Supplementary Information

Phosphorylation of TRAF2 within its RING domain inhibits stress-induced cell death by promoting IKK and suppressing JNK activation

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Supplementary Methods

In vivo ^{32}P-orthophosphate labeling

Cells cultured in 6-well or 100-mm culture plates were transfected with 1.0 μg or 2.0 μg of Flag-TRAF2. 36 hrs after transfection, cells were incubated for 1 hr in phosphate-free DMEM containing 10% dialyzed fetal bovine serum (FBS), followed by incubation for 90 min in the same medium supplemented with 0.5 mCi/ml of ^{32}P-orthophosphate. Cells were then washed with ice-cold tris-buffered saline (TBS) and lysed in TNE buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1.0 % NP-40, 2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 x Halt protease and phosphatase inhibitor cocktail) on ice for 30 min, followed by centrifugation in screw-capped tubes at 12,500g for 20 min at 4°C. ^{32}P-labeled Flag-TRAF2 was immunoprecipitated using anti-Flag antibody, separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was then stained with Ponceau S and exposed to X-ray film. The same membrane was then immunoblotted with anti-Flag antibody to visualize the level of Flag-TRAF2.

Two-dimensional separation of phosphoamino acids on TLC plate

293T cells (two 100-mm plates) were co-transfected with Flag-TRAF2 (2.0 μg) and CA-PKCα (1.0 μg) or pCDNA3 (1.0 μg), and labeled with ^{32}P-orthophosphate (1.0 mCi/ml) for 90 min. ^{32}P-labeled Flag-TRAF2 was immunoprecipitated, separated by SDS-PAGE and transferred onto PVDF membrane. The ^{32}P-Flag-TRAF2 band was then excised and digested in 200μl of 6N HCl in a screw-capped tube at 110°C for 1 hr followed by SpeedVac drying for 2 hrs. Hydrolyzed and washed ^{32}P-Flag-TRAF2 amino acids were dissolved in 8 μl of pH1.9 buffer containing phosphoamino acid standards, and separated (horizontally with pH1.9 buffer and vertically with pH3.5 buffer) on a glass-backed thin-layer cellulose chromatography (TLC) plate using a Hunter Thin Layer Peptide Mapping Electrophoresis System (HTLE-7002, CBS Scientific, Del Mar,

CA). The TLC plate was baked at 60°C for 20 min and sprayed with 0.25% of ninhydrin (w/v) in acetone, and then baked again at 60°C for 15 min to visualize the position of phosphoamino acid standards. The TLC plate was then exposed to X-ray film for a week.

Luciferase reporter gene assays

HeLa cells and MEFs cultured in 6-well plates were transfected with either an NF-κB or a c-Jun firefly luciferase reporter plasmid (NF-κB-Luc or Jun2-Luc; 0.2 μg), together with a control Renilla luciferase reporter plasmid (pRL-TK; 0.01 μg) and wild-type or phosphomutant Flag-TRAF2 (0.2 μg), using Lipofectamine 2000 reagents according to the manufacturer's protocol. 36 hrs after transfection, test cells were treated with hTNFα (10 ng/ml) or mTNFα (5 ng/ml), and protein samples were prepared at 6 (HeLa) or 4 (MEFs) hrs after treatment. The firefly and Renilla luciferase activities were then measured using the Dual-luciferase assay system according to the manufacturer's instructions (Promega).

Cytotoxicity assay

TRAF2/5 DKO and WT MEFs were plated on 6-well plates at a density of $2x10^4$cells/well. The next day, cells were left untreated, treated with mTNFα (10ng/ml) or treated with H_2O_2 (0.075 mM). At 24 and 48 hrs after treatment, all cells including those floating in the medium were harvested, and total cell death was assessed via the trypan blue exclusion assay. Data shown represent the results of three experiments performed in triplicate.

Colony formation assay

MEFs were plated (at 500 cells/well) in 6-well plates and treated the next day with vehicle (0.1% DMSO), H_2O_2 (0.1 mM), hydroxyurea (400 μM/ml) or etoposide (10 μg/ml) for 6 hrs. Cells were then washed twice with PBS and maintained in complete medium under standard conditions. The medium was replaced every 4 days. 12-14 days later, cells were fixed with 3% paraformaldehyde and stained with 0.25% crystal violet in 50% methanol for 30 min. Colonies containing more than 50 cells were counted and relative survival levels were calculated as the ratio of the number of colonies on the treated plates to the number of colonies on the corresponding untreated plates.

JNK and IKK immunokinase assays

MEFs were treated with mTNFα (10 ng/ml) and protein samples were extracted using kinase lysis buffer (20 mM HEPES, pH 7.4, 350 mM NaCl, 0.5 % Triton X-100, 1mM DTT, 1mM EDTA, 20% glycerol and a cocktail of protease and phosphatase inhibitors). Endogenous JNK1 or IKK complexes were immunoprecipitated using anti-JNK1 or anti-IKKγ antibody, and then subjected to *in vitro* kinase assays in which GST-Jun$^{1–87}$ (for JNK) or GST-IκBα$^{1–55}$ (for IKK) served as substrate, as described previously (11).

Real-time RT-PCR

MEFs were treated with mTNFα (10 ng/ml), and total RNA was prepared using the RNeasy Mini Kit (Qiagen). Five μg of total RNA were treated with RQ1 RNase-free DNase for 30 min at 37 °C, and then reverse transcribed using an oligo dT-primer. The resulting cDNA was subjected to quantitative real-time PCR using the Power SYBR Green AB Master Mix and an ABI Prism 7700 Sequence Detector (Applied Biosystems). Mouse GAPDH-specific primers were used to generate an internal control, and the average threshold cycle (C_T) calculated for samples in triplicate was used in the subsequent calculations. Relative expression levels of NF-κB target genes were calculated as the ratio with respect to GAPDH levels. The mean ± S.E. of four independent experiments was considered statistically significant at $p < 0.05$. Real-time PCR products were also separated on an agarose gel to confirm the presence of single bands.

Supplementary Data

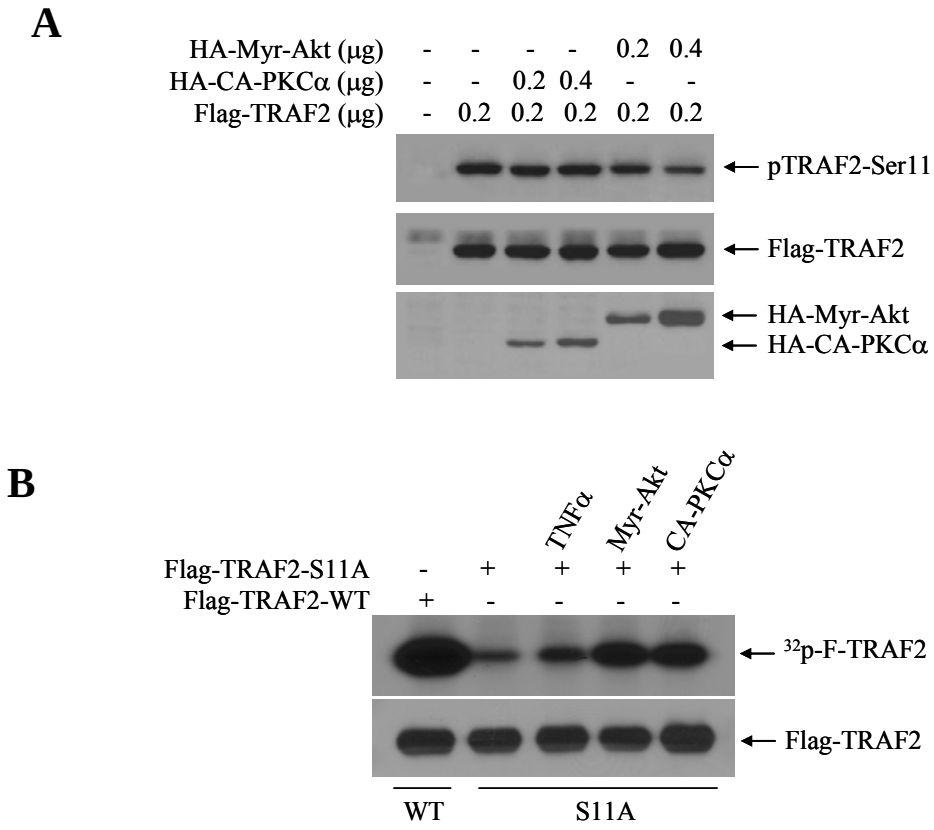


Figure S1. CA-PKCα induces TRAF2 phosphorylation at a site different from Ser-11

A, NIH 3T3 cells were co-transfected with Flag-TRAF2 and either HA-Myr-Akt1 or HA-CA-PKCα as indicated. 36 hrs after transfection, phosphorylation at TRAF2 Ser-11 was monitored by Western blotting using a phosphospecific antibody (pTRAF2-Ser11). The expression of Flag-TRAF2, HA-Myr-Akt1 and HA-CA-PKCα was detected by Western blotting using anti-FLAG or anti-HA antibody, as appropriate.

B, NIH 3T3 cells were co-transfected with either Flag-TRAF2-WT (1.0 μg) or -S11A (1.0 μg) and HA-Myr-Akt1 (0.5 μg) or HA-CA-PKCα (0.5 μg) as indicated. 36 hrs after transfection, cells were labeled with ^{32}P-orthophosphate and either mock treated or treated with TNFα for 15 min. ^{32}P-labeled Flag-TRAF2 was then immunoprecipitated, separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was exposed to X-ray film for 6 hrs (upper panel), and was then probed with anti-Flag antibody (lower panel).

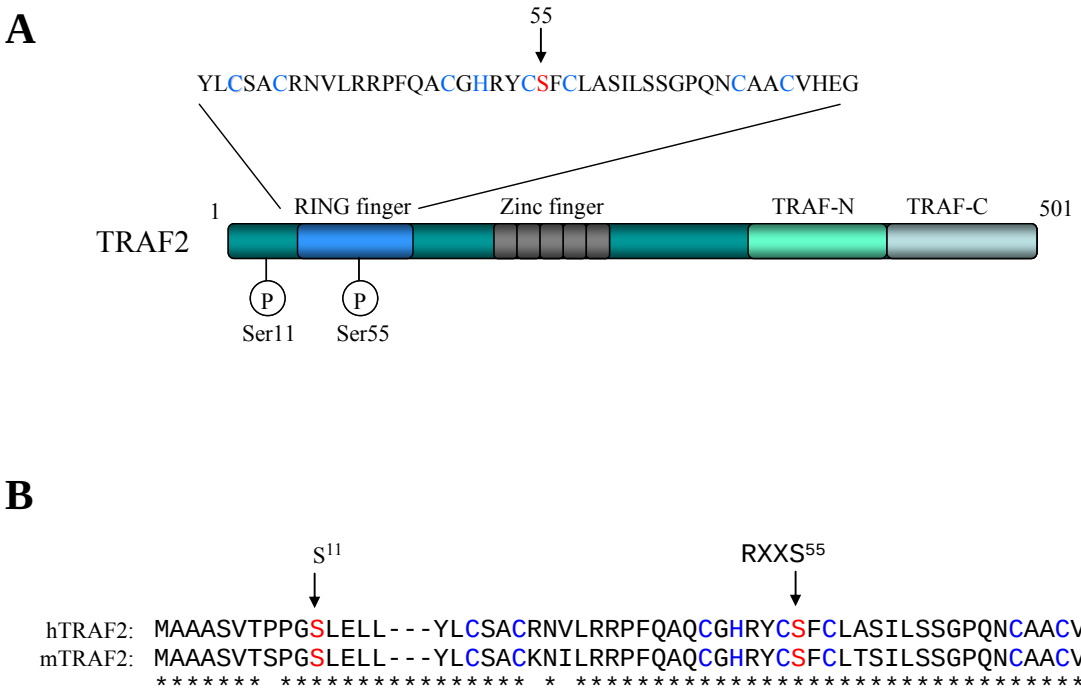
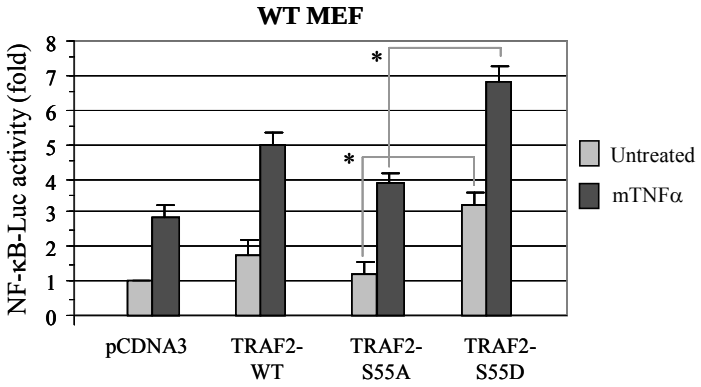


Figure S2. TRAF2 phosphorylation sites are conserved between human and mouse

A, A diagram showing schematically the TRAF2 domains, phosphorylation sites and amino acid sequence of the RING domain. TRAF2 has a C3HC4-type RING domain. The consensus C and H residues in the RING domain were shown in blue.

B, Amino acid sequence alignment of the human and mouse TRAF2 N-terminal region, with the conserved and phosphorylated serine residues shown in red.

A



B

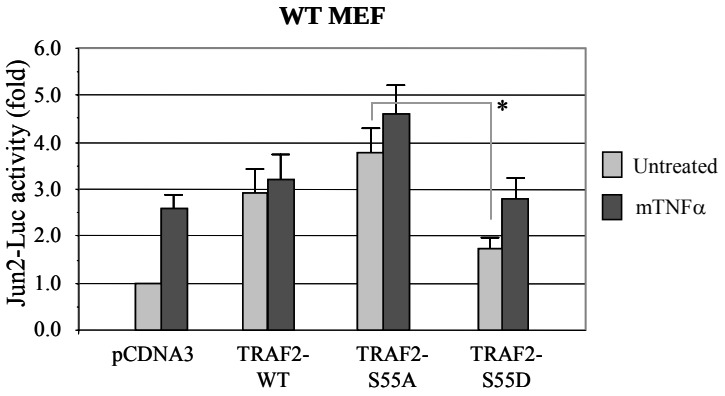


Figure S3. TRAF2 Ser-55 phosphorylation increases NF-κB activity but decreases c-Jun activity.

A, WT MEFs were co-transfected with NF-κB-Luc, pRL-TK and pCDNA3, TRAF2-WT, -S55A or -S55D as indicated. 36 hrs after transfection, the cells were either mock treated or treated with mTNFα (5ng/ml) for 4 hrs, after which the NF-κB-Luc activity was measured and normalized to pRL-TK activity. Data shown are the mean ± SD of three experiments that were done in triplicate. "*" indicates $p<0.05$.

B, WT MEFs were co-transfected with Jun2-Luc, pRL-TK and pCDNA3, TRAF2-WT, -S55A or -S55D as indicated. 36 hrs after transfection, the cells were either mock treated or treated with mTNFα (5ng/ml) for 4 hrs, after which the Jun2-Luc activity was measured and normalized to pRL-TK activity. Data shown are the mean ± SD of three experiments that were done in triplicate. "*" indicates $p<0.05$.

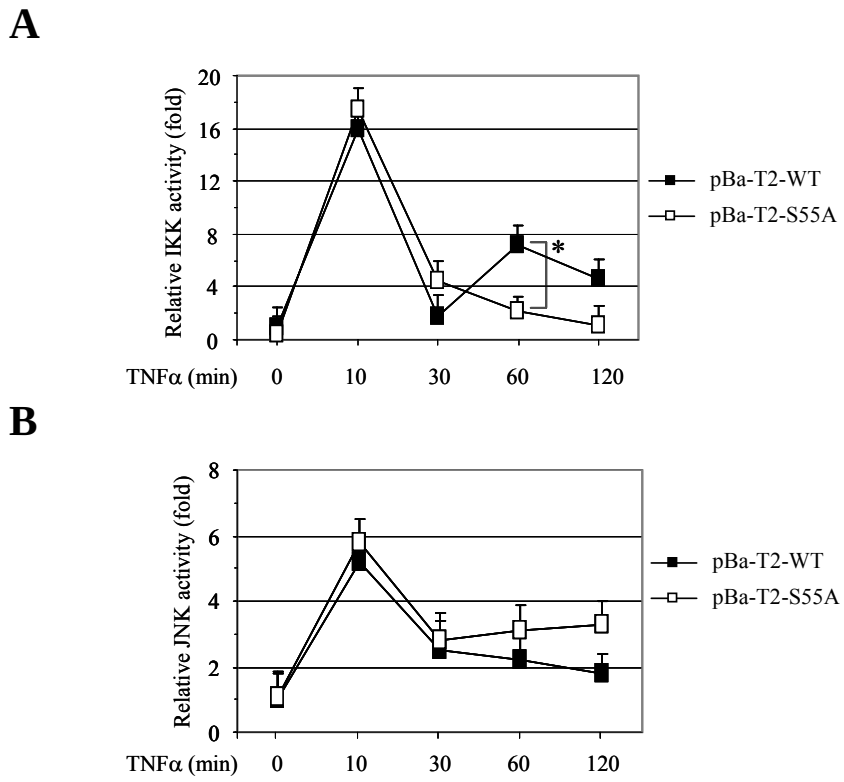


Figure S4. TRAF2 Ser-55 phosphorylation regulates prolonged phases of IKK and JNK activation in response to TNFα stimulation.

A, B, TRAF2/5 DKO cells reconstituted with TRAF2-WT (pBa-T2-WT) or -S55A (pBa-T2-S55A) were treated with mTNFα (10ng/ml) for the indicated times. The IKK complex or JNK1 was immunoprecipitated with anti-IKKγ or anti-JNK1 antibody, respectively, and subjected to *in vitro* kinase assays in which GST-IκBα$^{1-55}$ served as substrate for IKK and GST-jun$^{1-87}$ served as substrate for JNK1. Reaction mixtures were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and exposed to X-ray film for 6 hrs (^{32}p-G-IκBα or ^{32}p-G-jun). The average IKK and JNK activities from three independent kinase assays were then summarized. "*" indicates $p<0.05$.

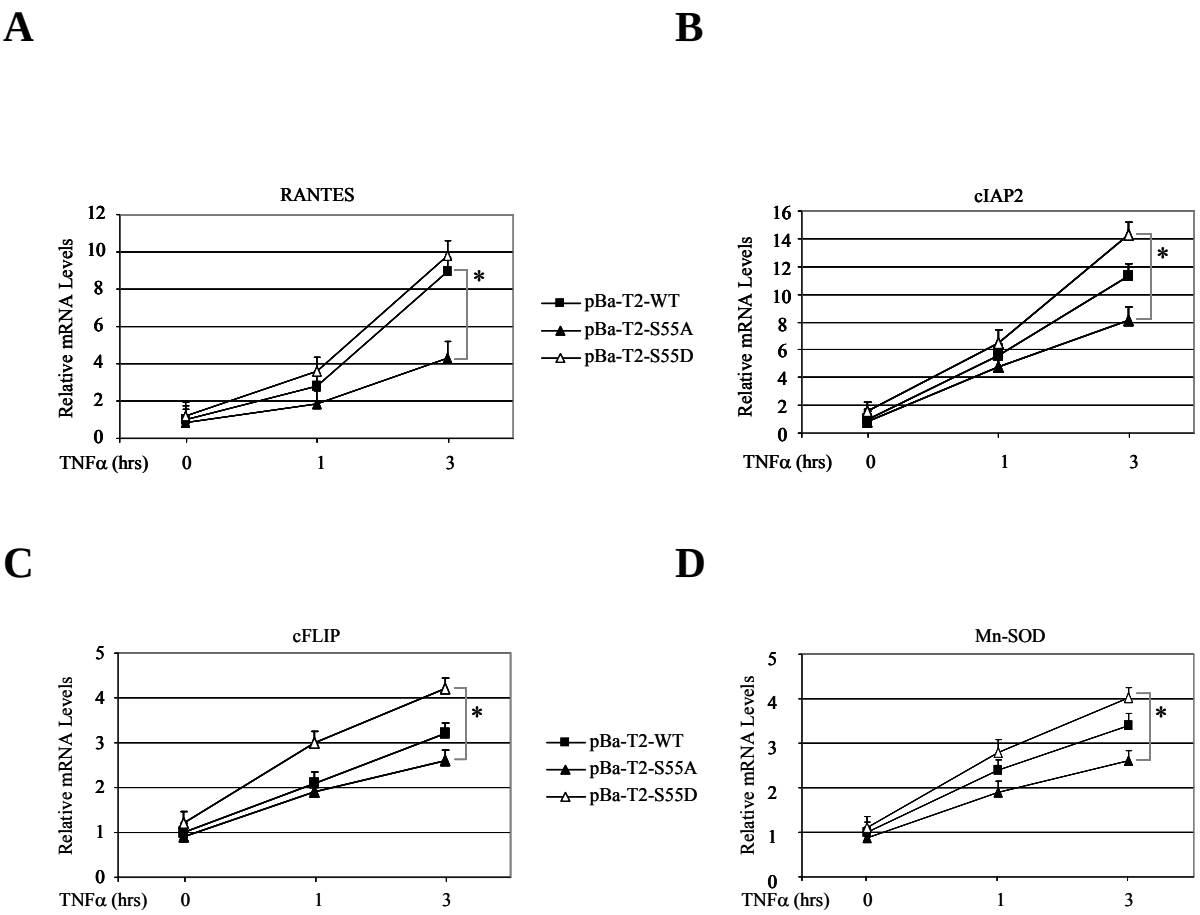


Figure S5. TRAF2 Ser-55 phosphorylation is essential for efficient TNFα-induced expression of a subset of NF-κB target genes.

A—D, TRAF2/5 DKO cells reconstituted with TRAF2-WT (pBa-T2-WT), -S55A (pBa-T2-S55A) or -S55D (pBa-T2-S55D) were treated with mTNFα (10ng/ml) as indicated, and the expression levels of RANTES, cIAP2, cFLIP and Mn-SOD were determined by real-time PCR. The relative expression level of each gene is presented as the ratio between it and the reference gene GAPDH, as an average from four independent experiments. "*" represents $p<0.05$.

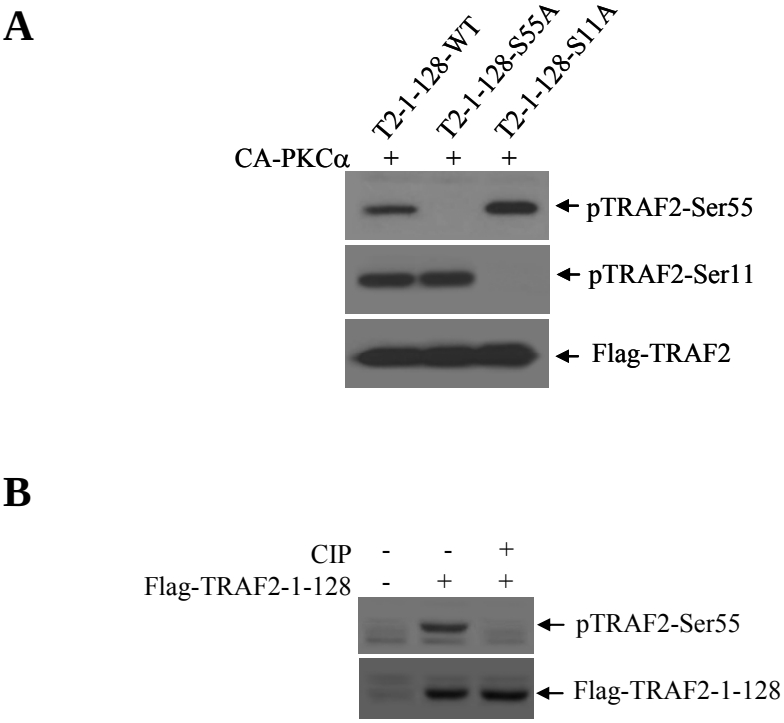


Figure S6. The anti-phospho-TRAF2 antibody pTRAF2-Ser55 specifically recognizes Ser-55-phosphorylated TRAF2.

A, NIH 3T3 cells were co-transfected with Flag-TRAF2-1-128-WT, -S11A or -S55A and CA-PKCα. At 36 hrs after transfection, TRAF2 phosphorylation was detected by Western blotting using a phosphospecific antibody directed against either TRAF2 Ser-11 (pTRAF2-Ser11) or Ser-55 (pTRAF2-Ser55). One of the membranes was then stripped and reprobed with an anti-Flag antibody.

B, NIH 3T3 cells were co-transfected with Flag-TRAF2-1-128-WT and CA-PKCα, and subjected to immunoprecipitation with an anti-Flag antibody. Immunopurified Flag-TRAF2-1-128-WT was then mock treated or treated with 5 units of calf intestinal alkaline phosphatase (CIP) for 30 min at 30°C, and subjected to Western blotting for an assessment of the specificity of TRAF2 Ser-55-specific phosphoantibody.

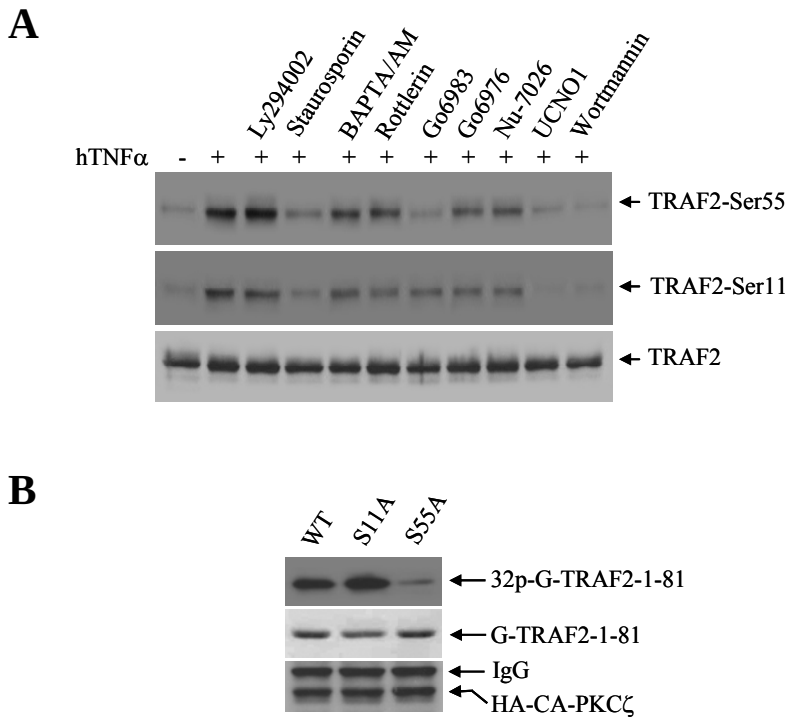


Figure S7. TNFα-induced TRAF2 Ser-55 phosphorylation is mediated by PKCζ.

A, HeLa cells were pretreated with inhibitors of PKC (staurosporine [200 nM] or UCN01 [10 μM]), PI3K/Akt (Ly294002 [40 μM] or Wortmannin [10 μM]), classical and atypical PKC (Go6983 [10 μM]), classical PKC (Go6976 [10 μM]), PKCδ (rottlerin [10 μM]), DNA-PK (Nu-7026 [10 μM]),) or a membrane-permeable Ca^{++} chelator (BAPTA/AM [10 μM]) for 60 min before being stimulated with hTNFα (20 ng/ml). TRAF2 Phosphorylation was then detected by Western blotting using TRAF2 phosphoantibodies.

B, GST-TRAF2-1-81-WT, -S11A and -S55A were subjected to an *in vitro* kinase assay in the presence of HA-CA-PKCζ purified from 293T cells. Reaction mixtures were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and exposed to X-ray film overnight (^{32}p-G-TRAF2-1-81). The same membrane was stained with Ponceau S (G-TRAF2-1-81) and then immunoblotted with anti-HA antibody (HA-CA-PKCζ).

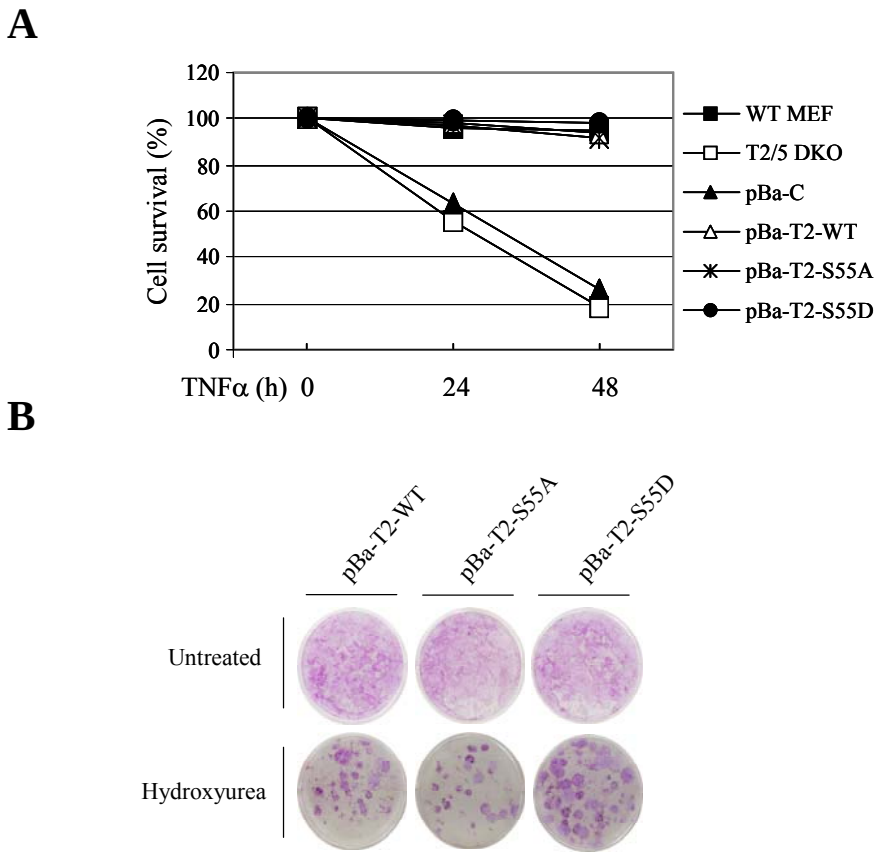


Figure S8. TRAF2 Ser-55 phosphorylation inhibits hydroxyurea-induced apoptosis

A, WT and TRAF2/5 DKO cells and TRAF2/5 DKO cells reconstituted with TRAF2-WT (pBa-T2-WT), -S55A (pBa-T2-S11A) or -S55D (pBa-T2-S11D) were treated with mTNFα (10ng/ml). At 24 or 48 hrs after treatment, total cell death was assessed via the trypan blue exclusion assay, and data shown represent the average of three experiments performed in triplicate. "*" indicates $p<0.05$.

B, TRAF2/5 DKO cells reconstituted with TRAF2-WT (pBa-T2-WT), -S55A (pBa-T2-S11A) or -S55D (pBa-T2-S11D) were plated in 6-well plates at a density of 500 cells/well. The next day, cells were left untreated or treated with 0.4 mM hydroxyurea for 6 hrs. 14 days later, colonies were visualized by crystal violet staining.

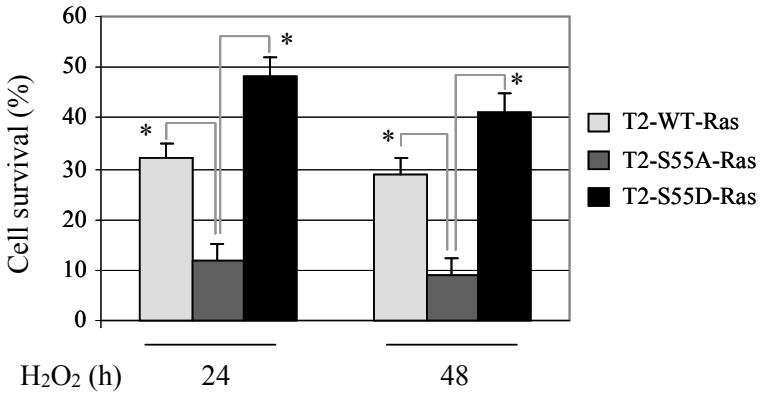


Figure S9. TRAF2 Ser-55 phosphorylation inhibits H_2O_2-induced ell death. Ha-Ras-V12-transformed pBa-T2-WT, -S55A and -S55D cells were treated with H_2O_2 (0.075mM) as indicated. At 24 or 48 hrs after treatment, total cell death was assessed via the trypan blue exclusion assay, and data shown represent the average of three experiments performed in triplicate. "*" indicates $p<0.05$.

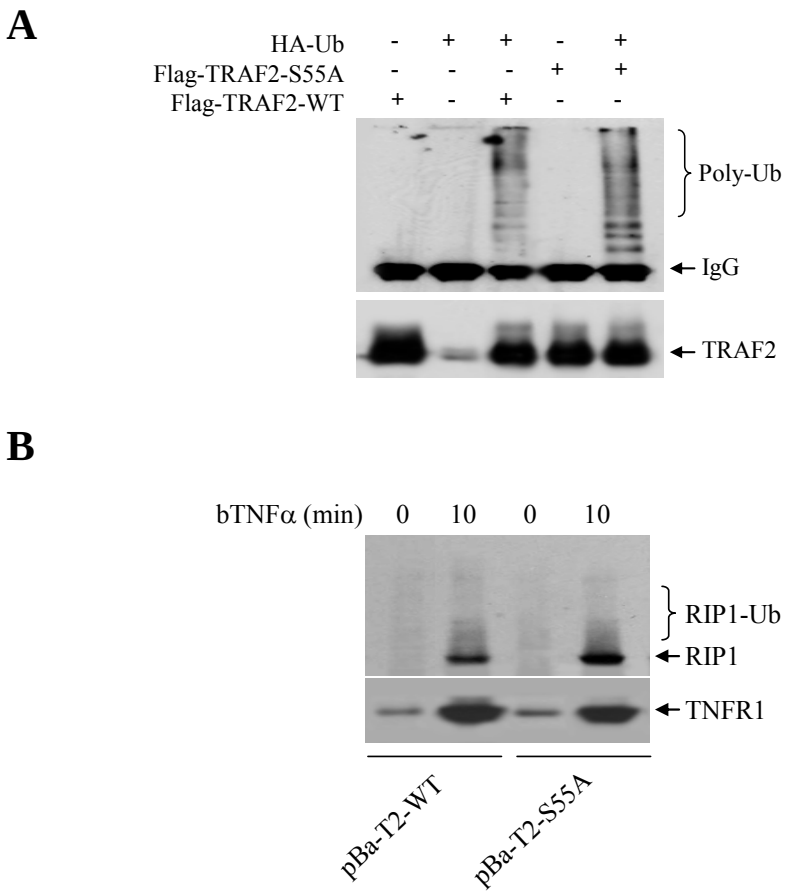


Figure S10. TRAF2 phosphorylation at Ser-55 has no effect on its self-ubiquitination or its ubiquitination of RIP1.

A, HeLa cells were co-transfected with Flag-TRAF2-WT (1.0 μg) or -S55A (1.0 μg) and HA-Ub (2.0 μg). At 36 hrs after transfection, proteins were extracted with 2% SDS/TBS, heat denatured at 95°C for 8 min and then diluted with 9 vol. 1% Triton X-100/TBS before being subjected to immunoprecipitation with anti-Flag Ab. Ubiquitination of TRAF2 was then detected by Western blotting with anti-HA Ab. The same membrane was stripped and reprobed with anti-TRAF2 polyclonal Ab (lower panel).

B, TRAF2/5 DKO MEFs reconstituted with TRAF2-WT (pBa-T2-WT) and -S55A (pBa-T2-S55A) were left untreated or treated with biotinylated TNFα (bTNFα; 100ng/ml) for 10 min, after which the TNFR1 complexes were pulled down with the aid of Dynabeads Streptavidin (Invitrogen). RIP1 ubiquitination was then monitored by Western blotting using anti-RIP1 antibody. The same membrane was stripped and reprobed with anti-TNFR1 antibody.

Table S1. Primers used for real-time RT-PCR

Gene	5' Primer	3' Primer	PCR amplicon size	Flanking intron?
GAPDH	CAGCAAGGACACTGAGCAAG	GGGTGCAGCGAACTTTATTG	157	Yes
IP-10	TCATCCTGCTGGGTCTGAGT	CCTATGGCCCTCATTCTCAC	106	Yes
RANTES	GTGCCCACGTCAAGGAGTAT	CCACTTCTTCTCTGGGTTGG	110	Yes
ICAM-1	TGGAGACGCAGAGGACCTTA	GTGGGCTTCACACTTCACAG	110	Yes
IκBα	AGACCTGGCCTTCCTCAACT	GCTTTCAGAAGTGCCTCAGC	102	Yes
MnSOD	TCAATGGTGGGGGACATATT	GCTTGATAGCCTCCAGCAAC	99	Yes
cFLIP	AGAGCAAGCCCCTAGGAATC	ATGATAGCCCAGGAAGTGA	92	Yes
cIAP1	TTTTGAATGCTGAAGATGAGAGA	CTGTTGAAAGAGGGCCATTC	91	Yes
cIAP2	TCCCCAGAAGATGAGAATGC	TCCAAGGCTTTAACCAC	93	Yes