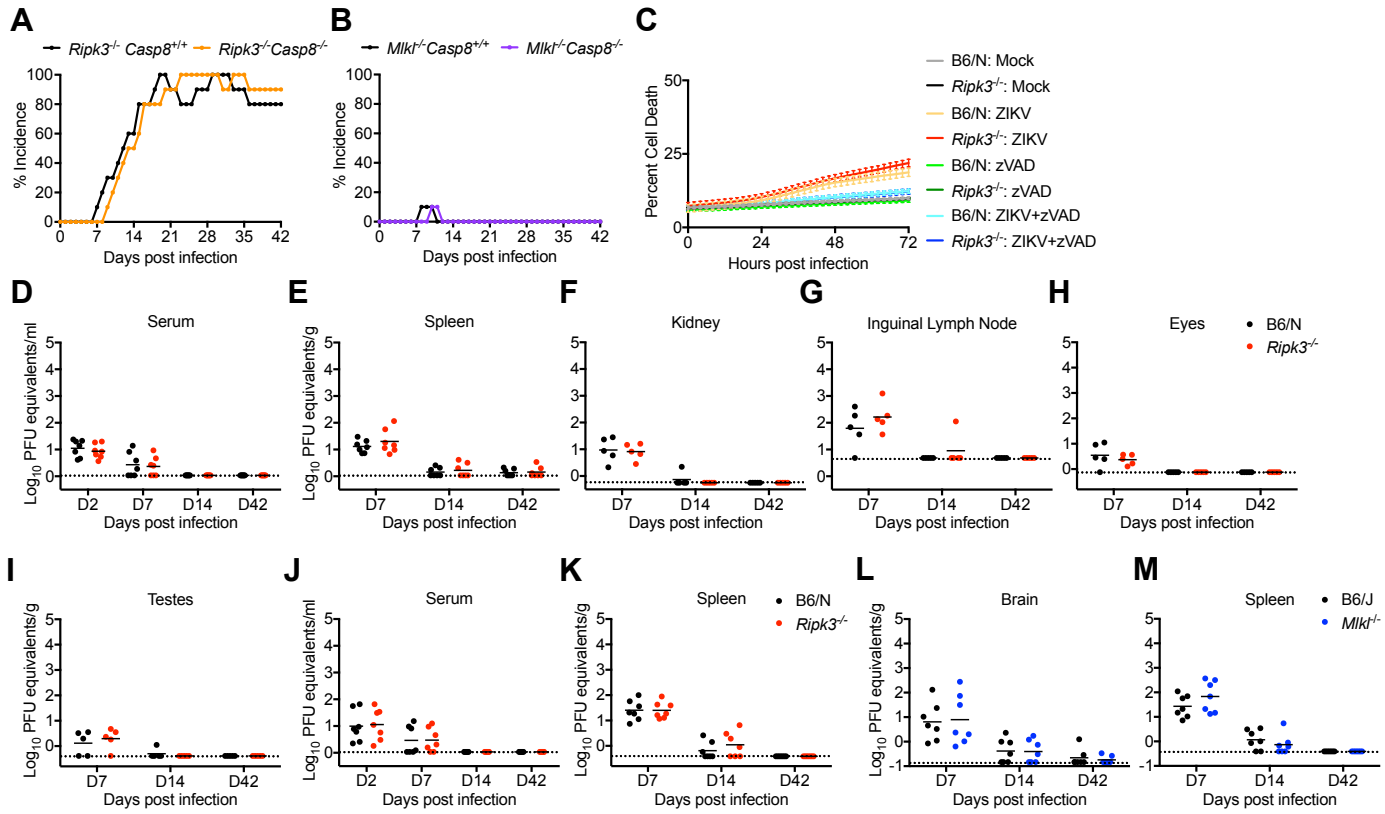

Figure S1 (Related to Figure 1): RIPK3 does not induce cell death during ZIKV infection and is dispensable for virologic control in peripheral tissues.

(A-B) Percentages of 5-6 week old *Ripk3*$^{-/-}$ *Casp8*$^{-/-}$ (A) or *Mlkl*$^{-/-}$ *Casp8*$^{-/-}$ (B) mice and *Casp8*$^{+/+}$ littermate controls exhibiting clinical signs of paresis following subcutaneous inoculation with 10^3 PFU ZIKV-MR766. Signs of paresis included loss of tail tone, impaired righting reflex, irregular gait, and/or kinetic tremor. N= 10 mice/genotype.

(C) Kinetics of cell death in primary cerebral cortical neuron cultures generated from *Ripk3*$^{-/-}$ mice or congenic WT controls. Cultures were infected with 0.1 MOI ZIKV-MR766 with or without cotreatment with the pan-caspase inhibitor zVAD. Cell death was identified by Sytox Green incorporation. N= 3 independent replicates.

(D-I) 5-6 week old B6/N or *Ripk3*$^{-/-}$ mice were infected subcutaneously with 10^3 PFU ZIKV-MR766. On indicated days following infection, the indicated tissues were harvested, weighed, homogenized, and assayed for ZIKV titers via qRT-PCR. qRT-PCR data are normalized against a standard curve of known viral titers to generate PFU equivalents.

(J-K) 5-6 week old B6/N or *Ripk3*$^{-/-}$ mice were infected subcutaneously with 10^3 PFU ZIKV-Fortaleza. Viral RNA levels in indicated tissues were assessed as in (D-I).

(L-M) 5-6 week old B6/J or *Mlkl*$^{-/-}$ mice were infected subcutaneously with 10^3 PFU ZIKV-MR766. Viral RNA levels in indicated tissues were assessed as in (D-I).

Error bars represent SEM. Dotted lines indicate limits of detection. All data are pooled from two or three independent experiments.

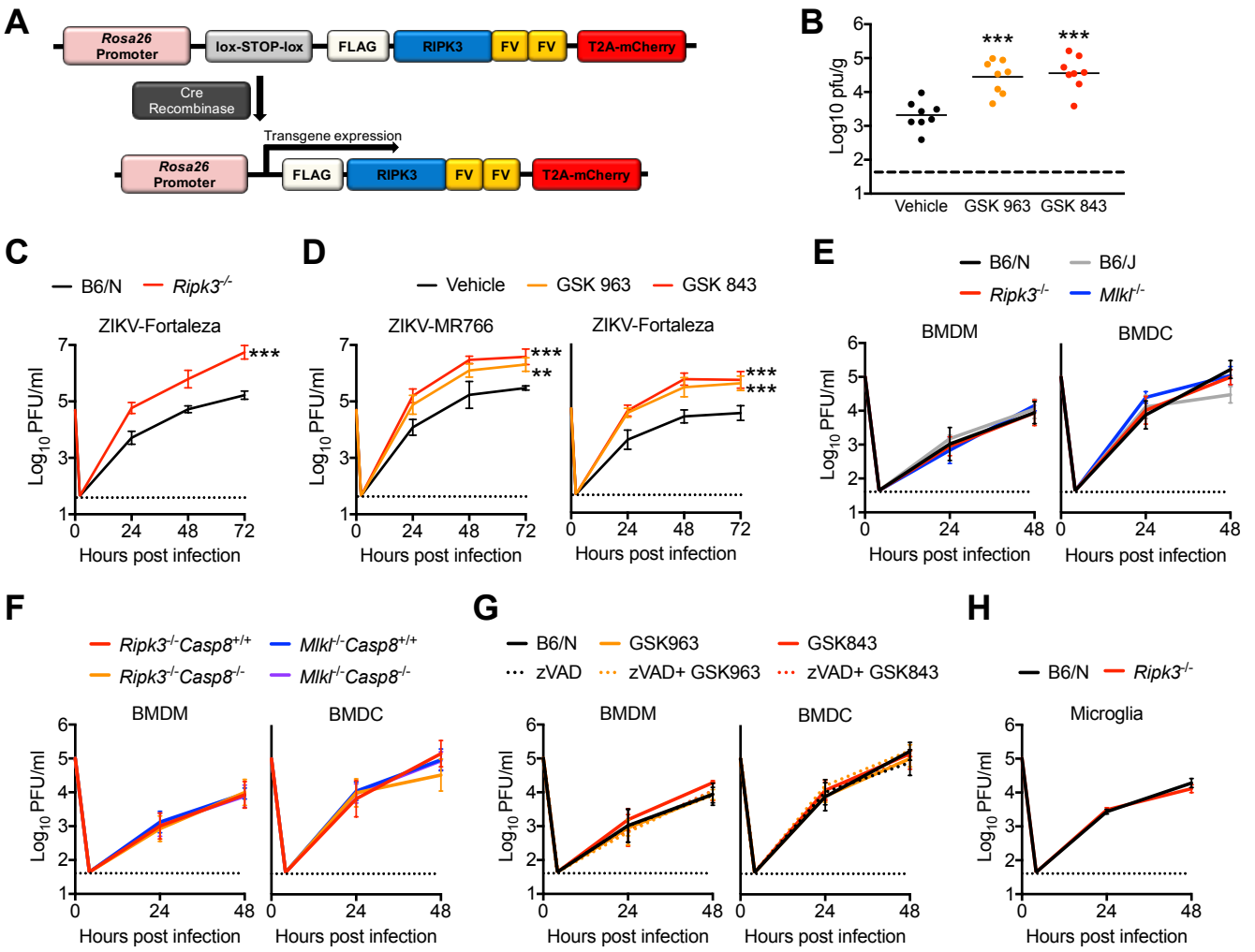

Figure S2 (Related to Figure 2): RIPK signaling restricts ZIKV infection in neurons, but not myeloid cells.

(A) Schematic of *Ripk3*-2xFV transgene.

(B) 5-6 week old WT (B6/N) mice were infected intracranially with 10^3 PFU ZIKV-MR766. Viral inoculum was delivered concurrently with the RIPK1 inhibitor GSK 963, the RIPK3 inhibitor GSK 843, or a DMSO vehicle solution. Two days following infection, whole brains were harvested, weighed, homogenized, and assayed for ZIKV titers via plaque assay.

(C) Multistep growth curve analysis in B6/N or *Ripk3*$^{-/-}$ cultures of primary cerebral cortical neurons following infection with ZIKV-Fortaleza (MOI 0.1). N= 6 independent replicates.

(D) Multistep growth curve analysis in WT B6/J cultures of primary cerebral cortical neurons pretreated for 2h with GSK 963, GSK 843, or a DMSO vehicle solution prior to infection with indicated strain of ZIKV. (MOI 0.1). N= 6 independent replicates.

(E-F) Multistep growth curve analysis in bone marrow derived macrophage (BMDM) and dendritic cell (BMDC) cultures of the indicated genotypes following infection with ZIKV-MR766 (MOI 1.0). N= 3 independent replicates.

(G) Multistep growth curve analysis in BMDM and BMDC cultures receiving 2h pretreatment with the indicated RIPK inhibitors and/or the pan-caspase inhibitor zVAD prior to infection with ZIKV-MR766 (MOI 1.0). N= 3 independent replicates.

(H) Multistep growth curve analysis in primary microglial cultures generated from *Ripk3*$^{-/-}$ mice or congenic WT controls (MOI 1.0). N= 6 independent replicates.

**p<0.01, ***p<0.001. Error bars represent SEM. All data are pooled from two or three independent experiments.

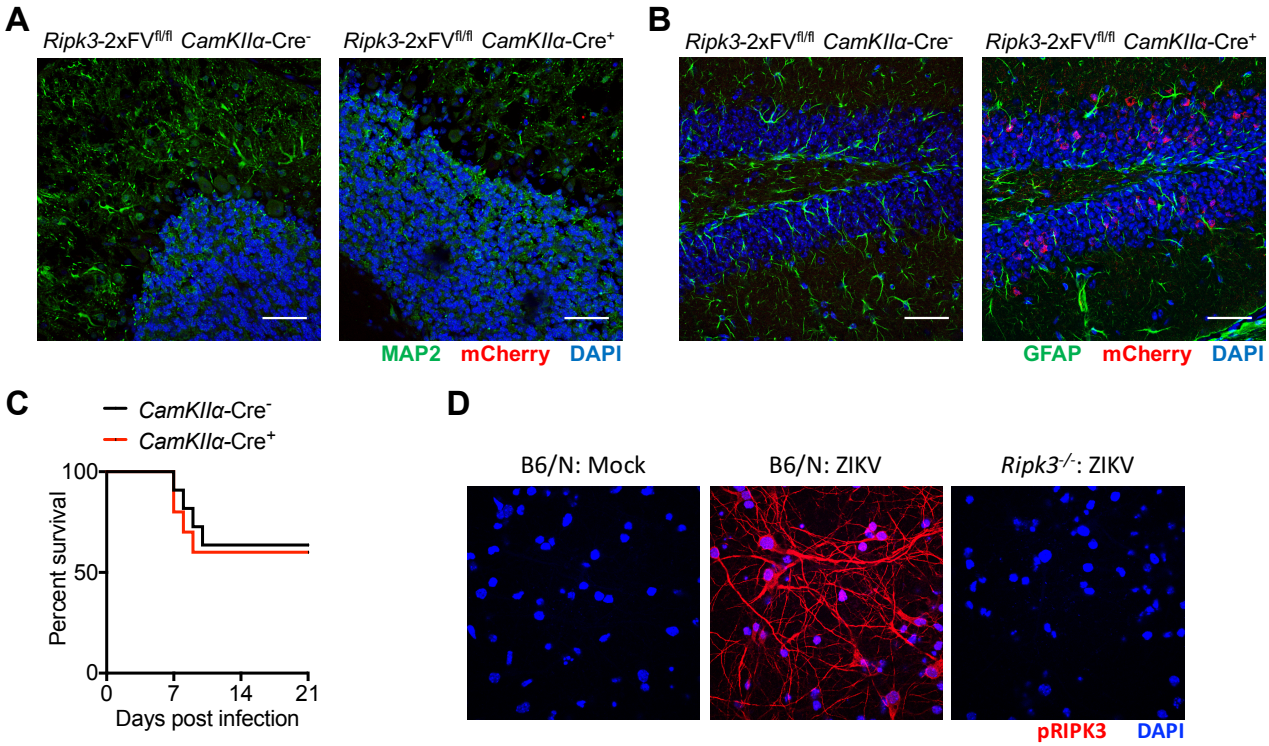

Figure S3 (Related to Figures 3 and 4): *Ripk3*-2xFV$^{fl/fl}$ *CamkIIα*-Cre^{+} mice do not exhibit Cre-mediated recombination in the hindbrain or in GFAP^{+} cells.

(A) Fluorescent immunohistochemical detection of mCherry (red) and the neuronal marker MAP2 (green) in cerebella of a *Ripk3*-2xFV$^{fl/fl}$ *CamkIIα*-Cre^{+} mouse and a Cre^{-} littermate control.

(B) Fluorescent immunohistochemical detection of mCherry (red) and the astroglial marker GFAP (green) in hippocampi of a *Ripk3*-2xFV$^{fl/fl}$ *CamkIIα*-Cre^{+} mouse and a Cre^{-} littermate control.

Images in (A-B) are representative of at least 2 images acquired per brain region from each of 3 independent mice per genotype. Nuclei are stained with DAPI (blue). Scale bar: 50μm.

(C) Survival analysis in 5-6 week old *CamKIIα*-Cre^{+}mice and littermate Cre^{-} controls following intracranial inoculation with 10^{3} PFU ZIKV-MR766. N= 10-11 mice/genotype.

(D) Fluorescent immunocytochemical detection of phosphorylated RIPK3 (red) in primary cerebral cortical neuron cultures isolated from B6/N or *Ripk3*$^{-/-}$ mice 6h following infection with ZIKV-MR766 (MOI 10.0). Images are representative of at least 3 independent replicates per condition.

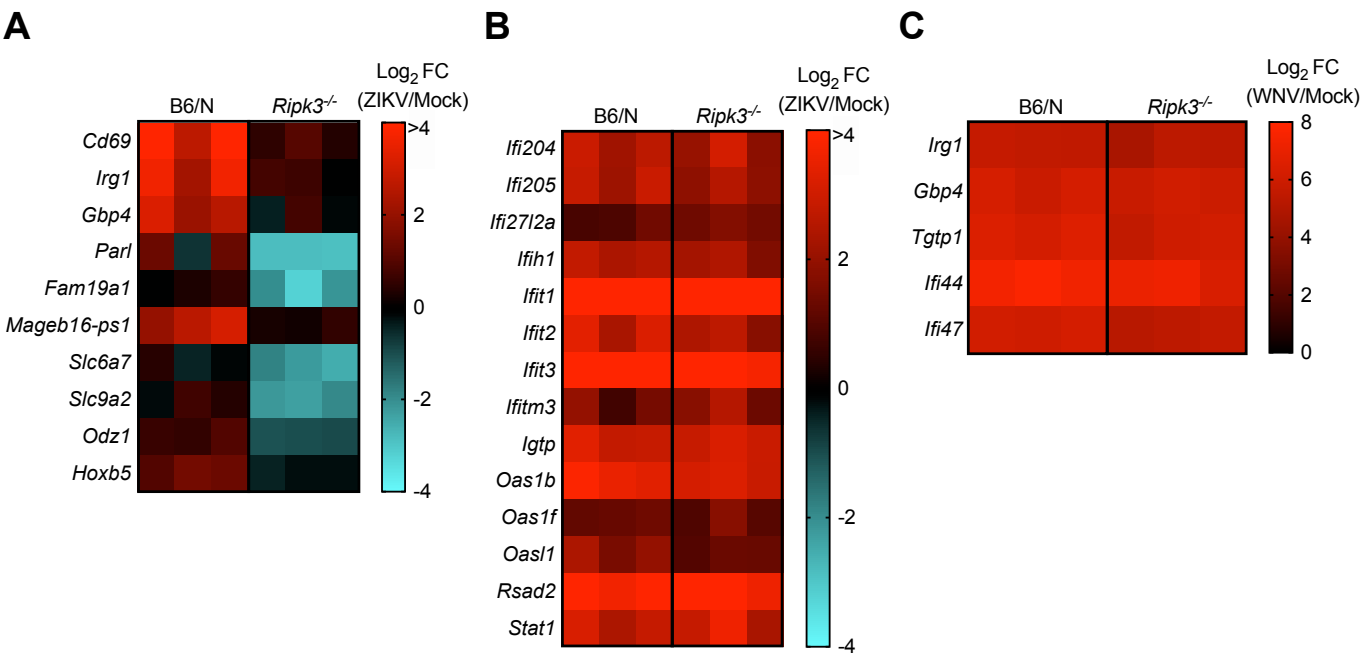

Figure S4 (Related to Figure 5) Additional microarray expression analysis in flavivirus infected neurons

(A) Heat map depicting relative expression values of the top 10 differentially expressed genes in a microarray analysis of *Ripk3*$^{-/-}$ cultures of primary cerebral cortical neurons compared to WT controls 24h following ZIKV-MR766 infection (MOI 0.1).

(B) Heat map depicting expression values of conventional interferon stimulated genes in neuronal cultures described in (A).

(C) Heat map depicting expression values of the 5 candidate antiviral molecules identified in Figure 5A in B6/N and *Ripk3*$^{-/-}$ cultures of primary cerebral cortical neurons 24h following infection with WNV-TX (MOI 0.001).

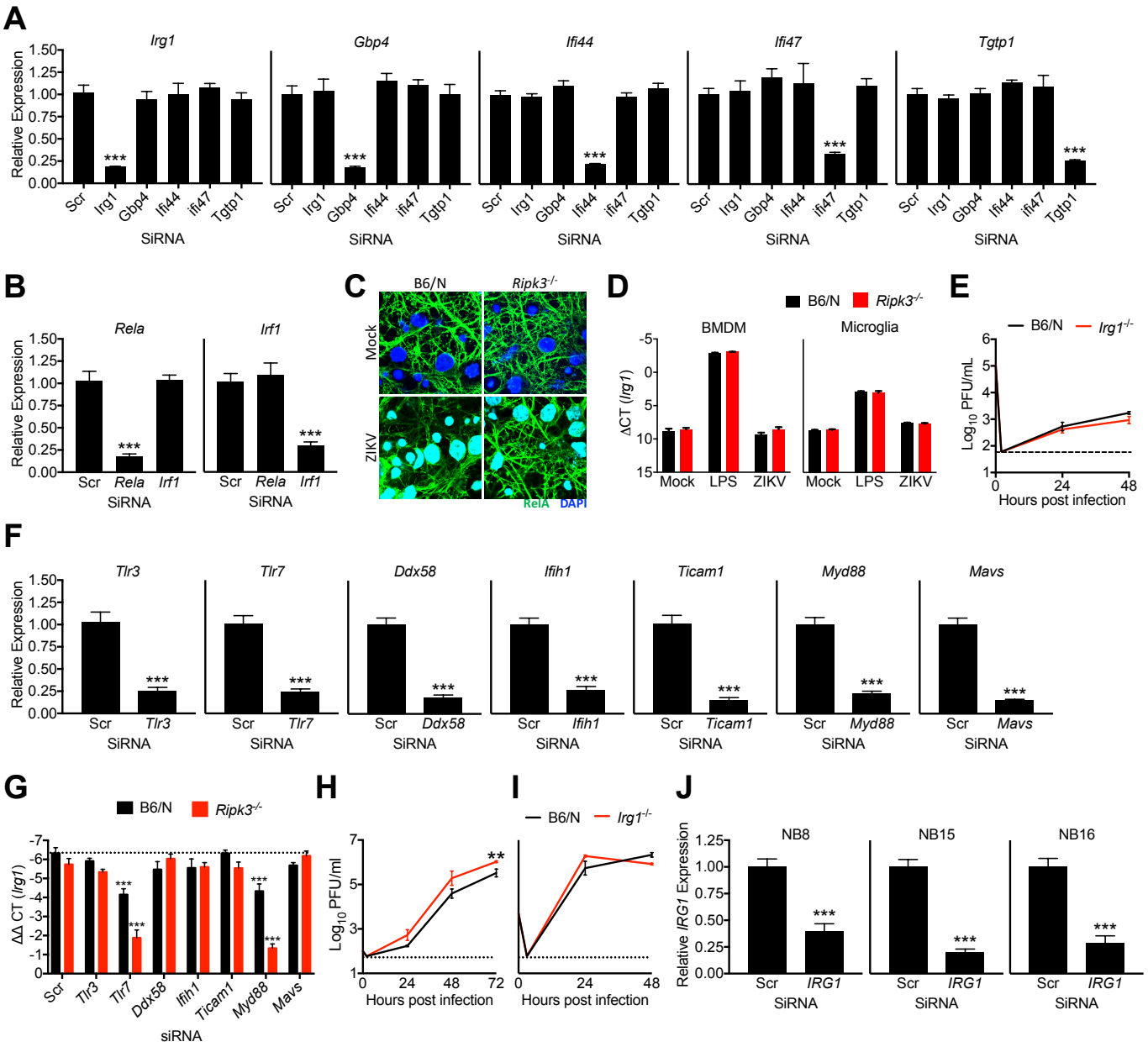
A
Irg1
Gbp4
Ifi44
Ifi47
Tgtp1
Relative Expression
Scr
Irg1
Gbp4
Ifi44
ifi47
Tgtp1
SiRNA
B
Rela
Irf1
Relative Expression
Scr
Rela
Irf1
SiRNA
C
B6/N
Ripk3-/-
Mock
ZIKV
RelA
DAPI
D
B6/N
Ripk3-/-
BMDM
Microglia
ΔCT (Irg1)
Mock
LPS
ZIKV
E
B6/N
Irg1-/-
Log10 PFU/mL
Hours post infection
F
Tlr3
Tlr7
Ddx58
Ifih1
Ticam1
Myd88
Mavs
Relative Expression
Scr
SiRNA
G
B6/N
Ripk3-/-
ΔΔ CT (Irg1)
siRNA
H
Log10 PFU/ml
Hours post infection
I
B6/N
Irg1-/-
Hours post infection
J
NB8
NB15
NB16
Relative IRG1 Expression
Scr
IRG1
SiRNA

Figure S5 (Related to Figure 6): Confirmation of siRNA knockdown of candidate antiviral genes, and lack of IRG1 induction in myeloid cells

(A-B) WT B6/N cultures of primary cerebral cortical neurons were treated for 48h with 25nM siRNA pools (4 siRNAs per pool) targeted against indicated genes or a nontargeting control pool (Scr). Knockdown was assessed by qRT-PCR analysis of the indicated transcripts (x axis). N= 4 independent replicates.

(C) *Ripk3*$^{-/-}$ or B6/N cortical neuron cultures were infected at MOI 10.0 with ZIKV-MR766 for 4h. Nuclear translocation of RelA was assayed via immunocytochemical staining. Images are representative of at least 3 high power fields taken from each of 4 independent replicates.

(D) Primary BMDM or microglial cultures were generated from B6/N or *Ripk3*$^{-/-}$ mice. Expression of *Irg1* mRNA was assessed via qRT-PCR following 4h treatment with 1μg/ml LPS or 24h post infection with ZIKV-MR766 (MOI 1.0). N= 4 (BMDM) or 6 (microglia) independent replicates.

(E) Multistep growth curve analysis in B6/N or *Irg1*$^{-/-}$ BMDM cultures following infection with ZIKV-MR766 (MOI 1.0). N= 4 independent replicates.

(F) Cultures as in (A) were treated with indicated siRNA pools and knockdown was assessed by qRT-PCR. N= 4 independent replicates.

(G) *Ripk3*$^{-/-}$ or B6/N cortical neuron cultures were treated with indicated siRNA pools for 48h, followed by infection for 24h with 0.001 MOI WNV-TX. *Irg1* mRNA expression was assessed via qRT-PCR. N = 4 independent replicates.

(H-I) Multistep growth curve analysis in B6/N or *Irg1*$^{-/-}$ cortical neuron (H) or BMDM (I) cultures following infection with WNV-TX (MOI 0.001 for cortical neurons; MOI 0.01 for BMDM). N= 3 independent replicates per genotype for each cell type.

(J) Differentiated primary human neuroblastoma lines were treated with a 25nM siRNA pool targeted against human *IRG1* or a nontargeting control pool (Scr) for 48h. Knockdown was assessed by qRT-PCR analysis. N= 6 independent replicates.

**p<0.01, ***p<0.001. Error bars represent SEM. All data are pooled from two or three independent experiments.

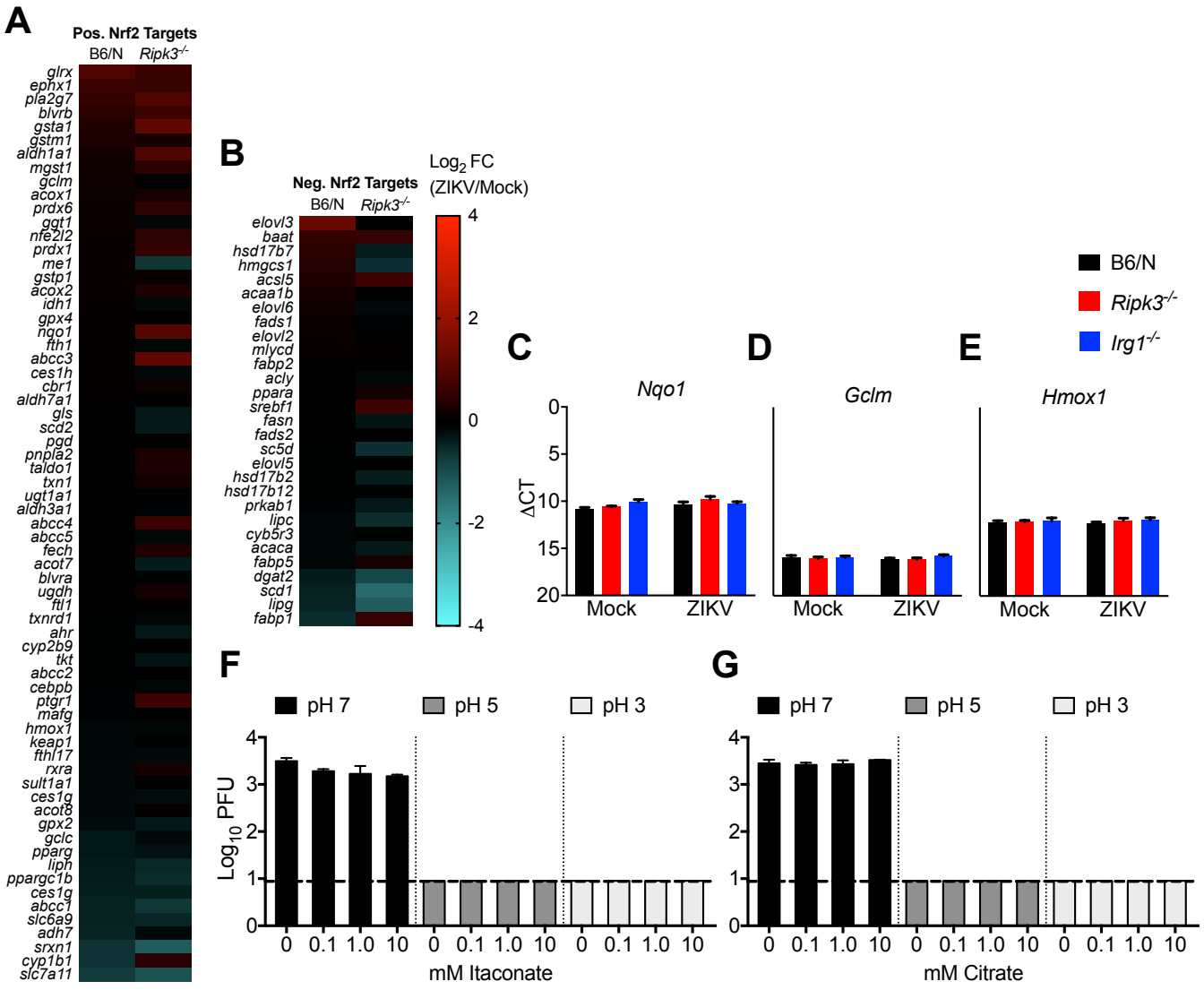

Figure S6 (Related to Figure 7): IRG1/itaconate do not engage Nrf2 transcriptional targets during ZIKV infection of neurons, nor exhibit direct neutralizing activity against ZIKV particles.

(A-B) Heat map depicting relative expression values of target genes known to be positively (A) or negatively (B) regulated by Nrf2. Expression values derived from microarray analysis of *Ripk3*$^{-/-}$ cultures of primary cerebral cortical neurons compared to WT controls 24h following ZIKV-MR766 infection (MOI 0.1). Individual cells represent average values for three independent replicates.

(C-E) B6/N, *Ripk3*$^{-/-}$, or *Irg1*$^{-/-}$ primary cerebral cortical neuron cultures were infected with ZIKV-MR766 (MOI 0.1) and assayed via qRT-PCR for expression of the canonical Nrf2 targets *Nqo1* (C), *Gclm* (D), and *Hmox1* (E). N= 6 independent replicates.

(F-G) 5x10^3 PFUs of ZIKV were incubated at 37C for 2h in DMEM supplemented with indicated concentrations of itaconate (F) or citrate (G) buffered to standard pH values. Samples were then analyzed for infectivity via plaque assay. N= 3 independent replicates.

Error bars represent SEM. Data in (C-G) pooled from two or three independent experiments.

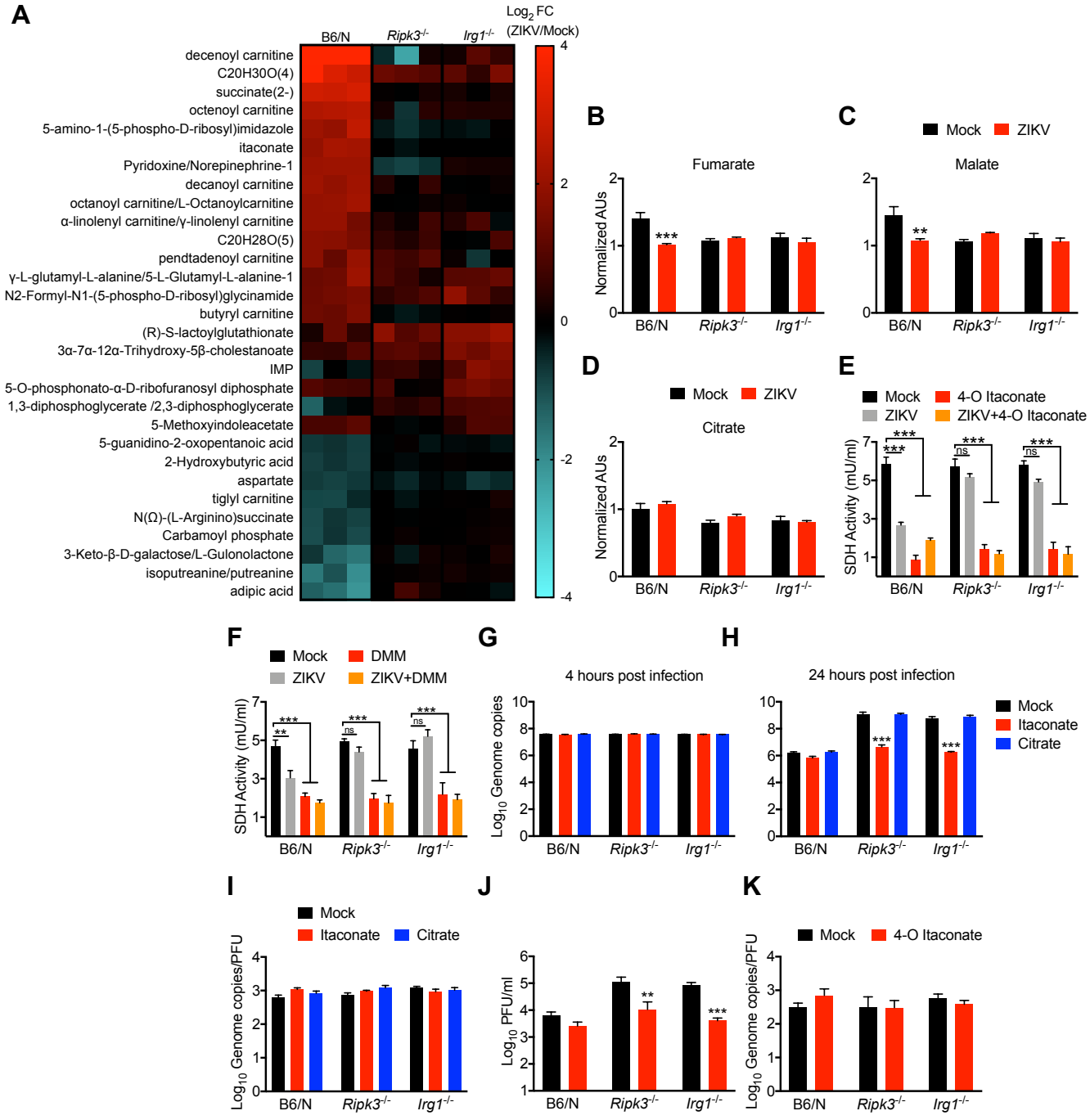
A
B6/N
Ripk3-/-
Irg1-/-
Log2 FC (ZIKV/Mock)
decenoyl carnitine
C20H30O(4)
succinate(2-)
octenoyl carnitine
5-amino-1-(5-phospho-D-ribosyl)imidazole
itaconate
Pyridoxine/Norepinephrine-1
decanoyl carnitine
octanoyl carnitine/L-Octanoylcarnitine
α-linolenyl carnitine/γ-linolenyl carnitine
C20H28O(5)
pendtadenoyl carnitine
γ-L-glutamyl-L-alanine/5-L-Glutamyl-L-alanine-1
N2-Formyl-N1-(5-phospho-D-ribosyl)glycinamide
butyryl carnitine
(R)-S-lactoylglutathionate
3α-7α-12α-Trihydroxy-5β-cholestanoate
IMP
5-O-phosphonato-α-D-ribofuranosyl diphosphate
1,3-diphosphoglycerate /2,3-diphosphoglycerate
5-Methoxyindoleacetate
5-guanidino-2-oxopentanoic acid
2-Hydroxybutyric acid
aspartate
tiglyl carnitine
N(Ω)-(L-Arginino)succinate
Carbamoyl phosphate
3-Keto-β-D-galactose/L-Gulonolactone
isoputreanine/putreanine
adipic acid
B
Fumarate
Normalized AUs
C
Mock
ZIKV
Malate
D
Citrate
E
4-O Itaconate
ZIKV+4-O Itaconate
SDH Activity (mU/ml)
ns
F
DMM
ZIKV+DMM
G
4 hours post infection
Log10 Genome copies
H
24 hours post infection
Itaconate
Citrate
I
Log10 Genome copies/PFU
J
Log10 PFU/ml
K

Figure S7 (Related to Figure 7): RIPK3/IRG1 inhibition of succinate dehydrogenase alters neuronal metabolism and suppresses ZIKV replication. (legend cont. on next page)
(A) Heat map depicting relative abundance of metabolites as assessed by metabolomic profiling of primary cerebral cortical neuron cultures isolated from *Ripk3*$^{-/-}$ or *Irg1*$^{-/-}$ mice or congenic WT controls at 24h post infection with ZIKV-MR766 (MOI 0.1).
(B-D) Relative abundance of fumarate (B), malate (C), and citrate/isocitrate (D) in neurons as described in (A). (AUs= arbitrary units) N= 3 independent replicates.
(E-F) Succinate dehydrogenase activity in lysates of B6/N, *Ripk3*$^{-/-}$, or *Irg1*$^{-/-}$ primary cerebral cortical neuron cultures infected for 24h with ZIKV-MR766 (MOI 0.1) following 2h pretreatment with 1mM 4-octyl (4-O) itaconate (E), 1mM dimethyl malonate (DMM, F) or a DMSO vehicle solution. N= 4 independent replicates.
(G-H) B6/N, *Ripk3*$^{-/-}$, or *Irg1*$^{-/-}$ primary cerebral cortical neuron cultures were infected with ZIKV-MR766 (MOI 10.0) following 2h pretreatment with 1mM itaconate, 1mM citrate, or DMEM vehicle. Viral RNA in cell pellets was assessed by qRT-PCR at 4h (G) or 24h (H) post infection. N= 4 independent replicates.
(I) Neuronal cultures were generated and treated as described in (G-H). Genome copy/PFU ratios of virus in supernatants 24h after infection were calculated from simultaneous qRT-PCR analysis of viral RNA and plaque assay analysis of infectious particles using matched supernatant samples. N= 4 independent replicates.
(J-K) Neuronal cultures as in (G-I) were infected with ZIKV-MR766 (MOI 10.0) following 2h pretreatment with 1mM 4-O itaconate and assayed for viral titers (J) and genome copy/PFU ratios (K) in supernatants at 24h.
**p<0.01, ***p<0.001. Error bars represent SEM. Data in (E-K) are pooled from two or three independent experiments.

Table S1 (Related to STAR Methods): Primer sequences for qRT-PCR studies

Target	Note	Sequence
Ddx58	Forward	GAGAGTCACGGGACCCACT
	Reverse	CGGTCTTAGCATCTCCAACG
Gbp4	Forward	TGGGGGACACAGGCTCTACA
	Reverse	GCCTGCAGGATGGAACTCTCAA
Gclm	Forward	GACAAAACACAGTTGGAACAGC
	Reverse	CAGTCAAATCTGGTGGCATC
Hmox1	Forward	TCAGGCAGAGGGTGATAGAA
	Reverse	GCTCCTGCAACTCCTCAAA
Ifi44	Forward	TTCGATGCGAAGATTCACTG
	Reverse	CCCTTGGAAAACAGACCTCA
Ifi47	Forward	GTTAGAACCAAGGTGGATAGTGAC
	Reverse	CGAGATATTGGGCAAGAGCA
Ifih1	Forward	TGATGCACTATTCCAAGAACTAACA
	Reverse	TCTGTGAGACGAGTTAGCCAAG
Irf1	Forward	TGTGTCGTCAGCAGCAGTCTCT
	Reverse	GTCTTCGGCTATCTTCCCTTCCT
IRG1 (human)	Forward	CTACCCACTGGGTGGCA
	Reverse	TCCTCCTGGCTCAGTGG
Irg1 (mouse)	Forward	GCGAACGCTGCCACTCA
	Reverse	ATCCCAGGCTTGGAAGGTC
Myd88	Forward	CCCAACGATATCGAGTTTGT
	Reverse	TTCTTCATCGCCTTGTATTT
Nqo	Forward	GCTGCAGACCTGGTGATATT
	Reverse	ACTCTCTCAAACCAGCCTTT
Rela	Forward	GACCAACAATAACCCCTTTCAC
	Reverse	GTTTGAGATCTGCCCTGATGG
Tgtp1	Forward	TGCACAGATGGGGATGAATTTC
	Reverse	TCACTGTCGAGAGACTCCTGA
Ticam1	Forward	GTCTGTCAGGAGGTGCTC
	Reverse	CGTTCCGGACATGCTCTTTCTC
Tlr7	Forward	ACAGAAATCCCTGAGGGCATT
	Reverse	CAGATGGTTCAGCCTACGGAAG
Zbp1	Forward	AAGAGTCCCCTGCGATTATTTG
	Reverse	TCTGGATGGCGTTTGAATTGG
ZIKV	Forward	CCGCTGCCCAACACAAG
	Reverse	CCACTAACGTTCTTTTGCAGACAT