AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VIRUS-CELL INTERACTIONS

Variable Inhibition of Zika Virus Replication by Different *Wolbachia* Strains in Mosquito Cell Cultures

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ABSTRACT Mosquito-borne arboviruses are a major source of human disease. One strategy to reduce arbovirus disease is to reduce the mosquito's ability to transmit virus. Mosquito infection with the bacterial endosymbiont *Wolbachia pipientis w*Mel is a novel strategy to reduce *Aedes* mosquito competency for flavivirus infection. However, experiments investigating cyclic environmental temperatures have shown a reduction in maternal transmission of *w*Mel, potentially weakening the integration of this strain into a mosquito population relative to that of other *Wolbachia* strains. Consequently, it is important to investigate additional *Wolbachia* strains. All Zika virus (ZIKV) suppression studies are limited to the *w*Mel *Wolbachia* strain. Here we show ZIKV inhibition by two different *Wolbachia* strains: *w*AlbB (isolated from *Aedes albopictus* mosquitoes) and *w*Stri (isolated from the planthopper *Laodelphax striatellus*) in mosquito cells. *Wolbachia* strain *w*Stri inhibited ZIKV most effectively. Single-cycle infection experiments showed that ZIKV RNA replication and nonstructural protein 5 translation were reduced below the limits of detection in *w*Stri-containing cells, demonstrating early inhibition of virus replication. ZIKV replication was rescued when *Wolbachia* was inhibited with a bacteriostatic antibiotic. We observed a partial rescue of ZIKV growth when *Wolbachia*-infected cells were supplemented with cholesterol-lipid concentrate, suggesting competition for nutrients as one of the possible mechanisms of *Wolbachia* inhibition of ZIKV. Our data show that *w*AlbB and *w*Stri infection causes inhibition of ZIKV, making them attractive candidates for further *in vitro* mechanistic and *in vivo* studies and future vector-centered approaches to limit ZIKV infection and spread.

IMPORTANCE Zika virus (ZIKV) has swiftly spread throughout most of the Western Hemisphere. This is due in large part to its replication in and spread by a mosquito vector host. There is an urgent need for approaches that limit ZIKV replication in mosquitoes. One exciting approach for this is to use a bacterial endosymbiont called *Wolbachia* that can populate mosquito cells and inhibit ZIKV replication. Here we show that two different strains of *Wolbachia*, *w*AlbB and *w*Stri, are effective at repressing ZIKV in mosquito cell lines. Repression of virus growth is through the inhibition of an early stage of infection and requires actively replicating *Wolbachia*. Our findings further the understanding of *Wolbachia* viral inhibition and provide novel tools that can be used in an effort to limit ZIKV replication in the mosquito vector, thereby interrupting the transmission and spread of the virus.

KEYWORDS *Wolbachia*, Zika virus, arthropod vectors, vector biology

Received 2 March 2017 **Accepted** 10 April 2017

Accepted manuscript posted online 26 April 2017

Citation Schultz MJ, Isern S, Michael SF, Corley RB, Connor JH, Frydman HM. 2017. Variable inhibition of Zika virus replication by different *Wolbachia* strains in mosquito cell cultures. J Virol 91:e00339-17. https://doi.org/10.1128/JVI.00339-17.

Editor Michael S. Diamond, Washington University School of Medicine

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Arboviruses are zoonotic viruses transmitted by arthropods. A large number of arboviruses are human pathogens, including dengue virus (DENV), West Nile virus (WNV), Chikungunya virus (CHIKV), and yellow fever virus (YFV). Among arboviruses that cause human disease, 90% are transmitted by mosquitoes (1). Mosquitoes are an effective vector for rapidly disseminating arboviral diseases around the globe. This has been evidenced by the spread of DENV, WNV, and CHIKV into the Western Hemisphere. Zika virus (ZIKV) is the fourth arbovirus to cause a Western Hemisphere epidemic in the last 20 years (2). Trying to control the spread of these diseases through control of the mosquito vector has long been attempted, with mixed results. Traditional attempts to suppress arboviruses include mosquito population control through insecticides (1, 3). There have been many successes, but eradication is limited by the mosquito's resistance to insecticides and widespread population dynamics (3). The irregular success of insecticide-based arbovirus eradication strategies has highlighted the need for alternative strategies.

One promising alternative strategy is to limit arbovirus transmission and spread through the use of endosymbiotic bacteria, such as *Wolbachia pipientis* (4). *Wolbachia* organisms are obligate intracellular bacterial endosymbionts of arthropods and nematodes. *Wolbachia* bacteria are maternally transmitted and affect host reproductive phenotypes. This allows efficient integration into a population (5, 6). As a result, it is estimated that up to 40% of all insects are infected with diverse strains of *Wolbachia* (7). *Wolbachia* strains which have been investigated for the ability to inhibit arboviruses span two major phylogenetic clades (supergroup A and supergroup B) (8). *Wolbachia* strains from both clades cultured in *Aedes* mosquito cells have been shown to inhibit the replication of viral pathogens (9–14).

Wolbachia-infected *Aedes* mosquitoes have a strong resistance to infection with various arboviruses. *w*MelPop is a pathogenic strain of *Wolbachia* native to *Drosophila melanogaster* which broadly inhibits DENV (11), CHIKV (11, 15), YFV (15), and WNV (12). However, the extreme density of *w*MelPop has too large of a fitness cost to the mosquito to successfully integrate into the mosquito population (13). This led to the investigation of other *Wolbachia* strains that do not overgrow in the mosquito host.

Wolbachia strain *w*Mel, also indigenous to *D. melanogaster*, does not have the fitness costs incurred by *w*MelPop (6, 16, 17). Nevertheless, introduction of *w*Mel into the *Aedes* mosquito host (10, 16, 18) also limits DENV (10, 19, 20), ZIKV (21, 22), and CHIKV (23) infections. *w*Mel's lower density causes a limited reduction in fitness of the *Aedes* host, but the strain is less effective at reducing viral titers than *w*MelPop (10, 15, 16, 18). Field trials investigating the integration of *w*Mel and suppression of DENV are currently being conducted (13, 20, 24). While some models predict successful control of arboviruses by *Wolbachia* strain *w*Mel (6), concerns have been raised that over time, ongoing evolutionary adaptations of *Wolbachia*/vector/virus interactions may undermine the long-term effectiveness of *w*Mel to control arboviruses (25). For example, recent studies suggest that *w*Mel may struggle to integrate into large mosquito populations (26). This is due to a loss of maternal transmission and density and a reduced ability to induce cytoplasmic compatibility at tropical cyclic temperatures simulated in laboratory experiments (26). As a result, strategies employing alternative *Wolbachia* strains, which effectively reduce viral titers without large host fitness costs, have been suggested to improve the efforts of a *Wolbachia*-mediated suppression strategy (27).

In this study, we investigated two *Wolbachia* strains belonging to supergroup B, *w*AlbB and *w*Stri, to determine if they effectively inhibit ZIKV *in vitro*. *w*AlbB has been reported to have opposing phenotypes for different viruses in different mosquito hosts (28–31), making it an interesting *Wolbachia* strain for study. *w*Stri of the leafhopper *Laodelphax striatella* has also been established in *Aedes albopictus* culture (32), making it potentially useful for future vector suppression approaches, yet it has never been studied in the context of arboviruses. Our results show that both *Wolbachia* strains inhibit ZIKV in *A. albopictus* cells. Our data for *w*Stri-infected cells demonstrate the early inhibition of virus infection prior to or at transcription and translation of virus. We also provide evidence that competition for cholesterol and/or other lipids plays a partial role

in the suppression of viral infection in *Aedes* cells harboring the endosymbiont *Wolbachia*. We conclude that *w*Stri and *w*AlbB are effective inhibitors of ZIKV.

RESULTS

Phylogenetically distinct *Wolbachia* strains, *w*Stri and *w*AlbB, successfully established in mosquito cell cultures inhibit ZIKV *in vitro*. Previous work showed that *Wolbachia* strain *w*Mel, belonging to supergroup A (33), inhibits ZIKV *in vivo* (21, 22). To increase the repertoire of *Wolbachia* strains available for ZIKV control and to develop an *in vitro* system amenable to high-throughput approaches, we investigated whether the *w*AlbB and *w*Stri strains of *Wolbachia* were capable of restricting ZIKV infection in mosquito cells. These strains are phylogenetically distant from the *w*Mel and *w*MelPop strains (Fig. 1A). *w*Stri and *w*AlbB form a clade with *w*Pip, consistent with previous reports defining each of these strains as group B *Wolbachia* strains (34) (Fig. 1A). Because *w*AlbB and *w*Stri are adapted to *Aedes* cell culture, we investigated them further to determine if they are candidates for ZIKV control.

While no investigations have pursued *w*Stri, *w*AlbB has been shown to inhibit DENV, a relative of ZIKV, in *Aedes* mosquitoes (27). Group B *Wolbachia* strains have been shown to have an inhibitory effect on DENV growth in *A. albopictus* (14, 24). Thus, we hypothesized *w*AlbB and *w*Stri may inhibit ZIKV. To investigate this hypothesis, we utilized *A. albopictus* cells with persistent *Wolbachia* infection. Aa23 *w*AlbB-infected cells and their respective *Wolbachia*-free cell line (Aa23 W^-), as well as C/*w*Stri-infected cells and their respective *Wolbachia*-free line (C710 W^-), were infected with ZIKV at a low multiplicity of infection (MOI), i.e., 0.01. After 5 days of incubation, titers were determined by focus forming assay (FFA). *Wolbachia* strain *w*AlbB significantly inhibited titers of African strain ZIKV MR766, by approximately 1 log (93%), from a mean of 3.5×10^5 to 2.4×10^4 focus-forming units (FFU) per ml (Fig. 1B). *w*AlbB-containing mosquito cells were also resistant to infection with a clinical isolate of the Asian lineage of ZIKV. Puerto Rican strain PRVABC59, isolated in 2016, produced fewer infectious virions in *w*AlbB-infected cells than in *w*AlbB-free cells, from 7.6×10^7 to 4.4×10^3 FFU/ml (Fig. 1C). Cells infected with *Wolbachia* strain *w*Stri also showed significantly less replication of ZIKV MR766, with a titer from *w*Stri-free cells of 2.0×10^5, compared to a titer of 7.3×10^1 from cells containing *w*Stri (Fig. 1B). There was a similar decrease in replication of ZIKV PRVABC, from 7.8×10^5 in *w*Stri-free cells to 4.9×10^1 in cells containing *w*Stri (Fig. 1C). Three independent experiments showed ZIKV titers in *w*Stri-infected cells of $\sim 10^2$, representing repression close to or at the limit of detection in this assay. *w*Stri consistently reduced titers below the limit of detection, while *w*AlbB cells always grew low levels of virus (Fig. 1B and C).

We confirmed these findings by quantitative reverse transcription-PCR (qRT-PCR) of cells to determine the production of viral genome copies. We first looked at viral genome copies by infecting *Wolbachia*-free and *Wolbachia*-infected cells with ZIKV at a low multiplicity of infection (MOI = 0.01). After 5 days of incubation, cell lysates were collected and analyzed by quantitative RT-PCR. ZIKV MR766 was only nonsignificantly reduced, from a cycle threshold (CT) of ~19 in *Wolbachia*-free Aa23 cells to a CT of ~22 in Aa23 cells infected with *w*AlbB, consistent with the 1-log reduction observed by focus forming assay (Fig. 1B and D). ZIKV MR766 RNA was significantly reduced, from a CT of ~17 in *Wolbachia*-free C710 cells to a CT of 27 or equivalent to that of the no-template control (termed undetectable) in C/*w*Stri cells (Fig. 1D). ZIKV MR766 genome copies were significantly restricted in C/*w*Stri cells compared to C710 W^- cells, consistent with the reduction observed by focus forming assay (Fig. 1B and D). *Wolbachia* *w*AlbB and *w*Stri had a robust inhibitory effect on ZIKV PRVABC59 (Fig. 1E). ZIKV PRVABC59 RNA was significantly reduced, from a CT of ~17 in Aa23 cells to a CT of ~25 in *w*AlbB-infected cells. Likewise, a reduction of CT of ~16 in C710 cells to CT of 29 or undetectable in C/*w*Stri cells was observed (Fig. 1E). Both *w*AlbB-infected cells and *w*Stri-infected cells showed significantly reduced ZIKV PRVABC59 viral genome copies, to below the limit of detection, showing a viral-strain-specific effect (Fig. 1C and

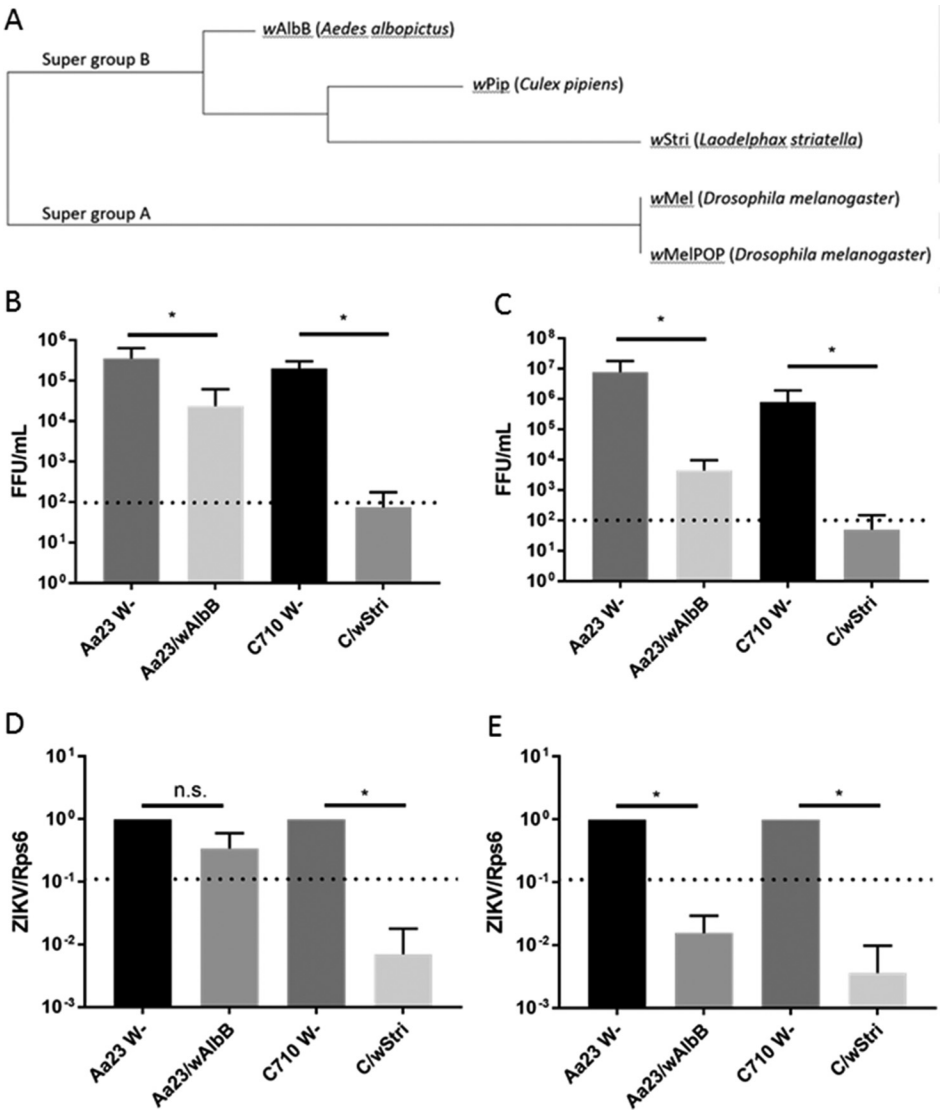

FIG 1 Phylogenetically distinct *Wolbachia* strains, *w*Stri and *w*AlbB, significantly inhibit ZIKV production of infectious virions in *A. albopictus* cells. (A) Phylogenetic analysis was performed on five concatenated multilocus sequence typing genes (*coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*) (33) by MUSCLE alignment and the Tamura-Nei model to produce a maximum likelihood tree in MEGA6 (48). (B) *A. albopictus* cells (Aa23 W^- and C710 W^-) produced $>10^5$ infectious units/ml after initial infection at an MOI of 0.01. *Wolbachia* strain *w*AlbB significantly reduced ZIKV MR766 in each experiment ($P < 0.013$ for each experiment). *Wolbachia* strain *w*Stri inhibited ZIKV MR766 in each experiment ($P < 0.016$ for each experiment). Statistical significance was determined using the Holm-Sidak method, with an alpha value of 0.05. Each experiment was analyzed individually, without assuming a consistent standard deviation. Statistical tests were calculated by GraphPad Prism. Data shown are means and standard deviations of three independent experiments with a minimum of two technical replicates each. (C) *Wolbachia* strain *w*AlbB significantly reduced ZIKV PRVABC FFU in each experiment ($P < 0.05$ for each experiment). *Wolbachia* strain *w*Stri significantly inhibited ZIKV PRVABC in each experiment ($P < 0.01$ for each experiment). Statistical significance was determined using the Holm-Sidak method, with an alpha value of 0.05. Each experiment was analyzed individually, without assuming a consistent standard deviation. Statistical tests were calculated by GraphPad Prism. Data shown are means and standard deviations from three independent experiments with a minimum of two technical replicates each. The dotted line represents the limit of detection. (D) After infection at an MOI of 0.01, cells were incubated for 5 days. Cells were assayed for viral genome by qRT-PCR. The limit of detection was determined based on a no-input control. *w*AlbB did not significantly reduce ZIKV MR766 viral genome copies ($P > 0.05$). *w*Stri reduces ZIKV MR766 genome copies to undetectable levels ($P < 0.05$ for C710 compared to C/*w*Stri). Statistical significance was determined by a ratio-paired *t* test. Statistics were calculated on the collective of three independent experiments. Statistical tests were calculated by GraphPad Prism. Data are means from three independent experiments with no less than two technical replicates each. n.s., not significant. (E) After infection with PRVABC59 at an MOI of 0.01, cells were incubated for 5 days and assayed for viral genome by qRT-PCR. *w*AlbB and *w*Stri significantly reduced ZIKV PRVABC59 below the limit of detection (dotted line) determined by a no input control ($P < 0.05$ for both strains compared to their respective *Wolbachia*-free lines). Statistical significance was determined by a ratio-paired *t* test. Statistics were calculated on the collective of three independent experiments. Statistical tests were calculated by GraphPad Prism. Data are means from three independent experiments with no less than two technical replicates each. *, $P < 0.05$.

TABLE 1 Frequency of *Wolbachia* infections in cell lines[a]

Cell line	No. of cells infected/total (%) as determined with:	
	Probe 1	Probe 2
C/*w*Stri	125/129 (96.9)	48/51 (94.1)
Aa23 *w*ALbB	112/114 (98.2)	161/162 (99.4)

[a]Using the DIC channel to delineate cell membranes, cells were counted and recorded as *Wolbachia* infected or *Wolbachia* free to determine the frequency of *Wolbachia* infection in each cell line. Two different *Wolbachia* 16S rRNA probes were used to confirm the reliability of this technique across two independent experiments.

E). Overall, the data suggest that *w*Stri is more effective at reducing ZIKV MR766 and PRVABC59 titers than *w*AlbB.

***Wolbachia* *w*AlbB and *w*Stri cell lines are infected at similar frequencies yet different densities.** To better characterize the *Wolbachia* *w*AlbB- and *w*Stri-infected cells, we determined the frequency of *Wolbachia* infection in each cell line by imaging. Fluorescent *in situ* hybridization by two different probes showed that each *Wolbachia* strain infected 94% or more cells in each cell line (Table 1; Fig. 2A). *Wolbachia* density has been demonstrated to be a determinant of DENV inhibition (35). Thus, we hypothesized that the stronger inhibition by *w*Stri might be attributed to the higher density of this *Wolbachia* strain than of *w*AlbB in cells. We quantitated *Wolbachia* density by Hsp60 band intensity (Fig. 2B and C). *w*AlbB-infected cells showed a density 2 to 3 times lower than that of *w*Stri-infected cells (Fig. 2C). *Wolbachia* density in a host is prede-

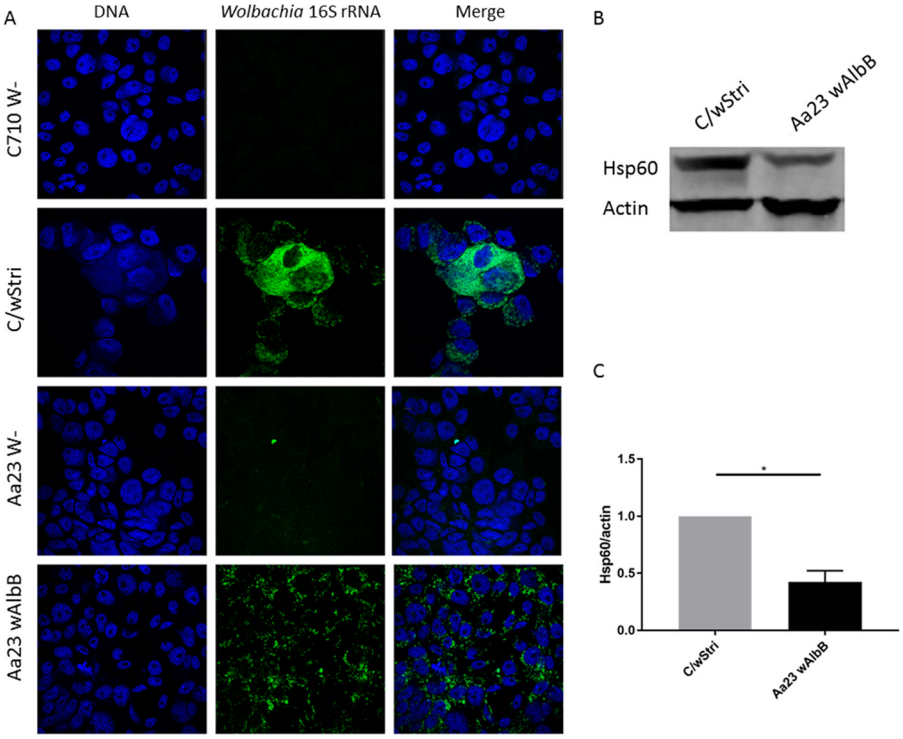

FIG 2 *Wolbachia* *w*AlbB and *w*Stri lines are infected at similar frequencies but different densities. (A) Fluorescent *in situ* hybridization of Aa23 cells with and without *w*AlbB and C710 cells with and without *w*Stri. Using the differential interference contrast (DIC) channel to delineate cell barriers, cells were counted and recorded as *Wolbachia* infected or *Wolbachia* free to determine the frequency of *Wolbachia* infection in each cell line. Data are recorded in Table 1. (B) Western blot to quantitate *Wolbachia* density (Hsp60) relative to host (actin) proteins. (C) Hsp60 normalized to actin band intensity was quantified for three independent experiments to compare *w*Stri density to *w*AlbB density in *A. albopictus* cells (significance, $P < 0.05$). *w*Stri density is normalized to 1 for each experiment to compare *w*AlbB density. Statistical significance was determined by paired *t* test (one-tailed; alpha = 0.05) on the natural log of the (Hsp60/actin) ratio accounting for nonnormal distribution of fluorescent intensities. Statistical tests were calculated by GraphPad Prism.

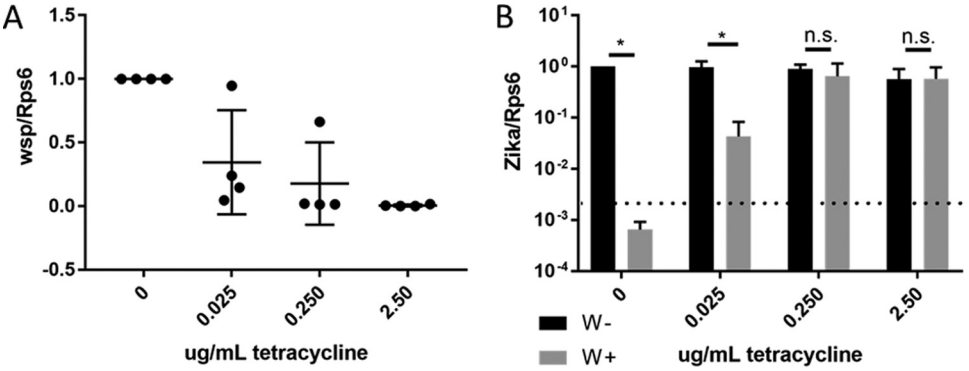

FIG 3 Removal of *Wolbachia* from C/*w*Stri cells rescues ZIKV growth. (A) Tetracycline dose response of *Wolbachia* as determined by *Wolbachia* surface protein, quantified by qRT-PCR. *Wolbachia* concentration was normalized to nontreatment conditions. Data shown are means from three independent experiments with no less than two technical replicates each. (B) Tetracycline dose response of C/*w*Stri cells treated with tetracycline after infection with ZIKV PRVABC. ZIKV infection is significantly reduced in *w*Stri-infected cells at 0 and 0.025 μg/ml of tetracycline ($P < 0.001$ and $P < 0.005$, respectively). At tetracycline concentrations of 0.25 μg/ml and 2.5 μg/ml, ZIKV infection is not significantly different from W- comparable treatment ($P > 0.05$), demonstrating a *Wolbachia*-specific inhibition in C/*w*Stri cells. Statistical significance was independently calculated by Student's *t* test to determine if ZIKV was reduced in *Wolbachia*-infected cells under each dose of tetracycline. Discovery was determined using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli, with a Q value of 1%. Statistical tests were calculated by GraphPad Prism. Each tetracycline concentration was analyzed individually, without assuming a consistent standard deviation. Data were normalized to C710 W^{-} (no treatment). Data are the means and standard deviations from three independent experiments.

termined by the genetics of the *Wolbachia* strain (36). We therefore hypothesize that *Wolbachia* strain density caused by strain-specific interactions with their host confers an antiviral phenotype. Reduction of *w*Stri density may reduce viral protection, while increasing *w*AlbB density may increase the antiviral phenotype to that observed in *w*Stri-infected cells.

Tetracycline treatment reduces *Wolbachia* titer, rescuing ZIKV growth. To further investigate the role of *Wolbachia*, we asked if the rescue of ZIKV growth is dependent on the density of *Wolbachia* present in ZIKV-challenged cells. Treatment of *w*Stri-containing cells with increasing concentrations of tetracycline demonstrated a reduction in *Wolbachia* titers from a mean of six copies of *Wolbachia* surface protein (*wsp*) per host ribosomal protein (Rps6) to less than one copy of *wsp* per 20 copies of Rps6 (Fig. 3A). When these cells were infected with ZIKV, there was an inverse dose-dependent increase in ZIKV replication rescue (Fig. 3A and B). ZIKV RNA copies in C/*w*Stri cells treated with tetracycline increased from undetectable concentrations to CTs of 18 to 22, similar to the *Wolbachia*-free C710 control CT of 20 to 22 (Fig. 3B). Thus, ZIKV inhibition in the C/*w*Stri line is due to the presence of *Wolbachia* directly, rather than a result of epigenetic or genetic changes caused by *Wolbachia* that altered the cells' permissiveness.

***Wolbachia w*Stri inhibits ZIKV early in viral infection.** We next wanted to investigate the stage of viral infection which is blocked by *Wolbachia* to gain mechanistic insight. Toward this goal, we chose the strain with the most robust effect on virus growth, *w*Stri. We divided the virus life cycle into two phases to investigate a stage of viral inhibition: (i) viral entry, transcription, and translation and (ii) assembly and release of mature virions (Fig. 4A). The previous results demonstrated *Wolbachia* inhibition of the production of viral genome and infectious particles at a low multiplicity of infection (MOI = 0.01) (Fig. 1). To determine if C/*w*Stri cells were highly resistant to ZIKV infection, we next infected cells at a high multiplicity of infection (MOI = 10) and assayed the production of infectious units by FFA. We found a consistent significant reduction in FFU in C/*w*Stri cells compared to C710 cells ($P < 0.05$) (Fig. 4B). We investigated differences in virus production upstream of the production of infectious particles to determine if there is an early block of virus production by infecting C710 and C/*w*Stri cells with ZIKV PRVABC59 at a high multiplicity of infection (MOI =10) and

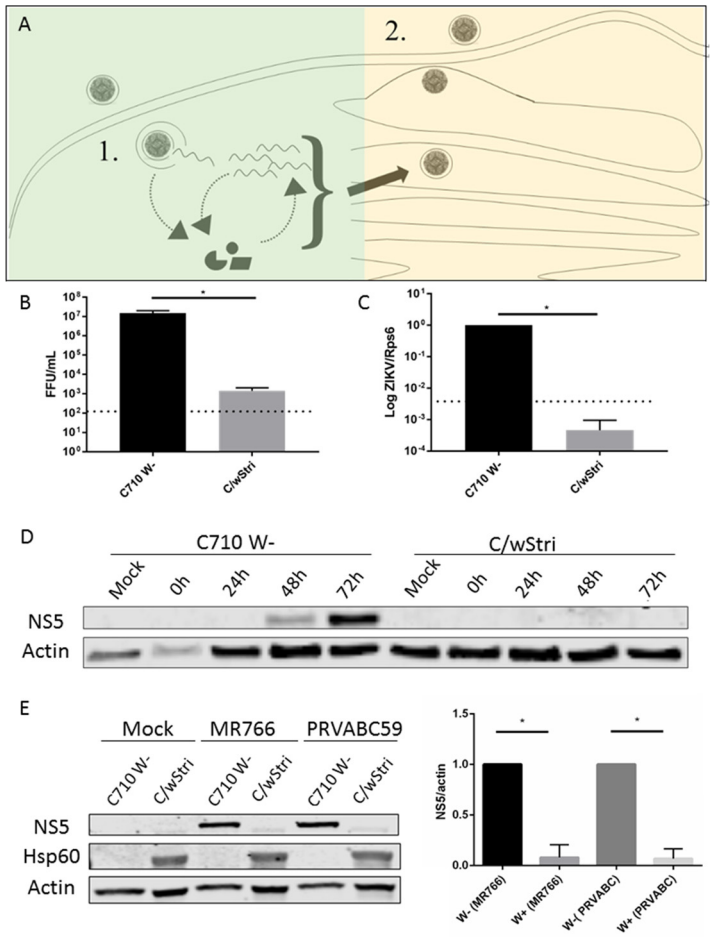

FIG 4 *w*Stri inhibits ZIKV early in virus infection. (A) Isolation of ZIKV life cycle: viral entry, transcription, and translation (1) and assembly and release of mature virions (2). Quantitative RT-PCR and Western blotting were used to assess early infection. Focus forming assay assessed late infection. (B) Infectious virus was assayed by focus forming assay 3 days postinfection at a high multiplicity of infection (MOI = 10) ($P < 0.05$). Statistical significance was determined by a paired *t* test. Statistics were calculated on the collective of three independent experiments. Statistical tests were calculated by GraphPad Prism. (C) After infection at an MOI of 10, cells were incubated for 3 days. Cells were assayed for viral genome by qRT-PCR. *w*Stri reduces ZIKV PRVABC59 genome copies to undetectable levels ($P < 0.05$). Statistical significance was determined by a ratio-paired *t* test on the collective of three independent experiments calculated by GraphPad Prism. Data are means from three independent experiments with no less than two technical replicates each. (D) C710 and C/*w*Stri cells were assayed for NS5 production by Western blotting 1, 2, and 3 days postinfection at MOI = 10. (E) ZIKV MR766 and PRVABC NS5 expression is significantly reduced following a high multiplicity of infection (MOI = 10) in *w*Stri-infected cells ($P < 0.05$ for both ZIKV strains). Statistical significance was determined by a one-tailed ratio-paired *t* test under the assumption that samples are from a population where the log of the ratios follows a Gaussian distribution. Statistics were calculated on the collective of three independent experiments. Statistical tests were calculated by GraphPad Prism. Data are means and standard deviations from three independent experiments.

analyzing viral genome copies by qRT-PCR 3 days postinfection. We observed a consistent significant reduction in viral genome copies, from a CT of ~14 in C710 cells to a CT of ~26 in C/*w*Stri cells (Fig. 4C).

Viral RNA is replicated by nonstructural protein 5 (NS5), a viral protein not packaged in the infectious virion. We assessed early virus infection by analyzing NS5 translation by Western blotting. Cells were infected with ZIKV PRVABC59 at a high MOI (MOI = 10), restricting our analysis to fewer rounds of virus amplification, and collected after 1, 2, and 3 days. C/*w*Stri cells infected with ZIKV MR766 translated undetectable amounts of NS5 at all time points (Fig. 4D). *Wolbachia*-free C710 cells produced significantly more NS5, with the most robust signal at 3 days postinfection (Fig. 4D). We repeated this

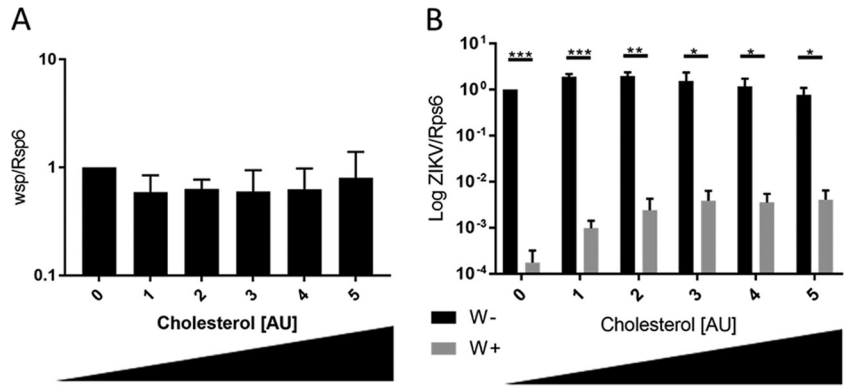

FIG 5 Addition of cholesterol-lipid supplement partially rescues ZIKV growth in C/*w*Stri cells. (A) *Wolbachia* density quantitated by *wsp* is unchanged by the addition of cholesterol concentrate. Data are normalized to no-treatment control. (B) ZIKV RNA is partially rescued in *w*Stri-infected cells relative to *Wolbachia*-free (C710) cells. Statistical significance was determined using the Holm-Sidak method, with an alpha value of 0.05. Each experiment was analyzed individually, without assuming a consistent standard deviation. Statistical tests were calculated by GraphPad Prism. Data are the means and standard deviations from three independent experiments with a minimum of two technical replicates each.

analysis with both ZIKV strains MR766 and PRVABC59 for three independent experiments to quantitate ZIKV NS5 in C710 and C/*w*Stri cells. Consistent with the time course, ZIKV NS5 was significantly reduced in C/*w*Stri cells (Fig. 4E). Hsp60 indicated *Wolbachia* infection in the C710 cells (Fig. 4E). In conclusion, *Wolbachia w*Stri inhibits ZIKV at or prior to translation and genome replication.

Addition of cholesterol-lipid supplement partially rescues ZIKV growth in C/*w*Stri cells. *Wolbachia* inhibition of *Drosophila* C virus (DCV) has been shown to be mediated by competition for cholesterol (37). When cholesterol is added to the flies' diet, DCV grows to lethal titers in the presence of *Wolbachia* (37). Cholesterol is important for several steps in flavivirus infection, including entry early in infection (38). To determine if the *Drosophila* observed phenotype translates to a human pathogen, we investigated if the addition of cholesterol would rescue ZIKV growth in *Wolbachia*. We infected cells at an MOI of 0.01 with ZIKV PRVABC59 for 1 h. A cholesterol-lipid supplement designed for cell culture was added in increasing concentrations. After 6 days, we observed no change in *Wolbachia* density quantitated by qRT-PCR (Fig. 5A). However, ZIKV RNA was increased $>$1 log with the addition of the cholesterol-lipid solution (Fig. 5B). This result strongly implies a role for the competition for cholesterol and/or other lipids in limiting ZIKV infection in cells as a result of persistent *Wolbachia* infection.

DISCUSSION

The data presented here show that ZIKV replication is compromised by two different *Wolbachia* strains. *w*AlbB reduced virus growth by 1 to 3 logs. *w*Stri was more effective, consistently ablating growth below detection. This could be due to the different cell types the *Wolbachia* strains are transinfected into, C710 and Aa23. However, C710 and Aa23 cells are equally permissive to ZIKV growth, suggesting a *Wolbachia* strain-specific phenotype. This is to our knowledge the first report of *w*Stri inhibition of any flavivirus.

An alternative hypothesis for the weaker inhibition by *w*AlbB is that *w*AlbB is native to *A. albopictus*. Nonnative *Wolbachia* strains have been reported to be more effective at pathogen inhibition than native *Wolbachia* strains in the native host. *w*AlbB restricts DENV dissemination in its native host, *A. albopictus* (29, 39), albeit to a lesser extent than *w*AlbB restricts DENV in *A. aegypti*, a nonnative host (27). Consistent with this hypothesis, transinfection of nonnative *w*Mel into *A. albopictus* or *A. aegypti* induces a strong antiviral phenotype (17). Our data show a similar trend such that *w*Stri, a *Wolbachia* strain nonnative to *A. albopictus*, is more effective at reducing ZIKV titers than *w*AlbB, a native *Wolbachia* endosymbiont of *A. albopictus*.

Differences in the native versus nonnative virus growth phenotype trends with

different *Wolbachia* densities. *w*AlbB inhibition of DENV has been shown to be dependent on *Wolbachia* titer (35). *w*AlbB grows to a higher per-cell density in nonnative *Aedes aegypti* hosts and lower densities in its native *A. albopictus* host (35). We observed reduced titers of *w*AlbB relative to *w*Stri in *A. albopictus*, consistent with a density-dependent phenotype. Higher densities, like that demonstrated by *w*MelPop (24) than for *w*Mel, are more effective at reducing viral titers. Too high a *Wolbachia* titer comes with a fitness cost (13). It is important to next investigate if *w*Stri reduces ZIKV *in vivo* and with limited transinfection fitness cost.

Mechanism of action. The stage of flavivirus life cycle that is inhibited in *Wolbachia*-infected cells has not been identified. Our studies suggest that *Wolbachia* restriction of ZIKV occurs at an early step of infection. It is likely that many flaviviruses are restricted at this step based on flavivirus similarities. One study suggested that the alphavirus Semliki Forest virus is inhibited by *Wolbachia* early in viral inhibition (40), consistent with our data. Thus, *Wolbachia* may block many positive RNA viruses by the same mechanism.

Previous investigations into a mechanism of *Wolbachia*-mediated virus suppression have focused on molecular pathways in the immune system (41, 42) and on metabolic competition (37). We chose to look at the impact of cholesterol on virus growth in *Wolbachia*-infected cells because *Wolbachia* bacteria have been shown to compete with the host for cholesterol, lipids, and amino acids (43, 44). *Wolbachia* protection from *Drosophila* C virus is dependent on cholesterol in *Drosophila* (37). We found an increase in ZIKV growth in *Wolbachia*-infected cells but not *Wolbachia*-free cells when cholesterol-lipid concentrate was added to cell media.

This suggests a partial dependence on cholesterol and/or other lipids in *Wolbachia*-mediated virus inhibition. This competition may play a role in inhibiting viral entry since increased cholesterol has a strong impact on virus entry (45). However, additional mechanisms must also contribute to viral inhibition.

***In vitro* studies.** Cell lines offer a valuable tool to dissect molecular aspects of virus-host interactions. While C6/36 mosquito cells are permissive to ZIKV, other mosquito cell lines, such as CCL-125s, do not allow ZIKV growth (46). Often ZIKV is propagated in C6/36 cell lines, because they are defective in the antiviral RNA interference (RNAi) response (47). However, this also renders them limited for vector competence and RNAi screens. Here we demonstrate that two additional insect cell lines, Aa23 and C710, are competent for ZIKV. The availability of a cell line system to investigate *Wolbachia* ZIKV suppression offers a platform for future RNAi screens and other high-throughput approaches aimed at determining the mechanism and pathways of viral suppression by *Wolbachia*. This is the first evidence that *Wolbachia* bacteria block ZIKV in cell culture, showing that blockage occurs in a cell-autonomous manner independent of systemic immunity.

***In vivo* application.** Our results have implications for using *Wolbachia* to control arbovirus. The current *Wolbachia*-based strategy to inhibit arboviruses employs only *w*Mel (4, 21). Multiple models have predicted successful establishment of *w*Mel control (6, 19). Field trials are currently being conducted. However, there are concerns that *w*Mel will adapt to its new *Aedes* host (25). This may cause loss of virus inhibition (25). Investigation of additional strains will provide additional tools to *w*Mel to improve upon disease control strategies. Our data suggest that *w*Stri and *w*AlbB to a lesser extent could also be used to inhibit ZIKV replication in mosquitoes.

Our study highlights two important aspects that support improving the utilization of *Wolbachia* to limit ZIKV transmission. The first is that the robust inhibition of ZIKV by *w*Stri is optimal for the investigation of *Wolbachia*-arbovirus interaction. Understanding this mechanism of *Wolbachia*-caused virus suppression might allow direct targeting of arboviruses *in vivo*. Second, this work provides precedence for the use of *w*Stri *in vivo*. Studies will need to be conducted to assess if *w*Stri can successfully inhibit ZIKV in *A. aegypti*. Future studies should include the transinfection of *w*Stri into *A. aegypti in vitro* to adapt to *A. aegypti* for *in vivo* approaches.

MATERIALS AND METHODS

Insect cell culture. *A. albopictus* C710 and C/*w*Stri1 cells were a kind gift from Ann Fallon. C/*w*Stri cells were derived from C710 cells transinfected with *Wolbachia pipientis w*Stri from the planthopper *Laodelphax striatellus* (32). C710 and C/*w*Stri cells were grown at 28°C with 5% CO_2 and subcultured weekly at a 1:5 dilution in E-5 medium as previously described (32).

A. albopictus Aa23 cells with and without a stable *Wolbachia pipientis w*AlbB infection were a kind gift from Zhiyong Xi. Aa23 cells are derived from *A. albopictus* with a natural *Wolbachia pipientis w*AlbB infection. Aa23TET cells were treated with tetracycline to remove *Wolbachia* as previously described (35). *w*AlbB-infected cells were subcultured weekly at a 1:5 dilution. Cells were grown in Schneider's medium with 10% tetracycline-tested fetal bovine serum (FBS), 50 μg/ml of penicillin, and 50 μg/ml of streptomycin at 28°C with 5% CO_2.

A. albopictus C6/36 cells were cultured in minimal essential medium (MEM) with 10% fetal bovine serum, 1× nonessential amino acids, and 2 mM glutamine. C6/36 cells were subcultured weekly at a 1:10 dilution.

Mammalian cell culture. *Macaca mulatta* kidney LLC-MK2 cells were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum and 2 mM glutamine. LLC-MK2 cells were subcultured weekly at a 1:10 dilution.

Phylogenetic analysis. Phylogenetic analysis was performed by generating a concatenated sequence using five multilocus sequence typing genes (*coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*) (33). *w*Mel sequences identified in the assembled genome are available on the NCBI website (accession number AE017196.1). All other genes were identified by BLAST search of unassembled genome sequence contigs. *w*Stri sequences were identified from GenBank accession numbers LRUH01000003.1 (*coxA* and *gatB*), LRUH01000022.1 (*ftsZ*), LRUH01000049.1 (*fbpA*), and LRUH01000065.1 (*hcpA*). *w*AlbB sequences were identified from GenBank accession numbers CAGB01000095.1 (*coxA* and *gatB*), CAGB01000132.1 (*ftsZ*), CAGB01000163.1 (*fbpA*), and CAGB01000012.1 (*hcpA*). *w*MelPop sequences were identified from GenBank accession numbers AQQE01000026.1 (*coxA*), AQQE01000016.1 (*gatB*), AQQE01000047.1 (*ftsZ*), AQQE01000005.1 (*fbpA*), and AQQE01000033.1 (*hcpA*). *w*Pip sequences were identified from GenBank accession numbers CACK01000072.1 (*coxA*), CACK01000072.1 and CACK01000085.1 (*gatB*), CACK01000123.1 (*ftsZ*), CACK01000089.1 (*fbpA*), and CACK01000050.1 (*hcpA*). Sequences were aligned by MUSCLE and the Tamura-Nei model to produce a maximum likelihood tree in MEGA6 (48).

Virus stocks. ZIKV strains MR766 and PRVABC59 were obtained from the Biodefense and Emerging Infections Research Resources Repository. Virus was grown in C6/36 cells infected at an MOI of 0.01 and harvested at 7 days. Virus supernatant was filtered and aliquoted. To concentrate virus for high-MOI experiments, 8% polyethylene glycol 8000 was incubated with virus overnight at 4°C. Virus was pelleted at a relative centrifugal force (RCF) of 30,000 and resuspended in NaCl-Tris-EDTA (NTE) buffer. Infectious virus units were quantified by focus forming assay.

Focus forming assay for infectious virus production. Cells were infected at an MOI of 0.01 for 1 h at 28°C. The virus inoculum was removed and complete medium was added to each well. Cells were incubated at 28°C with 5% CO_2 for 5 days. After incubation, medium was removed for quantitation by focus forming assay. The focus forming assay protocol was adapted from that previously described by Paul et al. (49). Briefly LLC-MK2 cells were inoculated with serial dilutions of supernatant and incubated for 1 h. Virus was removed and cells were rinsed one time before addition of 1% agar in MEM with 10% FBS. Plates were incubated for 72 h, followed by fixation in 10% formalin for 1 h at room temperature and then permeabilization with 70% ethanol for 30 min. Cell were stained with cross-reactive primary human anti-dengue E virus protein antibody (D11C) (50) in phosphate-buffered saline (PBS) with 0.01% Tween 20 and 5% nonfat dry milk (NFDM), followed by goat anti-human horseradish peroxidase (HRP). Foci were developed with 0.5 mg/ml diaminobenzidine in 25 mM Tris-HCl, pH 7.2. Foci were counted and graphed in GraphPad Prism.

Tetracycline treatment. Cells were infected at an MOI of 0.01 for 1 h at 28°C. The virus inoculum was removed and complete medium with either 0.0235, 0.25, or 2.5 μg/ml of tetracycline was added to each well. Cells were incubated at 28°C with 5% CO_2 for 5 days. After incubation, medium was removed and cells were rinsed one time with PBS. Cellular RNA was extracted with a Qiagen RNeasy kit per manufacturer recommendations. Quantitative RT-PCR was carried out with a Roche one-step SYBR green kit as described below.

Fluorescent *in situ* hybridization. Fluorescent *in situ* hybridization was performed with minor modifications to a method previously described (51). Briefly, cells were fixed with 4% paraformaldehyde in serum-free medium with 0.1% Triton X-100 and 0.1% Tween 20 for 1 h. Cells were then incubated in hybridization buffer (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 250 mg/liter of salmon sperm DNA, 0.5× Denhardt's solution, 20 mM Tris HCl, 0.1% SDS) at 37°C for 1 h. Probes were added in *in situ* buffer at a concentration of 1 ng/μl (probe 1) or 7.5 pg/μl (probe 2). Fluorescent *in situ* hybridization utilized two *Wolbachia* 16S rRNA probes: probe 1 (5′ Cy3-ATCTTGCGA CCGTAGTCC 3′ and probe 2 (5′ Alexa Fluor 488-ACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATT 3′). Probes were incubated with cells for a minimum of 3 h at 37°C and then washed in buffer 1 (1× SSC, 20 mM Tris-HCl, and 0.1% SDS) followed by wash buffer 2 (0.5× SSC, 20 mM Tris-HCl, and 0.1% SDS) at 37°C for 15 min each. Cells were stained with Hoechst at 0.5 μg/ml in wash buffer 2 for 30 min at room temperature. Finally, cells were rinsed with wash buffer 2 twice and mounted in ProLong gold for imaging on an Olympus FV100 FluoView confocal microscope.

Quantitative RT-PCR of virus genome copies. Cells were infected at an MOI of 0.01 for 1 h at 28°C in serum-free medium. The virus inoculum was removed, and complete medium was added to each well. Cells were incubated at 28°C with 5% CO_2 for 5 days. After incubation, medium was removed and cells

were rinsed one time with PBS. Cellular RNA was extracted with a Qiagen RNeasy kit per manufacturer recommendations. Quantitative RT-PCR was carried out with a Roche one-step SYBR green kit. ZIKV primers (ZIKV_For [AARTACACATACCARAACAAAGTGGT] and ZIKV_Rev [TCCRCTCCCYCTYTGGTCTTG]) were previously published (52). *w*Stri primers (*w*Stri_For [TCAAGCAAAAGCTGGTGTTAGC] and *w*Stri_Rev [CAGCATCATCCTTAGCTGCC]) and *w*AlbB primers (*w*AlbB_For [AGCATCTTTTATGGCTGGTGG] and *w*AlbB_Rev [AATGTTGCACCACCAACGTC]) were made against *Wolbachia* surface protein. All reactions were annealed at 55°C.

Western blots. Cells were infected with ZIKV at an MOI of 10 for 1 h at 28°C in serum-free medium. The virus inoculum was removed and complete medium was added to each well. Cells were incubated at 28°C with 5% CO_2 for 1, 2, or 3 days. After incubation, medium was removed and cells were rinsed one time with PBS. Protein was extracted by NP-40 buffer with protease inhibitor on ice for 5 min. Fifteen micrograms of protein was loaded per well in a 10% SDS TGX minigel and subsequently transferred to low-fluorescence polyvinylidene difluoride (PVDF) paper (Bio-Rad). After blocking for 1 h in Odyssey PBS blocking buffer, proteins were probed for with Genetex polyclonal rabbit anti-Zika virus NS5, Abcam mouse anti-Hsp60 LK-2, and Millipore mouse anti-actin in Odyssey blocking buffer with 0.01% Tween 20 overnight at 4°C. Blots were then incubated with Licor secondary antibodies, IRDye 680LT donkey anti-mouse IgG (H+L), and IRDye 800CW donkey anti-rabbit IgG (H+L) at a dilution of 1:5,000 in Odyssey blocking buffer with 0.01% Tween 20 and 0.01% SDS. Membranes were visualized with the Licor Odyssey Clx. Quantitation of band intensity was performed with Image Studio and graphed in GraphPad Prism. To determine the appropriate statistical test, we consulted a computational biologist/statistician (Tom Kepler, Microbiology, Mathematics & Statistics, Boston University), who advised us to log transform the intensity ratios, which typically produces normally distributed errors and ameliorates heteroscedasticity in data.

Cholesterol supplementation. Cells were infected at an MOI of 0.01 for 1 h at 28°C in serum-free medium. The virus inoculum was removed and complete medium with or without cholesterol-lipid concentrate (Thermo Fisher; catalog number 12531018). Incubation took place at 28°C with 5% CO_2 for 5 days. The supernatant was removed. Cells were rinsed one time with PBS and then lysed with Qiagen RLT buffer. RNA was extracted with an RNeasy kit per the manufacturer's recommendations. Quantitative RT-PCR was carried out as described above.

ACKNOWLEDGMENTS

We thank Ann Fallon and Zhiyong Xi for kindly sharing cell lines infected and not infected with *Wolbachia*. We thank Tom Kepler (Microbiology, Mathematics & Statistics, BU) for statistical consultation for Western blot quantitation.

Funding was provided by the following: The Directors Fund (BU NEIDL) and the National Institute of Allergy and Infectious Diseases (R21 NS101151, 1R56AI097589, and 1R56AI097589-01A1).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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