Supporting Information

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SI Materials and Methods

Plasmids and siRNA Oligos. The cDNA of HSV-1 gene ICP6 was amplified by RT-PCR from total RNA of MEF cells infected with HSV-1. For the RIP3 lentiviral expression construct, mouse RIP3 was cloned into the pCAG-MCS-IRES vector, which was a gift from the laboratory of Yun Zhao (Soochow University). For the ICP6 retroviral expression construct, ICP6 was cloned into the PQCXIP vector. Lentiviral expression construct containing the RIP3 kinase dead mutant (RIP3 K51A) or RIP3 RHIM domain mutant (RIP3 RHIM Mut, residues from 448 to 451 are mutated to four alanine residues), and retroviral expression construct containing ICP6 RHIM domain mutant (ICP6 RHIM Mut, residues from 73 to 76 are mutated to four alanine residues) were generated using a Quik-Change Lightning Site-Directed Mutagenesis Kit (Stratagene). Mouse RIP3, MLKL, RIP1, and CYLD siRNAs were synthesized by Shanghai GenePharma Co., Ltd.: RIP3 (cccgacgaugucuucugucaa), CYLD-1 (uccauugaggauguaaauaaa), CYLD-2 (aaggguugaaccauuguuaaa), MLKL-1 (gagauccaguucaacgaua), MLKL-2 (uaccaucaaaguauucaacaa), and control siRNA (aacguacgcggaauacuucga), RIP1 (ccacuagucugacugauga), nectin-1–1 (ccggatgactcggatgatgaa), and nectin-1–2 (caggtggaggtcaatatcaca). RNA interference was performed using INTERFERin (Polyplus).

Cell Culture and Virus. HEK-293T, African green monkey kidney (Vero), and mouse fibrosarcoma L929 cells were obtained from ATCC. MEFs were isolated from day 14.5–15.5 embryos. HEK-293T, Vero, L929, and MEFs were cultured in DMEM containing 10% (vol/vol) FBS (Gibco) and 100 units/mL penicillin/streptomycin (HyClone). WT and mutant HSV-1 were grown in Vero cells.

Western-Blot Analysis. The cells were harvested by scraping and centrifugation at 16,000 × *g* for 1 min. The harvested cells were washed once with PBS and then resuspended in lysis buffer containing 20 mM Tris·HCl (pH 7.4), 150 mM NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM Na_3VO_4, 25 mM β-glycerol-phosphate, 0.1 mM PMSF, a complete protease inhibitor set (Roche), and a phosphatase inhibitor set (Sigma). The cell pellet was vortexed for 20 seconds. After lysis on ice for 20 min, the total cell lysates were spun down at 20,000 × *g* for 20 min. The soluble fraction was collected and used for Western-blot analysis or immunoprecipitation studies. For nonreducing gel analysis, cells were lysed in lysis buffer without DTT and separated by SDS/PAGE without β-mercaptoethanol.

Immunoprecipitation and Silver Staining. For Flag pull-down, cell lysates were incubated with anti-Flag agarose beads (Sigma) overnight at 4 °C. The following day, beads were washed with lysis buffer, and the immunoprecipitants were eluted off the beads with a low pH elution buffer (Pierce) or 0.5 mg/mL 3× Flag peptide (Sigma) at 4 °C for 4 h. HA immunoprecipitation was performed using anti-HA agarose beads (Sigma). Acid elution was neutralized using 1/20 volume of 1 M Tris·HCl (pH 9.4). The silver staining assay was performed using a Silver Stain Kit according to the manufacturer's instructions (Sigma).

RT-PCR and qPCR. Total RNA was extracted with TRIzol (Invitrogen) and reverse-transcribed into cDNA with a RevertAid First Strand cDNA kit (Thermo). Gene expression was determined by qPCR analysis using Power SYBR Green PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7500 Fast Real-Time PCR System. The following primers were used: For HSV-1 TK, 5′-CAA TCG CGA ACA TCT ACA CC-3′ (forward) and 5′-TAT CTG GGC GCT TGT CAT TA-3′ (reverse); for HSV-1 gB, 5′-ACA CAA GGC CAA GAA GAA GG-3′ (forward) and 5′-TTG TTG GGA ACT TGG GTG TA-3′ (reverse).

PI Staining. MEF cells were cultured in six-well plates and infected with HSV-1.Ten hours postinfection, cells were stained with PI (Biouniquer) and then evaluated by fluorescence microscopy (Olympus).

Lentivirus and Retrovirus Expression System. For lentivirus packaging, pCAG-MCS-IRES plasmid harboring the target gene (RIP3 WT, RIP3 K51A, RIP3 RHIM Mut or MLKL), ΔR, VSV-G and Rev were cotransfected into 293T cells, respectively. Eight hours post transfection, the medium was changed to fresh complete medium. The supernatants were collected 32–56 h post transfection. To generate RIP3 KO MEFs stably expressing RIP3 WT, RIP3 K51A or RIP3 RHIM Mut, RIP3 KO MEFs were infected with 0.5 mL of supernatant containing lentivirus particles expressing the corresponding target gene. To generate MEFs stably expressing MLKL, MEFs were infected with lentivirus particles expressing MLKL. Three days post infection, the infected cells were sorted by fluorescence-activated cell sorting (FACS) for GFP fluorescence and then used as stable lines. For retrovirus packaging, the PQCXIP plasmid harboring the target gene, VSV-G and gagpol were cotransfected into 293T cells. The supernatants were collected 36 h post transfection. To concentrate the retrovirus, the supernatants were centrifuged at 105,000 × *g* and the pellet at the bottom of the tube was then resuspended with DMEM containing Dnase I. MEFs and L929 cells were infected using the retrovirus concentrate.

Generation of MLKL-shRNA and DAI KO cells. To generate MLKL-shRNA expression cells, MLKL-shRNA construct was transfected into MEFs. Forty-eight hours posttransfection, the cells were cultured in complete medium containing 1 mg/mL neomycin. Clones were tested for expression of MLKL. We designed two DAI single guide RNAs (sgRNA): sgRNA-1 (GAGCATAGGCGGGGCTGCTTC) and sgRNA-2 (CTCTGTCCTCCTTCTTCAGG). Each DAI sgRNA was cloned to a pLKO-GFP vector, which was a gift from Zhirong Shen (National Institute of Biological Sciences, Beijing, China). These two plasmids were cotransfected with D10A Cas9 mutant into MEFs. Forty-eight hours posttransfection, the cells were sorted by FACS for GFP fluorescence. After 2 wk, 60 single clones were picked up, and genomic DNAs of these clones were used for PCR examination. Two DAI KO clones (32# and 40#) were further confirmed by DNA sequencing (Figs. S4 and S5).

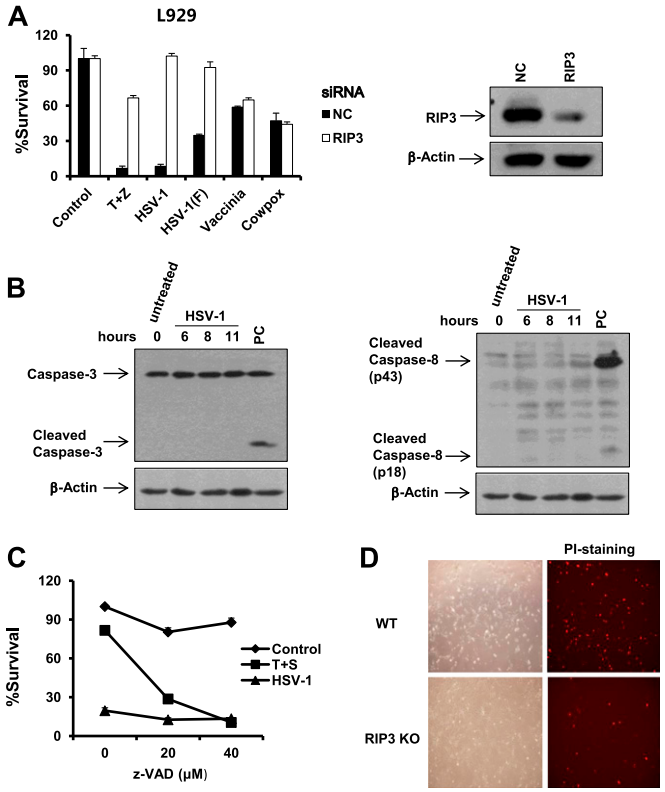

Fig. S1. HSV-1 infection activates RIP3-dependent necrosis. (*A*) L929 cells were transfected with NC or RIP3 siRNA oligos. Forty-eight hours posttransfection, cells were treated with the indicated viruses (MOI = 5) for an additional 16–18 h. Identical MOI was used in L929 cells in later experiments unless otherwise stated. Cell-survival rate was determined by measuring ATP levels. Cell lysates were collected 48 h posttransfection and subjected to Western-blot analysis of RIP3 and β-Actin levels. (*B*) Proteins extracted from MEFs infected with HSV-1 at indicated times were analyzed by Western blot using cleaved-caspase-3, cleaved-caspase-8, and β-Actin antibodies. PC, MEFs were treated with staurosporine at 200 nM for 10 h. (*C*) MEFs were treated with z-VAD 1 h before the treatment of control, TNF-α/Smac mimetic, or HSV-1 for 16–18 h, and cell viability was determined by measuring ATP levels. (*D*) WT or RIP3 KO MEFs were infected with GFP-labeled HSV-1 F strain for 16 h. Then cells were analyzed for PI staining by inverted microscope.

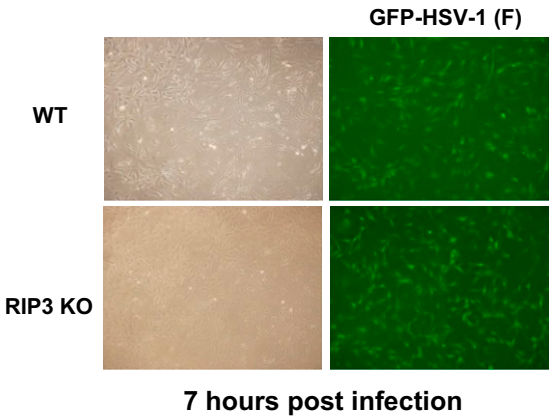

Fig. S2. RIP3 is dispensable for HSV-1 entry and replication. WT or RIP3 KO MEFs were infected with GFP-labeled HSV-1 F strain for 7 h. Then cells were analyzed for GFP by inverted microscope.

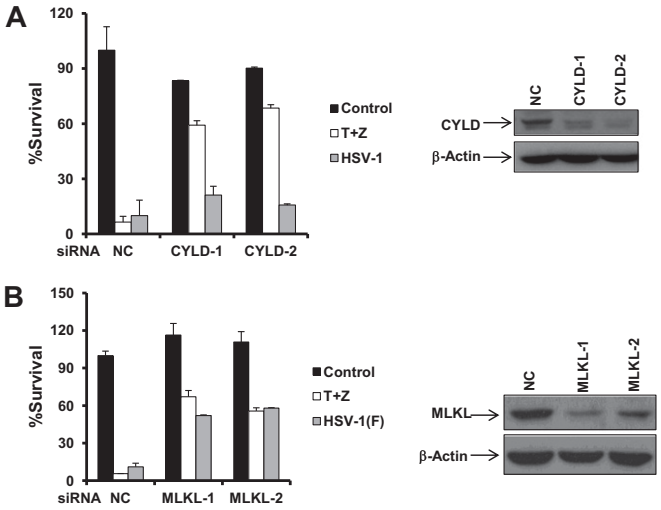

Fig. S3. HSV-1 infection-induced necrosis requires MLKL, but not CYLD. (*A*) L929 cells were transfected with NC or CYLD siRNA oligos. Forty-eight hours posttransfection, cells were treated as indicated for an additional 16–18 h. Cell-survival rate was determined by measuring ATP levels. Cell lysates were collected 48 h posttransfection and subjected to Western-blot analysis of CYLD and β-Actin levels. (*B*) L929 cells were transfected with NC or MLKL siRNA oligos. Forty-eight hours posttransfection, cells were treated as indicated for an additional 16–18 h. Cell-survival rate was determined by measuring ATP levels. Cell lysates were collected 48 h posttransfection and subjected to Western-blot analysis of MLKL and β-Actin levels.

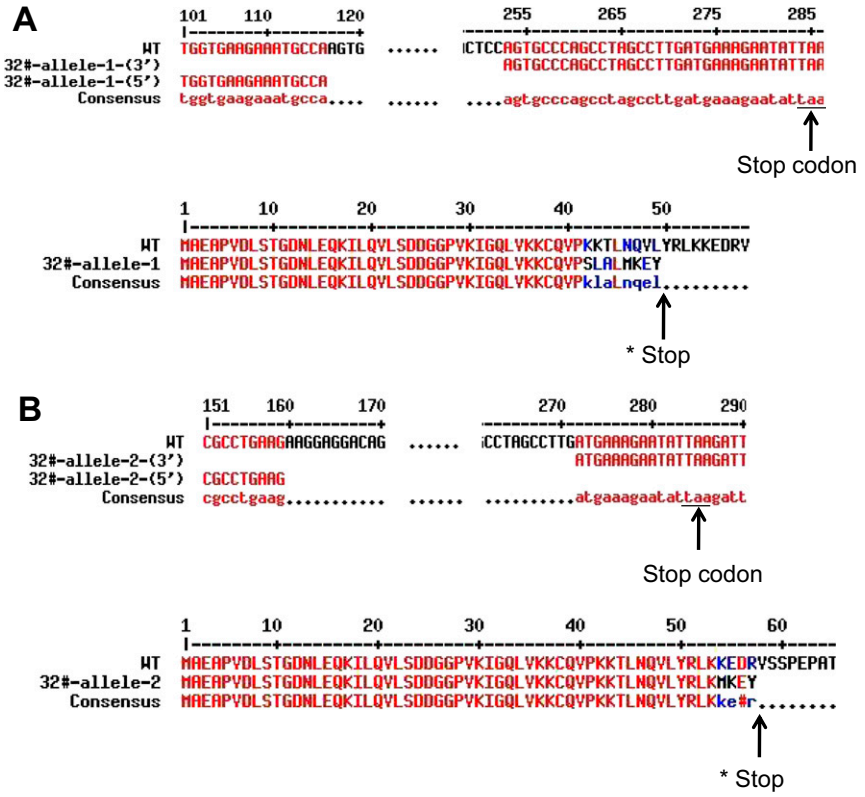

Fig. S4. Altered DAI DNA sequence shown in DAI KO clone 32#. (*A*) One allele of DAI KO clone 32# showed the inserted stop at residue 50. 32#-allele-1-(5′), N-terminal DNA sequence (1–116 bp) of DAI; 32#-allele-1(3′), C-terminal DNA sequence (117–1,110 bp) of DAI. (*B*) The other allele of DAI KO clone 32# showed the inserted stop at residue 58. 32#-allele-2-(5′), N-terminal DNA sequence (1–159 bp) of DAI; 32#-allele-2-(3′), C-terminal DNA sequence (160–1,124 bp) of DAI.

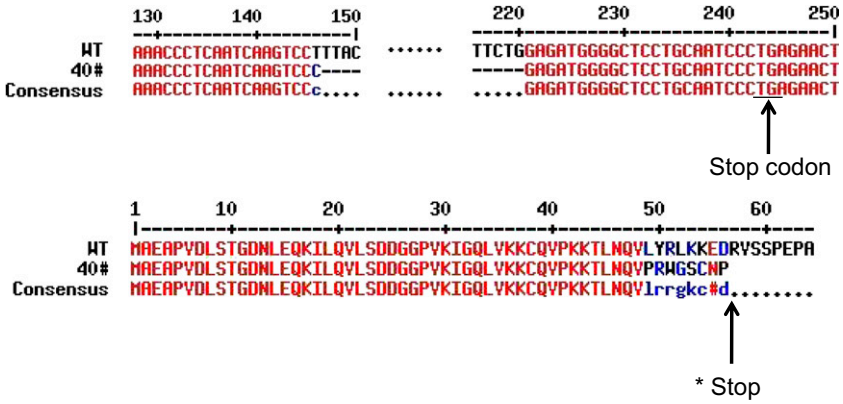

Fig. S5. Altered DAI DNA sequence shown in DAI KO clone 40#. This clone is a homozygous mutant with the inserted stop at residue 57.

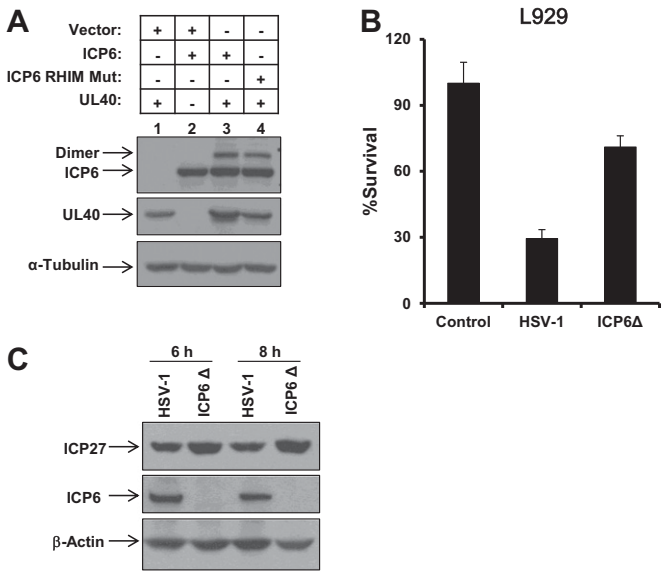

Fig. S6. Viral protein ICP6 interacts with RIP3 through its RHIM domain and is required for HSV-1–induced necrosis. (*A*) The 293T cells were transfected with DNA plasmids as indicated for 48 h. Cell lysates were collected and subjected to Western-blot analysis of ICP6, UL40, and β-Actin levels. (*B*) L929 cells were infected with HSV-1 or HSV-1 ICP6Δ for 16–18 h. Cell-survival rate was determined by measuring ATP levels. (*C*) L929 cells were infected with HSV-1 or HSV-1 ICP6Δ for the indicated time. Cell lysates were collected and subjected to Western-blot analysis of ICP6, ICP27, and β-Actin levels.

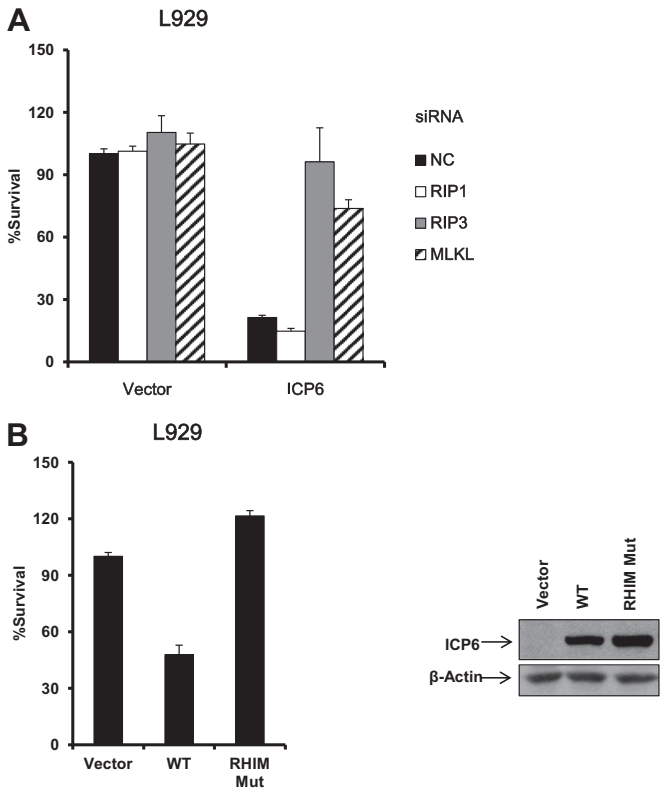

Fig. S7. ICP6 is sufficient to activate RIP3/MLKL-mediated necrosis. (*A*) L929 cells were transfected with NC, RIP3, RIP1, or MLKL siRNA oligos. Forty-eight hours posttransfection, cells were infected with retrovirus expressing vector or ICP6 for 48 h. Cell-survival rate was determined by measuring ATP levels. (*B*) L929 cells were infected with retrovirus expressing WT ICP6 or ICP6 RHIM Mut for about 48 h. The cell-survival rate was determined by measuring ATP levels. Cell lysates collected from cells infected for 24 h with retrovirus as indicated were subjected to Western-blot analysis for ICP6 and β-Actin levels.

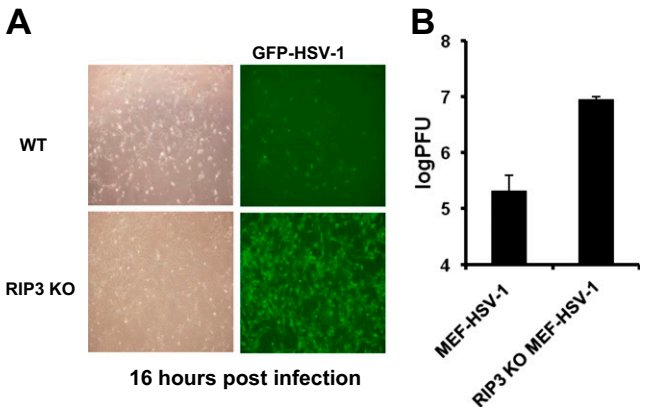

Fig. S8. After GFP-HSV-1 F strain infection, RIP3 KO MEFs survived and showed increased GFP signal intensity compared with WT cells. (*A*) WT or RIP3 KO MEFs were infected with GFP-labeled HSV-1 F strain for 16 h. Then cells were analyzed for GFP fluorescence by inverted microscope. (*B*) We infected WT or RIP3 KO MEFs with HSV-1 for 16 h and collected the culture medium and cells. The mixed culture medium and cells were thawed and frozen three times, followed by spin at 600 × *g* for 3 min. The supernatant was collected for the analysis of viral titers by plaque assay. MEF-HSV-1, HSV-1 was collected from WT MEFs infected with HSV-1 for 16 h. RIP3 KO MEF-HSV-1, HSV-1 was collected from RIP3 KO MEFs infected with HSV-1 for 16 h.