Wolbachia uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*

Guangmei Zhang[a], Mazhar Hussain[a], Scott L. O'Neill[b,c], and Sassan Asgari[a,1]

[a]Australian Infectious Disease Research Centre, School of Biological Sciences, University of Queensland, St. Lucia, QLD 4072, Australia; [b]School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia; and [c]Institute of Molecular Biosciences, University of Queensland, St. Lucia, QLD 4072, Australia

Edited by Nancy A. Moran, Yale University, West Haven, CT, and approved May 7, 2013 (received for review February 25, 2013)

The endosymbiont *Wolbachia* is common among insects and known for the reproductive manipulations it exerts on hosts as well as inhibition of virus replication in their hosts. Recently, we showed that *Wolbachia* uses host microRNAs to manipulate host gene expression for its efficient maintenance in the dengue mosquito vector, *Aedes aegypti*. Cytosine methylation is mediated by a group of proteins called DNA (cytosine-5) methyltransferases, which are structurally and functionally conserved from prokaryotes to eukaryotes. The biological functions of cytosine methylation include host defense, genome stability, gene regulation, developmental promotion of organs, and lifespan regulation. *Ae. aegypti* has only one DNA methyltransferase gene (*AaDnmt2*) belonging to the cytosine methyltransferase family 2, which is the most deeply conserved and widely distributed gene among metazoans. Here, we show that in mosquitoes the introduced endosymbiont, *Wolbachia*, significantly suppresses expression of *AaDnmt2*, but dengue virus induces expression of *AaDnmt2*. Interestingly, we found that aae-miR-2940 microRNA, which is exclusively expressed in *Wolbachia*-infected mosquitoes, down-regulates the expression of *AaDnmt2*. Reversely, overexpression of AaDnmt2 in mosquito cells led to inhibition of *Wolbachia* replication, but significantly promoted replication of dengue virus, suggesting a causal link between this *Wolbachia* manipulation and the blocking of dengue replication in *Wolbachia*-infected mosquitoes. In addition, our findings provide an explanation for hypomethylation of the genome in *Wolbachia*-infected *Ae. aegypti*.

endosymbiosis | Wolbachia pipientis

Dengue is endemic in more than 110 tropical countries, with 2.5–3.0 billion people living at risk for infection. Each year, it is reported to infect more than 50 million people worldwide (1). Dengue virus (DENV) from the genus *Flavivirus* is a positive-strand RNA virus, which is primarily transmitted to humans by female *Aedes* mosquitoes. Because there are currently neither specific antiviral drugs nor approved vaccines against DENV, insect vector control is still considered the best approach to prevent dengue transmission. In recent years, *Wolbachia* infections of mosquitoes have been proposed as a novel approach for area-wide dengue control that is inexpensive to implement and self-sustaining (2, 3).

Wolbachia, maternally inherited Gram-negative endosymbiotic bacteria, are estimated to occur naturally in more than 40% of all insect species and 28% of surveyed mosquito species (4, 5). However, with some exceptions, they have not been found in important species that are the vectors of human pathogens such as dengue and malaria. Recently, several strains of *Wolbachia* have been successfully introduced into *Aedes aegypti* (6, 7). Mosquitoes with *w*MelPop strain transinfection showed about a 50% reduction in adult female lifespan (8). Interestingly, *Ae. aegypti* infected with *Wolbachia* induces very strong resistance to several arboviruses including DENV, yellow fever, and Chikungunya viruses, *Plasmodium*, and filarial nematodes (9–12).

MicroRNAs (miRNAs) are an evolutionarily conserved class of small ~22 nucleotides noncoding RNAs that regulate gene expression via partially or completely matching to their target genes (13). They play important roles in cellular processes including development, differentiation, apoptosis, and immunity. Until now, more than 25,000 miRNA sequences have been identified from different organisms including protozoans, plants, animals, and viruses (miRBase v19) (14). It has been reported that the expression levels of cellular miRNAs dramatically change in response to bacterial and viral infections in animals and plants (15–20). In human, a liver-specific miRNA, miR-122, can target the 5′ UTR of hepatitis C virus to enhance viral replication (21). Huang et al. (2007) (17) reported that HIV also uses host miRNAs to target its genes to its own advantage. Recently, we reported differential expression of several miRNAs in *Wolbachia*-infected *Ae. aegypti* mosquitoes with an miRNA, aae-miR-2940, which is exclusively expressed in *Wolbachia*-infected mosquitoes. By using the host aae-miR-2940, *Wolbachia* up-regulates the expression of a host metalloprotease gene, *metalloprotease m41 ftsh*, which is essential for the endosymbiont's maintenance (18).

In this study, we identified another target gene of aae-miR-2940, *AaDnmt2*. AaDnmt2 belongs to cytosine methyltransferase family 2, which is the only DNA methyltransferase found in Diptera. In *Drosophila*, dDnmt2 is primarily localized in the cytoplasm and much less in the nucleus and could mediate both DNA and RNA methylations (22, 23). Here, we aimed to investigate the impact of *Wolbachia* and consequently up-regulation of aae-miR-2940 on AaDnmt2. In addition, we investigated the possible impact of regulation of the target gene on DENV replication. Our results suggest that AaDnmt2 plays an important role not only in the maintenance of *Wolbachia* infection in mosquito cells, but also in the resistance of mosquito cells to DENV infection.

Results

Expression Profile of AaDnmt2 in *Ae. Aegypti*. By using National Center for Biotechnology Information (NCBI) BLAST, RNAHybrid, and RNA22 software, a putative 5-cytosine DNA methyltransferase (AaDnmt2, GenBank ID: XM_001657505) from *Ae. aegypti* was identified as another target of a differentially expressed miRNA, aae-miR-2940, which was previously shown to up-regulate the transcript levels of a metalloprotease gene in *Wolbachia*-infected mosquitoes (18). Further analysis of the sequence showed that AaDnmt2 is a homolog of pmt1 from fission

Author contributions: G.Z., M.H., and S.A. designed research; G.Z. and M.H. performed research; S.L.O. and S.A. contributed new reagents/analytic tools; G.Z. and S.A. analyzed data; and G.Z., M.H., S.L.O., and S.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: s.asgari@uq.edu.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1303603110/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1303603110

yeast (24) and Dnmt2 from human, *Drosophila*, and other organisms (25–27). AaDnmt2 is a 344-aa protein with 47% identity to the *Drosophila* Dnmt2 protein. Like other Dnmt2 proteins, AaDnmt2 contains all 10 diagnostic and catalytic motifs of active DNA methyltransferases. In addition, similar to *Drosophila*, there is only one Dnmt gene present in *Ae. aegypti* genome.

In *Drosophila*, human, and zebrafish, the expression of Dnmt2 is developmentally and tissue-specifically regulated (22, 23, 25, 28). In this study, we investigated the expression pattern of *AaDnmt2* in *Ae. aegypti* by RT-PCR using *AaDnmt2* gene–specific primers. We found that the transcript levels of *AaDnmt2* gene were not detectable in any of the larval stages, but could be detected in adults (Fig. 1*A*). The transcript levels of *AaDnmt2* were lower in 1-d-old adults compared with 4-d-old adults (Fig. 1*A*). We also found that *AaDnmt2* is mainly expressed in the abdomen of both male and female mosquitoes, which suggests that *AaDnmt2* could be specifically expressed in some organs in the abdomen (Fig. 1*B*). Tissue-specific RT-quantitative PCR (qPCR) analysis of four tissues (ovary, salivary glands, midgut, and thoracic muscles) from *Ae. aegypti* females at 4 and 12 d after emergence confirmed that the highest transcript levels of *AeDnmt2* were found in the ovaries (see below).

Ae. aegypti is the host and vector of an important human pathogen, DENV. Recently, the insect endosymbiont *Wolbachia* was successfully introduced into *Ae. aegypti* and found to inhibit the ability of pathogens such as DENV to infect this mosquito species (10, 11). Based on this, we investigated the expression profile of *AaDnmt2* in mosquitoes infected with *Wolbachia* or DENV. In *Wolbachia*-infected mosquitoes at 4 d after emergence, *AaDnmt2* was not detected by RT-PCR, but it could be detected in its tetracycline-cured (tet-cured) counterpart mosquitoes (Fig. 2*A*). *AaDnmt2* was mainly expressed in the abdomen of tet-cured mosquitoes (Fig. 2*B*). Interestingly, in 3-d-old females infected with *Wolbachia*, *AaDnmt2* could not be detected in either the head and thorax or the abdomen (Fig. 2*B*). Consistently, when specific tissues from 4- and 12-d-old female mosquitoes were analyzed using RT-qPCR, in general, about twofold less *AeDnmt2* transcripts were found in *Wolbachia*-infected mosquito tissues (Fig. 3). In tet-cured mosquitoes infected with DENV, RT-PCR results showed that *AaDnmt2* was expressed at much higher levels compared with the mosquitoes without DENV (Fig. 2*C*).

aae-miR-2940 Down-regulates the Expression of *AaDnmt2*. The target sequences of aae-miR-2940 were predicted in the coding region of *AaDnmt2* (nucleotides 1011–1035) with significant complementarity to aae-miR-2940 (Fig. 4*A*). In *Ae. aegypti* Aag2 cells as well as *w*MelPop-CLA–infected Aag2 cells (aag2.*w*MelPop-CLA), *AaDnmt2* transcripts could not be detected (Fig. 2*B*). The reason for lack of detection of *AaDnmt2* in Aag2 cells, compared with *Wolbachia*-free mosquitoes (Fig. 2), is the expression of aae-miR-2940 in noninfected mosquito cell lines compared with whole mosquitoes, although at a lower level compared with *Wolbachia*-infected cells (18). To confirm this, the *Dicer-1* gene was silenced in Aag2 cells using RNAi. After confirming gene silencing, RT-PCR results showed that the transcript levels of *AaDnmt2* were considerably higher compared with mock-transfected Aag2 cells (Fig. 4*B*), which confirmed that inhibition of *AaDnmt2* expression in Aag2 cells is mediated by miRNAs. To further confirm that aae-miR-2940 contributes in this inhibition, we transfected *Ae. aegypti* Aag2 cells with synthetic aae-miR-2940–specific and aae-miR-2940*–specific inhibitors, respectively. RT-PCR results showed increased transcript levels of *AaDnmt2* in cells transfected with aae-miR-2940–specific inhibitor compared with cells transfected with control aae-miR-2940*–specific inhibitor and mock-transfected cells (Fig. 4*B*). These results suggest that aae-miR-2940 down-regulates the transcript levels of *AaDnmt2*, which is consistent with the expression pattern of *AaDnmt2* gene in mosquitoes with or without *Wolbachia* (Figs. 2 *A* and *B* and 3).

AaDnmt2 Suppresses *Wolbachia* Replication. In a previous study, we reported that aae-miR-2940 up-regulates the transcript levels of the host metalloprotease, which is required for *Wolbachia* replication and maintenance in mosquito cells (18). Considering that aae-miR-2940 down-regulates the transcript levels of *AaDnmt2*, we investigated whether AaDnmt2 has any effect on *Wolbachia* replication. For this, AaDnmt2 was overexpressed in aag2.*w*MelPop-CLA cells and the density of *Wolbachia* was analyzed by PCR. Two different amounts of pIZ/AaDnmt2 were used to transfect aag2.*w*MelPop-CLA cells resulting in one line expressing the gene at a higher level (L1) compared with the other (L2) (Fig. 5*A*). PCR results with *wsp* gene–specific primers revealed that *Wolbachia* density was considerably lower in cells transfected with the pIZ/AaDnmt2 vector compared with cells transfected with the empty vector (pIZ) (Fig. 5*B*). This effect was found to be dose-dependent because PCR results showed that *Wolbachia* density was much lower in L1, where AaDnmt2 is expressed at a higher level compared with L2, the lower overexpressing AaDnmt2 line (Fig. 5*B*). However, *Wolbachia* density was lower in L2 compared with pIZ empty vector transfected cells (Fig. 5*B*). A qPCR analysis further confirmed that overexpression of AaDnmt2 leads to lower *Wolbachia* density (Fig. 5*C*). These results suggest that AaDnmt2 suppresses *Wolbachia* replication in the cell line and the suppression is concentration-dependent, which is consistent with the expression profile that AaDnmt2 is detected at considerably lower levels in *Wolbachia*-infected mosquitoes compared with tetracycline-cured counterpart mosquitoes (Figs. 2*A* and 3).

AaDnmt2 Enhances DENV Replication. In both *Ae. aegypti* and mosquito cell lines, *Wolbachia* was found to limit replication of DENV (10, 11), which could be due to manipulation of the host genes expression via miRNAs by *Wolbachia*. To explore the effect of AaDnmt2 on DENV replication, we overexpressed AaDnmt2 in Aag2 cells that were subsequently infected with DENV. Total RNA at 72 h after viral infection was isolated and analyzed by RT-PCR with DENV-specific primers. The results showed that the relative abundance of DENV was significantly higher in the cells overexpressing AaDnmt2 compared with cells transfected with the pIZ empty vector (Fig. 6*A*). This result was also confirmed by RT-qPCR (Fig. 6*B*). These results suggest that AaDnmt2 enhances replication of DENV in cells, which is consistent with the expression profile that *AaDnmt2* transcripts levels were induced in mosquitoes infected with DENV (Fig. 2*C*). However, when

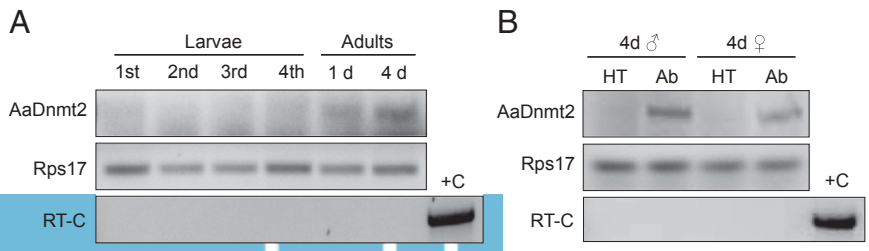

Fig. 1. AaDnmt2 expression profile. (*A*) RT-PCR analysis was performed using the total RNA samples from *Ae. aegypti* mosquito larvae and adults. (*B*) RT-PCR analysis was performed with head + thorax (HT) and abdomen (Ab) of mosquito females and males. *Rps17* gene was used as control to show the integrity of RNA. +C, positive control using Aag2 genomic DNA (gDNA); RT-C, RT-PCR control reactions in which reverse transcriptase was not added.

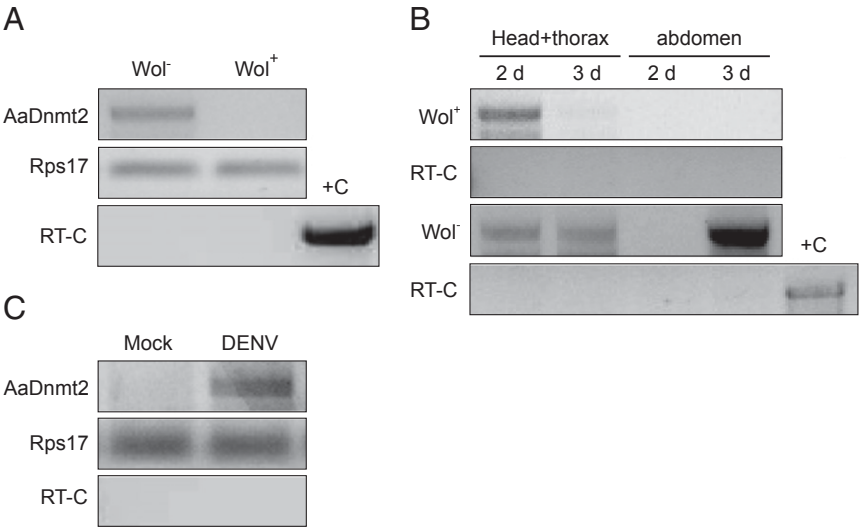

Fig. 2. AaDnmt2 expression profile in *Wolbachia* or DENV-infected mosquitoes. (*A*) RT-PCR analysis was performed using total RNA samples from 4-d-old *Wolbachia*-infected (Wol+) and uninfected (Wol–) female mosquitoes. (*B*) RT-PCR analysis was performed using total RNA samples from Wol+ and Wol– head + thorax (HT) and abdomen (Ab) of female mosquitoes at 2 and 3 d after emergence. (*C*) RT-PCR analysis was performed using total RNA samples from 3-d DENV-infected and uninfected mosquitoes (without *Wolbachia*). *Rps17* gene was used as control to show the integrity of RNA. +C in *A* serves as positive control for *C* because the reactions were done at the same time.

aag2.*w*MelPop-CLA cells were transfected with pIZ/AaDnmt2 and then infected with DENV, virus replication was not enhanced by AaDnmt2 compared with cells transfected with the pIZ empty vector (Fig. 6*C*). Consistently, when *w*MelPop-CLA cells were transfected with aae-miR-2940–specific inhibitor and subsequently infected with DENV, virus replication increased over time compared with *w*MelPop-CLA cells transfected with the control inhibitor; however, the effect was moderate (1.5 times at 72 h) and not statistically significant ($P > 0.05$; Fig. 7). The moderate effect could be due to regulation of *AeDnmt2* transcripts by more than one miRNA; therefore, when aae-miR-2940 is specifically inhibited, another miRNA may still down-regulate *AeDnmt2* transcript levels leading to only a partial up-regulation of *AeDnmt2* transcript levels resulting from aae-miR-2940 inhibition. In fact, when *Dicer-1* was silenced in Aag2 cells, which affects miRNA biogenesis in general, considerably more *AeDnmt2* transcripts were amplified compared with when aae-miR-2940 was specifically inhibited using the miRNA-specific inhibitor (Fig. 4*B*; compare Dicer 1 RNAi with 2940 inhibitor), which further supports the assumption. It has already been shown that one mRNA can be a target of more than one miRNA (29–31). Our preliminary bioinformatics search showed that a number of other *Ae. aegypti* miRNAs (Fig. S1), which are differentially expressed in *Wolbachia*-infected mosquitoes (18), could potentially target *AeDnmt2*; however, their interactions with *AeDnmt2* need to be experimentally validated.

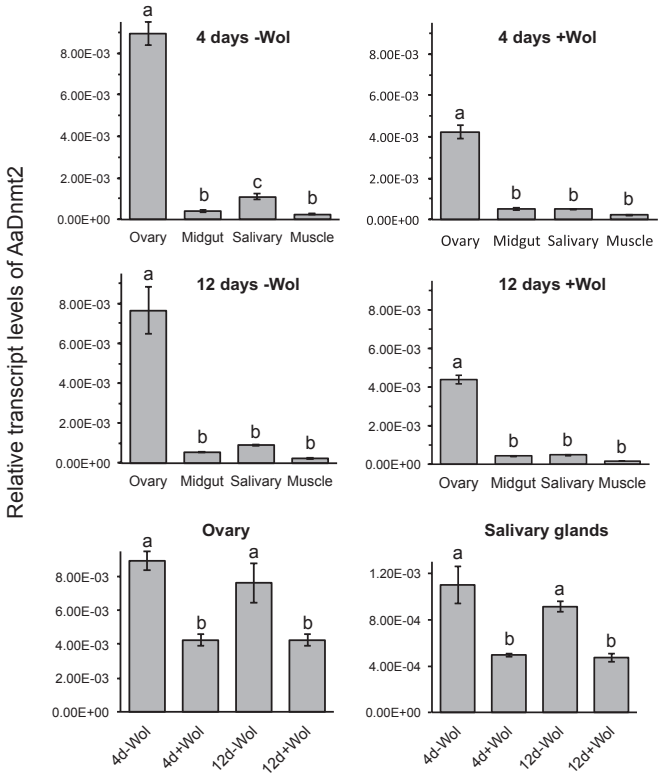

Fig. 3. Tissue-specific expression of *AeDnmt2* in female *Ae. aegypti* mosquitoes. RT-qPCR analysis of *AeDnmt2* transcript levels in the ovary, midgut, salivary glands, and thoracic muscle tissues (muscle) of 4- and 12-d-old Wol+ and Wol– female mosquitoes. Transcript levels of *AeDnmt2* were also compared in the ovaries and salivary glands in the samples (bottom two graphs). There are statistically significant differences between groups with different letters at $P < 0.0001$ (one-way ANOVA with Tukey's post-hoc test).

Discussion

In a recent study, we reported that *w*MelPop strain of *Wolbachia* altered the expression of a number of host miRNAs, including aae-miR-2940 in the host mosquito *Ae. aegypti* (18). In turn, aae-miR-2940 up-regulated the transcript levels of a target gene, *metalloprotease ftsh*. Metalloprotease was found to be crucial for efficient replication and maintenance of the endosymbiont in *Ae. aegypti* (18). However, the role of induced miRNAs in the immune response to DENV and other microorganisms has not been explored. The present study identified *AaDnmt2*, a methyltransferase, as another target gene of aae-miR-2940 in *Ae. aegypti*, which is highly expressed in the ovaries. We found that in the absence of the miRNA, either by *Dicer-1* silencing or specifically using a synthetic inhibitor of aae-miR-2940, AeDnmt2 was up-regulated, suggesting that aae-miR-2940 negatively regulates the transcripts of *AeDnmt2*. In addition, by investigating the expression patterns, we found that the transcription levels of *AaDnmt2* were significantly suppressed by *Wolbachia* in mosquitoes and by aae-miR-2940 in the mosquito cell line. This trend was also observed in specific tissues of the mosquitoes tested. The transcription levels of *AaDnmt2* were increased by DENV infection in mosquitoes. Interestingly, overexpression of AaDnmt2 in aag2.*w*MelPop-CLA cells resulted in a significant decline in *Wolbachia* density. Overexpression and coinfection with DENV in Aag2 cells showed a significant increase in DENV replication, but not in aag2.*w*MelPop-CLA cells. These results

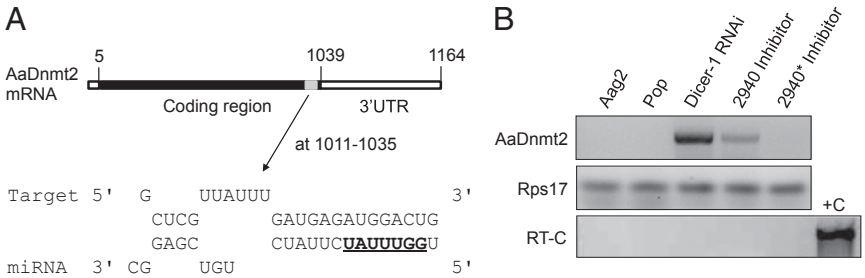

Fig. 4. *AaDnmt2* transcription is down-regulated by aae-miR-2940. (*A*) Schematic diagram showing *AaDnmt2* mRNA and its target sequences with complete complementarity of aae-miR-2940 seed region (bold and underlined) with the sequences. (*B*) RT-PCR analysis of RNA extracted from mock-transfected Aag2 and aag2.*w*MelPop-CLA (Pop) cells, and Aag2 cells transfected with *Dicer-1* dsRNA, aae-miR-2940, and aae-miR-2940* (control) synthetic inhibitors. *Rps17* gene was used as control to show the integrity of RNA.

suggest that by inducing the expression of aae-miR-2940, *Wolbachia* could down-regulate AaDnmt2, which in turn inhibits replication of DENV. This describes a potential mechanism of suppression of DENV replication by *Wolbachia*.

Insects use a variety of immune responses such as RNA interference, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) (JAK/STAT), Toll, and Immune Deficiency (IMD) pathways and autophagy to contain viral infections (32–36). In their original host *Drosophila*, some strains of *Wolbachia* could induce resistance to various viruses such as *Drosophila* C virus, cricket paralysis virus, Nora virus, flock house virus, and West Nile virus (37–39). *Drosophila* synergistically uses several pathways to control viral infections (40–42). Recently, some strains of *Wolbachia* were successfully introduced into various mosquito species such as *Ae. aegypti*, *Aedes albopictus*, and *Anopheles gambiae* (somatic infection) in an effort to limit replication and spread of various vector-borne pathogens such as DENV and malaria (6, 7, 43). *Wolbachia* effectively suppress DENV, Chikungunya virus, yellow fever virus, and *Plasmodium* in their new hosts (9–12, 43, 44). Studies have shown that in *Ae. aegypti*, the symbionts up-regulate the expression of a large number of host immune genes such as defensins, cecropins, JAK-STAT, and Toll pathway genes (10, 33, 35, 43, 44). These suggest that similar to *Drosophila*, *Wolbachia*-infected *Ae. aegypti* may also use multiple pathways activated by *Wolbachia* to control pathogen infections. AaDnmt2 down-regulation might be used synergistically to suppress DENV by *Ae. aegypti*.

Reductions in AaDnmt2, the only methyltransferase in *Ae. aegypti*, by *Wolbachia* via induction of aae-miR-2940, would imply hypomethylation of the genome in *Wolbachia*-infected mosquitoes, which has significant implications in epigenetic regulation of gene expression. Role of miRNAs in regulation of methylation via controlling DNA methyltransferases has been documented. For example, miR-148 down-regulates Dnmt3B in human HeLa cells by targeting its transcripts in the coding region (45) and Dnmt1 in both human cholangiocarcinoma and systemic lupus erythematosus cell lines by targeting either 3′UTR or the coding region (46, 47). Overexpression of miR-148 decreased Dnmt1, which in turn promoted DNA hypomethylation and enhanced the expression of various methylation-sensitive genes. Considering substantial changes in the transcriptome profile of *Wolbachia*-infected mosquitoes relative to noninfected ones (12), it is conceivable that some of these changes are due to differential/hypo methylation of the mosquito genome. Indeed, a recent report demonstrated that in *w*MelPop-infected *Ae. aegypti* mosquitoes the pattern of cytosine methylation of the genome is widely disrupted leading to an overall hypomethylation of the genome in *Wolbachia*-infected mosquitoes compared with noninfected mosquitoes (48).

Taken together, our studies suggest that aae-miR-2940 plays an important role not only in the maintenance of *Wolbachia* infection in mosquito cells, but also contributes to the resistance to DENV infection in mosquito cells. Our findings describe a mechanism for *Wolbachia* to regulate host gene expression by affecting methylation and limit DENV infection by using host miRNAs in *Ae. aegypti*. Our results demonstrated a critical functional link between *Wolbachia*, miRNAs, and DENV, which furthers our understanding of *Wolbachia*–virus–mosquito interactions.

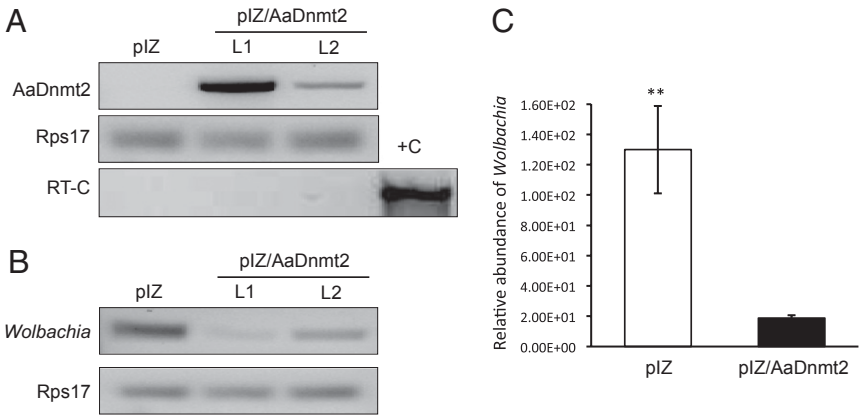

Fig. 5. AaDnmt2 reduces *Wolbachia* replication. (*A*) RT-PCR analysis was performed to confirm that *AaDnmt2* was overexpressed in pIZ/AaDnmt2 plasmid-transfected aag2.*w*MelPop-CLA cells. L1 and L2 refer to two transfected lines established using two different concentrations of pIZ/AeDnmt2; L1 reflects a concentration of 2 μg; L2 a concentration of 1 μg. *Rps17* primers were used as control to show the integrity of RNA. (*B*) PCR analysis of *Wolbachia* density 72 h after transfection with pIZ or pIZ/AaDnmt2 plasmid in L1 and L2 (as in *A*) in aag2.*w*MelPop-CLA cells using primers specific to the *Wolbachia wsp* gene. *Rps17* primers were used as control to show the integrity of DNA. (*C*) qPCR analysis of DNA extracted from aag2.*w*MelPop-CLA cells 72 h after transfection with pIZ or pIZ/AaDnmt2. Three biological with three technical replicates were carried out for each transfection. Asterisks indicate a significant difference between mock and AaDnmt2 transfections ($P < 0.001$; *t* test).

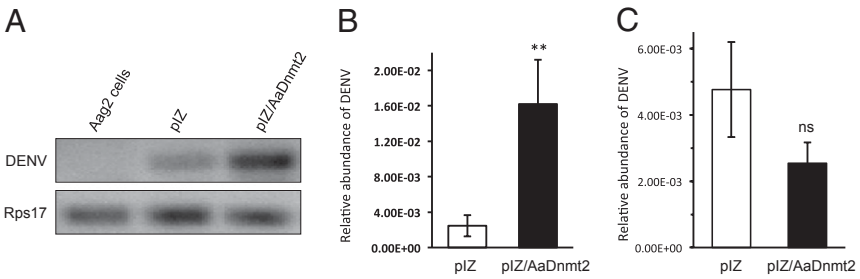

Fig. 6. AaDnmt2 enhances DENV replication in cells without *Wolbachia*. (*A*) RT-PCR analysis of RNA using DENV-specific primers 72 h after transfection with pIZ or pIZ/AaDnmt2 in Aag2 cells. Rps17, primers to the *Rps17* gene showing the integrity of RNA. (*B*) RT-qPCR analysis of DENV abundance 72 h after transfection with pIZ or pIZ/AaDnmt2 in Aag2 cells. (*C*) RT-qPCR analysis of RNA using DENV-specific primers in aag2.*w*MelPop-CLA cells 72 h after transfection with pIZ or pIZ/AaDnmt2. For *B* and *C*, three biological replicates with three technical replicates were carried out for each transfection. Asterisks indicate a significant difference between mock and AaDnmt2 transfections ($P < 0.001$; *t* test); NS indicates no significant differences between mock and pIZ/AaDnmt2 transfections ($P > 0.05$; *t* test).

Materials and Methods

Mosquitoes, Insect Cell Lines, and DENV Infection. *Ae. aegypti* infected with *w*MelPop-CLA strain of *Wolbachia* (Wol+) and a *Wolbachia*-free strain, tet-cured line (Wol–), were the stocks as previously described (7). Adults and larvae were reared at 25 °C with 80% relative humidity and a 12-h light regimen. Larvae were maintained with fish food pellets (Tetramin, Tetra) at a density of 50 larvae per liter of water in flat trays; adults were offered 10% (wt/vol) sucrose solution, ad libitum. *Ae. aegypti* Aag2 cells and those infected with *Wolbachia* (denoted as aag2.*w*MelPop-CLA) (49) were maintained in a media with a 1:1 mixture of Mitsuhasgi-Maramorosch and Schneider's insect media (Invitrogen) supplemented with 10% FBS.

RNA Extraction, cDNA Synthesis, and PCR. Total RNA from mosquitoes and cell lines was isolated using Tri-Reagent (Ambion Inc.), and subsequently treated with DNase I before being used for reverse transcription. The first-strand cDNA for *AaDnmt2* was synthesized by RT with *AaDnmt2* gene-specific primers (forward: 5′-ATGAGTGCTACCGACGTA-3′; reverse: 5′-TCACCAGTCCATCTCATC-3′). In each RT reaction, ~2 μg of total RNA was used as template in a total volume of 20 μL. Amplification following cDNA synthesis was carried out in a total reaction volume of 25 μL with 2 μL of RT products. The amplification was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 68 °C for 1 min, and a final extension at 68 °C for 10 min. The ribosomal protein S17 (*RPS17*) gene was used for normalizing data as described previously (18).

Quantitative PCR of *Wolbachia* Density. Total genomic DNA was extracted from cells as described previously (50). *Wolbachia* density in cells was determined by qPCR using the *wsp* gene-specific primers as described previously (18). qPCR was performed using Platinum SYBR Green Mix (Invitrogen) with 20 ng of total genomic DNA in a Rotor-Gene thermal cycler (Qiagen) under the following conditions: 95 °C hold for 30 s, then 40 cycles of 95 °C for 15 s, 50 °C for 15 s, and 72 °C for 20 s, following by the melting curve (68–95 °C). For this experiment, three biological replicates with three technical replicates were analyzed. The *RPS17* gene was used for normalization of DNA templates. The Student *t* test was used to compare the differences in means between different treatments.

qRT-PCR. Total RNA was extracted and reverse transcribed as described previously using a specific reverse primer to DENV (reverse, CCATCCCGTACCAGCATCCG). Following RT, qPCR with DENV gene-specific primers (forward, GTGGTGGTGACTGAGGACTG) was performed to determine DENV density in cells. This amplified a 138-nt fragment from nucleotides 3273–3409 of the DENV genome. Platinum SYBR Green Mix (Invitrogen) was used for qPCR with 1 μL of RT products as described previously. For this experiment, three biological replicates with three technical replicates were analyzed. The *RPS17* gene was used for normalization of RNA templates. The Student *t* test was used to compare the differences in means.

For tissue-specific analysis of *AaDnmt2* transcript levels, total RNA was extracted from ovaries, thoracic muscle tissue, salivary glands, and midgut dissected from 4- and 12-d-old Wol+ and Wol– female mosquitoes as described previously. RT-qPCR reactions were carried out as described previously using primers specific to *AaDnmt2* gene (forward, GTAGTATCAGCAATTGACATCA; reverse, TGGGGGTGACATGAGAATAAG). Similarly, three biological replicates with three technical replicates were analyzed for each tissue and mosquito type. Each biological replicate consisted of a pull of total RNA extracted from specific tissues of five mosquitoes. The *RPS17* gene was also used for normalization of RNA templates.

Overexpression of AaDnmt2 in Insect Cell Lines. The cDNA of *AaDnmt2* gene (GenBank accession no. XM_001657505) was synthesized by RT-PCR using gene-specific primers with KpnI and SacII restriction sites (forward: 5′-GGTACCATGGGGAGTGCTACCGACGTA-3′; reverse: 5′-CCGCGGCCAGTCCATCTCATC-3′; restriction sites are underlined). *AaDnmt2* was cloned into pIZ/V5-His vector containing an insect-specific promoter (Life Technologies). Two micrograms of the produced plasmid with *AaDnmt2* gene was transfected into Aag2 or aag2.*w*MelPop-CLA cell lines using Cellfectin (Life Technologies) as a transfection reagent. Cells transfected with the empty pIZ/V5-His vector were used as controls. Cells were collected at 72 h after transfection, and total RNA was isolated and analyzed by RT-PCR and/or RT-qPCR with gene-specific primers as required.

Inhibition of aae-miR-2940 in *Wolbachia*-Infected Cells. aae-miR-2940 (5′-GCCUCGACAGAUAAGAUAAACCA-3′) and aae-miR-2940* (5′-AGUGAUUUAUCUCCCUGUCGAC-3′, as a control) inhibitors were synthesized by Genepharma.

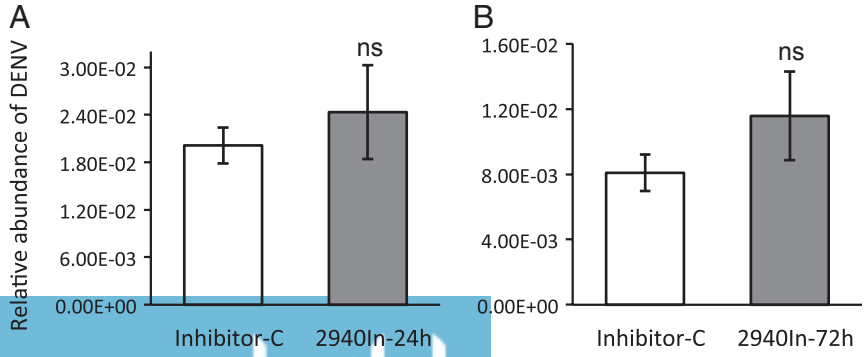

Fig. 7. Effect of inhibition of aae-miR-2940 on DENV replication. aag2.*w*MelPop-CLA cells were transfected with synthetic aae-miR-2940 inhibitor (2940In) and control inhibitor (Inhibitor-C) 48 h before infection with DENV. RNA samples were extracted from cells at (*A*) 24 h and (*B*) 72 h after infection and analyzed by RT-qPCR using specific primers to DENV. Three biological with three technical replicates were carried out for each transfection. NS, no significant differences between Inhibitor-C and 2940In ($P > 0.05$; *t* test).

In this experiment, 100 ng of the aae-miR-2940 and aae-miR-2940* inhibitors were transfected into aag2.*w*MelPop-CLA cells using the Cellfectin transfection reagent. Cells were collected at 72 h after transfections, total RNA was extracted, and RT-PCR analyses were performed with *AaDnmt2* gene–specific primers as previously.

RNA-Mediated Gene Silencing. For RNAi-based experiments, dsRNA was synthesized in vitro by using the T7 Megascript transcription kit according to the manufacturer's instructions (Ambion Inc.). T7 promoter sequences (TAATACGACTCACTATAGGG) were incorporated in both forward and reverse primers designed to amplify a 450 bp of the *Ae. aegypti Dicer-1* gene (forward: 5′-CCCGGACCAAGTCCTAGTA-3′; reverse: 5′-CAACTCTTTCGGCACGTAA-3′). About 1 μg of the PCR product was used in a 4-h incubation at 37 °C for dsRNA synthesis. After DNase treatment, RNA was precipitated by lithium chloride. About 500 ng of dsRNA was used for transfection of Aag2 cells, which were transfected again at 48 h after the first transfection. Cells were collected for RNA isolation for analysis 48 h after the second transfection. Gene silencing was confirmed by RT-qPCR with *Dicer-1*–specific primers.

Prediction of Cellular Targets of aae-miR-2940. NCBI BLAST, RNAHybrid, and RNA22 software were used to identify the potential targets of aae-miR-2940. tBLASTN was performed to search for homologous sequences. The BioEdit program was used to align sequences and to calculate the identities and similarities of sequences.

ACKNOWLEDGMENTS. We thank Guangjin Lu from Brian Kay's laboratory (Queensland Institute of Medical Research) for providing mosquito larval samples. This work was supported by Australian Research Council Discovery Grant DP110102112 (to S.A.), Australian Research Council Discovery Early Career Researcher Award fellowship DE120101512 (to M.H.), and a grant from the Foundation of the National Institutes of Health through the Grand Challenges in Global Health initiative of the Bill and Melinda Gates Foundation (to S.L.O.).

1. WHO (2009) *Dengue and Dengue Haemorrhagic Fever*. Fact Sheet No. 117. (World Health Organization, Geneva).
2. Hoffmann AA, et al. (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 476(7361):454–457.
3. Walker T, et al. (2011) The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* 476(7361):450–453.
4. Zug R, Hammerstein P (2012) Still a host of hosts for *Wolbachia*: Analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS ONE 7*: e38544.
5. Jeyaprakash A, Hoy MA (2000) Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* 9(4): 393–405.
6. Xi ZY, Khoo CCH, Dobson SL (2005) *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science* 310(5746):326–328.
7. McMeniman CJ, et al. (2009) Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* 323(5910):141–144.
8. Moreira LA, et al. (2009) Human probing behavior of *Aedes aegypti* when infected with a life-shortening strain of *Wolbachia*. *PLoS Negl Trop Dis* 3(12):e568.
9. van den Hurk AF, et al. (2012) Impact of *Wolbachia* on infection with chikungunya and yellow fever viruses in the mosquito vector *Aedes aegypti*. *PLoS Negl Trop Dis* 6(11):e1892.
10. Moreira LA, et al. (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell* 139(7):1268–1278.
11. Bian G, Xu Y, Lu P, Xie Y, Xi Z (2010) The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathog* 6(4):e1000833.
12. Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* 326(5949): 134–136.
13. Asgari S (2013) MicroRNA functions in insects. *Insect Biochem Mol Biol* 43(4):388–397.
14. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008) miRBase: Tools for microRNA genomics. *Nucleic Acids Res* 36(Database issue):D154–D158.
15. Fassi Fehri L, et al. (2010) *Helicobacter pylori* induces miR-155 in T cells in a cAMP-Foxp3-dependent manner. *PLoS ONE* 5(3):e9500.
16. Gao CP, et al. (2010) Reduced microRNA-218 expression is associated with high nuclear factor kappa B activation in gastric cancer. *Cancer* 116(1):41–49.
17. Huang JL, et al. (2007) Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. *Nat Med* 13(10):1241–1247.
18. Hussain M, Frentiu FD, Moreira LA, O'Neill SL, Asgari S (2011) *Wolbachia* uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector Aedes aegypti. *Proc Natl Acad Sci USA* 108(22):9250–9255.
19. Lu F, et al. (2008) Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J Virol* 82(21):10436–10443.
20. Tili E, et al. (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol* 179(8):5082–5089.
21. Jopling CL (2008) Regulation of hepatitis C virus by microRNA-122. *Biochem Soc Trans* 36(Pt 6):1220–1223.
22. Kunert N, Marhold J, Stanke J, Stach D, Lyko F (2003) A Dnmt2-like protein mediates DNA methylation in Drosophila. *Development* 130(21):5083–5090.
23. Schaefer M, Steringer JP, Lyko F (2008) The *Drosophila* cytosine-5 methyltransferase Dnmt2 is associated with the nuclear matrix and can access DNA during mitosis. *PLoS ONE* 3(1):e1414.
24. Wilkinson CRM, Bartlett R, Nurse P, Bird AP (1995) The fission yeast gene pmt1+ encodes a DNA methyltransferase homologue. *Nucleic Acids Res* 23(2):203–210.
25. Okano M, Xie SP, Li E (1998) Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res* 26(11):2536–2540.
26. Tweedie S, et al. (1999) Vestiges of a DNA methylation system in *Drosophila melanogaster? Nat Genet* 23(4):389–390.
27. Yoder JA, Bestor TH (1998) A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. *Hum Mol Genet* 7(2):279–284.
28. Rai K, et al. (2007) Dnmt2 functions in the cytoplasm to promote liver, brain, and retina development in zebrafish. *Genes Dev* 21(3):261–266.
29. Thomson DW, Bracken CP, Goodall GJ (2011) Experimental strategies for microRNA target identification. *Nucleic Acids Res* 39(16):6845–6853.
30. Vo NK, Dalton RP, Liu N, Olson EN, Goodman RH (2010) Affinity purification of microRNA-133a with the cardiac transcription factor, Hand2. *Proc Natl Acad Sci USA* 107(45):19231–19236.
31. Wu S, et al. (2010) Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3′ untranslated region. *Oncogene* 29(15):2302–2308.
32. Ding S-W (2010) RNA-based antiviral immunity. *Nat Rev Immunol* 10(9):632–644.
33. Pan X, et al. (2012) *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. *Proc Natl Acad Sci USA* 109(1):E23–E31.
34. Sabin LR, et al. (2009) Ars2 regulates both miRNA- and siRNA-dependent silencing and suppresses RNA virus infection in *Drosophila*. *Cell* 138(2):340–351.
35. Souza-Neto JA, Sim S, Dimopoulos G (2009) An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proc Natl Acad Sci USA* 106(42): 17841–17846.
36. Xi Z, Gavotte L, Xie Y, Dobson SL (2008) Genome-wide analysis of the interaction between the endosymbiotic bacterium *Wolbachia* and its *Drosophila* host. *BMC Genomics* 9:1.
37. Glaser RL, Meola MA (2010) The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. *PLoS ONE* 5(8):e11977.
38. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) *Wolbachia* and virus protection in insects. *Science* 322(5902):702.
39. Teixeira L, Ferreira A, Ashburner M (2008) The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol* 6(12):e2.
40. Dostert C, et al. (2005) The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *drosophila*. *Nat Immunol* 6(9):946–953.
41. Tanji T, Hu XD, Weber ANR, Ip YT (2007) Toll and IMD pathways synergistically activate an innate immune response in *Drosophila melanogaster*. *Mol Cell Biol* 27(12): 4578–4588.
42. Zambon RA, Nandakumar M, Vakharia VN, Wu LP (2005) The Toll pathway is important for an antiviral response in *Drosophila*. *Proc Natl Acad Sci USA* 102(20): 7257–7262.
43. Blagrove MSC, Arias-Goeta C, Failloux A-B, Sinkins SP (2012) *Wolbachia* strain *w*Mel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. *Proc Natl Acad Sci USA* 109(1):255–260.
44. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL (2011) *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS Pathog* 7(5):e1002043.
45. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R (2008) miR-148 targets human DNMT3b protein coding region. *RNA* 14(5):872–877.
46. Braconi C, Huang N, Patel T (2010) MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. *Hepatology* 51(3):881–890.
47. Pan W, et al. (2010) MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. *J Immunol* 184(12):6773–6781.
48. Ye YH, et al. (2013) Infection with a virulent strain of *Wolbachia* disrupts genome wide-patterns of cytosine methylation in the mosquito *Aedes aegypti*. *PLoS ONE*, 10.1371/journal.pone.0066482.
49. Frentiu FD, Robinson J, Young PR, McGraw EA, O'Neill SL (2010) *Wolbachia*-mediated resistance to dengue virus infection and death at the cellular level. *PLoS ONE* 5(10): e13398.
50. Glatz R, et al. (2004) Lectin-induced haemocyte inactivation in insects. *J Insect Physiol* 50(10):955–963.