Orozco et al. Supplementary Material

→3 Supplementary Figures with legends

→Legends for Supplementary Movies 1&2, which are available online.

Orozco et al. Supplementary Figure Legends:

Fig. S1: A) Jax cells, which express endogenous RIPK3, or NIH-3T3 cells, which do not, were treated with 10ng/ml recombinant TNF along with inhibitors as indicated. **B)** Lysates from Jax cells, or NIH-3T3 cells stably expressing indicated constructs, were resolved by Western blot using the indicated antibodies. Note that the RIPK3 antibody used recognizes an epitope in the C-terminus, which is lacking in the RIPK3$^{\Delta C}$ construct. **C&D)** NIH-3T3 cells stably expressing RIPK3-1xFV were treated with recombinant TNF, 30μM Nec1, 50μM zVAD, or 200ng/ml TNFR1-Fc as indicated. **p* =0.0002. **E-H)** NIH-3T3 cells stably expressing catalytically inactive RIPK3^{K51A}-1xFV, phosphorylation site mutant RIPK3^{T231A,S232A}-1xFV, RHIM domain point mutant RIPK3$^{\Delta RHIM}$-1xFV, or RHIM-truncated RIPK3$^{\Delta C}$-1xFV were treated as indicated. **I)** NIH-3T3 cells stably expressing the indicated constructs were lysed and resolved by western blotting. Jax cells are included as a control for endogenous RIPK3 expression.

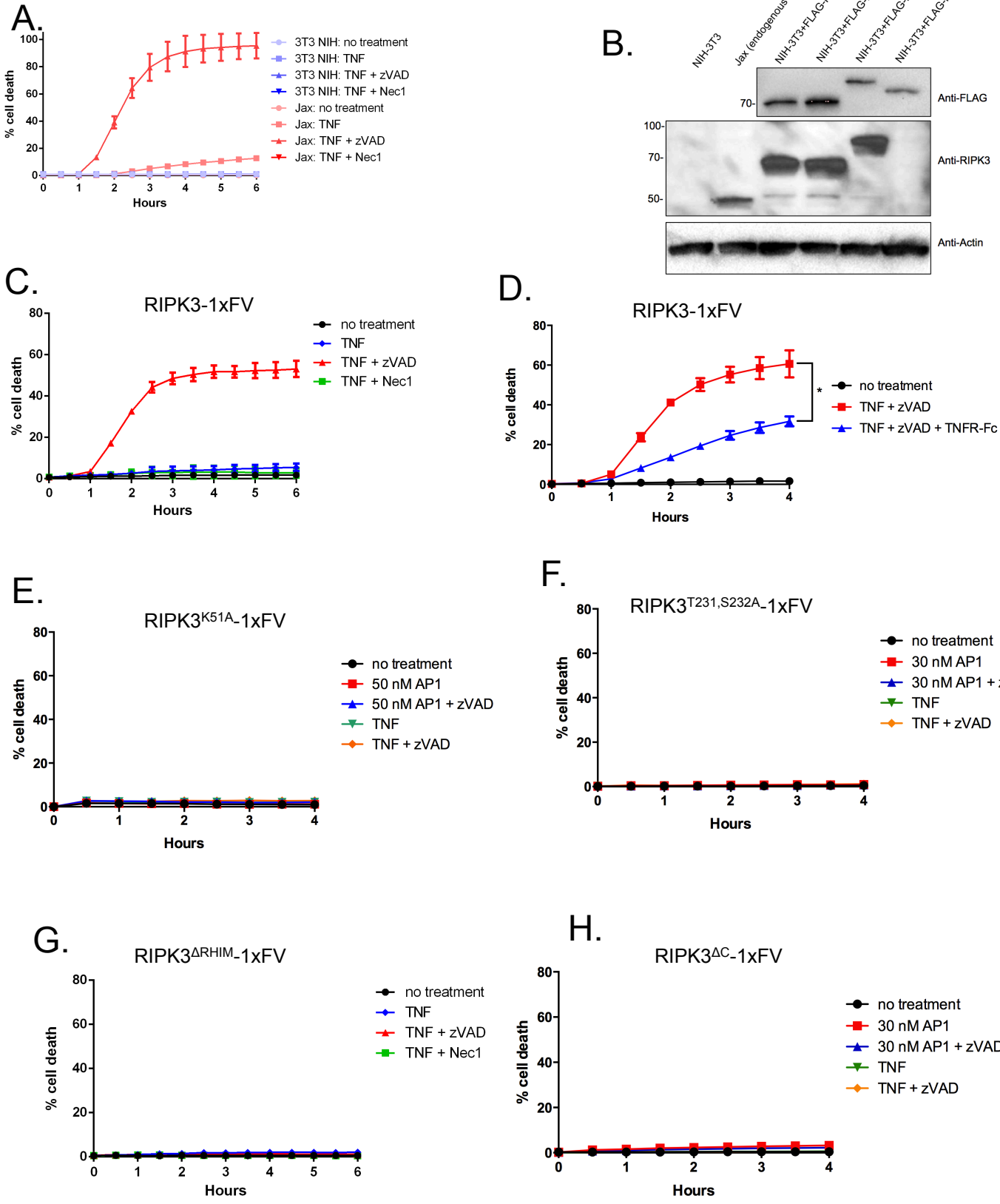
Fig. S2: A) NIH-3T3 cells were transfected with indicated siRNAs. Seventy-two hours later, lysates were collected and expression of indicated proteins was assessed by western blot. **B)** NIH-3T3 cells stably expressing RIPK3-1xFV were treated with 30nM AP1, 50μM zVAD and 200ng/ml TNFR1-Fc as indicated. **C)** Densitometric analysis of the RIPK3 dimer and oligomer bands depicted in Fig. 2D. **D)** 3T3-NIH cells stably expressing RIPK3-1xFV were treated with AP1, then lysates were collected and subjected to DSS-mediated chemical crosslinking. These complexes were then resolved by western blotting using the indicated antibodies. **E)** RIPK1-associated immunocomplexes were purified as described in Fig. 1E, and co-precipitation of FADD was assessed by western blotting. **F)** NIH-3T3 cells stably expressing RIPK3$^{\Delta C}$-2xFV were transfected with indicates siRNAs. Seventy-two hours later these cells were treated as indicated. All cell death measurements were performed using an IncuCyte bioimager as described.

Fig. S3: A) Schematic representation of the destabilization domain (DD)-RIPK3 construct used. A DD-RIPK3 chimeric open reading frame was created by recombinant

PCR, then cloned upstream of a T2A-GFP-T2A-PURO sequence. Of note, both DD-RIPK3 and GFP protein include a C-terminal 2A epitope. **B)** NIH-3T3 cells stably expressing DD-RIPK3 were pre-treated with 1μM Shield drug for 8 hours to stabilize RIPK3 expression, then treated with 1ng/ml TNF and 50μM zVAD as indicated. *P=0.0024 **C)** NIH-3T3 cells stably expressing DD-RIPK3 were treated with 1μM Shield drug and 200ng/ml TNFR-Fc as indicated. **p* =0.0004 **D)** NIH-3T3 cells stably expressing catalytically inactive DD-RIPK3^{K51A} were treated with 1μM Shield drug as indicated. All cell death measurements were performed using an IncuCyte bioimager as described.

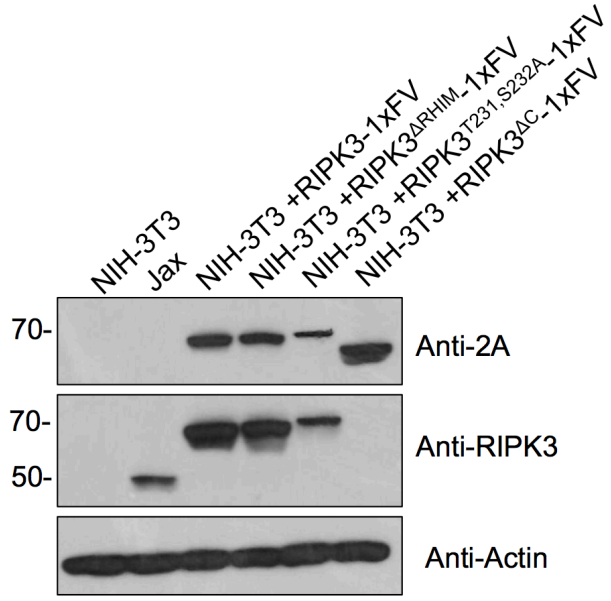
Supplemental Movies 1&2: A representative image set acquired from the IncuCyte imager. These images are NIH-3T3 cells stably expressing RIPK3-1xFV, treated with 10nM AP1 (see **Fig. 1B** for a quantified graphical representation of this cell death). **Movie 1** shows one field of these cells undergoing RIPK3-dependent cell death; the media contains the cell impermeable DNA binding dye Sytox Green, which marks cells that have lost membrane integrity in green. **Movie 2** depicts the same image set, following analysis by the IncuCyte software package. This software counts each green (dead) cell in each image; counted cells are surrounded by purple halos. Each trace shown in this manuscript depicts data averaged from at least 3 independently treated wells of cells, each of which is imaged in at least 4 separate fields. Each experiment shown is representative of at least 3 separate replicates. All experiments on stably transduced cells is representative of at least three separate replicates performed on each of at least two distinct, independently-derived stable lines.

Orozco et al. Supplementary Fig.1

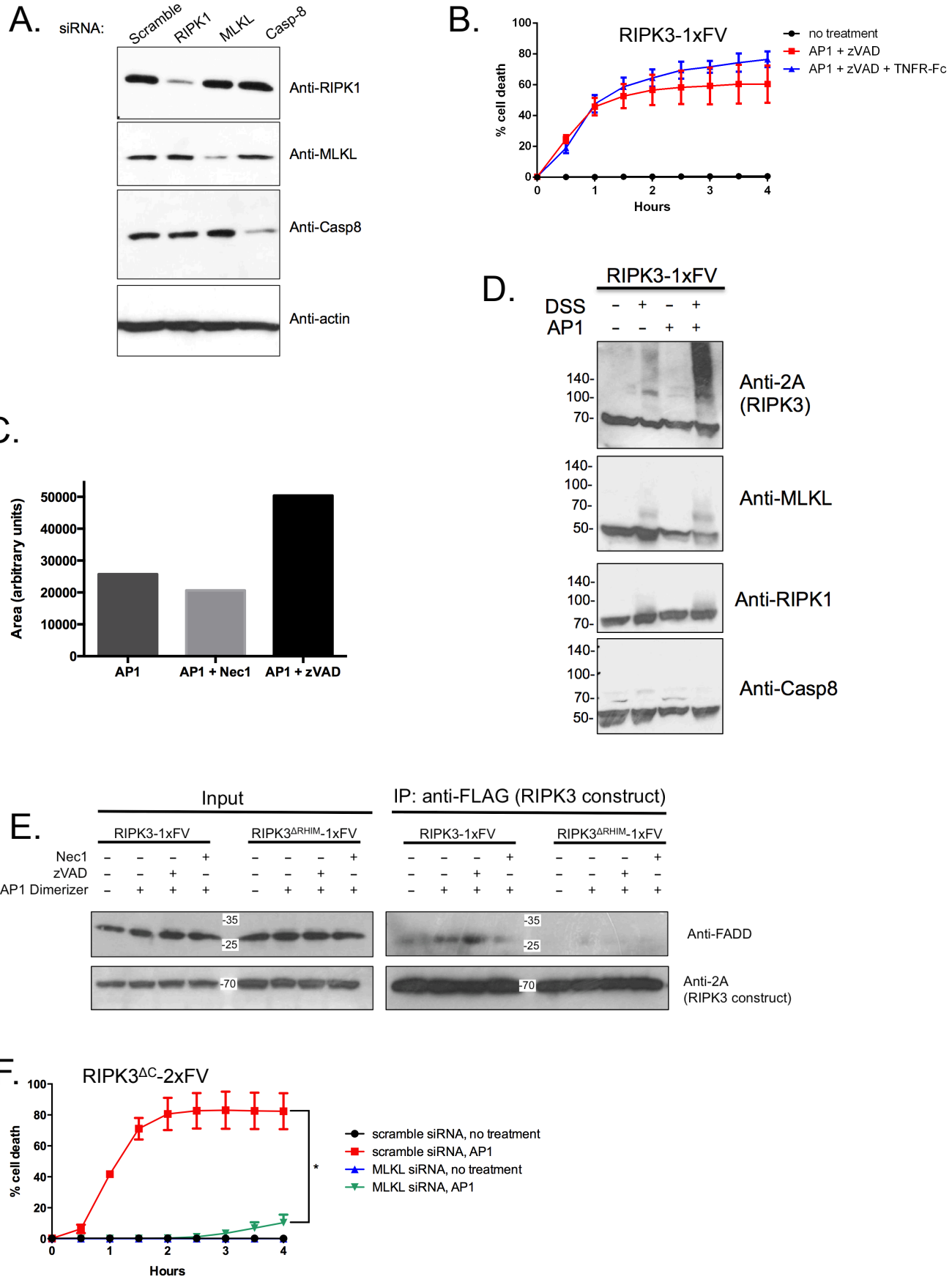


Orozco et al. Supplementary Fig.1, cont.

I.



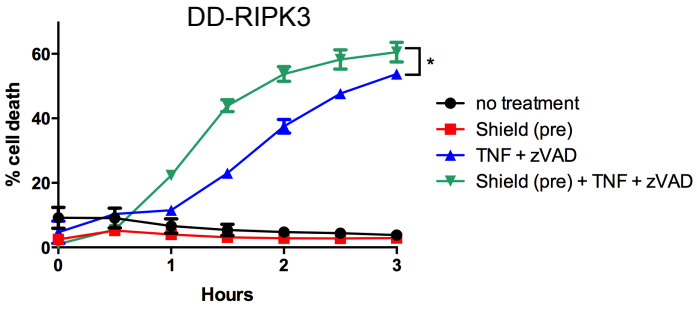
Orozco et al, Supplementary Fig. 2



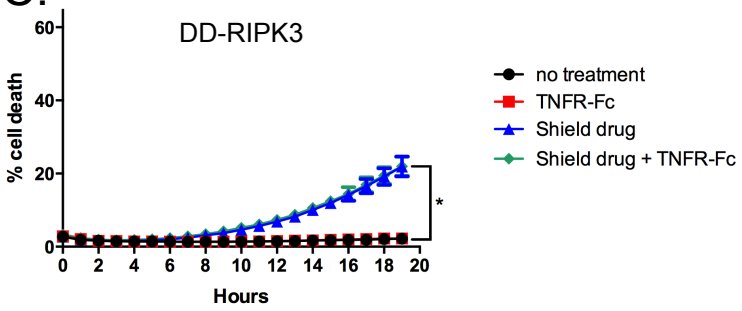
Orozco et al., Supplementary Fig. 3

A.

B.



C.



D.