Supplementary Information for

Differential viral RNA methylation contributes to pathogen blocking in *Wolbachia*-colonized arthropod

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Supplementary Materials and Methods

Drosophila melanogaster Cell Culture

JW18 *Drosophila melanogaster* cells with and without *Wolbachia w*Mel were grown at 24 ºC in Shields and Sang M3 insect media (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 1% each of L-Glutamine (Corning), non-essential amino acids (Corning) and penicillin-streptomycin-antimycotic (Corning).

Virus infection in cells and progeny virus production

Virus stocks were generated from RML12, C7-10 or JW18 cells, either with or without *Wolbachia* or overexpressing DNMT2 orthologs by infecting naïve cells with virus at a MOI of 10. In all cases, serum-free media was used for downstream virus purification. Media containing virus was collected 5 days post-infection for alphaviruses SINV (SINV-nLuc, TE12-untagged, TE3'2J-GFP and TE3'2J-mCherry) and CHIKV (CHIKV18125-capsid-mKate). Virus stocks were subsequently purified and concentrated by ultracentrifugation (43K for 2.5 h) over a 27% (w/v) sucrose cushion dissolved in HNE buffer. Viral pellets were stored and aliquoted in HNE buffer before being used for all subsequent experiments.

DNMT2 inhibition in mosquito cells

Inhibition of *Aedes* DNMT2 activity in C7-10 cells was achieved using RNA and DNA cytosine methyltransferase inhibitors, 5-aza-cytidine (5-AZAC, Sigma-Aldrich) and 5-deoxy-azacytidine (DAC-5, Sigma-Aldrich). In each case, *Aedes albopictus* C7-10 cells were treated overnight with media containing either 5μM inhibitor diluted in Dimethyl sulfoxide (DMSO) or DMSO alone. Due to poor stability of 5-AZAC, media containing fresh inhibitor was added everyday post infection (1).

Drosophila melanogaster DNMT2 overexpression

Drosophila Mt2 (FBgn0028707) cDNA clone (GM14972) obtained from DGRC (https://dgrc.bio.indiana.edu/) was cloned into the pAFW expression vector (1111) with an engineered SalI site (Gateway Vector Resources, DGRC) downstream of and in-frame with the 3X FLAG tag using Gibson assembly (HiFi DNA assembly mix, NEB). Expression of FLAG-tagged

DNMT2 in fly cells was confirmed using qRT-PCR and Western Blots using an anti-FLAG monoclonal antibody (SAB4301135 - Sigma-Aldrich). Catalytically inactive *Mt2* (*Mt2* C78A) variant was generated via site-directed mutagenesis (NEB, Q5 Site-Directed Mutagenesis Kit) using primers listed in the primer table (Table S1).

Virion RNA extraction and transfection

Virion encapsidated RNA was extracted from viruses (SINV-nLuc) were purified over a 27% sucrose cushion using TRiZOL reagent (Sigma Aldrich) using manufacturer's protocol. Post extraction, RNAs were DNase (RQ1 RNase-free DNase, NEB) treated using manufacturer's protocol to remove cellular contaminants and viral RNA copies were quantified via quantitative RT-PCR using primers probing for SINV nsP1 and E1 genomic regions (Supplementary Table 1) and a standard curve comprised of linearized SINV infectious clone containing the full-length viral genome. To maximize production of infectious units, equal mass (1 µg) of virion (SINV-nLuc) isolated RNA derived from JW18 fly cells was transfected into BHK-21 cells. Transfection was carried out for 6 hours before the transfection inoculum was removed and overlay was applied. Cells were fixed 48 (SINV) or 72 (CHIKV) hours post transfection using 10% (v/v) formaldehyde and stained with crystal violet to visualize plaque forming units.

Live cell imaging

Live cell imaging experiments were carried out using a setup similar to our previous study (2). Growth of fluorescent reporter viruses in *Aedes albopictus* (C7-10) cells were monitored using Incucyte live cell analysis system (Essen Biosciences, USA). *Aedes albopictus* C7-10 cells were grown under standard conditions as described earlier under 5% ambient CO_2 at 27 ºC. Cells were plated to 75-80% confluency in 96-well plates to allow distinct separation between adjacent cells and preserve cell shape for optimal automated cell counting. Cells per well were imaged and averaged across four distinct fields of view, each placed in one quarter of the well, every 2 hours over the course of the infection. For every sample, total fluorescence generated by cells expressing the red fluorescent reporter mKate was calculated and normalized by the cell number. A manual threshold was set to minimize background signal via automated background correction at the time of data collection. Following acquisition, data was analyzed real-time using the native Incucyte ® Base Analysis Software.

Data availability

Full Incucyte dataset is available in the form of an excel sheet available through Dryad.

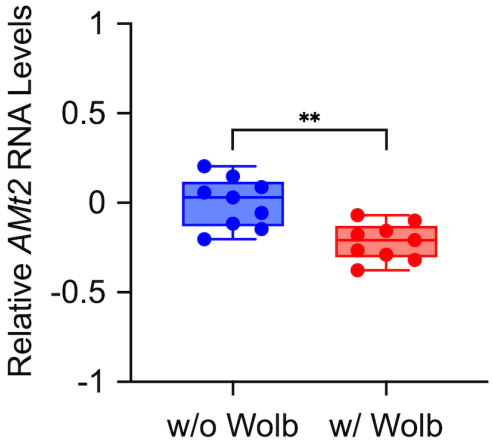

Fig. S1. Presence of *Wolbachia* reduces MTase expression *ex vivo.* Relative *AMt2* expression in the presence (w/ Wolb) and absence (w/o Wolb) of *Wolbachia* in *Aedes albopictus* cells. Quantitative RT-PCR was used to measure relative mRNA levels of *AMt2* in *Aedes albopictus* cells colonized with *w*Mel strain of *Wolbachia* (RML12). Welch's t-test on log-transformed values. Error bars represent standard error of mean (SEM) of independent experimental replicates. Primer details are available in Table S1. **P < 0.01.

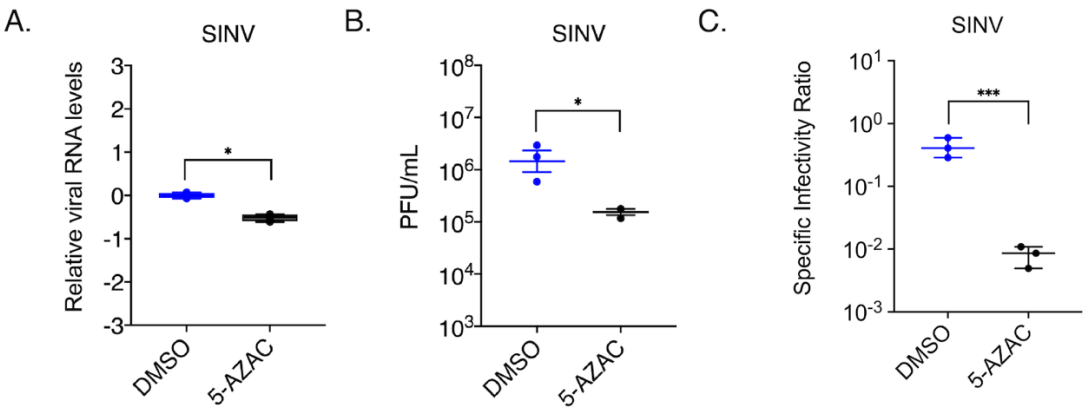

Fig S2. Pharmacological inhibition of mosquito DNMT2 reduces virus replication and per-particle infectivity. Inhibition of mosquito DNMT2 in *Wolbachia*-free *Aedes albopictus* derived C7-10 cells was carried out using MTase inhibitors 5-Azacytidine (5-AZAC). Dimethyl-sulfoxide (DMSO) was used as the negative control. In each case, cells were pretreated with 5 μM inhibitors overnight prior to infections with SINV at MOI of 10. Cell lysates and supernatants were harvested at 24 hours post infection to quantify cellular viral RNA levels and infectious titer, respectively. (A) Levels of SINV RNA in mosquito cells treated with MTase inhibitor 5-AZAC were determined using quantitative RT-PCR. (B) Infectious SINV titers produced from mosquito cells treated with MTase inhibitor 5-AZAC were determined using plaque assays on BHK-21 cells. (C) Specific infectivity ratios of progeny SINV was calculated as the ratio of infectious plaque forming units (B) over total viral genome copies present in collected cell supernatants as quantified by qRT-PCR. Error bars represent standard error of mean (SEM) of three independent experimental replicates. *P < 0.05; ****P < 0.0001.

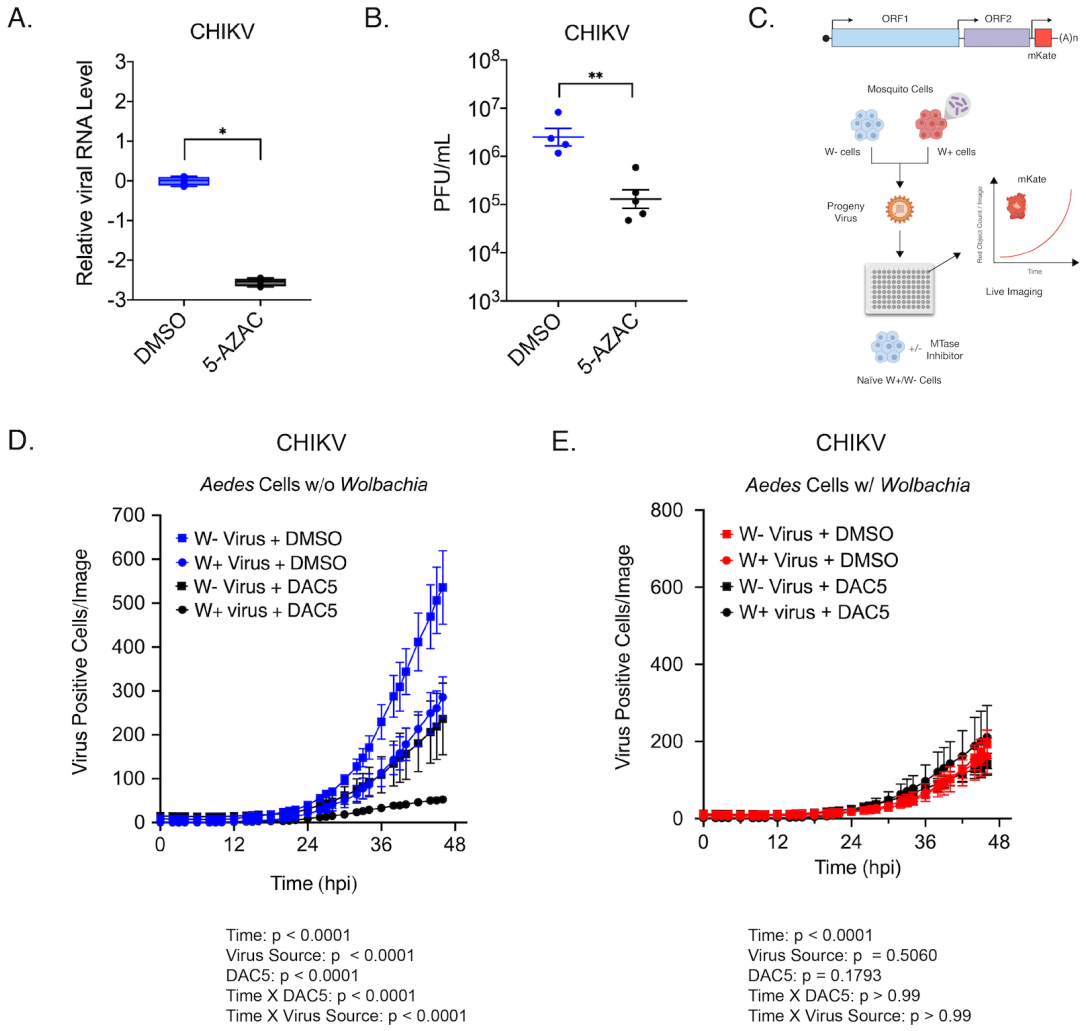

Fig S3. Pharmacological inhibition of mosquito DNMT2 reduces virus replication and spread in mosquito cells. Inhibition of mosquito DNMT2 in *Wolbachia*-free *Aedes albopictus* derived C7-10 cells was carried out using MTase inhibitors 5-Azacytidine (5-AZAC), or 5-Deoxyazacytidine (DAC5). Dimethyl-sulfoxide (DMSO) was used as the negative control. In each case, cells were pretreated with 5 μM inhibitors overnight prior to infections with CHIKV-mKate virus at MOI of 10. Cell lysates and supernatants were harvested at 48 hours post infection to quantify cellular viral RNA levels and infectious titer, respectively. (A) Levels of CHIKV RNA in mosquito cells treated with MTase inhibitor 5-AZAC were determined using quantitative RT-PCR. (B) Infectious CHIKV titers produced from mosquito cells treated with MTase inhibitor 5-AZAC were determined using plaque assays on BHK-21 cells. (C) Schematic representation of live cell experiments. (D) CHIKV expressing mKate fluorescent reporter protein was grown in C7-10 *Aedes albopictus* cells in the presence (W+ virus) or absence (W- virus) of *Wolbachia (*strain *w*Stri). These progeny viruses were then used to infect naïve C7-10 cells without (D) and with (E) *Wolbachia (*strain *w*Stri) pretreated with MTase inhibitor (depicted in color) or DMSO (depicted in black) synchronously at a MOI of 1 particle/cell. Virus growth in cells was measured in real time by imaging and quantifying the number of red cells (Virus Positive Cells/Image) expressing the virus encoded mKate protein over a period of forty-eight hours, using live cell imaging. Color of the data points distinguish treatment conditions; blue represent C7-10 *Wolbachia*-free cells treated with DMSO, red represent C7-10 *Wolbachia*-colonized cells treated with DMSO, black represent both C7-10 cell types treated with 5 μM DAC5. Shape of data points represent the

progeny virus type used to initiate infection; boxes represent viruses derived from W- cells, circles represent viruses derived from W+ cells. The Y-axis label Red object count/Image represent virus-positive cells in a single field of view, four of which were collected and averaged/sample at each two-hour time point over the course of infection. Three-way ANOVA with Tukey's post hoc test for multivariate comparisons. Error bars represent standard error of mean (SEM) of independent experimental replicates (n=3). *P < 0.05; **P < 0.01, ****P < 0.0001.

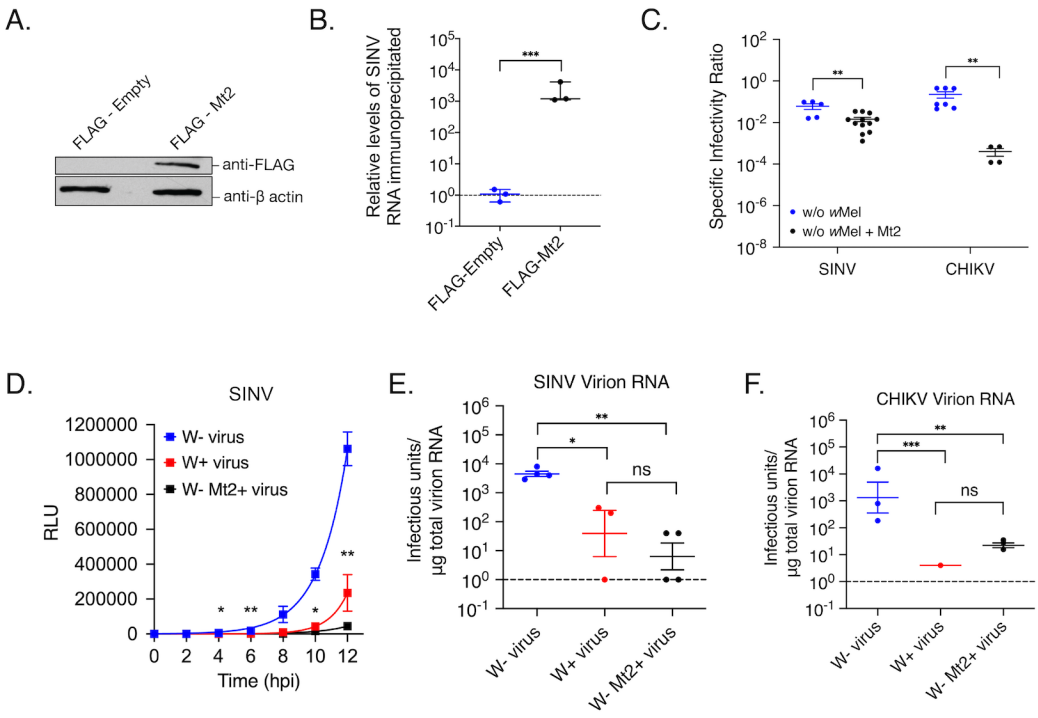

Fig S4. *Drosophila melanogaster* DNMT2 functions as an antiviral. (A) Western Blot of fly DNMT2 in *Wolbachia*-free *Drosophila melanogaster* derived JW18 cells transfected with expression vector constructs with (FLAG-Mt2) or without (FLAG-empty) *Mt2.* Cytoplasmic lysates of cells were collected 72 hours post transfection and probed with anti-FLAG and anti-β actin antibodies. (B) Specific Infectivity Ratios of progeny viruses derived from *Drosophila melanogaster* cells colonized with native *Wolbachia* strain *w*Mel. Fly cells without *Wolbachia* were transfected with expression vectors FLAG-empty (w/o Wolb) or FLAG-Mt2 (w/o Wolb + Mt2) for 48 hours prior to infection with SINV-nLuc or CHIKV (MOI=10). Specific Infectivity Ratios of the progeny viruses generated 96 hours post infection were calculated as before. (C) Relative levels of viral RNA recovered following AZA-IP of Mt2 in fly cells was quantified using qRT-PCR. JW18 fly cells without *Wolbachia* were transfected with expression vectors FLAG-empty or FLAG-Mt2 for 72 hours prior to infection with SINV at MOI of 10. Cells were treated for approximately 18h with 5 µM 5-Azacytidine to covalently trap Mt2 with its target cellular RNA prior to RNA immunoprecipitation using anti-FLAG antibody. (D) Progeny SINV-nLuc derived from fly cells with (W+ virus), without (W- virus) *Wolbachia* or overexpressing Mt2 (W- Mt2+ virus) were subsequently used to synchronously infect naïve BHK-21 cells at equivalent MOIs of 5 particles/cell. Cell lysates were collected at indicated times post infection and luciferase activity (RLU), was used as a proxy for viral replication. Two-way ANOVA with Tukey's post hoc multiple comparisons test. (E) Overexpression of *Drosophila* DNMT2 ortholog reduces infectivity of progeny virion RNA. Approximately 1µg of virion encapsidated RNA extracted from the aforementioned W+, W- and W- Mt2+ SINV or CHIKV viruses were transfected into naïve BHK-21 cells and infectious titer was determined by the counting the number of plaques produced after 48 hours post transfection. One-way ANOVA with Tukey's post hoc test for multivariate comparisons. Dotted line at Y=0 indicate the limit of detection. . Error bars represent standard error of mean (SEM) of independent experimental replicates. *P < 0.05; **P < 0.01, ***P < 0.001, ns = non-significant.

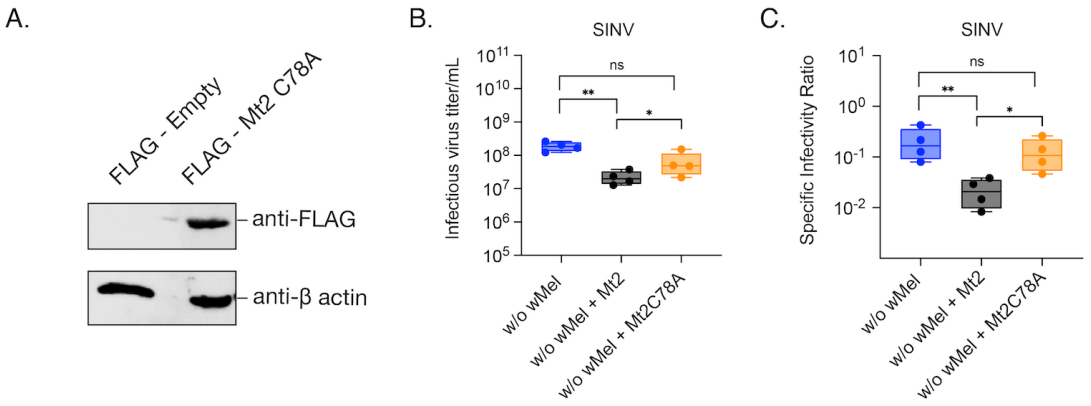

Fig S5. Antiviral effect of fly MTase is dependent on its catalytic activity. (A) Expression of the catalytic mutant of fly DNMT2 in *Wolbachia*-free *D. melanogaster* JW18 cells was assessed by Western Blot 72 hours post transfection with either the epitope tagged Mt2 mutant (FLAG-Mt2 C78A) or the empty control vector (FLAG-Empty) plasmid. (B) 72 hours after *Wolbachia*-free *D. melanogaster* JW18 cells were transfected with plasmids carrying either the wild-type (FLAG-Mt2), catalytic mutant (FLAG-Mt2 C78A) or the empty control vector (FLAG-Empty), they were challenged with SINV at MOI of 10. Cell supernatants were harvested 48 hours post infection, clarified and used to assess Infectious SINV titer by standard plaque assay on vertebrate BHK-21 cells. One-way ANOVA with Tukey's post hoc test for multivariate comparisons. Error bars represent the standard error of mean of independent experimental replicates. (C) Specific Infectivity Ratios of progeny viruses produced 48 hours post infection was measured as the ratio of infectious virus titer (presented in B) and viral genome copies present in the cell supernatant, quantified using qRT-PCR using primers probing SINV E1 gene (see Materials and Methods for more details on the procedure and Table S1 for primer details). One-way ANOVA with Tukey's post hoc test for multivariate comparisons. Error bars represent the standard error of mean of independent experimental replicates. *P < 0.05, **P < 0.01, ns = non-significant.

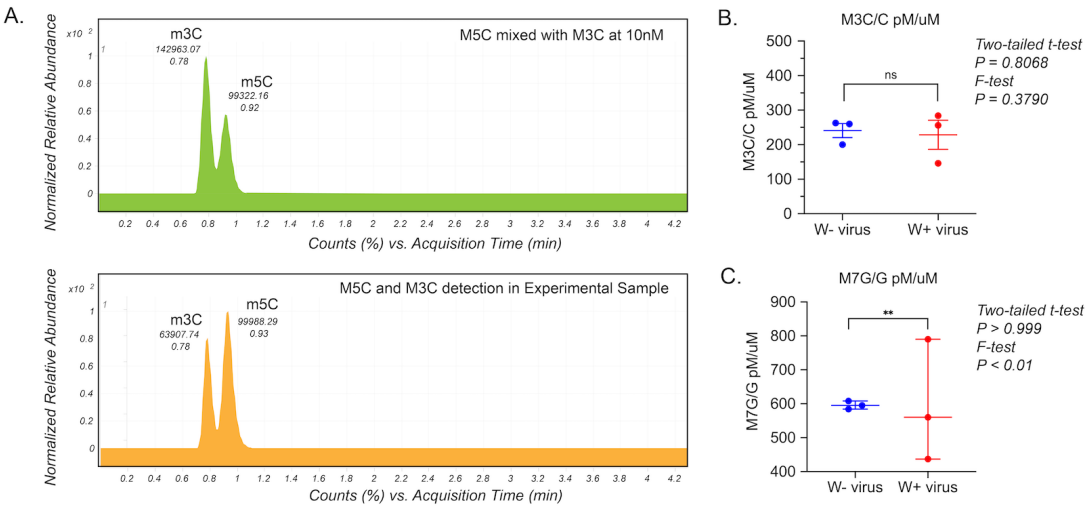

Fig S6. LC-MS/MS detection of methylated cytosine residues. (A) Chromatograms representing simultaneous detection of 3-methyl cytosine (m3C) and 5-methyl cytosine (m5C) groups in mixture comprised of 10nM of each standard (Top) and one representative experimental sample (Bottom). (B) Normalized 3-methyl cytosine content of RNA isolated from W- and W+ viruses represented as a ratio of total unmodified cytosine content. (C) Normalized 7-methyl guanosine content of RNA isolated from W- and W+ viruses represented as a ratio of total unmodified guanosine content. Error bars represent standard error of mean (SEM) of three independent virus preps from each cell type. Unpaired t-test with Welch's correction and F-test to compare variances. F-test results: **P < 0.01, ns = not-significant.

Table S1. Primers used in this study. Primers were purchased from Integrated DNA Technologies (IDT). All primers were used at a final concentration of 10μM for quantitative PCR and RT-PCR reactions. Recommended primer concentrations according to manufacturer's protocol were used for cloning experiments.

Primer Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
CHIKV E2	GGAATAAAGACGGATGATAGC	GGTCGGGAATGAAATTTTTCC
SINV E1	TCAGATGCACCACTGGTCTCAACA	ATTGACCTTCGCGGTCGGATACAT
SINV nsP1	AAGGATCTCCGGACCGTACTTG	CATGAACTGGGTGGTGTCGAAGC
Aedes 18S	CGAAAGTTAGAGGTTCGAAGGCGA	CCGTGTTGAGTCAAATTAAGCCGC
WSP	CATTGGTGTTGGTGTTGGTG	ACCGAAATAACGAGCTCCAG
Aedes GAPDH	CCGCTGATCTGCTAAACATAGA	GTTCTTCCGGGAGGATTCATTAG
Fly Mt2	CCGTGGCGTGAAATAGCG	ACACCGCTTTCGGAGGACG
Aedes AMt2	TATCAATCCGGTGGCCAATAC	CGGCGGTGACATGAGAATAA
pAFW-Mt2 QC_SalI	ACAAGGATGACGATGACAAGGTCCGAC	GGGTCGGCGCGCCCACCCTTGTCGAC
pAFW-Mt2_GA_Insert	AGGATGACGATGACAAGGTCATGGTATTTCGGGTCTTAGA	TCGGCGCGCCCACCCTTGTCTCATTTTATCGTCAGCAATT
pAFW-AMt2	GCAACCGGTTTATGAGTGTTACCGACGGA	GCAGCTAGCTCAGTCCATCTCATCAAACAACGAACTC
Mt2-C78A_QC	GTCCCCGCCAGCTCAGCCCCACAC	ATCAGCAGCATGTTGGCC
AMt2-C78G_QC	GTCACCGGGCCAACCATTCA	ATGAGAGTAACGTTCACACCAAGCTTCTGAATG

Dataset S1 Legend: Excel spreadsheet containing raw data collected from Incucyte S3 Live Cell Imaging Platform.

Dataset S1 (separate file). Data has been uploaded to Dryad. Link is available to reviewers upon request

SI References

1. Z. H. Israili *et al.*, The disposition and pharmacokinetics in humans of 5-azacytidine administered intravenously as a bolus or by continuous infusion. *Cancer Res* **36**, 1453-1461 (1976).
2. T. Bhattacharya, I. L. G. Newton, R. W. Hardy, Viral RNA is a target for Wolbachia-mediated pathogen blocking. *PLoS Pathog* **16**, e1008513 (2020).