Published in final edited form as:

Dev Comp Immunol. 2014 March ; 43(1): 1–9. doi:10.1016/j.dci.2013.10.002.

***Aedes aegypti* ML and Niemann-Pick type C family members are agonists of dengue virus infection**

Natapong Jupatanakul¹, Shuzhen Sim^{1,2}, and George Dimopoulos^{1,*}

¹W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe St., Baltimore, Maryland, USA 21205

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.

Keywords

dengue virus; *Aedes aegypti*; myeloid differentiation 2-related lipid recognition gene; Niemann Pick-type C gene

1. Introduction

Dengue fever and dengue hemorrhagic fever are the most important arthropod-borne viral diseases, with an estimated 390 million infections occurring annually across more than 100 countries in the tropics and subtropics (Bhatt et al., 2013). A dramatic increase in dengue incidence in recent decades has resulted from an increased global prevalence of its primary vector, *Aedes aegypti*, along with its secondary vector, *Ae. albopictus* (Guzman et al., 2010; Whitehead et al., 2007). Dengue can be caused by any of four antigenically distinct serotypes (DENV serotype 1 to 4), and there are currently no anti-DENV drugs or vaccines available. Thus, disease control relies mainly on mosquito-targeted intervention programs.

© 2013 Elsevier Ltd. All rights reserved.

*Correspondence should be addressed to G. D. (gdimopou@jhsph.edu, +1- 443-287-0128).

²Current address: Genome Institute of Singapore, Singapore

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

However, the conventional mosquito elimination programs depend on the use of insecticides and environmental management, which raise ecological, environmental, and effectiveness concerns (Ault, 1994; K. Dong, 2007; Gubler, 1998; Rivero et al., 2010). For this reason, the development of novel vector and disease control strategies is essential, and a molecular understanding of the mosquito's immune responses against these viruses is needed.

DENV is transmitted from infected humans to other individuals through mosquito bites. After mosquitoes feed on infectious blood, the virus infects the mosquito midgut epithelium and propagates to establish the infection (Black et al., 2002). Virus levels in the midgut generally peak at 7–10 days, with the virus then disseminating to other parts of the body through the trachea. The virus finally infects the salivary glands, from which it can be transmitted to another host through a mosquito blood meal, which typically occurs about 10 days after the original infectious blood meal (10 dpbm) (Salazar et al., 2007).

The publication of the *Ae. aegypti* genome in 2007 (Nene et al., 2007) has opened new avenues for the study of the mosquito's response to DENV infection. 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 (Souza-Neto et al., 2009; Xi et al., 2008). 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 (Inohara and Nunez, 2002). Members of this family have diverse functions associated with lipid recognition, including pathogen recognition, lipid trafficking and metabolism, and pheromone perception (Chang et al., 2006; Gruber et al., 2004; Horácková et al., 2010; Starostina et al., 2009). 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) (Shimazu et al., 1999). *Drosophila* MD2 homologs have been shown to mediate the activation of the immune deficiency (IMD) immune signaling pathway upon exposure to lipopolysaccharide (LPS) (Shi et al., 2012). The *An. gambiae* homolog of ML, AgMDL1, is involved in the mosquito's immune defense against *Plasmodium falciparum* infection (Y. Dong et al., 2006). 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 (Chang et al., 2006; W. S. Garver and Heidenreich, 2002). NPC1 has been shown to be required for the Ebola virus to escape from the vesicular compartment (Carette et al., 2011; Côté et al., 2011), but the immune function of the NPC1 family in the 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 suggested roles for an ML and a NPC1 protein 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.

2. Materials and methods

2.1 Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University (Permit Number: M006H300). Commercial anonymous human blood was used for DENV infection experiments. The Johns Hopkins School of Public Health Ethics Committee has approved this protocol.

2.2 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 (Lawson et al., 2009; Waterhouse et al., 2007). To compare sequence similarity, a multiple sequence alignment (MSA) was generated using t-coffee software (<http://www.tcoffee.org/>) (Di Tommaso et al., 2011). The MSA was then used to generate a phylogenetic tree using MEGA 5.05 software (Tamura et al., 2011). 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 (Sim et al., 2012; Xi et al., 2008) Dimopoulos group, unpublished data).

2.3 Mosquito strains and mosquito maintenance

The mosquitoes used for most of the experiments were of the *Ae. aegypti* Rockefeller/UGAL strain. The other mosquito strain used to confirm that the results were common among different mosquito strains was a recently colonized *Ae. aegypti* population obtained from the Caribbean island of Saint Kitts (sixth to seventh generation) (Sim et al., 2013). The mosquitoes were maintained on a 10% sugar solution at 27°C and 95% humidity with a 12-hr light/dark cycle, following the protocol described previously (Xi et al., 2008).

2.4 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% CO₂.

The baby hamster kidney (BHK) cell line was used for plaque assays. It was 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% CO₂.

2.5 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 (L. Garver and Dimopoulos, 2007). 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.

2.6 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 (Das et al., 2007). In brief, DENV stock was added to a 75-cm² 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 CO₂ and a 37°C water bath, centrifuged at 800g for 10 min, and mixed 1:1 with commercial human blood. The infectious blood meal (~10⁶–10⁷ 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.

2.7 DENV titration by plaque assay

Virus titers in the midguts were determined at 7 dpbm according to an established protocol (Das et al., 2007; Xi et al., 2008). 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, 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% CO₂. 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.

2.8 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 Table S3.

2.9 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).

3. Results

3.1 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>)) (Huang et al., 2007; Waterhouse et al., 2007). Of these

26 members, 24 contain the ML domains and are annotated as 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 *Drosophila* NPC2 genes will be considered as ML gene family genes (as per the annotation in immunoDB database) (Table S1). All six cysteines are conserved in all the ML genes used for analysis, suggesting their importance for protein structure and function across organisms (data not shown). Phylogenetic analysis of the ML genes suggests four groups of *Ae. aegypti* ML genes, with groups 2 being unique to *Ae. aegypti* (Figure 1A). The amino acid sequence of AegML13 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 (Ko et al., 2003), were mutated in some of *Aedes* ML genes (data not shown).

While the human genome contains only one NPC1, the mosquito genomes (*C. pipiens quinquefasciatus*, *Ae. gambiae*, and *Ae. aegypti*) contain two NPC1 genes (NPC1a and NPC1b), as in *Drosophila* (Figure 1B) (Huang et al., 2005). 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.

3.2 *Ae. aegypti* ML and NPC1 family members are agonists of 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 (Sim et al., 2012; Xi et al., 2008, Dimopoulos group, unpublished data), suggesting possible roles for these proteins as DENV host or restriction factors. Upon DENV infection, the transcript abundance of AegML13 and AegML33 increased in the mosquito midgut (2.2 fold) and carcass (2.8 fold), respectively, and the mRNA abundance of AegNPC1b increased in the midgut compartment (1.9 fold). We further investigated whether any of these genes are 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 2). Silencing of AegNPC1b and AegML33 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 AegML13, 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 AegNPC1b and AegML33 since the silencing of these genes resulted in phenotypic change.

3.3 AegNPC1b and AegML33 are dengue agonists in field-derived strains 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 AegNPC1b and AegML33 on DENV infection is conserved in field-derived mosquitoes. Silencing these genes in the recently colonized *Ae. aegypti* strain from Saint Kitts (Sim et al., 2013) prior to feeding on DENV-infected blood resulted in significantly lower midgut DENV titers than in the controls, as we had observed for the laboratory strain (Figure 3).

3.4 *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 S1). 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). 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.

3.5 AegNPC1b and AegML33 may regulate *Ae. aegypti* immune pathways

Since the transient silencing of AegNPC1b and AegML33 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 AegNPC1b and AegML33 gene silencing. Activation of the Toll, Imd, and JAK/STAT pathways results in increased antimicrobial peptide (AMP) gene expression (Shi et al., 2012; Souza-Neto et al., 2009; Xi et al., 2008). We hypothesized that if AegNPC1b and AegML33 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 AegNPC1b and AegML33. 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 (Xi et al., 2008). 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 5). The AMPs analyzed in this study were defensin E, cecropin E, lysozyme C, dipterin, and gambicin. Although they do not belong to the AMP class, we also assayed SOCS36E and DVRF1 because they are regulated by the anti-dengue JAK/STAT pathway (Y. Dong et al., 2012; Ramirez and Dimopoulos, 2010; Souza-Neto et al., 2009; Xi et al., 2008) 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 S2.

Overall, the transcript abundance of several AMP genes was influenced by AegNPC1b and AegML33 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 AegNPC1b 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 (Xi et al., 2008) to be induced after Toll pathway activation. The up-regulation of DVRF1 transcripts after AegNPC1b silencing also suggested the possible activation of the JAK/STAT pathway (Souza-Neto et al., 2009). The silencing of AegML33 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 (Souza-Neto et al., 2009). The transcript abundance of both SOCS36E and DVRF1 in fat body tissue was decreased at 3 days post-silencing of AegML33 (Figure 5D). Defensin E transcripts were enriched in the midgut tissue after the silencing of both AegNPC1b and AegML33, suggesting an activation of the Imd pathway, since this AMP has been shown to be induced by silencing of the Imd pathway negative regulator Caspar (Xi et al., 2008).

3.6 Expression patterns and tissue tropisms of AegNPC1b and ML33

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 AegNPC1b and AegML33 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 6). 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 midgut increased at 3 dpbm and peaked at 7 dpbm (a 2.15-fold increase), then decreased at the later time points (Figure 7A). AaegML33 transcripts were enriched in the midgut throughout the infection (a 1.99- to 18.85-fold increase) (Figure 7B). 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 7C).

4. Discussion

The ML family comprises of proteins that contain the MD2 lipid-binding (ML) domain, which has generally been studied in the MD2 protein because of its LPS-binding property and its role as a TLR4 co-receptor (Shimazu et al., 1999). The immune-related function of the ML gene family was recently characterized in the insect model organism, *D. melanogaster* (Shi et al., 2012). Three of the eight *Drosophila* ML genes (DmNPC2a, DmNPC2e, and DmNPC2h) were shown to bind to LPS, peptidoglycan, and lipoteichoic acid. Overexpression of recombinant DmNPC2a and DmNPC2e in the *Drosophila* S2 cell line activated the promoter of the antimicrobial peptide dipterin, which is controlled by the IMD immune signaling pathway (Huang et al., 2007; Shi et al., 2012). The *An. gambiae* ML protein AgMDL1 is also implicated in the anti-*Plasmodium* response (Y. Dong et al., 2006). 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 (Waterhouse et al., 2007). 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 (Chang et al., 2006). 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 (Chang et al., 2006; Huang et al., 2005; R. Niwa and Y. S. Niwa, 2011).

Multiple sequence alignment of the *Ae. aegypti* ML family revealed variations in ML conserved amino acids, as reported for the mouse ML gene (Ko et al., 2003). 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 (Huang et al., 2007), *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 1A). The *Drosophila* NPC2 a-h genes were grouped as in previous studies, indicating the accuracy of the tree (Huang et al., 2007; Shi et al., 2012). The DmNPC2a mutant has been shown to possess physiological defects and disordered cholesterol metabolism, similar to that observed with the human NPC2 mutant (Huang et al., 2007; R. Niwa and Y. S. Niwa, 2011).

Thus, AegML13, 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. AegML1 clustered together with AgMDL1, an anti-*Plasmodium* ML gene (Y. Dong et al., 2006), 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 (AegML33) resulted in lower DENV midgut titers (Figure 2), suggesting that they facilitate DENV infection and underscoring the potential role of the ML gene family members as DENV agonists. AegML33 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 (Huang et al., 2005; McQuilton et al., 2012; Voght et al., 2007). 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 1B). NPC1a is an insect NPC1 gene more closely related to human NPC1 and is required for molting and sterol homeostasis in *Drosophila* (Chang et al., 2006; Huang et al., 2005; R. Niwa and Y. S. Niwa, 2011). NPC1a mutant larvae have abnormally high levels of accumulated sterol in cells and are unable to molt (Huang et al., 2005). 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 (Voght et al., 2007). 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 AegNPC1 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. AegNPC1b 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 (Carette et al., 2011; Côté et al., 2011). Silencing of AegNPC1b resulted in lower midgut DENV titers, emphasizing its potential role in DENV infection (Figure 2).

The functions of AegNPC1b and AegML33 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 recently colonized field mosquitoes (Figure 3). Simultaneous silencing of AegNPC1b and AegML33 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).

Based on the infection data and previous studies of ML genes, we hypothesized that AegNPC1b or AegML33 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 (Y. Dong et al., 2012; Ramirez and Dimopoulos, 2010; Souza-Neto et al., 2009; Xi et al., 2008). 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 5).

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 S2) (Souza-Neto et al., 2009; Xi et al., 2008). 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 (Arbouzova and Zeidler, 2006). A prominent up-regulation of defensin E in the midgut occurred after the silencing of both AaegNPC1b and AaegML33 (5A 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 (Luplertlop et al., 2011). Different expression pattern of Defensin E and other immune effector genes in the midgut and fat body also suggested that transcription of these genes are controlled differently in different mosquito tissues and cell types as has been shown previously (Manfrulli et al., 1999). Immune genes can be controlled by multiple immune pathways, and the transcriptional regulation by a 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 (Sim and Dimopoulos, 2010). Changes in AMPs 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 (Gray et al., 2010).

Tissue-specific transcript abundance analysis revealed that in uninfected mosquitoes, AaegML33 was constitutively expressed in the midgut and fat body-containing carcass (Figure 6). In contrast, AaegNPC1b expression was limited mainly to the mosquito midgut, which corresponds to the expression of NPC1b in *Drosophila* (Voght et al., 2007), 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 DENV-infected blood meal (Figure 7) 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank members of the Dimopoulos lab, the Johns Hopkins Malaria Research Institute Mosquito and Parasitology core facilities and the Johns Hopkins School of Public Health Genomic Analysis and Sequencing core facility. We would also like to thank Dr. Anna P. Durbin Dr. Hamish Mohammed for providing technical support and materials (DENV strains and C6/36 cell line and field-derived *Aedes aegypti*) and Dr. Deborah McClellan for editorial assistance. This work has been supported by National Institutes of Health/National Institute of Allergy and Infectious Disease grants R R21AI090188, R01AI101431, a fellowship from the Agency for Science, Technology and Research (A*STAR), Singapore to SS, a Royal Thai Government fellowship NJ. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

- Arbouzova NI, Zeidler MP. JAK/STAT signalling in *Drosophila*: insights into conserved regulatory and cellular functions. *Development*. 2006; 133:2605–2616. [PubMed: 16794031]
- Ault SK. Environmental management: a re-emerging vector control strategy. *Am J Trop Med Hyg*. 1994; 50:35–49. [PubMed: 8024083]
- Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GRW, Simmons CP, Scott TW, Farrar JJ, Hay SI. The global distribution and burden of dengue. *Nature*. 2013; 496:504–507. [PubMed: 23563266]
- Black WC, Bennett KE, Gorrochategui-Escalante N, Barillas-Mury CV, Fernández-Salas I, de Lourdes Muñoz M, Farfán-Alé JA, Olson KE, Beaty BJ. Flavivirus susceptibility in *Aedes aegypti*. *Arch Med Res*. 2002; 33:379–388. [PubMed: 12234528]
- Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N, Kuehne AI, Kranzusch PJ, Griffin AM, Ruthel G, Cin PD, Dye JM, Whelan SP, Chandran K, Brummelkamp TR. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature*. 2011; 477:340–343. [PubMed: 21866103]
- Chang TY, Chang CCY, Ohgami N, Yamauchi Y. Cholesterol Sensing, Trafficking, and Esterification. *Annu Rev Cell Dev Biol*. 2006; 22:129–157. [PubMed: 16753029]
- Côté M, Misasi J, Ren T, Bruchez A, Lee K, Filone CM, Hensley L, Li Q, Ory D, Chandran K, Cunningham J. Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. *Nature*. 2011; 477:344–348. [PubMed: 21866101]
- Das S, Garver L, Ramirez JR, Xi Z, Dimopoulos G. Protocol for dengue infections in mosquitoes (*A. aegypti*) and infection phenotype determination. *J Vis Exp*. 2007:220. [PubMed: 18979018]
- Di Tommaso P, Moretti S, Xenarios I, Orobittg M, Montanyola A, Chang JM, Taly JF, Notredame C. T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res*. 2011; 39:W13–7. [PubMed: 21558174]
- Dong K. Insect sodium channels and insecticide resistance. *Invert Neurosci*. 2007; 7:17–30. [PubMed: 17206406]
- Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G. *Anopheles gambiae* immune responses to human and rodent Plasmodium parasite species. *PLoS Pathog*. 2006; 2:e52–525. [PubMed: 16789837]
- Dong Y, Morton JC, Ramirez JL, Souza-Neto JA, Dimopoulos G. The entomopathogenic fungus *Beauveria bassiana* activate toll and JAK-STAT pathway controlled effector genes and anti-

- dengue activity in *Aedes aegypti*. *Insect Biochem Mol Biol*. 2012; 42:126–132. [PubMed: 22198333]
- Garver L, Dimopoulos G. Protocol for RNAi assays in adult mosquitoes (*A. gambiae*). *J Vis Exp*. 2007:230. [PubMed: 18979027]
- Garver WS, Heidenreich RA. The Niemann-Pick C proteins and trafficking of cholesterol through the late endosomal/lysosomal system. *Curr Mol Med*. 2002; 2:485–505. [PubMed: 12125814]
- Gray P, Michelsen KS, Sirois CM, Lowe E, Shimada K, Crother TR, Chen S, Brikos C, Bulut Y, Latz E, Underhill D, Arditi M. Identification of a novel human MD-2 splice variant that negatively regulates Lipopolysaccharide-induced TLR4 signaling. *J Immunol*. 2010; 184:6359–6366. [PubMed: 20435923]
- Gruber A, Mancek M, Wagner H, Kirschning CJ, Jerala R. Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. *J Biol Chem*. 2004; 279:28475–28482. [PubMed: 15111623]
- Gubler DJ. Dengue and dengue hemorrhagic fever. *Clin Microbiol Rev*. 1998; 11:480–496. [PubMed: 9665979]
- Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, Hunsperger E, Kroeger A, Margolis HS, Martínez E, Nathan MB, Pelegrino JL, Simmons C, Yoksan S, Peeling RW. Dengue: a continuing global threat. *Nat Rev Microbiol*. 2010; 8:S7–16. [PubMed: 21079655]
- Horácková J, Rudenko N, Golovchenko M, Grubhoffer L. Der-p2 (*Dermatophagoides pteronyssinus*) allergen-like protein from the hard tick *Ixodes ricinus* - a novel member of ML (MD-2-related lipid-recognition) domain protein family. *Parasitology*. 2010; 137:1139–1149. [PubMed: 20388233]
- Huang X, Suyama K, Buchanan J, Zhu AJ, Scott MP. A *Drosophila* model of the Niemann-Pick type C lysosome storage disease: dnpc1a is required for molting and sterol homeostasis. *Development*. 2005; 132:5115–5124. [PubMed: 16221727]
- Huang X, Warren JT, Buchanan J, Gilbert LI, Scott MP. *Drosophila* Niemann-Pick Type C-2 genes control sterol homeostasis and steroid biosynthesis: a model of human neurodegenerative disease. *Development*. 2007; 134:3733–3742. [PubMed: 17804599]
- Inohara N, Nunez G. ML - a conserved domain involved in innate immunity and lipid metabolism. *Trends Biochem Sci*. 2002; 27:219–221. [PubMed: 12076526]
- Ko DC, Binkley J, Sidow A, Scott MP. The integrity of a cholesterol-binding pocket in Niemann-Pick C2 protein is necessary to control lysosome cholesterol levels. *Proc Natl Acad Sci USA*. 2003; 100:2518. [PubMed: 12591949]
- Lawson D, Arensburger P, Atkinson P, Besansky NJ, Bruggner RV, Butler R, Campbell KS, Christophides GK, Christley S, Dialynas E, Hammond M, Hill CA, Konopinski N, Lobo NF, Maccallum RM, Madey G, Megy K, Meyer J, Redmond S, Severson DW, Stinson EO, Topalis P, Birney E, Gelbart WM, Kafatos FC, Louis C, Collins FH. VectorBase: a data resource for invertebrate vector genomics. *Nucleic Acids Res*. 2009; 37:D583–7. [PubMed: 19028744]
- Luplertlop N, Surasombatpattana P, Patramool S, Dumas E, Wasinpiyamongkol L, Saune L, Hamel R, Bernard E, Sereno D, Thomas F, Piquemal D, Yssel H, Briant L, Missé D. Induction of a Peptide with Activity against a Broad Spectrum of Pathogens in the *Aedes aegypti* Salivary Gland, following Infection with Dengue Virus. *PLoS Pathog*. 2011; 7:e1001252. [PubMed: 21249175]
- Manfrulli PP, Reichhart MJ, Steward RR, Hoffmann JAJ, Lemaitre BB. A mosaic analysis in *Drosophila* fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. *EMBO J*. 1999; 18:3380–3391. [PubMed: 10369678]
- McQuilton P, St Pierre SE, Thurmond J, FlyBase Consortium. FlyBase 101--the basics of navigating FlyBase. *Nucleic Acids Res*. 2012; 40:D706–14. [PubMed: 22127867]
- Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu ZJ, Loftus B, Xi Z, Megy K, Grabherr M, Ren Q, Zdobnov EM, Lobo NF, Campbell KS, Brown SE, Bonaldo MF, Zhu J, Sinkins SP, Hogenkamp DG, Amedeo P, Arensburger P, Atkinson PW, Bidwell S, Biedler J, Birney E, Bruggner RV, Costas J, Coy MR, Crabtree J, Crawford M, deBruyn B, Decaprio D, Eiglmeier K, Eisenstadt E, El-Dorri H, Gelbart WM, Gomes SL, Hammond M, Hannick LI, Hogan JR, Holmes MH, Jaffe D, Johnston JS, Kennedy RC, Koo H, Kravitz S, Kriventseva EV, Kulp D, Labutti K, Lee E, Li S, Lovin DD, Mao C, Mauceli E, Menck CFM, Miller JR, Montgomery P, Mori A,

- Nascimento AL, Naveira HF, Nusbaum C, O'leary S, Orvis J, Pertea M, Quesneville H, Reidenbach KR, Rogers YH, Roth CW, Schneider JR, Schatz M, Shumway M, Stanke M, Stinson EO, Tubio JMC, Vanze JP, Verjovski-Almeida S, Werner D, White O, Wyder S, Zeng Q, Zhao Q, Zhao Y, Hill CA, Raikhel AS, Soares MB, Knudson DL, Lee NH, Galagan J, Salzberg SL, Paulsen IT, Dimopoulos G, Collins FH, Birren B, Fraser-Liggett CM, Severson DW. Genome sequence of *Aedes aegypti*, a major arbovirus vector. *Science*. 2007; 316:1718–1723. [PubMed: 17510324]
- Niwa R, Niwa YS. The Fruit Fly *Drosophila melanogaster* as a Model System to Study Cholesterol Metabolism and Homeostasis. *Cholesterol*. 2011; 2011:176802. [PubMed: 21512589]
- Ramirez JL, Dimopoulos G. The Toll immune signaling pathway control conserved anti-dengue defenses across diverse *Ae. aegypti* strains and against multiple dengue virus serotypes. *Developmental and Comparative Immunology*. 2010; 34:625–629. [PubMed: 20079370]
- Rivero A, Vézilier J, Weill M, Read AF, Gandon S. Insecticide control of vector borne diseases: when is insecticide resistance a problem? *PLoS Pathog*. 2010; 6:e1001000. [PubMed: 20700451]
- Salazar MI, Richardson JH, Sánchez-Vargas I, Olson KE, Beaty BJ. Dengue virus type 2 replication and tropisms in orally infected. *BMC Microbiol*. 2007; 7:9–13. [PubMed: 17263893]
- Shi XZ, Zhong X, Yu XQ. *Drosophila melanogaster* NPC2 proteins bind bacterial cell wall components and may function in immune signal pathways. *Insect Biochem Mol Biol*. 2012; 42:545–556. [PubMed: 22580186]
- Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med*. 1999; 189:1777–1782. [PubMed: 10359581]
- Sim S, Dimopoulos G. Dengue virus inhibits immune responses in *Aedes aegypti* cells. *PLoS ONE*. 2010; 5:e10678. [PubMed: 20502529]
- Sim S, Jupatanakul N, Ramirez JL, Kang S, Romero-Vivas CM, Mohammed H, Dimopoulos G. 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.
- Sim S, Ramirez JL, Dimopoulos G. Dengue Virus Infection of the *Aedes aegypti* Salivary Gland and Chemosensory Apparatus Induces Genes that Modulate Infection and Blood-Feeding Behavior. *PLoS Pathog*. 2012; 8:e1002631. [PubMed: 22479185]
- Souza-Neto JA, Sim S, Dimopoulos G. An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *P Natl Acad Sci Usa*. 2009; 106:17841–17846.
- Starostina E, Xu A, Lin H, Pikielny CW. A *Drosophila* protein family implicated in pheromone perception is related to Tay-Sachs GM2-activator protein. *J Biol Chem*. 2009; 284:585–594. [PubMed: 18952610]
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*. 2011; 28:2731–2739. [PubMed: 21546353]
- Voght SP, Fluegel ML, Andrews LA, Pallanck LJ. *Drosophila* NPC1b promotes an early step in sterol absorption from the midgut epithelium. *Cell Metab*. 2007; 5:195–205. [PubMed: 17339027]
- Waterhouse RM, Kriventseva EV, Meister S, Xi Z, Alvarez KS, Bartholomay LC, Barillas-Mury C, Bian G, Blandin S, Christensen BM, Dong Y, Jiang H, Kanost MR, Koutsos AC, Levashina EA, Li J, Ligoxygakis P, Maccallum RM, Mayhew GF, Mendes A, Michel K, Osta MA, Paskewitz S, Shin SW, Vlachou D, Wang L, Wei W, Zheng L, Zou Z, Severson DW, Raikhel AS, Kafatos FC, Dimopoulos G, Zdobnov EM, Christophides GK. Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *Science*. 2007; 316:1738–1743. [PubMed: 17588928]
- Whitehead SS, Blaney JE, Durbin AP, Murphy BR. Prospects for a dengue virus vaccine. *Nat Rev Microbiol*. 2007; 5:518–528. [PubMed: 17558424]
- Xi Z, Ramirez JL, Dimopoulos G. The *Aedes aegypti* toll pathway controls dengue virus infection. *PLoS Pathog*. 2008; 4:e1000098. [PubMed: 18604274]

Highlights

- Members of the lipid binding gene families, ML and NPC1, function as DENV host factors in *Aedes aegypti* mosquitoes.
- The role(s) of ML and NPC1 in DENV infection is conserved among *Ae. aegypti* strains.
- AaegML33 and AaegNPC1b are potential negative regulators of immune signaling pathways.
- DENV induce the expression of the mosquito AaegML33 and AaegNPC1b genes, and this facilitates infection.

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

Figure 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 two to three 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 (* $p < 0.05$, ** $p < 0.01$).

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 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 (* $p < 0.05$, ** $p < 0.01$).

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. *Ae. aegypti* NPC1b and ML33 may influence DENV infection through the same mechanisms

Midgut DENV titers were assayed upon single- and double-silencing of AegNPC1b and AegML33 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 (* $p < 0.05$, ** $p < 0.01$).

Figure 5. Immune-related gene expression changes after AagNPC1b and AagML33 silencing
 Expression of immune-related gene transcripts was assayed by quantitative PCR of cDNA from AagNPC1b- and AagML33-silenced mosquito tissues. Gene expression levels were assayed from three biological replicates. Error bars represent the standard error of the mean (SEM).

Figure 6. Transcript abundance of AaegNPC1b and AaegML33 in the midgut, and fat body in uninfected mosquitoes

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).

Figure 7. AegNPC1b and AegML33 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).