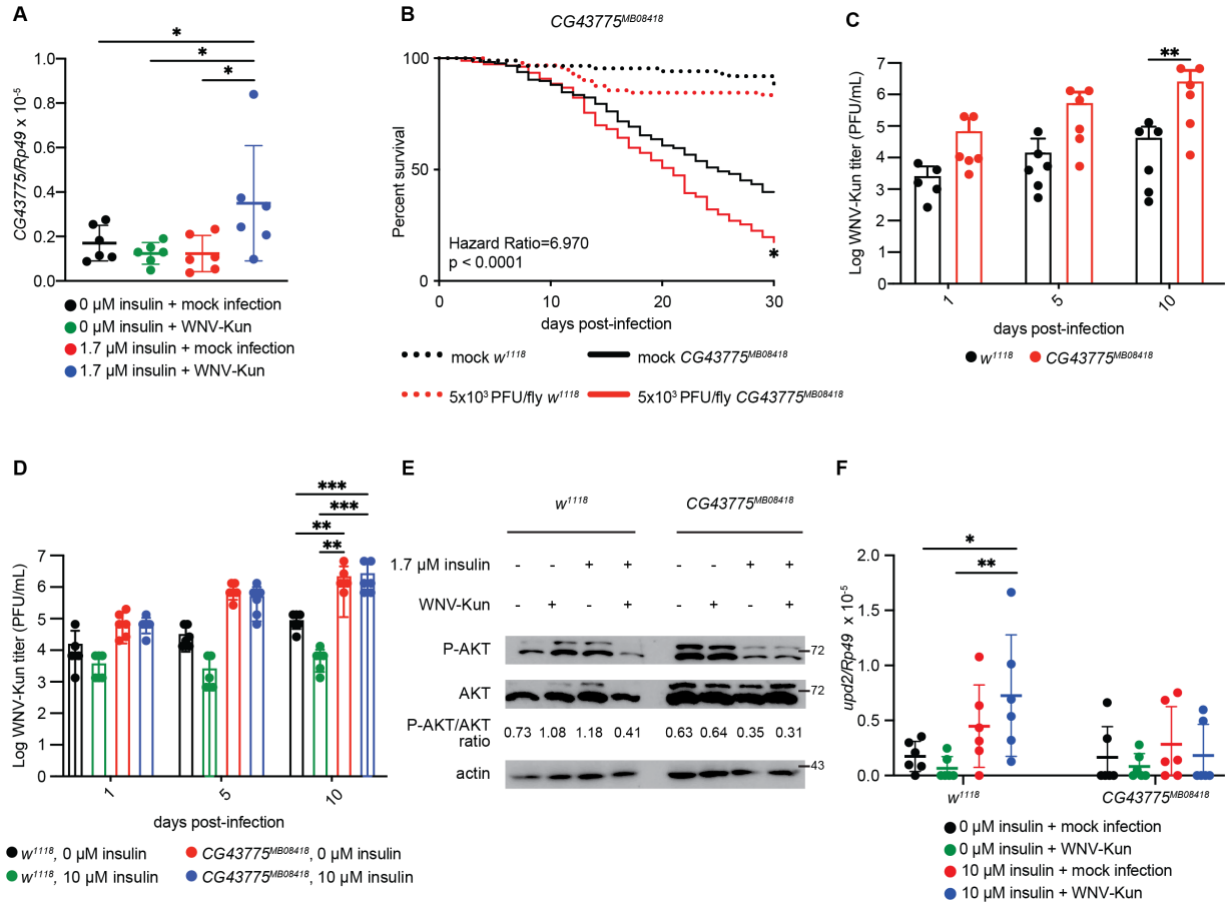

Fig. 2: *CG43775* mutant flies are more susceptible to WNV-Kun infection due to deficient insulin-mediated antiviral protection. (A) *CG43775* is induced in *D. melanogaster* S2 cells that were insulin-treated and WNV-Kun infected (*p < 0.05, One-way ANOVA). (B) Flies with mutations in *CG43775* (solid red line) have higher mortality to WNV-Kun infection compared with the w^{1118} genetic control (dotted red line). (C) WNV-Kun titer is higher in $CG43775^{MB08418}$ mutant flies relative to w^{1118} parental control by 10 d p.i. (**p < 0.01, 2-way ANOVA). (D) Insulin treatment reduces WNV-Kun titer in control w^{1118} flies but not in $CG43775^{MB08418}$ mutant

flies (**p < 0.01, ***p < 0.001, 2-way ANOVA). (E) Akt is phosphorylated and active in the presence of insulin for w^{1118} flies but not in $CG43775^{MB08418}$ mutant flies at 5 d p.i. (F) $CG43775^{MB08418}$ mutant flies have impaired induction of *upd2* compared to parental control w^{1118} flies. For qRT-PCR results, each circle represents individual biological replications consisting of individual well (A) or pooled collection of 3 flies (F). For titer results each circle represents individual biological replications consisting of pooled collection of 5 flies. Titer and qRT-PCR results (B-D, F) are representative of triplicate independent experiments western blot results are representative of duplicate independent experiments (E).

Upon establishing that *CG43775* impacts host survival and WNV-Kun replication, we sought to examine the role of *CG43775* in insulin-mediated antiviral immunity. We fed mutant and control flies 0 or 10 μM insulin two days prior to and during infection and collected flies at 1-, 5-, and 10 d p.i to measure virus replication (Fig. 2D). Similar to the previous results, we observed that mutant flies had higher viral titers relative to the genetic control. We also observed that while insulin-treated control flies had a reduction in viral titers, there was no difference between 0 or 10 μM insulin-treated *CG43775* mutant flies. These results indicate that loss of *CG43775* expression results in a loss of insulin-mediated reduction in viral replication.

To further dissect the role that *CG43775* has on insulin-mediated antiviral immunity, we sought to evaluate the impact that *CG43775* expression has on insulin signaling and JAK/STAT activation. Previous results demonstrate that insulin treatment of S2 cells activates AKT and JAK/STAT signaling, leading to the reduction of WNV-Kun (Ahlers et al., 2019). At 5 d p.i, we observed increased AKT phosphorylation in insulin-treated w^{1118} flies compared to *CG43775*

mutant flies (Fig. 2E) and quantified using densitometry analysis (Fig. S2). This leads us to conclude that *CG43775* mutant flies have a dysfunctional insulin signaling response that may impact insulin-mediated induction of antiviral JAK/STAT signaling. However, in the presence of insulin treatment and WNV-Kun infection, AKT phosphorylation was similar between genotypes, which may be due to virus-induced inhibition of AKT activation (Ohno et al., 2020). Furthermore, w^{1118} flies that were treated with insulin and infected with WNV-Kun had diminished AKT phosphorylation compared to flies that received either insulin or WNV-Kun. This may be caused by a secondary physiological signaling pathway which is absent *in vitro* and results in diminished AKT phosphorylation regardless of insulin treatment but remains sufficient to protect against WNV disease (Xu et al., 2013; DiAngelo et al. 2009; Sansone et al., 2015). Insulin treatment in S2 cells leads to the induction of *unpaired* (*upd*) cytokines and JAK/STAT activation (Ahlers et al., 2019). Thus, we examined *upd2* induction in control and *CG43775* mutant flies. At 5 d p.i, we observed significant induction of *upd2* in insulin-treated control flies, but not in *CG43775* mutant flies (Fig. 2F). Collectively these data suggest that *CG43775*, a previously uncharacterized gene that was identified within in the endothelin signaling gene set cluster, contributes to antiviral immunity during WNV-Kun infection through canonical insulin and JAK/STAT signaling.

Insulin and endothelin signaling reduce WNV-Kun replication in human HepG2 cells. The endothelin signaling pathway is not well-characterized in *D. melanogaster*; however, the pathway has been heavily dissected in mammals and permits us to investigate its potential role as an antiviral mediator to WNV-Kun in human cells. We first evaluated the extent to which insulin-mediated antiviral immunity functions in this model system. Human HepG2 liver cells

were treated with either 0 or 1.7 μM bovine insulin and infected with WNV-Kun (MOI 0.01 PFU/cell). Viral titer was measured at 1, 2, 3, and 5 d p.i. (Fig. 3A). As previously observed in fruit fly and mosquito cells (Ahlers et al., 2019), we observed a significant reduction in viral titer in cells treated with insulin. We followed up viral titer analysis by comparing insulin-treated cells to cells that received interferon (IFN)-β or -γ treatment (Fig. S3), following the protocol of Diamond et al. IFN treatment is known to reduce WNV replication in human cells (Keller et al., 2006; Laurent-Rolle et al., 2010; Lazear et al., 2011, 2015, Samuel and Diamond, 2005), so this comparison was to determine the efficacy of insulin in reducing WNV-Kun replication. We observed that insulin had a similar efficacy in reducing virus replication as IFN-γ and IFN- β at 2 d p.i..

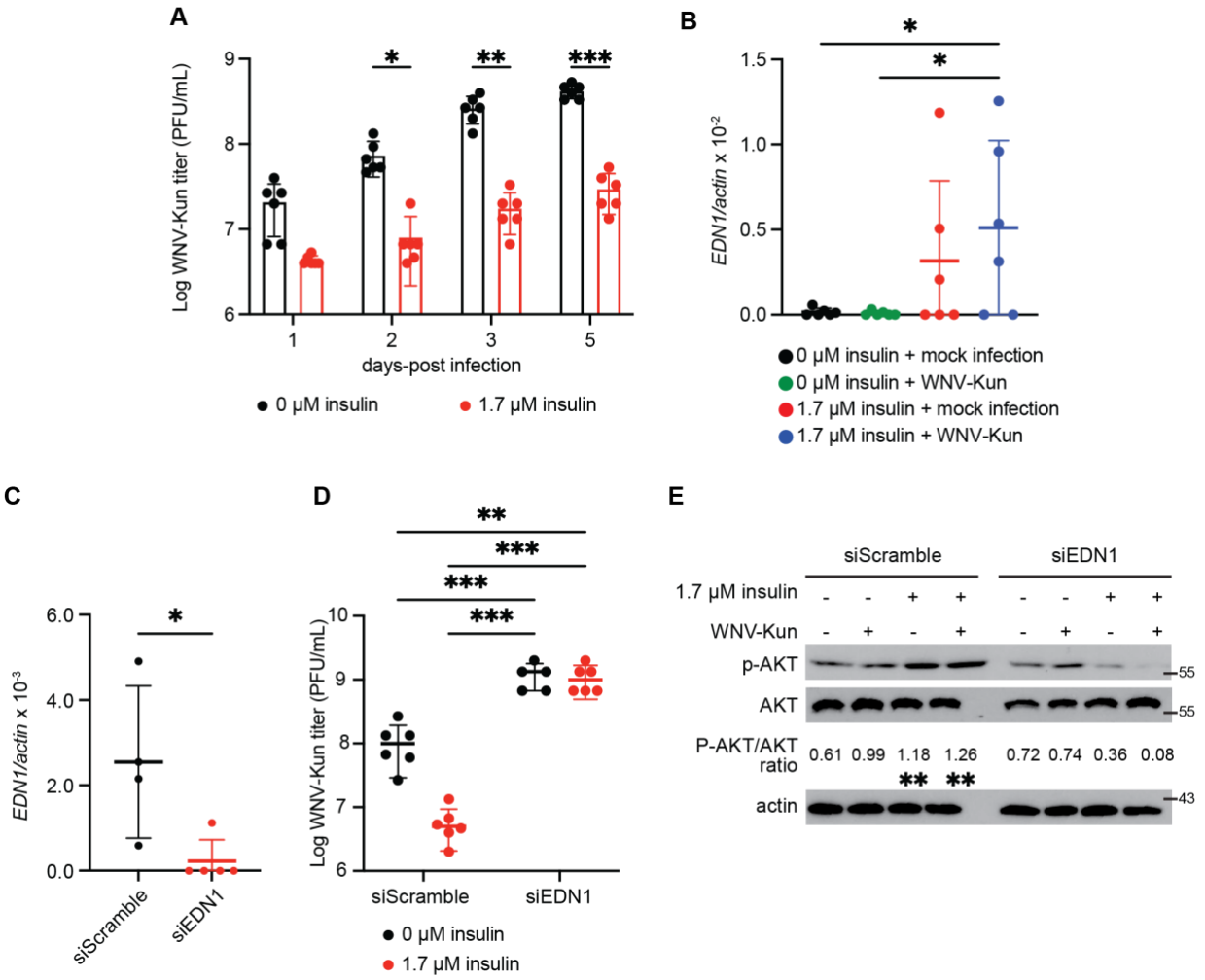

Fig. 3: Endothelin signaling is antiviral to WNV-Kun through an insulin-dependent mechanism in human HepG2 cells. (A) Insulin-treatment of HepG2 cells reduces WNV-Kun titer (MOI 0.01 PFU/cell) (*p < 0.05, **p < 0.01, ***p < 0.001, 2-way ANOVA). (B) *EDN1* is induced in insulin-treated and WNV-Kun-infected HepG2 cells (*p < 0.05, One-way ANOVA). (C-E) *EDN1* was knocked down in HepG2 cells (C) (*p < 0.05, upaired t-test) 48h prior to insulin-treatment and WNV-Kun infection and viral titer was measured by standard plaque assay at 2 days-post infection (D) **p < 0.01, ***p < 0.001, 2-way ANOVA). (E) Insulin-mediated Akt phosphorylation is decreased in the absence of *EDN1*. Circles represent individual biological replications. Horizontal bars represent the mean. Error bars represent SDs. Titer and qRT-PCR

results (A-D) are representative of triplicate independent experiments western blot results are representative of duplicate independent experiments (E).

To investigate endothelin signaling during insulin-mediated antiviral immunity, we measured induction of the ligand *endothelin 1* (*EDN1*) in HepG2 cells during WNV-Kun infection and insulin treatment (Fig. 3B). We observed significant induction of *EDN1* induction in the presence of insulin and WNV-Kun infection. This indicates that like our previous observations in *D. melanogaster*, endothelin signaling may be involved in insulin-mediated antiviral immunity in human cells. To further evaluate this hypothesis, we transfected HepG2 cells with either non-targeting control siRNA (siScramble) or EDN1 siRNA (siEDN1) (Fig. 3C). We observed a 91% reduction in *EDN1* expression in cells transfected with siEDN1. In cells knocked-down for EDN1 and treated with insulin, we observed that while the siScramble control cells maintain a reduction in WNV-Kun replication in the presence of insulin, we lose this insulin-mediated antiviral protection when EDN1 expression is diminished (Fig. 3D). We also observed a significant increase in overall WNV-Kun replication even in the absence of insulin treatment. Taken together, endothelin signaling may be connected with the insulin-mediated antiviral response previously observed by others in the mammalian system (Shives et al., 2014; Li et al., 2014; Wang et al., 2017; Wang et al., 2016).

We next tested the role that *EDN1* expression has on insulin signaling by measuring phosphorylation of AKT in HepG2 cells following insulin treatment and WNV-Kun infection at 2 d p.i. These cells were also transfected with siScramble or siEDN1 (Fig. 3E). We observed that control cells had higher expression of P-AKT in the presence of insulin and infection while the

loss of EDN1 had diminished P-AKT expression regardless of insulin-treatment (Fig. 3E) and quantified using densitometry analysis (Fig. S4). This further connects endothelin as a mediator of antiviral protection through an insulin-specific mechanism.

Insulin- and endothelin-mediated signaling is antiviral to virulent WNV-NY99. Previous analysis of insulin-mediated antiviral immunity in an insect (Ahlers et al., 2019) and present mammalian context has used the attenuated Kunjin subtype of WNV. While useful in dissecting and evaluating host immunity to WNV in a general context, a present limitation is that this strain causes limited disease in immune-competent human hosts. This is due to a number of factors including increased sensitivity to type I interferon responses (Daffis et al., 2011) and decreased efficacy in antagonizing JAK/STAT signaling due to a mutation in the NS5 protein (Laurent-Rolle et al., 2010). Because of this limitation regarding clinical relevance, we sought to evaluate whether insulin-mediated antiviral protection was present against more virulent flaviviruses and if so the impact that endothelin signaling possesses for regulating viral replication. Like previous experiments, we used HepG2 cells that received either 0 or 1.7 μM insulin treatment 24 h prior to and during WNV-NY99 (MOI 0.01 PFU/cell) infection and measured viral titer at 1, 2, 3, and 5 d p.i (Fig. 4A). We observed that WNV-NY99 titer was reduced in cells that received insulin treatment. We also observed a higher virus titer in WNV-NY99 infected cells compared to WNV-Kun infected cells. Because of the established link that insulin signaling induces JAK/STAT in mammals (Frias and Montessuit, 2013; Gual et al., 1998) and insects (Ahlers et al., 2019), this increase in overall viral titer is likely due to the enhanced antagonism WNV-NY99 can successfully initiate as opposed to the attenuated WNV-Kun strain (Laurent-Rolle et al., 2010; Daffis et al., 2011). We followed up this analysis by measuring WNV-NY99 titer in

HepG2 cells that received either non-targeting or EDN1 siRNA (Fig. 4B). We observed a similar loss of insulin-mediated protection and increased viral load in siEDN1-transfected cells that was previously observed during WNV-Kun infection. This observation ultimately leads us to conclude that downstream components of insulin-mediated antiviral immunity, specifically endothelin signaling, plays a role in reducing WNV replication for both attenuated and more virulent strains that may be a potential target for future clinical or therapeutic research.

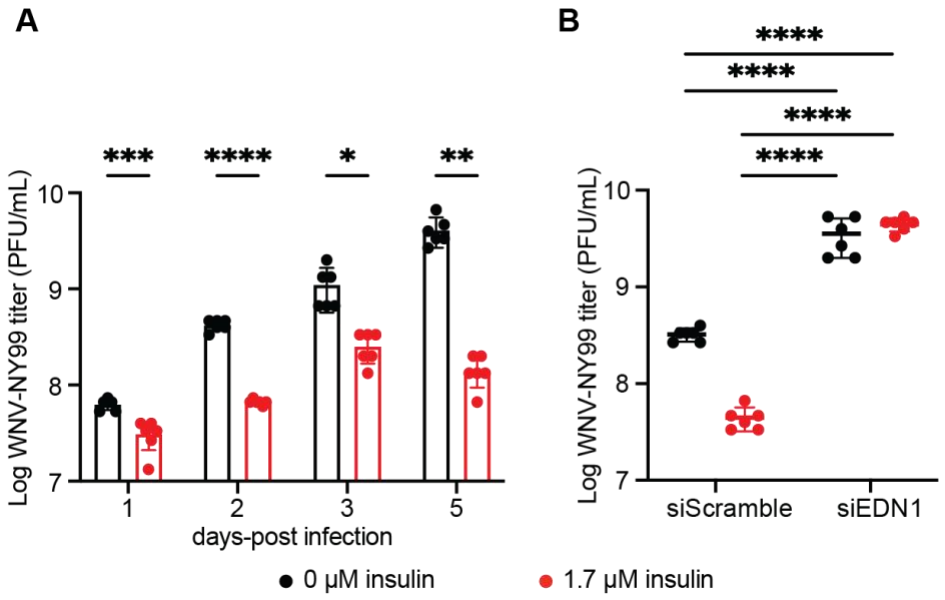

Fig. 4: Endothelin and insulin-mediated signaling is conserved against more virulent WNV-NY99 strain in HepG2 cells. (A) Insulin-treatment reduces WNV-NY99 titer (MOI=0.01 PFU/cell) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, 2-way ANOVA). (B) siRNA silencing of EDN1 results in increased WNV-NY99 viral replication and loss of insulin-mediated protection compared to non-specific siScramble control at 2 days post-infection (****p < 0.0001, 2-way ANOVA). Circles represent individual biological replications. Horizontal bars represent the mean. Error bars represent SDs. Results are representative of triplicate independent experiments.

DISCUSSION

Arbovirus infections are a growing health threat that require more effective means of intervention at both the vector transmission and clinical level. While our ability to develop more effective vector control protocols has improved, the ability to understand and clinically address human infections and severe disease remains underdeveloped. As WNV, along with other mosquito-borne diseases, continue to expand in both global distribution and incidence (Gorris et al., 2021; Ludwig et al., 2019; Hahn et al., 2015; Holcomb, 2022), the need for effective preventatives and treatments is more necessary than ever. Human vaccine development against WNV has made limited progress (Saiz, 2020), so development of effective antivirals post exposure is necessary.

In the study presented here, we highlight the genetic power of *D. melanogaster* to advance the study of antiviral immunity and identify components of insect and mammalian host responses that regulate WNV infection. We demonstrate that insulin induces a number of genes and signaling pathways that are both canonical and previously unidentified antiviral mediators (Fig. 1). Our study using the *D. melanogaster* model system expands upon the limited knowledge pertaining to the endothelin signaling pathway in insects and how it pertains to host survival and viral replication in the insect (Fig. 2). We also demonstrate that we can use these results to translate our findings into the more pertinent human model (Fig. 3). In addition, we demonstrate that our findings are applicable to a related yet more virulent and clinically relevant WNV strain (Fig. 4).

In our study, we show that dysfunctional endothelin signaling results in increased host mortality and WNV replication. However, further investigation is also necessary to evaluate its role during infection. Like insulin, endothelins are linked to various physiological processes like cardiovascular health so induction of this pathway, while potentially antiviral, may impact other off-target processes. Increased production and secretion of EDN1 has been used as an indicator for oncogenic and virus-induced hepatocellular carcinoma (Notas et al., 2001; Ersoy et al., 2006; Elbadry et al., 2020) as a promoter of cell growth and proliferation while inhibiting pro-apoptotic signaling (Shi et al., 2017). EDN1 expression is also proposed as a biomarker for patients receiving interferon-α treatment as elevated levels can be used to infer progression to interferon induced pulmonary toxicity (George et al., 2012). In relation to insulin sensitivity and signaling, serum EDN1 is elevated in diabetic individuals who later develop diabetic microangiopathy and nephropathy that progresses to more advanced insulin resistance (Kalani, 2008; Lenoir et al. 2014). Additional concerns are apparent as endothelin signaling, while antiviral in this study, may promote or enhance infection against other pathogens. *Mycobacterium tuberculosis* secretes the protease enzyme Zmp1 that cleaves EDN1 and activates endothelin signaling that promotes survival within the lungs (Correa et al. 2014). Thus, further investigation is needed to understand how targeting this pathway influences other pathogens individually or during co-infection with WNV. Because of the importance that endothelin signaling has in humans beyond antiviral immunity, further investigation is necessary to ensure that its proposed therapeutic potential is not at the cost of other host processes.

Further investigation is needed to determine the overall effect that insulin-mediated protection and endothelin signaling has in a clinical context and its ability to be an intervention target for

WNV disease. It is unlikely that administering insulin to a patient alone is a viable approach for treating WNV since it can influence a number of off-target physiological processes and may lead to further insulin resistance or disease pathology (Campo et al., 2012; Yu et al., 2021). Instead, we propose through our study that by targeting pathways downstream of insulin signaling, we can effectively and directly induce more potent antiviral responses with limited toxicity to the host. While our study focused on endothelin signaling, there were a number of other gene sets and associated pathways identified in our RNAseq screen that are worth further investigation regarding their potential role in antiviral immunity.

Taken together, our study identifies a novel component of insect and human antiviral immunity and expands our current understanding regarding insulin-mediated responses to infection. Previous investigation demonstrated that a variety of viruses including influenza (Ohno et al., 2020), WNV (Shives et al., 2014, 2016), and ZIKV (Liang et al., 2016; Harsh et al., 2018) target and disrupt host processes associated with insulin signaling. Typically, insulin signaling disruption results in metabolic dysfunction that can cause more severe morbidity and mortality. Here we demonstrate that targeting insulin signaling protects fruit flies and humans from increased viral replication. Additionally, we show that endothelin signaling is provides antiviral immunity to WNV. While endothelins have been heavily dissected as a regulator of cardiovascular health and vasoconstriction (Jiang et al., 1999; Chen, Edvinsson, and Xu, 2009; George et al., 2012), they also possess a role in hepatic (Cifarelli et al., 2012; Notas et al., 2001; Ersoy et al., 2006; Elbadry et al., 2020) and neuronal (Jin et al., 2020; Adams et al., 2020; Koyama, 2013; Swire et al., 2019) regulation and health. WNV disease is heavily associated with encephalitis and neurodegenerative disease (Sejvar, 2014; Briese et al., 1999). Because EDN1

has been linked to virus-induced demyelinating disease (Jin et al., 2020) and promotes anti-inflammatory signaling in circulating immune cells (Elisa et al., 2015), endothelin signaling may also function as an antiviral target and determinant in severe WNV disease progression and is worth further investigation.

Given the conservation of insulin signaling and its activation during viral infection across insect and mammalian species, it would be worth investigating the potential that downstream targets of insulin or endothelin signaling has in a broader antiviral context. If possible, it may provide a means of more effectively responding to these growing pathogens of concern while also limiting potential complications associated with current intensely robust antiviral therapeutics.

MATERIALS AND METHODS

Fly lines and rearing. Flies used in this study are listed in Table S2. Flies were maintained on standard cornmeal food (Genesee Scientific #66-112) at 25°C and 65% relativity humidity, and a 12 h/ 12 h light/dark cycle. Flies are negative for *Wolbachia* infection. Female adult flies that were 2-7 d post-eclosion were used for all experiments. For insulin treatment, cornmeal food was supplemented with 10 μM bovine insulin (Sigma-Aldrich I6634) and flies were maintained on food 48 h prior and during infection as described (Ahlers et al., 2019).

Cells and virus. Vero cells (ATCC, CRL-81) were provided by A. Nicola and cultured at 37 °C/5% CO_2 in DMEM (ThermoFisher 11965) supplemented with 10% FBS (Atlas Biologicals FS-0500-A) and 1x antibiotic-antimycotic (ThermoFisher 15240062). S2 cells were cultured as described (Ahlers et al., 2016) and are negative for Flock House virus. HepG2 cells (ATCC, HB-

8065) were provided by M. Konkel and cultured at 37 °C/5% CO_2 in DMEM supplemented with 10% FBS. For insulin treatment, culture media with 2% FBS were supplemented with 1.7 μM bovine insulin as described (Zhang et al., 2011). For interferon-β and -γ treatment, 2% FBS in DMEM media was supplemented with 10 units/mL of either IFN-β or IFN-γ for 24 h prior to infection as described (Diamond et al., 2000).

West Nile virus-Kunjin strain MRM16 (WNV-Kun) was gifted by R. Tesh, and propagated in Vero cells. West Nile virus strain 385-99 (WNV-NY99) was obtained by BEI Resources, NIAID, NIH (NR-158), and propagated in Vero cells. All experiments with a specific virus type utilized the same stock.

RNA isolation, library preparation, and RNA-sequencing. *D. melanogaster* S2 cells were treated with 0 or 1.7 μM bovine insulin for 24 h. Cells were then either mock-infected for infected with WNV-Kun (MOI 1 PFU/cell) for 8 h. Total RNA was extracted from three individual wells using Direct-zol (Zymo Research, Irvine, CA) following the manufacturer's instructions. Following total RNA extraction, the integrity of total RNA was assessed using Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA) with the High Sensitivity RNA Analysis Kit. RNA Quality Numbers (RQNs) from 1 to 10 were assigned to each sample to indicate its integrity or quality. "10" stands for a perfect RNA sample without any degradation, whereas "1" marks a completely degraded sample. RNA samples with RQNs ranging from 8 to 10 were used for RNA library preparation with the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA). Briefly, mRNA was isolated from 2.5 μg of total RNA using poly-T oligo attached to magnetic beads and then subjected to fragmentation, followed by cDNA

synthesis, dA-tailing, adaptor ligation, and PCR enrichment. The sizes of RNA libraries were assessed by Fragment Analyzer with the High Sensitivity NGS Fragment Analysis Kit. The concentrations of RNA libraries were measured by StepOnePlus Real-Time PCR System (ThermoFisher Scientific, San Jose, CA) with the KAPA Library Quantification Kit (Kapabiosystems, Wilmington, MA). The libraries were diluted to 2 nM in10 mM Tris-HCl, pH 8.5 and denatured with 0.1 N NaOH. Eighteen pM libraries were clustered in a high-output flow cell using HiSeq Cluster Kit v4 on a cBot (Illumina). After cluster generation, the flow cell was loaded onto HiSeq 2500 for sequencing using HiSeq SBS kit v4 (Illumina). DNA was sequenced from both ends (paired-end) with a read length of 100 bp. The raw bcl files were converted to fastq files using software program bcl2fastq2.17.1.14. Adaptors were trimmed from the fastq files during the conversion. On average, 40 million reads were generation for each sample. RNA-sequencing was performed at the Spokane Genomics CORE at Washington State University-Spokane in Spokane, WA, USA.

Bioinformatics Analysis. RNA-seq reads were imported and aligned using Qiagen CLC Genomics Workbench 11.0.1 to the *D. melanogaster* genomic reference sequence. Reads for each biological replicate within an experimental condition were pooled and averaged. Differential expression of transcript levels for each experimental condition (WNV-Kun infection, insulin treatment, or both infection and treatment) were normalized to reads for cells that received neither treatment nor infection. Transcripts were filtered for p-values less than or equal to 0.05 and a $\log_2$(fold change) > ± 1.5 for at least one experimental condition.

Filtered transcripts were imported into Tibco Spotfire for gene clustering and heatmap generation. Gene clustering was performed using hierarchical clustering using UPGMA (unweighted pair group method with arithmetic mean) with Euclidean distance with ordering weight set to average value and normalization by mean. Gene set enrichment analysis (GSEA) was performed as previously described (Goodman et al., 2009) using a cutoff of $p < 0.05$ for at least one experimental condition for gene ontology (GO) classifications (Subramanian et al., 2005). Highlighted classifications are shown in Figure 1B. *Drosophila* gene ontologies were imported from FlyBase (version fb_2016_04) as previously described (Martin et al., 2018). Further GO analysis for genes clustered and presented in Figure 1E used PANTHER GO-Slim (Version 14.0) to identify endothelin signaling pathway as an overrepresented GO category.

Fly infections. 2-7 day old adult female *D. melanogaster* were anesthetized with CO_2 and injected intrathoracically with WNV-Kun with 5,000 PFU/fly, as previously described (Yasunaga et al., 2014; Martin et al., 2018). Mock infected-flies received equivalent volume of PBS. For mortality studies, groups of 30-50 flies were injected and maintained on cornmeal food for 30 days. All survival studies were repeated three times and survival data were combined. Fly food vials were changed every 2-3 days. For viral titration experiments, three groups of 4-5 flies were collected, homogenized in PBS, and used as individual samples for plaque assay as described in (Ahlers et al., 2019). For qRT-PCR and Western blot experiments, three groups of 3-5 flies were collected, homogenized in Trizol or RIPA, respectively, and centrifuged to isolate and remove cuticle. Supernatant was collected and used for further analysis.

***In vitro* virus replication.** HepG2 cells were seeded into a 24-well plate at a confluency of 1.25 x 10^5 cells/well with 6 independent wells for each experimental condition. The following day, cells were treated with either 1.7 μM bovine insulin or acidified water in 2% FBS in DMEM for 24 h prior to infection. For measuring viral replication following interferon treatment, 2% FBS in DMEM media was supplemented with 10 units/mL of either IFN-β or IFN-γ for 24 h prior to infection as described (Diamond et al., 2000). Cells were then infected with WNV-Kun or WNV-NY99 at MOI of 0.01 PFU/cell for 1 h. Virus inoculum was removed, and fresh experimental media was added. Supernatant samples were collected at 1, 2, 3, and 5 d p.i. for later titration. WNV titer were determined by standard plaque assay on Vero cells.

Immunoblotting. Protein extracts were prepared by lysing cells or flies with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1mM Na_3VO_4, 1 mM NaF, 0.1 mM PMSF, 10 μM aprotinin, 5 μg/mL leupeptin, 1 μg/mL pepstatin A). Protein samples were diluted using 2x Laemmli loading buffer, mixed, and boiled for 5 minutes at 95 °C. Samples were analyzed by SDS/PAGE using a 10% acrylamide gel, followed by transfer onto PVDF membranes (Millipore IPVH00010). Membranes were blocked with 5% BSA (ThermoFisher BP9706) in Tris-buffered saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl) and 0.1% Tween-20 for 1 h at room temperature.

Primary antibody labeling was completed with anti-P-Akt (1:1,000; Cell Signaling 4060), anti-Akt (pan) (1:2,000) (Cell Signaling 4691), or anti-actin (1:10,000; Sigma A2066) overnight at 4 °C. Secondary antibody labeling was completed using anti-rabbit IgG-HRP conjugate (1:10,000; Promega W401B) by incubating membranes for 2 h at room temperature. Blots were imaged

onto film using luminol enhancer (ThermoFisher 1862124). P-AKT/AKT ratio for each experimental condition was determined using densitometry analysis using BioRad Image Lab comparing band intensity of P-AKT to AKT. Reported P-AKT/AKT ratio is the mean of duplicate independent experiments.

RNA interference *in vitro*. Double-stranded RNA (dsRNA) targeting human *EDN1* (Horizon Discovery J-016692-05-005) and non-targeting control (siScramble) dsRNA (Horizon Discovery D-001810-10-05) was transfected into HepG2 cells for 48 h prior to insulin treatment and infection as described (Ahlers et al., 2016). Total RNA was extracted and purified to confirm reduced expression by qRT-PCR.

Quantitative reverse transcriptase PCR. qRT-PCR was used to measure mRNA levels in *D. melanogaster* S2 cells, adult flies, and human HepG2 cells. Cells or flies were lysed with Trizol Reagent (ThermoFisher 15596). RNA was isolated by column purification (ZymoResearch R2050), DNase treated (ThermoFisher 18068), and cDNA was prepared (BioRad 170–8891). Expression of *D. melanogaster CG43775* and *upd2* were measured using SYBR Green reagents (ThermoFisher K0222) and normalized to *Rp49* to measure endogenous gene levels for all treatment conditions. Expression of human *EDN1* was measured using the probe for *EDN1* (Hs00174961_m1 ThermoFisher 4331182) and primers and normalized to *β-actin* (Hs01060665_g1 ThermoFisher 4331182) using TaqMan Universal Master Mix (ThermoFisher 4304437). The reaction for samples included one cycle of denaturation at 95 °C for 10 minutes, followed by 50 cycles of denaturation at 95 °C for 15 seconds and extension at 60 °C for 1 minute, using an Applied Biosystems 7500 Fast Real Time PCR System. ROX was used as an

internal control. qRT-PCR primer sequences are listed in Table 3 Table (Ahlers et al., 2019; Spellberg and Marr, 2015; Torres et al., 2021).

Quantification and Statistical Analysis. Results presented as dot plots show data from individual biological replicates (n=2-6), the arithmetic mean of the data shown as a horizontal line, and error bars representing standard deviations from the mean. Biological replicates of adult *D. melanogaster* (n=6-40) consisted of triplicate pooled flies. Results shown are representative of at least duplicate independent experiments, as indicated in the figure legends. All statistical analyses of biological replicates were completed using GraphPad Prism 9 and significance was defined as $p < 0.05$. Ordinary one-way ANOVA with uncorrected Fisher's LSD for multiple comparisons was used for qRT-PCR analysis. Two-way ANOVA with Šidák correction for multiple comparisons was used for multiday viral titer analysis and for siRNA viral titer analysis. One-way ANOVA with Šidák correction for multiple comparisons was used for single day viral titer in the presence of insulin and interferon-β and -γ analysis. Two-tailed unpaired t test was used for qRT-PCR validation of knocked-down expression of *EDN1*. Repeated measures one-way ANOVA with uncorrected Fisher's LSD for multiple comparison was used for densitometry analysis. All error bars represent standard deviation (SD) of the mean. Outliers were identified using a ROUT test (Q=5%) and removed.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.E.T., L.R.H.A, and A.G.G.; Methodology, C.E.T., L.R.H.A, and A.G.G.; Validation, C.E.T., E.H.R., B.J.J., A.B.C., S.F., and A.G.G..; Investigation, C.E.T., E.H.R., and A.G.G; Resources, A.G.G.; Writing – Original Draft, C.E.T.; Writing – Review and Editing, E.H.R., B.J.J., L.R.H.A., A.B.C., S.F., and A.G.G.; Visualization, C.E.T. and A.G.G.; Funding Acquisition, C.E.T., B.J.J., L.H.R.A, and A.G.G.

DECLARATION OF INTERESTS

The authors have declared that no competing interests exist.

SUPPLEMENTAL INFORMATION

Table S1, Sheet 1: Summary of RNAseq reads (related to Figure 1).

Experiment Condition	Sample #	Average Total Reads	Average Total Mapped	Average % Mapped
mock-mock	1, 2, 3	78500232.67	73303857.33	93.43
insulin-mock	4, 5, 6	80376152.67	74634122.67	92.89333333
mock-KUNV	7, 8, 9	77176451.33	73079306.67	94.59333333
insulin-KUNV	10, 11, 12	84974130	77950122.67	91.94666667
	Average:	80256741.67	74741852.33	93.21583333

Table S1, Sheet 2: PANTHER GO analysis results (related to Figure 1E).

PANTHER Pathways	Endothelin signaling pathway	Unclassified
(REF #)	79	12625
# genes	1	11
expected	0.7	10.96
Fold Enrichment	14.58	1
(+ / -)	+	+
P-value	6.73E-02	1.00E+00

Table S1, Sheet 3: Expression values of selected gene cluster (Sheet 3) (related to Figure 1E).

Gene Name	0 uM insulin + WNV-Kun	1.7 uM insulin + mock	1.7 uM insulin + WNV-Kun
CG8180	-0.31	4.32	4.24
Cyp4p1	-0.27	4.67	4.62
betaNACtes6	-0.1	5.4	4.09
CG13043	-0.08	5.63	3.35
gudu	-0.02	5.4	3.66
CG5326	-0.05	3.95	5.3
Acp53Ea	-0.03	3.69	5.37
CG7299	-0.01	3.96	5.26
TotF	-0.00296	2.55	5.19
CG42507	-0.03	5.81	6.68
CG13670	-0.01	5.9	5.82
CG43775	-0.03	5.4	5.08
ac	0.04	4.73	5.29
Ugt36Bc	0.06	5.62	5.49
CG31769	0.08	4.48	4.39
CG42460	0.11	5.22	4.35
MtnB	0.11	5.95	5.47
CG2962	0.15	5.51	5.76
CG14354	0.1	3.28	-2.9
CG46275	0.17	7.48	-0.31
Amt	0.27	4	3.71
MtnD	0.28	4.41	3.67
CG32695	0.27	3.38	3.32

Table S2: Fly lines and reagents used in this study.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-phospho-Akt (Ser473)	**Cell Signaling**	**Cat#4060 RRID:AB_2315049**
Rabbit monoclonal anti-Akt (pan) (C67E7)	**Cell Signaling**	**Cat#4691 RRID:AB_915783**
Rabbit polyclonal anti-actin	**Sigma**	**Cat#A2066 RRID:AB_476693**
Anti-rabbit IgG (H+L) HRP conjugate	**Promega**	**Cat#4011 RRID:AB_430833**
Virus Strains		
West Nile virus-Kunjin	**Laboratory of Robert Tesh**	**MRM16 strain**
West Nile virus-NY99	**BEI Resources**	**385-99 strain**
Experimental Models: Organisms/Strains		
***D. melanogaster*: wild-type line: w^{1118}**	**Bloomington Drosophila Stock Center**	**BDSC: 5905; Flybase: FBst0005905**

***D. melanogaster*: *CG43775* mutant: w[1118]; Mi{GFP[E.3xP3]=ET1}CG3257[MB08418] CG43775[MB08418] CG43776[MB08418] CG43777[MB08418]**	**Bloomington Drosophila Stock Center**	**BDSC: 26113; Flybase: FBst0026113**
Experimental Models: Cell Lines		
Insulin from bovine pancreas	**Sigma-Aldrich**	**I6634**
Chemicals, Peptides, and Recombinant Proteins		
***Cercopithecus aethiops*: Cell line Vero**	**ATCC**	**CCL-81**
***D. melanogaster:* Cell line S2: S2-DGRC**	**Laboratory of Lucy Cherbas**	**FlyBase: FBtc0000006**
***Homo sapiens:* Cell line HepG2**	**ATCC**	**HB-8065**
Oligonucleotides		
***DmRp49* qRT-PCR: Forward: CCACCAGTCGGATCGATATGC Reverse: CTCTTGAGAACGCAGGCGACC**	**Integrated DNA Technologies**	**Spellberg and Marr, 2015**
***DmUpd2* qRT-PCR: Forward: CCTATCCGAACAGCAATGGT Reverse: CTGGCGTGTGAAAGTTGAGA**	**Integrated DNA Technologies**	**Ahlers et al., 2019**

***CG43775* qRT-PCR:** **Forward: CTGCAACAACAAGACGCACA** **Reverse: GAACTTGGTCGAGTTCCCGT**	**Integrated DNA Technologies**	**This study**
***HsEDN1* qRT-PCR:** **Forward:** **CAGGGCTGAAGACATTATGGAGA** **Reverse: CATGGTCTCCGACCTGGTTT**	**Integrated DNA Technologies**	**Torres et al., 2021**
Software and Algorithms		
Prism	**GraphPad**	**Version 9**
CLC Genomics Workbench	**Qiagen**	**Version 11.0.1**
Image Lab	**Bio-Rad**	**Version 6.1**
PANTHER Classification System	**Mi et al., 2019** **Thomas et al., 2022**	**Version 14.0**
Gene Set Enrichment Analysis (GSEA)	**Subramanian et al., 2005** **Goodman et al., 2009**	**N/A**
***D. melanogaster* gene ontology categories**	**Flybase**	**Version fb_2016_04**
Adobe Illustrator 2021	**Adobe**	**Version 25.2.3**
TIBCO Spotfire Analytics	**TIBCO**	**Version 1.1.3**

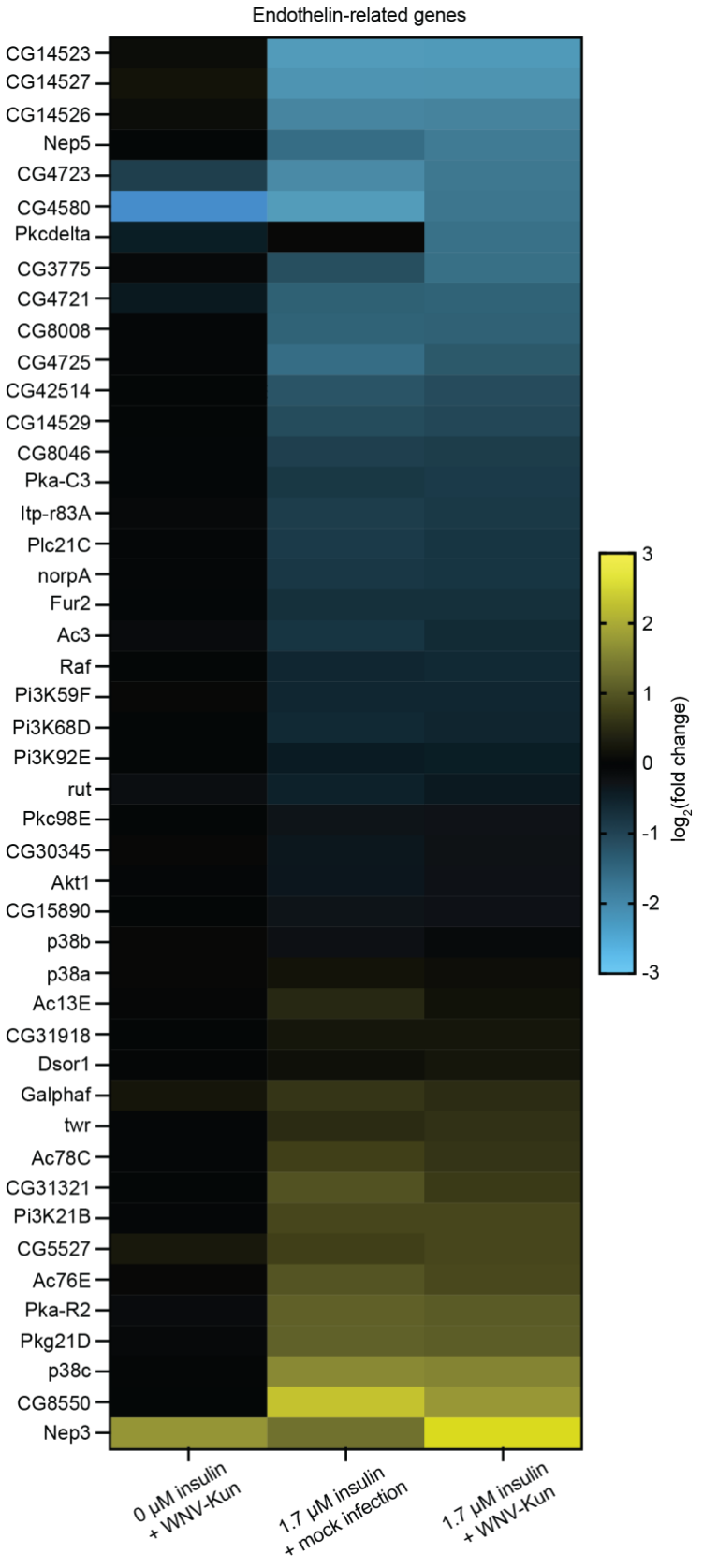
Endothelin-related genes
CG14523
CG14527
CG14526
Nep5
CG4723
CG4580
Pkcdelta
CG3775
CG4721
CG8008
CG4725
CG42514
CG14529
CG8046
Pka-C3
Itp-r83A
Plc21C
norpA
Fur2
Ac3
Raf
Pi3K59F
Pi3K68D
Pi3K92E
rut
Pkc98E
CG30345
Akt1
CG15890
p38b
p38a
Ac13E
CG31918
Dsor1
Galphaf
twr
Ac78C
CG31321
Pi3K21B
CG5527
Ac76E
Pka-R2
Pkg21D
p38c
CG8550
Nep3
0 µM insulin + WNV-Kun
1.7 µM insulin + mock infection
1.7 µM insulin + WNV-Kun
3
2
1
0
-1
-2
-3
log2(fold change)

Fig. S1: Heat map expression of genes transcriptionally enriched or suppressed as identified in Fig. 1E. PANTHER-GO analysis identifies this gene set associated with the endothelin signaling pathway.

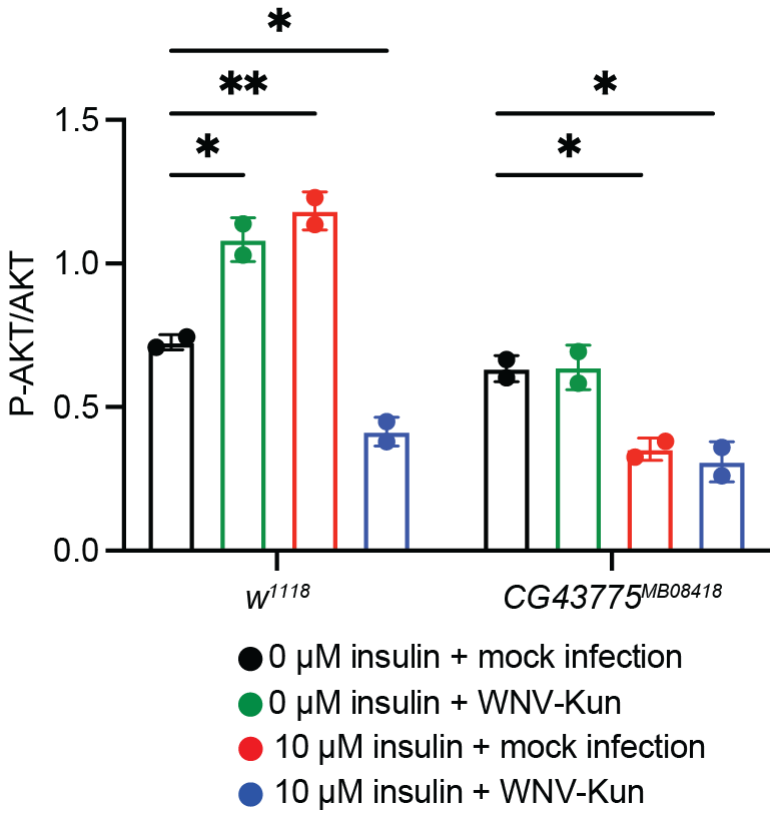

Figure S2: AKT phosphorylation is diminished in insulin-treated *CG43775* mutant flies but not control flies as analyzed in Fig. 2E. Densitometry analysis of western blots measuring P-AKT abundance relative to AKT shows reduced activation in *CG43775* mutants compared to control flies. (*p < 0.05, **p < 0.01, One-way ANOVA). Circles represent individual experimental replications. Horizontal bars represent the mean. Error bars represent SDs. Results are of pooled duplicate independent experiments.

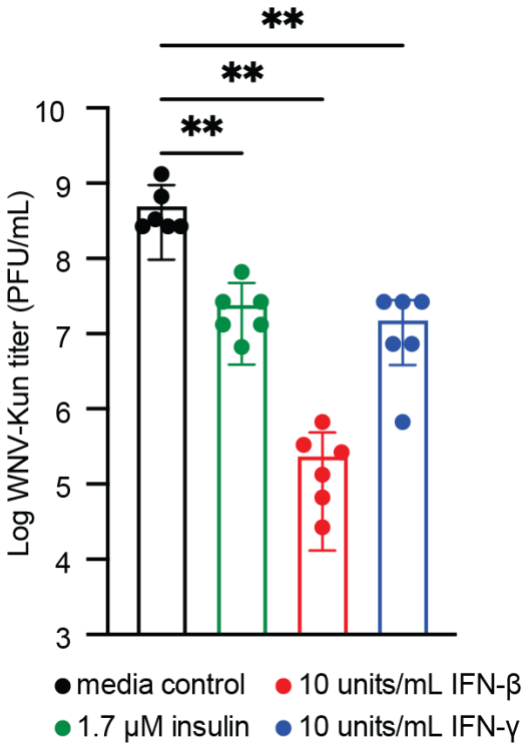

Fig. S3: Insulin reduces WNV-Kun titer in HepG2 cells to similar levels as IFN-β or IFN-γ treatment. WNV-Kun titer at 2 d p.i is reduced in cells that received either 1.7 μM insulin, 10 units/mL IFN-β, or 10 units/mL IFN-γ treatment 24 h prior to infection (MOI=0.01 PFU/cell) (**$p < 0.01$, One-way ANOVA). Circles represent individual biological replications. Horizontal bars represent the mean. Error bars represent SDs. Results are representative of duplicate independent experiments.

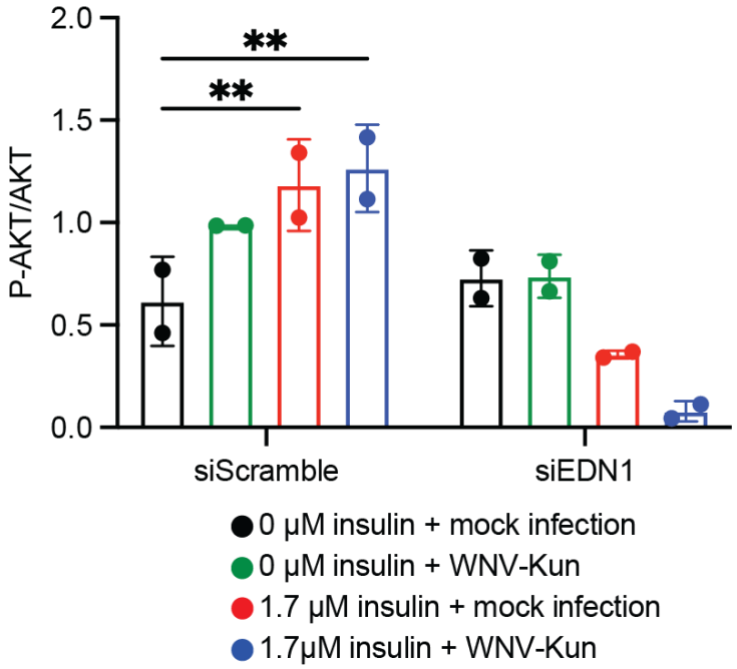

Fig. S4: AKT phosphorylation is enhanced in HepG2 cells following insulin treatment and WNV-Kun infection but diminished following siEDN1 transfection as analyzed in Fig. 3E. Densitometry analysis of western blots measuring P-AKT abundance relative to AKT shows reduced activation in siEDN1 transfected HepG2 cells compared to siScramble transfected cells. (**$p < 0.01$, One-way ANOVA). Circles represent individual experimental replications. Horizontal bars represent the mean. Error bars represent SDs. Results are of pooled duplicate independent experiments.

CHAPTER FOUR: EMERGING MECHANISMS OF INSULIN-MEDIATED ANTIVIRAL IMMUNITY IN DROSOPHILA MELANOGASTER

Trammell, C.E., and Goodman, A.G. (2019). Emerging Mechanisms of Insulin-Mediated Antiviral Immunity in Drosophila melanogaster. Front Immunol *10*, 2973.

Attributions:

C.E. Trammell wrote and revised the text and figures of this manuscript.

A.G. Goodman was consulted and assisted in the revisions of this manuscript.

ABSTRACT

Arboviruses (arthropod-borne viruses), such as Zika (ZIKV), West Nile (WNV), and dengue (DENV) virus, include some of the most significant global health risks to human populations. The steady increase in the number of cases is of great concern due to the debilitating diseases associated with each viral infection. Because these viruses all depend on the mosquito as a vector for disease transmission, current research has focused on identifying immune mechanisms used by insects to effectively harbor these viruses and cause disease in humans and other animals. *Drosophila melanogaster* are a vital model to study arboviral infections and host responses as they are a genetically malleable model organism for experimentation that can complement analysis in the virus' natural vectors. *D. melanogaster* encode a number of distinct mechanisms of antiviral defense that are found in both mosquito and vertebrate animal systems, providing a viable model for study. These pathways include canonical antiviral modules such as RNA interference (RNAi), JAK/STAT signaling, and the induction of STING-mediated immune responses like autophagy. Insulin signaling plays a significant role in host-pathogen interactions. The exact mechanisms of insulin-mediated immune responses vary with each virus type, but nevertheless ultimately demonstrates that metabolic and immune signaling are coupled for antiviral immunity in an arthropod model. This mini review provides our current understanding of antiviral mechanisms in *D. melanogaster*, with a focus on insulin-mediated antiviral signaling, and how such immune responses pertain to disease models in vertebrate and mosquito species.

INTRODUCTION

Mosquitoes are a prominent vector for various arboviruses including West Nile virus (WNV), Zika virus (ZIKV), and dengue virus (DENV). These viruses pose a significant concern to

human populations as the mosquitoes' continual encroachment into previously unexposed regions expands (Kraemer et al., 2015; Leta et al., 2018). This habitat expansion renders more individuals at risk of exposure with limited, if any, treatments available. Climate change has also resulted in alterations in mosquito seasonal activity (Muttis et al., 2018) and feeding behavior (Hagan et al., 2018) resulting in increasing frequency and severity of arboviral cases. There is a direct correlation between the expansion of vector-competent mosquitoes and disease incidence within afflicted regions [reviewed in (Cucunawangsih and Lugito, 2017; Deseda, 2017)] indicating that vector activity is a significant risk factor for arboviral disease.

Figure 1 outlines the transmission cycle of various arboviruses as they move within host populations and how *Drosophila melanogaster* can be used to study arboviral immunity for each system. Transmission from mosquito to vertebrates requires a bloodmeal exchange where infected saliva is ejected into the new host. Viral replication then permits the spread of virus from infected host to mosquito to continue the transmission cycle (Figure 1). Research is required to identify the signaling responses used in regulating these viruses at the vector and human level. Studies regarding immune responses initiated during the initial bloodmeal exchange (Uraki et al., 2019) are important as this event is a key determinant whether transmission occurs (Sim, Ramirez, and Dimopoulos, 2012). An emphasis as to how immune and nutritional signaling interact with one another is of particular interest as both would be active during ingestion of an infected bloodmeal. Insulin-mediated signaling regulates numerous viruses by inducing activation of canonical immune pathways (Xu et al., 2013). Because insulin is ingested during the bloodmeal, a recent study has shown that vertebrate insulin is able to

regulate the type of innate immune response that occurs during viral infection in insect vector hosts (Ahlers et al., 2019).

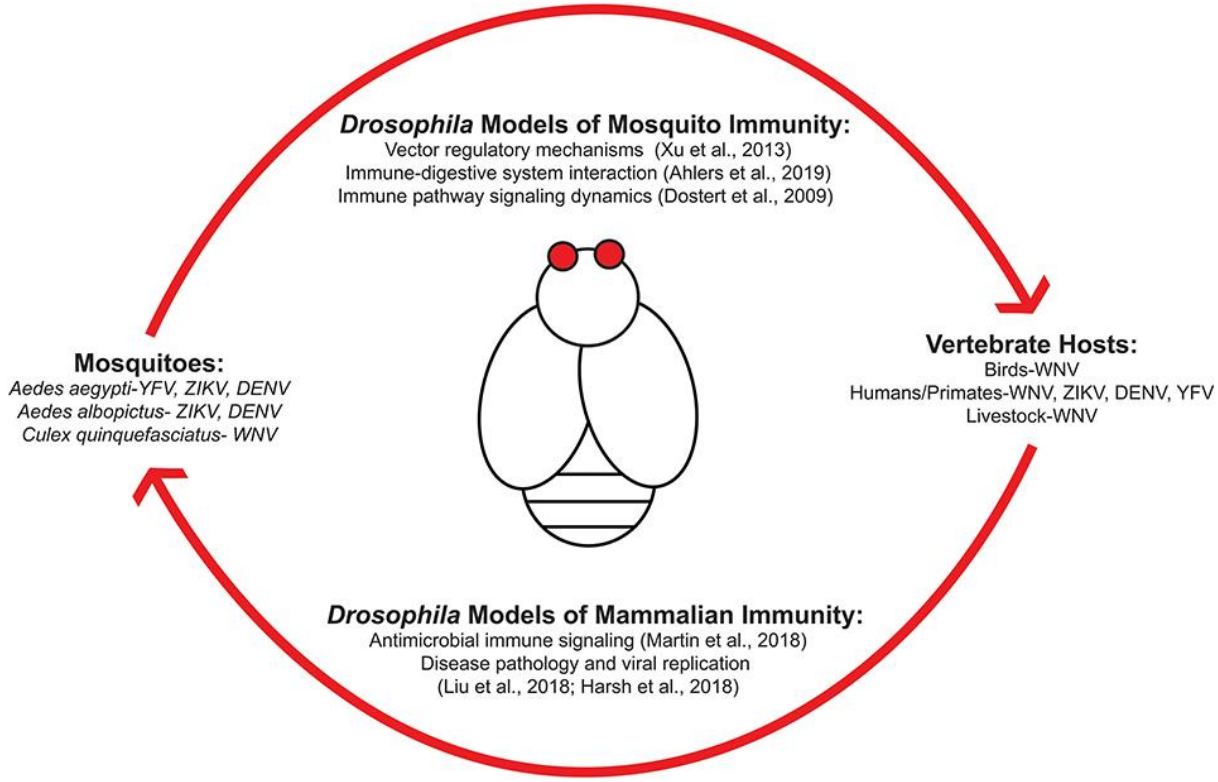

Figure 1. *Drosophila melanogaster* are an ideal model organism for studying host-arboviral interactions. Various arboviruses utilize mosquitoes as reservoirs and vectors for transmission into vertebrate hosts. This can include species that are either involved in viral replication and spread (such as bird populations for West Nile virus) or dead-end host that become infected without being able to properly propagate viral replication for further spread (i.e., humans). Transmission is accomplished via a bloodmeal exchange. *Drosophila* possess orthologous host response pathways found in mosquitoes and humans, making it an ideal model organism for studying transmission dynamics and host-pathogen interactions at both vector and human level. Previous work has identified the signaling pathways that respond to arboviral infection and their significance with respect to disease outcome and severity (Burdette et al., 2011; Dostert et al.,

2005; Galiana-Arnoux et al., 2006). *Drosophila* have proven to be a significant model organism for studying arboviruses as many of the signaling pathways identified are conserved amongst insect species [reviewed in (Hillyer, 2016)]. These studies have utilized the genetic power provided by the *Drosophila* system to demonstrate the effect that nutritional status poses on host immunity.

Immune responses during various arboviral infections are evolutionarily conserved among insects and include the canonical RNA interference (RNAi) (Galiana-Arnoux et al., 2006; Goic et al, 2013; Saleh et al., 2009), JAK/STAT (Ahlers et al., 2019; Dostert et al., 2005), and STING-mediated signaling (Liu et al., 2018). These pathways are associated with the insulin/insulin-like growth factor signaling (IIS) pathway and have been established as key determinants in vector competency and disease outcome (Xu et al., 2013; Spellberg and Marr, 2015). It has been demonstrated that ingestion of vertebrate insulin regulates whether an RNAi- or JAK/STAT-mediated response is active during infection against WNV (Ahlers et al., 2019). STING-mediated immunity has been previously linked to induce JAK/STAT signaling (Chen et al., 2011) and affects nutritional homeostasis during infection (DiAngelo et al., 2009; Péan et al., 2017) implying that it may be regulated by insulin as well. Since insulin-mediated signaling appears to have a broad impact on insect immunity, recent studies have sought to establish how insulin connects each antiviral pathway to respond to different arboviruses. Because vector competency and transmission is so closely dependent on gut-associated immune signaling, the connection that nutrition and immunity has is indicative of their importance in regulating infection (Xu et al., 2013; Sansone et al., 2015). This mini review presents a condensed understanding regarding the major responses that occur during arboviral infection using

Drosophila, the role that insulin signaling plays, and a summation of current and future efforts taken within this field.

RNA INTERFERENCE PATHWAY

One of the most broadly restricting antiviral responses used by insects is the RNAi pathway (Galiana-Arnoux et al., 2006; Saleh et al., 2009). RNAi signaling occurs in response to detection of viral nucleic acids within the cytosol of infected cells. In *Drosophila*, recognition of viral nucleic acids by the endonuclease Dicer-2 results in the recruitment of proteins Argonaute-2 (AGO2) and r2d2 to form an RNA-induced silencing complex (RISC) (Galiana-Arnoux et al., 2006; Deddouche et al., 2008). This results in the cleavage and degradation of bound viral nucleic acids (Galiana-Arnoux et al., 2006; Deddouche et al., 2008; Kemp et al., 2013). While this antiviral response is a significant component of insect immunity against RNA viruses like Sindbis virus (SINV) (Kemp et al., 2013) and ZIKV (Harsh et al., 2018), an arthropod-borne alphavirus and flavivirus, respectively, RNAi has also been shown to respond to DNA viruses like Invertebrate iridescent virus 6 (IIV-6) (Bronkhorst et al., 2012) (Figure 2A). While Harsh et al. showed that the loss of the RNAi component, Dicer-2, resulted in increased ZIKV replication and mortality, they linked the increased susceptibility of these flies to dysregulated homeostasis of the gut and fat body. Moreover, studies have shown that another RNAi component, namely AGO2, is dispensable for an antiviral response against ZIKV (Liu et al., 2018; Harsh et al., 2018). Additionally, there are flaviviruses that encode viral suppressors of RNAi (VSR) (Schnettler et al., 2012). Not only do VSRs function in *Drosophila* model systems (van Cleef et al., 2011), but they also function during WNV, DENV, and Yellow fever virus infections in *Culex* mosquitoes (Göertz et al., 2016; Samuel et al., 2016). Thus, the antiviral role of RNAi

depends not only on the host but also virus type. Further studies are needed to clarify the antiviral role of RNAi in model and vector organisms, especially with respect to flaviviruses and the VSRs they may encode. Mosquitoes become infected and spread disease via bloodmeal exchanges. Because of the direct role that nutritional acquisition has during infection, its role in antiviral immunity is mediated in part by regulating RNAi.

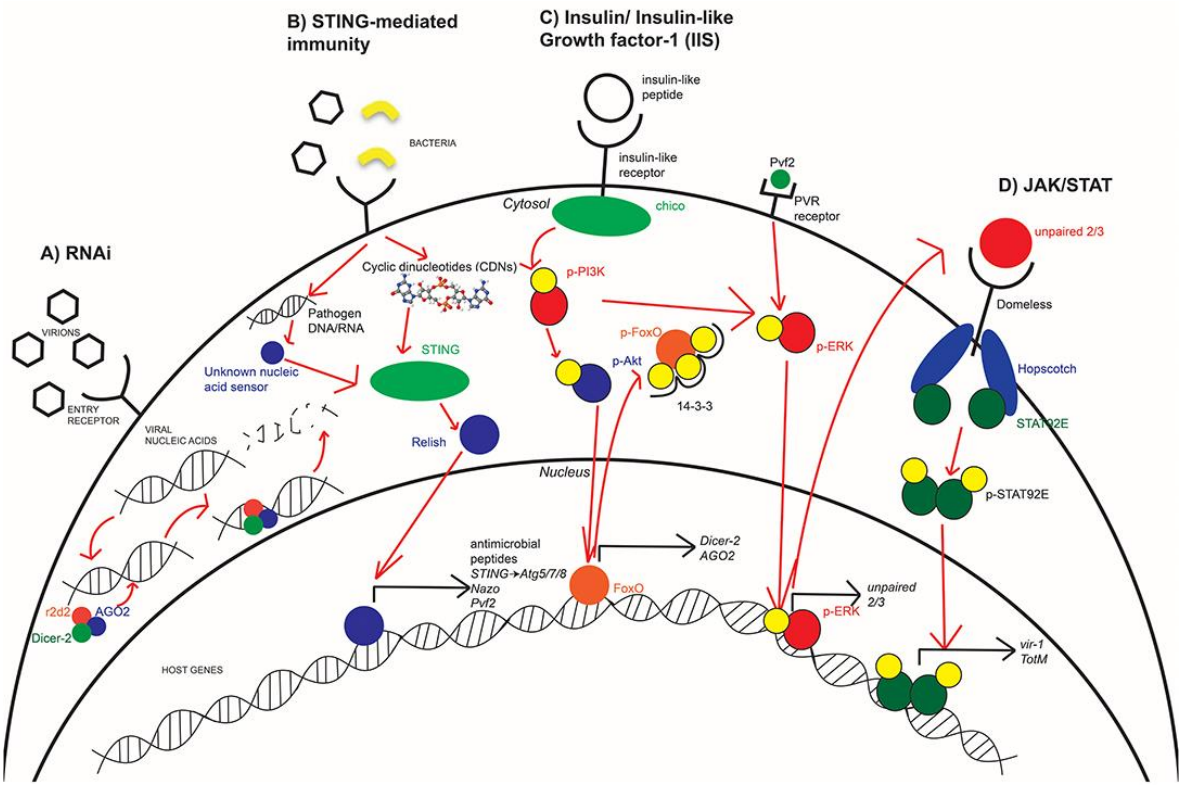

Figure 2. Innate immune antimicrobial pathways are conserved in arthropods. Insects utilize RNAi **(A)**, STING-mediated immunity **(B)**, and JAK/STAT signaling **(D)** in order to effectively respond to various arboviruses at different stages of infection. The IIS pathway **(C)** is an important mediator in host immunity as it regulates which immune responses are active or suppressed. During times of starvation, RNAi is more active while the bloodmeal provides the needed insulin to suppress RNAi and activate JAK/STAT. STING-mediated immunity has not yet been directly linked to IIS but may be affected by broader nutritional signaling. Each of these

pathways, to varying degrees, are conserved in fly, mosquito, and human systems with different efficiencies in responding to viral infection.

In both insect and mammalian systems, IIS regulates the transcription factor forkhead box O (FOXO), and FOXO is predominately associated with longevity and nutritional signaling as it induces *dInR* (*insulin-like receptor*) in *Drosophila* (DiAngelo and Birnbaum, 2009; Puig et al., 2003). FOXO possesses a secondary role in host immunity through its induction of RNAi-specific genes. Specifically, FOXO regulates the transcription of *Dicer-2* and *AGO2* in *Drosophila* and is demonstrated to enhance RNAi signaling during Cricket paralysis viral infection (Spellberg and Marr, 2015). Since IIS regulates FOXO transcriptional activity, there is a direct connection between RNAi immunity and insulin signaling. In *Drosophila*, the IIS pathway is induced by insulin-like peptides (ILPs) binding to dInR (Puig and Tijan, 2005). Upon binding, a phosphorylation cascade commences that includes phosphorylation of PI3K and Akt. This results in the phosphorylation of nuclear FoxO at three residues, its association with the 14-3-3 chaperone protein, and export into the cytosol (Puig et al., 2003; Nielsen et al., 2008) (Figure 2C). Insulin treatment is demonstrated to result in transcriptional suppression of *Dicer-2* and *AGO2* to reduce RNAi signaling (Ahlers et al., 2019). This insulin-mediated suppression of RNAi proposes that mosquitoes have evolved multiple immune responses to pathogens that is dependent on its nutritional status.

RNAi is evolutionarily conserved across organisms; however, its role in antiviral immunity varies between insects and mammals. While the signaling cascade and proteins involved are conserved, mammals have evolved other sensing mechanisms to detect and respond to viruses.

RIG-I and MDA5 RNA sensing are canonical pathways for vertebrate innate immunity and have developed from RNA sensors like Dicer (Sarkar, DeSalle, and Fisher, 2008). In particular, RIG-I and MDA5 are shown to be critical immune regulators in response to flaviviruses like ZIKV (Riedl et al., 2019) and WNV Errett et al., 2013) and alphaviruses like SINV (Akhrymuck, Frolov, and Frolova, 2016). This would imply that while RNAi is still fully functional in mammals, its role in responding to viruses is less stringent than in insect systems [reviewed in (Cullen, 2014; Schuster, Miesen, and van Rij, 2019)]. Other invertebrate and plant species utilize RNAi signaling as a means of antiviral immunity [reviewed in (Baulcombe, 2004; Boisvert and Simar, 2008)]. Researchers interested in studying RNAi immunity against arboviruses utilize *Drosophila* as the signaling cascades described are well-conserved in the more relevant mosquito vector. While the functional antiviral role of RNAi varies in mammalian systems, the proteins and signaling events involved are conserved. Because of this, studies utilizing *Drosophila* have proven it to be a viable model for study in host immunity and other regulatory functions.

JAK/STAT PATHWAY

Innate immunity uses various responses to different viruses. These responses include phagocytosis of viral particles or by inducing production of downstream cytokines and antiviral effectors (Lamiable et al., 2016). Whereas, the RNAi pathway provides a broad means of protection through the degradation of viral nucleic acids (Saleh et al., 2009), the JAK/STAT pathway is a signaling cascade where detection of infection or other stimuli results in the induction of antiviral effectors like *vir-1*, *Vago*, and *TotM* (Dostert et al., 2005; Kemp et al., 2013; Paradkar et al., 2012). In *Drosophila* immunity, this pathway is activated upon detection of infection (Boutros, Agaisse, and Perrimon, 2002), resulting in induction of the *unpaired* ligands

(Agaisse et al., 2003; Wright et al., 2011). Unpaired ligands bind to the receptor domeless (Ghiglione et al., 2002), resulting in the Janus kinase (JAK) ortholog hopscotch to be phosphorylated and form docking sites for phosphorylation and dimerization of the STAT92E transcription factor (Dostert et al., 2005; Binari and Perrimon, 1994). The activated STAT92E protein complex is imported into the nucleus to induce transcription of downstream antiviral effectors. This includes *TotM* during early stages of infection (Kemp et al., 2013) and *vir-1* during later stages (Dostert et al., 2005) (Figure 2D). JAK/STAT has been shown to be involved in the immune response against the insect virus *Drosophila* C virus but not SINV (Kemp et al., 2013) in *Drosophila*, and JAK/STAT is antiviral against WNV in both *Drosophila* and *Culex* mosquitoes (Ahlers et al., 2019; Paradkar et al., 2015).

The connection between JAK/STAT and IIS is not as direct as insulin's effect on the RNAi pathway, but recent research has demonstrated that insulin signaling in insects controls a switch between RNAi- and JAK/STAT-dependent responses. Specifically, when insulin treatment causes transcriptional suppression of RNAi, insects induce enrichment of JAK/STAT (Ahlers et al., 2019). This is mediated by insulin's phosphorylation and activation of downstream Akt and ERK proteins during SINV and DENV infection (Xu et al., 2013; Sansone et al., 2015) that was further evaluated to induce immunity through JAK/STAT against WNV (Ahlers et al., 2019). Insulin-mediated induction of JAK/STAT then induces the transcription of downstream antiviral effector proteins *vir-1* and *TotM* (Ahlers et al., 2019; Dostert et al., 2005; Kemp et al., 2013). JAK/STAT immunity responds to pathogens during early stages of infection in the mosquito which corresponds to ingestion of an infected bloodmeal and escape from the midgut to distal tissues (Taracena et al., 2018). This association between IIS and JAK/STAT signaling is

indicative that vector-competent insects have evolved immune mechanisms that are responsive to nutritional acquisition. While mosquitoes use an RNAi-dependent response during times of starvation, the bloodmeal provides the insulin needed to activate a JAK/STAT-dependent response during infection. While the ability to regulate immune responses based on nutritional status and how it impacts viral efficacy and transmission has yet to be established, it is plausible that insulin-mediated signaling could be targeted in future vector-control protocols. Its potential as a target depends on whether insulin's antiviral activity in the salivary glands is just as important as it is in midgut or fat body, critical digestive and immune organs (Sim, Ramirez, and Dimopoulos, 2012; Xu et al., 2013).

JAK/STAT signaling is an evolutionarily conserved immune response utilized in both insect and mammalian systems. Induction of JAK/STAT in mammals results in a Type I interferon (IFN) response against viral infection (Keller et al., 2006). The JAK/STAT pathway is similarly regulated by RIG-I-like receptor (RLR) signaling in mammalian systems (Sarkar, DeSalle, and Fisher, 2008; Stone et al., 2019). This pathway is an important means of responding to WNV in both insects (Ahlers et al., 2019; Paradkar et al., 2012) and mammals (Keller et al., 2006; Stone et al., 2019). Because JAK/STAT is related to other regulatory processes like cell proliferation and differentiation [reviewed in (Herrera and Bach, 2019)], researchers have also used the *Drosophila* model to study the pathway's role in a non-immunological context like cellular growth (Wright et al., 2011), differentiation (Ghiglione et al., 2002), polarization (Binari and Perrimon, 1994), and oogenesis (Wittes and Schüpbach, 2019). *Drosophila* provide a unique system for studying JAK/STAT signaling at various levels of complexity in both an immune and regulatory context to provide clarity regarding the pathway's significance in the organism.

STING-MEDIATED IMMUNITY

Mediators of mammalian immunity have evolved from established signaling pathways that are present in invertebrate systems (Sarkar, DeSalle, and Fisher, 2008; Martin et al., 2018). One such set of responses is stimulator of IFN genes (STING)-mediated immunity. Upon detection of viral nucleic acids in the cytosol, the DNA sensor cGAS metabolizes cyclic dinucleotides that bind to and activate STING (Burdette et al., 2011), which induces phosphorylation and activation of various transcription factors like TBK1, IRF3, and STAT6 (Chen et al., 2011; Ishikawa and Barber; 2008). These transcription factors regulate the induction of Type I IFN responses through the secretion of IFN-α and β (Burdette et al., 2011; Chen et al, 2011; Ishikawa and Barber, 2008). STING-mediated immunity has been heavily studied within mammalian systems; however, it is only recently that STING and its role in innate immunity have been identified in insects and other invertebrates (Liu et al., 2018; Martin et al., 2018; Goto et al., 2018; Hua et al., 2018; Kranzusch et al., 2015) (Figure 2B).

In *Drosophila*, STING signaling provides immunity against both bacterial and viral infections. During infection with *Listeria monocytogenes*, cyclic dinucleotides are produced which results in STING-mediated signaling and nuclear import of Relish, the fly ortholog of mammalian NF-κB (Martin et al., 2018). This immune response induces transcription and secretion of IMD-characteristic antimicrobial peptides to reduce bacterial burden (Martin et al., 2018). STING-mediated antiviral immunity also occurs through Relish and IKKβ, which regulate expression of the antiviral factor *Nazo* (Goto et al., 2018). In the silkmoth, *Bombyx mori*, STING signaling activates antiviral activity through Dredd and IMD, leading to Relish signaling and induction of antimicrobial peptides against nucleopolyhedrovirus (NPV) (Hua et al., 2018). Other studies

using the *Drosophila* system have further evaluated STING-mediated immunity by autophagy (Liu et al., 2018).

Autophagy is a cellular process in which intracellular structures and proteins are degraded in a lysosomal-dependent manner [reviewed in (Levine, Mizushima, and Virgin, 2011)]. Because viruses are obligate intracellular pathogens, autophagy is an established antiviral response that is partially regulated by nutritional and STING-mediated signaling (Moretti et al., 2017). While STING-mediated autophagy has been established in responding to numerous viruses in mammals, recent studies using *Drosophila* have demonstrated that insects can utilize autophagy to respond to ZIKV in neuronal tissues (Liu et al., 2018). Specifically, ZIKV infection results in a pro-inflammatory response in *Drosophila* brains which induces STING-mediated activation of autophagy and immunity (Liu et al., 2018). This form of antiviral immunity is indicative that neuronal protection against arboviruses is mediated through STING-mediated signaling. This study provides another example of the versatility provided in using the *Drosophila* system as the fly model is a viable means for studying antiviral immunity conserved between invertebrates and vertebrates.

Unlike the RNAi and JAK/STAT pathways, which are both regulated in part by insulin signaling, a direct link between IIS and STING has yet to be shown. However, since autophagy is partially regulated by nutritional status [reviewed in (Jung et al., 2010)] and STING has been previously shown to induce *STAT6* in the mammalian system (Chen et al., 2011), it is plausible that STING-mediated immunity may also be partially regulated by insulin signaling. Since STING has only recently been discovered in the insect model, future research is needed to

further evaluate how STING connects to other canonical immune and nutritional pathways and its involvement in vector competency.

PROSPECTIVE

Both the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) agree that mosquito-borne arboviruses will be of great concern in the following years due to the expansion of mosquitoes' habitation range and activity into previously unexposed regions (Rosenberg et al., 2018; World Health Organization; 2019). Research using *Drosophila* have permitted investigators to identify the key signaling events that occur during infection and develop more effective vector control protocols that target viral replication and likelihood of transmission. Recently, *Drosophila* have been used to identify ingestion of mammalian insulin as a key regulator in controlling WNV replication in the insect model. The genetic screen to identify insulin receptor was performed using *Drosophila*, and the role of insulin signaling was then validated in the mosquito model (Ahlers et al., 2019).

While *Drosophila* have been an invaluable tool in the study of arboviruses in place of the more relevant arthropod vector, there are limitations. For example, JAK/STAT signaling, while protective in the mosquito model against arboviruses such as WNV and DENV (Ahlers et al., 2019; Paradkar et al., 2012; Souza-Neto, Sim, and Dimopoulos, 2009), may not be protective in *Drosophila* during SINV or vesicular stomatitis virus infections (Dostert et al., 2005; Kemp et al., 2013). Because *Drosophila* are not the natural host for these arboviruses, there are limitations surrounding whether the responses observed are indeed what occurs in natural hosts. As such,

work involving *Drosophila* must ensure that their findings are further evaluated in the more relevant model, whether that be the insect vector or human host.

Developing disease response protocols that aim at preventing transmission from arthropods to humans would be the most beneficial in terms of cost-efficiency and alleviating disease burden at a global scale. Research efforts also aim to identify novel therapeutics that are effective at treating humans post-exposure. Because many of the immune pathways discussed here are also present in humans (Figure 2), future research aimed at identifying novel human-specific antiviral therapeutics could benefit from the use of *Drosophila*. The use of *Drosophila* to model host immunity at both the mammalian and vector level during arboviral infection has provided a greater depth of knowledge regarding which signaling pathways are involved during infection and how they can be targeted in the arthropods that transmit disease.

AUTHOR CONTRIBUTIONS

CT wrote the first draft of the manuscript. AG revised the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FOOTNOTES

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CHAPTER FIVE: COUPLED SMALL MOLECULES TARGET RNA INTERFERENCE AND JAK/STAT SIGNALING TO REDUCE ZIKA VIRUS INFECTION IN AEDES AEGYPTI

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Attributions:

C.E. Trammell was responsible for experimental design and execution. They completed experiments with assistance of G. Ramirez, I. Sánchez-Vargas, L.A. St Clair, and O.C. Ratnayake. They analyzed data and interpretation and wrote the text of this manuscript in consultation of R. Perera and A.G. Goodman.

R. Ramirez assisted in mosquito survival and titer studies presented in Figure 4, Figure S3, and Figure S6 in consultation with C.E. Trammell.

I. Sánchez-Vargas was responsible for optimization of mosquito assays and maintenance. She assisted in completing titer studies presented in Figure 4 and Figure S6 in consultation with C.E. Trammell.

L.A. St Clair and O.C. Ratnayake were responsible for mosquito survival and sample collection presented in Figure 2 and Figure S2 in consultation with C.E. Trammell.

S. Luckhart assisted in optimization of western blots presented in Figure 1 in consultation with C.E. Trammell.

R. Perera and A.G. Goodman were responsible for funding acquisition and writing in consultation with C.E. Trammell. They also assisted in experimental design and optimization in consultation with C.E. Trammell and I. Sánchez-Vargas.

ABSTRACT

The recent global Zika epidemics have revealed the significant threat that mosquito-borne viruses pose. There are currently no effective vaccines or prophylactics to prevent Zika virus (ZIKV) infection. Limiting exposure to infected mosquitoes is the best way to reduce disease incidence. Recent studies have focused on targeting mosquito reproduction and immune responses to reduce transmission. Previous work has evaluated the effect of insulin signaling on antiviral JAK/STAT and RNAi in vector mosquitoes. Specifically, insulin-fed mosquitoes resulted in reduced virus replication in an RNAi-independent, ERK-mediated JAK/STAT-dependent mechanism. In this work, we demonstrate that targeting insulin signaling through the repurposing of small molecule drugs results in the activation of both RNAi and JAK/STAT antiviral pathways. ZIKV-infected *Aedes aegypti* were fed blood containing demethylasterriquinone B1 (DMAQ-B1), a potent insulin mimetic, in combination with AKT inhibitor VIII. Activation of this coordinated response additively reduced ZIKV levels in *Aedes aegypti*. This effect included a quantitatively greater reduction in salivary gland ZIKV levels up to 11 d post-bloodmeal ingestion, relative to single pathway activation. Together, our study indicates the potential for field delivery of these small molecules to substantially reduce virus transmission from mosquito to human. As infections like Zika virus are becoming more burdensome and prevalent, understanding how to control this family of viruses in the insect vector is an important issue in public health.

AUTHOR SUMMARY

Arboviruses pose a significant threat to humans and are an increasing concern as a result of climate change and expanding vector-competent populations. The recent Zika outbreaks

demonstrate that mosquito-borne illnesses caused by viral infection remain a prominent and evolving threat that must be actively addressed. As there are currently no post-exposure therapeutics available for Zika virus infection, reducing transmission and, in turn, the likelihood of infection would provide sizeable benefit for human populations most at risk. Here, we show that readily available small molecules can be repurposed to effectively reduce viral replication and likelihood of transmission for a clinically relevant strain of Zika in vector competent mosquitoes. Furthermore, we show how two insulin-mediated canonical antiviral pathways are simultaneously activated in our drug treatment regimen to reduce virus levels in mosquito saliva, through which virus is transmitted to humans. Together, we demonstrate the viability of targeting insulin signaling as a means of reducing the rate of mosquito infection and decreased transmission of Zika virus.

INTRODUCTION

Mosquito-borne viruses pose a significant global health threat, and this threat is increased by dynamic ecological and human factors. Global warming and urbanization have permitted mosquitoes and arboviruses to spread into regions that previously lacked mosquito infection and mosquito-to-human virus transmission (Alaniz et al., 2018; Samy, Thomas, et al., 2016). This occurred during the 2015-17 Zika virus (ZIKV) Western hemisphere epidemic that originated in South America and spread into North America, resulting in 538,451 suspected cases, 223,477 confirmed cases, and 3,720 congenital syndrome cases (Pan American Health Organization, 2015, 2016). Subsequent outbreaks have followed that establishes ZIKV as an active pathogen of concern that requires intervention (Bhargavi and Moa, 2020). Current efforts have focused on strategies to reduce virus transmission to and from the mosquito vector, including the use of

insecticides and biological/genetic manipulation of primary vector species. The introduction of the bacterial symbiont *Wolbachia* to reduce flavivirus infection in the major arbovirus vector species *Aedes aegypti* (Aliota et al., 2016; Dutra et al., 2016; Haqshenas et al., 2019) and the release of genetically modified individuals to reduce transmission-competent progeny of this species (Waltz, 2021) have been included among the latter strategies. There is evidence to suggest that *Wolbachia*, while effective in reducing ZIKV and dengue virus (DENV) infection in targeted species may inadvertently enhance replication of West Nile virus (WNV) (Dodson et al., 2014). It is also not known how effective or advantageous genetically modified mosquito populations are compared to wild type populations or to other various viruses (Evans et al., 2019; Resnik, 2017). Because of these challenges, additional strategies to reduce vector transmission of these important viral pathogens are critically needed.

As an alternative strategy, it may be possible to reduce or block arborvirus transmission through mosquito-targeted delivery of bioactive small molecules at attractive sugar bait stations, a modification of the successful delivery of toxic baits for mosquito control (Dong and Dimopoulos, 2021). To this end, it is necessary to identify druggable mosquito antiviral effectors and their upstream regulatory factors. The insulin/insulin-like growth factor signaling (IIS) cascade regulates RNA interference (RNAi) and JAK/STAT antiviral immunity against West Nile virus (WNV), dengue virus (DENV), and ZIKV (Ahlers et al., 2019; Trammell and Goodman, 2019). In *Drosophila melanogaster*, the IIS-dependent transcription factor forkhead box O (FOXO) induces expression of RNAi transcripts *AGO2* and *Dicer2* (Spellberg and Marr, 2015). We demonstrated that ingestion of exogenous insulin reduced expression of these RNAi components in WNV-infected *Culex quinquefasciatus* (Ahlers et al., 2019) and that manipulation

of IIS-dependent extracellular-signal regulated kinases (ERK) activation reduced WNV infection in this mosquito host (Ahlers et al., 2019). Further, insulin treatment suppressed the activation of RNAi, while activating ERK-dependent JAK/STAT induction of unpaired (upd) ligands to control WNV replication *in vitro* and *in vivo* (Ahlers et al., 2019). Previous studies established that both JAK/STAT and RNAi antiviral pathways are independently involved in arthropod antiviral immunity to ZIKV (Angleró-Rodríguez et al., 2017; Harsh et al., 2018, 2020; Xu et al., 2019). To date, however, no mechanism(s) have been established whereby both antiviral pathways are induced simultaneously in response to arthropod infection.

In this study, we repurposed small molecules that target IIS pathway proteins to induce simultaneous activation of RNAi and JAK/STAT signaling in *Ae. aegypti*. Specifically, we used the potent insulin mimetic demethylasterriquinone B1 (DMAQ-B1), an activating ligand of the insulin receptor (InR) (Zhang et al., 1999) and Protein kinase B (AKT) inhibitor VIII, which reduces AKT phosphorylation (Lindsley et al., 2005) (Fig. 1A). Small molecule treatment induced activation of JAK/STAT via ERK and blocked inhibition of RNAi via the AKT/FOXO signaling axis. Combined treatment with DMAQ-B1 and AKT inhibitor VIII significantly lowered ZIKV titers in *Ae. aegypti* cells and adult female mosquitoes relative to single treatment and vehicle control. Combined treatment also additively reduced salivary gland virus titers, a surrogate measure of reduced transmission efficacy (Ferguson et al., 2015; Raquin and Lambrechts, 2017). Accordingly, we argue that activation of both antiviral pathways resulted in enhanced defenses that lowered viral titers to non-detectable levels. This work demonstrates the feasibility of strategically targeting mosquito immunity via IIS as a means of reducing a clinically relevant strain of ZIKV infection and transmission at the vector level.

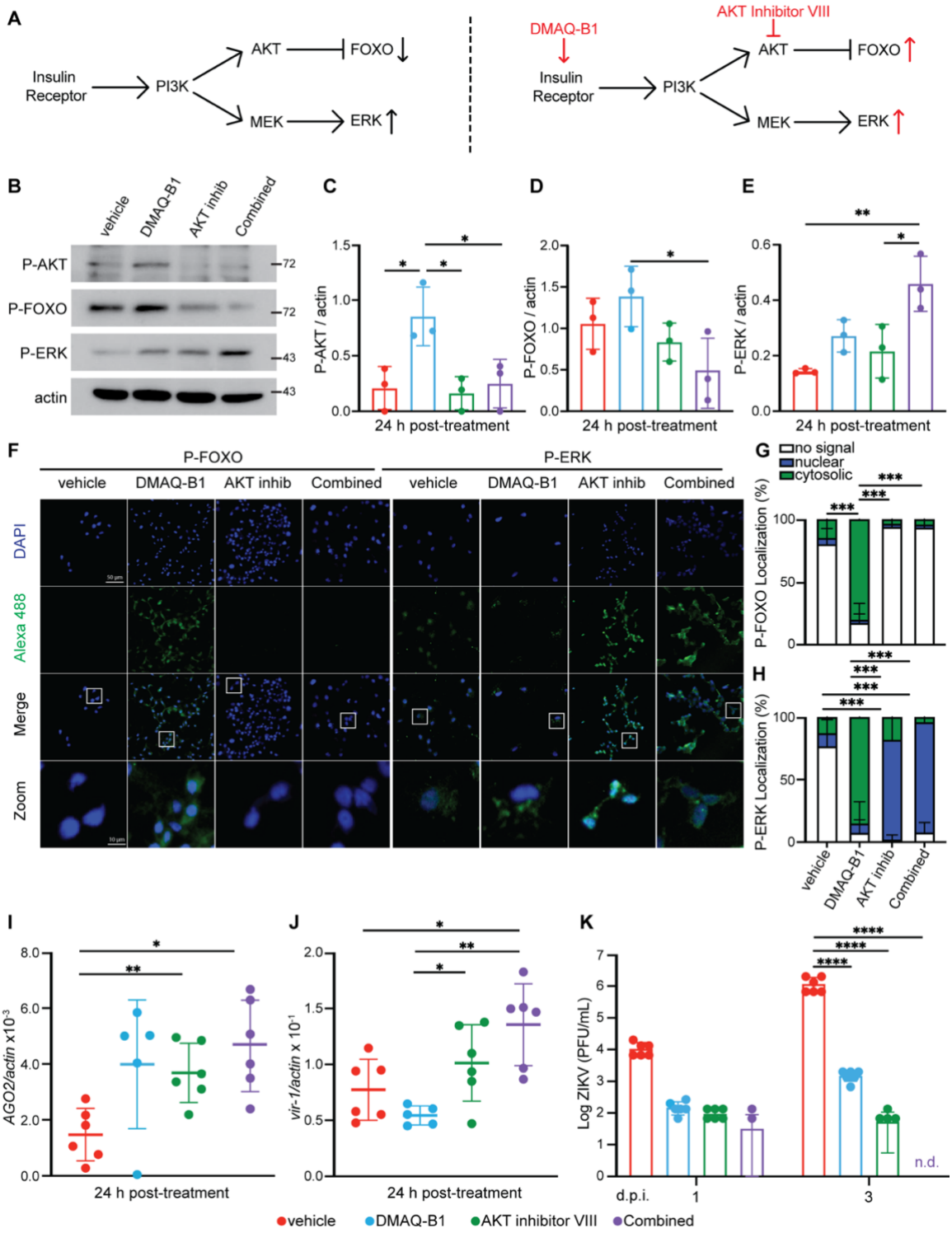

Figure 1: DMAQ-B1 and AKT inhibitor VIII activated RNAi and JAK/STAT *in vitro*. (A) Insulin/insulin-like growth factor I signaling (IIS) pathway schematic and proposed effect of DMAQ-B1 and AKT inhibitor VIII on downstream transcription factor activity. (B-E) Aag2

cells were treated with vehicle (DMSO), 1 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or combined drugs for 24 h. Phosphorylation of AKT, FOXO, and ERK were measured by western blot and phosphorylation was quantified for (B) P-AKT, (C) P-FOXO, and (D) P-ERK by densitometry and normalized to actin (*$p < 0.05$, One-way ANOVA with Tukey's test correction for multiple comparisons). (F) P-FOXO and P-ERK abundance and localization were visualized in DAPI-stained Aag2 cells by immunofluorescence microscopy. (G-H) Protein localization of (F) P-FOXO and (G) P-ERK was quantified in microscopy images to evaluate whether fluorescent-tagged proteins was cytosolic or nuclear within individual cells by manually counting cells in images (***$p<0.001$, Two-way ANOVA with Tukey's correction). (I-J) Induction of RNAi and JAK/STAT signaling was evaluated as transcript levels of (I) *AGO2* and (J) *vir-1* by qRT-PCR (*$p < 0.05$; **$p<0.01$, Unpaired t test with Welch's correction for multiple comparisons). (K) Aag2 cells that received vehicle, individual, or combined drug treatment for 24 h were infected with ZIKV (MOI 0.01) and supernatant was collected at 1 and 3 d p.i. Supernatant virus was titered by standard plaque assay (****$p<0.0001$, Two-way ANOVA with Tukey's correction). Closed circles represent individual replicates. Horizontal bars represent mean and error bars represent SD. Results are representative of triplicate independent experiments.

RESULTS

DMAQ-B1 and AKT inhibitor VIII activated *Aedes aegypti* insulin and antiviral signaling pathways. Since the JAK/STAT and RNAi antiviral pathways are linked to IIS, we sought to test the activity of small molecules against phosphorylation of key IIS protein targets and activity of these antiviral pathways. Given that phosphorylation of AKT and ERK correlate with

activation of IIS and JAK/STAT signaling (Ahlers et al., 2019; Boulton et al., 1991), respectively, and that FOXO phosphorylation is consistent with suppression of RNAi (Biggs et al., 1999; Brunet et al., 1999; Spellberg and Marr, 2015), we used these readouts to evaluate the efficacy of DMAQ-B1 and AKT inhibitor VIII control of RNAi and JAK/STAT.

Protein lysates from *Ae. aegypti* Aag2 cells treated with 1% DMSO vehicle, 1 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or these combined drug treatment for 24 hours were analyzed by western blot for phosphorylation of AKT, FOXO, and ERK (Fig. 1B). Drug concentrations were based on prior toxicity analysis (Fig. S1). DMAQ-B1 treatment was associated with the highest levels of AKT and FOXO phosphorylation; this phosphorylation was significantly reduced when combined with AKT inhibitor VIII (Fig. 1C-D). Single drug and combined drug treatments were associated with increased ERK phosphorylation relative to vehicle control (Fig. 1E). We validated these findings by immunofluorescence microscopy of 24 h treated cells probed for phospho-FOXO (P-FOXO) and P-ERK. Consistent with western blot analyses, we observed increased P-FOXO only in the DMAQ-B1-treated cells and P-ERK in both individual and combined-treated cells (Fig. 1F). Further, P-FOXO localization was primarily cytosolic (Fig. 1G) in DMAQ-B1 treated cells and P-ERK localization was primarily nuclear (Fig. 1H) in AKT inhibitor and combined treated cells, confirming that the transcription factors involved in RNAi and JAK/STAT induction are both nuclear and transcriptionally active under the expected treatment conditions. We also observed increased transcript expression of *AGO2* and *virus-induced RNA-1* (*vir*-1) which are indicative of RNAi and JAK/STAT activation, respectively, in cells treated with the combined drugs (Fig. 1I-J). Collectively, these data indicated that DMAQ-B1 and AKT inhibitor VIII treatment alter IIS phosphorylation in *Ae. aegypti* cells in a pattern

consistent with the activation of FOXO- and ERK-dependent antiviral signaling independent of viral infection.

Based on these effects on RNAi and JAK/STAT signaling in the absence of virus, we sought to determine the effects of single and combined drugs on ZIKV replication in *Ae. aegypti* cells. Aag2 cells were primed with individual and combined drugs for 24 h prior to infection with the clinically isolated PRVABC59 strain of ZIKV (Fig. 1H). We observed significant reductions in ZIKV titer in cells treated with individual and combined drugs by 3 days post-infection (d p.i.). Most notably, ZIKV titers were undetectable by 3 d p.i. in cells treated with the combined drugs (Fig. 1H). Patel and Hardy (2012) showed that AKT inhibitor VIII was antiviral in Sindbis virus (SINV)-infected *Aedes albopictus* C6/36 cells (Patel and Hardy, 2012), but the dysfunctional RNAi response in these cells (Brackney et al., 2010) precluded the confirmation of mechanism in its entirety. Accordingly, we concluded that DMAQ-B1 and AKT inhibitor VIII treatments induced an antiviral response that was increased to the point of non-detectable ZIKV titers when these treatments were combined.

DMAQ-B1- and AKT inhibitor VIII-supplemented sucrose water induces activation of RNAi and JAK/STAT signaling in *Aedes aegypti*. To model established sugar-bait strategies implemented in the field (Dong and Dimopoulos, 2021) and translatability of our *in vitro* findings, we next evaluated the effect of DMAQ-B1 and AKT inhibitor VIII treatment against the IIS-dependent antiviral response in adult female *Ae. aegypti*. Aged-matched 6-9-day old female mosquitoes were continuously exposed to 10% sucrose water supplemented with either 1% DMSO vehicle control, individual, or combined 10 μM DMAQ-B1 and 10 μM AKT

inhibitor VIII. Survival was measured over a 14-day treatment period with no difference between treatment conditions (Fig. S2). Whole mosquitoes were collected at 3 (Fig. 2A-D), 7 (Fig. 2E-H), and 11 day (Fig. 2I-L) for analysis of RNAi and JAK/STAT gene induction by qRT-PCR. *AGO2* and *p400* were examined as markers of RNAi (Figs. 2A-B, 2E-F, 2I-J) (Bernhardt et al., 2012; McFarlane et al., 2020), while *Vago2* and *vir-1* were examined as downstream effectors of JAK/STAT (Figs. 2C-D, 2G-H, 2K-L) (Asad, Parry, and Asgari, 2018; Diop et al., 2019).

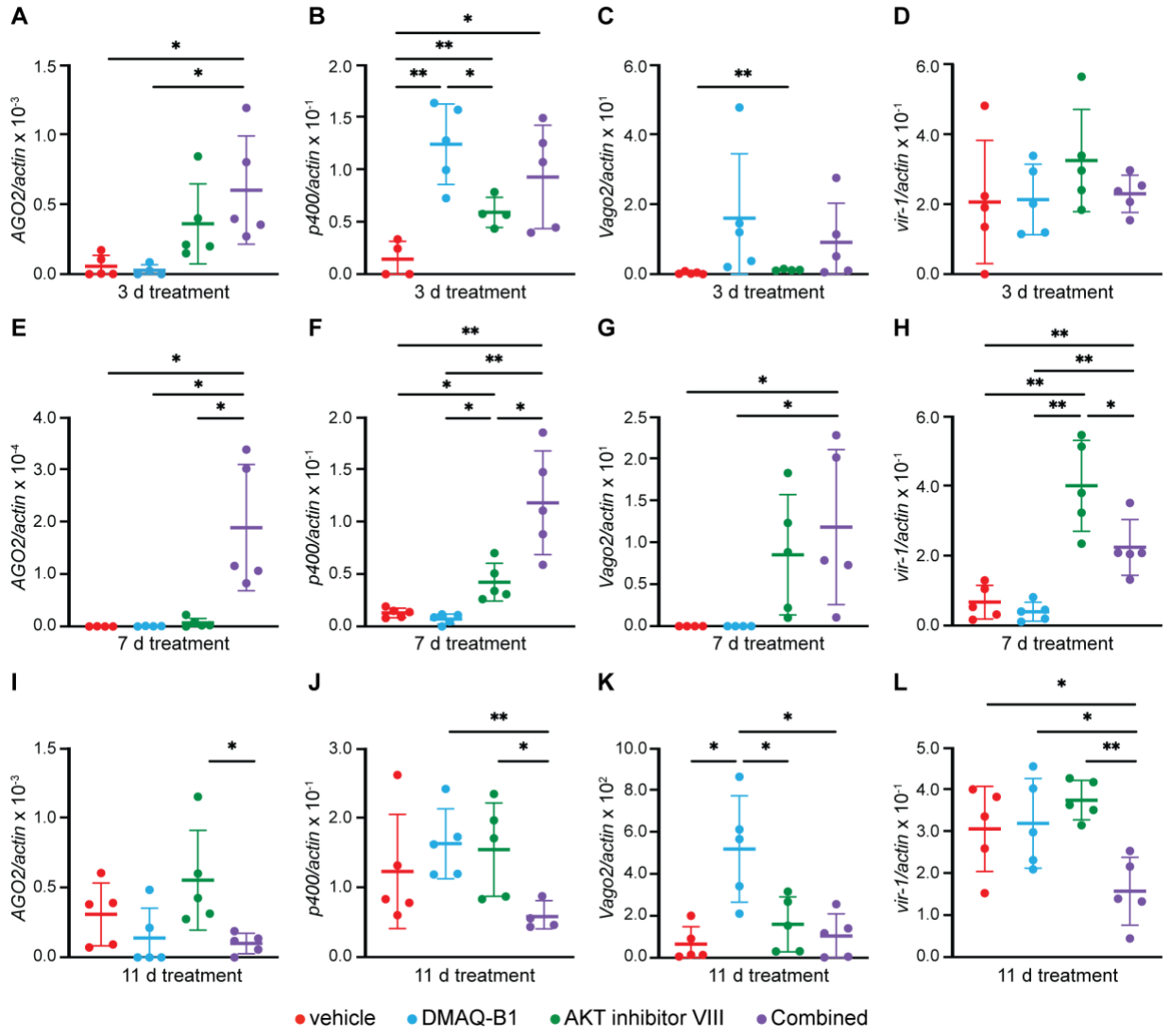

Figure 2: Continuous drug treatment via sucrose water results in induction of RNAi and JAK/STAT signaling in *Aedes aegypti* mosquitoes. Induction of RNAi and JAK/STAT gene

transcripts at (A-D) 3 d, (E-H) 7 d, and (I-L) 11 d treatment was measured in whole mosquitoes receiving continuous treatment of vehicle, 10 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or combined drugs via sucrose water. Transcripts were measured for (A, E, I) *AGO2,* (B, F, J) *p400,* (C, G, K) *Vago2*, and (D, H, L) *vir-1* by qRT-PCR (*p < 0.05; **p<0.01, Unpaired t test with Welch's correction for multiple comparisons). Closed circles represent individual replicates. Outliers were identified using a ROUT test (Q = 5%) and removed. Horizontal bars represent mean and error bars represent SD. Results represent duplicate independent experiments.

At day 3 we observed higher expression of RNAi-related genes in combined drug-treated mosquitoes compared to our vehicle control (Fig. 2A-B) but no difference in JAK/STAT-related genes (Fig. 2C-D). At day 7, however, we saw that RNAi and JAK/STAT gene induction were significantly higher in our combined drug-treated mosquitoes compared to our vehicle control (Fig. 2E-H). By day 11 we observed a loss in immune gene induction on the combined drug-treated mosquitoes (Fig. 2I-L). This leads us to infer that the effectiveness and activity of DMAQ-B1 and AKT inhibitor VIII, at least in combination, induces RNAi and JAK/STAT genes is optimal up to 7 days of continuous treatment but gene induction is reduced by 11 days, possibly due to negative feedback that inhibits the induction of these genes during continuous feeding. Because of this limited toxicity, transcript profile, and range of efficacy, we conclude that DMAQ-B1 and AKT inhibitor VIII induces RNAi and JAK/STAT signaling in a similar manner as we observed *in vitro*.

Bloodmeal treatment of DMAQ-B1 and AKT inhibitor VIII in ZIKV-infected *Aedes aegypti* induced simultaneous activation of RNAi and JAK/STAT signaling. Based on IIS-dependent

antiviral activity of DMAQ-B1 and AKT inhibitor VIII *in vitro* and *in vivo*, we sought to evaluate whether similar drug effects could be detected in *Ae. aegypti* adult females during ZIKV infection. Aged-matched 6–9-day old female mosquitoes were fed a ZIKV-containing bloodmeal including vehicle, individual, or combined 10 μM DMAQ-B1 and 10 μM AKT inhibitor VIII. Drug concentrations were selected based on mortality studies to measure drug lethality to mosquitoes over a dose range (Fig. S3). Mosquitoes were collected at 3, 7, and 11 d p.i., timepoints that corresponded with complete digestion of the blood meal, progression of viremia into distal tissues, and virus infection of the salivary glands (Roundy et al., 2017; Weger-Lucarelli et al., 2016; Williams et al., 2020). Expression levels of RNAi and JAK/STAT signaling gene products were quantified by qRT-PCR at 7 d p.i. (Figs. 3A-D) and 11 d p.i. (Fig. 3E-H). *AGO2* and *p400* were examined as markers of RNAi (Figs. 3A-B, 3E-F) (Bernhardt et al., 2012; McFarlane et al., 2020), while *Vago2* and *vir-1* were examined as downstream effectors of JAK/STAT (Figs. 3C-D, 3G-H) (Asad, Parry, and Asgari, 2018; Diop et al., 2019). Similar to previously discussed findings in Aag2 cells and non-infected mosquitoes, we observed that the combination drug treatment resulted in higher expression of RNAi and JAK/STAT signaling gene products at 7d p.i. (Figs. 3A-D). Interestingly, at 11 d p.i., only transcript levels for *AGO2* remained significantly higher for individual drug- and combination drug-treated mosquitoes (Figs. 3E-H). This gene expression profile is similar to mosquitoes that were continuously treated with individual or dual-drug supplemented sucrose water. Based on high transcript expression at 3 d p.i. in mosquitoes treated with the drug combination (Fig. S4), we demonstrate loss of gene induction between 7 and 11 d p.i. suggesting that drug treatment may have a limited efficacy by 11 d post-bloodmeal regardless if DMAQ-B1 and AKT inhibitor VIII is introduced in a single blood-feed or a multiday exposure. Expression levels of other related RNAi genes

including *Dicer2* and *Ppo8* (Almire et al., 2021; Bernhardt et al., 2012) and JAK/STAT *dome* (Souza-Neto, Sim, and Dimopoulos, 2009) were enhanced in combined drug-treated mosquitoes 7 d p.i. (Fig. S5) which affirms the effect that DMAQ-B1 and AKT inhibitor VIII have for RNAi and JAK/STAT signaling in *Ae. aegypti* mosquitoes.

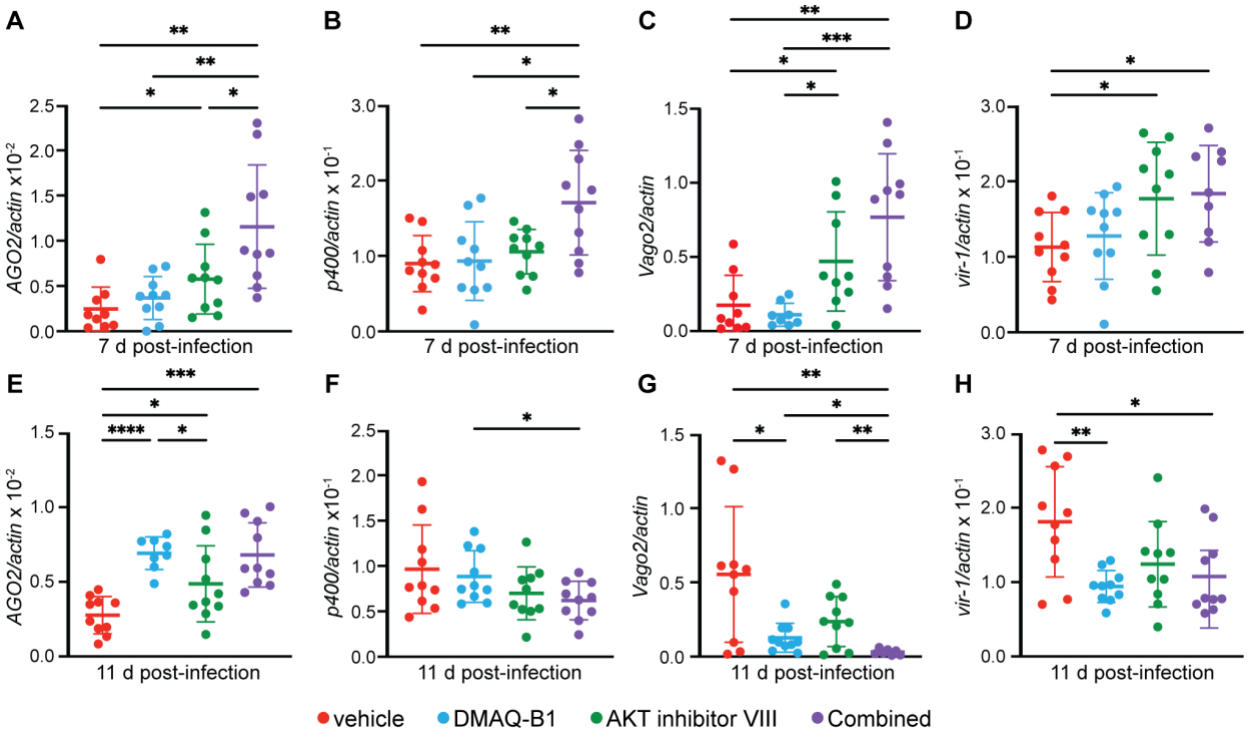

Figure 3: Combined drug treatment induced activation of RNAi and JAK/STAT in *Aedes aegypti* at 7 d p.i. that was reduced by 11 d p.i. Induction of RNAi and JAK/STAT gene transcripts at (A-D) 7 d p.i. and (E-H) 11 d p.i. was measured in whole mosquitoes infected with ZIKV and treated with vehicle, 10 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or combined drugs. Transcripts were measured for (A, E) AGO2, (B, F) p400, (C, G) Vago2, and (D, H) vir-1 by qRT-PCR (*p < 0.05; **p<0.01; ***p<0.001; **** p<0.0001, Unpaired t test with Welch's correction for multiple comparisons). Closed circles represent individual replicates. Outliers were identified using a ROUT test (Q = 5%) and removed. Horizontal bars represent mean and error bars represent SD. Results represent duplicate independent experiments.

DMAQ-B1 and AKT inhibitor treatment reduced infection prevalence and ZIKV titer in *Aedes aegypti*. We next sought to evaluate the effects of individual and combined drug treatments on infection prevalence and ZIKV titers in adult mosquitoes. Mosquitoes were fed a ZIKV-containing bloodmeal treated with vehicle, DMAQ-B1, AKT inhibitor VIII, or combined drug treatment as described. We collected mosquitoes at 3, 7, and 11 d p.i. and analyzed individual midguts, pairs of salivary glands, and carcasses for ZIKV titers (n=30). There were no differences in virus infection prevalence or viral titers at 3 d p.i. (Fig. S6). At 7 d p.i. infection prevalence was significantly reduced midgut and salivary gland tissues but not carcasses from combined drug-treated mosquitoes (Figs. 4A-C). The titer of ZIKV-positive mosquitoes at 7 d p.i., however, was only reduced in the midguts of combined drug-treated mosquitoes with an approximate 30-fold reduction. There was no difference in carcass and salivary gland titers (Figs. 4D-F)**.** By 11 d p.i., both infection prevalence (Figs. 4G-I), and viral titer (Figs. 4J-L) were significantly reduced across tissues in mosquitoes treated with individual and combined drug treatment relative to vehicle control. Titers in ZIKV-positive mosquitoes were reduced by ~42-fold in carcass, ~15-fold in midgut, and ~24-fold in salivary glands (Figs. 4J-L). Notably, infection prevalence and salivary gland viral titers were reduced in combined drug-treated mosquitoes at 11 d p.i., a time consistent with virus transmission during feeding (Armstrong et al., 2020; Sánchez-Vargas et al., 2018). Mosquitoes that received the combined drug treatment were not only less likely to be ZIKV-positive, but salivary gland viral load was also substantially reduced in positive mosquitoes. These observations suggested that combined drug treatment and coordinated activation of RNAi and JAK/STAT provides antiviral immunity against ZIKV that effectively reduced infection prevalence and viral load compared to vehicle controls. These

effects of combined drug treatment would be predicted, therefore, to reduce mosquito transmission of ZIKV.

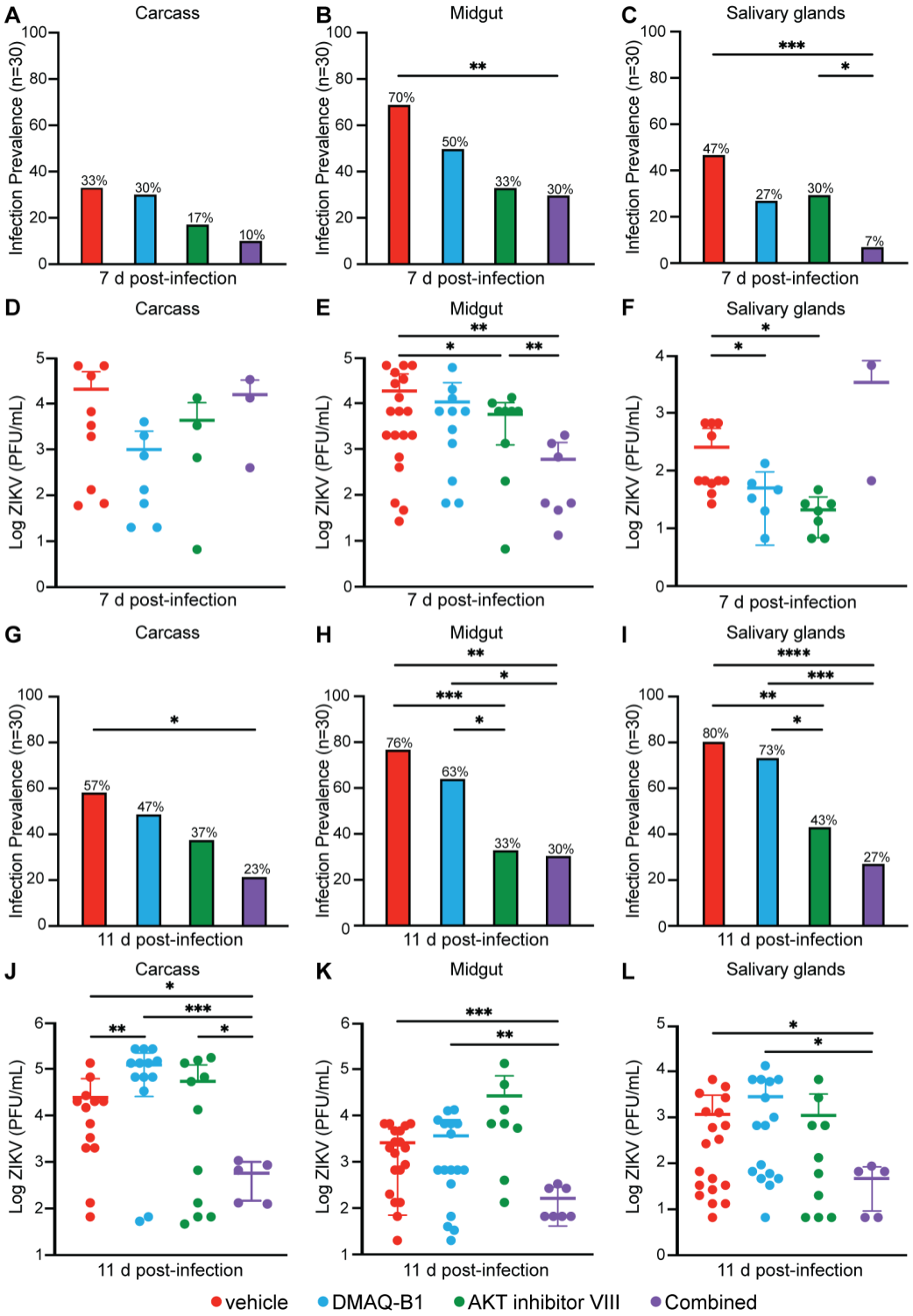

Figure 4: Individual and combined drug treatments reduced infection prevalence and ZIKV titers in *Aedes aegypti*. Individual mosquito midguts, pairs of salivary glands, and carcasses (n = 30) were titered for ZIKV by standard plaque assay at (A-F) 7d p.i. and (G-L) 11 d p.i. (A-C, G-I) Infection prevalence was calculated as the ratio of ZIKV-positive samples to the total sample size (*p < 0.05; **p<0.01; ***p<0.001, Two-tailed Fisher's exact test). (D-F, J-L) Viral titers were determined in ZIKV-positive mosquitoes (*p<0.05; **p<0.01; ***p<0.001; **** p<0.0001, Unpaired t test with Welch's correction for multiple comparisons). Closed circles represent individual replicates. Outliers were identified using a ROUT test (Q = 5%) and removed. Horizontal bars represent mean and error bars represent SD. Results represent duplicate independent experiments.

Inhibition of RNAi and JAK/STAT signaling resulted in loss of drug-mediated antiviral protection. To confirm that DMAQ-B1 and AKT inhibitor VIII mediated-antiviral protection is via RNAi- and JAK/STAT-dependent responses, we transfected Aag2 cells with siRNA constructs to knockdown expression of *AGO2* and *vir-1*. Cells were transfected with siRNAs that targeted either gene individually (siAGO2, siVir-1) or stacked gene expression (siAGO2 + siVir-1) (Terradas, Joubert, and McGraw, 2017). We measured *AGO2* and *vir-1* expression at 48, 72, and 120 h in accordance with siRNA removal, when cells were infected, and when supernatant was collected, respectively (Fig. 5A).

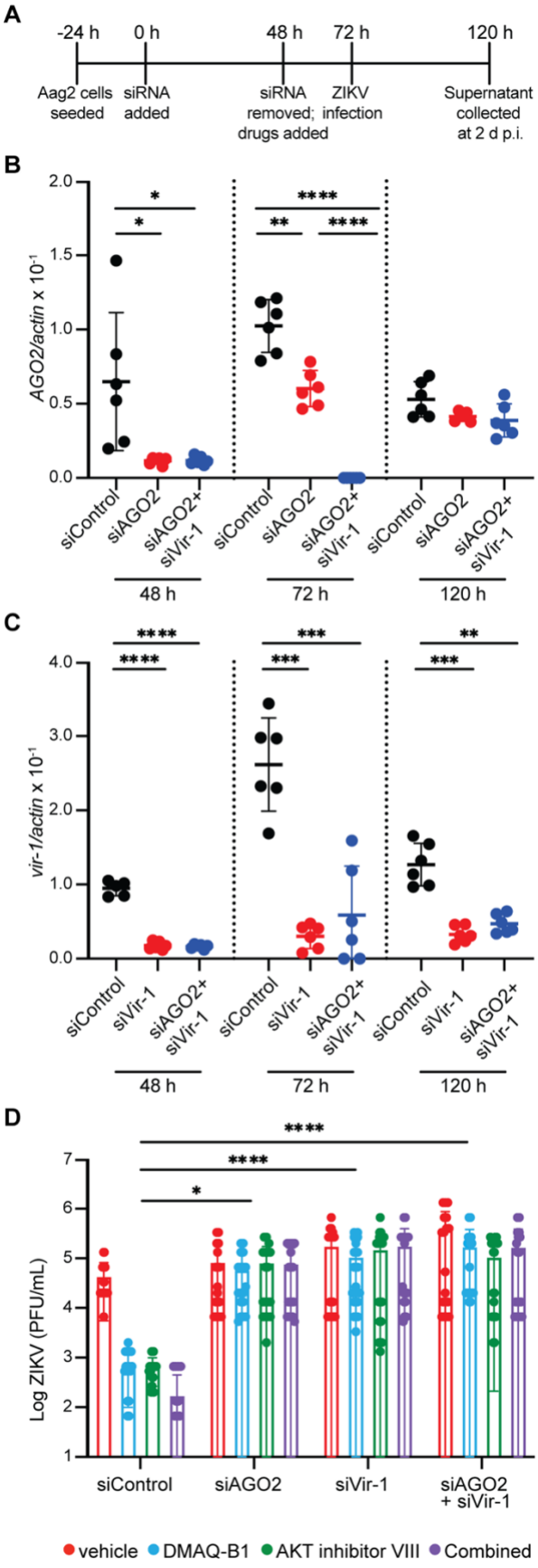
A
-24 h
0 h
48 h
72 h
120 h
Aag2 cells seeded
siRNA added
siRNA removed; drugs added
ZIKV infection
Supernatant collected at 2 d p.i.
B
AGO2/actin x 10-1
siControl
siAGO2
siAGO2+ siVir-1
48 h
72 h
120 h
C
vir-1/actin x 10-1
siControl
siVir-1
siAGO2+ siVir-1
48 h
72 h
120 h
D
Log ZIKV (PFU/mL)
siControl
siAGO2
siVir-1
siAGO2 + siVir-1
vehicle
DMAQ-B1
AKT inhibitor VIII
Combined

Figure 5: Knockdown of RNAi and JAK/STAT signaling resulted in loss of drug-mediated antiviral protection. (A) Experimental schematic illustrating process in which cells are transfected with siRNA for 48 h, drug-treated for 24 h prior to infection (72 h), and viral titer measured at 2 d p.i. (120 h). (B-C) AGO2 and vir-1 were knocked down in Aag2 cells and transcript levels were determined for (A) *AGO2* and (B) *vir-1* by qRT-PCR for cells transfected with scramble control (siControl), individual siRNA construct, or stacked siRNA (siAGO2+siVir-1) at 48 h, 72 h, and 120 h (**p<0.01; ***p<0.001; **** p<0.0001, Unpaired t test with Welch's correction). (D) 48 h following transfection, cells were primed with DMAQ-B1 or AKT inhibitor VIII for 24 h prior to infection with ZIKV (MOI = 0.01 PFU/cell). Supernatant was collected at 2 d p.i. and virus was titered by standard plaque assay (*p<0.05; **** p<0.0001,Two-way ANOVA with uncorrected Fisher's LSD test). Closed circles represent individual well replicates. Outliers were identified using a ROUT test (Q = 5%) and removed. Results are presented as pooled data of triplicate, independent experiments.

We observed significantly reduced gene expression at 48 h and 72 h post transfection for both individual and stacked siRNA treatments compared to cells that were treated with non-targeting siRNAs as a control (Clemons et al., 2011). Individual siAGO2 treated cells exhibited a 68% and 41% reductions in *AGO2* expression at 48 h and 72 h, respectively, whereas stacked siRNA treated cells exhibited 84% and 99% reduction at the same timepoints (Fig. 5B). We observed that *AGO2* expression returned to control levels in our siAGO2 and siAGO2 + siVir-1 transfected cells by 120 h. Expression levels of *vir-1* in siVir-1- and stacked transfected cells were reduced at all timepoints measured (Fig. 5C). In siVir-1-transfected cells, *vir-1* expression was reduced by 69% at 48 h, 88% at 72 h, and 74% at 120 h. In stacked-transfected cells, *vir-1*

expression was reduced by 82% at 48 h, 77% at 72 h, and 63% at 120 h. Finally, we treated cells at 48 h post siRNA transfection with vehicle, individual, or combined drug treatments for 24 h prior to ZIKV infection. Viral titers were measured in supernatants collected at 2 d p.i. to determine if drug-mediated antiviral protection was impacted in the absence of antiviral RNAi, JAK/STAT, or both. We observed that both individual and combined *AGO2* and *vir-1* knockdowns resulted in significant losses of drug-mediated antiviral protection relative to drug-treated controls (Fig. 5D). Interestingly, while we observed a loss in antiviral protection when comparing all data among gene knockdown groups (Table S2, Sheet1)**,** we observed a significant increase in viral titers only among the vehicle control groups when comparing siControl to siVir-1 or combined knockdown (Table S2, Sheet2). One explanation for why we did not observe differences between the siControl and siAGO2 group during vehicle treatment may be that while AGO2/RNAi is inhibited at the time of infection, its activity is restored by the time of sample collection (Fig. 5B). Another possibility is that while IIS-dependent RNAi and JAK/STAT signaling are sufficient to significantly reduce ZIKV titers, other pathways may also contribute to this biology. For example, despite the reduction in ZIKV to undetectable levels via IIS-dependent antiviral immunity, Toll signaling (Angleró-Rodríguez et al., 2017) and autophagy (Liu et al., 2018) could contribute to control of ZIKV replication. Collectively, our data suggest that repurposing small molecule drugs to target mosquito IIS can induce antiviral responses that significantly reduce ZIKV infection prevalence and transmission potential in *Ae. aegypti* through activation of RNAi and JAK/STAT signaling as inhibition of either or both pathways result in a loss of drug-mediated antiviral protection.

DISCUSSION

Global climate change has enabled the expansion of mosquito populations into new ranges with concomitant increases in the variety and incidence of mosquito-borne diseases. Recent ZIKV epidemics have demonstrated the need for research focused on identifying novel and more effective targets at the vector level. Current vector control efforts involving microbiota or genetic manipulations, while promising, could be enhanced by the addition of antiviral drug strategies to ongoing control efforts.

In the present work, we evaluated the potential of IIS-targeted small molecules to reduce ZIKV infection prevalence and titers in *Ae. aegypti*. We demonstrated that the potent insulin mimetic DMAQ-B1 and the AKT inhibitor VIII synergized IIS-mediated antiviral immunity in *Ae. aegypti* to reduce ZIKV infection prevalence and titers in infected mosquitoes (Fig. 6). While this study is not the first to identify IIS regulation of antiviral immunity, we have advanced this field by demonstrating that readily available and potent IIS-targeted small molecules induced substantial and significant antiviral immunity in *Ae. aegypti* against a clinically virulent strain of ZIKV. By targeting IIS as a mediator of two independent antiviral pathways, we reduced both infection prevalence and virus titers, outcomes predicted to reduce the likelihood of transmission. Both of these effects would be predicted to reduce ZIKV transmission by the primary vector *Ae. aegypti*. In demonstrating these effects, we have also provided a foundation for future translation of our findings to the field. We have demonstrated that exposure of DMAQ-B1 and AKT inhibitor VIII through either continuous exposure (Fig. 2) or a single treatment (Fig. 3) was sufficient to induce activation of both antiviral pathways independently of ZIKV infection. More importantly, even with the limited time range in which these drugs appear to induce pathway activation, there was a significant reduction in ZIKV positive- and vector competent-mosquitoes

following IIS pathway activation (Fig. 4). Specifically, we seek to advance small molecule delivery via attractive bait stations to induce IIS-mediated, broad antiviral immunity in mosquitoes that ingest these compounds.

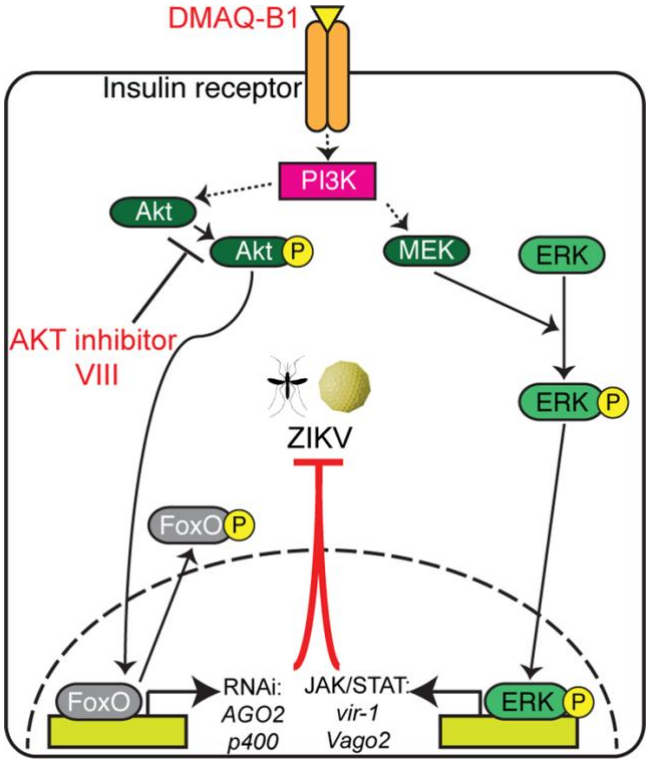

Figure 6: Selective targeting of insulin-signaling in *Ae. aegypti* impacts canonical antiviral responses that can effectively reduce ZIKV replication and likelihood of transmission. Schematic of proposed mechanism of antiviral action mediated by DMAQ-B1 and AKT inhibitor VIII during ZIKV infection through simultaneous induction of antiviral RNAi and JAK/STAT signaling.

Of particular interest for a potential field-based strategy is the broad impact that IIS appears to have across species. Previous studies have confirmed that exogenous treatment with or the endogenous effects of insulin in *D. melanogaster* and *Culex* spp. reduced replication of both WNV and DENV (Ahlers et al., 2019; Xu et al., 2013). While this broad-antiviral effect is

promising, further investigation is necessary to elucidate the full impact our proposed small molecules may have when introduced as a field-based strategy. Because of the limited studies on the effects of DMAQ-B1 and AKT inhibitor VIII on insects, it is unknown if these drugs may be hazardous to beneficial species that could be exposed during mosquito treatment (Dong and Dimopoulos, 2021; Kapaldo, Carpenter, and Cohnstaedt, 2018).

Interestingly, the antiviral effects of IIS during ZIKV infection are not limited to arthropod species. In mammalian models, ZIKV NS4A/NS4B activates PI3K-AKT signaling that is associated with neurogenetic dysregulation (Liang et al., 2016). Further, the broadly antiviral celecoxib kinase inhibitor AR-12 and AKT inhibitor VIII have been shown to reduce ZIKV replication and pathogenesis in mice by blocking PI3K-AKT activation (Chan et al., 2018). It is also established that diabetic individuals with dysfunctional IIS are more susceptible to severe disease during WNV (Kumar et al., 2012, 2014), DENV (Lee et al., 2020), and ZIKV infection (Nielsen and Bygbjerg, 2016).

Given the variety of mosquito species that deploy IIS-dependent immunity against notable major arboviruses, it would be worth investigating whether similarly broad IIS regulation of antiviral responses can be detected in mammalian hosts. If so, it may be possible to develop IIS-targeted transmission blocking therapeutic drugs that mitigate Zika disease and, when delivered in blood from treated patients to *Ae. aegypti*, reduce infection in and transmission by the mosquito vector.

MATERIALS AND METHODS

Mosquito rearing. *Aedes aegypti* strain Poza Rica , from the state of Veracruz, Mexico were originally collected in 2012, and maintained as previously described (Weger-Lucarelli et al., 2016). Adult mosquitoes were provided continuous access to water and 10% sucrose *ad libitum* using soaked cotton balls that were replaced every other day prior to and following bloodmeal feeding. Females were allowed to feed on defibrinated sheep blood (Colorado Serum Company 31123) supplemented with 1mM ATP using an artificial feeding system to stimulate oogenesis. Larvae were reared and maintained under constant 28 °C, 70% humidity, and 12-hour light, 12 hour dark diurnal cycle. 6-9 day old adult female mosquitoes were deprived of sucrose 24 hours prior to experimental feedings as described (Weger-Lucarelli et al., 2016). Mosquito infections, maintenance, and plaque assays were performed under BSL3 and ACL3 facilities, approved by Colorado State University's Institutional Biosafety Committee 16-074B.

Cells and virus. Vero cells (ATCC, CRL-81) were provided by A. Nicola and cultured at 37 °C/5% CO_2 in DMEM (ThermoFisher 11965) supplemented with 10% FBS (Atlas Biologicals FS-0500-A) and 1x antibiotic-antimycotic (ThermoFisher 15240062). *Ae. aegypti* Aag2 cells (*Wolbachia*-free) (Terradas, Joubert, and McGraw, 2017) were gifted by S. O'Neill and cultured as described in (Terradas, Joubert, and McGraw, 2017). For drug treatment, culture media with 2% FBS were supplemented with 1% DMSO, 1 μM Demethylasterriquinone B1 (DMAQ-B1) (R&D Systems 1819/5), 10 μM AKT inhibitor VIII (Sigma Aldrich 124018), or combined drugs. Concentrations of DMAQ-B1 and AKT inhibitor VIII were selected at non-cytotoxic levels for both cell culture (Figure S1) and adult mosquitoes (Figure S2-S3). ZIKV strain PRVABC59 (Accession # KU501215) was obtained from the CDC and was isolated in 2015 from a clinical case in Puerto Rico and prepared as described (Weger-Lucarelli et al., 2016).

***In vitro* virus replication.** Aag2 cells were seeded into a 24-well plate at a confluency of 5×10^5 cells/well with 6 independent wells for each experimental condition. The following day, cells were treated with 1% DMSO, 1 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or combined drugs in 2% FBS media as described (Ahlers et al., 2019) for 24 h prior to infection. Cells were then infected with ZIKV at MOI of 0.01 PFU/cell for 1 h. Virus inoculum was removed, and fresh experimental media was added. Supernatant samples were collected at 1 and 3 d p.i. for later titration. ZIKV titers were determined by standard plaque assay on Vero cells (Baer and Kehn-Hall, 2014; Sánchez-Vargas, Olson, and Black, 2021).

Cytotoxicity of DMAQ-B1 and AKT inhibitor VIII. Cytotoxicity of DMAQ-B1 and AKT inhibitor VIII was evaluated in both cell culture and in adult female *Ae. aegypti*. DMAQ-B1 and AKT inhibitor VIII was added to a monolayer of 2.5×10^5 cells/well in 48-well plates at various concentrations (100 μM, 10 μM, 1 μM, 0.1 μM). Cells were collected at 1, 2, and 3 d post-treatment, stained with trypan blue (ThermoFisher 15250-061) and scored as live or dead as described (Ahlers et al., 2016). Combined DMAQ-B1 and AKT inhibitor VIII cytotoxicity was evaluated using the maximum individual concentrations that corresponded to minimal cytotoxicity. A total of eight technical replicates were averaged for each biological replicate. 1% DMSO treated and 1% Triton X-100 treated cells were also scored as negative and positive controls, respectively. Toxicity was evaluated similarly in 6-9 day old female mosquitoes treated by either drug-treated sucrose water or drug-treated bloodmeal. Sucrose water-treated mosquitoes were provided 10% sucrose water with either 10 μM DMAQ-B1, 10 μM AKT VII inhibitor, combined treatment of both 10 μM DMAQ-B1 and 10 μM AKT inhibitor, or 1%

DMSO vehicle control *ad libitum*. Mosquitoes were maintained for 14 d to monitor mortality. Bloodmeal-treated mosquitoes were fed a bloodmeal containing small molecule drugs (100 μM, 10 μM, 1 μM), 1% DMSO vehicle control, or blood only. Following 1 h of feeding, engorged females were kept and maintained on sucrose for 14 d to monitor mortality. Combined small molecule drug treatment was evaluated using observed lethal and nonlethal individual concentrations. Each experimental group contained approximately 70-100 mosquitoes.

Immunoblotting. Protein extracts were prepared by lysing cells with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1mM Na_3VO_4, 1 mM NaF, 0.1 mM PMSF, 10 μM aprotinin, 5 μg/mL leupeptin, 1 μg/mL pepstatin A). Protein samples were diluted using 2x Laemmli loading buffer, mixed, and boiled for 5 minutes at 95 °C. Samples were analyzed by SDS/PAGE using a 10% acrylamide gel, followed by transfer onto PVDF membranes (Millipore IPVH00010). Membranes were blocked with 5% BSA (ThermoFisher BP9706) in Tris-buffered saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl) and 0.1% Tween-20 for 1 h at room temperature.

Primary antibody labeling was completed with anti-P-Akt (1:1,000; Cell Signaling 4060), anti-P-ERK (1:1000; Sigma M8159), anti-P-FOXO (1:1000; Millipore 07-695), or anti-actin (1:10,000; Sigma A2066) overnight at 4 °C. Secondary antibody labeling was completed using anti-rabbit IgG-HRP conjugate (1:10,000; Promega W401B) or anti-mouse IgG-HRP conjugate (1:10,000; Promega W402B) by incubating membranes for 2 h at room temperature. Blots were imaged onto film using luminol enhancer (ThermoFisher 1862124). Densitometry analysis was

completed using three independent blots using BioRad Image Lab and GraphPad Prism 9 with bands normalized to actin.

RNA interference *in vitro*. Long dsRNA targeting *Ae. aegypti AGO2*, *vir-1*, and non-targeting control dsRNA was synthesized as described (Terradas, Joubert, and McGraw, 2017). Targeted sequences and primers are listed in Table S1. dsRNA was transfected into Aag2 cells as described (Terradas, Joubert, and McGraw, 2017) for 48 h prior to small molecule treatment and infection. RNA was extracted and purified to confirm reduced expression by qRT-PCR at 48 h, 72 h, and 120 h post transfection. Viral concentration was confirmed by standard plaque assay at 2 d p.i.

Quantitative reverse transcriptase PCR. qRT-PCR was used to measure mRNA levels in *Ae. aegypti* Aag2 cells and adult females. Cells or mosquitoes were lysed with Trizol Reagent (ThermoFisher 15596). RNA was isolated by column purification (ZymoResearch R2050), DNA was removed (ThermoFisher 18068), and cDNA was prepared (BioRad 170–8891). Expression of *Ae. aegypti AGO2*, *p400, Vago2*, and *vir-1* were measured using SYBR Green reagents (ThermoFisher K0222) and normalized to *actin* to measure endogenous gene levels for all treatment conditions. The reaction for samples included one cycle of denaturation at 95 °C for 10 minutes, followed by 45 cycles of denaturation at 95 °C for 15 seconds and extension at 60 °C for 1 minute, using an Applied Biosystems 7500 Fast Real Time PCR System. ROX was used as an internal control. qRT-PCR primer sequences are listed in Table S1.

Immunofluorescence microscopy. *Ae. aegypti* Aag2 cells were seeded onto coverslips in 12-well plates at a confluency of approximately $1x10^6$ cells/well. Cells were then treated for 24 hours with 1% DMSO, 1 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or combined small molecule treatment supplemented in 2% FBS media as described (Ahlers et al., 2019). Coverslips were fixed in 4% paraformaldehyde for 10 minutes at room temperature, permeabilized in 0.1% Triton-X-100 for 30 minutes at room temperature and blocked in 1% BSA in TBS for 30 min at 37 °C. Primary antibody labeling was completed with anti-P-FOXO (1:100) and anti-P-ERK (1:100) for 2 h at humified room temperature. Secondary antibody labeling was completed using anti-rabbit (Life Technologies A11034) or anti-mouse (Life Technologies A11029) Alexafluor 488 (1:300) by incubating membranes for 1 h at room temperature in the dark. Samples were stained with DAPI (1:100; Cell Signaling 4083), mounted onto coverslips using ProLong Diamond Antifade Mountant (Invitrogen P36961), and imaged using a Leica Sp8X confocal microscope. Localization percentages were determined using Adobe Illustrator 2021 by counting the total number of cells and evaluating if green-fluorescent signal was cytosolic, nuclear, or no signal in relation to DAPI-stained nuclei.

Mosquito infections. Fresh ZIKV virus stock was made from Vero cells infected at MOI of 0.1 PFU/cell at 72 hours prior to bloodmeal feed of 6-9 day old female mosquitoes as described (Weger-Lucarelli et al., 2016; Williams et al., 2020). Mosquitoes were fed a bloodmeal supplemented with 1 mM ATP and infected with the fresh virus inoculum that was back-titrated to 2.4×10^6 PFU/mL. Bloodmeals included 1% DMSO vehicle control, 10 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or combined drugs. Following 1 h of feeding, mosquitoes were anesthetized on ice and engorged mosquitoes were moved into new cartons and maintained on sucrose.

Mosquitoes were collected at 3, 7, and 11 d p.i. in which the midgut, salivary glands, and carcass were separated, homogenized, and filtered for infection determination and viral titer. Whole mosquitoes were collected at the same timepoints for qRT-PCR analysis.

Quantification and Statistical Analysis. Results presented as dot plots show data from individual biological replicates (n=3-18), the arithmetic mean of the data, shown as a horizontal line. Biological replicates of adult mosquitoes (n=2-24) consisted of two pooled mosquitoes. Results shown are representative of at least duplicate independent experiments, as indicated in the figure legends. All statistical analyses of biological replicates were completed using GraphPad Prism 9 and significance was defined as $p<0.05$. One-way ANOVA with Tukey's correction for multiple comparisons was used for densitometry analysis. Two-way ANOVA with Tukey's correction for multiple comparisons was used for microscopy localization analysis. Unpaired t test with Welch's correction was used for qRT-PCR and *in vivo* viral titer analysis. Two-way ANOVA with Tukey's correction was used for analysis of multiday *in vitro* viral titer. Two-tailed Fisher's exact test was used to compare infection prevalence. Two-way ANOVA with Tukey's correction for multiple comparisons was used for analysis of small molecule cytotoxicity *in vitro* and *in vivo*. All error bars represent standard deviation (SD) of the mean. Outliers were identified using a ROUT test (Q=5%) and removed.

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Infectious Diseases, for assistance with mosquito husbandry and feedback regarding experimental design. We also thank L.R.H Ahlers for critical reading of our manuscript.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.E.T. and A.G.G.; Methodology, C.E.T., G.R., I.S.V., L.A.S., O.C.R., S.L., R.P., and A.G.G.; Validation, C.E.T., G.R., I.S.V, L.A.S., and O.C.R.; Investigation, C.E.T., G.R., I.S.V., R.P., and A.G.G; Resources, I.S.V., S.L., R.P., and A.G.G.; Writing – Original Draft, C.E.T.; Writing – Review and Editing, G.R., I.S.V., L.A.S., O.C.R., S.L., R.P., and A.G.G.; Visualization, C.E.T. and A.G.G.; Funding Acquisition, C.E.T., I.S.V., S.L., R.P., and A.G.G.

DECLARATION OF INTERESTS

The authors have declared that no competing interests exist.

SUPPLEMENTAL INFORMATION

Table S1: Primers for qRT-PCR and siRNA synthesis

Gene	Forward qRT-PCR primer	Reverse qRT-PCR primer	Citation
Ae Actin	GAACACCCAGTCCTGCTGACA	TGCGTCATCTTCTCACGGTTAG	Diop et al., *Viruses* 2019
Ae AGO2	CAACTTCGGTATCCTTCT	TTCCCGTCTTGTAATCTCC	Bernhardt et al., *PLOS One* 2012
Ae Vir-1	GCCAAAGTCCGGTATTCTTC	TTCACGAGATCGTCAAGGTAA	Diop et al., *Viruses* 2019
Ae P400	GGAACCAGTCCAGCCATGAA	CGATCGCTCCTGCATTTGTG	McFarlane et al., *mSphere* 2020
Ae Vago2	CGACCCGGAATGTGTGAAGA	GCAGCATTGTGGGTAGTCCT	Asad, Parry, and Asgari, *Insect Biochem and Mol Bio* 2018
Ae Dicer2	GTGTAATCGGTCTTTCTG	ACGCCAGTCTTAGCATTG	Bernhardt et al., *PLOS One* 2012
Ae Ppo8	GCTTTGCTATGTCCGCCAAT	CGCATTCGGAGACAATGATG	Almire et al. *PLOS Path* 2021
Ae Dome	AAACGGTGGCAAAATGAACT	CATACAGCCGGCTTTCTTCT	Souza-Neto et al., *Proc Natl Acad Sci USA* 2009
Ae AGO2 siRNA	ACAACAGCAACAATCCCAGA	GTGGACGTTGATCTTGTTGG	Terradas, Joubert, and McGraw, *Sci Rep* 2017
Ae Vir-1 siRNA	GCCAAAGTCCGGTATTCTTC	TTCACGAGATCGTCAAGGTAA	Terradas, Joubert, and McGraw, *Sci Rep* 2017

Gene	Sense/ Antisense	Reverse dsRNA primer	Start	Target Sequence	Citation
Ae AGO2	sense	CCUAAAGCAGGGUGUCCAAdTdT	1836	CCTAAAGCAGGGTGTCCAA	Terradas, Joubert, and McGraw, *Sci Rep* 2017
	antisense	UUGGACACCCUGCUUUAGGdTdT	1836	TTGGACACCCTGCTTTAGG	Terradas, Joubert, and McGraw, *Sci Rep* 2017
Ae Vir-1	sense	CGGAAGAUACCCAGACCAAdTdT	404	CGGAAGATACCCAGACCAA	Terradas, Joubert, and McGraw, *Sci Rep* 2017
	antisense	UUGGUCUGGGUAUCUUCCGdTdT	404	TTGGTCTGGGTATCTTCCG	Terradas, Joubert, and McGraw, *Sci Rep* 2017
Ae Scramble	sense	GATTAGACGAATACCACTA			Clemons et al., *PLOS One* 2011
	antisense	CTAATCTGCTTATGGTGAT			Clemons et al., *PLOS One* 2011

Table S2: Statistical analysis of data presented in Figure 5D.

Compare row means (overall drug effect)					
Number of families	1				
Number of comparisons per family	6				
Alpha	0.05				
Uncorrected Fisher's LSD	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Individual P Value
siCt vs. siAGO2	-64961	-123257 to -6666	Yes	*	0.0291
siCt vs. siVir-1	-143391	-201687 to -85095	Yes	****	<0.0001
siCt vs. siCombined	-198649	-256945 to -140354	Yes	****	<0.0001
siAGO2 vs. siVir-1	-78430	-136725 to -20134	Yes	**	0.0086
siAGO2 vs. siCombined	-133688	-191984 to -75392	Yes	****	<0.0001
siVir-1 vs. siCombined	-55258	-113554 to 3037	No	ns	0.0631

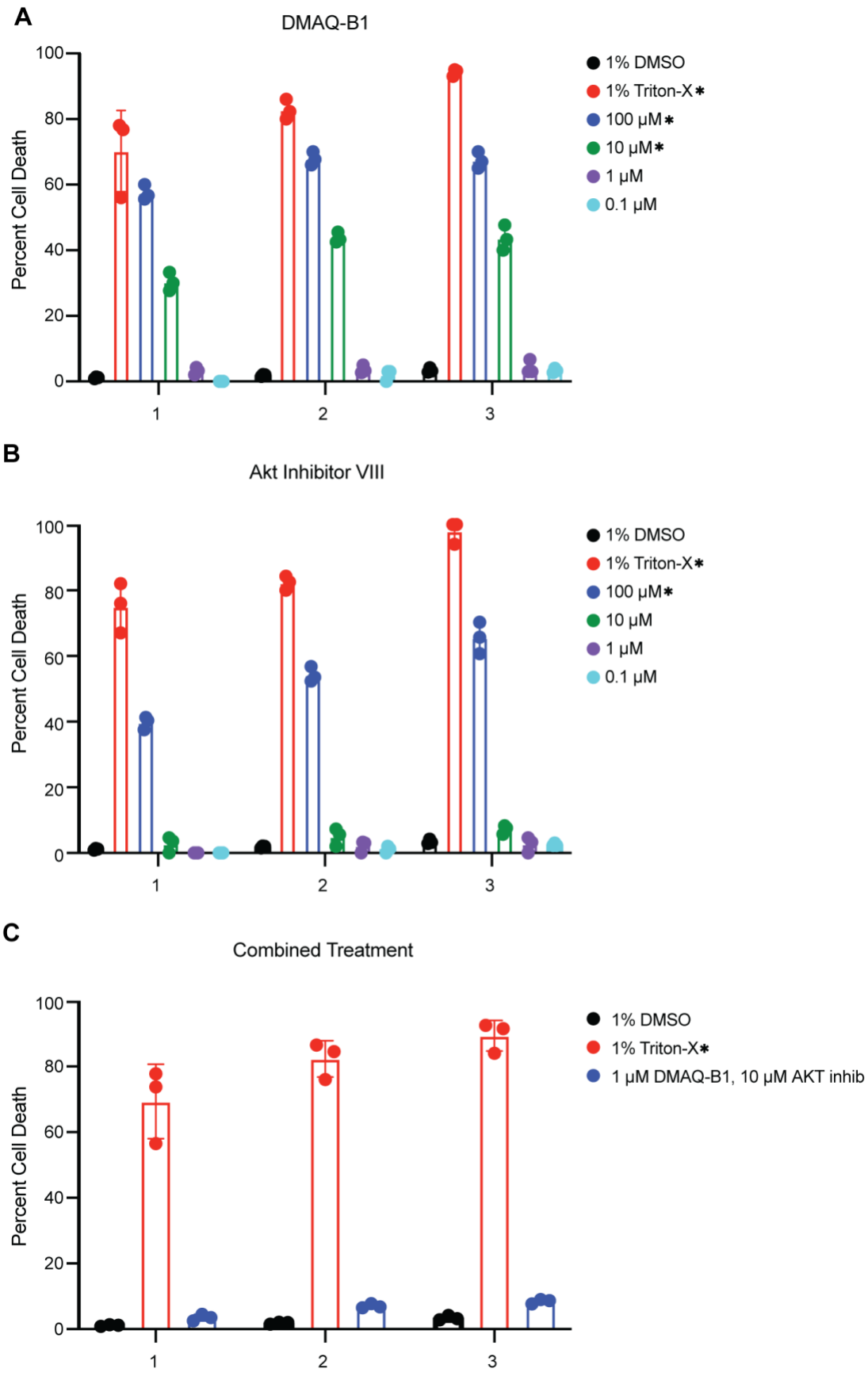
A
DMAQ-B1
Percent Cell Death
100
80
60
40
20
0
1
2
3
1% DMSO
1% Triton-X*
100 µM*
10 µM*
1 µM
0.1 µM
B
Akt Inhibitor VIII
Percent Cell Death
100
80
60
40
20
0
1
2
3
1% DMSO
1% Triton-X*
100 µM*
10 µM
1 µM
0.1 µM
C
Combined Treatment
Percent Cell Death
100
80
60
40
20
0
1
2
3
1% DMSO
1% Triton-X*
1 µM DMAQ-B1, 10 µM AKT inhib

Figure S1: DMAQ-B1 and AKT inhibitor VIII exhibited dose-dependent cytotoxicity in Aag2 cells. Aag2 cells were treated with various concentrations of (A) DMAQ-B1, (B) AKT inhibitor VIII, (C) combined drugs, or DMSO vehicle control and cell viability was measured by trypan blue exclusion. Cells that received 1% Trixton-X-100 treatment were used as a positive, 100% lethality control. Closed circles represent biological replicates measured in technical triplicate. Horizontal black bars represent the mean. Error bars represent SD. Significance was measured by Two-Way ANOVA with 1% DMSO vehicle control (*$p<0.01$). Data are representative of triplicate independent experiments.

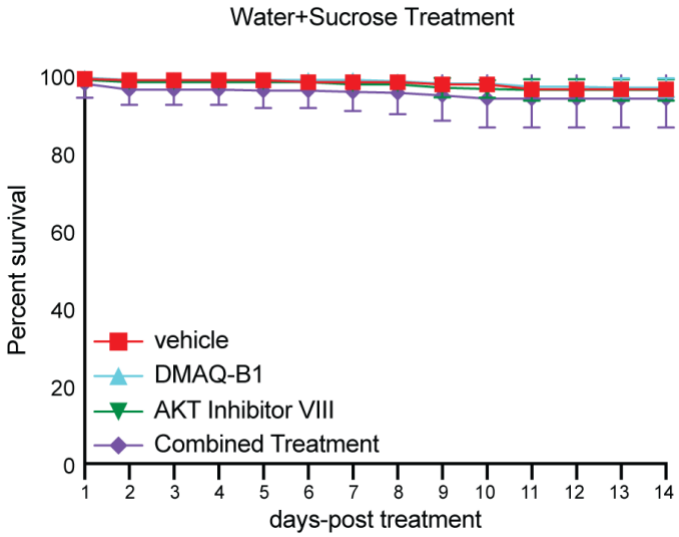

Figure S2: Continuous DMAQ-B1 and AKT inhibitor treatment via sucrose and water does not impact mosquito survival. Adult female *Ae. aegypti* were given water and sucrose supplemented with 1% DMSO (vehicle), 10 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or combined drugs *ad libitum* and toxicity was measured by survival over 14 days. Closed circles represent percent survival of mosquitoes (n=60-100) measured in triplicate. Horizontal black bars represent the mean. Error bars represent SD. Significance was measured by Two-Way ANOVA with 1% DMSO vehicle control. Data are pooled triplicate independent experiments.

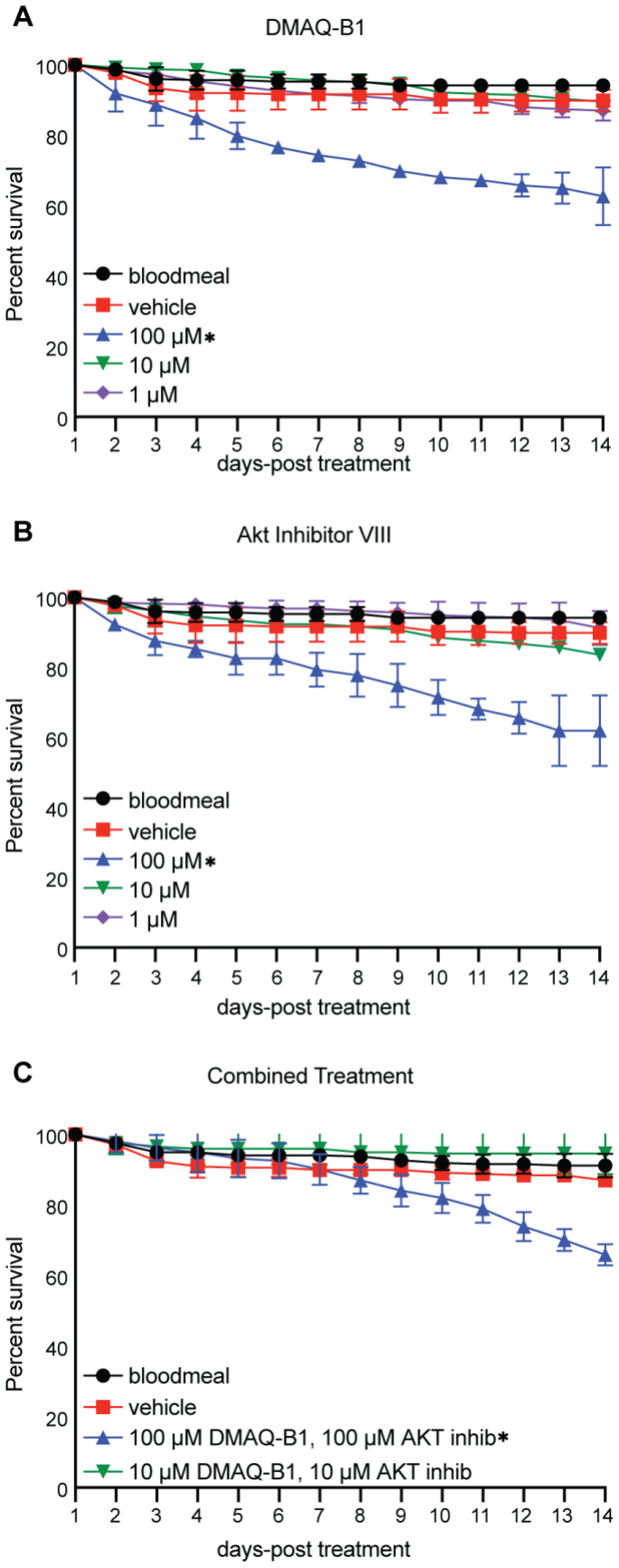
A
DMAQ-B1
Percent survival
days-post treatment
bloodmeal
vehicle
100 µM*
10 µM
1 µM
B
Akt Inhibitor VIII
Percent survival
days-post treatment
bloodmeal
vehicle
100 µM*
10 µM
1 µM
C
Combined Treatment
Percent survival
days-post treatment
bloodmeal
vehicle
100 µM DMAQ-B1, 100 µM AKT inhib*
10 µM DMAQ-B1, 10 µM AKT inhib

Figure S3: DMAQ-B1 and AKT inhibitor VIII exhibited minimal, dose-dependent toxicity to *Ae. aegypti* in bloodmeal. Adult female *Ae. aegypti* were treated with various concentrations of (A) DMAQ-B1, (B) AKT inhibitor VIII, (C) combined drugs and toxicity was measured by survival over 14 days. Closed circles represent percent survival of mosquitoes (n=60-100) measured in triplicate. Horizontal black bars represent the mean. Error bars represent SD. Significance was measured by Two-Way ANOVA with 1% DMSO vehicle control (*p<0.05). Data are representative of duplicate independent experiments.

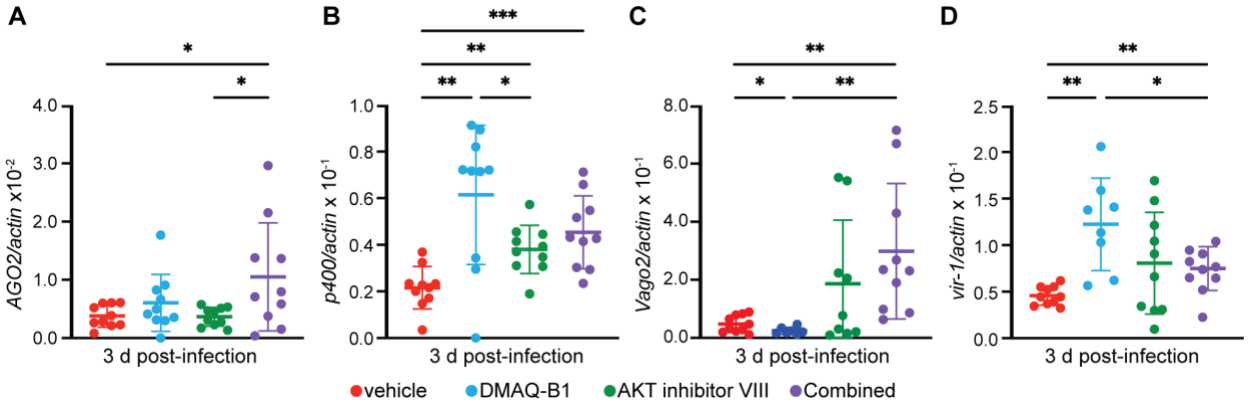

Figure S4: RNAi and JAK/STAT signaling was induced in small molecule treated mosquitoes at 3 d p.i. Induction of (A) *AGO2,* (B) *p400,* (C) *Vago2,* and (D) *vir-1* in adult female *Ae. aegypti* was measured by qRT-PCR 3 d p.i. of ZIKV- and drug-containing bloodmeal. (*p<0.05; **p < 0.01; ***p < 0.001). Open circles represent individual biological replicates. Outliers were identified using a ROUT test (Q=5%) and removed. Horizontal black bars represent the mean. Error bars represent SDs. Data are representative of duplicate independent experiments.

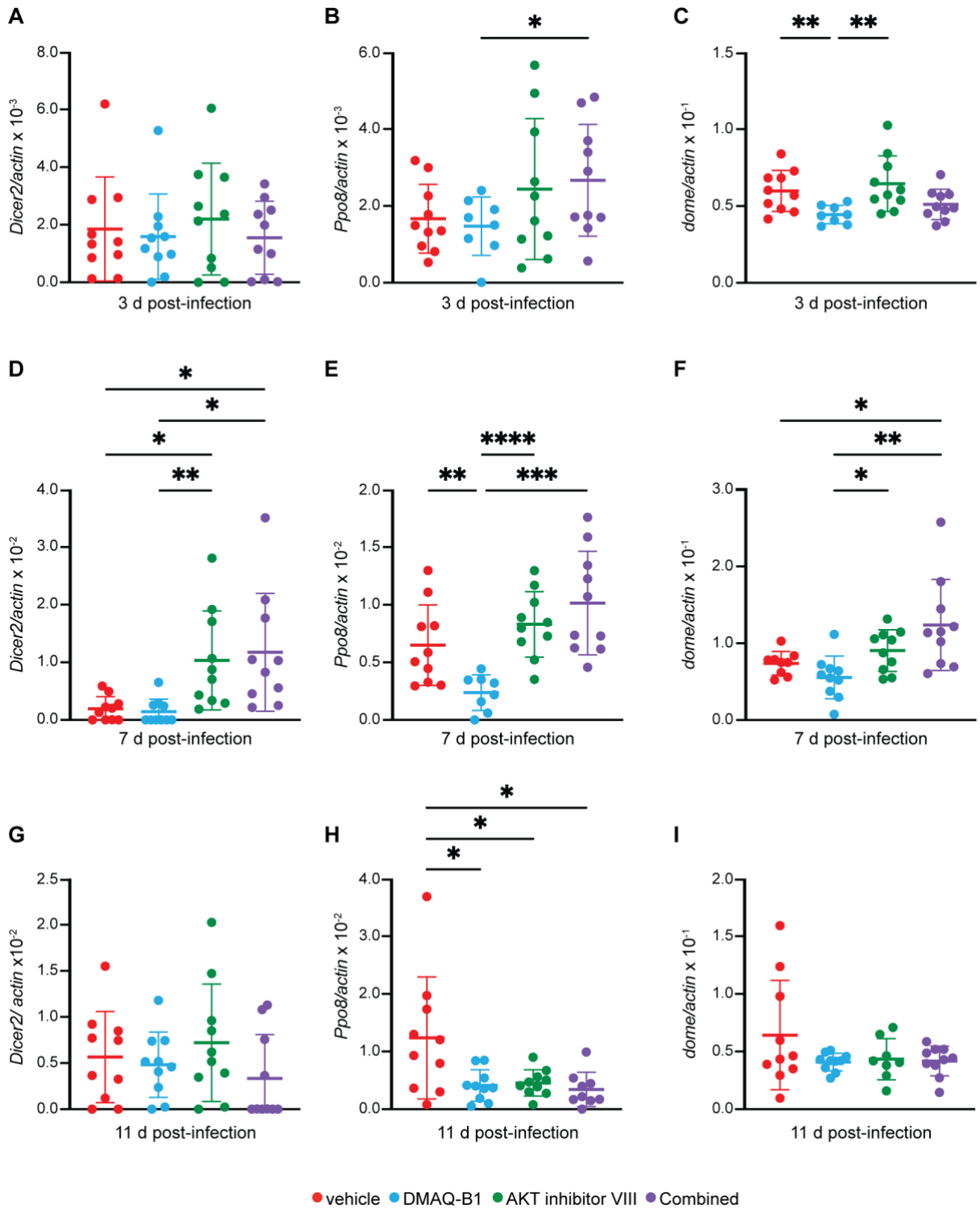

Figure S5: Additional RNAi and JAK/STAT genes were induced in small molecule treated mosquitoes at 3, 7, and 11 d p.i. Induction of additional immune genes at (A-C) 3, (D-F) 7, and (G-I) 11 d p.i. in adult female *Ae. aegypti* was measured by qRT-PCR. RNAi associated genes

(A, D, G) *Dicer2* and (B, E, H) *Ppo8* and JAK/STAT (C, F, I) *dome* were measured. (*p < 0.05; **p<0.01; ***p<0.001; **** p<0.0001, unpaired t test with Welch's correction for multiple comparisons). Closed circles represent individual replicates. Outliers were identified using a ROUT test (Q=5%) and removed. Horizontal bars represent mean and error bars represent SD. Results represent duplicate independent experiments.

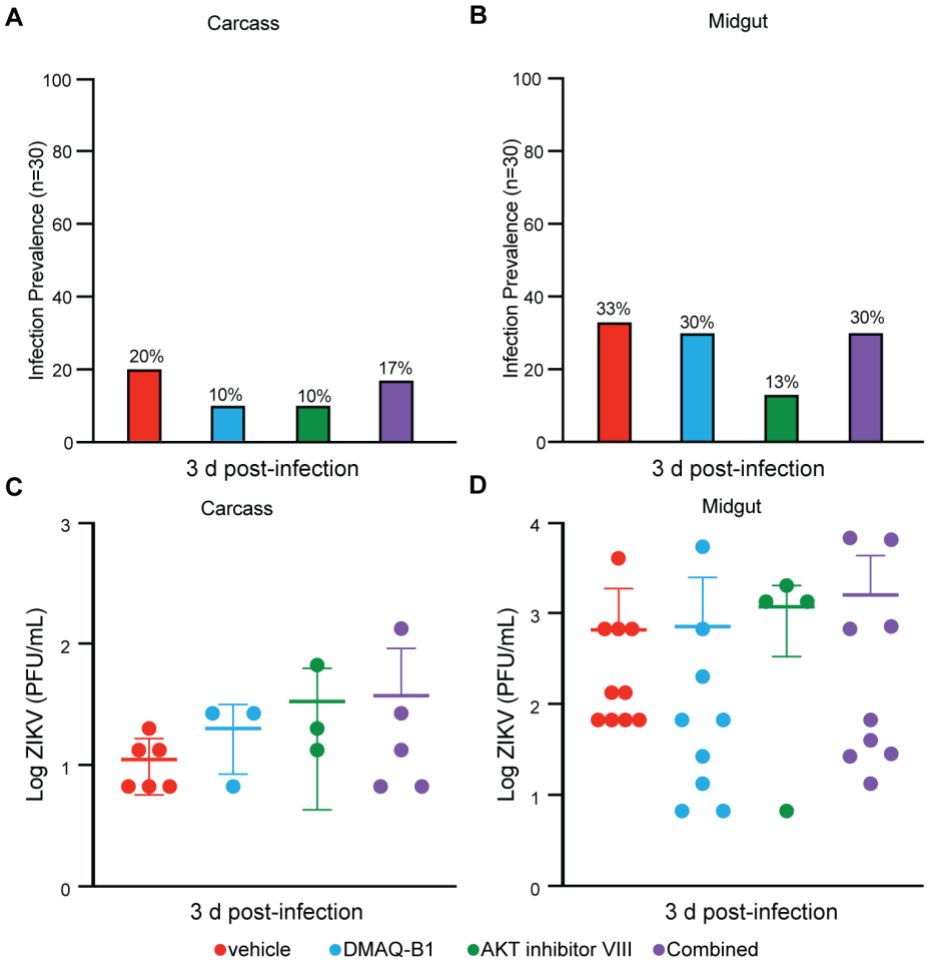

Figure S6: Infection prevalence and ZIKV titers were not different among small molecule-treated and control *Ae aegypti* at 3 d p.i. *Ae. aegypti* were primed with 1% DMSO, 10 μM DMAQ-B1, 10 μM AKT inhibitor VIII, or combined drugs and infected with ZIKV by bloodmeal. Mosquitoes (n=30) were collected at 3 d p.i. and individual midguts, pairs of salivary

glands, and carcasses were prepared and titered by standard plaque assay. Infection prevalence was determined by comparing the number of mosquitoes with detectable virus to the total mosquitoes in the sample. Viral titer was measured in mosquitoes that were positive for ZIKV. There were no differences in infection prevalence or viral titers among conditions. Open circles represent biological replicates. Outliers were identified using a ROUT test (Q=5%) and removed. Bars represent the mean. Error bars represent SDs. Data are representative of duplicate independent experiments.

CHAPTER SIX: DISCUSSION

The growing expansion of mosquito-borne viruses like WNV and ZIKV makes it more necessary than ever to have effective and variety of means to address these pathogens at both the vector transmission and clinical manifestation level. This dissertation demonstrates that targeting downstream effectors of the insulin signaling PI3K/AKT axis controls viral replication: Chapter 3 demonstrates that a multitude of both canonical and novel signaling pathways, specifically endothelin signaling, controls WNV replication in both *Drosophila* and humans, and Chapter 5 evaluates how small molecule treatment targets different signaling events in the insulin signaling pathway that results in reduced ZIKV replication and likelihood of transmission in *Aedes* mosquitoes. Collectively, this work further expands how insulin-mediated antiviral immunity functionally controls viral replication and its potential as an intervention target in mosquitoes and humans.

To address mosquito-borne viruses effectively, there needs to be a variety of avenues and protocols in place that mitigates exposure to infectious mosquitoes and enhances the ability to prevent severe disease for individuals that do become infected. Vaccine development would significantly alleviate disease burden at the clinical level; however, there remains no approved human vaccine for a variety of mosquito-borne viruses at this time. For WNV and ZIKV, there are numerous factors that contribute to the delay in an available vaccine including lack of funding, general need without ongoing outbreaks (Ulbert, 2019; Castanha and Marques, 2021), and complications regarding antibody-dependent enhancement (ADE) that can be detrimental to patient health (Castanha and Marques, 2021; Charles and Christofferson, 2016; Khandia et al., 2018). This is especially the case for individuals who live in hot zones where there is active

transmission of virus or co-circulation of related viruses like DENV (Chaves et al., 2018; Göertz et al., 2017; Vogels et al., 2019). Additionally, while vaccines and vector control are the most effective means of preventing disease emergence, there is also a necessity for the development of specific post-exposure treatments to limit morbidity and mortality. There are no clinically approved antivirals against WNV or ZIKV. Currently the best that clinicians can do is addressing symptoms and pain relief. Priming host antiviral immune responses, whether in mosquitoes or humans, would alleviate viral replication and protect against transmission and progression to severe disease, respectively.

Insulin treatment, as described in Chapter 3, impacts the induction of many genes that are beyond the PI3K/AKT axis discussed within this dissertation. It is worth noting that while these other pathways may also be involved in host antiviral immunity, these off-target effects indicate that insulin treatment exclusively is not an ideal or direct treatment option for addressing clinical cases in humans. Instead, targeting the downstream pathways that insulin treatment induces that directly impact viral replication and host immunity, as shown in Chapters 3 and 5, would be the most effective and viable approach. Chapter 3 demonstrates that endothelin signaling is induced by insulin treatment and when dysfunctional results in increased WNV replication in both *Drosophila* and humans. More importantly, this insulin-induced endothelin signaling is antiviral to both WNV-Kun, an attenuated strain that causes limited disease, and WNV-NY99, which is a clinically relevant strain that is more prone to cause neuroinvasive disease. Using endothelin signaling as a biomarker or as a potential intervention target may alleviate disease burden for individuals that progress to more severe symptoms. While this dissertation focused on insulin-mediated signaling in the context of whole organism or hepatic environment *in vitro*, previous

investigation has also connected endothelin signaling with neuronal function (Schinelli et al., 2001; Adams et al., 2020) and repair (Koyama, 2013; Swire et al., 2019; Jin et al., 2020). This would indicate that endothelin signaling, while primarily associated with cardiovascular and hepatic health, may also play a role in neuronal health that strengthens its role in protecting against neuroinvasive disease like WNV. Further investigation is required to evaluate the role endothelin has in WNV-mediated encephalitis, but there is potential that insulin-mediated induction of endothelin signaling may perform a novel role in antiviral immunity in neuronal tissues. Measuring expression and activation of endothelin signaling in neuronal tissue *in vitro* or from clinical patients that are exhibiting either asymptomatic, mild, or severe neurodegenerative WNV disease would better define its role in disease progression or role as a biomarker. *EDN1* expression was one of many genes differentially induced in WNV-infected human primary macrophages *in vitro* (Qian et al., 2013) so it's plausible that expression may also be induced in other infected tissues. Additionally, it would be valuable to test whether endogenous EDN1 treatment reduces WNV replication in hepatic and neuronal tissues to better define its therapeutic potential.

It is also important to note that while small molecule targeting of insulin signaling in the mosquito vector may help alleviate disease burden and transmission in the field, there remains certain gaps in knowledge pertaining to its efficacy and direct translatability in the field. The work in Chapter 5 demonstrates that specific small molecule treatment of the PI3K/AKT axis to prime RNAi and JAK/STAT in *Aedes* mosquitoes reduces ZIKV replication when administered prior to or at time of infection. However, it is currently not known how effective these small molecules are as a prophylactic in reducing ZIKV replication in mosquitoes already infected.

Further investigation is necessary to evaluate if and how these small molecules function in mosquitos' post-infection, but it is possible that administration of these drugs via sucrose feeding, which is independent of the feeding schedule associated with bloodmeals, may induce antiviral signaling sooner than untreated mosquitoes and better respond to infection and later transmission events. It is also necessary to investigate the efficacy of these small molecules against other mosquito species and viruses. While insulin treatment, whether introduced in cell culture media *in vitro* or during mosquito ingestion *in vivo*, shows reduced viral replication against a range of related mosquito-borne viruses in a range of species (Ahlers et al., 2019; Xu et al., 2013), it is possible that this specific small molecule targeting of the PI3K/AKT axis has a limited antiviral effect specific for a virus or mosquito species (Patel and Hardy, 2012). Clarification is needed to determine whether this proposed targeted approach possesses similar antiviral potency against viruses that similarly are reduced by insulin like WNV and DENV and other vector competent mosquito species like *Culex quinquefasciatus* and *Aedes albopictus* (Ahlers et al., 2019). It is also important to investigate the potency and potential toxicity that these small molecules possess for off target species that may be exposed in the field. Other drug or insecticide-based field methods, while effective in killing mosquitoes, have also been shown to impact other native insect species like honeybees (Dong and Dimopoulos, 2021). This approach may alleviate this concern as antiviral immunity was achieved within nontoxic ranges, but further investigation is needed to clarify its impact on other organisms that may also be exposed given the evolutionarily conserved nature that is insulin signaling. Small molecule treatment of native insect or vertebrate species, like hummingbirds, and analyzing the compounds' toxicity, efficacy, and effects on host behavior would determine whether this proposed approach in the field would pose an off-target effect if implemented.

Collectively, understanding host responses to viral infection, whether that be at the mosquito or human level, is a necessary area of research that would provide greater insight into how to more effectively respond to the growing problem that is mosquito-borne viruses. The work presented in this dissertation demonstrates that an evolutionarily conserved pathway like the PI3K/AKT axis in insulin signaling plays an important role in antiviral immunity across species and may be targeted in reducing viral replication for related viruses like WNV and ZIKV. Insulin-mediated regulation of endothelin signaling in humans impacts WNV in both attenuated and clinically virulent strains. As induction of endothelin signaling is a proposed marker for interferon-induced pulmonary toxicity (George et al., 2012) and disease progression against *Mycobacterium tuberculosis* (Correa et al., 2014) and hepatitis B and C viruses (HBV/HCV) (Ersoy et al., 2006), it may be possible to consider endothelin signaling as a marker for WNV disease as well. Additionally, small molecule treatment that selectively targets the insulin receptor and AKT activation in *Aedes* mosquitoes more effectively primes antiviral immune signaling to respond to and reduce ZIKV replication more effectively. DMAQ-B1 and AKT inhibitor VIII are effective in activating RNAi and JAK/STAT signaling regardless of administration route, indicating that use of these small molecules could be used as a potential preventative or prophylactic measure in addressing vector control (Fig. 1).

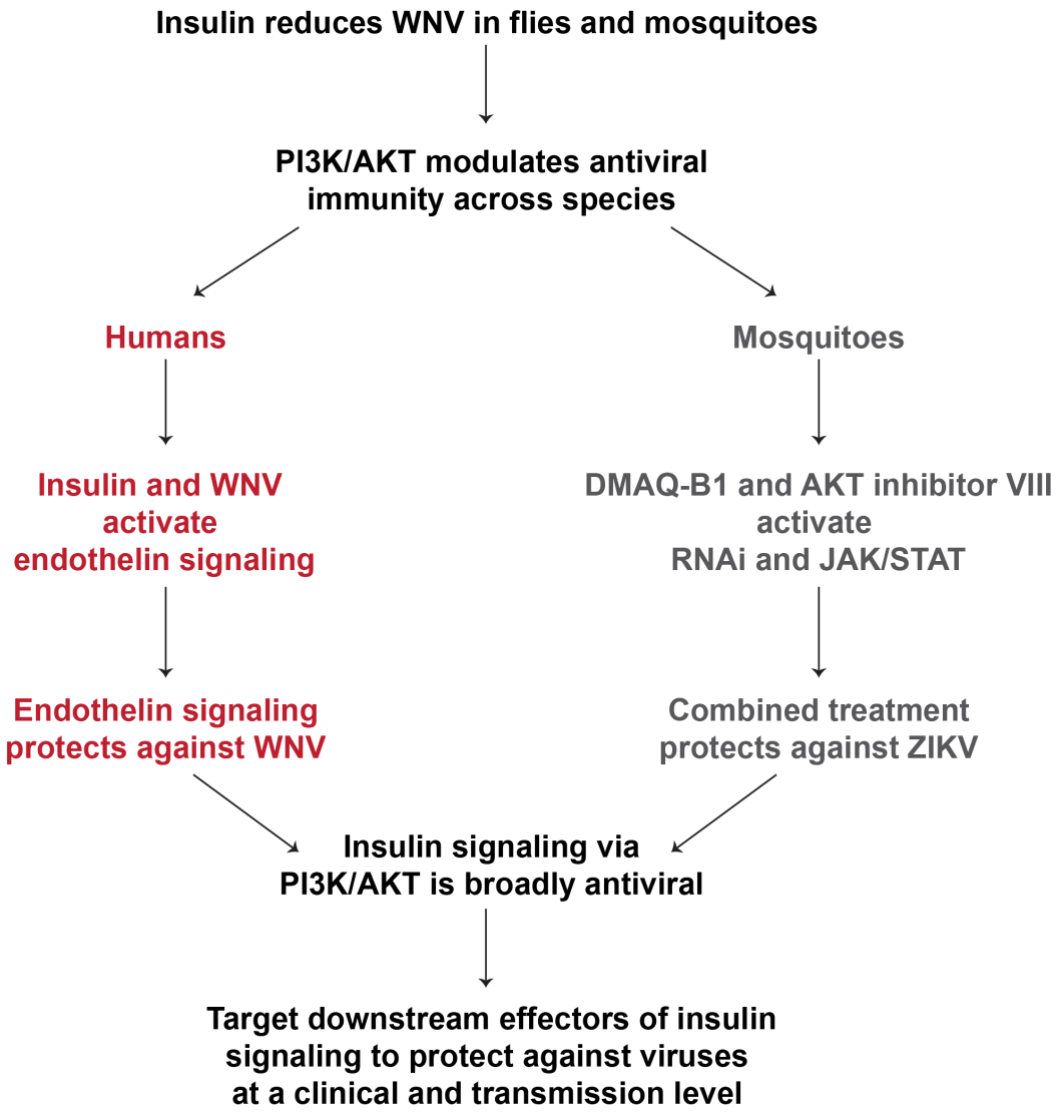

Figure 1: Selective targeting of insulin-signaling downstream effectors in humans and mosquitoes provides protection against related flaviviruses. Summation of findings presented within this dissertation and evaluating its therapeutic and vector control potential.

Overall, this dissertation expands upon our current understanding regarding insulin-mediated antiviral immunity against related flaviviruses like WNV and ZIKV, identifies and characterizes the pathways evolutionarily conserved nature across *Drosophila melanogaster*, *Aedes aegypti*, and humans, and demonstrates its potential as a clinical marker and vector control target. As

more mosquito-borne viruses grow from neglected tropical diseases to global pathogens of concern, it is important to continue to develop more effective and targeted means of responding to infection from initial transmission to the clinical case manifestation.

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