Supplemental Information

Zika virus NS3 mimics a cellular 14-3-3-binding motif to antagonize RIG-I- and MDA5-mediated innate immunity

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Supplementary Figures S1-S5

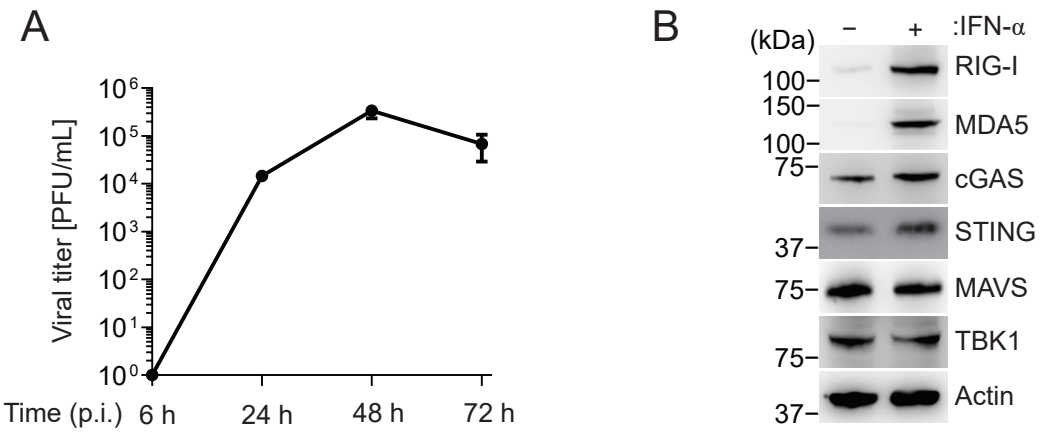


Figure S1. SVGA cells support ZIKV replication and express key innate immune sensors, Related to Figure 1.

(A) Viral titers in the supernatant of SVGA cells infected with ZIKV (recombinant MR 766, MOI 0.01) for the indicated times, determined by plaque assay and presented as PFU/mL ± SD (n = 3).

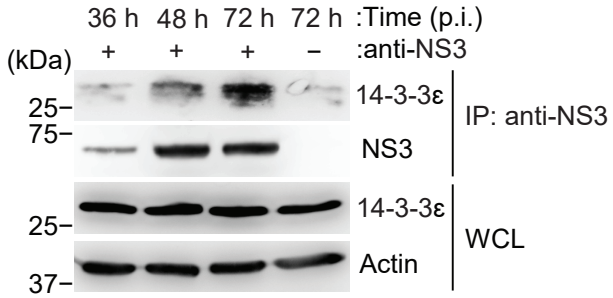
(B) Endogenous expression of intracellular innate immune sensor and signaling proteins in SVGA cells that were stimulated with IFN-α (1,000 U/mL) for 16 h or left unstimulated, determined by immunoblot (IB) with anti-RIG-I, anti-MDA5, anti-cGAS, anti-STING, anti-MAVS, anti-TBK1, and anti-Actin (loading control).

Data are representative of two (**A, B**) independent experiments.

A

Virus	# Sequences analyzed	# Sequences encoding (E/D) in position 3
ZIKV	673	673
WNV	1,798	1,797
DENV	3,280	3,279
HCV	2,132	0
TBEV	199	0
YFV	155	0
JEV	327	0

C



B

14-3-3ε
12 Unique Peptides (Total of 21)

K.LAEQAERYDEM*VESM*KK.V
K.LAEQAERYDEM*VESM*K.K
R.YDEM*VESM*K.K
K.KVAGM*DVELTVEER.N
K.VAGM*DVELTVEER.N
R.IISSIEQK.E
R.QM*VETELK.L
K.HLIPAANTGESK.V
R.YLAEFATGNDR.K
R.KEAAENSLVAYK.A
K.AASDIAM*TELPPTHPIR.L
K.EALQDVEDENQ.

14-3-3η
7 Unique Peptides (Total of 9)

K.AVTELNEPLSNEDRNLLSVAYK.N
K.AVTELNEPLSNEDR.N
R.YLAEVASGEKK.N
R.YLAEVASGEK.K
R.LAEQAERYDDM*ASAM*K.A
K.KNSVVEASEAAYK.E
K.NSVVEASEAAYK.E

Figure S2. Presence of a Rx(E/D)P motif in flavivirus NS3 as well as binding of ZIKV NS3 to 14-3-3ε and 14-3-3η, Related to Figure 2.

(A) Summary of sequence analysis of the indicated flavivirus NS3 proteins for the presence of a phosphomimetic (E or D) amino acid in position 3 of the Rx(E/D)P motif at amino acid position 64-67 in ZIKV NS3, or the equivalent position in other NS3s, using complete genomes of viruses from the NIAID Virus Pathogen Database and Analysis Resource (ViPR). Data for DENV NS3 have been published in (Chan and Gack, 2016). ZIKV, Zika virus; WNV, West Nile virus; DENV, dengue virus; HCV, hepatitis C virus; TBEV, tick-borne encephalitis virus; YFV, yellow fever virus; JEV, Japanese encephalitis virus.

(B) Unique peptides of 14-3-3ε and 14-3-3η identified by MS analysis to be in complex with purified FLAG-NS3 (ZIKV, H/PF/2013) from transiently transfected HEK 293T cells (related to data shown in Figure 2B).

(C) Interaction of endogenous 14-3-3ε with ZIKV NS3 in SVGA cells that were infected with ZIKV (BRA/2015; MOI 1) for the indicated times, determined by immunoprecipitation (IP) with anti-NS3, followed by IB with anti-14-3-3ε and anti-NS3. Whole cell lysates (WCLs) were further probed with anti-14-3-3ε and anti-Actin (loading control).

Data are representative of one (**B**) or three (**C**) independent experiments.

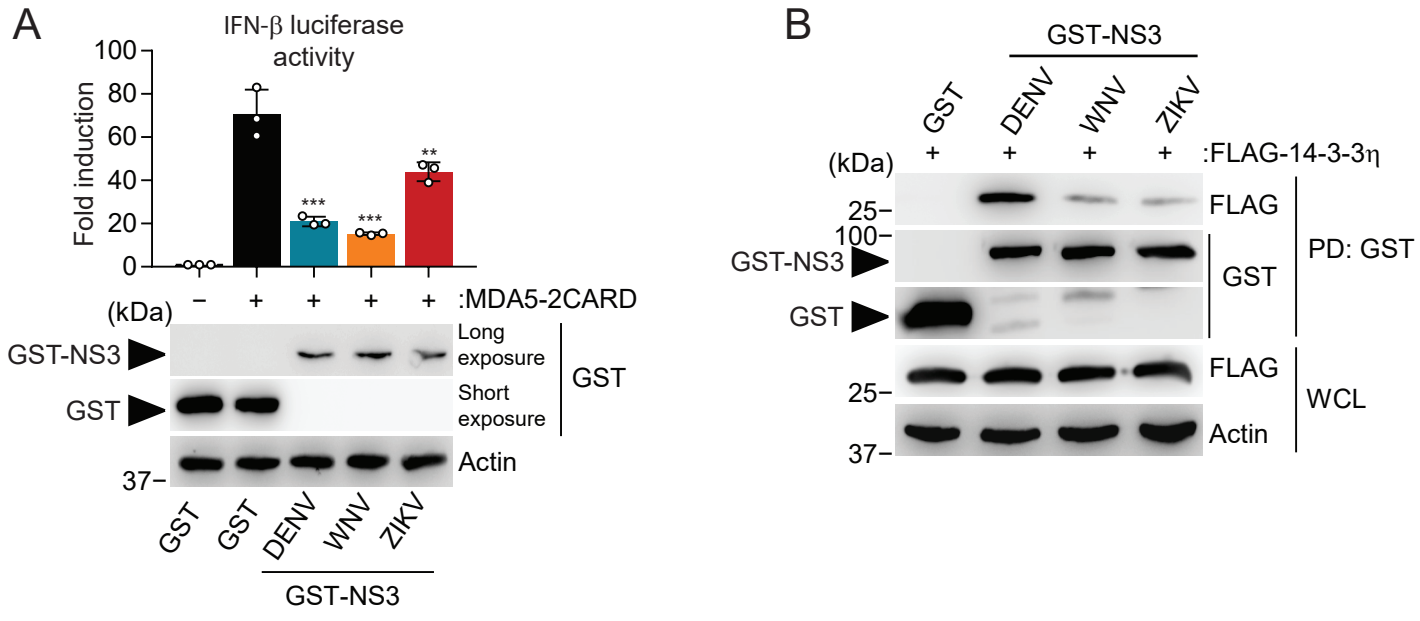


Figure S3. The NS3 proteins of DENV, WNV, and ZIKV bind to 14-3-3η and antagonize MDA5-mediated signaling, Related to Figure 4.

(A) Upper panel: IFN-β luciferase activity in HEK 293T cells transfected for 48 h with an IFN-β luciferase reporter plasmid together with MDA5-2CARD and GST (negative control) or GST-NS3 from DENV (NGC), WNV (NY99), or ZIKV (BRA/2015). Luciferase values were normalized to values for co-transfected β-galactosidase. Results are presented relative to those of GST-expressing cells, set as 1. Lower panel: IB analysis of WCLs with anti-GST and anti-Actin. Data are expressed as means ± SD (n = 3). **p < 0.001, ***p < 0.0001 (ANOVA).

(B) Interaction of GST (negative control) or GST-NS3 of DENV (NGC), WNV (NY99), or ZIKV (BRA/2015) with FLAG-14-3-3η in HEK 293T cells transfected for 42 h to express those proteins, determined by GST-PD and IB using anti-FLAG and anti-GST. WCLs were further probed with anti-FLAG and anti-Actin (loading control).

Data are representative of four (**A**) or three (**B**) independent experiments.

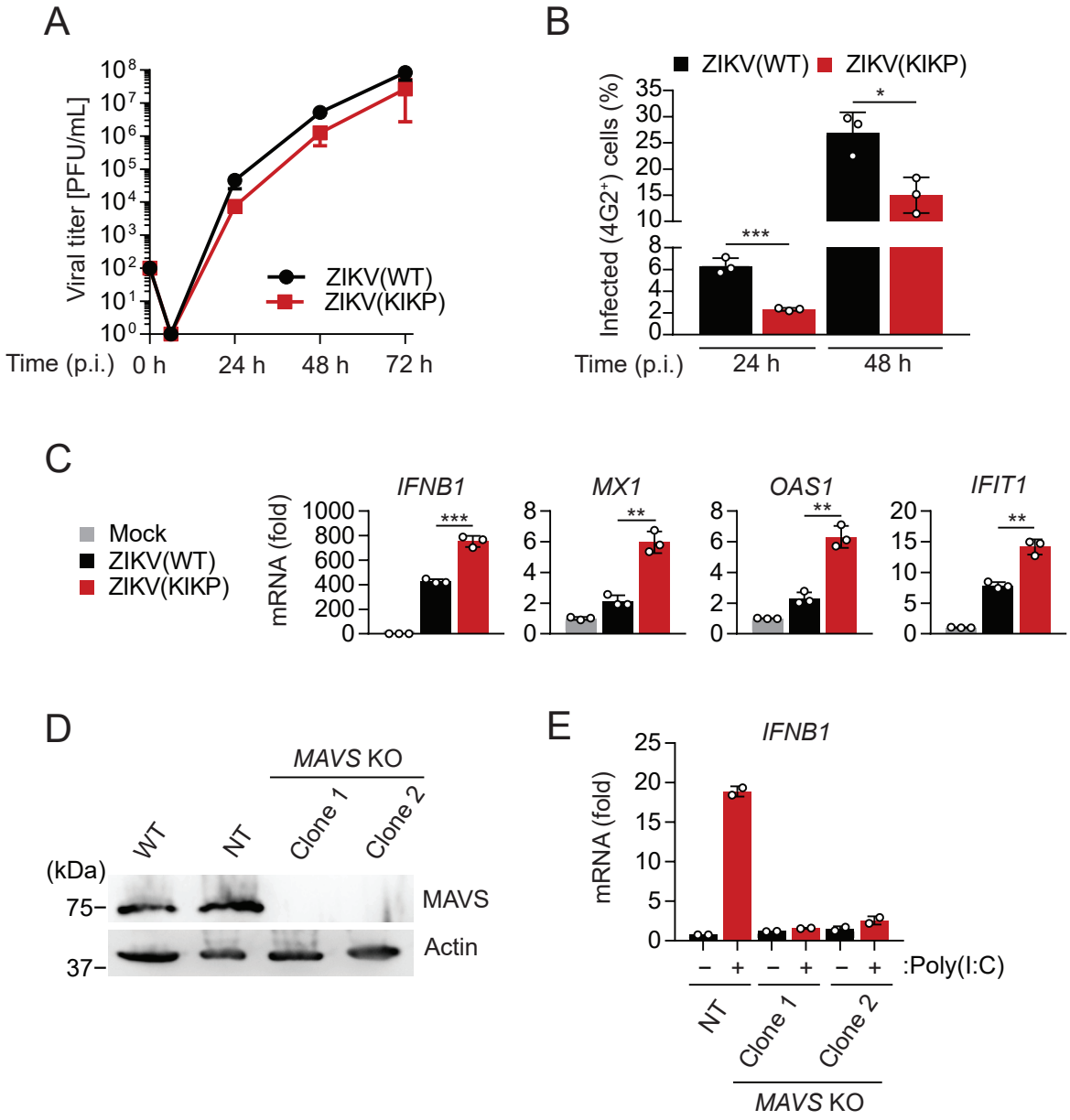


Figure S4. ZIKV(KIKP) replication and its ability to induce IFN/ISG expression as well as validation of *MAVS* KO SVGA cells, Related to Figure 5.

(A) Viral titers in the supernatant of Vero cells infected with ZIKV(WT) or ZIKV(KIKP) (MOI 0.001 for each) for the indicated times, determined by plaque assay (limit of detection: 10^2) and presented as PFU/mL ± SD (n = 3).

(B) Frequency of ZIKV-positive A549 cells that were infected for 24 h or 48 h with ZIKV(WT) or ZIKV(KIKP) (MOI 0.01 for each), assessed by flow cytometry using an anti-envelope (4G2) antibody. Data are presented as means ± SD (n = 3). *p < 0.05, ***p < 0.001 (Student's *t*-test).

(C) qRT-PCR analysis of the indicated transcripts (normalized to cellular *GAPDH*) in HMC3 cells that were mock-treated or infected with ZIKV(WT) or ZIKV(KIKP) (MOI 0.5 for each) for 48 h. Data are presented as means ± SD (n = 3). **p < 0.01, ***p < 0.001 (Student's *t*-test).

(D) Endogenous MAVS protein abundance in the WCLs of normal (WT), CRISPR nontargeting (NT) or *MAVS* KO SVGA cells (clones 1 and 2) used for the experiments shown in Figure 5F and 5G, determined by IB with anti-MAVS. WCLs were further probed with anti-Actin (loading control).

(E) Functional validation of *MAVS* KO SVGA cells (clones 1 and 2) by analyzing *IFNB1* transcript induction upon stimulation with 500 ng/mL HMW-poly(I:C)/Lyovec for 24 h using qRT-PCR. Data are expressed as means ± SD (n = 2).

Data are representative of three (**A, B**) or two (**C–E**) independent experiments.

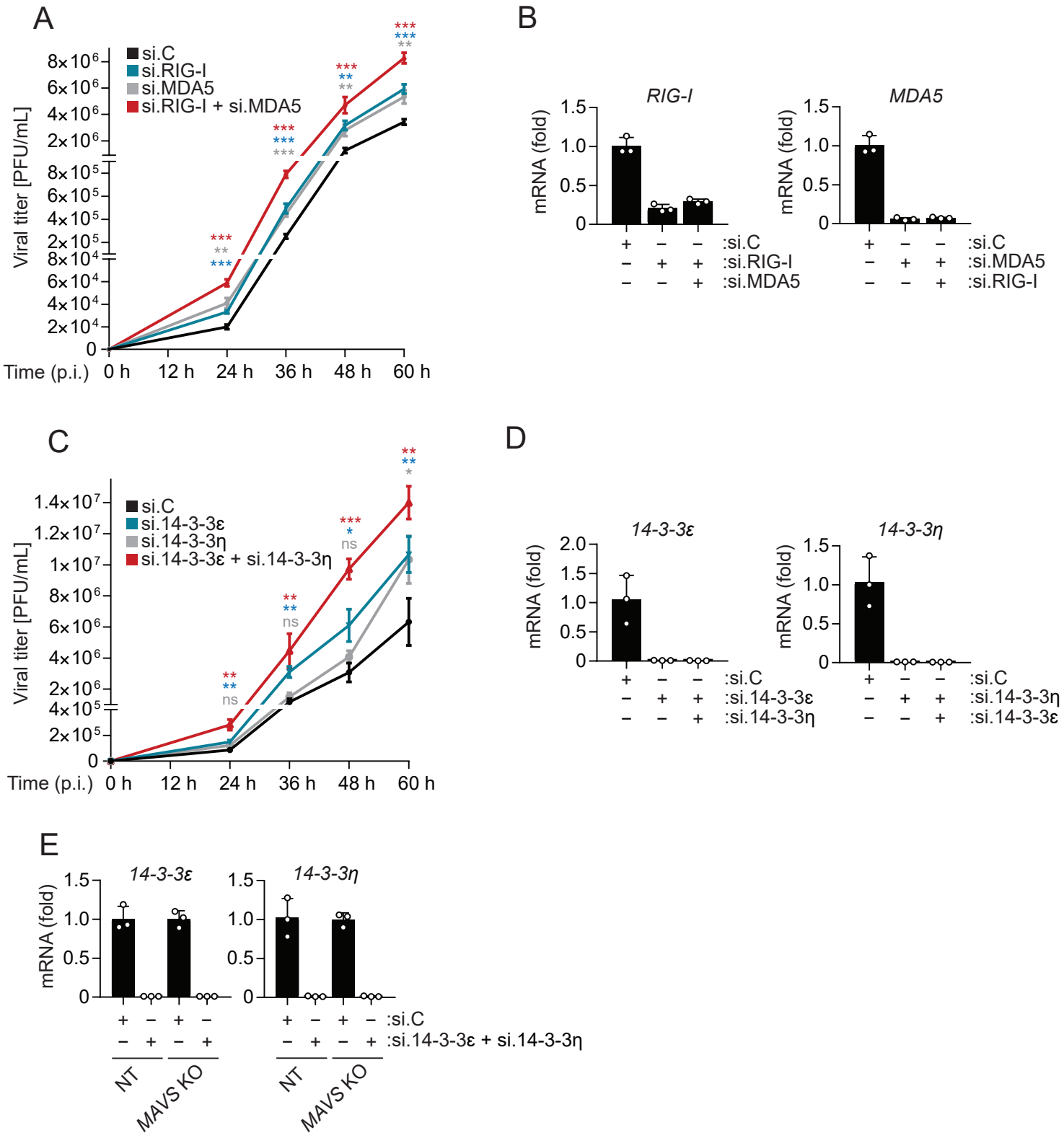


Figure S5. ZIKV replication in RLR or 14-3-3 protein knockdown SVGA cells, Related to Figure 5.

(A) Viral titers in the supernatant of SVGA cells transfected with the indicated siRNAs for 30 h and then infected with ZIKV(KIKP) (MOI 0.01) for the indicated times, determined by plaque assay and presented as PFU/mL ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (Student's *t*-test; pairwise comparisons to si.C).

(B) Representative knockdown efficiency of the indicated genes (normalized to cellular *GAPDH*) in SVGA cells at 36 h post-transfection with the indicated siRNAs for the experiment in (A), determined by qRT-PCR.

(C) Viral titers in the supernatant of SVGA cells transfected with the indicated siRNAs for 30 h and then infected with ZIKV(KIKP) (MOI 0.01) for the indicated times, determined by plaque assay and presented as PFU/mL ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (Student's *t*-test; pairwise comparisons to si.C). ns, not statistically significant.

(D) Representative knockdown efficiency of the indicated genes (normalized to cellular *GAPDH*) in SVGA cells at 36 h post-transfection with the indicated siRNAs for the experiment in (C), determined by qRT-PCR.

(E) Knockdown efficiency of the indicated genes at day 3 post-transfection for the experiment shown in Figure 5G, determined by qRT-PCR.

Data are expressed as means ± SD (n = 3) and are representative of two independent experiments.