MOLECULAR CHARACTERIZATION OF DENGUE VIRUS HOST AND RESTRICTION FACTORS IN *AEDES AEGYPTI* MOSQUITOES

by

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ABSTRACT

Despite decades of attempts at disease control, dengue remains one of the most significant mosquito-borne arboviral diseases, causing an estimated 390 million infections annually. While studies of molecular interactions between DENV and *Ae. aegypti* have paved a way for the development of alternative DENV control strategies, this field is still relatively understudied. Here, we used multiple molecular tools to study interactions between the virus and *Ae. aegypti*, as well as to identify DENV host and restriction factors. First, we have developed genetically modified mosquitoes with increased activity of the JAK/STAT pathway, and showed that these transgenic mosquitoes could inhibit DENV infection. Through microarray-based transcriptomic comparisons, we identified candidate DENV host and restriction factors and confirmed their function through RNAi. Second, we compared transcriptomic profiles of a panel of field-derived and laboratory *Ae. aegypti* strains with different DENV susceptibility. Through RNAi-mediated gene silencing, we have shown that basal level of immune activity, and expression level of host factors are important determinants for DENV susceptibility. Lastly, through a study of transcriptomic datasets comparing DENV-infected and uninfected *Ae. aegypti*, we identified and characterized lipid binding protein families, ML and NPC1, as host factors for DENV replication in *Ae. aegypti*.

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CHAPTER 1 Introduction

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Global burden of dengue

Dengue is the most important arthropod-borne viral disease, with an estimated 390 million infections annually across over a hundred countries in tropical and subtropical areas (Figure 1.1) [1]. The disease has become major global public health concerns, with increasing incidence in recent decades as a result of the geographical expansion of its primary vector, *Aedes aegypti*, and secondary vector, *Aedes albopictus*, as well as global transport, unplanned urbanization, and climate change [2-10].

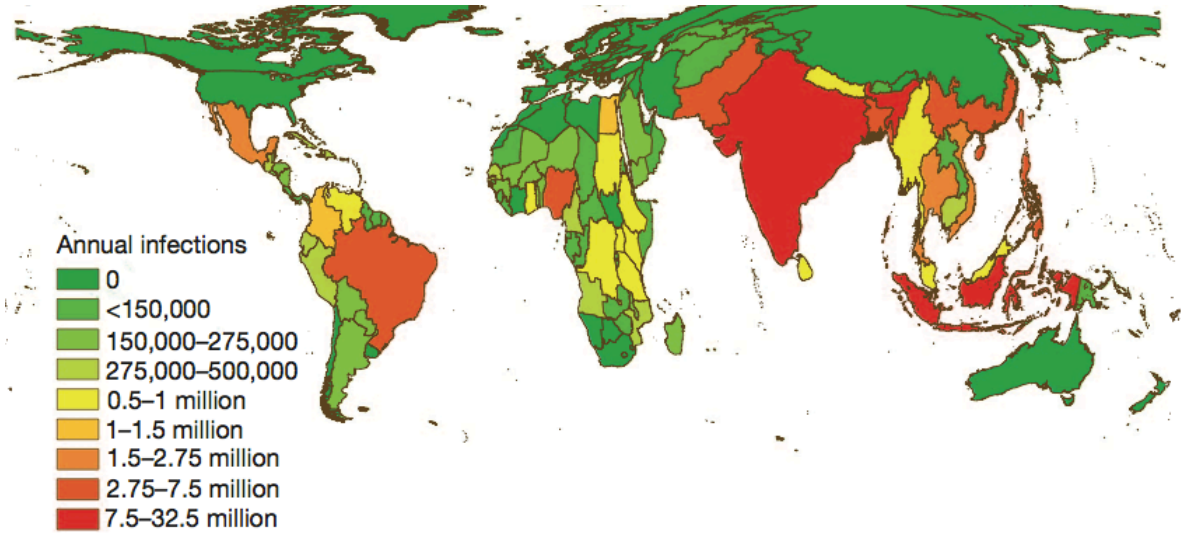


Figure 1.1. Global distribution of dengue. Cartogram of the annual number of infections for all ages. Figure was obtained from [1]. Data were from the year 2010.

Dengue is caused by any of the four serologically distinct dengue viruses (DENV serotype 1 to 4) [11]. The genomes of the four serotypes of DENV share only 65% similarity at the nucleotide level, yet they have similar life cycles and clinical manifestations in humans [9,10,12]. The infection by one serotype of DENV is thought to yield life-long antibody protection against symptomatic disease with that serotype; however, these neutralizing antibodies do not provide life-long protection from infections by other serotypes [11,13]. Conversely, epidemiological studies have demonstrated that

the highest risk for developing severe dengue is previous infection with a different dengue serotype, thought to be due to the antibody-dependent enhancement (ADE) [14,15]. This problem made vaccine development for DENV challenging as a balanced antibody response to all four serotypes is required for protection and to reduce the risk of severe disease due to ADE. Although these pathogens can cause serious diseases in humans, they rarely cause mosquito pathology and can persistently infect the mosquito vector for life [16].

Due to difficulties in developing dengue vaccines, only Dengvaxia (developed by Sanofi Pasteur), has been approved for use in three countries: Mexico, the Philippines, and Brazil. However, this vaccine has not been approved for use in children under 9 years of age, a group that is most vulnerable to severe disease [17]. Clinical studies have shown that efficacies of Dengvaxia was estimated at 65.6% for participants who were 9 years of age or older, and only 44.6% in participants under the age of 9 years [18]. Because Dengvaxia was approved for use in limited group of population, the mosquito vector control remains an essential strategy to reduce disease burden in the general population. However, conventional vector control methods such as insecticide spraying and the removal of mosquito breeding sites have in many cases proven to be unsustainable solutions for a variety of reasons, including lack of adequate funds to sustain the vector control program, ecological concerns, as well as the development of insecticide resistance [11,19]. In addition, vectors such as *Ae. aegypti* are extremely well adapted to urban environments, laying their eggs in clean water in artificial containers, and displaying a preference for staying indoors. For this reason, the development of novel

vector and disease control strategies is essential, and a molecular understanding of mosquito immune responses against these viruses is necessary.

DENV replication and tissue tropism in *Ae. aegypti*

Human and non-human primates are hosts for DENV; however, the virus does not require an enzootic cycle (replication in non-human hosts) to sustain epidemic transmission in humans [9]. DENV are maintained in a human population through horizontal transmission cycle between *Ae. aegypti* mosquitoes and humans. Vertical transmission of DENV from infected female mosquitoes to their offspring has also been reported in the laboratory and in the field with efficiencies around 1-4% [20], which is not an important factor for long-term virus persistence in an endemic situation according to mathematical model [21].

After the mosquito ingests an infectious blood meal, the DENV must pass through various infection barriers [2]. First, they have to infect and replicate in the midgut epithelium (midgut infection barrier), then escape from the midgut to spread throughout the insect body and infect other tissues (midgut escape barrier). In order to transmit dengue, the viruses then have to infect and replicate in the salivary glands, where they disseminate into mosquito saliva (salivary gland infection and escape barriers) [2]. The extrinsic incubation period (EIP), i.e., the time from virus ingestion until its dissemination in mosquito saliva where it can be transmitted to naïve humans, can vary depending on conditions such as mosquito strain, virus strain, and temperature but it generally ranges from 10-14 days [16]. Virus levels in the salivary glands will remain high throughout the infection which means that once the mosquito salivary glands get infected by the virus, it can be transmitted for life of the mosquito [16].

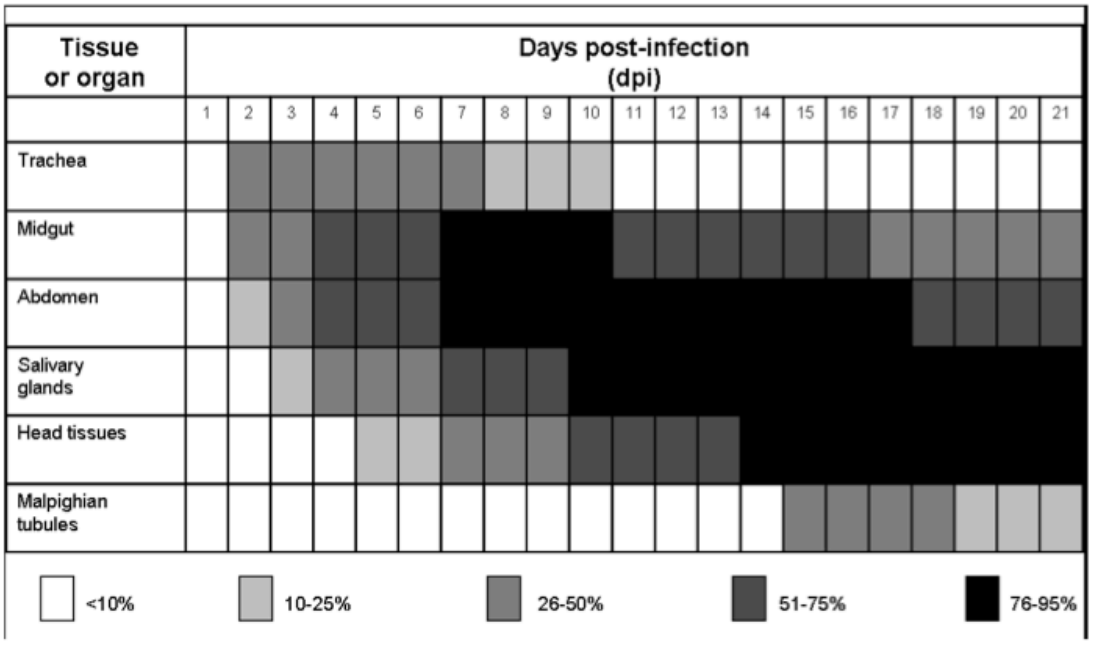


Figure 1.2. Temporal tissue tropism of dengue virus type 2 in the *Ae. aegypti* mosquito. The infection rate in the respective tissues is shown in grey scale [16].

Mosquito immune responses to DENV infection

Mosquitoes, like other organisms, are exposed to a wide range of microbes from their environments, and also during blood feeding. Knowledge of the mosquito immune responses has been largely based on research in the insect model organism, *Drosophila melanogaster*, which in contrast to vertebrate immunity, do not have adaptive immunity and rely mainly on their innate immune system. The insect innate immune system is comprised of cellular and humoral components [22]. Mechanisms involved in cellular immune responses include phagocytosis, encapsulation, and nodule formation, and they are mediated by hemocytes [23-26]. Humoral immune responses are mechanisms to prevent systemic infection, which include systemic immune signaling, melanization, and the production of anti-microbial peptides (AMPs). Innate immune signaling is triggered by specific pathogen recognition receptors (PRRs) that recognize conserved molecular

patterns among microbes, termed Pathogen-associated molecular patterns (PAMPs). These include lipopolysaccharides, peptidoglycans, mannans, and dsRNA [22,27]. Upon pathogen recognition, PRRs activate different signaling cascades, which regulate the transcription of effector molecules [28,29].

The mosquito's innate immune system mounts potent immune responses against microbial challenge and is capable of distinguishing among broad classes of microorganisms. The availability of the *Ae. aegypti* genome in 2007 [30,31] has facilitated the study of mosquito immunity in response to DENV infection. In this section, we focus on the major mosquito immune signaling pathways that have been implicated in the antiviral defense, namely the Toll, immune deficiency (IMD), and Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathways. In addition, we will consider the RNA interference (RNAi) pathway; though not a classical innate immune pathway, it also plays a key role in antiviral defense. A summary of these immune pathways is presented in Figure 1.3.

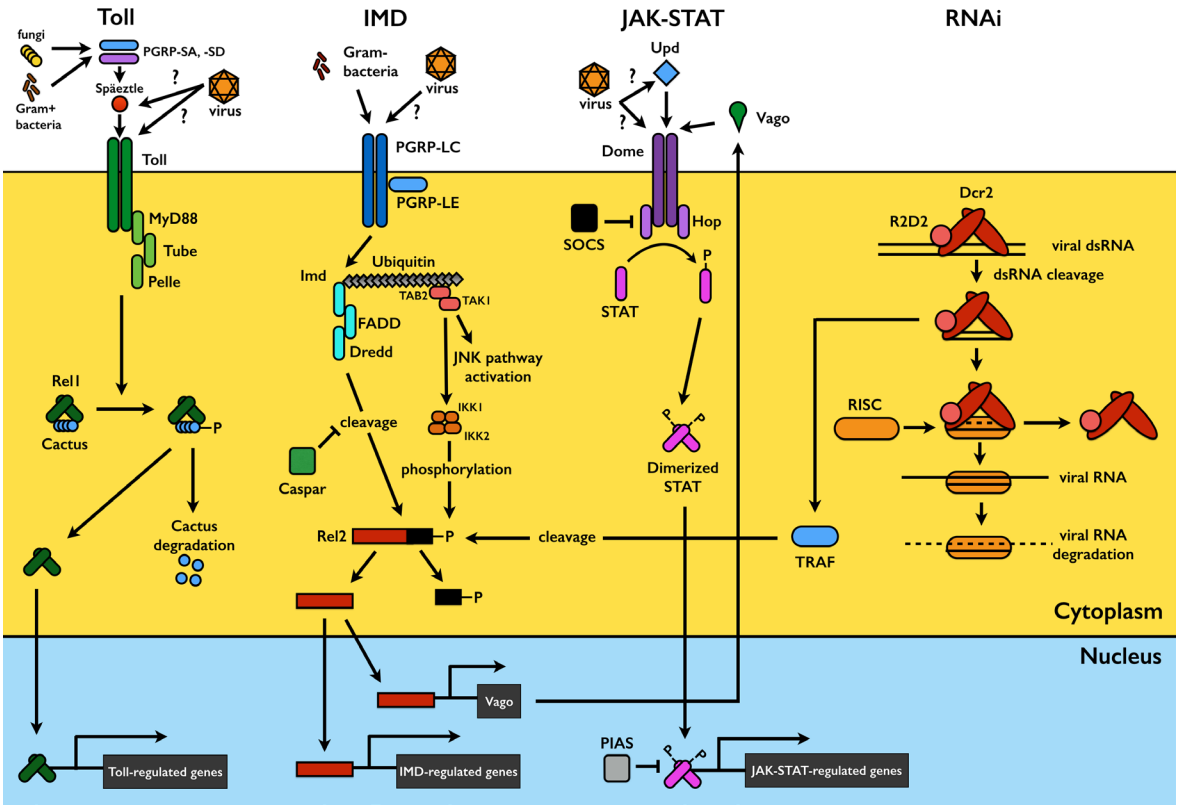


Figure 1.3 Mosquito immune signaling and RNAi pathways [32]. Mosquitoes use classical innate immune pathways such as the Toll, Imd, and JAK/STAT pathway to deal with pathogen infections. The RNAi pathway, eventhough not a classical immune pathway, is also important for controlling viral infections.

The Toll pathway

The Toll pathway is an NF-kB signaling pathway, which was first characterized for its role in *Drosophila* development and subsequently shown to play a role in insect immune responses against Gram-positive bacteria, fungi, and virus [25,33-37]. Unlike the vertebrate TLR, the insect Toll pathway does not directly interact with PAMPs. Instead, the recognition of pathogens by peptidoglycan recognition proteins (PGRPs), such as peptidoglycan recognition proteins (PGRP)-SA and -SD, triggers a proteolytic cascade that cleaves a cytokine Spätzle (Spz) [35]. Activated Spz bound to the Toll receptor and

triggers signaling through the associated adaptor proteins MyD88 and Tube and the kinase Pelle [35]. This leads to the degradation of the negative regulator, Cactus, which binds the NF-kB transcription factor Dorsal (Rel1 in mosquitoes). After being released from Cactus, Dorsal is translocated to the nucleus and binds to cis-acting elements of the promoters of antimicrobial peptides and other immune effector genes [35,38,39].

The Toll pathway is conserved in mosquitoes and also plays a key role in antiviral defense in these insects. DENV infection of the *Ae. aegypti* midgut, carcass, and salivary gland activates the transcription of Toll pathway components and putative effectors such as Späetzle, Toll, Rel1A, and multiple AMPs [12,40,41]. The activation of the Toll pathway through the RNAi-mediated gene silencing of Cactus resulted in a reduction of midgut DENV titers, while inactivation of Toll pathway signaling by silencing the adaptor protein MyD88 resulted in higher midgut DENV titers [12,42]. The DENV-infected mosquito transcriptome and that of Cactus-silenced (or Rel1-activated) mosquitoes also overlap considerably in terms of the magnitude and direction of gene regulation [12,43]. Subsequent experiments revealed that the role of the Toll pathway in controlling DENV was conserved in field-derived *Ae. aegypti*, and against different DENV serotypes [44,45].

Stable transinfection of *Ae. aegypti* with the endosymbiont bacterium *Wolbachia* greatly limits infection of the mosquito vector with a range of human pathogens, including DENV and Chikungunya virus (CHIKV) [46-51]. The inhibition occur via several mechanisms [30,52,53], one of which is the induction by *Wolbachia* of reactive oxygen species (ROS) production by the mosquito, resulting in Toll pathway activation

and the subsequent production of the AMPs cecropin and defensin, which hinder DENV replication [42,44,54-56].

The JAK/STAT pathway

The Janus kinase-signal transduction and activation of transcription (JAK/STAT) pathway was discovered in a vertebrate model as an interferon (IFN)-induced signaling pathway important for development [46,48,57], and was later found to be important for anti-viral immunity [52,53,58]. In *Drosophila*, the JAK/STAT pathway plays a crucial role as a signaling pathway in insect development and in the immune response against pathogenic bacteria and viruses [42,54-56].

The canonical *Drosophila* JAK-STAT pathway is triggered by the binding of the activated cytokine-like Unpaired ligand (Upd) to the extracellular domain of the Domeless receptor (Dome) [42,57]. The binding of Upd to the receptor triggers a conformational change and dimerization of the Dome receptor [43,58]. This dimerization then triggers the Janus kinase Hopscotch (Hop) to phosphorylate the cytosolic tail of the Dome receptor, which in turn activates STAT [55,59]. The activated STAT is dimerized and translocated to the nucleus and triggers the transcription of JAK/STAT pathway-regulated genes [42,59]. The JAK/STAT pathway is negatively regulated by the protein inhibitors of activated STAT (PIAS), and suppressors of cytokine signalling (SOCS) repressor proteins to prevent its over-activation [43,60-62].

The antiviral role of the JAK-STAT pathway is conserved in the *Ae. aegypti* defense against DENV. DENV replication in the mosquito midgut is significantly increased when the pathway is transiently suppressed by RNAi-mediated depletion of the receptor Dome or the JAK ortholog Hop, and the opposite effect on virus replication is

observed when the pathway is activated by silencing of protein inhibitor of activated STAT (PIAS), a negative regulator [59,63]. However, JAK-STAT pathway-activated anti-DENV mechanisms are poorly understood. Two DENV-induced, JAK-STAT-regulated putative effector genes that restrict DENV replication in midgut tissues have been identified but remain uncharacterized [59,60,64,65]. These genes were named as dengue virus restriction factors (DVRFs) 1 and 2. DVRF1 is a predicted transmembrane protein, which potentially function as a pathway receptor. DVRF2 contains antifreeze and allergen domains and might have a function in virus recognition.

The IMD Pathway

The immune deficiency (IMD) pathway is well known to play crucial roles in insect defense against bacteria [60-62,66,67]. In *Drosophila*, activation of the IMD pathway, like that of the Toll pathway, is initiated by PRR-mediated recognition of microbial PAMPs (reviewed in [61-63]). Intracellular signaling through the adaptor IMD protein and various caspase-like proteins and kinases then leads to a functional split in the pathway into two downstream branches [60,64,65,68,69]. One branch, similar to the mammalian c-Jun/JNK pathway, activates the transcription factor AP-1 via JNK signaling [66,67,70], while the other branch culminates in the processing and activation of the NF-kB transcription factor Relish (Rel2 in mosquitoes) via caspase-mediated cleavage of its carboxy-terminal end [61,62,71-74]. Activated Relish is then translocated to the nucleus to promote the transcription of anti-microbial effectors [68,69,75,76]. The human Fas-associated factor 1 ortholog Caspar negatively regulates Relish activation, possibly by interfering with the enzymes involved in its cleavage [12,70]. In mosquitoes,

the IMD pathway also plays important roles in the antibacterial defense, and it also directs immune responses against *Plasmodium* parasites [71-74,77].

The antiviral role of the IMD pathway has more recently been investigated, and in flies it has been found to be active against SINV and cricket paralysis virus (CrPV) [75,76,78]. In mosquitoes, up-regulation of IMD components and effectors in response to DENV and SINV infection has been observed [24,34], but transient activation of the pathway by RNAi-mediated gene silencing of Caspar has no effect on midgut DENV titers [12,79,80].

RNA interference

The RNAi antiviral mechanism is not a classical pathogen-stimulated immune response, but plays an important role in insects' antiviral responses. RNAi is a mechanism that can target foreign RNA for degradation, and it has long been recognized to be a key player among the mechanisms of anti-viral immunity in insects. This process relies on the Dicer2 (Dcr2) enzyme, which contains the DExD/H-Box RNA helicase domain and acts as a pattern recognition receptor in RNAi's recognition of exogenous dsRNAs [77,81]. Once they are recognized, Dcr2 cleaves long exogenous dsRNAs to generate 21–22 basepair small-interfering RNAs (siRNAs). The siRNAs, together with Dcr2, can be loaded onto the RNA-induced silencing complex (RISC). During the effector stage of the pathway, RISC unwinds the siRNAs, degrades one of the RNA strands, and then guides it to the complementary RNA. Argonaut 2 (Ago2), a protein in the RISC complex that contains endonuclease activity, then degrades the target RNA strand [78,82,83]. RNAi was previously characterized as an antiviral mechanism, but recent studies have shown that it can also function as a PRR for immune signaling

pathways. In the *Drosophila* system, in addition to degrading target RNA, RNAi can also induce the expression of antiviral effectors, in a manner similar to RIG-I in mammals [79,80,84]; for example, recognition of DCV by the DExD/H-Box RNA helicase domain of Dcr2 can induce the expression of the anti-viral effector Vago [77,81,84].

Recent studies have demonstrated that the RNAi pathway also serves as an anti-DENV mechanism in *Ae. aegypti*. The very first evidence of a role for RNAi in modulating DENV infection was obtained with the transformation of the plasmid expressing inverted repeat DENV RNA (irRNA) in mosquito cells [82,83,85]. Later, transgenic mosquitoes expressing inducible irRNA were also used to confirm the importance of RNAi [84,85]. These transgenic mosquitoes had lower DENV titers when compared with wild-type mosquitoes, suggesting a role for siRNA in anti-DENV responses. Knockdown of Ago2 in the transgenic mosquitoes negated the protective effect of the irRNA, confirming the importance of the RNAi mechanism [84,85]. However, the role of the RNAi mechanism in the anti-DENV defense in wildtype mosquitoes was not confirmed until 2009 [79,85]. DENV infection in mosquito cell lines and adult female mosquitoes resulted in the production of siRNAs that could inhibit virus replication [81,85]. On the other hand, transient silencing of the RNAi pathway components (Dcr2, R2D2, and Ago2) resulted in an increase in DENV titres and a reduction in the DENV extrinsic incubation period in mosquitoes [82,85].

Characterization of the role of RNAi in the systemic immune response is also important for our understanding of how the mosquito systemically controls virus infection. Previous studies of *Drosophila* C virus (DCV) in *Drosophila* have found a systemic spread of RNAi through the uptake of dsRNA from the cellular environment

[79,86,87]. However, a similar mechanism has not yet been identified in the mosquito's anti-DENV response. It is complicated to confirm this phenomenon in mosquitoes because systemic RNAi was originally discovered in a *Drosophila* mutant that is deficient in the dsRNA uptake pathway, and no such mutant is available in the mosquito system.

Another study from the *Drosophila* model suggests a role for insect-encoded reverse transcriptase (RT) enzymes and the RNAi machinery in maintaining the persistence of RNA viruses. Here, viral genome fragments are reverse-transcribed and inserted into the insect genome by retrotransposon elements; these insertions later serve as templates for RNAi responses against the virus [81,88-90]. Given that the *Ae. aegypti* genome also contains RTs and transposable elements [82,91], and that flavivirus and rhabdovirus sequence fragments have been detected in the genomes of *Aedes* species [86,87,92], it would be intriguing to study this phenomenon in mosquitoes.

Arbovirus interactions with host cell processes and host factors

Arboviruses are obligate intracellular pathogens that exploit the host's cellular machinery in order to replicate. The intracellular replication cycle for DENV has been well studied and is likely to be similar in insects and vertebrates. DENV enters cells via clathrin-dependent receptor-mediated endocytosis, and uncoating of the positive-strand RNA viral genome requires trafficking through an acidic endosomal compartment [13-15,88-90]. The receptors and proteins of the mosquito midgut that interact with the virus during early infection stages (reviewed in [91,93,94]) are poorly characterized. Translation of viral RNA (vRNA) occurs on endoplasmic reticulum (ER)-derived

membranes, producing a single polypeptide that is then processed into individual structural and non-structural proteins. vRNA replication occurs through the production of a negative-strand intermediate that serves as a template for the synthesis of multiple copies of positive-sense vRNA. The structural proteins C, prM, and E are then produced in large quantities through successive rounds of translation and assembled with vRNA in the ER. Virions mature in the Golgi and exit the cell via the host's secretory pathway. Host genes that facilitate DENV replication and infection are called DENV host factors.

The Vacuolar ATPase Complex

The vacuolar ATPase (vATPase) is a multisubunit enzyme located in the membranes of endosomes, lysosomes, and secretory vesicles. The vATPase complex brings about the acidification of these organelles via an ATP-dependent rotary mechanism that drives proton transport [92,95]. This process is important for DENV replication, since an acidic pH in the late endosome is required for DENV membrane fusion and RNA genome entry into cells [13-15,88,96,97]. Bafilomycin, a specific inhibitor of vATPases, has been reported to inhibit flaviviruses in both mammalian and insect cells [92-94], and a recent study found that chemical inhibition of vATPase by injecting or feeding adult *Ae. aegypti* with bafilomycin also restricts DENV replication in the midgut and salivary glands [95,98-100]. Various vATPase subunits have been found to be transcriptionally upregulated in DENV-susceptible strains of *Ae. aegypti*, when compared to refractory strains [16,96,97,101,102]. In yeast, individual deletion of all of the subunit genes results in either a complete loss of assembly of the complex or an inactive vATPase [92,103]. Taken together, these pieces of evidence indicate the importance of a functional vATPase complex for DENV replication in mosquitoes,

making this complex a promising target for chemical interventions such as treatment with small-molecule inhibitors of DENV replication.

The Myeloid Differentiation 2-Related and Niemann-Pick Type C1 Proteins

The myeloid differentiation 2-related lipid recognition (ML) and Niemann-Pick type C1 (NPC1) gene families encode proteins with diverse roles related to their lipid-binding domains. ML proteins are involved in processes such as lipid trafficking and metabolism, pheromone perception, and pathogen recognition [98-100,104]: mammalian MD2, for example, is a co-receptor for Toll-like receptor 4 (TLR4) binding to bacterial lipopolysaccharide [16,101,102,105,106], and silencing of *An. gambiae* AgMDL1 significantly increases midgut *Plasmodium falciparum* infection levels [1,103]. NPC1 proteins are involved in cholesterol transport and homeostasis in the late endosome, and function together with NPC2, a member of ML family [2,100,104]. NPC1 proteins also play roles in host–pathogen interactions, for example, Ebola virus requires mammalian NPC1 for membrane fusion and escape from the endosome [16,97,105-109]. The roles of these protein families in DENV infection in *Ae. aegypti*; however, has yet to be studied.

Study Objectives

The main aim of this thesis research is to study molecular interactions between DENV and *Ae. aegypti* mosquito, specifically to identify and characterize genes that play roles in DENV infection in the insect vector. We can classify genes that play roles in DENV infection into two categories; DENV restriction factors which are genes that inhibit virus replication in the vector, and DENV host factors which are genes that facilitate or required for virus to replicate in the insect vector. In this thesis research,

different approaches and tools were used to identify such factors, and can be summarized into following specific aims:

- Aim1 (Chapter 2): To characterize anti-DENV mechanisms of the JAK/STAT pathway in *Ae. aegypti* using transgenic approach.
- Aim2 (Chapter 3): To identify and characterize candidate DENV host and restriction factors from a panel of field-derived and laboratory strains *Ae. aegypti* with different degrees of susceptibility.
- Aim3 (Chapter 4): To use previously published microarray dataset to identify and characterize host factor functions of two lipid binding protein families, ML and NPC1.

CHAPTER 2

Engineered *Aedes aegypti* JAK/STAT pathway-mediated immunity to dengue virus

ABSTRACT

The JAK/STAT pathway is an evolutionary conserved pathway involved in anti-dengue defense in *Ae. aegypti* mosquitoes. Here, our data have shown that we can induce activation of the JAK/STAT pathway through over-expression of the JAK/STAT pathway receptor Dome, as well as the Janus kinase Hop, under the control of a blood meal-inducible fat body-specific Vg promoter. Activation of the JAK/STAT pathway prior to exposure to dengue virus (DENV) inhibited DENV replication in the midguts and limited the spread of the virus from the midgut to other parts of mosquito body, including the salivary glands. The JAK/STAT pathway could inhibit different dengue serotypes, suggesting a conserved function of the pathway. These transgenic VgDome and VgHop lines had only a minimal longevity disadvantage, but their fecundity was compromised, partly as a result of their lower expression level of the vitellogenin gene. We also used these transgenic mosquitoes to dissect the molecular interactions between the DENV and its mosquito vector and found that the greater resistance to DENV in the transgenic lines was the result of a combination of a higher transcript abundance of DENV restriction factors and a lower transcript abundance of DENV host factors.

INTRODUCTION

Despite decades of attempts at disease control, dengue remains a major mosquito-borne arboviral disease, causing an estimated 390 million infections annually [1,59]. With vaccine recently licensed for use only in three countries and only among people from the age of 9 to 45 years, vector control has remained the most important way to reduce disease transmission in the general population.

Dengue virus (DENV) is maintained in a population through a horizontal transmission cycle between *Aedes* mosquitoes and humans. The DENV replication cycle begins when mosquitoes take an blood meal from a dengue-infected individual. DENV in the blood meal infects the mosquito and propagates in its midgut epithelial cells, then disseminates to other organs. DENV eventually infects the salivary glands, from which the virus can be injected into a human host through the mosquito's saliva, thus resulting in virus transmission [2,59]. The replication cycle of DENV from midgut to salivary glands in *Aedes* mosquitoes takes 10-14 days but can vary depending on different factors such as the mosquito, the virus strains, and the temperature [16,54,97,107-109].

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is a conserved immune signaling pathway that regulates developmental processes and antiviral immunity in both mammals and insects. We have previously shown that the JAK/STAT pathway controls DENV infection in *Ae. aegypti* [43,59]. Transient activation of the JAK/STAT pathway through RNAi-mediated gene silencing of the protein inhibitor of activated STAT (PIAS) renders mosquitoes more resistant to DENV infection of the mosquito midgut, whereas silencing of the receptor Dome or the Janus kinase Hop renders the mosquitoes more susceptible to DENV infection [59,110].

The JAK/STAT pathway controls DENV infection as early as 3 days post-infectious blood meal (dpibm) ingestion, suggesting that genetic engineering of the pathway for earlier activation after a blood meal may result in a DENV resistance phenotype, and may therefore be a likely strategy for dengue control. Activation of the JAK/STAT pathway is triggered by cytokine binding to the extracellular domain of the receptor, Dome. The binding changes the conformation of Dome, resulting in a dimerization of the receptor and self-phosphorylation of the Janus kinase Hop. Activated Hop then phosphorylates the cytoplasmic tail of Dome to generate a docking site for the transcription factor STAT. Once STAT is recruited to the receptor, it is phosphorylated, which leads to dimerization. Dimerized STAT is then translocated to thenucleus to activate the transcription of JAK/STAT pathway-regulated genes [54,111]. The JAK/STAT pathway is also negatively regulated at different steps by the suppressor of cytokine signalling (SOCS) and PIAS proteins [43,97].

We hypothesized that if we activated the JAK/STAT pathway prior to or immediately upon DENV infection, the infection would be significantly limited, perhaps to a degree that could adversely affect DENV transmission. To modify the expression pattern of the JAK/STAT pathway, we generated genetically modified *Ae. aegypti* that expressed Dome or Hop under the control of the blood meal-inducible, fat body-specific vitellogenin (Vg) promoter. These transgenic *Ae. aegypti* showed greater resistance to DENV infection than did wild-type (WT) mosquitoes, and they have enabled the further characterization of the molecular interactions between DENV and *Ae. aegypti.*

MATERIALS AND METHODS

Generation of transformation vector constructs

A schematic of the gene constructs used to generate the VgDome and VgHop transgenic *Ae. aegypti* lines is shown in Figure 2.1. *Ae. aegypti* Dome and Hop genes were PCR-amplified from *Ae. aegypti* cDNA using the primers listed in Appendix1, and cloned downstream of the vitellogenin promoter [12,59,110]. The terminator sequence from the *An. gambiae* trypsin gene was cloned downstream of Dome or Hop. The gene cassettes were then cloned into the piggy-Bac-based transformation vectors using either the EGFP or DsRed selection marker driven by the eye-specific 3xP3 promoter [97,111], pBac-3xP3-EGFPafm and pBac-3xP3-DsRedafm.

Generation of transgenic *Ae. aegypti*

Embryo microinjections and initial screening for transformants were performed by the Insect Transformation Facility at the University of Maryland Biotechnology Institiute. Two transgenic *Ae. aegypti* lines expressing Dome or Hop under the control of the blood meal-inducible and fat body-specific vitellogenin (Vg) promoter were generated in the background of the Orlando (Orl) laboratory strain of *Ae. aegypti.* PCR confirmation of the inserts was performed using the primers in Appendix1.

Because the Orl strain of *Ae. aegypti* used to generate transgenic mosquitoes was highly refractory to DENV infection [97], the VgDome and VgHop lines were subsequently introgressed to the DENV-susceptible Rockefeller/UGAL (Rock) strain *Ae. aegypti* for five generations. After crossing to Rock, each line was mated within the same strain for another five generations to ensure homogeneity.

In an attempt to increase the induction of the JAK/STAT pathway, we crossed homozygous transgenic VgDome male mosquitoes with homozygous transgenic VgHop female mosquitoes in a ratio of 1:5 to generate a heterozygous hybrid VgDomexVgHop line overexpressing both Dome and Hop after a blood feeding. All adult mosquitoes were maintained on 10% sucrose solution in a controlled environment at 27°C and 80% humidity with a 12 h light/dark cycle.

Cell culture and DENV strains

The *Ae. albopictus* C6/36 cells were maintained in MEM media (Gibco, USA) supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 1% penicillin-streptomycin, and 1% MEM non-essential amino acids at 32°C and 5% CO2.

The Baby hamster kidney cells (BHK-21) were maintained on DMEM media supplemented with 10% FBS, 1% penicillin-streptomycin, and 5 ug/ml Plasmocin at 37°C and 5% CO_2.

DENV serotype 2 New Guinea C strain (DENV2), and DENV serotype 4 strain Dominica/814669 (DENV4) were propagated in C6/36 cells as previously described [12,74].

Oral DENV infections in *Ae. aegypti* and virus titration

Mosquitoes were orally infected with DENV via artificial membrane feeding, as previously described [12,59,112]. Briefly, DENV2 was infected to C6/36 cells seeded to 80% confluence at a multiplicity of infection (MOI) of 3.5 and incubated at 32°C and 5% CO_2 for 6 days. The infected cells were then harvested and lysed through 3 cycles of freezing and thawing between dry ice and 37°C water bath. The propagation yielded a virus titers of between 10^6 and 10^7 PFU/ml. Then DENV was mixed 1:1 v/v with

commercial human blood and supplemented with 10% human serum and 1 mM ATP. The bloodmeal was then offered to mosquitoes via an artificial membrane feeding system. Each experiment was performed using at least three biological replicates. DENV2 titers were determined by plaque assay using the BHK cell line, and plaques were visualized by staining with 1% crystal violet. Because DENV4 cannot lyse and form plaque in BHK cells, DENV4 titers were determined by focus-forming assay (FFA) in C6/36 cells and visualized using peroxidase immunostaining with monoclonal antibody 4G2 as a primary, and a goat anti-mouse horseradish peroxidase (HRP) conjugate as a secondary antibody. All procedures involving DENV infections were performed in a BSL2 environment.

Genome-wide oligonucleotide microarray transcriptomic analyses

Fat body transcriptomes of transgenic lines were compared to the WT at 24 hpbm using Agilent-based oligonucleotide microarrays, as previously described [97,113]. In brief, pools of abdominal fat body tissue from 10-15 WT or transgenic mosquitoes were collected at 24 hours post-naïve blood meal. We used 200 ng of total RNA from each pool to generate cy3- and cy5-labeled dCTP probes. Hybridizations were performed according to the manufacturer's instructions, and the arrays were scanned with an Agilent SureScan microarray scanner; spot intensity was extracted using Agilent Feature extraction software. The expression data were processed and analyzed as described previously [97,114-116]. Numeric gene expression data are presented in Table S1.

RNA interference (RNAi)-mediated gene silencing

We used RNAi to study the function of candidate host factors (HFs) and restriction factors (RFs) in WT mosquitoes as previously described [12,74,117], and the

primers used to generate the dsRNAs are listed in Table S2. GFP dsRNA was used as a negative control for all experiments, and gene silencing efficiency was determined three days after dsRNA injection by using real-time PCR with gene specific primers presented in Appendix1.

Mosquito fitness assays

Mosquito longevity and fecundity assays were performed in three biological replicates as previously described [74,110]. Because male and female mosquitoes have a different life span, longevity assays were performed with three- to four-day-old adult male or female mosquitoes maintained on 10% sucrose solution. For the longevity assays involving JAK/STAT pathway activation, mosquitoes were provided a single naïve human blood meal, followed by maintenance on 10% sucrose solution. The number of dead mosquitoes was then monitored daily.

For the fecundity assays, three- to four-day-old adult female mosquitoes were fed on human blood via an artificial membrane feeding. The fed mosquitoes were individually transferred to oviposition tubes, and the number of eggs laid was monitored until five days post-blood meal.

Bacterial challenge

Pantoea spp. and Bacillus cereus isolated from a field site in Zambia [111,112] were used to represent Gram-negative and Gram-positive bacteria, respectively. Bacteria were cultured in Luria-Bertani (LB) medium at 30°C at 250 rpm for 12-14 h . Overnight cultures were washed twice with 1xPBS buffer, then resuspended in 1xPBS buffer to OD_{600}=0.01. For bacterial challenge, we blood-fed mosquitoes with naïve blood meal to

activate the JAK/STAT pathway, then injected 69 nl of resuspended bacteria (approximately 400 bacteria per injection) into the thorax of each cold-anesthetized mosquito. Mosquitoes were also injected with 1xPBS as the negative control for this experiment.

RESULTS

Generation of JAK/STAT pathway transgenic *Ae. aegypti*

To conditionally activate the JAK/STAT pathway when the female *Ae. aegypti* acquires the virus through an infected blood meal, we generated the homozygous transgenic *Ae. aegypti* lines VgDome and VgHop, which over-express the pathway receptor Dome or Janus kinase Hop under the control of the bloodmeal-inducible, fat body-specific vitellogenin promoter (Figure 2.1). The Vg promoter has been shown to be activated after a blood meal and to reach its highest level of promotion at 24-48 h after blood ingestion [59,113]. *Aedes* mosquitoes usually take multiple blood meals during their gonadotropic cycle, especially when blood feeding is interrupted by a physical response from the host or probing in a non-optimal skin area [114-116,118,119], and we therefore hypothesized that transgene-mediated activation of the immune pathway by the selected promoter would likely prime the mosquito's JAK/STAT-mediated anti-DENV defense for the next potentially infectious blood meal.

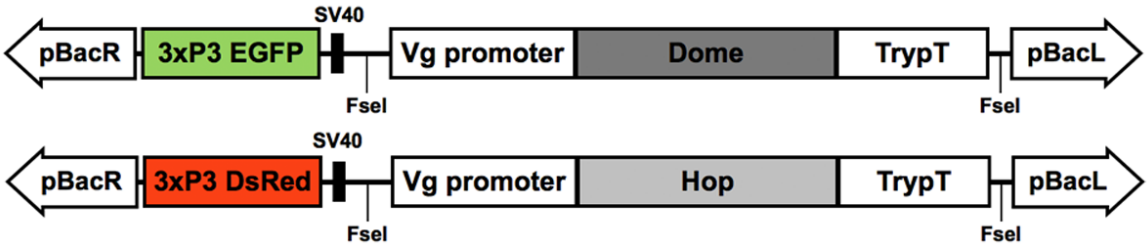


Figure 2.1 Schematic of the transgene constructs used to generate VgDome and VgHop lines.

Ae. aegypti Dome (AAEL012471) was PCR-amplified from cDNA in two segments: bp 1-1531 and bp 1532–3432; full-length Dome was then obtained through PCR using the Dome1F_PstI and Dome2R_*Pst*I primers, with equal proportions of each segment as template. Full-length Dome was cloned into the pBluescript II KS vector (Stratagene) at the *EcoR*V site. A 392-bp sequence from the putative terminator region of *Anopheles gambiae* trypsin was PCR-amplified from the vector pENTR-carboxypeptidase P-antryp1T [74,117,120-122] and cloned into pBluescript downstream of Dome at the *Xho*I/Klenow-filled site. A 2085-bp fragment from the promoter region of *Ae. aegypti* vitellogenin (Vg) [74,110,119] was PCR-amplified from genomic DNA and cloned into pBluescript at the *Sma*I site upstream of Dome. The AeVg-Dome-TrypT cassette was digested from pBluescript with *Fse*I and cloned into the *Fse*I site of the pBac[3xP3-EGFPafm] vector [59,111]. The resulting vector was used for embryo microinjections to generate the VgDome line.

Ae. aegypti Hop (AAEL012533) was PCR-amplified from cDNA in two segments: bp 1-1516 and bp 1517-3408. Each segment was separately cloned into pBluescript at the *EcoR*V site. The 5' and 3' segments were cleaved with *EcoR*I/*Sac*I and *Sac*I/*Sal*I, respectively, and re-ligated into pBluescript at the *EcoR*I/*Sal*I sites to obtain full-length Hop. The trypsin terminator sequence was cloned at the *Xho*I/Klenow-filled

site downstream of Hop, and the AeVg promoter sequence was cloned at the *Xba*I/Klenow-filled site upstream of Hop. The AeVg-Hop-TrypT cassette was digested from pBluescript with *Fse*I and cloned into the *Fse*I site of the pBac[3xP3-DsRedafm] vector. The resulting vector was used for embryo microinjections to generate the VgHop line.

To generate the VgDome transgenic line, 565 embryos were injected with the transformation vector and the phsp-pBac helper plasmid. Of these, 279 survived to adulthood and were backcrossed to WT Orl adults in 19 pools. G1 larvae were screened for GFP eye fluorescence (Figure 2.2), and one pool was found to contain positives.

To generate the VgHop transgenic line, 613 embryos were injected with the transformation vector and the phsp-pBac helper plasmid. Of these, 132 survived to adulthood and were backcrossed to WT Orl adults in 10 pools. G1 larvae were screened for DsRed eye fluorescence (Figure 2.2), and one pool was found to contain positives.

Positive larvae were reared to adulthood and then intercrossed to G5 to ensure homozygosity of the transgene. PCR confirmation of each line was performed with the VgPro R and ITRR2' primers for the VgDome line and the AeVgPro R and DsRed S primers for the VgHop line (Figure 2.3).

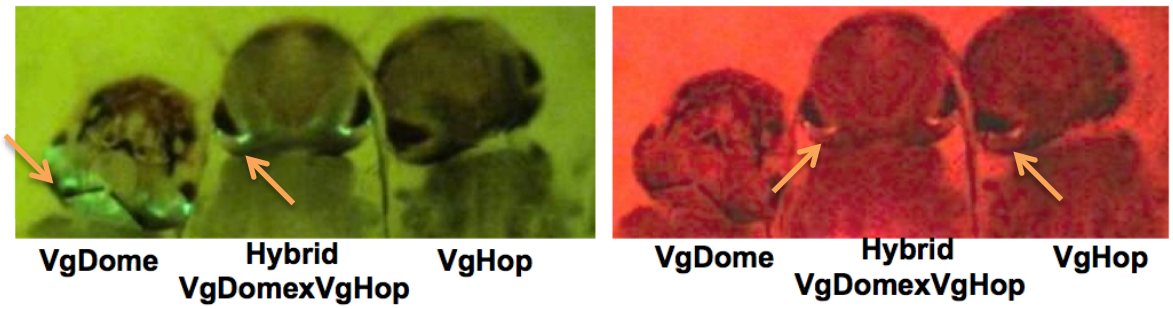


Figure 2.2 Fluorescence screening of VgDome, VgHop, and hybrid VgDomexVgHop transgenic lines. VgDome line contains an eye-specific EGFP marker. VgHop line contains an eye-specific DsRed marker. The hybrid line contains eye-specific EGFP and DsRed markers. Fluorescence signals in the eyes of all the strains are indicated by orange arrows.

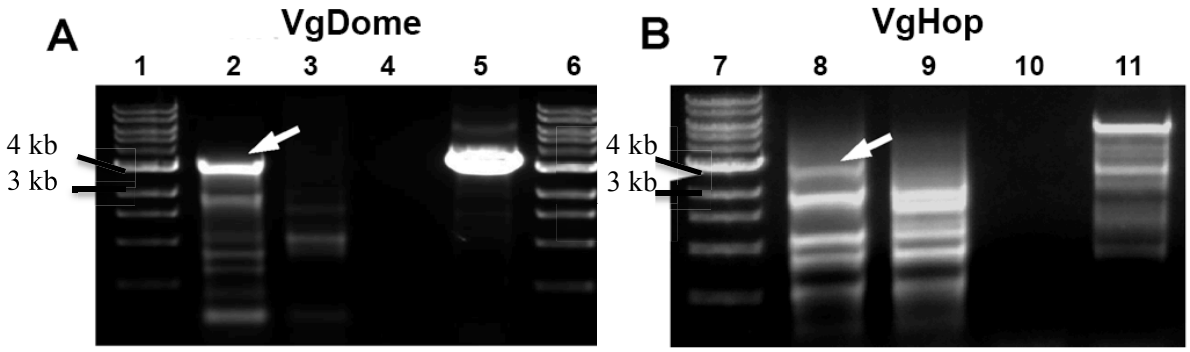


Figure 2.3 PCR confirmation of the transgenic *Ae. aegypti* VgDome and VgHop lines. (A) PCR confirmation of the VgDome transgenic line. The arrow indicates an expected band at 3.6 kb. (B) PCR confirmation of the VgHop transgenic line. The arrow indicates an expected band at 2.9 kb. The following templates were used: Lane 2: Genomic DNA from the VgDome line; Lanes 3 and 9: Genomic DNA from WT *Ae. aegypti*; Lanes 4 and 10: No template; Lane 5: pBac[3xP3-EGFPafm-AeVg-Dome-TrypT] plasmid; Lane 8: Genomic DNA from the VgHop line; Lane 11: pBac[3xP3-DsRedafm-AeVg-Hop-TrypT] plasmid. Lanes 1, 6, 7: 1-kb ladder.

To test susceptibility to DENV infection, both the VgDome and VgHop transgenic lines were introgressed with DENV-susceptible Rockefeller/UGAL (Rock) strain *Ae. agypti* for five generations. After five generations of outcrossing with the Rock strain, both the VgDome and VgHop transgenic lines were bred within the same strain for

another five generations to ensure homogeneity. The WT Orl strain was mated with the Rock strain in parallel to serve as a control.

To generate a hybrid transgenic line over-expressing Dome and Hop simultaneously, male homozygous VgDome and female homozygous VgHop were mated in a ratio of 1:5. The offspring were then screened for the expression of both GFP and DsRed (Figure 2.2) and used for subsequent experiments to test their susceptibility to DENV.

In the VgDome line, fat body expression of Dome was rapidly induced relative to WT, peaking as early as 6 hours post bloodmeal (hpbm) and again at 48 hpbm. Dome induction in the hybrid line followed a similar pattern, albeit with an approximately two-fold higher peak at 6 and 24 hpbm (Figure 2.4). In the VgHop line, Hop expression was induced more gradually, peaking at 24 hpbm. Hop induction in the hybrid line followed a similar pattern, but with an earlier peak at 12 hpbm (Figure 2.4).

Dengue virus restriction factor 1 (DVRF1; AAEL008492) is transcriptionally regulated by the JAK/STAT pathway, and encodes a putative anti-DENV effector molecule [12,59]. In the VgDome and VgHop lines, DVRF1 expression relative to WT peaked at 24 hpbm (Figure 2.4), indicating pathway activation. Interestingly, DVRF1 expression was induced to similar levels in the hybrid line (Figure 2.4), suggesting that there might be limiting factors acting downstream of Dome and Hop.

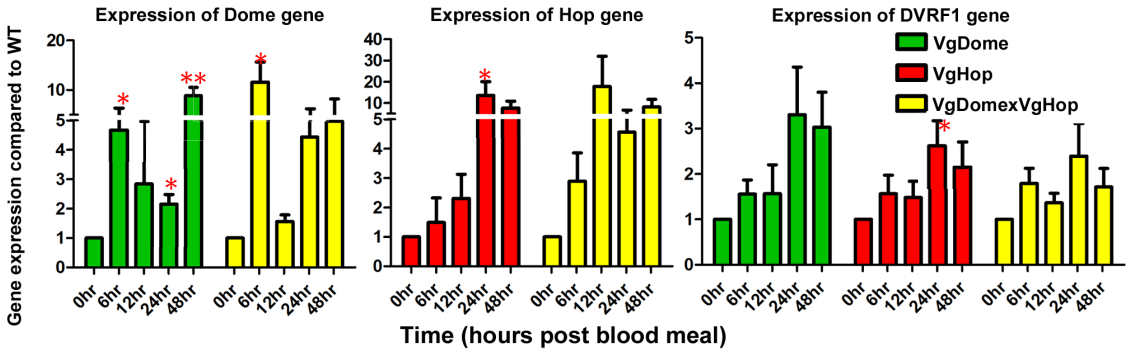


Figure 2.4 Transcript abundance of transgenes and effector genes in the fat body of VgDome and VgHop lines from before blood feeding (0 hr) up to 48 hpbm. Each bar represents relative fold change of Dome, Hop or DVRF1 gene compared between transgenic lines and WT *Ae. aegypti*. The S7 ribosomal gene was used to normalize cDNA templates. Error bars indicate standard error of the mean. Statistical analyses were performed using t-test using Prism software, *: $p < 0.05$, **: $p < 0.01$ compared to WT before blood feeding.

Transgenic activation of the JAK/STAT pathway inhibits virus replication throughout the mosquito's body

We next investigated the effect of the transgene-mediated activation of the JAK/STAT pathway on DENV infection in the transgenic lines. Mosquitoes were first fed a naïve bloodmeal to activate the JAK/STAT pathway; two days later, they were orally infected with DENV2 via a second (infectious) bloodmeal. We determined DENV2 titers in the midguts at 7 day-post infectious blood meal (dpibm) (Figure 2.5A), in the carcasses (whole mosquito except midgut) at 14 dpibm (Figure 2.5B), and in the salivary glands at 21 dpibm (Figure 2.5C). VgDome and VgHop mosquitoes showed significantly lower midgut DENV2 titers than did the WT mosquitoes (a 78.18% and 83.63% reduction in median titers for VgDome and VgHop, respectively). The VgDome and VgHop lines displayed a 87.37% and 94.21% reduction in median carcass DENV2 titers, and more importantly, the transgenic mosquitoes also had a lower DENV2 titers in

the salivary glands (100% reduction in median salivary gland DENV2 titers for both lines when compared to WT).

We orally infected VgDome and VgHop mosquitoes without prior activation of the JAK/STAT pathway in order to determine whether pathway activation at the time of infection was enough to grant systemic resistance. We found that the VgDome mosquitoes had comparable median midgut DENV2 titers, whereas the VgHop strain showed a 42.86% reduction in median midgut DENV2 titers (Figure 2.5D). We saw DENV2 resistance in the VgHop strain but not VgDome, suggesting that overexpression of the downstream component of the pathway can provide earlier protection against DENV infection. However, reduction of DENV titers in VgHop strain was not as strong as when compared to the VgHop mosquitoes that were given naïve blood meal before DENV infection. This result suggested that a naïve blood meal is required before the infectious blood meal to provide higher resistance to DENV infection, further suggesting that systemic immune activation by the JAK/STAT pathway is delayed.

Although the hybrid line also displayed significantly lower DENV2 titers in the carcass compared to WT, these were not significantly different from the VgDome and VgHop lines (Figure 2.5E). Since no difference in DENV2 susceptibility was seen between the hybrid and the VgDome and VgHop transgenic lines, we chose to use only the VgDome or VgHop lines for subsequent experiments.

To confirm that the inhibitory activity of the JAK/STAT pathway on DENV infection is conserved among different DENV serotypes, we also challenged the VgDome and VgHop lines with DENV4 as we did with DENV2 (Figure 2.5F). Both lines were

more refractory to DENV4 infection compared to the WT (100% reduction in median carcass titers).

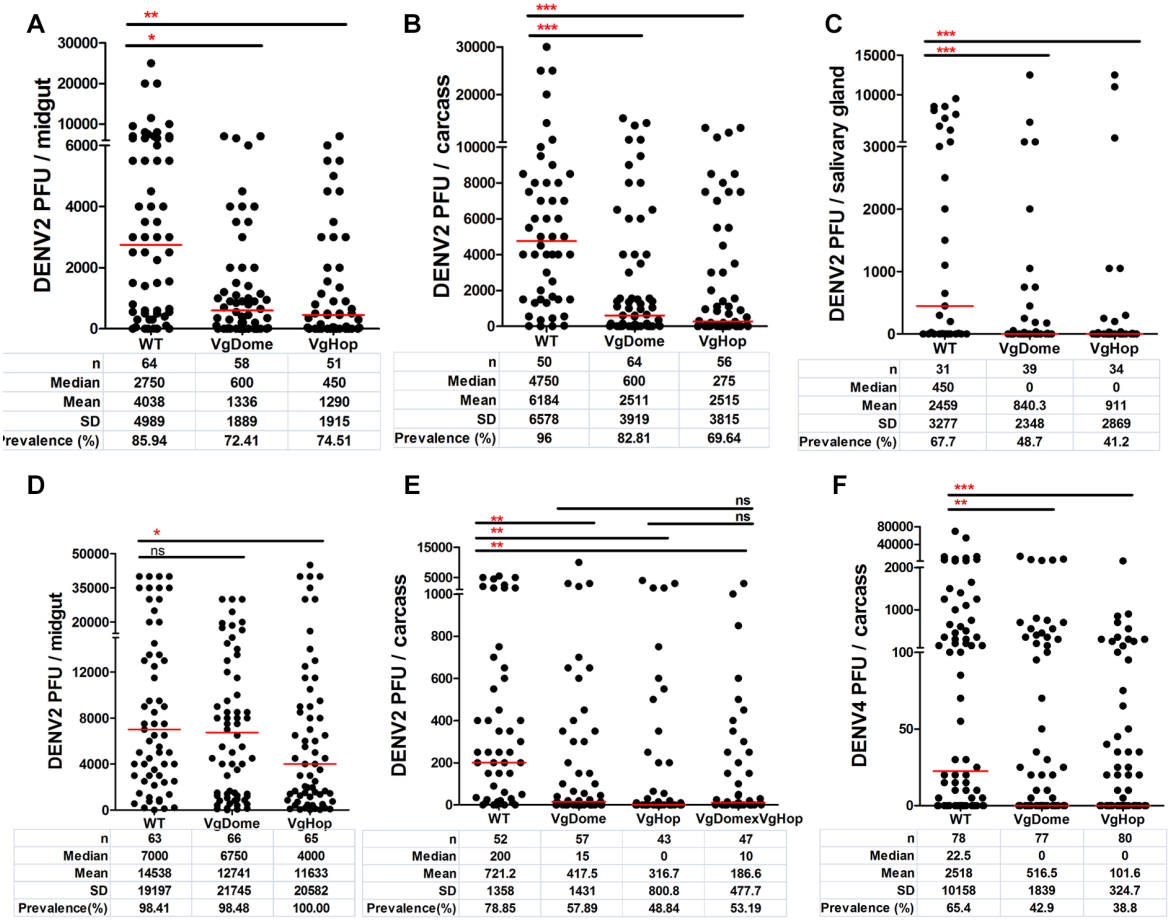


Figure 2.5 Effect of JAK/STAT pathway activation on DENV infection in transgenic *Ae. aegypti*. The JAK/STAT pathway was induced in the transgenic lines by providing them a naïve blood meal; two days later, JAK/STAT-activated mosquitoes were orally infected with DENV2 or DENV4. DENV2 titers of the VgDome and VgHop lines were determined (A) in the midguts at 7 dpibm, (B) the carcasses at 14 dpibm, and (C) the salivary glands at 21 dpibm. (D) DENV2 titers in the mosquito midguts at 7 dpibm without prior activation of the JAK/STAT pathway through a naïve blood meal. (E) DENV2 titers of the JAK/STAT-activated hybrid VgDomexVgHop line in carcasses at 14 dpibm. (F) DENV4 titers of the JAK/STAT-activated VgDome and VgHop lines in carcasses at 14 dpibm. WT mosquitoes were used as a control in parallel in all experiments. Horizontal red lines indicate medians. Statistical analyses were performed using either the Mann-Whitney test or Kruskal-Wallis test with Dunn's post-test using Prism software, *: $p < 0.05$, **: $p < 0.01$, ***:$p < 0.001$ compared to WT.

Fitness impact of transgenic Dome and Hop mediated JAK/STAT pathway activation

Activation of the immune system and transgenic over-expression of certain immune-related genes has been associated with fitness trade-offs [118,119,123] that could compromise the utility of a transgenic *Ae. aegypti* as a dengue control strategy. Transgenic activation of the JAK/STAT pathway may be particularly prone to fitness costs because it also functions in insect development [120-122,124]. For this reason, we examined the impact of introduction and expression of Dome or Hop under Vg promotor on the fitness of our transgenic lines.

First we studied the impact of the introduction of Dome and Hop gene cassettes by measuring the longevity of male and female transgenic mosquitoes maintained on 10% sucrose solution without a blood meal that would induce the transgene. In male mosquitoes, we found that the longevity of the VgDome line was comparable to that of the WT strain, while the longevity of the male VgHop line was slightly longer (by 4 days) than that of the WT (Figure 2.6). The female VgDome and VgHop lines had longevities comparable to that of the WT, suggesting a minimal impact of these transgenes on the mosquito life span in the absence of a blood meal. Next, we examined the effect of blood meal-inducible transgene expression on female *Ae. aegypti* longevity. The longevity of the female VgDome and VgHop lines after blood feeding was comparable to that of the WT strain, suggesting minimal fitness effects on the mosquito life span when the JAK/STAT pathway is transiently activated. This minimal effect on longevity after transient activation of immune pathways was in concordance with previous studies, which activated the immune deficiency pathway (Imd) pathway in *Drosophila* and *An. gambiae* mosquitoes [74,119,125].

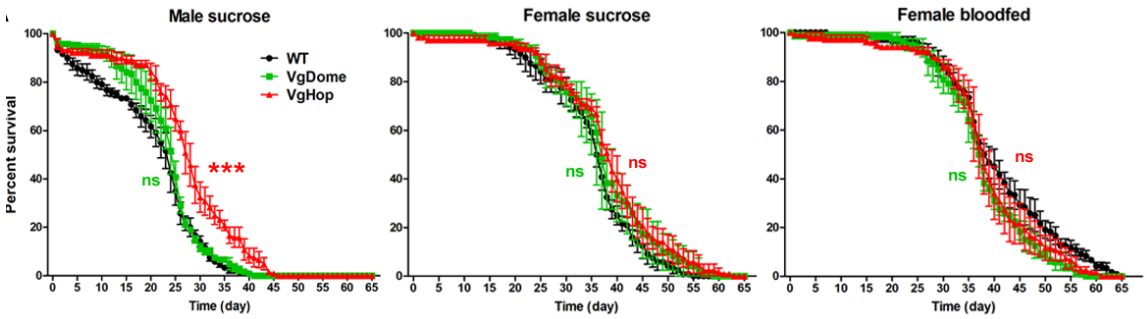


Figure 2.6 Effect of transgenes introduction and expression on mosquito longevity. Lifespans of male and female mosquitoes maintained on 10% sucrose solution or of female mosquitoes that were provided a blood meal to induce transgene expression. Statistical analyses of survival curve was performed using Log rank test with Prism software. ***: $p < 0.001$.

Both VgDome and VgHop lines produced significantly fewer eggs compared to WT (Figure 2.7), suggesting that transgene introduction or expression compromises fecundity. The lower egg production is likely, at least in part, due to the competition between the vitellogenin promoter of the transgenes and the endogenous vitellogenin gene, as indicated by the lower expression level of the vitellogenin gene after blood feeding in the transgenic mosquitoes when compared to WT (Figure 2.8).

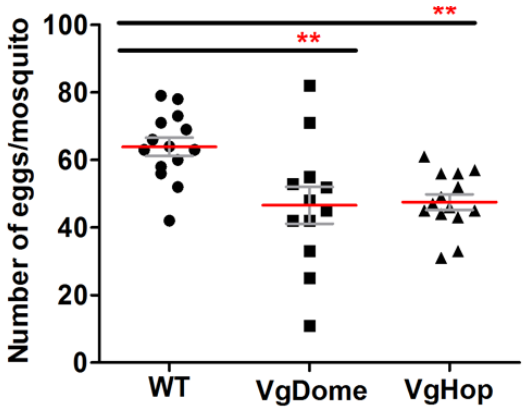


Figure 2.7 Fecundity of the WT and transgenic *Ae. Aegypti,* as represented by number of eggs produced by each female mosquito. Statistical analyses were performed using the Mann-Whitney test with Prism software **: $p < 0.01$ as compared to WT

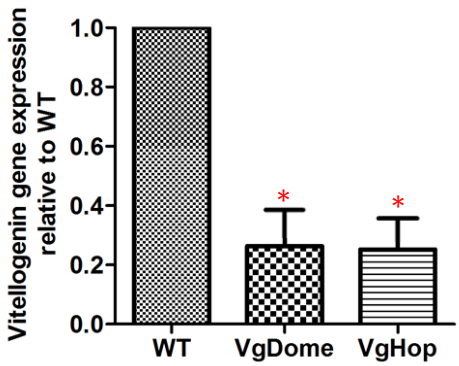


Figure 2.8 Expression of vitellogenin in the transgenic lines as compared to WT. mRNA levels were measured by real-time PCR, with ribosomal gene S7 as the normalization control. Error bars indicate standard error of the mean. Statistical analyses were performed using t-test using Prism software, *: $p < 0.05$ compared to vitellogenin gene expression in WT at 24 hours post blood meal.

Immune-related transcripts are enriched upon JAK/STAT activation

The JAK/STAT pathway-regulated antiviral effectors responsible for suppressing DENV infection are largely unknown, except for two genes, DVRF1 and DVRF2, that encode putative secreted and membrane-bound proteins, respectively, of unknown function [59,125]. To identify possible JAK/STAT pathway-regulated antiviral effectors and to assess the impact of transgenic JAK/STAT pathway activation on mosquito physiology at the molecular level, we used whole-genome oligonucleotide microarrays to compare the fat body transcriptomes of WT, VgDome, and VgHop lines at 24 hpbm (17,346 genes in the *Ae. aegypti* transcriptome). We selected the 24-h post-blood meal time point because of the DVRF1 peak expression at that time, suggesting the peak activity of the JAK/STAT. Genes that showed at least 1.68-fold (0.75 on a $\log_2$-scale) compared to WT were considered to be significantly differentially regulated. The $\log_2$-fold difference in transcript abundance for each gene between VgDome or VgHop and WT mosquitoes is listed in Appendix 2, and the number and percentage of transcripts significantly regulated in each category are presented in Figure 2.9A and 2.9B. Genes

commonly- or differentially-regulated in VgDome and Hop was represented in figure 2.9C. As expected, DVRF1 transcripts were enriched in both lines relative to WT (see Appendix 2), an indication of pathway activation. In VgDome, 130 transcripts (0.75% of the whole transcriptome) were enriched compared to WT, and 71 (0.47%) were depleted. In VgHop, 254 transcripts (1.46%) were enriched compared to WT, and 204 (1.18%) were depleted.

In both lines, IMM transcripts made up the largest specific class of enriched transcripts (excluding those with diverse (DIV) and unknown (UKN) functions). Of the 659 immune-related genes (IMM) in the *Ae. aegypti* transcriptome, 17 genes (2.58% of the total IMM genes) had a higher transcript abundance and 10 genes (1.52% of total IMM) had a lower transcript abundance in the VgDome line. In the VgHop line, 29 genes (4.40% of the total IMM) had a higher transcript abundance, and 15 genes (2.28% of total IMM) had a lower transcript abundance. The IMM had at least a 3-fold higher percentage of genes with higher transcript abundance when compared to the average percentage of regulated genes in the whole transcriptome. These results emphasize the importance of the JAK/STAT pathway in mosquito immune regulation and corroborate the fact that the VgDome and VgHop lines had higher immune activity than did the WT. IMM transcripts that are enriched upon JAK/STAT activation may encode potential DENV restriction factors (RFs) - gene products that inhibit DENV replication in the mosquito.

Fifty transcripts were enriched and 18 were depleted in both VgDome and VgHop compared to WT (Figure 2.9 A, and C). Again, the IMM category was the largest specific class of transcripts that were enriched in both lines (9 genes, 1.37% of the total IMM). These were: three C-type lectins (CLECs; AAEL005482, AAEL011610, and

AAEL014390), three fibrinogen and fibronectin-related proteins (FBNs; AAEL006704, AAEL011400, and AAEL013417), two transferrins (TFs; AAEL015458, and AAEL015639), and a cathepsin b (CatB; AAEL015312).

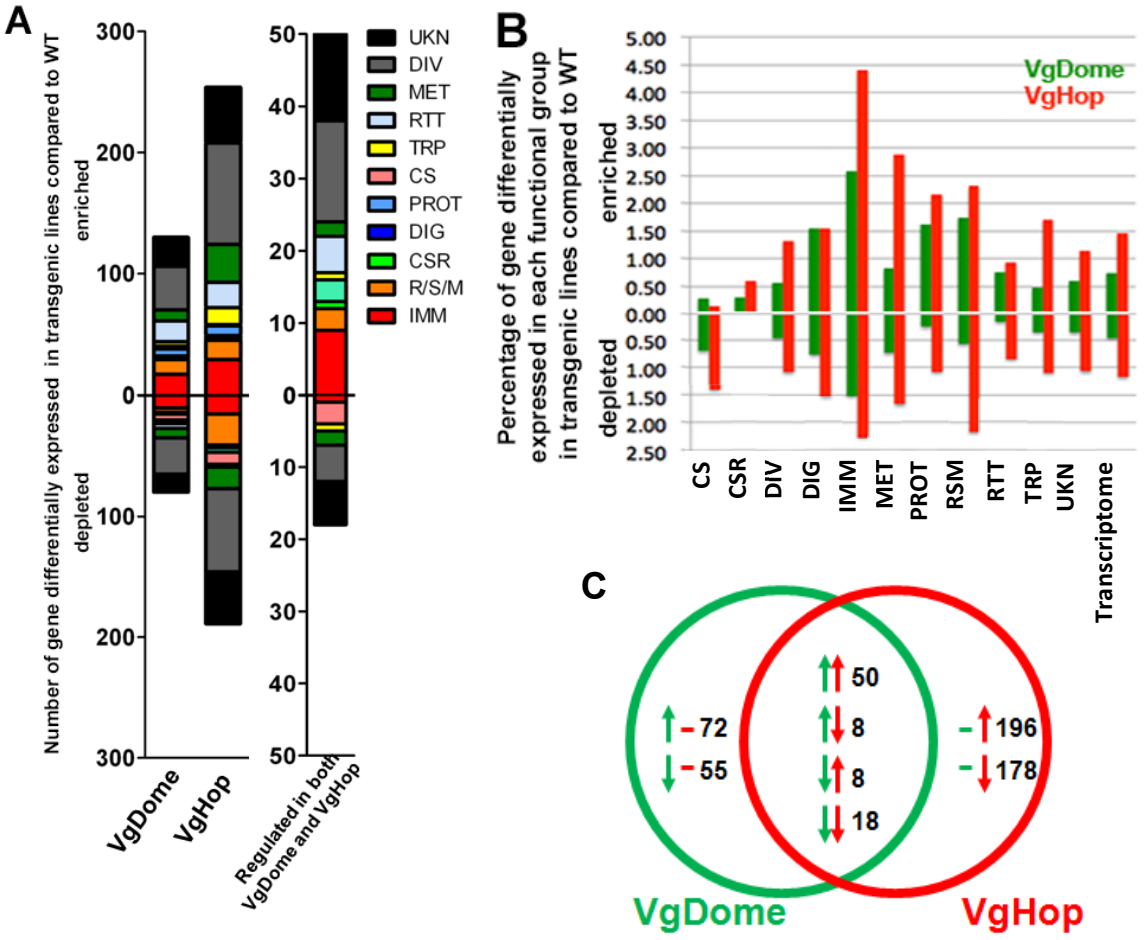


Figure 2.9. Transcriptomic profiles of the VgDome and VgHop mosquitoes. (A) Number of differentially expressed transcripts, classified according to functional groups as previously described [12,59,126]. Abbreviations: CS, cytoskeletal and structural; CSR, chemosensory reception; DIV, diverse functions; DIG, blood and sugar food digestive; IMM, immunity; MET, metabolism; PROT, proteolysis; RSM, redox, stress and mitochondrion; RTT, replication, transcription, and translation; TRP, transport; UKN, unknown functions. (B) Percentage of genes enriched or depleted in each functional group for the VgDome or VgHop line as compared to WT. (C) Venn diagram shows genes significantly regulated in VgDome and VgHop mosquitoes. Green arrows and circle represent VgDome strain, and red arrows and circle represent VgHop strain. Upward arrows represent genes significantly enriched, downward arrows represent genes significantly depleted in each strain compared to the WT.

Table 2.1 List of top ten enriched and depleted transcripts shared between VgDome and VgHop lines compared to WT

Gene ID	Description	Functional group	VgDome	VgHop
AAEL007703	conserved hypothetical protein	U	3.215	3.352
AAEL011400	conserved hypothetical protein	I	2.634	2.21
AAEL005482	conserved hypothetical protein	I	2.369	2.205
AAEL010196	trypsin	PROT	2.031	2.224
AAEL000986	NADH-ubiquinone oxidoreductase ashi subunit	RSM	2.027	2.067
AAEL002860	conserved hypothetical protein	D	2.016	1.633
AAEL012605	conserved hypothetical protein	D	2.008	1.699
AAEL005106	conserved hypothetical protein	U	1.749	0.958
AAEL008492	conserved hypothetical protein	U	1.721	1.275
AAEL013417	fibrinogen and fibronectin	I	1.584	1.708
AAEL015337	neutral alpha-glucosidase ab precursor (glucosidase ii alpha subunit) (alpha glucosidase 2)	M	-0.967	-2.407
AAEL010097	nuclein acid binding	D	-1.149	-1.591
AAEL014937	hypothetical protein	U	-0.945	-1.289
AAEL002554	anosmin, putative	D	-1.466	-1.099
AAEL002652	hypothetical protein	U	-1.234	-1.081
AAEL017491	hypothetical protein	U	-1.251	-1.001
AAEL013734	hypothetical protein	U	-1.287	-0.987
AAEL009962	hypothetical protein	D	-1.241	-0.96
AAEL008595	conserved hypothetical protein	D	-1.162	-0.953
AAEL007458	amino acid transporter	TRP	-0.833	-0.945

Pattern recognition receptors (PRRs) are important molecules responsible for binding and recognition of pathogen-associated molecular patterns (PAMPs). PRR for DENV in *Ae. aegypti* mosquitoes has yet to be identified. Transcriptomic analyses showed that transcripts of CLEC and FBN, gene families with potential function as PRRs, were enriched in VgDome and VgHop mosquitoes compared to the WT. CLECs have carbohydrate binding properties, and serve as PRR molecules. In *Drosophila*, CLECs are PRRs for *E. coli* [123,127] and play a role in the melanization and

encapsulation processes [124,128]. In *Ae. aegypti,* several CLEC have been reported to function as receptors for DENV entry into cells [125,129]. However, none of the CLEC identified in our study were reported as a receptor for virus entry. Silencing of AAEL005482 had no effect on DENV infection, whereas silencing of a homolog of AAEL014390 resulted in a non-significant increase in DENV loads, by 1.48-fold [40,125]. AAEL011610 has not been tested for its role in DENV replication, but has been reported to be up-regulated in transgenic *Ae. aegypti* over-expressing Rel2, transcription factor for the Imd pathway, under the control of the vitellogenin promoter [126,130]. FBNs are thought to serve as PRRs in *Drosophila* and in *Anopheles* mosquitoes [127,131] [126,128]. but their function in *Ae. aegypti* has yet to be elucidated.

CatB is a family of lysosomal cysteine proteases with functions in TLR signaling as well as T and B cell apoptosis [12,59,129]. One of the CatBs regulated in our study (AAEL007585) has been reported to facilitate DENV infection in *Ae. aegypti* salivary glands [40,41,44]; it was hypothesized that CatB-mediated apoptosis may facilitate cell-to-cell spread of the virus.

Over-expression of Dome and Hop also regulated specific subsets of IMM transcripts (Appendix2). Eight IMM genes were enriched in the VgDome but not in the VgHop, including three serine proteases (AAEL003279, AAEL000030, and AAEL006434), two Niemann-Pick Type C2 molecules (AAEL012064, and AAEL004120), a cathepsin b (AAEL007599), and a lysozyme C (AAEL017132). Twenty IMM transcripts were enriched in VgHop but not in VgDome. These included four cathepsin b genes (AAEL009637, AAEL009642, AAEL007585, and AAEL012216), four serine proteases (AAEL007969, AAEL007006, AAEL015430, and AAEL003625), a

thioester-containing protein (TEP22; AAEL000087), and several anti-microbial peptides (AMPs) such as cecropins (AAEL000621, AAEL000625), defensins (AAEL003832, AAEL003841), a gambicin (AAEL004522), and a lysozyme P (AAEL003723). These line-specific transcripts suggest complexities in JAK/STAT pathway regulation, and different as-yet unknown branches of the pathway and fine-tuning mechanisms may come into play to regulate different subsets of genes.

TEPs, which encode complement factor-like proteins belonging to the alpha-2-macroglobulin family, play important roles in insect immunity [12,130]. In *Ae. aegypti,* TEP22 was previously reported to be regulated by the CTL CLSP2 (AAEL011616), and to be involved in the mosquito's anti-fungal response [54,131-133]. TEP22 was also up-regulated in transgenic *Ae. aegypti* over-expressing the Toll pathway transcription factor Rel1 [93,126], suggesting that there may be crosstalk between these two immune pathways, both of which both play an important role in anti-DENV responses [12,59,97]. In further support of this, several AMPs belonging to the defensin and cecropin families were up-regulated in our JAK/STAT transgenic strains and have been previously studied with regard to their anti-DENV properties [41,44,134]. Gambicin was previously described to be regulated by the Toll pathway [12,134,135]; however, it has never been tested for anti-DENV activity.

Because we observed regulation of several AMPs, in both the VgDome and VgHop lines, that might provide protection against bacterial infection, we challenged these mosquitoes with a Gram-negative bacterium, *Pantoea spp*., and a Gram-positive bacterium, *Bacillus cereus.* We found no resulting differences in mortality between the VgDome or VgHop lines and WT (Figure 2.10).

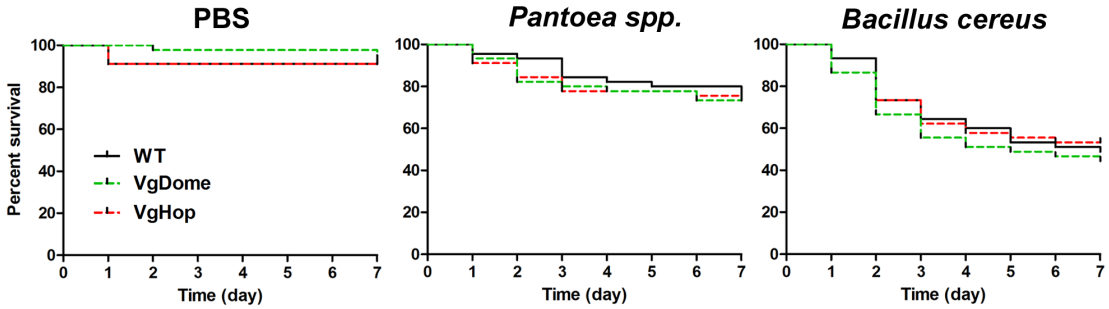


Figure 2.10. Mortality of the VgDome and VgHop lines from bacterial infection. Mosquitoes were challenged with *Pantoea spp.* or *Bacillus cereus*, with PBS as a negative control. Survival analysis was performed using Data are from three independent replicates.

Transcript abundances of potential DENV host factors were depleted upon JAK/STAT activation

Other than the IMM genes, hundreds of transcripts in other functional classes were differentially expressed in VgDome and VgHop compared to the WT. (Figure 2.9A). This result is not unexpected since the JAK/STAT pathway plays important roles in other biological processes such as cell development and homeostasis, as well as lipid metabolism [54,95,132,133]. We also found that genes with unknown function contributed to a large proportion of top shared enriched and depleted genes shared between VgDome and VgHop compared to WT (3 out of 10 enriched transcripts, and 4 out of 10 depleted transcripts). These data show that our knowledge is still limited on the function of the JAK/STAT pathway in *Ae. aegypti*.

The transcript abundances of several previously reported putative DENV host factors (HFs; genes that facilitate virus replication in the host) were significantly depleted in the transgenic lines compared to WT; a pattern that could potentially result in a reduced virus replication in mosquito.

Glucosidase 2 alpha subunit (aGluc; AAEL015337) transcripts were depleted in VgDome and VgHop (0.967- and 2.407-fold in $\log_2$ scale, respectively). aGluc has been identified in a high-throughput screen as a DENV HF in both HuH-7 human and D.Mel-2 *Drosophila* cells [93,136,137]. We previously identified aGluc as putative HF from transcriptomic comparisons between DENV-refractory and -susceptible strains, but silencing of aGluc had no effect on DENV infection in *Ae. aegypti* [97,132]. Nevertheless, previous studies have suggested that aGluc is required for proper glycosylation of the viral glycoproteins PrM and E [134,138], and chemical inhibition of aGluc results in lower virus production from infected cells [134,135,139]. It is possible that the transient silencing of aGluc by RNAi in the previous study was not sufficient to deplete aGluc at the protein level, and therefore no effect was observed.

Transcripts of vacuolar ATP synthase subunit ac39 (vATPase-ac39; AAEL0011025) were depleted in VgHop compared to WT (2.707-fold in $\log_2$ scale). Cell entry, a crucial step in DENV infection, requires acidification of endosomes by vATPase enzymes. Knockdown of vATPase-ac39 and several other vATPase subunits, as well as chemical inhibition of vATPase activity with bafilomycin, have been shown to inhibit DENV replication in *Ae. aegypti* [95,140,141].

Lipid homeostasis and trafficking play important roles in the replication of DENV, an enveloped virus. DENV is thought to facilitate its replication by altering the expression of lipid binding proteins and enzymes involved in lipid biosynthesis, such as fatty acid synthases and Niemann-Pick type C protein family members [136,137,142,143]. The JAK/STAT pathway has previously been shown to influence lipid metabolism in mammals, and it may have a similar function in mosquitoes

[132,144,145]. In VgHop, sterol carrier protein 2 (SCP2; AAEL012697) transcripts were depleted compared to WT (3.624-fold in $\log_2$ scale). SCP2 encodes an intracellular sterol carrier protein that facilitates cholesterol uptake in *Ae. aegypti* cells [138,146]; knockdown or chemical inhibition of SCP2 was recently shown to inhibit DENV replication in *Ae. aegypti* Aag2 cells [44,47,139].

Transcripts of the DEAD-box ATP-dependent RNA helicase (DDX; AAEL004978) gene were depleted in both VgDome and VgHop (0.70- and 0.79-fold in $\log_2$ scale, respectively). DDX gene family plays important cellular functions in transcription, mRNA transport, and translation [140,141,147]. DDX gene family members are required for viral replication in hepatitis C virus (HCV) [142,143,148,149], retroviruses [59,144,145,150], and Japanese encephalitis virus [74,146,151]. DDX proteins are used by these viruses to regulate the translational machinery and for viral RNA transport to favor virus replication. However, the role of this gene family in DENV replication has not yet been studied in *Ae. aegypti*.

Functional analysis of JAK/STAT pathway-regulated putative DENV restriction factors and DENV host factors using RNA interference

Our transcriptomic analysis of the VgDome and VgHop lines yielded candidate genes with potential function as DENV restriction factors (RFs) or host factors (HFs). We selected candidate genes based on their expression patterns and previous reports of their gene function. Based on their expression patterns (enriched in transgenic lines) and previous reports of their gene function, we selected five candidate RFs from enriched genes (FBN, TEP22, gambicin, and two genes of unknown function (Ukn7703, and

Ukn566)) and two candidate HFs from depleted genes (DDX and SCP2) for further characterization using RNAi-mediated gene silencing assays (Table 2.2).

Table 2.2. List of candidate RFs and HFs for functional confirmation by RNAi

Accession no.	Gene name	Functional group	Abbreviation	Putative role	Log2-fold difference compared to WT	
					VgDome	VgHop
AAEL013417	fibrinogen and fibronectin	I	FBN	RF	1.584	1.708
AAEL000087	macroglobulin/complement (TEP22)	I	TEP22	RF	0.414	0.752
AAEL004522	gambicin	I	GAMB	RF		1.796
AAEL007703	conserved hypothetical protein	U	UKN7703	RF	3.215	3.352
AAEL000566	conserved hypothetical protein	U	UKN566	RF	0.946	1.748
AAEL004978	DEAD box ATP-dependent RNA helicase	RTT	DDX	HF	-0.699	-0.79
AAEL012697	sterol carrier protein-2, putative	M	SCP2	HF		-3.624

Ukn7703 (AAEL007703) transcripts were very highly enriched in the transgenic lines compared to WT (3.215-, and 3.352-$\log_2$ fold higher in VgDome and VgHop respectively). The gene has also been reported to be induced in two strains of *Wolbachia*-infected *Ae. aegypti* [44,47,59,152], and is conserved among *Aedes*, *Culex*, and *Anopheles* mosquitoes (Figure 2.11), suggesting an important role in mosquito biology. Ukn7703 encodes a putative secreted protein with a C-terminal beta-propeller domain distantly related to WD-40 repeats (predicted using NCBI conserved domain search [147,153]). WD-40 domains are involved in protein-protein interactions in several biological processes, including signal transduction [148,149,154].

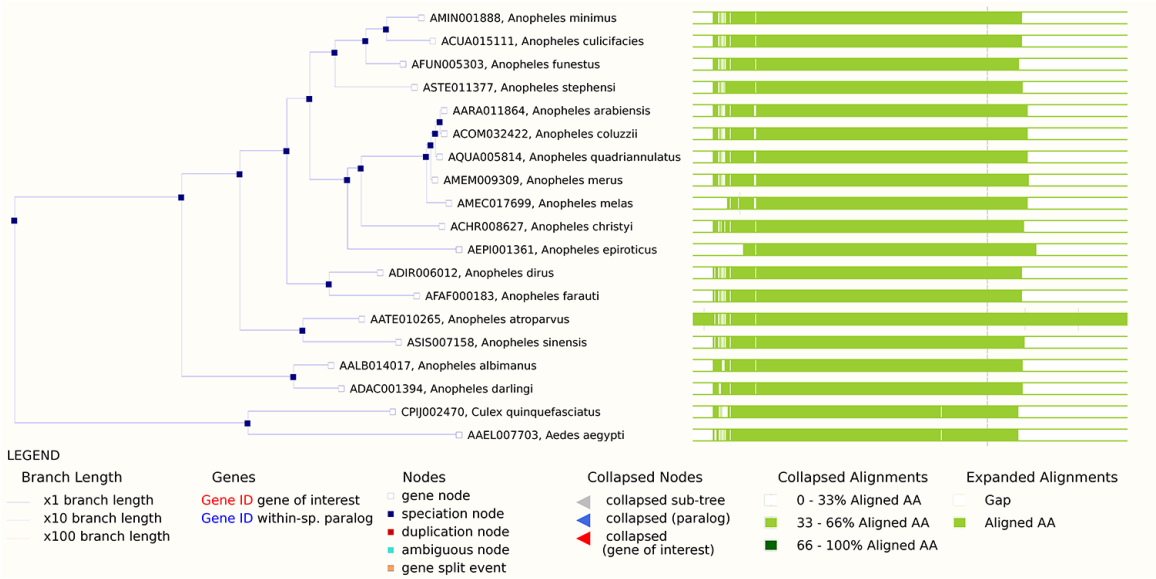


Figure 2.11. Phylogenetic tree of orthologs of AAEL007703 gene obtained from Vector base.
URL: https://www.vectorbase.org/Multi/GeneTree/Image?gt=VBGT00190000016830

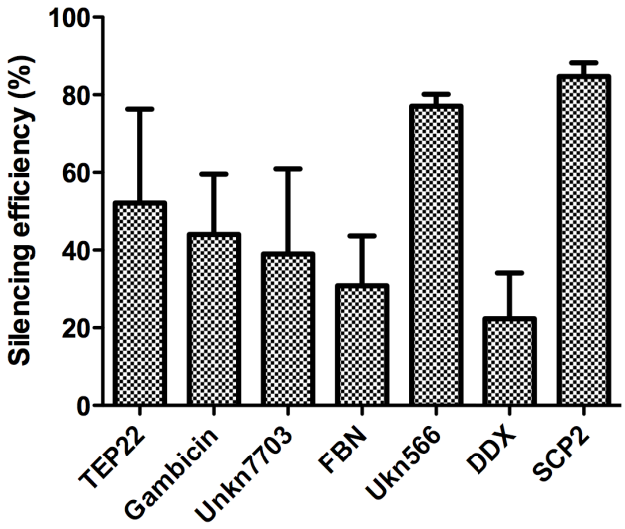
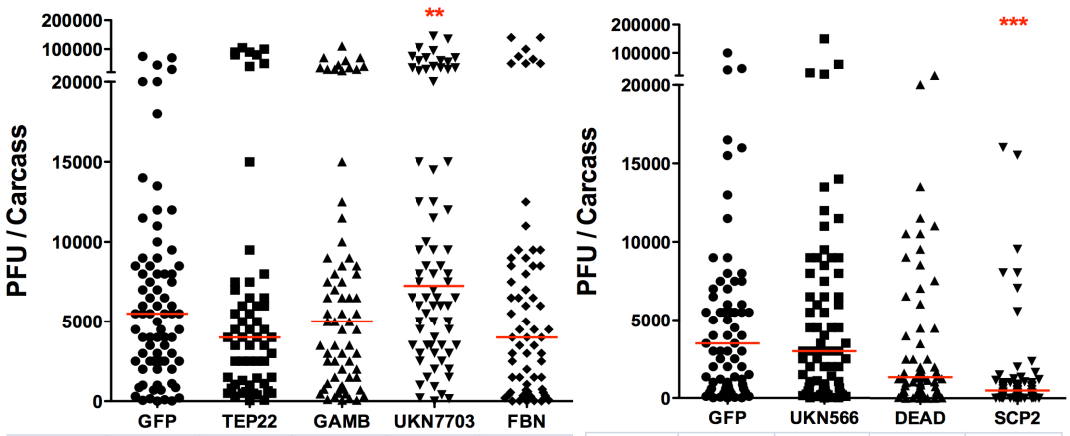


Figure 2.12 Silencing efficiencies for candidate RFs and HFs.

Ukn566 (AAEL000566) transcripts were enriched in both VgDome and VgHop compared to WT (0.946- and 1.748-$\log_2$ fold, respectively); this gene was also induced in *Wolbachia*-infected *Ae. aegypti* [59,148,150]. Ukn566 is predicted to be a transmembrane

protein using TMHMM software [59,74,151]. While no predicted protein domains were detected, Ukn566 and its orthologs share conserved cysteine positions. Cysteine repeats have previously been reported to be important for the three-dimensional structure and function of receptor proteins such as LDL [59,74,152] and scavenger receptors [59,153]. Potential functions of the remaining candidate genes have been discussed in the previous section.

Across the candidate genes, silencing efficiencies varied from 22% to 85%, as shown in Figure 2.12. Our screen confirmed Ukn7703 as a putative RF (31.82% increase in median DENV titer when compared to the GFP dsRNA-injected group), and SCP2 as a putative HF (85.71% decrease in DENV titer when compared to the GFP dsRNA-injected group) (Figure 2.13). Silencing of DDX also reduced the DENV2 titers in the carcass by 61.43%, although this result was not statistically significant by a small margin ($p=0.0555$). The lack of statistical significance may be a result of the lower silencing efficiency (22%) achieved for this gene. Because of the limitation of RNAi in failing to completely knock down a gene of interest, the lack of effects on DENV infection for the other candidate RFs does not necessarily exclude their potential involvement in anti-dengue defense



	GFP	TEP22	GAMB	UKN7703	FBN
n	76	57	66	72	65
Median	5500	4000	5000	7250	4000
Mean	8191	14224	12293	19988	13711
SD	12689	27920	20234	30717	29546

	GFP	UKN566	DEAD	SCP2
n	69	72	60	63
Median	3500	3000	1350	500
Mean	6616	7263	3475	1563
SD	13707	19046	4710	3293

Figure 2.13 Effect of putative host and restriction factor silencing on DENV susceptibility. DENV2 titers at 14 dpibm in the carcasses (whole mosquito body except midgut) of the WT *Ae. aegypti* after silencing of the putative HFs or RFs, compared to GFP dsRNA-injected control. Data are a pool of three biological replicates, and statistical analyses were performed using the Mann-Whitney test, **: $p < 0.01$, ***: $p < 0.001$ vs.WT**.**

DISCUSSION

We have demonstrated that we can induce activation of the JAK/STAT pathway by over-expressing JAK/STAT pathway receptor Dome and the Janus kinase Hop under the control of Vg promoter. Overexpression of Dome and Hop prior to exposure to DENV systemically controls DENV infection. Since Dome and Hop were over-expressed specifically in the fat body, the reduction of DENV titers in the midgut suggested that the JAK/STAT pathway functions as a systemic anti-viral defense system in *Ae. agypti*, and may possibly prime uninfected cells and other immune cells to assume an antiviral state. This type of systemic activation in the mosquito is analogous to the mammalian JAK/STAT pathway's role as a Type I interferon-induced immune pathway [154,155], or

the systemic immune response function of the JAK/STAT pathway in *Drosophila* [97,148].

A comparable level of DENV infection was found in the hybrid line compared to VgDome or VgHop, suggesting the existence of a limiting factor downstream of Dome and Hop, a possibility that is in agreement with the results concerning the induction of the DVRF1. It will be an interesting attempt to further reduce the vector competence for DENV by generating transgene constructs expressing both receptor and downstream pathway molecules such as STAT. Availability of JAK/STAT pathway downstream components might not be the only limiting factor of pathway activation. The JAK/STAT pathway is negatively regulated by PIAS or suppressor of cytokine signaling (SOCS) proteins to prevent overactivation of the pathway [54], and we have previously shown that silencing PIAS activates the JAK/STAT pathway and inhibits DENV infection [59,156]. To improve JAK/STAT pathway activation in transgenic mosquitoes, we can generate a transgene construct that contains the receptor gene together with a hairpin double-stranded RNA sequence complementary to PIAS or SOCS to reduce the level of negative regulator molecules.

We also showed that both transgenic lines were refractory to DENV2 and DENV4 infection compared to the WT, which suggests that the anti-viral function of the JAK/STAT pathway is conserved across different DENV serotypes. It will be interesting to determine if JAK/STAT pathway activation is also effective against other mosquito-borne flaviviruses such as the West Nile, zika, and yellow fever virus, as well as against alphaviruses such as chikungunya virus.

Analyses of the fitness impact from transgene expression in the transgenic lines showed that there was a minimal disadvantage to the longevity of these mosquitoes, but there was a negative impact on fecundity for both transgenic lines. It should be noted that these mosquitoes were maintained under laboratory conditions with an abundant food supply and minimal environmental stress. The assays may therefore not reflect the effect of transient JAK/STAT pathway activation on mosquito longevity in a natural setting where a myriad of stressors can apply. Additional experiments will be necessary to fully evaluate the effect of transient JAK/STAT pathway activation on longevity in natural settings. Reduced egg production and lower expression of vitellogenin gene have also been observed in transgenic *An. stephensi* lines using the Vg promoter to drive gene expression of Imd pathway component [74,97]. This phenomenon; however, was not observed in transgenic *An. stephensi* that overexpressed Imd pathway component under a regulation of bloodmeal inducible, gut-specific carboxypeptidase promoter. These results suggest that the use of Vg promoter compromises mosquito's fecundity, which suggests that alternative fat body-specific promoters may be required to help to minimize fitness disadvantages.

Transcriptomic comparisons between the fat body of blood-fed transgenic mosquitoes and WT allowed us to identify genes that influence DENV infection in *Ae. Aegypti*. The greater resistance to DENV infection displayed by the transgenic lines was a result of a combination of a higher transcript abundance of RFs and a lower transcript abundance of HFs. These multifactorial factors for DENV resistance make it harder for DENV to develop counter-measures to be able to efficiently replicate in our transgenic mosquitoes.

Even though several AMPs were enriched in the transgenic lines compared to the WT, we did not observe differences in mosquitoes' mortality from bacterial infection This result was similar to what we observed in a previous study: that the transient silencing of PIAS, a negative regulator of the JAK/STAT pathway, has no effect on mosquito mortality resulting from bacterial infection [59,97]. It is also possible that the regulated AMPs may have more specialized anti-DENV function or may not have anti-microbial activity against the particular bacteria used in our study. A previous study of defensins from humans has also suggested that the anti-bacterial activity of certain AMPs is highly specific [85,155]. Future extensive study of the anti-bacterial function of these AMPs will require recombinant expression of individual AMPs so they can be tested with a wide panel of bacteria.

In summary, our study is the first to provide a proof-of-concept that genetic engineering of the mosquitoes' JAK/STAT immune pathway can be used to render the insect more resistant to dengue virus infection and possibly to block transmission of the disease.

CHAPTER 3

Identification of putative host and restriction factors that contributes to refractoriness to DENV infection among laboratory and field-derived *Aedes aegypti* mosquitoes

Parts of this work were published in:

Sim S, **Jupatanakul N**, Ramirez JL, Kang S, Romero-Vivas CM, Mohammed H, et al. Transcriptomic profiling of diverse *Aedes aegypti* strains reveals increased basal-level immune activation in dengue virus-refractory populations and identifies novel virus-vector molecular interactions. Plos Neglect Trop D. 2013;7: e2295–e2295. doi:10.1371/journal.pntd.0002295

Kang S, Shields AR, **Jupatanakul N**, Dimopoulos G. Suppressing dengue-2 infection by chemical inhibition of *Aedes aegypti* host factors. Plos Neglect Trop D. 2014;8: e3084. doi:10.1371/journal.pntd.0003084

ABSTRACT

Our lab has previously established a panel of laboratory and field-derived *Ae. agypti* strains from different geographical origins. We have shown that these mosquito strains vary in susceptibility to DENV infection, and comparative genome-wide gene expression microarray-based analysis revealed higher basal levels of numerous immunity-related gene transcripts in DENV-refractory mosquito strains compared to susceptible strains. Here we used RNA interference-mediated gene silencing assays to further confirm functions of the Toll, Imd, JAK/STAT, and RNAi-pathway in contribution to refractoriness in different strains. By correlating transcript abundance patterns with DENV susceptibility, we also identified new candidate modulators of DENV infection in the mosquito, and we provide functional evidence for vATPase subunits as DENV host factors. Our comparative transcriptome dataset thus not only provides valuable information about immune gene regulation and usage in natural refractoriness of mosquito populations to dengue virus but also allows us to identify new molecular interactions between the virus and its mosquito vector.

INTRODUCTION

Mosquitoes, like other organisms, are exposed to a variety of microbes in their natural habitats and possess an innate immune system that is capable of mounting a potent response against microbial challenge. However, studies of mosquito immune responses to DENV and other human pathogens have largely been performed in laboratory strains of *Ae. aegypti*, which have been maintained under insectary conditions for many generations. As compared to natural mosquito populations, laboratory mosquito strains are exposed to lower doses and a much narrower range of microbes, constant temperature and humidity; together with the genetic bottleneck of a small initial parental population size, this often results in a loss of genetic variability. However, differences between laboratory-maintained mosquitoes and field populations have been poorly studied.

In this study, we collected *Ae. aegypti* from different DENV-endemic geographical locations as well as laboratory-maintained *Ae. aegypti* strains [20,97], and showed that they have a wide range of DENV susceptibility and different transcriptomic profiles. These mosquito strains and transcriptomic dataset served as powerful tools to dissect interactions between DENV and to identify novel modulators of DENV infection in *Ae. aegypti*.

MATERIALS AND METHODS

Mosquito rearing and cell culture conditions

Mosquitoes were maintained on a 10% sucrose solution in insectary condition at 27°C and 95% humidity with a 12 h light/dark cycle. The C6/36 *Aedes albopictus* cell

line was maintained in MEM (Gibco) supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 1% non-essential amino acids, and 1% penicillin-streptomycin at at 32°C and 5% CO_2. BHK-21 hamster kidney cells were maintained on DMEM (Gibco) supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin, and 5 ug/ml Plasmocin (Invivogen) at 37°C and 5% CO_2.

Oral DENV infections in *Ae. aegypti*

Mosquito infections with DENV were carried out by oral infection as previously described [97,156]. The New Guinea C strain of DENV2 was propagated in C6/36 cells as previously described [12]. Briefly, DENV2 was infected to C6/36 cells seeded to 80% confluence at a multiplicity of infection (MOI) of 3.5 and incubated at 32°C and 5% CO_2 for 6 days. The infected cells were then harvested and lysed through 3 cycles of freezing and thawing between dry ice and 37°C water bath. The propagation yielded a virus titer of between 10^6 and 10^7 PFU/ml. Then DENV was mixed 1:1 v/v with commercial human blood and supplemented with 10% human serum and 1 mM ATP. The bloodmeal was then offered to mosquitoes via an artificial membrane feeding system. Midguts were dissected at 7 days post-blood meal (dpbm) and stored individually in DMEM at -80°C until titrated by plaque assay.

Gene silencing assays

Silencing of candidate hotst and restriction factor genes was performed through RNA interference (RNAi)-mediated gene silencing as previously described. Field-derived mosquitoes used in these assays were from generations F5 to F13, depending on the strain. Each experiment was performed with mosquitoes from the same generation to ensure valid comparison between experimental and control groups. In brief, three-day-old

female mosquitoes were cold-anesthetized and individually injected with 200 ng of dsRNA specific for the target gene of interest, and GFP dsRNA was used as control in all experiments. The dsRNA-injected mosquitoes were then orally infected with DENV2-supplemented blood at 3-4 days post-dsRNA injection. Midguts were dissected at 7 dpbm for DENV titration by plaque assay. dsRNA was synthesized using the HiScribe T7 *in vitro* transcription kit (New England Biolabs). Primer sequences used for dsRNA synthesis and to confirm gene silencing by real-time PCR are presented in the Appendix1. Due to high sequence identity, it was unavoidable that dsRNA against AAEL010429 also targeted AAEL013577 and AAEL010436 (also putative insect allergen family members), and dsRNA against AAEL015337 also targeted AAEL010599 (also a neutral alpha-glucosidase ab precursor). We cannot however exclude the possibility that these seemingly different transcripts represent the same gene, due to possible genome sequence annotation errors.

DENV titration by plaque assay

DENV2 titers in the mosquito midguts were determined by plaque assay on BHK-21 cells. Individual midguts were homogenized in DMEM using glass beads with a Bullet Blender homogenizer (NextAdvance), 10-fold serially diluted, and then inoculated onto BHK cells seeded to 80% confluence in 24-well plates. Plates were rocked for 15 min at room temperature, and then incubated for 45 min at 37°C and 5% CO_2. Each well was then overlayed with 1 ml of DMEM containing 2% FBS and 0.8% methylcellulose and plates were incubated for 5 days at 37°C and 5% CO_2. Plates were fixed with a methanol / acetone mixture (1:1 volume/volume) for at least 1 h at 4°C, and plaque-forming units

were visualized by staining with 1% crystal violet solution for at least 10 min at room temperature.

RESULTS

Laboratory and field-derived *Ae. aegypti* strains have different degrees of DENV2 susceptibility

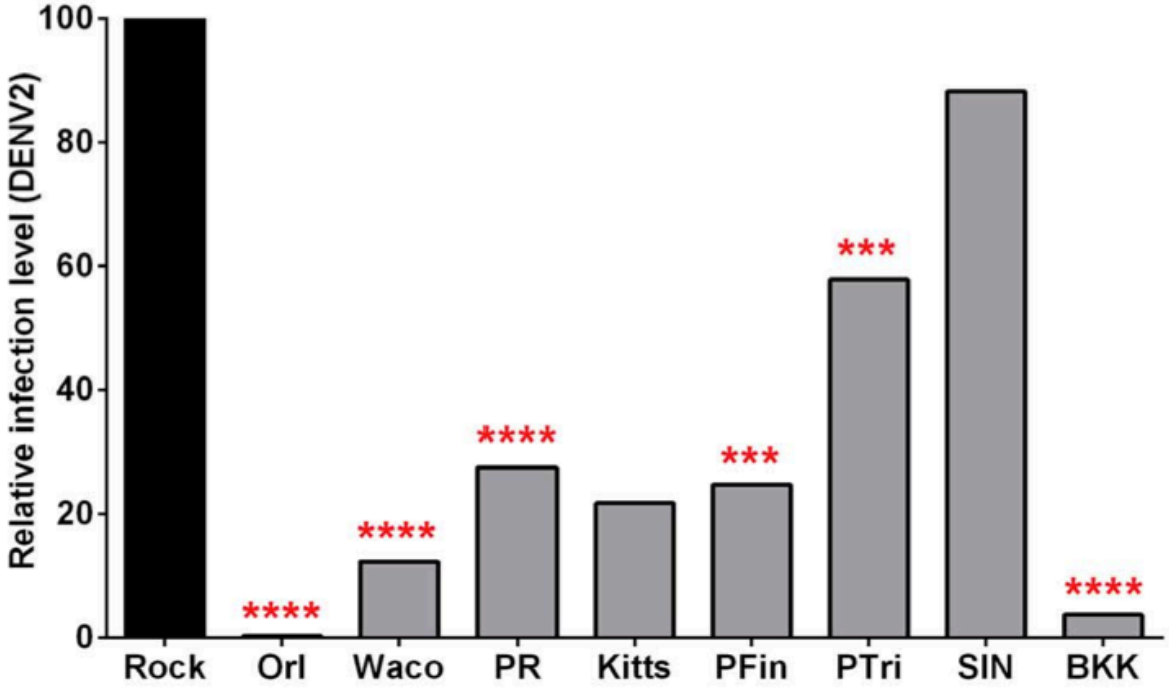
Our lab has established a panel of field-derived and laboratory strains *Ae. aegypti* strains obtained either through generous contributions from collaborators or our own field collections (Table 3.1). Field locations were selected to represent geographically distinct dengue-endemic regions spanning South America, the Caribbean, and Southeast Asia, and laboratory strains were included to allow us to compare DENV susceptibility between laboratory maintained and natural mosquito populations.

To assess their susceptibility for DENV, we orally infected each mosquito strain with DENV2 and assessed midgut virus titers at 7 dpbm. The highly susceptible Rockefeller (Rock) laboratory strain typically used in our group's experiments served as a basis for comparison. To compare susceptibility to DENV2 infection, we calculated the relative infection level of each strain by calculating its median DENV2 titer as a percentage of the median titer of its respective Rock control. We found that Orl, Waco, PFin, PR, PTri, and BKK had significantly lower midgut DENV2 titers as compared to Rock, and median virus titers in Kitts were four-fold lower than in Rock (Figure 3.1). SIN was the only strain with midgut DENV2 titer comparable to Rock strain. Among these *Ae. aegypti* strains, the SIN and PTri strains were the most susceptible, while BKK and Orl were the most refractory to DENV2 infection.

Table 3.1 Origins and name abbreviations of laboratory and field-derived *Ae. aegypti* strains [93,97]**.**

Strain	Abbreviation	Lab/Field	Origin/Approx. date of colonization	GPS coordinates of collection site
Rockfeller	Rock	Lab	Caribbean/~1930s	Unknown
Orlando	Orl	Lab	Orlando, Florida/~1940s	Unknown
Waco	Waco	Lab	Waco, Texas/~1987	Unknown
Puerto Rico	PR	Field	Carolina, Puerto Rico/2010	18°27′6″N, 66°4′8″W
Saint Kitts	Kitts	Field	Saint Kitts/2010	17°17′27″N, 62°41′27″W
Por Fin	PFin	Field	Por Fin, Barranquilla, Colombia/2010	10°58′23″N, 74°49′43″W
Puerto Triunfo	PTri	Field	Puerto Triunfo, Colombia/2010	5°52′0″N, 74°39′0″W
Singapore	SIN	Field	Singapore, Singapore/2010	1°22′12.6″N, 103°50′44.23″E
Bangkok	BKK	Field	Bangkok, Thailand/2011	13°39′12″N, 100°24′19″E

Figure 3.1 Susceptibilities of *Ae. aegypti* strains to DENV2 infection. Relative median DENV2 midgut infection levels at 7 days post-bloodmeal (dpbm) for each *Ae. aegypti* strain, compared to the Rockefeller strain. Statistical analyses performed by using Mann-Whitney test using Prism software, ***:$p < 0.001$, ****: $p < 0.0001$ compared to Rock.



Mosquito immune signaling pathways and the RNAi pathway control DENV2 infection to different degrees in various *Ae. aegypti* strains

Transcriptomic analyses by whole genome microarray assays revealed that differences in basal levels of immune related gene (IMM) transcript abundance among mosquito strains is an important factor in determining susceptibility to DENV [93,97]. These IMM are likely to be regulated by major immune signaling pathways, the Toll, IMD, and JAK/STAT pathways, which regulate the mosquito immune response to a variety of pathogens [32]. We examined the contributions of these pathways to refractoriness in these strains. In addition, since the RNAi pathway is an important controller of DENV infection in *Ae. aegypti* [85,92], we also examined its role in this regard. Each pathway was inhibited or activated through the RNAi-mediated knockdown of a key pathway component or regulator, and the effect of this manipulation on midgut DENV2 titers was assessed.

In the Orl strain, individually compromising the Toll, IMD, JAK/STAT, and RNAi pathways via knockdown of MyD88, IMD, Dome, and Dcr2, respectively, resulted in a significant and dramatic increase in midgut DENV2 titers to a level that was comparable to what typically seen in Rock (Figure 3.2A), suggesting that each of these pathways is a major contributor to the refractoriness seen in Orl. In the BKK strain, compromising the Toll, IMD, JAK/STAT or RNAi pathways only resulted in a non-significant 3- to 5-fold increase in midgut virus titers (Figure 3.2A), suggesting the action of BKK strain-specific DENV restriction factor(s) that operate independently of these pathways (Figure 3.2A).

Conversely, activating immune signaling pathways by silencing pathway negative regulators should render the susceptible Rock and SIN strains more refractory to DENV

infection (Figure 3.2B). In agreement with our previous studies [20,93,94], silencing the Toll and JAK/STAT pathway negative regulators Cactus and PIAS in the Rock strain resulted in a significant decrease in midgut DENV titers, while silencing the IMD pathway negative regulator Caspar resulted in a non-significant decrease (Figure 3.2B). As expected, silencing Cactus in the SIN strain also resulted in a significant decrease in virus titers, but no effect was seen for PIAS or Caspar. It is possible that the JAK/STAT and IMD pathways are already operating at maximum capacity, especially given the high viral load observed in this strain, or that the SIN strain possesses factors acting independently of these pathways that facilitate DENV infection. From these data, we speculate that the basal activation levels of immune pathways may be higher in refractory mosquitoes, and that these elevated levels in refractory strains may contribute to refractoriness by increasing the transcript abundance of various immune effectors.

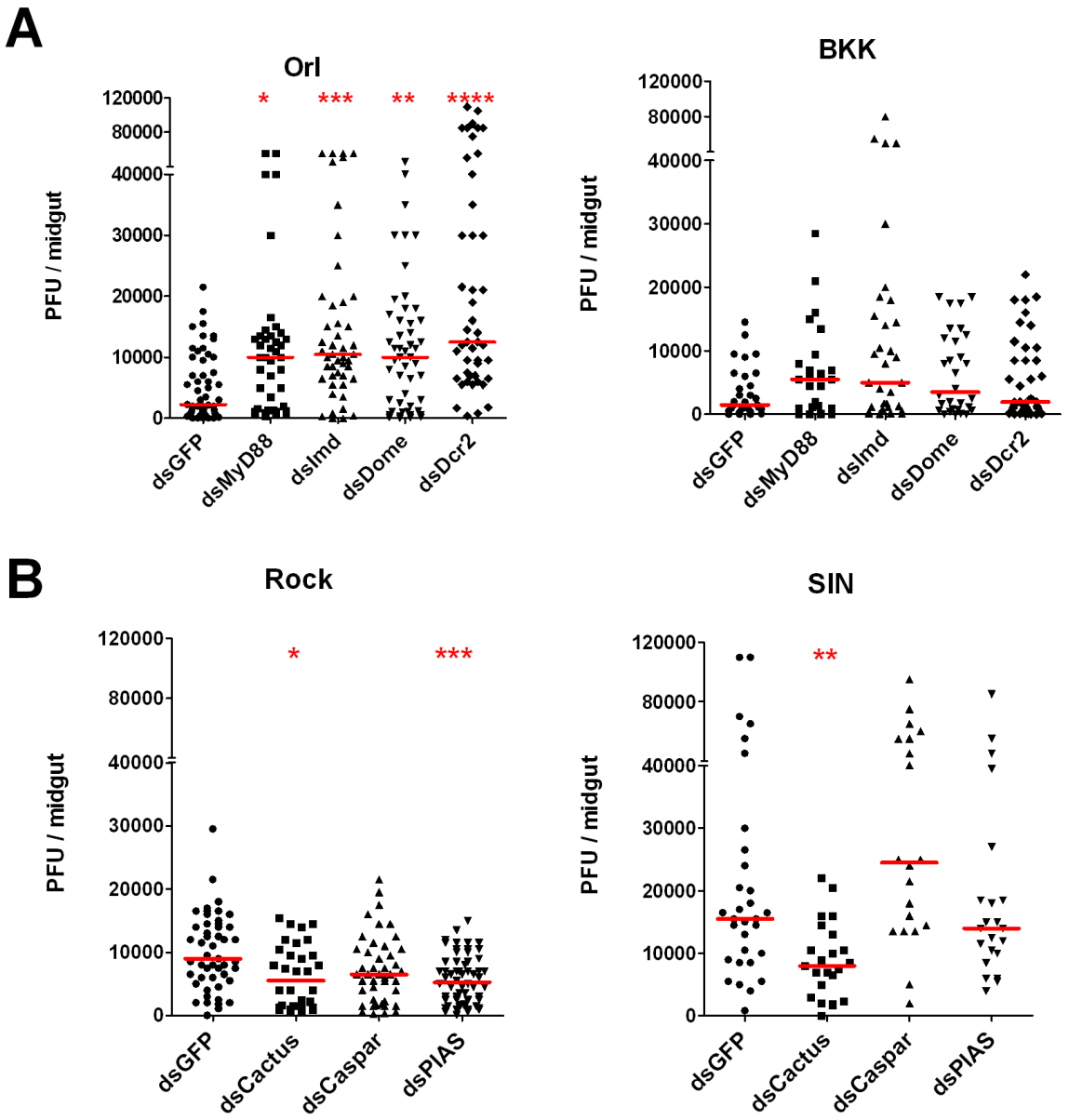


Figure 3.2 Contributions of the Toll, Imd, JAK-STAT, and RNAi pathways to the control of DENV2 in refractory and susceptible mosquito strains. Midgut DENV2 titers at 7 dpbm in (A) MyD88, Imd, Dome, and Dcr2-silenced Orl and BKK mosquitoes, and (B) Cactus, Caspar, and PIAS-silenced Rock and SIN mosquitoes. Data are a pool of at least three independent biological replicates. ****, $p<0.0001$; ***, $p<0.001$; **, $p<0.01$ *, $p<0.05$ compared to dsGFP-treated mosquitoes in Dunn's post-test after Kruskal-Wallis test.

Transcriptomic comparison between refractory and susceptible *Ae. aegypti* reveals candidate DENV restriction and host factors

Other than immune-related transcripts, genes in other functional groups can also influence DENV infection in different fashion. DENV host factors (HFs) are host genes that the virus uses to facilitate its infection or are required to complete its replication cycle. To identify putative HFs, hierarchical cluster analyses of the transcriptomes were performed between the two most refractory (Orl, BKK) and the two most susceptible (PTri, SIN) mosquito strains (Figure 3.3). In addition to expanding our limited knowledge of molecular interactions between DENV and *Ae. aegypti* mosquitoes, this analysis also offered the potential to identify molecular determinants that affect vector competence in field mosquitoes.

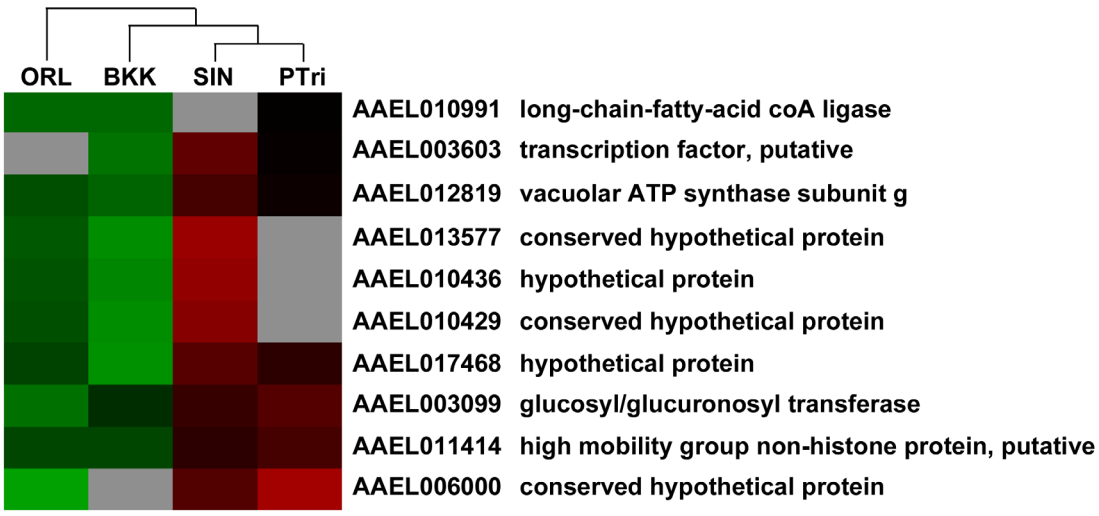


Figure 3.3 Identification of novel candidate DENV host factors through hierarchical clustering. Selected gene clusters obtained through hierarchical clustering of the midgut transcriptomes of the two most refractory (Orl, BKK) and two most susceptible (PTri, SIN) *Ae. aegypti* strains. Genes that displayed differentially abundant transcripts in at least two of the four strains were included in the analysis. Red and green indicate enriched and depleted transcript abundance, respectively, compared to the Rock strain. [97]

Hierarchical clustering in the midgut revealed potential gene cluster for host factors that had lower transcript abundance in the refractory Orl and BKK strains but higher transcript abundance in the susceptible PTri and SIN strains (Figure 3.3A). The cluster consisted of a vacuolar ATP synthase (vATPase) subunit G (AAEL012819), a glucosyl/gluronosyl transferase (AAEL003099), a putative high mobility group non-histone protein (AAEL011414), and three hypothetical protein genes that encoded insect allergen repeats domain structure (AAEL013577, AAEL010436, and AAEL010429).

Functional characterization of selected candidate DENV host factors

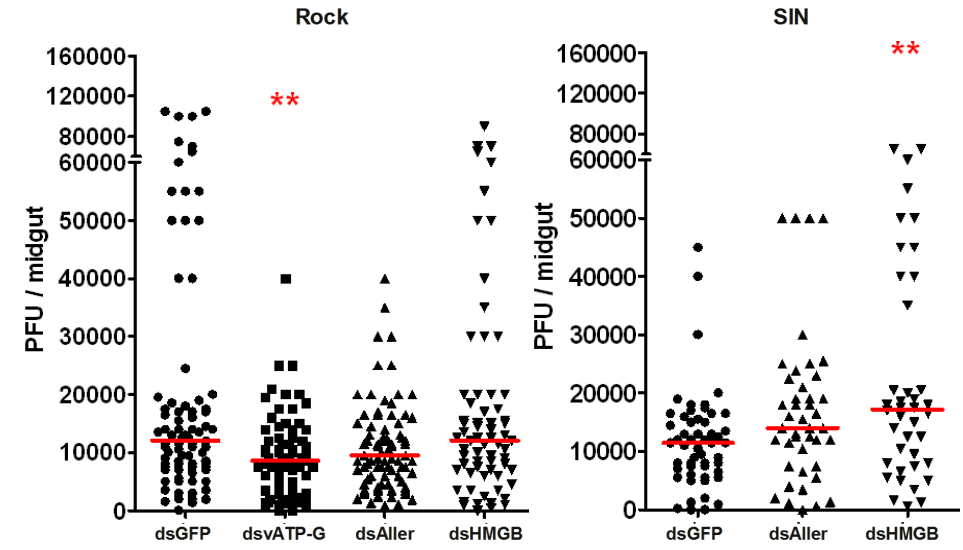
We selected three candidate DENV host factors identified through hierarchical clustering for functional analysis in the susceptible Rock, PTri, and SIN mosquito strains (Table 3.2) and hypothesized that knockdown of these genes via RNAi-mediated gene silencing would render mosquitoes more refractory to DENV.

Table 3.2 Candidate DENV host factors selected for functional characterization via RNAi-mediated gene silencing.

Gene ID	Name	Functional group	Orl	BKK	P Tri	SIN
AAEL011414	high mobility group non-histone protein	M	-0.83	-0.83	0.82	0.53
AAEL003099	glucosyl/glucuronosyl transferases	M	-1.34	-0.54	1.01	0.66
AAEL016980	Hypothetical protein	U	-0.77	-0.29	1.71	1.92
AAEL017468	hypothetical protein	U	-0.79	-1.72	0.54	1.03
AAEL012819	vacuolar ATP synthase subunit g	TRP	-0.92	-1.18	0.15	0.82

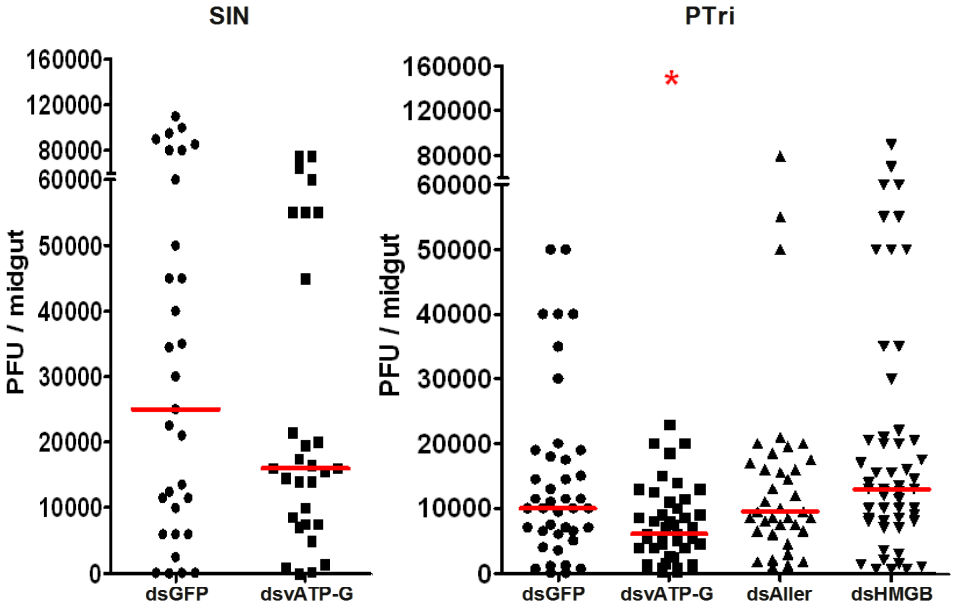
Knockdown of the vATPase subunit G gene (AAEL012819) resulted in significantly reduced midgut DENV titers in both the Rock and PTri strains and a non-significant decrease in the SIN strain, suggesting that it does indeed function as a DENV host factor (Figure 3.4). Surprisingly, knockdown of the gene encoding a HMGB protein (AAEL011414) resulted in a significant increase in SIN strain DENV titers, but it had no effect in the other two mosquito strains (Figure 3.4). Knockdown of the putative insect allergen had no effect in any of the strains.

Our panel of *Ae. aegypti* strains is also an excellent platform for characterizing or validating candidate DENV host factors identified through other screening methodologies. Sessions *et al. (*2009) identified numerous candidate DENV host factors by performing a high-throughput RNAi screen in *Drosophila* cells [12,59,93]. We selected the *Ae. aegypti* orthologs of three hits from this study for functional characterization in our susceptible mosquito strains. Only the knockdown of AAEL002430, which codes for an N-acetylglucosamine-6-phosphate deacetylase, resulted in significantly decreased DENV titers in the Rock strain but not in the SIN or PTri strains (Figure 3.5).



	dsGFP	dsvATP-G	dsAller	dsHMGB
n	83	74	83	67
Median	12000	8500	9500	12000
Mean	21317	9197	10961	17959
SD	25088	7143	7647	19310

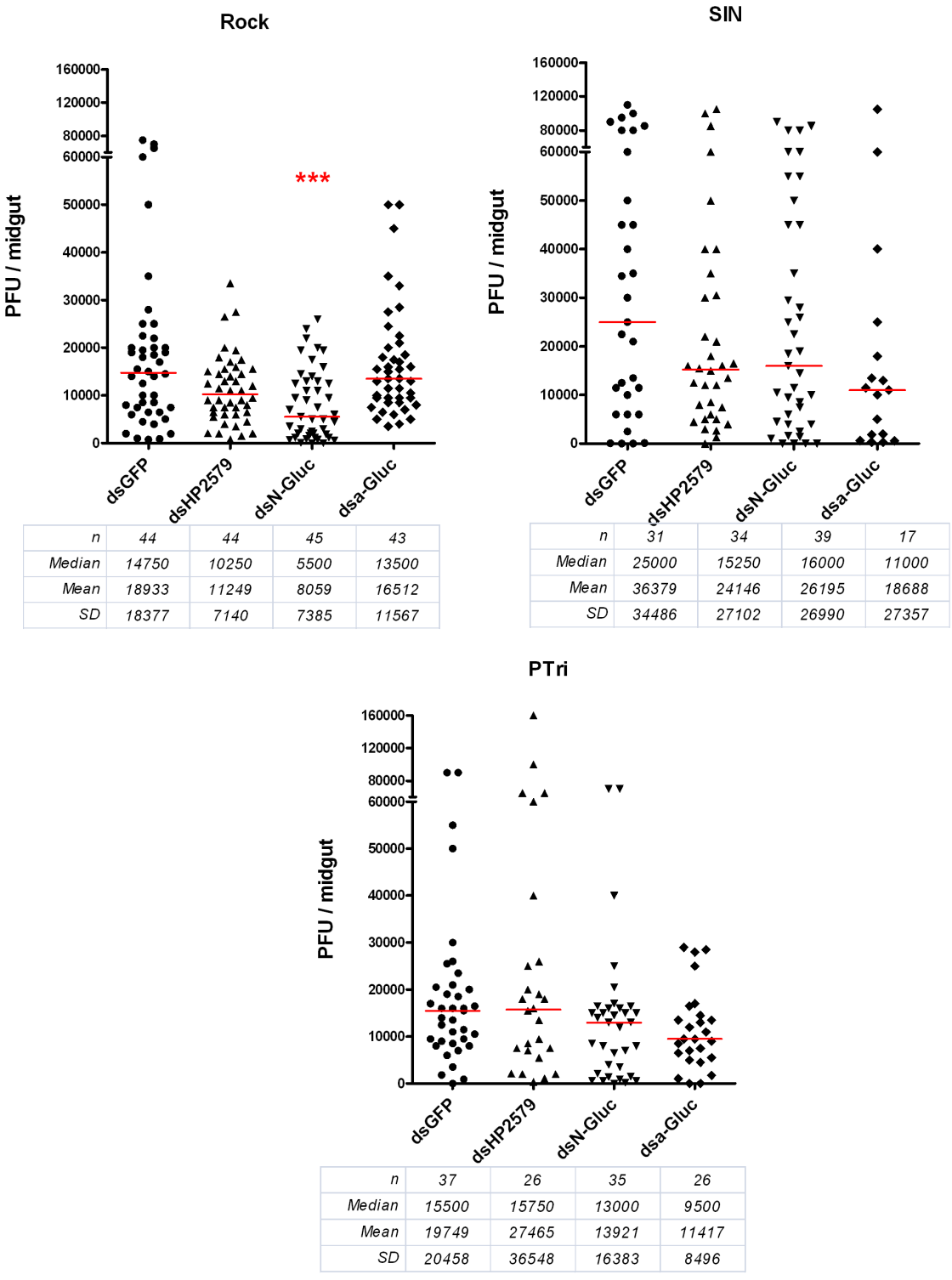
	dsGFP	dsAller	dsHMGB
n	54	43	40
Median	11500	14000	17250
Mean	11851	17031	22390
SD	8449	13055	18795



	dsGFP	dsvATP-G
n	31	29
Median	25000	16000
Mean	36379	24762
SD	34486	23852

	dsGFP	dsvATP-G	dsAller	dsHMGB
n	41	45	37	54
Median	10000	6000	9500	13000
Mean	14489	7400	14501	20244
SD	13484	5690	15818	20140

Figure 3.4 Effect of candidate DENV host factor knockdown on midgut DENV2 titers in susceptible *Ae. aegypti* strains. Midgut DENV2 titers at 7 dpbm, and data are a pool of at least three independent biological replicates. **, $p \leq 0.01$ *, $p \leq 0.05$ compared to dsGFP-treated mosquitoes in Dunn's post-test after Kruskal-Wallis test.



n	44	44	45	43
Median	14750	10250	5500	13500
Mean	18933	11249	8059	16512
SD	18377	7140	7385	11567

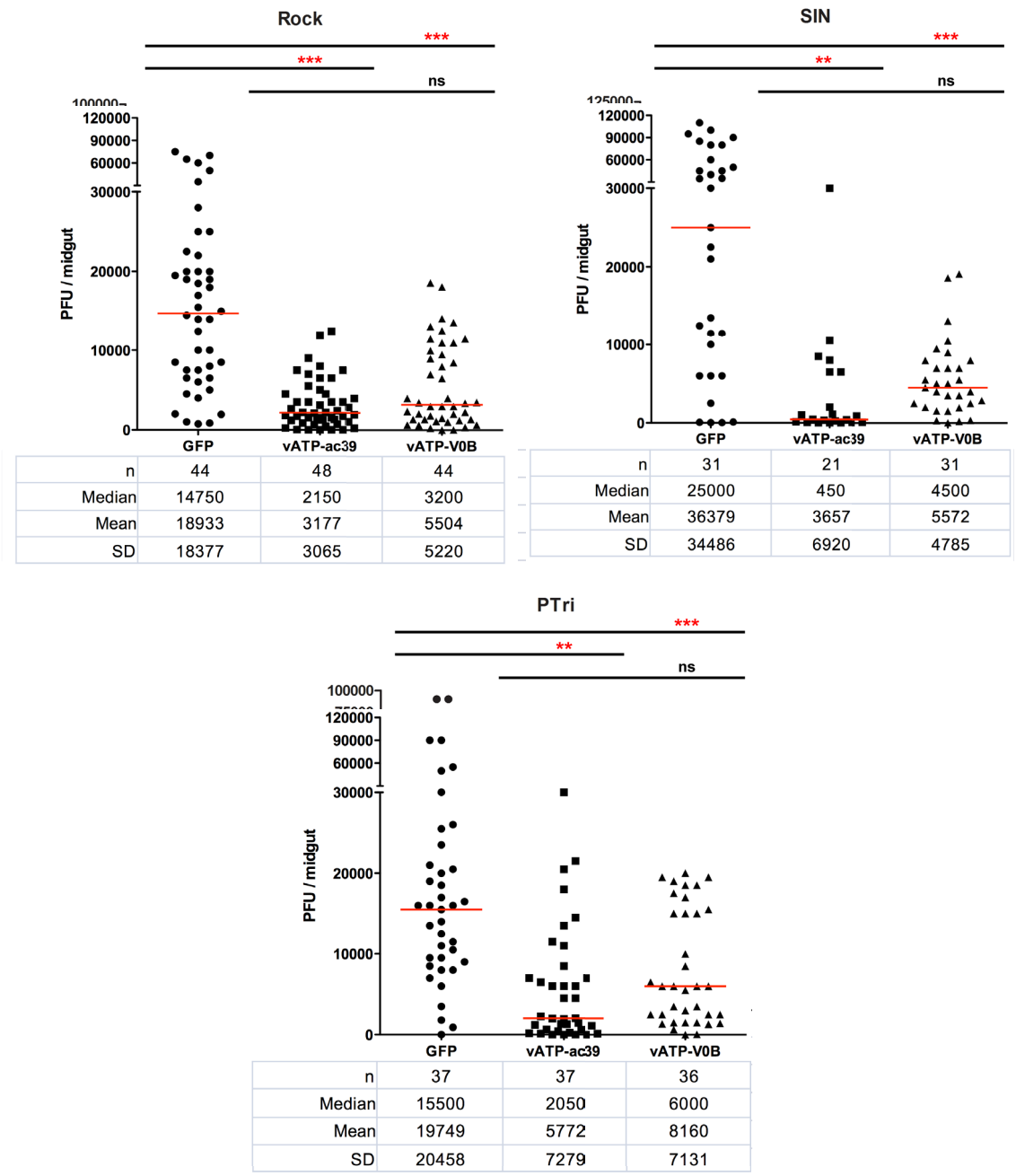
n	31	34	39	17
Median	25000	15250	16000	11000
Mean	36379	24146	26195	18688
SD	34486	27102	26990	27357

n	37	26	35	26
Median	15500	15750	13000	9500
Mean	19749	27465	13921	11417
SD	20458	36548	16383	8496

Figure 3.5 Effect of candidate DENV host factor selected from high-througput screen knockdown on midgut DENV2 titers in susceptible *Ae. aegypti* strains. Data are a pool of at least three independent biological replicates. **, $p \leq 0.01$ *, $p \leq 0.05$ compared to dsGFP-treated mosquitoes in Dunn's post-test after Kruskal-Wallis test.

vATPase subunits are important DENV host factors in *Ae. aegypti*

In addition to our results, previous high-throughput screening for insect and human DENV host factor suggested that several vATPase subunits might serve as DENV host factors in the mosquitoes [93,97,157]. In the previous section, vATPase subunit g (vATP-g: AAEL012819) was experimentally confirmed as a host factor; however, the requirement of other subunits on DENV replication was unknown. We performed RNA-mediated gene silencing on more vATPase subunits to confirm that the function of the whole vATPase enzyme complex, not just the vATP-g as DENV host factors. The knock down of vATPase subunits ac39 (vATP-ac39), and vATPase subunits V0B (vATP-V0B) resulted in a reduction in midgut DENV titers in all the mosquito strains tested ranging from 61-98% (Figure 3.6).



	GFP	vATP-ac39	vATP-V0B
n	44	48	44
Median	14750	2150	3200
Mean	18933	3177	5504
SD	18377	3065	5220

	GFP	vATP-ac39	vATP-V0B
n	31	21	31
Median	25000	450	4500
Mean	36379	3657	5572
SD	34486	6920	4785

	GFP	vATP-ac39	vATP-V0B
n	37	37	36
Median	15500	2050	6000
Mean	19749	5772	8160
SD	20458	7279	7131

Figure 3.6 Effect of vATPase subunits knockdown on midgut DENV2 titers in susceptible *Ae. aegypti* strains. Data are a pool of at least three independent biological replicates. **, $p \leq 0.01$ *, $p \leq 0.05$ compared to dsGFP-treated mosquitoes in Dunn's post-test after Kruskal-Wallis test.

DISCUSSION

A major finding of this study is that we confirmed the role of the Toll, JAK-STAT, and IMD pathways in controlling DENV infection in refractory *Ae. aegypti* strains. Our results indicate that basal levels of mosquito immunity influence vector competence, in addition to other factors, such as the availability of receptors or host factors required for virus infection and replication. While these may seem to be obvious conclusions, determinants of vector competence in natural mosquito populations have been very poorly studied, and the idea of a basal level of immunity is not necessarily a given.

The importance of classical immune pathways varies among strains as we observed that activation of the JAK/STAT pathway resulted in a decreased midgut DENV2 titers in Rock but not SIN strain. Similarly, inhibition of immune pathways in BKK strain also results in a weaker increase of DENV2 midgut titers when compared to the inhibition of the immune pathways in Orl strain. These results emphasized that there is a natural variation in how each immune pathway contributes to DENV susceptibility.

Our data also suggest that while the major immune signaling pathways play a key role in determining DENV susceptibility in both laboratory- and field-derived mosquitoes, strain-specific factors acting independently of these pathways are also likely to make important contributions. This panel of *Ae. aegypti* strains also allowed us to functionally characterize several candidate DENV host factors, which were selected because they displayed increased transcript abundance in susceptible strains but decreased transcript abundance in refractory strains.

RNAi-mediated knockdown of the vATPase subunit G gene (vATP-g, AAEL012819) rendered susceptible mosquito strains more refractory to midgut DENV2 infection, suggesting that it does indeed function as a DENV host factor. In the vATPase complex, subunit G is part of a peripheral stalk connecting the peripheral domain (which catalyzes ATP hydrolysis) and the integral domain (through which protons are translocated), and it may play the role of stator in the rotary machinery. In yeast, deletion of subunit G leads to complete loss of assembly of the complex [92]. In our dataset, vATP-G was the only subunit to show a clear pattern of enriched basal-level transcript abundance in susceptible strains. However, functional assays of other vATPase subunits as DENV host factors by RNAi-mediated gene silencing suggested that the function of vATPase enzyme as a whole complex is required for efficient DENV infection in *Ae. aegypti*. Moreover, we observed the effect across laboratory-adapted and field-derived mosquito strains emphasizing a crucial role of this enzyme complex in facilitating DENV infection. vATPases are multisubunit enzymes found in the membranes of endosomes, lysosomes, and secretory vesicles that bring about the acidification of these organelles via an ATP-dependent rotary mechanism that drives proton transport [92,98,99,158,159]. This is an important step in the DENV replication cycle, since an acidic pH in the late endosome is required for DENV fusion and entry. Although bafilomycin, a specific inhibitor of vATPases, has been reported to inhibit flaviviruses in both mammalian and insect cells [93,94,101], this is to our knowledge the first functional evidence in adult *Ae. aegypti* for the role of the vATPase complex as a DENV host factor, and it suggests that this class of enzyme could be a promising target for chemical interventions, such as the development of small-molecule inhibitors of DENV replication.

The knockdown of a candidate DENV host factor, HMGB gene (AAEL011414), unexpectedly resulted in a significant increase in SIN midgut DENV titers, suggesting that it may function as a restriction factor instead in this strain. This is in agreement with the relatively well-studied role of this gene family in mammalian cells, in which human HMGB1 translocates out of the nucleus and is released from DENV-infected epithelial and dendritic cells, triggering a pro-inflammatory antiviral response [160-162]. While our functional data suggest that *Ae. aegypti* HMGB may play a similar antiviral role at least in the SIN strain, the transcript abundance pattern of this gene across our panel runs counter to this possibility. Since HMGB family members are also abundant in the nucleus, where they regulate chromatin structure, transcription, and DNA repair and replication[163], the transcription pattern we observed may have more to do with one or more of these functions than with a response to DENV. A recent study characterizing a separate *Ae. aegypti* HMGB family member (AAEL011380) confirmed that it, like human HMGB1, effectively binds and alters the topology of DNA. The authors suggest diverse regulatory roles for mosquito HMGB family members, for example in vitellogenesis and molting, in addition to innate immunity[164]. This example illustrates the idea that the transcriptome is shaped by multiple environmental factors, and it underscores the importance of performing functional assays to validate any predictions drawn from transcriptomic data.

High-throughput RNAi screens have proved to be a powerful method for identifying candidate flavivirus host and restriction factors in vertebrate and invertebrate systems [93]. Our panel of laboratory and field-derived *Ae. aegypti* strains with a range of DENV susceptibilities is a valuable tool for functionally characterizing these candidates.

We tested the *Ae. aegypti* orthologs of three candidate DENV host factors identified through an RNAi screen in *Drosophila* cells [93] for ability to modulate resistance to infection by silencing them in three susceptible mosquito strains. However, only the knockdown of a gene encoding an N-acetylglucosa- mine-6-phosphate deacetylase (N-Gluc, AAEL002430) significantly decreased midgut DENV2 titers, and this effect was only seen in the Rock strain and not in the SIN or PTri strains. This result was somewhat unexpected, given the strength of these hits in the initial high-throughput RNAi screen [93], but it again illustrates the utility of performing functional assays in adult mosquitoes.

CHAPTER 4

Molecular characterization of *Aedes aegypti* ML and Niemann-Pick type C family members as dengue virus host factors

Parts of this work has been published in:

Jupatanakul N, Sim S, Dimopoulos G. *Aedes aegypti* ML and Niemann-Pick type C family members are agonists of dengue virus infection. Developmental and Comparative Immunology. 2014;43: 1–9. doi:10.1016/j.dci.2013.10.002
Jupatanakul N. *Aedes aegypti* ML and Niemann-Pick type C family members are agonists of dengue virus infection. Master thesis. 2012.

ABSTRACT

Upon exposure to dengue virus, the *Aedes aegypti* mosquito vector mounts an anti-viral immune defense by activating the Toll, JAK/STAT, and RNAi pathways, thereby limiting infection. While these pathways and several other factors have been identified as dengue virus antagonists, our knowledge of factors that facilitate dengue virus infection is limited. Previous dengue virus infection-responsive transcriptome analyses have revealed an increased mRNA abundance of members of the myeloid differentiation 2-related lipid recognition protein (ML) and the Niemann Pick-type C1 (NPC1) families upon dengue virus infection. These genes encode lipid-binding proteins that have been shown to play a role in host-pathogen interactions in other organisms. RNAi-mediated gene silencing of a ML and a NPC1 gene family member in both laboratory strain and field-derived *Ae. aegypti* mosquitoes resulted in significantly elevated resistance to dengue virus in mosquito midguts, suggesting that these genes play roles as dengue virus agonists. In addition to their possible roles in virus cell entry and replication, gene expression analyses suggested that ML and NPC1 family members also facilitate viral infection by modulating the mosquito's immune competence. Our study suggests that the dengue virus influences the expression of these genes to facilitate its infection of the mosquito host.

INTRODUCTION

Through genome-wide transcriptomic analyses, in conjunction with RNAi-mediated gene silencing, we have identified the Toll and JAK-STAT pathways as key DENV antagonists that act by controlling virus restriction factors [12,59,103]. DENV infection-responsive transcriptome analyses have revealed that the transcript abundance of five members of two lipid-binding protein gene families, the myeloid differentiation 2-related lipid recognition protein (ML) and Niemann Pick-type C1 (NPC1) families, is increased in response to DENV infection. Since DENV is an enveloped virus and its outer shell is lipid-based, these lipid-binding proteins are likely to play a role(s) in mosquito-virus interactions.

The ML domain is a lipid recognition protein domain found in several proteins with lipid-binding properties [100,157,158]. Members of this family have diverse functions associated with lipid recognition, including pathogen recognition, lipid trafficking and metabolism, and pheromone perception [98,99,105,106,158,159]. A role for the ML domain in immune recognition has been described for the vertebrate MD2 protein and its insect homologs. MD2 is a secreted glycoprotein that mediates the activation of the vertebrate Toll-like receptor 4 (TLR4) upon exposure to bacterial lipopolysaccharide (LPS) [101,165,166]. *Drosophila* MD2 homologs have been shown to mediate the activation of the immune deficiency (IMD) immune signaling pathway upon exposure to lipopolysaccharide (LPS) [160,167]. The *An. gambiae* homolog of ML, AgMDL1, is involved in the mosquito's immune defense against *Plasmodium falciparum* infection [103,168]. Niemann-Pick disease type C1 (NPC1) is another class of lipid-binding proteins that is responsible for cholesterol transport and homeostasis; these

proteins function together with the NPC2 proteins in the late endosomal/lysosomal system [12,40,100,158]. NPC1 has been shown to be required for the Ebola virus to escape from the vesicular compartment [97,105,106], but the function of the NPC1 family in DENV infection in mosquito has yet not been investigated.

Although these lipid-binding protein families have been shown to be involved in virus-host interaction and immune responses in various systems, little is known about their function in the *Ae. aegypti*-DENV interaction. Here, we investigated the role of *Ae. aegypti* ML and NPC1 gene family members in modulating DENV infection in the mosquito by conducting RNAi-mediated gene silencing and gene expression studies. Our results suggest roles for the ML and a NPC1 proteins as agonists of DENV in the mosquito. Furthermore, our data suggest that the virus might influence the expression of these genes to facilitate its infection, emphasizing the importance of lipid-binding proteins in viral infection of insects.

MATERIALS AND METHODS

Bioinformatics analyses and genes selection

The gene sequences and gene annotations for the insect ML and NPC gene families were obtained from the ImmunoDB (http://cegg.unige.ch/Insecta/immunodb) and Vectorbase (http://aaegypti.vectorbase.org/) databases [12,165,166]. To compare sequence similarity, a multiple sequence alignment (MSA) was generated using t-coffee software (http://www.tcoffee.org/) [156,167]. The MSA was then used to generate a phylogenetic tree using MEGA 5.05 software [168,169]. ML and NPC genes that potentially play a role in DENV infection were suggested from the transcriptional changes of these genes in previous microarray-based transcriptome studies [12,40,169], Dimopoulos group, unpublished data).

Mosquito strains and mosquito maintenance

The mosquitoes used for most of the experiments were of the *Ae. aegypti* Rockefeller/UGAL strain. A second mosquito strain was used to confirm that the results were common among different mosquito strains. This strain was a recently colonized *Ae. aegypti* population obtained from the Caribbean island of Saint Kitts (sixth to seventh generation) [97]. The mosquitoes were maintained on a 10% sugar solution at 27^{o}C and 95% humidity with a 12-hr light/dark cycle, following the protocol described previously [12].

Cell culture

The *Ae. albopictus* C6/36 cell line was used to propagate DENV. It was maintained in complete minimum essential medium (MEM) supplemented with 10%

heat-inactivated FBS, 1% L-glutamine, 1% MEM non-essential amino acids, 10 unit/mL penicillin, and 10 μg/mL streptomycin at 32°C and 5% CO_2.

The baby hamster kidney (BHK) cell line was used for plaque assays to determine DENV titter. BHK cells were maintained in complete Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 10 unit/mL penicillin, 10 μg/mL streptomycin, and 5 μg/mL plasmocin at 37°C and 5% CO_2.

Genes silencing by RNA interference

The role of the ML and NPC1 genes in DENV infection in *Ae. aegypti* was assessed using RNA interference-mediated gene silencing as described previously [156]. In brief, dsRNAs were constructed using in vitro transcription with the HiScribe™ T7 In Vitro Transcription Kit (New England Biolabs). Approximately 200 ng of dsRNA was injected into the thorax of cold-anesthetized 3- to 4-day-old female mosquitoes using a nano-injector. The dsRNA-injected mosquitoes were kept in the insectary under the conditions mentioned above. Gene silencing efficiency, evaluated using real-time PCR, was determined by comparison to the GFP dsRNA-injected group at 3 days after dsRNA injection, the time when we infected mosquito with DENV.

DENV propagation and viral infection in the mosquito

The DENV strain used in these experiments was DENV serotype 2 (New Guinea C strain, DENV-2). The virus was propagated in the C6/36 cell line according to a protocol previously described [169]. In brief, DENV stock was added to a 75-cm^2 flask of C6/36 cells at 80% confluence to yield a multiplicity of infection of 1. The virus-infected cells were harvested 6 days after infection. The virus was extracted from the cells by freezing and thawing for two cycles in dry CO_2 and a 37°C water bath, centrifuged at

800g for 10 min, and mixed 1:1 with commercial human blood. The infectious blood meal (~10^6-10^7 colony-forming units [cfu]/ml) was maintained at 37°C for 30 min prior to membrane feeding to 5- to 7-day-old or 3-day post-dsRNA-injected mosquitoes. Blood-fed mosquitoes were separated on ice and maintained under the conditions mentioned above.

DENV titration by plaque assay

Virus titers in the midguts were determined at 7 dpbm according to an established protocol [12,169]. Mosquito midguts were dissected in sterile 1XPBS and stored in complete DMEM medium at -80°C until used. Midgut samples were homogenized using a homogenizer (Bullet Blender, Next Advance) with 0.5-mm glass beads. The virus-containing homogenates were 10-fold serially diluted in DMEM media, then inoculated onto 80% confluent BHK cells in 24-well plates. The cell monolayer was then overlaid with 0.8% methylcellulose in complete DMEM medium, and kept for 5–7 days at 37 °C and 5% CO_2. Plaque forming units (PFUs) were visualized by crystal violet staining. All procedures involving DENV-2 infections were carried out in a Biological Safety Level 2 laboratory.

Gene expression and silencing efficiency analysis by quantitative PCR

Gene silencing efficiency and expression of the genes of interest were assayed using real-time PCR. Whole mosquito samples were used for the silencing efficiency analysis while different mosquito organs were used for gene transcript abundance analyses. Mosquito samples were dissected in 1XPBS, collected in RLT buffer (QIAGEN), and then stored at -80°C until extraction. Total RNA was extracted from tissue samples using the RNeasy Mini Kit (QIAGEN). To construct cDNA, RNA samples

were treated with Turbo DNase (Ambion) before reverse transcription with a MMLV Reverse Transcriptase kit (Promega) according to the manufacturer's instructions. The cDNA was then used to determine gene expression by quantitative PCR using SYBR® Green PCR Master Mix (Applied Biosystem). Transcript abundance of genes was compared to the expression of the ribosomal protein gene S7 as a normalization control. The primers specific for each gene are presented in Appendix 1.

Statistical analysis of midgut DENV titer and gene expression level

The DENV midgut titers of GFP dsRNA and experimental groups were compared using the plaque assay results from at least two biological replicates, with the elimination of outliers (data outside the median plus 2SD). The significance values were determined using either the Kruskal-Wallis test with Dunn's post-test or the Mann-Whitney test (* $p<0.05$, ** $p<0.01$).

RESULTS

ML and NPC1 gene families are distinct and expanded in *Ae. aegypti*

The *Ae. aegypti* genome contains 26 ML-like genes, suggesting an expansion of this gene family when compared to other insects (19 in *Culex pipiens quinquefasciatus*, 15 in *Anopheles gambiae*, and 8 in *Drosophila melanogaster*) (immunoDB database (http://cegg.unige.ch/Insecta/immunodb)) [166,170]. Of these 26 members, 24 contain the ML domains and are annotated as Niemann-Pick C 2 (NPC2) genes, while the remaining two are annotated as Niemann-Pick C1 (NPC1) proteins. However, further sequence analysis revealed that the putative NPC1 does not contain the MD2 domain. Thus, the *Ae. aegypti* NPC2 genes will be considered as ML gene family genes (as per

the annotation in immunoDB database). All six cysteine residues are conserved in all the ML genes used for analysis, suggesting their importance for protein structure and function across organisms. Phylogenetic analysis of the ML genes suggests four groups of *Ae. aegypti* ML genes, with groups 2 being unique to *Ae. aegypti* (Figure 4.1A). The amino acid sequence of AaegML13 is the most conserved when compared to the human NPC2 gene. The conserved cholesterol-binding pocket amino acids of the mouse NPC2 proteins, F66 V96 and Y100 [171], were mutated in some *Aedes* ML genes (Appendix 4).

While the human genome contains only one NPC1, the mosquito genomes of *C. pipiens quinquefasciatus, Ae. gambiae, and Ae. aegypti* contain two NPC1 genes (NPC1a and NPC1b), as in *Drosophila* (Figure 4.1B) [172]. The alignment revealed the presence of all 13 conserved transmembrane domains and a sterol-sensing domain in NPC1b; in contrast, NPC1a has approximately 150 amino acids truncated at N-terminus.

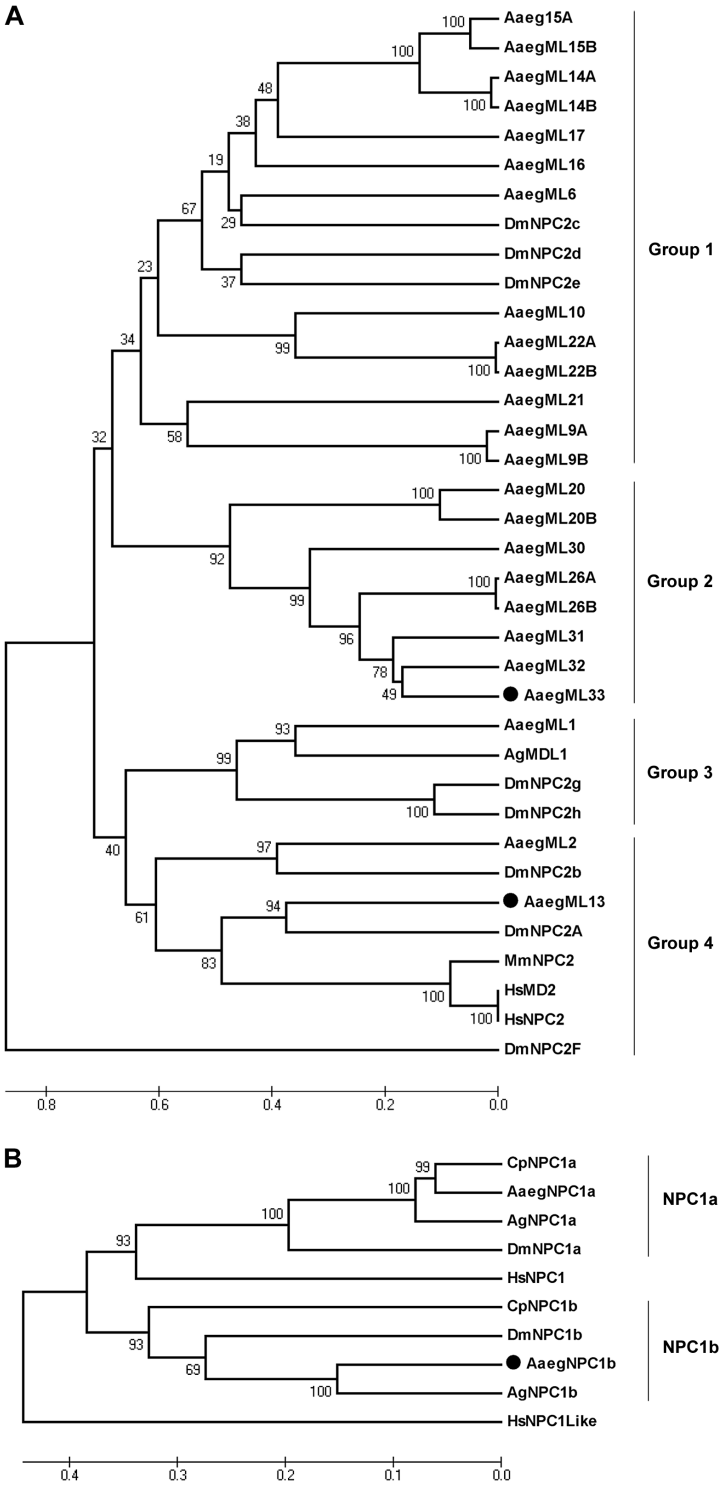
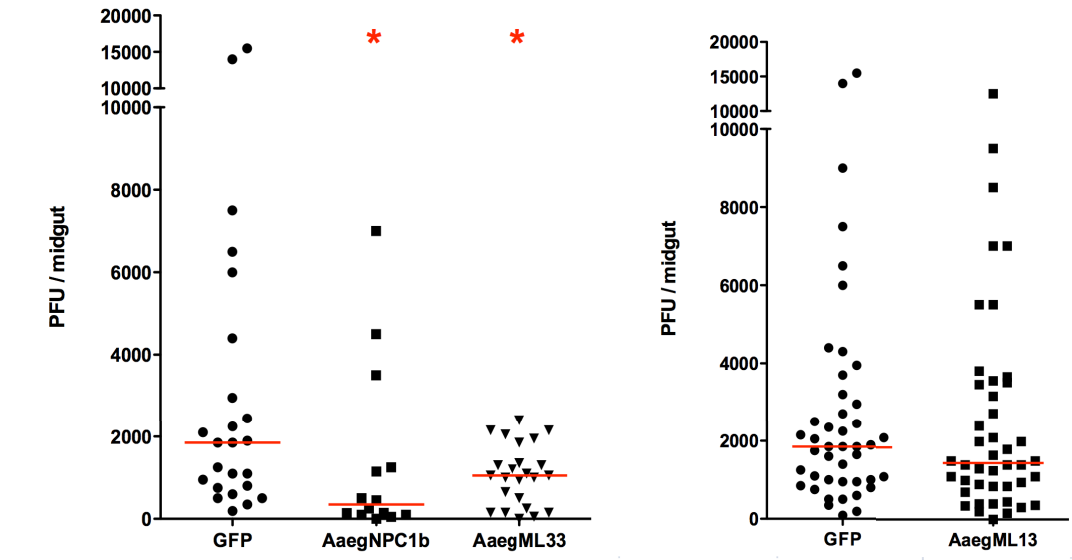


Figure 4.1 Phylogenetic tree of the ML and NPC1 gene family in insects. (A) All the *Ae. aegypti* ML family members were compared to all eight *D. melanogaster* ML genes (DmNPC2 A–F), *An. gambiae* ML1 (AgMDL1), *Homo sapiens* MD2 (HsMD2), *H. sapiens* NPC2 (HsNPC2), and *Mus musculus* NPC2 (MmNPC2). (B) The *Ae. aegypti* NPC1 genes were compared to *D. melanogaster* (Dm), *Anopheles gambiae* (Ag), C*ulex*

pipiens quinquefasciatus (Cp), and *H. sapiens* (Hs). The genes selected for further study are indicated by filled circles.

Ae. aegypti ML and NPC1 family members facilitate DENV infection

Using genome-wide transcriptome analyses, we have previously shown that the mRNA abundance of members of the *Ae. aegypti* NPC1 and ML gene families (3 of 24 members) were found to be modulated by DENV infection in the mosquito midgut and carcass tissue compartments [12,40], suggesting possible roles for these proteins as DENV host or restriction factors (data is presented in Appendix3). Upon DENV infection, the transcript abundance of AaegML13 and AaegML33 increased in the mosquito midgut (2.2 fold) and carcass (2.8 fold), respectively, and the mRNA abundance of AaegNPC1b increased in the midgut compartment (1.9 fold). We further investigated whether any of these genes is involved in modulating DENV infection in the midgut by using RNAi-mediated gene silencing in laboratory Rockefeller/UGAL strain *Ae. Aegypti,* followed by infection with DENV via blood feeding (Figure 4.2). Silencing of AaegNPC1b and AaegML33 resulted in a significant reduction in the median DENV titer in mosquito midguts by 81.1% and 43.24%, respectively, at 7 dpbm. This result suggests that these genes represent potential DENV agonists that could facilitate virus infection of the mosquito midgut. The silencing of AaegML13, which is closely related to the human ML gene, had no significant effect on midgut DENV infection. The following experiments will focus only on the characterization of the AaegNPC1b and AaegML33 since the silencing of these genes resulted in phenotypic change.



n	24	14	24
p-value	-	0.012	0.0372
Prevalence (%)	100	92.86	95.83
Median	1850	350	1050
Mean	3223	1367	1073
SD	4079	2127	739

n	44	44
p-value	-	0.4351
Prevalence (%)	100	97.73
Median	1850	1450
Mean	2850	2525
SD	3267	2746

Figure 4.2 Effect of *Ae. aegypti* ML and NPC1 gene knockdown on midgut DENV titers. Members of the ML and NPC1 gene families were silenced in *Ae. aegypti* mosquitoes, and midgut DENV titers at 7 dpbm were determined by plaque assay. Data represent a pool of 2–3 biological replicates with the elimination of outliers (data outside the median plus 2SD). p-values were determined by comparing the experimental group with the dsGFP-treated group in the Mann–Whitney test (*$p < 0.05$, **$p < 0.01$).

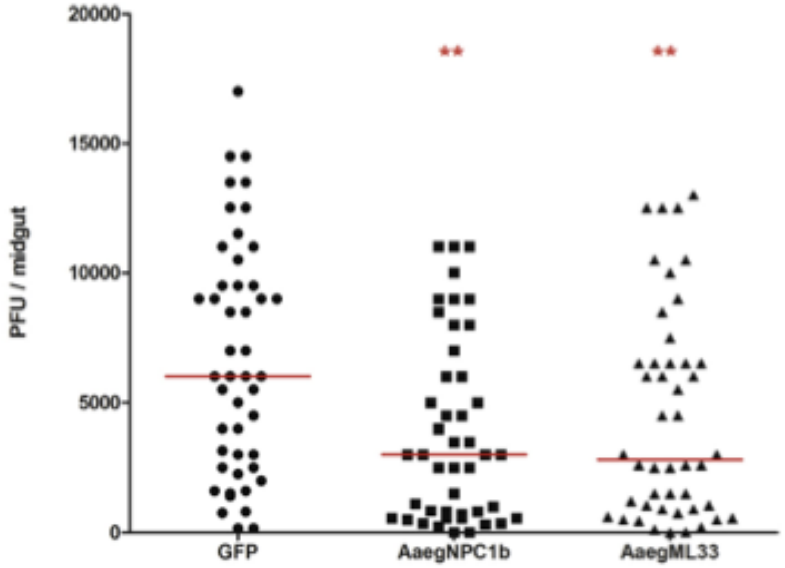
Functions of AaegNPC1b and AaegML33 as DENV host factors are conserved in field-derived strain of *Ae. aegypti*

Since the introduction and maintenance of mosquitoes into a laboratory environment can impose a selection bias that can lead to genetic differences between laboratory-adapted mosquitoes and the natural mosquito population, we investigated whether the influence of AaegNPC1b and AaegML33 on DENV infection is conserved in field-derived mosquitoes. Silencing these genes in the recently colonized *Ae. aegypti*

strain from Saint Kitts, [97] prior to feeding on DENV-infected blood, resulted in significantly lower midgut DENV titers than in the controls, just as we had observed for the laboratory strain (Figure 4.3).

Ae. aegypti NPC1 and ML genes may influence DENV infection through the same mechanism or pathway

Although silencing of some ML and NPC1 genes significantly reduced DENV infection in the mosquito midgut, how these genes function as DENV agonists was still unknown. To investigate whether these genes were acting as DENV agonists through independent mechanisms or the same mechanism/pathway, we compared the effects of their independent and combined gene silencing on mosquito susceptibility to virus infection. The silencing efficiency of each gene was similar in both single- and double-silencing experiments (Figure 4.4). The double-silencing of these genes lowered DENV infection of the midgut to the same level as did independent silencing of each gene, without an apparent synergistic effect (Figure 4.5). This result suggested that these two genes influence DENV infection through a similar or same mechanism, as no additive effect was observed upon double-silencing.



n	47	45	46
p-value	-	0.0013	0.0097
Prevalence (%)	100.00	95.56	97.83
Median	6000	3000	3550
Mean	6731	3860	4426
SD	4473	3540	4004

Figure 4.3 AaegNPC1b and AaegML33 silencing resulted in lower midgut DENV titers in field-derived mosquitoes from Saint Kitts. Data represent a pool of four biological replicates with the elimination of outliers (data outside the median plus 2SD). p-values were determined by comparing the experimental group with the dsGFP- treated group in the Mann–Whitney test (*$p < 0.05$, **$p < 0.01$

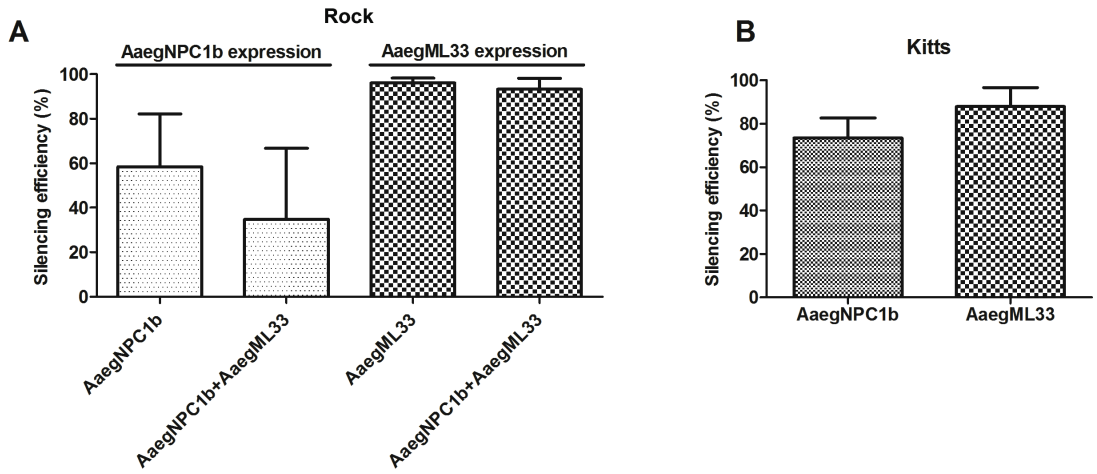
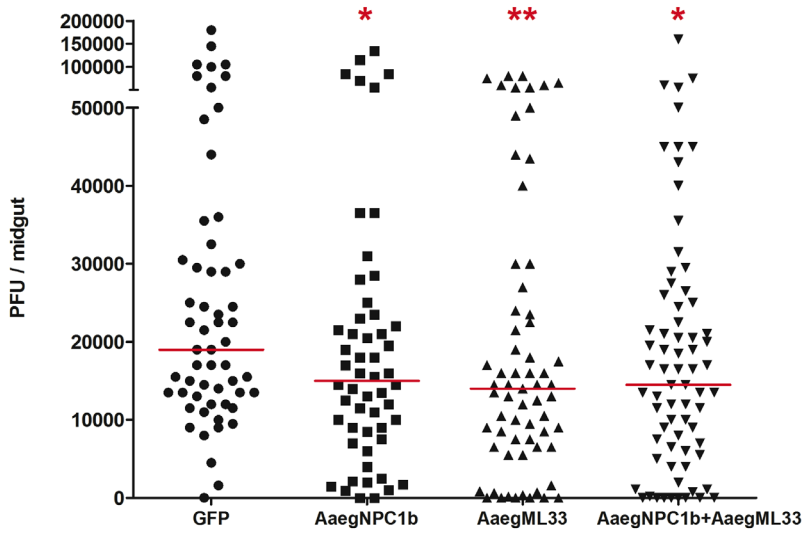


Figure 4.4 Silencing efficiency for AaegNPC1b and ML33 in Rock and Kitts strains *Ae. aegypti.* (A) Silencing efficiency in Rockefeller/UGAL strain. (B) Silencing efficiency in Kitts strain



n	57	54	65	71
p-value	-	0.0306	0.0081	0.0114
Prevalence (%)	98.25	96.30	92.31	92.96
Median	19000	15000	14000	14500
Mean	31686	22617	20490	19798
SD	34987	27453	21194	23205

Figure 4.5 *Ae. aegypti* NPC1b and ML33 may influence DENV infection through the same mechanisms. Midgut DENV titers was assayed upon single- and double-silencing of AaegNPC1b and AaegML33 at 7 dpbm. Data represent a pool of three biological replicates with the elimination of outliers (data outside the median plus 2SD). p-values were determined by comparing the experimental group with dsGFP-treated group in the Kruskal–Wallis one-way ANOVA test (*$p < 0.05$, **$p < 0.01$).

AaegNPC1b and AaegML33 may regulate *Ae. aegypti* immune pathways

Since the transient silencing of AaegNPC1b and AaegML33 resulted in a lower midgut DENV level, we wanted to investigate whether this resistance could have resulted from altered Toll, Imd, and/or JAK/STAT pathway activity in response to AaegNPC1b and AaegML33 gene silencing. Activation of the Toll, Imd, and JAK/STAT pathways results in increased antimicrobial peptide (AMP) gene expression [12,59,160]. We hypothesized that if AaegNPC1b and AaegML33 play a role in regulating these immune pathways (as potential negative regulators), silencing these genes would result in changes in the transcript abundance of AMPs and other effector genes. To test this hypothesis, we assayed the transcript abundance of AMPs and other effector molecules after silencing AaegNPC1b and AaegML33. We measured transcript abundance of immune effector genes in the midgut and fat body since they represent major immune tissues and the midgut is the first mosquito immune-barrier that the virus encounters [12]. The fat body is mainly responsible for humoral and systemic immune responses. Mosquito midguts and fat bodies were collected at 1 and 3 days post-dsRNA injection to assay the transcript abundance of AMPs and other effector molecules (Figure 4.6). The AMPs analyzed in this study were defensin E, cecropin E, lysozyme C, diptericin, and gambicin. Although they do not belonging to the AMP class, we also assayed SOCS36E and DVRF1 because they are regulated by the anti-dengue JAK/STAT pathway [12,45,59,173] and can be used as markers of JAK/STAT activation. The transcript abundance patterns of these proteins upon immune pathway activation, as determined by previous studies, are presented in Table 4.1.

Overall, the transcript abundance of several AMP genes was influenced by AaegNPC1b and AaegML33 silencing, either in the midgut or fat body, and the differential transcript abundances were greater at 3 days than at 1 day post-gene silencing. The silencing of AaegNPC1b resulted in an increase in cecropin E and lysozyme C transcript abundance, suggesting a possible activation of the Toll pathway, since these genes have been shown in previous studies [12] to be induced after Toll pathway activation. The up-regulation of DVRF1 transcripts after AaegNPC1b silencing also suggested the possible activation of the JAK/STAT pathway [59]. The silencing of AaegML33 resulted in an increase in SOCS transcript abundance at 1 day post-dsRNA injection, as has been seen for activation of the JAK/STAT pathway [59]. The transcript abundance of both SOCS36E and DVRF1 in fat body tissue was decreased at 3 days post-silencing of AaegML33 (Figure 4.6D). Defensin E transcripts were enriched in the midgut tissue after the silencing of both AaegNPC1b and AaegML33, suggesting an activation of the Imd pathway, since this AMP was induced by silencing of the Imd pathway negative regulator Caspar [12].

Table 4.1 Expression pattern of the selected immune genes from previous microarray datasets in dengue virus infected midgut (DV MG), dengue virus infected carcass (DV Car), Cactus silenced (Toll activated), Caspar silenced (Imd activated), and PIAS silenced (JAK/STAT activated).

Name	Accession#	DV MG	DV Car	Cactus	Caspar	PIAS
Defensin E	AAEL003849		0.824	-0.811	1.697	-0.525
Diptericin	AAEL004833			-0.546		
Cecropin E	AAEL000611			1.33		-1.127
Lysozyme C7B	AAEL015404	0.935	1.007	1.105		
Gambicin	AAEL004522	1.118	0.851	-1.406	0.85	
SOCS36E	AAEL000393		0.909			0.539
DVRF1	AAEL008492	0.559	2.517			0.79

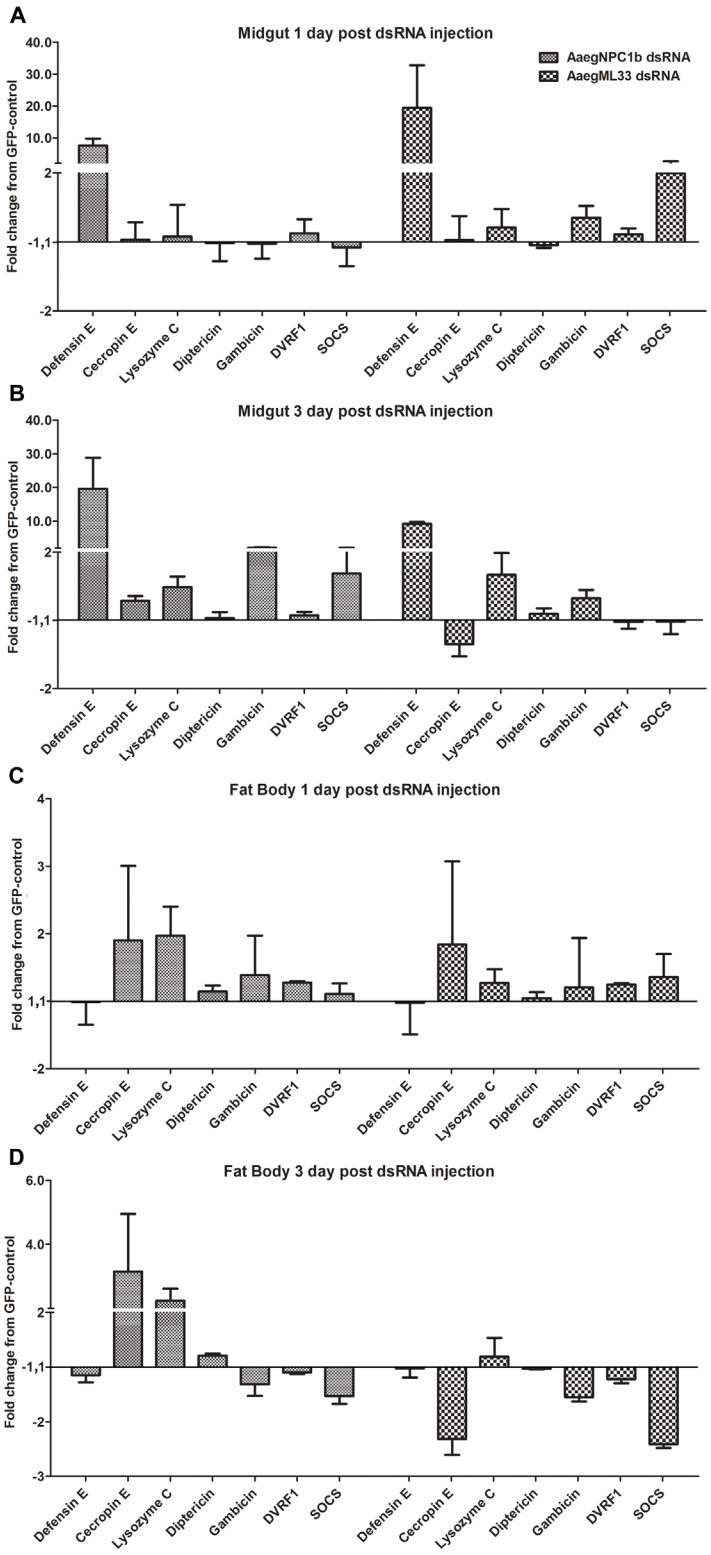


Figure 4.6 Immune-related gene expression changes after AaegNPC1b and AaegML33 silencing. Expression of immune-related gene transcripts was assayed by quanti- tative PCR of cDNA from AaegNPC1b- and AaegML33-silenced mosquito

tissues. Gene expression levels were assayed from three biological replicates. Error bars represent the standard error of the mean (SEM)

Expression patterns and tissue tropisms of AaegNPC1b and AaegML33

Tissue-specific infection-responsive gene expression patterns can provide information about the putative functions of genes during the course of infection. We used real-time PCR to compare the transcript abundance of AaegNPC1b and AaegML33 in the midgut and fat body. AaegNPC1b was highly expressed almost exclusively in the midgut tissue, whereas AaegML33 was expressed at similar levels in both tissues (Figure 4.7).

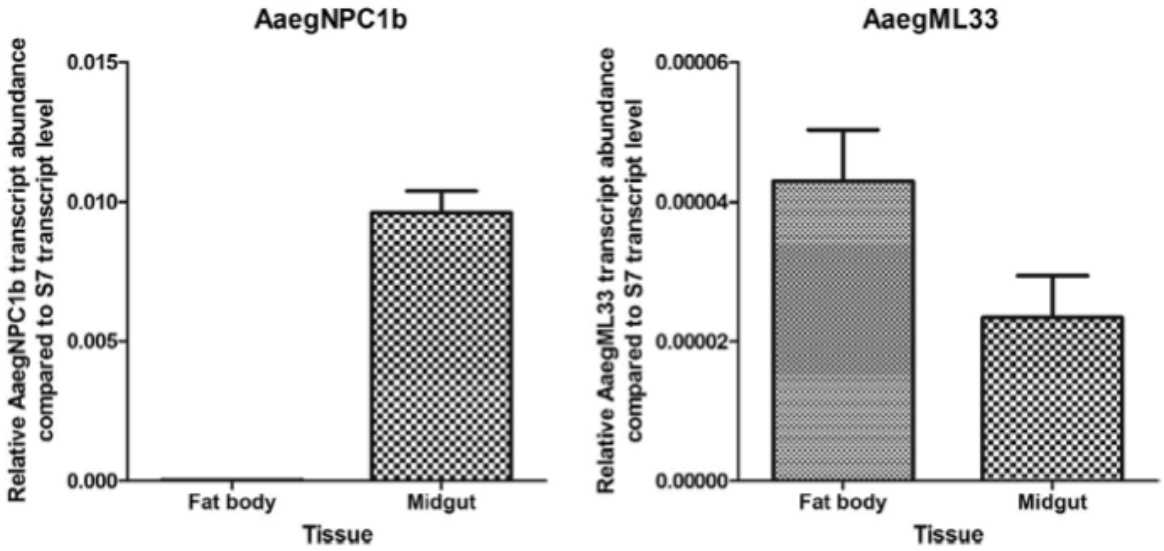


Figure 4.7 Transcript abundance of AaegNPC1b and AaegML33 in the midgut, and fat body in uninfected mosquitos. The graphs show a transcript abundance of AaegNPC1b and AaegML33 relative to the transcript abundance of ribosomal S7 gene as a normalization control. The data was an average of three pools of 14-day old mosquitoes. Transcript abundances were assayed from three biological replicates. Error bars represent the standard error of the mean (SEM).

We then investigated the infection-responsive changes in expression of these genes by comparing their transcript abundance between DENV-infected and naïve blood-fed mosquitoes at 1, 3, 7, 10, and 14 dpbm. After DENV bloodmeal, at 1 dpbm,

AaegNPC1b transcript abundance in the midgut was decreased in comparison to uninfected controls (a 1.59-fold decrease). The abundance of AaegNPC1b transcripts in the midgut increased at 3 dpbm and peaked at 7 dpbm (a 2.15-fold increase), then decreased at the later time points (Figure 4.8A). AaegML33 transcripts were enriched in the midgut throughout the infection (a 1.99- to 18.85-fold increase) (Figure 4.8B). The AaegML33 transcripts in the fat body were also up-regulated at 1, 7, 10, and 14 dpbm (3.23-, 6.40-, 1.82-, and 3.99-fold increases, respectively) (Figure 4.8C).

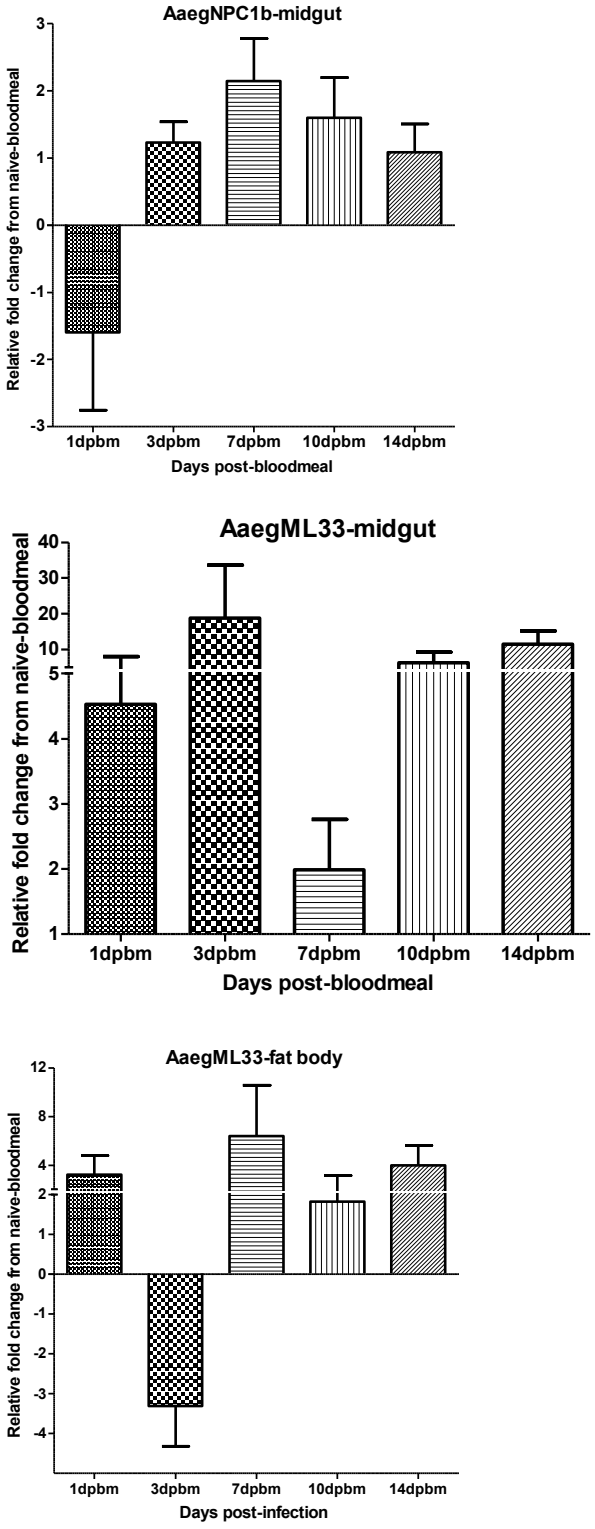


Figure 4.8 AaegNPC1b and AaegML33 gene expression in the midgut, and fat body over the time course of DENV infection. Relative expression of these two genes was compared between DENV-bloodfed and naïve-bloodfed mosquitoes. The gene expression

was compared relative to the ribosomal S7 gene, and was assayed from three biological replicates. Error bars represent the standard error of the mean (SEM).

DISCUSSION

The ML family comprises of proteins that contain the MD2-related lipid-binding (ML) domain, which has been extensively studied in the humansfor its LPS-binding property and its role as a TLR4 co-receptor [101]. The immune-related function of the ML gene family was recently characterized in the insect model organism, *D. melanogaster* [160]. Three of the eight *Drosophila* ML genes (DmNPC2a, DmNPC2e, and DmNPC2h) were shown to bind to LPS, peptidoglycan, and lipoteichoic acid. Over expression of recombinant DmNPC2a and DmNPC2e in the *Drosophila* S2 cell line activates the promoter of the anti-microbial peptide diptericin, which is controlled by the IMD immune signaling pathway [160,170]. The *An. gambiae* ML protein AgMDL1 is also implicated in the anti-*Plasmodium* response [103]. Here we investigated the function of the ML and NPC genes in the *Ae. aegypti* response to DENV infection. Since DENV is an enveloped virus, these lipid-binding proteins might be involved in interactions with the DENV and modulate immune responses.

According to the immunoDB database, the *Ae. aegypti* ML gene family consists of 26 members [166]. Our analysis of *Ae. aegypti* amino acid sequences, however, suggested that only 24 genes actually contain the ML domain. The other two ML genes reported in the database belong to the Nieman-Pick C1 protein family. This discrepancy in annotation might be due to the similar names of NPC1 and NPC2, but in fact, these two protein families have different protein structures. NPC2 is a soluble protein containing an ML domain, whereas NPC1 is a transmembrane protein containing 13

transmembrane domains, with a sterol-sensing domain located between the third and seventh transmembrane domains [158]. Both the NPC1 and NPC2 proteins reside and work closely together in the late endosomal/lysosomal compartment of the cells and facilitate cholesterol trafficking and metabolism [158,172,174].

Multiple sequence alignment of the *Ae. aegypti* ML family revealed variations in ML conserved amino acids, as reported for the mouse ML gene [171]. This variation implies that the insect ML family, and especially *Ae. aegypti*, is under high selective pressure to expand its spectrum of biological functions and lipid-binding properties. According to the phylogenetic analysis, as well as previous classification of the DmNPC2 [170], *Ae. aegypti* ML genes can be classified into four different groups. There are eight ML genes in group 2 that are unique to *Ae. aegypti* and lack *Drosophila* homologs, suggesting an expansion of these ML genes during evolution (Figure 4.1A). The *Drosophila* NPC2 a-h genes were grouped as in previous studies, indicating the accuracy of the tree [160,170]. The DmNPC2a mutant has been shown to possess physiological defects and disordered cholesterol metabolism, similar to that observed with the human NPC2 mutant [170,174]. Thus, AaegML13, which clustered together with DmNPC2a and human NPC2, may have a similar function, but this relationship was not the focus of our study and still needs to be experimentally confirmed. AaegML1 clustered together with AgMDL1, an anti-*Plasmodium* ML gene [103], but its transcript abundance was not influenced by DENV infection in any tissue compartment, suggesting that it might not play a role in DENV infection. This result is not entirely unexpected, since the mosquito uses different mechanisms to combat different pathogens.

The data from previous genome-wide transcriptomic analyses revealed that the transcript abundance of several ML genes was influenced by DENV infection in the midgut and carcass tissue compartments. The differences in transcript abundance suggest that the *Ae. aegypti* ML genes might have diverse functions in different tissue compartments. Functional screening by RNAi-mediated gene silencing of one of the two selected ML genes (AaegML33) resulted in lower DENV midgut titers (Figure 4.2), suggesting that they facilitate DENV infection, underscoring the potential role of the ML gene family members as DENV agonists. AaegML33 was also of particular interest, since it belongs to the distinct *Ae. aegypti* ML group 3, suggesting that it might have a specialized DENV-related function in *Ae. aegypti*.

Studies of the *Drosophila* NPC1 gene revealed two NPC1 genes in its genome, NPC1a and NPC1b [172,175,176]. All the genomes of insects used for phylogenetic analysis also contain two NPC1 genes, suggesting a duplication of the gene to NPC1a and NPC1b in a common insect ancestor (Figure 4.1B). NPC1a is an insect NPC1 gene more closely related to human NPC1 and is required for molting and sterol homeostasis in *Drosophila* [158,172,174]. NPC1a mutant larvae have abnormally high levels of accumulated sterol in cells and are unable to molt [172]. Hence, the *Ae. aegypti* NPC1a might have a similar lipid-related function. The other NPC1 gene, NPC1b, has also been studied in *Drosophila* and found to be involved in sterol absorption in the midgut epithelium. The study of DmNPC1a and DmNPC1b revealed that they have non-redundant roles in sterol homeostasis and are not interchangeable; DmNPC1a is important for sterol trafficking, while DmNPC1b is important for sterol absorption [176]. However, the function(s) of *Ae. aegypti* NPC1 in both lipid homeostasis and immunity

are yet to be determined. In *Ae. aegypti*, the transcript abundance of AaegNPC1 genes has been shown to be altered by DENV infection in both midgut and carcass tissue compartments, suggesting a possible role for *Ae. aegypti* NPC1 genes in DENV infection. AaegNPC1b was of particular interest to us because it is involved in lipid absorption in the midgut and might interact with DENV during that cellular process. The role of NPC1 as a host factor for virus infection has also been suggested in mammals, since Ebola virus can hijack human NPC1 for viral entry into the cytosol [105,106,177]. Silencing of AaegNPC1b resulted in lower midgut DENV titers, emphasizing its potential role in DENV infection (Figure 4.2).

The functions of AaegNPC1b and AaegML33 with regard to DENV infection are likely to be conserved across *Ae. aegypti* strains and populations, since a similar level of resistance was observed after silencing of these genes in a strain of recently colonized field mosquitoes (Figure 4.3). Simultaneous silencing of AaegNPC1b and AaegML33 was conducted to provide an indication as to whether these genes might affect DENV infection through a similar mechanism, and indeed, we saw comparable levels of resistance when they were silenced individually (Figure 4.5).

Based on the infection data and previous studies of ML genes, we hypothesized that AaegNPC1b or AaegML33 could be involved in modulating mosquito immune responses to DENV infection. The silencing of these genes resulted in the activation of immune pathways, of which the Toll and JAK/STAT pathways are of particular interest, since they have been reported to control DENV infection in the mosquito [12,45,59,173,178]. To investigate this hypothesis, we measured the expression of immune pathway-regulated gene transcripts after transient silencing of AaegNPC1b and

AaegML33. Our study showed that the transcript abundance of AMPs and other JAK/STAT-regulated genes was influenced by AaegNPC1b/AaegML33 silencing, suggesting that they playing a role as negative regulators of these anti-DENV innate immune pathways (Figure 4.6).

The expression pattern of the pathway-regulated genes upon AaegNPC1b and AaegML33 gene silencing was similar to the pattern obtained when the Toll, Imd, and JAK/STAT pathways were activated (Table 4.1) [12,59,176]. We observed an increase of SOCS gene transcript abundance, which suggested an activation of the JAK/STAT pathway. The dynamic changes in SOCS gene expression likely reflects feedback loops in the respective JAK/STAT pathway transcription circuits, as has been shown for the *Drosophila* SOCS36E [31,54]. A prominent up-regulation of defensin E in the midgut occurred after the silencing of both AaegNPC1b and AaegML33 (Figure 4.6A and B). This increased transcript abundance of defensin E in the midgut suggests that the activation of the Imd pathway, or some other unknown immune pathway, might also influence DENV infection, as has been shown in *Ae. aegypti* salivary glands [41,179]. Different expression pattern of Defensin E and other immune effector genes in the midgut and fat body also suggested that transcription of these genes is controlled differently in different mosquito tissues and cell types, as has been shown previously [12,59,84,180]. Immune genes can be controlled by multiple immune pathways, and the transcriptional regulation by an immune signaling pathway can also be fine-tuned by different factors. AaegNPC1b and AaegML33 might serve as alternative regulators of the immune signaling pathways. A previous study has shown that DENV can suppress immune responses in a mosquito cell line, but the mechanism was not investigated

[97,177]. Changes in AMP expression after silencing AaegNPC1b and AaegML33 emphasize their role as immune pathway antagonists, and the up-regulation of the AaegNPC1b and AaegML33 by DENV infection is likely reflecting one of the mechanisms the virus uses to suppress mosquito immune responses. The function of the ML gene as negative regulator was also shown in humans; a splice variance of the MD2 protein (MD2s) can inhibit TLR4 signaling by competing with normal MD2 for binding to TLR4 [12,40,178].

Tissue-specific transcript abundance analysis revealed that in uninfected mosquitoes, AaegML33 was constitutively expressed in the midgut and fat body-containing carcass (Figure 4.7). In contrast, AaegNPC1b expression was limited mainly to the mosquito midgut, which corresponds to the expression of NPC1b in *Drosophila* [97,176], suggesting that the biological role of the NPC1b gene may be conserved among insects. The increase in the transcript abundance of the AaegNPC1b and AaegML33 genes after the ingestion of a DENV-infected blood meal (Figure 4.8) suggests that DENV may up-regulate the expression of both genes to prevent immune activation or suppress mosquito immune responses. The dynamic temporal changes of their expression patterns likely reflect variations in virus titer and propagation of virus infection through different host cells and tissues during the course of infection.

This is the first report to show that two lipid-binding protein families, ML and NPC1, play a role as DENV agonists in *Ae. aegypti*. The silencing of these genes resulted in higher resistance to DENV in the mosquito midgut, likely through altered regulation of immune pathways. These effects were conserved between laboratory-adapted and field-derived *Ae. aegypti*. Moreover, our study suggests that DENV might influence the

expression of these genes to facilitate efficient virus infection. The direct interaction of the ML and NPC1 genes with DENV and how these molecules modulate immune signaling pathways are yet to be investigated.

CHAPTER 5

Conclusion and General Discussion

Dengue has remained a significant public health concern for a number of decades; unfortunately, current dengue controls strategies, such as vector population reduction using insecticides, have been shown to be both inefficient and costly, and novel disease control strategies are needed to reduce burden of dengue. Dengue transmission-blocking through self-propagating genetically-modified mosquitoes has been deemed as a logistically simpler and likely cheaper disease control strategy compared to the use of vcaccines and drugs that, nevertheless, still under development. However, little is known about the interactions between the dengue virus and its insect vector for the development of transgenic strategies, and there is therefore a great need for further research in this area.

The recent availability of the *Ae. aegypti* genome [31,95] together with genomic analyses tools, such as Vectorbase [136,179], ave accelerated research on dissecting interactions between DENV and mosquito vector. Previous studies identified the Toll, JAK/STAT, and RNAi pathways as anti-DENV defense systems in *Ae. aegypti* [12,59,84,139]; however, molecular the mechanisms of how these pathways inhibit DENV were still largely unknown. It was also unknown whether these pathways could be used for DENV transmission control. To identify and characterize DENV restriction factors and host factors in *Ae. aegypti*, this thesis research has used different approaches and tools, such as genetically modified mosquitoes with inducible JAK/STAT pathway activation, a panel of field-derived and laboratory strains of *Ae. aegypti* with different

DENV susceptibility [97], as well as previous microarray-based transcriptomic datasets [12,40].

First we studied the interactions between DENV and *Ae. aegypti* by using a transgenic approach to manipulate the activation of the mosquitoes' immune pathway, specifically the JAK/STAT pathway. We generated transgenic *Ae. aegypti* overexpressing the JAK/STAT pathway components, Dome and Hop, under the control of a blood meal-inducible fat body-specific vitellogenin promoter. These genetically modified mosquitoes showed an increased resistance to DENV infection, perhaps because they had higher expression of dengue virus restriction factors and lower expression of DENV host factors than did wild-type mosquitoes. DENV, as RNA virus, is prone to mutations which allows the virus to evade control strategies. The multiple factors contributing to DENV resistance in our transgenic lines reduce the chance of DENV to mutate to overcome the resistance.

We also showed that these transgenic mosquitoes were useful for studying the molecular interactions between DENV and *Ae. aegypti*. Through transcriptomic comparisons by microarray-based methodology together with functional confirmation by RNAi, we identified a gene with unknown function (AAEL007703) as a putative DENV restriction factor, and the SCP2 and DDX genes as putative DENV host factors.

This study is the first to provide a proof-of-concept that genetic engineering of the mosquitoes' JAK/STAT immune pathway can be used to render the insect more resistant to DENV infection and possibly to block transmission of the disease through further development of the technology. Mosquito transgenesis technique has been employed as a *Ae. aegypti* population suppression approach through the sterile insect technique (SIT) in

several countries including Brazil, Panama, Malaysia, and the Cayman islands [181-183]. A biotechnology company, Oxitec, has released genetically modified sterile male *Ae. aegypti* in the field and genetically modified *Ae. aegypti* carries a lethal gene that prevents offsprings of the cross between the genetically modified *Ae. aegypti* and wild mosquitoes to survive to adulthood. One advantage that a transgenic *Ae. aegypti* with increased DENV resistance has over Oxitec's technology is that it will not require constant production and release of transgenic mosquitoes into the field., but function as a logistically simpler and more cost-effective self-propagating system. Offsprings the transgenic mosquitoes can survive to adulthood and spread the transgene to next generations of mosquitoes through a genetic drive mechanism.

Our lab has previously established a panel of laboratory and field derived strains with various degrees of DENV susceptibility, and transciptomic profiles of these mosquitoes suggested that basal level of immune activity might contribute to the differences in DENV susceptibility [97]. In this study we confirmed this hypothesis by using RNAi-mediated gene silencing to activate or inhibit the Toll, Imd, JAK/STAT, and RNAi pathways, and confirm that these pathway contribute to the natural differences in DENV susceptibility for different strains to certain degrees. Transciptomic profiles of these mosquitoes also showed differential expression of hundreds of genes belongoing to various functional groups which play potential roles in DENV infection in *Ae. aegypti*. Hierarchical cluster analyses revealed differential transcript abundance of several DENV host and restriction factors among DENV-susceptible and -refractory strains. Functional confirmation by RNAi-mediated gene silencing assays emphasized an importance of the vATPase enzyme as a DENV host factor in natural *Ae. aegypti* populations, suggesting it

may be a candidate intervention to decrease DENV transmission. The function of vATPase genes as a DENV host factor confirmed in this study also support our findings from Study 1 that the higher DENV resistance in VgHop mosquitoes might be a result from lower expression of vATPase genes. Subsequent experiments by our group applied this knowledge in a more translational fashion using a chemical inhibitor of vATPase, bafilomycin, to inhibit DENV infection in *Ae. aegypti* [95].

Previous studies have shown that DENV perturbs lipid homeostasis [136], and have also shown that a lipid carrier protein is important for efficient infection of mosquitoes with DENV [139]. This thesis used microarray-based transcriptomic datasets of DENV infected *Ae. aegypti* to identify members of two lipid binding protein families, ML and NPC1, that play important roles in DENV infection in *Ae. aegypti*. Transient silencing of NPC1b and ML33 resulted in lower DENV infection in the mosquito midgut, suggesting that they were required for efficient infection. We have also shown that the silencing of these genes resulted in an induction of AMP gene expression, and DENV influenced expression of these genes in a fashion that suggested it would facilitate infection. However, the direct interaction between ML and NPC1 genes with DENV and how these molecules modulate immune signaling pathways remain to be investigated.

In recent years, arboviruses other than DENV such as Chikungunya virus and Zika virus have also become global public health concerns. However, knowledge of interactions between *Ae. aegypti* and these arboviruses is still limited. It is possible that the JAK/STAT pathway is involved in mosquito immunity against these arboviruses since the pathway is an evolutionary conserved immune signaling pathway which have been shown to control virus infection in several insects such as *Drosophila* and *Culex*

[184,185]. The VgDome and VgHop transgenic mosquito strains should allow further characterization of the role of JAK/STAT pathway in arboviruses infection. Our recently colonized and laboratory *Ae. aegypti* strains can also be used to study genes that can modulate infection of other arboviruses. The natural variations in the basal level of immune activity and expression level of host factor genes should allow identification of factors contributing to refractoriness against specific arboviruses.

Collectively, this thesis has used multiple tools available in the lab such as the JAK/STAT transgenic mosquitoes, a panel of field-derived and laboratory *Ae. aegypti* strains with various degrees of DENV susceptibility, as well as published microarray datasets to broaden our knowledge on the molecular interactions between DENV and *Ae. aegypti* mosquito. We have identified several novel DENV host factors involved in lipid trafficking and homeostasis such as SCP2, ML, and NPC1 genes as well as genes that facilitate cell entry such as vATPases. Tools and knowledge generated by this thesis research may prove to be useful for the development of alternative dengue transmission control approaches.

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APPENDICES

1. Primer sequences

Chapter 2

Gene / Segment	Primer Name	Sequence	RE sites / Notes
Dome 1-1531	Dome1F_PstI	TAGTCTGCAGATGGTACAGAG ACAAGTATT	PstI
	Dome1R_AclI	AGCTAACGTTGTTCAGTTCATA GCT	AclI
Dome 1532-3432	Dome2F_AclI	GAACAACGTTAGCTCGACGTTA AATTTTGC	AclI
	Dome2R_PstI	TAGTCTGCAGTTACTGCATTTT CAGACCAT	PstI
Hop 1-1516	Hop1F_EcoRI	TATGAGAATTCATGTCGGAGCA TGAGAACAAAT	EcoRI
	Hop1R_SacI	GAAAGAGCTCAGCTCTTGTCCT TTCAAAGA	SacI
Hop 1517-3408	Hop2F_SacI	AGCTGAGCTCTTTCTGCCGAAT AATACCAA	SacI
	Hop2R_EcoRI	TATGAGAATTCTTAGAAAAGTT GAATTGATT	EcoRI
Trypsin terminator	Tryp-Ter-F	TGAATACTAGTTAGGTAGCTGA GCGCATGCGATCTC	SpeI
	Tryp-Ter-R	TAAGTGCGGCCGC***GGCCGGCC*** GGTCGGCGCGCCCACCCTTGAG	NotI, ***FseI***
AeVg promoter	AeVgPro F	TAGTCTCGAG***GGCCGGCC***GAAT TCCACCACCAGG	XhoI, ***FseI***
	AeVgPro R	TAGT**GTCGAC**CTTCAAGTATCC GGCAGCTG	SalI
Transgenic verification	ITRR2'	GGGGTCCGTCAAAACAAAACA	used with VgProR
FBN	T7FBN13417-F436	TAATACGACTCACTATAGGG ACCCTGGTTCCCGACAAATC	dsRNA synthesis
	T7FBN13417-R845	TAATACGACTCACTATAGGG TCCAAAGCATCACGAGCAGT	
	qFBN13417-F168	AGCAGTGAACGCAGACATGA	realtime PCR
	qFBN13417-R261	GCGATGCGTGATCGTTGTTT	
GAMB	T7GAMB4522-F58	TAATACGACTCACTATAGGG ACCGATGCTTTGGTGTTTGTT	dsRNA synthesis
	T7GAMB4522-R249	TAATACGACTCACTATAGGG GTAGCATTCGGTGATGGCAC	
	qGAMB4522-F13	ACAGTGTGTATTTTGCTGGCAC	realtime PCR
	qGAMB4522-R65	GCATCGGTATAGGCAGCTGAT	
	T7-EGFP-R	CGACGATAATACGACTCACTAT AGGGCTGGTAGTGGTCGGCGA	

		G	

Gene / Segment	Primer Name	Sequence	RE sites / Notes
UKN7703	T7UKN7703-F718	TAATACGACTCACTATAGGG GGTCGGCTATCGGCAGTATC	dsRNA synthesis
	T7UKN7703-R1164	TAATACGACTCACTATAGGG CTCCAATCCCAGTTGGCTGT	
	qUKN7703-F144	CGCTCGGAACTCGCTATCTT	realtime PCR
	qUKN7703-R269	GAATACACACCTCCCGCCAA	
UNK566	T7UKN566-F135	TAATACGACTCACTATAGGGCA GACCTTCAGACGCTGCTA	dsRNA synthesis
	T7UKN566-R413	TAATACGACTCACTATAGGGAC GTATGCCTTGCACCAATC	
	qUKN566-F5	CGCAGACAATCAAGATAAGCGG	realtime PCR
	qUKN566-99	CAGCAACAGAACCCCTAGCA	
DDX	T7DDX-F272	TAATACGACTCACTATAGGGATG GCCACGAGAACGGATTT	dsRNA synthesis
	T7DDX-R480	TAATACGACTCACTATAGGGATC CGTGCCGTTCTCATTGT	
	qDDX-F1041	TCGTCTGTTGGACTTCGTCG	realtime PCR
	qDDX-R1150	CAACCGATGGCATGAAACCC	
SCP2	T7SCP2-F71	TAATACGACTCACTATAGGGAG GTTCTGGGAGTGTTCCAGT	dsRNA synthesis
	T7SCP2-R233	TAATACGACTCACTATAGGGAA GGTCTTTCCGCTGATGGC	
	qSCP2-F22	GAGAGAATCAAGGCTCGCGT	realtime PCR
	qSCP2-R107	GCGGTCTTGATGTTCAACTGG	
TEP22	T7TEP22-87-F2302	TAATACGACTCACTATAGGGTTG GGGGAAATCGCGATCAA	dsRNA synthesis
	T7TEP22-87-R2769	TAATACGACTCACTATAGGGGTT CCATTGACCAAACGCCC	
	qTEP22-87-F291	TGTCAACGACGGTGGTAGTG	realtime PCR
	qTEP22-87-R400	CGCCTGGTTTGTAGACAGGT	
Vitellogenin	qAeVg1 F1-6145	TCTTCGGAATTCAGCTCTCTGG	realtime PCR
	qAeVg1 R1-6260	ACTGGGCATTCCTTCATGCT	
S7	qS7-F	GCAGACCACCATTGAACACA	realtime PCR
	qS7-R	CACGTCCGGTCAGCTTCTTG	
Dome	Dome 2F	AAACGGTGGCAAAATGAACT	realtime PCR
	Dome 2R	CTCCAGACCGGTGAGATTGT	
Hop	Hop 2F	CCGGACTTTATCGAGCTGTC	realtime PCR
	Hop 2R	ATCTGGTTCACTCCGTCGTC	
DVRF1	qDVRF1-F226	CAGGCCAAATCGTGGGAAAC	realtime

	qDVRF1-R319	TGGGCTGTTCATAGAATGGGG	PCR
GFP	T7-EGFP-F	CGACGATAATACGACTCACTATA GGGTTCATCTGCACCACCGGC	dsRNA synthesis
	T7-EGFP-R	CGACGATAATACGACTCACTATA GGGCTGGTAGTGGTCGGCGAG	

Chapter 3

Accession no.	Primer Name	Sequence
AAEL007768	dsMyD88 F	TAATACGACTCACTATAGGGGGCGATT GGTGGTTGTTATT
AAEL007768	dsMyD88 R	TAATACGACTCACTATAGGGTTGAGCGC ATTGCTAACATC
AAEL010083	dsIMD F	TAATACGACTCACTATAGGGACCGAAG AAGACCGCACAAGGC
AAEL010083	dsIMD R	TAATACGACTCACTATAGGGTGCCGAG CGTTGGTTCGTCG
AAEL012471	dsDomeF	TAATACGACTCACTATAGGGCCATCTCC ACCACGAAACTT
AAEL012471	dsDome R	TAATACGACTCACTATAGGGCCGGTGGT TGCCATATAATC
AAEL006794	dsDcr2 F	TAATACGACTCACTATAGGGGCATTGAC GACGAAATCATCGTCCGATG
AAEL006794	dsDcr2 R	TAATACGACTCACTATAGGGACCATGG CATCCGCCGGTGTCTTGTCC
AAEL011414	dsHMBG F	TAATACGACTCACTATAGGGCGCCAAG CGAGGTGGTGAGC
AAEL011414	dsHMBG R	TAATACGACTCACTATAGGGGTCCTTGC CGCCGCCATTTC
AAEL010429	dsAller10-13 F	TAATACGACTCACTATAGGGAGACCAG CCCCGAGTTCAAGG
AAEL010429	dsAller10-13 R	TAATACGACTCACTATAGGGCCCCAGCC GAAGAAACCGGC
AAEL012819	dsvATP-G F	TAATACGACTCACTATAGGGAAGGCCG CCGAAAAGGTCGG
AAEL012819	dsvATP-G R	TAATACGACTCACTATAGGGGTGAGAG CTGAGGGCCCGGT
AAEL012092	dsLRRprot F	TAATACGACTCACTATAGGGCCTGCCTC GGTTGGCACTGG
AAEL012092	dsLRRprot R	TAATACGACTCACTATAGGGGACCAGC TTGCCGGACGTGA
AAEL015458	dsTsf F	TAATACGACTCACTATAGGGTAGGAGG CGCCCCAGCCAAA
AAEL015458	dsTsf R	TAATACGACTCACTATAGGGAGGCCGG ACGGACATCACGA

AAEL002833	dsCatL F	TAATACGACTCACTATAGGGACTGCGG CTCGTGTTGGTCG
AAEL002833	dsCatL R	TAATACGACTCACTATAGGGGCGCTTCC TCGTCACCCTGG

Accession no.	Primer Name	Sequence
AAEL007768	RT MyD88 F	GGCGAGGGTTGTTTCAAGTA
AAEL007768	RT MyD88 R	TCCCATCTGTCGATTAAGCC
AAEL010083	RT IMD F	TCATTCCGCGAAGGGCTGGC
AAEL010083	RT IMD R	AGCGCAGAAACATCGTTCGCA
AAEL012471	RT Dome 2F	AAACGGTGGCAAAATGAACT
AAEL012471	RT Dome 2R	CTCCAGACCGGTGAGATTGT
AAEL006794	RT Dcr2 F	CAATTGCTACCGTTGGGAGT
AAEL006794	RT Dcr2 R	ATTGATCCCCCAAAAAGACC
AAEL011414	RT HMBG F	CTGTGGCTCAACTCTGCCCGC
AAEL011414	RT HMBG R	AGCTCACCACCTCGCTTGGC
AAEL010429	RT Aller10-13 2F	ACGAAACGGTTGCTTTATTGCCTCT
AAEL010429	RT Aller10-13 2R	GCCATCCACATCGAGTCCGTAGC
AAEL012819	RT vATP-G F	CAGCTGCTGGCCGCTGAGAA
AAEL012819	RT vATP-G R	TTTAGACGGCGGGCCTTGCG
AAEL012092	RT LRRprot F	CCGGAGGTACCGAGAGCCCA
AAEL012092	RT LRRprot R	TCGTCCCCTAGCGGCTTCCA
AAEL015458	RT Tsf F	TCGTGATGTCCGTCCGGCCT
AAEL015458	RT Tsf R	CGCTGGTGGATGTTGCGGGT
AAEL002833	RT CatL F	TGGAGCGACCGACAAGGGCT
AAEL002833	RT CatL R	CCCCAGGTGGTTCCCCACGA
AAEL009496	RT S7 F	GCAGACCACCATTGAACACA
AAEL009496	RT S7 R	CACGTCCGGTCAGCTTCTTG

Chapter 4

Gene name	Primer name	Sequence
NPC1b	T7NPC1b2-1122	TAATACGACTCACTATAGGGTCCC GAAAGCCGATCACGCG
	T7NPC1b2-1558	TAATACGACTCACTATAGGGAGGG ACCGAAGCAAGCCGGA
	NPC1b2870F	ACACCTTTTGCGAATCCTGCCC
	NPC1b3061R	CATGGACGTTCAGATGACCGGC
ML13	T7ML13F	TAATACGACTCACTATAGGGAAAA TTGCGGTGAC
	T7ML13R	TAATACGACTCACTATAGGGAATG ACGTCCTTATC
	ML13-150F	GGATGAACCAGCTTGCGTCCTG
	ML13-325R	GGCATTCCAAACCGCTGTCCTT
ML33	T7ML33F	TAATACGACTCACTATAGGGAACT TCCGAGTATG
	T7ML33R	TAATACGACTCACTATAGGGACCC AAAGCTACGC
	ML33-235F	ATGGACCTCGGTTTCAGGACCC
	ML33-385R	TTTCGACCGGCATATTGACCGC
SOCS36E	qAeSOCS36E F1-81	CCACTGTTTGGTGCCGGATTTGC
	qAeSOCS36E R1-266	GCGTGCAGCGACCGGTTGTA
Defensin E	DEFE-F	AACGTCGAAAGCGCATCTCA
	DEFE-R	CGGTAGCGCCAGCTTATGG
DVRF1	DVRF1-F	TCTTCATGCGGCATACTCAG
	DVRF1-R	AGGAATGTTTCCGAGGGTTT
LYSC7B	LYSC-F	CCACGGCAACTGGATATGTCT
	LYSC-R	TCTGCGTCACCTTGGTGGTAT
Cecropin E	CecE-F	CGAAGCCGGTGGTCTGAAG
	CecE-R	ACTACGGGAAGTGCTTTCTCA
Gambicin	GAM-F	CGGACCATCAAGCATTTCTCAA
	GAM-R	CCAGACGGTGGGTAGAACA

2. Log2-fold values and functional groups of transcripts that were up- or down-regulated in the fat body of VgDome or VgHop lines relative to WT

Gene ID	Description	Functional group	VgDome	VgHop
AAEL001904	arp2/3	CS		-0.773
AAEL002185	cuticle protein, putative	CS	-0.61	-0.853
AAEL002495	conserved hypothetical protein (mucin-like protein)	CS		-1.65
AAEL002759	tropomyosin invertebrate	CS		-0.991
AAEL004798	conserved hypothetical protein (mucin-like protein)	CS	0.65	-1.569
AAEL005417	annexin x	CS	0.194	0.964
AAEL010094	cyclin b	CS	0.505	-1.224
AAEL013984	structural constituent of cuticle	CS	0.389	-1.924
AAEL005146	conserved hypothetical protein	CS	-1.023	-0.652
AAEL005426	annexin x	CS	-1.442	-0.606
AAEL012644	conserved hypothetical protein	CS	2.702	
AAEL017334	Conserved hypothetical protein (chitin-binding domain type 2)	CS	2.267	
AAEL000335	lamin	CS	-0.971	-0.84
AAEL006726	innexin	CS	-1.513	-0.835
AAEL009572	cyclin B3	CS	-0.784	-0.818
AAEL003593	hypothetical protein	CSR		0.851
AAEL005772	Odorant-binding protein 99c, putative	CSR	1.051	0.893
AAEL000005	hypothetical protein	D		-0.76
AAEL000079	hypothetical protein	D		-0.769
AAEL000105	beta-alanine synthase, putative	D	0.322	-1.513
AAEL000115	conserved hypothetical protein	D		0.864
AAEL000125	hypothetical protein	D		-0.762
AAEL000147	single-stranded DNA binding protein, putative	D		-0.903
AAEL000159	nipsnap	D	0.715	1.073
AAEL000262	conserved hypothetical protein	D		-0.848
AAEL000428	tryptophan 2,3-dioxygenase	D		-1.939
AAEL000551	hypothetical protein (pacifastin light chain [Culex quinquefasciatus])	D	-0.516	-1.886
AAEL000807	Tetratricopeptide repeat protein, putative	D	-0.165	0.829
AAEL000923	conserved hypothetical protein	D	-0.725	-0.829

Gene ID	Description	Functional group	VgDome	VgHop
AAEL001087	synaptic vesicle protein	D	0.724	1.109
AAEL001100	phosphoserine phosphatase	D	0.417	1.23
AAEL001287	conserved hypothetical protein	D		0.861
AAEL001293	conserved hypothetical protein	D	-0.214	1.057
AAEL001307	SEC14, putative	D		1.183
AAEL001352	scaffold attachment factor b	D	-0.575	-0.803
AAEL001401	conserved hypothetical protein	D		1.052
AAEL001627	UDP-n-acteylglucosamine pyrophosphorylase	D		1.085
AAEL001795	orfY, putative	D	0.615	0.818
AAEL002048	histidyl-tRNA synthetase	D	0.337	0.969
AAEL002125	conserved hypothetical protein	D		-0.762
AAEL002194	uricase	D	-0.48	-1.086
AAEL002261	GTP cyclohydrolase i	D		0.968
AAEL002501	protein disulfide isomerase	D		2.191
AAEL002675	arginase	D	0.186	0.775
AAEL002764	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase	D		0.764
AAEL002920	hypothetical protein	D		-1.949
AAEL002948	frataxin, putative	D		-0.761
AAEL003039	nonsense-mediated mrna decay protein	D	-0.355	1.211
AAEL003109	atlastin	D		1.988
AAEL003165	low molecular weight protein-tyrosine-phosphatase	D	0.221	1.078
AAEL003179	protein arginine n-methyltransferase 1, putative	D		-0.857
AAEL003312	hypothetical protein	D		1.425
AAEL003371	f-box and wd-40 domain protein	D		-0.816
AAEL003413	f-spondin	D		1.129
AAEL003509	smap1	D	-0.37	-0.805
AAEL003581	amidophosphoribosyltransferase	D	-0.447	0.803
AAEL003599	DNA binding, sulfiredoxin activity, oxidation reduction	D		0.778
AAEL003606	purine biosynthesis protein 6, pur6	D	-0.542	2.35
AAEL003980	component of oligomeric golgi	D	-0.678	-1.022

Gene ID	Description	Functional group	VgDome	VgHop
	complex			
AAEL004023	Juvenile hormone-inducible protein, putative	D		1.056
AAEL004237	vacuolar protein sorting 18 (deep orange protein)	D	-0.385	1.032
AAEL004278	conserved hypothetical protein	D		1.592
AAEL004335	secreted ferritin G subunit precursor, putative	D		1.225
AAEL004392	IAP-antagonist michelob-X-like protein, pro-apoptotic protein	D		1.038
AAEL004404	HIG1 domain family member 2A, putative	D		0.751
AAEL004480	cell division cycle 20 (cdc20) (fizzy)	D	0.248	1.017
AAEL004503	conserved hypothetical protein	D	0.3	1.134
AAEL004547	conserved hypothetical protein	D	-0.355	0.937
AAEL004566	myo inositol monophosphatase	D		-1.415
AAEL004575	beta-galactosidase	D		0.809
AAEL004613	phenylalanyl-tRNA synthetase beta chain	D	0.674	1.109
AAEL004701	argininosuccinate synthase	D	0.615	0.976
AAEL004813	M-phase phosphoprotein, putative	D		0.885
AAEL005199	hypothetical protein	D	0.607	1.41
AAEL005289	ornithine aminotransferase	D		1.273
AAEL005384	phosphoribosylformylglycinamidine synthase, putative	D	0.475	2.203
AAEL005457	conserved hypothetical protein	D	-0.656	0.937
AAEL005558	conserved hypothetical protein	D	-0.184	1.717
AAEL005760	hypothetical protein	D	0.037	1.508
AAEL006023	Vanin-like protein 1 precursor, putative	D	-0.541	-0.98
AAEL006279	hypothetical protein	D		-0.955
AAEL006446	trehalose-6-phosphate synthase	D	-0.398	-0.862
AAEL006518	cytidine deaminase, putative	D		-2.892
AAEL006544	nucleoporin P54	D		-1.09
AAEL006602	hypothetical protein	D	0.467	0.752
AAEL006712	serine/threonine protein kinase	D	-0.662	-0.897
AAEL006909	hypothetical protein	D		-0.928
AAEL007072	conserved hypothetical protein	D		-0.751

Gene ID	Description	Functional group	VgDome	VgHop
AAEL007114	conserved hypothetical protein	D	-0.566	-0.79
AAEL007226	nidogen	D		1.601
AAEL007383	secreted ferritin G subunit precursor, putative	D	-0.388	-0.768
AAEL007621	conserved hypothetical protein	D		2.049
AAEL007686	conserved hypothetical protein	D		0.755
AAEL007701	conserved hypothetical protein	D		-0.85
AAEL007767	conserved hypothetical protein	D	-0.624	0.977
AAEL007783	centromere protein-A, putative	D	-0.437	-1.078
AAEL007828	palmitoyl-protein thioesterase	D		2.194
AAEL008007	conserved hypothetical protein	D	0.391	0.955
AAEL008320	conserved hypothetical protein	D		-0.754
AAEL008473	cysteinech venom protein, putative	D		-1.328
AAEL008753	conserved hypothetical protein	D	0.331	-1.126
AAEL008863	protein regulator of cytokinesis 1 prc1	D		-1.226
AAEL008953	conserved hypothetical protein	D		-1.559
AAEL009037	GTP-binding protein (i) alpha subunit, gnai	D	-0.553	-0.792
AAEL009309	lipid depleted protein	D	0.207	1.381
AAEL009508	zinc finger protein	D		-0.852
AAEL009629	endoU protein, putative	D	0.452	-0.818
AAEL009636	conserved hypothetical protein	D		0.876
AAEL009719	conserved hypothetical protein	D		-1.359
AAEL009931	arsenite inducuble RNA associated protein aip-1	D	-0.473	1.379
AAEL009968	hypothetical protein	D		-0.987
AAEL010028	sarcosine dehydrogenase	D		0.922
AAEL010204	dihydropyrimidine dehydrogenase	D	-0.661	-1.28
AAEL010280	conserved hypothetical protein	D	0.666	0.874
AAEL010455	cxyorf1	D		-0.827
AAEL010520	conserved hypothetical protein	D	0.17	-0.778
AAEL010572	late endosomal/lysosomal MP1 interacting protein, putative	D		-1.297
AAEL010656	conserved hypothetical protein	D		1.414
AAEL010879	conserved hypothetical protein	D	0.484	-1.093
AAEL011063	tumor endothelial marker 7 precursor	D	0.567	0.817
AAEL011088	conserved hypothetical protein	D	0.448	0.757

Gene ID	Description	Functional group	VgDome	VgHop
AAEL011135	conserved hypothetical protein	D		-0.774
AAEL011159	cartilage associated protein	D		-0.836
AAEL011168	GTP-binding protein (i) alpha subunit, gnai	D	-0.181	-0.75
AAEL011341	apyrase, putative	D		-0.98
AAEL011529	late endosomal/lysosomal MP1 interacting protein, putative	D		-1.047
AAEL011580	conserved hypothetical protein	D		0.786
AAEL011853	conserved hypothetical protein	D	-0.376	-0.865
AAEL011892	receptor for activated C kinase, putative	D	-0.497	-1.134
AAEL011980	hypothetical protein	D	-0.594	-0.84
AAEL012233	hypothetical protein	D		1.117
AAEL012417	conserved hypothetical protein	D		1.117
AAEL012464	alanine-glyoxylate aminotransferase	D	-0.743	1.099
AAEL012502	conserved hypothetical protein	D	0.108	-1.179
AAEL012856	hypothetical protein	D		1.083
AAEL013078	glycosyltransferase	D	0.595	0.764
AAEL013334	conserved hypothetical protein	D	0.192	1.068
AAEL013338	lethal(2)essential for life protein, l2efl	D		-0.949
AAEL013590	conserved hypothetical protein	D		-0.854
AAEL013596	phosphatidylinositol 3-kinase regulatory subunit	D		-0.957
AAEL013822	protein binding	D		-0.927
AAEL014199	dihydropyrimidine dehydrogenase	D		-1.669
AAEL014275	molybdopterin cofactor sulfurase (mosc)	D	0.428	0.787
AAEL014310	hypothetical protein	D		-0.852
AAEL014561	conserved hypothetical protein	D	-0.189	1.955
AAEL015375	serine/threonine protein kinase	D		-0.905
AAEL015658	conserved hypothetical protein	D		-1.281
AAEL000016	conserved hypothetical protein	D	1.064	0.748
AAEL000315	pigeon protein (linotte protein)	D	0.838	
AAEL000442	conserved hypothetical protein	D	-0.931	-0.656
AAEL000776	conserved hypothetical protein	D	-0.846	
AAEL000973	conserved hypothetical protein	D	-0.789	-0.431
AAEL001666	nucleic acid binding, zinc ion binding	D	-1.265	-0.696

Gene ID	Description	Functional group	VgDome	VgHop
AAEL001682	nuclear movement protein nudc	D	1.58	0.221
AAEL002473	hypothetical protein	D	-0.777	-0.633
AAEL002559	conserved hypothetical protein	D	1.526	-0.335
AAEL003213	guanine deaminase	D	0.813	0.527
AAEL003237	low molecular weight protein-tyrosine-phosphatase	D	0.958	0.295
AAEL003345	argininosuccinate lyase	D	-1.081	-0.382
AAEL003385	conserved hypothetical protein	D	-0.914	
AAEL003877	ubiquitin	D	-0.916	-0.582
AAEL004860	acireductone dioxygenase	D	1.969	
AAEL005348	hypothetical protein	D	-0.928	
AAEL005790	malic enzyme	D	-0.935	
AAEL005976	adenine phosphoribosyltransferase, putative	D	1.025	
AAEL006353	sulfotransferase (sult)	D	1.528	0.18
AAEL006972	hepatocellular carcinoma-associated antigen	D	1.463	0.23
AAEL007130	leucyl-tRNA synthetase	D	-1.176	
AAEL007477	ubiquitin-conjugating enzyme E2 i	D	-0.967	-0.585
AAEL007702	chaperonin	D	-0.868	-0.201
AAEL008076	PIWI	D	-2.045	
AAEL008598	conserved hypothetical protein	D	1.055	
AAEL009652	activin receptor type ii	D	0.955	
AAEL009654	hypothetical protein	D	-1.179	-0.669
AAEL009859	nucleolar GTP-binding protein	D	-1.567	
AAEL010065	protein disulfide-isomerase A6 precursor	D	2.233	
AAEL010943	conserved hypothetical protein	D	-0.772	0.358
AAEL011105	adducin	D	1.201	
AAEL011264	phosphatidylethanolamine-binding protein	D	1.128	-0.242
AAEL011448	conserved hypothetical protein	D	1.044	
AAEL011452	conserved hypothetical protein	D	0.913	
AAEL011478	cytoplasmic dynein light chain	D	-0.928	-0.489
AAEL011849	hypothetical protein	D	-0.79	-0.598
AAEL012260	wdpeat protein	D	1.298	-0.709
AAEL012632	hypothetical protein	D	0.93	

Gene ID	Description	Functional group	VgDome	VgHop
AAEL012939	gamma-subunit,methylmalonyl-CoA decarboxylase, putative	D	-1.029	
AAEL013510	smaug protein	D	-0.972	
AAEL013844	diazepam binding inhibitor, putative	D	1.276	-0.564
AAEL014715	67 kDa polymerase-associated factor PAF67, putative	D	0.883	0.661
AAEL014852	hypothetical protein	D	-0.862	
AAEL001607	galactose-1-phosphate uridylyltransferase	D	1.168	1.245
AAEL001667	multicopper oxidase	D	1.553	1.086
AAEL002554	anosmin, putative	D	-1.466	-1.099
AAEL002860	conserved hypothetical protein	D	2.016	1.633
AAEL005308	pyruvate dehydrogenase	D	0.933	1.36
AAEL005458	carnitine o-acyltransferase	D	-0.783	0.776
AAEL006625	conserved hypothetical protein	D	-1.063	2.651
AAEL006662	hypothetical protein	D	1.212	2.29
AAEL007484	protein transport protein sec23	D	0.809	-0.805
AAEL007494	calcineurin b subunit	D	0.771	0.791
AAEL007557	asparagine synthetase	D	1.038	1.431
AAEL007868	ubiquinol-cytochrome c reductase complex 14 kd protein	D	0.816	1.358
AAEL008431	a kinase anchor protein	D	-1.011	-0.919
AAEL008595	conserved hypothetical protein	D	-1.162	-0.953
AAEL008789	apolipophorin-III, putative	D	-0.792	0.865
AAEL009962	hypothetical protein	D	-1.241	-0.96
AAEL010097	nuclein acid binding	D	-1.149	-1.591
AAEL011881	conserved hypothetical protein	D	0.897	0.867
AAEL012605	conserved hypothetical protein	D	2.008	1.699
AAEL012851	wdpeat protein	D	0.903	1.017
AAEL012855	hypothetical protein	D	1.019	3.375
AAEL013851	conserved hypothetical protein (acetyltransferase (GNAT) family domain)	D	1.233	2.448
AAEL015631	asparagine synthetase	D	1.071	1.418
AAEL002969	brain chitinase and chia	DIG	0.452	1.381
AAEL003060	serine-type enodpeptidase, putative	DIG		2.319
AAEL005481	alpha-glucosidase	DIG	-0.062	-0.964

Gene ID	Description	Functional group	VgDome	VgHop
AAEL006121	Trypsin, putative	DIG		-0.788
AAEL008080	trypsin-eta, putative	DIG	1.369	
AAEL013262	conserved hypothetical protein	DIG	-1.001	
AAEL014361	amidase	DIG	0.782	
AAEL000064	dopachrome-conversion enzyme (DCE) isoenzyme, putative	I	0.357	0.764
AAEL000087	macroglobulin/complement	I	0.414	0.752
AAEL000621	antibacterial peptide, putative	I		1.547
AAEL000625	antibacterial peptide, putative	I		1.659
AAEL002276	serine protease, putative	I	-0.461	-1.607
AAEL002592	hypothetical protein	I	-0.523	-0.792
AAEL003625	clip-domain serine protease, putative	I	0.452	0.892
AAEL003723	lysozyme P, putative	I	0.691	0.94
AAEL003832	conserved hypothetical protein	I		1.168
AAEL003841	conserved hypothetical protein	I		1.435
AAEL004401	peroxinectin	I		-0.836
AAEL004522	gambicin	I		1.796
AAEL005431	clip-domain serine protease, putative	I	0.495	-1.312
AAEL006168	serine carboxypeptidase, putative	I	-0.372	-0.822
AAEL007006	serine protease	I	0.7	0.834
AAEL007969	serine protease	I	0.665	0.789
AAEL008607	tep3	I	0.43	-1.084
AAEL009637	cathepsin b	I	-0.462	1.513
AAEL009642	cathepsin b	I	-0.371	1.828
AAEL011446	galactose-specific C-type lectin, putative	I	-0.433	1.316
AAEL012092	leucinech repeat	I	0.601	1.094
AAEL012251	low-density lipoprotein receptor (ldl)	I	-0.371	-0.805
AAEL012471	protein tyrosine phosphatase, putative	I		-0.979
AAEL012711	trypsin, putative	I		1.502
AAEL014238	aromatic amino acid decarboxylase	I	-0.15	-0.795
AAEL014349	serine protease	I	-0.357	-1.681
AAEL014755	tep2	I	0.07	-0.957
AAEL015430	serine protease, putative	I	0.373	0.905

Gene ID	Description	Functional group	VgDome	VgHop
AAEL017325	Clip-Domain Serine Protease, family B. (Truncated Protease). [Source:Aedes_ManualAnnotation;Acc:AAEL800831]	I	-0.095	-0.978
AAEL000030	clip-domain serine protease, putative	I	1.079	0.496
AAEL000598	antibacterial peptide, putative	I	-0.877	0.719
AAEL000611	antibacterial peptide, putative	I	-1.488	
AAEL003279	clip-domain serine protease, putative	I	0.953	0.686
AAEL004120	Niemann-Pick Type C-2, putative	I	0.832	-0.144
AAEL006434	serine protease, putative	I	0.803	-0.39
AAEL006586	serine protease	I	-0.976	-0.482
AAEL007599	cathepsin b	I	1.229	0.703
AAEL011616	serine protease, putative	I	-0.956	-0.501
AAEL012064	Niemann-Pick Type C-2, putative	I	0.808	0.241
AAEL014004	clip-domain serine protease, putative	I	-0.761	0.461
AAEL014385	conserved hypothetical protein	I	-1.179	-0.298
AAEL017536	Holotricin, Glycine Rich Repreat Protein (GRRP), Anti-Microbial Peptide. [Source:Aedes_ManualAnnotation;Acc:AAEL800434]	I	0.801	0.186
AAEL005482	conserved hypothetical protein	I	2.369	2.205
AAEL006271	superoxide dismutase	I	-0.853	-0.821
AAEL006704	fibrinogen and fibronectin	I	0.794	1.227
AAEL006830	yellow protein precursor	I	-0.839	1.7
AAEL007585	cathepsin b	I	-0.853	1.184
AAEL011400	conserved hypothetical protein	I	2.634	2.21
AAEL011610	galactose-specific C-type lectin, putative	I	0.767	0.796
AAEL012216	cathepsin b	I	-0.776	1.181
AAEL013417	fibrinogen and fibronectin	I	1.584	1.708
AAEL014390	galactose-specific C-type lectin, putative	I	0.76	0.763
AAEL015312	cathepsin b	I	1.105	0.823
AAEL015458	transferrin	I	0.874	2.292
AAEL015639	transferrin	I	1.24	1.726

Gene ID	Description	Functional group	VgDome	VgHop
AAEL017132	C-Type Lysozyme (Lys-C). [Source:Aedes_ManualAnnotation;Acc:AAEL800171]	I	1.146	-1.13
AAEL010125	mitotic protein phosphatase 1 regulator, putative	I(LRR)	-0.382	-1.094
AAEL000006	phosphoenolpyruvate carboxykinase	M	0.511	1.422
AAEL000059	proacrosin, putative	M		0.944
AAEL000080	phosphoenolpyruvate carboxykinase	M		1.135
AAEL000101	AMP dependent coa ligase	M		1.032
AAEL000111	nitrilase, putative	M		0.928
AAEL001423	acid phosphatase-1	M	0.676	0.76
AAEL001586	glucosyl/glucuronosyl transferases	M	0.481	1.056
AAEL001593	glycerol-3-phosphate dehydrogenase	M		-0.756
AAEL002304	porphobilinogen synthase	M		-0.816
AAEL002964	brain chitinase and chia	M	-0.248	1.046
AAEL004059	cystathionine beta-lyase	M		0.769
AAEL004126	sterol desaturase	M	0.548	1.252
AAEL004127	acyl-coa dehydrogenase	M	0.656	1
AAEL004313	fk506-binding protein	M	-0.434	1.016
AAEL004739	acyl-coa dehydrogenase	M	-0.486	1.391
AAEL004757	cleavage and polyadenylation specificity factor	M	-0.518	0.768
AAEL005732	acyl-coa dehydrogenase	M		1.331
AAEL005740	AMP dependent ligase	M		0.94
AAEL006085	methylenetetrahydrofolate dehydrogenase	M	-0.108	0.758
AAEL006171	n-myc downstream regulated	M		0.786
AAEL006354	epoxide hydrolase	M		-0.851
AAEL007201	glutamyl aminopeptidase	M		-0.888
AAEL007707	malate dehydrogenase	M		-1.012
AAEL007880	ornithine decarboxylase	M	-0.091	-0.782
AAEL008006	3-hydroxyacyl-coa dehyrogenase	M	-0.732	-1.513
AAEL008302	glutamine-dependent nad(+) synthetase	M		-0.961
AAEL008330	hexaprenyldihydroxybenzoate methyltransferase	M	0.394	1.062

Gene ID	Description	Functional group	VgDome	VgHop
AAEL009503	4-nitrophenylphosphatase	M	0.686	1.389
AAEL009911	rotamase	M	0.38	1.465
AAEL010366	glucosyl/glucuronosyl transferases	M	0.592	0.918
AAEL010590	aldose-1-epimerase	M		-1.259
AAEL010691	ribonucleoside-diphosphate reductase small chain	M		-0.945
AAEL010938	l-asparaginase	M		0.842
AAEL011126	alcohol dehydrogenase	M		-0.794
AAEL011130	alcohol dehydrogenase	M		-0.852
AAEL012312	proliferation-associated 2g4 (pa2g4/ebp1)	M	-0.613	0.841
AAEL012341	lysosomal acid lipase, putative	M	-0.557	-1.067
AAEL012430	AMP dependent ligase	M	-0.509	-1.048
AAEL012697	sterol carrier protein-2, putative	M		-3.624
AAEL013245	proacrosin, putative	M	0.574	1.485
AAEL013458	glutamine synthetase 1, 2 (glutamate-amonia ligase) (gs)	M	-0.11	-2.122
AAEL014662	AMP dependent coa ligase	M		1.081
AAEL017039	Conserved hypothetical protein (alcohol dehydrogenase 2 [Culex quinquefasciatus] _	M	-0.364	1.013
AAEL017299	AMP dependent coa ligase, putative	M		0.973
AAEL007097	4-nitrophenylphosphatase	M	-1.064	-0.357
AAEL007883	fk506-binding protein	M	0.798	
AAEL008144	AMP dependent ligase	M	0.918	-0.327
AAEL008467	cysteine synthase	M	1.265	
AAEL009038	prolylcarboxypeptidase, putative	M	-1.184	-0.387
AAEL009462	hydroxyacylglutathione hydrolase	M	1.342	0.13
AAEL011624	granzyme A precursor, putative	M	0.894	
AAEL012179	methylthioadenosine phosphorylase	M	0.834	0.444
AAEL012825	bifunctional purine biosynthesis protein	M	-1.089	
AAEL013521	tryptophanyl-tRNA synthetase	M	0.771	0.183
AAEL013967	Methylmalonyl-CoA carboxyltransferase 12S subunit, putative	M	-1.655	

Gene ID	Description	Functional group	VgDome	VgHop
AAEL015143	glycine rich RNA binding protein, putative	M	-1.01	
AAEL001548	glucosyl/glucuronosyl transferases	M	0.975	1.273
AAEL002422	cytoplasmic polyadenylation element binding protein (cpeb)	M	-1.192	-0.861
AAEL009246	glycoside hydrolases	M	0.878	1.662
AAEL014709	methionine-tRNA synthetase	M	-1.072	2.089
AAEL015337	neutral alpha-glucosidase ab precursor (glucosidase ii alpha subunit) (alpha glucosidase 2)	M	-0.967	-2.407
AAEL005638	conserved hypothetical protein	PROT		1.034
AAEL006563	retinoid-inducible serine carboxypeptidase (serine carboxypeptidase	PROT		1.62
AAEL008862	conserved hypothetical protein	PROT	-0.523	-1.014
AAEL009771	hypothetical protein	PROT		0.816
AAEL014350	hypothetical protein	PROT		-0.83
AAEL014353	conserved hypothetical protein	PROT		-0.957
AAEL015527	conserved hypothetical protein	PROT		0.768
AAEL017451	proteolysis, metallopeptidase activity, peptidyl-dipeptidase activity, membrane.	PROT	0.431	0.789
AAEL009406	n(4)-(beta-n-acetylglucosaminyl)-l-asparaginase	PROT	1.252	
AAEL011658	plasma glutamate carboxypeptidase	PROT	1.067	0.403
AAEL015432	Trypsin, putative	PROT	-1.16	-0.491
AAEL000252	hypothetical protein	PROT	0.845	0.766
AAEL006323	hypothetical protein	PROT	0.797	-3.277
AAEL006542	retinoid-inducible serine carboxypeptidase (serine carboxypeptidase	PROT	0.932	1.054
AAEL010196	trypsin	PROT	2.031	2.224
AAEL010634	hypothetical protein	REDOX	-0.295	0.938
AAEL010592	esterase, putative	REDOX	1.749	0.442
AAEL000546	carboxylesterase	RSM		-1.14
AAEL001960	cytochrome P450	RSM		-1.033
AAEL002046	cytochrome P450	RSM	-0.741	-0.82
AAEL002886	thioredoxin reductase	RSM	-0.025	-0.8

Gene ID	Description	Functional group	VgDome	VgHop
AAEL003380	cytochrome P450	RSM		1.003
AAEL004643	mitochondrial ribosomal protein L1	RSM	-0.644	-0.805
AAEL005178	juvenile hormone esterase	RSM	0.616	1.172
AAEL005305	conserved hypothetical protein	RSM		-0.824
AAEL005946	NADH-ubiquinone oxidoreductase subunit B14.5b	RSM	-0.268	0.956
AAEL006230	gonadotropin inducible transcription factor	RSM		-0.758
AAEL006824	cytochrome P450	RSM	-0.314	2.468
AAEL007046	mitochondrial brown fat uncoupling protein	RSM	0.193	-0.854
AAEL007355	mitochondrial ribosomal protein, S18A, putative	RSM	0.258	0.95
AAEL008128	mitochondrial inner membrane protein translocase, 13kD-subunit, putative	RSM	0.426	0.781
AAEL008397	glutathione peroxidase	RSM		-1.617
AAEL010075	oxidoreductase	RSM		1.014
AAEL011016	carboxypeptidase m	RSM	0.662	1.25
AAEL012427	metabolic proces, oxidoreductase activity, oxidation reduction	RSM		-0.863
AAEL013066	checkpoint kinase	RSM	-0.473	-1.071
AAEL013555	cytochrome P450	RSM	-0.246	1.358
AAEL014019	cytochrome P450	RSM		0.918
AAEL014673	NADH:ubiquinone dehydrogenase, putative	RSM		1.103
AAEL014830	cytochrome P450	RSM		1.882
AAEL015578	alpha-esterase	RSM	0.108	-1.038
AAEL017071	Alpha-esterase, putative	RSM		-1.378
AAEL001210	NADH ubiquinone oxidoreductase subunit, putative	RSM	1.049	0.578
AAEL002683	aldehyde oxidase	RSM	1.058	
AAEL003890	cytochrome P450	RSM	-1.064	-0.396
AAEL007752	cytochrome c oxidase, subunit VIIA, putative	RSM	1.135	-0.688
AAEL008757	juvenile hormone esterase	RSM	0.861	0.392
AAEL009225	mitochondrial ribosome recycling factor	RSM	-0.807	-0.64

Gene ID	Description	Functional group	VgDome	VgHop
AAEL012845	mitochondrial import inner membrane translocase subunit tim44	RSM	0.777	
AAEL013744	NADH:ubiquinone dehydrogenase, putative	RSM	-1.282	
AAEL014893	cytochrome P450	RSM	-1.374	
AAEL015635	mitochondrial ribosomal protein, S10, putative	RSM	1.038	0.216
AAEL000986	NADH-ubiquinone oxidoreductase ashi subunit	RSM	2.027	2.067
AAEL003423	NADH dehydrogenase, putative	RSM	1.189	-1.074
AAEL004450	cytochrome b5, putative	RSM	0.84	0.826
AAEL007946	glutathione-s-transferase theta, gst	RSM	0.919	-0.797
AAEL010181	mitochondrial ribosomal protein, L51, putative	RSM	1.064	0.868
AAEL000032	ribosomal protein S6	RTT	0.606	0.965
AAEL000497	histone h2a	RTT	0.678	-1.106
AAEL002103	histone H1, putative	RTT		-0.901
AAEL002879	heterogeneous nuclear ribonucleoprotein r	RTT	-0.27	0.895
AAEL003352	ribosomal protein l7ae	RTT	0.534	0.751
AAEL003427	ribosomal protein S9, putative	RTT	-0.559	-0.751
AAEL003646	conserved hypothetical protein	RTT		0.867
AAEL003685	histone H3	RTT	0.449	-1.192
AAEL003818	histone h2a	RTT		-1.202
AAEL003820	histone h2a	RTT		-1.126
AAEL003826	histone h2a	RTT		-1.197
AAEL003851	histone h2a	RTT		-0.976
AAEL003942	60S ribosomal protein L44 L41, putative	RTT		1.133
AAEL004978	DEAD box ATP-dependent RNA helicase	RTT	-0.699	-0.79
AAEL005368	transcription initiation factor TFIIB	RTT		-0.92
AAEL007078	eukaryotic translation initiation factor 3, theta subunit	RTT	0.186	1.249
AAEL007928	eukaryotic translation initiation factor 4 gamma	RTT		-0.827
AAEL008266	hypothetical protein	RTT		-0.847
AAEL008500	DEAD box ATP-dependent	RTT		-0.956

Gene ID	Description	Functional group	VgDome	VgHop
	RNA helicase			
AAEL009653	40S ribosomal protein S30	RTT		2.336
AAEL010085	DNA polymerase epsilon subunit, putative	RTT		-1.042
AAEL010787	DEAD box ATP-dependent RNA helicase	RTT	-0.278	1.474
AAEL011150	RNA-binding protein precursor, putative	RTT		-0.775
AAEL012185	ribosome biogenesis regulatory protein	RTT	0.619	1.255
AAEL012684	conserved hypothetical protein	RTT	0.006	1.378
AAEL012877	homeobox protein extradenticle, putative	RTT	0.126	0.978
AAEL013221	60S ribosomal protein L10a	RTT	0.505	1.023
AAEL014764	acidic ribosomal protein P1, putative	RTT	-0.184	0.929
AAEL014838	60S ribosomal protein L27e	RTT	-0.444	-0.751
AAEL017595	5S ribosomal RNA [Source: RFAM 9.0]	RTT		0.76
AAEL017630	5S ribosomal RNA [Source: RFAM 9.0]	RTT		0.782
AAEL017779	5S ribosomal RNA [Source: RFAM 9.0]	RTT	0.435	0.757
AAEL002534	60S ribosomal protein L10	RTT	-2.644	0.277
AAEL003071	tRNA pseudouridine synthase D	RTT	0.986	0.527
AAEL003396	60S ribosomal protein L32	RTT	-0.765	
AAEL005127	ribonuclease UK114, putative	RTT	0.872	0.448
AAEL006698	60S ribosomal protein L31	RTT	0.812	0.431
AAEL007005	histone h2a	RTT	0.795	
AAEL010821	60S acidic ribosomal protein P0	RTT	1.649	-0.269
AAEL011251	RNA binding motif protein	RTT	-0.934	-0.681
AAEL012074	conserved hypothetical protein	RTT	2.387	0.194
AAEL013964	ribosomal protein L20, putative	RTT	1.411	
AAEL014106	ATP-dependent RNA helicase	RTT	-0.763	-0.358
AAEL015244	splicing factor 3a	RTT	1.212	
AAEL017685	Nuclear RNase P [Source: RFAM 9.0]	RTT	1.164	0.367
AAEL000518	histone h2a	RTT	0.826	-1.071
AAEL000525	histone h2a	RTT	0.768	-0.969
AAEL003659	histone H3	RTT	1.049	-1.216

Gene ID	Description	Functional group	VgDome	VgHop
AAEL005129	40S ribosomal protein S30	RTT	0.859	2.634
AAEL011447	60S ribosomal protein L14	RTT	1.526	1.235
AAEL012686	ribosomal protein S12, putative	RTT	1.388	1.085
AAEL017742	5S ribosomal RNA [Source: RFAM 9.0]	RTT	0.846	0.766
AAEL017760	5S ribosomal RNA [Source: RFAM 9.0]	RTT	0.888	0.76
AAEL000471	monocarboxylate transporter	TRP		0.809
AAEL001308	CRAL/TRIO domain-containing protein	TRP	-0.269	1.065
AAEL002063	cationic amino acid transporter	TRP	0.565	0.984
AAEL002576	sodium/solute symporter	TRP		0.931
AAEL003548	sulfate transporter	TRP	-0.641	-0.859
AAEL003626	sodium/shloride dependent amino acid transporter	TRP		0.832
AAEL004247	Sialin, Sodium/sialic acid cotransporter, putative	TRP		-0.758
AAEL005769	glucose dehydrogenase	TRP		-0.836
AAEL006138	hypothetical protein	TRP		1.353
AAEL008406	cationic amino acid transporter	TRP	0.647	0.955
AAEL008635	abc transporter	TRP	-0.293	0.907
AAEL009832	exocyst complex protein exo70	TRP	-0.255	-1.481
AAEL010102	tetraspanin, putative	TRP		1.164
AAEL010434	conserved hypothetical protein	TRP		1.519
AAEL010481	sugar transporter	TRP	-0.343	-1.701
AAEL010485	sugar transporter	TRP		1
AAEL010584	vesicular mannose-binding lectin	TRP	0.18	-0.972
AAEL011025	vacuolar ATP synthase subunit ac39	TRP		-2.707
AAEL011244	surfeit locus protein	TRP	-0.425	1.09
AAEL014927	sodium/chloride dependent transporter	TRP		-1.124
AAEL015549	calcineurin b subunit	TRP	0.387	0.769
AAEL002555	sodium/solute symporter	TRP	0.771	
AAEL002726	D7 protein, putative	TRP	-0.869	
AAEL004855	adp,atp carrier protein	TRP	-0.778	-0.628
AAEL005496	zinc/iron transporter	TRP	0.884	0.703
AAEL012674	d-amino acid oxidase	TRP	1.387	
AAEL007458	amino acid transporter	TRP	-0.833	-0.945
AAEL009863	sodium/dicarboxylate	TRP	1.445	1.056

Gene ID	Description	Functional group	VgDome	VgHop
	cotransporter, putative			
AAEL000309	hypothetical protein	U	0.318	-1.223
AAEL000619	conserved hypothetical protein	U		1.291
AAEL001325	conserved hypothetical protein	U	0.188	-1.639
AAEL001414	conserved hypothetical protein	U	-0.064	0.884
AAEL001718	conserved hypothetical protein	U	0.397	0.776
AAEL001880	conserved hypothetical protein	U		1.242
AAEL001885	conserved hypothetical protein	U		1.258
AAEL001888	hypothetical protein	U	0.555	1.118
AAEL001892	conserved hypothetical protein	U	-0.484	-1.661
AAEL001897	conserved hypothetical protein	U	-0.323	-1.378
AAEL002719	conserved hypothetical protein	U	0.362	0.81
AAEL002758	conserved hypothetical protein	U	0.286	1.46
AAEL002815	conserved hypothetical protein	U		1.197
AAEL002828	hypothetical protein	U		-0.805
AAEL002900	conserved hypothetical protein	U		0.774
AAEL003029	hypothetical protein	U	-0.316	0.81
AAEL003067	conserved hypothetical protein	U	0.11	0.997
AAEL003766	hypothetical protein	U	-0.211	0.901
AAEL003842	hypothetical protein	U	0.267	0.838
AAEL003944	conserved hypothetical protein	U		1.086
AAEL004498	hypothetical protein	U	0.384	0.806
AAEL004670	conserved hypothetical protein	U		1.172
AAEL004809	conserved hypothetical protein	U	0.317	0.858
AAEL004826	conserved hypothetical protein	U		-1.156
AAEL005215	conserved hypothetical protein	U		-0.759
AAEL005620	conserved hypothetical protein	U		1.81
AAEL005755	hypothetical protein	U	-0.25	1.331
AAEL006792	conserved hypothetical protein	U		-0.865
AAEL006848	conserved hypothetical protein	U	-0.606	-0.876
AAEL006863	hypothetical protein	U		3.042
AAEL006971	conserved hypothetical protein	U		0.876
AAEL007259	conserved hypothetical protein	U	-0.382	-0.914
AAEL007342	conserved hypothetical protein	U		-0.814
AAEL007847	conserved hypothetical protein	U		1.119
AAEL008025	conserved hypothetical protein	U	0.214	1.749
AAEL008039	conserved hypothetical protein	U		-1.212
AAEL008100	conserved hypothetical protein	U		-0.992
AAEL008274	conserved hypothetical protein	U	-0.158	-1.427

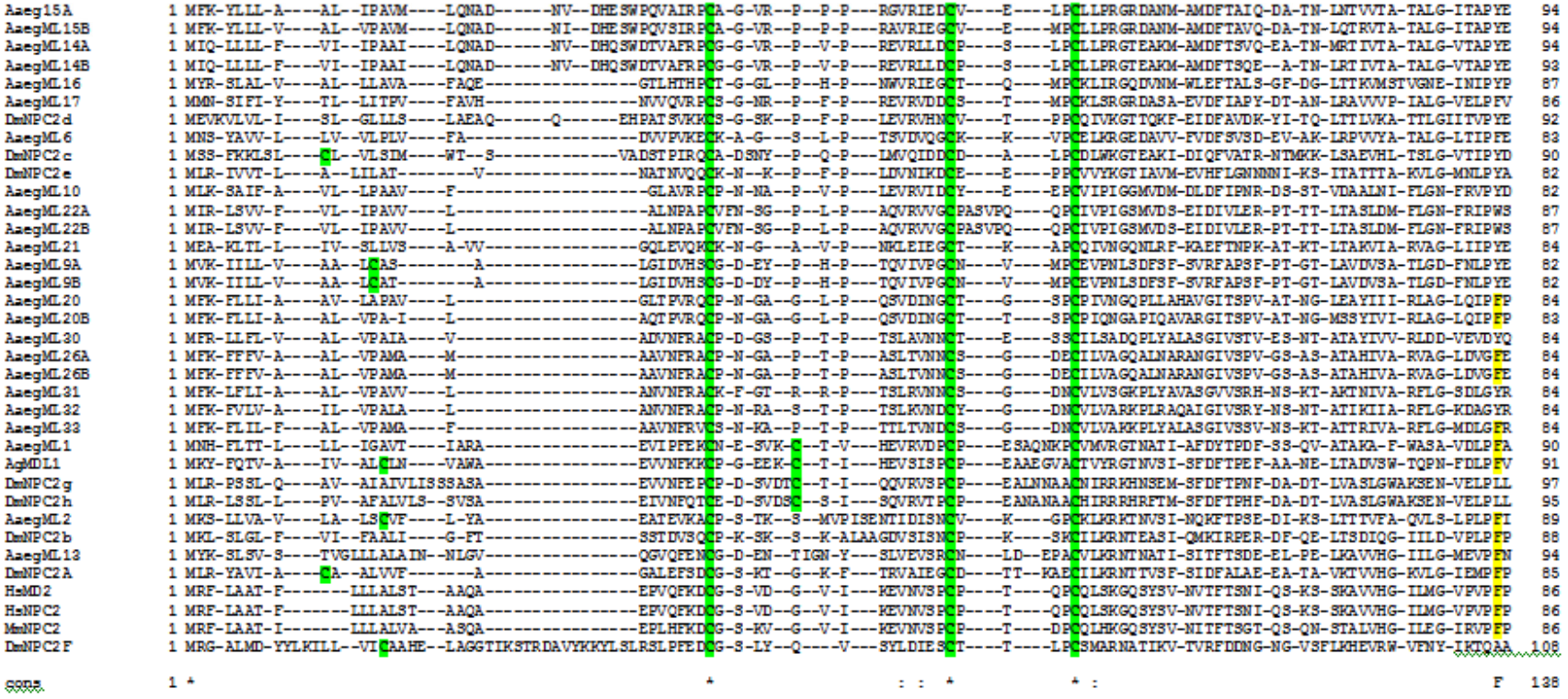
Gene ID	Description	Functional group	VgDome	VgHop
AAEL008286	conserved hypothetical protein	U		-1.18
AAEL008365	conserved hypothetical protein	U	0.262	-1.158
AAEL008485	conserved hypothetical protein	U		-1.848
AAEL008771	conserved hypothetical protein	U		1.584
AAEL008802	conserved hypothetical protein	U	-0.479	0.829
AAEL009177	conserved hypothetical protein	U	-0.399	-0.864
AAEL009487	hypothetical protein	U	0.282	0.893
AAEL009519	hypothetical protein	U		-0.785
AAEL010752	hypothetical protein	U		-1.077
AAEL011010	conserved hypothetical protein	U	0.325	1.191
AAEL011330	conserved hypothetical protein	U		-0.807
AAEL011388	conserved hypothetical protein	U	-0.136	0.756
AAEL011456	conserved hypothetical protein	U		1.762
AAEL011532	hypothetical protein	U	-0.694	-0.827
AAEL011665	hypothetical protein	U		-0.762
AAEL011884	hypothetical protein	U	0.395	-2.065
AAEL012208	hypothetical protein	U	-0.332	-1.004
AAEL012454	conserved hypothetical protein	U	0.456	1.13
AAEL012858	hypothetical protein	U		-0.952
AAEL012860	conserved hypothetical protein	U	0.573	0.912
AAEL012867	conserved hypothetical protein	U	0.632	1.242
AAEL013300	conserved hypothetical protein	U		-0.786
AAEL013484	hypothetical protein	U		-0.754
AAEL013486	hypothetical protein	U		-1.061
AAEL013800	conserved hypothetical protein	U	-0.652	-0.864
AAEL014171	conserved hypothetical protein	U		-1.184
AAEL014388	conserved hypothetical protein	U		-0.913
AAEL014511	predicted protein	U		-0.883
AAEL014565	hypothetical protein	U		-0.957
AAEL017034	Hypothetical protein	U		-1.584
AAEL017190	Hypothetical protein	U	0.227	-1.305
AAEL017530	hypothetical protein	U		-1.363
AAEL000019	conserved hypothetical protein	U	1.278	
AAEL001032	conserved hypothetical protein	U	-0.753	-0.462
AAEL001323	conserved hypothetical protein	U	0.933	
AAEL001511	conserved hypothetical protein	U	-0.769	-0.653
AAEL002889	hypothetical protein	U	2.243	
AAEL004100	hypothetical protein	U	1.055	0.384
AAEL004591	hypothetical protein	U	1.108	-0.655

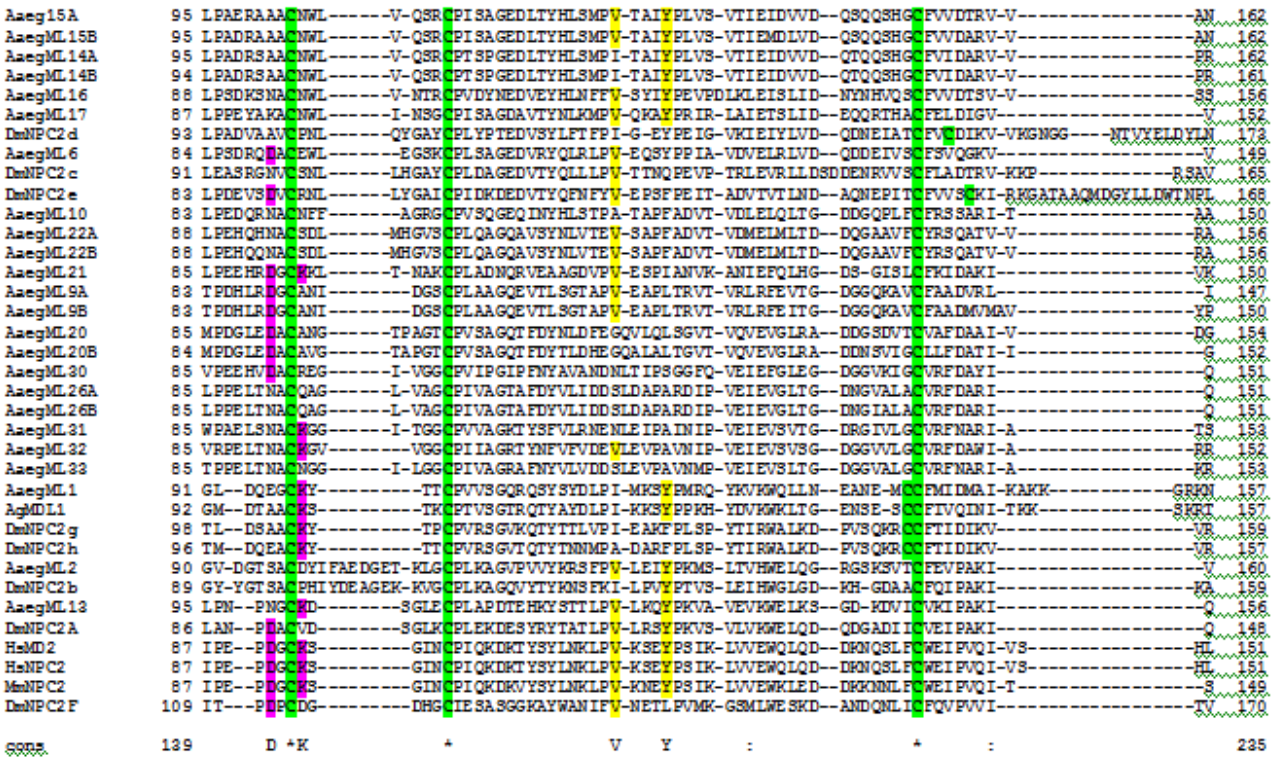
Gene ID	Description	Functional group	VgDome	VgHop
AAEL005968	conserved hypothetical protein	U	1.69	
AAEL006131	hypothetical protein	U	0.861	0.426
AAEL006585	predicted protein	U	-0.898	
AAEL006629	conserved hypothetical protein	U	-0.79	
AAEL006676	conserved hypothetical protein	U	0.891	0.455
AAEL006969	conserved hypothetical protein	U	1.265	
AAEL008182	conserved hypothetical protein	U	-1.003	-0.717
AAEL012293	conserved hypothetical protein	U	1.193	0.317
AAEL012859	conserved hypothetical protein	U	-0.75	0.589
AAEL013287	conserved hypothetical protein (cystatin-like domain; cysteine-type endopeptidase inhibitor activity)	U	-1.37	0.33
AAEL013843	conserved hypothetical protein	U	1.543	-0.24
AAEL015379	conserved hypothetical protein	U	-0.888	
AAEL017144	Hypothetical protein	U	1.364	
AAEL000566	conserved hypothetical protein	U	0.946	1.748
AAEL001107	hypothetical protein	U	-0.837	-0.924
AAEL002652	hypothetical protein	U	-1.234	-1.081
AAEL003482	hypothetical protein	U	1.257	1.157
AAEL005106	conserved hypothetical protein	U	1.749	0.958
AAEL007703	conserved hypothetical protein	U	3.215	3.352
AAEL008492	conserved hypothetical protein	U	1.721	1.275
AAEL008729	hypothetical protein	U	-0.788	-0.835
AAEL009201	conserved hypothetical protein	U	0.754	1.139
AAEL011928	conserved hypothetical protein	U	1.037	0.87
AAEL012710	conserved hypothetical protein	U	0.905	0.831
AAEL012862	hypothetical protein	U	1.151	2.434
AAEL013734	hypothetical protein	U	-1.287	-0.987
AAEL014068	conserved hypothetical protein	U	1.351	0.863
AAEL014300	hypothetical protein	U	0.842	0.868
AAEL014937	hypothetical protein	U	-0.945	-1.289
AAEL017016	Conserved hypothetical protein	U	0.828	0.958
AAEL017455	hypothetical protein	U	-0.981	1.642
AAEL017491	hypothetical protein	U	-1.251	-1.001

3. Summary of the changes in transcript abundance of ML and NPC1 genes after dengue virus infection in mosquito tissues. Dengue virus infected midgut at 7 day post ingestion of dengue virus infected blood (DV MG 7dpi), dengue virus infected midgut at 10 day post ingestion of dengue virus infected blood (DV MG 10dpi) and carcasses at 10 dpi (DV Car 10dpi) was performed previously [12], dengue virus infected salivary glands at 14 day post ingestion of dengue virus infected blood (DV SG 14dpi) and carcasses at 14 day post ingestion of dengue virus infected blood (DV Car 14dpi) was from Sim et al, 2012. Cactus silenced (Toll activated), Caspar silenced (Imd activated), and PIAS silenced (JAK/STAT activated) were also included. The genes selected for further studies are in bold

Name	Accession#	DV MG 7dpi	DV MG 10dpi	DV Car 10dpi	DV SG 14 dpi	DV Car 14dpi	Cactus	Caspar	PIAS
ML1	AAEL004120								
ML10	AAEL015135								
ML13	**AAEL006854**	**1.138**	**1.143**						
ML14A	AAEL009553								
ML14B	AAEL015516								
ML15A	AAEL009555								
ML15B	AAEL009556								
ML16	AAEL015140								
ML17	AAEL009557						1.77		
ML2	AAEL012064								
ML20	AAEL015137								
ML20B	AAEL007592		0.931				-0.719		
ML21	**AAEL009760**				**2.12**				
ML22A	AAEL015139								
ML22B	AAEL009954								
ML26A	AAEL007591								
ML26B	AAEL013835								
ML30	AAEL001654								
ML31	AAEL001661								
ML32	AAEL001634								
ML33	**AAEL001650**			**1.51**				**0.615**	
ML6	**AAEL015136**				**1.60**		**-1.38**		
ML9A	AAEL015138								
ML9B	AAEL009953								
NPC1a	AAEL003325		0.85	-0.949					
NPC1b	**AAEL009531**	**0.89**				**1.43**		**-0.83**	

4. Multiple sequence alignment of amino acid sequences from insect ML genes. Cysteine residues are labeled with green color. The yellows are conserved amino acids required for cholesterol binding and magenta is the conserved amino acid required for NPC2 function but not cholesterol binding.




Aaeg15A 95 LPAERAAACNWL------V-QSRCPISAGEDLTYHLSMPV-TAIYPLVS-VTIEIDVVD--QSQQSHGCFVVDTRV-V---------------AN 162
AaegML15B 95 LPADRAAACNWL------V-QSRCPISAGEDLTYHLSMPV-TAIYPLVS-VTIEMDLVD--QSQQSHGCFVVDARV-V---------------AN 162
AaegML14A 95 LPADRSAACNWL------V-QSRCPTSPGEDLTYHLSMPI-TAIYPLVS-VTIEIDVVD--QTQQSHGCFVIDARV-V---------------PR 162
AaegML14B 94 LPADRSAACNWL------V-QSRCPTSPGEDLTYHLSMPI-TAIYPLVS-VTIEIDVVD--QTQQSHGCFVIDARV-V---------------PR 161
AaegML16 88 LPSDKSNACNWL------V-NTRCPVDYNEDVEYHLNFFV-SYIYPEVPDLKLEISLID--NYNHVQSCFVVDTSV-V---------------SS 156
AaegML17 87 LPPEYAKACNWL------I-NSGCPISAGDAVTYNLKMPV-QKAYPRIR-LAIETSLID--EQQRTHACFELDIGV---------------V 152
DmNPC2d 93 LPADVAAVCPNL------QYGAYCPLYPTEDVSYLFTFPI-G-EYPEIG-VKIEIYLVD--QDNEIATCFVSDIKV-VKGNGG----NTVYELDYLN 173
AaegML6 84 LPSDRQDACEWL-------EGSKCPLSAGEDVRYQLRLPV-EQSYPPIA-VDVELRLVD--QDDEIVSCFSVQGKV---------------V 149
DmNPC2c 91 LEASRGNVCSNL------LHGAYCPLDAGEDVTYQLLLPV-TTNQPEVP-TRLEVRLLDSDDENRVVSCFLADTRV-KKP----------RSAV 165
DmNPC2e 83 LPDEVSDVCRNL------LYGAICPIDKDEDVTYQFNFYV-EPSFPEIT-ADVTVTLND--AQNEPITCFVVSCKI-RKGATAAQMDGYLLDWTNPL 168
AaegML10 83 LPEDQRNACNFF-------AGRGCPVSQGEQINYHLSTPA-TAPFADVT-VDLELQLTG--DDGQPLFCFRSSARI-T---------------AA 150
AaegML22A 88 LPEHQHNACSDL------MHGVSCPLQAGQAVSYNLVTEV-SAPFADVT-VDMELMLTD--DQGAAVFCYRSQATV-V---------------PA 156
AaegML22B 88 LPEHQQNACSDL------MHGVSCPLQAGQAVSYNLVTEV-SAPFADVT-VDMELMLTD--DQGAAVFCYRSQATV-V---------------PA 156
AaegML21 85 LPEEHRDGCWKL------T-NAKCPLADNQRVEAAGDVPV-ESPIANVK-ANIEFQLHG--DS-GISLCFKIDAKI---------------VK 150
AaegML9A 83 TPDHLRDGCANI--------DGSCPLAAGQEVTLSGTAPV-EAPLTRVT-VRLRFEVTG--DGGQKAVCFAADVRL---------------I 147
AaegML9B 83 TPDHLRDGCANI--------DGSCPLAAGQEVTLSGTAPV-EAPLTRVT-VRLRFEITG--DGGQKAVCFAADMVMAV---------------VP 150
AaegML20 85 MPDGLEDACANG------TPAGTCPVSAGQTFDYNLDFEGQVLQLSGVT-VQVEVGLRA--DDGSDVTCVAFDAAI-V---------------DG 154
AaegML20B 84 MPDGLEDACAVG------TAPGTCPVSAGQTFDYTLDHEGQALALTGVT-VQVEVGLRA--DDNSVIGCLLFDATI-I---------------G 152
AaegML30 85 VPEEHVDACREG------I-VGGCPVIPGIPFNYAVANDNLTIPSGGFQ-VEIEFGLEG--DGGVKIGCVRFDAYI---------------Q 151
AaegML26A 85 LPPELTNACQAG------L-VAGCPIVAGTAFDYVLIDDSLDAPARDIP-VEIEVGLTG--DNGVALACVRFDARI---------------Q 151
AaegML26B 85 LPPELTNACQAG------L-VAGCPIVAGTAFDYVLIDDSLDAPARDIP-VEIEVGLTG--DNGIALACVRFDARI---------------Q 151
AaegML31 85 WPAELSNACWGG------I-TGGCPVVAGKTYSFVLRNEMLEIPAINIP-VEIEVSVTG--DRGIVLGCVRFNARI-A---------------TS 153
AaegML32 85 VRPELTNACWGV--------VGGCPIIAGRTYNFVFVDEVLEVPAVNIP-VEIEVSVSG--DGGVVLGCVRFDAWI-A---------------PR 152
AaegML33 85 TPPELTNACNGG------I-LGGCPIVAGRAFNYVLVDDSLEVPAVNMP-VEIEVSLTG--DGGVALGCVRFNARI-A---------------PR 153
AaegML1 91 GL--DQEGCKY----------TTCPVVSGQRQSYSYDLPI-MKSYPMRQ-YKVKWQLLN--EANE-MCCFMIDMAI-KAKK----------GRKN 157
AgMDL1 92 GM--DTAACWS----------TKCPTVSGTRQTYAYDLPI-KKSYPPKH-YDVKWKLTG--ENSE-SCCFIVQINI-TKK----------SKRT 157
DmNPC2g 98 TL--DSAACKY----------TPCPVRSGVKQTYTTLVPI-EAKFPLSP-YTIRWALKD--FVSQKRCCFTIDIKV---------------VR 159
DmNPC2h 96 TM--DQEACKY----------TTCPVRSGVTQTYTNNMPA-DARFPLSP-YTIRWALKD--FVSQKRCCFTIDIKV---------------VR 157
AaegML2 90 GV-DGTSACDYIFAEDGET-KLGCPLKAGVPVVYKRSFPV-LEIYPKMS-LIVHWELQG--RGSKSVTCFEVPAKI---------------V 160
DmNPC2b 89 GY-YGTSACPHIYDEAGEK-KVGCPLKAGQVYTYKNSFKI-LPVYPTVS-LEIHWGLGD--KH-GDAACFQIPAKI---------------KA 159
AaegML13 95 LPN--PNGCWD--------SGLECPLAPDTEHKYSTTLPV-LKQYPKVA-VEVKWELKS--GD-KDVICVKIPAKI---------------Q 156
DmNPC2A 86 LAN--PDACVD--------SGLKCPLEKDESYRYTATLPV-LRSYPKVS-VLVKWELQD--QDGADIICVEIPAKI---------------Q 148
HsMD2 87 IPE--PDGCWS---------GINCPIQKDKTYSYLNKLPV-KSEYPSIK-LVVEWQLQD--DKNQSLFCWEIPVQI-VS---------------HL 151
HsNPC2 87 IPE--PDGCWS---------GINCPIQKDKTYSYLNKLPV-KSEYPSIK-LVVEWQLQD--DKNQSLFCWEIPVQI-VS---------------HL 151
MmNPC2 87 IPE--PDGCWS---------GINCPIQKDKVYSYLNKLPV-KNEYPSIK-LVVEWKLED--DKKNNLFCWEIPVQI-T---------------S 149
DmNPC2F 109 IT---PDPCDG---------DHGCIESASGGKAYWANIFV-NETLPVMK-GSMLWESKD--ANDQNLICFQVPVVI---------------TV 170
conS 139 D *K * V Y : * : 235

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EDUCATION

2012-Present Johns Hopkins Bloomberg School of Public Health, expected May, 2016
Doctor of Philosophy (Molecular Microbiology and Immunology)
Advisor: George Dimopoulos

2010-2012 Johns Hopkins Bloomberg School of Public Health, May, 2012
Master of Science in Molecular Microbiology and Immunology
Advisor: George Dimopoulos

2007-2009 Mahidol University
Master of Science in Biotechnology, August, 2009
Advisors: Timothy W. Flegel, Boonsirm Withyachumnarnkul, Kallaya Sritunyalucksana

2003-2007 Mahidol University
Bachelor of Science in Biotechnology with Second class honor, May, 2007

RESEARCH EXPERIENCE

August 2012- present
PhD student, *Laboratory of Dr George Dimopoulos, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health*

December 2010- May 2012
ScM student, *Laboratory of Dr George Dimopoulos, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health*

August 2010-2015
Research assistant, Laboratory of Dr. Fidel Zavala, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health

August 2009-July 2010
Research assistant, Shrimp-Virus Interaction Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.
Under the supervision of Dr. Kallaya Sritunyalucksana.

June 2007-August 2009
Master degree student, Department of Biotechnology, Faculty of Science, Mahidol University Thailand.

Under the supervision of Dr.Timothy W. Flegel, Dr.Boonsirm Withyachumnarnkul, and Dr.Kallaya Sritunyalucksana.

August 2006-May 2007
Senior Project research, Department of Biotechnology, Faculty of Science, Mahidol University Thailand.
Under the supervision of Dr.Timothy W. Flegel, and Dr.Kallaya Sritunyalucksana.

April 2006-May 2006
Research internship, Armed Forces Research Institute of Medical Sciences (AFRIMS), Thailand.
Under the supervision of Dr. Thippawan Chuenchitr.

TEACHING EXPERIENCE

January 2015
Teaching assistant in PH. 260.613: Techniques in Molecular Biology
- *Prepared materials and teach basic molecular biology techniques*

September 2011
Teaching assistant in PH.260.852: Molecular Biology literature
- *Assisted Dr. Jelena Levitskaya in class discussion, and encouraged student participation*

October 2009
Teaching assistant in SCBT 609: Biology and Pathology of shrimp
- *Prepared materials for lab section in the course*
- *Assisted students and demonstrated lab in Shrimp total hemocyte count and Bacterial isolation and identification using biochemical and bioinformatics analysis.*

November 2008-March 2009
Teaching assistant in SCBT 203: Bacteriology
- *Assisted and demonstrated students in lab section of the course, held office hours to discuss the lab report and homework*

October 2008
Teaching assistant in SCBT 609: Biology and Pathology of shrimp
- *Prepared materials for lab section in the course*
- *Assisted students and demonstrated lab in Shrimp total hemocyte count and Bacterial isolation and identification using biochemical analysis.*

HONORS AND AWARDS

2010-present	*Ministry of Science and Technology , Royal Thai Government Scholarship for Master and PhD study*
2014	*James Stuart Porterfield Prize in International Virology*
2012-2013	*Dr.Lioyd and Mae Rozeboom Scholarship for academic excellence and research potential in molecular microbiology and immunology*
2011-2012	*Tuition Scholarship from Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health.*

2009	*Honorable mention: student presentation at the 11th International congress of International Society of Developmental and Comparative Immunology, Prague, Czech Republic*
2009	*Travel award from the Department of Biotechnology, Faculty of Science, Mahidol University.*
2007-2009	*Thailand Graduate Institute of Science and Technology (TGIST) scholarship from the National Science and Technology Development Agency (NSTDA), Thailand* *cover tuition fee and stipend*
2007	*Best Presentation Award from The 8th Science Project Exhibition, Faculty of Science, Mahidol University, Thailand*
2003-2007	*Sritang Thong Scholarship from the Faculty of Science, Mahidol University to cover tuition fee and stipend.*

ORAL AND POSTER PRESENTATIONS

Poster 2014 JAK/STAT super-immune *Aedes aegypti* as a tool for dengue disease control. **Jupatanakul N, Sim S, Anglero Y, Souza-Neto J, Dimopoulos G.** The forth pan-american dengue research network meeting, Belem, Brazil.

Oral 2013 *Aedes aegypti* laboratory adaptation leads to global transcriptomic down-regulation and dengue vector capacity changes. **Jupatanakul N, Bahia AC, Sim S, Dimopoulos G.** The 62nd ASTMH annual meeting, Washington DC, USA.

Oral 2013 Laboratory adaptation leads to global transcriptomic down-regulation and dengue vector capacity changes in *Aedes aegypti*. **Jupatanakul N, Bahia AC, Sim S, Dimopoulos G.** The Third International Conference on Dengue and Dengue Hemorrhagic fever, Bangkok, Thailand.

Poster 2012 *Aedes aegypti* ML and Niemann-Pick type C proteins are agonists of dengue virus infection. **Jupatanakul N, Sim S, Dimopoulos G.** The Third pan-american dengue research network meeting, Cartagena, Colombia.

Oral 2009 PmRab7 and its interacting partners are involved in white spot syndrome virus infection in the black tiger shrimp, *Penaeus monodon*, a WSSV binding protein. **Jupatanakul N**, Wannapapho W, Ongvarrasopone C, Saksamerprome W, Senapin S, Sritunyalucksana K. The 11th International congress of International Society of Developmental and Comparative Immunology, Prague, Czech Republic.

Poster 2008 Yeast two-hybrid system identification of shrimp proteins that interacts with PmRab7, a WSSV binding protein of shrimp. **Jupatanakul N**, Areechon N, Wannapapho W, Senapin S, Ongvarrasopone C, Sritunyalucksana K. Poster presentation, The 7th Symposium on Diseases in Asian Aquaculture (DAA VII), Taipei, Taiwan.

Oral 2008 Recombinant VP28 and PmRab7 production and their use in protection against White spot syndrome virus. Sritunyalucksana K, Wannapapho W, **Jupatanakul N**, Eurwilaichitr L, Withyachumnarnkul B. Oral presentation, The 7th Symposium on Diseases in Asian Aquaculture (DAA VII), Taipei, Taiwan.

Poster 2008 Yeast feed additive for shrimp viral protection. **Jupatanakul N**, Wannapapho W,Sritunyalucksana K, Withyachumnarnkul B, and Flegel TW. Poster presentation, NSTDA Annual Conference, Thailand Science Park, Thailand.

Oral 2007 PmRab7 in yeast system: Protein production and protein-protein interaction.**JupatanakulN**, Wannapapho W, Sritunyalucksana K, and Flegel TW.

Oral presentation, The 8th Science Project Exhibition. Faculty of Science, Mahidol University, Thailand.

Poster 2007 Production of recombinant PmRab7 in *Pichia pastoris*. **Jupatanakul N**, Wannapapho W, Sritunyalucksana K, and Flegel TW. Poster presentation, The 6th National Symposium on Marine Shrimp, Thailand.

PUBLICATIONS

Bottino-Rojas V, Talyuli OAC, **Jupatanakul N**, Sim S, Dimopoulos G, Venancio TM, et al. Heme Signaling Impacts Global Gene Expression, Immunity and Dengue Virus Infectivity in *Aedes aegypti*. PLoS ONE. 2015;10: e0135985. doi:10.1371/journal.pone.0135985

Sim S, J**upatanakul N**, Dimopoulos G. Mosquito Immunity against Arboviruses. Viruses. 2014 Nov 19;6(11):4479-4504.

Jupatanakul N, Sim S, Dimopoulos G. The Insect Microbiome Modulates Vector Competence for Arboviruses. Viruses. 2014 Nov 11;6(11):4294-4313.

Dennison NJ, **Jupatanakul N**, Dimopoulos G, The mosquito microbiota influences vector competence for human pathogens, Current Opinion in Insect Science, Volume 3, September 2014, Pages 6-13, ISSN 2214-5745, http://dx.doi.org/10.1016/j.cois.2014.07.004.

Kang S, Shields AR, **Jupatanakul N**, Dimopoulos G (2014) Suppressing Dengue-2 Infection by Chemical Inhibition of *Aedes aegypti* Host Factors. PLoS Negl Trop Dis 8(8): e3084. doi:10.1371/journal.pntd.0003084

Jupatanakul N, Sim S, Dimopoulos G, *Aedes aegypti* ML and Niemann-Pick type C family members are agonists of dengue virus infection, Developmental & Comparative Immunology, Volume 43, Issue 1, March 2014, Pages 1-9,

Sim S, **Jupatanakul N**, Ramirez JL, Kang S, Romero-Vivas CM, et al. (2013) Transcriptomic Profiling of Diverse Aedes aegypti Strains Reveals Increased Basal-level Immune Activation in Dengue Virus-refractory Populations and Identifies Novel Virus-vector Molecular Interactions. PLoS Negl Trop Dis 7(7): e2295. doi:10.1371/journal.pntd.0002295

Jupatanakul N, Wannapapho W, Eurwilaichitr L, Flegel TW, Sritunyalucksana K. Cloning and expression of recombinant shrimp PmRab7 (a virus-binding protein) in *Pichia pastoris*. Protein Expr Purif. 2011 Mar;76(1):1-6. Epub 2010 Nov 4. PubMed PMID: 21056104.

BOOK CHAPTER

Jupatanakul N, and Dimopoulos G. 2016. Chapter 8. "Molecular Interactions Between Arboviruses and Insect Vectors: Insects' Immune Responses to Virus Infection" In: Arboviruses: Molecular Biology, Evolution and Control, Caister Academic Press, D. Gubler and N. Vasilakis (eds), in press. ISBN 978-1-910190-21-0

PATENT

Production of PmRab7 in yeast and its use for WSSV protection in shrimp. Sritunyalucksana K, **Jupatanakul N**, Flegel TW, Eurwilaichitr L, patent filing no. 0801004402, Thailand.

PROFESSIONAL MEMBERSHIPS

- Member of International Society of Developmental and Comparative Immunology
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- Member of American Society of Tropical Medicine and Hygine

REFERENCES

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