Supplementary Appendix

Engineered resistance to Zika virus in transgenic *Ae. aegypti* expressing a polycistronic cluster of synthetic small RNAs

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Supplementary Appendix Contents

- ➢ **SI Appendix Table S5.** Primer sequences and small RNA target sites utilized to generate synthetic small RNA constructs used in this study.
- ➢ **SI Appendix Table S6.** Primers used to assemble plasmid OA959C (the anti-ZIKV transgene).
- ➢ **Supplementary Table S7.** Diagnostic primers used for inverse PCR (iPCR) assays, zygosity confirmation, ZIKV NS5 RT-qPCR, and *w*Mel infection confirmation.

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SI Materials and Methods

Synthetic anti-ZIKV small RNAs design and construction

The *Drosophila melanogaster* miR6.1 stem-loop, which has been previously validated in *D. melanogaster* [1], was modified to target eight unique sites in the ZIKV polyprotein region as previously described [2]. The eight target sites corresponded to regions of capsid (C), membrane precursor (prM), and envelope (E) structural genes, RNA-directed RNA polymerase NS5 (which contained three target sites), and non-structural proteins NS1 and NS2A, of ZIKV strain H/PF/2013 (GenBank: KJ776791.2) [3]. These sites were highly conserved in ZIKV strain FSS13025 (Cambodia 2010, Genbank KU955593)[4] and in ZIKV strain PRVABC59 (isolated from US traveller to Puerto Rico in 2015, GenBank KU501215) (SI Appendix Fig. S1). To generate miR6.1 stem-loop backbones that create mature synthetic small RNAs complementary to each of these target sites, pairs of primers were annealed and products were utilized for two subsequent rounds of PCR and cloned into the pFusA backbone (from the Golden Gate TALEN and TAL Effector Kit 2.0, Addgene #1000000024) in sets of four using Golden Gate assembly [5] to generate plasmids OA959A and OA959B. Assembled small RNAs were then digested with either PmeI/BglII (vector OA959A) or with BamHI/PacI (vector OA959B) and were subcloned into a PacI/PmeI-digested final vector OA959C (the anti-ZIKV transgene). The ZIKV target sequences and sequences of primers used in the small RNA cloning are listed in SI Appendix Table S5.

Plasmid Assembly

To generate vector OA959C (the anti-ZIKV transgene), several components were cloned into the *piggyBac* plasmid pBac[3xP3-DsRed] [6] using Gibson assembly/EA cloning [7]. First, a *Drosophila* codon optimized tdTomato marker was amplified with primers 959C.10A and 959C.10B from a gene synthesized vector (GenScript, Piscataway, NJ) and cloned into a XhoI/FseI digested pBac[3xP3-DsRed] backbone using EA cloning. The resulting plasmid was

digested with AscI, and the following components were cloned in via EA cloning: the predicted *Aedes aegypti* carboxypeptidase promoter [8] amplified from *Ae. aegypti* genomic DNA using primers 959C.11A and 959C.11B, a GFP sequence amplified from vector pMos[3xP3-eGFP] [9] with primers 959C.12A and 959C.12B, and a 677 bp p10 3' untranslated region (UTR) amplified with primers 959C.13A and 959C.13B from vector pJFRC81-10XUAS-IVS-Syn21-GFP-p10 (Addgene plasmid #36432). Assembled small RNA fourmers were then subcloned into final plasmid OA959C using PacI and PmeI using traditional cloning. All primer sequences are listed in SI Appendix Table S6. Complete annotated plasmid sequence and DNA is available via Addgene (plasmid #104968).

Generation of Transgenic Mosquitoes

Germline transformations were carried out largely as described [10]. Briefly, 0-1 hr old Higgs and Liverpool strain *Ae. aegypti* pre-blastoderm embryos were injected with a mixture of vector OA959C (200 ng/ul) and a source of *piggyBac* transposase (200 ng/ul) [9]; the injected embryos were hatched in deoxygenated H_2O. A total of 52 surviving Higgs adult males and 64 surviving Higgs adult females, and 61 surviving adult Liverpool males and 75 surviving adult Liverpool females, respectively, were recovered after the injection. Higgs adults were assigned to 35 pools and Liverpool adults were assigned to 39 pools, and outcrossed to Higgs or Liverpool adults, respectively, of the opposite sex in cages. Larvae were fed ground fish food (TetraMin Tropical Flakes, Tetra Werke, Melle, Germany) and adults were fed with 0.3M aqueous sucrose. Adult females were blood fed three to five days after eclosion using anesthetized mice. All animals were handled in accordance with the guide for the care and use of laboratory animals as recommended by the National Institutes of Health and supervised by the local Institutional Animal Care and Use Committee (IACUC). A total of 8,189 Higgs and 10,949 Liverpool G_1s were screened. Larvae with positive fluorescent signals (3xp3-tdTomato) were selected under the fluorescent stereomicroscope (Leica M165FC) and were crossed to establish stable transgenic lines. Four independent lines (termed TZIKV-A, B, and D recovered from Liverpool G_1s, and TZIKV-C recovered from Higgs G_1s) with the strongest fluorescence expression patterns were selected for further characterization. To determine whether these lines represented single chromosomal insertions, we backcrossed single individuals from each of the lines for four generations to wild-type stock, and measured the Mendelian transmission ratios in each generation; in all cases, we observed a 50% transmission ratio, indicating insertion into single chromosomes. For one of the four lines (TZIKV-C), transgenic mosquitoes were inbred for at least 12 generations to generate a homozygous stock. Mosquito husbandry was performed under standard conditions as previously described [11].

Characterization of Transgene Genomic Insertion Sites

To characterize the insertion site of vector OA959C in transgenic mosquitoes, we adapted a previously described inverse polymerase chain reaction (iPCR) protocol [12] as follows.

Genomic DNA (gDNA) was extracted from 10 transgenic *Ae. aegypti* fourth instar larvae of each line using the DNAeasy Blood & Tissue Kit (Qiagen #69504) per the manufacturer's protocol. Two separate restriction digests were performed on diluted gDNA to characterize the 5' and 3' ends of the insertion using Sau3AI (5' reaction) or HinP1I (3' reaction) restriction enzymes. A ligation step using NEB T4 DNA Ligase (NEB #M0202S) was performed on the restriction digest products to circularize digested gDNA fragments, and two subsequent rounds of PCR were carried out per ligation using corresponding *piggyBac* primers listed in SI Appendix Table S7. Final PCR products were cleaned up using the MinElute PCR Purification Kit (Qiagen #28004) in accordance with the manufacturer's protocol, and sequenced via Sanger sequencing (Source BioScience, Nottingham, UK). To confirm transgene insertion locus and orientation via PCR, primers were designed based on iPCR mapped genomic regions and used in tandem with *piggyBac* primers based on their location as listed in SI Appendix Table S7. Sequencing data was then blasted to the AaegL5.0 reference genome (NCBI). An alignment of the sequencing data was carried out with SeqManPro (DNASTAR, Madison, WI) to determine orientation of the transgene insertion site. Analysis of the sequencing data indicated that the insertion sites were on chromosome 2 (at approximate position 167,899,561) for line TZIKV-A, on chromosome 3 (at approximate position 402,525,313) for line TZIKV-B, on chromosome 3 (at approximate position 173,647,938) for line TZIKV-C, and on chromosome 1 (at approximate position 228,972,549) for line TZIKV-D. These insertion locations were also confirmed by PCR and sequencing performed on genomic DNA from the transgenic mosquitoes.

Small RNA Extraction, Isolation, Sequencing, and Bioinformatics

Total RNA was extracted from midguts of 30 ZIKV-C transgenic and WT (Higgs strain) non-blood-fed adult females as well as midguts of 30 ZIKV-C transgenic and WT (Higgs strain) adult females 24 hours post blood-feeding using the Ambion mirVana mRNA Isolation Kit (ThermoFisher Scientific #AM1560). Following extraction, RNA was treated with Ambion Turbo DNase (ThermoFisher Scientific #AM2238). The quality of RNA was assessed using RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies #5067-1513) and a NanoDrop 1000 UV–vis spectrophotometer (NanoDrop Technologies/Thermo Scientific, Wilmington, DE). Small RNA was then extracted and prepared for sequencing with QIAseq miRNA Library Kit (Qiagen #331502). Libraries were quantified with Qubit dsDNA HS Kit (ThermoFisher Scientific #Q32854) and High Sensitivity DNA Kit for Bioanalyzer (Agilent Technologies #5067- 4626) and sequenced on Illumina HiSeq2500 in single read mode with the read length of 75 nt following manufacturer's instructions. After adapter trimming and UMI extraction, reads were aligned to mature *Ae. aegypti* miRNAs downloaded from miRBase (release 22, [13]) and to each synthetic small RNA's passenger, loop, and guide sequences using bowtie2 in 'very-sensitive-local' mode. (We assumed, based on the design of the synthetic small RNAs, that they are processed as miRNAs; however, it remains possible that they are instead processed as endogenous small RNAs (esiRNA) or some other small RNA species.) Custom Perl scripts were

used to quantify the number of reads that mapped to each target. 5 out of 8 target sites were reliably detected at TPM values between 2 and 91. Sites 3, 5 and 7 were not detected above background in either of the transgenic samples (Table S1). Correlation coefficients of TPM values between WT and transgenic animals were calculated in R[14]. Differential expression analysis was performed with R package DESeq2 using two factor design (design= ~ feeding + genotype). TPM values and MA plots were generated with R package ggplot2 (SI Appendix Fig. S3). Quantification data are shown in SI Appendix Table S1. All sequencing data can be accessed at NCBI SRA (accession ID: SRP150144; BioProject ID: PRJNA475410).

RT-PCR confirmation of anti-ZIKV transgene expression

To assay synthetic small RNA expression in mosquitoes, total RNA was separately extracted from 50 dissected midguts and 6 carcasses (midguts and heads removed) of Higgs WT and ZIKV-C non blood fed females, as well as 30 dissected midguts and 6 carcasses (midguts and heads removed) of Higgs WT and ZIKV-C females 24 hours post blood-feeding using the Ambion mirVana mRNA Isolation Kit (ThermoFisher Scientific #AM1560). Following extraction, total RNA was treated with Ambion Turbo DNase (ThermoFisher Scientific #AM2238). RNA was then converted to cDNA using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific #K1631) using a mix of oligo$(dT)_{18}$ and random hexamer primers. PCR was then performed on the resulting cDNA using standard procedures. To confirm presence of synthetic small RNA transcripts, primers 959.S7 and 959.S8 were used to amplify a fragment from the 5'UTR region of the carboxypeptidase A promoter (downstream of the transcription start site) to the loop-guide strand region of small RNA 1. As a positive control, primers 959.S10 and 959.S11 were used to amplify a short sequence of the *Actin1* gene (AAEL011197)[15]. Expression of the anti-ZIKV transgene transcript was observed in both TZIKV-C midgut and carcass tissues regardless of mosquito blood meal state, but was completely absent in Higgs WT mosquito tissues (SI Appendix Figure S4), while *Actin1* positive control transcripts were present in all samples. PCR products were sequenced to confirm product identity. All primer sequences are listed in SI Appendix Table S7.

ZIKV Infection of Mosquitoes, Virus Determination and Longevity

All experiments were performed under biosafety level 3 (BSL-3) conditions in the insectary at the Australian Animal Health Laboratory. Insectary conditions were maintained at 27.5℃ and 70% in relative humidity with a 12hr light/dark cycle. ZIKV strain FSS13025 (Cambodia 2010, Genbank KU955593)[4] or PRVABC59 (Puerto Rico 2015, GenBank KU501215) were used for viral challenge experiments. Both belong to the Asian/Pacific/American clade and were passaged once in C6/36 cells and twice in Vero cells before using for mosquito infections. WT (Higgs strain for TZIKV-C experiments, Liverpool strain for TZIKV-A, B, and D experiments) and transgenic (confirmed by red fluorescence in the eye) mosquitoes were infected with ZIKV as previously described [16]. Briefly, female mosquitoes were challenged with a chicken blood

meal spiked with ZIKV ($TCID_{50}$ 10^6/mL) through chicken skin membrane feeding. Blood-fed female mosquitoes were sorted and maintained at standard conditions in an environmental cabinet with sugar *ad libitum*. For infection rate and virus titer, mosquito midguts were collected at 4 dpi. For dissemination and transmission rate, mosquito saliva, midguts, and carcasses were collected at 14 dpi. Mosquito saliva was used to determine viral titers using $TCID_{50}$ assay on Vero cells. Midguts and carcasses were used to determine presence of viral RNA using RT-qPCR against ZIKV NS5 [16] (SI Appendix, Table S7). Mosquito viral challenge, processing, saliva testing, and molecular analyses of infection and dissemination were carried out as previously described [16]. ZIKV infection rate was defined by the number of midguts (4 dpi) found positive for viral nucleic acid over tested midguts. Similarly, the dissemination rate was calculated by the number of carcasses (14 dpi) testing ZIKV positive by qPCR. Transmission rate was defined by the number of $TCID_{50}$ positive saliva samples over the number tested. For each experiment, data from three replicates was pooled. The average $TCID_{50}$ values were compared by two-tailed unpaired t test. To measure fitness after infection, blood-fed ZIKV-infected females were quickly sorted out after CO_2 anaesthesia and housed in waxed cardboard cup 250 ml containers with a maximum of 25 mosquitoes. Mosquitoes were maintained at standard conditions for 14 days with 10% sugar solution *ad libitum*. Dead mosquitoes were counted daily. Females surviving at day 14 were marked as censored (status=0) in the database for survival analysis, which was performed using the GraphPad Prism software (GraphPad Software, La Jolla California, USA). The Mantel-Cox test was used to compare the survival of infected mosquitoes at 14 dpi.

Generation of *w*Mel *Wolbachia* Line and Infection Assay

Eggs of *Ae. aegypti* infected with the *Wolbachia* strain *w*Mel were obtained from the World Mosquito Program (Prof. Scott O'Neill, Monash University). Higgs mosquitoes infected with *w*Mel were generated by crossing *w*Mel+ females with males from the Higgs line, and the resulting offsprings were used for ZIKV infections experiments. At the end of the experiment, *Wolbachia* infection status of these mosquitoes was tested using PCR with primers specific for *w*Mel detection [17] (SI Appendix, Table S7). The PCRs indicated presence of *w*Mel in >90% of mosquitoes, and only results from these positive mosquitoes were used for further analysis.

Mouse Transmission Assays

All experiments were performed under biosafety level 3 (BSL-3) conditions in the insectary at NHRI. Insectary conditions were maintained at 29°C and 80% relative humidity with a 12 hr light/dark cycle, and mosquitoes were maintained as previously described [18]. For experimental assays, transgenic anti-TZIKV-C mosquitoes were outcrossed to WT (Higgs strain) for a generation to obtain heterozygotes. Non-transgenic sibling mosquitoes from the above cross were used as Higgs WT controls. ZIKV strain PRVABC59 (Puerto Rico 2015, GenBank KU501215) was used for viral challenge experiments. It was obtained from the Taiwan Center

for Disease Control, and maintained/amplified as previously described [18]. For direct ZIKV infection, 7–10 day-old female TZIKV-C and Higgs WT mosquitoes were inoculated with 200 plaque forming units (pfu) of ZIKV by thoracic injection as previously described [18] and maintained under standard housing conditions for 7 days prior to their use in assays. Infection via artificial membrane blood feeding was carried out as described above, and infected mosquitoes were then maintained under standard conditions for 14 days prior to their use in transmission assays. Viral titers were measured at 7 dpi (for thoracic injection infections) or 14 dpi (for membrane blood feeding infection) by plaque assay as previously described [18,19]. Briefly, $2x10^5$ cells/well of Vero cells (a kind gift from Dr. Guann-Yi Yu) were incubated for one day (in serum-free 1xDMEM medium (HyClone, SH30022), at 37℃) before being infected with ZIKV. At two hours post infection, unbound virus particles were removed, and cells were gently washed by PBS and overlaid with 3 ml of 1xDMEM medium containing 2% FBS (Gibco, 16000044), 10 mM HEPES, 10nM sodium pyruvate, 2mM L-Glutamine (Gibco, 25030081), 1x Penicillin-Streptomycin (Gibco, 15140122), and 1% Methyl cellulose (Sigma, M0512-250G). The infected cells were then incubated at 37℃ and 5% CO_2 for 4 days until plaque formation. Cells were fixed and stained with 0.5mL crystal violet/methanol mixed solution (ASK®Gram Stain Reagent) for 2 hours, and washed with H_2O. Number of plaques was then calculated, and viral titers were determined as plaque forming units per mosquito and were compared by one-way ANOVA.

All mouse-related experiments were conducted in compliance with the guidelines of the Laboratory Animal Center of NHRI. The animal protocol (NHRI-IACUC-105111) was approved by the Institutional Animal Care and Use Committee of NHRI, according to the Guide for the Care and Use of Laboratory Animals (NRC 2011). Management of animal experiments and animal care and use practices of NHRI have been accredited by the AAALAC International. *Stat1*-/- (C57BL/6 background) mice were provided by Dr. Guann-Yi Yu (NTU, Taiwan). Both male and female mice between the ages of 11-12 weeks were used in the study.

Mosquito-mediated ZIKV mouse infections were carried out as previously described [18,19]. Briefly, mice were anesthetized with Ketalar (100 mg/Kg, Pfizer, New York, NY) via intraperitoneal injection, and their ventral surfaces were shaved. Then, mice were placed on top of a polyester mesh covering a mosquito-housing cage that permitted female mosquitoes to take a blood meal. Female mosquitoes were starved for 10h before they were allowed to take blood meals from mice, and each mouse was fed on by 6–11 mosquitoes. Mouse body weight and mortality were then recorded for 6-30 days. Mouse weights were compared by the Mann Whitney test to evaluate for significant weight loss.

Fitness Assessment and Conditions

To determine if the anti-ZIKV transgene confers a fitness cost, several fitness parameters were evaluated in Higgs WT and TZIKV-C mosquitoes. For these experiments, homozygous TZIKV-C mosquito stock obtained after 12 generations of inbreeding (see above) and the Higgs

WT stock utilized to obtain transgenic lines were used. Evaluation of all experimental and control replicates were performed simultaneously. Insectary conditions were maintained at 28℃ and 70-80% in relative humidity with a 12hr light/dark cycle. To assess larval to pupal development time, eggs were vacuum hatched and larvae were distributed into pans (50 larvae per pan) containing 2.5L of ddH_2O and 0.6mL of fish food slurry. To determine the development time of TZIKV-C and Higgs WT control mosquitoes, 4th instar larvae were sorted according to fluorescence phenotype and reared until pupation. Pupae were collected and counted every day until no pupae were left. To assess female fertility and fecundity, 90 TZIKV-C or Higgs WT females were mated to 20 Higgs WT males in a cage. After four days, females were blood fed and individually transferred into plastic vials filled with water and lined with egg paper. After three days, egg papers were collected, and eggs were counted and vacuum hatched in 9-ounce plastic cups. Starting on the fourth day, larvae were counted every day until no larvae were present. Female fecundity refers to the number of eggs laid per female, and fertility reflects the number of eggs hatching to produce larvae. To measure male mating success, fecundity, and fertility, one TZIKV-C or Higgs WT male was mated to five Higgs WT females in a single cup filled with water and lined with egg paper. Three days post blood meal, cups were checked for the presence of eggs, which were collected, counted, and hatched. Hatched larvae were then counted every day until no larvae were present. Male mating success was calculated as the percentage of single male outcrosses that produced larvae. Fecundity was measured as the number of eggs laid per cup; fertility was determined by the number of hatching larvae in each cup. To asses wing length as a proxy for body size, images of TZIKV-C and Higgs WT mosquito wings were taken with a Leica M165 FC microscope (Leica Microsystems). Wing length measurements were done by using the measurement tool on the Leica Application Suite X, measuring from the axial incision to the intersection of the R 4+5 margin. Finally, to assess mosquito longevity, equal numbers of male and female TZIKV-C or Higgs WT mosquitoes were placed in medium sized cages (in triplicate). Mosquitoes that died were counted and removed daily until all mosquitoes had died. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, La Jolla California, USA). Means were compared using unpaired t tests with Welch's correction except for male mating success (no Welch's correction). Analyses of mosquito survivorship utilized the Mantel-Cox test. *P*-values>0.05 were considered non-significant.

Confirmation of Transgene Zygosity

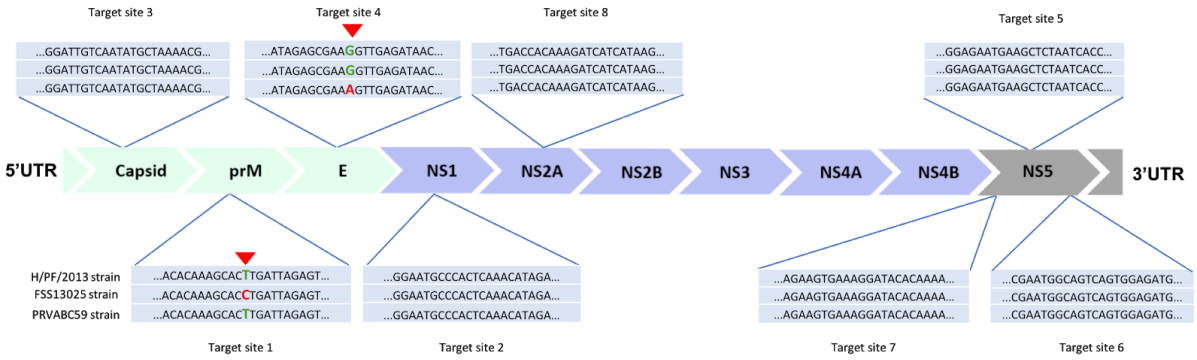
To molecularly confirm zygosity of transgenic mosquitoes, mosquito heads were homogenised using bead-beater for DNA extraction in 30 ul extraction buffer (1x Tris-EDTA, 0.1M EDTA, 1M NaCl and 2.5 uM proteinase K), and incubated at 56°C for 5 minutes and then at 98°C for 5 minutes. PCR was then performed on each line to detect the presence of the transgene by pairing a *piggyBac* primer with a genomic primer as follows: primers 1018.S46 and 991.5R2 for TZIK-A, 1018.S26 and 991.3F2 for TZIK-B, 1018.S8 and 991.5R1 for TZIK-C, and 1018.S50

and 991.3F2 for TZIK-D (SI Appendix Table S7). To determine zygosity, we amplified the WT locus of each transgenic line using corresponding forward and reverse primers listed in SI Appendix Table S7. WT mosquitoes (Higgs strain for TZIKV-C assays, Liverpool for TZIKV-A, B, and D assays) served as controls to ensure that the WT locus was successfully amplified in each genetic background. A PCR kit (ThermoFisher Scientific #F553S) with 57°C annealing temperature and standard protocols was used for all PCRs.

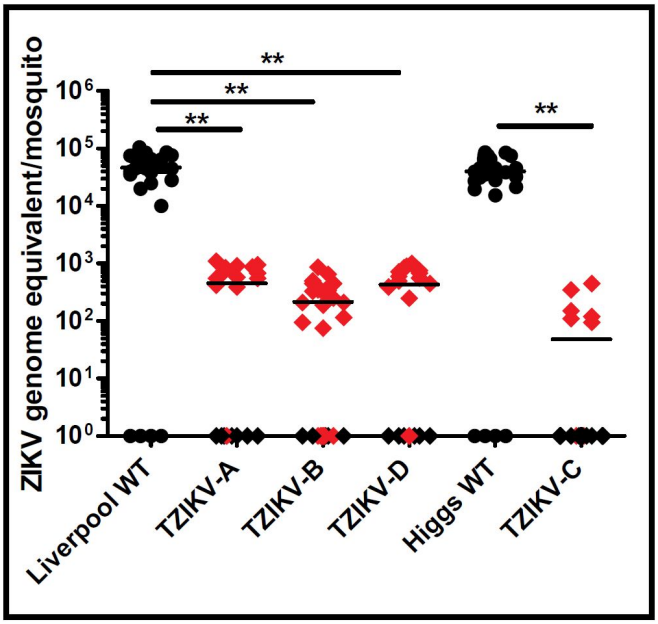
Data Availability Statement

All sequencing data associated with this study are available from NCBI sequence read archive (SRA) accession ID: SRP150144; BioProject ID: PRJNA475410. Complete annotated plasmid sequence and DNA is publically available at Addgene (plasmid #104968). Transgenic mosquitoes will be made available by corresponding author upon request.

SI Appendix Figures and Tables

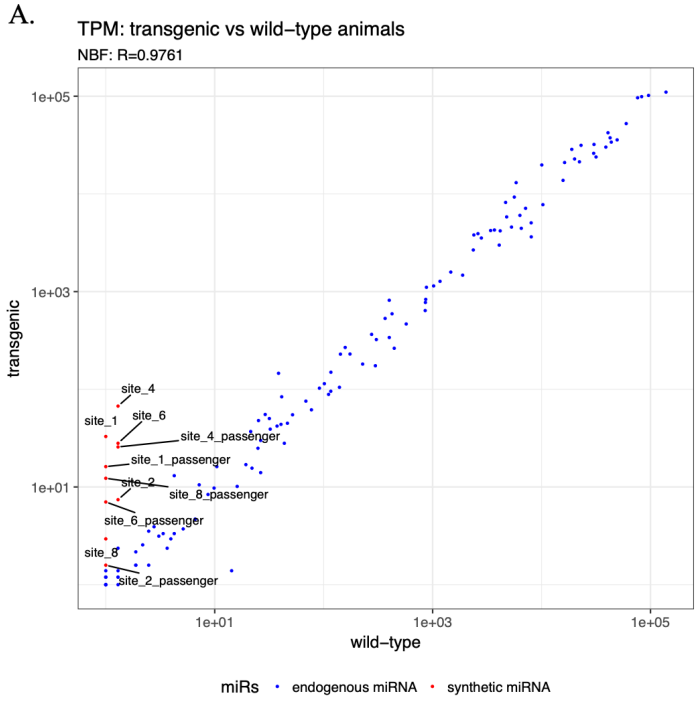


SI Appendix Fig. S1. small RNA target site conservation between ZIKV strains H/PF/2013, FSS13025, and PRVABC59. small RNA target sites between the ZIKV strain used for small RNA target selection (H/PF/2013, top sequence) and the strains used for mosquito challenges (FSS13025, middle sequence; PRVABC59, bottom sequence) are highly conserved, with only one base pair mismatch in one target site in each strain (shown in red).



SI Appendix Fig. S2: Effect of anti-ZIKV transgene on ZIKV titres in four independent mosquito lines. ZIKV virus titres in wildtype (Liverpool WT and Higgs WT), anti-ZIKV transgenic mosquito lines (TZIKV-A, TZIKV-B, TZIKV-C, TZIKV-D) following a blood meal infected with a Cambodian (FSS13025) are shown. ZIKV genome equivalent from mosquito midgut (day 4 post infection) of Liverpool WT, Higgs WT, and transgenic mosquitoes were determined using real-time RT-qPCR and calculated using previously published methods. Circles represent WT mosquitoes; black diamonds represent anti-ZIKV Hm transgenic mosquitoes; red colored diamonds represent anti-ZIKV Ht transgenic mosquitoes. Horizontal bars represent the mean virus titer. Mantel-Cox test was used for statistical analysis. **represents $p<0.001$.

A.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NBF: R=0.9761
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wild−type
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1e+03
1e+05
site_4
site_1
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site_4_passenger
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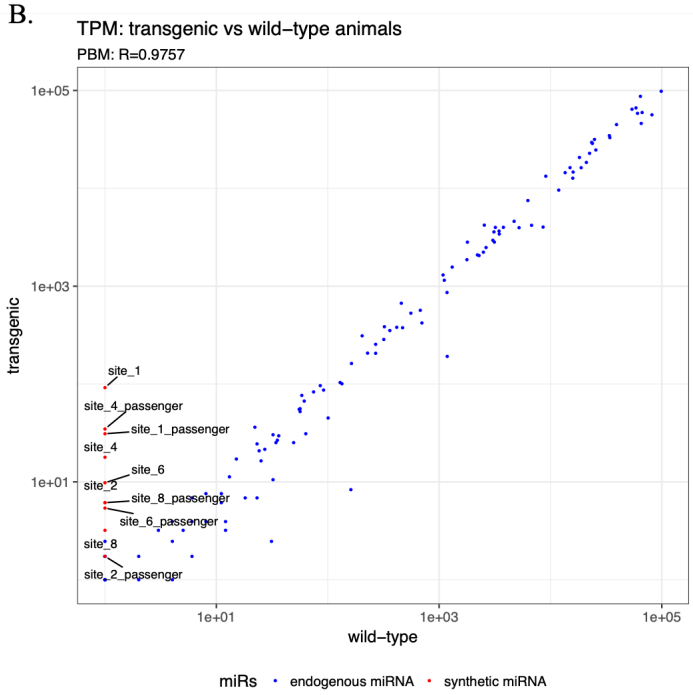
miRs
endogenous miRNA
synthetic miRNA

B.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PBM: R=0.9757
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wild−type
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1e+03
1e+05
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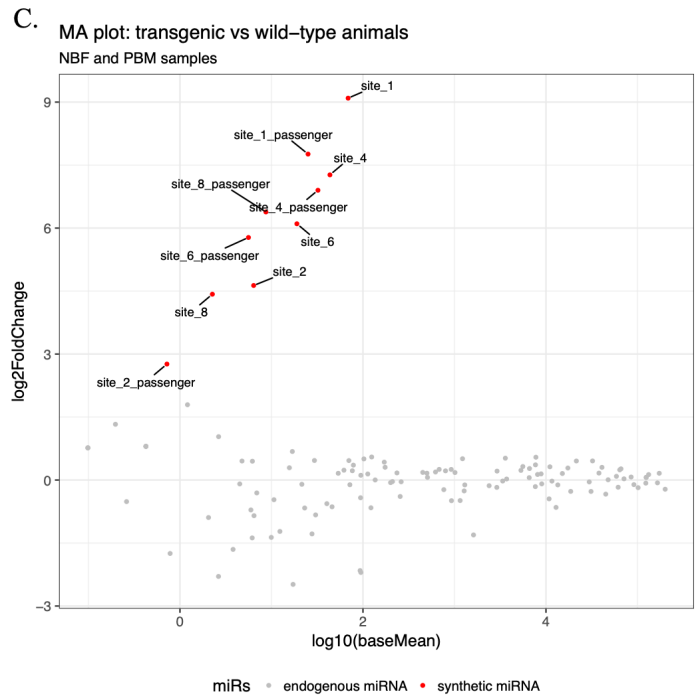
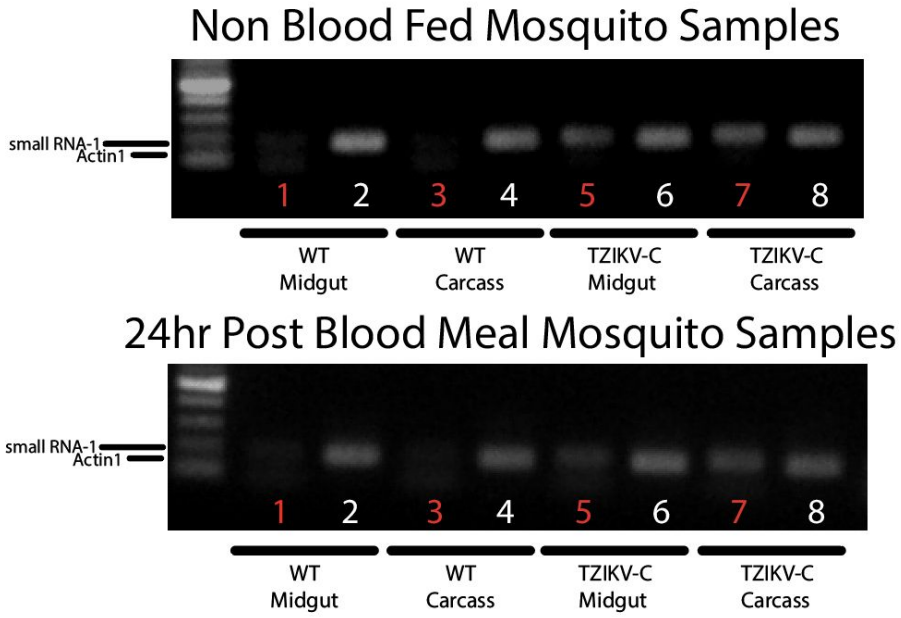
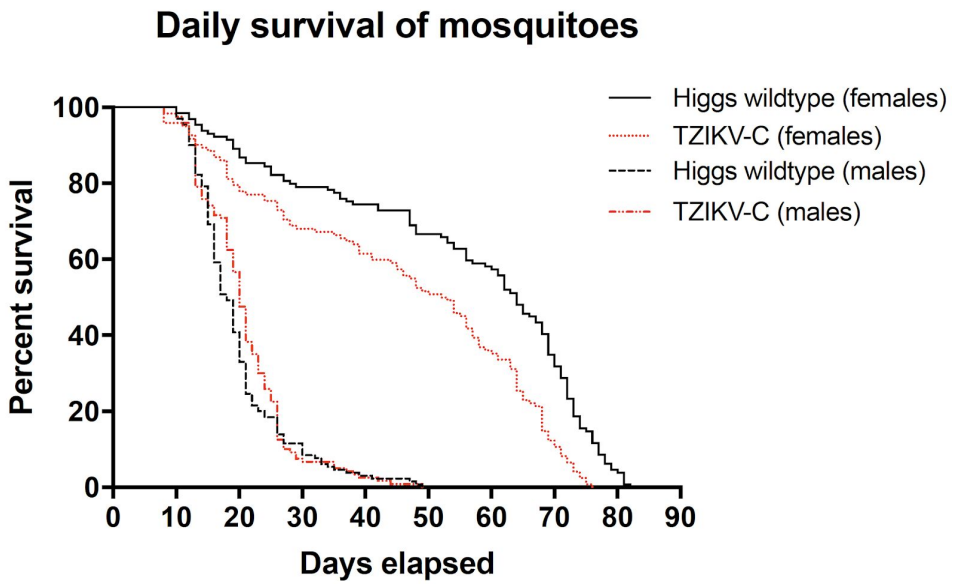
synthetic miRNA



SI Appendix Fig. S3. Differential expression analysis of small RNAs from Higgs WT and TZIKV-C mosquito midguts. TPM (transcripts per million) values for transgenic versus Higgs WT animals without a blood meal (**A**) and 24 hours after a blood meal (**B**) are shown. Expression of synthetic small RNAs does not affect expression levels of endogenous miRNAs significantly (correlation coefficients of 0.9761 and 0.9757, respectively). MA (log2FoldChange vs. baseMean) (**C**) plot demonstrates that detected synthetic small RNAs are strongly differentially expressed between Higgs WT and transgenic animals.



SI Appendix Fig. S4. RT-PCR analysis on non blood fed and 24-hr post blood fed Higgs WT and TZIKV-C female midgut and carcass samples. A 195bp region of the anti-ZIKV transgene, from the 5'UTR region of the carboxypeptidase A (AAEL010782) promoter to the loop-target site-1 region, was amplified to confirm expression of the anti-ZIKV transgene (odd numbered lanes, labeled in red). A 175bp region of the *Actin1* gene was amplified as a control (even numbered lanes, labeled in white). Higgs WT midgut (lanes 1 and 2), Higgs WT carcass (lanes 3 and 4), TZIKV-C midgut (lanes 5 and 6), and TZIKV-C carcass (lanes 7 and 8) samples were assayed in both a non blood fed (top panel) and 24-hr blood fed (bottom panel) state. All PCR products were sequenced to confirm product identity.



SI Appendix Fig. S5. Survivorship curve of Higgs WT and TZIKV-C male and female mosquitoes. The x-axis indicates the number of elapsed days after the start of the experiment, and the y-axis indicates the percent of mosquitoes surviving on each elapsed day. Each line represents accumulated results from 120-130 adult mosquitoes combined from 3 biological replicates.

SI Appendix Table S1. Quantification of endogenous and engineered small RNA expression on read and UMI (Unique Molecular Identifiers) levels in Higgs WT and TZIKV-C mosquitoes prior to blood meal (NBF) and 24 hr post blood feeding (PBM). Both raw read or UMI counts and normalized TPM (Transcripts Per Million) values are shown.

ID	NBF.WT.counts	NBF.transgenic.counts	PBM.WT.counts	PBM.transgenic.counts	NBF.WT.TPM	NBF.transgenic.TPM	PBM.WT.TPM	PBM.transgenic.TPM
target_site_1	0	164	0	124	0	31.87937467	0	90.86865303
target_site_1_passenger	0	78	0	41	0	15.16214161	0	30.04528044
target_site_2	1	33	0	7	0.295439041 6	6.41475222	0	5.129682026

target_site_2_passenger	0	3	0	1	0	0.5831592927	0	0.732811718
target_site_3	0	5	0	0	0	0.9719321545	0	0
target_site_3_passenger	0	97	0	24	0	18.8554838	0	17.58748123
target_site_4	1	341	0	23	0.295439041 6	66.28577293	0	16.85466951
target_site_4_passenger	1	127	0	46	0.295439041 6	24.68707672	0	33.70933903
target_site_5	0	3	0	1	0	0.5831592927	0	0.732811718
target_site_5_passenger	0	1	0	0	0	0.1943864309	0	0
target_site_6	1	139	0	12	0.295439041 6	27.01971389	0	8.793740615
target_site_6_passenger	0	31	0	6	0	6.025979358	0	4.396870308
target_site_7	0	1	0	2	0	0.1943864309	0	1.465623436
target_site_7_passenger	0	4	0	0	0	0.7775457236	0	0
target_site_8	0	10	0	3	0	1.943864309	0	2.198435154
target_site_8_passenger	0	58	0	7	0	11.27441299	0	5.129682026
loop	0	2	0	0	0	0.3887728618	0	0

aae-bantam-3p	78005	161675	33520	47139	23045.72244	31427.42621	33832.20357	34544.01157
aae-bantam-5p	2967	5690	1779	3843	876.5676365	1106.058792	1795.569515	2816.195432
aae-let-7	147717	174426	63660	118851	43641.36891	33906.0476	64252.926	87095.40549
aae-miR-1	167227	183838	38859	61155	49405.38461	35735.61268	39220.93075	44815.10061
aae-miR-10	2923	4276	1172	260	863.5683187	831.1963785	1182.915948	190.5310467
aae-miR-100	27174	26008	6706	5715	8028.260517	5055.602295	6768.459343	4188.018968
aae-miR-1000	1006	891	671	772	297.2116759	173.1983099	677.2496599	565.7306463
aae-miR-11-3p	68020	117231	13429	19718	20095.76361	22788.11568	13554.07702	14449.58145
aae-miR-11-5p	1501	1348	319	524	443.4540015	262.0329088	321.9711498	383.9933402
aae-miR-1174	324904	524151	58076	90526	95989.32638	101887.8421	58616.91691	66338.51358
aae-miR-1175-3p	19772	67074	11759	13075	5841.420731	13038.27547	11868.52273	9581.513212
aae-miR-1175-5p	24143	36627	6218	10236	7132.784782	7119.791804	6275.914136	7501.060745
aae-miR-12-3p	3955	6549	1167	1175	1168.46141	1273.036736	1177.869379	861.0537686
aae-miR-12-5p	107093	122897	15726	17317	31639.45328	23889.5092	15872.47116	12690.10052
aae-miR-124	0	0	0	1	0	0	0	0.732811718
aae-miR-125-5p	15829	42090	8513	5481	4676.50459	8181.724876	8592.289649	4016.541026
aae-miR-13-3p	14115	21530	3081	4892	4170.122072	4185.139857	3109.696277	3584.914924
aae-miR-13-5p	1351	1738	453	912	399.1381452	337.8436169	457.219219	668.3242868
aae-miR-133	62	82	17	8	18.31722058	15.93968733	17.15833714	5.862493744

aae-miR-137	1934	2393	694	573	571.3791065	465.1667291	700.4638807	419.9011144
aae-miR-14	280675	509250	33835	44969	82922.35301	98991.28993	34150.13747	32953.81014
aae-miR-184	131686	155037	59981	79673	38905.18563	30137.08909	60539.65998	58385.308
aae-miR-1889-3p	86	149	22	32	25.40775758	28.9635782	22.20490688	23.44997497
aae-miR-1889-5p	144086	193001	18703	22167	42568.62975	37516.77555	18877.19879	16244.23735
aae-miR-1890	2903	3977	412	517	857.6595378	773.0748357	415.837347	378.8636582
aae-miR-1891	82	241	31	40	24.22600141	46.84712985	31.28873242	29.31246872
aae-miR-190	1027	1656	161	220	303.4158957	321.9039296	162.4995458	161.2185779
aae-miR-193	0	1	0	0	0	0.1943864309	0	0
aae-miR-210	0	0	0	1	0	0	0	0.732811718
aae-miR-219	1	0	5	8	0.295439041 6	0	5.046569746	5.862493744
aae-miR-252-3p	9	7	5	4	2.658951375	1.360705016	5.046569746	2.931246872
aae-miR-252-5p	0	0	0	1	0	0	0	0.732811718
aae-miR-263a-3p	0	1	0	0	0	0.1943864309	0	0
aae-miR-263a-5p	469	534	132	136	138.5609105	103.8023541	133.2294413	99.66239364
aae-miR-263b-3p	1	1	0	0	0.295439041 6	0.1943864309	0	0
aae-miR-263b-5p	172	277	48	33	50.81551516	53.84504136	48.44706956	24.18278669
aae-miR-275-3p	53314	70791	97920	133917	15751.03706	13760.80983	98832.0219	98135.94683

aae-miR-275-5p	144	139	1103	1568	42.54322199	27.01971389	1113.273286	1149.048774
aae-miR-276-3p	64126	146999	64764	62800	18945.32398	28574.61095	65367.2086	46020.57589
aae-miR-276-5p	530	1374	465	512	156.5826921	267.086956	469.3309863	375.1995996
aae-miR-2765	14	14	3	4	4.136146583	2.721410033	3.027941847	2.931246872
aae-miR-277-3p	16190	29921	3012	4019	4783.158084	5816.236399	3040.053615	2945.170294
aae-miR-277-5p	340	582	23	27	100.4492742	113.1329028	23.21422083	19.78591638
aae-miR-278-3p	8826	20161	3747	5450	2607.544981	3919.024833	3781.899367	3993.823863
aae-miR-278-5p	768	927	201	423	226.897184	180.1962214	202.8721038	309.9793567
aae-miR-279	17933	23540	3439	4652	5298.108333	4575.856583	3471.030671	3409.040112
aae-miR-281-3p	257677	496005	80788	76980	76127.84593	96416.64166	81540.45532	56411.84605
aae-miR-281-5p	229	384	127	140	67.65554053	74.64438946	128.1828715	102.5936405
aae-miR-282-3p	21	49	7	9	6.204219874	9.524935114	7.065197644	6.595305462
aae-miR-282-5p	258	312	62	41	76.22327274	60.64856644	62.57746485	30.04528044
aae-miR-283	469526	566444	65983	81269	138716.3115	110109.0275	66597.5623	59554.87551
aae-miR-285	45	2	73	112	13.29475687	0.3887728618	73.67991829	82.07491241
aae-miR-286a	0	0	0	0	0	0	0	0
aae-miR-286b	0	0	0	1	0	0	0	0.732811718
aae-miR-2940-3p	3456	5875	2477	3035	1021.037328	1142.020282	2500.070652	2224.083564
aae-miR-2940-5p	34818	39921	20831	25104	10286.59655	7760.100708	21025.01887	18396.50537

aae-miR-2941	3	3	30	2	0.886317124	0.5831592927	30.27941847	1.465623436
aae-miR-2942	1350	4188	552	721	398.8427062	814.0903726	557.1412999	528.3572486
aae-miR-2943	0	0	0	0	0	0	0	0
aae-miR-2944a-3p	0	0	0	0	0	0	0	0
aae-miR-2944a-5p	5	3	1	1	1.477195208	0.5831592927	1.009313949	0.732811718
aae-miR-2944b-3p	1	1	1	0	0.295439041 6	0.1943864309	1.009313949	0
aae-miR-2944b-5p	3	6	3	0	0.886317124 9	1.166318585	3.027941847	0
aae-miR-2945-3p	929	1872	266	346	274.4628697	363.8913986	268.4775105	253.5528544
aae-miR-2945-5p	134	220	60	90	39.58883158	42.765014 8	60.55883695	65.95305462
aae-miR-2946	4	8	31	13	1.181756166	1.555091447	31.28873242	9.526552333
aae-miR-2a-3p	12501	21971	2264	2804	3693.283459	4270.864273	2285.086781	2054.804057
aae-miR-2a-5p	390	486	84	130	115.2212262	94.47180541	84.78237173	95.26552333
aae-miR-2b	1433	3044	357	479	423.3641466	591.7122956	360.3250798	351.0168129
aae-miR-2c	1237	2729	315	388	365.4580945	530.4805699	317.933894	284.3309466
aae-miR-305-3p	372	451	2616	3386	109.9033235	87.66828033	2640.365291	2481.300477
aae-miR-305-5p	2890	3285	23599	39219	853.8188303	638.5594255	23818.79989	28740.14277
aae-miR-306-3p	104	253	35	39	30.72566033	49.17976702	35.32598822	28.579657

aae-miR-306-5p	55076	107795	18050	28210	16271.60066	20953.88532	18218.11678	20672.61856
aae-miR-307	5	13	3	2	1.477195208	2.527023602	3.027941847	1.465623436
aae-miR-308-3p	6	15	2	3	1.77263425	2.915796463	2.018627898	2.198435154
aae-miR-308-5p	11533	21698	2524	5739	3407.298467	4217.796778	2547.508408	4205.606449
aae-miR-309a	1	7	7	4	0.295439041 6	1.360705016	7.065197644	2.931246872
aae-miR-309b-3p	1	2	0	2	0.295439041 6	0.3887728618	0	1.465623436
aae-miR-309b-5p	0	0	0	0	0	0	0	0
aae-miR-31	22048	22854	5178	5405	6513.83999	4442.507492	5226.227629	3960.847336
aae-miR-315-3p	0	0	0	0	0	0	0	0
aae-miR-315-5p	11	12	11	3	3.249829458	2.332637171	11.10245344	2.198435154
aae-miR-316	19022	47740	3106	3851	5619.84145	9280.008211	3134.929126	2822.057926
aae-miR-317	34006	101790	15839	20012	10046.70005	19786.5948	15986.52364	14665.0281
aae-miR-33	95	279	54	74	28.06670895	54.23381422	54.50295325	54.22806713
aae-miR-34-3p	86	67	26	28	25.40775758	13.02389087	26.24216268	20.5187281
aae-miR-34-5p	137865	217512	25325	33646	40730.70347	42281.38136	25560.87576	24656.18306
aae-miR-375	19	19	11	4	5.613341791	3.693342187	11.10245344	2.931246872
aae-miR-7	27224	18691	9001	18145	8043.032469	3633.27678	9084.834856	13296.86862
aae-miR-71-3p	9479	18187	1761	2542	2800.466676	3535.306019	1777.401864	1862.807387

aae-miR-71-5p	6381	7572	1299	2137	1885.196525	1471.894055	1311.09882	1566.018641
aae-miR-79-3p	136	426	55	75	40.17970966	82.80861956	55.5122672	54.96087885
aae-miR-79-5p	154	226	21	48	45.49761241	43.93133338	21.19559293	35.17496246
aae-miR-8-3p	202571	269854	53468	87714	59847.3821	52455.95592	53965.99823	64277.84703
aae-miR-8-5p	7995	13719	3171	5439	2362.035138	2666.787445	3200.534533	3985.762934
aae-miR-87	391	764	90	117	115.5166653	148.5112332	90.83825542	85.738971
aae-miR-927	30	45	12	14	8.863171249	8.74738939	12.11176739	10.25936405
aae-miR-929	0	0	0	0	0	0	0	0
aae-miR-92a-3p	587	1176	14853	22191	173.4227174	228.5984427	14991.34009	16261.82483
aae-miR-92a-5p	11	62	10	9	3.249829458	12.05195872	10.09313949	6.595305462
aae-miR-92b-3p	81	123	225	281	23.93056237	23.909531	227.0956386	205.9200927
aae-miR-92b-5p	71	75	24	21	20.97617195	14.57898232	24.22353478	15.38904608
aae-miR-932-3p	3	3	5	1	0.886317124 9	0.5831592927	5.046569746	0.732811718
aae-miR-932-5p	8	12	10	7	2.363512333	2.332637171	10.09313949	5.129682026
aae-miR-957	51	47	22	8	15.06739112	9.136162252	22.20490688	5.862493744
aae-miR-965	479	1173	266	280	141.5153009	228.0152834	268.4775105	205.187281
aae-miR-970	21420	31008	4673	6275	6328.304271	6027.534449	4716.524084	4598.39353
aae-miR-980-3p	69	185	34	35	20.38529387	35.96148972	34.31667427	25.64841013

aae-miR-980-5p	10	10	4	3	2.954390416	1.943864309	4.037255796	2.198435154
aae-miR-981	32	78	14	22	9.454049332	15.16214161	14.13039529	16.12185779
aae-miR-988-3p	127	745	99	60	37.52075829	144.817891	99.92208096	43.96870308
aae-miR-988-5p	306	523	57	103	90.40434674	101.6641034	57.5308951	75.47960695
aae-miR-989	26	38	159	10	7.681415082	7.386684374	160.4809179	7.32811718
aae-miR-993	0	1	0	0	0	0.1943864309	0	0
aae-miR-996	8091	19550	2188	2846	2390.397286	3800.254724	2208.378921	2085.582149
aae-miR-998	106	196	55	70	31.31653841	38.09974046	55.512672	51.29682026
aae-miR-999	13825	15387	3423	4986	4084.44475	2991.024012	3454.881648	3653.799226
aae-miR-9a	75376	109615	22259	31092	22269.0132	21307.66862	22466.31919	22784.58193
aae-miR-9b	101849	133902	23240	40224	30090.17095	26028.73187	23456.45618	29476.61854
aae-miR-9c-3p	4966	8146	1075	1774	1467.150281	1583.471866	1085.012495	1300.007988
aae-miR-9c-5p	102564	165125	24637	43109	30301.40986	32098.0594	24866.46776	31590.78035
aae-miR-iab-4-3p	7	11	0	1	2.068073291	2.13825074	0	0.732811718
aae-miR-iab-4-5p	123	211	33	33	36.33900212	41.01553692	33.30736032	24.18278669

SI Appendix Table S2. Anti-ZIKV transgene effect on ZIKV infection, dissemination, and transmission rates. ZIKV infection rates were quantified in the midgut at 4 days post infection (dpi). Dissemination rates were quantified in both the midgut and carcass at 14 dpi. Transmission rates were calculated by measuring prevalence of ZIKV in the saliva at 14 dpi. For each experiment, data from three replicates is pooled.

Mosquito Line	Zygosity	Viral Strain	Infection (Midgut 4 dpi)	Dissemination		Transmission (Saliva 14 dpi)
				Midgut (14 dpi)	Carcass (14 dpi)	
Higgs WT	**N/A**	**FSS13025**	**42/50 (84%)**	**52/65 (80%)**	**52/65 (80%)**	**48/65 (73.8%)**
TZIKV-C	**Heterozygote**	**FSS13025**	**28/32 (87.5%)**	**29/39 (74.4%)**	**29/39 (74.4%)**	**29/39 (74.4%)**
TZIKV-C	**Homozygote**	**FSS13025**	**0/32 (0%)**	**0/46 (0%)**	**0/46 (0%)**	**0/46 (0%)**
Higgs WT	**N/A**	**PRVABC 59**	**28/32 (87.5%)**	**53/70 (75.7%)**	**53/70 (75.7%)**	**53/70 (75.7%)**
TZIKV-C	**Heterozygote**	**PRVABC 59**	**26/32 (81.25%)**	**49/70 (70%)**	**49/70 (70%)**	**49/70 (70%)**
TZIKV-C	**Homozygote**	**PRVABC 59**	**0/32 (0%)**	**0/70 (0%)**	**0/70 (0%)**	**0/70 (0%)**
Higgs-*w*Mel	**N/A**	**PRVABC 59**	**42/50 (84%)**	**38/50 (76%)**	**38/50 (76%)**	**38/50 (76%)**

SI Appendix Table S3. Fitness evaluation of Higgs WT and TZIKV-C mosquitoes. Comparisons of several fitness parameters (leftmost column) between Higgs WT (second column from left) and TZIKV-C mosquitoes (third column from left) suggest that there are few significant differences (rightmost column) between the two groups, indicating that the anti-ZIKV transgene does not have a major impact on mosquito fitness.

	Mosquito Strain		
Fitness Parameter	**Higgs WT (N)**	**TZIKV-C (N)**	***P*-value**
Female fecundity□†§	**102.5 ± 3.8 (65;**	**113.2 ± 4.4 (65; 7,117)**	**0.0708**

	6,648)		
Egg hatchability□ ‖§	**70.9 ± 2.7 (63; 4,684)**	**58.5 ± 2.8 (63; 4,130)**	**0.0019**
Male mating success□ º¶	**92 ± 0.05 (25)**	**92 ± 0.05 (25)**	**>0.9999**
Male fecundity□ ¹§	**118.2 ± 11.8 (25; 2,846)**	**119.2 ± 12.7 (25; 3,089)**	**0.9580**
Egg hatchability□ ‖‖§	**61 ± 5.8 (23; 1,562)**	**60.7 ± 4.8 (23; 1,771)**	**0.9709**
Larval to pupal development in days□ §	**10.35 ± 0.07 (1,224)**	**9.836 ± 0.10 (904)**	**0.002**
Female wing length□ §	**3.65 ± 0.02 (56)**	**3.62 ± 0.08 (58)**	**0.1489**
Male wing length□ §	**2.76 ± 0.01 (54)**	**2.79 ± 0.01 (55)**	**0.0600**
Female median survival in days††	**64 (124)**	**52 (124)**	**<0.0001**
Male median survival in days††	**18 (130)**	**20 (120)**	**0.2195**
% Survival at 14 dpi with ZIKV□ ‡††	**80.6 ± 3.5 (129)**	**75 ± 4.6 (88)**	**0.3636**

SI Appendix Table S4. The survivorship of ZIKV-infected TZIKV-C mosquitoes at 14 days post infection (dpi). Higgs WT, Higgs *w*Mel+, and TZIKV-C mosquitoes infected with ZIKV strain FSS13025 or PRVABC59 were assessed for survival at 14 dpi. The mean percentage±SEM of surviving mosquitoes and number of mosquitoes tested (in parentheses) are reported. No assay was performed for Higgs *w*Mel mosquitoes infected with strain FSS13025. The Mantel-Cox test was used to compare the survival of infected Higgs WT, Higgs *w*Mel (for PRVABC59 strain only), and TZIKV-C mosquitoes.

	Mosquito strains			
Virus strain	**Higgs WT**	**Higgs WT**	**TZIKV-C**	**P-value**

	*w*Mel			
FSS13025 (Cambodia)	64.1% ± 6.6 (53)	--	73.6% ± 6.1 (53)	0.2528
PRVABC59 (Puerto Rico)	92.1% ± 3.0 (76)	86.9% ± 5.0 (46)	77.1% ± 7.0 (35)	0.0817

SI Appendix Table S5. Primer sequences and small RNA target sites utilized to generate synthetic small RNA constructs used in this study. Self annealing primers are listed first, and consist of forward and reverse target site sequences flanking the stem loop region of the synthetic small RNA. Primers amplifying flanking regions, BsaI cut sites, and multiple cloning sites are listed below.

Primer	Primer Sequence, 5' to 3'	Source
small RNA 1 959C.1A	ATCACAGCCTTTAATGTACACAAAGCACTTGATTAGCGTTAAGTTAATATACCATATCTA	Self annealing primers
959C1.B	TTAGGCACTTTAGGTACACACAAAGCACTTGATTAGAGTTAGATATGGTATATTAACTTA	
small RNA 2 959C.2A	ATCACAGCCTTTAATGTGGAATGCCCACTCAAACATCGATAAGTTAATATACCATATCTA	Self annealing primers
959C.2B	TTAGGCACTTTAGGTACGGAATGCCCACTCAAACATAGATAGATATGGTATATTAACTTA	
small RNA 3 959C.3A	ATCACAGCCTTTAATGTGGATTGTCAATATGCTAAACCGTAAGTTAATATACCATATCTA	Self annealing primers
959C.3B	TTAGGCACTTTAGGTACGGATTGTCAATATGCTAAAACGTAAGTTAATATACCATATCTA	

small RNA 4 959C.4A 959C.4B	 ATCACAGCCTTTAATGTATAGAGCGAAGGTTGAGATCACTAAGTTAAT ATACCATATCTA TTAGGCACTTTAGGTACATAGAGCGAAGGTTGAGATAACTAGATATGG TATATTAACTTA	Self annealing primers
small RNA 5 959C.5A 959C.5B	 ATCACAGCCTTTAATGTGGAGAATGAAGCTCTAATCCCCTAAGTTAAT ATACCATATCTA TTAGGCACTTTAGGTACGGAGAATGAAGCTCTAATCACCTAGATATGG TATATTAACTTA	Self annealing primers
small RNA 6 959C.6A 959C.6B	 ATCACAGCCTTTAATGTCGAATGGCAGTCAGTGGAGCTGTAAGTTAAT ATACCATATCTA TTAGGCACTTTAGGTACCGAATGGCAGTCAGTGGAGATGTAGATATGG TATATTAACTTA	Self annealing primers
small RNA 7 959C.7A 959C.7B	 ATCACAGCCTTTAATGTAGAAGTGAAAGGATACACATAATAAGTTAAT ATACCATATCTA TTAGGCACTTTAGGTACAGAAGTGAAAGGATACACAAAATAGATATG GTATATTAACTTA	Self annealing primers
small RNA 8 959C.8A 959C.8B	 ATCACAGCCTTTAATGTTGACCACAAAGATCATCATCAGTAAGTTAAT ATACCATATCTA TTAGGCACTTTAGGTACTGACCACAAAGATCATCATAAGTAGATATGG TATATTAACTTA	Self annealing primers
720C 720D	TTTAAAGTCCACAACTCATCAAGGAAAATGAAAGTCAAAGTTGGCAGC TTACTTAAACTTAATCACAGCCTTTAATGT AAAACGGCATGGTTATTCGTGTGCCAAAAAAAAAAAAAAATTAAATAA TGATGTTAGGCACTTTAGGTAC	Self-annealed products of 959C.#A/959C .#B
small RNAs 1,5 959C.C1 720F.1	 TCTAGAGGTCTCGCTATCGGCGGCCGCGCGTTTAAACAACCGGATCCT TTAAAGTCCACAACTCATC CTCGAGGGTCTCCCATGGTCAAAACGGCATGGTTATTCGTG	Self-annealed products of 720C/720D-1 and 720C/720D-5

small RNAs 2,6 720E-2 720-F2	 TCTAGAGGTCTCCCATGGCTTTAAAGTCCACAACTCATCAAGGA CTCGAGGGTCTCGGTCCTGAAAACGGCATGGTTATTCGTGTGC	Self-annealed products of 720C/720D-2 and 720C/720D-6
small RNA 3,7 720-E3 720-F3	 TCTAGAGGTCTCAGGACCATTTAAAGTCCACAACTCATCAAGGAA CTCGAGGGTCTCCCTGGCAAAAACGGCATGGTTATTCGTGTGC	Self-annealed products of 720C/720D-3 and 720C/720D-7
small RNA 4,8 720-E4 959C.C2	 TCTAGAGGTCTCGCCAGGATTTAAAGTCCACAACTCATCAAGGA CTCGAGGGTCTCCCGCCACTGCGGCCGCTTAATTAATGGCCGGCCAAA GATCTAAAACGGCATGGTTATTCG	Self-annealed products of 720C/720D-4 and 720C/720D-8
small RNA target site	**Target Sequence (5' to 3')**	**Viral Region Targeted**
small RNA target site 1	ACACAAAGCACTTGATTAGAGT	Membrane glycoprotein precursor M
small RNA target site 2	GGAATGCCCACTCAAACATAGA	Nonstructural protein NS1
small RNA target site 3	GGATTGTCAATATGCTAAAACG	Capsid protein C
small RNA target site 4	ATAGAGCGAAGGTTGAGATAAC	Envelope protein E

small RNA target site 5	GGAGAATGAAGCTCTAATCACC	RNA-dependent RNA polymerase NS5
small RNA target site 6	CGAATGGCAGTCAGTGGAGATG	RNA-dependent RNA polymerase NS5
small RNA target site 7	AGAAGTGAAAGGATACACAAAA	RNA-dependent RNA polymerase NS5
small RNA target site 8	TGACCACAAAGATCATCATAAG	Nonstructural protein NS2A

SI Appendix Table S6. Primers used to assemble plasmid OA959C (the anti-ZIKV transgene).

Fragment and Primers	Primer Sequence, 5' to 3'	Source
tdTomato marker 959C.10A 959C.10B	 GACGGTACGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGGTCATCAAAGAGT TGGTATGGCTGATTATGATCTAGAGTCGCGGCCGCCTACTTGTACAGCTCGTCCATGCCG	Gene synthesized vector
Carboxypeptidase promoter 959C.11A 959C.11B	 ATGGTTAATTCGAGCTCGCCCGGGGTCCTAGGGAATTCGTCAATAAAAAAATACGTTCAA CTCCTCGCCCTTGCTCACCATGTTTAAACTTTCCCAACTAACCGATACACACTAACCTG	Genomic *Ae. aegypti* DNA
GFP marker 959C.12A 959C.12B	 AGTGTGTATCGGTTAGTTGGGAAAGTTTAAACATGGTGAGCAAGGGCGAGGAGCTGTTCAC TGATTTGTTATTTTAAAAACGATTCATTCTAGTTAATTAATTACTTGTACAGCTCGTCCATGCC	pMos[3xP3-eGFP]
p10 3' UTR 959C.13A 959C.13B	 GGACGAGCTGTACAAGTAATTAATTAACTAGAATGAATCGTTTTTAAAATAACAAAT TCCCCGGGCGAGCTCGAATTGGCGCGCCCGGCCGTTAACTCGAATCGCTATCCAAGC	pJFRC81-10XUAS-IVS-Syn21-GFP-p10

Supplementary Table S7. Diagnostic primers used for inverse PCR (iPCR) assays, zygosity confirmation, ZIKV NS5 RT-qPCR, and *w*Mel infection confirmation.

***piggyBac* Primers**			
Reaction	**Primer Name**		**Primer Sequence, 5' to 3'**
5' (1st Round PCR)	991.5F1		GACGCATGATTATCTTTTACGTGAC
	991.5R1		TGACACTTACCGCATTGACA
5'(2nd Round PCR)	991.5F2		GCGATGACGAGCTTGTTGGTG
	991.5R2		TCCAAGCGGCGACTGAGATG
3' (1st Round PCR)	991.3F1		CAACATGACTGTTTTTAAAGTACAAA
	991.3R1		GTCAGAAACAACTTTGGCACATATC
3' (2nd Round PCR)	991.3F2		CCTCGATATACAGACCGATAAAAC
	991.3R2		TGCATTTGCCTTTCGCCTTAT
Zygosity Primers			
Reaction	**Line**	**Primer Name**	**Primer Sequence, 5' to 3'**
Forward primer	TZIK-A	1018.S46	GTACTGGCACCCATAGCTCG
	TZIK-B	1018.S27	GCAAATCCTAAAACCTCATCGAACCG
	TZIK-C	1018.S8	TTTCCACGAAATGAACTCAAACGC
	TZIK-D	1018.S48	ACGTTATGCAAATCTCTCGGAT

Reverse primer	TZIK-A	1018.S47.1	CGAAAATTGCGTTTACCTTATACGT
	TZIK-B	1018.S26	AATCGAATGAACGAATTTCTGATTAAATCC
	TZIK-C	1018.S10	AGAAGAACAGAGGCAATCAACTACATTGA
	TZIK-D	1018.S50	CCATCTTTCGATGGAAATGCATT
ZIKV NS5 RT-qPCR Primers			
Primer name			**Primer Sequence, 5' to 3'**
NS5-F			GAACGAGGATCACTGGATGG
NS5-R			CTCCTGGTATGCGACTCATC
***w*Mel Primers**			
Primer name			**Primer Sequence, 5' to 3'**
*w*Mel-F			CAAATTGCTCTTGTCCTGTGG
*w*Mel-R			GGGTGTTAAGCAGAGTTACGG
RT-PCR Primers			
Primer name			**Primer Sequence, 5' to 3'**
Actin1-F 959.S10			CGTTCGTGACATCAAGGAAA
A*ctin1*-R 959.S11			GAACGATGGCTGGAAGAGAG
Synthetic small RNA-F 959.S7			ATGGCACCTGATTGCAATTGGC

Synthetic small RNA-R 959.S8	AGTTAGATATGGTATATTAACTTAACGCTAATCAAGTG

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