Supplementary Information for

Small Molecule Activators of TAK1 Promotes Its Activity-Dependent Ubiquitination and TRAIL-mediated Tumor Cell Death

Author list

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This PDF file includes:

Supplementary Methods

High-Throughput Screen (HTS) for Sensitizers of Necroptosis

The primary HTS was performed using the small molecule libraries (including NP800, DMIC, 1280, 3050 and L2000 compounds libraries) at 10μM on L929 cells treated with TNFα in 384-well plate. Hits exhibiting > 20% promotion of cell death in the primary screen were cherry-picked and tested in two independent confirmation runs. Identical bioactive compounds of the necroptosis-inducing agents were used in subsequent experiments.

Cell Culture

MEFs, 661W, BMDM, L929, E0771, LLC cells and HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% (v/v) FBS (Gibco) and 100 units/mL penicillin/streptomycin. FADD deficient Jurkat, NCI-H975 and NCI-H1299 cells were cultured in RPMI-1640 (Gibco) with 10% (vol/vol) FBS (Gibco) and 100 units/mL penicillin/ streptomycin. HT-29 cells were cultured in McCoy's 5A (Gibco) with 10% (vol/vol) FBS (Gibco) and 100 units/mL penicillin/ streptomycin. All cells were cultured in 37 °C with 5% CO_2. Sf-9 insect cells were cultured in ESF-921 medium at 27 °C with a shaking rate of 120 rpm.

Construction and Transfection of Plasmids

Full-length cDNAs for mouse IKKβ, mouse/human TAK1 and RIPK1 were PCR-amplified from the plasmid library and cloned into pcDNA3.1 using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd) with appropriate tags. Retroviral virus production used Flag/HA tagged pMSCV-puromycin/pLenti vector. pMSCV/pLenti were used as the plasmid backbone for all of the constructs used for reconstitution study. Mouse IKKβ, TAK1 and RIPK1 mutants were generated using MutExpress™ II mutagenesis kit (Vazyme Biotech Co., Ltd). cDNA encoding ZsGreen-Luciferas was cloned into pLenti-blasticidin plasmid for E0771 expression. For protein purification, cDNA encoding truncated mTRAIL (aa 118–291) were cloned into pET-32a plasmid for expression in Escherichia coli using ClonExpress™ II One Step Cloning Kit (Vazyme Biotech Co., Ltd), cDNA encoding mTAK1 (aa 1–303), mTAK1(aa 1-303)-TAB1(466-502), hTAK1(aa 15-303)-TAB1(468-504) and hTAK1(aa 31-303)-TAB1(468-504) with 6× His-tagged and HRV 3c protease cutting sites (LEVLFQGPGS) at N-terminus were cloned into the XhoI/EcoRI sites in pFAST plasmid for Sf-9 expression. All plasmids were verified by DNA

sequencing and the details of the plasmid sequences are available upon request. In brief, cells were plated at a density of 5×10^4 cells per well in a 12-well plate and transfected with a total of 1 μg DNA per well for 24 h. Medium was changed the day after transfection.

Analysis of Cytotoxicity and Viability

The rates of cell death were measured in triplicate or quadruplicate in a 384-well plate using SYTOX Green Nucleic Acid Stain (Invitrogen). The intensity of luminescence was determined in an EnSpire Multimode Plate Reader (PerkinElmer). Data were collected using PerkinElmer EnVision Manager Version 1.13 software. Cytotoxicity was expressed as percentages of cell death per well after deducting the background signal in non-induced cells and compared to that of the maximal cell death with 100% Lysis Reagent. The rates of cell viability were determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol and the results are expressed as percentages of luminescence intensity per well after deducting the background signal in blank wells and compared to that of the viability in the non-treated wells.

Immunoprecipitation

Cells were lysed with Nonidet P-40 buffer (150 mM NaCl, 50 mM Tris·HCl at pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 10% glycerol) supplemented with 1 mM PMSF, 1× protease inhibitor mixture (Roche), 10 mM β-glycerophosphate, 5 mM NaF, 1 mM Na_3VO_4 and 50 mM N-Ethylmaleimide. The lysate was incubated with an antibody overnight at 4°C. The immunocomplex was captured by Protein A/G Agarose (Life Technologies) with the appropriate antibodies for 2–4h at 4°C. Beads were washed four times, and the immunocomplex was eluted from beads by loading buffer.

***In Vitro* Kinase Assay**

Flag-tagged RIPK1 and Flag-tagged IKKβ–K44A was immunoprecipitated from HEK293T cells and washed with high-salt lysis buffer (5% glycerol, 0.5% NP-40, 500mM NaCl and 50mM Tris-HCl at pH7.4) for 4 times and then with water for 4 additional times. 20 μL beads with Flag-RIPK1 or Flag-IKKβ–K44A were resuspended in 24μL 1×kinase buffer (20 mM $MgCl_2$,100 μM ATP, 2 mM DTT, 10 μM compounds as indicated and 40 mM Tris-HCl at pH7.5) after the final wash step.

TAK1 kinase assays were performed with 200ng TAK1-TAB1 protein expressed and purified from Sf-9 cells in 24 μL 1×kinase buffer. Kinase reactions were performed for 15min, 30min or 60min at 25 °C or 30 °C and quenched by the addition of 6 μL 5×sample loading buffer and boiling at 100 °C for 10min.

Ni-NTA Pull Down

Cells were lysed with 8 M urea lysis buffer (150 mM NaCl, 50 mM Tris·HCl at pH 8.0, 8 M Urea, 10 mM Imidazole, 0.5% TritonX-100) supplemented with 1 mM PMSF, 1× protease inhibitor mixture (Roche), 2 mM Na_3VO_4 and 50 mM N-Ethylmaleimide. The lysate was incubated with Ni-NTA His-Bind Resin overnight at 4°C. Beads were washed four times, and the complex was eluted from beads by loading buffer.

Quantitative PCR (qPCR) Analysis

Total RNA was extracted and purified using RNAiso Plus Peagent (Takara). Reverse transcription reactions were performed with M-MLV reverse transcriptase (Takara; catalog no. 2640A). For qPCR, SYBR Green Master Mix (Biotool; catalog no. 2120) was used in the QuantStudio 7 Real-Time PCR System (Applied Biosystems), and the PCR conditions were 95°C for 10min, and 40 cycles of 95°C for 15s and 60°C for 1min. Fold induction of gene expression was calculated using ΔΔCt (cycle threshold) method. Namely, Ct values for genes of interest were normalized to Ct values of β–actin (mouse) and then to those of DMSO groups. The following primers were used: *TNFα*, forward, 5'-CCCTCACACTCAGATCATCTTCT-3'; reverse, 5'-GCTACGACGTGGGCTACAG-3'; *Cxcl1*, forward, 5'-CTGGGATTCACCTCAAGAACATC-3'; reverse, 5'-CAGGGTCAAGGCAAGCCTC-3'; *Cxcl2*, forward, 5'-ACAGAAGTCATAGCCACTCTC-3'; reverse, 5'-TTAGCCTTGCCTTTGTTCAG-3'; *Ccl2*, forward, 5'-GGGATCATCTTGCTGGTGAA-3'; reverse, 5'-AGGTCCCTGTCATGCTTCTG-3'; *Ccl5*, forward, 5'- CCAATCTTGCAGTCGTGTTTGT-3'; reverse, 5'-CATCTCCAAATAGTTGATGTATTCTTGAAC-3'; *Actin*, forward, 5'-ATGGAGGGGAATACAGCCC-3'; reverse, 5'-TTCTTTGCAGCTCCTTCGTT-3'.

Mass Spectrometry and Data Analysis

Flag-RIPK1 MEFs were treated with TNFα for 30 min and then lysed with NP-40 buffer. After IP against Flag-RIPK1, the binding proteins of RIPK1 were trypsin digested on beads. The resulting peptides were subjected to the enrichment of phosphorylated peptides by using TiO_2. The enriched phosphorylated peptides were analyzed on the Q Exactive HF-X mass spectrometer (Thermo Scientific). The identification and quantification of phosphorylated peptides was done by MaxQuant 2.0 (1). The tandem mass spectra were searched against UniProt human protein database together with a set of commonly observed contaminants. The precursor mass tolerance was set as 20 ppm, and the fragment mass tolerance was set as 0.1 Da. The cysteine carbamidomethylation was set as a static modification, and the methionine oxidation as well as serine, threonine and tyrosine phosphorylations were set as variable modifications. The FDR at peptide spectrum match level and protein level were controlled below 1%.

Quantitative mass spectrometry analysis of ubiquitination sites in TAK1 overexpressed in 293T cells. Immunoprecipitated TAK1 was trypsin-digested on beads. The resulting peptides were subjected to enrichment of diGly peptides using antibody against ubiquitin remnant motif (K-ε-GG) (PTM biolabs, lnc). The enriched diGly peptides were analyzed on the Q Exactive HF-X mass spectrometer, and the data were acquired using xCalibur3.1 from Thermo Fisher Scientific. DiGly peptides were identified and quantified using MaxQuant 2.0 (1). The tandem mass spectra were searched against UniProt mouse protein database together with a set of commonly observed contaminants. The precursor mass tolerance was set as 20 ppm, and the fragment mass tolerance was set as 0.1 Da. The cysteine carbamidomethylation was set as a static modification, and the methionine oxidation as well as lysine with a diGly remnant were set as variable modifications. The FDR at peptide spectrum match levels were controlled below 1%.

Protein Expression and Purification

DNA sequence of mTRAIL(118-291) was cloned into a modified pET32a vector (Novagen) to make a plasmid that encode an N-terminally GB1-6×His–tagged recombinant protein with a HRV 3c protease cutting site (LEVLFQGPGS) between His-tag and mTRAIL(118-291). For protein expression, *E. coli* BL21(DE3) cells transformed with the plasmid are cultured to OD600 ~ 0.8 at 37 ºC and transferred to 16 ºC incubator to be cooled down for 30 min. Then protein expression was induced by adding 0.1 mM IPTG and culturing overnight at 16 ºC. Bacteria were harvested and disrupted in binding buffer (20 mM Tris-HCl at pH 8.0, 500 mM NaCl, 5 mM imidazole) with

a high-pressure homogenizer (Shanghai Litu Mechanical Equipment Engineering Co., Ltd.,). The lysate was centrifugated at 15,000 rpm at 4 ºC for 30 min with a Backman JA20 rotor. Supernatant was applied to Ni^{2+} affinity resin (SA036005, Smart-Lifesciences, Changzhou, China) and incubated for 30 min with stirring at room temperature. After thorough washing the resin with binding buffer, the His-tagged protein was eluted with elution buffer (20 mM Tris-HCl at pH 8.0, 250 mM NaCl, 250 mM imidazole). The eluted GB1-6×His-mTRAIL(183-291) protein was further polished by gel-filtration on a HiLoad Superdex 75 16/600 column (Cytiva) in a buffer containing 20 mM Tris-HCl pH at 7.5, 100 mM NaCl, 1 mM DTT. The peak fraction was collected and digested with home-made HRV 3c protease. The GB1-6×His tag was removed by another round of gel-filtration in PBS buffer on a HiLoad Superdex 75 16/600 column. The residual endotoxin in mTRAIL(118-291) protein sample was removed by Pierce™ High Capacity Endotoxin Removal Spin Column (Thermo-Fisher) following manufacturer's protocol.

The mTAK1(1-303), mTAK1(1-303)-TAB1(466-502), hTAK1(15-303)-TAB1(468-504), hTAK1(31-303)-TAB1(468-504) proteins were expressed in Sf-9 cells with the Bac-to-Bac overexpression system (Invitrogen) according to the protocol of vendor. The cDNAs encoding the proteins were cloned into a bacmid which can produce an N-terminally 6× His-tagged recombinant protein with a HRV 3c protease cutting site (LEVLFQGPGS) between His-tag and proteins. Bacmids were generated in DH10Bac cells, and the resulting baculoviruses were generated by three rounds of amplification in Sf-9 insect cells. After infection by baculoviruses for 48 h, Sf-9 cells were collected by centrifugation at 4,000 rpm for 10min with a Backman JA9.1 rotor. Cells were disrupted in buffer A (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 5 mM imidazole, 1 mM DTT and 10% [v/v] glycerol) with a high-pressure homogenizer (Shanghai Litu Mechanical Equipment Engineering Co., Ltd.,). The lysate was centrifugated at 15,000 rpm at 4 ºC for 50 min with a Backman JA20 rotor. Supernatant was applied to Ni^{2+} affinity resin (SA036005, Smart-Lifesciences, Changzhou, China) and incubated for 1 hour with stirring at 4 ºC. After thorough washing the resin with buffer A, the His-tagged protein was eluted with buffer B (buffer A without glycerol but with 250 mM imidazole) and further separated by gel-filtration on a HiLoad Superdex 200 26/600 column (Cytiva) in buffer B (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1 mM DTT). The target fraction was collected and digested with HRV 3c protease and dephosphorylated by lambda protein phosphatase overnight at 4 ºC. The TAK1-TAB1 fusion protein was further separated by ion-exchange column HiTrap QFF (Cytiva) in buffer B with a linear gradient of NaCl

from 100 mM to 1,000 mM. The main peak fraction was collected and further polished by gel-filtration in buffer B with a HiLoad Superdex 200 16/600 column (Cytiva).

Analytical Ultracentrifugation

Sedimentation velocity experiments were performed on a Beckman XL-I analytical ultracentrifuge equipped with an eight-cell rotor under 142,250×*g* at 20 °C. The partial specific volume of protein samples and the buffer density were calculated using the program SEDNTERP (http://www.rasmb.org/). The final sedimentation velocity data were analyzed and fitted to a continuous sedimentation coefficient distribution model using the program SEDFIT (2).

Thermal Shift Assay

Recombinant hTAK1-TAB1 (TAK1 residues 15–303, TAB1 residues 468-504, 2 μM) was mixed with the SYRO Orange Protein dye (final concentration 10×) in a final reaction volume of 10μL. The protein thermal stability was analyzed by differential scanning calorimetry on a QuantStudioTM 7 Flex Real-Time PCR System according to the manufacturer's recommendations using a melting protocol of 25 to 95 °C at a 1% ramp rate (equivalent to 0.015 °C/s). Data were analyzed using the Protein Thermal ShiftTM Software v1.3. (3).

Photo-Affinity Labeling and Click Chemistry

Cells were pretreated with 20 μM photo-affinity probe #5-27 or photostable control compound 8-56 for 2 h, and then lysates were prepared at a concentration of 2 mg/mL total protein in Nonidet P-40 buffer. The whole-cell lysates, or the purified hTAK1-TAB1 (TAK1 residues 31-303, 50μg) proteins were individually treated with 200μM #8-56 or #5-27 for 60min, and all samples were photo-crosslinked at ∼350 nm on ice for 30 min using a UV crosslinker (energy: 1200). After photo-affinity labeling, click chemistry was preformed to allow the linkage of Biotin-PEG3-Azide to enable pulldown (4). A master mix of the catalyst (final concentration) was prepared immediately before use by combining: 100 μM Biotin-PEG3-Azide (TCI (Shanghai) Development Co.,Ltd.), 100 μM TBTA (TCI (Shanghai) Development Co.,Ltd.), 1 mM $CuSO_4$ (Sinopharm Chemical Reagent Co.,Ltd.), 1 mM CuBr (Shanghai Macklin Biochemical Technology Co.,Ltd.), 1 mM TCEP (Sun Chemical Technology (Shanghai) Co.,Ltd.). The samples were vortexed and incubated for 1h at room temperature and TCEP was added one more time after incubation for 30

min. Chloroform-methanol precipitation was preformed next to isolate proteins. The biotin-labeled proteins were isolated by incubating with streptavidin-coupled beads overnight at 4 °C.

Photo-Crosslinking-Coupled Mass Spectrometry

The recombinant hTAK1-TAB1 (IIe31-Gln303, His468-Pro504) protein was pulled down using streptavidin beads, and then digested by trypsin on beads. After washing out free peptides, the biotinylated peptides were eluted from beads with 30% acetonitrile and 2% formic acid. The resulting peptides were analyzed on a Q Exactive HF-X mass spectrometer (Thermo Scientific) in a data-dependent mode. The MS/MS data were subjected to the database search against a UniProt human protein database in Proteome Discoverer 1.4 (Thermo Scientific). The precursor mass tolerance was set as 10 ppm, and the fragment mass tolerance was set as 0.1 Da. The cysteine carbamidomethylation was set as a static modification. The mass shift of 1000.4865Da was set as a variable modification on four or five amino acids in every round database search until all 20 proteinogeic amino acids were covered. The FDR at peptide spectrum match level was controlled below 1%.

LiP-MS

Recombinant hTAK1-TAB1 (TAK1 residues 15-303, TAB1 residues 468-504, 20 μg) were treated with vehicle (0.4% DMSO), R788 (200 μM), respectively, for 2 h at room temperature. Samples were then digested with proteinase K at 1:100 (proteinase K:protein, w/w) for 3 min at 25 °C, and incubated at 98 °C for 3 min to inactivate proteinase K. Denaturation was performed by addition of 10% sodium deoxycholate at 1:1 ratio. Samples were further digested with Trypsin (Promega) at 1:100 (Trypsin:protein, w/w) overnight at 37 °C. Peptide mixtures were desalted with mono-spin C18 column (GL Sciences). Equal amount peptide of each sample was loaded onto a homemade 30 cm-long pulled-tip analytical column (ReproSil-Pur C18 AQ 1.9 μm particle size, Dr. Maisch GmbH, 75 μm ID × 360μm OD) connected to an Easy-nLC1200 UHPLC (Thermo Scientific) for mass spectrometry analysis.

The acquired MS/MS data were analyzed against a reconstructed database (UniProtKB Spodoptera frugiperda database (database released on Jul 1st, 2020) with recombinant hTAK1-TAB1 sequence) by Andromeda algorithm built-in MaxQuant engine (v1.6). Trypsin was defined as cleavage enzyme. A decoy database containing the reversed sequences of all the proteins was

appended to the target database to accurately estimate peptide probabilities and false discovery rate (FDR), and FDR was set at 0.01. Linear fit model was used to fit the intensity of every peptide across all the samples for sake of good performance at filtering out significantly changed peptides. This experiment aimed at identifying conformotypic peptides that significantly change their abundance between the control and compound-treated groups. Each condition was tested with three biological replicates and statistically tested for differential conformotypic peptide abundances between conditions applying a logarithmic fold-change cutoff of 1 and adjusted P-value cutoff of 0.05. Peptide abundance statistics are obtained by grouping different precursor ions of the same peptide sequences.

HDX-MS

Recombinant hTAK1-TAB1 (TAK1 residues 15-303, TAB1 residues 468-504, 100μg) were treated with vehicle (0.4% DMSO) and R788 (200 μM), respectively for 2 h at room temperature. Samples were processed automatically by a LEAP Technologies Hydrogen Deuterium Exchange PAL system (Carrboro, NC). HDX measurements were taken at 0 s, 30 s, 100 s, 300 s, 1000 s, 3000 s and 10000 s at 4 °C. After each time point, an aliquot of sample was transferred to a vial in a 0.5 °C chamber and quenched by addition of an equal volume of quench buffer (200 mM citric acid, 4 M guanidine-HCl, 500 mM TCEP in H_2O, pH 2.3) for 0.5 min prior to online digestion. The complete HDX-MS procedure was repeated three times for each sample and each time point. Online digestion, trapping, desalting process and separation process was then performed in the temperature-controlled compartment of the HDX PAL system. Data were acquired using a Thermo LTQ Orbitrap-Elite mass spectrometer (San Jose, CA) with a Thermo H-ESI II probe. For peptide identification, mass spectra were acquired in a data-dependent scan using FTMS mode in MS1 (one microscan, 100 ms max injection time, 60 k resolution at 400 m/z) at the m/z range of 300–1500 followed by ten CID MS2 scans in the ion trap with a ± 2.0 m/z isolation width. Once the peptides were identified, the deuterium uptake in HDX experiments was conducted using FTMS mode in MS1.

The spectra generated were searched in PEAKS Studio X against a homemade database including all target proteins with a precursor mass tolerance of ≤ 20 ppm and MS/MS fragment ≤ 0.02 Da. Retention time and sequence information for each peptide were exported to Excel for HDX data processing. HDX data analysis was carried out using HDExaminer 2.0 (Sierra Analytics

Inc., Modesto, CA). The number of deuterium taken up by each peptide at each exchange time was calculated by the software algorithm for matching the best theoretical isotope distribution pattern to the observed isotope distribution pattern. Deuterium uptake was plotted as a function of exchange time. Triplicate runs were compared using Student's t-test at the 95% confidence level to confirm the consistency of the analytical results obtained. Deuterium uptake was converted to %D for each peptide based on the theoretical number of D; %D was used to generate heat maps. In addition, H/D-ex analysis was also carried out on non-deuterated and fully deuterated samples to correct back-exchange.

Surface Plasmon Resonance

The binding affinity between R788 and TAK1-TAB1 was analysed at 25 °C on a BIAcore T200 machine with CM5 chips (GE Healthcare). PBS-P buffer (GE Healthcare) was used for all measurements. For SPR measurements, His-tagged TAK1-TAB1 protein was purified from Sf-9 cells. The protein was further purified by size exclusion chromatography on a Superdex 75 column (GE Healthcare) in a buffer containing 20 mM imidazole (pH 6.5), 200 mM NaCl, 20 mM DTT. The protein was dialysed into PBS and diluted to a final concentration of 60 μg/ml in NaOAc buffer (pH 4.5) before immobilization on a CM5 chip. About 5,000 response units of protein were immobilized on the chip with a running buffer composed of PBS-P. A reference was used to normalize the response unit (RU) values of protein. A series of compound concentrations ranging from 1.875 to 60 μM was tested at 30 μl/min flow rate. The contact time was 100 s and the dissociation time was 120 s. When the data collection was finished in each cycle, the sensor surface was regenerated with PBS-P buffer. DMSO solvent correction was performed following the BIAcore T200 guide. Binding curves were displayed, and equilibrium binding constants (K_D) for the interaction were determined using the steady-state affinity method incorporated in the BIAEVALUATION 4.1 software (GE Healthcare).

***In Vivo* Tumor Growth and Treatments in Mice**

C57BL/6 (6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed under pathogen-free conditions for 1 week before the experiments. Mice were injected subcutaneously in the right flank with different cell lines diluted in 100 μl of phosphate-buffered saline. R788 (Selleck, S2625) was administered to the mice at 50 mg/kg daily by oral

gavage. mTRAIL was administered intraperitoneally to the mice at 10mg/kg daily. Vehicle treatment was performed using sodium carboxymethyl cellulose (0.5% in water). Tumor volume was measured using a caliper every other day and estimated as follows: volume (cm^3) = $(width)^2$ × length × 0.5. Mice that did not develop tumors or developed tumors larger than the threshold defined in the in vivo experimentation protocols approved by the animal welfare committee of LIH (volume, ≥2000 mm^3) were excluded.

Bioluminescent Tumor Cell Tracking

For *in vivo* whole-animal imaging, anesthetized mice received 150 mg/kg of D-luciferin (MKBio) via intraperitoneal injection (i.p.). Luminescence readings were collected on a Bruker Xtreme, starting at 10 min post i.p. injection for 10 min. Analysis was conducted using the Bruker Xtreme Living Image software package.

Histology and Immunochemistry

Tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 8 μm sections and dewaxed, rehydrated, and then stained with hematoxylin & eosin or AB/PAS. For immunohistochemistry of Cleaved caspase-3, Ki67 and CD31, paraffin-embedded sections (7 μm thick) were dewaxed and rehydrated, before incubated in 0.01 M sodium citrate buffer (pH 6.0) for heat-induced antigen retrieval. The sections were blocked with 3% H_2O_2 and then with 5% goat serum in PBST (PBS with 0.1% Triton X 100) and incubated with primary antibodies at 4 °C overnight and then washed 3 times with PBST before incubating with secondary antibodies. The signals were detected by SignalStain® DAB Substrate kit (CST, 8059). Primary antibodies for immunohistochemistry used: anti-Ki67 (CST, 12202), CD31 (CST, 92841) and Cleaved caspase-3 (CST, 9661). Secondary antibodies for immunohistochemistry used: goat anti-rabbit IgG H&L (Abcam, ab214880).

Cellular Thermal Shift Assay

The procedure was modified from the reported procedure by Molina (3). HEK293T cells were transfected with expression vectors for Flag-TAK1, Flag-TAK1(1-303) or Flag-TAK1-TAB1 fusion expression plasmids for 24h. 3μM R406 and 500nM 5Z-7 was treated at 20h after transfection, and then harvested and resuspended in 1/10 volume of pre-warmed PBS containing

protease inhibitor cocktail. The cell suspensions were then divided into Eppendorf tubes (100μl / tube) and transiently heated to different temperatures ranging from 43 to 52 °C for 3min using thermal-mixer. The heat-treated cell suspensions were freeze-thawed three times using liquid nitrogen, and centrifuged at 15000rpm for 15min at 4 °C to separate the soluble proteins from the cell debris and aggregates. The supernatant containing the remaining soluble proteins was transferred to new tubes and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by western blotting.

ADP-Glo^{TM} kinase assay

The kinase reactions were initiated by ATP in 1× in vitro kinase assay buffer containing 50 mM HEPES, 50 mM NaCl, 30 mM $MgCl_2$, 1 mM DTT, 0.05% BSA, 0.02% CHAPS, and the reactions were carried out at 30 °C for 10 min, 20 min, 30min, 40min, 50 min and 60 min, separately. After the kinase reaction, the assay was performed in two steps: (1) ADP-Glo^{TM} Reagent was added to terminate the kinase reaction and deplete the remaining ATP, and (2) the Kinase Detection Reagent was added to convert ADP to ATP and allowed the newly synthesized ATP to be measured using a luciferase reaction.

NMR Spectroscopy

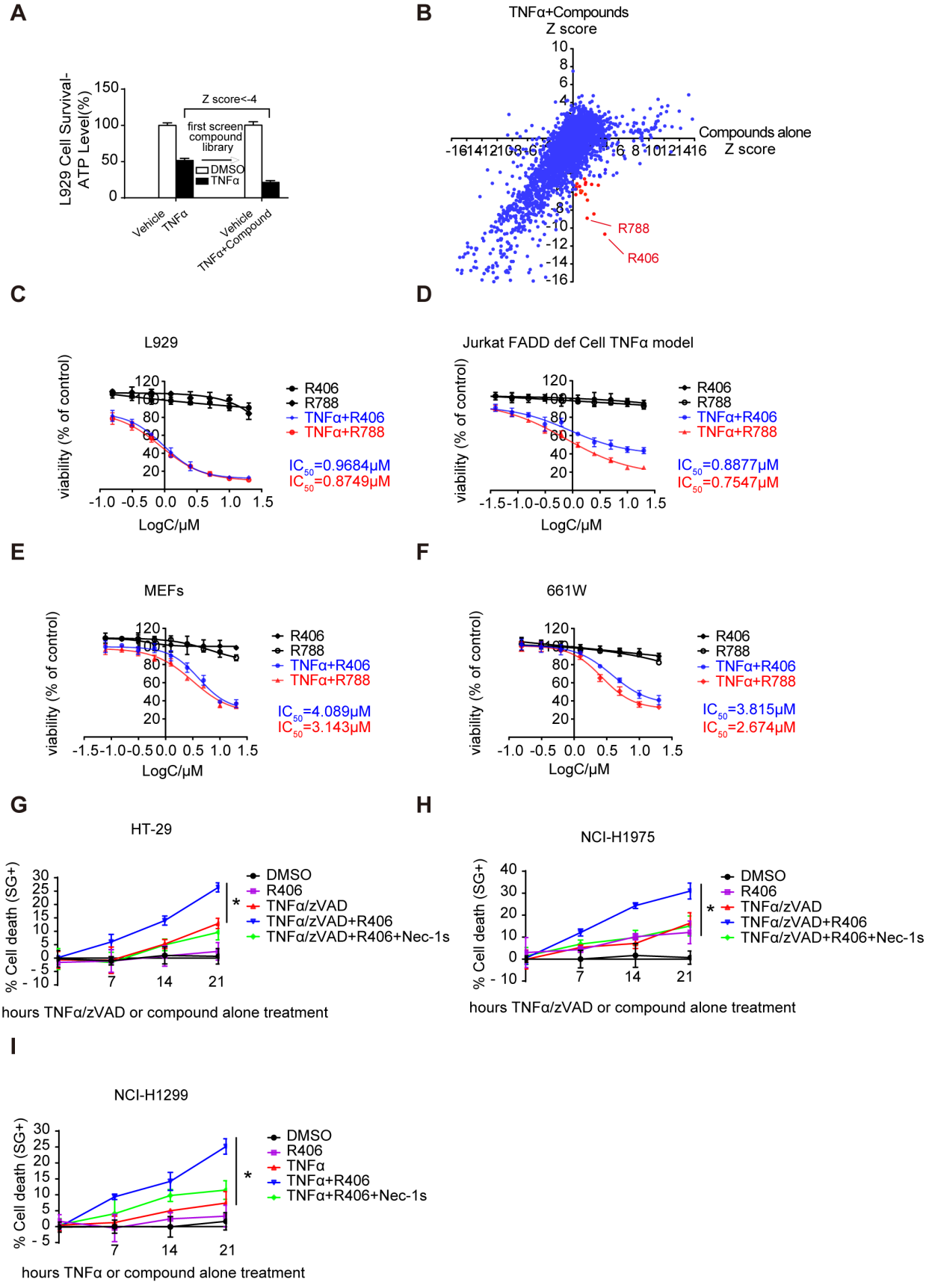
Saturation transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy was used to detect the interaction of compounds with TAK1-TAB1 fusion protein. The STD spectra were acquired at 25 °C on an Agilent 800MHz spectrometer. Samples used for STD-NMR experiments were prepared as 5 mM R788 in PBS with 10% (v/v) D_2O, 1mM R406 in PBS with 65% (v/v) D^6-DMSO, 30% (v/v) D_2O, with additional 15 μM TAK1-TAB1 fusion protein (in PBS, constituting 5% volume of the NMR sample). Reference spectra were recorded with the same sample of R788 or R406 without TAK1-TAB1 fusion protein. The on-resonance irradiation of TAK1-TAB1 was set at a chemical shift of -0.5 ppm, whereas the off-resonance irradiation was conducted at 20 ppm. Spectra were acquired using the following parameters: spectral window of 12 kHz, 512 scans, acquisition time of 2s, and repetition time of 3s. The signal in STD spectrum, resulting from the transfer of saturation from the protein to the ligand, was evaluated by subtracting the on-resonance spectrum from the off-resonance spectrum. This subtraction yielded a positive signal from an interacting ligand. The asterisks indicate the signals of the compounds.

Molecular docking

The docking was performed in program glide-dock from Schrodinger suite, using the active conformation of TAK1-TAB1 structure from PDB entry 2EVA. The missing loops were rebuilt in the docking program.

References

1. J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* **26**, 1367-1372 (2008).
2. P. Schuck, Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys J* **78**, 1606-1619 (2000).
3. D. Martinez Molina *et al.*, Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science* **341**, 84-87 (2013).
4. A. L. Mackinnon, J. Taunton, Target Identification by Diazirine Photo-Cross-linking and Click Chemistry. *Curr Protoc Chem Biol* **1**, 55-73 (2009).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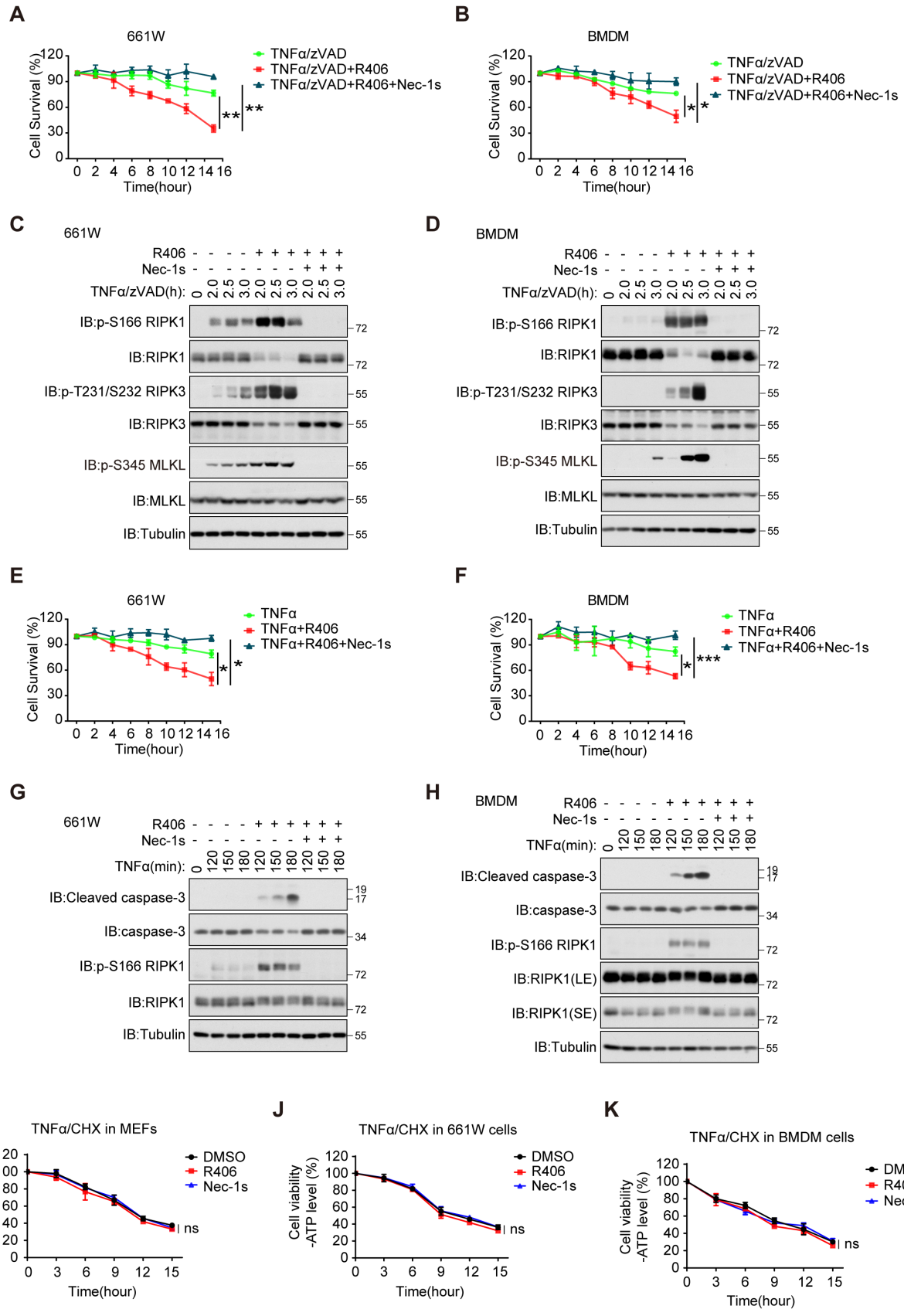
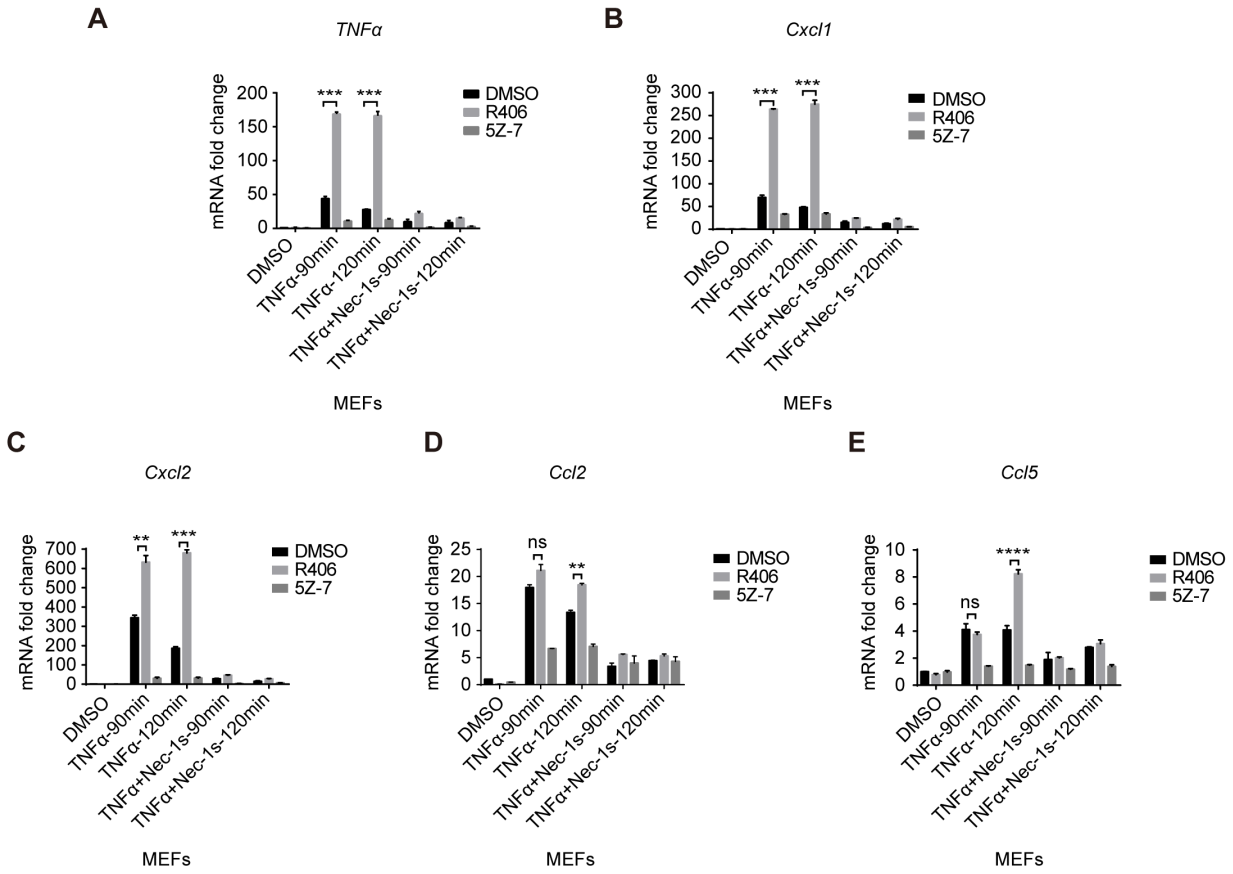
TNFα+R406+Nec-1s
hours TNFα or compound alone treatment

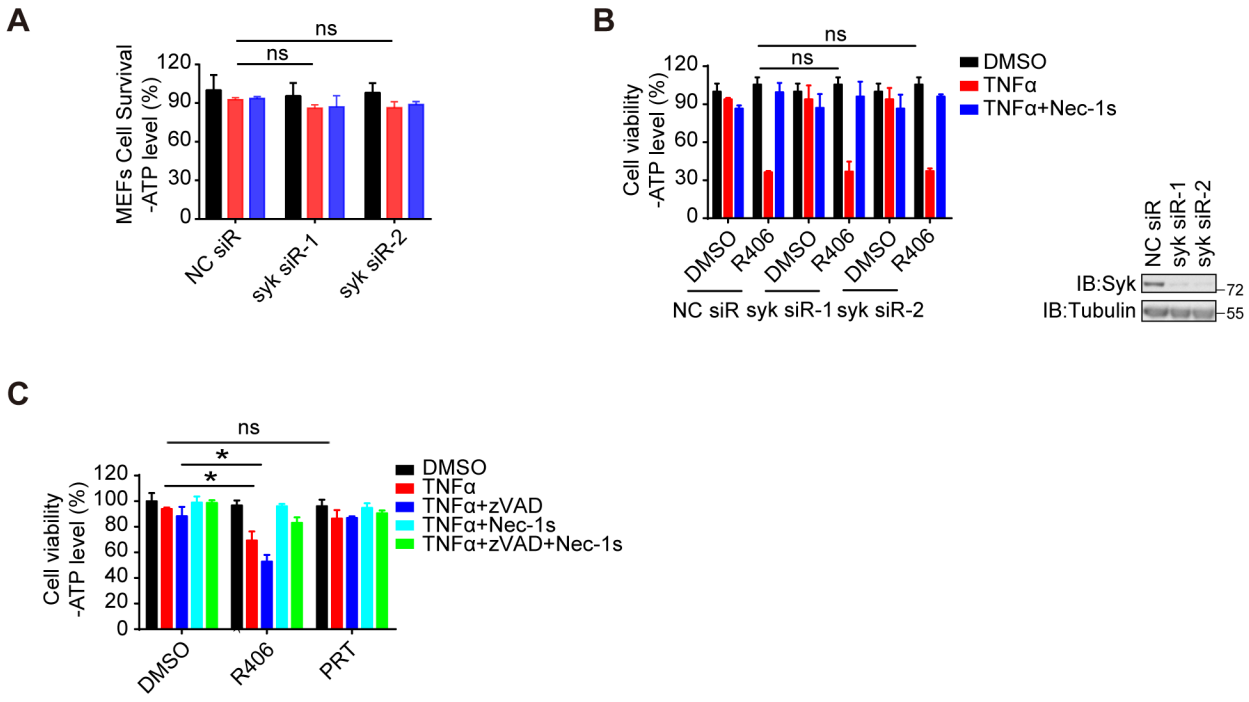
Supplementary Figure 1. Isolation of R406 and R788 for apoptosis and necroptosis enhancers. (A) High-throughput screen design as described in Experimental Procedures. The number of surviving cells was normalized to control cells that were treated with vehicle only (0.1% DMSO). Necroptosis was induced by adding the final concentration of 10ng/ml TNFα to the L929 cells which led to ~50% of cell viability. Identical bioactive compounds of the necroptosis-inducing agents were used in subsequent experiments. The cell survival was measured by CellTiter-Glo assay. The data are represented as the mean ± SEM of duplicate wells. (B) The number of surviving cells was normalized to control cells that were treated with vehicle. Low levels of necroptosis were induced by adding the final concentration of 10ng/ml TNFα to the L929 cells, with or without bioactive compounds. The cell survival was measured by CellTiter-Glo assay. The data are reresented as the mean ± SEM of duplicate wells. The z-score of 7263 small molecule compounds was plotted as a scatter plot. The x-coordinate is the z-score corresponding to the mono-addition compound, the y-coordinate is the z-score corresponding to the TNFα and the compound co-treatment. (C) IC_{50} of R406 and R788 promoting TNFα-induced necroptosis (10ng/ml) in L929 cells treated with indicated compound at 15h and cell viability was measured by CellTiter-Glo assay. (D) IC_{50} of R406 and R788 promoting TNFα-induced cell death (2ng/ml) in FADD deficient Jurkat cells treated with indicated compound at 11h and cell viability was measured by CellTiter-Glo assay. (E) IC_{50} of R406 and R788 promoting TNFα-induced cell death (20ng/ml) in MEFs treated with indicated compound at 15h and cell viability was measured by CellTiter-Glo assay. (F) IC_{50} of R406 and R788 promoting TNFα-induced cell death (20ng/ml) in 661W cells treated with indicated compound at 15h and cell viability was measured by CellTiter-Glo assay. (G-H) Necroptosis was induced in HT-29 (G) or NCI-H1975 cells (H) by the treatment with TNFα (20ng/ml) and zVAD.fmk (20μM) together with R406 (3μM) at indicated times and cell death was determined by STOX Green. Mean ± SEM of n=3. *P< 0.05. (I) RDA was induced in NCI-H1299 cells by the treatment with TNFα (20ng/ml) together with R406 (3μM) at indicated times and cell death was determined by SYTOX Green. Mean ± SEM of n=3. *P< 0.05.


A
661W
Cell Survival (%)
Time(hour)
TNFα/zVAD
TNFα/zVAD+R406
TNFα/zVAD+R406+Nec-1s
B
BMDM
C
661W
R406
Nec-1s
TNFα/zVAD(h):
IB:p-S166 RIPK1
IB:RIPK1
IB:p-T231/S232 RIPK3
IB:RIPK3
IB:p-S345 MLKL
IB:MLKL
IB:Tubulin
D
BMDM
E
661W
TNFα
TNFα+R406
TNFα+R406+Nec-1s
F
BMDM
G
661W
TNFα(min):
IB:Cleaved caspase-3
IB:caspase-3
IB:p-S166 RIPK1
IB:RIPK1
IB:Tubulin
H
BMDM
IB:RIPK1(LE)
IB:RIPK1(SE)
I
TNFα/CHX in MEFs
Cell viability -ATP level (%)
DMSO
R406
Nec-1s
ns
J
TNFα/CHX in 661W cells
K
TNFα/CHX in BMDM cells

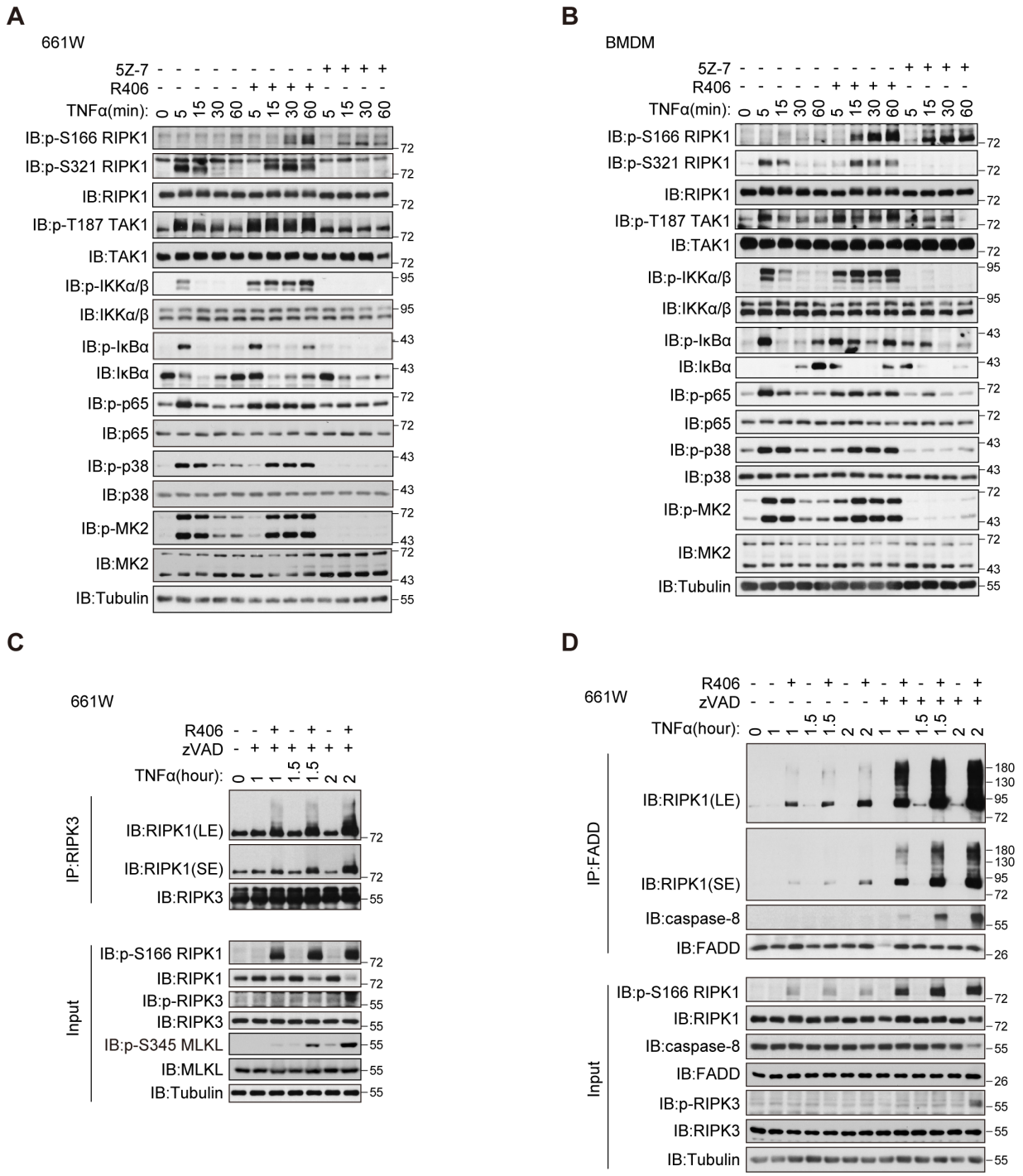
Supplementary Figure 2. R406 had no effect on RIPK1 independent cell death. (A-B) 661W (A) and BMDM cells (B) were pretreated with or without 3μM R406 or 10μM Nec-1s for 30min as indicated and then treated with 20ng/ml TNFα and 20μM zVAD.fmk for 2, 4, 6, 8, 10, 12, and 15h, separately. The cell survival was measured by CellTiter-Glo assay. Mean ± SEM of n=3. *P < 0.05; **P < 0.01. (C-D) 661W (C) and BMDM cells (D) were pretreated with or without 3μM R406 or 10μM Nec-1s for 30min as indicated and then 20ng/ml TNFα with 20μM zVAD.fmk were added for various time points, separately. The cell lysates were analyzed by phosphorylated- and total RIPK1, RIPK3, MLKL and Tubulin as indicated. (E-F) 661W (E) and BMDM cells (F) were pretreated with or without R406 (3μM), R406 and Nec-1s (10μM) for 30min and then TNFα (20ng/ml) was added for indicated periods of time, separately. The cell survival was measured by CellTiter-Glo assay. Mean ± SEM of n=3. *P < 0.05; ***P < 0.001. (G-H) 661W (G) and BMDM cells (H) were pretreated with or without 3μM R406 or 10μM Nec-1s for 30min as indicated and then 20ng/ml TNFα was added for various time points, separately. The cell lysates were analyzed by phosphorylated- and total RIPK1, Cleaved caspase-3, caspase-3 and Tubulin as indicated. (I-K) MEFs (I), 661W (J) and BMDM cells (K) were pretreated with or without 3μM R406 or 10μM Nec-1s for 30min as indicated and then treated with 20ng/ml TNFα and 2μg/ml Cycloheximide (CHX) for 3, 6, 9, 12 and 15h, separately. The cell survival was measured by CellTiter-Glo assay. Mean ± SEM of n=3. n.s. not significant.



Supplementary Figure 3. R406 promotes TNFα-induced inflammatory cytokine production. (A-E) MEFs were treated as indicated. The transcription level of TNFα, Cxcl1, Cxcl2, Ccl2, and Ccl5 were analyzed by RT-qPCR assay. Data were represented as Mean ± SEM of triplicates. ***P*< 0.01; ****P*< 0.001; *****P*< 0.0001; n.s. not significant.

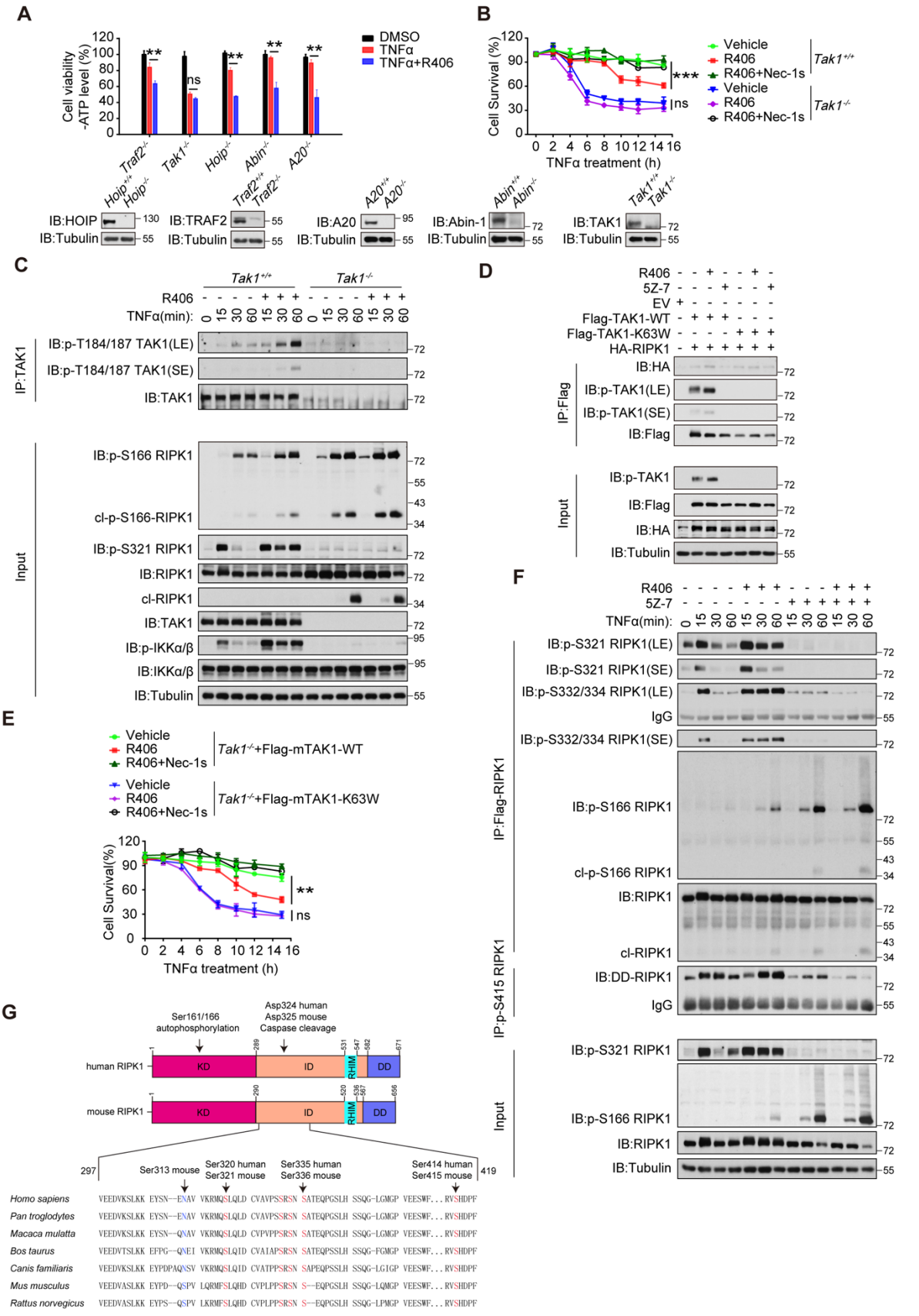


Supplementary Figure 4. Inhibiting Syk has no effect on cell death. (A-C) MEFs were transfected with 50nM NC, Syk-1 and Syk-2 siRNA using Lipofectamin RNAiMax, respectively (siRNAs targeting mouse *Syk* (5'-CCAUCGAGAGGGAACUUAATT-3', 5'-CCGGGUGGAAUAAUCUCAATT-3')). The cells were pretreated with or without 3μM R406, 10μM Nec-1s and 10μM PRT (Syk inhibitor) and then stimulated with 20ng/ml TNFα or TNFα and 20μM zVAD.fmk for 15h. The cell survival was measured by CellTiter-Glo assay. The data are represented as the Mean ± SEM of n=3 biologically independent samples. *P< 0.05; n.s. not significant.

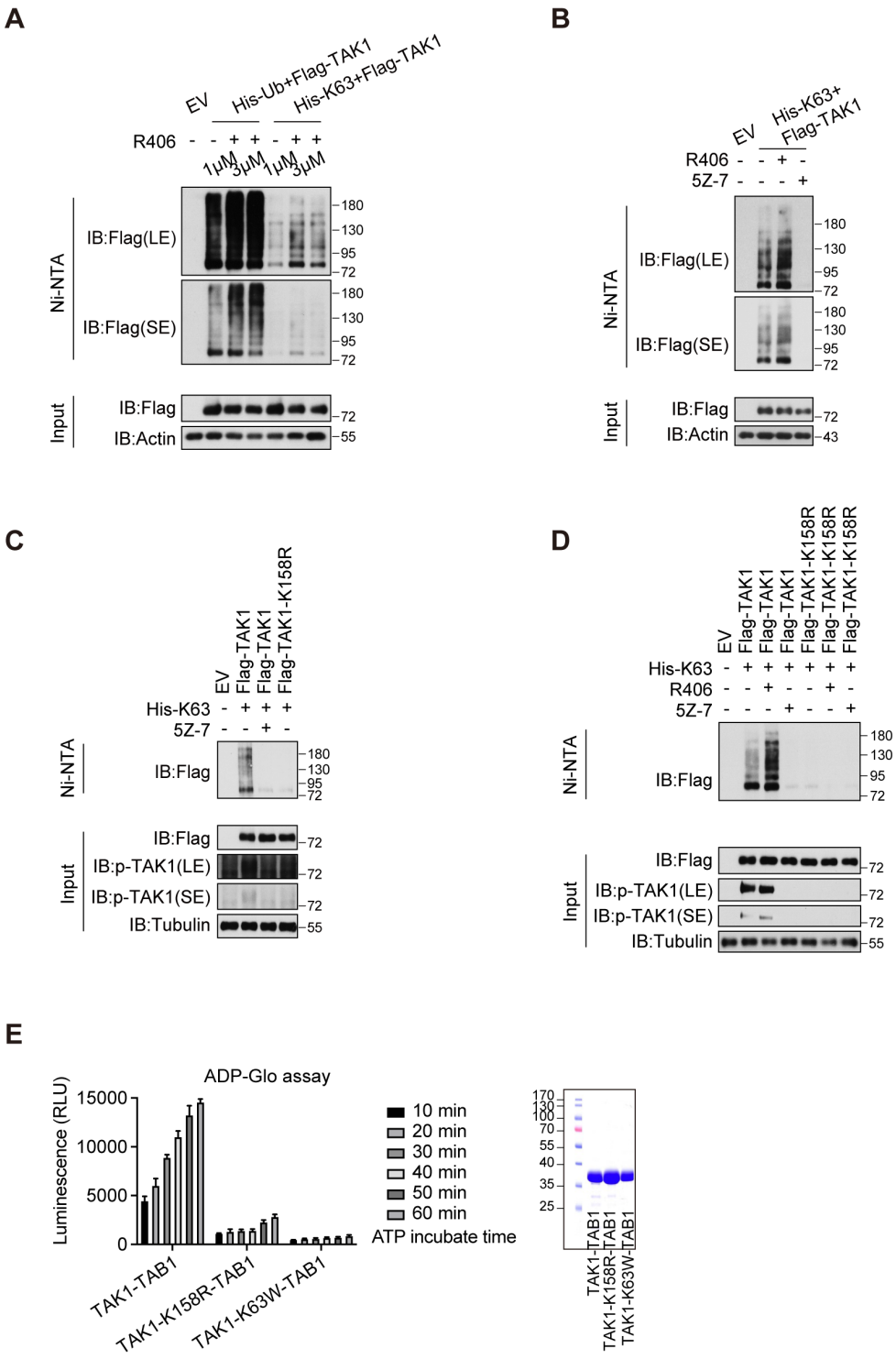


Supplementary Figure 5. R406 promotes the activation of TAK1 kinase. (A-B) 661W (A) and BMDM cells (B) were treated for 30min with R406 (3μM) or 1h with 5Z-7 (0.5μM), and then stimulated with 20ng/ml TNFα for indicated time points, separately. The cells were lysed with RIPA buffer. The cell lysates were analyzed by western blotting with the indicated antibodies. (C)

661W cells were pretreated with or without R406 (3μM) and Nec-1s (10μM) for 30min, then 20ng/ml TNFα and 20μM zVAD.fmk were added for indicated time points. The cells were lysed with 0.5% Nonidet P-40 buffer and cell lysates were immunoprecipitated with anti-RIPK3 antibody. All immunoprecipitated complexes and whole-cell lysates were analyzed by western blotting with the indicated antibodies. (D) 661W cells were pretreated with or without 3μM R406 for 30min, then 20ng/ml TNFα and 20μM zVAD.fmk were added for indicated time points. The cells were lysed with 0.5% Nonidet P-40 buffer and cell lysates were immunoprecipitated with anti-FADD antibody. All immunoprecipitated complexes and whole-cell lysates were analyzed by western blotting with the indicated antibodies.

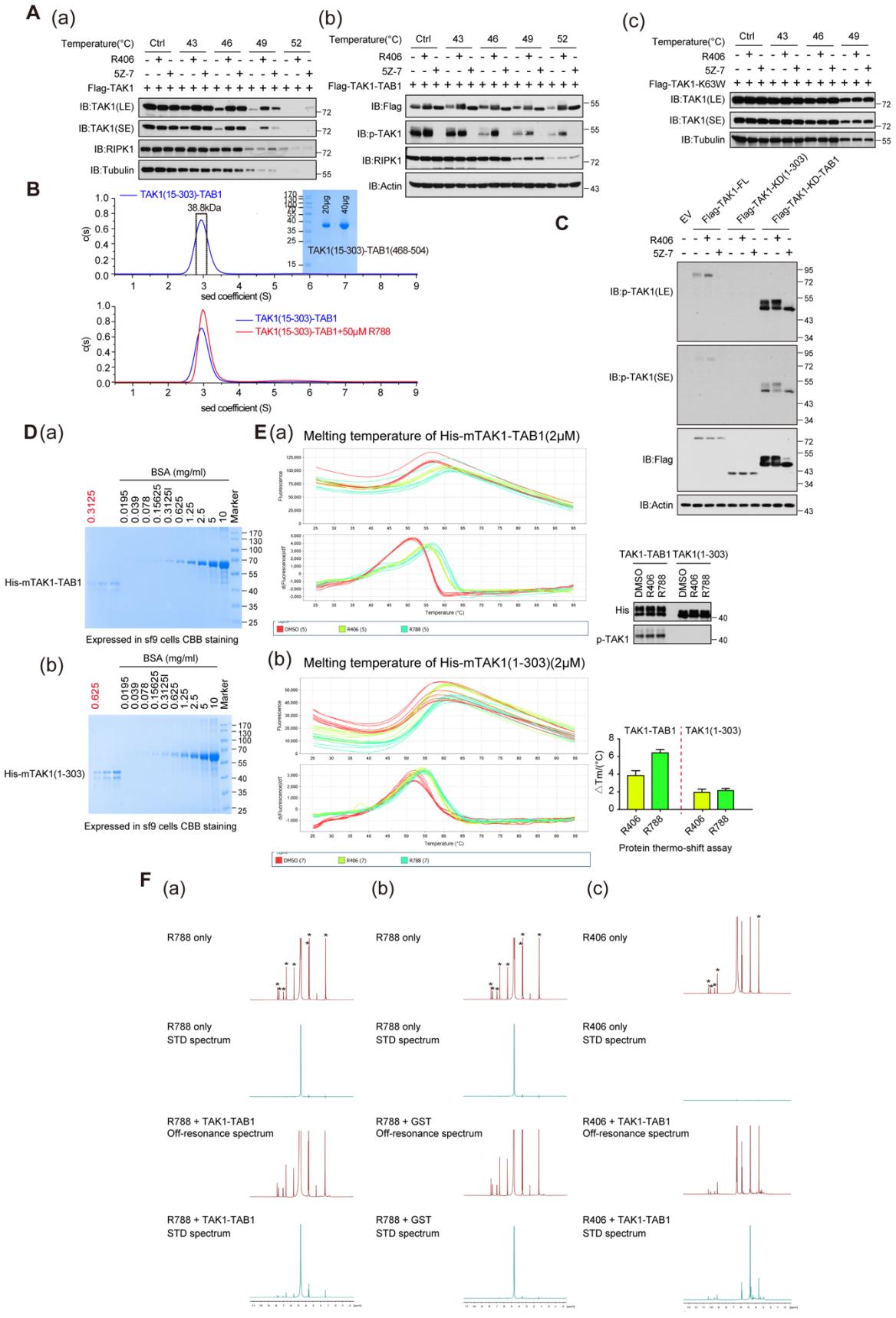

A
Cell viability -ATP level (%)
DMSO
TNFα
TNFα+R406
Traf2-/-
Tak1-/-
Hoip-/-
Abin-/-
A20-/-
IB:HOIP
IB:TRAF2
IB:A20
IB:Abin-1
IB:TAK1
IB:Tubulin
B
Cell Survival (%)
TNFα treatment (h)
Vehicle
R406
R406+Nec-1s
Tak1+/+
Tak1-/-
C
IP:TAK1
IB:p-T184/187 TAK1(LE)
IB:p-T184/187 TAK1(SE)
IB:TAK1
Input
IB:p-S166 RIPK1
cl-p-S166-RIPK1
IB:p-S321 RIPK1
IB:RIPK1
cl-RIPK1
IB:p-IKKα/β
IB:IKKα/β
D
R406
5Z-7
EV
Flag-TAK1-WT
Flag-TAK1-K63W
HA-RIPK1
IP:Flag
IB:HA
IB:p-TAK1(LE)
IB:p-TAK1(SE)
IB:Flag
IB:p-TAK1
E
Tak1-/-+Flag-mTAK1-WT
Tak1-/-+Flag-mTAK1-K63W
F
IP:Flag-RIPK1
IP:p-S415 RIPK1
IB:p-S321 RIPK1(LE)
IB:p-S321 RIPK1(SE)
IB:p-S332/334 RIPK1(LE)
IB:p-S332/334 RIPK1(SE)
IgG
IB:DD-RIPK1
G
Ser161/166 autophosphorylation
Asp324 human
Asp325 mouse
Caspase cleavage
human RIPK1
mouse RIPK1
KD
ID
RHIM
DD
Ser313 mouse
Ser320 human
Ser321 mouse
Ser335 human
Ser336 human
Ser414 human
Ser415 mouse
Homo sapiens
Pan troglodytes
Macaca mulatta
Bos taurus
Canis familiaris
Mus musculus
Rattus norvegicus

Supplementary Figure 6. R406 targets TAK1 to regulate RIPK1 by phosphorylation. (A) Identification of the R406 target from screening Complex I components such as *A20*$^{-/-}$, *Hoip*$^{-/-}$, *Tak1*$^{-/-}$, *Traf2*$^{-/-}$ and *Abin-1*$^{-/-}$ cells. The cells were pretreated with 3μM R406 for 30min and then treated with 20ng/ml TNFα for 11h. The cell survival was measured by CellTiter-Glo assay. The data are represented as the Mean ± SEM of n=3 biologically independent samples. $^{**}P< 0.01$; n.s. not significant. (B) *Tak1*$^{+/+}$ or *Tak1*$^{-/-}$ MEFs were treated for 30min with or without R406 (3μM) and Nec-1s (10μM), and then stimulated with 20ng ml^{-1} TNFα for the indicated time points. The cell survival was measured by CellTiter-Glo assay. Mean ± SEM of n=3. $^{***}P< 0.001$; n.s. not significant. (C) *Tak1*$^{+/+}$ or *Tak1*$^{-/-}$ MEFs were treated for 30min with or without R406 (3μM) and then stimulated with 20ng ml^{-1} TNFα for the indicated time points. The cells were lysed with 0.5% Nonidet P-40 buffer for immunoprecipitation with anti-TAK1 antibody. All immunoprecipitated complexes and whole-cell lysates were analyzed by western blotting with the indicated antibodies. (D) HEK293T cells were transfected with expression vectors for empty control, HA-tagged RIPK1, Flag-tagged TAK1 full length and the catalytic dead mutant Flag-TAK1(K63W) expression plasmids for 24h. 3μM R406 and 500nM 5Z-7 was added at 20h after transfection. The cells were lysed with 0.5% Nonidet P-40 buffer and cell lysates were immunoprecipitated with anti-Flag antibody. All immunoprecipitated complexes and whole-cell lysates were analyzed by western blotting with the indicated antibodies. (E) *Tak1*$^{-/-}$ MEFs were retrovirally reconstituted with the Flag-tagged TAK1 (Flag-TAK1) and the catalytic dead mutant Flag-TAK1(K63W). The cells were subsequently pretreated with or without 3μM R406 for 30min and then stimulated with TNFα for indicated time points. The cell survival was measured by CellTiter-Glo assay. Mean ± SEM of n=3. $^{**}P< 0.01$; n.s. not significant. (F) Flag-RIPK1 MEFs were pretreated for 30min with R406 (3μM) and 1h with 5Z-7 (0.5μM) when indicated and then stimulated with 20ng/mL TNFα for indicated time points. The cells were lysed with 0.5% Nonidet P-40 buffer and divided equally into two parts for immunoprecipitation with anti-Flag and anti-p-S415 RIPK1, respectively. All immunoprecipitated complexes and whole-cell lysates were analyzed by western blotting with the indicated antibodies. (G) Domain organization of RIPK1 showing additional residues of functional significance. Sequence alignment of key phosphorylation sites (STY) within RIPK1 orthologs from different species.

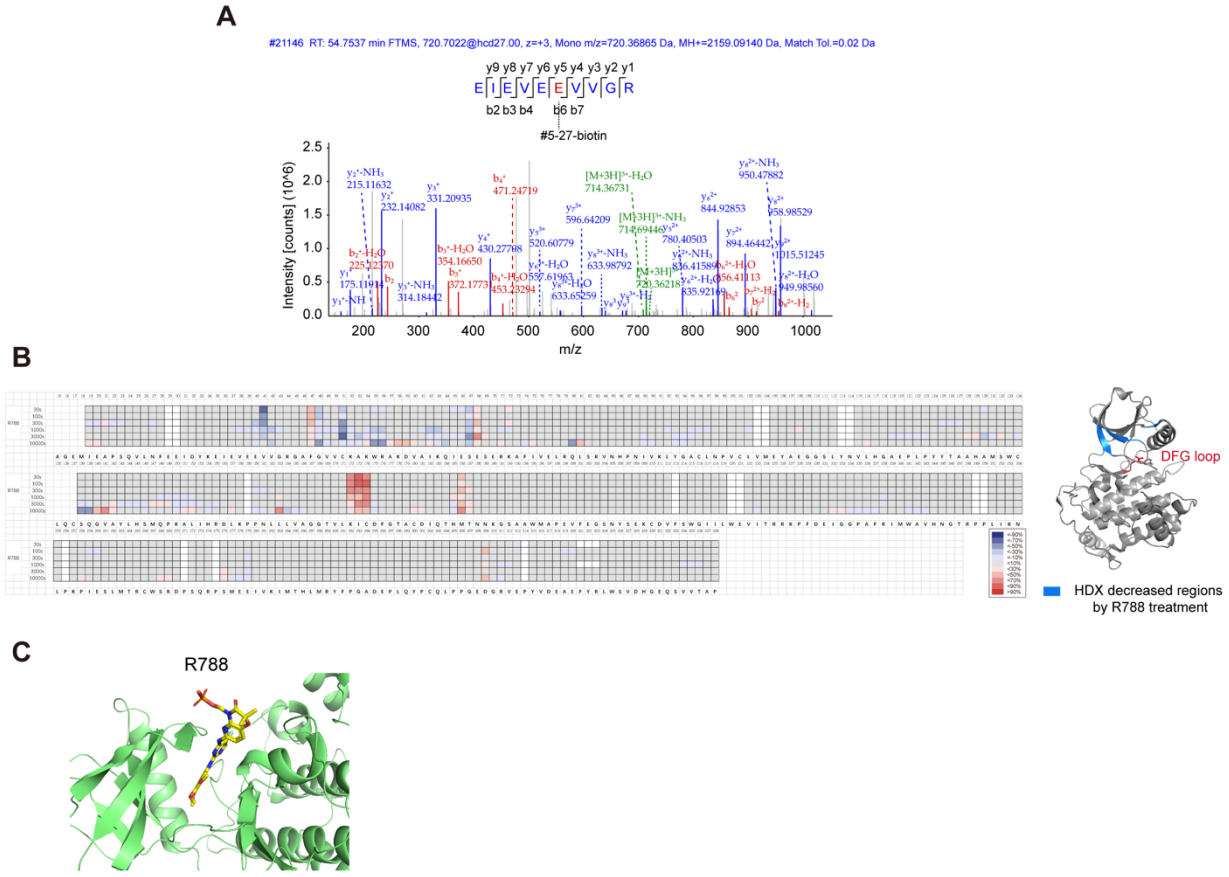


Supplementary Figure 7. R406 promotes Lys63-linked TAK1 polyubiquitination at the Lys158 residue within the kinase domain. (A) Expression vectors harbouring Flag-tagged TAK1 (0.5μg) and His-Ub (0.5μg) or His-K63 (0.5μg) were transfected into HEK293T cells, two different concentrations of R406 were added at 18h after transfection. Cell lysates were then subjected to

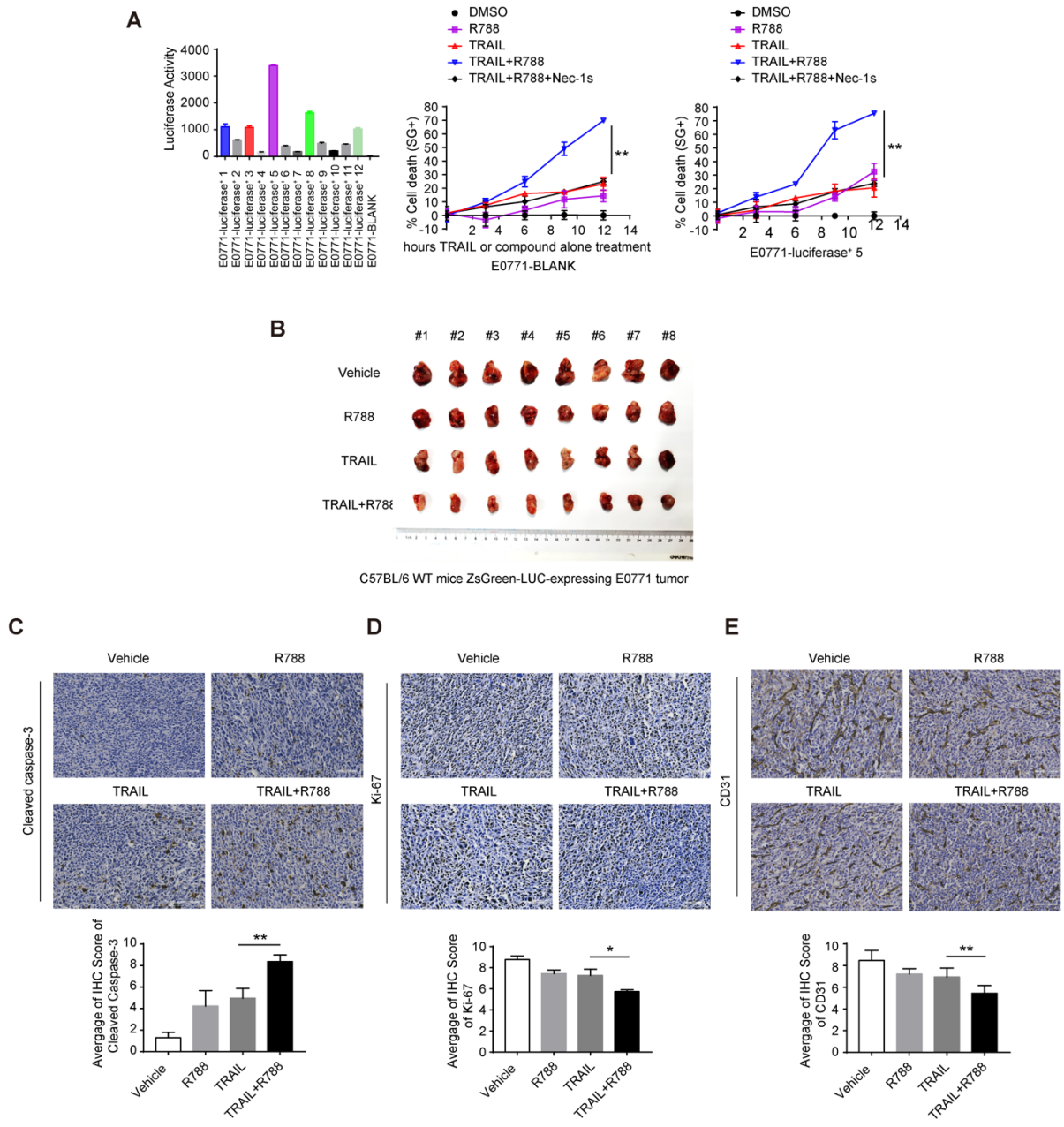
pull-down via Ni-NTA and analyzed by western blotting with antibodies as indicated. (B) HEK293T cells were co-transfected with the indicated expression vectors (Flag-TAK1, 0.5μg; His-K63, 0.5μg) for 24h. 3μM R406 and 500nM 5Z-7 was added at 18h after transfection. Cell lysates were then subjected to pull-down via Ni-NTA and analyzed by western blotting with antibodies as indicated. (C) HEK293T cells were co-transfected with expression vectors of His-K63 (0.5μg) and Flag-TAK1 (0.5μg) or the mutant Flag-TAK1(K158R) (0.5μg) for 24h. Cells were pretreated with or without 5Z-7 (250 nM) for 6h. Cell lysates were then subjected to pull-down via Ni-NTA and analyzed by western blotting with antibodies as indicated. (D) Expression vectors harbouring His-K63 (0.5μg) and Flag-tagged TAK1 (0.5μg) or the mutant Flag-TAK1(K158R) (0.5μg) were transfected into HEK293T cells, 3μM R406 and 500nM 5Z-7 was added at 18h after transfection. Cell lysates were then subjected to pull-down via Ni-NTA and analyzed by western blotting with antibodies as indicated. (E) ADP-Glo™ Kinase Assay of TAK1-TAB1, TAK1-K158R-TAB1 and TAK1-K63W-TAB1. Recombinant hTAK1-TAB1 (1μM), hTAK1(15-303)-K158R-TAB1 (1μM) and hTAK1(15-303)-K63W-TAB1 fusion protein (1μM) purified from Sf-9 cells were initiated by 10μM ATP, and the reactions were carried out at 30°C for 10min, 20min, 30min, 40min, 50min and 60min as indicated, and then the amount of ADP produced during the kinase reaction was detected by ADP-Glo™ Reagent.


A (a)
Temperature(°C) Ctrl 43 46 49 52
R406
5Z-7
Flag-TAK1
IB:TAK1(LE)
IB:TAK1(SE)
IB:RIPK1
IB:Tubulin
(b)
Flag-TAK1-TAB1
IB:Flag
IB:p-TAK1
IB:RIPK1
IB:Actin
(c)
Flag-TAK1-K63W
IB:TAK1(LE)
IB:TAK1(SE)
IB:Tubulin
B
TAK1(15-303)-TAB1
38.8kDa
c(s)
sed coefficient (S)
20μg
40μg
TAK1(15-303)-TAB1(468-504)
TAK1(15-303)-TAB1
TAK1(15-303)-TAB1+50μM R788
C
EV
Flag-TAK1-FL
Flag-TAK1-KD(1-303)
Flag-TAK1-KD-TAB1
IB:p-TAK1(LE)
IB:p-TAK1(SE)
IB:Flag
IB:Actin
D (a)
BSA (mg/ml)
0.3125
0.0195
0.039
0.078
0.15625
0.3125l
0.625
1.25
2.5
5
10
Marker
His-mTAK1-TAB1
Expressed in sf9 cells CBB staining
(b)
0.625
His-mTAK1(1-303)
Expressed in sf9 cells CBB staining
E (a) Melting temperature of His-mTAK1-TAB1(2μM)
Fluorescence
-dFluorescence/dT
Temperature (°C)
DMSO (5)
R406 (5)
R788 (5)
TAK1-TAB1 TAK1(1-303)
DMSO R406 R788 DMSO R406 R788
His
p-TAK1
(b) Melting temperature of His-mTAK1(1-303)(2μM)
DMSO (7)
R406 (7)
R788 (7)
TAK1-TAB1 TAK1(1-303)
△Tm(°C)
R406 R788 R406 R788
Protein thermo-shift assay
F (a)
(b)
(c)
R788 only
R406 only
R788 only STD spectrum
R406 only STD spectrum
R788 + TAK1-TAB1 Off-resonance spectrum
R788 + GST Off-resonance spectrum
R406 + TAK1-TAB1 Off-resonance spectrum
R788 + TAK1-TAB1 STD spectrum
R788 + GST STD spectrum
R406 + TAK1-TAB1 STD spectrum

Supplementary Figure 8. R406 binds to TAK1. (A) Cellular thermal stability. HEK293T cells were transfected with expression vectors for Flag-tagged TAK1 (a), TAK1-TAB1 fusion expression plasmids (b) and kinase dead of TAK1 (K63W) plasmids (c) for 24h. 3μM R406 and 500nM 5Z-7 was added at 20h after transfection. The HEK293T cells were harvested and resuspended with PBS and then were incubated in 0, 43, 46, 49, 52°C for 3min, and then frozen in liquid nitrogen quickly. The cells were subject to repeated freeze-thaw three times, and then centrifuged at 15000rpm for 15min. The products were analyzed by western blotting using indicated antibodies. (B) The analysis results of AUC, in which the molecular weight is marked on the peak of sed coefficient. (C) HEK293T cells were transfected with expression vectors for empty control, Flag-tagged TAK1 full length, Flag-tagged TAK1 kinase domain (1-303) and TAK1-TAB1 fusion expression plasmids for 24h, respectively. 3μM R406 and 500nM 5Z-7 was added at 20h after transfection. The HEK293T cells were lysed with RIPA buffer and analyzed by western blotting with indicated antibodies. (D) CBB staining of His-mTAK1-TAB1 fusion protein (a) and mTAK1 kinase domain (1-303AA) (b) purified from Sf-9 cells. (E) Thermal stability profiles of protein thermal shift assay. Recombinant mTAK1-TAB1 fusion protein (2μM) (a) and mTAK1 kinase domain (1-303AA) (2μM) (b) were purified from the Bac-to-Bac^{TM} Baculovirus Expression System and treated with 160μM R406, R788, 5Z-7 for 2h. The protein thermal stability were analyzed using differential scanning calorimetry by real-time PCR and the melting temperatures were calculated by Protein Thermal Shift Software. Five or seven replicates for each reaction were performed. (F) (a-c) TAK1-TAB1/R788 (a), GST/R788 (b), TAK1-TAB1/R406 (c) samples used for STD NMR experiments were prepared as 5mM R788 in PBS with 10%(v/v) D_2O, 1mM R406 in PBS with 65%(v/v) D^6-DMSO, 30%(v/v) D_2O, with additional 15μM TAK1-TAB1 fusion protein (in H_2O, constituting 5% volume of the NMR sample). Reference spectra were recorded with the same sample of R788 or R406 without TAK1-TAB1 fusion protein. The on-resonance irradiation of TAK1-TAB1 was set at a chemical shift of -0.5 ppm, whereas the off-resonance irradiation was conducted at 20 ppm. Spectra were acquired using the following parameters: spectral window of 12 kHz, 512 scans, acquisition time of 2s, and repetition time of 3s. The signal in STD spectrum, resulting from the transfer of saturation from the protein to the ligand, is evaluated by subtracting the on-resonance spectrum from the off-resonance spectrum. This subtraction yields a positive signal from an interacting ligand. The asterisks indicate the signals of the compounds.

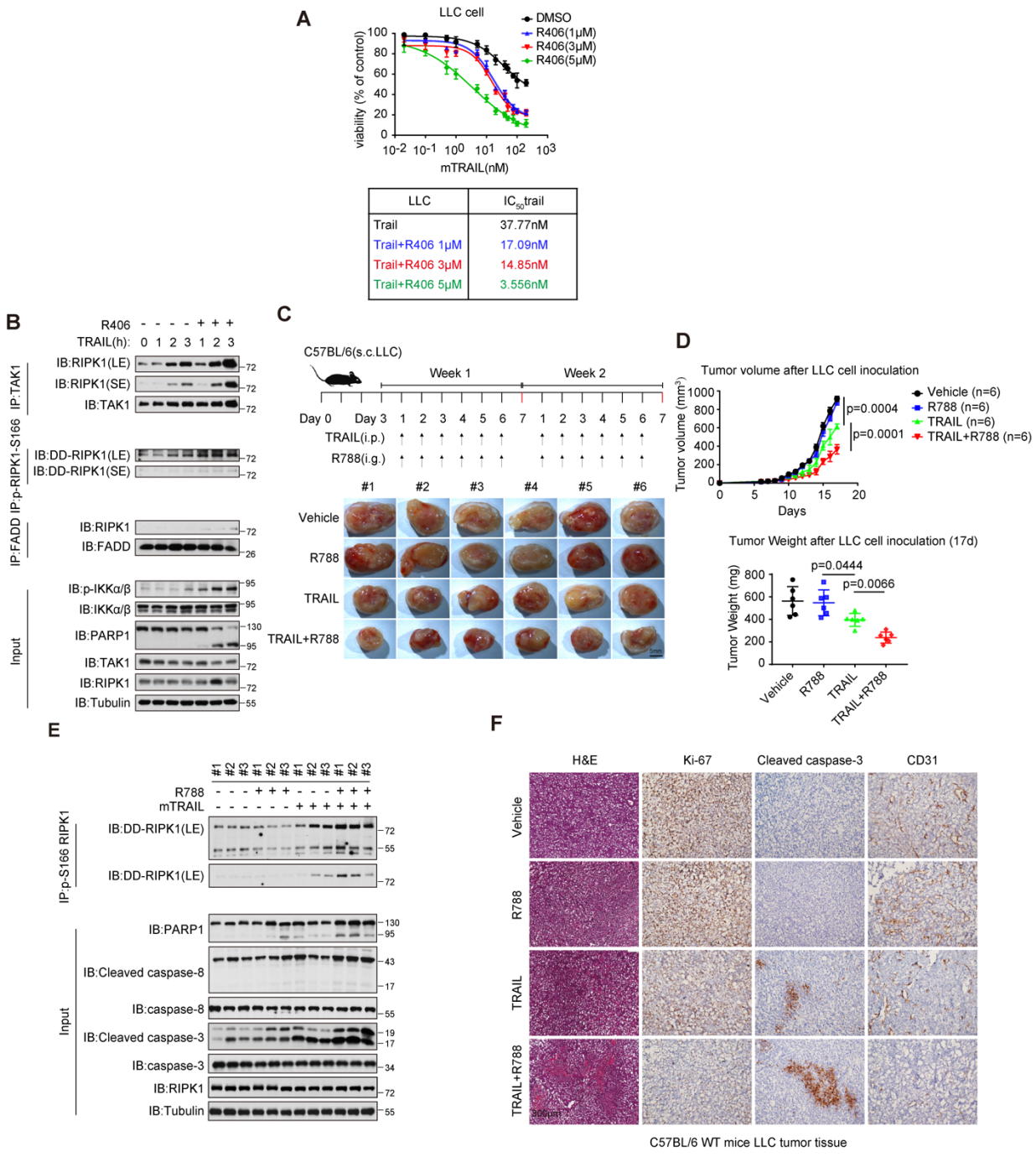


Supplementary Figure 9. A distinct conformation of activated TAK1 induced by R788. (A) Identification of R406-binding sites by mass spectrometry. TAK1-TAB1 (TAK1 residues 31-303, 50μg) was labeled by incubating with photo-affinity probe #5-27 before UV-crosslinking. After click-chemistry reaction and biotin-streptavidin affinity purification, the enriched peptides of TAK1 that bound with #5-27 were digested by trypsin and identified by mass spectrometry. MS/MS of a TAK1-TAB1 peptide 35-EIEVEEVVGR-44 crosslinked with biotinylated compound #5-27. The ion b2, b3, b4, b6, b7 are annotated with a mass shift+1000.48650 Da. (B) HDX-MS analysis of R788 binding sites. Summarized data for deuterium uptake differences between R788 bound TAK1-TAB1 and free TAK1-TAB1 at 30s, 100s, 300s, 1000s, 3000s and 10000s were indicated by a color gradient from blue (decreased deuterium uptake) via white (unchanged) to red (increased deuterium uptake). (C) Molecular docking of R788 to TAK1 kinase domain. The active conformation of TAK1-TAB1 fusion protein from PDB entry 2EVA is used in the docking simulation performed in Schrodinger software.



Supplementary Figure 10. R406/R788 promote the E0771 cancer-killing activity of TRAIL. (A) E0771 cells were transduced with vector for ZsGreen-Luciferase. The cells were selected with Blasticidin S. Twelve monoclonal cells were selected and tested for luciferase activity. E0771-BLANK cells and E0771-luciferase^{+}5 cells (the strongest luciferase activity) were treated with or without R788 (3μM) and Nec-1s (10μM) for 30min, and then treated with 10ng/ml TRAIL for 3h, 6h, 9h and 12h. Cell death was determined by SYTOX Green. Mean ± SEM of *n*=4. ***P*< 0.01. (B) ZsGreen-LUC-expressing E0771 tumor cells were implanted in 6-week-old C57BL/6 WT

female mice. After sixteen days, collected the tumor tissues of female mice. (C) Representative images of IHC staining for cleaved caspase-3. Brown elements, representative cells bearing cleaved caspase-3. Positive cells are more frequent in TRAIL+R788 groups. Mean ± SEM of n=4. Two-tailed t-test. Scale bars, 200μm. **P< 0.01. (D) Ki-67 staining in tumors derived from ZsGreen-LUC-expressing E0771 cells was assessed using immune histochemistry staining. Brown elements, represent the proliferation of cells. Positive cells are more frequent in Vehicle and R788 groups, in particular, around ZsGreen-LUC-expressing E0771tumor tissue proliferative areas. Mean ± SEM of n=4. Two-tailed t-test. Scale bars, 200μm. *P< 0.05. (E) Representative CD31 endothelial staining for vasculature in tumors derived from. ZsGreen-LUC-expressing E0771 cells. Positive cells are more frequent in Vehicle and R788 groups, in particular, around ZsGreen-LUC-expressing E0771 tumor tissue endothelial cells proliferative areas. Mean ± SEM of n=4. Two-tailed t-test. Scale bars, 200μm. **P< 0.01.



Supplementary Figure 11. R406/R788 promote the LCC cancer-killing activity of TRAIL. (A) LLC cells treated with TRAIL, with or without three different concentrations of R406, for 24h. Representative dose-response curves, tabular listing of IC_{50} values for trail with or without increasing concentrations of R406. (B) LLC tumor cells were treated with 3μM R406 for 30min and then 10ng/ml mTRAIL was added for various time points. The cell lysates were analyzed by

immunoblotting. (C) Schematic diagram of the experimental schedule. LLC tumor cells were implanted in 6-week-old C57BL/6 WT mice. After three days, mice were injected with vehicle and mTRAIL (10mg/kg intraperitoneally [i.p.]) once a day for 2 weeks. At the same time, R788 alone (50mg/kg p.o.administration [i.g.]) or the combination (R788: 50mg/kg i.g. + mTRAIL: 10mg/kg i.p.) one times a day for 2 weeks. Pictures of representative tumors from each experimental group (n=6). (D) LLC tumor parameters in C57BL/6 mice during indicated treatment. Mean ± SEM of *n*=6. (E) The LLC tumor samples were analyzed by immunoblotting (n=3). (F) Representative images of LLC tumor sections from mice with the indicated. treatments stained with H&E or immunostained for Ki-67, CC3 and CD31. Scale bars, 300μm.

HEK293T_IP_Flag-mTAK1_K63-Ubi_GlyGly (K) sites						
TAK1 Ubi site	Localization prob	Intensity			Ratio	
		DMSO	5Z-7	R406	5Z-7/ DMSO	R406/ DMSO
227	1	7.99E+08	5.71E+08	1.02E+09	0.7146433	1.2765957
532	1	4.47E+07	1.06E+07	5.64E+07	0.2371364	1.2617449
447	1	6.68E+07	3.03E+07	8.88E+07	0.4535928	1.3293413
158	1	8.16E+06	1.82E+06	8.37E+06	0.2230392	1.0257353
351	1	3.61E+08	1.63E+08	4.55E+08	0.4515235	1.2603878
501	1	1.62E+08	1.27E+08	3.21E+08	0.7839506	1.9814815
563	1	1.39E+09	8.88E+08	1.94E+09	0.6388489	1.3956834
386	1	6.89E+07	4.77E+07	1.26E+08	0.6923076	1.8287373
209	1	2.89E+06	1.86E+06	3.71E+06	0.6435986	1.2837371
358	1	3.04E+07	1.71E+07	4.88E+07	0.5625	1.6052632
54	1	8.84E+07	3.65E+07	9.88E+07	0.4128959	1.1176471

Table S1.

Quantitative mass spectrometry analysis of ubiquitination sites in TAK1 overexpressed in 293T cells. HEK293T cells were transfected with expression vectors of Flag-TAK1 with His-Ub (K63) for about 24h. Cell lysates were immunoprecipitated with Flag M2 Agarose Affinity beads. Flag-TAK1 was trypsin-digested and subjected to enrichment of diGly peptides. The ubiquitination sites with localization probability lower than 75% were excluded in further analysis. The intensity of each ubiquitinated site (K) in TAK1 and the ratios of each ubiquitinated site (K) among indicated treatment were shown in the table.