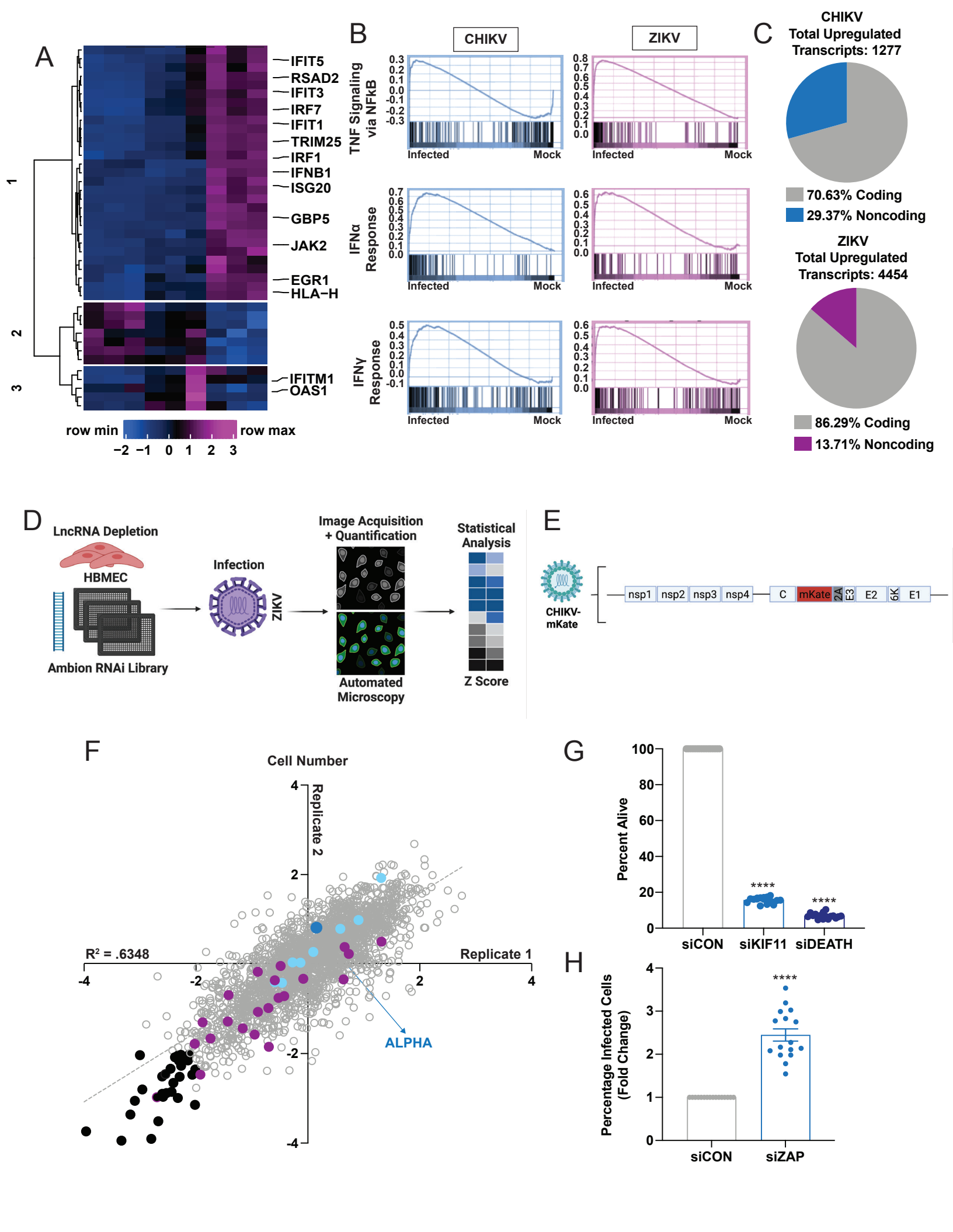
A
IFIT5
RSAD2
IFIT3
IRF7
IFIT1
TRIM25
IRF1
IFNB1
ISG20
GBP5
JAK2
EGR1
HLA−H
IFITM1
OAS1
1
2
3
row min
row max
−2 −1 0 1 2 3
B
CHIKV
ZIKV
TNF Signaling via NFkB
IFNα Response
IFNγ Response
Infected
Mock
C
CHIKV
Total Upregulated Transcripts: 1277
70.63% Coding
29.37% Noncoding
ZIKV
Total Upregulated Transcripts: 4454
86.29% Coding
13.71% Noncoding
D
LncRNA Depletion
HBMEC
Ambion RNAi Library
Infection
ZIKV
Image Acquisition + Quantification
Automated Microscopy
Statistical Analysis
Z Score
E
CHIKV-mKate
nsp1
nsp2
nsp3
nsp4
C
mKate
2A
E3
E2
6K
E1
F
Cell Number
Replicate 2
Replicate 1
R² = .6348
ALPHA
G
Percent Alive
siCON
siKIF11
siDEATH
****
H
Percentage Infected Cells (Fold Change)
siCON
siZAP
****

Figure S1, Related to Figure 1: High-throughput approaches reveal putative antiviral lncRNAs (A-C) HBMEC were uninfected or infected with CHIKV or ZIKV at MOI 5 for 24h in three independent experiments and subjected to total RNA-seq. Displayed are (A) Heat maps depicting IFN responsive gene expression with log_2 fold change greater than 1, read cutoff of 50, and adjusted p value of less than 0.05 in CHIKV and ZIKV-infected cells. (B) Gene set enrichment (GSEA) plots of enriched signatures induced by CHIKV or ZIKV in HBMEC. Y-axis represents enrichment score (ES). (C) Pie charts indicating the percentage of significantly upregulated transcripts which are coding vs. noncoding. Exact transcript numbers are shown. (D) Schematic describing the screen pipeline: a predesigned RNAi library targeting 2200 lncRNAs was transfected into HBMEC and knockdown was allowed to proceed for 3 days. The cells were then infected with CHIKV-mKate at MOI 0.05 for 24h, fixed and immunostained for nuclei (Hoechst). Images were acquired using automated microscopy and the percentage of cells infected was quantified. The screen was performed in duplicate and statistically analyzed using Z scores. (E) The structure of the CHIKV-mKate virus used in the screen is shown. (F) Cell number was measured using Hoechst staining and automated microscopy and Z scores were calculated. Data points with Z<-2 are highlighted in black and were removed from percent infection data shown in Figure 1B. The antiviral and proviral candidates from Figure 1B are highlighted in light blue and purple respectively. *ALPHA* is highlighted in dark blue. (G) siRNAs targeting the pro-mitotic *KIF11* and anti-apoptotic genes (siDEATH) were used as controls for RNAi efficiency within the screen. Cell number was measured by Hoechst staining and automated microscopy. (H) siRNAs targeting *ZAP* were included as a positive control for an antiviral effect. Percent infection was measured by automated microscopy. Data are presented as fold change relative to siCON. n=2-3; RNA-seq data are displayed as log_2 fold change with $p<0.05$. ****$p<0.001$; error bars represent S.E.M.; n=2; Statistical analyses were performed using one-way ANOVA with Dunnett correction for multiple comparisons (G), Student's (unpaired, two-tailed) *t* test (H).

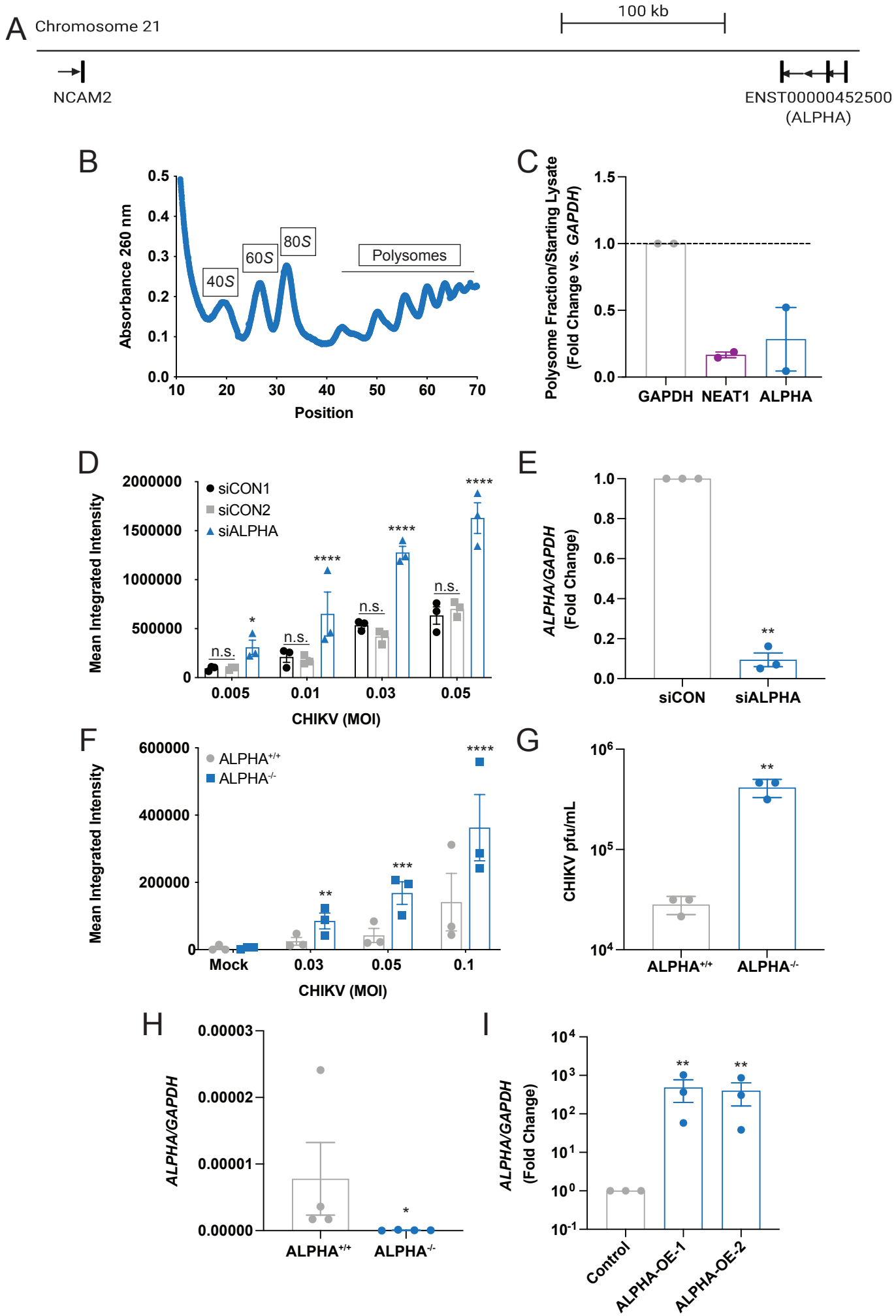
A
Chromosome 21
100 kb
NCAM2
ENST00000452500
(ALPHA)
B
Absorbance 260 nm
40S
60S
80S
Polysomes
Position
C
Polysome Fraction/Starting Lysate
(Fold Change vs. GAPDH)
GAPDH
NEAT1
ALPHA
D
Mean Integrated Intensity
siCON1
siCON2
siALPHA
n.s.
CHIKV (MOI)
E
ALPHA/GAPDH
(Fold Change)
siCON
siALPHA
F
Mean Integrated Intensity
ALPHA+/+
ALPHA-/-
Mock
CHIKV (MOI)
G
CHIKV pfu/mL
H
ALPHA/GAPDH
I
ALPHA/GAPDH
(Fold Change)
Control
ALPHA-OE-1
ALPHA-OE-2

Figure S2, Related to Figure 1: Characterization of *ALPHA* biology and function (A) The *ALPHA* locus is encoded in Chromosome 21 and is comprised of 3 exons spanning 42 kb transcribed in the antisense direction relative to the nearest coding gene. The most proximal coding gene is neural cell adhesion molecule (NCAM)2 which is ~500 kb away. (B) Polysome fractions were isolated from HBMEC using a sucrose gradient and collected using a Biocomp Piston Gradient Fractionator. The y-axis refers to A_{260} absorbance values and the x-axis refers to fraction position. (C) RNA was isolated from either unfractionated total lysate or polysome fractions (positions 45-70) and *GAPDH*, *NEAT1*, and *ALPHA* were quantified by qPCR. *GAPDH* was used a positive control for a translated mRNA and *NEAT1* was used as negative control for an untranslated ncRNA. Data are displayed as enrichment relative to total lysate. (D) *ALPHA* was depleted in HBMEC using pooled siRNAs and infected with CHIKV-mKate at the indicated MOIs for 24h. CHIKV protein was measured using immunofluorescence and automated microscopy. (E) Quantification of *ALPHA* in RNAi-depleted HBMEC by qPCR. (F-G) $ALPHA^{+/+}$ and $ALPHA^{-/-}$ HBMEC were (F) infected with CHIKV-mKate at the indicated MOIs for 24h and viral protein was quantified by immunofluorescence and automated microscopy or (G) infected with CHIKV MOI 0.2 for 30h and titers were quantified by $TCID_{50}$. *ALPHA* levels in (H) $ALPHA^{+/+}$ and $ALPHA^{-/-}$ HBMEC and (I) control and *ALPHA* overexpressing HBMEC as measured by qPCR. Data in (I) are represented as fold change vs. control cells. *GAPDH* was used as a loading control in all qPCR experiments unless otherwise specified. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; error bars are S.E.M.; n=2-4 as indicated; Statistical analyses were performed using two-way ANOVA with Sidak correction for multiple comparisons (D, F), Student's (unpaired, two-tailed) *t* test (E, G, H), one-way ANOVA with Dunnet correction for multiple comparisons (I).

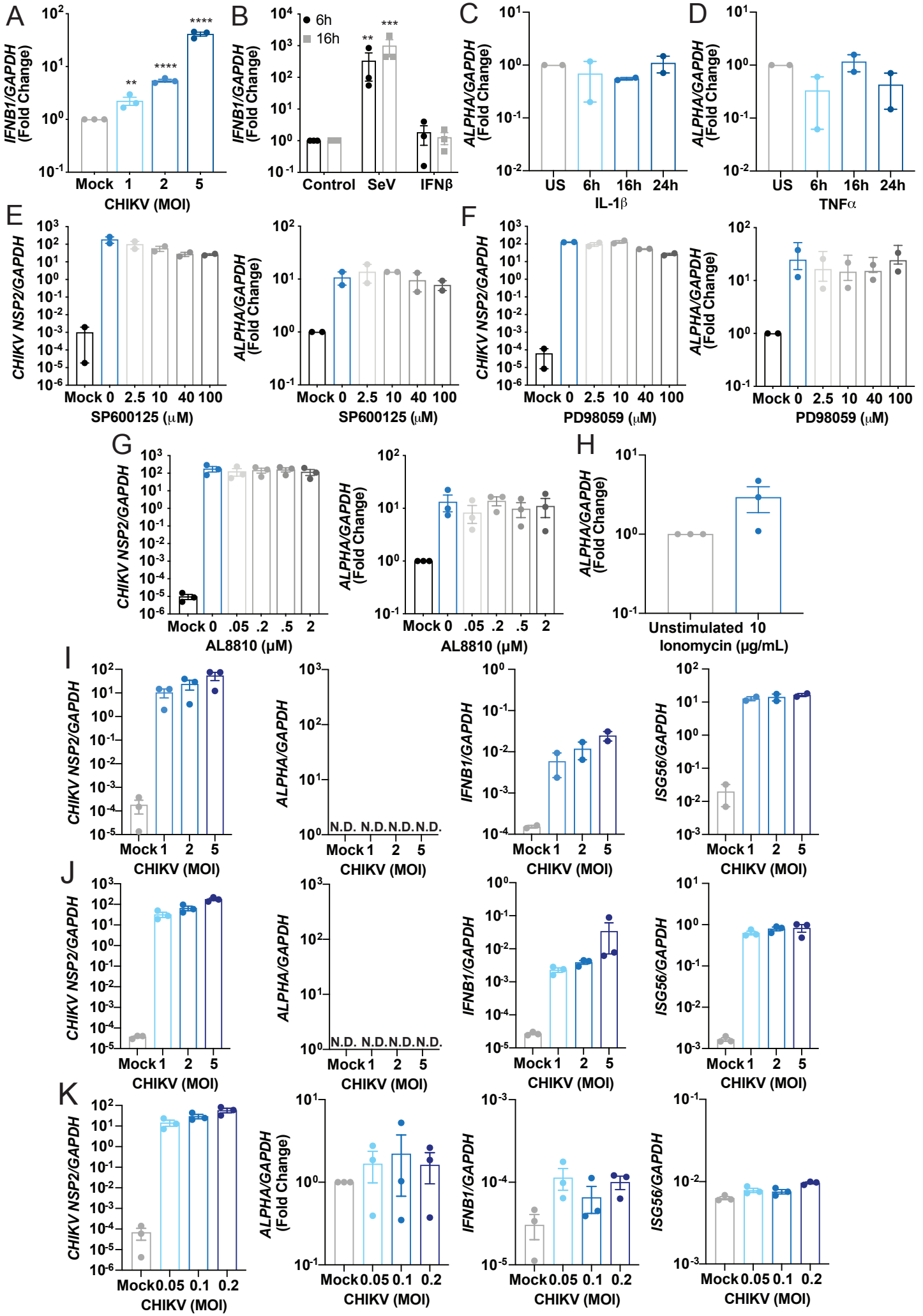
A
IFNB1/GAPDH (Fold Change)
Mock 1 2 5
CHIKV (MOI)
B
6h
16h
IFNB1/GAPDH (Fold Change)
Control SeV IFNβ
C
ALPHA/GAPDH (Fold Change)
US 6h 16h 24h
IL-1β
D
ALPHA/GAPDH (Fold Change)
US 6h 16h 24h
TNFα
E
CHIKV NSP2/GAPDH
Mock 0 2.5 10 40 100
SP600125 (μM)
ALPHA/GAPDH (Fold Change)
Mock 0 2.5 10 40 100
SP600125 (μM)
F
CHIKV NSP2/GAPDH
Mock 0 2.5 10 40 100
PD98059 (μM)
ALPHA/GAPDH (Fold Change)
Mock 0 2.5 10 40 100
PD98059 (μM)
G
CHIKV NSP2/GAPDH
Mock 0 .05 .2 .5 2
AL8810 (μM)
ALPHA/GAPDH (Fold Change)
Mock 0 .05 .2 .5 2
AL8810 (μM)
H
ALPHA/GAPDH (Fold Change)
Unstimulated 10
Ionomycin (μg/mL)
I
CHIKV NSP2/GAPDH
Mock 1 2 5
CHIKV (MOI)
ALPHA/GAPDH
N.D. N.D. N.D. N.D.
CHIKV (MOI)
IFNB1/GAPDH
CHIKV (MOI)
ISG56/GAPDH
CHIKV (MOI)
J
CHIKV NSP2/GAPDH
CHIKV (MOI)
ALPHA/GAPDH
N.D. N.D. N.D. N.D.
CHIKV (MOI)
IFNB1/GAPDH
CHIKV (MOI)
ISG56/GAPDH
CHIKV (MOI)
K
CHIKV NSP2/GAPDH
Mock 0.05 0.1 0.2
CHIKV (MOI)
ALPHA/GAPDH (Fold Change)
CHIKV (MOI)
IFNB1/GAPDH
CHIKV (MOI)
ISG56/GAPDH
CHIKV (MOI)

Figure S3, Related to Figure 2: CHIKV-induced *ALPHA* upregulation is independent of classical signaling pathways and is cell type-specific (A) HBMEC were infected with CHIKV for 24h at the indicated MOIs. *IFNB1* was measured by qPCR to assess activation of an innate immune response. Data are displayed as fold change vs. unstimulated cells. (B) HBMEC were treated with either Sendai virus (SeV, 100 HAU/mL) or recombinant IFNβ (10 ng/mL) for 6 or 16h and *IFNB1* was quantified by qPCR. Data are displayed as fold change vs. unstimulated cells. (C-D) HBMEC were stimulated with (C) IL-1β or (D) TNFα at 10 ng/mL for the indicated time points. *ALPHA* transcripts were measured by qPCR. Unstimulated cells are marked as US. (E-F) HBMEC were infected with CHIKV at MOI 5 for 24h and simultaneously treated with (E) SP600125 (JNK inhibitor) (F) PD98059 (MEK inhibitor) or (G) AL8810 ($PGF_{2\alpha}$ antagonist). CHIKV RNA and *ALPHA* were quantified in each of these experiments by qPCR. (H) Ionomycin (10 μg/mL) was used to stimulate Ca^{2+} signaling for 24h and *ALPHA* was quantified by qPCR. (I) Primary human peripheral blood monocytes, (J) A549 lung epithelial carcinoma cells, and (K) U2OS osteosarcoma cells were infected with CHIKV at the indicated MOIs for 24h. CHIKV RNA, *ALPHA*, *IFNB1*, and *ISG56* were quantified by qPCR. *ALPHA* expression is displayed as fold change relative to uninfected (mock) cells. *GAPDH* was used as a loading control in all experiments. **p<0.01, ***p<0.001, ****p<0.0001; error bars are S.E.M.; n=2-3 as indicated; Statistical analyses were performed using one-way ANOVA with Dunnett correction for multiple comparisons (A, G), two-way ANOVA with Tukey correction for multiple comparisons (B), Student's (unpaired, two-tailed) *t* test (H).

Fig S4

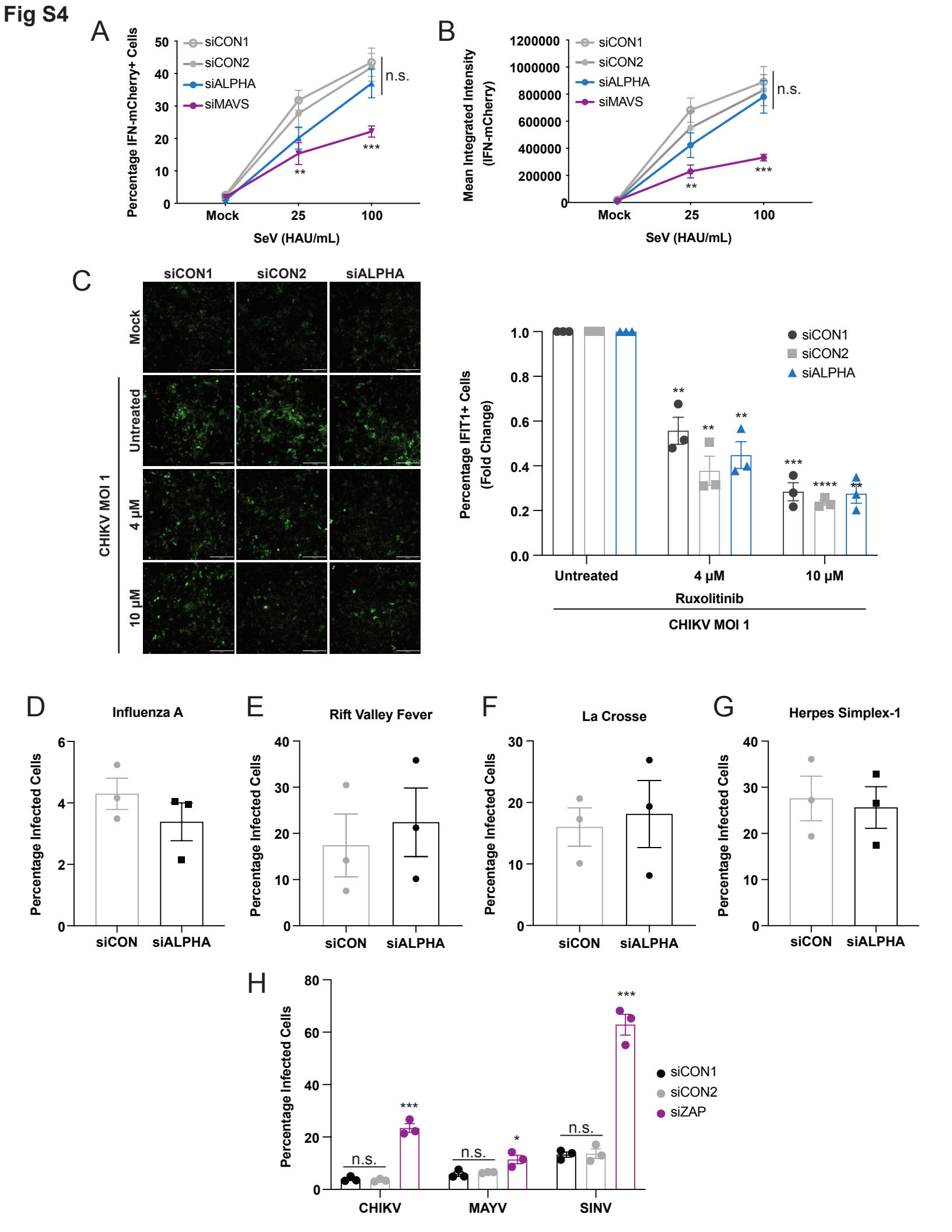
A
Percentage IFN-mCherry+ Cells
siCON1
siCON2
siALPHA
siMAVS
n.s.
**
***
Mock
25
100
SeV (HAU/mL)
B
Mean Integrated Intensity (IFN-mCherry)
siCON1
siCON2
siALPHA
siMAVS
n.s.
**
***
Mock
25
100
SeV (HAU/mL)
C
siCON1
siCON2
siALPHA
Mock
Untreated
4 μM
10 μM
CHIKV MOI 1
Percentage IFIT1+ Cells (Fold Change)
siCON1
siCON2
siALPHA
Untreated
4 μM
10 μM
Ruxolitinib
CHIKV MOI 1
D
Influenza A
Percentage Infected Cells
siCON
siALPHA
E
Rift Valley Fever
Percentage Infected Cells
siCON
siALPHA
F
La Crosse
Percentage Infected Cells
siCON
siALPHA
G
Herpes Simplex-1
Percentage Infected Cells
siCON
siALPHA
H
Percentage Infected Cells
n.s.
***
n.s.
*
n.s.
***
CHIKV
MAYV
SINV
siCON1
siCON2
siZAP

Figure S4, Related to Figures 2 and 3: *ALPHA* activity is IFN-independent and virus-specific
(A-B) HBMEC stably expressing an IFN-mCherry reporter were stimulated with SeV for 24h at the indicated concentrations following transfection with non-targeting siRNAs or siRNAs against *ALPHA* or *MAVS*. (A) Percent infection and (B) MFI were quantified by immunofluorescence and automated microscopy. (C) HBMEC were pre-treated for 2h with Ruxolitinib at the indicated concentrations followed by spin infection with CHIKV at MOI 1 for 24h. Representative images are shown (left). The percentage of IFIT1+ cells was quantified by immunofluorescence and automated microscopy (right). Data are displayed as fold change relative to the corresponding untreated control. Scale bars represent 200 μm. (D-G) Control or *ALPHA*-depleted HBMEC were infected with (D) Influenza A (IAV, MOI 0.02), (E) Rift Valley Fever Virus (RVFV, MOI 0.01), (F) La Crosse Virus (LACV, MOI 0.01), or (G) Herpes Simplex Virus 1-GFP (HSV-1-GFP, MOI 0.005). The percentage of infected cells was quantified by immunofluorescence and automated microscopy. (H) Control or *ZAP*-depleted HBMEC were infected with CHIKV (MOI 0.03), MAYV (MOI 0.1), or SINV (MOI 0.1) for 24h. The percentage of infected cells was quantified by immunofluorescence and automated microscopy. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; error bars are S.E.M.; n=3; Statistical analyses were performed using two-way ANOVA with Tukey correction for multiple comparisons. Significance shown is relative to siCON1 (A, B), Student's (unpaired, two-tailed) *t* test with Holm-Sidak correction for multiple comparisons (C, H), Student's (unpaired, two-tailed) *t* test (D-G).

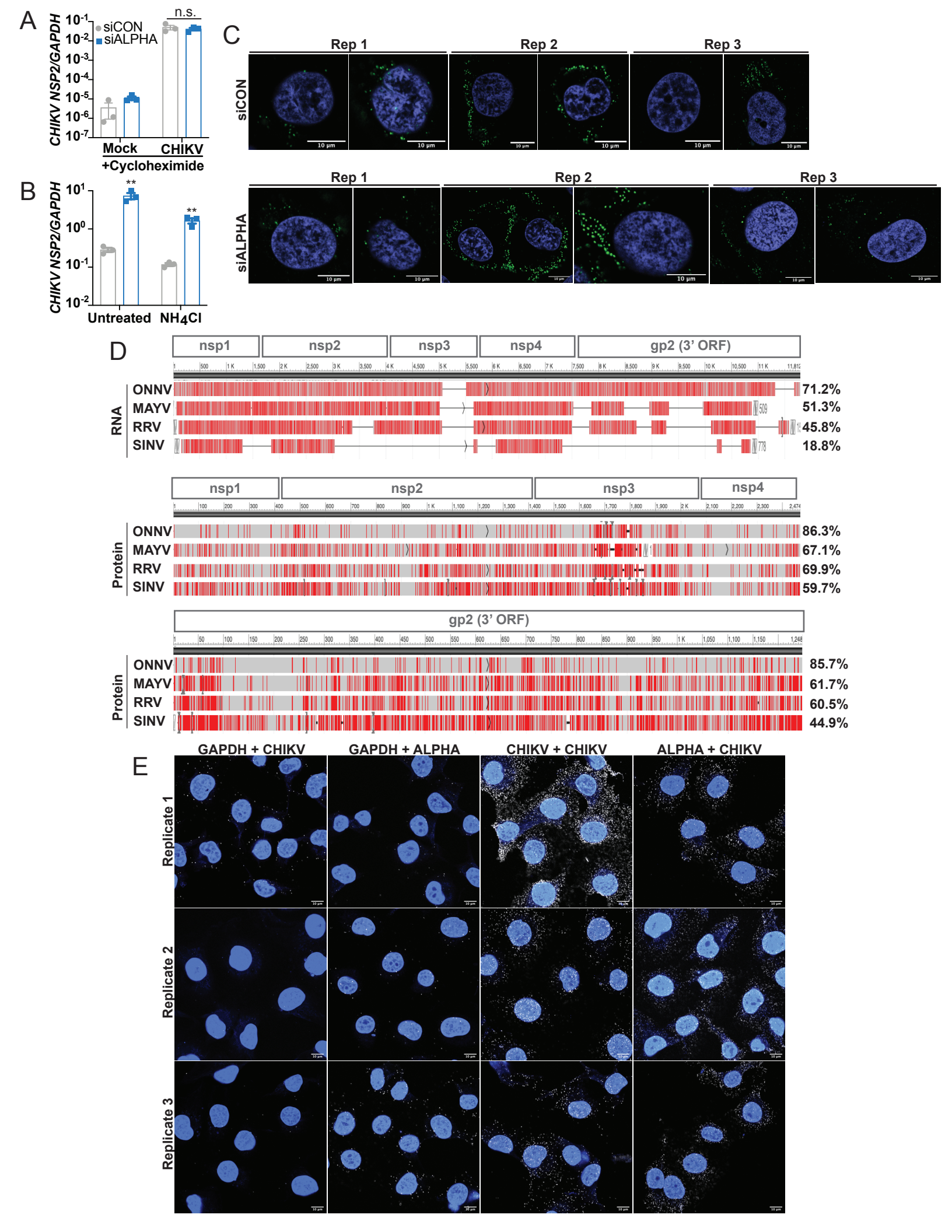
A
CHIKV NSP2/GAPDH
siCON
siALPHA
n.s.
Mock
CHIKV
+Cycloheximide
B
CHIKV NSP2/GAPDH
**
**
Untreated
NH4Cl
C
Rep 1
Rep 2
Rep 3
siCON
siALPHA
D
nsp1
nsp2
nsp3
nsp4
gp2 (3' ORF)
RNA
ONNV 71.2%
MAYV 51.3%
RRV 45.8%
SINV 18.8%
Protein
ONNV 86.3%
MAYV 67.1%
RRV 69.9%
SINV 59.7%
Protein
ONNV 85.7%
MAYV 61.7%
RRV 60.5%
SINV 44.9%
E
GAPDH + CHIKV
GAPDH + ALPHA
CHIKV + CHIKV
ALPHA + CHIKV
Replicate 1
Replicate 2
Replicate 3

Figure S5, Related to Figure 4: *ALPHA* does not regulate CHIKV entry or spread and interacts with CHIKV RNA in the cytoplasm (A) Control and *ALPHA*-depleted HBMEC were infected with CHIKV at MOI 20 for 4h in the presence of cycloheximide (10 μg/mL), extracellular virions were removed by trypsinization and intracellular CHIKV RNA was quantified by qPCR. (B) Control and *ALPHA*-depleted HBMEC were infected with CHIKV at MOI 0.05 for 4h followed by addition of either vehicle control or ammonium chloride (NH_4Cl) for 24h. Viral RNA levels were quantified by qPCR. *GAPDH* was used as a loading control for (A) and (B). (C) Additional representative images for each independent experiment in Figure 4A are shown. Scale bars represent 10 μm. (D) RNA and protein sequence alignments between related alphaviruses CHIKV (Ross), ONNV (SG650), MAYV (BeH407), RRV (T48), and SINV (Girdwood) generated using megaBLAST. The dark gray line at the top represents the CHIKV Ross reference sequence. Light gray rectangles represent regions of detected homology. Red lines demarcate nucleotides or amino acids which differ from CHIKV Ross. Thin black horizontal lines represent regions where there is no detectable homology. Percent identity compared to CHIKV Ross is indicated on the left. (E) HBMEC were infected with CHIKV at MOI 5 for 24h and subjected to PLA. Additional images corresponding to main Figure 4C-D from all three replicates are shown. Scale bars represent 10 μm. **$p<0.01$; error bars are S.E.M.; n=3; Statistical analyses were performed using two-way ANOVA with Sidak correction for multiple comparisons (A), Student's (unpaired, two-tailed) *t* test with Holm-Sidak correction for multiple comparisons (B).

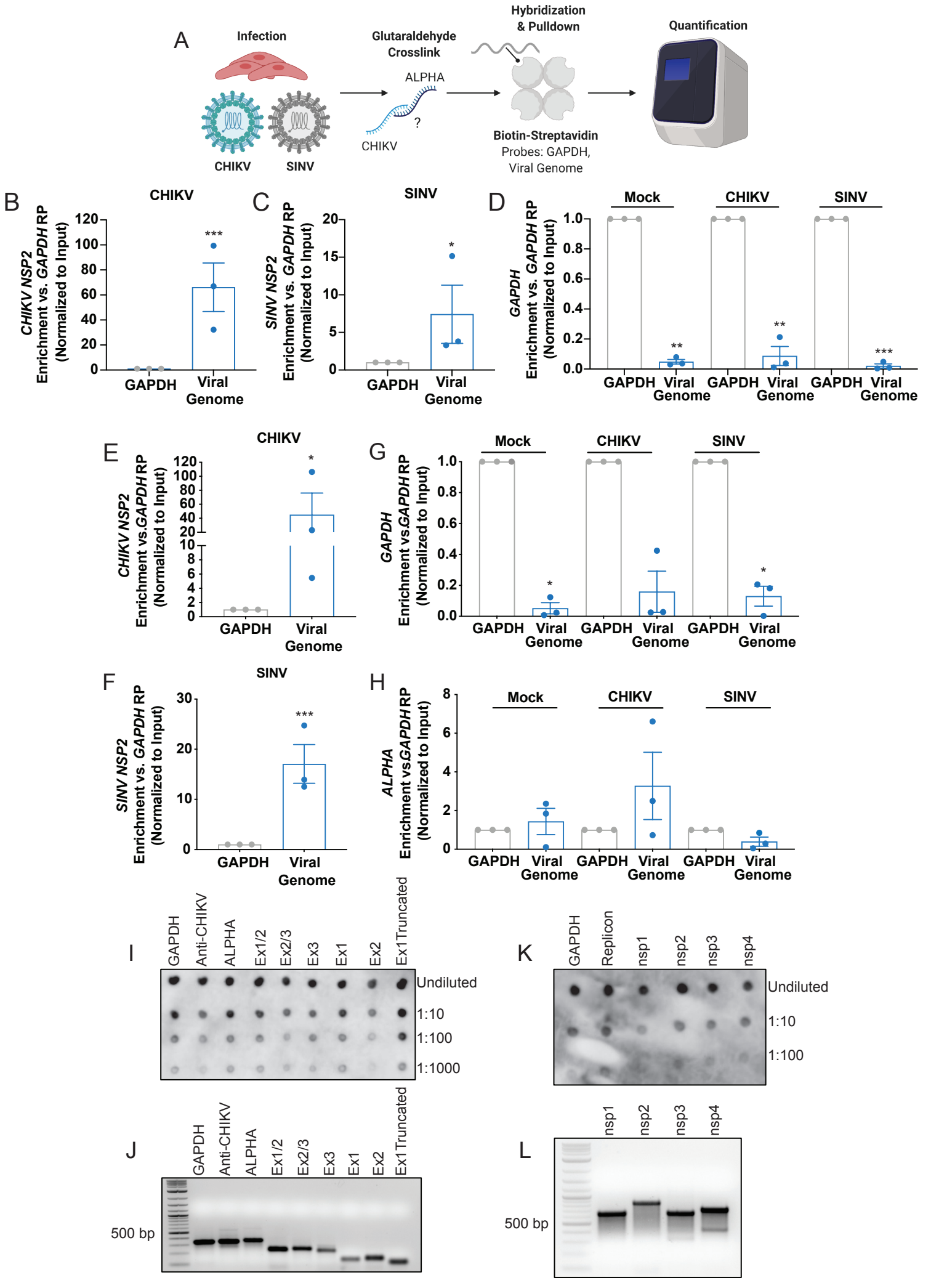
A
Infection
CHIKV
SINV
Glutaraldehyde Crosslink
ALPHA
?
CHIKV
Hybridization & Pulldown
Biotin-Streptavidin Probes: GAPDH, Viral Genome
Quantification
B
CHIKV
CHIKV NSP2 Enrichment vs. GAPDH RP (Normalized to Input)
GAPDH
Viral Genome
C
SINV
SINV NSP2 Enrichment vs. GAPDH RP (Normalized to Input)
D
Mock
CHIKV
SINV
GAPDH Enrichment vs. GAPDH RP (Normalized to Input)
E
CHIKV
CHIKV NSP2 Enrichment vs.GAPDH RP (Normalized to Input)
G
GAPDH Enrichment vs.GAPDH RP (Normalized to Input)
F
SINV
SINV NSP2 Enrichment vs. GAPDH RP (Normalized to Input)
H
ALPHA Enrichment vs.GAPDH RP (Normalized to Input)
I
GAPDH
Anti-CHIKV
ALPHA
Ex1/2
Ex2/3
Ex3
Ex1
Ex2
Ex1Truncated
Undiluted
1:10
1:100
1:1000
K
GAPDH
Replicon
nsp1
nsp2
nsp3
nsp4
Undiluted
1:10
1:100
J
500 bp
L
500 bp

Figure S6, Related to Figures 4 and 5: *ALPHA* interacts with CHIKV RNA in cells and *in vitro* (A) *ALPHA*-overexpressing HBMEC were left uninfected or infected with either CHIKV and SINV and crosslinked with glutaraldehyde. Lysates were generated and hybridized with 500mer, biotinylated, antisense, oligonucleotide probes followed by enrichment with streptavidin-conjugated beads. The relative enrichment of (B) CHIKV RNA, (C) SINV RNA, and (D) *GAPDH* were measured by qPCR. (E-H) Wildtype HBMEC were uninfected or infected with CHIKV or SINV and crosslinked with glutaraldehyde. Lysates were generated and hybridized with 500mer, biotinylated, antisense, oligonucleotide probes followed by enrichment with streptavidin-conjugated beads. The relative enrichment of (E) CHIKV RNA, (F) SINV RNA, (G) *GAPDH*, and (H) *ALPHA* were measured by qPCR. (I) Streptavidin dot blot demonstrating the relative biotinylation levels of each RNA used in the *in vitro* RNA interaction assays shown in Figure 5B-D. 200 ng of each RNA was spotted in the top row and diluted as indicated. (J) Agarose gel showing the sizes of each RNA used in the *in vitro* RNA interaction assays shown in Figure 5B-D. 1.5 μg of each RNA was visualized. (K) Streptavidin dot blot demonstrating the relative biotinylation levels of each RNA used in the *in vitro* RNA interaction assays shown in Figure 5E-F. 500 ng of replicon was used. Equal molar ratios relative to the replicon RNA were used for the remaining RNAs and diluted as indicated. (L) Agarose gel showing the sizes of each individual nsp RNA used in the *in vitro* RNA interaction assays shown in Figure 5E-H. 2.5 μg of each RNA was visualized. *p<0.05, **p<0.01, ***p<0.001; error bars are S.E.M.; n=3; Statistical analyses were performed using Student's (unpaired, two-tailed) *t* test (B, C, E, F), one-way ANOVA with Sidak correction for multiple comparisons (D, G, H).

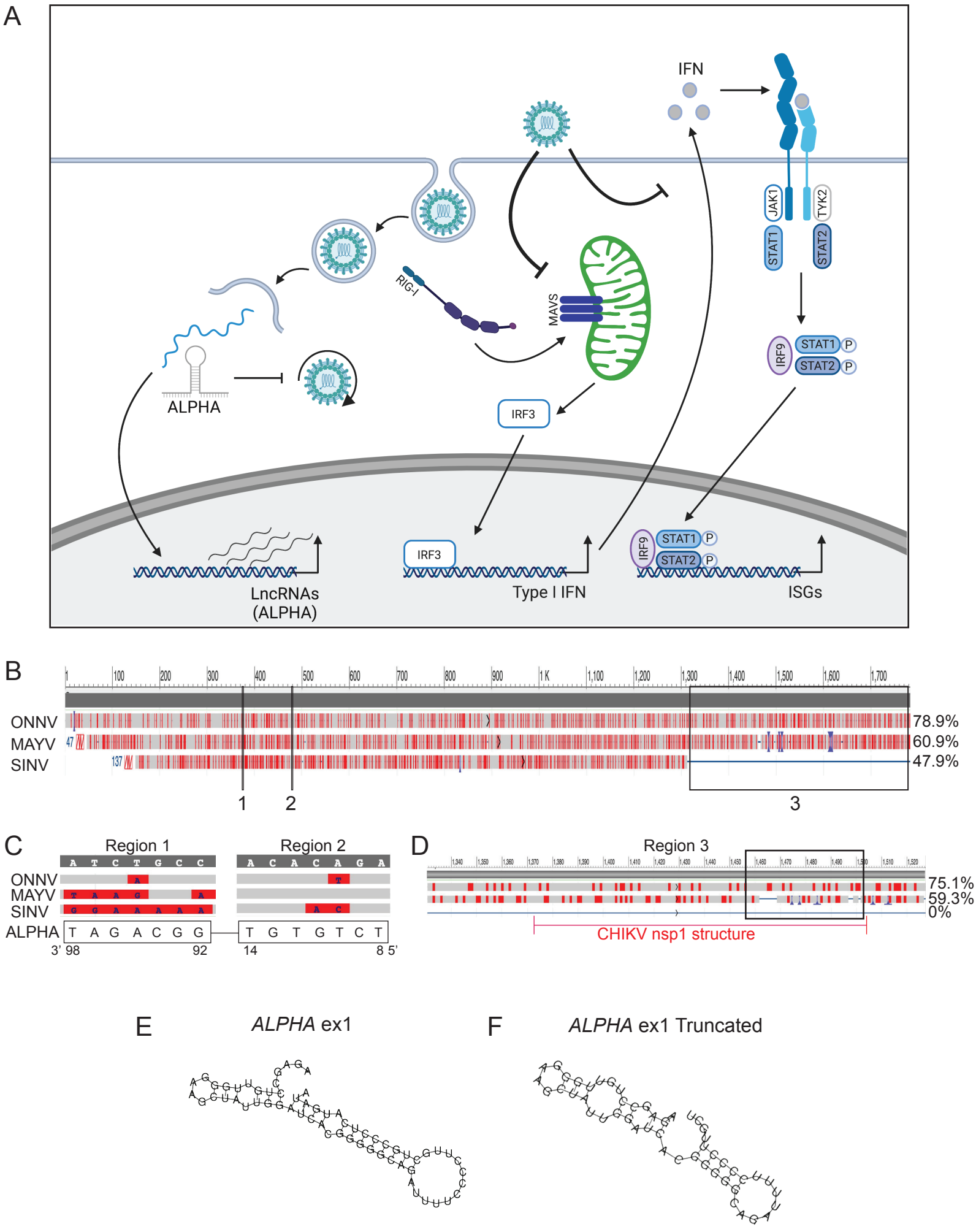
A
IFN
JAK1
TYK2
STAT1
STAT2
RIG-I
MAVS
IRF9
STAT1 P
STAT2 P
ALPHA
IRF3
LncRNAs
(ALPHA)
Type I IFN
ISGs
B
ONNV
MAYV
SINV
78.9%
60.9%
47.9%
1
2
3
C
Region 1
Region 2
ONNV
MAYV
SINV
ALPHA
T A G A C G G
T G T G T C T
3' 98
92
14
8 5'
D
Region 3
75.1%
59.3%
0%
CHIKV nsp1 structure
E
ALPHA ex1
F
ALPHA ex1 Truncated

Figure S7, Related to Figure 5 and Discussion: The functional role of *ALPHA* in innate immunity against CHIKV. (A) Schematic illustrating *ALPHA*'s antiviral function against CHIKV. CHIKV infection results in the activation of classical innate signaling pathways including IFN. However, this response is antagonized by CHIKV. In parallel, many lncRNAs are induced by CHIKV including *ALPHA*, which localizes to the cytoplasm and directly interacts with CHIKV genomic RNA to prohibit viral replication independently of IFN. (B-D) Genomic alignments of the nsp1 gene between related alphaviruses using megaBLAST. (B) Full-length nsp1 is shown with black boxes demarcating putative *ALPHA* binding regions: 1) nucleotides 380-386, 2) nucleotides 484-490, and 3) the 3' end of nsp1. (C) Zoomed in schematics of regions 1 and 2. The complementary sequence withing the *ALPHA* sequence is detailed in the black boxes in the 3' to 5' direction with nucleotide numbers indicated (D) Zoomed in schematic of region 3. The structural element described in Madden, et al. between nucleotides 1377-1506 is demarcated by a red line. Gaps in the MAYV genome are indicated by black lines and highlighted by a black box. (E-F) Secondary structure predications generated using RNAfold for (E) full-length *ALPHA* ex1 and (F) truncated *ALPHA* ex1.