Supplemental Experimental Procedures

Plasmids and retroviral transduction

pBabe Mito-tdTomato was generated by cloning amino-acids 1-54 of human Smac in frame with tdTomato into pBabe Puro. AcCaspase-8 was generated as previously described (Oberst et al., 2010). AcRIPK3 was generated by cloning full-length murine RIPK3 into the XbaI site of the $pC_4M\text{-}F_V2E$ vector, (a gift from Ariad Pharmaceuticals), to create RIPK3 with tandem FKBP domains fused to its C-terminus. The resulting FKBP-Caspase-8 and RIPK3-2xFKBP proteins were introduced into pBabe-Puro retroviral vectors by standard restriction-digest based sub-cloning. Phoenix Ecotropic cells (0.5 x 10^6 in a 10 cm dish) were transfected with LZRS mCherry-Parkin, LZRS YFP-Parkin, pBabe mito-td Tomato, pBabe-acCaspase-8, or pBabe-acRIPK3 (or vector controls, as indicated) using Lipofectamine 2000 (Invitrogen). Two days later virus-containing supernatant was harvested, filtered and used to infect target cells (SVEC, 3T3-SA or NIH-3T3, 1 x 10^5 cells) in the presence of polybrene (1ug/ml). Two days post-infection, stably expressing cells were selected by growth in Zeocin (200ug/ml, Invitrogen) or puromycin, as appropriate.

Microscopy

For imaging on the Marianas SDC imaging system images were acquired for Annexin V/TMRE staining with a Zeiss Plan- Neofluar 40x 1.3 NA DIC on a Cascade II 512 EMCCD (Photometrics, Tucson, AZ), using SlideBook 4.2 software (3i) or for Mitotracker Green analysis using a Zeiss 60x 1.4NA objective and Nikon-NIS elements software. For Tom20 immunostaining, cells were fixed with 4% formaldehyde for 20 minutes at 4^{o}C. Following fixation, cells were blocked and permeabilized in block buffer (1% BSA, 0.1% Triton in PBS) for 1 hour at RT. Cells were incubated overnight at 4^{o}C with rabbit anti-Tom20 antibody (Santa Cruz) diluted 1/300 in block buffer. Cells were washed extensively in TBS-Tween (Tris-buffered saline containing 0.05% Tween20) then incubated with AlexaFluor 568 goat anti-rabbit antibody (Invitrogen). Cells were extensively washed in TBS-Tween and mounted on slides with

DAPI-containing Vectashield (Vector Labs). Slides were analysed using an Olympus BX51 FL Microscope and CellF software. For live-cell imaging, SVEC cells were cultured in 3cm glass bottom dishes or 4 well-chamber slides (Mattek). Cells were incubated with Mitotracker Green FM (200nM, Invitrogen M7514) or TMRE (Invitrogen T669, 50nM final concentration), propidium iodide (PI,1ug/ml) and Annexin V Alexa Fluor 488 (Invitrogen A13201, 1% w/v) in DMEM containing 10% FCS, penicillin, streptomycin and 2.5mM $CaCl_2$. To mitigate phototoxicity and pH changes, cells were imaged in medium containing 55μM β-mercaptoethanol and 20mM Hepes. Cells were treated with murine TNF (10ng/ml) and zVAD (50μM) immediately prior to imaging. For electron microscopy, Parkin-expressing 3T3-SA cells were treated with CCCP for 48 hours on 100 mm plasma treated cell culture plastic dish. The cells were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer and were treated with en bloc heavy metal staining procedure (Ohno et al., 2011). The tissue was dehydrated through a graded series of alcohols and propylene oxide, followed by infiltration in 100% epon-araldite, then embedded and polymerized in a 70°C oven overnight. 70 nm sections were imaged at 80Kv with a CRX41 AMT camera (Advanced Technology Techniques Woburn, MA 01801), using an FEI Tecani 20 FEG 200Kv Transmission Electron Microscope (FEI Company, Hillsboro, OR 97124). After selecting the area of interest the blocks were trimmed to a 0.5 mm block face, and serial blockface SEM performed in a Zeiss Sigma VP SEM equipped with a Gatan 3View in-chamber ultramicrotome stage. Areas of 80 x 80 um were imaged at 2.25kV using the Gatan 3VBSE detector at a pixel resolution of ~10nm/pixel. Z-stacks of 330-360 slices were cut at 120nm/slice covering approx 40 μm depth. Stacks were registered using Image J software and cells traced and reconstructed using Reconstruct (Fiala, 2005) and Amira software. Percentage of the ratio "Area Mitochondria/Area Cytosol": The Area occupied by Mitochondria and the Area occupied by Cytosol was measured in 16 cells (or part of them) for each condition, chosen on single 3D-EM micrographs (each slide considered was 1.2 μm far from the other on the z axis). Percentage of the ratio "Volume Mitochondria/Volume Cytosol": Mitochondrial and cytosolic area was

measured in each of the 66 contiguous 8μmx8μm slides; the volume of the 8um cube was calculated integrating the area values previously obtained and given a slide thickness of 120nm. The analysis was performed on three cubes of 8μm side for each condition, chosen randomly in the main block of 80x80x45μm obtained after the 3D-EM scan analysis. The image analysis was performed using FIJI software (Fiji.sc/Fiji) an image-processing package based on ImageJ.

Clonogenicity assay, treatments and Cell Death Assays

Unless otherwise noted, cells were treated with the following reagents and concentrations: CCCP (Sigma C2759) at 12.5μM, TNF (Peprotech 900K25) at 10ng/ml, zVAD-FMK (Calbiochem 627610) at 50μM, ActD (Sigma A1410) at 3μM, and B/B homodimerizer (Clontech 635060) at 10nM, Necrostatin-1 (Sigma N9037) at 30μM, BHA (Butylated hydroxyanisole, Sigma W218308) at 30μM, NAC (N-Acetyl Cysteine - Sigma A9165) at 10mM. Viability was determined by propidium iodide exclusion (PI, Invitrogen P3566). Following treatment all cells were collected and resuspended in PBS containing 1ug/ml propidium iodide. Cells were analysed by flow-cytometry using a FACsCalibur and Cellquest Pro software (BD Biosciences). Alternatively, cell viability was analysed using an Incucyte FLR imaging system (Essen Bioscience). Cells were plated in medium containing the membrane impermeant dye, Sytox Green (Invitrogen S7020) or the membrane permeant dye Syto24 (Invitrogen S7559) both at 25nM. Cells were treated as described, continuously imaged and analysed using Incucyte image analysis software (Essen Bioescience).

Western blotting

Cell lysates were prepared using NP-40 lysis buffer (1% NP-40, 1mM EDTA, 150mM NaCl, 50mM Tris pH7.4, 1mM PMSF, Complete Protease Inhibitors [Roche]). Protein content was determined by Bio-Rad assay and proteins were separated by SDS-PAGE and blotted onto nitrocellulose. Membranes were probed with anti-Actin (MP Biomedicals, 1/10000), anti-Tom20 (Santa Cruz, 1/1000) anti-Hsp60 (1/1000) anti-cytochrome *c* (BD Pharmingen, 1/1000) anti-PGAM5 (Abcam, 1/1000) anti-Drp-1 (Cell Signaling, 1/1000),

anti-RIPK3 (Imgenex, 1/1000) or anti-GFP (gift of Dr. Jacques Neefjes, Netherlands Cancer Institute) antibodies followed by incubation with the appropriate HRP conjugated secondary antibody and detection of immunoreactive proteins by ECL.

Seahorse oxygen consumption, Mitochondrial DNA and ROS quantification

For Seahorse analysis respiration was measured sequentially after addition of 0.5mM of oligomycin (Sigma O4856), 0.5mM of FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, Sigma C2920) and 0.5 mM of rotenone (Sigma R 8875). After each injection OCR was measured for 3 min, the medium mixed and again measured for 3 min. Total cellular DNA was extracted using the DNeasy kit (Qiagen). The amount of mtDNA present per nuclear genome was determined by quantitative real-time PCR using the following primer pairs: mitochondrial ND2 forward primer, cccattccacttctgattacc; mitochondrial ND2 reverse primer, atgatagtagagttgagtagcg and nuclear 18s rRNA forward primer, tagagggacaagtggcgttc; nuclear 18s rRNA reverse primer, cgctgagccagtcagtgt as well as mitochondrial ND1 forward primer, cctatcacccttgccatcat; mitochondrial ND1 reverse primer, gaggctgttgcttgtgtgac and Pecam gene on chromosome 6 forward primer, atggaaagcctgccatcatg; Pecam gene on chromosome 6 reverse primer, tccttgttgttcagcatcac. Quantification of relative copy number differences was carried out by analyzing the difference in threshold amplification between mtDNA and nuclear DNA (delta delta C(t) method). For ROS quantification cells were treated to induce mitochondrial removal or cell death as described. Before analysis, cells were incubated for 20 minutes with 2μM of the cell-permeant dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Invitrogen C6827) before harvesting. The cell pellet was resuspended in 2μM propidium iodide in PBS, then washed in fresh PBS. Data were acquired using a FACScan cytometer (BD). The dichlorofluorescein (DCF, oxidized form of H_2DCF-DA) Mean Fluorescence Intensity (MFI) was calculated on the PI negative cell population.

Generation of cyclophilinD-ko/caspase-8 het mice and statistical analysis

From the crosses of the F1 generation (cyclophilinD$^{+/-}$, caspase-8$^{+/-}$, RIPK3$^{+/-}$), we used the cyclophilinD$^{-/-}$, caspase 8$^{+/-}$, RIPK3$^{+/+}$ males and females for further crossings (as described in Figure 1b). Primers for genotyping of cyclophilin D were wt-forward 5'-CTCTTCTGGGCAAGAATTGC-3', reverse common 5'-ATTGTGGTTGGTGAAGTCGCC-3' and ko-forward 5'-GGCTGCTAAAGCGCATGCTCC-3'. For genotyping of these mice, PCR reactions were performed under the following conditions (3 min at 94°C; 35 cycles, 30 sec min at 94°C, 60 sec at 65°C, 90 sec at 72°C; 10 min at 72°C). DNA products were visualized by ethidium bromide staining after electrophoresis in 1% Agarose. The two-tailed Student's t-test was applied to evaluate the statistical significance of differences measured throughout the data sets presented. For comparison of multiple groups, two-way Analysis of Variance (ANOVA) followed by Newman-Keuls Multiple Comparisons post-test were used. The differences in values obtained by two different groups were determined using unpaired Student's *t* test. Analyses were performed using Prism 6.0 software (GraphPad).

Supplemental References

Ohno, N., Kidd, G.J., Mahad, D., Kiryu-Seo, S., Avishai, A., Komuro, H., and Trapp, B.D. (2011). Myelination and axonal electrical activity modulate the distribution and motility of mitochondria at CNS nodes of Ranvier. J Neurosci *31*, 7249-7258.

Supplemental Movie Legends

Supplemental Movie 1 (Relating to Figure 1).

SVEC cells were incubated with TMRE (Red) and Annexin V AlexaFluor 488 (Green). Cells were treated with TNF in the presence of zVAD and imaged by live-cell confocal microscopy.

Supplemental Movie 2 (Relating to Figure 2).

3D-EM of non-treated 3T3-SA Parkin expressing cells. Cytosol is coloured blue and mitochondria red.

Supplemental Movie 3 (Relating to Figure 3).

3D-EM of CCCP treated 3T3-SA Parkin expressing cells. Cytosol is coloured blue and mitochondria red.

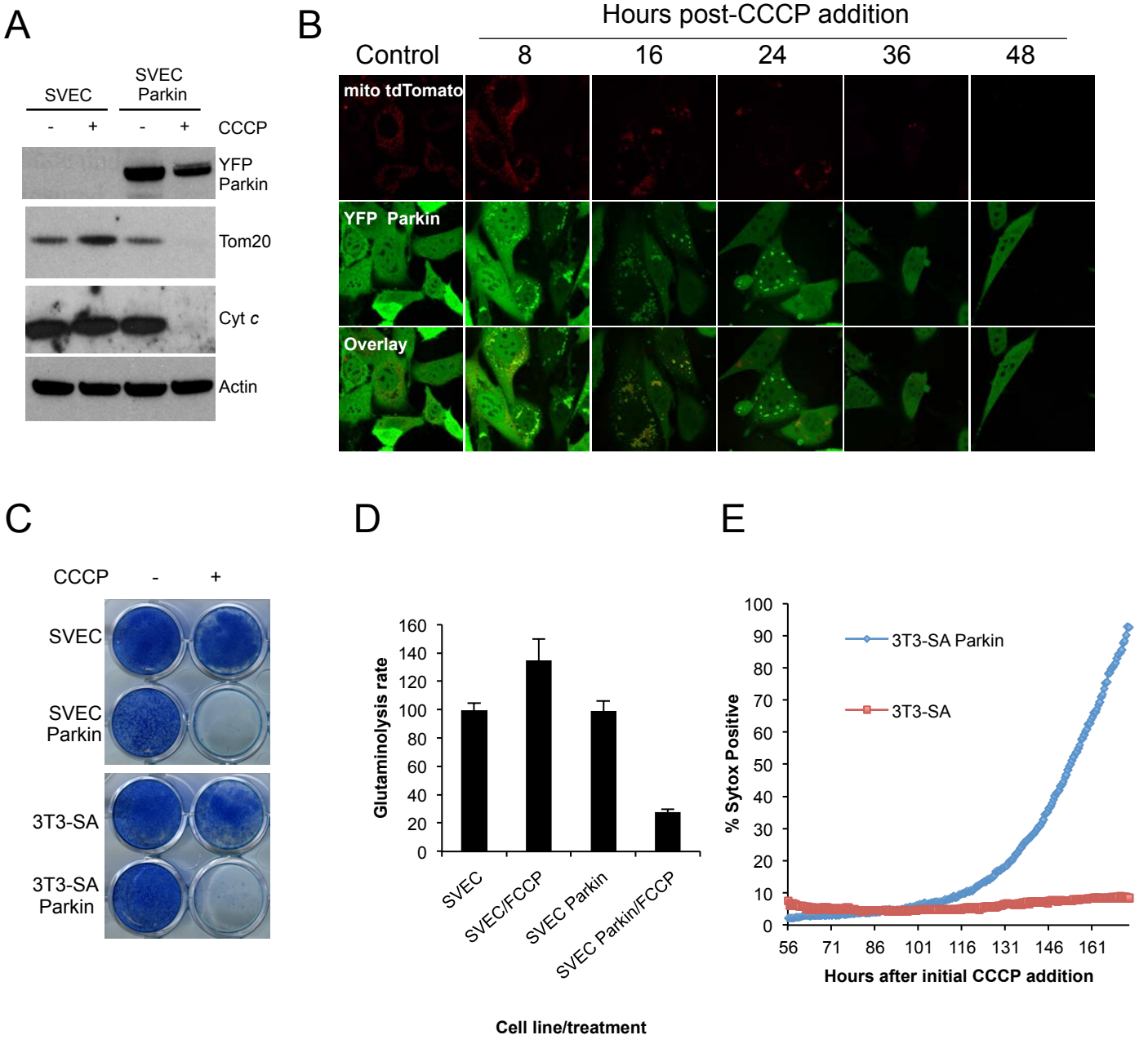


Figure S1 (relating to Figure 1). A. Control or Parkin expressing SVEC cells were treated with CCCP for 48 hours and protein expression was monitored by Western blot. Actin was used as a loading control. **B.** 3T3-SA cells expressing YFP Parkin and mitotdTomato were treated with CCCP and imaged by confocal microscopy at various times post-CCCP addition. Representative images are shown. **C.** Control or Parkin expressing 3T3-SA or SVEC cells were treated with CCCP for 24h, and assayed for clonogenic growth by methylene blue staining. **D.** Control or Parkin expressing SVEC cells were treated with FCCP for 48 hours and assayed for glutaminolysis. Error bars represent S.D. from the mean of triplicate samples. **E.** Control or Parkin expressing 3T3-SA cells were treated with CCCP for 48 hours then cell death was measured by Sytox Green uptake over time in an IncuCyte imager.

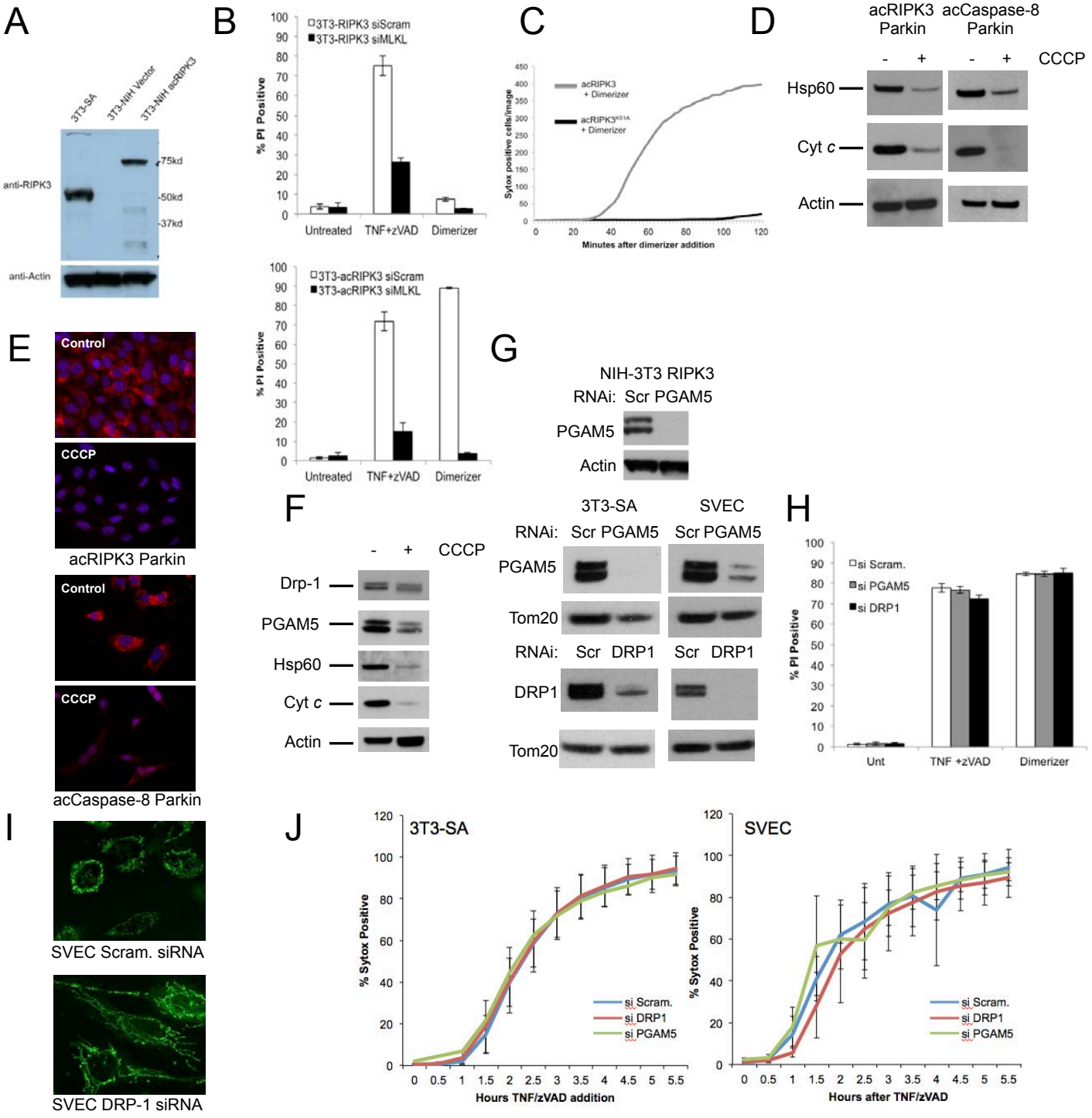
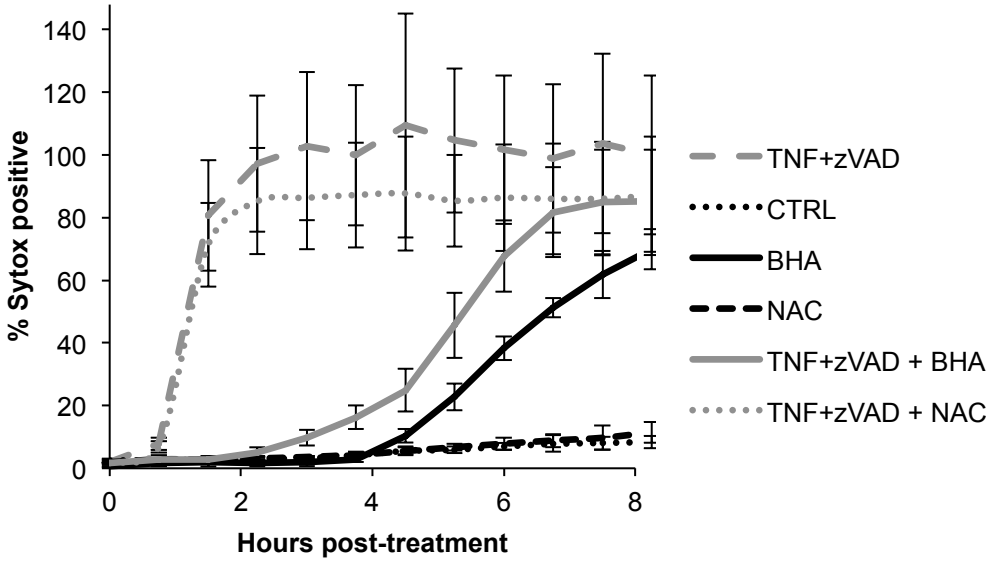


Figure S2 (relating to Figure 3). A. Expression levels of acRIPK3 in transduced NIH-3T3 cells were compared to endogenous RIPK3 in 3T3-SA cells by Western blot. **B.** NIH-3T3 cells stably expressing RIPK3 or acRIPK3 were treated with siRNA/dimeriser as indicated. PI positivity was quantified 4 hours later by flow-cytometry. **C.** NIH-3T3 cells stably expressing acRIPK3 or the catalytic mutant acRIPK3 K51A were treated with dimerizer and uptake of Sytox Green was quantified using an IncuCyte imager. Error bars represent the S.D. from 3 replicate experiments. **D and E.** NIH-3T3 cells expressing Parkin and acRIP3 or acCaspase-8 were treated with CCCP and examined for protein expression by Western blot (**D**) or immunonstained for Tom20 (**E**) **F.** NIH-3T3 cells expressing Parkin and acRIP3 were treated with CCCP then examined for protein expression by Western blot **G-J**. NIH-3T3 cells stably expressing acRIPK3, SVEC or 3T3- SA cells expressing endogenous RIPK3, were transfected with siRNAs as indicated and protein expression was quantified by Western blot (**G**); in parallel, cell death was induced by dimerizer or TNF+zVAD treatment in NIH-3T3 cells and quantified by flow-cytometry (**H**), or in case of the SVEC or 3T3-SA cells the cell death was induced by addition of TNF/zVAD, and kinetics were measured using an IncuCyte imager (**J**). Error bars represent the S.D. from the mean of 3 independent experiments. SVEC cells stained with Mitotracker Green were imaged 72 hours after transfection with DRP1 or control siRNA (**I**).

A



B

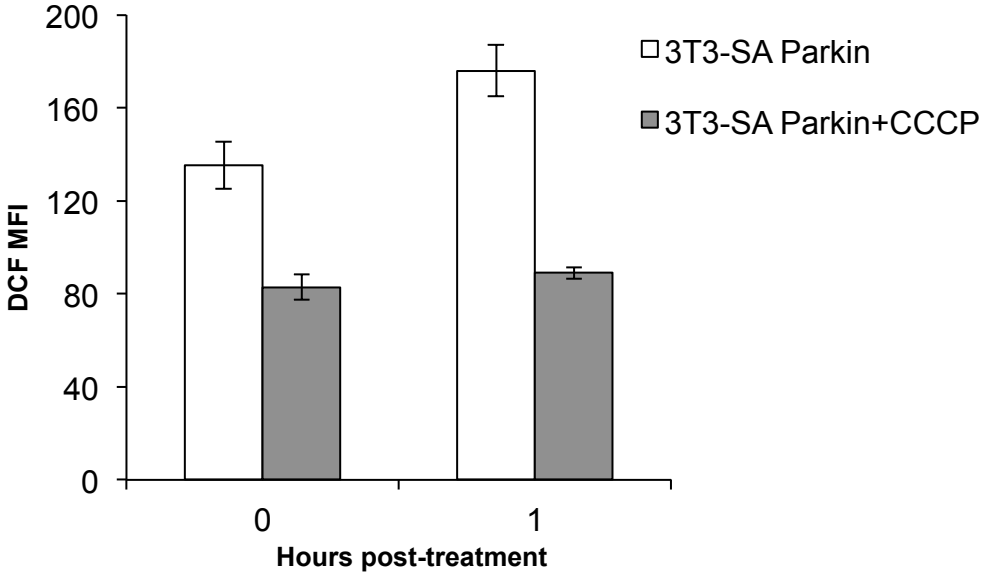


Figure S3 (Relating to Figure 4). A. NIH-3T3 cells stably expressing acRIPK3 were treated with TNF+zVAD in the presence of BHA and NAC as indicated. Cell death was assayed using Sytox Green uptake over time in an IncuCyte imager. Error bars represent the S.D. from one representative experiment in triplicate. **B.** 3T3-SA cells expressing Parkin were treated with CCCP to induce mitochondrial elimination, then treated with TNF+zVAD for 1 hour. ROS production was measured using the dye DCF. MFI=Mean Fluorescent Intensity. Error bars represent S.D. from the mean of triplicate samples. Data are representative of 3 independent experiments.