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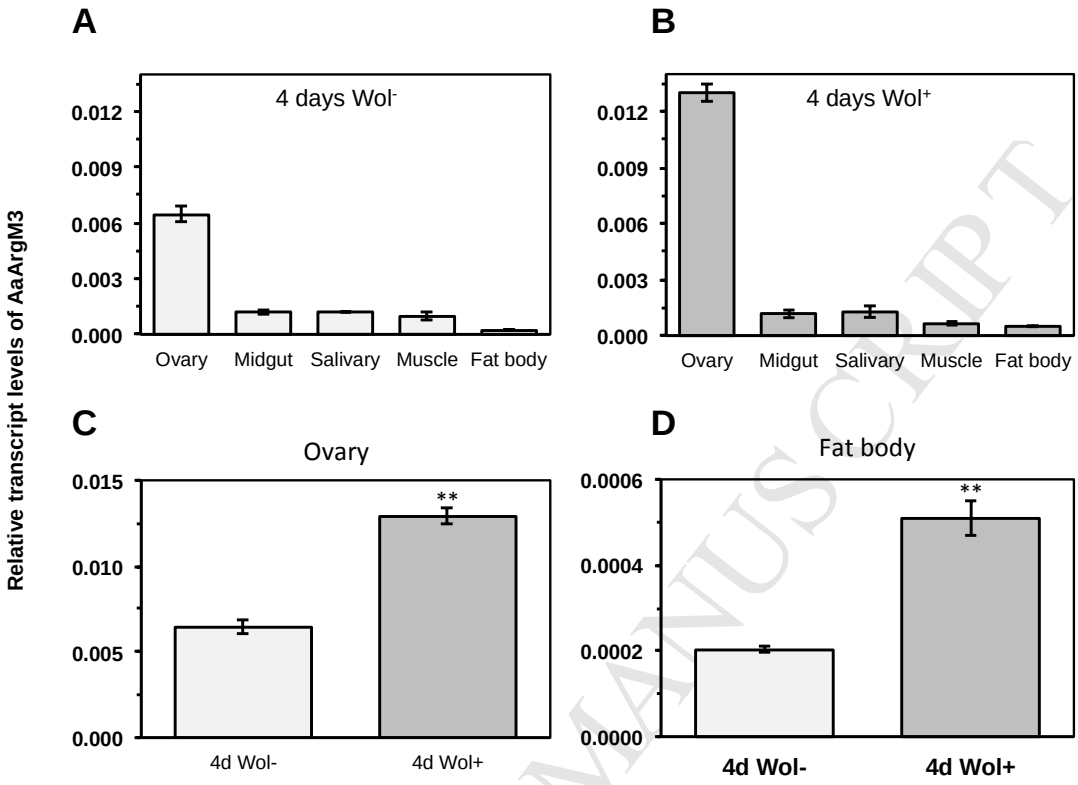
Graphical abstract



Regulation of arginine methyltransferase 3 by a *Wolbachia*-induced microRNA in *Aedes aegypti* and its effect on *Wolbachia* and dengue virus replication

Guangmei Zhang, Mazhar Hussain, and Sassan Asgari*

Australian Infectious Disease Research Centre, School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia

*Corresponding author: Sassan Asgari, Australian Infectious Disease Research Centre, School of Biological Sciences, The University of Queensland, Brisbane Qld 4072, Australia. Tel: +617 3365 2043; Fax: +617 3365 1655; s.asgari@uq.edu.au

Abstract

The gram-negative endosymbiotic bacteria, *Wolbachia*, have been found to colonize a wide range of invertebrates, including over 40% of insect species. Best known for host reproductive manipulations, some strains of *Wolbachia* have been shown to reduce the host life span by about 50% and inhibit replication and transmission of dengue virus (DENV) in the mosquito vector, *Aedes aegypti*. The molecular mechanisms underlying these effects still are not well understood. Our previous studies showed that *Wolbachia* uses host microRNAs (miRNAs) to manipulate host gene expression for its efficient maintenance and limiting replication of DENV in *Ae. aegypti*. Protein arginine methyltransferases are structurally and functionally conserved proteins from yeast to human. In mammals, it has been reported that protein arginine methyltransferases such as PRMT1, 5 and 6 could regulate replication of different viruses. *Ae. aegypti* contains eight members of protein arginine methyltransferases (AaArgM1-8). Here, we show that the *w*MelPop strain of *Wolbachia* introduced into *Ae. aegypti* significantly induces the expression of *AaArgM3*. Interestingly, we found that *Wolbachia* uses aae-miR-2940, which is highly upregulated in *Wolbachia*-infected mosquitoes, to upregulate the expression of *AaArgM3*. Silencing of *AaArgM3* in a mosquito cell line led to the inhibition of *Wolbachia* replication, but had no effect on the replication of DENV. These results provide further evidence that *Wolbachia* uses the host miRNAs to manipulate host gene expression and facilitate colonization in *Ae. aegypti* mosquito.

Key words: protein arginine methyltransferase 3; *Aedes aegypti*; *Wolbachia*; microRNA; dengue virus

1. Introduction

Wolbachia, the maternally inherited and gram-negative endosymbiotic bacteria, naturally occur in 40-65% of insect species (Hilgenboecker et al., 2008; Jeyaprakash and Hoy, 2000; Zug and Hammerstein, 2012). In the absence of naturally present strains of *Wolbachia* in the main vectors of

dengue virus (DENV; *Aedes aegypti*) and malaria (*Anopheles gambiae*), *Wolbachia* strains from *Drosophila melanogaster* and *Ae. albopictus* have recently been successfully introduced into *Ae. aegypti* and other mosquito species (Bian et al., 2013; McMeniman et al., 2009; Xi et al., 2005). Although a recent study found natural infections of *Wolbachia* in *An. gambiae* field populations in Burkina Faso, West Africa (Baldini et al., 2014). In some cases, transinfected *Wolbachia* strains have established stable inherited infections in the lab and the field (Frentiu et al., 2014; Walker et al., 2011). Similar to their original hosts, the newly introduced *Wolbachia* strains induce cytoplasmic incompatibility and life-shortening in adult mosquitoes by as much as 50% (McMeniman et al., 2009; Moreira et al., 2009; Xi et al., 2005). In addition, *Ae. aegypti* infected with *Wolbachia* possesses very strong resistance to several arboviruses including DENV and Chikungunya virus (Bian et al., 2013; Moreira et al., 2009), and *Plasmodium* (Moreira et al., 2009) and filarial nematodes (Kambris et al., 2009). Thus, the utilization of *Wolbachia* to control arbovirus transmission from mosquitoes to vertebrate hosts has become one of the most exciting approaches in vector-borne disease control.

The molecular mechanism(s) underlying suppression of replication of viruses in the presence of *Wolbachia* are thought to be complex and perhaps due to a combination of factors, but still largely unknown (see a recent review (Rainey et al., 2014). In its natural host, *D. melanogaster*, *Wolbachia* confer host resistance to RNA viruses and other pathogens via non-immune related mechanisms, since *Wolbachia* did not induce expression of innate immune genes (Bourtzis et al., 2000; Rances et al., 2013; Rancès et al., 2012). In *Ae. aegypti*, studies have shown that *Wolbachia* could use innate immune related mechanisms to suppress the replication of DENV by inducing the production of reactive oxygen species (ROS), overexpression of host immune genes and production of a variety of antimicrobial effectors (Bian et al., 2010; Kambris et al., 2010; Kambris et al., 2009; Moreira et al., 2009; Pan et al., 2012; Xi et al., 2008). Recently, our studies demonstrated that *Wolbachia* use host microRNAs (miRNAs) to manipulate the expression of several host genes such as the metalloprotease ftsh, MCT, MCM6 and AaDnmt2, which facilitate *Wolbachia* colonization and

some contribute to inhibition of DENV replication in *Ae. aegypti* (Hussain et al., 2011; Osei-Amo et al., 2012; Zhang et al., 2013).

miRNAs are an evolutionarily conserved class of small non-coding RNAs (~22 nucleotides), which down- or upregulate gene expression via partial or complete complementarity to their target gene sequences. They play important roles in cellular processes including development, differentiation, apoptosis, immunity and host-microorganism interactions (reviewed in Asgari, 2013; Bartel, 2009). miRNAs may bind to the 3'UTR, 5'UTR or coding region of target genes. Previous studies have shown that one miRNA could target several genes or several miRNAs could target one gene (e.g. Osei-Amo et al., 2012; Zhang et al., 2013). The expression levels of cellular miRNAs may substantially change in response to bacterial and viral infections in animals and plants (Fehri et al., 2010; Huang et al., 2007; Hussain et al., 2011; Lu et al., 2008; Tili et al., 2007). In our previous studies, we found differential expression of several miRNAs in *Wolbachia*-infected *Ae. aegypti* mosquitoes (Hussain et al., 2011) leading to up- or downregulation of a variety of host genes, which facilitate colonization and host resistance to DENV in *Ae. aegypti* (Hussain et al., 2011; Osei-Amo et al., 2012; Zhang et al., 2013).

In this study, we identified protein arginine methyltransferase 3 (*AaArgM3*) as another target gene of the *Wolbachia*-induced mosquito-specific aae-miR-2940-5p in *Ae. aegypti*. AaArgM3 belongs to protein arginine methyltransferase family, which includes eight members in *Ae. aegypti* (denoted AaArgM1-8). Arginine methyltransferases play diverse functions in cellular functions such as RNA processing and transcription (reviewed in Bedford and Clarke, 2009) and host-pathogen interactions (e.g. Duong et al., 2005; Souki et al., 2009; Yu et al., 2010). We investigated the effect of *Wolbachia* and DENV on these miRNAs and in turn their effect on replication of the two microorganisms. Our results suggest that AaArgM3 plays an important role in the maintenance of *Wolbachia* infection in mosquito cells but has no effect on DENV replication.

2. Materials and Methods

2.1. Mosquitoes and insect cell lines

Ae. aegypti infected with the *w*MelPop-CLA strain of *Wolbachia* (Wol^+) and a *Wolbachia*-free strain, tetracycline-cured line (Wol^-), were the stocks as previously described (McMeniman et al., 2009). *Ae. aegypti* was reared at 25 ºC with 80% relative humidity and a 12-h light regime. Larvae were maintained with fish food pellets (Tetramin, tetra) at a density of 50 larvae per litre water in flat trays. Adults were supplied 10% (W/V) sucrose solution, *ad libitum*. *Ae. aegypti* Aag2 cells and *w*MelPop infected Aag2 cells (denoted as aag2.*w*MelPop-CLA) (Frentiu et al., 2010) were maintained in a 1:1 mixture of Mitsuhashi-Maramorosch and Schneider's insect media (Invitrogen) supplemented with 10% FBS.

2.2. RNA extraction, cDNA synthesis and polymerase chain reaction (PCR)

Total RNA from female and male mosquitoes (separately) and mosquito cell lines was isolated using Tri-Reagent (Molecular Research Center). The RNA was treated with DNase I before used for reverse transcription (RT). The first strand cDNA was synthesized by RT with a Poly(dT) primer. In each RT reaction, approximately 2 µg of total RNA was used as template in a total volume of 20 µl. Following cDNA synthesis, 2 µl of RT products were used for each PCR in a total reaction volume of 25 µl with *AaArgM3* gene-specific primers (Forward: 5'-GTAGACGTAGACTGTCCC-3'; Reverse: 5'-ACCGGAATCGGTTCCTCG-3'). The amplification was performed at 94 ºC for 3 min, followed by 35 cycles of 94 ºC for 30 sec, 56 ºC for 30 sec, 68 ºC for 1 min, and a final extension at 68 ºC for 5 min. The ribosomal protein S17 (*RPS17*) gene was used as control.

2.3. Quantitative PCR (qPCR) of Wolbachia density

Total genomic DNA was extracted from aag2.*w*MelPop-CLA cells. *Wolbachia* density in cells was determined by qPCR using the *wsp* gene-specific primers as described previously (Zhang et al., 2013). qPCR was carried out by 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 ºC

hold for 30 sec, then 40 cycles of 95 ºC for 15 sec, 50 ºC for 15 sec and 72 ºC for 20 sec, followed by the melting curve analysis (68 ºC to 95 ºC). For this experiment, three biological replicates with three technical replicates were analysed. The *RPS17* gene was used for normalization of DNA templates. The student's *t* test was used to compare the differences in means between different treatments.

2.4. RT-qPCR

For RNA samples from mock and DENV-2 infected female mosquitoes, samples produced previously were utilized (Zhang et al., 2013). Following the RT reaction, qPCR with DENV gene-specific primers (forward: 5'-GTGGTGGTGACTGAGGACTG-3'; reverse: 5'-CCATCCCGTACCAGCATCCG-3') was carried out to determine DENV genomic RNA (gRNA) levels in cells. Platinum SYBR Green Mix (Invitrogen) was used for qPCR with 1 μl of RT products as described above. For this experiment, three biological replicates with three technical replicates were analysed. The *RPS17* gene was also used for normalization of RNA templates. The student's *t* test or ANOVA was used to compare the differences in means.

For tissue-specific analysis of *AaArgM3* transcript levels, total RNA was extracted from ovaries, salivary glands, thoracic muscle, midgut and fat body dissected from 4-day-old Wol^+ and Wol^- female mosquitoes (Zhang et al., 2013). RT-qPCR reactions were performed using *AaArgM3* gene-specific primers as described above. Similarly, three biological replicates with three technical replicates were analysed for each tissue of mosquito type. Each biological replicate consisted of a pool of total RNA extracted from different tissues of 10 female mosquitoes. The RPS17 gene was also used for normalization of RNA templates.

2.5. miRNA target prediction and validation

NCBI BLAST (http://www.ncbi.nih.gov/BLAST), RNAHybrid (Rehmsmeier et al., 2004) and RNA22 software (IBM) were used to identify the potential miRNAs induced in *Wolbachia*-infected female mosquitoes interacting with *AaArgM3* using the seed region complementarity and minimum

free energy (mfe) of -21 kcal/mol as the two main criteria.

To experimentally confirm the interaction between miRNAs and the target gene, *AaArgM3*, fragments of 200-500 bp long of *AaArgM3* 3'UTR containing the target sequences of aae-miR-2940, aae-miR-278, aae-miR-315, and aae-miR-1000 were amplified using primers with specific restriction sites XbaI and SacII. The fragments were then extracted from agarose gel, digested with XbaI and SacII, and ligated into pIZ/V5-His vector (Invitrogen) downstream of the *GFP* open reading frame. The right plasmids, confirmed by sequencing, were subsequently co-transfected into Sf9 cells (derived from *Spodoptera frugiperda*) together with control or miRNA mimics, respectively. All mimics were synthesized by Genepharma and used in transfection studies at a concentration of 100 μM/ml. Cells were collected at 72 h after transfections, total RNA was extracted and RT-qPCR analyses were performed to determine the expression levels of the *GFP* gene. Three biological replicates with three technical replicates were analysed.

2.6. RNAi-mediated gene silencing

For RNAi-based experiments, dsRNAs were synthesized *in vitro* using the T7 Megascript transcription kit according to the manufacturer's instruction (Ambion Inc., USA). T7 promoter sequences (TAATACGACTCACTATAGGG) were incorporated in both forward and reverse primers designed to amplify a ~500 bp fragment of the *Ae. aegypti Dicer-1* (forward: 5'-CCCGGACCAAGTCCTAGTA-3'; reverse: 5'-CAACTCTTTCGGCACGTAA-3'), *AaArgM3* (forward: 5'-ATGCTATCCTCGATAACG-3'; reverse: 5'-TGCTATGATGTTAGCATTG-3') and the jellyfish *GFP* genes. For dsRNA synthesis, 200-500 ng of PCR product was used for each reaction. Reactions were incubated for 12 h at 37 ºC, DNase-treated and precipitated by the lithium chloride method following the manufacturer's instructions. A total of 5 μg of dsRNA was used to transfect Aag2 or aag2.*w*MelPop-CLA cells with 5 μl of Cellfectin transfection reagent (Invitrogen). To reinforce silencing, cells were transfected again with the same reagent at 48 h after the first transfection. Cells were collected for RNA or DNA isolation as required for further analysis at 24 h after the second transfection. Gene silencing was confirmed by RT-qPCR using gene-

specific primers to *Dicer-1* and *AaArgM3* genes.

2.7. Western blotting

Cell samples were resuspended in PBS buffer to which 4×SDS-PAGE loading buffer was added. Proteins were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking the membrane, it was probed with anti-GFP antibody (Abcam) and subsequently with alkaline phosphatase conjugated anti-rabbit antibody (Sigma). The same blot was subsequently probed with anti-histone H3 antibody (Invitrogen) to confirm equal loading of samples.

3. Results

3.1. Expression profile of AaArgM3 in Ae. aegypti mosquito

By performing the NCBI BLAST, RNAHybrid and RNA22 software, a putative protein arginine methyltransferase 3 (*AaArgM3*, GeneBank ID: XM_001654962) from *Ae. aegypti* was identified as another target of a *Wolbachia* upregulated miRNA, aae-miR-2940-5p, which was previously confirmed to upregulate the transcript levels of the metalloprotease ftsh (*MetP*) gene (Hussain et al., 2011) and downregulate the transcript levels of *AaDnmt2* gene (Zhang et al., 2013). aae-miR-2940 is a mosquito-specific miRNA with its homolog absent in other insects (based on miRBase v.20). Sequence alignment showed that AaArgM3 is a homologue of protein arginine methyltransferases, PRMT3 from human and DART3 from *Drosophila* (Bedford and Clarke, 2009; Boulanger et al., 2004). PRMT3 is a type I PRMT, and has been shown to be a cytosolic protein. Alignment results showed that there is 48% amino acid identity between *Drosophila* DART3 and *Ae. aegypti* AaArgM3.

In *Drosophila*, human and other animals, the expression of ArgM3 is developmentally and tissue-specifically regulated (Bedford and Clarke, 2009; Boulanger et al., 2004). By using *AaArgM3* gene-specific primers, we first investigated the expression pattern of *AaArgM3* in different developmental stages of *Ae. aegypti* by RT-PCR. Results showed that the transcripts of *AaArgM3* were detectable in the first and fourth instar larvae and adult female mosquitoes, but hardly

detectable in the second and third instar larvae (Fig. 1A). Further analysis showed that *AaArgM3* was mainly expressed in the abdomen of both male and female mosquitoes (Fig. 1B), which suggests that *AaArgM3* could be specifically expressed in some organs in the abdomen. Tissue-specific RT-qPCR analyses of five tissues (ovary, midgut, salivary, muscles and fatty body) from 4-day-old female *Ae. aegypti* confirmed that AaArgM3 was mainly expressed in the ovary (Fig. 2A).

3.2. Wolbachia induces the expression of AaArgM3 by using host miRNAs

It has been shown that *Wolbachia* manipulates host gene expression by regulating miRNA expression in *Ae. aegypti*, which improves colonization and blockage of DENV replication in the host (Bian et al., 2010; Hussain et al., 2011; Moreira et al., 2009; Osei-Amo et al., 2012; Zhang et al., 2013). Based on these, we investigated the expression of *AaArgM3* in female mosquitoes infected with *Wolbachia* and DENV using RT-qPCR. Results showed about two-fold higher transcript levels of *AaArgM3* in *Wolbachia*-infected mosquito tissues compared with those of the tet-cured mosquitoes (without *Wolbachia*; Wol^-) (Fig. 2A-D). In *Ae. aegypti* mosquitoes infected with DENV, the transcript levels of *AaArgM3* did not significantly change compared with the mock-infected mosquitoes (Data not shown).

We also investigated the expression profiles of *AaArgM3* in *Ae. aegypti* cell lines infected with *w*MelPop-CLA (aag2.*w*MelPop-CLA) or without (Aag2) by RT-PCR. Results indicated that *AaArgM3* was expressed at much higher levels in aag2.*w*MelPop-CLA cells compared with Aag2 cells (Fig. 3A). To investigate whether miRNAs are involved in the regulation of *AaArgM3*, the Dicer-1 gene was silenced using RNAi in aag2.*w*MelPop-CLA cells. After confirming gene silencing, RT-PCR was carried out to explore the expression of *AaArgM3*. The expression levels of AaArgM3 were considerably decreased compared with mock-transfected aag2.*w*MelPop-CLA cells (Fig. 3B), which suggested that the upregulation of AaArgM3 expression in aag2.*w*MelPop-CLA cells could be mediated by miRNAs.

3.3. AaArgM3 is targeted by aae-miR-2940

The target sequences of aae-miR-2940-5p were predicted in the 3’ UTR of *AaArgM3* from

nucleotides 1991 to 2013 with significant complementarity to the miRNA's seed region (Fig. 4A). To confirm the interaction of aae-miR-2940-5p with *AaArgM3*, we transfected aag2.*w*MelPop-CLA cells with specific synthetic aae-miR-2940-5p and aae-miR-2940-3p inhibitors. RT-PCR results showed much lower transcript levels of *AaArgM3* in the cells transfected with aae-miR-2940-5p specific inhibitor compared with the cells transfected with the control aae-miR-2940-3p specific inhibitor (Fig. 4B). To further validate the positive interaction of aae-miR-2940 with *AaArgM3*, the target sequences were cloned downstream of the GFP gene in the pIZ/V5 vector (Fig. 5A). The plasmid was subsequently co-transfected into Sf9 cells together with aae-miR-2940 mimic and a control mimic (random sequences). The Sf9 cell line, which lacks the miRNA, provides an independent system to test the miRNA-target interaction. RT-qPCR analyses were carried out to assess the effect of miRNA-mRNA interaction on the transcript levels of the *GFP* gene. The results showed that there were significantly higher levels of *GFP* transcripts in cells transfected with aae-miR-2940 mimic compared to cells transfected with mock and control mimic (Fig. 5B). The upregulation was also confirmed at the protein level using an anti-GFP antibody (Fig. 5C). These results suggested that aae-miR-2940-5p upregulates the transcript levels of *AaArgM3*, which is consistent with the expression pattern of *AaArgM3* gene in mosquitoes with or without *Wolbachia* (Fig. 2).

Further bioinformatics analysis indicated that *AaArgM3* could also be a potential target of three other miRNAs, aae-miR-278, -315, and -1000. We investigated the interaction of *AaArgM3* gene with these predicted miRNAs by cloning their corresponding target sites in *AaArgM3* (Fig. 6A) downstream of the *GFP* gene. The constructs were co-transfected into Sf9 cells with their corresponding mimics. While aae-miR-278 and -1000 had no effect, aae-miR-315 mimic increased *GFP* transcript levels compared with mock and the control mimic (Fig. 6B). However, when we checked our previous microarray data (Hussain et al., 2011), we found that aae-miR-315 levels slightly increased in *Wolbachia*-infected female mosquitoes but the difference was not significant. Aae-miR-315 may regulate *AaArgM3* but perhaps not in the context of *Wolbachia*-mosquito

interaction.

3.4. AaArgM3 facilitates Wolbachia replication

In previous studies, we reported that *Wolbachia* infection leads to up- or downregulation of a number of host genes, which facilitate *Wolbachia* replication and maintenance in mosquito cells (Hussain et al., 2011; Osei-Amo et al., 2012; Zhang et al., 2013). Considering that aae-miR-2940-5p upregulates the transcript levels of *AaArgM3*, we first investigated whether AaArgM3 has any effect on *Wolbachia* replication in aag2.*w*MelPop-CLA cells. For this, *AaArgM3* was silenced in the cells and the density of *Wolbachia* was analysed by qPCR. RT-qPCR confirmed that the silencing efficiency was over 90% (Fig. 7A). qPCR results with *wsp* gene-specific primers revealed that *Wolbachia* density was significantly lower in *AaArgM3* silenced cells, when compared with cells transfected with dsGFP or mock (Fig. 7B). This result suggests that AaArgM3 enhances *Wolbachia* replication in the cell line, which is consistent with the expression profile that *AaArgM3* expression is considerably higher in the female mosquitoes with *Wolbachia*, compared with tet-cured counterpart mosquitoes (Fig. 2).

3.5. AaArgM3 does not regulate DENV-2 replication

In both *Ae. aegypti* mosquitoes and cell lines, *Wolbachia* was found to limit replication of DENV (Bian et al., 2010; Moreira et al., 2009), which could be due to manipulation of the host gene expression via miRNAs by *Wolbachia*. To explore the effect of AaArgM3 on DENV replication, we silenced AaArgM3 by transfecting Aag2 cells with *AaArgM3* dsRNA that were subsequently infected with DENV-2. Total RNA at 72 h after viral infection was isolated and analysed by RT-qPCR with DENV-specific primers. RT-qPCR confirmed that the silencing efficiency was about 85% (Fig. 8A). The results showed that the relative abundance of DENV was not significantly different in *AaArgM3* silenced cells compared with cells transfected with dsGFP or mock (Fig. 8B). Even silencing of the gene in aag2.*w*MelPop-CLA cells in which higher levels of *AaArgM3* are found, DENV replication was not different in mock, dsGFP or dsAaArgM3 cells (Fig. 8C). These results suggest that AaArgM3 might not regulate replication of DENV in the mosquito cells, which

is consistent with the expression profile that the transcript levels of *AaArgM3* were not different in non-infected and DENV-infected mosquitoes.

4. Discussion

Utilization of *Wolbachia* has appeared as a viable non-chemical control strategy to limit transmission of vector-borne pathogens since they block replication of a variety of pathogens, including arboviruses. *Wolbachia* strains have been successfully introduced into *Ae. aegypti* and *An. gambiae*, the important vectors of dengue fever and malaria, and others in an effort to suppress transmission of DENV and *Plasmodium* (Bian et al., 2013; Bian et al., 2010; Blagrove et al., 2012; McMeniman et al., 2009; Xi et al., 2005). To survive and persist in the new hosts, the endosymbiotic bacteria have to evade or overcome host immune responses. Hussain et al. (2011) have previously reported that *Wolbachia w*MelPop-CLA strain induces differential expression of a number of host miRNAs, including the mosquito-specific aae-miR-2940, in *Ae. aegypti*. In *Ae. aegypti*, aae-miR-2940 upregulates the expression of one target gene, metalloprotease ftsh (*MetP*), which is crucial for efficient replication and maintenance of the endosymbiont (Hussain et al., 2011). Osei-Amo et al. (2012) found that differentially expressed aae-miR-12 downregulates the expression of two target genes, *MCT1* and *MCM6*, which also play a role in *Wolbachia*'s fitness in the mosquito cells. In addition, the methyltransferase *AaDnmt2* was identified to be another target of aae-miR-2940 and plays an important role in the replication of *Wolbachia* and contributes to the inhibition of DENV replication in *Ae. aegypti* (Zhang et al., 2013). These findings have shed light on molecular mechanisms by which *Wolbachia* manipulate the host's environment in *Ae. aegypti*.

In the present study, *AaArgM3* was identified as another target gene of aae-miR-2940. The interaction of aae-miR-2940 with *AaArgM3* was confirmed and validated by using a synthetic inhibitor and mimic of aae-miR-2940 (Fig. 4B and 5). By examining the expression patterns, we found that the transcript levels of *AaArgM3* were significantly higher in *Wolbachia*-infected female mosquitoes (Fig. 2) and cells (Fig. 3A). Silencing of *AaArgM3* gene in aag2-*w*MelPop-CLA by RNAi showed a significant decline in *Wolbachia* density, but no effect on DENV (Fig. 7B, 8C).

Further, silencing of *AaArgM3* gene followed by DENV infection in Aag2 cells showed no significant effect on DENV replication. These results suggest that by inducing the expression of aae-miR-2940, *Wolbachia* upregulates the expression of *AaArgM3*, which in turn benefits *Wolbachia* in *Ae. aegypti*.

Methylation of arginine residues is a widespread posttranslational modification of proteins catalyzed by a conserved family of protein arginine methyltransferases. Protein arginine methyltransferases are classified into three types by methylated arginine residues including asymmetric ω-N^G, N^G-dimethylarginine (ADMA), symmetric ω-N^G, N^G-dimethylarginine (SDMA) and ω-N^G-dimethylarginine (MMA). Type I includes PRMT1, 2, 3, 4 and 8; type II includes PRMT5 and 7 and type III includes PRMT7 (Bedford and Clarke, 2009; McBride and Silver, 2001). They have diverse biological roles in the regulation of a large array of cell processes including signal transduction, subcellular localization, RNA processing and transcription (Bedford and Clarke, 2009; Krause et al., 2007; McBride and Silver, 2001). In recent years, PRMTs from mammals have been found to play essential roles in regulating the replication, production and infectivity of a variety of viruses. For example, PRMT1 negatively regulated Hepatitis Delta virus (Li et al., 2004), hepatitis B virus (Benhenda et al., 2013), hepatitis C virus (Duong et al., 2005) and Herpes Simplex virus (Souki et al., 2009; Yu et al., 2010). PRMT1 and PRMT5 together repressed HIV long terminal repeat transcription and consequently suppressed replication of the virus (Kwak et al., 2003). PRMT6 inhibited HIV-1 transcription through the methylation of Tat, Rev and nucleocapsid proteins (Boulanger et al., 2005; Invernizzi et al., 2007; Invernizzi et al., 2006; Singhroy et al., 2013; Xie et al., 2007). In our preliminary experiment, exposure of Aag2 cells to a protein arginine methyltransferase inhibitor (adenosine-2,3-dialdehyde) led to increased DENV replication (Zhang et al., unpublished data). In this study, we did not find that silencing of AaArgM3 had any effect on DENV replication, but we cannot exclude the possible role of AaArgM3 in regulating DENV replication. This is because in *Ae. aegypti* there are eight family members of protein arginine methyltransferases, which could have overlapping function probably

compensating the function of AaArgM3 when it was silenced. Further study is required to investigate which family member(s) play a role in regulating DENV replication.

miRNAs have been implicated as gene regulators controlling diverse biological processes including development, cancer, immunity and host–microorganism interactions. They usually downregulate their target genes by either degradation of the target mRNA or repression of translation (reviewed in Asgari, 2013; Bartel, 2009). A large number of miRNAs have been identified to control the DNA and RNA methylation machineries (Denis et al., 2011). However, very few miRNAs have been identified to regulate protein arginine methylation. Recently miR-181a, b, c, and d family members were found to directly regulate *CARM1* (*PRMT4*) expression in human embryonic stem cells (Xu et al., 2013). All the miR-181 family members target the 3' UTR of *CARM1*.

In our study, we identified and confirmed that aae-miR-2940, which is induced in the presence of *Wolbachia*, enhances the expression of a protein arginine methyltransferase, *AaArgM3*, in *Ae. aegypti*, which appears to be important for *Wolbachia* fitness. This suggests a positive feedback loop in which *Wolbachia* infection induces aae-miR-2940 that in turn positively regulates AaArgM3 leading to more *Wolbachia*. However, the mechanism by which the protein facilitates *Wolbachia* maintenance remains to be investigated. Our results suggest that *Wolbachia* manipulates host physiology and gene expression for colonization in mosquitoes using multiple targets of differentially regulated miRNAs.

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463 **Figure Legends**

464 **Fig. 1. AaArgM3 expression in *Ae. aegypti*. (A)** RT-PCR analysis was performed using the total
465 RNA samples from *Ae. aegypti* mosquito larvae and female adults. **(B)** RT-PCR analysis was
466 performed with head+thorax (HT) and abdomen (Ab) of mosquito females and males. *Rps17* gene
467 was used as control to show the integrity of RNA.

468 **Fig. 2. Tissue-specific expression of AaArgM3 in female *Ae. aegypti* mosquitoes.** RT-qPCR
469 analysis of transcript levels of *AaArgM3* in ovary, midgut, salivary glands (Salivary), thoracic
470 muscle tissues (Muscle) and fat body from 4-day-old **(A)** tetracycline-treated non-infected (Wol^-)
471 and **(B)** *Wolbachia*-infected (Wol^+) female mosquitoes. The transcript levels of *AaArgM3* were also
472 compared in **(C)** the ovaries and **(D)** fat body in the samples. Asterisks indicate a significant
473 difference between treatments (** $p < 0.001$).

474 **Fig. 3. *AaArgM3* expression in aag2-*w*MelPop-CLA and Aag2 cells. (A)** RT-PCR analysis of
475 RNA extracted from aag2-*w*MelPop-CLA **(**Pop) and Aag2 cells. **(B)** RT-PCR analysis of RNA
476 extracted from mock and dsDicer-1 transfected aag2-*w*MelPop-CLA cells. *Rps17* gene was used as
477 control to show the integrity of RNA.

478 **Fig. 4. *AaArgM3* transcript levels are upregulated by aae-miR-2940-5p. (A)** Schematic diagram
479 showing the *AaArgM3* mRNA and its target sequences with complete complementarity of aae-miR-
480 2940-5p seed region (bold and underlined) with the sequences. **(B)** RT-PCR analysis of *AaArgM3*
481 relative transcript levels using RNA extracted from aag2.*w*MelPop-CLA cells transfected with
482 mock, synthetic aae-miR-2940-5p or aae-miR-2940-3p (control) inhibitors. *Rps17* gene was used as
483 control to show the integrity of RNA.

484 **Fig. 5. Target validation of aae-miR-2940. (A)** Schematic diagram showing the cloning strategy
485 of *AaArgM3* target sequence complementary to the miRNA seed region from the *AaArgM3* 3'UTR
486 under the *GFP* reporter gene in the pIZ vector. **(B)** RT-qPCR analysis of *GFP* transcript levels
487 using the RNA extracted from Sf9 cells co-transfected with pIZ-GFP-target and mock, synthetic

control mimic or aae-miR-2940 mimic. *Actin* gene was used as the normalizing control. Asterisks indicate a significant difference between mock or control mimic and aae-miR-2940 mimic transfections ($p < 0.0001$). **(C)** Western blot analysis of Sf9 cells transfected with pIZ/GFP-target together with aae-2940-5p mimic (2940), control mimic (Cmimic), no mimic (Nmimic) or mock transfected without plasmid (Mock). The blot was probed with anti-GFP antibody and subsequently with anti-histone H3 to show equal loading of samples.

Fig. 6. Interactions of *AaArgM3* with predicted miRNAs. (A) *Ae. aegypti AaArgM3* was predicted to be the target of aae-miR-278, -315, and -1000 with complete complementarity of their seed regions (bold and underlined) with the sequences. **(B)** RT-qPCR analysis of GFP expression using RNA extracted from Sf9 cells transfected with pIZ-GFP-target and mock, synthetic control mimic, aae-miR-278, -315 or -1000 mimics. *Actin* gene was used as the normalizing gene. There are statistically significant differences between treatments with different letters at $p < 0.05$.

Fig. 7. AaArgM3 facilitates *Wolbachia* replication. RNAi-mediated silencing of *AaArgM3* gene was carried out in aag2.*w*MelPop-CLA cells for 72 h. **(A)** RT-qPCR analysis of *AaArgM3* gene relative to *RPS17* in aag2.*w*MelPop-CLA cells transfected with mock, GFP and *AaArgM3* dsRNAs. **(B)** qPCR analysis of *Wolbachia* density in aag2.*w*MelPop-CLA cells 72 h after transfection with mock, GFP and *AaArgM3* dsRNAs using primers specific to the *Wolbachia wsp* gene. Asterisks indicate a significant difference between transfection with *AaArgM3* dsRNA and other treatments (*** $p < 0.0001$; ** $p < 0.001$).

Fig. 8. AaArgM3 has no effect on DENV replication in Aag2 or *Wolbachia*-infected Aag2 cells. RNAi-mediated silencing of *AaArgM3* gene was carried out in Aag2 cells. 72 h after transfection with dsRNA, cells were infected with DENV-2. At 72 h after infection, total RNA was extracted from cells. **(A)** RT-qPCR analysis of *AaArgM3* gene relative to *RPS17* in Aag2 cells transfected with mock, GFP and *AaArgM3* dsRNAs and infected with DENV-2. **(B)** RT-qPCR analysis of RNA using DENV-specific primers in Aag2 cells transfected with mock, GFP and *AaArgM3*

dsRNAs and then infected with DENV-2. **(C)** RT-qPCR analysis of RNA using DENV-specific primers from aag2.*w*MelPop-CLA cells transfected with mock, GFP and *AaArgM3* dsRNAs and then infected with DENV-2 for 72 h. Silencing of *AaArgM3* in aag2.*w*MelPop-CLA cells was confirmed as shown in Fig. 7A. Asterisks indicate a significant difference between transfection with *AaArgM3* dsRNA and other treatments (*** $p < 0.0001$).

Figure 1

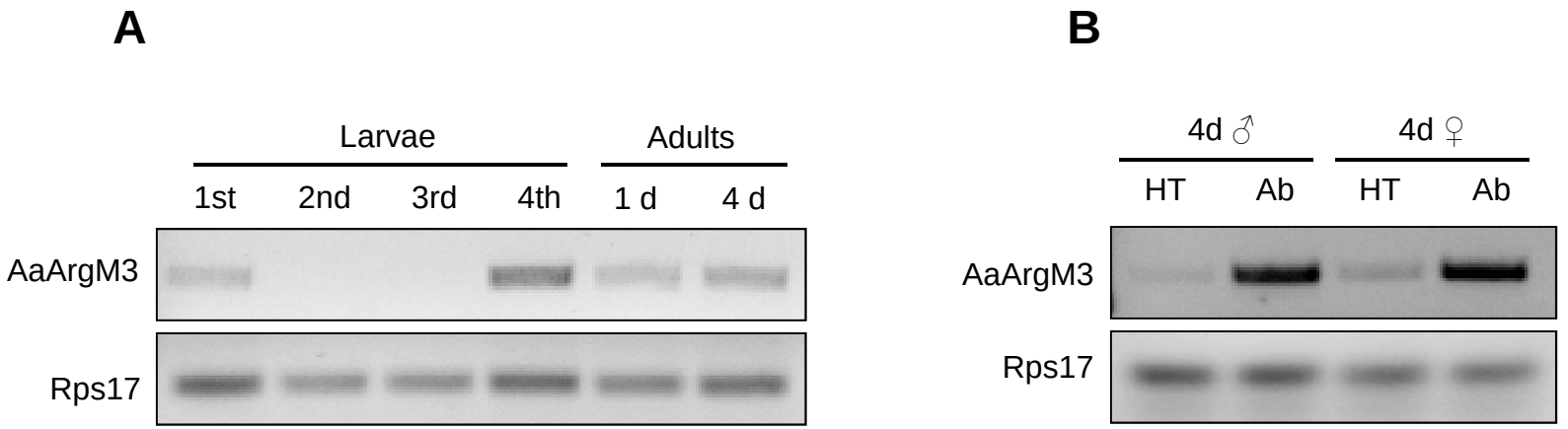


Figure 2

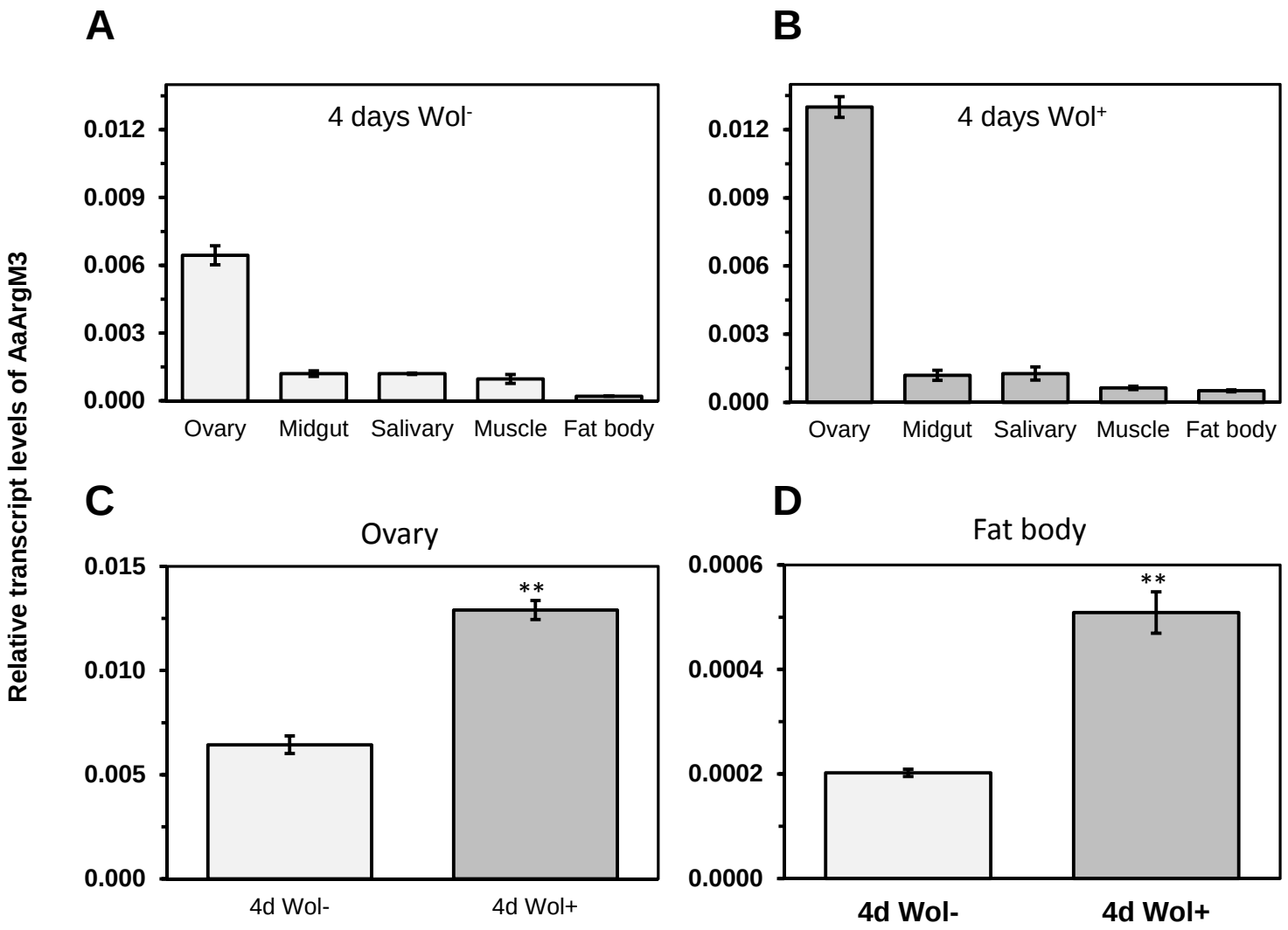


Figure 3

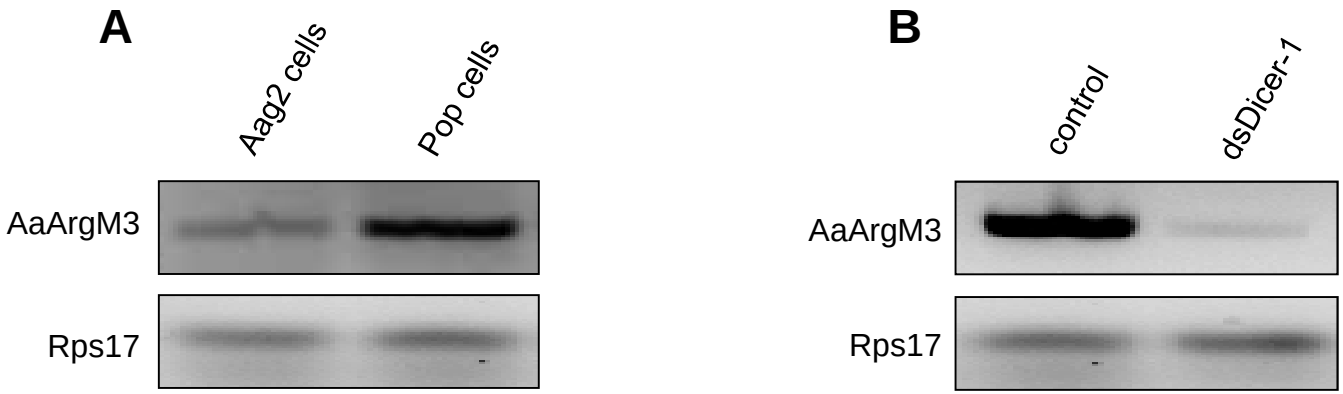


Figure 4

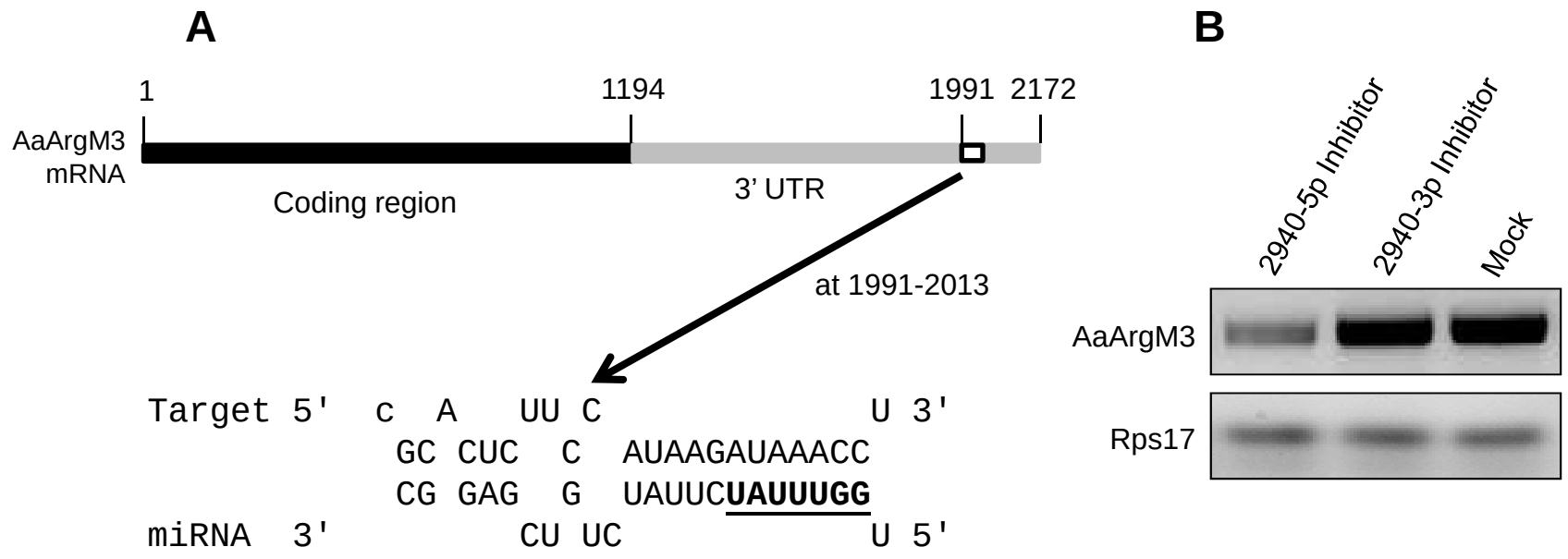
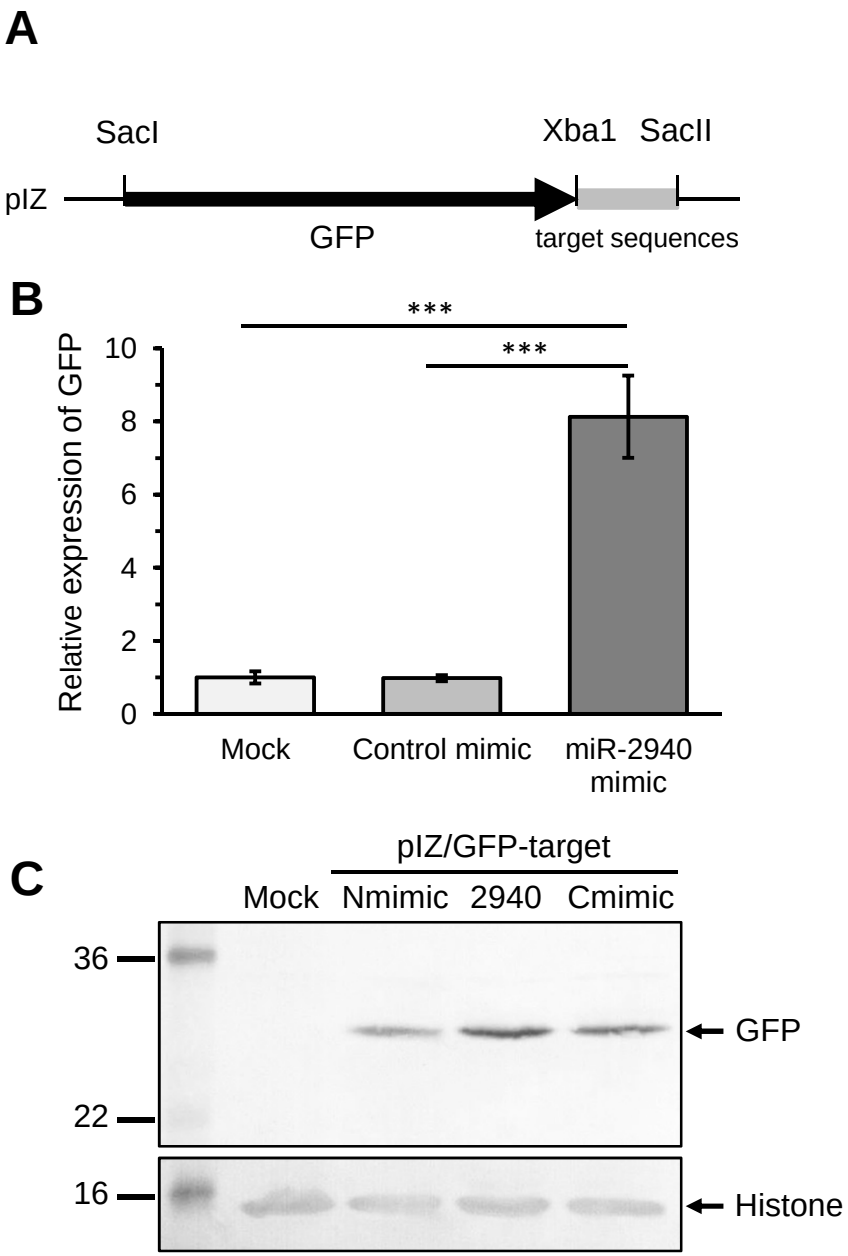


Figure 5


A
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Xba1
SacII
pIZ
GFP
target sequences
B
***
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Relative expression of GFP
10
8
6
4
2
0
Mock
Control mimic
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C
pIZ/GFP-target
Mock
Nmimic
2940
Cmimic
36
22
16
GFP
Histone

Figure 6

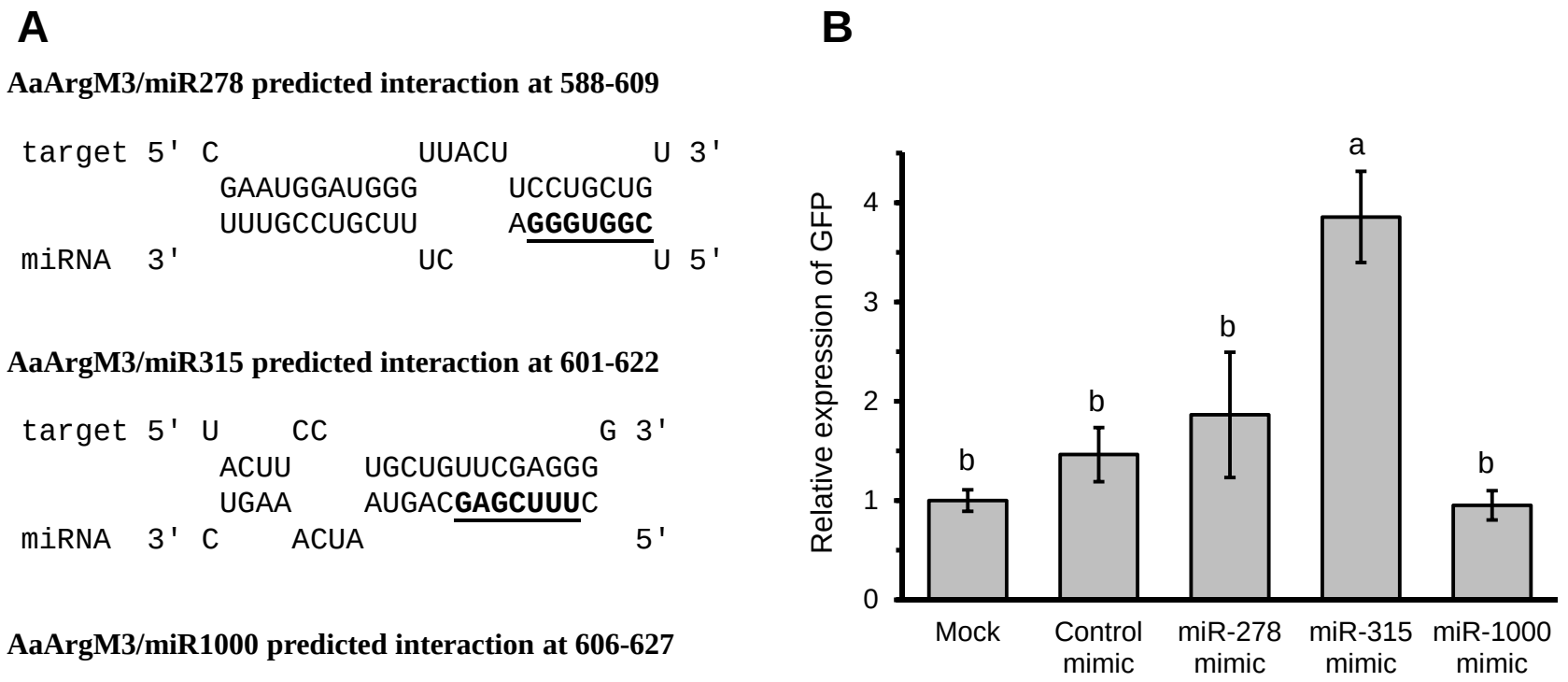
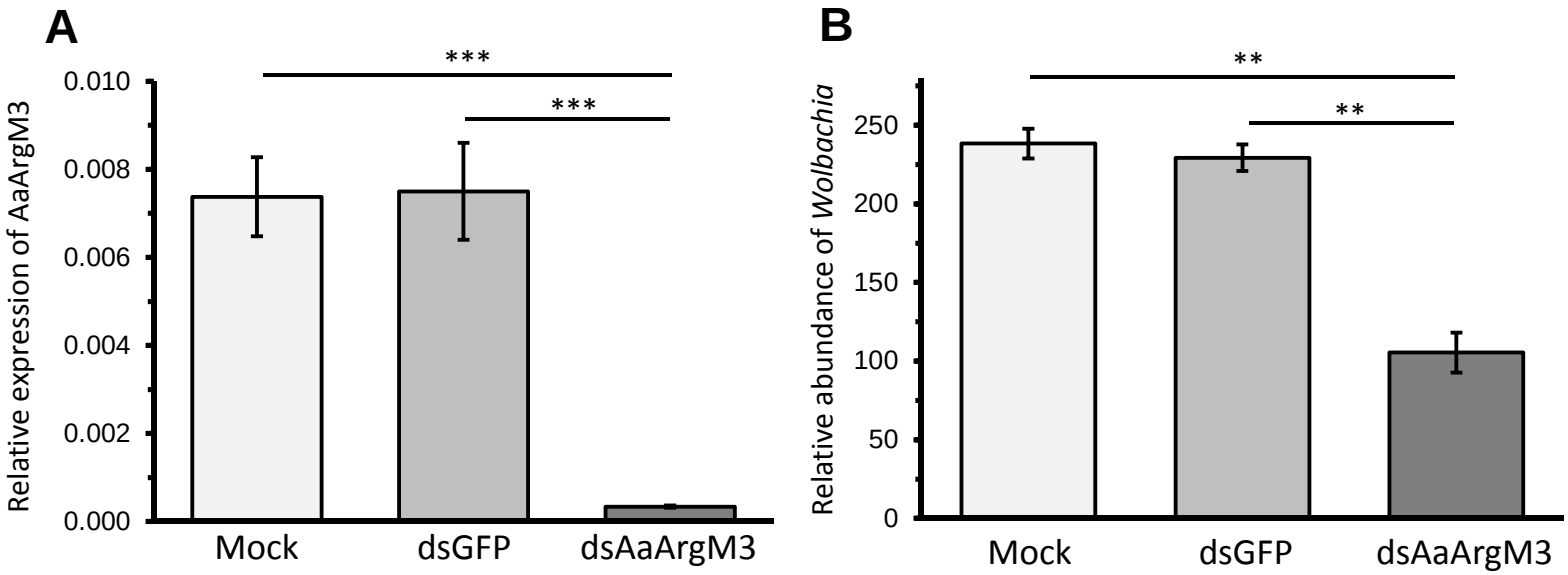


Figure 7


A
Relative expression of AaArgM3
0.010
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Mock
dsGFP
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B
Relative abundance of Wolbachia
250
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50
0
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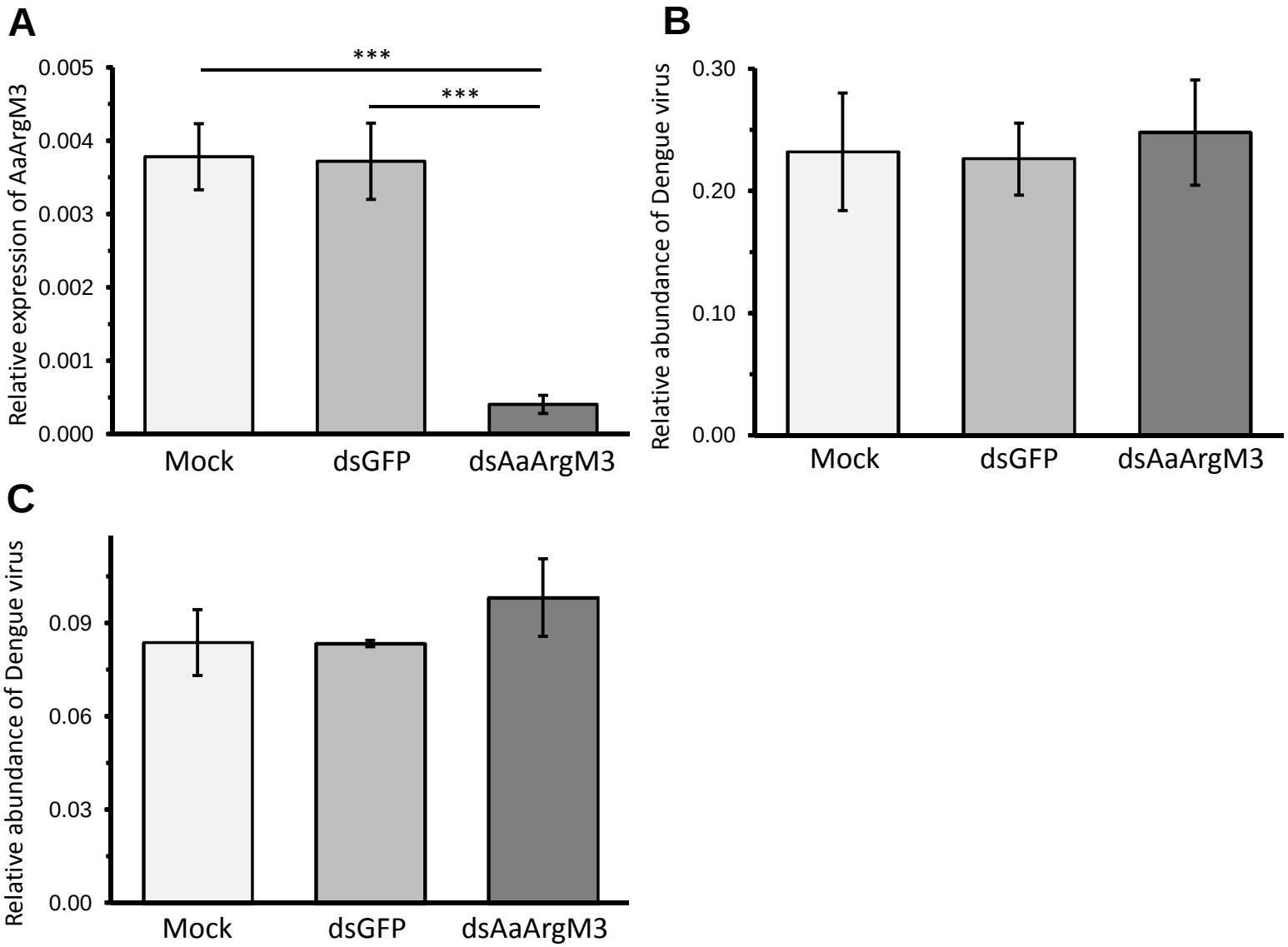
Mock
dsGFP
dsAaArgM3

Figure 8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Relative expression of AaArgM3
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dsGFP
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B
0.30
0.20
0.10
0.00
Relative abundance of Dengue virus
Mock
dsGFP
dsAaArgM3
C
0.09
0.06
0.03
0.00
Relative abundance of Dengue virus
Mock
dsGFP
dsAaArgM3

Highlights

- Arginine methyltransferase was found as another target of aae-miR-2940-5p, a mosquito-specific miRNA
- Arginine methyltransferase is induced in *Wolbachia*-infected *Aedes aegypti* mosquitoes and cells
- Arginine methyltransferase is positively regulated by aae-miR-2940-5p.
- Arginine methyltransferase contributes to replication/maintenance of *Wolbachia* but has no effect on dengue virus replication.