Exploring the molecular mechanism(s) involved in
Wolbachia-Aedes aegypti-dengue virus interactions

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Abstract

Dengue is one of the most significant health problems that has magnified its impact globally by affecting about 390 million people annually across 110 countries. The causative agent of this life-threatening disease is a positive single stranded RNA arbovirus known as dengue virus (DENV), which uses species of *Aedes* mosquitoes as vectors. To date, there is no effective available vaccine or cure for dengue, and the control options primarily rely on vector control strategies, mostly through the application of pesticides. However, reports of resistance in *Aedes* mosquitoes against pesticides has limited this option as well. Therefore, there is an urgent need for alternative approaches to control the spread of DENV. One of the novel options involves the use of the endosymbiotic bacterium *Wolbachia*, that has successfully limited the ability of *Aedes aegypti* mosquitoes to transmit a number of life-threatening mosquito-borne viruses such as DENV and Zika virus. Despite its effectiveness to inhibit replication of DENV, very little is known about the mechanism(s) that *Wolbachia* uses to impart this antiviral effect. In this study, we looked into *Ae. aegypti* host factors that affect DENV replication and their potential manipulations by *Wolbachia* to find molecular mechanism(s) that *Wolbachia* utilizes to limit DENV replication.

Recently, studies have shed light on the role of chromodomain DNA binding helicases in Human Immunodeficiency virus (HIV) and Influenza A virus. In Chapter 1, we have identified three *Ae. aegypti* homologs of chromodomain helicase DNA binding proteins (CHD) and determined their modulation in response to *Wolbachia* and DENV infections. We have found that among the three CHD members, *AeCHD7/Kismet* levels are significantly decreased in *Wolbachia* infection. Further investigations demonstrated that *AeCHD7* is significantly increased in the case of DENV replication suggesting that it may facilitate DENV replication. Knock down studies of *AeCHD7* confirmed this assumption as it resulted in significant reductions in DENV replication and virion production. In this study, we have identified *AeCHD7* as an *Ae. aegypti* pro-DENV host factor that is downregulated by *Wolbachia* which may contribute in limiting DENV replication.

Vago is an insect-specific secretory protein that has been identified in *Culex quinquefasciatus* to play an important role in the crosstalk between the mosquito’s immune pathways and reduce West Nile virus (WNV) replication. In Chapter 2, by *in-silico* identification of Vago characteristic SVWC domain and secretory signal, we identified two potential homologs of the Vago protein in *Ae. aegypti* and looked at their expression pattern in the case of *Wolbachia* infection to find that *AeVago1* is highly induced in *Ae. aegypti* upon *Wolbachia* infection.
However, we found no induction of \textit{AeVago1} expression in \textit{Ae. aegypti} mosquitoes infected with DENV. Further, \textit{AeVago1} knockdown studies demonstrated that there was a significant increase in DENV replication in \textit{Wolbachia} infected cells in \textit{AeVago1} deficient cells. However, there was no effect on \textit{Wolbachia} density in \textit{AeVago1} depleted cells. The outcomes of this study suggest that in the presence of \textit{Wolbachia} the immune gene \textit{AeVago1} is induced, which might also contribute towards inhibition of DENV replication.

Pelo has been recently reported as a positive regulator of Drosophila C virus (DCV) replication. However, its role in the case of DENV replication has not been elucidated yet. In Chapter 3, we looked into the possible involvement of pelo in the case of \textit{Wolbachia-Ae. aegypti}-DENV interactions. We found that the pelo protein levels increase during DENV replication. Silencing of \textit{pelo} led to severe reduction of DENV virion production, suggesting its important role in DENV replication. However, in the case of \textit{Wolbachia} infection, specifically in female \textit{Ae. aegypti} mosquitoes, there was a significant decrease in the transcript levels of \textit{pelo}. Further experiments confirmed that \textit{Wolbachia} changes the subcellular localization of the pelo protein, suggesting that it might be a novel \textit{Ae. aegypti} host factor that is used by \textit{Wolbachia} to limit DENV replication. In addition, we found that \textit{Wolbachia}-mediated down-regulation of \textit{pelo} transcripts might be regulated by aae-miR-2940-5p, which is highly induced by \textit{Wolbachia}. This study has identified a novel molecular mechanism that is used by \textit{Wolbachia} to limit DENV replication in \textit{Ae. aegypti} mosquitoes. Nevertheless, this mechanism of inhibition does not seem to be universally seen in \textit{Wolbachia}-host-virus interactions.

Overall, in this study we have identified three novel host genes that play important roles in DENV replication and that regulation of these genes in the presence of \textit{Wolbachia} may contribute towards virus blocking in \textit{Ae. aegypti} mosquitoes.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer-reviewed papers


Conference abstracts/talks


Asad S, Hussain M, Asgari S (2015) RNA activation in mosquito cells and its suppression by the dengue virus NS5 protein. 8th Australian Virology Society meeting 6-9 December 2015, Hunter valley, Australia. (Poster and oral presentation)
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<table>
<thead>
<tr>
<th>Contributor</th>
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<tr>
<td>Sultan Asad (Candidate)</td>
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# Table of Contents

Abstract .......................................................................................................................... ii  
Declaration by author ..................................................................................................... iv  
Publications during candidature .................................................................................. v  
  Peer reviewed papers .................................................................................................. v  
  Conferences abstracts/talks ....................................................................................... v  
Publication included in this thesis ................................................................................ vi  
Contributions by others to the thesis ........................................................................... vi  
Statement of parts of the thesis submitted to qualify for the award of another degree .. vi  
Acknowledgements ...................................................................................................... vii  
Australian and New Zealand Standard Research Classifications (ANZSRC) .......... ix  
Field of Research (FOR) Classifications ..................................................................... x  
Table of Contents .......................................................................................................... xi  
List of Figures ................................................................................................................ xv  
List of Tables ................................................................................................................ xvii  
List of Abbreviations .................................................................................................... xviii

## Chapter 1: Introduction and Literature Review ............................................................ 1  
  1. Introduction ............................................................................................................. 2  
  1.1. DENV Virus ........................................................................................................ 2  
  1.2. DENV Life Cycle ................................................................................................ 3  
    1.2.1. Viral Entry ..................................................................................................... 3  
    1.2.2. Viral Replication .......................................................................................... 4  
  1.3. DENV-Mosquitoes Interactions ........................................................................... 5  
    1.3.1. The RNA interference Pathway .................................................................... 6  
    1.3.2. The Toll Pathway ......................................................................................... 7  
    1.3.3. The Immune Deficiency (IMD) Pathway ....................................................... 7  
    1.3.4. The Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) Pathway .......................................................................................... 8  
    1.3.5. Crosstalk Between Innate Immunity Pathways ........................................... 9  
  1.4. Vector Control Strategies for DENV Prevention ............................................... 11  
  1.5. Wolbachia-Mosquitoes Interactions ................................................................... 11  
    1.5.1. Competition for Resources ......................................................................... 13  
    1.5.2. Immune Priming ............................................................................................ 14  
    1.5.3. Induction of miRNAs ..................................................................................... 15  
  1.6. Concluding Remarks ............................................................................................ 15
Chapter 2: Downregulation of *Aedes aegypti* chromodomain helicase DNA binding protein 7/Kismet by *Wolbachia* and its effect on dengue virus replication ........................................27

2.1. Abstract .................................................................................................................29
2.2. Introduction ............................................................................................................30
2.3. Results ....................................................................................................................31
  2.3.1. Screening of the CHD family genes during *Wolbachia* infection ....................31
  2.3.2. *AeCHD7* is ubiquitously expressed in all mosquito tissues .........................32
  2.3.3. Specific *Wolbachia*-mediated downregulation of *AeCHD7* in female *Ae. aegypti*33
  2.3.4. *AeCHD7* is upregulated upon DENV infection .............................................35
  2.3.5. *AeCHD7* is required for efficient DENV replication ....................................36
2.4. Discussion ..............................................................................................................38
2.5. Materials and Methods .........................................................................................41
  2.5.1. Mosquitoes and flies .........................................................................................41
  2.5.2. Cell culture .......................................................................................................42
  2.5.3. RT-PCR and qPCR analyses .............................................................................42
  2.5.4. RNAi-mediated gene silencing .........................................................................42
  2.5.5. Virus infection and plaque assay .......................................................................43
2.6. Acknowledgements ...............................................................................................43
2.7. Authors’ contributions ..........................................................................................43
2.8. Additional Information ..........................................................................................44
  2.8.1. Competing financial interests ..........................................................................44
2.9. References ..............................................................................................................44
2.10. Supplementary Material ......................................................................................49

Chapter 3: Upregulation of *Aedes aegypti Vago1* by *Wolbachia* and its effect on dengue virus replication ........................................................................................................52

3.1. Abstract ..................................................................................................................54
3.2. Introduction ............................................................................................................55
3.3. Materials and Methods ..........................................................................................57
  3.3.1. Cell lines, mosquitoes and flies used in study ..................................................57
  3.3.2. qPCR studies .....................................................................................................57
  3.3.3. Gene silencing using RNAi ..............................................................................58
3.4. Results ....................................................................................................................59
  3.4.1. Identification of secretory AeVago proteins .....................................................59
  3.4.2. *AeVago1* is upregulated in *Wolbachia* infection ..........................................59
Appendix 2 .............................................................................................................141
List of Figures

Chapter 1
Figure 1 DENV genome structure ................................................................. 3
Figure 2 DENV replication cycle ................................................................. 5
Figure 3 Mosquito innate immune signaling and RNAi pathway ......................... 10
Figure 4 Schematic representation of proposed hypotheses for Wolbachia-mediated antiviral defense ................................................................. 14

Chapter 2
Figure 1 Relative expression of AeCHD genes in uninfected and Wolbachia infected Ae. aegypti mosquitoes ................................................................. 32
Figure 2 Tissue-specific expression of AeCHD7 in Ae. aegypti mosquitoes ........... 33
Figure 3 Modulation of AeCHD7/Kismet by Wolbachia infection in male and female mosquitoes and flies, and mosquito cell lines .................................................. 34
Figure 4 Expression pattern of AeCHD7 in DENV infected female Ae. aegypti .......... 36
Figure 5 Depletion of AeCHD7 impairs DENV replication both at the genomic and the virion levels .............................................................................. 37
Figure S1 Diagramed illustration of AeCHD7 conserved domains ......................... 49
Figure S2 Phylogenetic tree for CHD from different insect species ..................... 50

Chapter 3
Figure 1 Differential expression of AeVago genes in Wolbachia-infected cell lines and mosquitoes .................................................................................. 60
Figure 2 Effect of DENV infection on AeVago1 expression in Ae. aegypti mosquitoes and D. melanogaster S2 cells ........................................................................ 61
Figure 3 Effect of DENV infection on AeVago1 activators (Dicer2, Rel2 and TRAF) in DENV-infected .................................................................................. 62
Figure 4 Silencing of AeVago1 affects DENV replication in Wolbachia-infected Ae. aegypti cells .................................................................................. 63
Figure S1 Identification of SVWC domain and secretory signal in AeVago1 protein ...... 71
Figure S2 Identification of SVWC domain and protein secretion signal in AeVago1 ....... 72
Figure S3 Effect of Wolbachia infection on Vago transcript levels in female D. melanogaster flies and Ae. albopictus cell lines .................................................. 73
Chapter 4

Figure 1 Tissue-specific expression of pelo in *Ae. aegypti* mosquitoes ........................................83

Figure 2 Relative expression of pelo in uninfected and *Wolbachia* infected *Ae. aegypti*
mosquitoes and cell lines ..................................................................................................................84

Figure 3 Tissue-specific modulation of *pelo* by *Wolbachia* .........................................................86

Figure 4 Effect of *Wolbachia* infection on the *pelo* gene expression in male and female
mosquitoes and flies, and cell line ..................................................................................................88

Figure 5 miRNA-mediated regulation of *pelo* .................................................................................90

Figure 6 aae-miR-2940-5p dependent regulation of *pelo* .............................................................91

Figure 7 Expression pattern of *pelo* in DENV challenged mosquito cells ....................................93

Figure 8 Silencing of *pelo* affects DENV virion production .........................................................94

Figure S1 Sequence complementarity of aae-miR-2940-5p with its predicted target *pelo* 105

Figure S2 Pelo is highly conserved among insects ....................................................................106

Figure S3 *Aedes aegypti* pelo is structurally similar to the human pelo .................................107
List of Tables

Chapter 2
Table S1 Primers used in this study ............................................................51

Chapter 3
Table S1 Primers used in this study ............................................................74

Chapter 4
Table S1 Primers used in this study ............................................................103
Table S2 Pelo protein sequences used for multiple sequence alignment ..........104
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
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<td><em>Aedes aegypti</em> chromodomain helicase DNA binding protein 7</td>
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<td>Ago</td>
<td>Argonaute</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<td>CD14</td>
<td>Cluster of differentiation 14</td>
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<td>CLR</td>
<td>C-type lectin receptors</td>
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<tr>
<td>HC-CLR</td>
<td>C-type lectin receptors</td>
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<td>DCV</td>
<td>Drosophila C virus</td>
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<td>DENV</td>
<td>Dengue virus</td>
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<td>DNA</td>
<td>Deoxy-ribonucleic acid</td>
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<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
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<td>HSP</td>
<td>Heat shock protein</td>
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<td>ICAM-3</td>
<td>Intracellular adhesion molecule 3</td>
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<td>Imd</td>
<td>Immune deficiency</td>
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<tr>
<td>JAK-STAT</td>
<td>Janus kinase signal transducer and activator of transcriptional</td>
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<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<td>Nf-kb</td>
<td>Nuclear factor kappa B</td>
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<td>ORF</td>
<td>Open reading frame</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PGRPs</td>
<td>Peptidoglycan recognition proteins</td>
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<tr>
<td>PIAS</td>
<td>Protein inhibitor of activated STAT</td>
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<td>PRRs</td>
<td>Pattern recognition receptors</td>
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<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNA</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>sfRNA</td>
<td>Subgenomic flavivirus RNA</td>
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siRNA  Small interfering RNA
Tet    Tetracycline
TRAF   TNF receptor associated factor
UTR    Untranslated region
WNV    West Nile virus
Wsp    Wolbachia surface protein
ZIKV   Zika virus
Chapter 1: Introduction and Literature Review
1. Introduction
Arthropod-borne diseases, such as malaria, leishmaniasis, filariasis, onchocerciasis and dengue, impose huge risks to human health (Hill et al., 2005). According to WHO report, there is an increased risk of emergence of a number of arthropod-borne diseases with more than half of the world population being at risk of getting one of these diseases (WHO, 2014). All of the aforementioned diseases need hematophagous arthropod vectors such as mosquitoes and ticks for transmission between humans and/or animals (Gubler, 1998; Kalluri et al., 2007). Among the arthropod-borne diseases, the ones caused by viruses are of immense importance and are generally referred to as arboviral diseases. Centre of Disease Control and Prevention has reported more than 600 arboviruses, among which 80 can infect humans (Conway et al., 2014). The majority of arboviruses belong to four viral families Togaviridae, Bunyaviridae, Reoviridae and Flaviviridae (Karabatsos, 1978). Flaviviridae is the most important arboviral family because it harbors highly pathogenic viruses to humans such as yellow fever virus, West Nile virus (WNV), Zika virus (ZIKV) and dengue virus (DENV) (Fernandez-Garcia et al., 2009).

1.1. Dengue Virus
DENV is a major arbovirus that uses Aedes spp. mosquitoes as vector to be transmitted to humans (Gubler, 2006). There are estimated 390 million DENV infections annually putting major burden on global health (Bhatt et al., 2013). Currently, there is no specific therapy or approved vaccine available while the treatment available is just supportive. Despite, substantial efforts to control DENV through vector control, it is still geographically expanding rapidly (Tatem et al., 2006).

DENV belongs to the Flaviviridae family that comprises lipid-enveloped, positive-sense single stranded ribonucleic acid (RNA) viruses (Clyde et al., 2006). DENV is classified into four antigenically distinct but closely related serotypes, represented as DENV-1 to DENV-4. The length of DENV genome is about 10.7 kilobases comprising a single open reading frame flanked by highly conserved 5’ and 3’ untranslated regions. A single ORF encodes a polyprotein of approximately 3391 amino acids, which is further processed by the host cellular proteases furin and signalase and viral NS2B/NS3 protease complex to generate three structural (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Bartenschlager and Miller, 2008) (Figure 1).
Figure 1. DENV Genome Structure. DENV RNA contains a single open reading frame encoding a polyprotein, which is cleaved by the host and viral proteases to produce 3 structural and 7 non-structural proteins, flanked by 5’ and 3’ UTRs. Conserved RNA secondary structures in the UTRs and in the coding region have been determined to function at various stages of the viral life cycle. Adapted from (Clyde et al., 2006).

1.2. DENV Life Cycle

1.2.1. Viral Entry

DENV is mostly introduced to humans by infected Aedes mosquitoes with the main vector being Aedes aegypti. After the introduction, DENV particularly targets mononuclear phagocyte lineage cells including the skin resistant Langerhans cells (Jessie et al., 2004; Wu et al., 2000). However, in mosquitoes DENV is thought to initially target midgut and then spread and replicate in other peripheral tissues (Rodenhuis-Zybert et al., 2010).

DENV can infect a very diverse range of cell lines including human, mosquito, monkey, hamster and murine cell lineages. This suggests that either DENV uses a ubiquitous receptor or it uses multiple receptors for its entry (Rodenhuis-Zybert et al., 2010). In mosquito cells, different independent groups have reported a number of potential DENV receptors such as prohibitin (Mishra et al., 2006), heat shock protein 70 (Hsp70), R80, R60 and a 45 kDa protein (Rodenhuis-Zybert et al., 2010). Recently, a group has demonstrated, both in vivo and in vitro, the interaction of Ae. aegypti C-type lectin 3 with DENV-2 envelope protein, and further suggested the possibility that DENV-2 may use multiple C-type lectins as receptors to gain entry into mosquito cells (Liu et al., 2014). In humans, heparan sulfate (Germi et al., 2002), Hsp90 (Reyes-Del Valle et al., 2005), CD14 (Chen et al., 1999), GRP78/BiP
(Jindadamrongwech et al., 2004), and a 37/67-kDa high-affinity laminin receptor (Thepparit and Smith, 2004) have been identified as potential receptors for DENV. C-type lectin receptors (CLR) are involved in the interaction of DENV particles with human myeloid cells (Fernandez-Garcia et al., 2009). These include DC-specific intracellular adhesion molecule 3 (ICAM-3)-grabbing no integrin (DC-SIGN, CD209) (Lozach et al., 2005; Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003), mannose receptor (MR) (Miller et al., 2008) and C-type lectin domain family 5, member A (CLEC5, MDL-1) (Chen et al., 2008).

Similar to other flaviviruses, after adsorption to receptors DENV is endocytosed by cells in a clathrin dependent manner and then is further transported into endosomes (Stiasny et al., 2011) (Figure 2). However, according to one study, DENV can adopt a clathrin-independent pathway as well to gain entry, in particular, into mammalian cells (Acosta et al., 2009).

### 1.2.2. Viral Replication

Following the entry, DENV nucleocapsid is released into the cytosol after acidic pH triggered rearrangement of its envelope protein in late endosomes leading to fusion of viral envelope and the cellular endosome membrane (Harrison, 2008; Modis et al., 2004). The nucleocapsid disassembles and viral genome is translated by ER-located ribosomes generating numerous copies of viral proteins. NS5 and other viral non-structural proteins along with various host proteins establish replication complexes (Welsch et al., 2009). At the replication complex, the viral polymerase (NS5) transcribes the negative strand viral RNA, which serves as a template for the synthesis of subsequent positive strand viral RNA copies. Viral replication is regulated by 5'-3' UAR sequences (upstream of AUG region) present in 5’ and 3’ UTRs, which are actively involved in the circularization of DENV RNA (Villordo and Gamarnik, 2009). These newly synthesized positive strand DENV RNAs interact with capsid proteins to assemble into nucleocapsids (Ivanyi-Nagy and Darlix, 2010). These nucleocapsids bud into the lumen of the endoplasmic reticulum (ER) thereby become enveloped in a membrane bilayer carrying the viral prM and E proteins (Welsch et al., 2009) (Figure 2). These immature particles are transported through the cellular secretory pathway, where the furin protease cleaves prM, resulting in the formation of mature virus particles capable of infecting naïve cells (Li et al., 2008).
Figure 2. DENV replication cycle. Virions bind to cell-surface attachment molecules/receptors such as heparan sulfate or DC-SIGN and are internalized through endocytosis. The low pH of the late endosomes triggers fusion of virions with endosomal membrane releasing viral RNA into the cytoplasm. Viral RNA is translated by the cellular machinery and viral non-structural proteins form the replication complexes where the viral RNA is amplified. Virions bud into the lumen of the ER. Virion maturation occurs during their transport through the secretory pathway. New round of infection can be initiated by the mature virions once they are released. Adapted from (Sampath and Padmanabhan, 2009).

1.3. DENV-Mosquito Interactions

In nature, almost every organism engages in ecological or molecular interactions with other organisms, whether in antagonistic or mutualistic associations, to thrive and excel. These interactions are major drivers of diversification and adaptive evolution. Among these interactions, the most fascinating examples are those involved in invertebrate susceptibility to pathogens (Schmid-Hempel and Ebert, 2003).

In the case of DENV, it is evident that despite the pathogenic nature of the virus in humans, its interaction with *Ae. aegypti* mosquito is somehow non-pathogenic. Studies have identified a specific RNA sequence present in the 3'UTR of DENV which is essential for virus replication
in mosquito cells but is not important in the case of virus replication in mammalian cells (Villordo and Gamarnik, 2013). Furthermore, it has been shown that DENV2 encodes a miRNA-like small RNA from its 3'UTR which targets the NS1 gene of DENV to autoregulate the DENV replication to keep the virus replication under check in the mosquitoes (Hussain and Asgari, 2014). DENV has evolved to overcome the host mosquito’s defenses and manipulate its cellular machinery by largely unknown mechanisms that facilitate its replication without interfering too much with the normal growth of the mosquitoes. Similar to other insects, mosquitoes generally lack an adaptive immune system to counter pathogens, thus innate immunity is the sole mechanism for defence against pathogen infections especially viruses. These defences commonly comprise of either RNA interference (RNAi) or antiviral protein-based responses.

1.3.1. The RNA Interference Pathway

RNAi is an evolutionary conserved defense response constituting a major component of the mosquito innate immune response to virus infections (Blair, 2011). RNAi response is generally triggered by detection of endogenous or exogenous (virus genome or virus replication intermediates) double stranded RNA (dsRNA) (Fire et al., 1998) by a ribonuclease III (RNase III) enzyme called Dicer, which processes dsRNA into 20-21 nucleotide short RNA duplexes (Hutvagner et al., 2001) (Figures 3). These small RNA duplexes are further unwound and one of the strands, having less thermostability, is loaded into the RNA-induced silencing complex (RISC) (Czech et al., 2009). The short interfering RNA (siRNA) along with the RISC complex is directed towards the target RNA sequences, based on sequence complementarity, which generally leads to the cleavage of the targeted RNA through slicer endonuclease activity of Argonaute-2 (Ago2) (Miyoshi et al., 2005; Schwarz et al., 2002).

In mosquitoes, RNAi plays an important role in antiviral defense, which was first discovered in Anopheles gambiae limiting the replication of O’nyong-nyong virus (ONNV) (Keene et al., 2004). In Ae. aegypti mosquitoes, silencing of key RNAi components Dicer-2 and R2D2 leads to significant increases in DENV titer (Sanchez-Vargas et al., 2009). It has been demonstrated that DENV elicits the RNAi response in Ae. aegypti, but it is unable to completely repress viral replication. Instead, it may just modulate virus replication to maintain persistent viral infection in order to ensure long-term survival of infected mosquitoes (Sanchez-Vargas et al., 2009). Given that DENV successfully develops persistent infection in mosquitoes despite their RNAi response, the precise mechanism of RNAi evasion is definitely a significant point of interest. Recent studies have shown that DENV might employ two different mechanisms to inhibit the RNAi pathway, involving RNAi suppressor protein NS4B and the subgenomic flavivirus RNA
(sfRNA). DENV encoded nonstructural protein NS4B has been reported to suppress the RNAi pathway in human Huh-7 cells by a mechanism that is still not fully understood (Kakumani et al., 2013). Furthermore, sfRNA produced by DENV is thought to reduce the RNAi response in both human and mosquito by inhibiting Dicer-mediated cleavage of dsRNA and through direct interaction with Ago2, which is an important component of the RISC complex (Moon et al., 2015; Schnettler et al., 2012). However, a recent study demonstrated that neither of those mechanisms could be involved in RNAi suppression, but the capsid protein, which sequesters dsRNA and protects it from cleavage by Dicer. The capsid protein, therefore, could be the true viral suppressor of RNAi in flaviviruses as this function of the protein seems to be conserved among a number of flaviviruses tested (Samuel et al., 2016).

1.3.2. The Toll Pathway

Generally, the Toll pathway has been associated with the infection of Gram positive bacteria and fungi (Lemaitre and Hoffmann, 2007). However, this pathway has been reported to play an important role in the antiviral defense in both mosquitoes (Christophides et al., 2002) and fruit flies (Zambon et al., 2005). The Toll pathway is triggered by the pattern recognition receptors (PRRs)-mediated activation of an insect cytokine Spätzle, leading to the activation of a cascade of events that result in the degradation of Cactus. Cactus, with the help of the adaptor protein MyD88, is a negative regulator of Nuclear factor kappa B (Nf-kb)-like transcription factor Rel1 (Bischoff et al., 2004; DeLotto and DeLotto, 1998; Michel et al., 2001) (Figure 3). Cactus degradation initiates nuclear translocation of Rel1 and transcriptional activation of effectors genes (Frolet et al., 2006; Nicolas et al., 1998).

In DENV infection, the Toll pathway plays an important role in antiviral response in the Ae. aegypti midgut. Studies have demonstrated activation of major Toll pathway genes such as Spätzle, Toll and Rel1a upon DENV infection suggesting that the pathway is activated as an antiviral response against DENV infection (Sim et al., 2012; Xi et al., 2008). In addition, a follow up study has further functionally characterized the role of important Toll pathway genes in the case of DENV infection by performing dsRNA-mediated silencing assays and found that in the midgut of Ae. aegypti mosquitoes silencing of Cactus significantly decreased the DENV titer, while silencing of MyD88 led to increases in DENV titer (Xi et al., 2008).

1.3.3. The Immune Deficiency (IMD) Pathway

The IMD pathway plays an important role in the insect defense system especially against gram negative bacteria (Georgel et al., 2001; Kaneko and Silverman, 2005). This pathway is similar to tumor necrosis factor signaling pathway in mammals (Aggarwal and Silverman,
In *Drosophila melanogaster*, activation of the IMD pathway occurs through pathogen detection by membrane bound class of peptidoglycan recognition proteins (PGRPs), leading to the activation of a signaling cascade through the adaptor IMD protein along with a number of caspases and kinases, which in turn process Relish 2 (Rel2) into its active form by removing its carboxyl end and exposing its nuclear localization signal for nuclear export (Stoven et al., 2003) (Figure 3). After translocation into the nucleus, Rel2 activates transcription of a number of antimicrobial peptides such as cecropin A and other defense related genes (Antonova et al., 2009; Lemaitre and Hoffmann, 2007). Recent studies have shed light on the antiviral role of the IMD pathway in *D. melanogaster* against Cricket paralysis virus and Sindbis virus (Avadhanula et al., 2009; Costa et al., 2009). Regarding the role of the IMD pathway in DENV infection, a study conducted on DENV-susceptible strains of *Ae. aegypti* by silencing key components of the IMD pathway showed no effect on DENV titer in the midgut (Xi et al., 2008). However, a recent study has demonstrated increase in DENV titers in IMD silenced *Ae. aegypti* mosquitoes of a DENV-resistant strain, which seems to suggest that the IMD pathway might be an important antiviral defense against DENV in the mosquito (Sim et al., 2013). This was further strengthened by a recent finding in *Culex quinquefasciatus*, which found that in Rel2 depleted cells there was a significant increase in WNV titer (Paradkar et al., 2014).

1.3.4. The Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) Pathway

The JAK/STAT pathway was initially identified in mammalian cells as an interferon-induced signaling pathway important for antiviral immunity and is named for the kinase (JAK) and the transcription factor (STAT) (Fu et al., 1992; Schindler et al., 1992). The key components of this pathway are conserved across most of the species, highlighting their evolutionary importance in antiviral immunity (Arbouzova and Zeidler, 2006). In *D. melanogaster*, the JAK/STAT pathway is generally activated by the attachment of Unpaired peptide ligand to the extracellular region of the transmembrane receptor Dome, leading to conformational changes and dimerization of Dome (Figure 3). Activation of Dome is followed by auto-phosphorylation of its associated JAK kinases which later phosphorylates the cytoplasmic tail of the Dome receptor as well to generate docking sites for recruitment of STAT proteins. After recruitment of the STAT proteins at the activated Dome-JAK complex, there is also a phosphorylation event of the STAT proteins resulting in their dimerization and activation. The activated STATs translocate into the nucleus and induce the expression of effector genes. Use of *D. melanogaster* has led to the identification of the antiviral role of the JAK/STAT pathway in
insects (Arbouzova and Zeidler, 2006; Hombria and Brown, 2002; Zeidler and Bausek, 2013). In the case of mosquitoes, there are several reports suggesting its major role in antiviral immunity. In *An. gambiae*, ONNV replication was significantly increased by silencing STAT-A, demonstrating its potential role in antiviral defense (Carissimo et al., 2015). Studies carried out to validate the role of the JAK/STAT pathway in *Ae. aegypti* have found that silencing of Dome and JAK significantly enhanced DENV replication in the midgut, while the opposite effect on DENV replication was observed when the pathway was activated through the silencing of protein inhibitor of activated STAT (PIAS), which is a negative regulator of the JAK/STAT pathway (Souza-Neto et al., 2009). In the same study, two JAK-STAT regulated DENV effector genes, DVFR1 and DRF2, were identified that were able to restrict DENV replication in the midgut tissue of *Ae. aegypti* (Souza-Neto et al., 2009).

### 1.3.5. Crosstalk Between Innate Immunity Pathways

The concept of a single type of pathogen leading to the activation of a single immune pathway is becoming obsolete. There is sufficient evidence to suggest that both the Toll and the IMD pathways perform dual functions in mounting immune response against both bacterial and viral infections (De Gregorio et al., 2002; Tanji et al., 2007). However, there lies a huge grey area of the potential crosstalk between the innate immunity pathways to activate a synchronized response to counter a pathogen challenge. Although there is limited data available to prove crosstalk between different immune pathways, one prime example is Dicer-2 (Dcr2) mediated activation of the antiviral peptide Vago in response to Drosophila C virus (DCV) and WNV infection in *D. melanogaster* and *Cx. quinquefasciatus*, respectively, in an RNAi independent manner (Deddouche et al., 2008; Paradkar et al., 2012). In *Cx. quinquefasciatus*, further elucidation of the mechanism of Vago activation revealed that upon sensing viral dsRNA, Dcr2 activates TNF receptor associated factor (TRAF) leading to Rel2 activation (transcription factor of the IMD pathway), which in return through binding to the NF-κb motif in the Vago promoter induces Vago transcription (Paradkar et al., 2014) (Figure 3). In contrast to *D. melanogaster*, *Cx. quinquefasciatus* Vago activates the JAK/STAT pathway to restrict WNV replication (Paradkar et al., 2012). Vago activation in *Cx. quinquefasciatus* presents a very novel example of a potential crosstalk between RNAi-IMD and JAK/STAT pathways, and provides a new way of studying insect immune response.
Figure 3. Mosquito innate immune signaling and RNAi pathways. In the Toll pathway signaling, detection of pathogen-derived ligands by pattern recognition receptors (PRRs) such as PGRP-SA and -SD triggers proteolytic cleavage of the cytokine Späetzle, which binds to and activates the Toll receptor. This triggers signaling through the adaptor proteins MyD88, Tube, and Pelle, resulting in the phosphorylation and degradation of Cactus, a negative regulator which binds to and sequesters the Rel1 transcription factor in the cytoplasm. Cactus degradation allows Rel1 translocation to the nucleus to activate transcription of Toll-pathway regulated genes. The IMD pathway is activated by ligand binding to PGRP-LCs and -LEs. This triggers signaling through IMD and various caspases and kinases, leading to a functional split in the pathway. One branch triggers JNK signaling to activate the transcription factor AP1, while the other results in the phosphorylation of the Rel2 transcription factor and its subsequent DREDD-mediated cleavage. Activated Rel2 translocates into the nucleus to activate IMD-regulated transcription. The JAK-STAT pathway is triggered by Unpaired (Upd) binding to the receptor Dome, activating the receptor-associated Hop Janus kinases, which phosphorylate each other and subsequently recruit and phosphorylate the STAT transcription factor. Phosphorylated STATs dimerize and translocate into the nucleus to activate JAK-
STAT-regulated transcription. The exogenous siRNA pathway is activated when virus-derived long dsRNA is recognized and cleaved by Dcr2 into siRNAs, usually 21 bases in length. siRNAs are loaded onto the multi-protein RISC complex, which degrades one strand of the duplex and uses the other for targeted degradation of complementary single stranded viral RNA. Sensing of viral dsRNA by Dcr2 also activates TRAF, leading to Rel2 cleavage and activation via a distinct pathway. Rel2 activates transcription of Vago, a secreted peptide which subsequently triggers the JAK-STAT signaling pathway. Adapted from (Sim et al., 2014).

1.4. Vector Control Strategies for DENV Prevention

Arbovirus-mediated diseases are very important medical ailments causing very high mortality and morbidity rates around the world, in particular, in developing countries. Recent increases in unplanned urbanization, ease of travel and environmental changes have led to increased risks of becoming infected by these viruses. In regards to DENV, this has been exacerbated by unavailability of potent vaccines and medicines against the virus, which has limited the disease prevention towards the control of the major vectors, Ae. aegypti and Ae. albopictus. So far, the widely-adopted methods to suppress vector populations have been the application of insecticides or reduction of the breeding sites of the mosquitoes. The aforementioned techniques have found limited success to control mosquito populations. In particular, the recent emergence of resistance in Ae. aegypti to available insecticides have urged the need for development of novel strategies (Marcombe et al., 2012). Currently, two of the important potential novel options are development of genetically modified mosquitoes with elevated immune/antiviral responses to make mosquitoes resistant to DENV infection (Beaty et al., 2010), and more recently the use of Wolbachia as a biological control agent to limit a number of arboviruses including DENV, ZIKV, and WNV (Bian et al., 2010; Kambris et al., 2009; Moreira et al., 2009). In addition, a non-radiation sterile male technique based on genetic manipulation of Ae. aegypti is being trialed by releasing insects that carry a dominant lethal gene (Carvalho et al., 2014). Amongst these, application of Wolbachia has been quite promising and more widespread.

1.5. Wolbachia-Mosquito Interactions

Wolbachia pipiens is an obligate endosymbiotic bacterium which is known to infect 40-60% of insect species belonging to diverse insect families, as well as other arthropods and nematodes (Hilgenboecker et al., 2008; Zug and Hammerstein, 2012). Wolbachia is a maternally transmitted bacterium and is usually associated with manipulation of host
reproduction such as cytoplasmic incompatibility (CI) (Hornett et al., 2010), male killing (Riparbelli et al., 2012) and feminization (Werren et al., 2008). Among reproduction manipulations, CI is considered as the highly prevalent one. *Wolbachia*-infected female mosquitoes can produce viable offspring if they mate with uninfected males or the males infected with similar strain of *Wolbachia*, whereby infected male mosquitoes can only give rise to viable offspring when mated with female mosquitoes infected with a similar strain of *Wolbachia* (Stouthamer et al., 1999). Although the exact mechanism that leads to CI is still elusive, recent studies have demonstrated that two *Wolbachia* prophage genes CiF-A and CiF-B are the main contributors towards CI (LePage et al., 2017). CI gives *Wolbachia* means to rapidly spread across an uninfected insect population (Hoffman and Turelli, 1997). Apart from imposing fitness costs on the host, *Wolbachia* protects its hosts from viral infections, especially against RNA viruses (reviewed in Johnson, 2015). This antiviral property of *Wolbachia* has provided an ideal opportunity to use this bacterium in order to limit the transmission of life threatening arboviruses including DENV by introduction of *Wolbachia* to *Ae. aegypti*. *Wolbachia* is not a natural host of *Ae. aegypti*, so there was a lot of challenges to transinfect the highly proliferating *Wolbachia* strain wMelPop from *D. melanogaster* directly into *Ae. aegypti*. In order to overcome these challenges, wMelPop was first transinfected into *Ae. albopictus* cell line Aa23.T, and after adapting wMelPop to the cells for up to 2.5 years, the wMelPop isolated from infected Aa23.T cells (now called cell line adapted wMelPop; wMelPop-CLA) was used to successfully infect the RML12 cell line (originally thought to be an *Ae. aegypti* cell line but turned out to be from *Ae. albopictus* (Voronin et al., 2010) (McMeniman et al 2008). After adapting wMelPop to mosquito cell lines, wMelPop-CLA was successfully introduced into *Ae. aegypti* mosquitoes by embryonic injections (Walker et al., 2011), reducing the life span of female *Ae. aegypti* by 50% (McMeniman et al., 2009), and successfully inhibited replication of several pathogens including DENV (Moreira et al., 2009). Using a similar strategy, another *Wolbachia* strain (wMel) was transinfected into *Ae. aegypti* mosquitoes. Unlike wMelPop-CLA strain, wMel showed minimal fitness cost on mosquitoes, but lesser viral protection as compared to wMelPop-CLA (Walker et al., 2011). Recently, superinfected *Ae. aegypti* mosquitoes with two *Wolbachia* strains (wMel and wAlbB) has been produced, which showed less fitness cost on the mosquitoes and higher viral protection as compared to mosquitoes infected only with wMel (Joubert et al., 2016). The exact mechanism with which *Wolbachia* produces this antiviral effect is still not well understood. Recently, a study carried out in a *D. melanogaster* cell line (JW18) demonstrated that *Wolbachia*-mediated restriction of Semliki Forest virus occurs at the early stage of infection (Rainey et al., 2016). Nonetheless, there is a good amount of evidence suggesting that in some *Wolbachia*
host-virus associations only tolerance to virus replication is established without much of an effect on virus titer (Osborne et al., 2009; Teixeira et al., 2008).

In *Ae. aegypti* mosquitoes, a number of studies have linked the increased reactive oxygen species (ROS), differential microRNA (miRNA) expression and DNA methylation patterns in the presence of *Wolbachia* with the antiviral effect against DENV replication (Hussain et al., 2011; Pan et al., 2012; Ye et al., 2013; Zhang et al., 2013). However, due to the limitations of these studies to one particular host species, *Ae. aegypti*, which is a transinfected host, there is still a lack of understanding of the exact molecular mechanism(s) that cause *Wolbachia*-mediated antiviral effect in general.

So far, there are two major possible hypotheses that are perceived to impart *Wolbachia*-mediated antiviral defense, which are competition for resources and induction of immune response commonly known as immune priming (Figure 4).

### 1.5.1. Competition for Resources

*Wolbachia* like other members of *Rickettsiales*, is unable to synthesize its own cholesterol, so it solely relies on its host cholesterol reserves for its efficient growth and reproduction (Molloy et al., 2016). On the other hand, almost all mosquito-borne flaviviruses and alphaviruses also depend on the host cholesterol for their successful, infectivity, replication, virion assembly, and release from the infected cells (Fernandez-Garcia et al., 2009; Kielian et al., 2010). Given that the *Wolbachia*-mediated antiviral protection is density dependent, it seems logical that at higher density *Wolbachia* consume more host resources, thus making the host cells’ environment non-conducive for viruses to propagate. A study conducted in *D. melanogaster* has shown that cholesterol levels modulated by *Wolbachia* play an important role in the antiviral protection of *D. melanogaster* flies against DCV (Caragata et al., 2013). Another evidence of involvement of *Wolbachia* in cholesterol competition comes from the study that has demonstrated that *Wolbachia* induces autophagy, which is well known to modulate lipid profile of host cells (Molloy et al., 2016; Voronin et al., 2012). Furthermore, flaviviruses, in particular DENV, also depend on the autophagy pathway for successful replication (Heaton and Randall, 2010). Thus, it seems that there could be direct competition of resources between *Wolbachia* and viruses for the host cholesterol.
1.5.2. Immune Priming

Immune response is the main line of defense of any organism against pathogen invasion. *Wolbachia* has been reported to activate immune genes in transinfected hosts such as *Ae. aegypti*. This induction involves upregulation of immune genes belonging to all the three main innate immune pathways including the Toll, IMD and JAK/STAT pathways (Kamtchum-Tatuene et al., 2016; Rainey et al., 2014; Rances et al., 2012). Studies have also shown the activation of reactive oxygen species (ROS) in response to *Wolbachia* infection in both flies and mosquitoes. The above study further demonstrated the ROS-mediated modulation of the Toll pathway which led to restricted DENV replication in *Ae. aegypti* mosquitoes (Pan et al., 2012). However, this is not the case in *D. melanogaster* flies, which show no change in immune genes in the case of *Wolbachia* infection. Although immune priming is not universal in *Wolbachia*-host interactions, in particular in natural *Wolbachia* hosts, it might still aid in mounting antiviral response against viruses specially in transinfected mosquitoes.
1.5.3. Induction of MiRNAs

miRNAs represent one of the shortest functional classes of non-coding RNAs (ncRNAs) depicting the hottest area of gene regulation research. Discovered in 1993 in the roundworm *Caenorhabditis elegans* (Lee et al., 1993), these 20 - 24 nucleotide-long ncRNAs have been demonstrated to be encoded by a diverse range of organisms including plants, humans, insects and even viruses (Almeida et al., 2011). Historically, miRNAs were thought to regulate gene expression either by degradation of particular target mRNA or inhibition of translation (Baek et al., 2008; Guo et al., 2010), however, recent studies have revealed that these small ncRNAs can also activate target gene expression through mRNA stabilization (Fabian et al., 2010; Vasudevan et al., 2007). Recently, studies have shown miRNAs as critical effectors in the case of bacterial infections (Staedel and Darfeuille, 2013). In insects, studies have demonstrated changes in the expression profile (Hussain et al., 2011), structure and localization of miRNAs in response to *Wolbachia* infection in transinfected *Ae. aegypti* mosquitoes (Mayoral et al., 2014). In particular, *Wolbachia* upregulates aae-miR-2940-5p in *Ae. aegypti* mosquitoes which leads to upregulation of metalloprotease m41 FtsH (Hussain et al., 2011) and downregulation of DNA methyltransferase Dnmt2 (Zhang et al., 2013) both of which are essential for high density of *Wolbachia*, which is important for viral inhibition.

1.6. Concluding Remarks

Given that there is an increasing impact of DENV on global health along with lack of publically available vaccines and antiviral treatment, there is an urgent need for greater understanding of vectors and devising novel vector control strategies that can be employed to limit DENV transmission. Currently, several studies have pointed out towards the potential use of *Wolbachia* as an invaluable tool for limiting DENV replication in mosquitoes, and thus reducing the risk of virus transmission to humans. Despite its potential, there is a lack of understanding of the exact mechanism(s) that *Wolbachia* uses to limit viral replication. In this project, we aimed to deepen our understanding of the potential mechanism(s) that *Wolbachia* employs in general, or in *Ae. aegypti* in particular, to induce the antiviral effect, especially against DENV.

1.7. References


Anopheles gambiae is highly compartmentalized, with distinct roles for RNA interference and gut microbiota. Proc Natl Acad Sci U S A 112, E176-185.


Chapter 2: Downregulation of *Aedes aegypti* chromodomain helicase DNA binding protein 7/Kismet by *Wolbachia* and its effect on dengue virus replication

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Downregulation of *Aedes aegypti* chromodomain helicase DNA binding protein 7/Kismet by *Wolbachia* and its effect on dengue virus replication

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2.1. Abstract

Dengue virus (DENV) is a mosquito-transmitted virus imposing a significant burden on human health around the world. Since current control strategies are not sufficient, there is an urgent need to find alternative methods to control DENV transmission. It has been demonstrated that introduction of *Wolbachia pipientis* in *Aedes aegypti* mosquitoes can impede DENV transmission with the mechanism(s) not fully understood. Recently, a number of studies have found the involvement of chromodomain DNA binding helicases in case of Human Immunodeficiency virus (HIV) and Influenza A virus infection. In this study, we have identified three chromodomain helicase DNA binding protein (CHD) genes in *Ae. aegypti* and looked at their response in the case of *Wolbachia* and DENV infections. Foremost amongst them we have found that *AeCHD7/Kismet* is significantly downregulated in the presence of *Wolbachia* infection only in female mosquitoes. Furthermore, *AeCHD7* levels showed significant increase during DENV infection, and *AeCHD7* depletion led to severe reduction in the replication of DENV. Our data have identified *AeCHD7* as a novel *Ae. aegypti* host factor that is important for DENV replication, and *Wolbachia* downregulates it, which may contribute towards the mechanism(s) of limiting DENV replication.
2.2. Introduction

Among arboviruses, dengue virus (DENV) is one of the most important flaviviruses having the potential to affect two-thirds of the world’s population \(^1,2\). DENV is primarily transmitted to humans through the bite of mosquito vector *Aedes aegypti*, leading to dengue infection and potentially dengue haemorrhagic fever \(^3-5\). Lack of availability of an effective vaccine and proper medical care has narrowed DENV management strategies to vector control. One of the strategies used to overcome DENV vector *Ae. aegypti* is through the application of pesticides, but due to their severe consequences on the environment and the emergence of resistance to pesticides, their potential application seems bleak in the near future \(^6\). Therefore, new strategies for vector control are urgently needed. One of the novel options is the use of an endosymbiotic bacterium *Wolbachia* which has recently been demonstrated to limit DENV, West Nile virus (WNV), and Zika virus (ZIKV) replication in *Ae. aegypti* \(^7-10\).

*Wolbachia pipientis* is an alphaproteobacterium that naturally infects almost 40-60% of insect species \(^11,12\). This bacterium is maternally transmitted and is usually associated with manipulations of host reproduction, such as feminization \(^13\) and male killing \(^14\), to promote successful colonization of its host species. *Wolbachia* naturally infects several mosquito species, including *Aedes albopictus* and *Culex pipiens* \(^15\). However, there is no natural *Wolbachia* infection in the case of *Ae. aegypti*, which is the most notorious vector for several arboviruses. In order to exploit *Wolbachia*’s potential to limit arbovirus transmission, three strains of *Wolbachia*, wAlbB (from *Ae. albopictus*) \(^16\), wMel (from *Drosophila melanogaster*) \(^17\) and wMelPop-CLA (from *D. melanogaster*) \(^18\) have been successfully transinfected into *Ae. aegypti*. Among these three strains, wMel and wMelPop-CLA are the most promising ones for virus blocking \(^7-10,19,20\). However, the exact mechanism(s) by which *Wolbachia* blocks viral replication in *Ae. aegypti* mosquitoes is still elusive. Few studies that have looked into the transcriptional changes in *Ae. aegypti* mosquitoes upon *Wolbachia* infection have found increased redox and mitochondrial activity along with differential serine protease activity \(^21-23\). However, very little is known about the role of chromatin remodelers in the case of DENV-*Aedes-Wolbachia* molecular interactions.

Chromodomain helicase DNA binding proteins (CHD) represent a class of ATP-dependent chromatin remodelling enzymes that contribute towards invoking changes in the interaction between DNA and nucleosomes \(^24\), influencing a wide array of cellular processes such as replication, transcription, recombination, repair and development \(^25\). Members of the CHD family have been found to be involved in replication of Human Immunodeficiency virus (HIV)
and Influenza A virus. All the CHD protein family members have a pair of chromodomains at their N-terminus along with one sucrose non-fermenting (SNF2) domain in the centre. In humans, the CHD family has nine members. These are further classified, on the basis of additional motif features, into three subfamilies: CHD1-2 (class I), CHD3-5 (Class II) and CHD 6-9 (class III). In D. melanogaster, there are three well characterized CHD members named CHD1, Mi2 and Kismet/CHD7. CHD1 is essential for the fecundity of both males and females and is indirectly involved in transcriptional elongation, whilst Mi2 actively participates in transcriptional repression and is vital for expression of heat shock proteins. Drosophila Kismet, that is a homolog of human CHD7, mediates transcriptional elongation. Apart from characterization of the CHD family members’ role in development and chromatin modification, very little is known about their potential role in host-pathogen interactions.

In this study, we have identified functional homologs of the CHD family members in Ae. aegypti and looked at the effect of Wolbachia infection on their expression. There was significant reduction in the expression of all CHD family members in the presence of Wolbachia. Furthermore, we found that AeCHD7 is highly induced during DENV infection in Ae. aegypti mosquitoes. A silencing assay demonstrated that AeCHD7 is required for the efficient replication and virion production of DENV. This study will help to understand the role of AeCHD7 in DENV-Aedes-Wolbachia interactions.

2.3. Results

2.3.1. Screening of the CHD family genes during Wolbachia infection

Three CHD genes were identified in the Ae. aegypti genome using Vectorbase. Blastp was run to identify their homologs in D. melanogaster and Culex quinquefasciatus, and these were determined as AeCHD1 (AAEL004716) having 58% identity with D. melanogaster CHD1 protein (NP_477197.1), AeCHD3 (AAEL013136) that showed 70% identity with D. melanogaster CHD3 protein (AAD17276.1) and AeCHD7 (AAEL002230) showing 58% identity with D. melanogaster Kismet/CHD7 protein (NP_001245820.1). qPCR primers were designed for all the three AeCHD family members to experimentally validate their expression in Ae. aegypti mosquitoes by RT-qPCR, and the effect of Wolbachia (wMelPop) infection on their expression level. For this, we selected two age groups of Ae. aegypti mosquitoes, 4-day- and 12-day-old. While expression of all the three AeCHD genes was confirmed in the mosquitoes, they were all mostly downregulated in Wolbachia-infected mosquitoes (Figure 1A-F), except for AeCHD3, which was found to be non-significantly upregulated in 4-day-old Wolbachia-infected mosquitoes (Figure 1C). However, AeCHD7 showed the highest change of 2.9-fold.
downregulation in 4-day-old *Ae. aegypti* female mosquitoes (Figure 1E), which led us to further characterise the gene.

**Fig. 1**: Relative expression of *AeCHD* genes in uninfected and *Wolbachia*-infected *Ae. aegypti* mosquitoes. RT-qPCR based quantification of (A-B) *AeCHD1*, (C-D) *AeCHD3*, and (E-F) *AeCHD7* genes in both *Wolbachia*-infected (Pop) and uninfected (Tet) 4-day-old and 12-day-old female mosquitoes, respectively showing an overall reduction in the level of *AeCHD* genes in the presence of *Wolbachia* except *AeCHD3* which was found to be upregulated in the presence of *Wolbachia* in the 4-day-old mosquitoes. Error bars represent standard error of mean (SEM) from three biological replicates with two technical replicates (*, p<0.05; A-C and E unpaired t-test; D and F Wilcoxon test).
2.3.2. *AeCHD7* is ubiquitously expressed in all mosquito tissues

In order to determine the relative abundance of *AeCHD7* across different tissues, the salivary gland, midgut, muscle, ovary and fat body were isolated from 3-day-old female *Ae. aegypti* mosquitoes. Following RT-qPCR detection of *AeCHD7* mRNA transcripts, it was found that *AeCHD7* is ubiquitously expressed in all tissues with the highest expression level in the salivary gland, which was 2.1-fold higher than its expression level in the fat body which showed the lowest relative abundance of *AeCHD7* transcripts (Figure 2). These results are consistent with the previous findings which showed that *AeCHD7* is expressed in all human tissues.37

![Relative expression of CHD7](image)

**Fig. 2: Tissue-specific expression of *AeCHD7* in *Ae. aegypti* mosquitoes.** RT-qPCR results of *AeCHD7* transcript levels in the salivary gland, midgut, muscles, ovaries and fat body of 3-day-old tetracycline treated female mosquitoes showing that *AeCHD7* is ubiquitously expressed in all tested tissues. Error bars represent SEM of the mean in three biological replicates with two technical replicates.

2.2.3. Specific *Wolbachia*-mediated downregulation of *AeCHD7* in female *Ae. aegypti*

To find out whether *Wolbachia*-mediated downregulation of *AeCHD7* is gender specific, we evaluated the transcript levels of *AeCHD7* in 4-day-old female and male *Ae. aegypti* mosquitoes with and without *Wolbachia* infection. RT-qPCR results showed that *Wolbachia* downregulates *AeCHD7* only in female mosquitoes and not in their male counterparts (Figure 3A). This is interesting in the sense that *Wolbachia* has a gender specific effect on gene expression in the mosquitoes. To examine if the effect can consistently be seen in cell lines as well, we cross-validated the *AeCHD7* mRNA expression levels in *Ae. aegypti* cell lines, Aag2 and Aag2 infected with *wMelPop*-CLA (Pop) and found a similar trend of *AeCHD7* transcript downregulation in *Wolbachia*-infected cells (Figure 3B).
Fig. 3: Modulation of *AeCHD7/Kismet* by *Wolbachia* infection in male and female mosquitoes and flies, and mosquito cell lines. **A)** RT-qPCR analysis of *AeCHD7* transcript levels in 4-day-old female and male mosquitoes, both uninfected (Tet) and infected with *Wolbachia* (Pop) showing reduction in the transcript levels of *AeCHD7* in 4-day-old female mosquitoes only. **B)** Relative expression of *AeCHD7* in Aag2 and Aag2 cells infected with wMelPop-CLA (Pop) confirmed the downregulation of *AeCHD7* transcripts in *Wolbachia*-infected cells. **C)** Relative expression of the *D. melanogaster Kismet* gene, homolog of *AeCHD7*, in uninfected (Tet) and *Wolbachia*-infected (Pop) flies showing that there was no significant difference in the expression in the case of *Wolbachia* infection. Error bars represent
SEM from three biological replicates with two technical replicates with two technical replicates (**, p<0.01; ***, p<0.001; ns, not significant; A and B; One-way ANOVA, and C; unpaired t-test, respectively).

Furthermore, to evaluate if Wolbachia has a similar effect on the CHD7 gene in its natural host D. melanogaster, four 7-day-old male and female flies infected with Wolbachia (wMelPop strain) were examined for the relative expression of CHD7. RT-qPCR results confirmed that there was no significant change in the level of CHD7 mRNA in both male and female D. melanogaster flies infected with Wolbachia (Figure 3C).

### 2.3.4. AeCHD7 is upregulated upon DENV infection

Considering the virus blocking effect of Wolbachia in Ae. aegypti mosquitoes, we examined the transcript levels of AeCHD7 in the context of mosquito-DENV interaction. For this, the transcript levels of AeCHD7 in DENV-injected mosquitoes at three different time points of 2, 6 and 12 days post-infection (dpi) were analysed. To have a more consistent and high success rate of DENV infection, mosquitoes were injected rather than orally fed with the virus. The results revealed that there was an increase in the AeCHD7 transcript levels upon DENV infection at all the time points (Figure 4A-C); however, the upregulation was only significant at 2 and 12 dpi, which was 2-fold (Figure 4A) and 4-fold higher than that in uninfected mosquitoes (Figure 4C), respectively. The aforementioned findings were further confirmed in the Ae. aegypti cell line, Aa20. Cells were infected with DENV2 at 0.1 multiplicity of infection (MOI) and were harvested at two different time points that were 1 and 5 dpi. RT-qPCR analysis showed significant increase in AeCHD7 transcript levels in the case of DENV infection at both 1 and 5 dpi (Figure 4D). Infection was confirmed by relative quantification of DENV genomic RNA levels, which showed gradual increase in DENV genomic RNA over time (Figure 4E).
2.3.5. *AeCHD7* is required for efficient DENV replication

The upregulation of *AeCHD7* in DENV-infected cells suggested that the gene could be beneficial for the virus. To investigate whether *AeCHD7* is required for efficient DENV...
replication, we knocked down *AeCHD7* transcripts in Aa20 cells and challenged these cells with DENV at 1 MOI for 72 h. The effect of *CHD7* knockdown on DENV was evaluated both at the genomic and the virion levels using RT-qPCR and plaque assay. RT-qPCR results confirmed ~50% decrease in *AeCHD7* mRNA level (Figure 5A), which led to 2-fold reduction in DENV genomic RNA (Figure 5B). RT-qPCR results were further validated with plaque assay, which confirmed reduction in DENV virion production in *AeCHD7* knocked down cells as compared with dsGFP or mock-transfected Aa20 cells (Figure 5C). These results indicate that *AeCHD7* is a host factor that is used by DENV to facilitate its replication in *Ae. aegypti* female mosquitoes, and *Wolbachia* downregulates *AeCHD7* as shown above, which may contribute to restricting DENV replication.

**Fig. 5:** Depletion of *AeCHD7* impairs DENV replication both at the genomic and the virion levels. **A**) RT-qPCR analysis of Aa20 cells transfected with either no RNA (Mock) or with dsRNA against GFP as a control or with dsRNA against *AeCHD7* confirming significant knockdown of *AeCHD7* in dsAeCHD7 transfected cells as compared to controls. *RPS17* was
used to normalize the qPCR data. Error bars show SEM from three biological replicates with two technical replicates (*, p<0.05; **, p<0.01; One-way ANOVA). B) RT-qPCR analysis of Aa20 cells treated as in (A) followed by DENV infection at 1 MOI using DENV-specific primers to quantify viral genomic RNA showed a significant decrease in the genomic RNA level of DENV in dsAeCHD7 treated cells. Error bars show SEM from three biological replicates (**, p<0.01; One-way ANOVA). C) Viral plaque visualization by in vitro cell plaque assay conducted on the supernatant media from cells treated as in (A and B).

2.4. Discussion

There is accumulating experimental evidence showing the effectiveness of Wolbachia in suppressing the replication of several flaviviruses, including DENV, ZIKV, WNV, and the alphavirus chikungunya virus (CHIKV) in both mosquitoes and mosquitoes-derived cell lines 8,10,38. Perhaps the most well studied is the case of DENV replication that is severely compromised in the presence of Wolbachia 39-41. However, the exact mechanism(s) of how Wolbachia imparts this antiviral effect is not yet fully understood. In this study, we provide experimental evidence that chromodomain DNA binding helicase 7 (AeCHD7) is an Ae. aegypti host factor that is exploited by DENV to facilitate its replication, and its downregulation by Wolbachia may contribute to limit DENV replication. Wolbachia is an endosymbiontic bacterium infecting 40-60% of insect species naturally 11 by manipulating host reproduction 12. Despite fitness costs, Wolbachia may benefit its host by blocking a variety of RNA viruses15,42. However, Wolbachia has not been found naturally infecting the most notorious vector Ae. aegypti, that is responsible for transmitting multiple viral diseases 18. McMeniman et al transinfected different strains of Wolbachia into Ae. aegypti mosquitoes 43 and found that they successfully inhibited replication of DENV and CHIKV 38. Further studies also demonstrated Wolbachia’s ability to block WNV and ZIKV in the mosquito 8-10. Wolbachia’s potential to be used as an invaluable tool for disease control and prevention represents an increasingly promising approach to limit several mosquito-borne viral diseases, and it is fascinating to explore the exact mechanism(s) that induce the antiviral effect. Apart from one study shedding light on the effect of Wolbachia infection on the global DNA methylation pattern in mosquitoes 44, there lies a huge grey area of the role of chromatin remodelers, in Wolbachia-host interactions and possibly the Wolbachia-mediated antiviral effect.

CHD proteins represent a class of proteins that belong to SNF2 superfamily of ATP-dependent chromatin modifiers. In mammals, there are 1-9 CHD proteins; however, in D. melanogaster, there are only three CHD proteins named CHD1, Mi2 and Kismet 25. Members
of the CHD family are involved in conducting a wide array of functions, including ATPase activity to maintain chromosome structure and regulation of heterochromatic elements, nucleosome mobilization, transcriptional regulation and elongation, and development and differentiation. Despite extensive characterization of CHD family proteins, their role in shaping host-pathogen interactions has not been much investigated. Yet, there are few reports supporting the involvement of CHD1 in the case of influenza A virus, and both CHD1 and CHD2 in the case of HIV as positive regulators. Furthermore, RNAi screen carried out in D. melanogaster S2 cells identified the involvement of CHD7/Kismet in antimicrobial humoral response. The aforementioned facts led us to investigate the possible role of the CHD family in Wolbachia-Aedes-DENV interactions. Data mining in VectorBase resulted in the identification of three potential AeCHD proteins encoded in the Ae. aegypti genome. Protein blast results identified them as AeCHD1, AeCHD3/Mi2 and AeCHD7/Kismet. In order to find out whether Wolbachia regulates the AeCHD genes during infection, RT-qPCR was performed to examine the transcript levels of all the three AeCHDs with and without Wolbachia infection in whole mosquitoes. Our results showed that there was a uniform trend of downregulation of the transcript levels of the AeCHD genes in Wolbachia-infected mosquitoes (Figure 1A-F), except those of AeCHD3 in 4-day-old Wolbachia-infected female mosquitoes, which showed a non-significant upregulation (Figure 1C). The reduction in the CHD genes was more pronounced in 12-day-old mosquitoes, which could be due to increases in the Wolbachia load as the mosquitoes ages. In particular, the wMelPop strain is a virulent strain that may cause tissue damage and sickness. The reductions in the AeCHD genes at this late stage may not be of benefit in affecting DENV replication. However, AeCHD7 showed the highest fold change reduction (Figure 1E-F) in both 4- and 12-day-old Wolbachia-infected mosquitoes, which prompted us to further investigate this gene in the context of Wolbachia-Aedes-DENV interactions.

AeCHD7/Kismet belongs to subfamily III of the CHD proteins that comprises CHD5-9 proteins. This subfamily is defined by the presence of two chromodomains at the N-terminus, one SNF2-like ATPase domain located in the central region of the protein structure and a Brahma and Kismet (BRK) domain at the C-terminus. To find out whether the Ae. aegypti homolog fulfills this particular protein signature, NCBI conserved domain finder was used to detect the conserved domains. Results confirmed the presence of all the domains characteristic of CHD7/Kismet proteins (Figure S1). Furthermore, in order to check the conservation of CHD7 across species, CHD7/Kismet amino acids were retrieved from Uniprot (Figure S2A) and subjected to maximum likelihood phylogenetic tree construction.
Phylogenetic results showed that *Ae. aegypti* CHD7 is closely related to that of *Cx. quinquefasciatus* but not to that of *D. melanogaster* or *Homo sapiens* (Figure S2B). To find out the tissue-specific expression of *AeCHD7*, RT-qPCR was performed, which revealed that it is ubiquitously expressed across all main mosquito tissues (Figure 2), which is consistent with the findings in humans 37.

In this study we found that *AeCHD7* was significantly downregulated in *Wolbachia*-infected mosquitoes. To further investigate whether this *Wolbachia*-mediated downregulation of *AeCHD7* in female *Ae. aegypti* is gender specific and can also be seen in its natural host *D. melanogaster*, RT-qPCR was employed. Interestingly, we found that in the presence of *Wolbachia* *AeCHD7* was specifically downregulated in female *Ae. aegypti* only (Figure 3), and there was no change in CHD7/Kismet transcript levels in both female and male *D. melanogaster* with and without *Wolbachia* (Figure 3C). This difference in *Wolbachia*-mediated regulation of CHD7/Kismet in *Ae. aegypti* and *D. melanogaster* may be due to the fact that *Wolbachia* is a natural symbiont in *D. melanogaster* with a long association, while it has been recently transinfected into *Ae. aegypti* 52. In regards to the mechanism by which *Wolbachia* infection may affect expression of *AeCHD7*, one can only speculate at this stage as there is very little information in regards to how *Wolbachia* manipulates its host at the molecular level. Only very recently, some molecular data have become available showing that *Wolbachia* infection leads to changes in the transcriptome or small RNA profiles of infected mosquitoes 23,53. Regulation of host gene expression could be due to components secreted from the endosymbiont, including small non-coding RNAs 54, or host response to accommodating the endosymbiont, in particular in new associations. However, how *Wolbachia* infection leads to these changes in the host remains to be investigated.

Viruses are the master manipulators of their host environment for their own benefit. Recently, it has been reported that CHD1 and CHD2 proteins play a pivotal role in the replication of influenza and HIV viruses 26,27. Both viruses replicate inside the nucleus. Interestingly, it has further been demonstrated that CHD1 interacts with RNA polymerase II to facilitate the replication of influenza A virus 27. Little is known about the role of CHD7/Kismet in the context of virus infection. However, the presence of conserved chromodomains and a SNF2 domain makes it highly likely that all the CHDs share similar functions 25. We were intrigued to find what happens to *AeCHD7* during DENV infection. RT-qPCR performed in DENV-infected mosquitoes at different time points suggested a continuous trend of upregulation during DENV infection (Figure 4A-C), which points to the fact that it might play an important role in DENV replication. To investigate the role of *AeCHD7* in DENV replication further, *AeCHD7*
knockdown study was carried out, which revealed that AeCHD7 is vital for DENV replication and virion production (Figure 5A-C). Very few studies that have been carried out on the involvement of CHDs in virus replication have predominantly focused on viruses that replicate inside the nucleus. Therefore, the role of CHDs in the replication of viruses that multiply in the cytoplasm (such as DENV) is not known. However, it has been demonstrated that DENV capsid 55,56 and NS5 proteins go inside the nucleus 57,58 with NS5 known to be involved in disrupting nucleosome formation 59. Therefore, these viral proteins may play a role in modulating AeCHD7 expression during virus infection. While we have found the involvement of AeCHD7 in mosquito-DENV interaction, the exact mechanism(s) that govern the interaction need further investigation.

In summary, we have demonstrated that AeCHD7 facilitates DENV replication, and Wolbachia-mediated downregulation of AeCHD7 in female Ae. aegypti may contribute to restriction of DENV replication. However, this mechanism is highly specific to female Ae. aegypti mosquitoes and does not appear to be a universal mechanism which Wolbachia employs across different hosts to block viral replication.

2.5. Materials and Methods

2.5.1. Mosquitoes and flies

For Wolbachia studies, mosquitoes had been previously generated by McMeniman et al. (2009) by transinfecting wMelPop-CLA strain of Wolbachia (Pop) into Ae. aegypti embryos, and uninfected mosquitoes were obtained through tetracycline (Tet) treatment of the infected mosquitoes 43. w118 fly line stably infected with wMelPop-CLA and the tetracycline cured line were generated by Min et al. (1997) 60 and kindly provided by Dr Karyn Johnson from the University of Queensland.

For DENV infection studies, Ae. aegypti eggs were collected in Townsville in August 2015 and reared in insectary at Public Health Virology FSS. Five-day-old Ae. aegypti (F3) were used for the experiments. DENV2 NGC strain obtained from Prof. Roy Hall’s Lab (University of Queensland, School of Chemistry & Molecular Biosciences, Brisbane, Australia), was diluted in Opti-MEM (GIBCO Life Technologies, Grans Island, NY) supplemented with 3% foetal bovine serum (FBS, In Vitro Technologies, Australian origin) and intrathoracically injected (200μl) in 5-day-old mosquitoes at 105.8/mL (102.1 per dose). Mosquitoes were placed into netted 900 mL containers at 28°C with light:dark (L:D) 12:12 hours cycle and at high humidity. Mosquitoes were offered 15% honey water ad libitum. Mosquitoes were collected at 2, 6 and 12 dpi for downstream applications.
2.5.2. Cell cultures

*Ae. aegypti* Aag2 cell line and Aag2 cells infected with wMelPop-CLA, previously described by 61, were maintained in 1:1 Mitsuhashi-Maramorosch and Schneider’s insect medium (Invitrogen) supplemented with 5-10% FBS, while Aa20 cells were maintained in L15 medium (Invitrogen) supplemented with 10% tryptose phosphate broth (TPB) and 5% FBS (Bovogen Biologicals, French origin). All mosquito cell lines were kept at 28°C and passaged every 3-4 days.

Vero cells were maintained in OptiMEM medium supplemented with 2% FBS and were kept at 37°C in the presence of 5% CO₂.

2.5.3. RT-PCR and qPCR analyses

Total RNA was extracted from mosquitoes (5 mosquitoes per biological replicate) or flies (5 flies per biological replicate) using Qiazol (Qiagen) and then treated with Turbo DNase (Ambion) according to the manufacturers’ instructions. 750-1000 ng of total RNA was then used to make the 1st strand cDNA using Superscript III (Invitrogen) with either oligo-dT primer for cellular transcripts or with DENV-qR primer in order to amplify the DENV genomic RNA.

For qPCR, cDNA produced as above was diluted in 1:5 ratios with nuclease free water. 2μl of the diluted cDNA was used for downstream qPCR reaction. Both forward and reverse gene-specific primers were used to amplify the target genes (primer sequences in Table S1), using QuantiFast SYBR Green (Qiagen) in a Rotorgene qPCR machine (Qiagen). For *Ae. aegypti* samples, *RPS17* transcript levels were used for the normalization of RNA templates, while *RPL32* was used for normalization of *D. melanogaster* samples. Each qPCR reaction was performed in duplicates with at least three biological replicates. All qPCR data were normalized with Qiagen analysis templates. The average of normalized values of each group with at least three biological replicates was subjected to normal distribution test by using Shapiro-Wilk normality test in Prism 7.0. The data set passing the normality test was further analysed by Prism 7.0 using parametric tests. Unpaired t-test was used to determine statistical significance between two individual groups while One-way ANOVA with Tukey’s post-hoc test was performed to find statistical significance between more than two groups of data. The data sets which failed the normal distribution test were analysed by the non-parametric Wilcoxon test using JMP software to determine the significance level between groups.

2.5.4. RNAi-mediated gene silencing

In order to knockdown the *AeCHD7* gene for functional analysis in DENV life cycle, primers were designed to amplify a 586 bp product from the *AeCHD7* gene with the addition of the T7
promoter sequences at both ends (Table S1). MEGAscript T7 Transcription kit was then used according to the manufacturer’s instructions in order to synthesize dsRNA targeting the AeCHD7 transcripts. A similar approach was followed to synthesize dsRNA against GFP RNA. For knockdown experiments, Aa20 cells were double transfected with 2-5μg of dsRNA per well against the target gene. dsGFP RNA was used as non-specific control.

2.5.5. Virus infection and plaque assay

For virus inoculation experiments, Ae. aegypti Aa20 were seeded at the density of 3x10⁵ cells per well in 12-well plates. Cells were first double transfected with dsRNA against the target gene or GFP control and after 6 h cells were infected with DENV2 (New Guinea strain) at a multiplicity of infection (MOI) of 1. Media were collected 72 h post-infection for plaque assay. To perform plaque assay, Vero cells were seeded in a 96-well plate and were allowed to form monolayers. Virus containing media from the experiments were serially diluted into 10⁰,10¹ ,10²,10³ dilutions and added to Vero cells in duplicates. Cells were incubated with virus at room temperature with continuous shaking on shaker for 1 h and then incubated at 37ºC for one additional hour. After 2 h of incubation, media were aspirated and an overlay was added to the cells which comprised of 1.5% carboxymethyl cellulose (CMC) and 2.5% FBS in Opti-MEM medium (Sigma). Cells were then incubated for 72 h at 37ºC and 5% CO₂ and fixed with 80% ice-cold acetone in 1×PBS for 20 min at -20ºC. Plates were then air dried overnight and blocked with 5% skimmed milk in 1×PBST at 37ºC for 30 min. Cells were then incubated with the primary antibody against DENV2-Envelope (human) in 1:1000 dilution in 0.1% skimmed milk in 1×PBST for 2 h at 37ºC as described previously ⁶². Plates were washed 3 times with 1×PBST and incubated with the secondary antibody (IRDye 800CW goat anti-human LICOR) for 1 h at 37ºC. Plates were washed and dried as above and were dried and scanned on the Odyssey imager (LI-COR Biosciences) at 41μM resolution.

2.6. Acknowledgements

We are thankful to Dr Karyn Johnson and Verna Hearne from UQ for providing D. melanogaster wMelPop-infected and tetracycline cured flies, Prof Paul Young from UQ for providing the anti-DENV antibody, and Dr Andrew van den Hurk from Queensland Health for providing Ae. aegypti eggs. Special thanks to Solomon-Osei-Amo (UQ) for critical reading of the manuscript. This project was supported by a National Health Medical Research Council grant (APP1062983) to S Asgari and a UQ International scholarship to S Asad.

2.7. Authors’ contributions

SAsa conceived and designed research, carried out experiments, analysed the data and
drafted the manuscript. SHM carried out experiments and edited the manuscript. SAsg designed research and edited the manuscript. All authors read and approved the final manuscript.

2.8. Additional Information

2.8.1. Competing financial interests. The authors declare no competing financial interests.

2.9. References


2.11. Supplementary Material

Conserved Domains

Chromodomains (Red): 1781-1821; 1843-1885
SNF2 (Green): 1933-2220
BRK (Yellow): 4137-4174

Fig. S1 Diagramed illustration of AeCHD7 conserved domains: Conserved domains were found in AeCHD7 by putting AAEL002230-PA amino acid sequences as query in NCBI conserved domain finder, and the domains characteristic of CHD7 proteins are shown. Red color shows chromodomain, green color shows SNF2 domain and yellow color shows BRK domain.
Fig. S2: A) Table of protein sequences used in this study. B) Maximum likelihood phylogenetic tree computed for the protein sequences in (A) showing that AeCHD7 protein in *Ae. aegypti* is more closely related to *C. quinquefasciatus* than its homolog in *D. melanogaster.*
### Table S1. Primers used in this study.

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Chapter 3: Upregulation of *Aedes aegypti Vago1* by *Wolbachia* and its effect on dengue virus replication

(Presented as manuscript, under review)

Asad S and Asgari S, Upregulation of *Aedes aegypti Vago1* by *Wolbachia* and its effect on dengue virus replication
Upregulation of *Aedes aegypti* Vago1 by *Wolbachia* and its effect on dengue virus replication

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**Running title:** Vago1 in *Wolbachia* and dengue virus infection

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3.1. Abstract

Dengue infection along with its related disease conditions poses a significant threat to human health. The pathogen responsible for this infection is dengue virus (DENV) which is primarily transmitted to humans through the bites of *Aedes aegypti* mosquitoes. Unavailability of a potent vaccine has recently sparked renewed research endeavours aimed at vector control. To date, *Wolbachia* as an endosymbiotic bacterium has shown promise as a novel biocontrol agent to restrict DENV replication in the vector, although the underlying antiviral mechanism remains elusive. Recent studies have demonstrated the potential role of Vago as a novel secretory protein involved in cross-talk between the innate immune pathways in *Culex quinquefasciatus* mosquitoes to restrict West Nile virus replication. In this study, we have identified two homologs of the Vago protein in *Ae. aegypti* and looked into their modulation in the case of *Wolbachia* wMelPop strain infection. Furthermore, we have investigated the role of *AeVago1*, that is highly induced by *Wolbachia*, in the context of *Wolbachia*-mosquito-DENV interactions. Knockdown studies of the *AeVago1* gene in *Wolbachia*-infected cells led to significant increases in DENV replication, with no effect on *Wolbachia* density. Our results suggest that the *Wolbachia*-induced *AeVago1* in *Ae. aegypti* may function as a host factor to suppress DENV replication in the mosquito.

**Keywords:** *AeVago1; Aedes aegypti; Wolbachia; Dengue virus*
3.2. Introduction

Dengue is one of the most important arboviral diseases which affect 390 million people throughout the world (Bhatt et al., 2013; Tatem et al., 2006). It is caused by a flavivirus, dengue virus (DENV), which uses Aedes aegypti as its primary and Aedes albopictus as its secondary vector (Gubler, 2006; Ponlawat and Harrington, 2005). Limitations such as unavailability of a proper cure and publically available vaccines have compounded the disease impact, and the disease control measures almost exclusively revolve around vector control. There are a number of pesticides that are being used to limit Ae. aegypti populations, however, recent emergence of resistance in the mosquito to a number of pesticides has urged the need to find alternative vector control strategies (Marcombe et al., 2012). One of the unique options is using Wolbachia as a novel biological control agent in mosquitoes to limit their ability to transmit a number of flaviviruses including DENV and Zika virus (ZIKV) (Dutra et al., 2016; Iturbe-Ormaetxe et al., 2011). However, very little is known about the molecular mechanism(s) that govern this antiviral effect.

Wolbachia pipientis is an endosymbiotic bacterium that naturally infects 40-60% of insect species (Zug and Hammerstein, 2012). It is maternally inherited and is known to manipulate the host reproduction strategies to promote stable infection in its host (Caragata et al., 2016; Hoffmann et al., 1998). Despite infecting their hosts, a number of Wolbachia strains give their host protection against several viruses including DENV, Chikungunya virus, West Nile virus (WNV) and ZIKV (Dutra et al., 2016; Hussain et al., 2013; Moreira et al., 2009) in mosquitoes and Drosophila C virus (DCV) and flock house virus in Drosophila (Martinez et al., 2014). This protection is largely correlated with the density of different Wolbachia strains in host tissues (Martinez et al., 2014). Interestingly, there is no natural association of Wolbachia with Ae. aegypti mosquitoes. To exploit the potential of Wolbachia as a biological control agent against DENV transmission, recently two Wolbachia strains wMel and wMelPop have been isolated from Drosophila melanogaster and transinfected into Ae. aegypti mosquitoes (McMeniman et al., 2009; Walker et al., 2011). Both strains successfully inhibited the ability of Ae. aegypti mosquitoes to transmit DENV with the latter, being a more virulent strain, showing higher efficacy in limiting DENV replication (McMeniman et al., 2009; Walker et al., 2011). Despite the potential of Wolbachia as a novel biocontrol agent to target vector competence, little is known about the mechanism(s) that Wolbachia use to limit DENV replication. There are two potential theories in this regard: either competition for host resources between Wolbachia and viruses or activation of immune genes by Wolbachia to overcome viral replication (Caragata et al., 2016). The former theory is supported by a number of studies that have shown Wolbachia
modulates the host cholesterol levels in *D. melanogaster* which leads to protection against DCV. A recent study carried out in an *Ae. albopictus* cell line has reported depletion of sphingolipids in response to *Wolbachia* infection and suggested their potential role in limiting mosquito-borne viruses. Furthermore, studies have demonstrated that *Wolbachia*-mediated modulation of *AeCHD7* (Asad et al., 2016), reactive oxygen species (ROS) (Pan et al., 2012) and immune genes (Rances et al., 2012) may play important roles in limiting DENV replication in transinfected host *Ae. aegypti* suggesting that *Wolbachia* elicits host response making the environment unfit for viral replication. Although it has been reported that immune activation does not play any part in *Wolbachia*-mediated antiviral response in its natural host *D. melanogaster*, it might play a part in transinfected hosts, such as *Ae. aegypti* (Rances et al., 2012).

In the absence of adaptive immunity in insects, they solely rely on their innate immune response, comprising of RNA interference (RNAi) and activation of antiviral proteins, to counter viruses (Buchon et al., 2014). Studies have shown that there is no role of RNAi in *Wolbachia*-mediated antiviral effect (Hedges et al., 2012; Zhang et al., 2016), which narrows down the search towards *Wolbachia*-mediated modulation of antiviral proteins in order to protect its host against several viruses. The protein-based innate immunity response is broadly centred on three important pathways that are Toll, immune deficiency (IMD) and Janus kinase/signal transducer and activator of transcription pathway (JAK/STAT) (Dostert et al., 2005; Tanji and Ip, 2005; Valanne et al., 2011). The JAK/STAT pathway is known to play a critical role in overcoming several viruses in insects (Dostert et al., 2005; Souza-Neto et al., 2009). Recent studies carried out on *Culex quinquefasciatus* have found a new antiviral mechanism which involves Dicer2-Rel2-TRAF mediated activation of a secretory protein Vago that is able to limit WNV by activating the JAK/STAT pathway (Paradkar et al., 2014; Paradkar et al., 2012). In addition, Vago was shown to be antiviral against Drosophila C virus in *D. melanogaster* (Deddouche et al., 2008). However, the role of Vago is not very well understood in *Ae. aegypti*-DENV-*Wolbachia* interactions.

In this study, we have investigated the role of *Ae. aegypti* Vago homolog proteins in the case of *Wolbachia* and DENV infections. We have found that *AeVago1* is significantly upregulated in the presence of *Wolbachia* in young *Ae. aegypti* mosquitoes and Aag2 cell line, which might contribute to DENV inhibition. In addition, we studied the effect of DENV infection on *AeVago1* expression and found that the gene is not modulated. Furthermore, knockdown studies of *AeVago1* in *Wolbachia*-infected cells showed significant increases in DENV modulating
replication, confirming its antiviral property, which could be utilized by Wolbachia as one of the mechanism(s) to overcome viral replication in Ae. aegypti mosquitoes.

3.3. Material and Methods
3.3.1. Cell lines, mosquitoes and flies used in study

Ae. aegypti cell line Aag2 infected with wMelPop-CLA strain of Wolbachia (Pop) previously generated as described (Frentiu et al., 2014), and Ae. albopictus cell lines infected with wAlbB strain of Wolbachia (Aa23) (O'Neill et al., 1997), or treated with tetracycline (Aa23-Tet) (to ensure the removal of Wolbachia from cell lines) were maintained in 1:1 mixture of Mitsuhashi-Maramorosch and Schneider’s insect medium (Invitrogen) supplemented with 10% FBS (Bovogen Biologicals). For D. melanogaster cell line S2, Schneider’s insect medium supplemented with 10% FBS was used. All the aforementioned insect cell lines were maintained at 28°C.

Ae. aegypti mosquitoes infected with wMelPop-CLA strain of Wolbachia (Pop) and Wolbachia free mosquitoes (Tet) used in this study were generated previously (McMeniman et al., 2009). Recolonization of gut microflora was also ensured by growing larvae of Tet mosquitoes on water used to rear natural mosquito larvae (McMeniman et al., 2009). w1118 fly line stably infected with wMelPop along with tetracycline treated Wolbachia free (Tet) flies were generated earlier (Min and Benzer, 1997).

For DENV infection studies, 5-day-old Ae. aegypti mosquitoes were intrathoracically injected with DENV2 NGC strain as described earlier (Asad et al., 2016). Injection was used for DENV infection to ensure a more uniform and high infection rate. Mosquitoes were placed in 900mL containers at 28°C with 12:12 hours dark and light cycle at high humidity. Mosquitoes were fed on 15% honey water ad libitum. Mosquitoes were collected at 2, 6, and 12 days post-infection for downstream applications.

3.3.2. qPCR studies

Total RNA was isolated from whole mosquitoes (5 per biological replicate), flies (5 per biological replicate), tissues (10 mosquitoes per biological replicate) and cell lines using QIAzol (Qiagen) as per the manufacturer’s instructions. The extracted RNA was subjected to Turbo DNase (Ambion) treatment according to the manufacturer’s instructions. 1000 ng of total RNA was further used to synthesize complimentary DNA using Superscript III (Invitrogen) with oligo(dT) for host genes or DENV-qR (specifically targeting the NS2A gene of DENV-2)
for DENV according to the manufacturer’s instructions. In order to perform qPCR analysis, the cDNA was diluted 1:5 with RNase free water, then gene-specific primers (Table S1) along with QuantiFast SYBER Green (Qiagen) were used to quantify the relative expression of genes using a Rotorgene qPCR machine (Qiagen). The qPCR cycling conditions were 95°C for 5 min for initial denaturation and hot start Taq activation, followed by 40 cycles of 95°C for 10 sec, 60°C for 15 sec and 72°C for 20 sec. Additionally, melt curve analysis was carried out on default settings of the Rotorgene machine (Qiagen) to check the specificity of the qPCR products. For mosquito samples, ribosomal protein S17 (RPS17; AY927787.2) was used as the housekeeping gene to normalize the relative expression of target genes, while ribosomal protein L32 (RPL32; NM_079843) was used as the housekeeping gene for D. melanogaster samples. Each qPCR reaction was performed in duplicates with at least three biological replicates.

For Wolbachia density determination, DNA extraction was performed using DNeasy Blood & Tissue Kit (Qiagen) as per the manufacturer’s instructions. 100ng DNA was used in each qPCR reaction, using primers specific to the Wolbachia surface protein (WSP; AF338346.1) gene along with QuantiFast SYBER Green (Qiagen) in Rotorgene qPCR machine. RPS17 gene from Ae. aegypti was used to normalize the qPCR data. Each reaction was performed in triplicates with at least three biological replicates.

For qPCR analysis, all the CT values were normalized with Qiagen analysis templates and further processed with Prism 7.0. All the data sets were subjected to Shapiro-Wilk normality test in Prism 7.0 to confirm the normal distribution of the data. For data sets that passed the normality test, un-paired t-test was performed to determine statistical significance between two individual groups. One-way ANOVA with Tukey’s post-hoc test was used to find out statistical significance between more than two groups. In order to evaluate the statistical significance, two different time points in grouped data Two-way ANOVA along with Sidak multiple comparison test were used. For data sets that failed the normal distribution test, Wilcoxon non-parametric test was employed using JMP software to determine the statistical significance between the individual groups.

3.3.3. Gene silencing using RNAi

AeVago1 (AAEL000200) was knocked down using dsRNA to find out its functional role in terms of Wolbachia-Aedes-DENV interactions. Briefly, primers were designed to amplify a 198 bp product from the AeVago1 gene with the addition of T7 promoter sites at both ends. The PCR template was used to make dsRNA using the MEGAscript T7 Transcription kit (Ambion)
according to the manufacturer’s instructions. GFP-specific dsRNA was also synthesized and used as a non-specific control. For knockdown experiments, Pop cells were double transfected with 5 and 2μg of dsRNA, respectively, per well against the target gene and GFP as control.

3.4. Results

3.4.1. Identification of secretory AeVago proteins

Using BLASTp and the Vago protein from *Cu. quinquefasciatus* (XP_001842264.1) as template, we identified two homologs of the protein in the *Ae. aegypti* proteome. As an earlier report demonstrated the importance of secretion of the Vago protein in its antiviral activity (Paradkar et al., 2012), we subjected the potential Vago hits to SingalP (Petersen et al., 2011) to check whether they have a distinct secretory signal or not (Petersen et al., 2011). As a result, two potential Vago homologs in *Ae. aegypti*, which are 100-120 amino acids long with a distinct secretory signal as well as a von Willebrand factor type C (SVWC) domain, were identified (Figure S1 and S2). These were named AeVago1 (AAEL000200) and AeVago2 (AAEL000165) with 53% and 41% amino acid sequence identity to CuVago, respectively.

3.4.2. AeVago1 is upregulated in Wolbachia infection

To examine whether *Wolbachia* affects the expression of the *AeVago* genes, which could contribute in antiviral defence, we compared the transcript levels of the genes in *Wolbachia*-infected (Pop) and uninfected Aag2 cell lines. RT-qPCR results showed that there was more than 10-fold statistically significant increase in *AeVago1* in cells that were infected with *Wolbachia* (Figure 1A), while *AeVago2* was almost 3.8-fold significantly downregulated in *Wolbachia*-infected cells (Figure 1B).

As *Wolbachia* is well known to block virus replication with the mechanism(s) not well understood, and Vago is known to be antiviral, we followed up on *AeVago1*. For this, we first looked into the relative abundance of *AeVago1* transcript levels in 4 and 12-day-old female *Wolbachia*-infected (Pop) and tetracycline cured (Tet) *Ae. aegypti* mosquitoes. Our results showed that *AeVago1* was 4.1-fold significantly increased in the 4-day-old *Wolbachia*-infected mosquitoes, however, there was no significant change in the levels of *AeVago1* transcripts in the older mosquitoes (Figure 1C).

To investigate whether *Wolbachia*-mediated *AeVago1* induction is a conserved phenomenon in its natural host *D. melanogaster*, 7-day-old *D. melanogaster* flies infected with *Wolbachia*
(wMelPop) were used to check the levels of Vago in female flies. Results led us to the conclusion that Vago is not upregulated in wMelPop-infected D. melanogaster (Figure S3A).

To test the hypothesis that Wolbachia-mediated upregulation of AeVago1 could most probably be due to transinfection of Wolbachia to a new host, that is Ae. aegypti, we checked the levels of the Vago homolog in Ae. albopictus (KT799989.1) Aa23 cells naturally infected with the wAlbB strain of Wolbachia (Kitrayapong et al., 2002; O'Neill et al., 1997). The results revealed no significant modulation of the Vago transcript levels in Ae. albopictus cells infected with Wolbachia (Figure S3B).

**Figure 1.** Differential expression of AeVago genes in Wolbachia-infected cell lines and mosquitoes. RT-qPCR based analysis of (A) AeVago1 and (B) AeVago2 transcripts in uninfected (Aag2) and wMelPop-infected (Pop) Ae. aegypti Aag2 cells, showing that AeVago1 is significantly upregulated while AeVago2 is significantly downregulated during Wolbachia infection. C) By RT-qPCR quantification of the AeVago1 gene in wMelPop-infected (Pop) and uninfected (Tet), 4- and 12-day-old female Ae. aegypti mosquitoes we found that AeVago1 was upregulated only in the young mosquitoes i.e. in 4-day-olds but not in the older ones i.e. 12-day-old Wolbachia-infected mosquitoes. RPS17 was used to normalize the qPCR data. Error bars represent standard error of mean (SEM) from three biological replicates with two technical replicates (*, p<0.05; ***, p<0.001; A, unpaired t-test; B, Wilcoxon test; C, One-way ANOVA).

**3.4.3. AeVago1 is not induced after DENV infection**

Vago is an antiviral gene that has been demonstrated to inhibit WNV and DCV in Cu. quinquefasciatus and D. melanogaster, respectively (Deddouche et al., 2008; Paradkar et al., 2012). To determine the effect of DENV infection on the relative abundance of AeVago1 in Ae. aegypti, the transcript levels were measured in DENV-infected Ae. aegypti mosquitoes at
three different time points 2, 6 and 12 days post-infection. Results showed that there was no induction of \textit{AeVago1} during DENV challenge (Figure 2A). To test our experimental procedure, we investigated the levels of \textit{D. melanogaster Vago} in the case of both DENV and DCV infection (used as positive control) in S2 cells. Our results validated the previous findings that \textit{Vago} is induced upon DCV infection (Figure 2B) (Deddouche et al., 2008), but not in DENV infection (Figure 2C). Altogether these results confirmed that there is no significant change in the levels of \textit{Vago} upon DENV infection.

To further look into the lack of induction of \textit{AeVago1} by DENV, we examined the expression pattern of the known activators of \textit{Vago} reported in the earlier studies, that are \textit{Dicer2} (Deddouche et al., 2008), \textit{Rel2} and \textit{TRAF} (Paradkar et al., 2014). We found that there was no significant modulation of \textit{Dicer2} and \textit{Rel2} across all infection time points (Figure 3A and B), however, we observed a significant upregulation of \textit{TRAF} in DENV-infected mosquitoes during early infection that is 2 days post-infection (Figure 3C). Although there was a significant upregulation of \textit{TRAF} at 2 days post-infection, we earlier observed no significant induction of \textit{AeVago1} at all stages of DENV infection (Figure 2A), which suggests that TRAF might not be directly involved in the activation of \textit{AeVago1}.

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption{Effect of DENV infection on \textit{AeVago1} expression in \textit{Ae. aegypti} mosquitoes and \textit{D. melanogaster} S2 cell line. A) \textit{AeVago1} expression in DENV challenged mosquitoes at 2, 6 and 12 days post-infection as compared to uninfected controls was analysed through RT-qPCR to find that there was no significant induction of \textit{AeVago1} transcription during DENV infection. B) Relative expression of \textit{Vago} in S2 cells infected with DCV, and (C) DENV. \textit{RPS17} was used to normalize qPCR data for \textit{Ae. aegypti}, while \textit{Rpl32} was used for qPCR data normalization of \textit{D. melanogaster}. Each error bar represents SEM of the mean of three
\end{figure}
biological replicates with two technical replicates (****, p<0.0001; A, Two-way ANOVA; B and C, unpaired t-test).

Figure 3. Effect of DENV infection on AeVago1 activators (Dicer2, Rel2 and TRAF) in DENV-infected mosquitoes. Relative expression of (A) Dicer2, (B) Rel2 and (C) TRAF in DENV-infected mosquitoes as compared to controls at 2, 6 and 12 days post-infection. RPS17 was used to normalize qPCR data. Error bars represent SEM of mean of three biological replicates with two technical replicates (**, p<0.01; Two-way ANOVA).

3.4.4. Wolbachia-mediated activation of AeVago1 limits DENV replication

To further investigate whether AeVago1 is antiviral and consequently overexpression of AeVago1 in Wolbachia-infected mosquitoes could contribute in limiting DENV replication, we knocked down AeVago1 in Pop cells by using dsRNA against the gene, followed by DENV2 (NGC) infection at 1 multiplicity of infection (MOI). Knock down efficiency was confirmed by RT-qPCR, which showed ~86% knock down efficiency in the cells transfected with dsRNA specific to AeVago1 as compared to the controls (Figure 4A). The effect on DENV replication was quantified using DENV-specific primers in RT-qPCR. We found a significant increase in the genomic RNA levels of DENV in the cells which were depleted of AeVago1 (Figure 4B). In order to confirm that dsAeVago1 treatment of Pop cells has no effect on Wolbachia density itself that could influence antiviral effects of Wolbachia, the relative Wolbachia density was determined in dsAeVago1 silenced cells as compared to controls. By employing DNA based qPCR, we found there was no significant effect on Wolbachia density in AeVago1 depleted cells (Figure 4C). The results suggest that AeVago1 is an antiviral gene and its upregulation in the presence of Wolbachia may contribute in limiting DENV replication in Ae. aegypti.
Figure 4. Silencing of *AeVago1* affects DENV replication in *Wolbachia*-infected *Ae. aegypti* cells. A) Confirmation of *AeVago1* knockdown in Pop cells through RT-qPCR. Pop cells were transfected with mock, dsGFP or ds*AeVago1* as described in the methods followed by DENV infection for 3 days. B) RT-qPCR based relative quantification of the genomic RNA levels of DENV in Pop cells infected at 1 MOI of DENV and transfected either with no dsRNA, or with dsRNA against *GFP* (control) or *AeVago1*. *RPS17* was used to normalize qPCR data. Each error bar represents SEM of the mean of three biological replicates with two technical replicates (*, p<0.05; ***, p<0.001; One-way ANOVA). C) Relative abundance of *Wolbachia* in *AeVago1* silenced Pop cells as compared to *GFP* and mock transfected controls using *wsp*-specific primers normalized with the *RPS17* gene. Each error bar represents SEM of the mean of three biological replicates with two technical replicates (ns show not significant; One-way ANOVA).

3.5. Discussion

Recent increases in dengue outbreaks in geographically unreported locations throughout the world has led to more attention towards the severity of the disease, the virus responsible for it, and its vector control (Organization, 2009). Recent investigations have led to the identification of a novel biological control agent *Wolbachia*, which can successfully inhibit the replication of several RNA viruses including DENV in the mosquito vector (Moreira et al., 2009). However, the exact mechanism that leads to this antiviral effect remains largely unknown. In this study, we have found that the *AeVago1* gene is upregulated during *Wolbachia* infection, and it may contribute in limiting DENV replication in *Ae. aegypti* mosquitoes.

About 40-60% of insect species are naturally infected with different strains of *Wolbachia* (Hilgenboecker et al., 2008). *Wolbachia* usually has a symbiotic relationship with its host and it manipulates host reproduction to successfully establish a permanent association, with certain
fitness costs to the host (Werren et al., 2008). So far, no natural association between any *Wolbachia* strains with *Ae. aegypti* mosquitoes has been found. In order to use *Wolbachia* to overcome arboviruses, especially DENV, *wMel* and *wMelPop* strains were isolated from *D. melanogaster* and transinfected them into *Ae. aegypti*, finding significant reduction in DENV replication (Walker et al., 2011; McMeniman et al., 2009). Further studies have found that these strains can successfully inhibit other important RNA viruses such as WNV, Chikungunya virus and ZIKV (Dutra et al., 2016; Hussain et al., 2013; van den Hurk et al., 2012). However, still little is known about the mechanism(s) that *Wolbachia* use to overcome DENV replication.

Few studies that have tried to find possible mechanism(s) for the virus blocking have demonstrated differential expression of miRNAs (Hussain et al., 2011) and immune genes (Rances et al., 2012), and involvement of ROS (Pan et al., 2012) in *Ae. aegypti*, which might contribute towards limiting DENV replication.

Vago is an insect-specific antiviral protein (Dedouche et al., 2008; Paradkar et al., 2012), having a distinct single von Willebrand factor type C (SVWC) domain that is usually comprised of 8-10 cysteines residues (Chen et al., 2011). Vago has been found to suppress Drosophila C virus (DCV) in the fat body of *D. melanogaster* (Dedouche et al., 2008). In *Cx. quinquefasciatus*, it has been demonstrated that CuVago is a secretory protein, which performs a similar function as the mammalian interferon to block WNV replication by activating the JAK/STAT pathway (Paradkar et al., 2012). However, the role of Vago protein is not well studied in *Ae. aegypti* mosquitoes. In this study, we looked into the potential homologs of Vago in *Ae. aegypti*. Our analysis identified two potential Vago protein homologs in *Ae. aegypti*, named AeVago1 and AeVago2 with a distinct SVWC domain along with a secretory signal. We further looked into the regulation of AeVago genes in the case of *Wolbachia* infection. Our results revealed a significant upregulation of the *AeVago1* gene in *Wolbachia*-infected Aag2 cells, while *AeVago2* was significantly downregulated in the presence of *Wolbachia*. In a previous study in *Cx. quinquefasciatus*, involvement of the immune genes *Rel2* and *TRAF* in the activation of CuVago was shown (Paradkar et al., 2014). Examining the published transcriptome data from *Ae. aegypti* mosquitoes infected with *wMelPop-CLA* revealed that both genes are induced in the mosquitoes by 3.44 and 2.84 folds, respectively (Rances et al., 2012). This suggests that *AeVago1* is most likely induced through the activation of the immune system by *Wolbachia wMelPop*. Interestingly, *AeVago1* was found to be only induced in *Wolbachia*-infected young mosquitoes (4-day-old), however at the older age (12-day-old), there was no significant change in the expression of *AeVago1* gene. One possible explanation for this difference in the expression pattern could be that *wMelPop-CLA*
strain of *Wolbachia*, which is a virulent strain, might cause tissue damages as the mosquitoes age affecting the expression of *AeVago1* (Turley et al., 2009). It is known that wMelPop proliferates very rapidly as the host ages (Amuzu and McGraw, 2016; McGraw et al., 2002; McMeniman et al., 2008).

To examine whether *Wolbachia*-mediated upregulation of *Vago* is consistent in the natural host of *Wolbachia*, we looked into the expression pattern of the gene in the females of *D. melanogaster* flies that were infected with wMelPop as compared with uninfected ones. We found that there was no change in the expression of the *Vago* gene in the case of *Wolbachia* infection in *D. melanogaster* flies. The possible reason behind this different *Vago* regulation in different hosts could be that *Wolbachia* is a natural host of *D. melanogaster* with a long evolutionary association, while *Ae. aegypti* is an unnatural transinfected host that has just recently been exposed to *Wolbachia*. For example, it was shown that wMel and wMelPop strains do not induce immune genes in *D. melanogaster* (Bourtzis et al., 2000), whereas they do (wMel to a lesser extent) in transinfected *Ae. aegypti* mosquitoes (Rances et al., 2012). To further strengthen this assumption, we examined the levels of the *Vago* gene in *Ae. albopictus* cells infected with the natural strain of *Wolbachia wAlbB*, and found that there was no change in the expression of *Vago* in *Wolbachia*-infected cells. However, further in-depth investigations are needed to follow up the effect of other transinfected strains of *Wolbachia* on *AeVago1* in *Ae. aegypti* mosquitoes.

In *Cu. quinquefasciatus*, *Vago* is highly induced during WNV infection (Paradkar et al., 2012). In the same study, the investigators also showed that *Vago* was induced in the case of DENV2 infection of *Ae. albopictus* RML12 cells. In the absence of evidence for *Vago* modulation in *Ae. aegypti* mosquitoes when challenged with DENV, we investigated the levels of *AeVago1* expression in *Ae. aegypti* mosquitoes infected with DENV2 and found that despite the upward trend there was no significant upregulation of *AeVago1* in DENV-infected mosquitoes. Results were cross-validated in *D. melanogaster*, which has been used in several studies to find out the host factors involved in DENV infection (Hackett et al., 2015; Sessions et al., 2009), indicating that there was no significant change in the levels of *Vago* in the case of DENV challenge. However, a significant increase was found in the case of S2 cells challenged with DCV, which is consistent with the previous report showing *Vago* is induced upon DCV infection (Deddouche et al., 2008).

Several studies have looked into the possible mechanism(s) involved in *Vago* activation and found three possible mechanisms, involving Dicer2 (Deddouche et al., 2008), Rel2 and TRAF
Since we did not find activation of Vago in DENV2 infection, we looked at the potential activators of Vago in the case of DENV2 infected mosquitoes at different time points. Our results demonstrated that there was no change in the levels of Dicer2, Rel2 and TRAF, except a significant upregulation in the levels of TRAF during early infection. Overall, the results suggest that there was no significant effect of DENV on AeVago1 and the expression of its three inducer genes in Ae. aegypti mosquitoes.

To investigate the role of AeVago1 in relation to Wolbachia-mediated suppression of DENV replication, we knocked down the AeVago1 gene in Wolbachia-infected cells and examined its effect on DENV replication. Results showed a significant but modest increase in the genomic RNA levels of DENV in AeVago1 depleted Pop cells, but no significant effect on Wolbachia density. However, it will be interesting to examine AeVago1 effect on DENV replication in vivo.

In summary, we have found that, consistent with the literature, AeVago1 is antiviral against DENV, and the virus may inhibit AeVago1 induction to avoid the host antiviral response. Interestingly, in Ae. aegypti, in the presence of Wolbachia, AeVago1 is specifically upregulated in young female mosquitoes, which may contribute towards the mechanism(s) to overcome DENV replication at early stages of infection. However, no significant effect of Wolbachia infection on AeVago1 in older mosquitoes suggests that AeVago1 may play a minor part in Wolbachia-mediated antiviral response in Ae. aegypti. Furthermore, given this interaction was not observed in other Wolbachia-host interactions with an older established association, further studies are required to find the fundamental mechanism(s) that Wolbachia employ to overcome the replication of RNA viruses.

3.6. Acknowledgements

We are thankful to Dr Karyn Johnson and Verna Hearne from UQ for providing D. melanogaster wMelPop-infected and tetracycline cured flies along with DCV inoculum. We also thank Dr Sonja Hall-Mendelin from Queensland Health for providing DENV-injected mosquitoes and their non-infected matches. This project was supported by a National Health Medical Research Council grant (APP1062983) to S Asgari and a UQ International scholarship to S Asad.

3.7. References


3.8. Supplementary information

Upregulation of *Aedes aegypti* Vago1 by *Wolbachia* and its effect on dengue virus replication

Sultan Asad and Sassan Asgari

**Figure S1.** Identification of SVWC domain and secretory signal in AeVago1 protein. A) Conserved domain database (CDD) analysis of AeVago1 (XP_001658930.1) protein sequence detected a SVWC domain (36-103 amino acids, shown in red bar) which is a signature domain of Vago proteins. B) SignalP4.1 analysis was carried out on the amino acid sequence of the AeVago1 (XP_001658930.1) protein. The output figure showed a sharp peak (red and blue lines) at amino acid 20 predicting a distinct secretory signal between 19th and 20th amino acids.
Figure S2. Identification of SVWC domain and protein secretion signal in AeVago2. A) CDD server based analysis of AeVago2 (XP_001658928.1) amino acid sequence confirming the presence of the Vago signature SVWC domain between amino acid 35th-103rd. B) Detection of the secretory signal in AeVago2 was predicted between 18th and 19th amino acid (spike at 19th amino acid in the graph, red and blue lines) by analysing the amino acid sequence of AeVago2 using the SignalP4.1 server.
Figure S3. Effect of *Wolbachia* infection on Vago transcript levels in female *D. melanogaster* flies and *Ae. albopictus* cell lines. Relative expression of Vago in (A) female *D. melanogaster* infected with wMelPop and tetracycline cured line (Tet), and (B) in *Wolbachia*-infected (Aa23) and uninfected (Aa23-Tet) *Ae. albopictus* cells. *RPS17* was used to normalize qPCR data in the case of *Ae. albopictus* gene, while *Rpl32* was used for qPCR data normalization of the *D. melanogaster* Vago gene. Error bars represent SEM from three biological replicates with two technical replicates (A: $p$ value = 0.9; B: $p$ value = 0.11; unpaired *t*-test).

A  

**D. melanogaster**

B  

**Aa23**
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence (5’-3’)</th>
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<tbody>
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<td>AeVago1-qF</td>
<td>GCATTTGCCGGTCAGAGC</td>
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Chapter 4: Suppression of the pelo protein by *Wolbachia* and its effect on dengue virus in *Aedes aegypti*

(Presented as manuscript, under review)

Suppression of the pelo protein by *Wolbachia* and its effect on dengue virus in *Aedes aegypti*

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**Running title:** Pelo role in dengue virus and *Wolbachia* infection

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4.1. Abstract

The endosymbiont Wolbachia is known to block replication of several important arboviruses, including dengue virus (DENV), in the mosquito vector Aedes aegypti. So far, the exact mechanism of this viral inhibition is not fully understood. A recent study in Drosophila melanogaster has demonstrated an interaction between the pelo gene and Drosophila C virus. In this study, we explored the possible involvement of the pelo protein, that is involved in protein translation, in Wolbachia-mediated antiviral response and mosquito-DENV interaction. We found that pelo is upregulated during DENV replication and its silencing leads to reduced DENV virion production suggesting that it facilities DENV replication. However, in the presence of Wolbachia, specifically in female mosquitoes, the pelo protein is downregulated and its subcellular localization is altered, which could contribute to reduction in DENV replication in Ae. aegypti. In addition, we show that the microRNA aae-miR-2940-5p, which is highly upregulated in Wolbachia-infected mosquitoes, might mediate regulation of pelo. Our data reveals identification of pelo as a host factor that is positively involved in DENV replication, and its suppression in the presence of Wolbachia may contribute to virus blocking exhibited by the endosymbiont.

Keywords: pelo; Wolbachia; Aedes aegypti; dengue virus; microRNA
4.2. Introduction

Dengue virus (DENV) is one of the major medically important arboviruses (Bhatt et al., 2013; Gubler, 2006). It belongs to the *Flaviviridae* family that comprises lipid-enveloped, positive-sense single stranded RNA viruses (Clyde et al., 2006). DENV is classified into four antigenically distinct but closely related serotypes, represented as DENV-1 to DENV-4. The bite of an infected female of either *Aedes aegypti* or *Aedes albopictus* is the most common mode of DENV transmission to humans (Diosa-Toro et al., 2013). Infected humans may suffer from dengue fever (DF), dengue shock symptom (DSS) or dengue hemorrhagic fever (DHF), leading to fatality (Gubler, 1998; Guzman et al., 2013). Currently, there is no specific therapy or approved vaccine available and the treatments available are palliative in nature. Despite substantial efforts to control DENV through vector control, it is still geographically expanding and alternate vector control strategies and therapeutic options are urgently needed (Tatem et al., 2006). One such strategy involves the use of a bacterial endosymbiont *Wolbachia* in transinfected *Aedes* spp. mosquitoes which limits DENV replication (Iturbe-Ormaetxe et al., 2011; Lu et al., 2012).

*Wolbachia* is an endosymbiotic, vertically transmitted bacterium that infects more than 40% of insect species in addition to other terrestrial arthropods (Zug and Hammerstein, 2012). However, it is not a natural symbiont of *Aedes aegypti*, which is the primary vector of DENV transmission. Recently, several different types of *Wolbachia* strains have been successfully introduced into *Ae. aegypti* mosquitoes, among which *Wolbachia wMel*-Pop-CLA (Pop) and *wMel* strains are the most promising ones (Moreira et al., 2009; Walker et al., 2011). Both strains produce cytoplasmic incompatibility (CI) thus facilitate replacement of the wild populations through CI (McMeniman et al., 2009; Min and Benzer, 1997). Furthermore, introduction of *Wolbachia* has induced viral resistance in *Ae. aegypti* mosquitoes to a variety of arboviruses including dengue, Zika, West Nile and chikungunya viruses (Dutra et al., 2016; Hussain et al., 2013; Moreira et al., 2009; van den Hurk et al., 2012). However, the exact mechanism which causes this antiviral effect is not fully understood. Despite a small number of studies that have elucidated the potential role of microRNAs (miRNAs) (Hussain et al., 2011; Zhang et al., 2013), reactive oxygen species (ROS) (Pan et al., 2012; Wong et al., 2015), and competition for resources (Caragata et al., 2013) in *Wolbachia*-mediated antiviral response, the fundamental molecular mechanism(s) underlying virus blocking are yet to be explored. One of the possible strategies to discover molecular interactions between DENV-*Wolbachia*-*Ae. aegypti* is to find host factors that facilitate replication of arboviruses and
examine their relative abundance in the presence of *Wolbachia* infection. A recent study in *Drosophila melanogaster* has identified pelo as an important host factor required for efficient replication of Drosophila C virus (DCV) providing DCV genome greater access to ribosomes for high level synthesis of viral structural proteins (Wu et al., 2014).

Pelo is an evolutionary conserved protein which plays an important role in the regulation of *D. melanogaster* germ cell meiosis. In the pelo mutant males of *D. melanogaster*, cell cycle in spermatogenesis is arrested at the late prophase stage (Lin et al., 1996; Shamsadin et al., 2000). In contrast, in mutant female flies, mitotic division during oogenesis is affected along with impaired development of the eyes (Eberhart and Wasserman, 1995). Functional studies performed for pelo’s homolog, Dom34, in yeast mutants led to the accumulation of free ribosomes and decrease in the number of polysomes suggesting its involvement in translational regulation (Davis and Engebrecht, 1998).

In this study, we aimed to characterize *Ae. aegypti*’s pelo protein in the case of *Wolbachia* and DENV infection. We found that the pelo protein facilitates DENV replication and *Wolbachia* suppresses the protein which may contribute to the inhibition of DENV replication in *Wolbachia*-infected cells. In addition, we explored the subcellular localization of the pelo protein in response to *Wolbachia* and DENV infection. Furthermore, we demonstrate an indirect involvement of aae-miR-2940-5p in regulating pelo in response to *Wolbachia* infection.

4.3. Materials and Methods

4.3.1. Mosquitoes, flies and cell lines

All mosquitoes used in this study had been previously generated by McMeniman et al. (2009) by infecting *Ae. aegypti* mosquitoes with wMelPop-CLA strain of *Wolbachia* (Pop), and uninfected mosquitoes were obtained through tetracycline (Tet) treatment to ensure the complete removal of *Wolbachia* infection (McMeniman et al., 2009). To ensure the availability of functional gut microflora the Tet mosquitoes were allowed to feed for two generations on water used to raise natural mosquito’s larvae (McMeniman et al., 2009). *Ae. aegypti* Aag2 and Pop cell lines previously described by Frentiu et al. (2010) were maintained in 1:1 Mitsuhashi-Maramorosch and Schneider’s insect medium (Invitrogen) supplemented with 10% FBS (Bovogen Biologicals) (Frentiu et al., 2010). For microRNA direct interaction analysis, *Spodoptera frugiperda* Sf9 cells were used which were maintained in Sf900II medium (Life Technologies). All the above-mentioned cell lines were kept at 28°C and passaged after every 3-4 days.
4.3.2. RT-PCR and qPCR analyses

Total RNA was extracted from either cell lines, mosquitoes or flies using Qiazol (Qiagen) and then treated with Turbo DNase (Ambion) according to the manufacturers’ guidelines. 1000 ng of total RNA was then used to make the 1\textsuperscript{st} strand cDNA using Superscript III (Invitrogen) with the help of either oligo(dT) primer for cellular transcripts or DENV-qR primer in order to amplify the DENV genomic RNA. The full-length \textit{pelo} gene was amplified by Taq DNA polymerase (New England Biolabs) using 1\,\mu l of cDNA in 25\,\mu l reaction together with \textit{pelo}-specific primers (all primer sequences are listed in Table S1).

For qPCR, cDNA produced as above was diluted in 1:5 ratio with water. 2\,\mu l of the diluted cDNA was used for downstream qPCR reaction. Both forward and reverse gene-specific primers were used to amplify the target genes, using QuantiFast SYBR Green (Qiagen) in a Rotorgene qPCR machine (Qiagen). For mosquito samples, ribosomal protein S17 (\textit{RPS17}; AY927787.2) transcripts were used for normalization of RNA templates, while ribosomal protein L32 (\textit{RPL32}; NM_079843) was used for normalization in the case of \textit{D. melanogaster}. Each qPCR reaction was performed in duplicates with at least three biological replicates with the exception for the 12-day tissue-specific qPCRs in which we used two biological replicates. All qPCR data were normalized with Qiagen analysis templates and were further analysed by Prism 7.0. The qPCR cycling profile included 95°C for 5 min for the initial denaturation and hot start Taq activation, and 40 cycles of 95°C for 10 sec, 60°C for 15 sec and 72°C for 20 sec. Melt curve analysis was carried out on default settings of the Rotorgene machine to check the specificity of the qPCR products. Shapiro-Wilk normality test was performed first to check the normal distribution of data. Unpaired t-test was used to identify statistical significance between two individual groups while One-way ANOVA with Tukey’s post hoc test was performed to find statistical significance between more than two groups of data.

4.3.3. Nuclear and cytoplasmic fractionation

Nuclear and cytoplasmic fractions of Aag2 and Pop cells were separated using the PARIS kit (Ambion) according to manufacturer’s instructions. Briefly, cells were washed with 1xPBS, centrifuged at 1500 rpm at 4°C, followed by resuspension of the cell pellet in 300\,\mu l ice-cold cell fractional buffer, incubated at 4°C for 10 min, centrifuged at 2000 rpm at 4°C for 5 min. The supernatant was collected as the cytoplasmic fraction, and the pellet was washed five times with 200\,\mu l ice-cold cell fractionation buffer to ensure removal of traces of the cytoplasmic faction and considered as the nuclear fraction. The pellet containing the nuclear
fraction was resuspended in 300μl of cell disruption buffer. 300μl of 4xSDS-PAGE buffer was added to both nuclear and cytoplasmic fractions.

4.3.4. Western blot analysis

RIPA buffer (Sigma) was used to get total cell lysates from cells and mosquitoes. Briefly, samples were homogenised with the help of 2mm glass beads using a TissueLyser II (Qiagen) at 30,000 frequency for 90 sec. The lysate was then centrifuged at 4°C at full speed for 5 min and supernatant was collected for protein analysis. Protein concentrations were determined by measuring absorption at 280 nm.

Protein samples were run on 10% polyacrylamide gels, transferred to nitrocellulose membrane and then blocked with 5% skimmed milk. After 3 times washing with 1xTBST, membrane was incubated with either anti-rabbit pelo (Wu et al., 2014), anti-GAPDH (Sigma), anti-HSP70 (Sigma) or anti-DENV envelope protein antibody (Abmart) overnight at 4°C on shaker. For secondary antibody incubation, the blot was washed three times with 1xTBST and then incubated with secondary antibodies either conjugated with alkaline phosphatase (Sigma) or infrared detection system (IRDye®800CW goat anti-human LICOR). After 1 h of incubation, the blot was again washed three times with 1xTBST and then it was further incubated with BCIP/NBT ready to use solution (Thermoscientific) or scanned with Odyssey imager LI-COR IR detection system.

4.3.5. Oversupply and inhibition of aae-miR-2940-5p

Aag2 cells were plated in a 12-well plate and transfected with 100 μM of artificially synthesized mimic or inhibitor of either aae-miR-2940-5p, aae-miR-2940-3p or non-specific control mimic/inhibitor (GenePharma) (sequences in Table S1). Cellfectin was used as the transfection reagent (Invitrogen). Cells were collected 72 h post-transfection for downstream analyses.

4.3.6. RNAi-mediated gene silencing

In order to knockdown the pelo gene for functional analyses in DENV life cycle, primers were designed to amplify a 552 bp product from the pelo gene with the addition of the T7 promoter sequences at both ends. MEGAscript T7 Transcription kit (Ambion) was then used according to the manufacturer’s instructions in order to synthesize dsRNA targeting the pelo transcripts. A similar approach was followed to synthesize dsRNA against Ae. aegypti Argonaut 1 (Ago1;
XP_001662554), Ago2 (FJ979880.1), and Green fluorescent protein (GFP; X83960). For knockdown experiments, cells were double transfected with 5 and 2μg of dsRNA, respectively, per well against the target gene. dsGFP RNA was used as a non-specific control.

4.3.7. Virus infection and plaque assay

For virus inoculation experiments, Ae. aegypti Aa20 cells were seeded at the density of 3x10⁵ cells per well in 12-well plates. Cells were first double transfected with dsRNA against the target gene and after 6 h they were infected with DENV-2 (New Guinea strain) at the multiplicity of infection (MOI) indicated in the text. Media were collected 72 h post-infection for plaque assay.

To perform plaque assay, Vero cells were seeded in a 96-well plate and were allowed to form monolayers. Virus containing media from the experiments were serially diluted into 10⁰, 10¹, 10², 10³ dilutions and added to Vero cells in duplicates. Cells were incubated with virus at room temperature with continuous shaking for 1 h and then incubated at 37°C for one additional hour. After 2 h of incubation, media were aspirated and an overlay was added to the cells which comprised of 1.5% carboxymethyl cellulose (CMC) and 2.5% FBS in OptiMEM medium (Sigma). Cells were then incubated for 72 h at 37°C and 5% CO₂ and fixed with 80% ice-cold acetone in 1XPBS for 20 min at -20°C. Plates were then air dried overnight and then blocked with 5% skimmed milk in 1xPBST at 37°C for 30 min. Cells were then incubated with the primary antibody against DENV-2-Envelope (human) in 1:1000 dilution in 0.1% skimmed milk in 1xPBST for 2 h at 37°C as described previously (Watterson et al., 2016). After that, plates were washed three times with 1xPBST and then incubated with the secondary antibody (IRDye®800CW goat anti-human LICOR) for 1 h at 37°C. Plates were washed and dried as above and were dried and scanned on the Odyssey imager (LI-COR Biosciences) at 41μM resolution. Plaques were counted and viral titre was calculated accordingly. Plaque numbers obtained for compound titration were performed in triplicates.

4.4. Results

4.4.1. Pelo is ubiquitously expressed in mosquito tissues

Pelo has been demonstrated to be ubiquitously expressed in human and D. melanogaster tissues (Chintapalli et al., 2010; Shamsadin et al., 2000). In order to investigate the tissue-specific expression of Ae. aegypti pelo, we dissected 3-day-old Ae. aegypti female mosquitoes into different tissues, including salivary glands, midgut, muscles, ovaries, and fat
body. The *pelo* transcript sequence (XM_001658653.1) was retrieved from NCBI in order to design primers. RT-qPCR results demonstrated that *pelo* is also ubiquitously expressed in all the *Ae. aegypti* tissues tested, with the highest expression in the salivary glands and the lowest in the fat body (Figure 1).

![Relative expression of Pelo](image_url)

**Fig. 1: Tissue-specific expression of pelo in Ae. aegypti mosquitoes.** RT-qPCR analysis of *pelo* transcript levels in the salivary gland, midgut, muscles, ovaries and fat body of 3-day-old female mosquitoes (five mosquitoes per biological replicate) showed *pelo* expression in all the tested tissues. Error bars represent standard deviation (SD) of the mean in three biological replicates with two technical replicates.

### 4.4.2. Wolbachia suppresses pelo in Ae. aegypti

In order to find the effect of *Wolbachia* on *pelo* gene expression, RT-qPCR was carried out using *pelo* gene-specific primers to examine its expression in tetracycline (Tet) treated and *Wolbachia* wMelPop-CLA infected (Pop) female mosquitoes at two different time points that were 7-days and 12-days post-emergence. Results showed that the *pelo* gene is significantly downregulated in Pop mosquitoes at both time points (Figure 2A). This result was also cross-validated in Aag2 and Aag2 cells infected with wMelPop-CLA (Figure 2B). To further investigate in which tissue(s) *Wolbachia* affect *pelo*, relative expression levels of the gene were assessed in different tissues of both uninfected and infected female mosquitoes at 12-days after emergence by dissecting midgut, muscles, ovaries and fat body from a total of 20
mosquitoes divided into two biological replicates. We found decreases in *pelo* transcript levels in almost all the tissues of Pop mosquitoes tested including midgut, muscles, ovaries and fat body (Figures 3A-D).

4.4.3. *Wolbachia* affects the subcellular localization of the pelo protein

Pelo has been reported to stay mainly in the cytoplasm in *D. melanogaster* (Xi et al., 2005). Although the pelo protein does have a conserved nuclear localization signal (PRKRRK), there is a lack of experimental evidence of its presence inside the nucleus (Shamsadin et al., 2000); therefore, we explored its subcellular localization in *Ae. aegypti* cells and whether *Wolbachia* affects the localization of pelo. Nuclear and cytoplasmic fractions of both Aag2 and Pop cells were separated as described in the methods section. Lysates were run on 5-15% Bis/Tris polyacrylamide gels, total proteins were transferred to nitrocellulose membranes and then probed with an anti-pelo antiserum along with other control antisera, including anti-histone H3 (to confirm the nuclear fraction), anti-GAPDH (to confirm cytoplasmic fraction), and anti-WSP
(to confirm *Wolbachia* infection). Here it is important to note that we detected the WSP protein in the nuclear fraction of Pop cells as well, although in smaller amount as compared to the cytoplasmic fraction, which is consistent with the earlier report demonstrating the presence of *Wolbachia* (wMelPop) in the nuclei of *D. melanogaster* cells with the help of electron microscopy (Min and Benzer, 1997). Nevertheless, the control antibodies show a very good fractionation of the two cellular compartments. Our results demonstrated that in Aag2 cells, pelo was only detectable in the cytoplasmic fraction, however, *Wolbachia* infection leads to the nuclear import of the protein thus affecting its subcellular localization as compared to uninfected Aag2 cells (Figure 3E). Further investigation is required to elucidate if the pelo protein plays any role inside the nucleus of *Wolbachia*-infected cells.
Fig 3: Tissue-specific modulation of pelo by Wolbachia. A-D) Relative expressions of the pelo transcripts in different tissues including midgut, muscles, ovaries and fat body of 12-day-old female mosquitoes in the absence (Tet) and presence of Wolbachia (Pop) showing consistent downregulation trend throughout the tested tissues in the presence of Wolbachia. Error bars show SD from two biological replicates each containing tissue samples of 10
mosquitoes with three technical replications. E) Western blot analysis to examine the effect of *Wolbachia* on subcellular localization of the pelo protein showing translocation of pelo into the nucleus during *Wolbachia* infection. Anti-histone H3 and anti-GAPDH antibodies were used to check the efficiency of nuclear and cytoplasmic fractionations, while anti-WSP antibody was used to confirm the presence of *Wolbachia*.

4.4.4. *Wolbachia*-mediated downregulation of pelo is highly specific to female *Ae. aegypti*

We checked the levels of *pelo* transcripts in both male and female 4-day-old Tet and Pop mosquitoes. RT-qPCR analysis showed that there was no significant change in the levels of *pelo* between Tet and Pop male mosquitoes (Figure 4A). Conversely, the level of *pelo* transcripts was significantly reduced in females in the presence of *Wolbachia* (Figure 4A). Although there is no evidence in *Ae. aegypti* mosquitoes to show differential host response to bacterial infections in the two sexes, a study carried out in the parasitoid *Asobara tabida* has highlighted differential immune response in males as compared to females challenged with *Wolbachia* strains *wAtab1, wAtab2,* and *wAtab3,* where males showed higher levels of immune gene expression upon *Wolbachia* infection as compared to females (Kremer et al., 2012). Therefore, our results also point out towards sex-specific response in *Ae. aegypti* mosquitoes during *Wolbachia* infection.

As *Wolbachia* is not a natural symbiont of *Ae. aegypti,* we were interested to test whether *Wolbachia* has the same suppressive effect on pelo in its natural host *D. melanogaster.* By measuring the relative levels of the *pelo* transcripts in Tet and *Wolbachia* wMelPop infected 4-day-old flies, we found that *Wolbachia*-mediated suppression of *pelo* is highly specific to *Ae. aegypti* females as there was no significant change in the levels of *pelo* in infected and uninfected male and female flies (Figure 4B). This pointed out to the fact that *Wolbachia*-mediated suppression of *pelo* could be due to the transinfection of *Wolbachia* strain wMelPop-CLA into a novel host that is *Ae. aegypti* in this case.
Fig. 4: Effect of *Wolbachia* infection on the *pelo* gene expression in male and female mosquitoes and flies, and cell line. A) RT-qPCR for the *pelo* transcripts levels in 4-day-old female and male mosquitoes, both uninfected (Tet) and infected with *Wolbachia* (Pop) (*, p<0.05; One-way ANOVA). B) Relative expression of the *pelo* gene in uninfected (Tet) and *Wolbachia*-infected (Pop) flies. Error bars represent SD from three biological replicates including five mosquitoes/flies per biological replicate (ns represents not-significant; One-way ANOVA).

4.4.5. *microRNA aae-miR-2940-5p* is involved in regulation of *pelo*

microRNAs (miRNA) are known to regulate different target genes. To find out whether the suppression of *pelo* in *Wolbachia*-infected mosquitoes is mediated by miRNA, we knocked down *Ago1* and *Ago2* genes in Pop cells, as both are important components of the RISC complex involved in miRNA functions (Okamura et al., 2004; Okamura et al., 2009). After confirming silencing of the Agos with dsRNA specific to the genes (Figure 5A and 5B), we employed RT-qPCR and found that after *Ago2* knockdown there was a significant increase in the expression of *pelo* (Figure 5C), which suggested that miRNAs might be involved in regulating *pelo* levels. Interestingly, we found that when *Ago1* was silenced, *Ago2* levels increased (Figure 5A), and conversely when *Ago2* was knocked down, *Ago1* transcript levels increased (Figure 5B). This is consistent with previous studies carried out in *D. melanogaster* that Ago proteins may compensate for each other (Yang et al., 2014).

As *Wolbachia*-mediated *pelo* suppression was found to be specific to *Ae. aegypti* mosquitoes,
in particular females, we aimed to determine if a mosquito-specific miRNA could be involved. Hussain et al (2011) provided evidence that in Wolbachia wMelPop-CLA infected female mosquitoes a mosquito-specific aae-miR-2940-5p was significantly up-regulated as compared to uninfected mosquitoes (Hussain et al., 2011). Although three targets of this miRNA have already been identified (Hussain et al., 2011; Zhang et al., 2014; Zhang et al., 2013), we tried to determine if it has any interaction with the pelo gene. RNAhybrid analysis showed potential aae-miR-2940-5p target sequences in the ORF of the pelo gene at positions 561-582 with significant seed region complementarity and high minimum free energy of -25.8 kcal/mol (Figure S1). In order for a miRNA to regulate a target mRNA, it is important that the mature miRNA and its target transcript co-localize in the same tissue. Aforementioned results showed that pelo is expressed in all the mosquito tissues that we examined and Wolbachia supresses its expression in all the tested tissues (Figure 3A-D). We were interested to find out the tissue-specific expression of aae-miR-2940-5p in mosquito tissues in the presence and absence of Wolbachia. Northern blot analysis of RNA isolated from various tissues of Pop and Tet Ae. aegypti mosquitoes confirmed induction of aae-miR-2940-5p in Pop mosquitoes, with substantial inductions in the midgut and fat body (Figure 6A). These inductions and their extents were in inverse correlation with reductions of the pelo transcript levels in the corresponding tissues examined (Figure 3A-D).

To verify the interaction between aae-miR2940-5p and pelo, Aag2 cells were transfected in triplicates with synthetic aae-miR2940-5p mimic, control mimic with random sequences. RT-qPCR results showed that there were significant reductions in the transcript levels of pelo in Aag2 cells transfected with aae-miR2940-5p mimic as compared to mock and control mimic transfected cells (Figure 6B). Furthermore, the effect of synthetic mimic and inhibitor of aae-miR-2940-5p on Pelo protein expression was analysed 72 h post-transfection. Western blot results confirmed downregulation of the pelo protein in the presence of aae-miR-2940-5p mimic while there was an increase in the level of the pelo protein in cells that were treated with aae-miR-2940-5p inhibitor (Figure 6C).

In order to validate the direct interaction between aae-miR-2940-5p and the predicted target sequences in pelo, we cloned the target sequences downstream of GFP in the pIZ/V5-His vector. The GFP-pelo target sequence clone was co-transfected with the control random sequence mimic and aae-miR-2940-5p mimic into Aag2 cells. RNA was collected after 72 h, and RT-qPCR was carried out to check the level of GFP transcripts. The results showed that there was no change in the levels of GFP in response to aae-miR-2940-5p mimic (Figure 6D),
which suggests that likely there is no direct interaction between *pelo* and aae-miR-2940-5p. This result is not surprising as most miRNAs are reported to target transcription factors or other proteins that in return fine tune the abundance of different transcripts (Gosline et al., 2016). To rule out the possibility of another target site for the miRNA in the *pelo* gene undetected by the prediction program, the full-length *pelo*, including its 3'UTR, was cloned into the pIZ/V5-His vector and co-transfected with aae-miR-2940-5p mimic in Sf9 cells. There was no downregulation of *pelo* in the presence of aae-miR-2940-5p mimic (data not shown), which confirmed our aforementioned observation.

**Fig. 5: miRNA-mediated regulation of *pelo*.** A-B) RT-qPCR showing dsRNA-mediated silencing of Ago1 and Ago2, respectively. C) Relative expression of the *pelo* transcripts in Ago1 and Ago2 silenced Aag2 cells by RT-qPCR showing increase in *pelo* expression at RNA level in Ago2 depleted cells. Each error bar shows SD from three biological replicates with two technical replicates (**, p<0.001; One-way ANOVA).
**Fig. 6: aae-miR-2940-5p dependent regulation of pelo.**

**A)** Northern blot analysis of RNA isolated from various tissues (OV, ovary; MG, midgut; FB, fat body; SG, salivary gland) of wMelPop (P) infected and tetracycline-treated (T) Ae. aegypti mosquitoes hybridized with a specific probe to aae-miR-2940-5p probe confirmed increase in the expression of aae-miR-2940-5p throughout the tested tissues in the case of Wolbachia infection. U6 is shown as control to confirm equal loading of RNA. **B)** RT-qPCR performed to confirm the effect of overexpression of aae-miR-2940-5p mimic on pelo transcript levels showing a significant decrease in pelo transcript levels in aae-miR-2940-5p mimic transfected cells. Error bars represent SD from three biological replicates with two technical replicates (**, p<0.001; ***, p<0.0001; One-way ANOVA). **C)** Western blot analysis to examine the effect of aae-miR-2940-5p mimic and inhibitor on pelo protein levels using an anti-pelo specific antibody (upper panel), while anti-beta-actin antibody was used as control to confirm equal loading of samples. **D)** RT-qPCR analysis of the effect of aae-miR-2940-5p mimic on the level of GFP transcripts in Sf9 cells transfected with plZ/GFP fused with the pelo target sequences along with the negative control (NC) or aae-miR-2940-5p mimic.

4.4.6. *Pelo* plays a role in DENV virion production

Recently, it has been reported that *pelo* is required for efficient replication of Drosophila C virus (DCV) (Wu et al., 2014). In order to examine if the pelo protein has a similar effect on
DENV replication, we infected *Ae. aegypti* Aa20 cells with DENV-2 (NGC strain) at 1.8 multiplicity of infection (MOI). Cells were collected at 1 and 5 days post-infection (dpi) from which RNA and proteins were extracted. RT-qPCR results showed no significant change in the *pelo* transcript levels during DENV infection at both 3 and 5 dpi (Figure 7A). However, western blot analysis of the cells using an antiserum against *D. melanogaster* pelo clearly showed an increase in the levels of the protein upon infection at both 3 and 5 dpi (Figure 7B). The same blot was re-probed with an anti-DENV-Envelope antiserum to confirm infection. This suggests that pelo could be regulated at the post-transcriptional level. To check the localization of the pelo protein during DENV infection, we fractionated mock and 5 dpi Aa20 cells (1 MOI) into nuclear and cytoplasmic fractions. Both nuclear and cytoplasmic lysates were run on 5-15% Bis/Tris polyacrylamide gels. After transfer to nitrocellulose membrane, the blot was probed with the pelo antiserum. Results showed that unlike *Wolbachia*, DENV infection has no effect on the subcellular localization of pelo, which remains in the cytoplasm in both uninfected and DENV-2 infected cells (Figure 7C).

The above results suggest that DENV might increase levels of the host pelo protein in order to facilitate its replication, similar to DCV in *D. melanogaster*. In order to further confirm this link, the *pelo* gene was knocked down in Aa20 cells using pelo specific dsRNAs (Figure 8A) and subsequently infected with DENV-2 at 1 MOI. We carried out DENV infection experiments in *Ae. aegypti* cell line Aa20, because Aag2 cell line is persistently infected with insect-specific flavivirus cell fusing agent virus (CFAV) (Stollar and Thomas, 1975), but not Aa20 cells. At 3 dpi, cells were harvested and subjected to RT-qPCR using DENV-2 specific primers to the *NS1* gene. Surprisingly, RT-qPCR results showed no change in the DENV-2 genomic RNA levels in pelo depleted Aa20 cells as compared to mock and dsGFP transfected cells (Figure 8B). However, plaque assay displayed a significant reduction in the virus titre in the medium collected from Aa20 cells treated with dspelo and infected with DENV-2 (Figure 8C). These results demonstrated that when pelo was knocked down the number of DENV infectious particles significantly declined, but there was no effect on the total genomic RNA of DENV, implicating that pelo might play an important role in the translation of the viral genomic RNA and/or assembly and release of DENV-2 virions.
Fig. 7: Expression pattern of pelo in DENV challenged mosquito cells. A) RT-qPCR analysis of *pelo* transcripts in the presence and absence of DENV challenge (1 MOI) in Aa20 cells at 3 and 5 dpi. Data was normalised using *RPS17* as the control gene. Error bars represent SD from three biological replicates with two technical replicates each (ns shows no significance; One-way ANOVA). B) Western blot analysis carried out using the anti-pelo specific antibody to check the pelo protein levels during DENV infection at 3 and 5 days post-infection (dpi), respectively, showing an increase in pelo protein expression in DENV infected cells at both 3 and 5 dpi. C) Western blot results showing no change in localization of the pelo protein at 5 dpi with DENV. Histone H3 detection only in nuclear fractions and GAPDH detection only in cytoplasmic fractions shows the success of subcellular fractionations, while DENV envelope protein only in the infected cells confirms DENV infection.
Fig. 8: Silencing of *pelo* affects DENV virion production. A) RT-qPCR based confirmation of knock down of *pelo* in Aa20 cells. B) RT-qPCR analysis to examine the genomic RNA level of DENV in Aa20 infected cells (1 MOI) transfected with either no dsRNA (Mock) or with dsRNA against *GFP* as control or with dsRNA against *pelo* to silence the gene. *RPS17* was used to normalize the qPCR data, showing no effect at the DENV genomic RNA level. C) Viral titre determination by plaque assay conducted on the media collected from cells treated as in B showed a significant reduction in DENV virion production in *pelo* depleted cells. Error bars represent SD from three biological replicates with two technical replicates (**, p<0.001; ***, p<0.0001; ns shows no significance; One-way ANOVA).

4.5. Discussion

Despite numerous efforts to unveil the mechanism by which *Wolbachia* manipulates its host environment to restrict virus replication, the exact mechanism(s) that govern this antiviral property are largely unknown. In this study, we have found that the *Wolbachia* supresses *pelo* which in turn may contribute towards restricting DENV virion production in *Ae. aegypti*.

*Wolbachia* is a facultative endosymbiont in many insect species and other invertebrates, however, the *wMelPop-CLA* strain, which most effectively suppresses DENV replication, has no natural association with the primary DENV vector *Ae. aegypti* and has been artificially transinfected into *Ae. aegypti* (McMeniman et al., 2009). Several reports have shown that *Wolbachia*-mediated activation of immune genes in the Imd and Toll pathways could be involved in host protection from various viruses in mosquitoes (Bian et al., 2010; Kambris et al., 2009; Moreira et al., 2009). Other reports, however, have shown that *Wolbachia* does not elicit the host immune response, in particular in hosts that are naturally infected with *Wolbachia* (Ferreira et al., 2014; Rances et al., 2013; Rances et al., 2012). A recent study investigating the role of *Wolbachia*-induced restriction of Semliki Forest virus within *D.*
melanogaster cells (JW18) suggested that interference occurs at a very early stage of infection and at the level of viral RNA translation or host RNA transcription (Rainey et al., 2016). However, in some Wolbachia-host-virus associations, viral replication is not inhibited but rather a tolerance to viral infection is conferred by Wolbachia (Osborne et al., 2009; Teixeira et al., 2008).

Recently, findings of Wu et al. (2014) and Lapidopt et al. (2015) have shed light on the role of the pelo protein as an important host factor for effective viral replication in the case of Drosophila C virus (DCV) and Tomato yellow leaf curl virus (TYLCV) (Lapidot et al., 2015; Wu et al., 2014). However, the potential role of pelo has not been characterized yet in the case of the medically important DENV and its controlling agent Wolbachia in the mosquito vector. RT-qPCR analyses revealed that the pelo gene is expressed ubiquitously throughout all the main tissues of Ae. aegypti with the highest expression in the salivary glands, which is consistent with the studies conducted on tissue localization of pelo in the case of human and D. melanogaster (Chintapalli et al., 2010; Shamsadin et al., 2000). Interestingly, we found that pelo is suppressed in the presence of Wolbachia in cell line, whole mosquitoes and all the main tissues such as the midgut, muscles, ovaries and fat body. However, this suppression is mainly female mosquito specific, which was not seen in the male mosquitoes or in either male or females of D. melanogaster infected with wMelPop. The difference in pelo regulation in Wolbachia-infected Ae. aegypti versus D. melanogaster could be due to natural infection in the fly versus transinfection of Wolbachia into the mosquito (Hughes and Rasgon, 2014).

Pelo is a highly conserved protein in mammals (Shamsadin et al., 2000), and using multiple sequence alignments for pelo sequences from different insect species we found that the protein is also highly conserved among insect species (Figure S2) with a distinct nuclear localization signal (PRKRK), and shows structural similarities with human pelo (Figure S3). However, it has been demonstrated that pelo mostly resides in the cytoplasm and there is a lack of evidence for the presence of pelo in the nucleus (Xi et al., 2005). This led us to investigate the localization of the pelo protein in mosquito cells and in particular in the instance of Wolbachia infection. Western blot results showed that the pelo protein is mainly found in the cytoplasm of mosquito cells, however, Wolbachia infection leads to a change in the subcellular localization of pelo by moving it into the nucleus. This change in the subcellular localization of pelo might help Wolbachia to make the protein less available in the cytoplasm, which is required for the translation of quickly synthesized viral proteins. This finding is in agreement with a previous study in which it was shown that pelo is required for the synthesis
of the capsid protein of DCV, which they described as a quickly synthesizing protein (Wu et al., 2014). Alternatively, the translocation of pelo into the nucleus may have an inhibitory effect on a host gene(s) that normally facilitates viral replication.

miRNAs are important regulators of different cellular processes including timing, cell differentiation, cell proliferation, cell death, metabolic control, transposon silencing and antiviral defence (Chang et al., 2008; Schickel et al., 2008; Wang et al., 2009). Recently, our group has shed light on the modulation of different cellular miRNAs in the case of infection with wMelPop strain of Wolbachia in female mosquitoes (Hussain et al., 2011). Along similar lines, in this study we have investigated whether pelo has an interaction with any miRNA by silencing both Argonaute 1 (Ago1) and Argonaute 2 (Ago2) genes, which are important components of the RISC complex and involved in miRNA function (Hock and Meister, 2008; Yang et al., 2014). Interestingly, we found that silencing of one Ago led to the upregulation of the other Ago. One possible reason might be class switching between the Agos (Yang et al., 2014). Increase in Pelo levels in Ago2 silenced cells suggested that pelo downregulation in Wolbachia-infected cells could possibly be mediated by miRNA. Hussain et al (2011) provided evidence that in Wolbachia wMelPop-CLA infected mosquitoes the mosquito-specific aae-miR-2940-5p was significantly upregulated as compared to uninfected mosquitoes (Hussain et al., 2011). While three targets of this miRNA have already been identified (Hussain et al., 2011; Zhang et al., 2014; Zhang et al., 2013), we explored whether it has any interaction with the pelo gene. Tissue-specific expression analysis showed inverse correlation of the pelo transcript levels with aae-miR-2940-5p abundance. The co-localization of both pelo transcripts and mature aae-miR-2940-5p highlights the possibility that in Wolbachia-infected cells aae-miR-2940-5p might be utilized to downregulate pelo transcripts. These results also further confirmed differential expression of this miRNA in Wolbachia-infected mosquitoes. Further investigation showed that pelo transcript levels were downregulated in the presence of the artificially synthesized mimic of aae-miR-2940-5p both at the transcript and the protein levels, while there was an increase in the pelo protein in the case of addition of an artificially synthesized inhibitor of aae-miR-2940-5p. However, target validation results using GFP as a reporter suggested that pelo may not be a direct target of aae-miR-2940. This could be due to regulation of a transcription factor(s) or other protein(s) by the miRNA that fine-tunes the abundance of the pelo transcripts (Gosline et al., 2016).

Viruses are well known to modulate transcripts of host cells for their own benefit. Recently, a research group examined D. melanogaster mutants that resist DCV replication through a
forward genetic screen and demonstrated a DCV-resistant mutant to be deficient of the pelo gene (Wu et al., 2014). Pelo protein has also been implicated in TYLCV resistance in tomato TY172 cultivar (Lapidot et al., 2015). However, its potential role in the case of DENV has not yet been explored. Our results suggest an increase in the levels of pelo in DENV-infected cells at the protein level without a change in its subcellular localization, which was mostly cytoplasmic. Furthermore, pelo knockdown studies revealed that it is vital for DENV virion production. The outcome of this study is in agreement with the previous findings that showed pelo is vital for the translation of the capsid protein of DCV thus positively affecting replication of DCV. A similar role of pelo was found in other viruses including Cricket Paralysis Virus, Double Drosophila X virus, and invertebrate iridescent virus 6 and TYLCV replication (Lapidot et al., 2015; Wu et al., 2014), suggesting that pelo might be an important host factor that is recruited by viruses to facilitate the translation of viral genome.

In summary, we have demonstrated that the pelo protein facilitates DENV replication, and in female Ae. aegypti mosquitoes, Wolbachia suppresses the pelo protein, which may consequently contribute to restriction of DENV replication. This effect could be due to relocalization of the pelo protein to the nucleus in Wolbachia-infected cells as compared to non-infected cells. In addition, regulation of pelo in Wolbachia-infected mosquito cells appears to be mediated by aae-miR-2940-5p. However, the regulation of pelo was found to be female mosquito specific and not observed in male mosquitoes or in D. melanogaster males or females. Therefore, while suppression of pelo in female mosquitoes may contribute to virus inhibition, this does not seem to be the universal mechanism of virus inhibition seen across different host-virus interactions.

4.6. Acknowledgements

We are thankful to Dr Jiahuai Han from the Xiamen University for the pelo antibody, Prof Paul Young from UQ for anti-DENV-2-Envelope protein antibody and plaque assay reagents, Dr Karyn Johnson and Verna Hearne from UQ for providing D. melanogaster wMelPop-infected and tetracycline cured flies, and Mr Syed Ali Naqi Raza Jaffary from Queensland University of Technology for his help in IR related western blotting. This project was funded by the Australian Research Council (DP150101782) and the National Health and Medical Research Council (APP1062983) to SASg, and a PhD scholarship to SAsa. All authors declare no conflict of interest.
4.7. References


### 4.8. Supplementary Material

**Table S1:** Primers used in this study.

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<th>Gene Name</th>
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<tr>
<td>Ae-pelo-qF</td>
<td>CAAGGCCTTCTACGGGCAAGA</td>
</tr>
<tr>
<td>Ae-pelo-qR</td>
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<tr>
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<td>Dm-pelo-qF</td>
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Table S2. Pelo protein sequences used for multiple sequence alignment.

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<td>1,946</td>
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<td><em>Drosophila melanogaster</em></td>
<td>P48612</td>
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<td><em>Culex quinquefasciatus</em></td>
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Fig. S1: RNA hybrid analysis of sequence complementarity between aae-miR-2940-5p and the *pelo* transcript (XM_001658653.1) showing very strong binding pattern and very low minimum free energy (mfe = -25.8 kcal/mol)
Fig. S2: Pelo is highly conserved among insects: CLC workbench was used to perform multiple sequence alignment to schematically represent the level of conservation between different species shown in Table S2. The nuclear localization signal is shown by putting a box around it.
Aedes aegypti pelo is structurally similar to the human pelo: A) I-Tasser predicted model of Ae. aegypti pelo protein; B) Ramachandran plot showing more than 90% of residues fall in the allowed region thus confirming the strength of the model. C) Experimentally verified C-terminus model of human pelo. D) Superimposition of Ae. aegypti predicted pelo protein model and human pelo shows that they are highly similar in structure.
Chapter 5: General Discussion and Conclusion
5.1. General Discussion

Exploiting *Wolbachia*'s antiviral potential to limit the transmission of DENV by targeting its primary host *Ae. aegypti* has shown tremendous promise (Dutra et al., 2016; Lambrechts et al., 2015). This antiviral effect is not just limited to DENV; it also decapitates the ability of *Ae. aegypti* to transmit other medically important arboviruses including ZIKV, WNV and Chikungunya virus (Dutra et al., 2016; Hussain et al., 2013; Moreira et al., 2009). However, the exact mechanism(s) that induce these antiviral effects are still not well understood. During this PhD project, we aimed to find a deeper understanding of potential molecular mechanism(s) underlying the virus blocking property exerted by *Wolbachia* in the particular context of *Ae. aegypti* and DENV. We were able to find three different possible molecular mechanism(s) that could contribute towards inhibition of DENV replication in the presence of *Wolbachia* in *Ae. aegypti*, which are discussed sequentially.

Chromodomain helicase DNA binding protein 7 plays an important role in *Wolbachia*-mediated suppression of dengue virus in *Ae. aegypti* mosquitoes

In this study (Chapter 1), we have characterized the role of one of the members of chromodomain helicase family named *AeCHD7* in the context of *Wolbachia*-mediated antiviral response in *Ae. aegypti* mosquitoes. We have found that in the case of *Wolbachia* infection there is a reduction in *AeCHD7* transcript levels in female mosquitoes suggesting its potentially important role in the *Wolbachia*-host relationship. To date, there has been no report on the involvement of *CHD7* in bacterial infections, however, recently a study has found that in *D. melanogaster* *CHD1* constitutes an important part of immunity and plays a key role in mounting resistance against the bacterium *Pseudomonas aeruginosa* (Sebald et al., 2012). Further study conducted in mice embryonic stem cells has revealed that both *CHD7* and *CHD1* perform a similar function by associating themselves with the regulation of similar genes including *Sox2*, *Oct4* and *Nanog* (Zentner et al., 2011). Aforementioned studies showing the potential role of the CHD family member in *D. melanogaster* immunity and the similar function of both *CHD1* and *CHD7* in mice suggest that *AeCHD7* might be an immune regulator that is altered upon *Wolbachia* infection that could confer resistance to DENV replication.

There is a whole body of evidence pointing towards the difference in gender specific modulation of host immune response (Duneau and Ebert, 2012). Studies conducted on the parasitiod *Asobara tabida* have demonstrated that there is a highly different immune response
in males as compared to females upon infection with *Wolbachia* strains *wAtab1*, *wAtab2*, and *wAtab3*, where males showed higher levels of immune genes expression upon *Wolbachia* infection as compared to females (Kremer et al., 2012). To investigate whether *Wolbachia*-mediated suppression of *AeCHD7* gene is gender specific or not, we analysed the transcripts levels of *AeCHD7* in *Wolbachia*-infected male and female *Ae. aegypti* mosquitoes. We found that there was no significant difference in the expression levels of *AeCHD7* in male mosquitoes (with or without *Wolbachia*) as compared to female mosquitoes that showed significant reduction upon *Wolbachia* infection, indicating that *Wolbachia*-mediated reduction of *AeCHD7* is highly specific to female *Ae. aegypti* mosquitoes.

In order to investigate the role of *AeCHD7* in DENV replication, we found that there was a significant increase in *AeCHD7* transcript levels in mosquitoes infected with DENV. We further characterized the functional role of *AeCHD7* in the case of DENV replication by silencing *AeCHD7* using RNAi. Subsequently, we employed two different techniques to assess virus replication: i) RT-qPCR to quantify the genomic RNA level, showing significant decrease in DENV genomic RNA level, and ii) plaque assay to find the role of *AeCHD7* in DENV virion production, demonstrating reduction in the number of plaques in *AeCHD7* depleted cells. There is lack of information regarding the role of *CHD7* with respect to viral infection and replication. However, only recently there seems an increase in exploring the role of CDH family members in the events of pathogen infection and their own replication. Few reports available on the involvement of CHD family members in viral replication includes studies on HIV (Rodgers et al., 2014) and Influenza A virus (Marcos-Villar et al., 2016) showing the essential role of CHD family members in the replication of the aforementioned viruses.

*Wolbachia* is not a native inhabitant of *Ae. aegypti*. The *wMelPop-CLA* strain of *Wolbachia* was therefore transinfected into the mosquito after its isolation from its native host *D. melanogaster* and its adaptation to a mosquito cell line (McMeniman et al., 2008). Our experimental results determined that the *Wolbachia*-mediated reduction in *AeCHD7* is highly specific to female *Ae. aegypti* and similar modulation was not observed in *D. melanogaster* flies and *Ae. albopictus* cells which are natural hosts of *wMelPop* and *wAlb* strains of *Wolbachia*, respectively. This finding suggests that *Wolbachia*-mediated suppression of *AeCHD7* is not a universal mechanism that *Wolbachia* uses across different host species to impart antiviral ability to its host, but could be an effect induced in a recently introduced host. Furthermore, although we have found that *AeCHD7* appears to be an important component of *Wolbachia-Ae. aegypti*-DENV interactions (Chapter 2), further in-depth studies are required to
find the exact mechanism(s) which leads to Wolbachia-mediated suppression of AeCHD7 and its consequent effect on DENV replication.

The antiviral AeVago1 gene is induced in the presence of Wolbachia

Vago is an insect-specific secretory protein that was discovered in D. melanogaster to play an important role in limiting DCV replication (Dedduche et al., 2008). Recent studies revealed its importance in mosquitoes as well by showing that the secreted Vago plays an antiviral role by suppressing WNV in Cu. quinquefasciatus (Paradkar et al., 2012). This led us to investigate the potential involvement of Vago homolog(s) in Wolbachia-mediated suppression of DENV in Ae. aegypti. We identified two potential Vago homologs in Ae. aegypti and examined their expression in Wolbachia-infected mosquitoes and cells. We found that upon Wolbachia infection there was a significant increase in the levels of AeVago1 transcripts in both mosquitoes and cells infected with Wolbachia suggesting that as an immune gene, it might contribute towards limiting DENV. However, the similar trend of Wolbachia-mediated increase of Vago was not observed in Wolbachia-infected D. melanogaster flies and Ae. albopictus mosquitoes. This difference in Vago modulation in the presence of Wolbachia might be due to the fact that Ae. aegypti is transinfected by Wolbachia and is not a natural host of the endosymbiont.

To characterize AeVago1 further, we investigated the tissue-specific expression of the gene in female Ae. aegypti mosquitoes to find that AeVago1 is highly expressed in the fat body of mosquitoes, which is consistent with a previous study in D. melanogaster (Dedduche et al., 2008). Fat body of mosquitoes represents a major site of DENV replication and is the hub of host immune response against pathogens (Salazar et al., 2007). This notion suggests that AeVago1 is one of the immune genes of mosquitoes that is upregulated in response to Wolbachia infection as a part of immune priming. Furthermore, fat body of mosquitoes is one of the somatic tissues of higher Wolbachia density (Moreira et al., 2009), suggesting that there might be a correlation between induction of AeVago1 and anti-viral response in the mosquito.

DENV has been reported to induce AeVago1 in Ae. albopictus RML12 mosquito cell line (Paradkar et al., 2014), however, we found that in Ae. aegypti whole mosquitoes infected with DENV there was no significant change in the expression of AeVago1. Looking at the possible reason(s), a study in D. melanogaster has shown Vago to be induced in a Dicer-2 dependent manner (Dedduche et al., 2008), while in Cu. quinquefasciatus, another study has looked into the mechanism of CuVago activation suggesting Rel2 and TRAF involvement (Paradkar
et al., 2014). Our experiments have demonstrated that in DENV-infected whole *Ae. aegypti* mosquitoes there was no clear activation of any of the above reported genes except TRAF that was found to be significantly induced at only one time point during early infection. This clearly indicates that DENV somehow is able to restrict the *AeVago1*-mediated antiviral response of *Ae. aegypti* mosquitoes to facilitate its replication and *Ae. aegypti* responds differently upon infection with DENV as compared to *Cu. quinquefasciatus* infected with WNV.

To further elucidate the role of *AeVago1* in the *Wolbachia*-mediated suppression of virus replication, we knocked down *AeVago1* in *Wolbachia*-infected *Ae. aegypti* cells finding a significant increase in the DENV titre without affecting the density of *Wolbachia* thus confirming its role as an antiviral host gene that is altered in the presence of *Wolbachia* to reduce DENV replication. However, this does not seem to be a widespread mechanism(s) by which *Wolbachia* imparts its virus blocking effect. In light of the abovementioned results further investigations are required to find out the exact mechanism(s) governing the DENV evasion of *AeVago1* induction and how *Wolbachia* infection leads to alteration of *AeVago1* to limit DENV replication.

**Decrease in Pelo upon *Wolbachia* infection and its possible effect on DENV replication**

Flaviviruses are well known to hijack the host cellular machinery, including proteins involved in translation. Pelo is a multifunctional protein that is mainly involved in translational regulation (Davis and Engebrecht, 1998) and regulation of germ cells meiosis (Lin et al., 1996) along with other cellular functions, but also participates in viral protein synthesis in the case of DCV (Wu et al., 2014), and TYLC replication (Lapidot et al., 2015). In this study (Chapter 3), we looked into the possible role of *pelo* in relation to *Wolbachia*-*Ae. aegypti*-*Wolbachia* interactions. Experiments conducted on *Wolbachia*-infected and uninfected mosquitoes and cells showed that there was a significant decrease in *pelo* expression in the case of *Wolbachia* infection in only female *Ae. aegypti* mosquitoes, but not in its natural host *D. melanogaster* (flies) and *Ae. albopictus* (cell line). This is similar to our observations in regards to *AeCHD7* and *AeVago1*. Further experiments carried out at the tissue level also demonstrated downregulation of *pelo* in all major tissues including salivary gland, midgut, muscles, ovary and fat body in *Wolbachia*-infected mosquitoes. Pelo is a highly-conserved protein that has a well distinct nuclear localization signal (Shamsadin et al., 2000), however, *pelo* is mainly found in the cytoplasm and there is lack of experimental evidence of its localization inside the nucleus (Xi et al., 2005). Here, we showed that *Wolbachia* infection
leads to a change in subcellular localization of the pelo protein resulting in its translocation into the nucleus, making it less available in its active location that is the cytoplasm.

To look into the mechanism(s) involved in the Wolbachia-mediated reduction of pelo, we examined the possible involvement of miRNA-mediated regulation by using in silico tools and found that aae-miR-2940-5p, that has been previously reported to be highly induced in the presence of Wolbachia (Hussain et al., 2011), could be a potential target of the miRNA. A series of experiments conducted by transient overexpression of aae-miR-2940-5p showed reduction of pelo at both transcript and protein levels, but target validation experiment using a reporter gene revealed that pelo is not a direct target of the miRNA. This suggests that pelo could be regulated by aae-miR-2940-5p in an indirect manner. This indirect regulation of pelo by miRNA could be due to regulation of transcription factor(s) that might fine-tune the abundance of pelo transcripts.

Further investigation of the effect of DENV on pelo protein expression revealed that there was a significant increase in the expression levels of the protein upon DENV infection without changing its subcellular localization, signifying the potential involvement of the pelo protein in DENV replication. Knock down studies of pelo revealed significant reductions in DENV virion production without significant reduction in the genomic RNA level, suggesting the potential important role of pelo in DENV virion production. This result is consistent with a previous finding that has demonstrated involvement of pelo in DCV (Wu et al., 2014) and TYLCV replication (Lapidot et al., 2015).

Although Wolbachia-mediated suppression of pelo appears to lead to reduction in DENV virion production in Ae. aegypti mosquitoes, this does not seem to be a general mechanism(s) which Wolbachia uses in different species to overcome viral replication. The abovementioned findings have identified a novel molecular mechanism that Wolbachia adopts in Ae. aegypti mosquitoes to suppress another pro-viral protein (pelo) to limit DENV virion production. However, further studies are required to gain a deeper understanding of the exact role of the pelo protein inside the nucleus, the transcription factor(s) or other protein(s) regulated by aae-miR-2940-5p which in turn could downregulate pelo transcript levels, and the potential Wolbachia proteins that might interact with the host pelo protein.

5.2. Conclusion

In this study, we have unravelled the novel molecular mechanism(s) that Wolbachia opts in Ae. aegypti mosquitoes to limit DENV replication. Our findings demonstrate that in the
presence of *Wolbachia* *Ae. aegypti* host factors *AeCHD7, AeVago1* and *Pelo*, which are important in regulating DENV replication, are altered. Although all the above said molecular mechanism(s) are specific to *Ae. aegypti* mosquitoes, these highlight the complexity of *Wolbachia* relations with different host species (Bourtzis et al., 2000; Martinez et al., 2014). Similar to our findings, a number of studies have highlighted unique effects of *Wolbachia* on transinfected *Ae. aegypti* including activation of ROS, Toll pathway and antimicrobial peptides contributing toward the inhibition of DENV (Pan et al., 2012; Rainey et al., 2014; Rances et al., 2012). The outcomes of this study will contribute toward better understanding of *Wolbachia-Ae. aegypti-DENV* molecular interactions.

5.3. References


Assessing the epidemiological effect of *wolbachia* for dengue control. Lancet Infect Dis 15, 862-866.


Appendix 1
Downregulation of *Aedes aegypti* chromodomain helicase DNA binding protein 7/Kismet by *Wolbachia* and its effect on dengue virus replication

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**Running title:** Role of CHD in dengue virus-*Wolbachia*-mosquito interaction

**Keywords:** *Aedes aegypti*; *Wolbachia*; chromodomain helicase; dengue virus

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Abstract
Dengue virus (DENV) is a mosquito-transmitted virus imposing a significant burden on human health around the world. Since current control strategies are not sufficient, there is an urgent need to find alternative methods to control DENV transmission. It has been demonstrated that introduction of Wolbachia pipiensis in Aedes aegypti mosquitoes can impede DENV transmission with the mechanism(s) not fully understood. Recently, a number of studies have found the involvement of chromodomain DNA binding helicases in case of Human Immunodeficiency virus (HIV) and Influenza A virus infection. In this study, we have identified three chromodomain helicase DNA binding protein (CHD) genes in Ae. aegypti and looked at their response in the case of Wolbachia and DENV infections. Foremost amongst them we have found that AeCHD7/Kismet is significantly downregulated in the presence of Wolbachia infection only in female mosquitoes. Furthermore, AeCHD7 levels showed significant increase during DENV infection, and AeCHD7 depletion led to severe reduction in the replication of DENV. Our data have identified AeCHD7 as a novel Ae. aegypti host factor that is important for DENV replication, and Wolbachia downregulates it, which may contribute towards the mechanism(s) of limiting DENV replication.
Introduction

Among arboviruses, dengue virus (DENV) is one of the most important flaviviruses having the potential to affect two-thirds of the world’s population. DENV is primarily transmitted to humans through the bite of mosquito vector *Aedes aegypti*, leading to dengue infection and potentially dengue haemorrhagic fever. Lack of availability of an effective vaccine and proper medical care has narrowed DENV management strategies to vector control. One of the strategies used to overcome DENV vector *Ae. aegypti* is through the application of pesticides, but due to their severe consequences on the environment and the emergence of resistance to pesticides, their potential application seems bleak in the near future. Therefore, new strategies for vector control are urgently needed. One of the novel options is the use of an endosymbiotic bacterium *Wolbachia* which has recently been demonstrated to limit DENV, West Nile virus (WNV), and Zika virus (ZIKV) replication in *Ae. aegypti*.

*Wolbachia* is an alphaproteobacterium that naturally infects almost 40-60% of insect species. This bacterium is maternally transmitted and is usually associated with manipulations of host reproduction, such as feminization and male killing, to promote successful colonization of its host species. *Wolbachia* naturally infects several mosquito species, including *Aedes albopictus* and *Culex pipiens*. However, there is no natural *Wolbachia* infection in the case of *Ae. aegypti*, which is the most notorious vector for several arboviruses. In order to exploit *Wolbachia*’s potential to limit arbovirus transmission, three strains of *Wolbachia*, *wAlbB* (from *Ae. albopictus*), *wMel* (from *Drosophila melanogaster*) and *wMelPop-CLA* (from *D. melanogaster*) have been successfully transinfected into *Ae. aegypti*. Among these three strains, *wMel* and *wMelPop-CLA* are the most promising ones for virus blocking. However, the exact mechanism(s) by which *Wolbachia* blocks viral replication in *Ae. aegypti* mosquitoes is still elusive. Few studies that have looked into the transcriptional changes in *Ae. aegypti* mosquitoes upon *Wolbachia* infection have found increased redox and mitochondrial activity along with differential serine protease activity. However, very little is known about the role of chromatin remodelers in the case of DENV-*Aedes-Wolbachia* molecular interactions.

Chromodomain helicase DNA binding proteins (CHD) represent a class of ATP-dependent chromatin remodelling enzymes that contribute towards invoking changes
in the interaction between DNA and nucleosomes 24, influencing a wide array of cellular processes such as replication, transcription, recombination, repair and development 25. Members of the CHD family have been found to be involved in replication of Human Immunodeficiency virus (HIV) and Influenza A virus 26,27. All the CHD protein family members have a pair of chromodomains at their N-terminus along with one sucrose non-fermenting (SNF2) domain in the centre 25. In humans, the CHD family has nine members. These are further classified, on the basis of additional motif features, into three subfamilies: CHD1-2 (class I), CHD3-5 (Class II) and CHD 6-9 (class III) 25,28. In D. melanogaster, there are three well characterized CHD members named CHD1 29, Mi2 30 and Kismet/CHD7 31. CHD1 is essential for the fecundity of both males and females and is indirectly involved in transcriptional elongation 32, whilst Mi2 actively participates in transcriptional repression and is vital for expression of heat shock proteins 33,34. Drosophila Kismet, that is a homolog of human CHD7, mediates transcriptional elongation 35. Apart from characterization of the CHD family members’ role in development and chromatin modification, very little is known about their potential role in host-pathogen interactions.

In this study, we have identified functional homologs of the CHD family members in Ae. aegypti and looked at the effect of Wolbachia infection on their expression. There was significant reduction in the expression of all CHD family members in the presence of Wolbachia. Furthermore, we found that AeCHD7 is highly induced during DENV infection in Ae. aegypti mosquitoes. A silencing assay demonstrated that AeCHD7 is required for the efficient replication and virion production of DENV. This study will help to understand the role of AeCHD7 in DENV-Aedes-Wolbachia interactions.

Results

Screening of the CHD family genes during Wolbachia infection

Three CHD genes were identified in the Ae. aegypti genome using Vectorbase 36. Blastp was run to identify their homologs in D. melanogaster and Culex quinquefasciatus, and these were determined as AeCHD1 (AAEL004716) having 58% identity with D. melanogaster CHD1 protein (NP_477197.1), AeCHD3 (AAEL013136) that showed 70% identity with D. melanogaster CHD3 protein (AAD17276.1) and AeCHD7 (Kismet) (AAEL002230) showing 58% identity with D.
melanogaster Kismet/CHD7 protein (NP_001245820.1). qPCR primers were designed for all the three AeCHD family members to experimentally validate their expression in *Ae. aegypti* mosquitoes by RT-qPCR, and the effect of Wolbachia (wMelPop) infection on their expression level. For this, we selected two age groups of *Ae. aegypti* mosquitoes, 4-day- and 12-day-old. While expression of all the three AeCHD genes was confirmed in the mosquitoes, they were all mostly downregulated in Wolbachia-infected mosquitoes (Figure 1A-F), except for AeCHD3, which was found to be non-significantly upregulated in 4-day-old Wolbachia-infected mosquitoes (Figure 1C). However, AeCHD7 showed the highest change of 2.9-fold downregulation in 4-day-old *Ae. aegypti* female mosquitoes (Figure 1E), which led us to further characterise the gene.

**AeCHD7 is ubiquitously expressed in all mosquito tissues**

In order to determine the relative abundance of AeCHD7 across different tissues, the salivary gland, midgut, muscle, ovary and fat body were isolated from 3-day-old female *Ae. aegypti* mosquitoes. Following RT-qPCR detection of AeCHD7 mRNA transcripts, it was found that AeCHD7 is ubiquitously expressed in all tissues with the highest expression level in the salivary gland, which was 2.1-fold higher than its expression level in the fat body which showed the lowest relative abundance of AeCHD7 transcripts (Figure 2). These results are consistent with the previous findings which showed that AeCHD7 is expressed in all human tissues.37 Specific Wolbachia-mediated downregulation of AeCHD7 in female *Ae. aegypti*

To find out whether Wolbachia-mediated downregulation of AeCHD7 is gender specific, we evaluated the transcript levels of AeCHD7 in 4-day-old female and male *Ae. aegypti* mosquitoes with and without Wolbachia infection. RT-qPCR results showed that Wolbachia downregulates AeCHD7 only in female mosquitoes and not in their male counterparts (Figure 3A). This is interesting in the sense that Wolbachia has a gender specific effect on gene expression in the mosquitoes. To examine if the effect can consistently be seen in cell lines as well, we cross-validated the AeCHD7 mRNA expression levels in *Ae. aegypti* cell lines, Aag2 and Aag2 infected with wMelPop-CLA (Pop) and found a similar trend of AeCHD7 transcript downregulation in Wolbachia-infected cells (Figure 3B).
Furthermore, to evaluate if Wolbachia has a similar effect on the CHD7 gene in its natural host D. melanogaster, four 7-day-old male and female flies infected with Wolbachia (wMelPop strain) were examined for the relative expression of CHD7. RT-qPCR results confirmed that there was no significant change in the level of CHD7 mRNA in both male and female D. melanogaster flies infected with Wolbachia (Figure 3C).

**AeCHD7 is upregulated upon DENV infection**

Considering the virus blocking effect of Wolbachia in Ae. aegypti mosquitoes, we examined the transcript levels of AeCHD7 in the context of mosquito-DENV interaction. For this, the transcript levels of AeCHD7 in DENV-injected mosquitoes at three different time points of 2, 6 and 12 days post-infection (dpi) were analysed. To have a more consistent and high success rate of DENV infection, mosquitoes were injected rather than orally fed with the virus. The results revealed that there was an increase in the AeCHD7 transcript levels upon DENV infection at all the time points (Figure 4A-C); however, the upregulation was only significant at 2 and 12 dpi, which was 2-fold (Figure 4A) and 4-fold higher than that in uninfected mosquitoes (Figure 4C), respectively. The aforementioned findings were further confirmed in the Ae. aegypti cell line, Aa20. Cells were infected with DENV2 at 0.1 multiplicity of infection (MOI) and were harvested at two different time points that were 1 and 5 dpi. RT-qPCR analysis showed significant increase in AeCHD7 transcript levels in the case of DENV infection at both 1 and 5 dpi (Figure 4D). Infection was confirmed by relative quantification of DENV genomic RNA levels, which showed gradual increase in DENV genomic RNA over time (Figure 4E).

**AeCHD7 is required for efficient DENV replication**

The upregulation of AeCHD7 in DENV-infected cells suggested that the gene could be beneficial for the virus. To investigate whether AeCHD7 is required for efficient DENV replication, we knocked down AeCHD7 transcripts in Aa20 cells and challenged these cells with DENV at 1 MOI for 72 h. The effect of CHD7 knockdown on DENV was evaluated both at the genomic and the virion levels using RT-qPCR and plaque assay. RT-qPCR results confirmed ~50% decrease in AeCHD7 mRNA level (Figure 5A), which led to 2-fold reduction in DENV genomic RNA (Figure 5B). RT-qPCR results were further validated with plaque assay, which confirmed
reduction in DENV virion production in *AeCHD7* knocked down cells as compared with dsGFP or mock-transfected Aa20 cells (Figure 5C). These results indicate that *AeCHD7* is a host factor that is used by DENV to facilitate its replication in *Ae. aegypti* female mosquitoes, and *Wolbachia* downregulates *AeCHD7* as shown above, which may contribute to restricting DENV replication.

**Discussion**

There is accumulating experimental evidence showing the effectiveness of *Wolbachia* in suppressing the replication of several flaviviruses, including DENV, ZIKV, WNV, and the alphavirus chikungunya virus (CHIKV) in both mosquitoes and mosquitoes-derived cell lines. Perhaps the most well studied is the case of DENV replication that is severely compromised in the presence of *Wolbachia*. However, the exact mechanism(s) of how *Wolbachia* imparts this antiviral effect is not yet fully understood. In this study, we provide experimental evidence that chromodomain DNA binding helicase 7 (*AeCHD7*) is an *Ae. aegypti* host factor that is exploited by DENV to facilitate its replication, and its downregulation by *Wolbachia* may contribute to limit DENV replication.

*Wolbachia* is an endosymbiotic bacterium infecting 40-60% of insect species naturally by manipulating host reproduction. Despite fitness costs, *Wolbachia* may benefit its host by blocking a variety of RNA viruses. However, *Wolbachia* has not been found naturally infecting the most notorious vector *Ae. aegypti*, that is responsible for transmitting multiple viral diseases. McMeniman et al transinfected different strains of *Wolbachia* into *Ae. aegypti* mosquitoes and found that they successfully inhibited replication of DENV and CHIKV. Further studies also demonstrated *Wolbachia*’s ability to block WNV and ZIKV in the mosquito. *Wolbachia*’s potential to be used as an invaluable tool for disease control and prevention represents an increasingly promising approach to limit several mosquito-borne viral diseases, and it is fascinating to explore the exact mechanism(s) that induce the antiviral effect. Apart from one study shedding light on the effect of *Wolbachia* infection on the global DNA methylation pattern in mosquitoes, there lies a huge grey area of the role of chromatin remodelers, in *Wolbachia*-host interactions and possibly the *Wolbachia*-mediated antiviral effect.
CHD proteins represent a class of proteins that belong to SNF2 superfamily of ATP-dependent chromatin modifiers. In mammals, there are 1-9 CHD proteins; however, in *D. melanogaster*, there are only three CHD proteins named CHD1, Mi2 and Kismet. Members of the CHD family are involved in conducting a wide array of functions, including ATPase activity to maintain chromosome structure and regulation of heterochromatic elements, nucleosome mobilization, transcriptional regulation and elongation, and development and differentiation. Despite extensive characterization of CHD family proteins, their role in shaping host-pathogen interactions has not been much investigated. Yet, there are few reports supporting the involvement of CHD1 in the case of influenza A virus, and both CHD1 and CHD2 in the case of HIV as positive regulators. Furthermore, RNAi screen carried out in *D. melanogaster* S2 cells identified the involvement of CHD7/Kismet in antimicrobial humoral response. The aforementioned facts led us to investigate the possible role of the CHD family in *Wolbachia*-Aedes-DENV interactions. Data mining in VectorBase resulted in the identification of three potential AeCHD proteins in the *Ae. aegypti* genome. Protein blast results identified them as AeCHD1, AeCHD3/Mi2 and AeCHD7/Kismet. In order to find out whether *Wolbachia* regulates the AeCHD genes during infection, RT-qPCR was performed to examine the transcript levels of all the three AeCHDs with and without *Wolbachia* infection in whole mosquitoes. Our results showed that there was a uniform trend of downregulation of the AeCHD genes transcript levels in *Wolbachia*-infected mosquitoes (Figure 1A-F), except those of AeCHD3 in 4-day-old *Wolbachia*-infected female mosquitoes, which showed a non-significant upregulation (Figure 1C). The reduction in the CHD genes was more pronounced in 12-day-old mosquitoes, which could be due to increases in the *Wolbachia* load as the mosquitoes ages. In particular, the *wMelPop* strain is a virulent strain that may cause tissue damage and sickness. The reductions in the AeCHD genes at this late stage may not be of benefit in affecting DENV replication. However, AeCHD7 showed the highest fold change reduction (Figure 1E-F) in both 4- and 12-day-old *Wolbachia*-infected mosquitoes, which prompted us to further investigate this gene in the context of *Wolbachia*-Aedes-DENV interactions.

AeCHD7/Kismet belongs to subfamily III of the CHD proteins that comprises CHD5-9 proteins. This subfamily is defined by the presence of two chromodomains at the N-terminus, one SNF2-like ATPase domain located in the central region of the protein.
structure (9,10) and a Brahma and Kismet (BRK) domain at the C-terminus 25. To find out whether the Ae. aegypti homolog fulfills this particular protein signature, NCBI conserved domain finder was used to detect the conserved domains 51. Results confirmed the presence of all the domains characteristic of CHD7/Kismet proteins (Figure S1). Furthermore, in order to check the conservation of CHD7 across species, CHD7/Kismet amino acids were retrieved from Uniprot (Figure S2A) and subjected to maximum likelihood phylogenetic tree construction. Phylogenetic results showed that Ae. aegypti CHD7 is closely related to Cx. quinquefasciatus but not to that of D. melanogaster or H. sapiens (Figure S2B). To find out the tissue-specific expression of AeCHD7, RT-qPCR was performed, which revealed that it is ubiquitously expressed across all main mosquito tissues (Figure 2), which is consistent with the findings in humans 37.

In this study we found that AeCHD7 was significantly downregulated in Wolbachia-infected mosquitoes. To further investigate whether this Wolbachia-mediated downregulation of AeCHD7 in female Ae. aegypti is gender specific and can also be seen in its natural host D. melanogaster, RT-qPCR was employed. Interestingly, we found that in the presence of Wolbachia AeCHD7 was specifically downregulated in female Ae. aegypti only (Figure 3A), and there was no change in CHD7/Kismet transcript levels in both female and male D. melanogaster with and without Wolbachia (Figure 3B). This difference in Wolbachia-mediated regulation of CHD7/Kismet in Ae. aegypti and D. melanogaster may be due to the fact that Wolbachia is a natural symbiont in D. melanogaster with a long association, while it has been recently transinfected into Ae. aegypti 52. In regards to the mechanism by which Wolbachia infection may affect expression of AeCHD7, one can only speculate at this stage as there is very little information in regards to how Wolbachia manipulates its host at the molecular level. Only very recently, some molecular data have become available showing that Wolbachia infection leads to changes in the transcriptome or small RNA profiles of infected mosquitoes 23,53. Regulation of host gene expression could be due to components secreted from the endosymbiont, including small non-coding RNAs 54 or host response to accommodating the endosymbiont, in particular in new associations. However, how Wolbachia infection leads to these changes in the host remains to be investigated.
Viruses are the master manipulators of their host environment for their own benefit. Recently, it has been reported that CHD1 and CHD2 proteins play a pivotal role in the replication of influenza and HIV viruses. Both viruses replicate inside the nucleus. Interestingly, it has further been demonstrated that CHD1 interacts with RNA polymerase II to facilitate influenza A virus. Little is known about the role of CHD7/Kismet in the context of virus infection. However, the presence of conserved chromodomains and a SNF2 domain makes it highly likely that all the CHDs share similar functions. We were intrigued to find what happens to AeCHD7 during DENV infection. RT-qPCR performed in DENV-infected mosquitoes at different time points suggested a continuous trend of upregulation during DENV infection (Figure 4A-C), which points to the fact that it might play an important role in DENV replication. To investigate the role of AeCHD7 in DENV replication further, AeCHD7 knockdown study was carried out, which revealed that AeCHD7 is vital for DENV replication and virion production (Figure 5A-C). Very few studies that have been carried out on the involvement of CHDs in virus replication have predominantly focused on viruses that replicate inside the nucleus. Therefore, the role of CHDs in the replication of viruses that multiply in the cytoplasm (such as DENV) is not known. However, it has been demonstrated that DENV capsid and NS5 proteins go inside the nucleus with NS5 known to be involved in disrupting nucleosome formation. Therefore, these viral proteins may play a role in modulating AeCHD7 expression during virus infection. While we have found the involvement of AeCHD7 in mosquito-DENV interaction, the exact mechanism(s) that govern the interaction need further investigation.

In summary, we have demonstrated that AeCHD7 facilitates DENV replication, and Wolbachia-mediated downregulation of AeCHD7 in female Ae. aegypti may contribute to restriction of DENV replication. However, this mechanism is highly specific to female Ae. aegypti mosquitoes and does not appear to be a universal mechanism which Wolbachia employs across different hosts to block viral replication.

Materials and Methods

Mosquitoes and flies
For *Wolbachia* studies, mosquitoes had been previously generated by McMeniman et al. (2008) by transinfecting *wMelPop-CLA* strain of *Wolbachia* (Pop) into *Ae. aegypti* embryos, and uninfected mosquitoes were obtained through tetracycline (Tet) treatment of the infected mosquitoes \(^{43}\). *wMel* fly line stably infected with *wMelPop-CLA* and the tetracycline cured line were generated by Min et al. (1997) \(^{60}\) and kindly provided by Dr Karyn Johnson from the University of Queensland.

For DENV infection studies, *Ae. aegypti* eggs were collected in Townsville in August 2015 and reared in insectary at Public Health Virology FSS. Five-day-old *Ae. aegypti* (F3) were used for the experiments. DENV2 NGC strain obtained from Prof. Roy Hall’s Lab (University of Queensland, School of Chemistry & Molecular Biosciences, Brisbane, Australia), was diluted in Opti-MEM (GIBCO Life Technologies, Grans Island, NY) supplemented with 3% foetal bovine serum (FBS, Bovogen Biologicals, France) and intrathoracically injected (200µl) in 5-day-old mosquitoes at \(10^{5.8}/\text{mL} \ (10^{2.1} \text{ per dose})\). Mosquitoes were placed into netted 900 mL containers at 28°C with light:dark (L:D) 12:12 hours cycle and at high humidity. Mosquitoes were offered 15% honey water *ad libitum*. Mosquitoes were collected at 2, 6 and 12 dpi for downstream applications.

**Cell cultures**

*Ae. aegypti* Aag2 cell line and Aag2 cells infected with *wMelPop-CLA*, previously described by \(^{61}\), were maintained in 1:1 Mitsuhashi-Maramorosch and Schneider’s insect medium (Invitrogen) supplemented with 5-10% FBS, while Aa20 cells were maintained in L15 medium (Invitrogen) supplemented with 10% tryptose phosphate broth (TPB) and 5% FBS. All mosquito cell lines were kept at 28°C and passaged every 3-4 days.

Vero cells were maintained in OptiMEM medium supplemented with 2% FBS and were kept at 37°C in the presence of 5% CO₂.

**RT-PCR and qPCR analyses**

Total RNA was extracted from mosquitoes (1-5 mosquitoes per biological replicate) or flies (1-5 flies per biological replicate) using Qiazol (Qiagen) and then treated with Turbo DNase (Ambion) according to the manufacturers’ instructions. 750-1000 ng of total RNA was then used to make the 1\(^{\text{st}}\) strand cDNA using Superscript III
(Invitrogen) with either oligo-dT primer for cellular transcripts or with DENV-qR primer in order to amplify the DENV genomic RNA.

For qPCR, cDNA produced as above was diluted in 1:5 ratios with nuclease free water. 2µl of the diluted cDNA was used for downstream qPCR reaction. Both forward and reverse gene-specific primers were used to amplify the target genes (primer sequences in Table S1), using QuantiFast SYBR Green (Qiagen) in a Rotorgene qPCR machine (Qiagen). For Ae. aegypti samples, RPS17 transcript levels were used for the normalization of RNA templates, while RPL32 was used for normalization of D. melanogaster samples. Each qPCR reaction was performed in duplicates with at least three biological replicates. All qPCR data were normalized with Qiagen analysis templates and were further analysed by Prism 7.0. Unpaired t-test was used to determine statistical significance between two individual groups while one-way ANOVA with Tukey’s post-hoc test was performed to find statistical significance between more than two groups of data.

RNAi-mediated gene silencing

In order to knockdown the AeCHD7 gene for functional analysis in DENV life cycle, primers were designed to amplify a 586 bp product from the AeCHD7 gene with the addition of the T7 promoter sequences at both ends (Table S1). MEGAscript T7 Transcription kit was then used according to the manufacturer’s instructions in order to synthesize dsRNA targeting the AeCHD7 transcripts. A similar approach was followed to synthesize dsRNA against GFP RNA. For knockdown experiments, Aa20 cells were double transfected with 2-5µg of dsRNA per well against the target gene. dsGFP RNA was used as non-specific control.

Virus infection and plaque assay

For virus inoculation experiments, Ae. aegypti Aa20 were seeded at the density of 3x10^5 cells per well in 12-well plates. Cells were first double transfected with dsRNA against the target gene or GFP control and after 6 h cells were infected with DENV2 (New Guinea strain) at the 1 multiplicity of infection (1 MOI). Media were collected 72 h post-infection for plaque assay.

To perform plaque assay, Vero cells were seeded in a 96-well plate and were allowed to form monolayers. Virus containing media from the experiments were
serially diluted into $10^0, 10^1, 10^2, 10^3$ dilutions and added to Vero cells in duplicates. Cells were incubated with virus at room temperature with continuous shaking on shaker for 1 h and then incubated at 37°C for one additional hour. After 2 h of incubation, media were aspirated and an overlay was added to the cells which comprised of 1.5% carboxymethyl cellulose (CMC) and 2.5% FBS in Opti-MEM medium (Sigma). Cells were then incubated for 72 h at 37°C and 5% CO₂ and fixed with 80% ice-cold acetone in 1 PBS for 20 min at -20°C. Plates were then air dried overnight and blocked with 5% skimmed milk in 1×PBST at 37°C for 30 min. Cells were then incubated with the primary antibody against DENV2-Envelope (human) in 1:1000 dilution in 0.1% skimmed milk in 1×PBST for 2 h at 37°C as described previously. Plates were washed 3 times with 1×PBST and incubated with the secondary antibody (IRDye 800CW goat anti-human LICOR) for 1 h at 37°C. Plates were washed and dried as above and were dried and scanned on the Odyssey imager (LI-COR Biosciences) at 41μM resolution.

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Authors’ contributions

SAsa conceived and designed research, carried out experiments, analysed the data and drafted the manuscript. SHM carried out experiments and edited the manuscript. SAsg designed research and edited the manuscript. All authors read and approved the final manuscript.

Additional Information

Competing financial interests. The authors declare no competing financial interests.
References


Figure legends

Fig. 1: Relative expression of AeCHD genes in uninfected and Wolbachia-infected Ae. aegypti mosquitoes. RT-qPCR based quantification of (A-B) AeCHD1, (C-D) AeCHD3, and (E-F) AeCHD7/Kismet genes in both Wolbachia-infected (Pop) and uninfected (Tet) 4-day-old and 12-day-old female mosquitoes, respectively. Error bars represent standard error of mean (SEM) from three biological replicates (*, p<0.05; **, p<0.01).

Fig. 2: Tissue-specific expression of AeCHD7/Kismet in Ae. aegypti mosquitoes. RT-qPCR results of AeCHD7/Kismet transcript levels in the salivary gland, midgut, muscles, ovaries and fat body of 3-day-old tetracycline treated female mosquitoes. Error bars represent SEM of the mean in three biological replicates.

Fig. 3: Modulation of AeCHD7/Kismet by Wolbachia infection in male and female mosquitoes and flies, and mosquito cell lines. A) RT-qPCR analysis of AeCHD7 transcript levels in 4-day-old female and male mosquitoes, both uninfected (Tet) and infected with Wolbachia (Pop). B) Relative expression of AeCHD7 in Aag2 and Aag2 cells infected with wMelPop-CLA (Pop). (C) Relative expression of the D. melanogaster Kismet gene in uninfected (Tet) and Wolbachia-infected (Pop) flies. Error bars represent SEM from three biological replicates (**, p<0.01; ***, p<0.001; ns, not significant).

Fig. 4: Expression pattern of AeCHD7 in DENV infected female Ae. aegypti. A-C) RT-qPCR quantification of AeCHD7 transcript levels at 2, 6 and 12 days post DENV infection of female Ae. aegypti mosquitoes. D) Relative transcript levels of AeCHD7 in Aa20 cells infected with 1 MOI of DENV2 analysed at 1 and 5 dpi. E) RT-qPCR quantification of DENV2 genomic RNA in samples used in (D) confirming virus infection and replication. Error bars show SEM from three biological replicates (*, p<0.05; ***, p<0.001; ****, p<0.0001; ns, not significant).

Fig. 5: Depletion of AeCHD7 impairs DENV replication both at the genomic and the virion levels. A) RT-qPCR analysis of Aa20 cells transfected with either no RNA (Mock) or with dsRNA against GFP as a control or with dsRNA against AeCHD7. RPS17 was used to normalize the qPCR data. Error bars show SEM from three biological replicates (*, p<0.05; **, p<0.01). B) RT-qPCR analysis of Aa20 cells treated as in (A) followed by DENV infection at 1 MOI using DENV-specific primers
to quantify viral genomic RNA. Error bars show SEM from three biological replicates (**, p<0.01). C) Viral plaque visualization by in vitro cell plaque assay conducted on the supernatant media from cells treated as in (A and B).
Ae. aegypti

B

Aag2 cells

D. melanogaster

relative expression of CHD7

relative expression of Kismet

Tet-females
Pop-females
Tet-males
Pop-males

ns

ns

ns

ns

**

***

ns
Appendix 2
Identification of Aedes aegypti long intergenic non-coding RNAs and their association with Wolbachia and dengue virus infection

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Abstract

Long intergenic non-coding RNAs (lincRNAs) are appearing as an important class of regulatory RNAs with a variety of biological functions. The aim of this study was to identify the lincRNA profile in the dengue vector *Aedes aegypti* and evaluate their potential role in host-pathogen interaction. The majority of previous RNA-Seq transcriptome studies in *Ae. aegypti* have focused on the expression pattern of annotated protein coding genes under different biological conditions. Here, we used 35 publically available RNA-Seq datasets with relatively high depth to screen the *Ae. aegypti* genome for lincRNA discovery. This led to the identification of 3,482 putative lincRNAs. These lincRNA genes displayed a slightly lower GC content and shorter transcript lengths compared to protein-encoding genes. *Ae. aegypti* lincRNAs also demonstrate low evolutionary sequence conservation even among closely related species such as *Culex quinquefasciatus* and *Anopheles gambiae*. We examined their expression in dengue virus serotype 2 (DENV-2) and *Wolbachia* infected and non-infected adult mosquitoes and *Aa20* cells. The results revealed that DENV-2 infection increased the abundance of a number of host lincRNAs, from which some of them suppress viral replication in mosquito cells. RNAi-mediated silencing of lincRNA_1317 led to enhancement in viral replication, which possibly indicates its potential involvement in the host anti-viral defense. A number of lincRNAs were also differentially expressed in *Wolbachia*-infected mosquitoes. The results will facilitate future studies to unravel the function of lncRNAs in insects and may prove to be beneficial in developing new ways to control vectors or inhibit replication of viruses in them.

**Key words:** non-coding RNA; *Aedes aegypti*; lincRNAs; *Wolbachia*; Dengue virus

Author summary

*Aedes aegypti* is a major vector of several viruses such as dengue and Zika viruses. Understanding the intricate interaction of viruses with mosquito vectors and the factors involved in virus replication are essential for developing effective arbovirus control strategies. In this study, we report a comprehensive list of long intergenic non-coding RNAs encoded by the genome of *Ae. aegypti* for the first time. In addition, we show that a number of these long non-coding RNAs are differentially expressed in mosquitoes infected with dengue virus, which could be involved in DENV-mosquito interaction. The outcomes provide a new avenue to explore mosquito biology and mosquito-virus interactions that may lead to the discovery of molecules that could be beneficial for vector manipulation.
Introduction

Dengue and Zika viruses are related mosquito-borne viruses that have a common vector, *Aedes aegypti* and infect millions of people worldwide [1,2]. Recent outbreaks of Dengue and Zika in South America pose a serious risk for other tropical regions in the world as *Ae. aegypti* is one of the most abundant mosquito species in these areas [2]. Although certain vaccines have been licensed in some countries, there are no efficient specific therapeutics available for either diseases, hence, the best protection against their global spreading is an efficient vector control program [3,4].

The genome sequence of *Ae. aegypti* is available, however, it has not been fully annotated. Only 2% of its large genome (1.376 Mb) has been annotated as protein coding genes and it reflects the presence of great proportions of non-coding transcripts as well as repetitive elements [5]. Transcriptomic changes, including those of non-coding transcripts, could provide a genome scale insight into host-pathogen interactions. Previous studies identified a series of small ncRNAs in *Ae. aegypti* and demonstrated their interaction with arboviruses [6-9], but our knowledge about their long ncRNAs is limited.

RNA transcripts longer than 200 nucleotides, which do not contain an open reading frame of longer than 100 amino acids, are simply defined as long ncRNA [10]. Generally, they are classified by their location relative to their neighboring protein-coding genes and include the long intergenic ncRNA (lincRNA), intronic lncRNA, antisense lncRNA and enhancer RNA [10]. Although a number of mammalian lncRNAs have been characterized and identified in the last few years, genome-wide identification of this class of ncRNAs has only recently become possible with the arrival of deep sequencing technologies. An expanding body of evidence reveals that lncRNAs, once described as dark matter, are involved in many biological processes such as genomic imprinting and cell differentiation [11]. They also play important roles in epigenetic and non-epigenetic based gene regulation [12]. Relatively, little is known about their involvement in activation and differentiation of immune cells, but new discoveries have revealed the involvement of lncRNA in defense systems [13]. Previous works have also outlined their quick responses to different stimuli and stress factors [14-17]. In addition, it has been shown that some lncRNAs enhance virus replication or decrease antiviral immunity [18].

Although in most host-virus interaction studies typically protein-coding genes have been the center of attention, there are few examples of virus and host lncRNA interactions in human and mouse models [18,19]. For instance, Hepatitis B virus (HBV) infection altered lncRNA profiles in patients, with about 4% of human lncRNAs showing more than 2-fold changes in HBV infected liver tissue [20]. Winterling *et al.* (2014) identified a virus inducible lncRNA, which is induced by vesicular stomatitis virus and several strains of Influenza A virus (IAV) [18].
The sequence and structure of lncRNAs are important in their function, in particular for their interaction with DNA, RNA, or proteins. In case of extensive base-pairing of lncRNA with target mRNA, translation can be stabilized, while partial base-pairing may accelerate mRNA decay or inhibit translation of the target mRNA [21]. It has been shown that some lncRNAs interact with other small ncRNAs such as miRNAs. For example, in silkworm, 69 lncRNAs originating from 33 gene loci, may serve as miRNA precursors, and 104 lncRNAs may function as competing endogenous RNAs (ceRNAs) [22]. LncRNAs are also targeted by miRNAs similar to mRNAs and reduce their stability. They may also act as sponge or decoy of miRNAs, and compete with miRNAs for binding to mutual target mRNAs [21].

In insects, only a few genes have been experimentally annotated as lncRNA. It has been estimated that more than 5000 loci potentially encode non-coding transcripts in Drosophila melanogaster, however, just seven loci (bxd, Hsro, pge, roX1, rox2, sphinx and yar) have been annotated as functional regulatory lncRNAs by experimentally derived empirical data [23,24]. We recently found that a number of lncRNAs in Plutella xylostella, a pest of cruciferous plants, were linked to the insect’s resistance to insecticides and might be involve in detoxification processes [14]. Jenkins et al (2015) identified 2,949 lncRNAs in the malaria mosquito vector, Anopheles gambiae, using RNA-Seq data [25]. They showed that in various Anopheles species, lncRNAs have considerably lower sequence conservation as compared with protein-coding genes. In another study, it has been shown that 43% of total midgut transcripts of An. gambiae are lncRNAs and 32% of them showed some level of homology to other species [26].

The current study generated a comprehensive list of Ae. aegypti lincRNAs, which will be a complement to the other ncRNAs (microRNAs and piRNAs) that have already been discovered in this medically important species. This work also helps to improve the present annotation of the genome of Ae. aegypti. We also examined the expression pattern of some selected lincRNAs in response to microbial challenge namely dengue virus serotype 2 (DENV-2) and Wolbachia infection to identify potential immune related lincRNAs in Ae. aegypti [27,28]. The results help better understanding of mosquito-pathogen interactions providing new insights on the potential role of lncRNAs as candidates for exploitation to inhibit replication of mosquito-borne viruses.

Methods
RNA-Seq Data preparation
Previously sequenced RNA-Seq raw data of Ae. aegypti were downloaded from NCBI Sequences Read Archive and ArrayExpress Archive with accession numbers SRA048559, SRA058076, SRA244067 and ERP002530 [29-32]. Raw data were stripped of adapters using CLC Genomic
Workbench version 7.5.1 and reads with quality score of above 0.05 and maximum 2 ambiguous sequences were retained for further analysis.

**Large gap mapping and transcript discovery**

The CLC Genomic workbench’s Transcript Discovery plugin was used for lincRNA discovery in the *Ae. aegypti* genome. New transcripts were identified by large gap mapping of 1,148,814,115 reads of 35 RNA-Seq libraries to the genomic reference (AaegL3.3). We implemented strict mapping criteria (mismatch, insertion and deletion costs: 2: 3: 3 respectively). The minimum similarity and length fraction of 0.9 between a mapped segment and the reference were allowed as part of the mapping criteria. The large gap mapper algorithm also requires each mapped segment to include at least 10% of the read with minimum length of 17 bases. We considered a gap with maximum of 50 Kbp distance between mapped read segments to span the introns from RNA-Seq data. The annotations were generated by inspecting mapping of reads and identifying likely regions corresponding to genes, including their exons and splice sites. The algorithm scans each gap in the read mapping to explore whether the gap is assigned to a valid splice site or can be relocated to a valid splice site without cost.

**lincRNA identification pipeline**

A rigorous filtering pipeline was developed to remove transcripts that may potentially encode proteins. The pipeline for *Ae. aegypti* lincRNA discovery is summarized in Figure 1. We identified 75,069 potential genes using the CLC Genomic Workbench transcript discovery algorithm. The genes that were annotated as known *Ae. aegypti* protein-coding genes were discarded and 30,865 potential genes were also checked for any exon or intron overlap with other known *Ae. aegypti* protein-coding genes. We selected 22,079 sequences, which were located more than 1kb away from any other known transcripts, for finding putative open reading frames (ORF). All possible six frames were produced for all selected sequences and then the translated sequences were subjected to a domain search to identify any putative conserved protein domains through Pfam v27.0 database [33]. We discarded 8,795 sequences with potential ORF above 100 aa or conserved protein domains. The remaining sequences were submitted to a coding potential assessment tool (CPAT), which utilizes a logistic regression model made with four sequence features: ORF size, ORF coverage, Fickett TESTCODE statistics and hexamer usage bias [34]. We applied the coding probability threshold of 0.3, which led to discarding 376 sequences as putative coding RNAs. We also implemented an expression threshold on our data to strengthen the identification pipeline. Sequences with more than 10 mappable reads in at least 17 out of 35 RNA-Seq libraries were considered as valid sequences and were kept for the next step. Any possible similarity with other
known proteins was found by using BLASTx algorithm against nr and Swiss port database (E-value cut off $10^{-5}$). Finally, 3,842 potential lincRNAs were identified and used for further study.

To identify *Ae. aegypti* putative lincRNAs that are regarded as small RNA associated lincRNAs, we used the Blast algorithm to search for *Ae. aegypti* precursor miRNA sequences in the predicted *Ae. aegypti* lincRNA dataset. We also used publicly available small RNA libraries from DENV-infected and non-infected samples (SRP026241) in this analysis for further characterization of lincRNA_1317. All known *Ae. aegypti* miRNA sequences were mapped to lincRNA_1317 for possible best fitting using RNAhybrid, which is a tool for finding the normalized minimum free energy (mfe) of RNA. We did not allow G:U pairing in the seed region (nucleotide 2-8) and required miRNA-lincRNA duplexes to have a helix in this region. Maximum 5nt were allowed as unpaired nucleotides in either side of an internal loop. LncTar algorithm [35] was used to explore any potential interaction between lncRNA_1317 and DENV-2 genome (accession no. NC_001474.2) by finding the normalized mfe joint structure of two RNA molecules based on base pairing.

**Identification of differentially expressed lincRNAs upon infection**

The *Ae. aegypti* genome was annotated with the final list of lincRNAs and used as reference for RNA-Seq analysis in CLC Genomic Workbench. To measure the lincRNA normalized expression value, RPKM (Reads Per Kilobase per Million reads) was assigned for each library [36]. To find the differential expression pattern in response to DENV infection, data from DENV-2 (Jam1409) infected midgut and carcass tissues in 4 days post-infection (dpi), were compared with their corresponding control groups [30]. Baggerley's test, a count based statistical analysis was done on the data. The samples were given weights depending on their total counts. Based on the test “the weights are obtained by supposing a Beta distribution on the proportions in a group, and estimating these, along with the proportion of a binomial distribution”. We selected 20 potential lincRNAs with more than 4-fold change for further analysis with RT-qPCR in DENV-2 (New Guinea C strain) infected *Ae. aegypti* cell line (Aa20) and screened their expression profile in *Wolbachia*-infected mosquitoes.

*Ae. aegypti* infected with the wMelPop-CLA strain of *Wolbachia* (+Wol) and without *Wolbachia* (-Wol, tetracycline-cured line) were stocks produced previously [37]. For the experiments in this work, 4-day-old female mosquitoes were used from which total RNA was extracted with 6-10 adult mosquitoes for each biological replicates.

**Expression analysis of *Ae. aegypti* lincRNAs**
Detection and validation of the relative abundance of selected lincRNAs was carried through lincRNAs’ specific primers using SYBR Green chemistry in real time PCR machine. Briefly, total RNA was extracted from cells using Qiazol reagent according to the manufacturer’s instructions (Qiagen). The TURBO DNA-free™ kit (Ambion, USA) was used to remove possible genomic DNA contamination in RNA samples. First strand cDNA was synthesized from 2.5 μg of RNA using a poly-dT primer and Superscript III reverse transcriptase (Life Technologies). qPCR primers were designed using primer design tool of NCBI [38]. QuantiFast SYBR Green PCR Master Mix with ROX was used to quantify the relative expression of lincRNAs between different treatments. Three independent biological replicates were considered along with three technical replicates for each treatment. Reactions were performed in a Rotor-Gene thermal cycler (Qiagen) under the following conditions: 95°C for 5 min, and 40 cycles of 95°C for 10s and 60°C for 30s, followed by the melting curve (68°C to 95°C). Melting curves were analysed to examine the specificity of amplification. Relative expressions were calculated using the Rotor-Gene software and the mosquito RPS17 as reference gene for normalization. Unpaired t-test was used to identify statistically significant differences.

**RNAi of selected lincRNAs and virus replication assay**

To check the functional importance of the identified novel lincRNAs, dsRNAs were synthesized to knockdown selected lincRNAs (2329, 1613 and 1317) to check their effect on DENV replication. Briefly, primers with added T7 promoter sequence (Table S1) were used to generate 250-600 bp PCR products from selected lincRNAs. Megascript T7 kit (Ambion) was used according to the manufacturer’s instruction to generate respective dsRNAs. To induce efficient RNA silencing, Ae. aegypti Aa20 cells were double transfected with dsRNAs against selected lincRNAs. Aa20 cells were re-suspended and ~3x10^5 cells were added to each well of a 12-well plate. Cell were allowed to settle for ~1 h, medium was removed and replaced with a transfection mixture consisting of 0.5 ml medium (1:1 Schneider medium and Mitsuhashi–Maramorosch with 10% FBS), 8 μl Cellfectin (Invitrogen), and 5 μg dsRNA either for selected lincRNAs or GFP as control. Cells were also treated with 3 μg dsRNA 72 h after the primary transfection to increase the silencing efficiency of selected lincRNAs. Six hours after the secondary transfection, cells were infected at 1 multiplicity of infection (MOI) with DENV2-NGC (New Guinea C strain). All the treatments were collected three days post-infection. RNA extraction and cDNA synthesis were carried out as above. qPCR was performed to confirm the knockdown and the effect of particular lincRNA knockdown on the genomic RNA of DENV-2. Each treatment was repeated three times. All data from three biological replicates were subjected to one-way ANOVA statistical analysis.
Brown-Forsythe test was used to check the equality of group variances and Tukey's multiple comparisons test was also used to examine significant statistical differences among treatments.

**Results and Discussion**

**Identification and characterisation of *Ae. aegypti* lincRNAs**

In total, 3,482 putative lincRNAs in 1,114 *Ae. aegypti* genome scaffolds were identified (Table S2). The *Ae. aegypti* lincRNA genes displayed a slightly lower GC content (mean: 40.1%) in comparison to 47.8% in their protein-coding gene sequences (Fig. 2A). The lower GC content or AT enrichment is a typical characteristic of lincRNAs and our findings are congruent with other predicted lincRNAs in other species [14,39,40]. The majority of *Ae. aegypti* predicted lincRNAs are smaller than 3000 bases and their length distribution is represented in Fig. 2B. These mosquito lincRNA candidates are notably shorter in length than protein-coding genes, demonstrating another well-known characteristic of lincRNA transcripts (Fig. 2C) [41,42]. The majority of *Ae. aegypti* genome scaffolds contain less than five lincRNA loci (~80%), however, 23 of scaffolds (2%) were enriched with more than 10 lincRNAs (Fig. 2D). The detailed information of these scaffolds, which contain the highest number of lincRNAs are summarized in Table 1.

We examined all the identified lincRNAs to determine their association with *Ae. aegypti* miRNA precursors and piRNA clusters. We found that the pre-miRNA sequences of aae-miR-2940 and aae-miR-285 are located in lincRNAs 1431 and 3299, respectively. We could not detect any other pre-miRNA sequences identified in *Ae. aegypti* in the lincRNAs. Also lincRNA 1978 and 792 are originated from two previously reported piRNA clusters [43] located at supercontig 1.478 and 1.98, respectively.

LincRNAs demonstrate low evolutionary sequence conservation even among closely related species [10,14]. We used the BLAST algorithm bit score to identify the level of similarity among *Ae. aegypti* lincRNA sequences with other closely related insect genomes such as *Aedes albopictus*, *Culex quinquefasciatus* and *Anopheles gambiae* (Fig. 3A). As expected, most of the identified lincRNAs showed high level of similarity with *Ae. albopictus* genome sequence and probably are genus specific. The E-value cut off $10^{-50}$ was applied to our screening with the BLAST algorithm to identify the conserved sequences. Although the *Ae. aegypti* lincRNAs shared high level of sequence similarity with the genome of *Ae. albopictus*, only 62 and 7 lincRNAs had sequence similarity with *C. quinquefasciatus* and *An. gambiae*, respectively (Fig. 3B). They were mostly limited to a single short region with high conservation.

*Ae. aegypti* lincRNAs change upon microbial challenge
Following the identification of *Ae. aegypti* lincRNAs, we analyzed their transcript levels in DENV-2 infected mosquito tissues. To produce the lincRNA profile of infected and non-infected mosquitoes, we re-analyzed previously published RNA-Seq data from *Ae. aegypti* midgut and carcass samples at 4 dpi [30] (Fig. 4). 248 and 203 lincRNAs with fold changes above four were identified in the RNA-Seq libraries of midgut and carcass, respectively (Table S2). The majority of differentially expressed lincRNAs were considerably overexpressed in both tissues. The abundance of only 32% of *Ae. aegypti* lincRNA candidates decreased in response to DENV-2 infection in the mosquito carcass sample. Thirty lincRNAs were differentially expressed in both examined tissues. The transcription levels of 72 lincRNAs increased after infection while their expression could not be detected in the non-infected midgut tissue sample.

We selected 20 candidates of those differentially expressed lincRNAs from RNA-Seq analysis data for further investigation. The relative expression of lincRNA candidates were examined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) upon DENV-2 infection in Aa20 mosquito cells. Only significantly overexpressed lincRNAs after DENV-2 infection are represented in Figure 5. Although we used Aa20 cells for the lincRNA expression assays, the expression patterns of almost all the examined lincRNAs (5 out of 6) were consistent with the RNA-Seq (adult mosquito carcass sample). We used a poly-dT primer to produce cDNA, which also confirmed that all of those identified transcripts have poly-A tails and therefore are true transcripts. Based on these results, significant increase in the transcription levels of a selected number of *Ae. aegypti* lincRNAs suggests their possible involvement in host-pathogen interaction but further investigations are required to confirm their roles in antiviral/immune responses.

We also examined the impact of an endosymbiotic bacterium, *Wolbachia*, on some selected *Ae. aegypti* lincRNAs, which showed significant changes in response to DENV-2 infection. This gram-negative bacterium is transmitted maternally and potentially infects more than 40% of all insect species, manipulating its hosts using strategies [44,45]. A fascinating aspect of *Wolbachia* infection is to limit replication of vector-borne pathogens in mosquitoes [45,46]. However, the mechanism(s) behind the inhibition of virus replication is largely unknown. Here, we found that the transcript levels of several lincRNA genes significantly increased in *Wolbachia*-infected *Ae. aegypti* mosquitoes (Fig. 6), which may lead to differential regulation of cellular protein-coding genes. Our previous studies showed that *Wolbachia* could manipulate host small ncRNA such as miRNAs and piRNAs [47]. An overall induction of small ncRNAs between 18 and 28 nucleotides was also observed in *Ae. aegypti* cell line infected with wMelPop-CLA strain of *Wolbachia* [48]. It was assumed that the upregulation of small ncRNAs in infected cells may result in an enhanced immune response and activated RNAi pathway. However, the role of these modifications in the host
lincRNA gene expression profile, and potentially in anti-viral responses, is unknown and may lead to the discovery of lincRNAs that could be utilized for inhibition of virus replication in mosquitoes.

A recent study on mouse bone marrow-derived macrophage (BMDM) model reported a significant upregulation in 72 lincRNAs after treatment with the synthetic bacterial lipoprotein Pam3CSK4, which acts through Toll-like receptor [49]. In another study, differential expression of approximately 500 annotated mouse IncRNAs was reported during infection with severe acute respiratory syndrome coronavirus [50]. Recently, it has been shown that honeybee’s lincRNAs are also differentially expressed during infection with various viruses such as sacbrood virus (SBV) and deformed wing virus (DWV), but the biological significance of these lincRNAs is completely unknown [51]. Although exploring the in vivo functions of immune-related lincRNAs is one exciting area for future studies, the differential expression of some lincRNAs could simply be byproducts of mRNA biogenesis or changes in global transcriptional profile due to microbial challenges [52,53]. Struhl (2007) believed that the transcriptional machinery is not perfect producing RNAs that serve no purpose or have no significant role in infection [54]. On the other hand, there are several examples which have shown that lincRNAs could be potentially important factors in host antimicrobial responses, and may represent a new class of signaling molecules involved in innate immunity or provide a new layer in gene regulation. For instance, two interferon (INF) induced IncRNAs, which were upregulated by influenza and vesicular stomatitis viruses, regulate the expression of the antiviral factor tetherin in human HuH7 cells [55].

**RNAi of selected lincRNAs and enhancement of DENV-2 replication**

To confirm the role of DENV-induced lincRNAs on viral replication, we used RNAi-mediated silencing of two selected lincRNAs (lincRNA_1317 and 1613) using dsRNA in Aa20 cells followed by DENV-2 infection. Only RNAi-mediated silencing of lincRNA_1317 led to enhancement of DENV-2 replication (Fig. 7A). Silencing of the lincRNA was confirmed by RT-qPCR (Fig. 7B). Interestingly, expression of *Ae. aegypti* lincRNA_1317 increased substantially following the progression of infection (Fig. 7C) suggesting that this lincRNA might be involved in antiviral response. This idea is consistent with the finding that lincRNA_1317 was also highly overexpressed (2.33 fold) in Wolbachia-infected mosquitoes as compared with non-infected mosquitoes (Fig. 6).

While there are no reports on the involvement of IncRNAs in host-pathogen interactions in insects, time-dependent over-expression of host lincRNAs in response to viral infection has been observed in humans. A recent study showed more than 80% of host cell IncRNAs were upregulated upon an adenovirus infection of human primary lung fibroblast cells [56]. Zhang et al. (2013) reported alterations of expression of cellular IncRNAs in HIV-1-infected T cells. Among
differentially expressed lncRNAs, NEAT1 expression notably increased in infected cells. When NEAT1 was silenced, virus production was enhanced by increasing the nucleus-to-cytoplasm export of HIV-1 transcripts containing Rev-dependent instability element [57]. A significant induction in this lncRNA expression in response to influenza virus and herpes simplex virus infection has also been shown [58].

To further investigate the potential role of lincRNA_1317 in mosquito-pathogen interaction, we determined its association with host endogenous small RNAs and its possible direct interaction with DENV. Although this lincRNA is not located in any of the known piRNA clusters, the majority of mappable small RNA reads to its sequence are in the range of 26-29 nt (Fig. S1). However, there was no difference in the mapping pattern and mapped read length distribution when reads from DENV-infected and non-infected small RNA libraries were mapped to lincRNA_1317 (Fig. S1). It has been shown that piRNA-like small RNAs have a large impact on lincRNA transcriptome [57], but our knowledge about the function of piRNA-mediated lncRNAs is still limited. Recently, it has been reported that piRNAs derived from transposons and pseudogenes facilitate the degradation of lncRNAs in mouse late spermatocytes [57].

Next, we hypothesized that *Ae. aegypti* lincRNA_1317 response to microbial challenge could be due to cross-regulation between miRNAs and lncRNA. *Ae. aegypti* miRNA recognition elements on lincRNA_1317 were identified by calculating the normalized minimum free energy (mfe) of hybridization for each *Ae. aegypti* miRNA and lincRNA_1317 using RNAhybrid core script. Binding site enrichment was detected for a few miRNAs with more than two recognition elements (Table 2). For instance, more than four recognition sites were predicted for miR-278-5p and miR-252-3p on lincRNA_1317. We also identified some hot spots for miRNA recognition sites on lincRNA_1317, which may allow multiple miRNAs to bind to the same regions (Fig. S2). miRNAs can reduce lincRNA stability by targeting their transcripts similar to mRNAs. Also, lincRNAs with multiple recognition sites may actually be competitive inhibitors of miRNA function and stopping them from binding to their genuine targets by sequestering them [21]. Although the mfe for some of those miRNA-lincRNA recognition sites suggests high probability of a binding event, further experimental investigations are required to validate this interface.

We also used LncTar algorithm to predict any direct interaction between lincRNA_1317 and DENV-2 genome. One potential interaction was predicted in the region 1-3370 of lincRNA_1317 and the region of 3210-6579 of DENV-2 genome with mfe of -61.73 (normalized dG -0.0184). This tool has accuracy rate of 80% [35], but does not consider the tertiary structure of RNA, which could play a role in RNA–RNA interactions and further studies are required to validate any potential interaction.
The involvement of lincRNA_1317 in host response to viral infection might be through its interactions with regulatory proteins that are involved in epigenetic changes by directly interacting with chromatin modifying enzymes or DNA binding proteins such as transcription factors. This interaction has been shown in several examples in mammalian systems, including host-virus interactions in which lncRNAs mediate antiviral responses by controlling the expression of immune-related genes (reviewed in [58]).

Although our knowledge of the biological function of this class of ncRNAs in mosquitoes is still limited, the results generated from this study will facilitate forthcoming explorations of lincRNA functions in insects. Clearly, further research is required to provide concrete experimental evidence to support the role of lincRNA_1317 or any other Ae. aegypti lincRNAs in host-pathogen interaction. With advances in technology, the mosquito lincRNA-protein interactions can be identified using high-throughput sequencing of immunoprecipitated RNA after cross-linking (CLIP-Seq). Further, functional studies could be carried out to characterize immune-related lincRNAs. The involvement of lincRNAs in pathways associated with responses to viral infection and cellular stress makes them interesting candidates as potential targets for manipulation to inhibit virus replication or control vector populations.

Acknowledgments
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References


Table 1. Distribution of potential *Ae. aegypti* lincRNAs in different genome scaffolds with more than 10 lincRNAs and their comparison with the number of protein-coding genes.

<table>
<thead>
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<th>Scaffold</th>
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Figure Legends

Figure 1. The lincRNA identification pipeline flowchart.

Figure 2. *Aedes aegypti* lincRNA characterization. A) Comparison of the GC content in protein-coding genes and the putative lincRNA genes. B) Sequence length distribution of *Ae. aegypti* lincRNA candidates. C) Comparison of gene length in protein-coding genes and putative lincRNA genes. D) lincRNA distribution among different *Ae. aegypti* genome scaffolds. The majority of scaffolds (~77%) only contain 1-4 lincRNAs, while only 23 *Ae. aegypti* genome scaffolds contain more than 10 lincRNAs (~2%).

Figure 3. *Ae. aegypti* lincRNAs share some conserved areas with other closely related species. A) The similarity bit score showed more similar sequences with high-degree of similarity were present in *Ae. albopictus*. B) The Venn diagram displays the number of *Ae. aegypti* lincRNAs with similarity score above the cut off (E-value above $10^{-50}$) in other species using the BLAST algorithm.

Figure 4. Volcano plot of differentially expressed *Ae. aegypti* lincRNAs in DENV-2 infected tissues (midgut and carcass) compared with their corresponding controls. Dots with red color represent lincRNAs with more than 4-fold changes due to DENV-2 infection.

Figure 5. DENV infection leads to changes in the abundance of *Ae. aegypti* lincRNAs. The relative transcript levels of selected numbers of *Ae. aegypti* lincRNAs were measured by RT-qPCR analysis of Aa20 cells infected with 1 MOI of DENV-2 for three days. Three biological replicates were used for each treatment with three technical replicates each. *, p < 0.05; **, p < 0.01; ****, p < 0.0001.

Figure 6. The transcript levels of *Ae. aegypti* lincRNAs were altered in Wolbachia-infected mosquitoes. RT-qPCR was used to analyze the relative transcript levels of selected numbers of *Ae. aegypti* lincRNAs in response to Wolbachia infection. For this, RNA from 4-day-old female mosquitoes from wMelPop (Pop)-infected and their tetracycline-cured line (Tet) mosquitoes were used in three biological replicates, each with three technical replicates. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

Figure 7. Possible involvement of lincRNA_1317 in DENV-2 replication in *Ae. aegypti* Aa20 cells. A) Aa20 cells were double transfected with the transfection reagent only (Mock), dsRNA to GFP
(dsCont) or dsRNA to lincRNA_1317 (dslncRNA) for three days followed by 1 MOI infection of the cells with DENV-2. Primers to the NS2A region (Table S1) were used for measuring the relative DENV-2 genomic RNA levels. B) RNAi silencing of lincRNA_1317 using dsRNA was confirmed by RT-qPCR. C) Changes in DENV-2 genomic RNA levels during the course of infection analyzed by RT-qPCR on RNA extracted from Aa20 cells at 1, 3 and 5 days post-infection (dpi). D) DENV-2 infection increased the transcript levels of *Ae. aegypti* lincRNA_1317 in 3rd and 5th days post-infection when RNA from cells were analyzed by RT-qPCR. In all the experiments shown in this figure, three biological replicates, each with three technical replicates were used. **, p < 0.01; ***, p < 0.001.
**Figure S1.** Length distribution of small RNA reads mapped to lincRNA_1317.

**Figure S2.** The miRNA recognition hot spot sites on lincRNA_1317.

**Table S1.** List of primers used in this study.

**Table S2.** Identified lincRNA candidates in *Ae. aegypti* and their genome coordinate.

**Table S3.** Differentially expressed lincRNAs in response to DENV-2 infection in midgut and carcass.
Mapping of 1,148,814,115 reads from 35 RNAseq libraries with CLC Genomic Workbench large gap mapper (version 7.5.2)

Transcript discovery: 75,069 seq. (>200nt)

Transcripts similar to known Ae. aegypti genes (44,204) Discarded

Exon or intron overlap with known genes (8,786) Discarded

ORF>100 aa and contain Pfam conserved domains (8,795) Discarded

Coding Potential Score CPAT > 0.3 (376) Discarded

Low expression & potential assembly error (8,826) Discarded

At least 1 hit in nr and RefSeq data base (870) Discarded

30,865 Novel Transcripts

No overlap with any known genes and located more than 1kb away from any other transcripts (22,079)

ORF <100 aa and no conserved domain detected through Pfam database and Swissport search (13,284) Discarded

Coding Potential Score <0.3 by CPAT algorithm (12,908) Discarded

Expressed with more than 10 reads and at least in 17 out of 35 RNA-seq libraries (4,713) Discarded

No hit for BLASTx against nr and RefSeq E-value cut off <10^-5 (3,843)

Ae. aegypti lincRNA potential candidates (3,842)
Midgut (4 dpi)

Carcass (4 dpi)