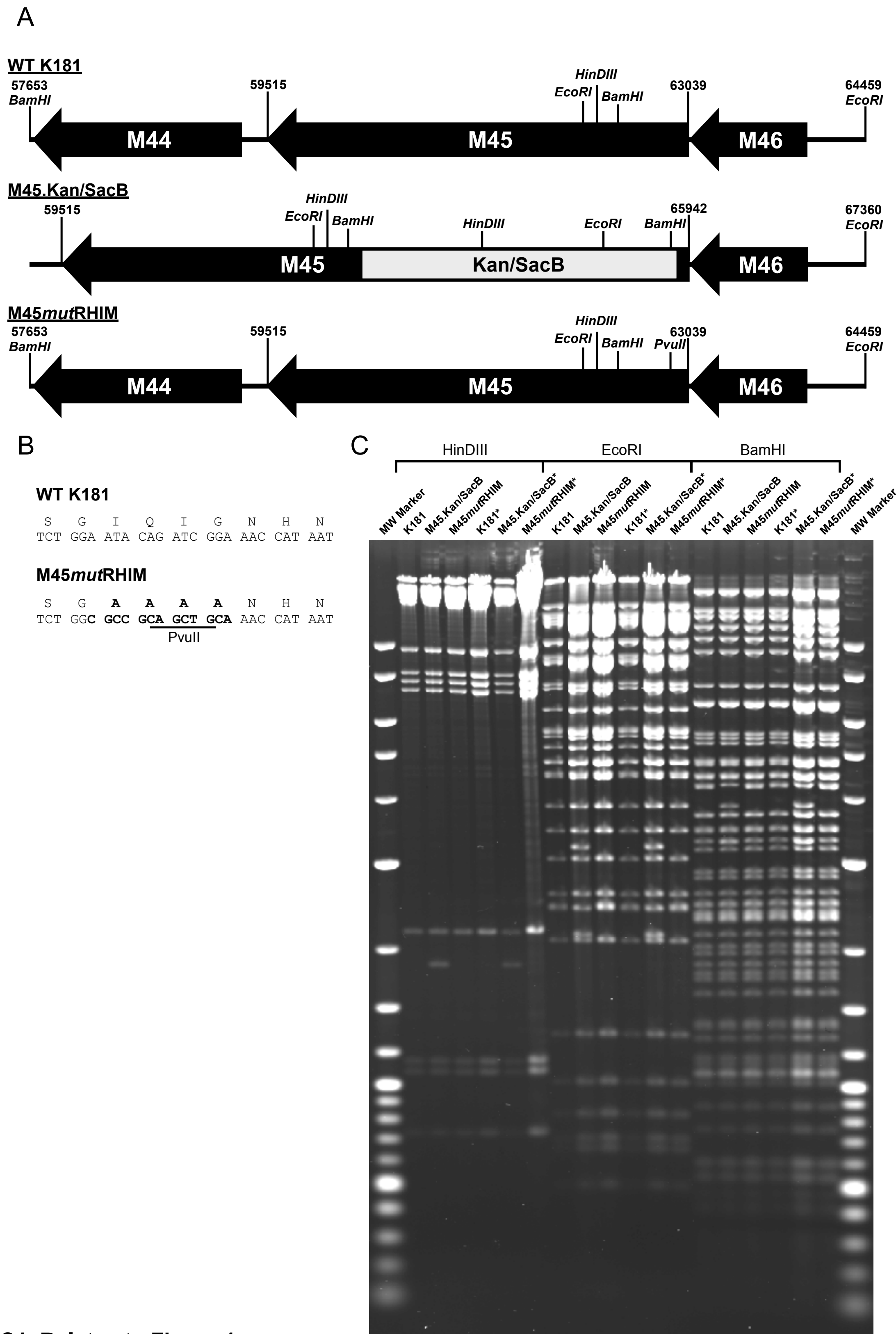

Figure S1. Relates to Figure 1

(A) Schematic representation of MCMV region encoding M45 (vIRA) and surrounding genes from bp 57653 to 64459 based on the K181 sequence (Accession# AM886412.1). The M45 ORF is encoded on the complementary strand from bp 63039 to 59515. Restriction enzyme sites shown were used for mutagenesis and/or diagnostics. Black arrows represent annotated ORFs. (B) Partial nucleotide and amino acid sequences of WT and mutant M45. Bold letters represent nucleotide changes. Underlined sequence represents introduced restriction enzyme site for mutant diagnostics. (C) WT and mutant BAC DNAs were isolated from bacteria and subjected to restriction enzyme digest with indicated enzyme. WT and WT* are independently isolated parental WT K181-MCMV bacmids individually used to derive M45sB/K or M45sB/K*, and M45mutRHIM or M45mutRHIM*, respectively. MW Marker, 2-Log Ladder (New England Biolabs).

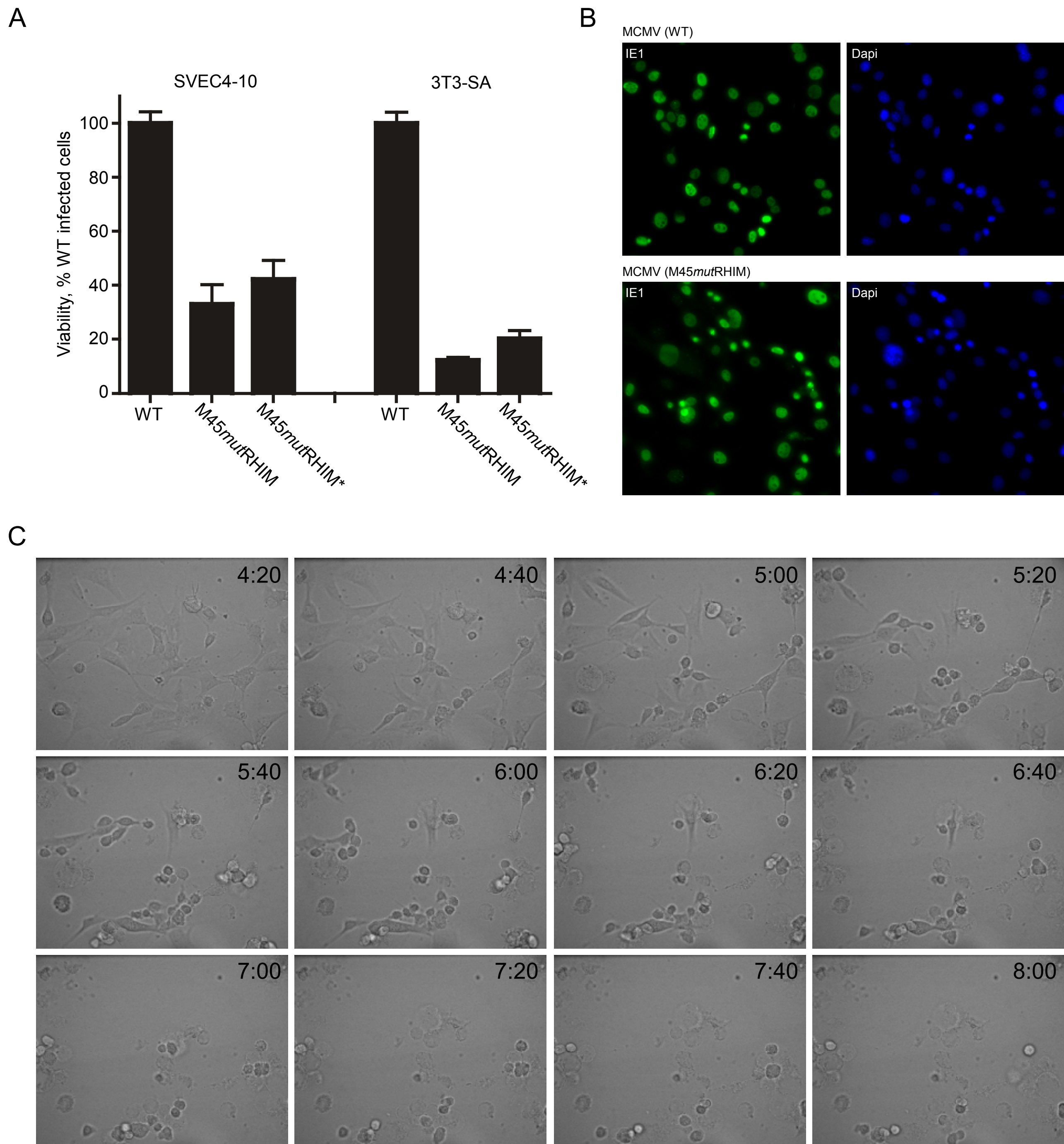

Figure S2. Relates to Figure 2
(A) SVEC4-10 or 3T3-SA cells were infected with the indicated virus, where the asterisk indicates an independent isolate of M45*mut*RHIM. Viability was assessed by measuring intracellular ATP levels at 18 hpi. (B) 3T3-SA cells are highly permissive to WT and M45*mut*RHIM virus. Microphotographs of IE1 positive cells at 6 h post infection with the indicated virus. (C) 3T3-SA cells were infected with M45*mut*RHIM. The time (h:mm) post infection an image was captured is shown in the right corner of each image. For all panels, cells were infected at an MOI of 10.

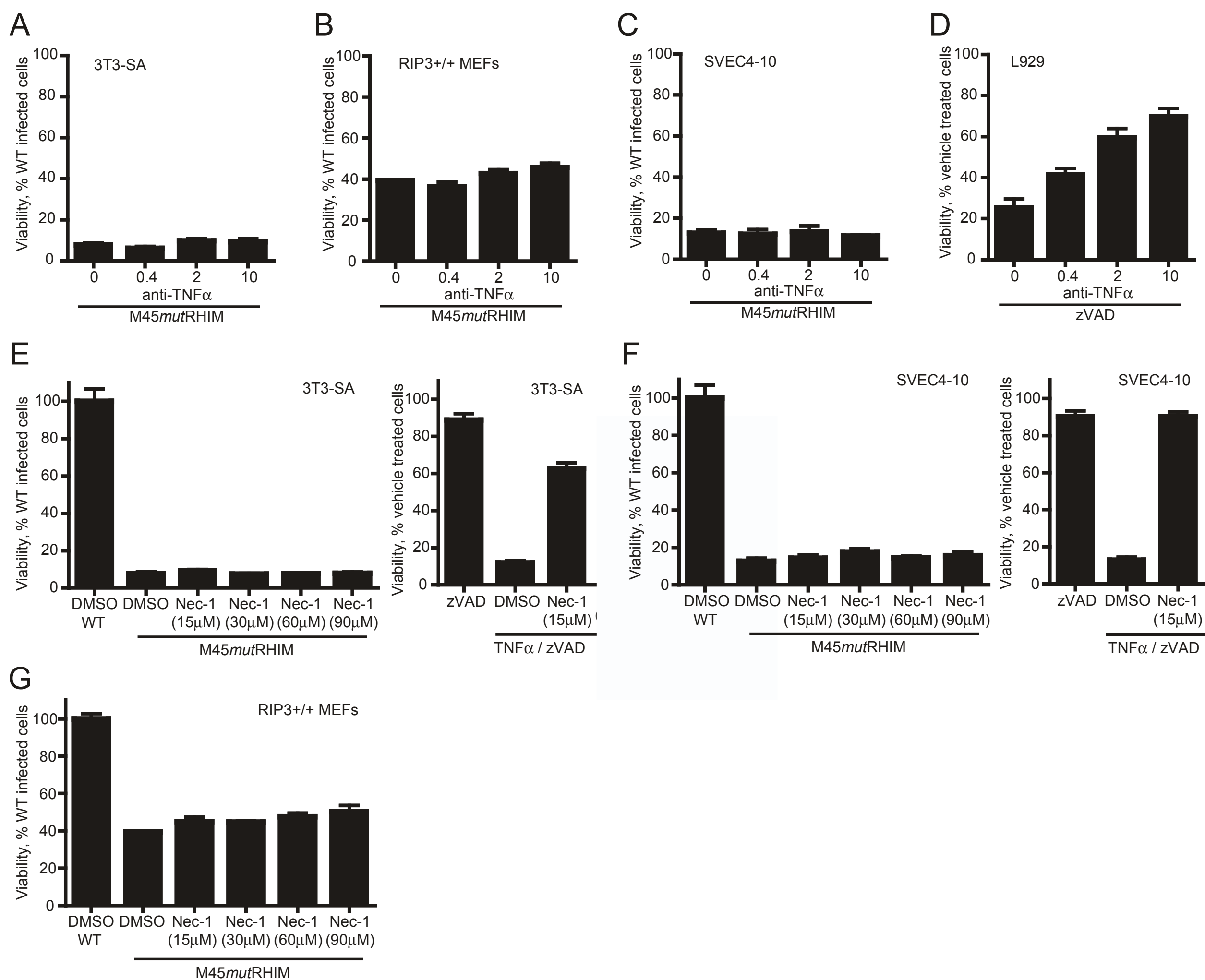

Figure S3. Relates to Figure 4

(A-D) Anti-TNFα neutralizing antibody blocks DR-induced necroptosis induced by zVAD-fmk but not MCMV-associated programmed necrosis. 3T3-SA (A), SVEC4-10 (B), RIP3+/+ MEFs (C) were infected with WT or M45*mut*RHIM virus (MOI of 10) for 18 h. L929 (D) cells were treated with zVAD-fmk (25 μM) for 18 h. Cells were pretreated with the indicated concentration of mouse anti-TNFα antibody at the indicated concentration. Antibody levels were maintained until cell viability was determined. (E-G) Necrostatin-1 blocks TNFα-induced necroptosis but not MCMV-associated programmed necrosis. 3T3-SA (E) or SVEC4-10 (F) cells were infected (left panels) with WT or M45*mut*RHIM virus (MOI of 10) for 18 h in the absence or presence increasing concentrations of Nec-1, or treated (right panels) with TNFα (25 ng/ml) and zVAD-fmk (25 μM) for 18 h in the presence or absence of Nec-1. (G) RIP3+/+ MEFs were infected with WT or M45*mut*RHIM virus (MOI of 10) for 18 h in the absence or presence increasing concentrations of Nec-1. The concentration of the Nec-1 vehicle, DMSO, was identical for all samples assayed. Cell viability was determined by measuring ATP levels.

Pathogen subversion of RIP3-dependent necrosis
Jason W. Upton, William J. Kaiser, and Edward S. Mocarski

Supplemental Experimental Procedures

BAC mutagenesis and recombinant viruses

The MCMV-K181 bacterial artificial chromosome, pARK25 (Redwood et al., 2005), and pSIM6, a plasmid encoding λ-RED recombination functions (a gift from Tim Barnett, Emory Children's Center), were introduced in DH10B by electroporation. Recombineering was performed essentially as previously described (Tandon et al., 2008). Briefly, multiple clones of pARK25/pSIM6 containing bacteria were grown to OD600 of 0.4 to 0.6, and recombination functions induced by incubation at 42°C. Bacteria were then rendered electrocompetent by multiple washes in ice-cold water. The kanamycin (Kan) resistance and levensucrase (SacB) genes and associated regulatory elements were PCR amplified from pTBE100 (another generous gift from Tim Barnett, Emory Children's Center) with 50nt overhangs corresponding to MCMV genomic sequence within the M45 ORF (primers; JUp045, 5'-GGGGAGCCTTCGGGGTGGGTGGGGGGCACAGCGTGCCCTACGTCAGGA-TCAATTCGAGCTCGGTACCCGG-3' and JUp046, 5'-GGCGATGCTCATAGCATTATGGTTTCCGATCTGT-ATTCCAGAGACTCCATATCCCGGGAAAAGTGCCACC-3'). PCR reactions were treated with DpnI to digest template DNA and the amplicons gel purified, then used to electroporate induced bacteria. Kanamycin-resistant, sucrose-sensitive clones were confirmed and analyzed for recombineered insertion and genomic integrity by RFLP analysis. Kan/SacB insertion deletes 4nt of MCMV sequence and introduces 2.9kb within the M45 open reading frame. Specific M45 mutations were introduced by a second round of recombineering with PCR amplicons encoding M45*mut*RHIM (Upton et al.,, 2008) (primers; JUp097, 5'-CCTCGTCGAGTTCGCGTGACATGGATCGCCAGCCCAAAGTC-3' and JUp098, 5'-TTGGCGACGAGTCCGCCGTCAGCGATAATTCACGGAAGGGG-3'). Colonies were screened for sucrose resistance and kanamycin sensitivity, and positive clones were further confirmed by RFLP and functional analyses. M45*mut*RHIM and M45*mut*RHIM* were each generated from independently isolated parental WT K181 bacmid clones. BAC DNA was isolated using Midi-prep columns (Qiagen), and cut with HindIII, EcoRI, or BamHI (New England Biolabs). Viruses were generated as described in Materials and Methods.

Time-lapse Microphotography

3T3-SA cells were seeded on a 35mm glass bottom culture dish (MatTek Corp.) and infected 18 h later. At 4 h post infection, cells were placed in a heated chamber with CO_2 perfusion. Live cell analysis of cells was performed with a Perkin Elmer Ultraview RS mounted on a Zeiss Axiovert 200m using a 10X objective.

Immunofluorescence Analysis At 6 h post infection with either MCMV WT or MCMV M45*mut*RHIM virus, 3T3-SA cells grown on coverslips were processed as described for immunofluorescence analysis (Kaiser et al., 2008). The anti-MCMV IE1 Ab (diluted 1/2000 in blocking buffer) was used as the primary Ab and AlexaFluor 488 (diluted 1/5000) was used as a secondary antibody.