Supplemental Figure Legends:

Figure S1: *Nlrc3* deficiency leads to enhanced IFN-I production in different littermate-matched MEF pairs, related to Figure 1. Different pairs of *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs were generated from siblings produced from independent heterozygous matings. Different MEF pairs were tested independently and yielded consistent result. (A) *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs were seeded in 24-well plate overnight and transfected with ISD at 4 μg/ml. Cells were harvested after 6 hours after transfection. Message RNA of *Il6* was determined by real-time PCR. (B-C) A pair of *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs other than those shown in Figure 1 were seeded in 24-well plate overnight and infected with HSV-1 at MOI of 0.2, 1 or 5. Cells were harvested at indicated time points. *Ifna4* and *Ifnb* level were determined by real-time PCR. (D) Supernatants were harvested from the same experiment and IFN-β ELISA was performed. Data are representative of at least three independent experiments.

Figure S2: *Nlrc3* deficiency does not alter SeV-induced IFN-I production, related to Figure 1. (A-B) *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs were seeded in 24-well plate overnight and infected with SeV at HA unit of 8, 80 and 800. Cells were harvested after 6 hours of infection. Message RNA of *Ifna4* and *Ifnb* were determined by real-time PCR. (C-J) Poly(I:C) were either added (extra-) or transfected (intra-) into *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs for indicated time point. Message RNA of *Ifna4, Ifnb, Tnf* and *Il6* were determined by real-time PCR. (K-N) *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs were seeded in 24-well plate overnight and infected with VSV at MOI of 1, 5 and 25. Cells were harvested after 6 or 16 hours of infection. Message RNA of *Ifna4, Ifnb, Tnf* and *Il6* were determined by real-time PCR. Data are representative of at least three independent experiments.

Figure S3: Deficiency of *Nlrc3* does not alter HSV-1, *L. monocytogenes* or *B. thaildensis*-induced cell death, related to Figure 1. (A) *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs were seeded in 24-well plate overnight and infected with HSV-1 at MOI of 0.2, 1 or 5. Supernatant were harvested at indicated time points. Cytotoxicity was determined by Cytotoxicity Detection Kit (Roche). (B) *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs were seeded in 24-well plate overnight and infected with *Listeria monocytogenes* at MOI of 10 or 50. Supernatant were harvested at 6 hours post infection. Cytotoxicity was determined by the Cytotoxicity Detection Kit. (C) *Nlrc3*$^{+/+}$ and *Nlrc3*$^{-/-}$ MEFs were seeded in 24-well plate overnight and infected *Burkholderia. thaildensis* at MOI of 10 or 50. Supernatant were harvested at 6 hours post infection. Cytotoxicity was determined by the Cytotoxicity Detection Kit. Data are representative of at least two independent experiments.

Figure S4: Domain Mapping of STING-TBK1 interaction regions, related to Figure 2. (A) HEK293T cells were transfected with STING truncation constructs. Cells were harvested 24 hours post transfection and fractionated using Subcellular Protein Fractionation Kit (Thermo) according to manufacturer's instruction. Western blot were performed using indicated antibodies. CE: cytosolic extract; ME: membrane extract; NE: nuclear extract. Constructs 41-379 and 81-379 were membrane associated, while 111-379 lost its membrane-association. (B) HEK293T cells were transfected with Flag-tagged TBK1 and HA-tagged STING/MITA truncation plasmids depicted above. The N-terminal N-240 residues are not sufficient for interaction, and only membrane-associated 41-379 and 81-379 associated with TBK1. Co-immunoprecipitation assays were performed 24 hours post-transfection with indicated antibodies. The kinase domain is necessary and

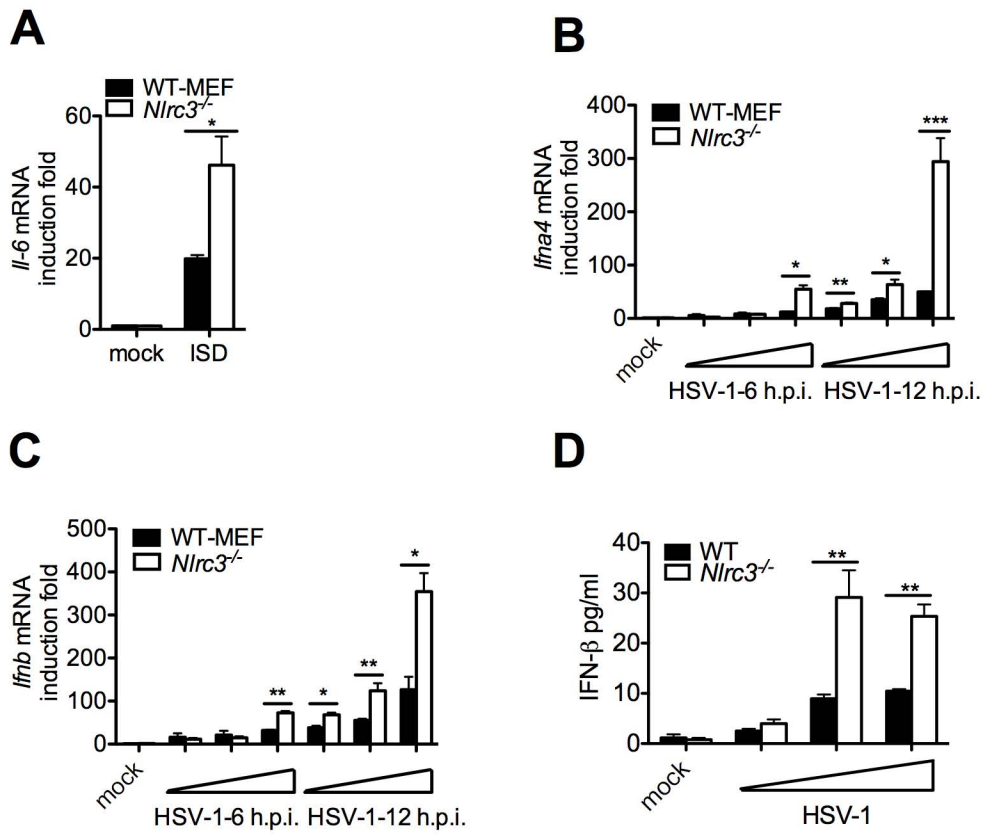
sufficient for interaction with NLRC3. Data are representative of at least two independent experiments.

Figure S5: TRAF6 is not required for HSV-1-induced IFN-I response, related to Figure 3. (A-B) *Traf6*$^{+/+}$, *Traf6*$^{+/-}$ and *Traf6*$^{-/-}$ MEFs were seeded in 24-well plate overnight and infected with HSV-1 at MOI of 1 or SeV at HA unit of 80. Cells were harvested after 6 hours of infection. Message RNA of *Ifna4* and *Ifnb* were determined by real-time PCR. (C) HEK293T cells were transfected with Flag-tagged STING/MITA and HA-tagged TBK1, HA-tagged NLRC3 and V5-tagged TRAF6. Co-immunoprecipitation assays were performed 24 hours post-transfection with indicated antibodies. Data are representative of at least two independent experiments.

Figure S6: Nlrc3 is not required for antiviral response against VSV *in vivo*, related to Figure 7. (A) WT and *Nlrc3*$^{-/-}$ mice were infected i.v. with VSV ($5x10^7$ pfu) and body weight was monitored. (B) WT and *Nlrc3*$^{-/-}$ mice were infected i.v. with VSV ($5x10^7$ pfu), serum were harvested 6 hours post-infection and measured for IFNβ.

Supplemental videos, , related to Figure 7: WT and *Nlrc3*$^{-/-}$ mice were infected i.v. with HSV-1 ($2x10^7$ pfu). A 30-seconds video recorded the mobility of WT (Movie S1) and *Nlrc3*$^{-/-}$ mice (Movie S2) 5 days post infection.

Figure S1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20
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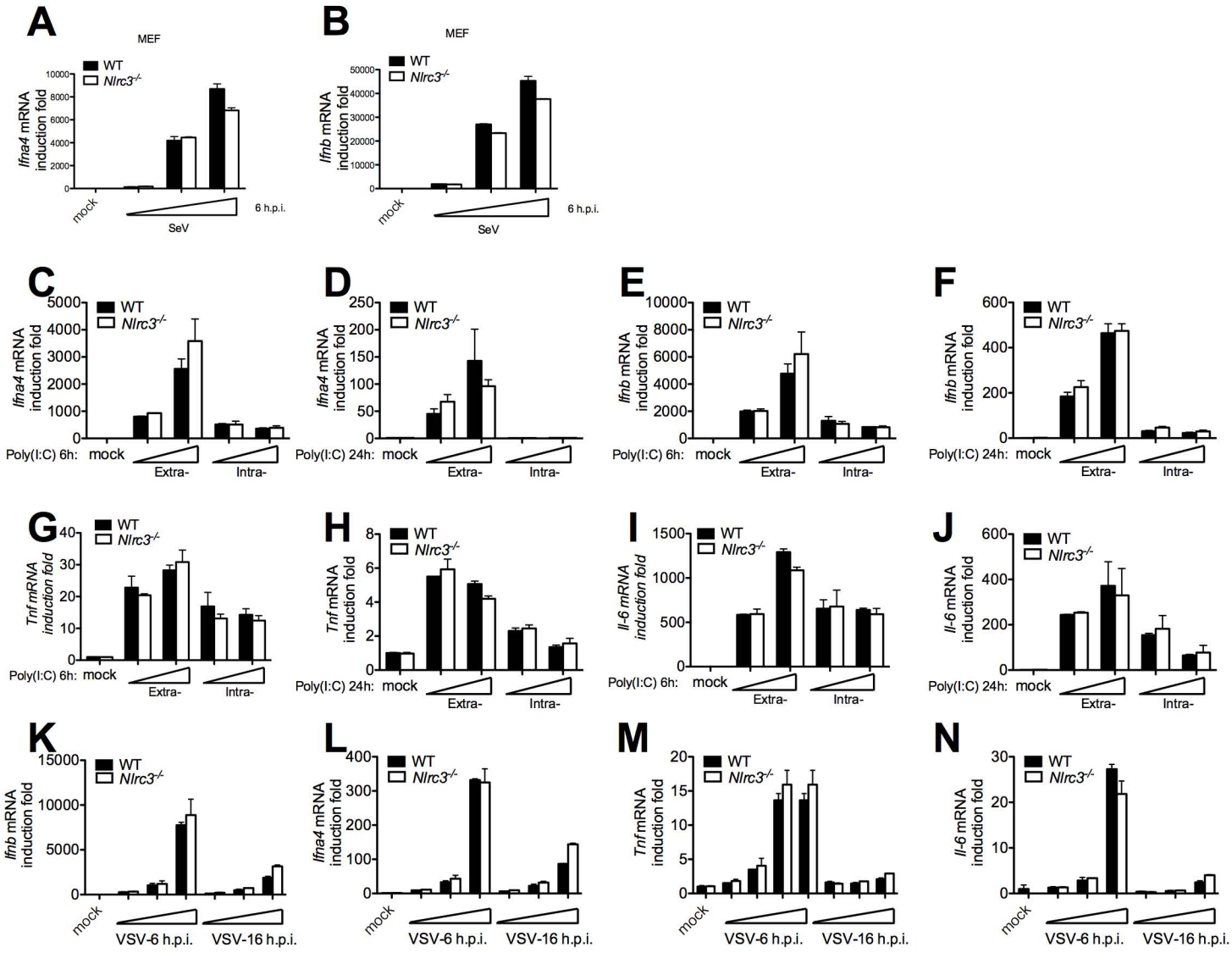
*
mock
ISD
B
WT-MEF
Nlrc3-/-
Ifna4 mRNA induction fold
400
300
200
100
0
*
**
*
***
mock
HSV-1-6 h.p.i.
HSV-1-12 h.p.i.
C
WT-MEF
Nlrc3-/-
Ifnb mRNA induction fold
500
400
300
200
100
0
**
*
**
*
mock
HSV-1-6 h.p.i.
HSV-1-12 h.p.i.
D
WT
Nlrc3-/-
IFN-β pg/ml
40
30
20
10
0
**
**
mock
HSV-1

Figure S2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MEF
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Nlrc3-/-
Ifna4 mRNA induction fold
mock
SeV
6 h.p.i.
B
MEF
WT
Nlrc3-/-
Ifnb mRNA induction fold
mock
SeV
6 h.p.i.
C
WT
Nlrc3-/-
Ifna4 mRNA induction fold
Poly(I:C) 6h: mock
Extra-
Intra-
D
WT
Nlrc3-/-
Ifna4 mRNA induction fold
Poly(I:C) 24h: mock
Extra-
Intra-
E
WT
Nlrc3-/-
Ifnb mRNA induction fold
Poly(I:C) 6h: mock
Extra-
Intra-
F
WT
Nlrc3-/-
Ifnb mRNA induction fold
Poly(I:C) 24h: mock
Extra-
Intra-
G
WT
Nlrc3-/-
Tnf mRNA induction fold
Poly(I:C) 6h: mock
Extra-
Intra-
H
WT
Nlrc3-/-
Tnf mRNA induction fold
Poly(I:C) 24h: mock
Extra-
Intra-
I
WT
Nlrc3-/-
Il-6 mRNA induction fold
Poly(I:C) 6h: mock
Extra-
Intra-
J
WT
Nlrc3-/-
Il-6 mRNA induction fold
Poly(I:C) 24h: mock
Extra-
Intra-
K
WT
Nlrc3-/-
Ifnb mRNA induction fold
mock
VSV-6 h.p.i.
VSV-16 h.p.i.
L
WT
Nlrc3-/-
Ifna4 mRNA induction fold
mock
VSV-6 h.p.i.
VSV-16 h.p.i.
M
WT
Nlrc3-/-
Tnf mRNA induction fold
mock
VSV-6 h.p.i.
VSV-16 h.p.i.
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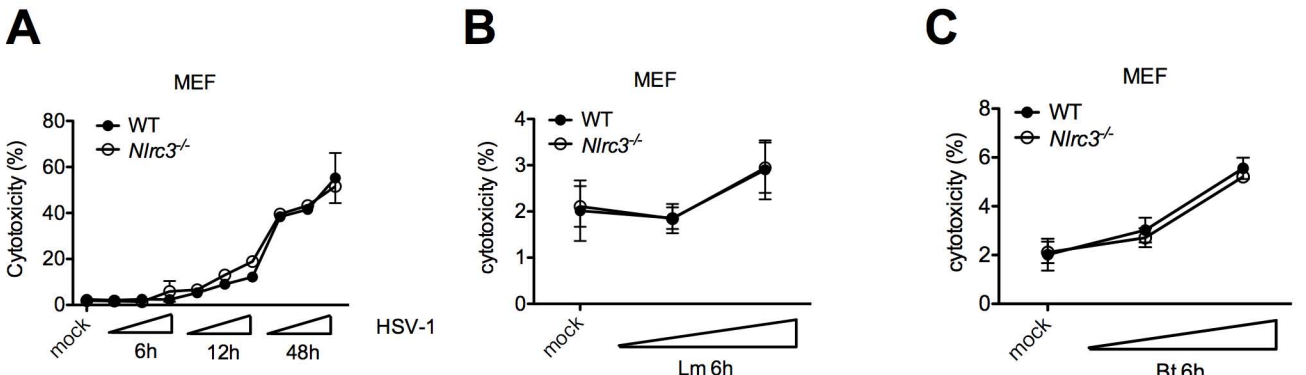
VSV-6 h.p.i.
VSV-16 h.p.i.

Figure S3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Nlrc3-/-
Cytotoxicity (%)
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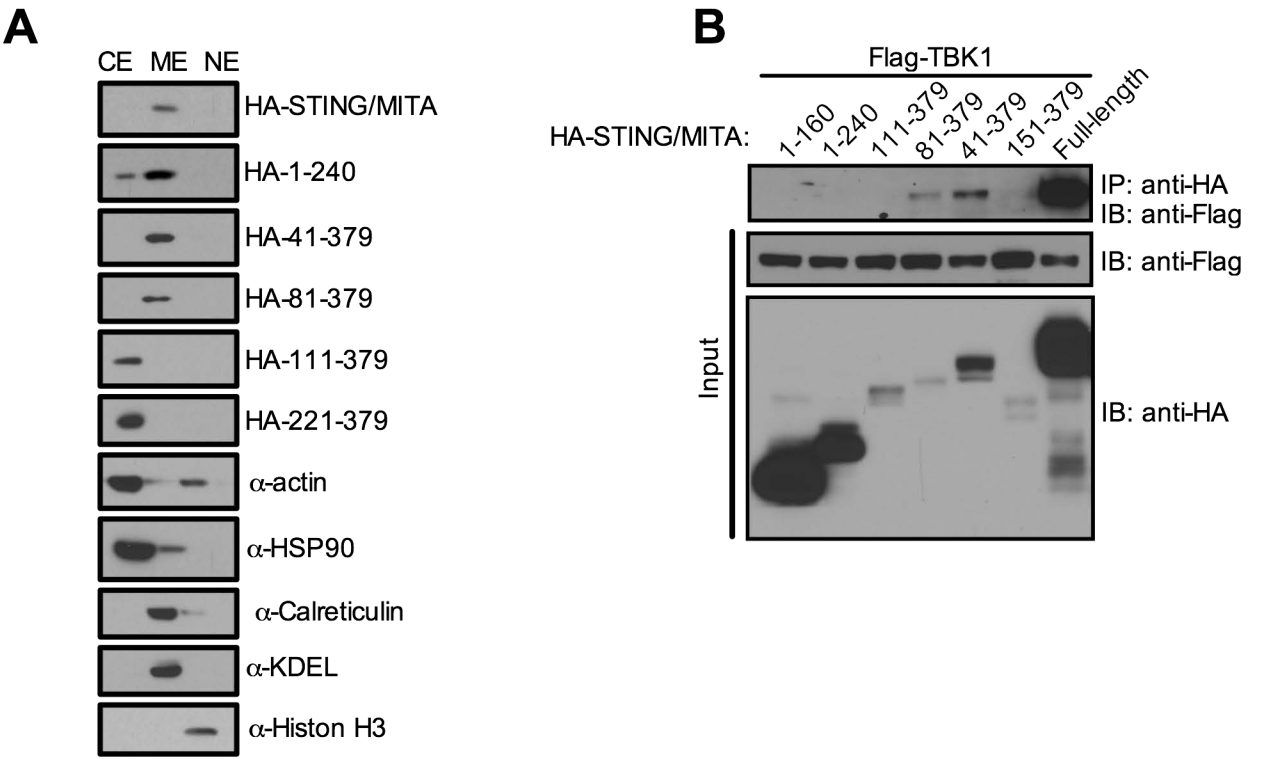
60
40
20
0
mock
6h
12h
48h
HSV-1
B
MEF
WT
Nlrc3-/-
cytotoxicity (%)
4
3
2
1
0
mock
Lm 6h
C
MEF
WT
Nlrc3-/-
cytotoxicity (%)
8
6
4
2
0
mock
Bt 6h

Figure S4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HA-41-379
HA-81-379
HA-111-379
HA-221-379
α-actin
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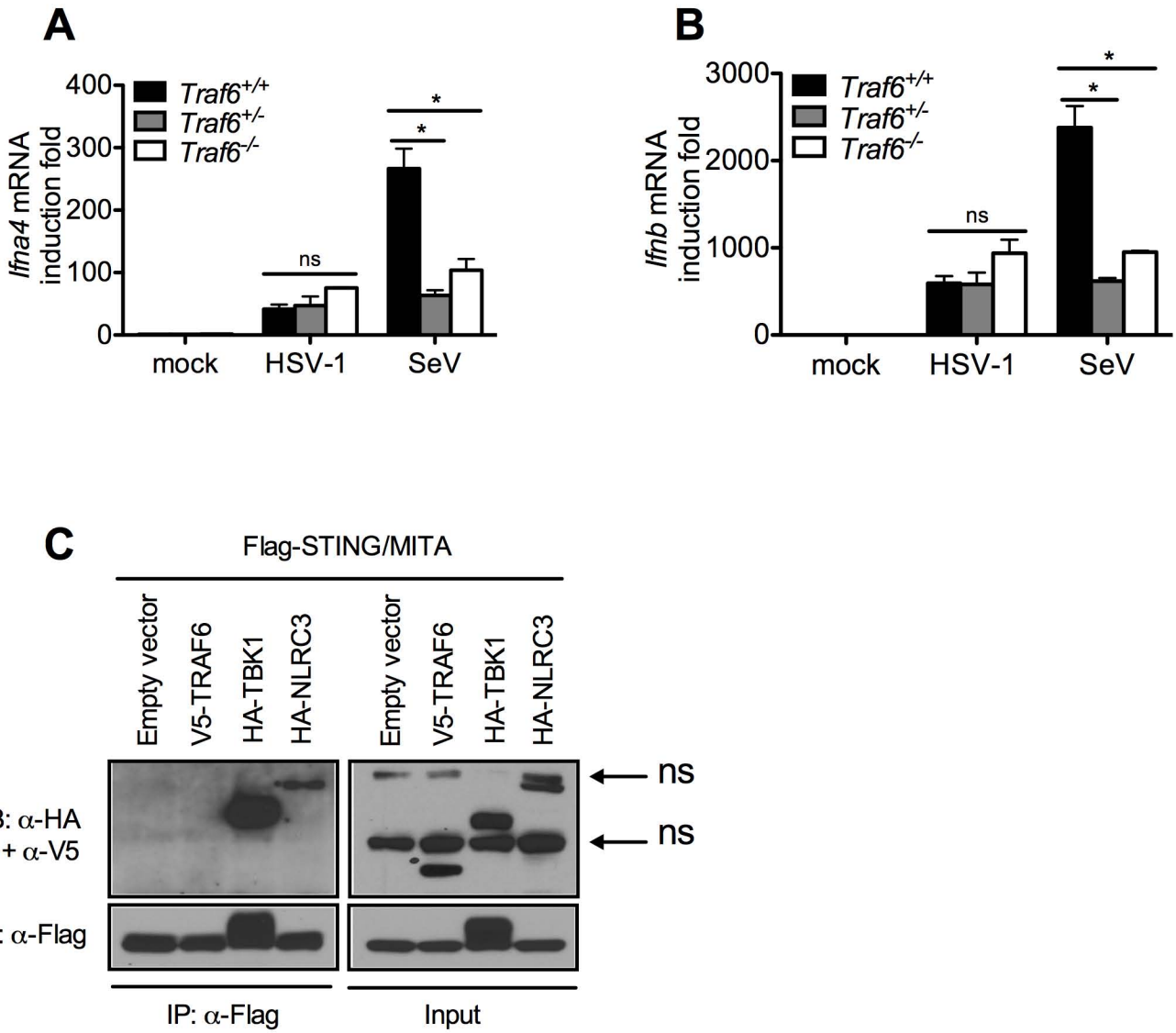
α-Calreticulin
α-KDEL
α-Histon H3
B
Flag-TBK1
HA-STING/MITA:
1-160
1-240
111-379
81-379
41-379
151-379
Full-length
IP: anti-HA
IB: anti-Flag
IB: anti-Flag
Input
IB: anti-HA

Figure S5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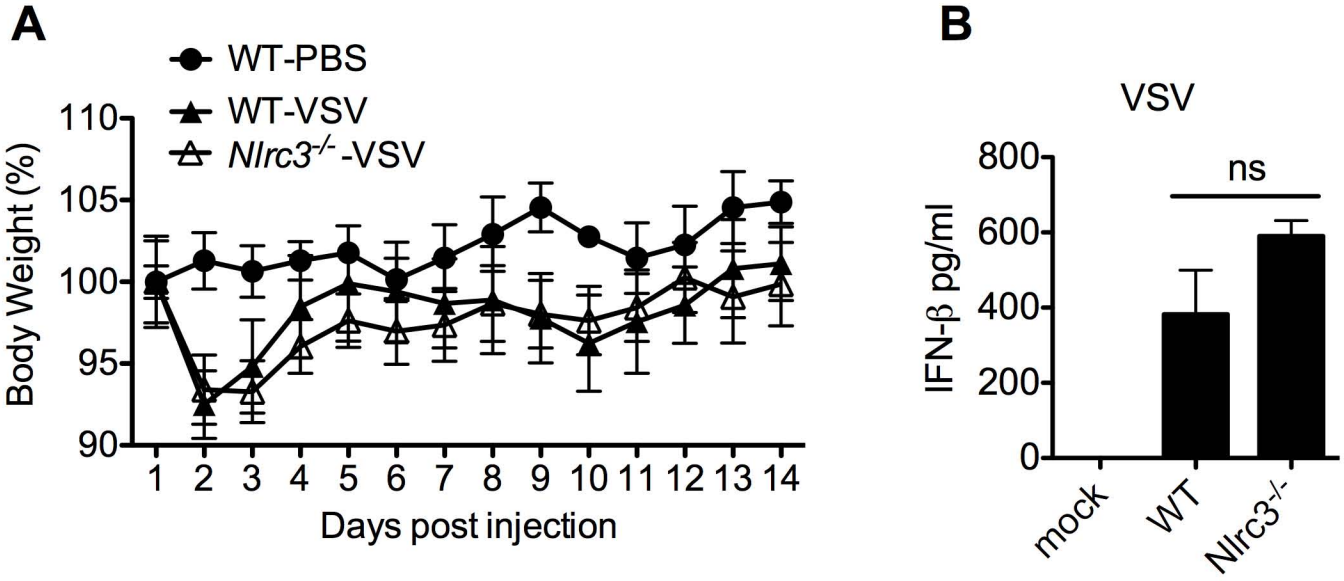
Ifna4 mRNA induction fold
400
300
200
100
0
Traf6+/+
Traf6+/-
Traf6-/-
ns
*
*
mock
HSV-1
SeV
B
Ifnb mRNA induction fold
3000
2000
1000
0
Traf6+/+
Traf6+/-
Traf6-/-
ns
*
*
mock
HSV-1
SeV
C
Flag-STING/MITA
Empty vector
V5-TRAF6
HA-TBK1
HA-NLRC3
Empty vector
V5-TRAF6
HA-TBK1
HA-NLRC3
IB: α-HA + α-V5
IB: α-Flag
ns
ns
IP: α-Flag
Input

Figure S6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WT-PBS
WT-VSV
Nlrc3-/- -VSV
Body Weight (%)
110
105
100
95
90
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post injection
B
VSV
ns
IFN-β pg/ml
800
600
400
200
0
mock
WT
Nlrc3-/-