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Supplementary Information for

Regulation of a distinct activated RIPK1 intermediate bridging complex I and complex II in TNFα-mediated apoptosis

Palak Amin, Marcus Florez, Ayaz Najafov, Heling Pan, Jiefei Geng, Dimitry Ofengeim, Slawomir A. Dziedzic, Huibing Wang, Vica Jean Barrett, Yasushi Ito, Mathew J. LaVoie and Junying Yuan.

Junying Yuan
Email: junying_yuan@hms.harvard.edu

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Supplementary Information Text

Extended Acknowledgments

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Materials

Antibodies.

The following commercial antibodies were used in this study: RIPK1-pS166, Cell Signaling Technology (31122); Caspase-3, BD Biosciences; Cleaved caspase-3, Cell Signaling Technology (9661); Caspase-8, Enzo (804-447-C100); MLKL-pS345, Abcam (ab196436); MLKL, Sigma (SAB1302339); TAK1, Novus Biologicals (NBP1-76441); RIPK1, Cell Signaling Technology (3493), and for immunoprecipitation, BD Biosciences (610459); PARP, Cell Signaling Technology (9542); p-IKKα/β, Cell Signaling Technology (2697); IKKα, BD Biosciences (556532); IKKß, Cell Signaling Technology (8943); IκBα, Santa Cruz (sc-371); CYLD, Cell Signaling Technology (8462); FADD, Abcam (ab124812) and for immunoprecipitation, Santa Cruz (6036); α-Tubulin, Sigma-Aldrich (T9026); β-actin, Santa Cruz (81178); anti-linear ubiquitin, Millipore (MABS199); anti-ubiquitin K11 linkage antibody, Millipore (MABS107); TNFR1, Cell Signaling Technology (13377) and for immunoprecipitation, R&D Systems (AF-425-PB); A20, Cell Signaling Technology (5630); RIPK3, Biorad (AHP1797); APC11, Cell Signaling Technology (14090S); HA-Tag, Abmart Inc (M20003); LRRK2, Cell Signaling Technology (5559) and Abcam (ab133474); c-Cbl, Cell Signaling Technology (2747); NEK1, Santa Cruz (sc-398813) and Gene Tex (GTX130828); APC10, Cell Signaling Technology (14807); CIN85, Cell Signaling Technology (12304); Phospho-RIPK3, Cell Signaling Technology (57220).

Compounds.

R-7-Cl-O-Nec-1 (Nec-1s): custom synthesis,10 μM. 5*Z*-7-Oxozeaenol: Sigma-Aldrich (O9890), 500 nM. zVAD.fmk: Sigma, 20 uM. SM-164 (1): custom made, 500 nM with at least 2 hours pretreatment.

Methods

Cell Line Generation.

Tak1$^{F/F}$ MEFs were immortalized spontaneously in culture and infected by lentivirus for the expression of Cre recombinase to generate *Tak1*$^{-/-}$ MEFs; WT MEFs refer to *Tak1*$^{F/F}$.

Animals and Primary Cell Generation.

All animal studies were performed according to ethical guidelines and procedures approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School. *Ripk1*^{D138N} mice (C57BL/6 strain) were provided by Dr. Manolis Pasparakis of U. Cologne, Germany and Dr. Michelle Kelliher of U. Mass and were back crossed to C57BL/6 mice for three generations. 8-10 weeks old *Ripk1*$^{D138N/+}$ (C57BL/6) were used for mating to generate littermate MEFs. Pregnancy was terminated at E13-14 stage. Embryos were homogenized individually, treated with trypsin, and sieved through a 70-micron filter to obtain primary MEFs. Genotyping was performed by standard methods, as previously described (2).

Primary CNS cell cultures were derived from wild type mice according to standard methods. Primary neurons were derived from E17-E18 embryos. Briefly, brains were removed from the embryo and cortices dissected, digested with trypsin, and sieved through a 70-micron filter to obtain primary neuronal cultures. Cells were plated on poly-D-lysine coated plates in DMEM + 10% FBS and media changed to Neurobasal supplemented with B27, Glutamax, and 1% penicillin-streptomycin after 3-4 hours. Half of the media was replaced every 4-5 days and cultures were used at 17 days *in vitro* for cell death assays.

Mouse primary astrocytes were isolated as previously described (3) with certain modifications. 0-3 day old pups were sacrificed by decapitation, the whole brains were isolated and the meninges were removed. The brains were trypsinized for 5 minutes at 37 ^{o}C followed by neutralization using DMEM F12 media supplemented with 10% FBS. The cell suspension was then homogenized using an 18 gauge needle and passed through a 40 μm filter, centrifuged at 500 x g for 5 minutes, resuspended in DMEM F12 media supplemented with 10% FBS and plated in two T75 flasks. On day 3, the media was changed. On day 7 the astrocytes were shaken at 180 rpm for 30 minutes followed by shaking at 240 rpm for 60 minutes at 37 ^{o}C. On day 8, the cells were trypsinized and plated at 100,000 cells per well of a 12 well plate.

For primary microglial culture, forebrains of 1 to 2 days old mouse pups were digested with 0.01% trypsin and cells plated in DMEM containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin onto poly-D-lysine coated T75 flasks and fed every 3 days for 7-10 days. Following an initial 1 hour shake of the culture, microglia were collected and cultured in DMEM + 10% FBS. The quality of purification was analyzed by FCS using CD11b as a marker for microglia (>90% CD11b+).

siRNA Screen.

Dharmacon siGENOME siRNA mouse library subsets used for the siRNA screen included the kinase/phosphatases library and a custom ubiquitin ligase/DUB list based on - Deubiquitinating Enzymes - SMARTpool # G-014705-01; Ubiquitin Conjugation Subset 1 - SMARTpool # G-015615-01; Ubiquitin Conjugation Subset 2 - SMARTpool # G-015625-01; Ubiquitin Conjugation Subset 3 - SMARTpool # G-015635-01. Kinases/phosphatases were screened in SMARTpool format followed by deconvolution while ubiquitin ligases/DUBs were screened as 4 individual duplexes per gene. TNFR1SF1a was used as a positive control for protection, and ABIN-1 (4) was used as a positive control for the sensitizer screen. 661W cells were plated in

white-opaque 384 well assay plates (Corning #3570) at 20 uL per well, for a final density of 1,000 cells per well. For each library plate, 6 assay plates were prepared. Cells were then incubated for 2.5 hours at 37 °C, 5% CO_2, during which time cells attached to the plate. 500 uL Lipofectamine-RNAiMAX was diluted into 29.3 mL Opti-MEM. 51 uL was then aliquoted into each well of a 384 well intermediate plate. Using robotics, 7 uL of 1 uM siRNA, from a library, cherry pick, or control siRNA plate, was then added to the 384-well intermediate plate and complexes allowed to form for 20 minutes. Using robotics, 8 uL of the complex was then added to each replicate assay plate, followed by 20 uL of full media. Cells were incubated at 37 °C, 5% CO_2 following transfection. 72 hours after transfection, 3 replicate plates were treated with 6 uL full media, and 3 replicate plates treated with a final concentration of 0.5 uM 5Z-7 + 0.2 ng/mL TNFα in 6 uL full media. After 9 hours, plates were removed from the incubator and allowed to come to room temperature for 30 minutes. CellTiter-Glo reagent was then added to each well at 18 uL per well, plates covered with foil, and incubated for 20 minutes at room temperature. Luminescence was read on an EnVision plate reader with crosstalk correction.

Data analysis: (A) For each replicate plate respectively, the average of negative controls (minimum of 16 wells) was calculated as well as their standard deviation (5-10%). Each experimental well in the plate was divided by the average of the negative controls to normalize values. (B) The normalized values of the 3 control replicate plates were averaged to generate an average control value for each siRNA. The normalized values of the 3 experimental replicate plates (5Z-7+TNF) were averaged to generate an average experimental value for each siRNA. (C) The average experimental value was divided by the average control value to calculate a ratio (exp/cntrl). Ratios > 1.5 are protector hits and ratios < 0.45 are sensitizer hits. (D) For the individual duplexes that were screened (DUBs and E3s), the ratio was calculated for each individual duplex and scored as in (C). A gene was considered a protector hit if (# duplex protecting - # duplex sensitizing) is 2 or more. A gene was considered a sensitizer hit if (# duplex sensitizing - # duplex protecting) is 2 or more. For follow up screens, genes were considered hits if 2 or more siRNAs showed protection or sensitization by greater than 1.5 fold over non-targeting control siRNA. Protector and sensitizer hits were used as inputs for STRING network analysis (5).

Cell Lysis.

The following buffer was prepared for immunoprecipitation (IP) or Western blotting, with the addition of either 0.2% NP-40 (for FADD M-19, RIPK1-pS166, RIPK1 immunoprecipitation), 0.5% NP-40 + 0.5% Triton X-100 (for TNFR1, FLAG-TNF, HA immunoprecipitation), 0.2% NP-40 + 1% Triton X-100 (for Western blotting), or 0.2% NP-40 + 6 M Urea (total cell lysates for Western blotting) depending on the application: 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM glycero-2-phosphate, 5 mM NaF, 10% Glycerol and 0.1% BME. Buffers were freshly supplemented with 1X protease inhibitor cocktail (Roche), 1 mM sodium orthovanadate, 1 mM PMSF, and 5 mM NEM (N-ethylmaleimide).

Immunoprecipitation.

For immunoprecipitations, cells were plated in 10 cm dishes, except for detection of c-Cbl in complex I and chain-specific ubiquitin immunoprecipitations, where 15 cm dishes were used. Cells were harvested in 700 uL immunoprecipitation buffer, rotated at 4 °C for 60 minutes, and centrifuged at 21,000 x g for 30 minutes at 4 °C. The supernatant was used for immunoprecipitation with incubation of antibody or antibody/bead conjugates overnight,

followed by addition of UltraLink Protein A/G beads for 3 hours with rotation at 4 °C. Beads were washed 4 times in immunoprecipitation buffer with rotation at 4 °C for 10 minutes for the last wash and immunocomplexes eluted with SDS-PAGE sample buffer at 90 °C for 10 minutes.

Ubiquitin Immunoprecipitation.

Chain-specific ubiquitin immunoprecipitation was performed with the 6 M urea fraction following fractionation of cell lysates. 6 M urea was diluted to 3 M urea with lysis buffer (for K11, K48, K63 immunoprecipitation), or left undiluted (for linear ubiquitin immunoprecipitation) and 0.5 ug of antibody added followed by Protein A beads, as previously described (6).

293T Transfection and RIPK1 Ubiquitination Assay.

293T cells were transfected with 2 ug GST-RIPK1 or GST alone and/or 5 ug HA-APC11, HA-c-Cbl, or control plasmid with polyethylenimine as previously described (7). After 36 hours, cells were harvested for HA immunoprecipitation as described. The lysate was split in half, with one half used for HA immunoprecipitation and the other for GST pulldown. For GST pulldown, 6 M urea and 4 M sodium chloride was added to the lysate to give a final concentration of 1 M urea and 500 mM sodium chloride to remove co-purifying and contaminating proteins prior to purification with glutathione sepharose.

Protein Purification and *in vitro* Ubiquitination Assay.

Human c-Cbl-HA and GST-RIPK1 were expressed and purified from HEK293T cells. Cells were transfected at 50% confluence in 15 cm dishes with 20 µg plasmids using 55 µl PEI diluted in 1 mL OptiMEM (the transfection mix was vortexed for 15 seconds and incubated at 25 ^{o}C for 20 minutes prior to addition to the cells in a 1 day-old medium). Cells were harvested 48 hours after transfection. Following a rinse with 10 mL PBS on ice, cells were scraped into 0.75 mL of NP-40 lysis buffer (25 mM HEPES (pH 7.5), 0.2% NP-40, 120 mM NaCl, 0.27 M sucrose, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 10 mM glycero-2-phosphate, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4 (fresh), 0.1% β-mercaptoethanol (fresh), 1mM PMSF (fresh), 2X Complete protease inhibitor cocktail (Roche)). HA immunoprecipitations and GST pull-downs were done from clarified lysates (centrifuge at 16,000 x g, 15 minutes, 4 ^{o}C and filter the supernatants through 0.45 µm filters) for 4 hours at 4 ^{o}C, on a rotating wheel. Beads were washed four times using the lysis buffer supplemented with NaCl to a final concentration of 0.5 M. GST-RIPK1 was eluted from beads using 40 mM GSH pH 7.5 in 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 % β-mercaptoethanol. Cbl-HA was eluted using 0.2 mg/mL HA peptide, using the same buffer. *In vitro* ubiquitination reactions were set up using Boston Biochem reagents (Ubiquitin Conjugation Initiation Kit K-995 and 1 µM E2 enzyme UbcH5), as described previously (8). Reaction was terminated with 5X SDS-PAGE sample buffer after 30 minutes at 30 ^{o}C and resolved on a 10% SDS-PAGE gel.

Electron Microscopy.

Cells were plated in 6 well dishes and treated with cell death inducers for 90 minutes. Fixative (1.25% formaldehyde, 2.5 % glutaraldehyde and 0.03% picric acid in 0.1 M Sodium cacodylate buffer, pH 7.4) was directly added 1:1 to the cell media followed by incubation at room temperature for 1 hour. Routine embedding protocol was used, with 1% Osmium tetroxide and 1.5% Potassium ferrocyanide incubation, followed by 1% Uranyl Acetate incubation, followed

by dehydration and embedding in Epon. Samples were imaged by transmission electron microscopy on a Tecnai G2 Spirit BioTWIN.

Cell Titer-Glo Assay for ATP Relase.

CellTiter-Glo luminescent cell viability kit was from Promega. Cells were plated in 6-well dishes and cell death induced 1-3 days later. For measuring ATP released by cells, conditioned media was centrifuged at 5000 x g at 4 °C, transferred to a 384 well plate, an equal volume of CellTiter-Glo reconstituted reagent added followed by incubation for 10 minutes and then luminescence measurement.

Supplemental Figures

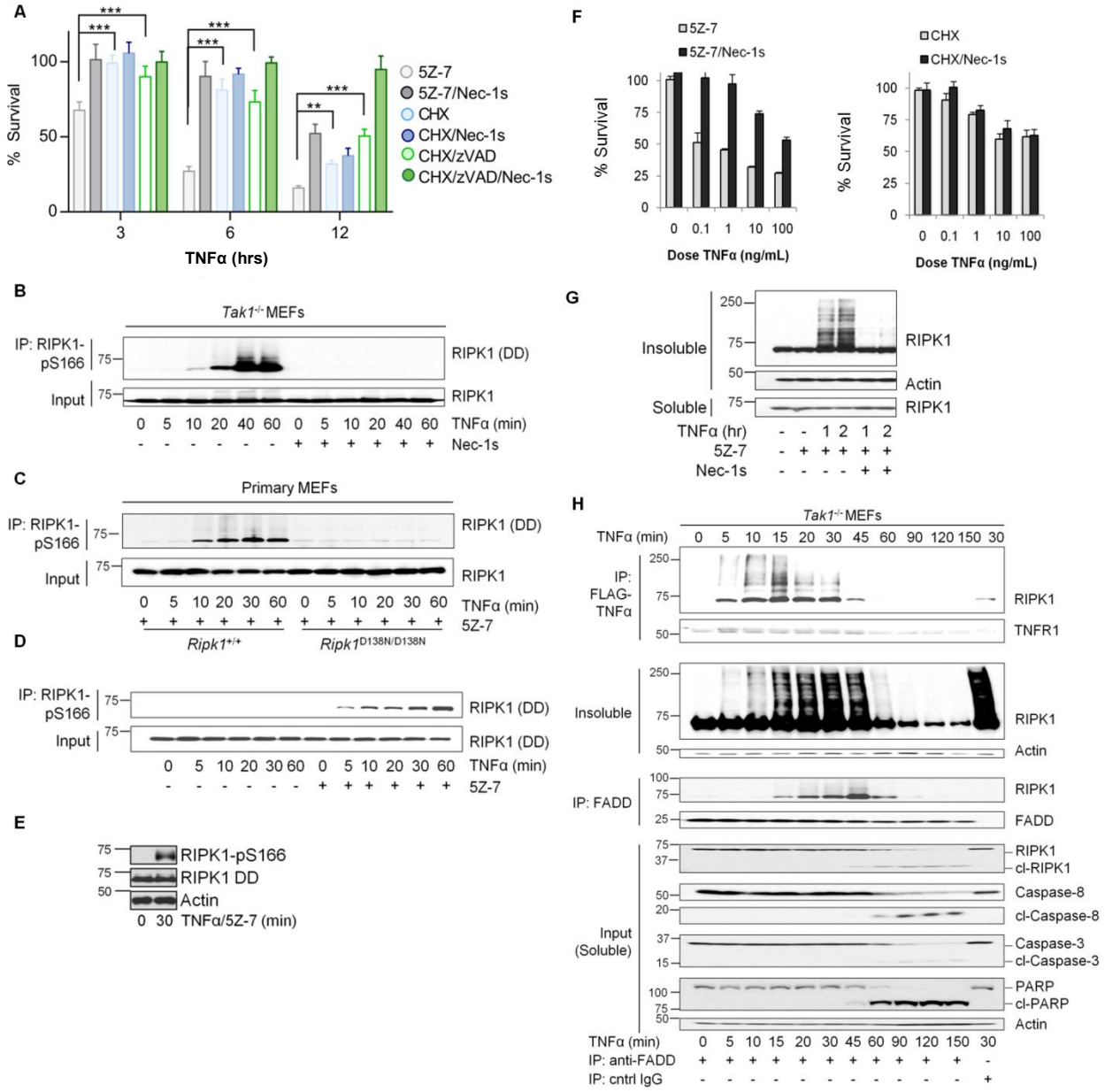

Figure S1. (A) WT MEFs treated with TNFα/5Z-7 to induce RDA, TNFα/CHX to induce RIPK1-independent apoptosis, or TNFα/CHX/zVAD to induce necroptosis in the presence or absence of Nec-1s. Cell viability determined by CellTiter-Glo. (B-E, G-H) Cell lysates analyzed by Western blotting with indicated antibodies. (B-D) Cells treated as indicated, cell lysates harvested in 0.2% NP-40 lysis buffer, and immunoprecipitated with RIPK1-pS166 ab. (B) *Tak1*$^{-/-}$ MEFs treated with TNFα in the presence or absence of Nec-1s. (C) *Ripk1*$^{+/+}$ or *Ripk1*$^{D138N/D138N}$ MEFs treated with TNFα/5Z-7. (D) 661W cells treated with TNFα in the presence or absence of 5Z-7. (E) Primary astrocytes treated with TNFα/5Z-7 for 30 min and total lysates Western blotted for RIPK1-pS166. (F) 661W cells treated with CHX or 5Z-7 with indicated concentrations of TNFα for 12 hours. Cell viability determined by CellTiter-Glo. EC50 for

TNFα/CHX: 904 pg/mL (95% CI 386 pg/mL – 1.83 ng/mL). EC50 for TNFα/5Z-7: < 100 pg/mL. (G) 661W cells treated with 5Z-7 and 1 ng/mL TNFα to induce RDA for indicated periods of times with or without Nec-1s. Cells harvested in buffer with 0.2% NP-40, and cleared by centrifugation to yield the soluble fraction. Pellet then resuspended in buffer with 1% Triton X-100 and cleared by centrifugation. Resulting pellet resuspended in buffer with 6 M urea and cleared by centrifugation to yield the detergent insoluble fraction. (H) *Tak1*$^{-/-}$ MEFs treated with FLAG-TNFα for indicated periods of time to induce RDA and then lysed as in (G). NP-40 soluble lysate used for complex I immunoprecipitation with anti-FLAG M2 beads or control protein A/G beads; or for complex II immunoprecipitation with anti-FADD (M-19) or control goat IgG. Concentrations of reagents: TNFα, 10 ng/mL (B-E), 1 ng/mL (G); FLAG-TNFα, 50 ng/mL; CHX, 1-2 μg/mL; 5Z-7, 500 nM; Nec-1s, 10 μM; zVAD.fmk, 20 μM. Identical concentrations of these compounds were used in subsequent experiments unless noted. All data shown are mean ± SD of 3 or more independent experiments. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ by one-way ANOVA.

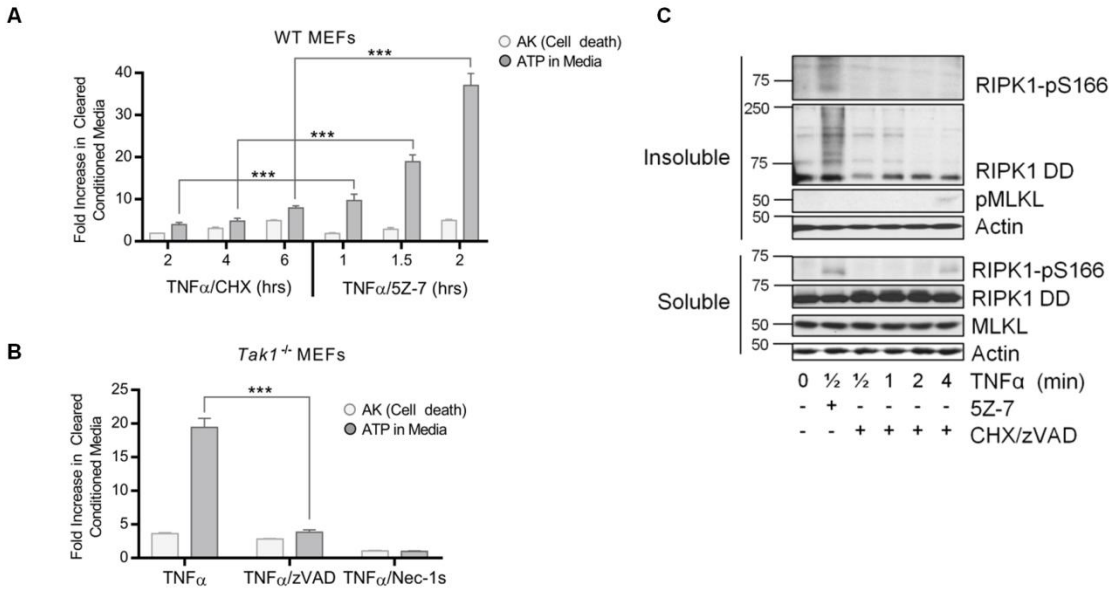

Figure S2. (A) WT MEFs treated with TNFα/CHX to induce RIPK1-independent apoptosis, or TNFα/5Z-7 to induce RDA. Different TNFα treatment times used to achieve comparable levels of cell death. Cleared conditioned media used to determine cell death using Toxilight and ATP release measured using CellTiter-Glo. (B) *Tak1*$^{-/-}$ MEFs treated with TNFα to induce RDA with or without Nec-1s or zVAD.fmk for 2 hours. Media analyzed as in (A). (C) 661W cells treated with 5Z-7 or zVAD/CHX and 10 ng/mL TNFα to induce RDA or necroptosis for indicated periods of times. Cells harvested in buffer with 0.2% NP-40, and cleared by centrifugation to yield the soluble fraction. Pellet then resuspended in buffer with 1% Triton X-100 and cleared by centrifugation. Resulting pellet resuspended in buffer with 6 M urea and cleared by centrifugation to yield the detergent insoluble fraction. The cell lysates were analyzed by Western blotting with indicated antibodies. All data shown are mean ± SD of 3 or more independent experiments. ***$P \leq 0.001$ by Student's *t* test.

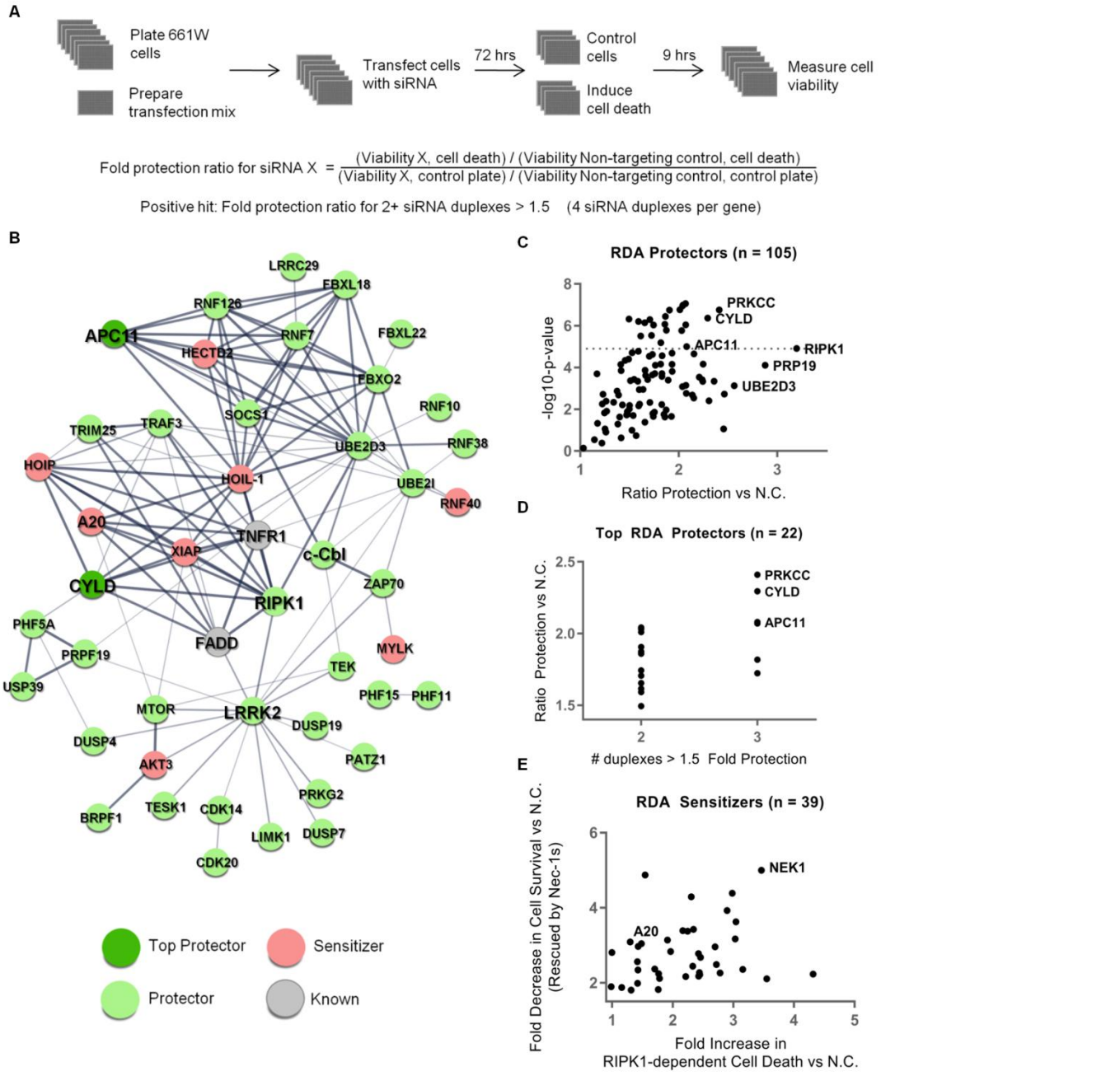

Figure S3. (A) Scheme of primary siRNA screen. (B) STRING (5) network of the 79 hits that protect or sensitize to RDA, and their connections to known pathway components TNFR1 and FADD. Disconnected nodes not shown. (C) Plot of average values of the top 2 duplexes per gene for the 105 potential RDA protectors identified from the primary screen. (D) Plot of average ratios of protection from the top 2 duplexes per gene for the 22 potential RDA protectors with p-values lower than RIPK1. (E) Plot of average-fold of sensitizations to RIPK1-dependent death induced by TNFα/5Z-7 of the top 2 duplexes per gene for the 39 potential RDA sensitizers identified from the primary screen.

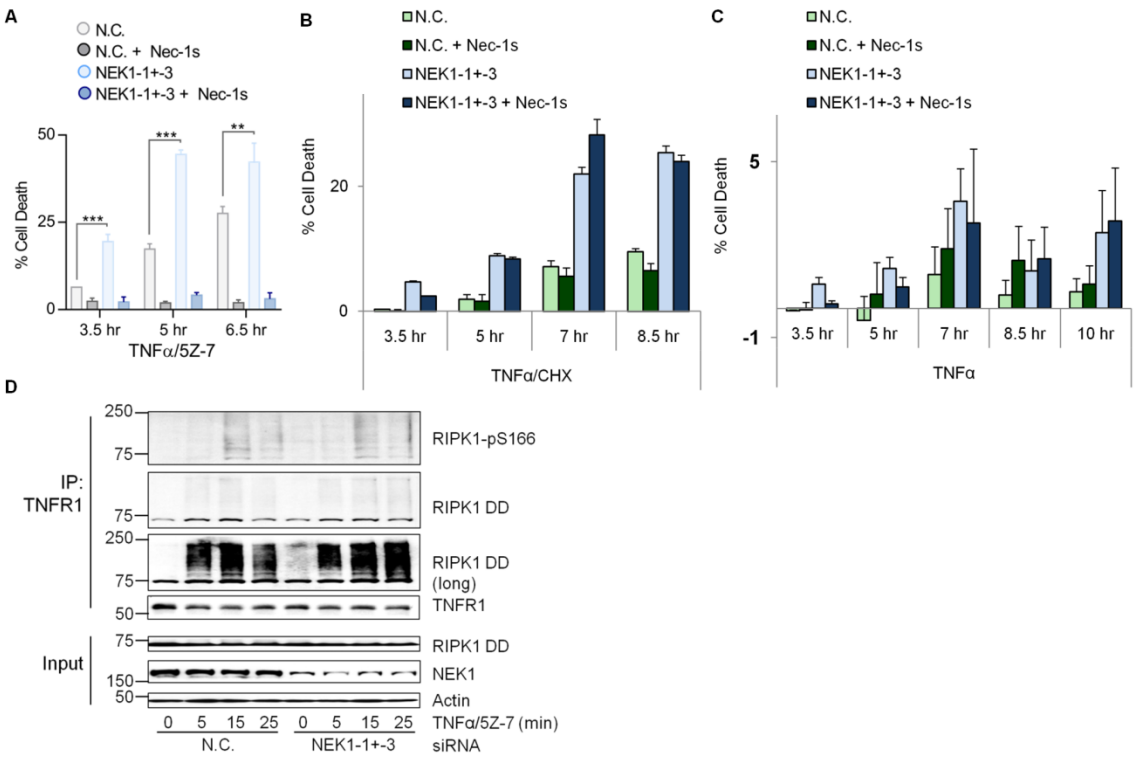

Figure S4. (A-D) 661W cells transfected with NEK1 siRNA or non-targeting control siRNA (N.C.) for 72 hours followed by induction of (A) RDA with TNFα/5Z-7 (B) RIPK1-independent apoptosis with TNFα/CHX or (C) treatment with TNFα alone and measurement of cell death by Toxilight assay at indicated time points. Knockdown efficiency is shown in (D). (D) 661W cells treated with TNFα/5Z-7 to induce RDA, harvested in 0.5% NP-40+0.5% Triton X-100 buffer, and complex I analyzed by anti-TNFR1 immunoprecipitation followed by Western blotting. The experiment was performed simultaneously with Fig. 3E and the same control (N.C.) lysates used. Concentrations of reagents: TNFα, 1 ng/mL (A, D), 10 ng/mL (B, C).

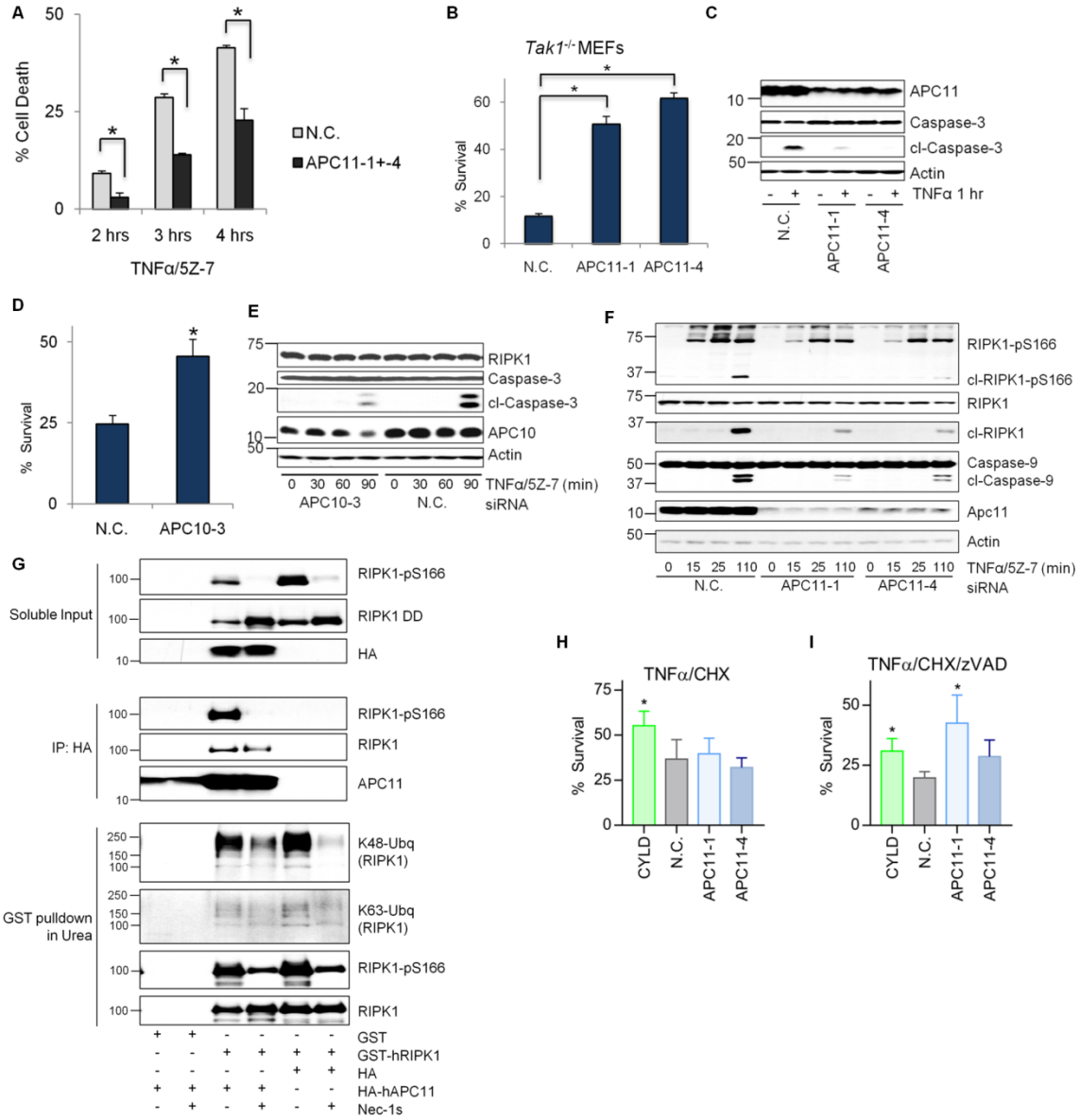

Figure S5. (A) 661W cells transfected with siRNAs for APC11 or non-targeting (N.C.) siRNA for 72 hours and treated with TNFα/5Z-7 to induce RDA. Cell death determined using Toxilight assay at indicated time point. (B) *Tak1*$^{-/-}$ MEFs transfected with indicated siRNA for 72 hours followed by treatment with TNFα for 4 hours and the cell viability determined by CellTiter-Glo. (C) Knockdown efficiency and caspase activation for (B) analyzed by Western blotting. (D) 661W cells transfected with siRNAs for APC10 or non-targeting control (N.C.) siRNA for 72 hours and treated with TNFα/5Z-7 to induce RDA. Viability determined using CellTiter-Glo. (E) Knockdown efficiency and caspase activation in RDA for (D) shown by Western blotting. (F) 661W cells transfected as in (A) to knockdown APC11 and then treated with TNFα/5Z-7 to induce RDA. Total cell lysates analyzed for RDA markers. (G) 293T cells transfected with indicated expression plasmids for GST-hRIPK1, HA-hAPC11 or control GST and HA alone for 36 hours and harvested in 0.2% NP-40 lysis buffer. HA-APC11 immunoprecipitated using HA affinity beads and RIPK1 purified by GST pulldown in 1 M urea. The experiment was performed

simultaneously with Fig. 6D and the same GST-RIPK1+HA alone controls used for each. (H-I) 661W cells transfected with siRNAs against APC11 or non-targeting control (N.C.) siRNA for 72 hours and then treated for 12 hours with TNFα/CHX (H) to induce RIPK1-independent apoptosis or TNFα/CHX/zVAD (I) to induce necroptosis. Cell survival determined by CellTiter-Glo. Concentrations of reagents: TNFα, 1 ng/mL (A-F), 10 ng/mL (H-I). All data shown are mean ± SD of 3 or more independent experiments. *$P \leq 0.05$ vs N.C. by Student's *t* test (A, D) or one-way ANOVA (B, H-I).

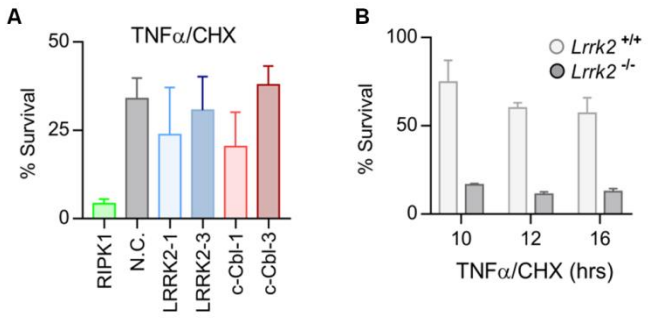

Figure S6. (A) 661W cells transfected with siRNAs against LRRK2, c-Cbl, RIPK1 (positive control), or non-targeting control (N.C.) siRNA for 72 hours. Knockdown efficiency shown in Fig 4D. Cells treated with TNFα/CHX for 8 hours to induce RIPK1-independent apoptosis and cell survival determined by CellTiter-Glo. (B) Littermate wild type or *Lrrk2* knockout MEFs treated with TNFα/CHX for indicated time to induce RIPK1-independent apoptosis and cell survival determined using CellTiter-Glo. Concentrations of reagents: TNFα, 10 ng/mL. All data shown are mean ± SD of 3 or more independent experiments.

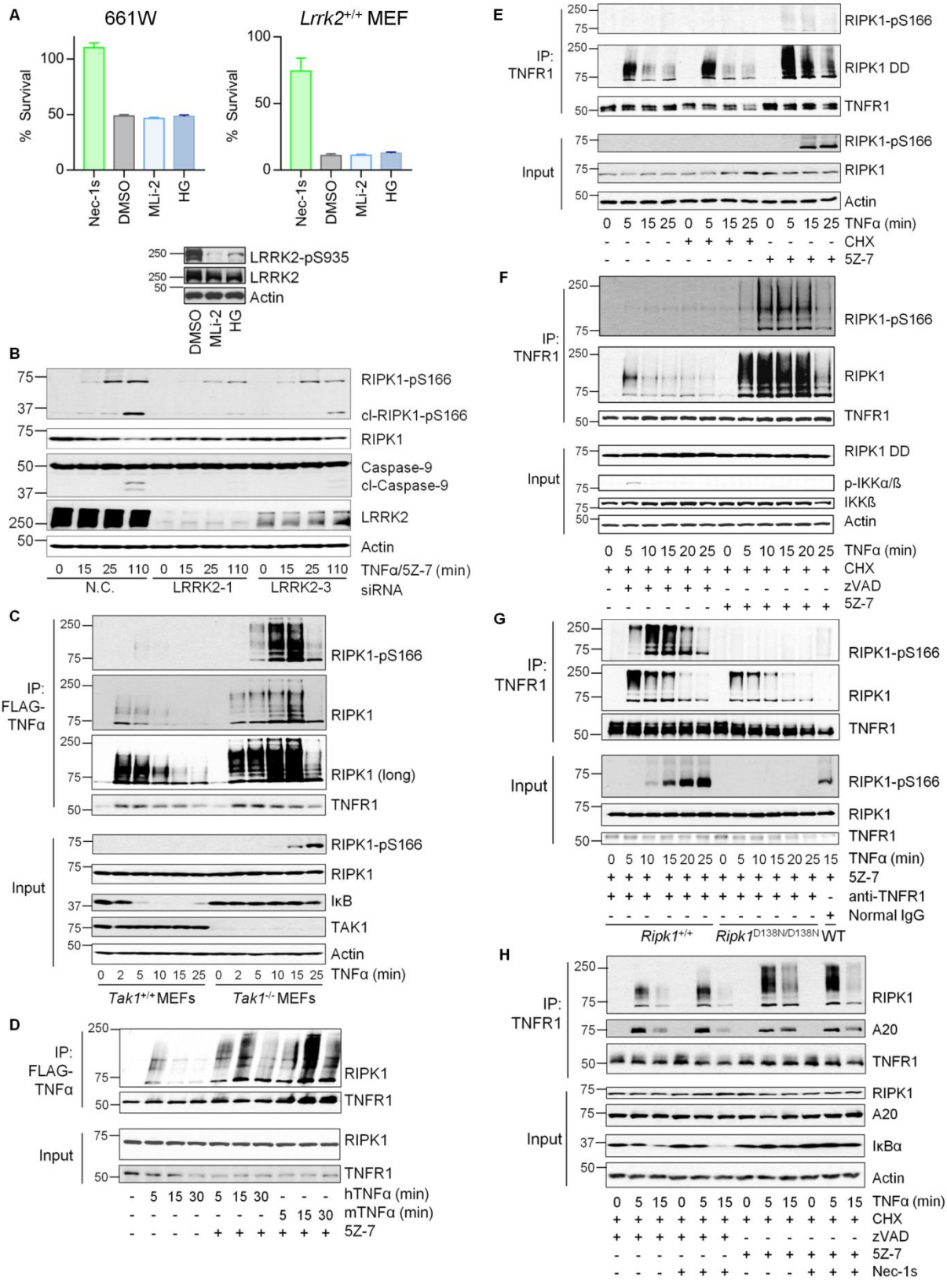
A
661W
Lrrk2+/+ MEF
% Survival
Nec-1s
DMSO
MLi-2
HG
LRRK2-pS935
LRRK2
Actin
B
RIPK1-pS166
cl-RIPK1-pS166
RIPK1
Caspase-9
cl-Caspase-9
LRRK2
Actin
TNFα/5Z-7 (min)
N.C.
LRRK2-1
LRRK2-3
siRNA
C
IP: FLAG-TNFα
RIPK1-pS166
RIPK1
RIPK1 (long)
TNFR1
Input
IκB
TAK1
TNFα (min)
Tak1+/+ MEFs
Tak1-/- MEFs
D
IP: FLAG-TNFα
RIPK1
TNFR1
Input
hTNFα (min)
mTNFα (min)
5Z-7
E
IP: TNFR1
RIPK1-pS166
RIPK1 DD
TNFR1
Input
RIPK1
Actin
TNFα (min)
CHX
5Z-7
F
IP: TNFR1
RIPK1-pS166
RIPK1
TNFR1
Input
RIPK1 DD
p-IKKα/ß
IKKß
Actin
TNFα (min)
CHX
zVAD
5Z-7
G
IP: TNFR1
RIPK1-pS166
RIPK1
TNFR1
Input
TNFα (min)
5Z-7
anti-TNFR1
Normal IgG
Ripk1+/+
Ripk1D138N/D138N
WT
H
IP: TNFR1
RIPK1
A20
TNFR1
Input
IκBα
Actin
TNFα (min)
CHX
zVAD
5Z-7
Nec-1s

Figure S7. (A) 661W cells or wild type *Lrrk2*$^{+/+}$ MEFs treated with indicated LRRK2 inhibitors, MLi-2 (15 nM) or HG (HG-10-102-01) (1 μM), Nec-1s, or vehicle control for 1 hour prior to induction of RDA by TNFα/5Z-7. Cell viability measured by CellTiter-Glo after 6 hours. Ability of LRRK2 inhibitors to reduce LRRK2 autophosphorylation assessed by Western blotting in wild type MEFs. (B-H) Samples analyzed by Western blotting with indicated antibodies. (B) 661W cells transfected with siRNAs against LRRK2 or non-targeting control (N.C.) siRNA for 72 hours. Total cell lysates assessed for biomarkers of RDA and knockdown efficiency. (C) WT and *Tak1*$^{-/-}$ MEFs treated with FLAG-TNFα for indicated periods of time and then lysed in 0.5% NP-40+0.5% Triton X-100 lysis buffer. Complex I immunoprecipitated with FLAG-M2 beads. (D) 661W cells treated with FLAG-hTNFα or FLAG-mTNFα with or without 5Z-7 as indicated and complex I analyzed as in (C). (E) 661W cells treated with TNFα/CHX or TNFα/5Z-7 for indicated periods of time and harvested as in (C). Complex I immunoprecipitated with anti-TNFR1. (F) 661W cells treated with TNFα/CHX/zVAD to induce necroptosis, or TNFα/5Z-7/CHX to induce RDA. Immunoprecipitation of complex I with anti-TNFR1. (G) *Ripk1*$^{+/+}$ or *Ripk1*$^{D138N/D138N}$ primary MEFs treated with TNFα/5Z-7 for indicated periods of time. Lysate immunoprecipitated with anti-TNFR1 or control normal IgG. (H) 661W cells treated with TNFα, CHX and zVAD with or without Nec-1s as indicated and complex I analyzed as in (E). Concentrations of reagents: TNFα, 1 ng/mL (A-B), 50 ng/mL (E-H), FLAG- TNFα, 50 ng/mL.

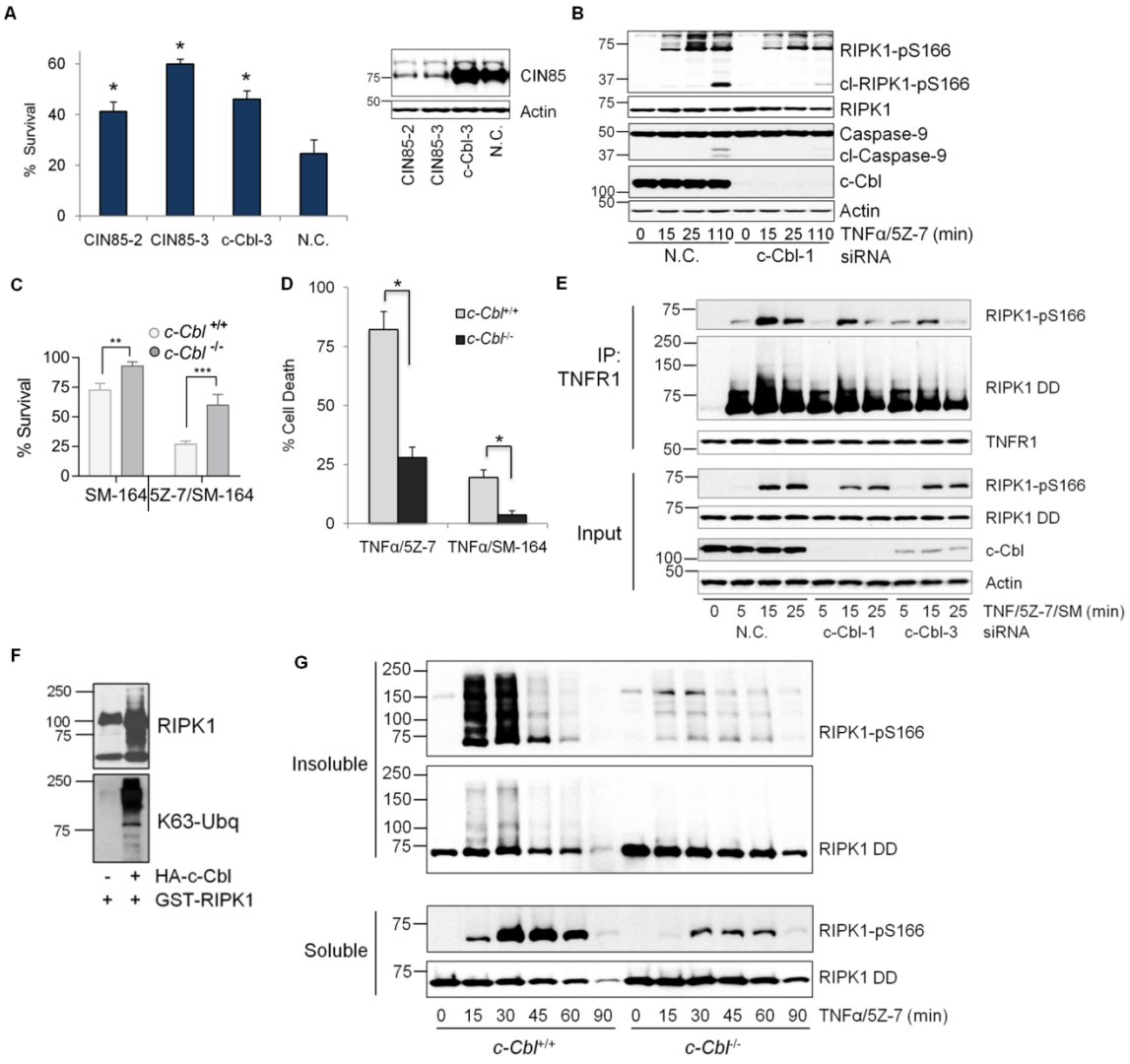

Figure S8. (A-B, E-G) Samples analyzed by Western blotting with indicated antibodies. (A) 661W cells transfected with indicated siRNAs for 72 hours and then treated with TNFα/5Z-7 for 8 hours to induce RDA. Cell survival measured with CellTiter-Glo. (B) 661W cells transfected with siRNA targeting c-Cbl or non-targeting control siRNA (N.C.) for 72 hours and RDA biomarkers and knockdown effeciency assessed in total lysates. (C) Littermate derived *c-Cbl*$^{+/+}$ and *c-Cbl*$^{-/-}$ MEFs treated for 4 hours and cell survival measured by CellTiter-Glo. (D) *c-Cbl*$^{+/+}$ and *c-Cbl*$^{-/-}$ MEFs treated as indicated with TNFα/5Z-7 or TNFα/SM-164 for 3.5 hours followed by Toxilight assay to measure cell death. (E) 661W cells transfected with indicated siRNA for 72 hours, treated with TNFα/5Z-7/SM-164 to induce RDA in the absence of cIAP1/2, harvested in 0.5% NP-40+0.5% Triton X-100 buffer, and complex I analyzed by anti-TNFR1 immunoprecipitation. (F) Human c-Cbl directly ubiquitinates hRIPK1 *in vitro*. Purified proteins were subjected to an *in vitro* ubiquitination assay with recombinant Ube1 and 1 μM E2 enzyme UbcH5 for 30 minutes. (G) *c-Cbl*$^{+/+}$ and *c-Cbl*$^{-/-}$ MEFs treated with TNFα/5Z-7 for indicated periods of time and harvested in lysis buffer with 0.5% NP-40+0.5% Triton X-100. The insoluble pellet was extracted using lysis buffer with 6 M urea. Concentrations of reagents:

TNFα, 1 ng/mL (A-D, G), 10 ng/mL (E). *$P \leq 0.05$ and **$P \leq 0.01$ by (A) one-way ANOVA or (C-D) Student's *t* test.

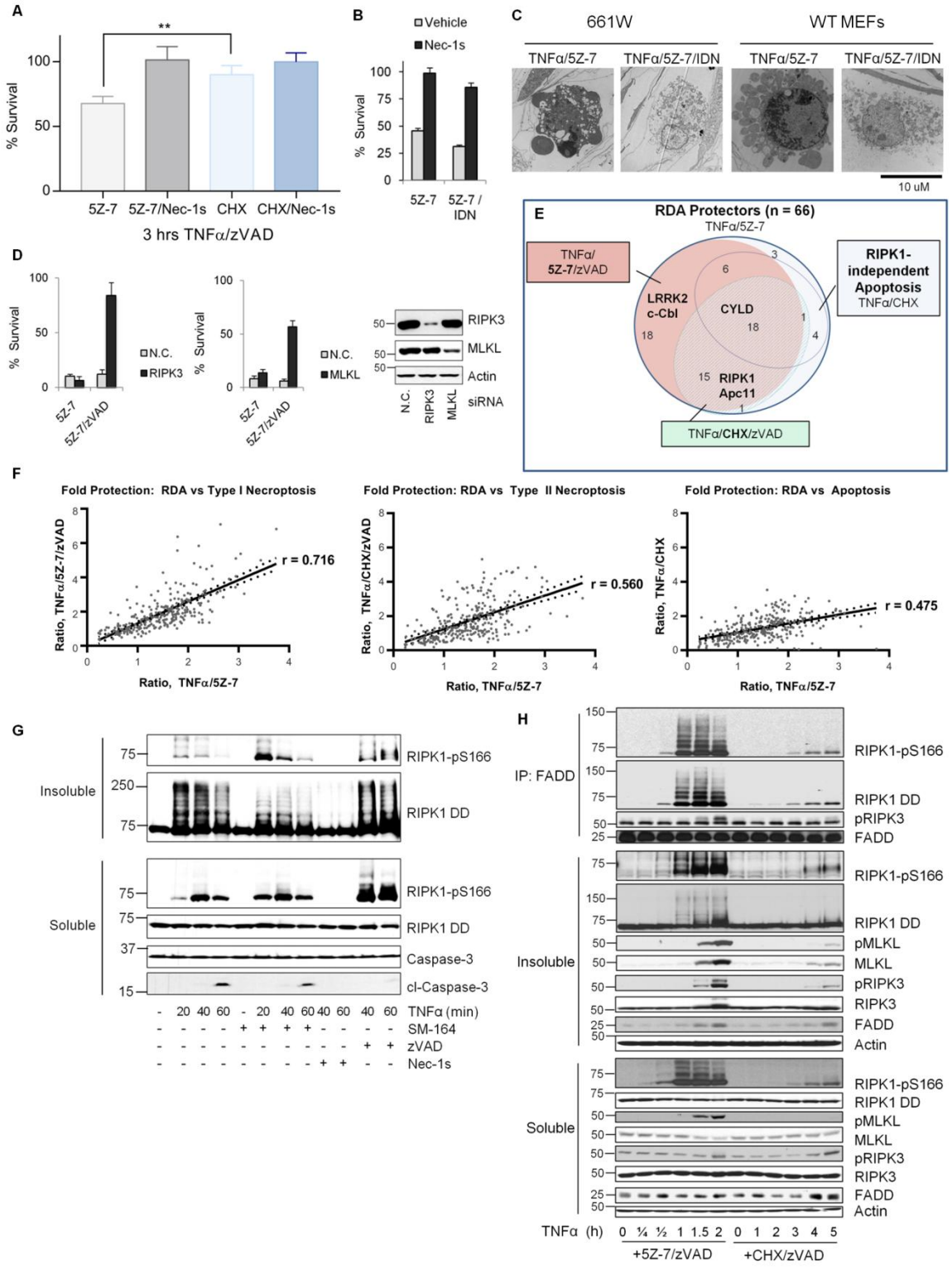

Figure S9. (A) WT MEFs treated with TNFα/zVAD for 3 hours in combination with either 5Z-7 or CHX to induce necroptosis in the presence or absence of Nec-1s. Cell viability determined by CellTiter-Glo. (B) 661W cells treated with pan caspase inhibitor IDN-5665 and TNFα for 8 hours. Cell viability determined by CellTiter-Glo. (C) 661W cells and WT MEFs treated with

TNFα/5Z-7 or TNFα/5Z-7/IDN and the cells fixed and processed for transmission electron microscopy. All images taken at 2900X. (D) 661W cells transfected with indicated siRNA (N.C.: non-targeting control) for 72 hours and then treated with TNFα/5Z-7 to induce RDA or TNFα/5Z-7/zVAD to induce necroptosis under RDA conditions. Cell viability measured by CellTiter-Glo and the knockdown efficiency determined by Western blotting. (E) Euler diagram of all of the tertiary screening results shows the overlapping genes whose knockdown protected against RDA (TNFα/5Z-7), different types of necroptosis (TNFα/5Z-7/zVAD or TNFα/CHX/zVAD), or RIPK1-independent apoptosis (TNFα/CHX). (F) The ability of an siRNA to protect against RDA is better correlated to its ability to protect against TNF/5Z-7/zVAD than RIPK1-independent apoptosis (TNF/CHX) or necroptosis induced by TNF/CHX/zVAD. The fold protection for each of the 4 siRNA duplexes for the 66 RDA protectors was plotted for TNF/5Z-7/zVAD vs RDA (TNF/5Z-7), TNF/CHX vs RDA, and TNF/CHX/zVAD vs RDA and the Pearson correlation r calculated, with $p < 0.001$ for all. Linear regression with 95% confidence interval shown. Lower limit for 95% confidence interval for TNF/5Z-7/zVAD (0.653) does not overlap with upper limit for 95% confidence interval for TNF/CHX (0.561) or TNF/CHX/zVAD, (0.635). (G) *Tak1*$^{-/-}$MEFs treated with TNFα or TNFα/SM-164 to induce RDA, or TNFα/zVAD to induce necroptosis under RDA conditions, in the presence or absence of Nec-1s for indicated periods of time. Cells lysed in buffer with 0.2% NP-40+1% Triton X-100, and the insoluble pellet resuspended in lysis buffer with 6 M urea. Samples analyzed by Western blotting using indicated antibodies. (H) WT MEFs treated and analyzed as in (G) with immunoprecipitation of complex II with anti-FADD from the soluble fraction. Concentrations of reagents: TNFα, 1 ng/mL (B-D), 10 ng/mL (A, G-H). $^{**}P \leq 0.01$ by one-way ANOVA.

Supplemental Tables

Table S1. Overlap of 661W RDA siRNA screen hits with previous L929 necroptosis (9) screen hits.

Overlap with L929+zVAD
CAMK1
CCL4
CYLD
DUSP19
LIMK1
PHF11
RIPK1

Table S2. RDA sensitizers.

RDA Sensitizers
A20
AKT3
HECTD2
HOIL-1
HOIP
MAPKAPK3
MOK
MYLK
NEK1
RNF40
TRIM31
TRIM32
XIAP

Table S3. RDA protectors.

RDA Protectors			
RDA Only	TNFα/CHX/zVAD	TNFα/CHX and TNFα/CHX/zVAD	TNFα/CHX
BDKRB2	APC11	AIRE	CCL4
CAMK2A	BRPF1	CYLD	NEK8
c-CBL	CAMK1	DTX2	RNF10
CDC42BPG	CAMKV	FBXL18	RNF13
DNAJC3B	CCRK	FBXL22	TESK1
DUSP7	DUSP19	FRAP1	TJP2
KHK	DUSP4	LIMK1	TRIM72
LRRC29	FBXO2	MAGI3	
LRRK2	FSD1	PHF11	
PFTK1	PATZ1	PHF5A	
PHF15	PRKCC	PRKCSH	
PHF16	PRKG2	PRP19	
PHF2	RIPK1	RNF126	
PHF6	RNF121	RNF7	
RNF38	SCEL	RSF1	
SOCS1	TSG101	TRIM25	
TEK	USP39	TWF2	
TRAF3	ZAP70	UBE2D3	
TTC3	ZFP185	UBE2I	
USP19			
ZFP592			

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