WOLBACHIA INDUCES RESISTANCE TO DENGUE VIRUS IN MOSQUITO *AEDES AEGYPTI*

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ABSTRACT

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Transmitted by the bite of mosquitoes, dengue virus (DENV) is the most important arboviral pathogen in humans. Currently no drug therapy or vaccine is available for dengue fever, leaving vector control as the major way to protect human populations. The endosymbiotic bacterium *Wolbachia* has been proposed as a potential agent for vector control, because it can not only spread within mosquito populations via manipulation of mosquito reproduction but also inhibit transmission of DENV in mosquitoes. In order to develop *Wolbachia*-based control strategies to prevent DENV infection, we have to better understand the interactions between DENV and *Wolbachia* in mosquitoes.

Aedes albopictus naturally carries *Wolbachia* infection, however, *Wolbachia*-mediated viral resistance was not observed in *Ae. albopictus*. In this study, we demonstrated that the native *Wolbachia* induce a resistance to DENV in *Wolbachia* density-dependent manner in *Ae. albopictus*. A decrease in *Wolbachia* density within the host cells results in increased dengue infection. We provide evidence that a very low *Wolbachia* density in mosquito tissues where DENV resides and travels could contribute to the absence of *Wolbachia*-mediated resistance to DENV in *Ae. albopictus*. We also investigated the impact of *Wolbachia* infection on DENV life cycle. We report here that *Wolbachia* is able to inhibit the intracellular accumulation of DENV in *Aedes. aegypti* mosquito cells. We showed that *Wolbachia* infection inhibits DENV binding to mosquito Aag2 cells. We then compared the DENV negative strand RNA levels in *Wolbachia*

infected and uninfected mosquito cells. *Wolbachia* infection also inhibits DENV replication in mosquito Aag2 cells.

To further investigate *Wolbachia*-DENV interactions in mosquito, we carried out microarray and real-time PCR analyses to define host cell transcriptional responses that are induced by *Wolbachia* infection. Our data indicates that sixteen previously identified DENV host factors were up-regulated in mosquito cells infected with *Wolbachia*. An RNA interference (RNAi) screen revealed eleven host factors that are associated with *Wolbachia*-mediated viral resistance. Our data provides several potential targets for interrupting dengue virus infection in mosquitoes.

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Chapter 1: Introduction

Dengue virus and Dengue fever

In recent decades, dengue fever, caused by infection with dengue virus (DENV), has become one of the most important emerging infectious diseases. The World Health Organization (WHO) estimates that there are 50-100 million dengue fever cases worldwide every year, and over 2.5 billion people are at risk of dengue infection (WHO, 2012). DENV infection is usually characterized by a high fever, severe joint pain, skin rash and flu-like symptoms, but a small proportion of infections develop into more serious conditions such as dengue hemorrhagic fever, or dengue shock syndrome. At present, no licensed vaccine or antiviral drug is available for dengue fever. Vector control is the primary method to reduce or prevent DENV transmission.

DENV is an RNA virus in the family *Flaviviridae*, genus *Flavivirus*. The DENV genome is a single strand positive sense RNA of approximately 11 kb in length. This RNA contains short untranslated regions (UTR) at both the 3' and 5' end, and a long open reading frame (ORF) encoding 10 genes. The whole viral genome is translated as one polypeptide chain that is then co- and post-translationally cleaved by viral and cellular proteases into three structural and seven nonstructural (NS) proteins (Murray et al., 2008). The structural proteins include a capsid (C) protein that binds and stabilizes the genomic RNA, a premembrane/membrane (PrM/M) protein that blocks immature virus fusion and may stabilize the envelope (E) protein conformation , and an E protein that facilitates virus binding, membrane fusion and viral assembly (Mukhopadhyay et al.,

2005). The NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) are involved in viral transcription, replication, assembly and attenuation of host immune responses (Murray et al., 2008, Bollati et al., 2010).

Dengue virions enter susceptible cells through clathrin-mediated endocytosis (van der Schaar et al., 2008, Acosta et al., 2008). After internalization, dengue virions are delivered to endosomes, where the low pH triggers the fusion of viral and endosomal membranes. Then the viral nucleocapsid, which consists of the viral genomic RNA and C protein, is released into the cytoplasm. In the cytoplasm, the viral RNA translation occurs at the rough endoplasmic reticulum (ER), giving rise to a single long polyprotein that is cleaved into 3 structural proteins and 7 NS proteins by host and viral proteases (Whitehorn and Simmons, 2011). The non-structural proteins form a replication complex that synthesizes new viral RNA. Viral replication starts with synthesis of the negative-strand RNA, which then serves as a template for the transcription of positive-strand RNA. The newly synthesized positive-sense RNAs are used for RNA replication, translation, or assembly of new Dengue virions, which most likely form on the ER membrane (Mackenzie and Westaway, 2001). New virions are further processed in the trans-Golgi network and are released from the cell through the cellular secretory pathway (Figure 1.1).

DENV is transmitted to humans by the bite of *Aedes* genus mosquitoes, primarily the urban-dwelling mosquito *Aedes aegypti* and, to a lesser extent, *Aedes albopictus*. After a mosquito bites a dengue-infected-person, DENV first infects the mosquito midgut epithelial tissue, then disseminates from the midgut to hemolymph and finally infects the salivary gland at approximately 7-14 days post-feeding. (Salazar et al., 2007). Once

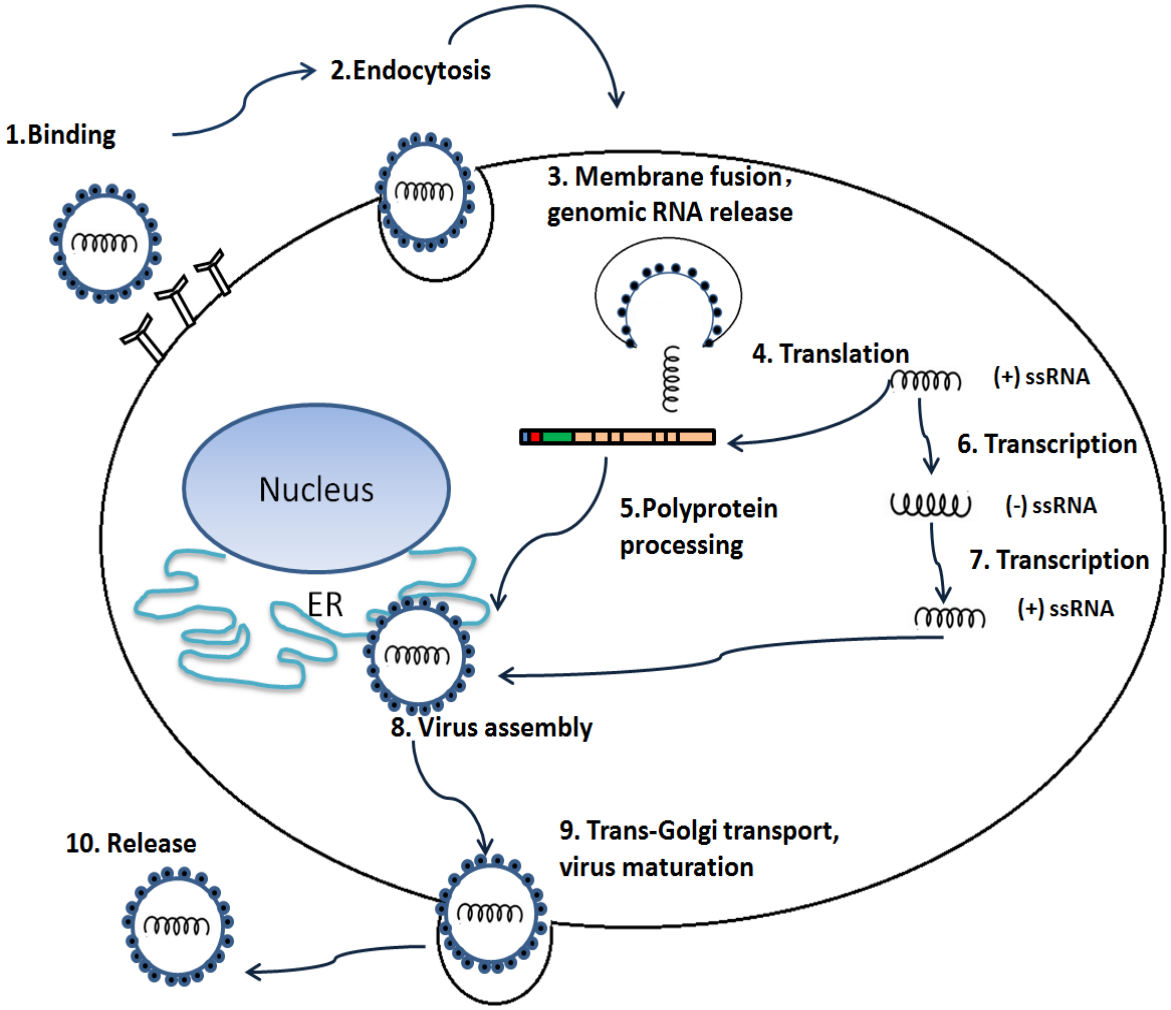

Figure 1.1: Overview of dengue virus life cycle (1) Virus particles attach to cell. (2) Dengue virions enter the cell by receptor-mediated endocytosis. (3) After fusion of the viral membrane with lysosomes, nucleocapsid enters into cytoplasm. (4) Viral genome is translated into single open reading frame. (5) Polyprotein is processed by host and viral proteases to produce individual proteins. (6) By using the positive-sense RNA as template, the viral RNA replication proteins synthesize negative-strand RNA. (7) The negative-strand RNA serves as template for synthesis of new positive-strand RNA. (8) Structural proteins and viral RNA assemble into immature viral particles on the ER membrane. (9) Newly assembled viral particles are further processed in trans-Golgi network, where they mature and change into infectious form. (10) Mature dengue virions are released into the extracellular environment.

Modified from Mukhopadhyay et al., 2005

DENV infects the salivary gland, it can be transmitted by the infected mosquito to a human host during blood feeding.

Wolbachia

Wolbachia is a maternally transmitted endosymbiotic bacterium that infects millions of arthropod and nematode species worldwide. Estimated frequencies of *Wolbachia* infected insect species is up to 65 percent (Hilgenboecker et al., 2008). *Wolbachia* belong to Rickettsiales, a group of obligate bacteria having mutualistic, commensalistic and parasitic interactions with various hosts. While the related genera *Anaplasma, Ehrlichia,* and *Rickettsia,* generally have life cycles including an invertebrate vector and mammalian host, *Wolbachia* does not infect vertebrates (Werren et al., 2008). Surrounding by a vacuole in cytoplasm, *Wolbachia* is bacilliform or coccoid in morphology with size ranging from 0.8 μm to 1.5 μm in length (Stouthamer et al., 1999).

Wolbachia was first described in the mosquito *Culex pipiens* in 1924 (Saridaki and Bourtzis, 2010), and has since been detected in many species of arthropods and filarial nematodes. Similar to most Rickettsiales, *Wolbachia* genome sizes are relatively small (1.08–1.7 Mb) (Werren et al., 2008), but they contain high numbers of mobile and repetitive elements (Saridaki and Bourtzis, 2010). Based on phylogenetic analysis of several genes including 16S ribosomal DNA, *wsp, ftsZ,* and others, *Wolbachia* has so far been taxonomically subdivided into 13 supergroups (namely A- N) (Doudoumis et al., 2013). The most common *Wolbachia* supergroups found in arthropods are A and B, while in filarial nematodes, supergroups C and D are dominant.

Wolbachia is obligate intracellular bacterium that reside within host-derived vacuoles. Typically, *Wolbachia* is transmitted vertically through the cytoplasm of eggs. The horizontal transmission of *Wolbachia*, which is fairly rare, allows those bacteria to move across species boundaries, resulting in the wide distribution of *Wolbachia* (Werren, 1997). *Wolbachia* establishes diverse symbiotic associations with their hosts, ranging from obligate mutualism in filarial nematodes, to commensal, or parasitic associations in arthropod hosts. To increase transmission frequencies, *Wolbachia* can induce host reproductive disorders including cytoplasmic incompatibility (CI), parthenogenesis, feminization and male-killing in arthropods.

CI, the most common phenotype in mosquitoes, occurs between infected males and uninfected females or infected females harboring different *Wolbachia* strains, and results in zygotic death (O'Neill and Karr, 1990, Zabalou et al., 2004). The CI mechanism is not yet fully understood, but it can be interpreted under a 'modification/rescue' model (Lassy and Karr, 1996, Callaini et al., 1997, Werren, 1997). According to this model, *Wolbachia* modifies sperm in infected males during early spermatogenesis, such that karyogamy failure occurs, leading to early embryo death (Xi et al., 2005). However, the modification can be rescued if the female carries a compatible *Wolbachia* strain, resulting in normal progeny development. There are two basic types of CI, unidirectional and bidirectional CI (Werren et al., 2008). Unidirectional CI occurs in mating between infected males and aposymbiotic females or single infected females and superinfected (infection with multiple *Wolbachia* strains) males (Figure 1.2 A, C). Bidirectional CI occurs in both directions when the individuals are infected by different *Wolbachia* strains (Figure 1.2 B).

Besides reproductive manipulation of arthropod hosts, *Wolbachia* is known to confer resistance against pathogens in insect hosts (Bian et al., 2010, Moreira et al., 2009). The phenotype was observed in *Drosophila*, where *Wolbachia* can induce resistance to DENV, Drosophila C virus (DCV), Flock house virus and Cricket Paralysis virus (Hedges et al., 2008, Teixeira et al., 2008). As observed in *Drosophila*, *Wolbachia* (*w*AlbB, *w*MelPop-CLA, and *w*Mel strains) was able to induce a strong inhibition to replication and dissemination of DENV in the transinfected *Ae. aegypti* (Bian et al., 2010, Walker et al., 2011). *Plasmodium* infection intensity was also significantly decreased in *Anopheles* mosquitoes transinfected with *Wolbachia* (*w*AlbB and *w*MelPop strain) (Bian et al., 2013, Hughes et al., 2011).

Recently, *Wolbachia* has attracted growing scientific and public attention as a control tool for arboviral diseases. The manipulation of hosts' reproductive systems, as well as vector pathogen-refractory phenotypes place *Wolbachia* among the most promising tools for mosquito-borne disease control. Several strategies for *Wolbachia*-based mosquito control have been proposed (Bourtzis, 2008). One such strategy is population replacement, in which wild mosquito populations would be replaced with *Wolbachia*-infected populations that are not competent to transmit pathogens. The first field release experiment utilizing *Wolbachia* (*w*Mel strain) transinfected *Ae. aegypti* was conducted in Australia. In the study, *w*Mel Wolbachia infection successfully invaded two natural *Ae. aegypti* populations within a few months following releases of infected adult mosquitoes (Hoffmann et al., 2011). One year post-release, field collected *w*Mel mosquitoes showed significantly reduced DENV replication and dissemination compared to *Wolbachia*-uninfected mosquitoes (Frentiu et al., 2014). Indeed, new field trials will be

carried out in other dengue-endemic countries, including China, Brazil, Indonesia, and Vietnam

In addition to a population replacement control strategy, another potential strategy is population suppression. Similar to Sterile Insect Technique (SIT), this strategy is also known as the incompatible insect technique (IIT) that uses Wolbachia-infected males to induce sterility within a mosquito population. Releases of incompatible males into a wild population successfully eliminated *Culex pipiens fatigans* in a village in Burma (Laven, 1967). A recent study also showed the potential of *Wolbachia* to eradicate *Aedes polynesiensis,* the primary vector of Lymphatic filariasis, in the South Pacific with population suppression (Brelsfoard et al., 2008).

Current understanding of *Wolbachia*-mediated Dengue virus inhibition in mosquitoes

The *Wolbachia*-mediated pathogen-refractory phenotype varies depending on the pathogen type, *Wolbachia* strain, infection type (natural, laboratory introduced) and host factors (Rainey et al., 2014). For example, *Wolbachia* (*w*AlbB strain) infected *Ae. albopictus* cell line Aa23 is more resistant to DENV compared to *Wolbachia*-cured controls. In contrast, *Wolbachia*-induced DENV resistance was not observed in *Ae. albopictus* mosquitoes, which naturally carry two *Wolbachia* strains (*w*AlbA and *w*AlbB) (Bian et al., 2010). *Wolbachia* (*w*Pip strain) native infected *Culex quinquefasciatus*

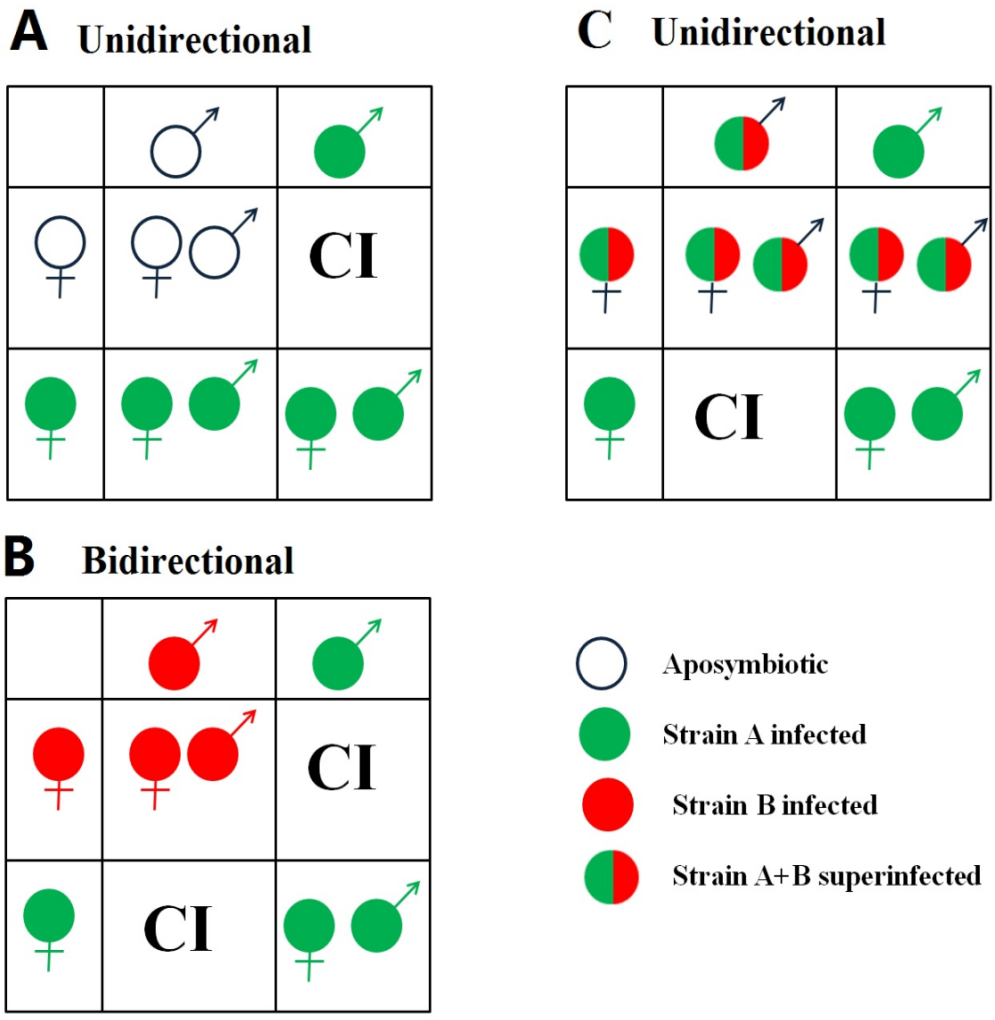

Figure 1.2: Examples of cytoplasmic incompatibility caused by *Wolbachia* (A) Unidirectional CI occurs in mating between infected males and aposymbiotic females. (B) Crosses of individuals infected with different, non-compatible *Wolbachia* strains result in bidirectional CI. (C) Unidirectional CI results from incompatible crosses between single infected and superinfected individuals.

Modified from Dobson, 2003, Sinkins and Gould, 2

mosquitoes are less susceptible to West Nile virus (WNV) infection compared to aposymbiotic controls (Glaser and Meola, 2010).

The underlying mechanism involved in *Wolbachia*-mediated DENV resistance in mosquitoes is still not clearly known; however, increasing numbers of studies suggest that the resistance is related to enhanced mosquito innate immunity. First, transcriptome analyses showed that *Wolbachia* infection activated a number of immune genes, for example cecropins, defensins, and several Toll and Imd pathway genes, in transinfected mosquitoes. Moreover, we found a positive relationship between *Wolbachia* density and the amount of defensin transcript, supporting the hypothesis that host immune level could contribute to *Wolbachia* induced viral resistance(Lu et al., 2012). Second, knockdown of the immune gene TEP1 by injection of specific dsRNA into *w*MelPop transiently infected *An. gambiae* has been shown to partially rescue the *Wolbachia*-mediated *Plasmodium* inhibition, which indicates up-regulation of innate immune responses contribute to this pathogen resistance phenotype(Kambris et al., 2010). In accordance with this observation, we found knockdown of antimicrobial peptides Defensin D and Cecropin C compromise resistance to DENV in *Wolbachia* transinfected *Ae. Aegypti*(Pan et al., 2012). Recently, we investigated the molecular mechanism of *Wolbachia*-mediated Toll pathway activation (Pan et al., 2012). In this study, we found *Wolbachia w*AlbB strain infection elevates the level of reactive oxygen species (ROS), and the increased level of ROS resulted in activation of the Toll pathway in *Ae. aegypti*. In addition, Pinto et al. reported that recombinant *Wolbachia* surface protein (WSP) stimulates immune gene expression in two different mosquito cell lines in a dosage dependent manner (Pinto et al., 2012).

In insects, RNA interference (RNAi) is an important innate immune response to virus infection. Mosquitoes have three major RNAi pathways: small interfering RNA (siRNA) pathway, microRNA (miRNA) pathway and Piwi RNA (piRNA) pathway (Ding and Voinnet, 2007). It is likely that siRNA pathway is not required for *Wolbachia*-induced antiviral protection. First, DENV replication was inhibited by *Wolbachia* in the C6/36 cell line that lacks a functional antiviral siRNA response (Frentiu et al., 2010, Brackney et al., 2010). Second, *Wolbachia* infection rendered siRNA mutant *D. melanogaster* more resistant to West Nile Virus infection (Glaser and Meola, 2010). Furthermore, Hedges et al. reported *Wolbachia*-mediated viral interference was present in all siRNA *Dropshilia* mutants (*dcr-z, r2de/CyO* and *AGO2*), indicating *Wolbachia*-mediated antiviral protection is independent of siRNA pathway (Hedges et al., 2012).

Although the siRNA pathway is not critical for *Wolbachia*-induced antiviral protection, the miRNA pathway may be involved in the interaction between *Wolbachia* and DENV (Zhang et al., 2013). Recently, Hussain et al. reported differential expression of miRNAs in *Ae. aegypti* mosquitoes transinfected with *Wolbachia* (*w*MelPop-CLA strain), and found a host miRNA (aae-miR-2940) was expressed only in *Wolbachia*-infected mosquitoes (Hussain et al., 2011, Zhang et al., 2013). Two target genes (metalloprotease m41 ftsh and AaDnmt2) of aae-miR-2940 were identified in *Ae. aegypti*. Metalloprotease m41ftsh gene was found to be up-regulated in the *Wolbachia*-infected host. Knockdown of this gene or inhibition of miR-2940 led to reduced *Wolbachia* density in mosquitoes and cells. *Wolbachia*-induced aae-miR-2940 negatively regulated the expression of the gene AaDnmt2, which in turn inhibited DENV replication. Conversely, overexpression of AaDnmt2 in mosquito cells led to an increase in DENV

replication, but a decrease in *Wolbachia* density (Zhang et al., 2013). These finding indicate the miRNA pathway is important for maintenance of *Wolbachia* density as well as the resistance to DENV in *Wolbachia* transinfected mosquitoes.

Evidence from recent transinfected hosts supports the hypothesis that *Wolbachia* interfere with viruses by elevating host basal immunity, while results from its natural host *Drosophila* suggest the up-regulation of immune genes is not the only mechanism responsible for *Wolbachia*-mediated viral interference (Rances et al., 2012). In *D. melanogaster*, *Wolbachia* (*w*MelPop and *w*Mel strains) could not induce transcription of antimicrobial peptides (AMPs), Toll pathway and melanization genes, but DENV inhibition occurred in the host (Rances et al., 2012). *D. simulans*, which naturally carries *Wolbachia*, showed no upregulation of immune effector genes compared to uninfected counterparts (Bourtzis et al., 2000, Wong et al., 2011). It has been hypothesized that the immune activation occurring in *Wolbachia* transinfected mosquitoes may be the consequence of the recent association with *Wolbachia*, and insects carrying long-term *Wolbachia* infections have evolved a certain degree of immune tolerance to *Wolbachia* (Blagrove et al., 2012).

Competition over common host nutrients such as cholesterol could also contribute to *Wolbachia*-mediated DENV resistance. Cholesterol is vital to a host, *Wolbachia*, and DENV. In insects, cholesterol plays a vital role in cellular signaling and membrane structure (Eaton, 2008). *Wolbachia* resides within cholesterol-rich Golgi-related vesicles, and requires cholesterol for its replication and propagation (Lin and Rikihisa, 2003, Cho et al., 2011). Both insect and *Wolbachia* are unable to synthesize cholesterol *de novo*,

thus both depend on and compete for dietary sterols as key nutrients (Clayton, 1964, Caragata et al., 2013). DENV is also dependent on host cholesterol for cellular entry and replication (Lee et al., 2008). Therefore, it is possible that changes in host cholesterol metabolism inflicted by *Wolbachia* influence DENV propagation. In *Ae. aegypti*, *Wolbachia* infection was found to reduce total cholesterol levels by 15-25% (Caragata et al., 2014). A recent study investigated whether competition for cholesterol is involved in *Wolbachia*-mediated pathogen resistance (Caragata et al., 2013). A significant increase in DCV titer was observed in both *w*MelCS- and *w*MelPop- infected *D. melanogaster* flies reared on cholesterol-enriched diets at five days post-infection, indicating the competition for cholesterol contributes to *Wolbachia*-mediated viral interference (Caragata et al., 2013).

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Chapter 2: *Wolbachia* Induces Density-dependent Inhibition to Dengue Virus in Mosquitoes

Introduction

Dengue fever and dengue hemorrhagic fever are emerging globally as a major public health problem in more than 100 countries. 2.5 to 3.0 billion people, or two fifths of the world's populations, are currently living in dengue-endemic areas. Each year there are an estimated 100 million new dengue cases and 22,000 deaths (Gubler, 1998). The number of cases reported to WHO has increased nearly nine times in the past 4 decades, and it continues to expand into temperate climates. Its fatal form, dengue hemorrhagic fever, has expended from Southeast Asia to 28 countries in the Western Hemisphere. Dengue virus (DENV) is transmitted to humans by the mosquitoes *Aedes aegypti* and *Aedes albopictus*. Currently no drug therapy or vaccines are available for dengue fever. Vector control is the primary intervention tool, but this barrier is weakened by increased pesticide resistance. This situation justifies seeking alternative and innovative approaches for the development of new prevention options. *Wolbachia*-based dengue control strategy is showing great potential because it could provide a solution that will be more sustainable, economical and environmental friendly than the other methods (Iturbe-Ormaetxe et al., 2011).

Wolbachia spp. are intracellular alpha-proteobacteria closely related to *Rickettsia*. Maternally inherited infections with *Wolbachia* occur in more than 65% of all the insect species and approximately 28% of the surveyed mosquito species (Werren et al., 2008, Kittayapong et al., 2000). Through the cytoplasmic incompatibility (CI) mechanism, *Wolbachia* can induce early embryo death when a *Wolbachia*-infected male mates with an uninfected female (Serbus et al.,

2008). Since infected females can successfully mate with uninfected males, *Wolbachia* can spread quickly in a population. This has been observed to occur naturally in *Drosophila simulans*(Turelli and Hoffmann, 1991), and demonstrated in *Ae. aegypti* through both laboratory cage studies and a recent field trial (Hoffmann et al., 2011, Xi et al., 2005).

Another important feature of *Wolbachia* is its ability to induce resistance to a number of human pathogens, including DENV, in its insect hosts (Walker et al., 2011, Hughes et al., 2011, Kambris et al., 2010, Hedges et al., 2008, Teixeira et al., 2008, Bian et al., 2010). In the transinfected *Ae. aegypti*, all the three different types of *Wolbachia*, *w*AlbB, *w*MelPop-CLA, and *w*Mel, show a significant inhibition to replication and dissemination of DENV, resulting in either complete or partial block of viral transmission (Walker et al., 2011, Bian et al., 2010, Moreira et al., 2009). Recent studies further show that *Wolbachia* induces production of reactive-oxygen species (ROS) which then activate Toll-pathway to induce expression of antiviral effectors (Pan et al., 2012). In the *Drosophila* host, native *Wolbachia* can also confer resistance to DENV and the other pathogens (Hedges et al., 2008). This resistance appears to be induced by the non-immune related mechanisms because the tested immune genes do not show differential expression in response to *Wolbachia*(Bourtzis et al., 2000, Rances et al., 2012). Inhibition of dengue virus replication was also observed in cell lines, and the extent of inhibition is related to bacterial density (Frentiu et al., 2010).

Ae. albopictus is generally considered as the secondary vector of dengue fever. This mosquito species naturally carries two types of *Wolbachia*, *w*AlbA and *w*AlbB, which distribute throughout both germ line and somatic tissues in mosquitoes (Dobson et al., 1999). However, *Wolbachia*-mediated viral interference was not observed in *Ae. albopictus* (Bian et al., 2010), consistent with the fact that it is a competent vector for at least 22 arboviruses (Gratz, 2004). The

recent study shows a transinfected *Ae. albopictus* line that carries *w*Mel is resistant to DENV (Blagrove et al., 2012), which excludes the possibility that *Ae. albopictus* lacks the genetic background for *Wolbachia* to induce viral interference. Thus, although both *w*AlbB and *Ae. albopictus* own the machinery to induce viral interference, the latter does not occur for unknown reasons.

In this work, we used an Aa23 cell line, initially established from eggs of *Ae. albopictus* (O'Neill et al., 1997), to study the mechanism underlying the lack of *Wolbachia*-mediated resistance to DENV. We confirmed that *w*AlbB induces a strong resistance to DENV in Aa23 cell line. Moreover, the levels of resistance strongly correlate with density of *Wolbachia* in Aa23 cells. Further comparison of genome copy of *w*AlbB in both somatic and germ line tissues between dengue resistant transinfected *Ae. aegypti* and susceptible *Ae. albopictus* suggests that *Wolbachia* density is too low to induce resistance to DENV in *Ae. albopictus*.

Methods

Mosquito rearing and cell culture maintenance

Houston (HOU) and HT1 strain of *Ae. albopictus* and WB1 strain of *Ae. aegypti* were maintained at 27 ℃ and RH 85% with a 12-hr light/dark cycle. *Ae. albopictus* Aa23, Aa23T cell lines (derived from Aa23 cells through tetracycline treatment) and *Ae. aegypti* Aag2 cell line were cultured as previously described (Dobson et al., 2002, Sim and Dimopoulos, 2010). Those cells were maintained in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS).

Introduction of *Wolbachia* into Aag2 cells

W-Aag2 cell line was generated by introducing *Wolbachia* from Aa23 cells into Aag2 cell line using shell vial technique as previously described (Dobson et al., 2002)with a slight modification. In brief, two 75-cm^2 flasks of confluent Aa23 cells were shaken, centrifuged at 1,000 x g for 10min, re-suspended in 1.5 ml Schneider's *Drosophila* Medium in a 50ml conical tube, and lysed by vortexing with 3-mm-diameter glass beads. The lysate was centrifuged at 2,500x g for 10min, and the supernatant was filtered through a 5μm syringe filter (Millipore). 500μl of the filtrate was overlaid on Aag2 cells grown in 12-well plate to 80% confluence. The plate was centrifuged at 2,000 x g at 15℃ for 1h and then incubated at 26 ℃ overnight. After that, the cells were transferred to a 25 ml cell flask with fresh medium. *Wolbachia* was stably maintained in W-Aag2 for more than 17 passages before used for dengue infection.

Density-dependent assay

To generate Aa23 cells with different *Wolbachia* densities, the cell culture was treated with rifampicin to make a final concentration at 5μg/ml, 0.5 μg/ml and 0.05μg/ml in 24-well plates. For each dose, cells were treated for four time periods: 5h, 10h, 40h and 70 hr. After treatment, the old medium was removed and cells were washed with fresh medium for one time. After adding new medium, cells were grown for another three days. Then, the cells were passaged to another 24 well-plate with new medium, and grown for one week. Two days after the 2^{nd} passage, cells were infected with DENV-2 at multiplicity of infection (MOI) of 0.1. At Day 5 post infection, cells were collected to measure the genome copy of *Wolbachia* and DENV-2. To study *Wolbachia*-density dependent expression of Defensin D (DEFD), cells were grown for 7 days in fresh medium after rifampicin treatment. Then, cells were collected to measure both the genome copy of *Wolbachia* and the expression of DEFD (Gao et al., 1999).

DENV-2 Infections in Mosquitoes

The New Guinea C strain of DENV serotype 2 (DENV-2) was propagated in C6/36 cells as previously described (Troyer et al., 2001). Virus was harvested 7 days post infection by collection of supernatants and centrifugation at 3,000 x g. The virus suspension was mixed in 1:1 with commercial ox defibrinated blood (Colorado Serum Company). The blood meal was warmed at 37 ℃ in water bath for 30 min and then used to feed 7-day-old mosquitoes as described previously(Das et al., 2007).

RNA Extraction, cDNA Synthesis and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The total RNA or viral genomic RNA were extracted from either cell lines or mosquitoes tissues by RNeasy Mini Kit (QIAGEN Sciences, Germantown, MD, USA) and then the cDNA were transcript using QuantiTect reverse Transcription Kit (QIAGEN Sciences, Germantown, MD, USA). Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN Sciences, Germantown, MD, USA) and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). DENV-2 genomic RNA was measured by qRT-PCR using primers directed to NS5 gene (Molina-Cruz et al., 2005). The dengue copy was normalized with host actin gene (Tortosa et al., 2008). A standard curve was generated for each of NS5 and actin by analyzing 10^1 to 10^8 copies/reaction of two plasmids containing each individual fragment (Tortosa et al., 2008). DENV-2 in mosquito head was diagnosed by RT-PCR with the same NS5 primers. DEFD was amplified with the primers: For (5′-GTCTGTTGCCAACTCTCTTT -3′) and Rev (5′- CACAAGCACTGTCACCAAC -3′).

***Wolbachia* Quantitative PCR (q-PCR)**

qPCR was performed to measure the *Wolbachia* density in the cell lines and mosquitoes (Tortosa et al., 2008). Genomic DNA was extracted from either Aa23 cells (two to six biological

replicates) or mosquito tissues (ten biological replicates). The primers specifically directed to *Wolbachia* surface protein (wsp) of *w*AlbA and *w*AlbB were used in PCR to measure the *Wolbachia* genome copy, which was normalized with the host actin for Aa23 cells (Tortosa et al., 2008) or ribosomal protein S6 (RPS6) gene for both *Ae. albopictus* and *Ae. aegypti* mosquitoes. RPS6 was amplified with the primers: For (5'-GAAGTTGAACGTATCGTTTC-3') and Rev (5'-GAGATGGTCAGCGGTGATTT-3'). A standard curve was generated for each of *w*AlbA, *w*AlbB and actin by analyzing 10^1 to 10^8 copies/reaction of the of the two plasmids containing each individual fragment (Tortosa et al., 2008). Another plasmid containing the RPS6 fragment was cloned to generate another standard curve for RPS6.

Susceptibility of the host cells to DENV infection

The susceptibility of Aa23, Aa23T, W-Aag2 and Aag2 cell lines for DENV-2 was measured by plaque assay with a slight modification (Das et al., 2007). DENV-2 virus stock (2x 10^7 PFU/ml; determined previously in C6/36 cells) was serially diluted in ten-fold increments (from 10^1 to 10^6) using cell medium, and then inoculated into each of the four mosquito cells in 24-well plates. After incubation at 26 ℃ for 7 days, the plates were assayed for plaque formation by peroxidase immunostaining, using mouse hyperimmune ascitic fluid and a goat anti-mouse HRP conjugate as the primary and secondary antibody.

Immunofluorescence

The viral antigen and *Wolbachia* wsp protein in Aa23 cells were detected simultaneously using an indirect immunofluorescence assay (IFA). Confluent rifampicin -treated Aa23 cells were grown on chamber slides (Thermo), and then were infected with DENV-2 at 0.1 MOI. After incubation for 2 days, cells were fixed for 30 min at 4 ℃ in 4% (w/v) paraformaldehyde in PBS, containing 0.5% (v/v) Triton X-100. Then, samples were incubated in PBS and 10% (v/v)

goat serum at room temperature for 1 h. Subsequently, the slides were incubated with a mouse anti-dengue complex monoclonal antibody at a 1:300 dilution and a rabbit anti-wsp polyclonal antibody (GenScript) at a 1:500 dilution in PBS at room temperature for 1 h. After wash, the slides were incubated with Alexa-488 anti-mouse and Alexa-594 anti-rabbit antibodies (Molecular Probes, Invitrogen) at a 1:1000 dilution in PBS at room temperature for 1 h. To stain cell nucleus, the samples were incubated with DAPI for 10 min. Immunostaining was examined under with an Olymupus Fluoview 1000 confocal microscope.

Results

The *Wolbachia w*AlbB induce strong resistance to DENV-2 in *Ae. albopictus* Aa23 cells

We previously reported that the *Wolbachia w*AlbB does not inhibit dissemination of DENV-2 to the mosquito head in the naturally infected *Ae. albopictus* (Bian et al., 2010). This could indicate that *w*AlbB does not induce resistance to DENV in the host genetic background of *Ae. albopictus*. To test that possibility, we compared susceptibility for DENV-2 between *w*AlbB-infected Aa23 cell line and its aposymbiotic Aa23T. Seven days after viral infection, the viral titer in Aa23T reached to 1.1×10^7 PFU/ml while no virus particle was detected in Aa23 cell line (Fig. 2.1A). A parallel assay was conducted to measure the *Wolbachia*-mediated viral interference in *Ae. aegypti* cell line Aag2. Like *Ae. aegypti* mosquito, Aag2 cell line is uninfected by *Wolbachia*. We introduced *w*AlbB from Aa23 into Aag2 through a shell-vial technique, generating a *Wolbachia*-infected cell line W-Aag2. As expected, viral titer in the Aag2 cell line (1.3×10^6 PFU/ml) is significantly higher than in the W-Aag2 cell line (12.5 PFU/ml; $P<0.01$, Student's t-Test; Fig. 2.1B). These results confirm that *w*AlbB can induce resistance to DENV-2 even in *Ae. albopictus* host background.

Generate Aa23 cell lines infected with *Wolbachia* at different densities

To study how the density of *w*AlbB can influence its induced resistance to DENV in Aa23 cell line, we used sub-lethal doses of rifampicin to partially remove *Wolbachia* and generated cell cultures with *Wolbachia* at different densities. Three dosages (0.05 μg/ml, 0.5 μg/ml and 5 μg/ml) with four treatment times (4 h, 10 h, 40 h and 70 h), resulting in a total of 12 treatments, were designed to make *Wolbachia* density cover a broad range. A mock treatment (control) was included to treat cells with only methanol solvent for the above four time periods. As a result (Fig. 2.2), we generated a serial of cell cultures with different *Wolbachia* densities, with a highest at 953.2 wsp/actin (from 0.05 μg/ml and 4 h) and a lowest at 18.0 wsp/actin (from 5 μg/ml and 70 h). *Wolbachia* density significantly decreased with an increase in treatment dose. Within each dosage, no significant difference in *Wolbachia* density is observed among four treatment times. This indicates a treatment for 4 h can effectively remove *Wolbachia* and a further increase in treatment time will not significantly enhance the inhibitory effect of rifampicin on *Wolbachia*. For example, a treatment at dosage of 5 μg/ml for 4 h can dramatically reduce *Wolbachia* to a very low level (75.2 wsp/actin), compared to the mock treatment (1,888.3 wsp/actin). Further increasing treatment time at this dose will not significantly change the *Wolbachia* density, resulting in 74.1 wsp/actin (10 h), 52.4 wsp/actin (40 h), and 18.0 wsp/actin (70 h, Fig. 2.2).

The *Wolbachia w*AlbB induces resistance to DENV in a density-dependent manner

To study the relationship between *Wolbachia* density and dengue infection level, cell cultures derived from the above 12 treatments were inoculated with DENV-2. Five days after infection, we measured genome copies of DENV-2 in the cells. *Wolbachia* density in the cells and its corresponding viral infection level were recorded in parallel to examine their interactions.

We observed that genome copy of DENV-2 in cells increased with a decrease in *Wolbachia* density. Moreover, there is a negative correlation between the genome copy of *Wolbachia* and DENV-2 ($r = -0.82$; $P<0.001$). The level of DENV-2 (y) can be predicted by the genome copy of *Wolbachia* (x) via a model: $y = -0.004x+3.847$ (Fig. 2.3). These results confirm that *Wolbachia* induces a density-dependent viral inhibition in Aa23 cells.

Wolbachia density in somatic tissues of _Ae. albopictus_ is too low to induce viral interference

Within mosquito vectors, DENV needs replicate and pass through midguts and salivary glands before transmission occurs. *Wolbachia*-mediated density-dependent viral inhibition in mosquito cells leads to a hypothesis that the *Wolbachia* density in the somatic tissues of *Ae. albopictus*is too low to induce viral interference in this mosquito species. Thus, we measured *Wolbachia* density in both somatic tissues (midgut, salivary gland, fatbody) and germ line tissue (ovary) of the HOU strain of *Ae. albopictus* (carrying *w*AlbA and *w*AlbB). As shown in Fig. 2.4A, *w*AlbB is significantly higher than *w*AlbA in all the four tissues. While none of *w*AlbA could be detected in midgut, salivary gland and fatbody, only 5.4 wsp/actin of wAlbA presents in ovary. There are 0.3, 5.3, and 12.3 wsp/actin of *w*AlbB in midgut, salivary gland and fatbody, respectively, compared to 57.9 wsp/actin of *w*AlbB in ovary.

To better compare *Wolbachia* density between the HOU strain of *Ae. albopictus* and the transinfected *Ae. aegypti* WB1 strain (carrying *w*AlbB), we designed one set of primers that can amplify a conserved region of Ribosomal protein S6 (RPS6) in both mosquito species, which was used to normalize the *Wolbachia* copy in qRT-PCR. We observed that *w*AlbB density is significantly lower in the midgut than in the ovary and salivary gland for both mosquito species ($P<0.05$, Student's t-Test). However, *w*AlbB density in fatbody is similar to that in salivary gland. Importantly, a significant lower density of *w*AlbB presents in midgut,

fatbody and salivary gland (P<0.001, Student's t-Test) of HOU mosquitoes as compared with that of WB1 mosquitoes. Specifically, the *Wolbachia* genome copies in midgut, fatbody and salivary gland of HOU are 80-, 18-, and 24-fold less than that of WB1, respectively (Fig. 2.4B). In ovary, *w*AlbB density is also 5.6-fold lower in HOU than in WB1 (P<0.001, Mann-Whiteney *U* test).

To confirm a lack of *Wolbachia*-mediated viral interference in *Ae. albopictus*, we compared mosquito susceptibility for DENV-2 at a serial diluted viral titers between the HOU and its aposymbiotic strain HT1 (initially derived from Houston strain through tetracycline treatment). We have previously observed that *Wolbachia* suppresses viral dissemination in the HOU mosquitoes after mosquitoes took infectious blood meal at a viral titer of 10^7 PFU/ml (Bian et al., 2010). However, it is possible that *Wolbachia* induces only a weak resistance to DENV in *Ae. albopictus* which could be overcome when a very high viral titer was used in the infection assay. As shown in Table 2.1, *Wolbachia* does not inhibit viral dissemination even mosquitoes took blood with a low viral titers. The head infection rate of both HOU and HT1 strain was dropped dramatically when mosquitoes fed with dengue-infected blood at diluted viral titers. No significant difference in head infection rate was observed between HOU and HT1 in all the three diluted viral titers (P>0.05, Fisher's exact test). These results confirm that *Wolbachia* does not suppress viral dissemination in the naturally infected *Ae. albopictus*.

Wolbachia induces expression of antimicrobial peptide DEFD in a density-dependent manner in _Ae. albopictus_ cells

We previously show that the boosted host immunity boosted plays important roles in *Wolbachia*-mediated viral inference and antimicrobial peptide defensin is involved in this anti-

dengue resistance (Pan et al., 2012).To test whether the above *Wolbachia*-density dependent viral inhibition in Aa23 cells relates to host immunity, we measured both *Wolbachia* density and the expression of DEFD in the antibiotic treated Aa23 cells. As shown in Fig. 2.5, there is a positive correlation between *Wolbachia* density and the amount of DEFD transcript ($r = 0.92$; $P = 0.0001$). This supports that host immune level could contribute to *Wolbachia*-density dependent viral inhibition.

Cells host both *Wolbachia* and DENV when *Wolbachia* density is low

To study whether the above antibiotic treatment leads to a uniform change in both *Wolbachia* and DENV at the cellular level, cells at two days post-DENV infection were assayed by double immunofluorescent staining to visualize the distribution of *Wolbachia* and DENV-2 using antibodies against the wsp of *Wolbachia* and envelop protein of DENV-2. As expected, we observed *Wolbachia* was largely removed by rifampicin treatment, resulting in more cells infected by DENV-2 (Fig. 2.6A–C). However, such a reduction in *Wolbachia* density appears not to be even, resulting in that certain cells host much more *Wolbachia* than the others (Fig. 2.6A–C). Importantly, we observed that colocalization of DENV-2 and *Wolbachia* can occur in the same cells when *Wolbachia* density is low in those cells (Fig. 2.6D–E). For those cells that were heavily infected by DENV-2, however, they typically do not contain *Wolbachia* (Fig. 2.6G–I).

	Head infection frequency, %	
PFU/ml	Houston	HT1
10^6	61.1 (11/18)	50.0 (9/18)
10^5	38.9 (7/18)	38.9 (7/18)
10^4	22.2 (4/18)	16.7 (3/18)

Table 2.1: The native *Wolbachia* does not inhibit dissemination of DENV-2 to mosquito heads in *Ae. albopictus.* The HOU strain of *Ae. albopictus* and its aposymbiotic strain HT1 were infected with the blood containing DENV-2 at titers of 10^6, 10^5, and 10^4 PFU/ml. At day 14 post infection, heads of ten mosquitoes were collected and used for diagnosis of DENV-2 by RT-PCR. Data from two experiments were pooled together.

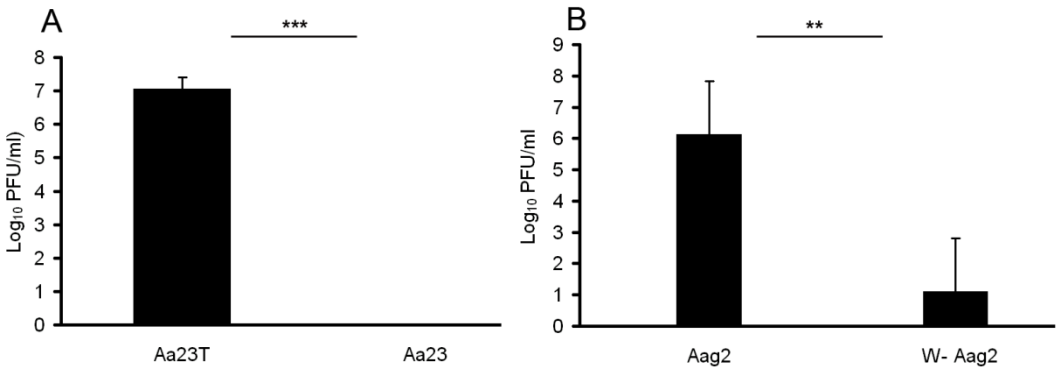

Figure 2.1: *w*AlbB induces a strong resistance to DENV-2 in both *Ae. albopictus* Aa23 (A) and *Ae. aegypti* Aag2 cell lines (B). *w*AlbB is a native infection in Aa23 cells while Aa23T cells were initially generated by tetracycline treatment of Aa23 cell to remove *Wolbachia* infection. There is no *Wolbachia* in Aag2 cells. W-Aag2 was generated from Aag2 cells by introducing *w*AlbB from Aa23 using a shell-vial technique. Five days after inoculated with DENV-2, the cells were tested for dengue infection by plaque assay. Error bars are standard errors. **, $P<0.01$; ***, $P<0.001$in Student's t-Test.

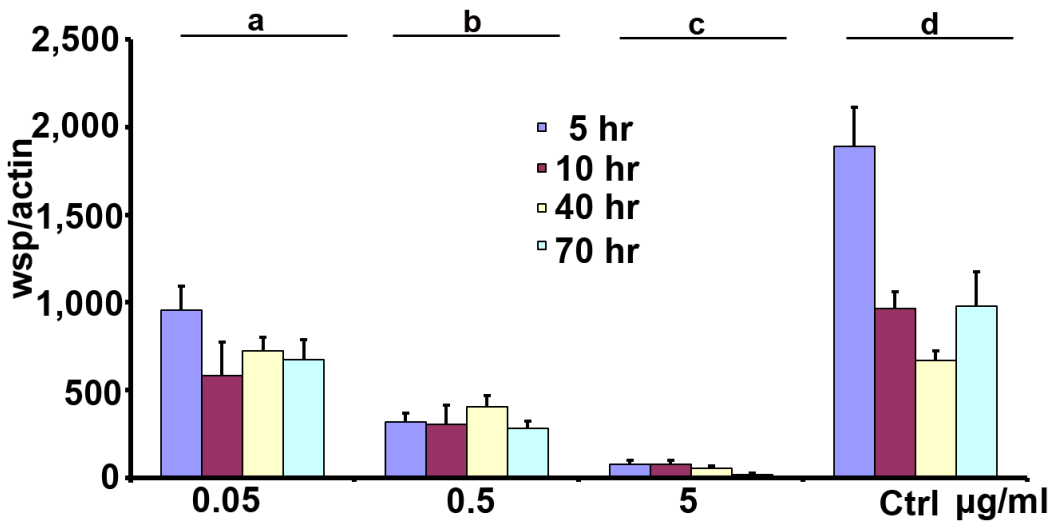

Figure 2.2: Generation of Aa23 cells with different *Wolbachia* density. Cells were treated using sub-lethal doses of rifampicin for a different time periods. Three dosages (0.05 µg/ml, 0.5 µg/ml and 5 µg/ml) and four time periods (4 h, 10 h, 40 h and 70 h) were used. The genome copies of wsp were measure by q-PCR, normalized by host gene actin. Error bars are standard errors of the mean of at least three biological replicates. Statistical significance is represented by letters above each column, with different letters signifying distinct statistical groups. Student's t test: a vs. b, $P<0.001$; b vs. c, $P<0.001$; d vs. a, $P<0.05$.

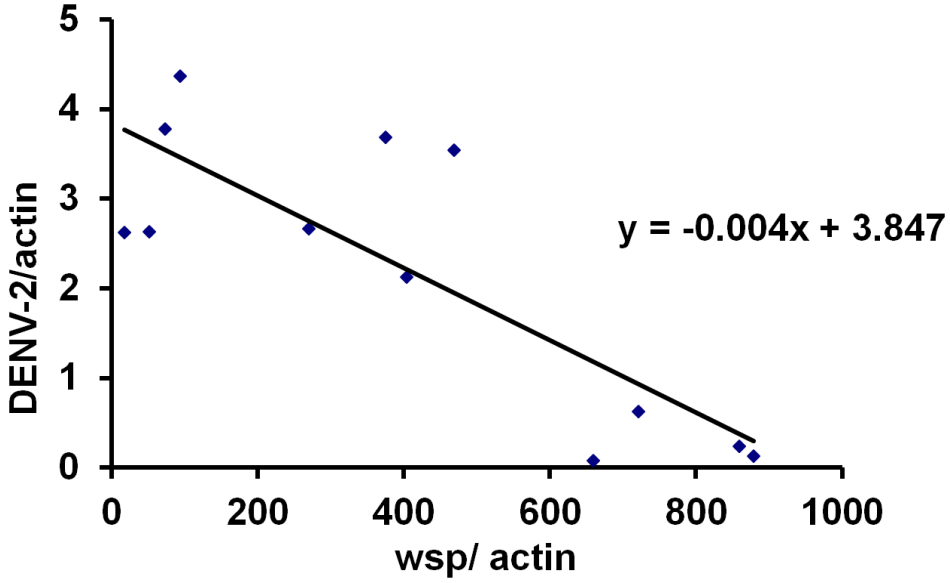

Figure 2.3: *Wolbachia* induces density-dependent inhibition to DENV-2 in Aa23 cell lines. Cell cultures derived from twelve different treatments in Fig. 2.2 were used in the assay. Five days after these cells were infected with DENV-2, viral genome copies were measured by qRT-PCR. Actin was used as a host gene to normalize the data. There is a negative linear correlation between *Wolbachia* density and DENV copy. Each point is the mean of at least three biological replicates**.**

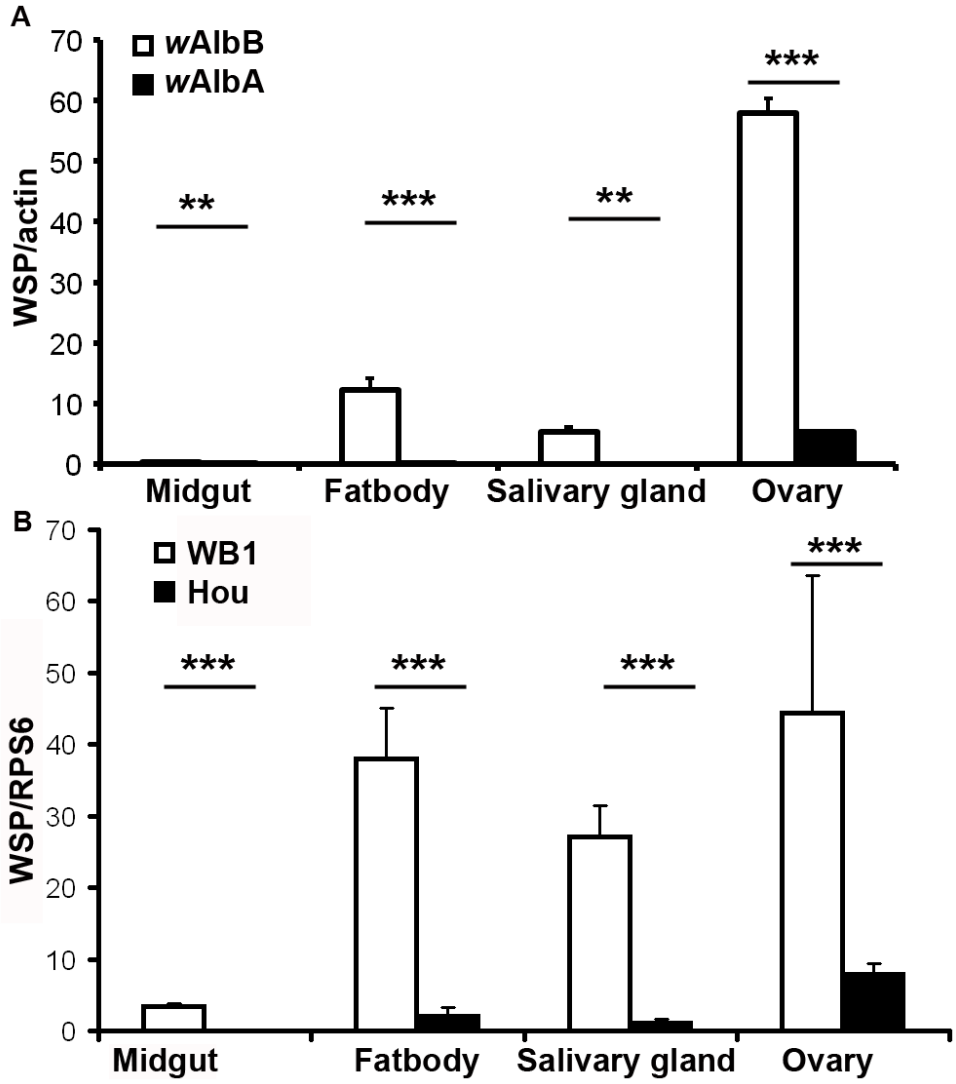

Figure 2.4: *Wolbachia* density in somatic tissues of *Ae. albopictus* is too low to induce resistance to DENV. (A) The density of *w*AlbA is significantly lower than *w*AlbB in midgut, fatbody, salivary gland and ovary of *Ae. albopictus*. The copy number of the *Wolbachia* wsp was normalized by *Ae. albopictus* actin; (B) *Wolbachia* density in somatic tissues is significantly lower in the *Ae. albopictus* HOU strain than in the transinfected *Ae. aegypti*WB1 strain. The copy number of the *Wolbachia* wsp was normalized by one conserved RPS6 in both *Ae. albopictus* and *Ae. aegypti*. In all the assays, midguts, salivary glands, fatbodies and ovaries of 7-day-old non blood fed females were dissected and used for extraction of total genomic DNA. ***, P<0.001; **, P<0.01 in Student's t-Test. Error bars are standard errors of the mean of ten biological replicates.

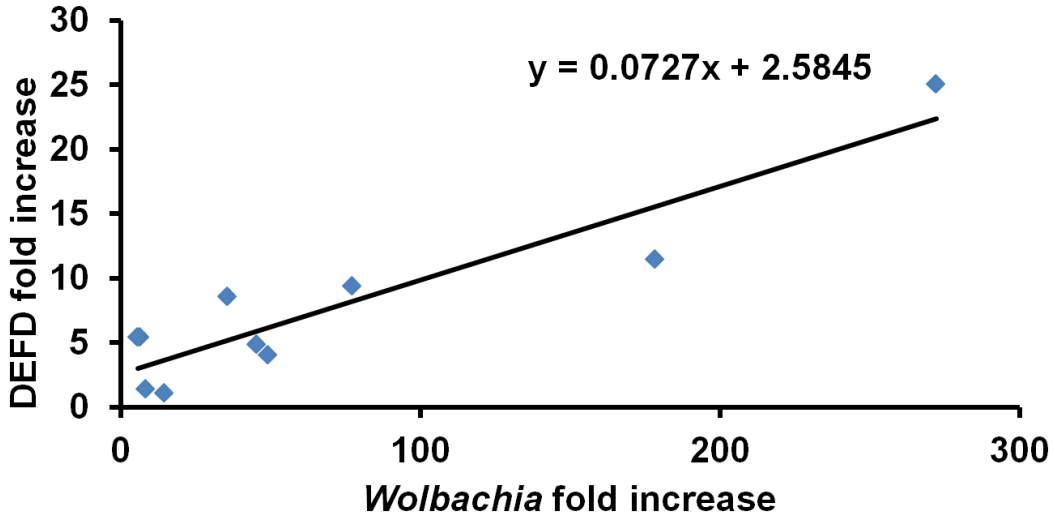

Figure 2.5: The expression of DEFD was induced by *Wolbachia* in density-dependent manner in Aa23 cell lines. Seven days after rifampicin treatment, cells were collected to measure *Wolbachia* densities and DEFD expression. Because treatment with 5 μg/ml of rifampicin for 70 h resulted in both *Wolbachia* infection and DEFD expression at lowest level, it serves as a reference for all the other treatments to calculate a fold increase in both *Wolbachia* density and DEFD expression. Each point is the mean of at least three biological replicate.

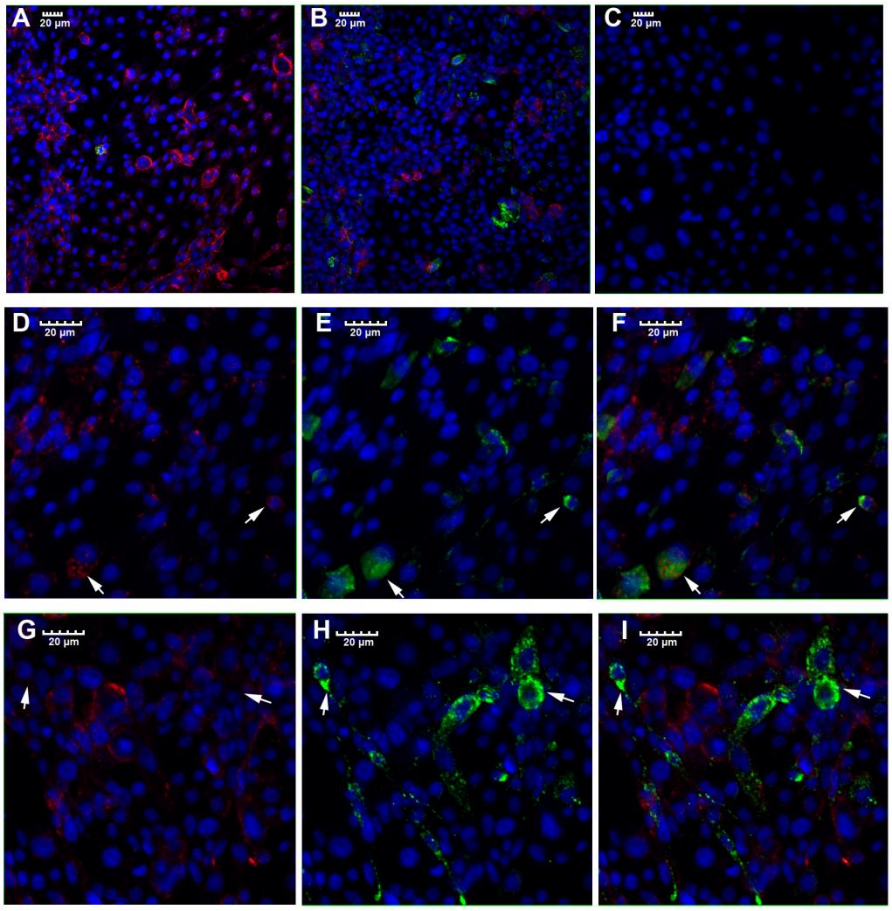

Figure 2.6: Localization of *Wolbachia* and DENV-2 in Aa23 cells. Double immunofluorescence staining of cells showing the localization of dengue virus (green) and *Wolbachia* (red). Cells were probed simultaneously with polyclonal anti-wsp antibody (*Wolbachia*) and monoclonal anti-DENV-2 antibody, followed by Alexa 594 (red) and Alexa 488 (green) conjugated antibodies, respectively. DNA (blue) is stained with DAPI. In panels (A, B, and C), the red, green and blue channels are merged. A and B show Aa23 cells with mock treatment and treatment with 5 µg/ml of rifampicin for 5 hr, respectively, followed by dengue infection. C is Aa23T cells without dengue infection (negative control). D to F or G to I is the same sample with different channel merged: D and G show only red and blue channel merged, E and H show only green and blue channel merged, F and I show all the red, green and blue merged. Aa23 cells treated with 5 µg/ml of rifampicin for 10 hr (D to F) and 40 hr (G to I), followed by dengue infection, are shown.

Discussion

Wolbachia can induce a resistance to DENV and other arboviruses in *Ae. aegypti* (Bian et al., 2010, Moreira et al., 2009). This raises a puzzle as to why mosquitoes that naturally carry *Wolbachia*, such as *Ae. albopictus*, can still serve as vectors for the arboviruses. Here, we demonstrate that *w*AlbB is able to induce resistance to DENV in *Ae. albopictus* Aa23 cell line, and such viral interference occurs in *Wolbachia* density-dependent manner. We also show that *Wolbachia* density in somatic tissues of *Ae. albopictus* is significantly lower than the transinfected *Ae. aegypti* WB1. Such a low density of *Wolbachia* is predicted not to induce viral interference in *Ae. albopictus*. Consistently, *Wolbachia* does not suppress viral dissemination in *Ae. albopictus* even when mosquitoes take an infectious blood meal at a very low viral titer. Moreover, we observed a positive correlation between *Wolbachia* density and the expression of the antimicrobial peptide DEFD. When *Wolbachia* density is low, DENV can colocalize with *Wolbachia* in the same cell hosts.

Consistent with previous studies, *Wolbachia* distributes in both somatic and germ line tissues in *Ae. albopictus* (Dobson et al., 1999). We further show that only *w*AlbB presents in midgut, salivary gland and fatbody while *w*AlbA was detected in only ovary. In this work, we focus on the above three somatic tissues because midgut and salivary gland are the two major sites for DENV to replicate and migrate through in order for mosquitoes to be infectious, and fatbody is the main immune organ to defend against foreign invaders (Beerntsen et al., 2000). Our results indicate that the *Wolbachia* infection in midgut and fatbody of *Ae. albopictus* may be too low to suppress viral replication in midgut and the subsequent dissemination into the other parts of mosquito body. This is supported by the lack of difference in the head infection rate between the HOU and HT1 strain. Comparison of *w*AlB infection between the dengue resistant

WB1 and the susceptible HOU strain shows that there is a general reduction in *Wolbachia* density in HOU strain and the magnitude of reduction in midgut is more than the other tissues. We also observed a significant higher *Wolbachia* density in salivary gland than in midgut in *Ae. albopictus*. This suggests that the future study should examine whether there is *Wolbachia*-mediated resistance to DENV in salivary gland or saliva.

We observe a linear negative correlation between *Wolbachia* density and genome copy of DENV-2 in Aa23 cell line. Based on the model ($y = -0.004x+3.847$), *Wolbachia* density at 961.8 wsp/actin is required to completely clean DENV in Aa23 cells. With the observed *Wolbachia* copy in midgut (0.3 wsp/actin) and salivary gland (5.3 wsp/actin) of *Ae. albopictus* HOU strain, *Wolbachia* would have no inhibition to DENV-2 in midgut and reduced DENV-2 by 0.6% in salivary gland in this mosquito species. However, it is possible that our model will be more appropriate to be used in the *in vitro* system. *Wolbachia* strains, host cells or tissues and environment may influence the viral inhibition, resulting in a modified linear correlation. Even that, a general pattern of *Wolbachia* density dependent viral inhibition should present in different systems. When *w*MelPop-CLA was introduced into two *Ae. albopictus* cell lines, RML12 and C6/36, the cell line with a high *Wolbachia* density (C6/36) shows a strong inhibition to DENV-2 (Frentiu et al., 2010). In *D. simulans*, *Wolbachia* strains that grow to high density provide the highest protection from virus infection (Osborne et al., 2009). All these indicate that *Wolbachia* density-dependent viral inhibition occurs for both native and non-native *Wolbachia* and cross different host cell types.

The linear negative correlation between *Wolbachia* density and dengue copy could lead to a simple hypothesis, in which *Wolbachia* secretes antimicrobial effector molecules into a host cell to directly inhibit pathogens. Such effectors should have a broad-spectrum activity against a

variety of pathogens, including virus, plasmodium and worm. Consistently, a bacterium was recently reported to produce ROS and induce resistance to malaria parasites in *Anopheles gambiae* mosquitoes (Cirimotich et al., 2011). The Type IV Secretion System, which presents within *Wolbachia* (Masui et al., 2000, Wu et al., 2004), was reported to secret different factors into host to mediate interactions between the other intracellular bacteria and their hosts (Stein et al., 2000). Our preliminary studies show that induction of DEFD expression occurs in *Wolbachia*-density dependent manner, providing us support to further test this hypothesis.

Currently there are two major modes to explain the mechanisms of *Wolbachia*-mediated viral interference (Teixeira et al., 2008, Rances et al., 2012). The first is *Wolbachia* boosts mosquito basal immunity against viral infection, while the second is that *Wolbachia* and virus compete for the same host resource. It appears that *Wolbachia* density dependent viral inhibition could be explained by both modes. In the first mode, a high *Wolbachia* density could cause high oxidative stress in host, leading to production of more ROS for a local antiviral immune response. The low *Wolbachia* density in somatic tissues of *Ae. albopictus* is consistent with the previous observation that *Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides in *Ae. albopictus*(Blagrove et al., 2012). In the second mode, more *Wolbachia* could lead to less host resource for virus. While it appears to be straightforward for the second mode to fit the density-dependent data, it will be challenging to identify the common host components needed by both *Wolbachia* and a variety of pathogens.

Our results indicate cell lines could be used as a simple *in vitro* system to study the mechanism of *Wolbachia*-mediated viral interference. DENV was strongly inhibited in *Wolbachia*-infected Aa23 and Aag-2 cells. Recent studies identified hundreds of host proteins that are required for DENV-2 propagation in cell lines (Sessions et al., 2009). Evidence indicates

that these host proteins form dengue-associated protein interaction networks, consisting of highly interconnected proteins with closely related functions in each of replication/transcription/translation, immunity, transport and metabolism (Guo et al., 2010). *Wolbachia* could perturb the above network to influence dengue infection. The cell culture provides us an ideal tool to study the interaction of *Wolbachia* with these dengue host proteins/networks.

In the field, a difference in *Wolbachia* density in mosquito vectors may directly influence the disease transmission. *Wolbachia* density was reported to differ largely among *Ae. albopictus* collected from the field in different locations in Thailand (Ahantarig et al., 2008). It is still unknown to what extent this will affect the vector status and vector competence of *Ae. albopictus*. However, conflicting results were reported on the relative susceptibility of *Ae. albopictus* versus *Ae. aegypti* to oral DENV infection (Lambrechts et al., 2010, Gratz, 2004). Some studies show that *Ae. albopictus* is more susceptible to DENV than *Ae. aegypti*, while the others show opposite results (Lambrechts et al., 2010, Rosen et al., 1985, Chen et al., 1993, Jumali et al., 1979, Vazeille et al., 2003). It was also reported a significant increase of susceptibility to DENV in *Ae. albopictus* with increasing generations in the laboratory (Lambrechts et al., 2010, Vazeille et al., 2003). Although generally considered a secondary vector of dengue, *Ae. albopictus* was the primary cause of dengue epidemics in a number of areas (Gratz, 2004, Hotta, 1998, Kobayashi et al., 2002, Metselaar et al., 1980, Effler et al., 2005). In China, *Ae. albopictus* has long been considered the primary dengue vector. Dengue epidemics frequently occurred without *Ae. aegypti*, with 22,122 new infections, including the fatal dengue hemorrhagic fever, recorded in single year (Gratz, 2004, Jin and Li, 2008, Kan, 1997). All the above suggest vector

competence and vector status of *Ae. albopictus* can vary in different locations. Future work should study the impacts of *Wolbachia* on vector status and vector competence of *Ae. albopictus*.

Our results could provide important implications for the future vector-borne disease control. First, novel control strategies can be developed to manually increase *Wolbachia* density in mosquitoes naturally carrying this bacterium. This could lead to block pathogen transmission to human but without a need to eradicate these mosquito species. Second, it is still unknown whether *Wolbachia* can persistently maintain infection at a level that effectively induces complete resistance to DENV in the transinfected *Ae. aegypti*. We could not exclude the possibility that adaption of a recent *Wolbachia* to a new host will finally lead to a low *Wolbachia* density, resulting in that what we see today in *Ae. albopictus* will be what happens tomorrow in those transinfected *Ae. aegypti*. Understanding how *Wolbachia* density is regulated by mosquito hosts and how the *Wolbachia* machinery controls its replication will facilitate the current effort to eliminate dengue through *Wolbachia*-based population replacement (Cook et al., 2008).

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Chapter 3: *Wolbachia* infection inhibits multiple stages of Dengue life cycle in mosquito *Aedes aegypti* cells

Introduction

Dengue viruses (DENV), a member of the family *Flaviviridae*, is the causative agent of dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. DENV infection is a major public health problem in the world, approximately 2.5 billion people are now at risk of dengue(WHO, 2012). DENV is transmitted to humans by *Aedes* mosquitoes. Currently, effective vaccines and antiviral therapies to prevent or treat DENV infections are unavailable. Vector control is the primary intervention method, but this tool is failing to prevent the global spread of dengue (Guzman et al., 2010). Innovative approaches are needed to control vector populations.

DENV is an enveloped positive-strand RNA virus with genome of approximately 11 kilobases. The DENV genome has a single open reading frame which encodes three structural proteins (capsid (C), membrane(M) and envelope (E) protein), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A and NS5). The infection process begins with the binding of the virions to the cellular receptors on the surface of susceptible cells. After receptor binding, DENV enters cells by receptor-mediated endocytosis. The acidification of endosome lumen promotes fusion of the viral membrane with the endosome membrane, thus delivering nucleocapsid into the cell cytoplasm. Following uncoating of the nucleocapsid in the cell cytoplasm, the viral RNA replication and translation start. Immature virions assembly occurs on the surface of the endoplasmic reticulum (ER) when the newly formed nucleocapsids bud into the lumen of ER. Subsequently, the immature virions are transported through the trans-Golgi network (TGN),

where they mature and from infectious particles. Finally, the mature viruses are released from the cell by exocytosis.

Wolbachia is a maternally transmitted intracellular symbiotic bacterium belonging to the α-proteobacteria. *Wolbachia* is believed to the most pervasive symbiont; it has been detected in most orders of insects and infects more than 60% of all insect species worldwide (Hilgenboecker et al., 2008). *Wolbachia* has been placed at the frontline of novel approaches to control Dengue infection. Through the cytoplasmic incompatibility (CI) mechanism(Werren, 1997), *Wolbachia* can spread quickly in a mosquito population. Another advantage of using *Wolbachia* for disease control is its ability to induce resistance to DENV in mosquitoes(Walker et al., 2011, Bian et al., 2010). However, the mechanism that underlying resistance remains poorly understood. In addition, the impact of Wolbachia infection on DENV life cycle has not been demostrated.

Our previous studies have shown that *Wolbachia* induce strong resistance to DENV serotype 2 (DENV-2) in *Wolbachia*-infected *Ae. aegypti* cell line W-Aag2. Here, we explored the impact of *Wolbachia* infection on DENV-2 life cycle in *Wolbachia* infected and uninfected mosquito cell lines, W-Aag2 and R-Aag2.

Materials and Methods

Cell culture and DENV-2 infection

Ae. aegypti W-Aag2 cell line was generated by introducing *Wolbachia* into Aag2 cells using centrifugation enhanced (shell vial) technique as previously described (Lu et al., 2012). W-Aag2 and R-Aag2 cell line (derived from W-Aag2 cells through rifampicin treatment) were

maintained at 25 ℃ in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies), and were passaged at 1:5 dilution every 6-7 days.

New Guinea C strain (NGC) of DENV-2 was used in this study. DENV-2 was grown in W-Aag2 and R-Aag2 cells as previously described (Sim and Dimopoulos, 2010). Briefly, cells were seeded in 48-well plates to a confluency of 80%. W-Aag2 and R-Aag2 monolayers were infected with DENV-2 at a desired multiplicity of infection (MOI) of DENV-2 in culture medium. Plates were incubated at 25 ℃ for the duration of the experiment.

DENV-2 binding assay

DENV binding assay was carried out to characterize the attachment of DENV to W-Aag2 and R-Aag2 cells. Prior to the binding experiment, the culture medium was removed, and cells were washed with cold Schneider's *Drosophila* Medium. Subsequently, DENV-2 was incubated with the cells at MOI of 1 or 10 for 1 hour at 4 ℃. The incubation medium was collected for measuring the titers of unbound viruses. Then, the cells were washed three times with cold PBS to remove the unbound viruses. 350 µl buffer RLT (Qiagen) was added to each well for RNA extraction. The gene copies of bound DENV-2 were quantified by real-time PCR.

RNA Extraction, cDNA synthesis and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cell lines by RNeasy Mini Kit (Qiagen), and then the cDNA transcript was produced using QuantiTect reverse Transcription Kit (Qiagen). Real-time PCR was conducted using the QuantiTect SYBR Green PCR Kit (Qiagen) and ABI Prism

7900HT Sequence Detection System (Applied Biosystems). DENV-2 genomic RNA was measured by qRT-PCR using primers directed to NS5 gene (Molina-Cruz et al., 2005).

Negative strand viral RNA was quantified using Tagged RT-PCR, a technique shown to be able to amplify specifically negative RNA by preventing false priming (Peyrefitte et al., 2003). The primer tagF (5′-CGGTCATGGTGGCGAATAAACAAGTAGAACAACCTGGTCCAT-3') was designed to contain the DENV-targeting sequence in its 3' end and a 19-mer-long non-DENV sequence in its 5' end. After RNA extraction, the RNAs were denatured at 65 ℃ for 3min in the presence of 20pmol of tagF primer for the negative strand-specific reverse transcription. cDNA was synthesized without addition of the RT primer mix. Real-time PCR was performed with a forward primer Tag (5'-CGGTCATGGTGGCGAATAA-3') and a DENV-targeting reverse primer, as previously described (Molina-Cruz et al., 2005). The host ribosomal protein S6 (RPS6) gene was used to normalize cDNA template.

DENV-2 RNA transfection

W-Aag2 and R-Aag2 cells were plated in 48-well plates 24 hours prior to transfection and were approximately 70-80% confluent at the time of transfection (1×10^5 cells/well). DENV-2 RNA was extracted from virus infected cell culture supernatant using RNeasy Mini Kit (Qiagen). The DNV infectious RNA was transfected using TransIT-mRNA Transfection Kit (Mirus) according to the manufacturer's instructions. Briefly, DNEV-2 RNA (0.5 μg) was incubated with 1 μl mRNA Boost Reagent and 1 μl *TranIT*-mRNA Reagent in 26 μl Schneider's *Drosophila* Medium for 5 minutes and then was transferred to 70-80% confluent W-Aag2 and R-Aag2 cells in a 48 well plate (1×10^5 cells/well) containing 260 μl complete medium.

Plaque assays for DENV-2 virus titration

The DENV-2 titers were measured by plaque assays, as previously reported(Das et al., 2007, Bian et al., 2010). Briefly, C6/36 cells were seeded in 48-well plates at a density of 4-8 × 10^4 cells/well and maintained for 2-3 days at 32 ℃ in 5% CO_2. The virus-containing culture medium was serially diluted and inoculated into C6/36 cell. After incubation for 5 d, plaque forming uinits (PFUs) were measured by the plates by peroxidase immunostaining, using mouse hyperimmune ascitic fluid (specific for DENV-2, CDC) as the primary antibody and a goat anti-mouse horseradish peroxidase(HRP) conjugate as the secondary antibody.

Results

Wolbachia inhibits the intracellular accumulation of DENV genome copies in Aag-2 cells

We previously reported that *Wolbachia w*AlbB induces density-dependent inhibition to DENV in mosquito cells. To further investigate the profile of DENV production in *Wolbachia* transinfected W-Aag2 cell line and its aposymiotic R-Aag2 (derived from W-Aag2 cells through rifampicin treatment), cells were infected with DENV-2 at an MOI of 1 and the genome copies of DENV-2 were measured by qRT-PCR at 8 time points over the course of 9 days post-infection (dpi). Results (Figure 3.1) showed that DENV RNA level declined in both R-Aag2 and W-Aag2 cells by 1 dpi, then it increased daily in R-Aag2 cells, while DENV genomic RNA level did not significantly change after 1 dpi in W-Aag2 cells. At 2 hours post infection, the mean DENV genomic RNA copies in R-Aag2 cells (0.0072 DENV/RPS6) was three times higher than in W-Aag2 cells (0.0023 DENV/RPS6) ($P<0.01$, Student's t-Test), indicating *Wolbachia* infection may interfere with early events DENV-2 life cycle. On 1 dpi, the mean of DENV genomic RNA copies in R-Aag2 cells (0.096 DENV/RPS6) was 55 fold higher than in W-Aag2 cells (0.0017

DENV/RPS6). On 9 dpi, the number of viral copies in R-Aag2 cells (4.3 DENV/RPS6) was 1500 times higher than in W-Aag2 cells (0.0028 DENV/RPS6). Those results indicate that *Wolbachia* infection inhibits intracellular accumulation of DENV genome copies in Aag-2 cells.

Wolbachia infection inhibits DENV binding to Aag-2 cells

To better understand of the mechanism behind *Wolbachia*-mediated viral interference, we analyzed the DENV binding to mosquito cells. R-Aag2 and W-Aag2 cells were incubated with DENV at multiplicity of infection (MOI) 1 and MOI 10 for 60 min at 4 ℃ in order to avoid virus penetration. Subsequently, cells were washed three time with ice-cold medium. RNA copies of DENV that bound to cells were determined by qRT-PCR. The amount of DENV binding to W-Aag2 cells (0.0033 DENV/RPS6) is significantly lower than the amount bound to R-Aag2 cells (0.013 DENV/RPS6) at a MOI of 10 ($P<0.0001$, Student's t-test) (Figure 3.2.A). Similar results were obtained when mosquito cells were exposed to a low level of input virus(MOI=1). The viral genomic copy number was only 0.0006 DENV/RPS6 in W-Aag2 cell line, as compared to 0.0019 DENV/RPS6 in R-Aag2 cells ($P<0.0001$, Student's t-test) (Figure 3.2.A). We also carried out plaque assays to measure titers of unbound DENV in incubation medium. When mosquito cells were incubated with DENV at 10 MOI, unbound DENV titer in the incubation medium of W-Aag2 cells (3.4×10^6 PFU/ml) was significantly higher than it in the incubation medium of R-Aag2 cells (1.6×10^6 PFU/ml, $P<0.05$, Student's t-test) (Figure 3.2.B). Similar results were observed when cells were infected with DENV at a MOI of 1. We next performed the same assay at 25 ℃. The viral genomic RNA level of W-Aag2 cells was 0.0036 DENV/RPS6 at 10 MOI, as compared to 0.022 DENV/RPS6 for the R-Aag2 cells ($P<0.0001$, Student's t-test). A similar pattern was also observed at a MOI of 1, where the viral genomic RNA copy number in W-Aag2 cells was 0.00058 DENV/RPS6, as compared to 0.0038

DENV/RPS6 ($P<0.0001$, Student's t-test). Those results indicate that *Wolbachia* infection inhibits DENV binding to mosquito cells at both high and low MOI.

Wolbachia inhibits DENV replication in Aag-2 cells

DENV negative sense antigenomic RNA is a hallmark of active DENV replication (Tuiskunen et al., 2010). To decipher whether *Wolbachia* infection interfere with DENV RNA replication, we first delivered infectious DENV RNA into cells by transfection, which could bypass the early events of DENV infection (binding, entry, nuclocapsid relase, and uncoating). Then we conducted tagged RT-PCR to achieve negative strand-specific amplification (Peyrefitte et al., 2003). DENV-2 RNA (0.5 μg) was transfected into W-Aag2 and R-Aag2 cells (1×10^5 cells/well) in 48 well plates. Cells were sampled at 4 hour, 1 day, 3 day and 7 day post RNA transfection, followed by RNA extraction, reverse transcription and tag-PCR amplification. No significant differences were observed in the relative amount of DENV-2 total RNA between W-Aag2 and R-Aa2 cells at 4 hours post-transfection ($P=0.13$, Student's t test) (Figure 3.3.A), indicating comparable amounts of DENV-2 RNA were transfected into two cell lines. As shown in Figure 3.3. B, negative strand RNA levels in R-Aag2 cell line were significantly higher than in W-Aag2 cell line at day1, day3 and day7 post transfection ($P<0.05$, Student's t test). DENV titer of supernatant was measured by plaque assay at 5 days post transfection. Viral titer in the supernatant of R-Aag2 cells (6×10^6 PFU/ml) was significantly higher than in W-Aag2 cells (2.6×10^3 PFU/ml, $P<0.05$, Mann Whitney test) at 5 day post-transfection (Figure 3.3.C). Those results indicate that *Wolbachia* infection inhibits DENV replication.

Wolbachia infection regulates expression of DENV binding proteins

In mosquitoes, several DENV binding proteins have been described (Colpitts et al., 2011, Munoz et al., 2013, Kuadkitkan et al., 2010). To better understand the *Wolbachia*-induced

DENV inhibition, we performed qRT-PCR to measure the expression profiles of 19 reported DENV binding proteins in W-Aag2 and R-Aag2 cells. Using ΔΔCt based fold change calculations with change >2 or <2 fold as cut off levels (Schmittgen and Livak, 2008), we found 6 genes were down-regulated and 5 genes were up-regulated in W-Aag2 cells (Table 3.1.). Among those differentially expressed proteins, beta tubulin ,cadherin and actin have been shown to associated with viral entry (Munoz et al., 2013); histone, enlogation factor, actin and beta are thought to involved in viral transcription and translation.

VectorBase ID	Average fold change	Gene name	Viral partner protein
AAEL003863	-24.5	Histone 4	Capsid
AAEL015390	-6.45	Histone 2A	Capsid
AAEL002851	-4.2	Beta tubulin	E, NS2A
AAEL017096	-4.23	Elongation factor 1-alpha	E,NS2A, NS4B
AAEL003670	-3.02	Myelinprotein expression factor	NS2A
AAEL001928	-2.92	Actin	Capsid,NS4B
AAEL001668	2.51	Enolase	Capsid, E
AAEL003594	2.67	Kinectin	Capsid
AAEL001411	2.81	Myosin heavy chain	E , NS2A
AAEL001196	2.95	Cadherin	E
AAEL000386	3.36	PI3 kinase	E

Table 3.1: Differentially expressed DENV binding proteins in W-Aag2 and R-Aag2 cells. qRT-PCR was used to assess the expression of DENV binding proteins. RPS6 was used as an internal reference control. $2^{-\Delta\Delta C}{}_{T}$ method was used to calculate fold change for each gene. A total of 11 genes were differentially expressed between *Wolbachia*-infected and uninfected cells with a fold change <-2 or >2.

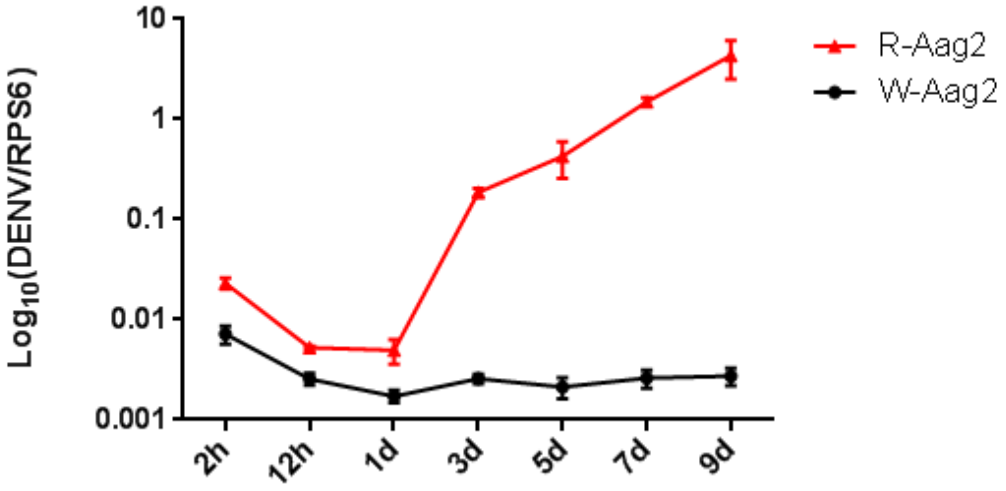

Figure 3.1: DENV infection of W-Aag2 and R-Aag2 cells. Time course of DENV RNA level in *Ae. aegypti* W-Aag2 and R-Aag2 cells. The DENV genome copies were measured by qRT-PCR. RPS6 was used as a host gene to normalize the data. Error bars are standard errors of the mean of three biological replicates.

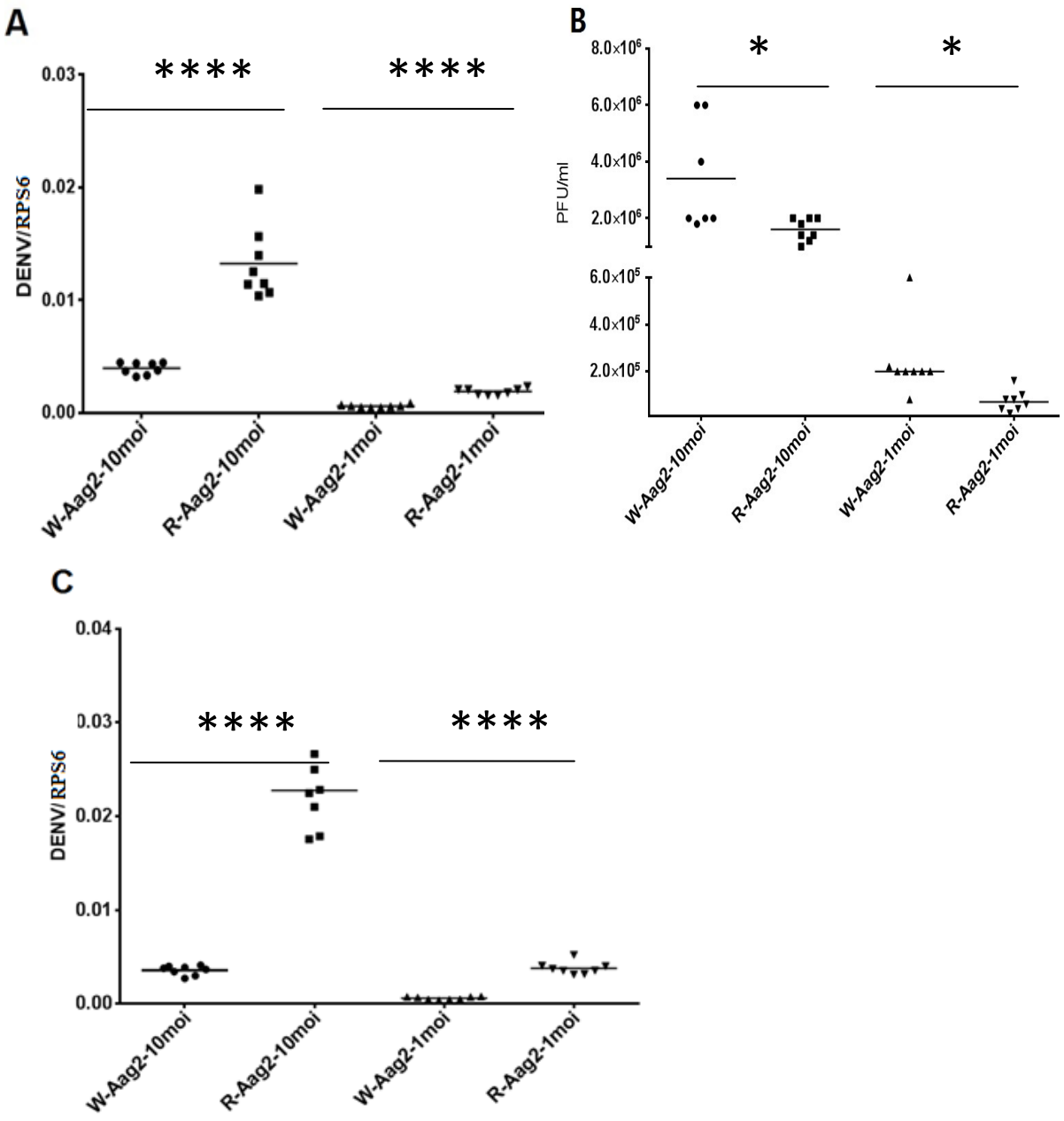

Figure 3.2: *Wolbachia* infection inhibits DENV binding to mosquito cells. (A) Binding of DENV to W-Aag2 and R-Aag2 cells at 4 ℃. W-Aag2 and R-Aag2 were incubated with DENV at 10 MOI and 1 MOI for 60 min at 4 ℃. Virus-cell binding was measured qRT-PCR analysis, and normalized by RPS6. (B) Unbound of DENV to W-Aag2 and R- Aag2 cells at 4 ℃. After incubation, took the DENV incubation medium out, followed by plaque assay to check the virus titer of unbound virus. (C) W-Aag2 and R-Aag2 were incubated with DENV at 10 MOI and 1 MOI for 60 min at 25 ℃. Viral genomic copies were measured by qRT-PCR, and normalized by RPS6. Line indicate the mean of 8 replicates *, P<0.05 ; ***, P<0.001; ****, p<0.0001in Student's t-Test.

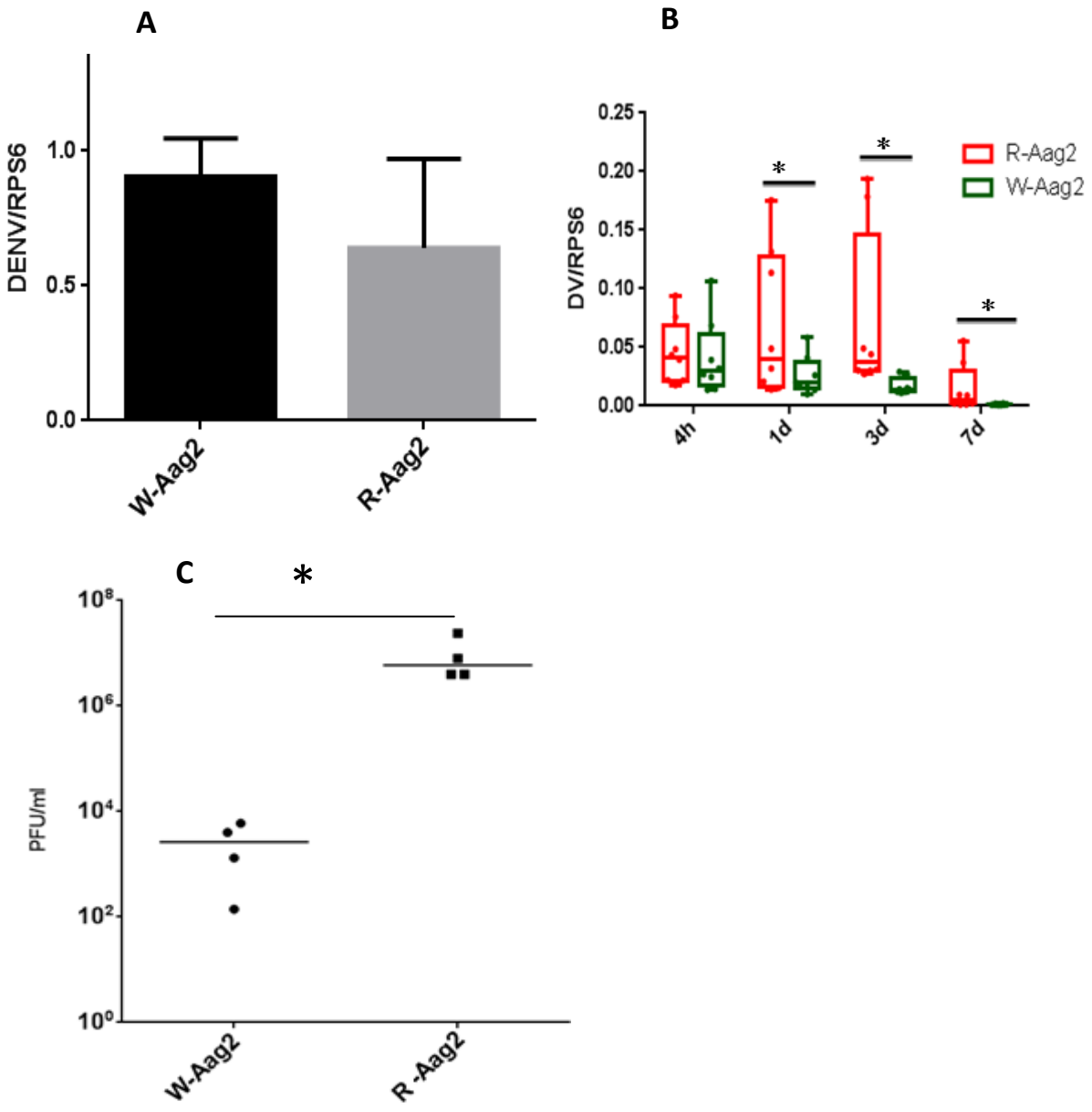

Figure 3.3: *Wolbachia* infection inhibits DENV replication in mosquito cells. (A) The relative copies of DENV-2 RNA in W-Aag2 and R-Aag2 cells at 4 hours post-transfection. (B) DENV-2 negative strand RNA levels in W-Aag2 and R-Aag2 cells after DENV-2 RNA transfection. Equivalent amount of purified DENV-2 RNA were transfected into cells, cells were sampled at 4 hour, 1 day, 3 day and 7 day post RNA transfection. Negative strand RNA were measured by Tag-PCR. *, $P<0.05$ in Student's t-Test. (C) DENV-2 titers in W-Aag2 and R-Aag2 cells supernatant at 5 days post DENV-2 RNA transfection. *, $P<0.05$ in Mann Whitney test.

Discussion

DENV is the most critical arboviral pathogen, over 2.5 billion people are at risk of DENV infection. Currently, no effective vaccines and antiviral therapies are available, making the development of novel DENV control approaches an important global public health priority. *Wolbachia*-based dengue control has emerged as a promising strategy because it provide a solution that is sustainable, inexpensive and green. Understanding the mechanism underlying the *Wolbachia*-mediated DENV resistance will facilitate the use of *Wolbachia*-based strategies to block the transmission of dengue viruses by mosquitoes. We have previously reported *Wolbachia* can induce resistance to DENV in *Ae. aegypti* in a density-dependent manner, and in this report, we further expand this research to investigate the effect of *Wolbachia* infection in DENV replication cycle. Here we show that *Wolbachia* is able to inhibit the intracellular accumulation of DENV genomic RNA in *Ae. aegypti* mosquito cells. We also demonstrate that *Wolbachia* infection inhibits DENV binding and lowers the negative strand RNA level.

The first step of DENV infection is binding. Direct binding assays were carried out at low temperature (4℃) to avoid viral entry (Salas-Benito and Del, 1997). We show that there are less DENV particles attached to *Wolbachia* infected W-Aag2 than to uninfected R-Aag2 at both high and low MOI. The amount of DENV bound to W-Aag2 is 3.34 times lower than to R-Aag2 at 10 MOI. Similar results were observed when cells incubated with DENV at 1 MOI, the amount of DENV bound to W-Aag2 is 3.18 times lower than to R-Aag2. Both results indicate *Wolbachia* infection inhibits DENV attachment to mosquito cells. Inhibition of viral binding might be caused by regulation of DENV receptors expression(Breiner et al., 2001), modification of DENV cellular receptors (e.g. Glycosylation; Phosphorylation; Alkylation, etc)(Lee et al., 1999, Lok et

al., 2008) or pre-saturation of DENV receptors (Chin et al., 2007) by *Wolbachia*-related small molecules.

The early phase of viral RNA synthesis begins in 30 minutes after infection. We also assayed the DENV binding at 25℃ for 60 minutes to investigate whether *Wolbachia* inhibits viral early events and RNA synthesis. We found the viral genomic RNA levels in W-Aag2 cells were significantly lower than in R-Aag2 cells at both 10 MOI and 1 MOI (6.36 fold and 6.51 fold, respectively), suggesting *Wolbachia* may also interfere with other viral early events (entry, nuclocapsid relase, and uncoating) and/or viral replication.

The presence of negative-sense RNA is a hallmark of DENV replication within mosquito cells, but detection and quantization of negative strand RNA is complicated by false-priming in regular qRT RCR. Tag qRT-PCR is a strand-specific amplification method which can accurately reflect amounts of a specific RNA strand(Tuiskunen et al., 2010). Using DENV-2 infectious RNA transfection, the viral binding ,entry and uncoating were bypassed. Since the time for *de novo* DENV-2 production in Aag-2 cells is 1 day(Sim and Dimopoulos, 2010), the negative strand RNA at 1 day post-transfection should come only from the initial round of infection. The relative negative sense RNA level in R-Aag2 cells (0.069 DENV/RPS6) was 2.65 times higher than in W-Aag2 cells (0.026DENV/RPS6) at 1 day post-transfection. Also, DENV titer in the supernatant of R-Aag2 cells (6×10^6 PFU/ml) was 2300 times higher than in W-Aag2 cells (2.6×10^3 PFU/ml). Because the difference between R-Aag2 and W-Aag2 cell line appears to be much more pronounced in viral titers than negative strand RNA levels, it is possible that *Wolbachia* interferes with downstream steps of RNA accumulation (for example, viral translation, assembly and trans-Golgi transport) as well.

After viral RNA replication, DENV RNA must go through translation, assembly, and trans-Golgi transport to generate new infectious virions. Viral translation, assembly and maturation occur in ER and Golgi apparatus(Yacoub et al., 2013). To facilitate replication DENV modifies ER membranes (Welsch et al., 2009). Interestingly, *Wolbachia* has also been found in the ER and Golgi-related vesicles (Cho et al., 2011, Voronin et al., 2004), which also suggests that *Wolbachia* could interfere with DENV translation, assembly and maturation by competition for space and host resources.

Several DENV binding proteins have been identified by protein-protein interaction assays. We measured the expression profile of 19 previously reported DENV binding proteins (Colpitts et al., 2011, Munoz et al., 2013), and found 11genes were differentially expressed in mosquito cells in response to *Wolbachia* infection. Among those genes, histone 4, histone 2A, beta tubulin, elongation factor-1 alpha, and actin were down-regulated by *Wolbachia* infection. Histone 4 is the most significantly down-regulated gene (-24.5 fold). DENV capsid protein has been found to bind and co-localize with histones, though the reasons for this are not clear(Colpitts et al., 2011). It has been hypothesized that DENV capsid protein may bind to histone proteins, preventing the inhibition of viral transcription and aiding in viral replication(Colpitts et al., 2011). *Wolbachia* infection also down-regulates elongation factor 1-alpha (-4.23 fold), which binds viral RNA and proteins(E, NS2A and NS4B) to aid in viral replication or translation (Blackwell and Brinton, 1997). In addition, two cytoskeleton genes (actin and beta tubulin) are down-regulated by *Wolbachia* infection. Actin has been shown to associated with viral endocytosis and replication (Acosta et al., 2008). It is reported that tubulin-like protein binding dengue envelope protein in mosquito cells, indicating tubulin could be a viral receptor for DENV entry (Chee and AbuBakar, 2004). Moreover, tubulin is required for Sendai virus and hepatitis C virus replication

(Lai et al., 2008, Ogino et al., 2001). We also found that the expression of PI3 kinase, cadherin, myosin, kinectin, and enolase were up-regulated in response to *Wolbachia* infection. It is likely that *Wolbachia* may up-regulate some host genes, which are down-regulated during DENV infection. For example, DENV infection down-regulates the expression of cadherin to increase permeability of human primary endothelial cells (Ong et al., 2013).

In summary, we have shown that *Wolbachia* can inhibit DENV binding at both high and low MOI. Moreover, we have also provided evidence that *Wolbachia* is able to lower negative strand viral RNA levels. Further studies will focus on identifying DENV cellular receptors associated with *Wolbachia* infection and the mechanism underlying *Wolbachia*-mediated DENV binding inhibition.

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Chapter 4: Identification of *Wolbachia*-mediated Host Factors Involved in Resistance to Dengue Virus Infection

Introduction

With more than one-third of the world's population living in areas at risk for transmission, dengue infection is among the most important reemerging infectious diseases in the tropics and subtropics. WHO estimates that 50 to 100 million cases with 22,000 deaths (mostly children) occur annually (WHO, 2014). Neither vaccine nor effective chemotherapy is currently available for dengue infection in humans; prevention of human cases relies almost exclusively on control of the mosquito vectors(WHO, 2012). The causative agent of dengue infection is the Dengue Virus (DENV), a member in the family *Flaviviridae*. DENV is an enveloped positive-sense RNA virus. The viral genomic RNA is approximate 11 kb in length.

Wolbachia is a maternally transmitted intracellular symbiotic bacterium belonging to the α-proteobacteria. *Wolbachia* is believed to the most pervasive symbiont; it has been detected in most orders of insects and infects more than 60% of all insect species worldwide (Hilgenboecker et al., 2008). *Wolbachia* is mainly localized in the reproductive tissues of arthropods and can induce host reproductive abnormalities including cytoplasmic incompatibility (CI), parthenogenesis and male-killing. CI between infected males and uninfected females, or females harboring a different *Wolbachia* type can cause early embryo death, which reduces egg hatching (Zabalou, et al., 2004). Since uninfected males can successfully mate with infected females, *Wolbachia* can spread quickly in a population. *Wolbachia* has recently been shown to confer resistance against pathogen infection in insects (Bian et al., 2010, Walker et al., 2011, Bian et al., 2013). Presently, *Wolbachia*-based control strategies merit great attention, because it could

provide a sustainable, economical, and environmentally friendly solution to prevent vector-borne diseases.

The underlying mechanisms of *Wolbachia*-mediated DENV resistance are unclear, however, two mechanisms have been proposed: host immune activation, and metabolic competition. Given both *Wolbachia* and DENV have relatively compact genomes (Saridaki and Bourtzis, 2010), they probably require a large number of host factors to survive. Host factors participate in most steps in the DENV life cycle, including entry, viral RNA replication, translation, assembly and maturation (Ahlquist et al., 2003). Meanwhile, *Wolbachia* infection has been shown to have a profound influence on the expression of host genes, including some immunity and reduction-oxidation (redox) pathway genes, which can regulate DENV replication (Hughes et al., 2011, Pan et al., 2012, Brennan et al., 2008). Thus, we hypothesized that *Wolbachia*-associated changes in expression of DENV host factors also contribute to DENV resistance.

Mosquito cell lines have been widely used as an *in vitro* system to dissect the immune responses and molecular interactions between microbe and host (Brennan et al., 2008, Salas-Benito and Del, 1997). Using the *Ae. aegypti* cell line system could avoid the physiological complexity of whole mosquitoes and provide a convenient and sensitive tool to study the interactions between *Wolbachia* and dengue virus in the host.

In this work, we used a *Wolbachia* infected W-Aag2 cell line to study the mechanism underlying the *Wolbachia*-mediated resistance to DENV. A total of 16 DENV host factors have been shown to be up-regulated using microarray and real-time PCR analyses. Functional RNA interference (RNAi) screen revealed that eleven DENV host factors may be involved in *Wolbachia*-induced resistance to DENV.

Materials and Methods

Cell-Culture Maintenance and DENV-2 infection

The *Ae. aegypti* Aag2 cell line was cultured as previously described. Those cells were maintained in Schneider's Drosophila Medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). *Ae. aegypti* W-Aag2 cell line was generated by introducing *Wolbachia* into Aag2 cells using centrifugation enhanced (shell vial) technique as previously described (Lu et al., 2012). W-Aag2 and R-Aag2 cell line (derived from W-Aag2 cells through rifampicin treatment) were maintained at 25 ℃ in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies), and were passaged at 1:5 dilution every 6-7 days.

The *Ae. albopictus* cell line C6/36 was grown in Eagle's minimal essential medium (MEM) with 10% (vol/vol) heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 100 μM nonessential amino acids at 32 °C and 5% (vol/vol) CO2.

New Guinea C strain (NGC) of DENV-2 was used in this study. DENV-2 was grown in W-Aag2 and R-Aag2 cells as previously described (Sim and Dimopoulos, 2010). Briefly, cells were seeded in 48-well plates to a confluency of 80%. W-Aag2 and R-Aag2 monolayers were infected with DENV-2 at a desired multiplicity of infection (MOI) of DENV-2 in culture medium. Plates were incubated at 25 ℃ for the duration of the experiment.

Microarray analysis

W-Aag2 and R-Aag2 cells were lysed by the addition of Buffer RLT(Qiagen). Total RNA was extracted using RNeasy Mini Kit (QIAGEN), according to the manufacturer's instruction. Transcription assays were conducted as reported previously (Nene et al., 2007) with

an *Ae. aegypti* full genome Agilent-based microarray platform. Briefly, 2-3 μg total RNA was used for probe synthesis of cy3- and cy5-labeled cRNA. Hybridizations were conducted using an *In Situ* Hybridization kit (Agilent Technologies) at 65 ℃. Hybridization intensities were measured using an Axon GenePix 4000B scanner, and images were analyzed with GenePix software. The expression data were processed and analyzed as described previously (Nene et al., 2007).

RNA Extraction, cDNA synthesis and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cell lines by RNeasy Mini Kit (Qiagen) and then the cDNA transcript was produced using QuantiTect reverse Transcription Kit (Qiagen). Real-time PCR was conducted using the QuantiTect SYBR Green PCR Kit (Qiagen) and ABI Prism 7900HT Sequence Detection System (Applied Biosystems). DENV-2 genomic RNA was measured by qRT-PCR using primers directed to NS5 gene (Molina-Cruz et al., 2005). The ribosomal protein S6 (RPS6) gene was used for normalization of cDNA templates.

RNAi-mediated gene silencing

The dsRNA was synthesized *in vitro* using the Megascript T7 high-yield transcription kit (Ambion). Transfection of dsRNA was carried out using Attractene Transfection Reagent (Qiagen) according to manufacturer's instruction. Briefly, W-Aag2 cells were seeded in 48-well plates 24 hours prior to transfection. 1 μg of dsRNA was incubated with 3.5 μl Attractene Transfection Reagent in 50 μl Schneider's *Drosophila* Medium for 10-15 minutes at room temperature and then was transferred to each well. 4 days after transfection, W-Aag2 cells were infected with DENV-2 at a multiplicity of infection (MOI) of 5 in infection medium (Schneider's *Drosophila* Medium with 2% FBS and 1% penicillin/streptomycin). At 12 hours post DENV

infection, dsRNA transfection was repeated. 4.5 days later, the supernatant was collected for detecting infectious viral particles by plaque assay, and W-Aag2 cells were harvested for measuring the DENV-2 RNA levels and *Wolbachia* densities.

Plaque assay

The DENV-2 titers were measured by plaque assays, as previously reported(Das et al., 2007, Bian et al., 2010). Briefly, C6/36 cells were seeded in 48-well plates at a density of 4-8 × 10^4 cells/well and maintained for 2-3 days at 32 ℃ in 5% CO_2. The virus-containing culture medium was serially diluted and inoculated into C6/36 cell. After incubation for 5 d, plaque forming uinits (PFUs) were measured from the plates by peroxidase immunostaining, using mouse hyperimmune ascitic fluid (specific for DENV-2, CDC) as the primary antibody and a goat anti-mouse horseradish peroxidase(HRP) conjugate as the secondary antibody.

Results

Differentially expressed profiles in *Wolbachia* infected and uninfected *Aedes aegypti* cells

To identify differentially expressed genes in mosquito cells in response to *Wolbachia* infection; we conducted microarray assays to compare transcript repertoires in *Wolbachia* infected *Ae. aegypti* Aag2 cells(W-Aag2) with those of corresponding Rifampicin-cured cells R-Aag2. Functional classification of significantly regulated genes was analyzed (Fig.4.1). *Wolbachia* infection significantly regulated 1860 genes in the cell line (910 up-regulated and 950 down-regulated). We observed that among the up-regulated genes, the most highly regulated *Wolbachia*-responsive transcripts were related to redox/stress/mitochondrion (R/S/M) group (128 genes), except for genes belonging to diverse functions (267 genes). Genes within the R/S/M group represented 14.1 percent of all highly regulated genes with predicted functions. The third

largest group included 123 genes with unknown function, followed by 114 genes involved in metabolism. Additionally, 56 genes were identified that are involved in immunity function. Among the down-regulated genes, the largest group contained 107 genes involved in replication/transcription/translation (R/T/T), except for genes with unknown or diverse functions.

The infection of *Wolbachia* significantly up-regulated several characterized immunity genes in W-Aag2 cells. Both Toll and IMD pathway genes were activated in W-Aag2 cells, including Gram-negative bacteria binding protein A1 (GNBPA1), Gram-negative binding protein B1 (GNBPB1), Relish-like protein 1A (Rel1A), and IMD pathway signaling NF-kappaB Relish-like transcription factor Rel2. Transcripts of the antimicrobial peptide genes defensin A, defensin D, cecropin E and cecropin N were highly elevated in W-Aag2 cells as well.

We also found a number of antioxidant transcripts were highly regulated in our microarray analysis, including glutathione peroxidase (AAEL012069), Cu,Zn-superoxide dismutase (*CuZnSOD*; AAEL011498), thioredoxin peroxidase (AAEL013528 and AAEL014548) and manganese superoxide dismutase ((*MnSOD*; AAEL004823). Moreover, both ferritin light chain(AAEL004335, AAEL010396, AAEL007383) and heavy chain(AAEL010393, AAEL007385) genes were up-regulated by *Wolbachia* in W-Aag2 cells as well.

Select candidate *Wolbachia*-mediated host factors involved in resistance to DENV infection

DENV is a positive-strand RNA virus that encodes 10 proteins. The small coding capacity of DENV demands that the virus use the host machinery for most, if not all, steps of its life cycle (Ahlquist et al., 2003). A large number of DENV host factors have been identified by using genomics techniques, computational approaches and protein-based techniques (Bonizzoni et al., 2012, Sim et al., 2012, Sim and Dimopoulos, 2010, Guo et al., 2010, Sessions et al., 2009, Doolittle and Gomez, 2011, Xi et al., 2008). Among those reported DENV host factors, some

were also regulated by *Wolbachia*. In order to further understand the mechanism of *Wolbachia*-mediated Dengue inhibition, we selected 40 DENV host factors, which were significantly up-regulated by *Wolbachia* in the microarray study (Table 4.1.). To verify the results of microarray analysis, the expression profiles of 40 candidate genes in W-Aag2 and R-Aag2 cells were measured by qRT-PCR. Using $2^{-\Delta\Delta Ct}$ method with fold change >2 as cut off level, a total of 16 genes were selected as candidate *Wolbachia*-mediated host factors involved in resistance to DENV infection (Table 4.1). Those candidate host factors include 5 genes belonging to R/S/M group, 4 genes with diverse functions, 2 immunity genes, 2 metabolism genes, 2 R/T/T genes and a transport gene.

Identify *Wolbachia*-associated DENV host factors by RNAi screen

To test whether differentially regulated host factors are involved in *Wolbachia*-mediated DENV inhibition, we performed RNAi screen in W-Aag2 cells. At 4 days post dsRNA transfection, cells were infected with DENV-2 at a MOI of 5. At day 5 post viral infection, cell supernatants were collected for detecting infectious viral particles by plaque assay, and W-Aag2 cells were harvested for determining the DENV-2 RNA levels and *Wolbachia* densities (Figure 4.2. A). Gene knock-down efficiency was evaluated using ΔΔCT method (Haimes and Kelley, 2010) to determine relative gene expression from qRT-PCR data with PRS6 as a reference gene. Except silencing of GPI and IMPase with 70.5% and 82.2% konck-down, dsRNA knock-down efficiency of all other genes were above 90%.

Comparing to dsGFP treatment, silencing of 10 genes, including 4 diverse function genes (STX4, Ferritin G, AKR and IMPase), 2 immunity genes (GNBPA1, PGRP-lc), 2 R/S/M genes (NADH-UQRP, ERCC),1 R/T/T gene (SFRS) and 1 metabolism gene (GPI), in W-Aag2 cells significantly increased DENV-2 genomic RNA levels ($P< 0.05$, Student's t-Test) (Figure 4.2. C).

To determine whether those candidate DENV host factors affected the generation of new infectious virions in W-Aag2, we assessed viral titers in cell medium by plaque assay after RNAi transcript depletion. Trends toward increased viral titers were observed in all RNAi silencing groups as compared with mock control (dsGFP), except for knock-down of STX4 and ATPD. DENV titers were increased significantly by silencing of Ferritin G, IMPase, MRPs-S18A, and ERCC as compared to the mock control ($P<0.01$, $P<0.01$, $P<0.05$ and $P<0.05$, respectively in Mann-Whitney *U* test) (Fig 4.2.D). Except for silencing of MRPs-S18A, knock-down of Ferritin G, IMPase, and ERCC resulted in increased viral genomic RNA levels (Fig 4.2.C).

DENV inhibition induced by *Wolbachia* is density-dependent, thus the increased level of DENV infection could be caused by a decrease in *Wolbachia* density (Lu et al., 2012, Osborne et al., 2012). We also measured *Wolbachia* density after RNAi screen. As shown in Fig 4.2.E, *Wolbachia* densities in W-Aag2 cells with silencing of ERCC, NADH-UQOR, HSP and ATPD were significantly decreased ($P< 0.05$, Student's t-Test) compared with mock-depleted (dsGFP) group, indicating that decreased *Wolbachia* densities may contribute to increased viral loads by knock-down of ERCC and NADH-UQOR.

Taken together, these results revealed eleven potential host factors involved in *Wolbachia*-induced DENV resistance. Those host factors include 4 diverse function genes (STX4, Ferritin G, AKR and IMPase), 2 immunity genes (GNBPA1, PGRP-lc), 3R/S/M genes (MRPs-S18A,NADH-UQRP and ERCC),1 R/T/T gene (SFRS) and 1 metabolism gene (GPI),

	Microarray average fold change	qRT-PCR average fold change	Functional group	Gene name	Reference
AAEL009471	8.55	24.77	D	syntaxin 4 knolle (STX4)	Bonizzoni et al.,2012
AAEL004335	2.35	7.44	D	secreted ferritin G subunit precursor,	Sim et al.,2010
AAEL004086	2.09	2.04	D	aldo-keto reductase (AKR)	Sim et al.,2010
AAEL004566	2.42	2.01	D	myo inositol monophosphatase (IMPase)	Sim et al.,2010
AAEL013943	2.04	1.8	D	mediator complex, 100kD-subunit	Sim et al.,2010
AAEL010119	1.99	-1.23	D	ER-derived vesicles protein ERV14	Bonizzoni et al.,2012
AAEL010119	1.99	-1.23	D	ER-derived vesicles protein ERV14	Bonizzoni et al.,2012
AAEL013338	1.8	-1.41	D	lethal(2)essential for life protein, l2efl	Sim et al.,2010, Bonizzoni et al.,2012
AAEL006948	3.91	-2.31	D	tomosyn	Doolittle et al.,2011
AAEL000541	1.83	-2.39	D	fasciclin, putative	Sim et al.,2010, Bonizzoni et al.,2012
AAEL009149	2.16	-2.97	D	kinectin, putative	Sim et al.,2010, Bonizzoni et al.,2012
AAEL013830	3.67	-3.43	D	bmp-induced factor	Doolittle et al.,2011
AAEL007626	4.11	6.06	I	gram-negative bacteria binding protein A1(GNBPA1)	Sim et al.,2010
AAEL009474	2.89	3.96	I	peptidoglycan recognition protein-lc isoform (PGRP-lc)	Sim et al.,2010

Table 4.1: Candidate *Wolbachia*-mediated host factors involved in resistance to DENV infection. We first selected 40 DENV reported host factors, which were significantly up-regulated by *Wolbachia*, using gene expression microarray data. After further analysis of relative gene expression using qRT-PCR and $2^{-\Delta\Delta Ct}$ method (fold-change cut-off=2), 16 host molecules (shaded rows) were selected as candidates. Functional group abbreviations: D, diverse functions; I, immunity; M, metabolism; R/S/M, redox, stress and mitochondrion; R/T/T, replication, transcription, and translation; TRP, transport.

Table 4.1 (cont'd)

	Microarray average fold change	qRT-PCR average fold change	Functional group	Gene name	Reference
AAEL003294	2.31	1.8	I	fibrinogen and fibronectin	Bonizzoni et al.,2012
AAEL000611	1.82	1.14	I	antibacterial peptide, putative	Sim et al.,2010,Sim et al.,2012
AAEL000621	1.89	-2.73	I	antibacterial peptide, putative	Sim et al.,2010
AAEL009384	5.89	-4.72	I	fibrinogen and fibronectin	Sim et al.,2010
AAEL003857	3.06	-4.8	I	Defensin	Sim et al.,2010, Bonizzoni et al.,2012
AAEL009237	10.07	5.46	M	glycoside hydrolases (GHs)	Sim et al.,2010
AAEL012994	2.76	3.51	M	glucose-6-phosphate isomerase (GPI)	Sim et al.,2010
AAEL002542	2.41	1.56	M	triosephosphate isomerase	Sim et al.,2010, Bonizzoni et al.,2012, Xi et al., 2008
AAEL011233	2.51	1.38	M	SM protein G	Sim et al.,2010, Bonizzoni et al.,2012 , Doolittle et al.,2011
AAEL011356	7.04	-4.02	M	alcohol dehydrogenase	Bonizzoni et al.,2012
AAEL002783	1.86	N/A	R/S/M	mitochondrial ribosomal protein, L37	Sim et al.,2010
AAEL008601	2.57	2.69	R/S/M	mitochondrial ribosomal protein, L28 (MRPs-L28)	Sim et al.,2010
AAEL007355	3	2.41	R/S/M	mitochondrial ribosomal protein, S18A (MRPs-S18A)	Sim et al.,2010

Table 4.1 (cont'd)

	Microarray average fold change	qRT-PCR average fold change	Functional group	Gene name	Reference
AAEL007355	3	2.41	R/S/M	mitochondrial ribosomal protein, S18A (MRPs-S18A)	Sim et al.,2010
AAEL013350	1.8	2.33	R/S/M	heat shock protein 26kD, putative (HSP)	Sim et al.,2010, Bonizzoni et al.,2012
AAEL005508	1.95	2.31	R/S/M	nadh-ubiquinone oxidoreductase 24 kda subunit (NADH-UQOR)	Sim et al.,2010, Bonizzoni et al.,2012
AAEL013693	2.02	2.14	R/S/M	excision repair cross-complementing 1 ercc1 (ERCC)	Sim et al.,2010
AAEL002523	2.03	1.61	R/S/M	mitochondrial inner membrane protein translocase	Sim et al.,2010, Bonizzoni et al.,2012
AAEL011463	4.56	1.1	R/S/M	cytochrome P450	Bonizzoni et al.,2012
AAEL011752	5.69	-1.98	R/S/M	glutathione-s-transferase theta, gst	Sim et al.,2010
AAEL013790	1.77	-9.5	R/S/M	mitochondrial ribosomal protein, L50	Sim et al.,2010
AAEL001280	1.94	2.19	R/T/T	mitochondrial ribosomal protein S15 (MRPs-S15)	Sim et al.,2010
AAEL006473	2.01	2.05	R/T/T	arginine/serine splicing factor (SFRS)	Sim et al.,2010, Xi et al., 2008
AAEL010823	1.79	2.32	TRP	atp synthase delta chain (ATPD)	Sim et al.,2010, Bonizzoni et al.,2012
AAEL000435	2.85	-1.24	TRP	THO complex, putative	Sim et al.,2010, Bonizzoni et al.,2012
AAEL014052	3.37	-1.65	TRP	endoplasmic reticulum protein erp29	Bonizzoni et al.,2012
AAEL015067	3.32	-4.08	TRP	multidrug resistance protein 2	Bonizzoni et al.,2012

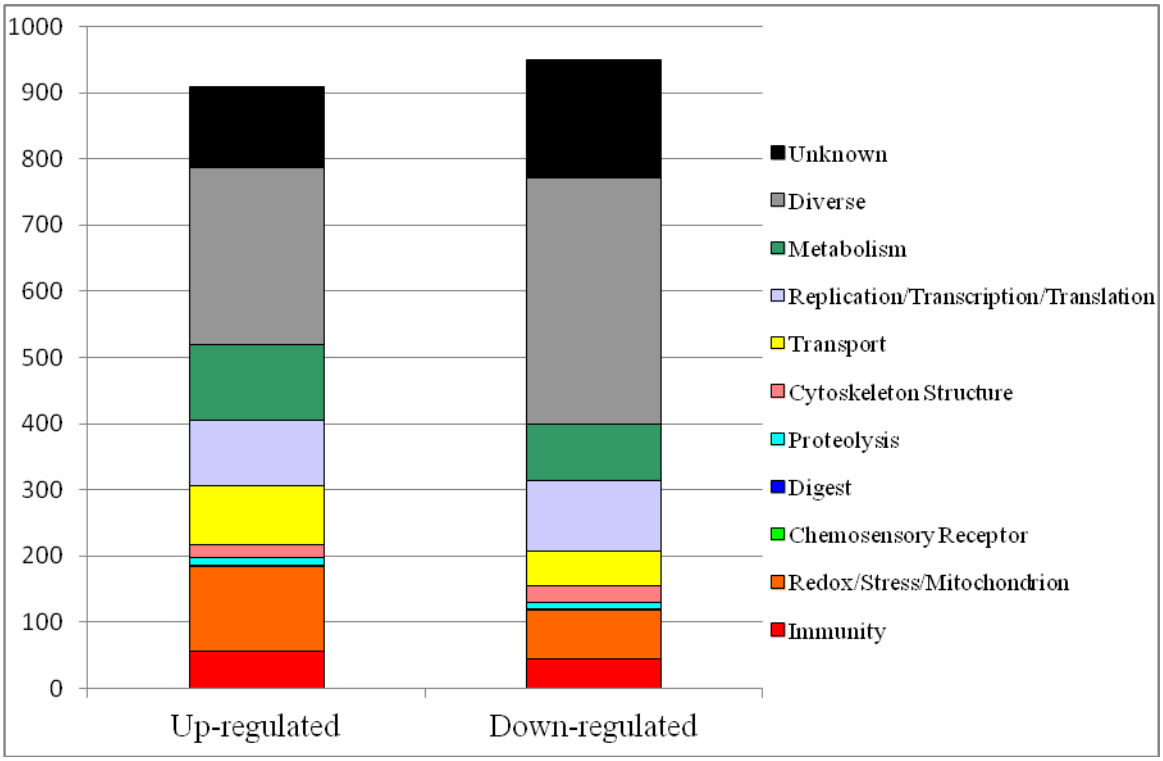


Figure 4.1: Functional classification of differentially expressed genes in the Aag2 cell line in response to *Wolbachia* infection. The graph shows the functional class distributions of genes that are regulated by *Wolbachia* infection.

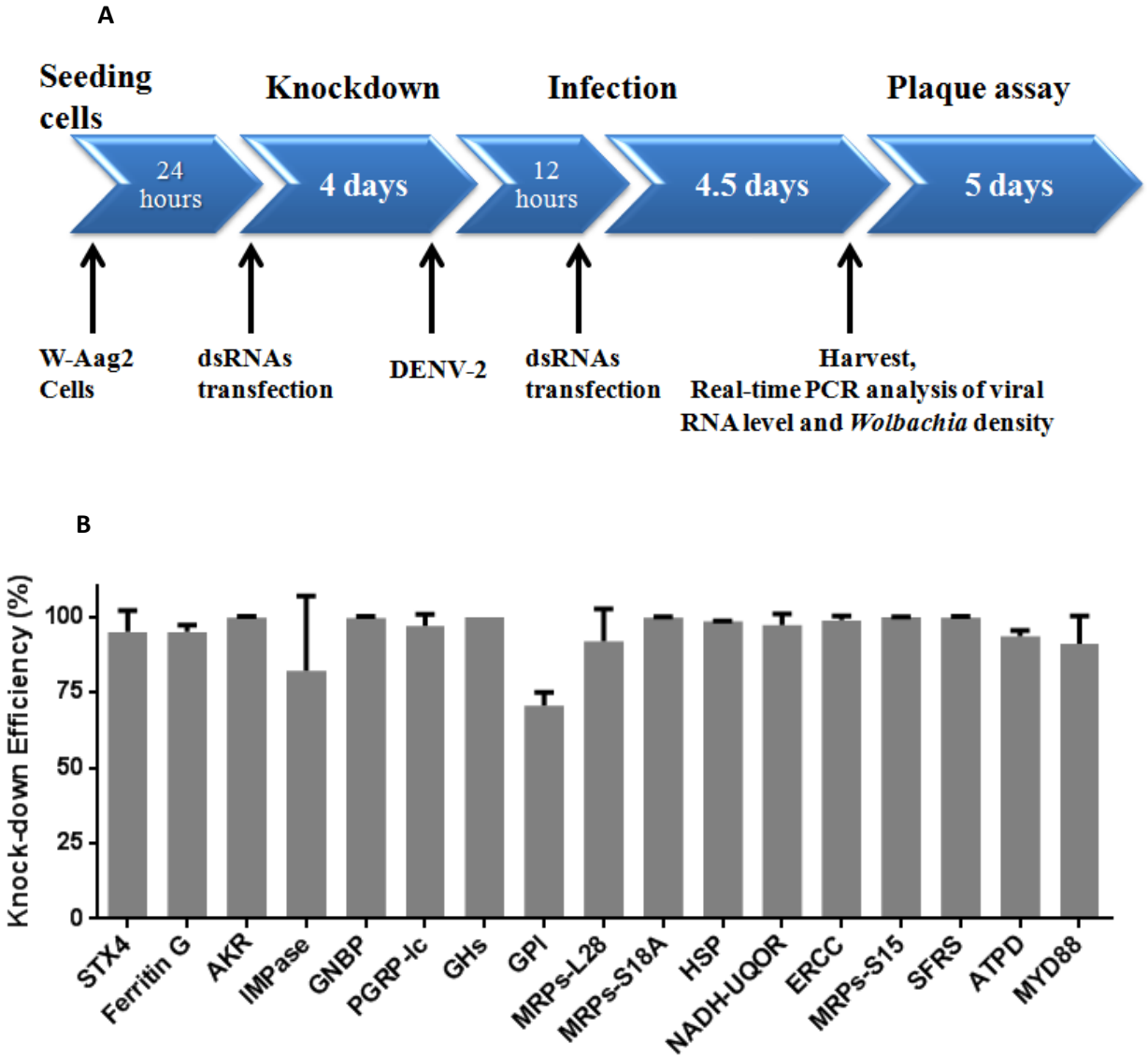


Figure 4.2: Characterizing *Wolbachia*-regulated DENV host factors by RNA interference (RNAi) screen. (A) Schematic of experimental design. (B) Knock-down efficiency for dsRNA treatment. Cells were sampled at 24 hours post dsRNA transfection. Gene silencing efficiency was assessed by ΔΔCT method with RPS6 as an endogenous reference gene. (C) Relative DENV-2 RNA copies in W-Aag2 cells treated with dsRNAs. W-Aag2 cells were infected DENV-2 at an MOI of 5 at day 4 post dsRNA transfection. Viral RNA was purified 5 days post infection. qRT-PCR was conducted using primers targeting NS5 gene. The DENV genomic RNA copy number was normalized with RPS6. Error bars represent standard error of 6 biological replicates. *, P<0.05; **,P<0.01; ***, P<0.001; ****, p<0.0001 in Student's t-Test comparing to GFP control. (D) Viral titer in the cell supernatant was measured after RNAi depletion of DENV host factors. dsRNA-mediated silencing of MYD88 was served as a positive control. Lines indicate the geometric mean of the four biological replicates.*, P<0.05; **,P<0.01 in Mann-Whitney *U* test.. (E) *Wolbachia* density in W-Aag2 cells after silencing of DENV host factors. Error bars represent standard error of 4 biological replicates, *, P<0.05 in Student's t-Test comparing to GFP control

Figure 4.2 (cont'd)

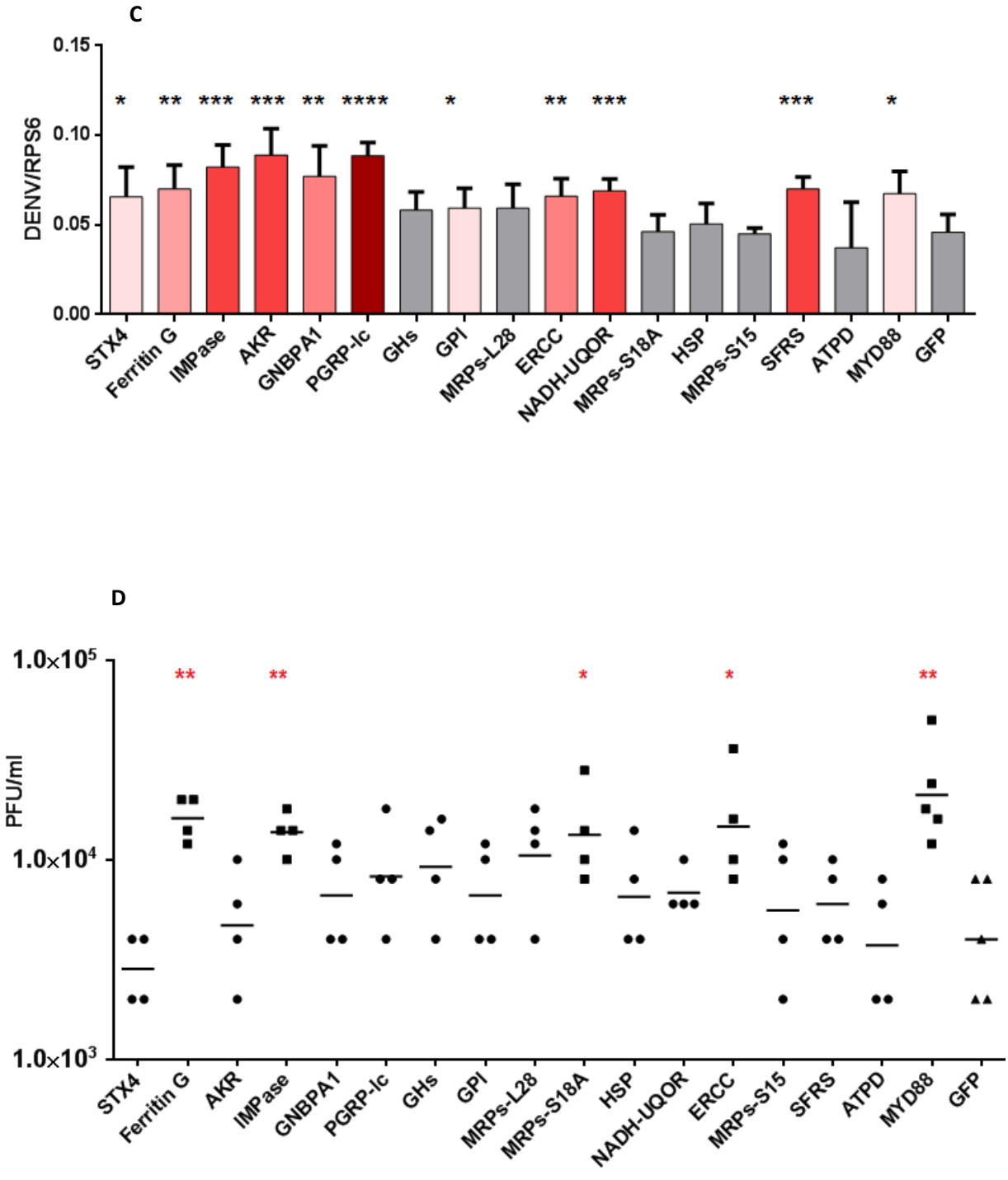
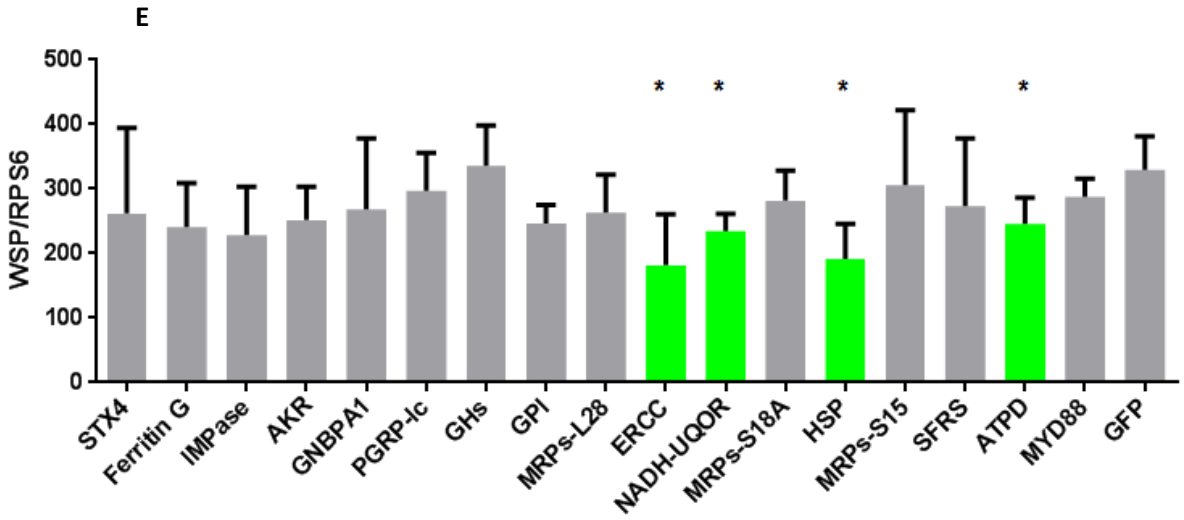


Figure 4.2 (cont'd)



Discussion

The *Wolbachia* genome is extremely reduced; consequently, many biosynthetic pathways are absent in *Wolbachia* (Kondorosi et al., 2013). To facilitate intracellular infection, *Wolbachia* needs to interact with numerous host proteins. It has been believed that *Wolbachia* Type IV secretion system (T4SS) secretes various effector macromolecules into the cytoplasm to alter host gene expression and affect the physiology of host cells (Rances et al., 2008). DENV also requires a number of host proteins at multiple stages of its life cycles. The viral replication is coordinated by a complex of viral and host factors. Thus, we hypothesized that *Wolbachia* may affect DENV host factors that are important in DENV infection. By comparing our microarray and real-time PCR data of mosquito transcripts changes to *Wolbachia* infection, we selected 16 candidate DENV host factors, which could be targeted by *Wolbachia* and then involved in *Wolbachia*-induced DENV inhibition. Furthermore, we identified eleven potential *Wolbachia*-mediated host factors involved in resistance to DENV infection with RNAi screen. Generally, the gene silencing effect could last from 5-7 days. Since W-Aag2 cells need to be grown for 9 days to observe phenotypes (Figure 4.2.A), we conducted dsRNA transfection twice to achieve long-term knock-down of target genes (McManus and Sharp, 2002).

DENV has to go through multiple stages, including binding, fusion, translation, transcription, assembly, maturation and budding to reproduce itself and create new infectious virus particles (Rodenhuis-Zybert et al., 2010). As shown in Chapter 3, *Wolbachia* infection affects multiple steps of DENV life cycle. Interestingly, knock-down of seven genes (STX4, AKR, GNBPA1, PGRP-lc, NADH-UQRP, SFRS and GPI) resulted in significant increased viral RNA levels compared with mock control, but did not induce changes in viral titers. It is likely that these seven host factors act on stages required for the accumulation of viral RNA (for

example, binding, fusion, translation, and transcription), and the *Wolbachia*-induced inhibition to stages downstream of RNA accumulation (for example, inhibition of assembly, maturation, and budding) is powerful enough to mask the effect of elevated DENV genomic copies. In contrast, viral titers were increased significantly by RNAi depletion of MRPs-S18A , but no change was observed in DENV RNA copies with the same treatment, suggesting MRPs-S18A acts at stages downstream of viral RNA accumulation (for example, assembly, maturation, budding) (Sessions et al., 2009).

Mosquitoes, like all insects, use immune mechanisms, including Toll, IMD, autophagy, JAK-STAT, and siRNA pathways, to protect against viral infection (Ding, 2010, Bronkhorst and van Rij, 2014, Costa et al., 2009, Zambon et al., 2005, Lamiable and Imler, 2014). In accordance with our previous microarray data from *Ae. aegyti* mosquitoes (Pan et al., 2012), we also found a number of *Wolbachia*-responsive transcripts were related to immunity and R/S/M group in our cell microarray. Recently evidence has shown that *Wolbachia* infection activated mosquito basal immunity, resulting in the suppression of DENV replication (Xi et al., 2008). Moreover, our previous study reported that *Wolbachia* could induce reactive oxygen species (ROS)-dependent activation of the Toll pathway to control DENV in *Ae. aegypti* (Pan et al., 2012). In this study, we identified 2 immunity genes (GNBPA1, PGRP-lc), and 3 R/S/M genes (NADH-UQOR, ERCC and MRPs-S18A) as potential *Wolbachia*-mediated DENV host factors.

Stable symbiotic interaction with *Wolbachia* in mosquito cells involves ROS generation and induction of antioxidant enzymes (Brennan LJ et al., 2008, Pan et al., 2011). ROS suppresses replication of Hepatitis C Virus in human hepatoma cells; whereas antioxidants tend to counter this suppression (Seronello et al., 2011). NADH-ubiquinone oxidoreductase (NADH-UQOR) is an enzyme of the mitochondrial electron transport chain, and it is a major source of ROS (Galkin

and Brandt, 2005). In this work, knock-down of NADH-UQOR resulted in decreased *Wolbachia* densities and increased DENV RNA levels, suggesting ROS plays an important role in *Wolbachia* propagation and *Wolbachia*-mediated DENV inhibition.

The female mosquito vector must take a blood meal in order to complete her life cycle. The blood meal provides a high level of iron that is required for egg development(Nichol et al., 2002). Although sufficient iron is present in the blood meal to provoke the formation of toxic free radicals, mosquitoes have developed mechanisms, partly by iron storage inside ferritin, that allow iron utilization while maintaining protection form iron-mediated oxidative stress (Geiser et al., 2006). Ferritin has also been shown to play an important role in host-pathogen interactions (Schaible and Kaufmann, 2004).The expression of ferritin heavy chains is under the control of the NF-kappaB signaling pathway in mammals (Bubici et al., 2006). It has been found recently that iron metabolism was influenced by the presence of *Wolbachia* in *Ae. aegypti* cells (Kremer et al., 2009). Iron is an essential element that is in limited supply in the cell, and it is also a highly toxic precursor of Reactive Oxygen Species (ROS). In accordance with previous work (Pan et al., 2011), we found genes involved in the iron/ferritin metabolism pathways were largely regulated by *Wolbachia* in mosquito cells. Furthermore, silencing of ferritin G protein resulted in elevated DENV RNA levels and viral titers, indicating ferritin plays an important role in *Wolbachia*-mediated viral resistance.

In conclusion, we have shown eleven potential host factors associated with *Wolbachia*-mediated DENV resistance. Identifying host factors involved in *Wolbachia*-induced antiviral protection may lead to new targets for dengue control, while understanding the details of *Wolbachia*, DENV and host interactions require further investigation.

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Chapter 5: Conclusions and future directions

Conclusions

The major goal of this dissertation study was to better understand the interactions between dengue virus (DENV) and *Wolbachia* in the mosquito host. First, we showed that *Wolbachia* induced a density-dependent viral inhibition in mosquito cells. With a decrease in *Wolbachia* density within the host cells, dengue infection increased dramatically. We provided evidence that the *Wolbachia* density in somatic tissues of *Ae. albopictus* was too low to induce resistance to dengue virus. Furthermore, we observed a positive correlation between *Wolbachia* density and the expression of the antimicrobial peptide Denfesin D, indicating host immunity could contribute to *Wolbachia*-density dependent viral inhibition

We then provided evidence that *Wolbachia* infection inhibited the intracellular accumulation of DENV genomic RNA in mosquito cells. DENV binding assay revealed that the attachment of virus particles was significantly inhibited by *Wolbachia* infection at both high and low multiplicity of infection (MOI). We also carried out a strand-specific tagged RT-PCR to compare the levels of the negative-strand RNA of DENV, a hallmark of viral replication, in *Wolbachia* infected and uninfected cells. The results showed that *Wolbachia* infection significantly decreased negative RNA levels in mosquito cells. Furthermore, we compared the gene expression profiles of identified DENV binding proteins between cells with and without *Wolbachia* infection, and found six genes were down-regulated and five genes were up-regulated in response to *Wolbachia* infection. Further studies should be conducted to study the molecular mechanisms of *Wolbachia*-induced inhibition to DENV binding and replication.

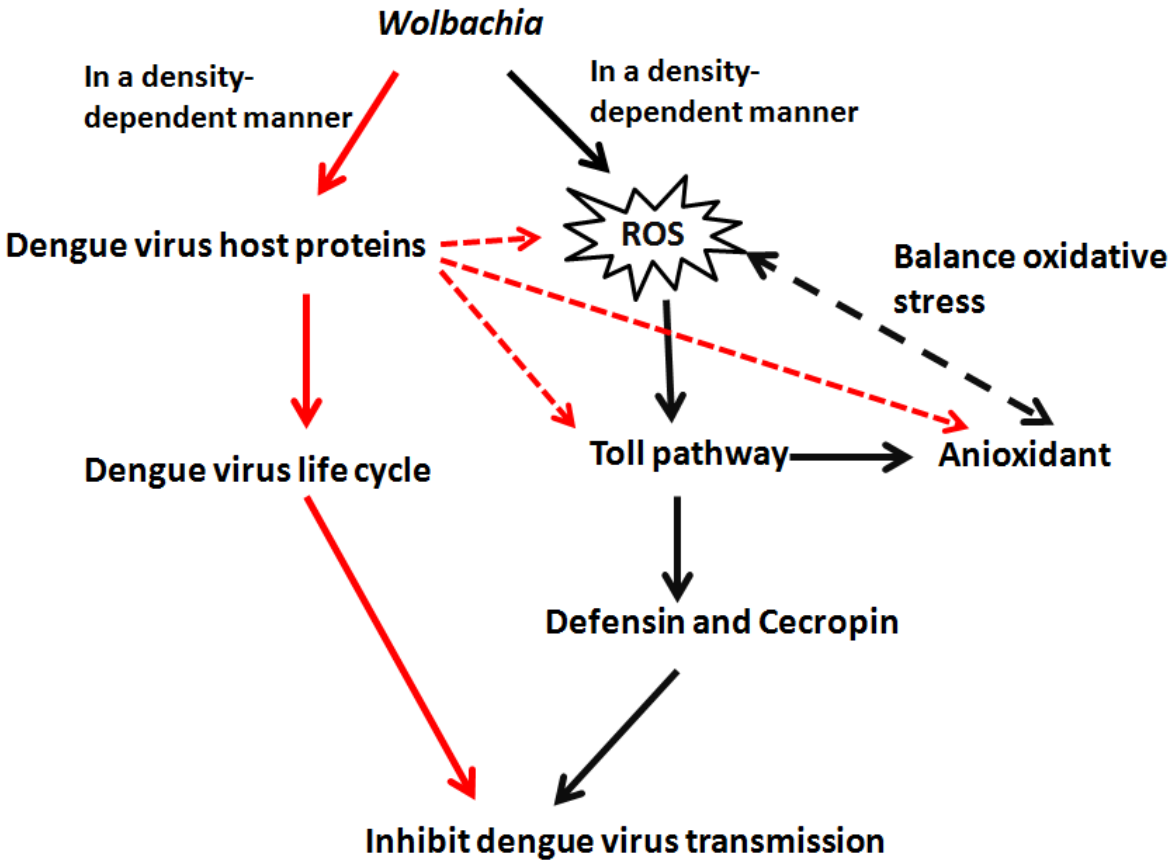

Figure 5.1: A current model of *Wolbachia*-mediated viral interference in *Ae. aegypti*.

Modified from Pan et al., 2012

Microarray assays indicated that *Wolbachia* infection significantly regulated 1,860 genes in mosquito cells. In order to further understand the underlying mechanisms of *Wolbachia*-mediated Dengue resistance, we selected 16 previously reported DENV host factors, which were up-regulated by *Wolbachia* infection. Functional RNA interference (RNAi) screen revealed that silencing of eleven DENV host factors resulted in increased DENV viral loads in *Wolbachia* infected mosquito cells. Those host factors include four genes with diverse function, three genes belonging to redox/stress/mitochondrion group, two immunity genes, a metabolism gene and a gene belonging to replication/transcription/translation group.

In our previous work, we proposed a model of *Wolbachia*-mediated resistance to DENV that is focusing on reactive oxygen species (ROS) and immunity pathways (Pan et al., 2012). Based on the results generated from the above studies, we further improve this model to better interpret *Wolbachia*-mediated viral interference (Fig. 5.1). *Wolbachia* infection influences the expression of several DENV host factors in a density-dependent manner. Some of those host factors are directly related to the activation of immunity pathways, the level of ROS and the expression of antioxidant, thus our previous model could be used to explain the viral interference. In addition, infection with *Wolbachia* also influences the expression of host proteins involved in DENV life cycle. Given its compact genome, DENV is highly dependent on host cell's machinery to propagate (Sessions et al., 2009). Therefore, *Wolbachia*-induced changes in the expression of host proteins, which are essential for DENV replication, could also contribute to viral inhibition (Fig. 5.1). For example, *Wolbachia* infection down-regulates the expression elongation factor 1-alpha (-4.23 fold), which binds to the 3'-untranslated region of DENV RNA to aid in viral replication (De Nova-Ocampo et al., 2002).

Future Directions

Wolbachia is emerging as a potential control agent to decrease transmission of vector-borne pathogens such as DENV(Cook et al., 2008, Moreira et al., 2009, Xi et al., 2005, Walker and Moreira, 2011, Bian et al., 2013) . Nevertheless, very little is known about the underlying mechanism of *Wolbachia*-mediated viral inhibition, limiting the capability of exploiting *Wolbachia* for vector control. A better understanding of interaction between *Wolbachia* and DENV in mosquito host will facilitate the current effort to eliminate dengue through *Wolbachia*-based strategies.

Our results indicate *Wolbachia* is able to induce resistance to DENV in host genetic background of *Ae. albopictus*, and highlight the importance of *Wolbachia* density in *Wolbachia*-mediated viral inhibition. The molecular mechanisms employed by the host to regulate *Wolbachia* density are not well understood. The within-host density of *Wolbachia* can be influenced by factors including insect age, temperatures, antibiotic treatment, length of symbiotic relationship, ROS and MicroRNAs (van Opijnen and Breeuwer, 1999, Unckless et al., 2009, Hussain et al., 2011, Bian et al., 2013). To block pathogen transmission to human, novel control strategies can be developed to increase *Wolbachia* density in mosquitoes carrying this bacterium. Consequently, there is a need to investigate how *Wolbachia* density is regulated by hosts and how the *Wolbachia* machinery controls its replication. Since the Drosophila genome-wide RNAi library is available for high-throughput screen (Sessions et al., 2009), the *Wolbachia*-infected *Drosophila* cell line can be used a simple model to study how insect hosts regulate *Wolbachia* density.

Mosquito host factors participate in most stages of DENV life cycle (Ahlquist et al., 2003). *Wolbachia* could interact with those DENV host proteins to influence dengue infection. Knowledge of those DENV host factors affected by *Wolbachia* not only informs us the

molecular mechanisms exploited by *Wolbachia* to induce viral protection but also provides potential targets that could be pursued for dengue control and antiviral drug development. There are several future directions associated with the *Wolbachia*-mediated host factors involved in resistance to DENV infection. First, future work should study molecular mechanisms of action of those eleven host factors involved in *Wolbachia*-mediated DENV resistance. Second, genome-wide RNAi screen could be carried out to uncover more host factors associated with this resistance. In addition, *Wolbachia* may down-regulate host factors required for DENV replication. However, we only investigated DENV host factors up-regulated in response to *Wolbachia* infection. Therefore, further study should be carried out to determine what are those DENV host factors down-regulated by *Wolbachia* and involved in *Wolbachia*-mediated antiviral protection.

Wolbachia infection inhibits DENV binding and replication. Further studies should be focused on the molecular mechanisms of *Wolbachia*-mediated inhibition to viral attachment and replication. Proteomics analysis of virus-receptor interaction can be used to study how *Wolbachia* inhibits DENV binding. DENV replicon with *Renilla* luciferase reporter can be used to study the molecular basis of *Wolbachia*-mediated inhibition to viral replication (Alcaraz-Estrada et al., 2010). In addition, we only investigated the impact of *Wolbachia* infection on DENV binding and replication stages, it is important to determine whether *Wolbachia* inhibits other stages of DENV life cycle, especially translation, assembly and maturation. The DENV reporter viruses will facilitate studies on the impact of *Wolbachia* infection on DENV life cycle (Schoggins et al., 2012, Mattia et al., 2011).

The *Wolbachia*-mediated antiviral protection varies depending on *Wolbachia* strain, infection type and host(Rainey et al., 2014). Theoretical modelling indicates that *Wolbachia*-

mediated protection has the potential to be a selective force on DENV evolution, perhaps resulting in increased virulence of DENV (Jones et al., 2011). Therefore, there is a clear need for studies of the impact of *Wolbachia* on DENV evolution in mosquito host. *Wolbachia* infected cell lines could be used as *in vitro* models for investigating the long-term effect of *Wolbachia* on DENV virulence.

Genetic analysis of *Wolbachia* has been hindered not only by the challenge in manipulating this obligate intracellular bacterium, but also by the lack of efficient tools for transformation of *Wolbachia*. Generation of transgenic *Wolbachia* will not only help us better understand the mechanisms underlying several *Wolbachia*-induced phenotypes such as cytoplasmic incompatibility and antiviral protection, but also provide an approach to utilize *Wolbachia* as a gene driver to spread anti-pathogen genes into mosquito vectors (Marshall, 2009). The recent progress in use of rifampin as an effective selectable marker for generating rickettsial transformation (Qin et al., 2004) and successful development of CRISPR/Cas9 technology in transformation of prokaryotic and eukaryotic organism is providing such an exciting opportunity to develop effective strategies for the transformation of *Wolbachia*.

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