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Supporting Information for

Necroptosis-based CRISPR knockout screen reveals Neuropilin-1 as a critical host factor for early stages of murine cytomegalovirus infection

Rebecca K. Lane, Hongyan Guo, Amanda D. Fisher, Jonathan Diep, Zhao Lai, Yidong Chen, Jason W. Upton, Jan Carette, Edward S. Mocarski, and William J. Kaiser.

Corresponding authors: William J Kaiser, Hongyan Guo
Email: wkaiser@inzentx.com; hguo@gsu.edu

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SI Materials and Methods

Cell culture and preparation of virus stocks

All cells were cultured in complete Dulbecco's Modified Eagle Medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C with 5% CO_2. The WT, M45mut, IE2-mCherry MCMV K181, and Mck-2mut viruses were propagated and titered as previously described [1]. HSV-1 F ICP6mut virus was grown as previously described [2].

Construction of MCMV IE2-mCherry

Recombineering for MCMV IE2-mCherry was generated using techniques previously described [3]. The kanamycin resistance and levensucrase (SacB) genes and associated regulatory elements were PCR amplified from pTBE100 overhangs corresponding to MCMV genomic sequence flanking HpaI sites surrounding IE2exon 1 (primers; JUp047, 5'-TGCATTTGGCATTAAAAACTATTGGTTCTAGTCATAAAACGGGCGGAGTTAATTCGAGCTCGGTACCCGG -3' and JUp048, 5'-AGCCCAATGCAACCTTACCCGGCCTGGGGGGCTCCGTTCACCCGCTCGTTATCCCGGGAAAAGTGCCACC -3'). Kanamycin-resistant, sucrose-sensitive clones were selected and confirmed by RFLP analysis. IE2sB/K insertion deletes 76 nt of MCMV sequence and introduces 2.9kb between the HpaI sites. The mCherry insertion mutation was introduced by a second round of recombineering with a PCR amplicon amplified from pmCherry-N1 (Clontech) (primers; U160, 5'-GGCATTAAAAACTATTGGTTCTAGTCATAAAACGGGCGGAGTTGCTATTACCATGGTGATGCGGTTTTG-3' and U161, 5'-

TGCAACCTTACCCGGCCTGGGGGGCTCCGTTCACCCGCTCGTTGGACAAACCACAAC TAGAATGCAGTG -3'). Colonies were screened for sucrose resistance and kanamycin sensitivity, and positive clones were further confirmed by RFLP and functional analyses. Genome integrity was confirmed by restriction digest and sequencing of the target region of IE2. BAC-derived recombinant virus was generated and purified as previously described [3].

CRISPR screen

The mouse GeCKOv2 pooled CRISPR gRNA library (Addgene pooled library #1000000052) was packaged into lentivirus according to Sanjana *et al* [4]. The library was amplified and packaged using a lentivirus expression system as previously described [5]. Briefly, 293FT cells were transfected using lipofectamine 2000 (Invitrogen). 48 hours after transfection, supernatants were collected, clarified by centrifugation (3,500 rpm for 10 min), filtered, and aliquoted for storage at -80°C. Lentivirus containing the library was then used to transduce SVEC4-10 endothelial cells at MOI 0.3. Three transductions 24 hours apart were carried out and cells selected with Puromycin for seven days. 2 x 10^8 library-containing cells were then infected with the MCMV M45mut or HSV-1 ICP-6mut viruses (MOI 5) for one hour, then cultured in the presence of 200 μg/mL phosphonoformic acid (PFA), an inhibitor of the herpes DNA polymerase. Resistant cells were allowed to grow to 80% confluency before being subjected to two additional rounds of infection and recovery. Genomic DNA was isolated from this population and unselected library-containing cells, gRNA sequences were amplified, and sequenced using an Illumina NextSeq 500 platform. The gRNA sequences against specific genes were recovered after removal of the tag sequences using the FASTX-Toolkit

(http://hannonlab.cshl.edu/fastx_toolkit/) and cutadapt 1.8.1. The cut-off for candidate gene 'hits' was made using a published computational tool (MAGeCK version 0.5.4) which determines the ranking of each hit by taking into account the number of sequencing reads for each unique guide, the number of unique guides per gene, and the enrichment of a guide compared to the untreated population [6].

Plasmids and transgenic cell lines

gRNA sequences against Nrp-1 were derived from the GeCKOv2 library, synthesized as oligonucleotides, and cloned into lentiCRISPRv2 (Addgene #52961) according to published protocol (http://genome-engineering.org/gecko/) [4]. Primers for cloning gRNA sequences were as follows: Nrp-1 fw: CACCGCTCTTCAGGCGTTCTATCCA; Nrp-1 rev: AAACTGGATAGAACGCCTGAAGAGC. gRNAs were transfected into HEK-293T cells with psPAX2 and VSV-G to generate lentivirus. Nrp-1 gRNA-containing lentivirus was then used to transduce SVEC4-10 or 3T3-SA cells. Cells were then selected with Puromycin. Nrp-1 KO cells were further sorted by flow cytometry to generate a clonal population. Murine Nrp-1 with N-terminal 6X-His and HA tags was obtained from Addgene (Pinco-mNrp1, plasmid #21937) and subcloned into pLV-EF1α-IRES-Puro (Biosettia) using MluI and NheI. Primers were used to recode the 20 bp stretch targeted by the Nrp-1 gRNA in order to make the transgene resistant to the gRNA. All constructs were made by amplifying two fragments and joining the two by assembly PCR. The Nrp-1 ΔC construct was cloned into pQCXIH (Takara) using AgeI and PacI. Primers used for cloning are detailed in the below table. Plasmids were introduced into Nrp-1 KO cells by lentiviral transduction and reconstitution was verified by western blot and flow cytometry for HA expression (Alexa Fluor 647-coupled anti-HA tag, R&D IC6875R).

92

Full-length Nrp-1	Nrp-1 MluI fw: CTCACGCGTCCAGCGTCTGCAGCATGG Nrp-1 NheI rev: TCTGCTAGCGCCGCTCACGCCTCTG Nrp-1 recode fw: CAGCAGCGGTGTCCTTAGTATGGTCTTTTACACTGACAGCG Nrp-1 recode rev: ACTAAGGACACCGCTGCTGGAGCGGATCCGGCCAG
Nrp-1 Δa	Nrp-1 Δa fw: GGAACCCTACCAGAGAATCATGGTGCTACAGAGCAGCATCTC Nrp-1 Δa rev: GAGATGCTGCTCTGTAGCACCATGATTCTCTGGTAGGGTTCC
Nrp-1 Δb	Nrp-1 Δb fw: GCCAACTACAGTGTGCTACAGGAAGTGGAAGCACCTACAGC Nrp-1 Δb rev: GCTGTAGGTGCTTCCACTTCCTGTAGCACACTGTAGTTGGC
Nrp-1 Δab	Nrp-1 Δab fw: GGAACCCTACCAGAGAATCATGGAAGTGGAAGCACCTACAGC Nrp-1 Δab rev: GCTGTAGGTGCTTCCACTTCCATGATTCTCTGGTAGGGTTCC
Nrp-1 ΔC	Nrp-1 AgeI fw: CTCACCGGTCCAGCGTCTGCAGCATGG Nrp-1 ΔC PacI rev: TCTTTAATTAAGGCACAGTACAGCACAAC

93

94 **Death assays**

95 5 x 10^3 cells/well were seeded into 96-well plates in triplicate. After 18 hours, cells were infected
96 at MOI 10 with the lytic MCMV M45mut or HSV-1 ICP6mut viruses, or treated with 25 ng/mL
97 TNFα (PeproTech) + 5 μM Emricasan (Adooq biosciences). Cell viability was assessed by
98 adding the live cell-impermeable dye Sytox Green (Molecular probes, 50 nM) and labeling
99 nuclei with Hoechst 33342 (Molecular probes, 2μM). Cells were imaged hourly with four frames
100 per well with a Cytation 5 Imaging Reader (Biotek). Cell death was quantified as the percentage
101 of Sytox Green positive cells divided by Hoechst+ cells. Alternatively, cell survival was
102 determined indirectly by measuring intracellular ATP levels using the Cell Titer-Glo
103 Luminescent Cell Viability Assay kit (Promega) on a Synergy HT Multi-Detection microplate
104 reader (Bio-Tek).
105
106 **Western blotting**
107 The following antibodies were used for immunoblotting: rabbit anti-Nrp-1 (Cell Signaling
108 Technology #3725S), mouse anti-MCMV IE1/Croma101 (gift from Stipan Jonjic, University of
109 Rijeka), mouse anti-β-actin (Sigma #A5441), and rabbit anti-HA (Cell Signaling Technology
110 #3724).
111
112 **Immunofluorescence**
113 For immunofluorescence to quantify viral infection, adherent cells were fixed in 4%
114 paraformaldehyde and incubated in blocking buffer containing 10% goat serum, then incubated
115 with anti-IE1 (MCMV) or anti-ICP0 (HSV-1) antibody followed by Alexa Fluor 488-coupled
116 goat anti-mouse antibody (Invitrogen). The cells were counterstained with DAPI (Invitrogen) for

nuclei. Images were acquired on an LSM 510 Meta confocal fluorescence microscope (Carl Zeiss).

Viral growth assays

WT MCMV viral yield in WT and Nrp-1 KO cells was determined by infecting cells at either a low (0.1) or high (10) MOI. Cells and media were harvested after 8 and 48 (MOI 10) or 72 (MOI 0.1) hours and titered as before. Viral yield was calculated as the titer at 48 or 72 h.p.i. divided by titer at 8 h.p.i. relative to the yield from WT cells.

Infection with mCherry reporter virus

Cells were infected in media containing Hoechst stain and either imaged at regular intervals with a Cytation 5 Imaging Reader or collected and analyzed by flow cytometry. Infection rates were quantified by counting mCherry+ cells as a percent of Hoechst+ cells using Image J. Rates of infection in WT cells were set as the maximum rate of infection and infection rates in Nrp-1 KO or soluble protein-treated cells are reported relative to the maximum rate. Quantification of mCherry fluorescence intensity was performed 24 h.p.i. on an LSRII analyzer and data analyzed by FlowJo software.

Soluble Nrp-1 and anti-Nrp-1 antibody inhibition experiments

Recombinant murine Nrp-1 (R&D Systems) and Nrp-2 (Sino Biological) were mixed with MCMV IE2-mCherry at varying concentrations as indicated in the text and incubated at 37°C for 1 hour. The viral inoculum was then added to cells (MOI 1) and analyzed after 20-24 hours with a Cytation 5 Imaging Reader or flow cytometry for mCherry+ cells.

For experiments using anti-Nrp-1 antibody to block infection, cells were cultured on coverslips and pre-incubated with anti-Nrp-1 antibody (R&D AF566, 4 μg/ml) at 37ºC for 30 minutes. In some instances, 20 μg/ml of heparin sodium salt (Sigma) was incubated with the virus either individually or in combination with anti-Nrp-1 antibody. Cells were then infected for six hours with MCMV or HSV-1 at MOI 10. The percentage of infected cells was assessed by immunofluorescence staining for IE1 or ICP0 in combination with DAPI.

To quantify differences in viral attachment to the cell surface, MCMV was pre-treated with recombinant protein or blocking antibody as before and added to cells for 30 minutes at 4ºC. Cells were then harvested by scraping, and unbound virions wished away with PBS, followed by three rounds of freezing at -80 ºC and thawing. Virus titer was then quantified by plaque assay on NIH3T3 cells.

Mice and preparation of BMDMs

Nrp-1$^{fl/fl}$ C57BL/6 (JAX #005247) and *Cx3cr1*-Cre^{ER} C57BL/6 (JAX #020940) mice were crossed to generate *Nrp-1*$^{fl/fl}$; *Cx3cr1*-Cre$^{ER+/-}$ mice. Bone marrow from the femurs of two 8- to 10-week- old *Nrp-1*$^{fl/fl}$;*Cx3cr1*-Cre$^{ER+/-}$ mice and *Nrp-1*$^{fl/fl}$ littermate controls was flushed with PBS. Harvested cells were then plated in complete DMEM supplemented with 20% L929-conditioned media and 2 μM 4-hydroxy-tamoxifen (Sigma). After seven days, 1 x 10^6 cells were collected for Western blot of Nrp-1 expression and 1.5 x 10^4 cells were seeded in a 96-well plate. Infection with MCMV M45mut was done as described above and survival quantified by Cell Titer-Glo Luminescent Cell Viability Assay. Alternatively, cells were seeded in 6-well plates and infected with WT MCMV. Six hours post infection, cells were collected, lysed, and whole-cell lysates analyzed by Western blot for IE1 expression.

SI References

1. Upton, J.W., W.J. Kaiser, and E.S. Mocarski, *Virus inhibition of RIP3-dependent necrosis.* Cell Host Microbe, 2010. **7**(4): p. 302-13.
2. Guo, H., et al., *Herpes simplex virus suppresses necroptosis in human cells.* Cell Host Microbe, 2015. **17**(2): p. 243-51.
3. Hilterbrand, A.T., et al., *Murine Cytomegalovirus Deubiquitinase Regulates Viral Chemokine Levels To Control Inflammation and Pathogenesis.* MBio, 2017. **8**(1).
4. Sanjana, N.E., O. Shalem, and F. Zhang, *Improved vectors and genome-wide libraries for CRISPR screening.* Nat Methods, 2014. **11**(8): p. 783-784.
5 Joung, J., et al., *Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening.* Nat Protoc, 2017. **12**(4): p. 828-863.
6. Li, W., et al., *MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens.* Genome Biol, 2014. **15**(12): p. 554.

SI Figures and Legends

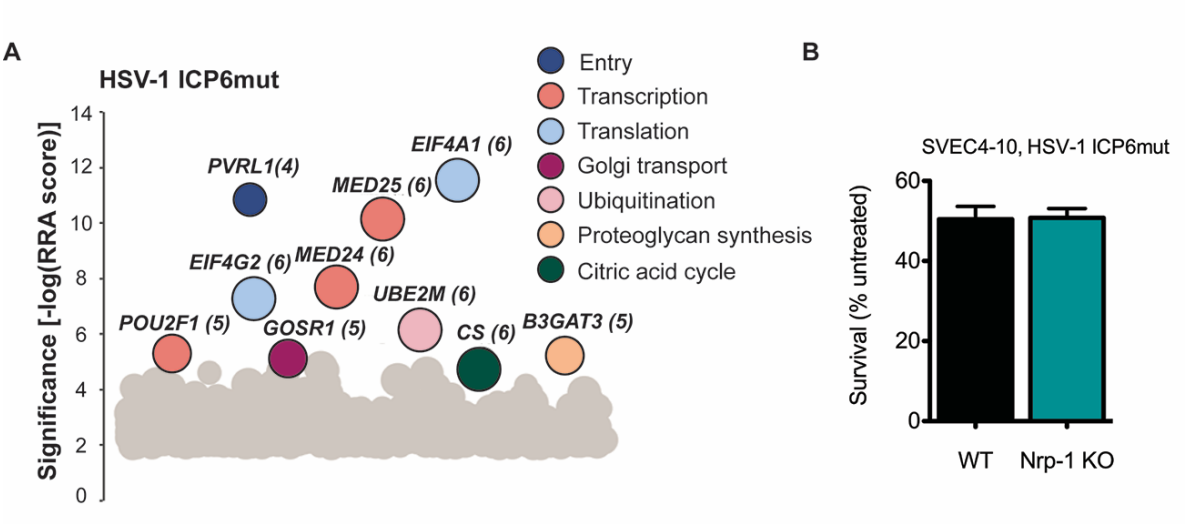
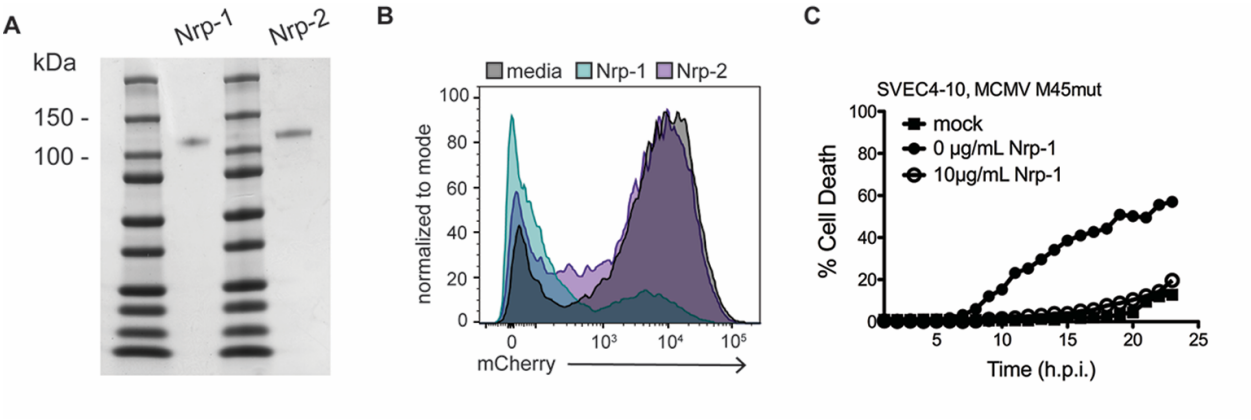


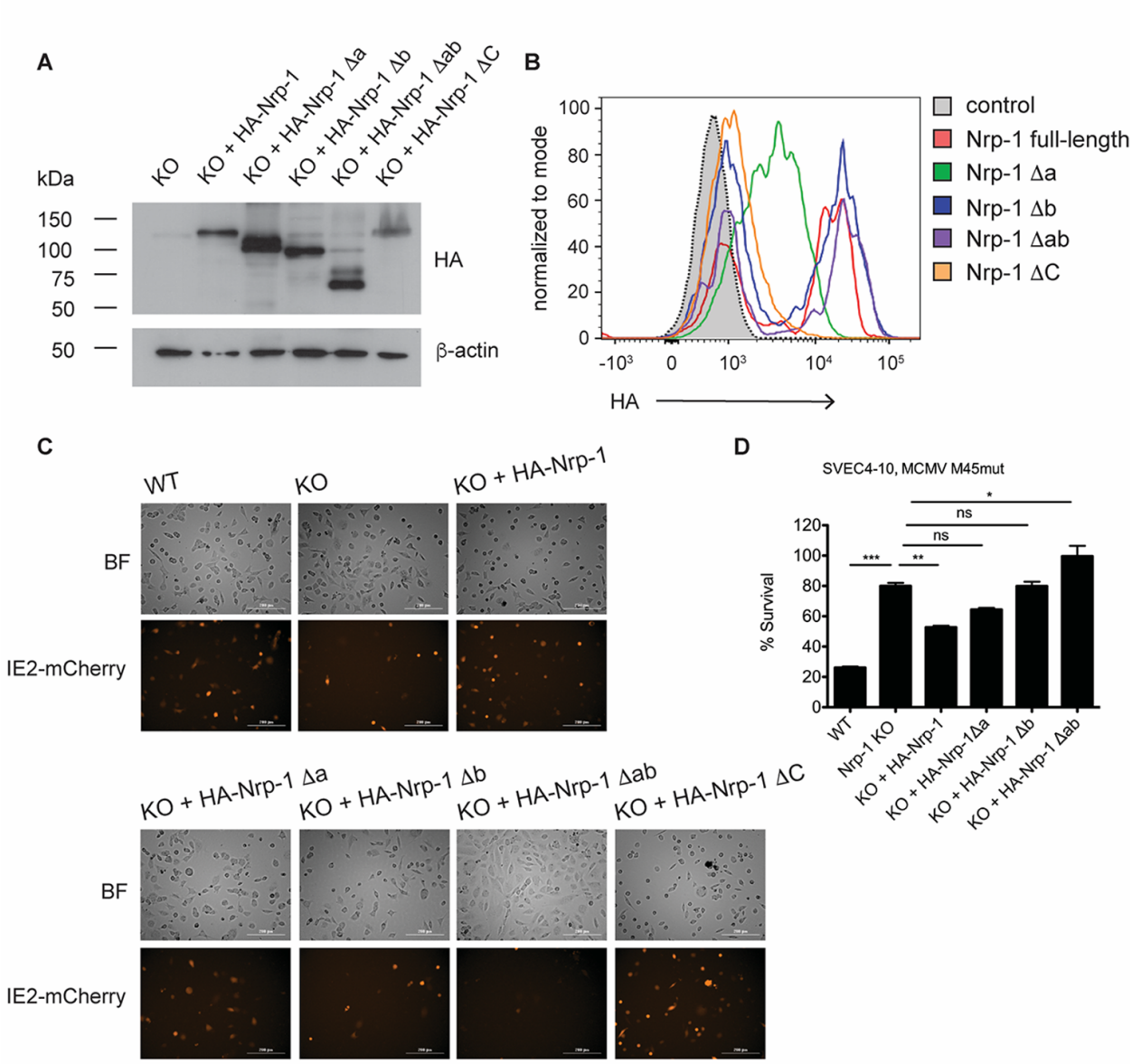
Fig S1. Nrp-1 is uniquely utilized for MCMV, not HSV-1 infection. (A) Screen results for HSV-1. Hits are stratified along a vertical axis according to significance and arbitrarily spread along the horizontal axis. The size of the circle and the number next to the gene name both indicate the number of gRNAs for that gene that were found in the resistant population. Colors indicate gene ontology. (B) Cell viability in WT and Nrp-1 KO SVEC4-10 cells infected with HSV-1 ICP6mut (MOI 10). After 24 hours, survival was measured by Cell Titer Glo Luminescent Cell Viability Assay. Infections were performed in triplicate with two independent experiments.



188

189 **Fig S2. Soluble Nrp-1 reduces infection rates in SVEC4-10 cells.** (A) Coomassie blue stain of
190 recombinant Nrp-1 and Nrp-2. Proteins were run on an SDS-PAGE gel under reducing
191 conditions. (B) MCMV IE2-mCherry was incubated with soluble recombinant Nrp-1, Nrp-2, or
192 media alone for one hour at 37°C before being added to WT SVEC4-10 cells at MOI 1. Cells
193 were analyzed by flow cytometry 20 h.p.i. (C) Cell death in cells infected in triplicate with
194 MCMV M45mut virus pre-treated with soluble Nrp-1 as in (B). Cell death was determined by
195 staining cells with Sytox and Hoechst and imaging every hour. At least two replicates were
196 performed per experiment.

197



198

199 **Fig S3. The extracellular domains of Nrp-1 facilitate MCMV infection.** Immunoblot (A) and

200 flow cytometry (B) detecting expression of HA-tagged Nrp-1 mutants in SVEC4-10 Nrp-1 KO cells.

201 (C) Fluorescence microscopy for IE2-mCherry expression in SVEC4-10 WT or Nrp-1 KO cells

202 reconstituted with full-length and deletion Nrp-1 mutants. Cells were infected with MCMV IE2-

203 mCherry (MOI 1) in triplicate and imaged after 20 h.p.i. (D) Quantification of survival of Nrp-1

204 mutant-expressing cells following MCMV M45mut infection (MOI 10). Infection was performed

205 in triplicate. After 24 hours, survival was measured by Cell Titer Glo Luminescent Cell Viability

Assay. At least two replicates were performed per experiment. Error bars indicate SEM. Significance was determined using a one-way ANOVA with Bonferroni's multiple comparisons test (*** $p<0.0001$ **$p<0.001$ * $p<0.01$).

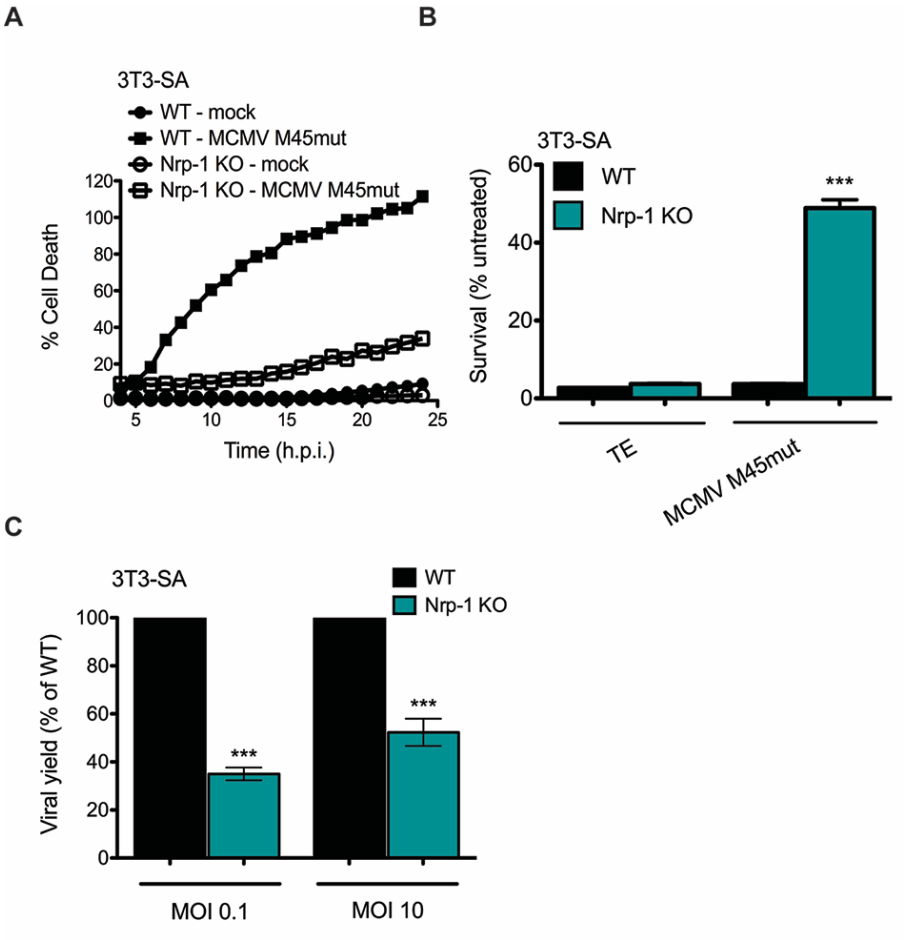
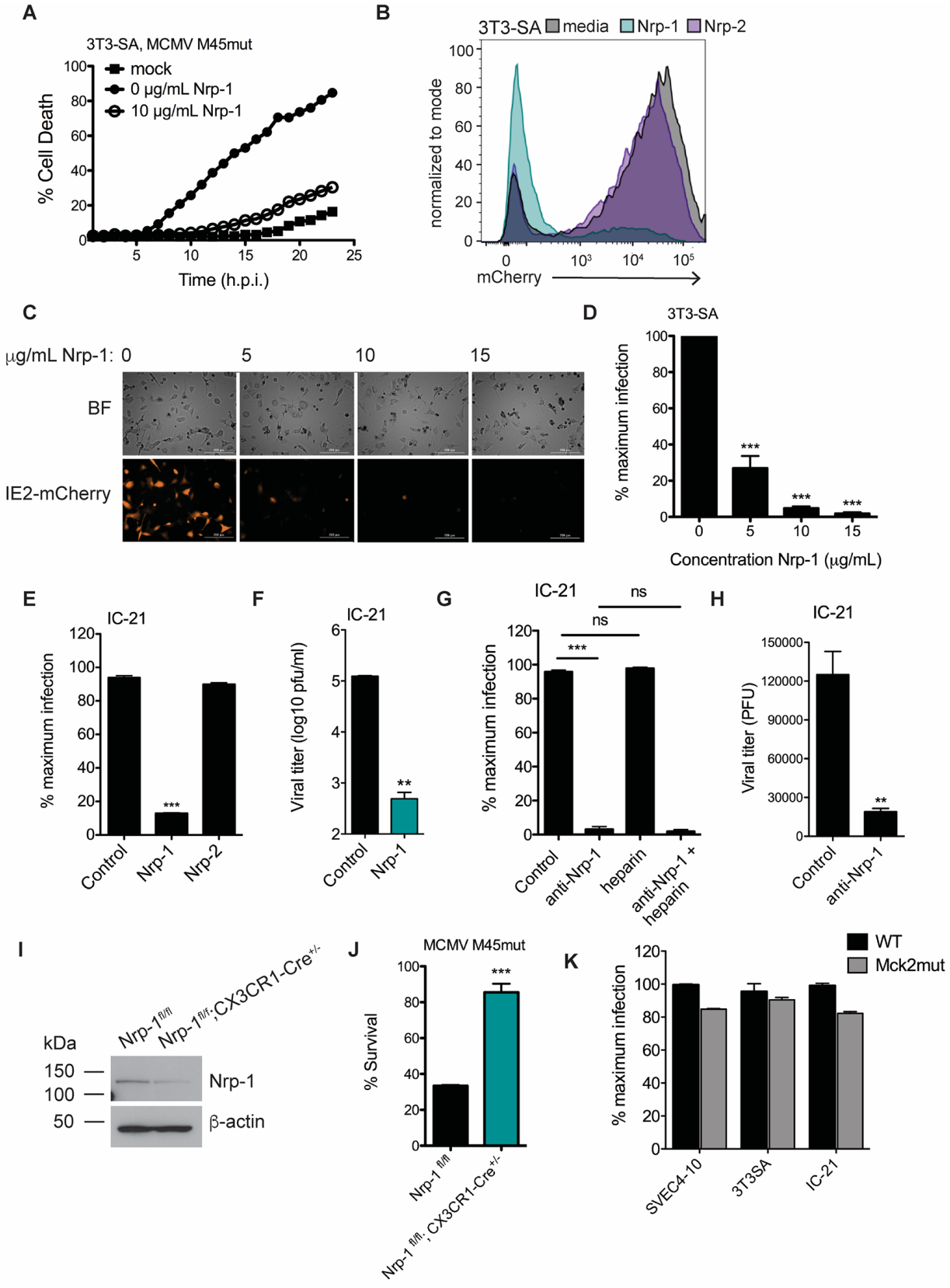


Fig S4. Cell death and viral titers are reduced in Nrp-1 KO 3T3-SA cells. (A) Cell death in 3T3-SA Nrp-1 KO cells was determined by Sytox uptake as described in Fig. 1F. (B) Cell survival in WT and Nrp-1 KO 3T3-SA cells infected with MCMV M45mut (MOI 10), or treated with 25 ng/mL TNFα + 5 μM Emricasan or media alone. After 24 hours, survival was determined by Cell Titer Glo Luminescent Cell Viability Assay. (C) WT or Nrp-1 KO SVEC4-10 cells were infected at the indicated MOI with WT MCMV for one hour and harvested after 48 (MOI 10) or 72 (MOI 0.1) h.p.i. and viral titer determined by plaque assay. Values shown are relative to WT counterparts. All infections were performed in triplicate and at least two replicates were performed per experiment. Error bars indicate SEM. Significance was determined using a student's t-test (*** $p<0.0001$).


A
3T3-SA, MCMV M45mut
mock
0 µg/mL Nrp-1
10 µg/mL Nrp-1
% Cell Death
Time (h.p.i.)
B
3T3-SA media Nrp-1 Nrp-2
normalized to mode
mCherry
C
µg/mL Nrp-1: 0 5 10 15
BF
IE2-mCherry
D
3T3-SA
% maximum infection
Concentration Nrp-1 (µg/mL)
E
IC-21
% maximum infection
Control
Nrp-1
Nrp-2
F
IC-21
Viral titer (log10 pfu/ml)
Control
Nrp-1
G
IC-21
ns
% maximum infection
Control
anti-Nrp-1
heparin
anti-Nrp-1 + heparin
H
IC-21
Viral titer (PFU)
Control
anti-Nrp-1
I
kDa
Nrp-1fl/fl
Nrp-1fl/fl;CX3CR1-Cre+/-
150
100
50
Nrp-1
β-actin
J
MCMV M45mut
% Survival
Nrp-1 fl/fl
Nrp-1 fl/fl; CX3CR1-Cre+/-
K
WT
Mck2mut
% maximum infection
SVEC4-10
3T3SA
IC-21

220

Fig S5. Soluble Nrp-1 inhibits MCMV infection in fibroblasts and macrophages. (A) MCMV M45mut virus was pretreated with soluble Nrp-1 for one hour before infecting 3T3-SA cells. Cell death was determined by staining cells with Sytox and Hoechst and imaging every hour. (B) Cells were infected with MCMV IE2-mCherry that had been pretreated for one hour with Nrp-1. Red fluorescence was quantified at 20 h.p.i. by flow cytometry. (C) 3T3-SA cells were infected with MCMV IE2-mCherry pre-treated with varying concentrations of soluble Nrp-1 and imaged 20 h.p.i. (D) Quantification of mCherry + cells from (C). Significance was determined by using a one-way ANOVA with Dunnett's multiple comparisons test. (E) Percent infection in IC-21 cells in the presence of soluble neuropilins. MCMV was pretreated with 10 μg/mL soluble Nrp-1 or 15 μg/mL Nrp-2 for one hour prior to infecting IC-21 cells. Cells were fixed six h.p.i. and percent infection quantified by staining for IE1 and DAPI. Significance was determined by using a one-way ANOVA with Dunnett's multiple comparisons test. (F) Viral yield in IC-21 cells infected with MCMV (MOI 1) in the presence or absence of 10 μg/mL Nrp-1 for 48 hours. Cell lysates were titered by plaque assay. (G) Percent infection in the presence of Nrp-1 blocking antibody. IC-21 cells were pre-incubated with anti-Nrp1 antibody (4 μg/ml), heparin (20 μg/ml), or both for 30 minutes and then infected for six hours with MCMV. The percentage of infected cells was assessed by staining for IE1 and DAPI. Significance was determined by using a one-way ANOVA with Bonferroni's multiple comparisons test. (H) Quantification of viral attachment at the cell surface in the presence of Nrp-1 blocking antibody. IC-21 cells were pre-incubated with anti-Nrp1 antibody (4 μg/ml) or media alone for one hour before being added to IC-21 cells in triplicate at MOI 1 and incubated for 30 minutes at 4°C. Cells were harvested by scraping and unbound virions washed away. The amount of adhered virus was then quantified by plaque assay. (I) Western blot for level of Nrp-1 knockout in

BMDMs collected from mice of the indicated genotypes and treated with 2 μM 4-hydroxy-tamoxifen for seven days to induce Cre activity. (J) Cell survival in BMDMs treated as in (I) and infected in triplicate with MCMV M45mut (MOI 10). 24 h.p.i., cell survival was quantified by Cell Titer Glo Luminescent Cell Viability Assay. (K) SVEC4-10, 3T3-SA, and IC-21 cells were infected with WT or MCK2-deficient virus (Mck2mut) at MOI 10 and 6 h.p.i. stained for IE1 expression by immunofluorescence. All infections were performed in triplicate and at least two replicates were performed per experiment. All microscopy data was quantified by taking the average from four images. Significance was determined using a student's t-test unless otherwise stated (*** $p<0.0001$, ** $p<0.001$).