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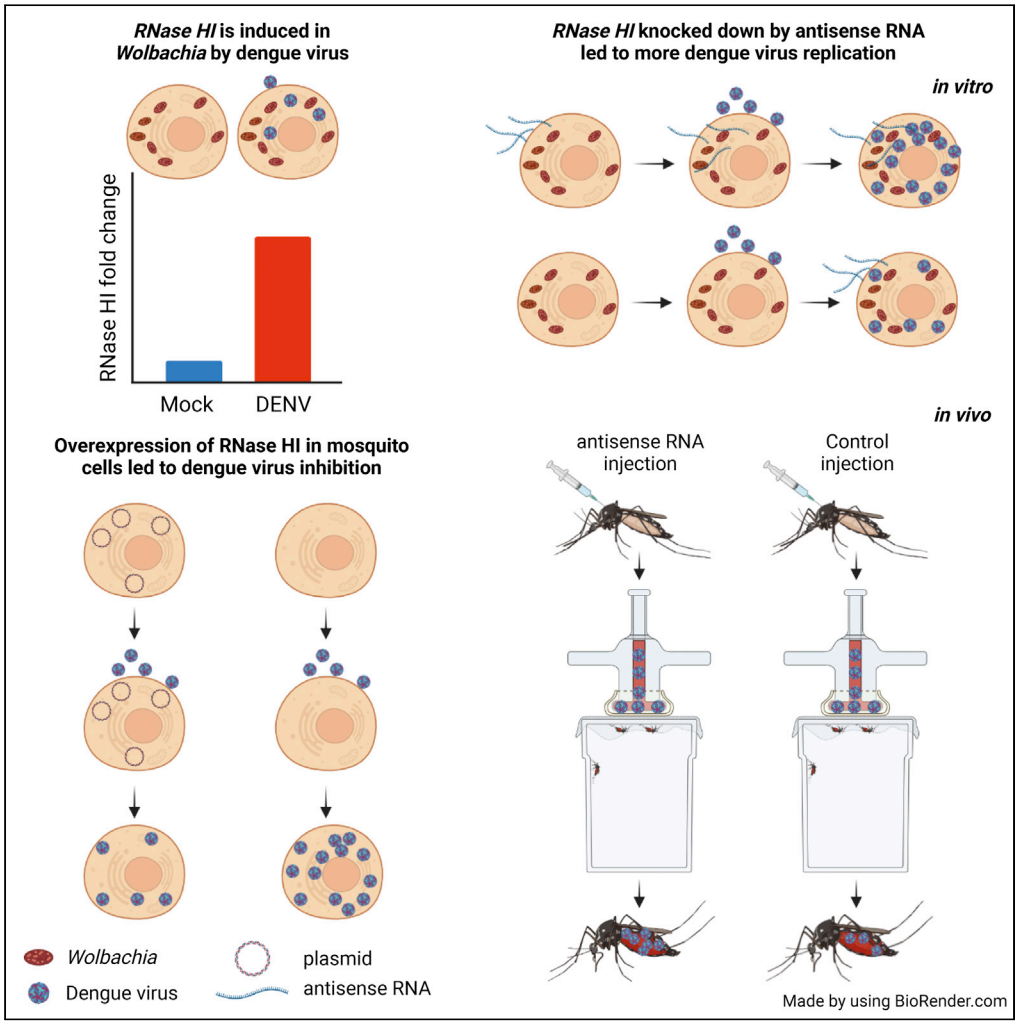

Mazhar Hussain, Guangmei Zhang, Michael Leitner, Lauren M. Hedges, Sassan Asgari

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Highlights

Wolbachia RNase HI was induced in mosquito cells following dengue virus infection

Overexpression of wRNase HI led to reduced replication of dengue virus

Antisense RNA (asRNA) can be used to silence *Wolbachia* genes in the host cells

Application of *wRNase HI* asRNA led to enhanced dengue virus replication

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Wolbachia RNase HI contributes to virus blocking in the mosquito *Aedes aegypti*

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SUMMARY

The endosymbiotic bacterium *Wolbachia pipientis* blocks replication of several arboviruses in transinfected *Aedes aegypti* mosquitoes. However, the mechanism of virus blocking remains poorly understood. Here, we characterized an *RNase HI* gene from *Wolbachia*, which is rapidly induced in response to dengue virus (DENV) infection. Knocking down *wRNase HI* using antisense RNA in *Wolbachia*-transinfected mosquito cell lines and *A. aegypti* mosquitoes led to increased DENV replication. Furthermore, overexpression of wRNase HI, in the absence of *Wolbachia*, led to reduced replication of a positive sense RNA virus, but had no effect on a negative sense RNA virus, a familiar scenario in *Wolbachia*-infected cells. Altogether, our results provide compelling evidence for the missing link between early *Wolbachia*-mediated virus blocking and degradation of viral RNA. These findings and the successful pioneered knockdown of *Wolbachia* genes using antisense RNA in cell line and mosquitoes enable new ways to manipulate and study the complex endosymbiont-host interactions.

INTRODUCTION

Wolbachia pipientis is an endosymbiotic bacterium that is estimated to infect more than 40% of insect species.[1] In addition to insects, it is found in other arthropods, and nematodes. There are several strains of *Wolbachia* that are classified into a number of supergroups (A-F and H-Q).[2] *Wolbachia* is mostly known for reproductive manipulations of its host to facilitate its spread in infected populations, and more recently also for blocking replication of a number of RNA viruses.[3,4] Although virus blocking was first discovered in *Drosophila*,[5,6] it was soon found that transinfected *Aedes aegypti* mosquitoes with different strains of *Wolbachia* efficiently block replication of several vector-borne viruses, such as dengue virus, Chikungunya virus, and Zika virus (e.g.[7–10]). This has led to deployment of *Wolbachia*-infected *A. aegypti* mosquitoes in various countries to reduce transmission of arboviruses.[11–15] The mechanism of virus blocking, however, remains elusive.

There have been a number of mechanisms suggested that contribute toward virus blocking, but none of them have been found to consistently explain the blocking phenotype. It is possible that there are several factors that work together in combination to exert virus blocking and that could vary depending on the host-*Wolbachia* strain combination. Among these mechanisms are induction of the immune system,[16–19] competition for metabolic resources[20–22] and space,[23] alteration of host pro- and anti-viral genes,[24–27] and inhibition of virus entry into host cells.[28] Nevertheless, it is evident that one of the main anti-viral responses in insects toward virus infections, the RNAi response targeting the virus through short-interfering RNAs, does not play a major role in *Wolbachia*'s virus blocking phenotype.[29–31]

It has been established that the presence of *Wolbachia* in the cell is required for virus blocking and the phenotype is not systemically conferred.[32] *Wolbachia* density has also been found to be critical for displaying the virus blocking phenotype in a few studies; however, it does not always hold. In a recent study, it was shown that the *w*Pip strain of *Wolbachia* transinfected into *A. aegypti* mosquitoes resides in high density, comparable to *w*Mel and *w*AlbB strains in the midgut and salivary glands, but it does not confer protection against DENV replication.[33] Another recent study also did not find a correlation between *Wolbachia* densities and viral titers.[34]

A couple of studies demonstrated that virus blocking occurs at the very early stages of virus infection.[35,36] In particular, it was suggested that viral RNA is the target, which encounters a rapid degradation following virus

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entry. In our recent study in which transcriptional response of *Wolbachia* wAlbB strain in *A. aegypti* Aag2 cells to DENV infection was assessed, an *RNase HI* gene (*wRNase HI*) was found induced as early as 1 h after DENV infection.[37] RNase H comprises a family of endoribonucleases that are mostly known for degradation of RNA in RNA/DNA hybrids and play various roles in prokaryotic and eukaryotic cells including genome stability and anti-viral response.[38–41] Based on the *wRNase HI* induction result, we formulated a hypothesis that *w*RNase HI could be involved in the virus blocking phenomenon. To test this hypothesis, we further analyzed the transcriptional response of *wRNase HI* to DENV infection in two strains of *Wolbachia*, and the RNase activity of *w*RNase HI in blocking DENV and other insect-specific RNA viruses using ectopic expression of the gene in mosquito cells. Importantly, we knocked down *wRNase HI* in an *A. aegypti* cell line and adult female mosquitoes and showed that DENV replication was significantly increased both *in vitro* and *in vivo*. As far as we know, knocking down of a gene in an endosymbiotic bacterium has not been shown so far. Our results open a new avenue in functional analysis of *Wolbachia* genes using gene knockdown and provide further insights into the virus blocking mechanism in mosquitoes.

RESULTS

Our recent RNA-Seq study showed induction of an *RNase HI* gene (NCBI: NP_966104.1) in *Wolbachia* wAlbB strain when Aag2.wAlbB cells were infected with DENV.[37] This induction occurred as early as 1 h after DENV infection. Here, we further characterized the *wRNase HI* gene in *Wolbachia* and investigated its possible role in blocking DENV in *Wolbachia*-infected mosquito cells.

Differential expression of *wRNase HI* in wMelPop- and wAlbB-infected mosquito cells

To examine the expression levels of *wRNase* HI in mosquito cells transinfected with *w*MelPop and *w*AlbB, we extracted total RNA from *Ades albopictus* wAlbB-infected Aa23 cells, and Aag2 cells transinfected with *w*AlbB (Aag2.*w*AlbB), or *w*MelPop (Aag2.*w*MelPop). RT-qPCR results using specific primers to *wRNase HI* demonstrated expression of the gene in all the *Wolbachia*-transinfected cells, however, significantly higher transcript levels (4– to 6-fold; Kruskal-Wallis test, $p < 0.0036$) of *wRNase HI* were found in Aag2.*w*MelPop cells compared to Aa23 and Aag2.*w*AlbB cells when data were normalized against the *Wolbachia 16S rRNA* gene (Figure 1A).

To find out whether the higher expression levels of *wRNase HI* in Aag2.*w*MelPop could be because of higher densities of *Wolbachia* in the cells or an actual higher expression of the gene in *w*MelPop, we analyzed a number of collected samples from Aag2.*w*AlbB and Aag2.*w*MelPop cells in which we determined *Wolbachia* density and *wRNase HI* transcript levels. At about comparable densities of *Wolbachia* in the two cell lines (131 and 137 *Wolbachia* per cell) (Figure 1B), the expression level of *wRNase HI* was significantly higher (about 2-fold; $p < 0.0001$) in Aag2.*w*MelPop cells (Figure 1C). Results suggested that *wRNase HI* is indeed expressed at higher levels in *w*MelPop compared to *w*AlbB.

Furthermore, we conducted expression analysis of *wRNase HI* in *w*AlbB transinfected mosquitoes at three different days post emergence (dpe). Of interest, RT-qPCR revealed the same transcript levels of *wRNase HI* at 2 and 6 dpe, which were significantly downregulated (average 2-fold overtime; One-way ANOVA, $p = 0.0016$) at 12 dpe (Figure 1D). However, qPCR using gDNA extracted from the same mosquitoes showed significant increases (average 2.5-fold overtime; One-way ANOVA, $p < 0.001$) in *Wolbachia* density with age (Figure 1E).

It is known that the *w*MelPop strain imposes a stronger virus blocking effect on virus replication than the other strains.[42] We confirmed this in our cells by infecting Aag2.*w*MelPop and Aa23 cells (infected with *w*AlbB *Wolbachia* strain) with dengue virus serotype 2 (DENV-2) and analyzed the RNA extracted from the cells five days post infection (dpi) using a specific probe to the virus short flavivirus RNA (sfRNA) in a northern blot analysis. The results showed a strong inhibition of DENV-2 replication in Aag2.*w*MelPop cells compared to Aa23 cells (Figure S1). These results indicated a correlation between the higher expression levels of *wRNase HI* by *w*MelPop with its stronger virus blocking property.

Rapid induction of *wRNase HI* upon DENV infection

To confirm that the expression levels of *wRNase HI* change on DENV infection, Aag2.*w*MelPop cells were infected with 1 MOI of DENV-2 and collected 0.5 h and 1 h following infection. RT-qPCR analysis of RNA extracted from the cells showed significant induction (about 3-fold; Kruskal-Wallis test, $p < 0.0036$) of

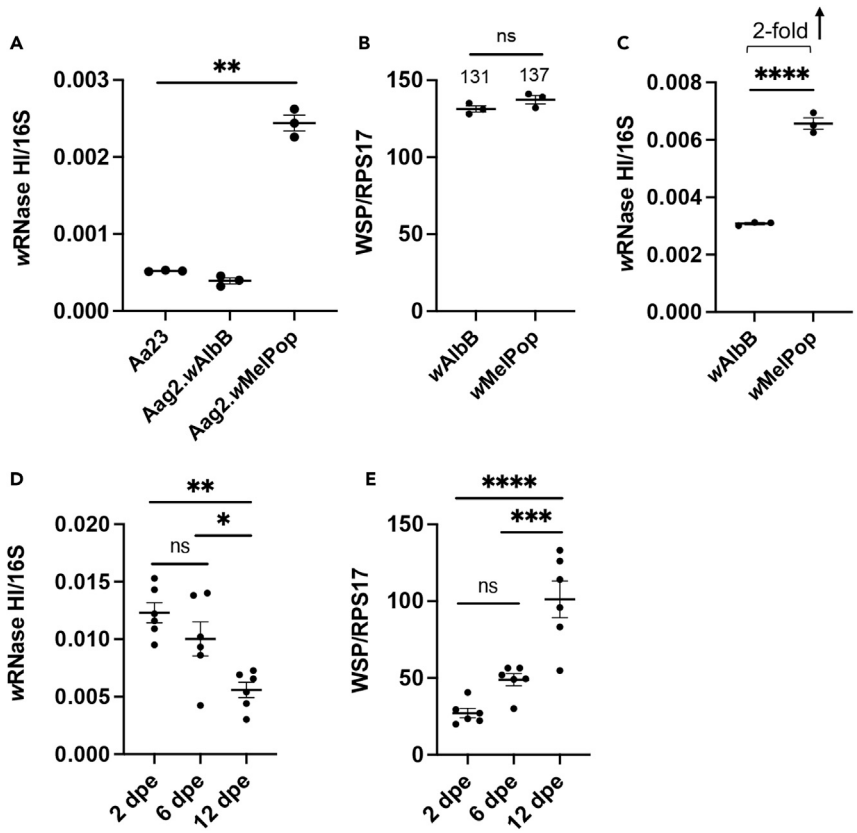

Figure 1. Differential expression of *wRNase HI* in *Wolbachia*-infected mosquitoes and cells

(A) Relative expression of *wRNase HI* in Aag23, Aag2.*w*AlbB, and Aag2.*w*MelPop cell lines measured using RT-qPCR in three biological replicates (N = 3 with a pool of several cells per N). Kruskal-Wallis test was carried out to determine statistical significance among groups.

(B) Relative density of *Wolbachia* in mosquito cell lines. The density of *Wolbachia* was determined by qPCR analysis using genomic DNA extracted from Aag2.*w*AlbB and Aag2.*w*MelPop cells. The numbers on each dataset show the average *Wolbachia* density of the biological replicates in each cell line. t-test was carried out to determine statistical significance between groups.

(C) Relative expression of *wRNase HI* in Aag2.*w*AlbB and Aag2.*w*MelPop cell lines in (B) confirming higher expression of *wRNase HI* in *w*MelPop compared to *w*AlbB. Fold change was calculated by comparing the average of the expression levels of *wRNase HI* in three replicates for each treatment. t-test was carried out to determine statistical significance between groups.

(D) *wRNase HI* expression in female *A. aegypti* *w*AlbB-transinfected mosquitoes at 2-, 6-, and 12-day post-emergence (dpe).

(E) Density of *Wolbachia* was quantified by qPCR in samples in (D). *A. aegypti RPS17* or *Wolbachia's 16S rRNA* genes were used as the normalizing genes as indicated on the y axis. One-way ANOVA test was carried out to determine statistical significance among groups in (D and E) where each data point represents one mosquito. The error bars in all the graphs represent SE of mean (SEM) of biological replicates. ns, not significant; *, p< 0.05; **, p< 0.01; ***, p< 0.001; ****, p< 0.0001.

wRNase HI in Aag2.*w*MelPop cells as early as 0.5 h after infection (Figure 2A). Similarly, *wRNase HI* was induced (about 1.5-fold; Kruskal-Wallis test, p =0.0036) in Aag2.*w*AlbB cells infected with 1 MOI of DENV-2 (Figure 2B). The results confirmed rapid induction *of wRNase HI* in *Wolbachia* on DENV infection.

Considering the presence of an *RNase H1* gene in the host *A. aegypti* genome (Vectorbase: AAEL017101), we investigated if the gene is induced on DENV infection in the same samples in which *wRNase HI* was induced. RT-qPCR results showed no induction (Kruskal-Wallis test, p = 0.1964) of the gene in Aag2.*w*MelPop or Aag2.*w*AlbB cells (Figures S2A and S2B). In fact, there was a significant reduction (Kruskal-Wallis test, p = 0.0036) in the host *RNase H1* transcript levels following DENV infection in Aag2.*w*AlbB cells (Figure S2B). The gene was not induced (Mann-Whitney, p = 0.7 and p = 0.1) even one day after DENV infection, either in Aag2 or Aag2.*w*AlbB cells (Figures S2C and S2D). Overall, these results demonstrate that the host *RNase H1* gene is not induced on DENV infection in the time points assessed.

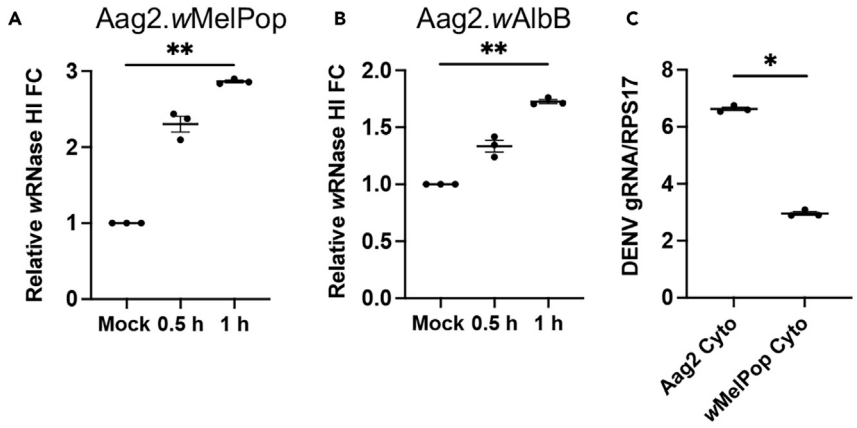

Figure 2. Rapid induction of *wRNase HI* upon DENV infection and higher viral RNA degradation in Aag2.*w*MelPop cytoplasmic fraction

(A and B) Expression of *wRNase HI* was induced by DENV infection in (A) Aag2.*w*MelPop, and (B) Aag2.*w*AlbB cells. RT-qPCR analysis of RNA extracted from mock, *w*MelPop, and *w*AlbB cells infected with DENV-2 collected at 0.5 and 1 h post-infection (hpi). *Wolbachia's 16S rRNA* was used as the normalizing gene. Kruskal-Wallis test was carried out to determine statistical significance among groups. FC, fold change.

(C) The stability of DENV genomic RNA was assessed in the cytoplasmic fractions from Aag2 and Aag2.*w*MelPop cells. DENV-2 genomic RNA was incubated with the cytoplasmic fractions of the respective cells. Mann-Whitney test was carried out to determine statistical significance between the two groups. The error bars in all the graphs represent SE of mean (SEM) of three biological replicates. *, $p< 0.05$; **, $p< 0.01$.

RNA degradation effect of the Aag2.*w*MelPop cytoplasmic fraction

To compare the cytoplasmic fractions of Aag2 and Aag2.*w*MelPop cells in degrading viral RNA, 3 μg of DENV-2 genomic RNA was incubated with the cytoplasmic fractions of the respected cells for 60 min at 37°C. Total RNA was extracted, and RT-qPCR was conducted to quantify DENV genomic RNA levels. Results showed significantly less (2.2-fold; Mann-Whitney test, $p = 0.05$) DENV RNA in reactions containing the cytoplasmic fraction from Aag2.*w*MelPop cells (Figure 2C) suggesting higher RNase activity in the cytoplasmic fraction of Aag2.*w*MelPop cells compared to control Aag2 cells.

*w*RNase HI degrades ssRNA and RNA/DNA hybrid, but not dsRNA *in vitro*

RNase HI is a ribonuclease mainly known for degrading RNA in RNA/DNA hybrids.[43] Amino acid sequence alignment of RNase HI from *w*MelPop and *Escherichia coli* (NCBI: NP_414750.1) showed 54% sequence identity between the two sequences and that *w*RNase HI contains the conserved RNase HI, but also the RNA/DNA hybrid binding sites (Figure S3). Here, we tested if *w*RNase HI has any RNase activity against single stranded RNA or dsRNA molecules. For this, the full-length gene from *w*MelPop was cloned into the bacterial expression vector pQE30 in fusion with an N-terminal 6×His tag. As a control, the coding region for *w*RNase III was cloned into the same vector. The expression of the proteins was induced by IPTG and confirmed by Western blot using an anti-His antibody (Figure S4A). The expressed proteins were purified and incubated with ssRNA and dsRNA of GFP. After 1 h of incubation, the reactions were run on an agarose gel and visualized with ethidium bromide staining. *w*RNase HI completely degraded ssGFP RNA compared to the control, whereas *w*RNase III degraded ssGFP RNA to a lesser extent (Figure 3A). Regarding dsGFP RNA, neither *w*RNase HI nor *w*RNase III degraded the dsRNA (Figure 3B). We repeated this experiment, but this time also included GFP RNA/DNA hybrid in addition to GFP ssRNA and dsRNA, and as control used an irrelevant 45 kDa protein (CrV1 from the wasp *Cotesia rubecula*[44]) cloned into the same vector and purified with the same procedure as *w*RNase HI (Figure S4B). Consistently, *w*RNase HI did not digest dsRNA, but it digested ssRNA as well as the RNA/DNA hybrid (Figure 3C). In addition, we compared the activity of *w*RNase HI with that of *E. coli* RNase HI using a purified commercial product. Consistent with *w*RNase HI, *E. coli* RNase HI did not digest dsRNA and digested the RNA/DNA hybrid, but unlike *w*RNase HI, it did not digest ssRNA (Figure 3D). When ethylenediaminetetraacetic Acid (EDTA) was added to the RNA/DNA hybrid reactions, both *w*RNase HI and *E. coli* RNase HI activities were inhibited (Figure 3D). Divalent metal ions are required for RNase HI activity,[45] which can be chelated by EDTA. The results suggest that similar to *E. coli* RNase HI *w*RNase HI degrades RNA/DNA hybrids and does not digest dsRNA, but unlike *E. coli* RNase HI, it digests ssRNA.

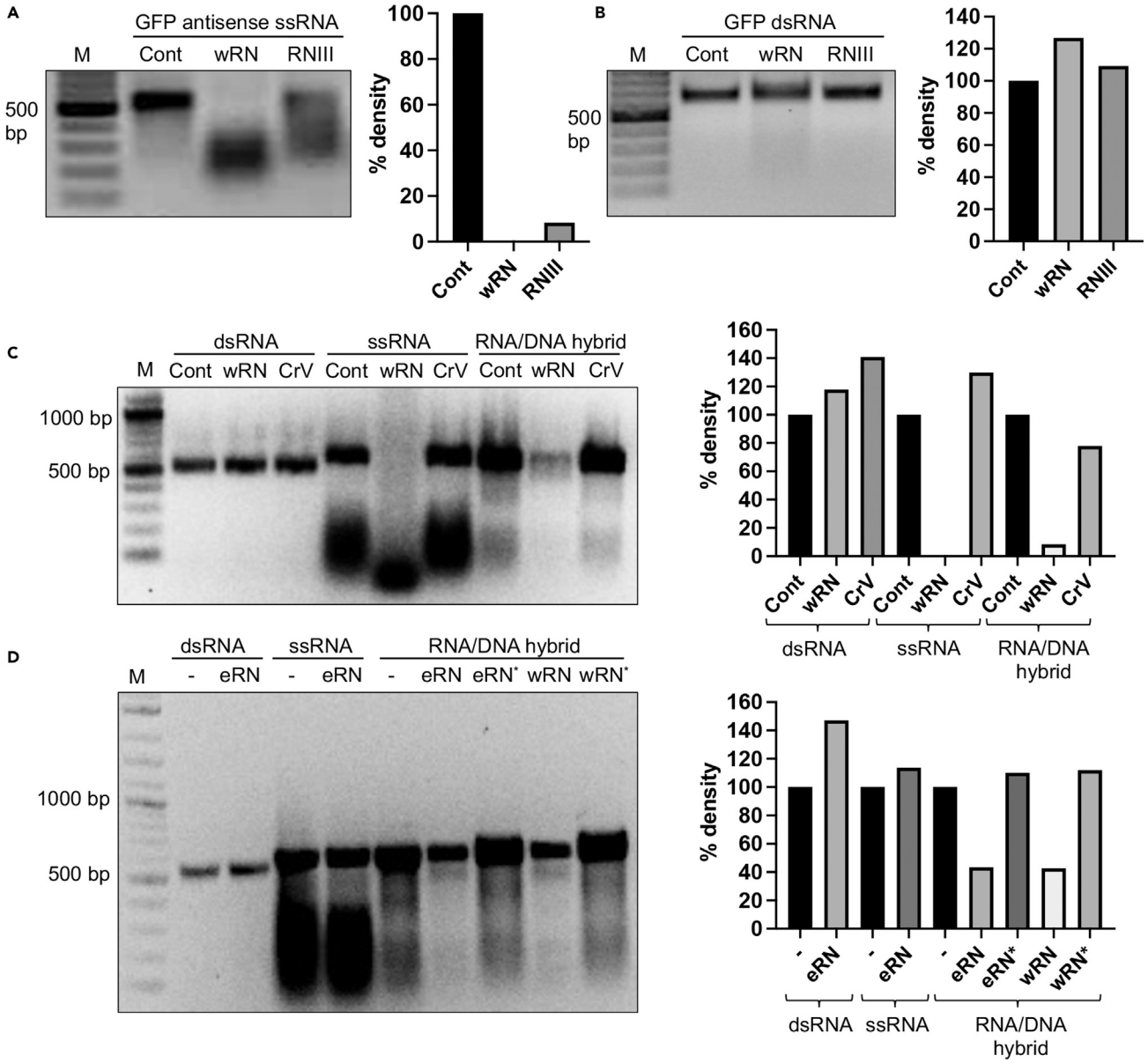

Figure 3. Confirmation of *w*RNase HI RNase activity

(A and B) Purified recombinant *w*RNase HI (wRN) and *w*RNase III (RNIII, as control) proteins expressed in *E. coli* were incubated with ssRNA and dsRNA for 60 min at 37°C, subsequently ran on an agarose gel, and visualized with ethidium bromide staining. *GFP* gene was used as the template to generate the ssRNA and dsRNA substrates. Cont, buffer only.

(C) GFP dsRNA (450 ng), ssRNA (1 μg), and RNA/DNA hybrid (1 μg) were incubated with purified *w*RNase HI or a control protein (CrV) expressed in *E. coli*, or buffer only (Cont) for 60 min at 37°C, subsequently ran on an agarose gel, and visualized with SYBR Safe staining.

(D) GFP dsRNA (450 ng), ssRNA (1 μg), and RNA/DNA hybrid (1 μg) were incubated with purified *w*RNase HI (wRN) or *E. coli* RNase HI (eRN). In eRN* and wRN* reactions, 50 mM EDTA was added to the reactions. (–), buffer only. The graphs next to each gel image show the relative density of bands determined by ImageJ, with the controls set at 100%.

*w*RNase HI suppressed DENV replication in mosquito cells

To explore the blocking effect of *w*RNase HI on DENV, the full-length gene coding for *wRNase HI* from *w*MelPop was cloned into the pIZ/V5-His vector. After confirmation of the sequence of the insert, the plasmid was transfected into *A. aegypti* Aa20 cells. Control cells were transfected with no plasmid, pIZ/V5-His empty vector (pIZ), and pIZ/V5-His with an insert coding for *wRNase III*. Three days after transfection, cells were infected with 1 MOI of DENV-2 and then collected 3 dpi. RT-qPCR analysis of RNA extracted from

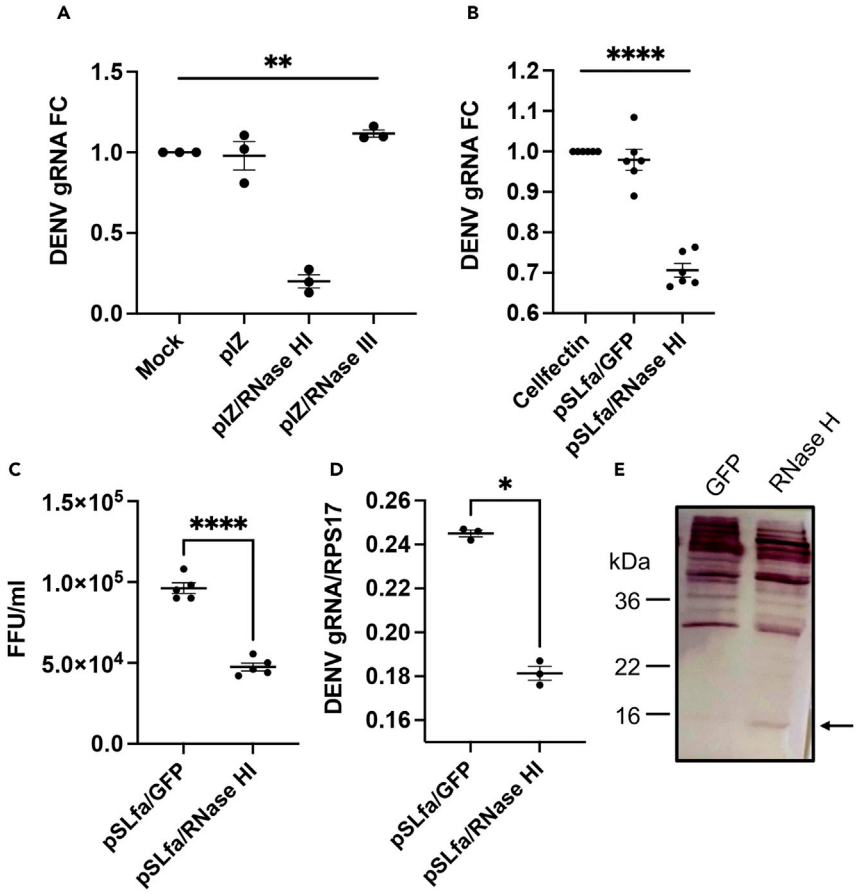

Figure 4. *w*RNase HI suppresses DENV replication in mosquito cell lines

(A) DENV genomic RNA levels were assessed 3 dpi (1 MOI) by RT-qPCR analysis of RNA from *A. aegypti* Aa20 cells transfected with pIZ/*w*RNase HI, pIZ/RNase III, empty pIZ vector, or no plasmid (mock). The error bars represent SE of mean (SEM) of three biological replicates. Kruskal-Wallis test was carried out to determine statistical significance among groups. **, $p < 0.01$; FC, fold change.

(B) DENV genomic RNA levels were assessed 3 dpi (1 MOI) by RT-qPCR analysis of RNA in Aag2 cells transfected with pSLfa/*w*RNase HI, pSLfa/GFP, or no vector (Cellfectin). RT-qPCR analyses of extracted RNA was performed using primers targeting the DENV-2 viral genome and *A. aegypti RPS17* as normalizing gene. One-way ANOVA test was carried out to determine statistical significance among groups. The error bars represent standard error of mean (SEM) of six biological replicates. ****, $p < 0.0001$; FC, fold change.

(C) Focus forming assay of the supernatants collected from the experiment described in (B). FFU, focus forming units. t-test was carried out to determine statistical significance between the two groups. The error bars represent standard error of mean (SEM) of five biological replicates. ****, $p < 0.0001$.

(D) RT-qPCR analysis of purified DENV RNA following incubation with cytoplasmic fractions of Aag2 cells transfected with pSLfa/*w*RNase HI or pSLfa/GFP. Mann-Whitney test was carried out to determine statistical significance between the two groups. The error bars represent SE of mean (SEM) of three biological replicates. *, $p < 0.05$.

(E) Confirmation of *w*RNase HI expression in pSLfa/*w*RNase HI and pSLfa/GFP transfected Aag2 mosquito cells. Extracted recombinant *w*RNase HI protein was detected by western blot analysis using a specific antibody to *w*RNase HI.

the cells revealed significant reductions (5-fold; Kruskal-Wallis test, $p < 0.0073$) in DENV genomic RNA levels in *w*RNase HI-expressing cells compared to the control cells (Figure 4A). Furthermore, we observed a similar blocking effect of *w*RNase HI on DENV in Aag2 cells after expressing the protein through cloning the gene from *w*MelPop in the pSLfa expression vector. RT-qPCR analysis revealed significant reductions (1.4-fold; One-way ANOVA, $p < 0.0001$) of DENV genomic RNA in cells transfected with pSLfa/*w*RNase HI construct compared to cells transfected with pSLfa/GFP or Cellfectin transfection reagent only (Figure 4B). A similar significant reduction (2-fold; t-test, $p < 0.0001$) in the number of DENV virions was obtained when the supernatants from the treated cells were subjected to focus forming assay (Figure 4C). We also compared viral RNA levels following incubation of purified DENV RNA with the cytoplasmic fractions

from Aag2 cells with and without *w*RNase HI overexpression. Results showed significant reductions (Mann-Whitney, p = 0.05) in DENV RNA levels in cells overexpressing *w*RNase HI compared to cell overexpressing GFP (Figure 4D). We confirmed expression of *w*RNase HI (~16 kDa) in the transfected cells and Aag2.*w*MelPop cells with western blot analysis using a specific antibody to *w*RNase HI (Figure 4E and S5A).

We also explored if host mRNAs could be affected by the overexpression of *w*RNase HI in Aag2 cells. Three *A. aegypti* genes that we had their primers available (*actin*, *RNase H1*, and *prohibitin*) did not show any change in their transcript levels assessed three days post transfection with pSLfa/*w*RNase HI, pSLfa/GFP, or Cellfectin transfection reagent only (Figure S5B; Kruskal-Wallis test; p = 0.0679, p = 0.8179, p = 0.1750, respectively). The expression of *RPS17* that was used as a normalizing gene also remained stable between the treatments. The results showed that although *w*RNase HI affected viral RNA, it did not affect host mRNAs.

Antisense RNA-mediated knockdown of *wRNase HI* increased DENV replication in *Wolbachia*-transinfected cells and mosquitoes

We utilized a gene-specific antisense RNA (asRNA) (195 nt) to knockdown *wRNase HI* in Aag2.*w*MelPop cell line. Although DENV did not induce the expression of *A. aegypti RNase H1* (*AeRNase H1*) (Figures S2A–S2D), we still checked the similarity of the gene with *wRNase HI* from *w*AlbB and *w*MelPop *Wolbachia* strains through sequence alignment. The overall similarity was found to be low with about 52% identity between *AeRNase H1* and *wRNase HI* genes (Figure S6). Furthermore, the asRNA made to *wRNase HI* does not have a continuous alignment with the *AeRNase H1* sequence, and also the similarity is very low. Consequently, formation of dsRNA by the asRNA and *AeRNase H1*, as a requisite for the host RNAi to target *AeRNase H1*, is unlikely. To ascertain this empirically, we assessed the transcript levels of *AeRNase H1* in Aag2.*w*MelPop cells transfected with asRNA to *wRNase HI* (as*w*RNaseH) compared to the controls three days post transfection. No change was found in the transcript levels of *AeRNase H1* (Figure S2E). Therefore, we assume there is no potential off-target effect of the asRNA on *AeRNase H1* expression.

Aag2.*w*MelPop cells were transfected with as*w*RNaseH or asGFP as control and after 24 h, they were infected with 1 MOI of DENV-2. Cells were collected three days after infection from which RNA was extracted. RT-qPCR analysis showed statistically significant downregulation (average 30%; One-way ANOVA, p< 0.0001) of *wRNase HI* in as*w*RNaseH transfected cells compared to cells transfected with asGFP or Cellfectin only (Figure 5A). To assess levels of DENV RNA, RT-qPCR analysis of RNA extracted from the cells showed significant increases (1.8-fold; One-way ANOVA, p< 0.0001) of virus genomic RNA in *wRNase HI* knocked down cells compared to the controls (Figure 5B). Similarly, titration of the supernatants collected in the experiment revealed significant increases (2.4-fold; t-test, p< 0.0001) in the number of DENV virions (Figure 5C). As a control, we also transfected Aag2 cells (without *Wolbachia*) with as*w*RNaseH, asGFP, and Cellfectin only to find out if as*w*RNaseH has any non-specific effect on DENV replication. Results showed no change (Kruskal-Wallis test, p = 0.3765) in replication of DENV between the treatments (Figure S7).

We were interested to assess if increases in DENV replication could be because of a possible decrease in *Wolbachia* density in *wRNase HI* knocked down cells. However, qPCR analysis of the cells showed no reduction in *Wolbachia* density (p = 0.075) (Figure 5D). We further carried out *wRNase HI* knockdown in Aag2.*w*MelPop and looked at *Wolbachia* density at 2 and 5 days after transfections. We found that although there was a significant decline (50%; Kruskal-Wallis test, p = 0.005) in *wRNase HI* levels at 2 days post transfection, the knockdown effect disappeared by 5 days post transfection (Figure S8A). Nevertheless, *Wolbachia* density was not affected (Kruskal-Wallis test, p = 0.7463) by *wRNase HI* knockdown (Figure S8B) as also shown in Figure 5D three days post transfection. Overall, the results suggest that DENV was able to replicate more efficiently in reduced amounts of *w*RNase HI even when *Wolbachia* density remained unchanged.

To explore if gene knockdown also works in another strain of *Wolbachia*, we knocked down *wRNase HI* in Aag2.*w*AlbB cells. RT-qPCR results showed significant reductions in the abundances of *wRNase HI* (50%; One-way ANOVA, p< 0.0001) in Aag2.*w*AlbB cells four days after transfection (Figure S9A). However, as*w*RNaseH transfection did not affect the transcript levels of three *Wolbachia* non-target genes (*wsp*, *M23 family peptidase* [DEJ70_04815], and *outer membrane protein assembly factor BamD* [DEJ70_04508]) suggesting specificity of the knockdown (Figures S9B–S9D). When the transfected cells with as*w*RNase HI or

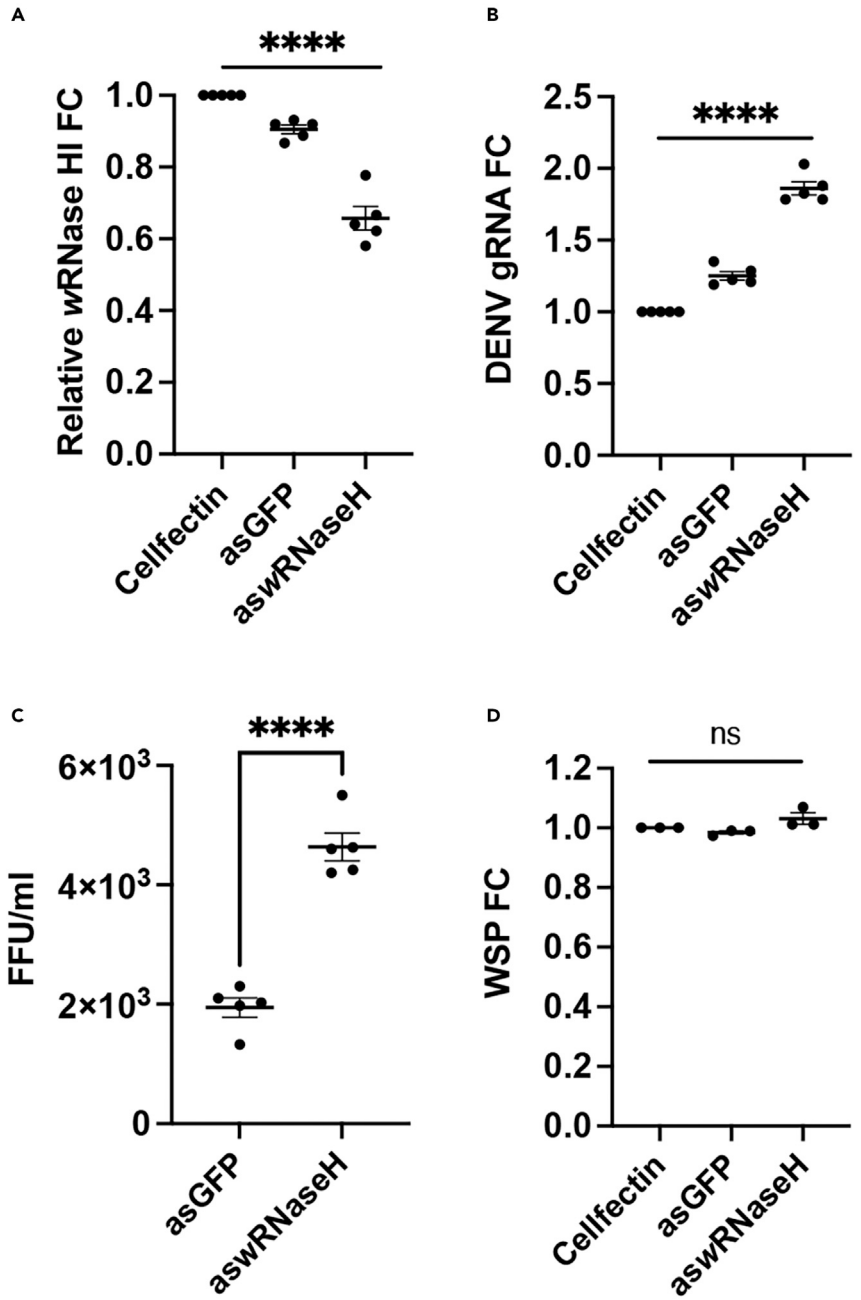

Figure 5. Antisense RNA-mediated knockdown of *wRNase HI* increased DENV replication in *Wolbachia*-transinfected cells

(A) RT-qPCR analysis of RNA extracted from Aag2.*w*MelPop cells transfected with antisense RNAs, aswRNase HI and asGFP as control, and infected with 1 MOI of DENV-2 and collected 3 dpi.

(B) Increased genomic DENV RNA levels as a result of *wRNase HI* knockdown. Cells transfected with asGFP and Cellfectin reagent only were used as controls. RT-qPCR analysis was performed using *Wolbachia's 16S rRNA* gene and *A. aegypti RPS17* as the normalizing genes in A and B, respectively.

(C) Focus forming assay of the supernatants collected from the experiment described in (B). FFU, focus forming units.

(D) Relative *Wolbachia* density following *wRNase HI* knockdown. qPCR analysis of extracted genomic DNA using primers to *Wolbachia*'s *wsp* gene and the host cell *RPS17* gene showed no reduction in *Wolbachia* density after *wRNase HI* knockdown when compared to controls 3 days post transfection. For statistical analysis, One-way ANOVA with Tukey's post-hoc test was carried out to determine statistical significance between groups in (A and B), t-test in (C), and Kruskal-Wallis test in (D). The error bars in all the graphs represent SE of mean (SEM) of biological replicates each represented by a data point. ns, not significant; ****, $p < 0.0001$. FC, fold change.

the controls were infected with DENV-2 (1 MOI), RT-qPCR analysis of the cells 3 dpi showed significant increases (One-way ANOVA, $p < 0.0001$) in genomic RNA levels of DENV in the *wRNase HI*-knocked down cells (Figure S9E), consistent with the results in Aag2.*w*MelPop cells. Although increases in virus replication in the case of knocking down *wRNase HI* in Aag2.*w*MelPop or Aag2.*w*AlbB was only about 2-fold, it is worth mentioning that the efficiency of knockdown was not very high (about 30–50%). Nevertheless, the effect on DENV was reproducible in several independent experiments. With further optimisation to increase the efficiency of knockdown, the effect on DENV replication could be stronger. Again, knocking down *wRNase HI* in Aag2.*w*AlbB cells had no effect on *Wolbachia* density (Figures S8C and S8D). Furthermore, similar to Aag2.*w*MelPop cells (Figure S8A), knockdown of *wRNase HI* was evident at 2 days post-transfection, but not at 5 days post-transfection (Figure S8C). As a control, we knocked down another *Wolbachia* gene (SPFH domain-containing protein) in Aag2.*w*AlbB cells (40% reduction; Kruskal-Wallis test, $p = 0.025$; Figure S9F), however, we did not find increases in DENV replication, but in fact some reductions in virus replication (Kruskal-Wallis test, $p = 0.025$; Figure S9G). These results confirmed specific blocking of DENV by *w*RNase HI.

Considering the availability of a specific polyclonal antibody against the *wsp* gene of the *w*MelPop strain of *Wolbachia* that we previously raised,[46] we examined the effect of knocking down *wsp* using asRNA at the protein level. For this, Aag2.*w*MelPop cells were transfected with Cellfectin only, asGFP, or asWSP. Knockdown of *wsp* with asWSP was confirmed by RT-qPCR when cells were examined 3 days post transfection (about 40% reduction; Figure S10A). Western blot analysis of cells also showed reductions in the wsp protein levels (Figures S10B and S10C). These results further provided proof-of-concept evidence for successful knockdown of *Wolbachia* genes using asRNA.

To find out if *Wolbachia* gene knockdown can be achieved *in vivo*, and if the effect of knocking down *wRNase HI* on DENV in cells can be replicated in mosquitoes, 2-day-old *w*AlbB *Wolbachia*-transinfected female mosquitoes were injected with as*w*RNaseH, asGFP, or buffer only. Two days after injection, mosquitoes were fed on blood containing 1×10^7/mL DENV-2. Unfed mosquitoes were discarded, and the remaining were maintained on sugar solution for four days. RT-qPCR analysis of RNA extracted from mosquitoes using primers to *wRNase HI* gene showed significant downregulation (50% reduction; One-way ANOVA, $p < 0.0001$) of the gene in as*w*RNaseH-injected mosquitoes compared to asGFP and buffer-only injected mosquitoes suggesting successful downregulation of the *Wolbachia* gene in the mosquitoes (Figure 6A). RT-qPCR showed significant increases (2.5-fold; One-way ANOVA, $p = 0.0022$) in DENV genomic RNA levels in as*w*RNaseH-injected mosquitoes compared to the controls (Figure 6B). In one of the as*w*RNaseH-injected mosquitoes in which there was no *w*RNaseH knockdown, the mosquito had the lowest DENV gRNA because of *Wolbachia* blocking (Figure 6A and 6B, red dots), whereas the mosquito with the highest DENV gRNA had about 50% *wRNase HI* knockdown (Figure 6A and 6B, blue dots). Altogether, the *in vitro* and the *in vivo* results suggest that *w*RNase HI limits DENV replication in mosquitoes contributing to *Wolbachia*'s virus blocking phenotype.

The effect of *w*RNase HI on insect-specific viruses

It has been established that *Wolbachia* blocks replication of positive sense RNA viruses, but not that of negative sense RNA viruses.[47–49] Aag2 cells are persistently infected with Cell fusing agent virus (CFAV, a positive sense flavivirus) and Phasi Charoen-like virus (PCLV, a negative sense bunyavirus).[48] To test the effect of *w*RNase HI on these viruses, Aag2 cells were transfected with Cellfectin transfection reagent only, pSLfa/GFP, or pSLfa/wRNase HI. Three days after transfection, total RNA was extracted from the cells and subjected to RT-qPCR using specific primers to each virus. Results showed significant reduction (One-way ANOVA; $p < 0.0001$) in CFAV gRNA levels in pSLfa/wRNase HI transfected cells compared to the controls (Figure 7A), whereas no statistically significant (One-way ANOVA; $p = 0.44$) effect was found on PCLV (Figure 7B). The results are consistent with the effect of *Wolbachia* on positive and negative sense RNA viruses.

DISCUSSION

Wolbachia has been shown to confer anti-viral protection by inhibiting the replication of positive sense RNA viruses in primary arbovirus mosquito vectors, however, the mechanism of blocking remains unknown.[8,10,50,51] Currently, these mechanisms revolve around host immune induction[8,16,18,31] and competition for resources and space.[8,10,52] Studies have also shown that virus blocking occurs in cells where *Wolbachia* is present, and the protection cannot be conferred to the adjacent cells.[10,32] Here, we

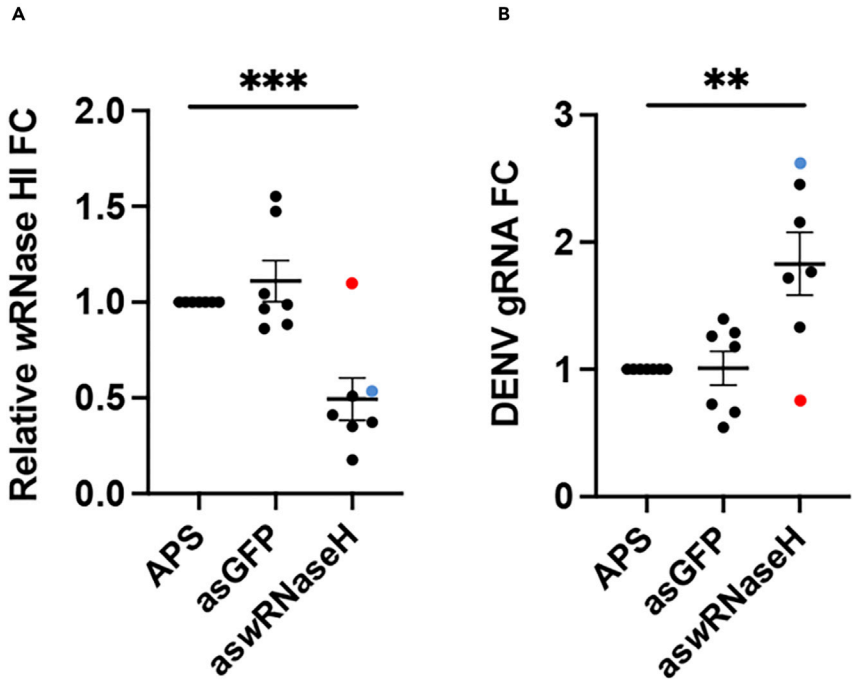

Figure 6. Antisense RNA-mediated knockdown of *wRNase HI* increased DENV replication in *Wolbachia*-transinfected mosquitoes

(A) RT-qPCR analysis of RNA extracted from mosquitoes injected with antisense RNAs, aswRNase HI and asGFP, as control, or APS buffer only. *Wolbachia's 16S rRNA* gene was used as the normalizing gene.

(B) Increased genomic DENV RNA levels as a result of *wRNase HI* knockdown in *w*AlbB-transinfected mosquitoes. Mosquitoes were injected with aswRNase HI, asGFP, or APS buffer only. Two days after injection, mosquitoes were fed on DENV-2 and analyzed four days following virus feeding. RT-qPCR analysis was performed using *A. aegypti RPS17* as the normalizing gene. For statistical analysis, One-way ANOVA test was carried out to determine statistical significance between groups. The error bars represent SE of mean (SEM) of biological replicates (individual mosquitoes). **, $p < 0.01$; ***, $p < 0.001$; FC, fold change. The red and the blue dots in (B) represent mosquitoes with the lowest and the highest DENV gRNA levels, respectively, corresponding to the same color dots and mosquitoes in (A) with no *wRNase HI* knockdown and about 50% *wRNase HI* knockdown, respectively.

demonstrate the ability of *Wolbachia's* RNase HI to degrade positive-sense single-stranded viral RNA, and as a result contributes to suppressing DENV replication and production of infectious virus particles.

wRNase HI is a gene that we recently found induced in Aag2.*w*AlbB cells as early as 1 h after DENV infection.[37] In this study, we confirmed this induction in both *w*AlbB and *w*MelPop strains of *Wolbachia* as early as half an hour after DENV infection. This induction does not seem to be because of introduction of foreign RNA (Figure 5A and S9; compare Cellfectin with asGFP). Of note, the host *RNase H1*, which does not show a high level of sequence similarity to *wRNase HI*, was not induced by DENV. Furthermore, we found the *w*MelPop strain of *Wolbachia* exhibited 2- to 3-fold higher expression of *wRNase HI* transcript levels in contrast to *w*AlbB-infected Aa23 and Aag2.*w*AlbB cells at comparable *Wolbachia* densities. This higher expression of *wRNase HI* by *w*MelPop is correlated with stronger virus blocking in Aag2.*w*MelPop cells. Consistently, studies have shown that the *w*MelPop strain confers a stronger virus blocking effect on virus replication compared to other *Wolbachia* strains.[42,51] Furthermore, we investigated the expression of *wRNase HI* in *w*AlbB mosquitoes and observed identical *wRNase HI* transcript levels at 2 and 6 dpe, however, these were significantly downregulated at 12 dpe. Despite the decline in *wRNase HI* expression over the lifetime of the mosquitoes, the *Wolbachia* density increased with age at 2, 6, and 12 dpe, respectively. This is consistent with studies showing that *Wolbachia*-conferred DENV blocking was neither *Wolbachia* density-dependent nor tissue specific in *A. aegypti*.[9,33,53] Lack of correlation between *wRNase HI* transcript levels and *Wolbachia* density may have different reasons. It could be because of *Wolbachia*'s response to changes in the host cellular environment as the mosquito ages, or that gene expression may not necessarily correlate with density depending on the functional role of the gene. It remains to be determined what exact role *wRNase HI* plays in *Wolbachia*'s biological processes.

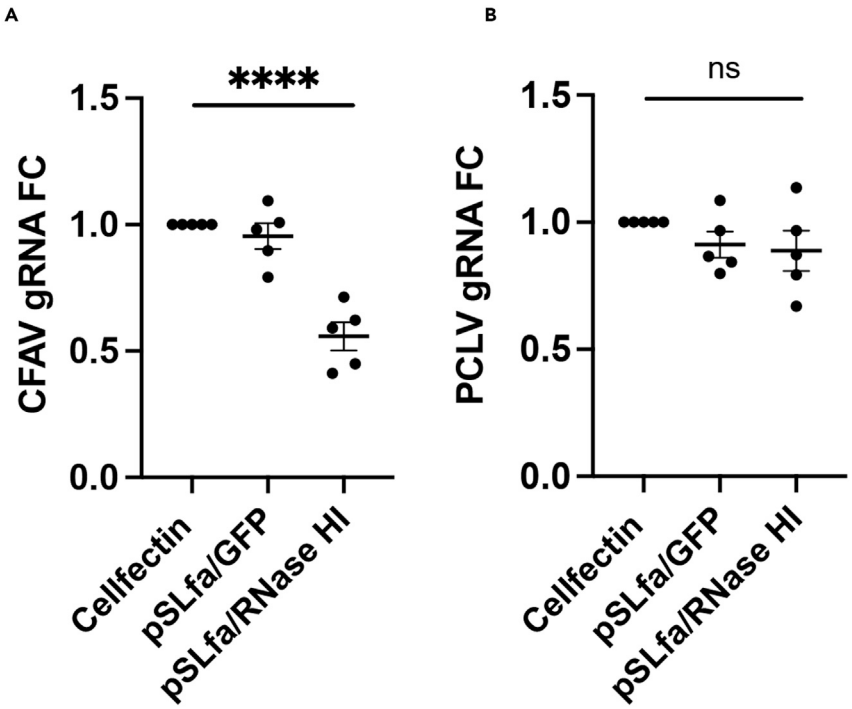

Figure 7. Effect of *w*RNase HI on insect-specific viruses CFAV and PCLV

(A and B) Aag2 cells were transfected with pSLfa/*w*RNase HI, and pSLfa/GFP, or Cellfectin reagent only as controls. Three days after transfection, total RNA was extracted from cells and subjected to RT-qPCR using primers specific to (A) CFAV and (B) PCLV. *A. aegypti RPS17* was used as the normalizing gene. One-way ANOVA was carried out to determine statistical significance between groups. The error bars represent SE of mean (SEM) of biological replicates each represented by a data point. ns, not significant; ****, $p < 0.0001$.

We found significantly higher RNase activity and consequent DENV RNA degradation in the cytoplasmic fraction of Aag2.*w*MelPop cells compared to Aag2 cells. This conforms with studies in Aag2.*w*Mel and RML-12.*w*Mel cells,[54] and *A. albopictus* and *Drosophila melanogaster* cells transinfected/colonized with *Wolbachia* *w*Stri and *w*Mel strains,[35] which reported that incoming DENV and Sindbis virus RNAs were almost immediately degraded in contrast to their *Wolbachia*-free counterparts.

By cloning the full-length *wRNase HI* gene into a bacterial expression vector, we confirmed the RNase activity of *w*RNase HI and that it specifically degrades ssRNA and RNA/DNA hybrid, but not dsRNA *in vitro*. This observation supports the hypothesis of degradation of the viral genome, that is in the form of ssRNA, by *w*RNase HI. Although *w*RNase HI appeared to digest ssRNA, *E. coli* RNase HI, which is a classic RNase HI enzyme that digests RNA in RNA/DNA hybrids did not digest ssRNA. The two proteins share conserved RNase HI and RNA/DNA hybrid binding residues, but identification of residues that contribute to the ssRNA activity of *w*RNase HI requires further investigation. Subsequently, overexpression of *w*RNase HI in transfected *A. aegypti* cell lines with insect-specific plasmid vectors, which were subsequently infected with DENV, showed a reduction in DENV genomic RNA levels and virion production, which further establishes the inhibitory functionality of *w*RNase HI on DENV.

Genetic manipulation of *Wolbachia* is not currently feasible. However, we were able to knockdown *Wolbachia*'s *RNase HI* gene by transfecting *Wolbachia*-transinfected mosquito cells with an antisense RNA (asRNA) to the gene. In addition to *wRNase HI*, we could also knockdown two other *Wolbachia* genes, SPFH and *wsp*, using asRNA, with knockdown of the latter confirmed at the protein level. Although it is unlikely that *wRNase HI* mRNA is transported outside *Wolbachia* into the host cytoplasm for interaction with asRNA, it is more likely that asRNA transverses into *Wolbachia*. This is not unlikely considering transfection of mitochondria with lipofectamine for RNAi has been achieved.[55] In addition, gene silencing/knockdown has been demonstrated in non-endosymbiotic bacteria with natural or artificial antisense nucleic acids, including RNA and DNA, which is distinct from gene silencing through RNAi observed in eukaryotic

cells.[56–58] Although Ago proteins are not essential for antisense gene silencing in bacteria,[57] recent research shows that Ago-like proteins are also present in bacteria that can facilitate gene silencing.[59,60] The interaction of the antisense nucleic acid with complementary target sequences leads to prokaryotic gene silencing through interference with transcription initiation or translation or combination of both (reviewed in[61]). Knocking down *wRNase HI* in Aag2.*w*MelPop and Aag2.*w*AlbB cells resulted in downregulation of *wRNase HI* expression, while concurrently increasing levels of DENV genomic RNA *in vitro* and *in vivo*. It is important to note here that knockdown of *wRNase HI* did not have any significant effect on *Wolbachia* density indicating that DENV replicates more efficiently in the absence or lower amounts of *w*RNase HI; although we cannot rule out the possibility of reduction in fitness of *Wolbachia* when *wRNase HI* is knocked down which could potentially aid DENV replication. We obtained similar results when quantifying infectious particles by focus forming assay where knocked down *wRNase HI* cells generated higher titers of DENV virions compared to control cells with a fully functioning *w*RNase HI. Furthermore, knocking down *wRNase HI* in *w*AlbB-transinfected mosquitoes, by injecting them with *wRNase HI* asRNA, led to higher amplification of DENV. The ability of RNase HI to target and degrade RNA/DNA hybrids generated during viral replication and its potential role in anti-viral defense is widely supported.[39–41]

We also investigated the effect of overexpressing *w*RNase HI on insect-specific viruses in the absence of *Wolbachia*, by transfecting Aag2 cells persistently infected with CFAV (a positive sense flavivirus) and PCLV (a negative sense bunyavirus), which resulted in significant reduction in CFAV gRNA levels, but no effect on PCLV. Our results are coherent with observations made involving the blocking of +ssRNA viruses belonging to *Flaviviridae* (DENV-2, CFAV) and *Togaviridae* (Sindbis virus, Chikungunya virus) families, but not -ssRNA viruses belonging to *Bunyavirales* (PCLV) and *Mononegavirales* (Aedes anphevirus).[35,47–49,54]

In summary, we demonstrate that *wRNase HI* is rapidly induced in *Wolbachia*-transinfected mosquito cells in response to DENV infection and is able to degrade the viral genomic RNA. Considering the higher degradation of DENV RNA in the cytoplasmic fraction from *Wolbachia*-infected cells compared to that of the control cells, *w*RNase HI is likely secreted from *Wolbachia* cells into the host cytoplasm. However, this needs to be experimentally confirmed. We show the possibility of knocking down *Wolbachia* genes using antisense RNA, which opens a significant opportunity for exploring functions of *Wolbachia* and perhaps other endosymbiotic bacterial genes in their interaction with hosts in the absence of tools to genetically manipulate them. Knockdown of *wRNase HI* led to significant increases in DENV replication both *in vitro and in vivo*. Furthermore, overexpression of *w*RNase HI in mosquito cells persistently infected with insect-specific viruses mimicked *Wolbachia*'s effect on inhibition of +ssRNA viruses and not-ssRNA viruses. Our findings reveal and provide compelling evidence for the missing link between early *Wolbachia*-mediated virus blocking, degradation of positive-sense single-stranded viral RNA, and inhibitory effect on the production of infectious viral particles. However, virus blocking is most likely due to a combination of different factors in which *w*RNase HI is one of the contributors. In regard to inhibition of dsRNA viruses by *Wolbachia* (only one example has been shown that was in planthoppers infected with *w*Stir strain[62]), *w*RNase HI could possibly target the +ssRNA that is produced during viral replication from which the-ssRNA is reverse transcribed forming a dsRNA genome,[63] which needs to be experimentally tested. This mechanism, however, does not explain the inhibition of eukaryotic microorganisms (e.g., *Plasmodium* or filarial worms) by *Wolbachia*, in which case other factors such as competition for space and resources could be more applicable. Future studies should also expand to other *Wolbachia* strains, including those that do not block virus replication, and other host species.

Limitations of the study

Our study investigated *w*RNase HI in two strains of *Wolbachia*, *w*MelPop and *w*AlbB, in the mosquito *A. aegypti*; the two strains known to block virus replication. Our study did not include a strain of *Wolbachia* that does not block virus replication, and experiments were done only in *A. aegypti*. Another limitation of the study is that the antibody raised against *w*RNase HI was not very specific and could not be used for immunofluorescence or immunoprecipitation studies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105836.

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AUTHOR CONTRIBUTIONS

M.H., G.Z., and S.A. designed the experiments. M.H., G.Z., L.M.H., and M.L. conducted the experiments with the majority done by M.H., and G.Z. S.A., and M.L. wrote the paper. M.H., M.L., and S.A. edited the paper.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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REFERENCES

1. Zug, R., and 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.

2. Bleidorn, C., and Gerth, M. (2018). A critical re-evaluation of multilocus sequence typing (MLST) efforts in *Wolbachia*. FEMS Microbiol. Ecol. *94*. https://doi.org/10.1093/femsec/fix163.

3. Johnson, K.N. (2015). The impact of *Wolbachia* on virus infection in mosquitoes. Viruses *7*, 5705–5717. https://doi.org/10.3390/v7112903.

4. Sinkins, S.P. (2013). *Wolbachia* and arbovirus inhibition in mosquitoes. Future Microbiol. *8*, 1249–1256.

5. Hedges, L.M., Brownlie, J.C., O'Neill, S.L., and Johnson, K.N. (2008). *Wolbachia* and virus protection in insects. Science *322*, 702.

6. Teixeira, L., Ferreira, A., and Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol. *6*, e2–e2763.

7. Ant, T.H., Herd, C.S., Geoghegan, V., Hoffmann, A.A., and Sinkins, S.P. (2018). The *Wolbachia* strain *w*Au provides highly efficient virus transmission blocking in *Aedes aegypti*. PLoS Pathog. *14*, e1006815. https://doi.org/10.1371/journal.ppat.1006815.

8. Bian, G., Xu, Y., Lu, P., Xie, Y., and Xi, Z. (2010). The endosymbiotic bacterium *Wolbachia* induces resistance to Dengue virus in *Aedes aegypti*. PLoS Pathog. *6*, e1000833.

9. Chouin-Carneiro, T., Ant, T.H., Herd, C., Louis, F., Failloux, A.B., and Sinkins, S.P. (2020). *Wolbachia* strain *w*AlbA blocks Zika virus transmission in *Aedes aegypti*. Med. Vet. Entomol. *34*, 116–119.

10. Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., Rocha, B.C., Hall-Mendelin, S., Day, A., Riegler, M., et al. (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and plasmodium. Cell *139*, 1268–1278.

11. Dutra, H.L.C., Rocha, M.N., Dias, F.B.S., Mansur, S.B., Caragata, E.P., and Moreira, L.A. (2016). *Wolbachia* blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes. Cell Host Microbe *19*, 771–774.

12. Nazni, W.A., Hoffmann, A.A., NoorAfizah, A., Cheong, Y.L., Mancini, M.V., Golding, N., Kamarul, G.M.R., Arif, M.A.K., Thohir, H., NurSyamimi, H., et al. (2019). Establishment of *Wolbachia* strain *w*AlbB in Malaysian populations of *Aedes aegypti* for Dengue control. Curr. Biol. *29*, 4241–4248.e5.

13. Pacidônio, E.C., Caragata, E.P., Alves, D.M., Marques, J.T., and Moreira, L.A. (2017). The impact of *Wolbachia* infection on the rate of vertical transmission of dengue virus in Brazilian *Aedes aegypti*. Parasit. Vectors *10*, 296.

14. Ryan, P.A., Turley, A.P., Wilson, G., Hurst, T.P., Retzki, K., Brown-Kenyon, J., Hodgson, L., Kenny, N., Cook, H., Montgomery, B.L., et al. (2019). Establishment of *w*Mel *Wolbachia* in *Aedes aegypti* mosquitoes and reduction of local dengue transmission in Cairns and surrounding locations in northern Queensland, Australia. Gates Open Res. *3*, 1547.

15. Tantowijoyo, W., Andari, B., Arguni, E., Budiwati, N., Nurhayati, I., Fitriana, I., Ernesia, I., Daniwijaya, E.W., Supriyati, E., Yusdiana, D.H., et al. (2020). Stable establishment of *w*Mel *Wolbachia* in *Aedes aegypti* populations in Yogyakarta, Indonesia. PLoS Negl. Trop. Dis. *14*, e0008157.

16. Kambris, Z., Cook, P.E., Phuc, H.K., and Sinkins, S.P. (2009). Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science *326*, 134–136.

17. Pan, X., Pike, A., Joshi, D., Bian, G., McFadden, M.J., Lu, P., Liang, X., Zhang, F., Raikhel, A.S., and Xi, Z. (2018). The bacterium *Wolbachia* exploits host innate immunity to establish a symbiotic relationship with the dengue vector mosquito *Aedes aegypti*. ISME J. *12*, 277–288.

18. Rances, E., Johnson, T.K., Popovici, J., Iturbe-Ormaetxe, I., Zakir, T., Warr, C.G., and O'Neill, S.L. (2013). The Toll and Imd pathways re not required for *Wolbachia*-mediated dengue interference. J. Virol. *8*, e1002548.

19. Zhang, D., Wang, Y., He, K., Yang, Q., Gong, M., Ji, M., and Chen, L. (2020). *Wolbachia* limits pathogen infections through induction of host innate immune responses. PLoS One *15*, e0226736.

20. Caragata, E.P., Rancès, E., Hedges, L.M., Gofton, A.W., Johnson, K.N., O'Neill, S.L., and McGraw, E.A. (2013). Dietary cholesterol modulates pathogen blocking by *Wolbachia*. PLoS Pathog. *9*, e1003459.

21. Geoghegan, V., Stainton, K., Rainey, S.M., Ant, T.H., Dowle, A.A., Larson, T., Hester, S., Charles, P.D., Thomas, B., and Sinkins, S.P. (2017). Perturbed cholesterol and vesicular trafficking associated with dengue blocking in *Wolbachia*-infected *Aedes aegypti* cells. Nat. Commun. *8*, 526.

22. Molloy, J.C., Sommer, U., Viant, M.R., and Sinkins, S.P. (2016). *Wolbachia* modulates lipid metabolism in *Aedes albopictus* mosquito cells. Appl. Environ. Microbiol. *82*, 3109–3120.

23. White, P.M., Serbus, L.R., Debec, A., Codina, A., Bray, W., Guichet, A., Lokey, R.S., and Sullivan, W. (2017). Reliance of *Wolbachia* on high rates of host proteolysis revealed by a genome-wide RNAi screen of *Drosophila* cells. Genetics *205*, 1473–1488.

24. Asad, S., Parry, R., and Asgari, S. (2018). Upregulation of *Aedes aegypti* Vago1 by *Wolbachia* and its effect on dengue virus replication. Insect Biochem. Mol. Biol. *92*, 45–52.

25. Asad, S., Hall-Mendelin, S., and Asgari, S. (2016). Downregulation of *Aedes aegypti* chromodomain helicase DNA binding protein 7/Kismet by *Wolbachia* and its effect on dengue virus replication. Sci. Rep. *6*, 36850.

26. Haqshenas, G., Terradas, G., Paradkar, P.N., Duchemin, J.B., McGraw, E.A., and Doerig, C. (2019). A role for the insulin receptor in the suppression of dengue virus and Zika virus in *Wolbachia*-infected mosquito cells. Cell Rep. *26*, 529–535.e3.

27. Zhang, G., Hussain, M., O'Neill, S.L., and Asgari, S. (2013). *Wolbachia* uses a host microRNA to regulate transcripts of a methyltransferase contributing to dengue virus inhibition in *Aedes aegypti*. Proc. Natl. Acad. Sci. USA *110*, 10276–10281.

28. Lu, P., Sun, Q., Fu, P., Li, K., Liang, X., and Xi, Z. (2020). *Wolbachia* inhibits binding of dengue and Zika viruses to mosquito cells. Front. Microbiol. *11*, 1750.

29. Hedges, L.M., Yamada, R., O'Neill, S.L., and Johnson, K.N. (2012). The small interfering RNA pathway is not essential for *Wolbachia*-mediated antiviral protection in *Drosophila melanogaster*. Appl. Environ. Microbiol. *78*, 6773–6776.

30. Sucupira, P.H.F., Ferreira, Á.G.A., Leite, T.H.J.F., de Mendonça, S.F., Ferreira, F.V., Rezende, F.O., Marques, J.T., and Moreira, L.A. (2020). The RNAi pathway is important to control Mayaro virus infection in *Aedes aegypti* but not for *Wolbachia*-mediated protection. Viruses *12*, E871.

31. Terradas, G., Joubert, D.A., and McGraw, E.A. (2017). The RNAi pathway plays a small part in *Wolbachia*-mediated blocking of dengue virus in mosquito cells. Sci. Rep. *7*, 43847.

32. Nainu, F., Trenerry, A., and Johnson, K.N. (2019). *Wolbachia*-mediated antiviral protection is cell-autonomous. J. Gen. Virol. *100*, 1587–1592.

33. Fraser, J.E., O'Donnell, T.B., Duyvestyn, J.M., O'Neill, S.L., Simmons, C.P., and Flores, H.A. (2020). Novel phenotype of *Wolbachia* strain *w*Pip in *Aedes aegypti* challenges assumptions on mechanisms of *Wolbachia*-mediated dengue virus inhibition. PLoS Pathog. *16*, e1008410. https://doi.org/10.1371/journal.ppat.1008410.

34. Kaur, R., Martinez, J., Rota-Stabelli, O., Jiggins, F.M., and Miller, W.J. (2020). Age, tissue, genotype and virus infection regulate *Wolbachia* levels in *Drosophila*. Mol. Ecol. *29*, 2063–2079.

35. Bhattacharya, T., Newton, I.L.G., and Hardy, R.W. (2020). Viral RNA is a target for *Wolbachia*-mediated pathogen blocking. PLoS Pathog. *16*, e1008513.

36. Rainey, S.M., Martinez, J., McFarlane, M., Juneja, P., Sarkies, P., Lulla, A., Schnettler, E., Varjak, M., Merits, A., Miska, E.A., et al. (2016). *Wolbachia* blocks viral genome replication early in infection without a transcriptional response by the endosymbiont or host small RNA pathways. PLoS Pathog. *12*, e1005536.

37. Leitner, M., Bishop, C., and Asgari, S. (2021). Transcriptional response of *Wolbachia* to dengue virus infection in cells of the mosquito *Aedes aegypti*. mSphere *6*, e0043321.

38. Cerritelli, S.M., and Crouch, R.J. (2009). Ribonuclease H: the enzymes in eukaryotes. FEBS J. *276*, 1494–1505.

39. Hartmann, G. (2017). Nucleic acid immunity. Adv. Immunol. *133*, 121–169.

40. Koonin, E.V. (2017). Evolution of RNA- and DNA-guided antivirus defense systems in prokaryotes and eukaryotes: common ancestry vs convergence. Biol. Direct *12*, 5.

41. Moelling, K., Broecker, F., Russo, G., and Sunagawa, S. (2017). RNase H as gene modifier, driver of evolution and antiviral defense. Front. Microbiol. *8*, 1745.

42. Joubert, D.A., Walker, T., Carrington, L.B., De Bruyne, J.T., Kien, D.H.T., Hoang, N.L.T., Chau, N.V.V., Iturbe-Ormaetxe, I., Simmons, C.P., and O'Neill, S.L. (2016). Establishment of a *Wolbachia* superinfection in *Aedes aegypti* mosquitoes as a potential approach for future resistance management. PLoS Pathog. *12*, e1005434.

43. Tadokoro, T., and Kanaya, S. (2009). Ribonuclease H: molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes. FEBS J. *276*, 1482–1493.

44. Asgari, S., Schmidt, O., and Theopold, U. (1997). A polydnavirus-encoded protein of an endoparasitoid wasp is an immune suppressor. J. Gen. Virol. *78*, 3061–3070.

45. Keck, J.L., Goedken, E.R., and Marqusee, S. (1998). Activation/attenuation model for RNase H. A one-metal mechanism with second-metal inhibition. J. Biol. Chem. *273*, 34128–34133.

46. Asad, S., Hussain, M., Hugo, L., Osei-Amo, S., Zhang, G., Watterson, D., and Asgari, S. (2018). Suppression of the pelo protein by *Wolbachia* and its effect on dengue virus in *Aedes aegypti*. PLoS Negl. Trop. Dis. *12*, e0006405.

47. Parry, R., and Asgari, S. (2018). Aedes anphevirus: an insect-specific virus distributed worldwide in *Aedes aegypti* mosquitoes that has complex interplays with *Wolbachia* and dengue virus infection in cells. J. Virol. *92*, e002244-18.

48. Schnettler, E., Sreenu, V.B., Mottram, T., and McFarlane, M. (2016). *Wolbachia* restricts insect specific flavivirus infection in *Aedes aegypti* cells. J. Gen. Virol. *97*, 3024–3029.

49. Zhang, G., Etebari, K., and Asgari, S. (2016). *Wolbachia* suppresses cell fusing agent virus in mosquito cells. J. Gen. Virol. *97*, 3427–3432.

50. Hussain, M., Lu, G., Torres, S., Edmonds, J.H., Kay, B.H., Khromykh, A.A., and Asgari, S. (2013). Effect of *Wolbachia* on replication of West Nile virus in mosquito cell line and adult mosquitoes. J. Virol. *87*, 851–858.

51. Walker, T., Johnson, P.H., Moreira, L.A., Iturbe-Ormaetxe, I., Frentiu, F.D., McMeniman, C.J., Leong, Y.S., Dong, Y., Axford, J., Kriesner, P., et al. (2011). The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature *476*, 450–453.

52. Caragata, E.P., Rancès, E., O'Neill, S.L., and McGraw, E.A. (2014). Competition for amino acids between *Wolbachia* and the mosquito host, *Aedes aegypti*. Microb. Ecol. *67*, 205–218.

53. Amuzu, H.E., and McGraw, E.A. (2016). *Wolbachia*-based dengue virus inhibition is not tissue-specific in *Aedes aegypti*. PLoS Negl. Trop. Dis. *10*, e0005145.

54. Thomas, S., Verma, J., Woolfit, M., and O'Neill, S.L. (2018). *Wolbachia*-mediated virus blocking in mosquito cells is dependent on XRN1-mediated viral RNA degradation and influenced by viral replication rate. PLoS Pathog. *14*, e1006879.

55. Gao, K., Cheng, M., Zuo, X., Lin, J., Hoogewijs, K., Murphy, M.P., Fu, X.-D., and Zhang, X. (2021). Active RNA interference in mitochondria. Cell Res. *31*, 219–228.

56. Lioliou, E., Romilly, C., Romby, P., and Fechter, P. (2010). RNA-mediated regulation in bacteria: from natural to artificial systems. N. Biotechnol. *27*, 222–235.

57. Good, L., and Stach, J.E.M. (2011). Synthetic RNA silencing in bacteria - antimicrobial discovery and resistance breaking. Front. Microbiol. *2*, 185.

58. Jani, S., Ramirez, M.S., and Tolmasky, M.E. (2021). Silencing antibiotic resistance with antisense oligonucleotides. Biomedicines *9*, 416.

59. Ryazansky, S., Kulbachinskiy, A., and Aravin, A.A. (2018). The Expanded universe of prokaryotic Argonaute proteins. mBio *9*, e01935-18.

60. Lisitskaya, L., Shin, Y., Agapov, A., Olina, A., Kropocheva, E., Ryazansky, S., Aravin, A.A., Esyunina, D., Murakami, K.S., and Kulbachinskiy, A. (2022). Programmable RNA targeting by bacterial Argonaute nucleases with unconventional guide binding and cleavage specificity. Nat. Commun. *13*, 4839.

61. Waters, L.S., and Storz, G. (2009). Regulatory RNAs in bacteria. Cell *136*, 615–628.

62. Gong, J.-T., Li, Y., Li, T.-P., Liang, Y., Hu, L., Zhang, D., Zhou, C.-Y., Yang, C., Zhang, X., Zha, S.-S., et al. (2020). Stable introduction of plant-virus-inhibiting *Wolbachia* into planthoppers for rice protection. Curr. Biol. *30*, 4837–4845.e5.

63. Tenorio, R., Fernández de Castro, I., Knowlton, J.J., Zamora, P.F., Sutherland, D.M., Risco, C., and Dermody, T.S. (2019). Function, architecture, and biogenesis of reovirus replication neoorganelles. Viruses *11*, 288.

64. Modhiran, N., Song, H., Liu, L., Bletchly, C., Brillault, L., Amarilla, A.A., Xu, X., Qi, J., Chai, Y., Cheung, S.T.M., et al. (2021). A broadly protective antibody that targets the flavivirus NS1 protein. Science *371*, 190–194.

65. Parry, R., Bishop, C., De Hayr, L., and Asgari, S. (2019). Density-dependent enhanced replication of a densovirus in *Wolbachia*-infected *Aedes* cells is associated with production of piRNAs and higher virus-derived siRNAs. Virology *528*, 89–100.

66. Hussain, M., Frentiu, F.D., Moreira, L.A., O'Neill, S.L., and Asgari, S. (2011). *Wolbachia* utilizes host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector *Aedes aegypti*. Proc. Natl. Acad. Sci. USA *108*, 9250–9255.

67. O'Neill, S.L., Pettigrew, M.M., Sinkins, S.P., Braig, H.R., Andreadis, T.G., and Tesh, R.B. (1997). In vitro cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. Insect Mol. Biol. *6*, 33–39.

68. Beebe, N.W., Pagendam, D., Trewin, B.J., Boomer, A., Bradford, M., Ford, A., Liddington, C., Bondarenco, A., De Barro, P.J., Gilchrist, J., et al. (2021). Releasing incompatible males drives strong suppression across populations of wild and *Wolbachia*-carrying *Aedes aegypti* in Australia. Proc. Natl. Acad. Sci. USA *118*. e2106828118.

69. Frentiu, F.D., Zakir, T., Walker, T., Popovici, J., Pyke, A.T., van den Hurk, A., et al. (2014). Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia*. PLoS Negl. Trop. Dis. *8*, e2688.

70. Bishop, C., Parry, R., and Asgari, S. (2020). Effect of *Wolbachia* wAlbB on a positive-sense RNA negev-like virus: a novel virus persistently infecting *Aedes albopictus* mosquitoes and cells. J. Gen. Virol. *101*, 216–225.

71. Pudney, M., Varma, M.G.R., and Leake, C.J. (1979). Establishment of cell lines from larvae of culicine (*Aedes* species) and anopheline mosquitoes. Tca. Manual *5*, 997–1002.

72. Ridley, A.W., Hereward, J.P., Daglish, G.J., Raghu, S., McCulloch, G.A., and Walter, G.H. (2016). Flight of *Rhyzopertha dominica* (Coleoptera: bostrichidae)—a spatio-temporal analysis with pheromone trapping and population genetics. J. Econ. Entomol. *109*, 2561–2571.

73. Watterson, D., Robinson, J., Chappell, K.J., Butler, M.S., Edwards, D.J., Fry, S.R., Bermingham, I.M., Cooper, M.A., and Young, P.R. (2016). A generic screening platform for inhibitors of virus induced cell fusion using cellular electrical impedance. Sci. Rep. *6*, 22791.

74. Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-wRNase HI	This paper	Anti-*w*RNase HI
Anti-DENV E protein	Modhiran et al., 2021[64]	Anti-DENV E
Anti-human antibody IRDYE 800CW	LI-COR	926–32232; RRID:AB_10806644
Anti-wsp antibody	Asad et al., 2018[46]	Anti-wsp
Anti-GAPDH	Sigma-Aldrich	G9545; RRID:AB_796208
Anti-guinea pig antibody conjugated with alkaline phosphatase	Sigma-Aldrich	A5062; RRID:AB_258221
Anti-polyhistidine conjugated with alkaline phosphatase	Sigma-Aldrich	A5588-.5 ML; RRID:AB_258251
Bacterial and virus strains		
Dengue virus serotype 2 ET-300 strain	GenBank EF440433.1	DENV
Chemicals, peptides, and recombinant proteins		
ethylenediaminetetraacetic Acid	Sigma-Aldrich	E4884
nitro blue tetrazolium chloride	Life Technologies	002209
5-Bromo-4-chloro-3-indolyl phosphate	Life Technologies	002209
Isopropyl ß-D-1-thiogalactopyranoside	ThermoFisher	R1171
Critical commercial assays		
QuantiFast SYBR Green PCR Kit	QIAGEN	217084
Turbo DNA-free kit	Invitrogen	AM1907
MEGAscript T7 transcription kit	Invitrogen	AM1334
Paris kit	ThermoFisher	AM1921
QIAamp viral RNA kit	QIAGEN	52904
RNeasy mini kit from cells	QIAGEN	74104
Experimental models: Cell lines		
Aedes aegypti Aag2.*w*AlbB cells	Parry et al., 2019[65]	Aag2.*w*AlbB
Aedes aegypti Aag2.*w*MelPop cells	Hussain et al., 2011[66]	Aag2.*w*MelPop
Aedes albopictus Aa23 cells infected with *w*AlbB	O'Neill et al., 1997[67]	Aa23
Experimental models: Organisms/strains		
Wolbachia-infected *Aedes aegypti* mosquitoes	Beebe et al., 2021[68]	WB2
Software and algorithms		
GraphPad Prism v 9.4.1	GraphPad	v 9.4.1

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sassan Asgari (s.asgari@uq.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The raw data related to each figure where quantification was performed can be found in the paper's supplemental information (Data S1).
- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mosquitoes

The *Wolbachia* wAlbB infected *A. aegypti* (strain *w*AlbB2-F4) mosquitoes[68] were reared by hatching eggs in 27°C water and feeding larvae daily with fish food (Hikari Cichlid Gold) *ad libitum*. Adults were maintained at 27°C, a 12 h:12h day/night cycle with relative humidity ranging between 65 and 75%, and allowed to feed *ad libitum* on 10% sucrose. All mosquito experiments were performed using female adults.

Mosquito cell lines

Aedes albopictus Aa23 cells persistently infected with *w*AlbB strain of *Wolbachia*,[67] *w*MelPop-infected Aag2 cells (Aag2.*w*MelPop),[69] *w*AlbB-infected Aag2 cells (Aag2.*w*AlbB),[70] and uninfected Aag2 cells were maintained as cell monolayers in flask in a 1:1 mixture of Mitsuhashi–Maramorosch and Schneider's Insect Media (Invitrogen), supplemented with 10% fetal bovine serum (FBS) at 27°C. *A. aegypti* Aa20 cells[71] were maintained in L-15 medium (Invitrogen) supplemented with 10% tryptose phosphate broth and 5% FBS at 27°C. For infection, cells were infected with DENV-2 (ET-300 strain) at the multiplicity of infection (MOI) of 1.

METHOD DETAILS

RNA extraction

Cell pellets collected from various experiments were subjected to total RNA extraction using Qiazol (QIAGEN) according to the manufacturer's instructions. Extracted RNA samples were treated with DNase using Turbo DNase (Ambion) according to the manufacturer's instructions and examined for quantification and quality by Epoch spectrophotometer (BioTek).

Reverse transcription qPCR (RT-qPCR)

RNA samples were reverse transcribed using M-MuLV reverse transcriptase (New England Biolabs) according to the manufacturer's instructions. Synthesized cDNAs were diluted 1:10 in Ultrapure DNase/RNase-free water (Invitrogen) and used in qPCR reactions with a Rotor-Gene Q machine (QIAGEN). PCR amplification was performed according to the instructions of QuantiFast SYBR Green PCR Kit (QIAGEN). qPCR was followed with melt curve analysis for all products. *A. aegypti* ribosomal protein S17 (*RPS17*) gene or *Wolbachia* 16S rRNA gene were used as the reference genes depending on the experiment. Primers used in this study are listed in Table S1. All qPCR experiments were run with at least three biological and two technical replicates.

Cell lysate preparation and incubation with DENV RNA

The cell lysate from Aag2 and Aag2.*w*MelPop cells were prepared by sonicating the cells. The cell lysate was centrifuged at 16,000 g for 5 min to pellet the cell debris, with the supernatant collected as the cytoplasmic fraction. Five hundred µL of the cytoplasmic fraction and 3 µg of DENV RNA were mixed and incubated at 37 °C for 60 min. RNA was extracted from the reactions and subjected to RT-qPCR as above.

Northern blotting

DENV genomic RNA was detected by northern blot analyses using 10 µg total RNA run on 1% agarose formaldehyde gels. DNA probes were labeled with ^{32}P-dCTP using a random primer DNA labeling kit (GE Healthcare) and all hybridization and washing steps were carried out at 65°C. Blots were then exposed to a phosphorimager screen for 2 h and radioactive signals were detected using a Storm phoshorimager scanner (GE Healthcare).

Overexpression of *Wolbachia* ribonucleases in *E. coli*

The full-length *w*RNase HI and *w*RNase III genes (NCBI: CP046921.1) were amplified from *w*MelPop and inserted into the pQE30 vector (QIAGEN) and transformed into *E. coli*. The expression of the proteins was induced by 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG). Samples were collected at 2 and 4 h after induction to confirm overexpression with western blotting using an anti-His antibody conjugated

with alkaline phosphatase (Sigma). The overexpressed proteins were purified with Ni-NTA resin beads from the soluble (RNase HI) and insoluble (RNase III) fractions according to the manufacturer's instructions (QIAGEN). pQE/CrV1 expressing the CrV1 protein from *C. rubecula* was also used as a control and purified similar to *w*RNase HI as CrV1 was previously found to be in the soluble fraction.[44]

Production of ssRNA, dsRNA, and RNA/DNA hybrid, and *in vitro* RNA cleavage

For production of RNA substrates for *w*RNase HI RNase activity, *GFP* gene was used in combination with forward and reverse GFP primers containing T7 promoter sequences. MEGAscript T7 transcription kit (Invitrogen) was used for *in vitro* synthesis of ssRNA (600 nt; using only the reverse primer) and dsRNA (500 nt; using both forward and reverse primers) according to the manufacturer's instructions. To produce GFP RNA/DNA hybrid, GFP ssRNA produced as above was reverse transcribed with M-MuLV reverse transcriptase without RNase H activity according to the manufacturer's instructions (New England Biolabs).

For *in vitro* cleavage assays, 450 ng dsRNA, 1 μg of ssRNA, or 1 μg of RNA/DNA hybrid substrates was incubated with 10 nmol of purified proteins or *E. coli* RNase HI in RNase HI buffer (New England Biolab) at 37°C for 1 h and analyzed on 1.5% agarose gel. To block the RNase HI activity, 50 mM EDTA was added to the reactions.

Wolbachia density

Genomic DNA was extracted from cells with EconoSpin silica membrane columns (Epoch Life Science) using a previously described protocol.[72] The relative densities of *w*AlbB and *w*MelPop in Aag2 cells were quantified by qPCR using specific primers for the relevant strain's *Wolbachia* surface protein (*wsp*) gene and the *A. aegypti RPS17* gene for normalizing the data. Primer sequences can be found in Table S1.

Extraction of DNA and RNA from single mosquitoes

Mosquitoes were ground to fine powder in liquid nitrogen individually in Eppendorf tubes with pestle. The ground material was resuspended thoroughly in 200 μL PBS and divided equally into two tubes for total RNA as well as genomic DNA extractions as described above.

Western blot analysis

Protein samples were run on 12% SDS-PAGE gels followed by western blot transfer using Mini-Cell Module III (BioRad). After blocking the blots with 5% non-fat dry milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h, they were washed three times in TBST. For detection, an anti-His antibody conjugated with alkaline phosphatase (1:5000; Sigma) was used for bacterially expressed proteins, an anti-*w*RNase HI antibody (1:2000) detected by anti-guinea pig antibodies (1:10,000; Sigma) conjugated with alkaline phosphatase for *w*RNase HI protein overexpressed in mosquito cells, polyclonal anti-wsp antibodies[46] (1:2000) detected by anti-rabbit goat antibodies (1:10,000; Sigma) conjugated with alkaline phosphatase to detect the wsp protein, and anti-GAPDH antibodies (1:5000; Sigma) as loading control. The anti-*w*RNase HI antibody was raised in guinea pigs against the peptide (RKDIYGREENTTNNK, *w*RNase HI aa 33–47) at Monash University. Blots with the antibodies were incubated in 1% non-fat dry milk in TBST for 2 h. Blots were then washed with TBST and developed using nitro blue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) reagents (Sigma).

Ectopic expression of *w*RNase HI in Aag2 cells and assessment of replication of DENV and insect-specific viruses

The full-length *wRNase HI* from *w*MelPop was cloned in the mosquito expression vector pSLfa-PUb-MSC (Addgene) under the *A. aegypti* polyubiquitin promoter (pSLfa/RNaseHI). For control, Green Fluorescent Protein (*GFP*) gene was cloned into the same vector (pSLfa/GFP). The cloned inserts were confirmed by Sanger sequencing. Plasmids were transfected into Aag2 cells in 12-well plates using Cellfectin II reagent (Invitrogen) according to the manufacturer's instructions. Cellfectin-only treated cells were used as additional control. Three days after transfections, cells were collected from which total RNA was extracted as above.

To find out if ectopic expression of *w*RNase HI in Aag2 cells has any effect on Cell fusing agent virus (CFAV), a positive sense flavivirus, and Phasi Charoen-like virus (PCLV), a negative sense bunyavirus, RNA was reverse transcribed using reverse primers specific to each virus (Table S1). Subsequently, cDNAs were

subjected to qPCR using forward and reverse primers specific to each virus (Table S1). Aag2 cells are persistently infected with CFAV and PCLV.[48]

We also assessed the effect of *w*RNase HI expression in Aag2 cells on DENV replication using pSLfa/RNase HI. Cells were transfected as above and after two days, they were infected with 1 MOI of DENV. They were then collected at 3 dpi and subjected to RT-qPCR. The effect of *w*RNase HI on DENV replication was also assessed in another *A. aegypti* cell line (Aa20) by overexpressing the gene using the pIZ/V5-His vector using a similar approach as above. Plasmids expressing the *GFP* gene were used as control in both experiments.

Cytoplasmic fraction of *w*RNase HI overexpressing cells and effect on DENV RNA

For overexpression of *w*RNase HI, pSLfa/RNase HI (4 μg/well) and pSLfa/GFP as control were transfected into Aag2 cells in a 6-well plate using Cellfectin II reagent (Invitrogen). Three days after transfections, cells were collected for preparation of cytoplasmic fractions using Paris kit (Thermofisher) according to the manufacturer's instructions. For viral genomic RNA (gRNA) isolation, Aag2 cells were infected with 1 MOI of DENV, and cells as well as supernatants were collected at 7 dpi. Viral RNA was isolated from 200 μL supernatant using QIAamp viral RNA kit (QIAGEN) and total RNA was extracted from cells with RNeasy mini kit from cells (QIAGEN) following the manufacturer's protocols. To find out the effect of overexpressed *w*RNase HI protein on DENV gRNA, 600 ng viral gRNA was mixed with 10 μL of the cytoplasmic fractions and incubated at 37°C for 1 h. Levels of DENV gRNA were analyzed by RT-qPCR as described above.

In vitro synthesis of antisense RNA and *wRNase HI* knockdown in *Wolbachia*

Primers were designed to the sequence of *wRNase HI* to amplify a PCR product for *in vitro* synthesis of antisense RNA (asRNA). While different forward primers were used for *w*MelPop and *w*AlbB *wRNase HI* genes, the same reverse primer was used for both genes due to high sequence similarity (Table S1; see Figure S11 for sequence alignment). *w*MelPop and *w*AlbB *wRNase HI* are 86 and 89% identical at the DNA and amino acid levels, respectively. Forward primer started from −15 position from ATG and reverse primer designed in the coding region ending at +180 nt. T7 promoter sequence was added to the reverse primer (Table S1). Similarly, for the control asRNA, primers were designed to amplify the first 200 bp of the GFP sequence. To make asRNA to the *wsp* gene, a similar strategy was adopted by amplifying a DNA fragment covering nucleotides from −11 position from ATG to +200 nt in the coding region. MEGAscript T7 transcription kit (Invitrogen) was used for *in vitro* synthesis of asRNAs according to the manufacturer's instructions.

To knockdown *wRNase HI* in *Wolbachia in vitro*, about 0.6 million Aag2.*w*MelPop cells in three replicates were transfected with 2 μg of asRNA. Control cells were transfected with asGFP. Cellfectin II transfection reagent was used for transfection according to the manufacturer's instructions (Invitrogen).

To knockdown *w*RNase HI in mosquitoes, the *Wolbachia w*AlbB-infected *A. aegypti* mosquitoes, strain *w*AlbB2-F4,[64] were used in this study. Mosquitoes were maintained at 27°C with about 75% humidity on 10% sugar solution. Thirty 2-day-old female mosquitoes per treatment were chilled on ice and subsequently intrathoracically injected with 200 nL of asRNAs (1 μg) to *wRNase HI* or *GFP*, or only *Aedes* physiological solution (APS; 150 mM sodium chloride, 4 mM potassium chloride, 0.1 mM sodium bicarbonate, 0.6 mM magnesium chloride, 1.7 mM calcium chloride, 25 mM HEPES Buffer at a pH of 7.0). A Nanoject III (Drummond) and pulled glass needles were used for injections. Two days after injection, mosquitoes were fed on human blood donated by Red Cross (ethics approval UQ 2016000870) containing 1×10^7/mL DENV-2 (ET-300 strain) using glass feeders. Those mosquitoes that did not take a blood meal were discarded and the remaining were maintained for four days as above. Subsequently, total RNA was extracted from individual mosquitoes and subjected to RT-qPCR.

Focus forming assay

To titrate DENV virions, focus forming assay was conducted according to a previously described method[73] with slight modifications by infecting C6/36 cells in 96-well plates with serial dilutions of media collected from experiments in triplicates. For infection, 10 μL/well of supernatants collected from DENV-infected Aag2 (*w*RNase HI ectopic expressing) or Aag2.*w*MelPop (knocked down *w*RNase HI) cell lines were used. Plates were first incubated at room temperature on a rocker for 1 h and then incubated at 37°C for an extra hour. At 3 dpi, cells were fixed in ice-cold 80% acetone in PBS for 20 min at −20°C, and then dried overnight. Then cells were blocked with 5% skimmed milk in PBST at 37°C for 30 min. This was followed by incubating cells with antisera specific to DENV-2 E protein, 4E11 (1:1000) in 0.1% skimmed milk in PBST for

2 h at 37°C. After that, plates were washed three times with PBST followed by probing with secondary antibody IRDYE 800CW Goat anti-Human (1:2500) for 1 h at 37°C. Plates were washed three times with PBST and dried as above and scanned for foci detection and counting by LI-COR Biosciences Odyssey infrared Imaging System according to manual instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism version 9 was used for all the statistical analyses and production of the graphs. Data were tested for normality using Shapiro-Wilk test and those that passed the test were considered for analysis. Non-parametric tests were used to determine significance levels between treatments for comparing two treatments (Mann-Whitney test) or more (Kruskal-Wallis) when samples sizes were smaller than six. One-way ANOVA was used to determine significance levels between three or more treatments when sample sizes were six or more. More details are provided in the relevant figure legends. qPCR data were analyzed using the relative expression ratio method (Ratio = $(E_{target})^{\Delta CP}{}_{target(control - sample)}/(E_{ref})^{\Delta CP}{}_{ref(control - sample)}$) as described previously.[74] Gene expression levels or DENV gRNA levels in controls were adjusted to 1 and the transcript levels in treatments are expressed as fold changes relative to the controls. The raw data related to each figure where quantification was performed can be found in the Data S1 file.