Supplemental Data

SPHINGOSINE-1-PHOSPHATE: A MISSING COFACTOR FOR THE E3 UBIQUITIN LIGASE TRAF2

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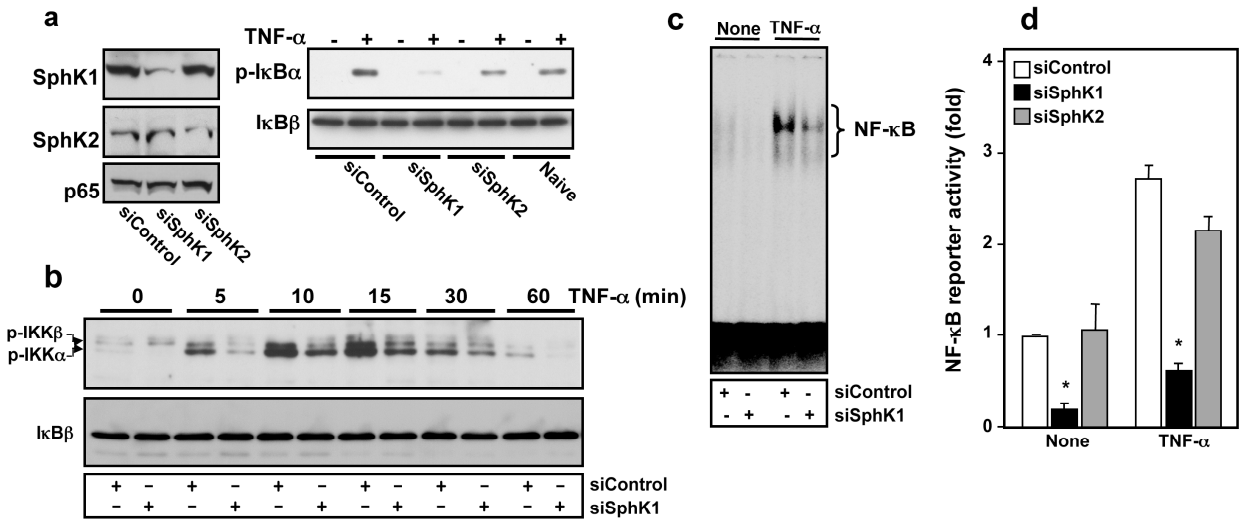
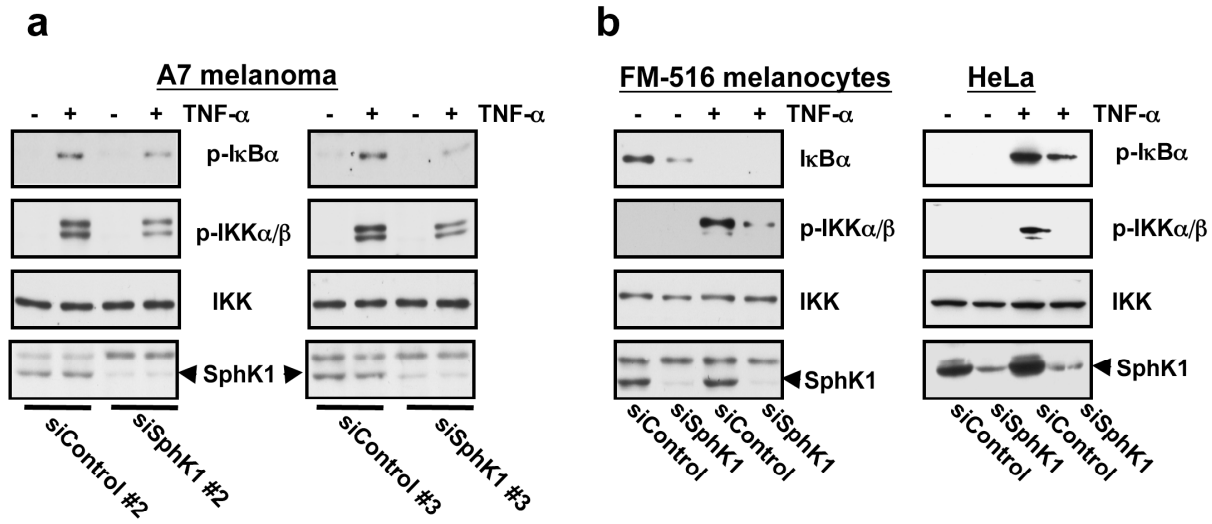
Supplementary Table 1. Cell sphingolipids and S1P bound to endogenous TRAF2.

Sphingolipid	Cell pmol/mg	TRAF2 immunoprecipitate pmol/mg
Sphingomyelin	4200 ± 250	ND
Monohexosylceramide	311 ± 18	ND
Ceramide	284 ± 16	ND
Sphingosine	28.6 ± 1.9	ND
Dihydrosphingosine	10.2 ± 0.6	ND
S1P	13.6 ± 1.8	0.07
Dihydro-S1P	5.4 ± 0.4	ND

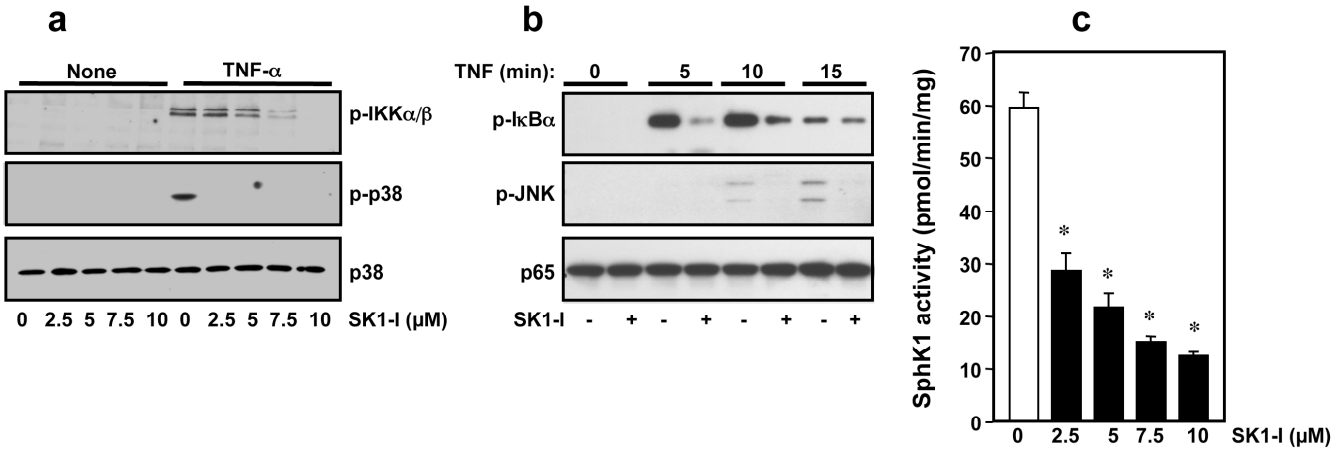
Lipids were extracted from HEK 293 cells and mass levels of sphingolipids were determined by LC-ESI-MS/MS. TRAF2 was immunoprecipitated with anti-TRAF2 antibody or control IgG, complexes captured with protein A/G agarose beads, washed extensively, lipids extracted, and sphingolipids analyzed by mass spectrometry. The data are averages of triplicate determinations and are expressed as pmol lipid per mg protein ± s.d. ND, not detected. Note that no sphingolipids were detected in control IgG immunoprecipitates (Fig. 4d,e).



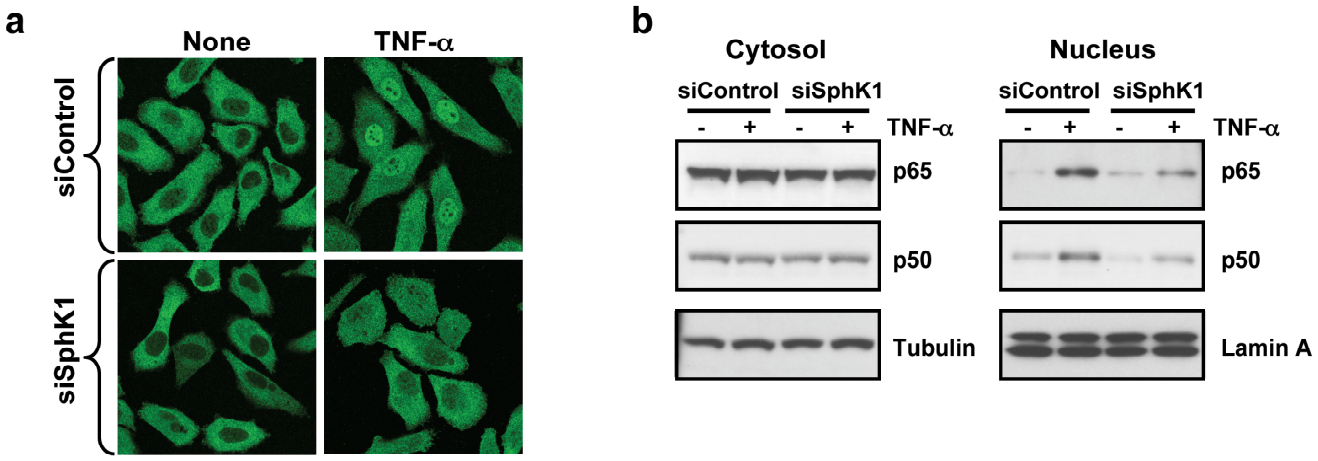
Supplementary Figure 1. SphK1 is required for TNF-α-stimulated phosphorylation of IKKα/β and IκBα, andNF-κB reporter and DNA binding activities. **a,** A7 cells transfected with siControl, siSphK1, or siSphK2, as indicated, were treated without or with TNF-α (10 ng/ml)Equal amounts of lysate proteins were analyzed by immunoblotting with the indicated antibodies. **b,c** A7 cells transfected with siControl or siSphK1 were stimulated without or with TNF-α for the indicated times (b) or for 30 min (c). Equal amounts of cell lysates were analyzed by immunoblotting with the indicated antibodies (b) and NF-κB DNA binding activity determined by EMSA (c). **d,** A7 cells were transfected with siControl, siSphK1, or siSphK2. 24 h later, cells were co-transfected with pNF-κB luciferase and β-galactosidase plasmids, and then stimulated without or with TNF-α (10 ng/ml) for 18 h. Luciferase was normalized to β-galactosidase activity measured with the Dual-Light reporter gene assay. Data are expressed as fold induction and are means ± s.e.m. from three independent experiments. * $P < 0.05$ compared to siControl.



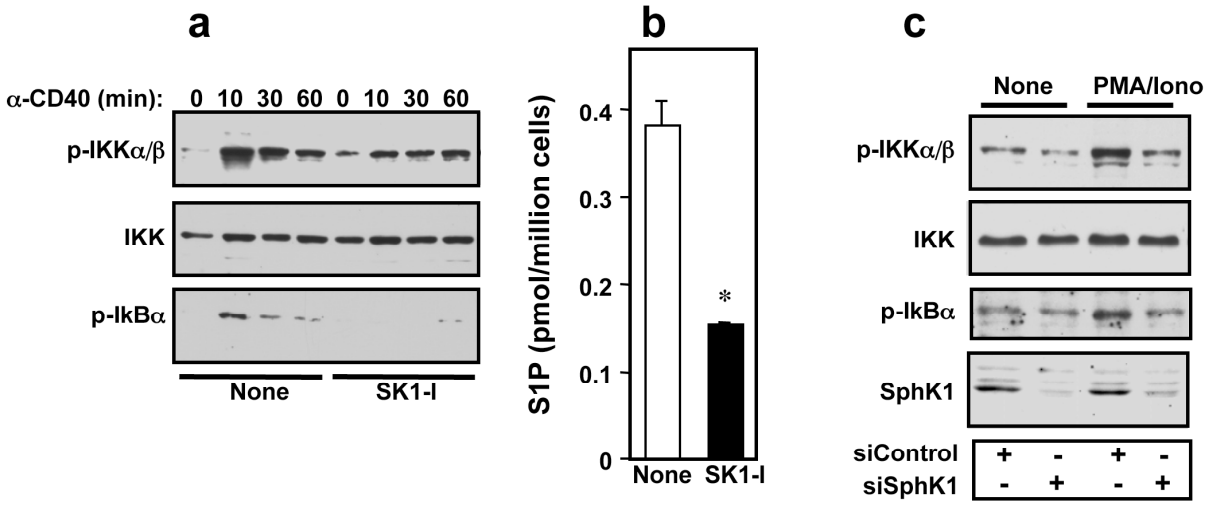
Supplementary Figure 2. A general role for SphK1 in the canonical NF-κB pathway triggered by TNF-α. **a**, A7 cells were transfected with siRNAs targeted to two additional SphK1 sequences (siSphK1 #2 and siSphK1 #3) or corresponding control siRNAs. **b**, FM-516 normal immortal melanocytes and HeLa cells were transfected with siControl or siSphK1 and stimulated without or with TNF-α (10 ng/ml) for 10 min. Equal amounts of cell lysates were analyzed by immunoblotting with the indicated antibodies. Immunoblotting with anti-IKK confirmed equal loading and transfer.



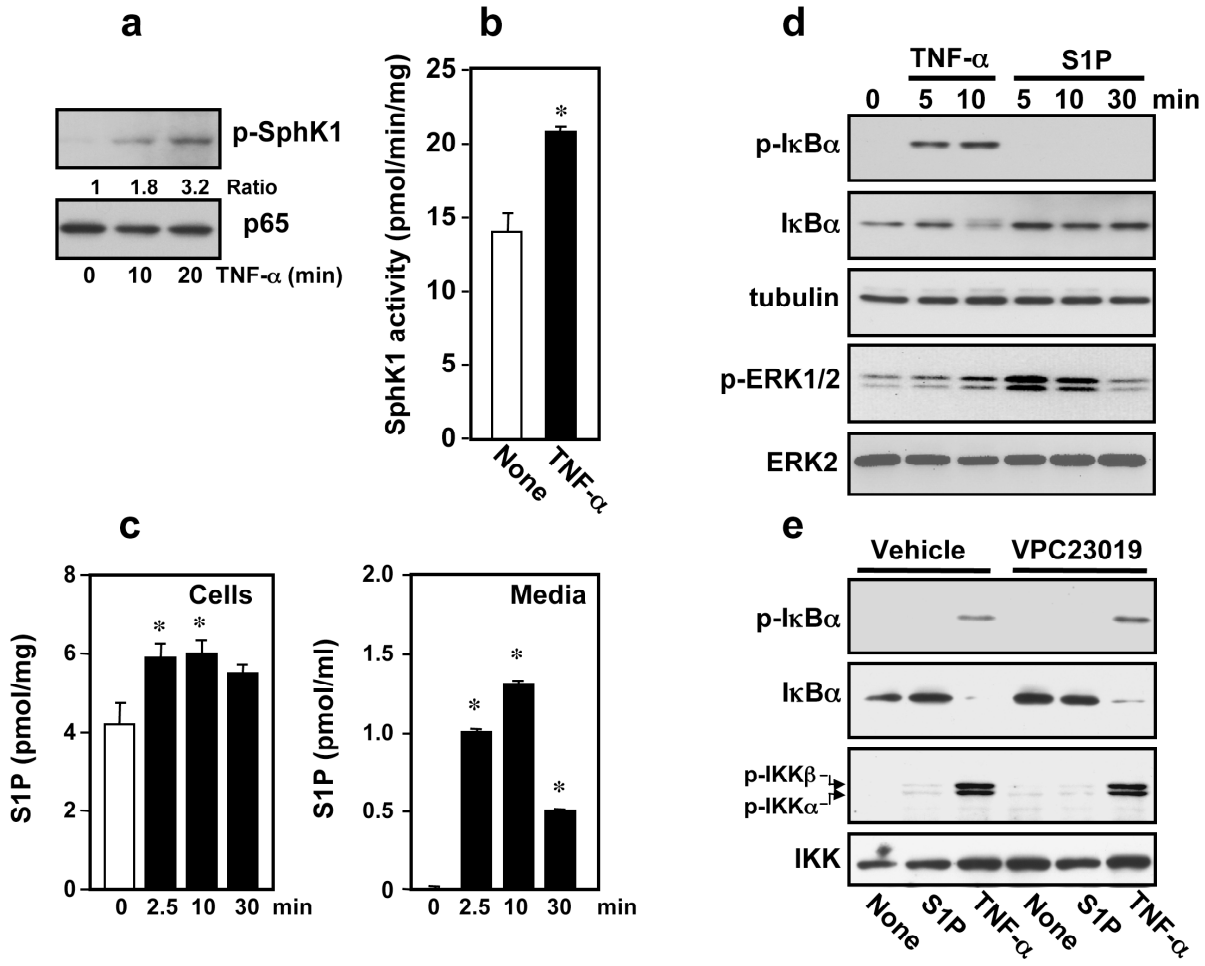
Supplementary Figure 3. Effect of a specific SphK1 inhibitor on TNF-α induced responses. a,b, A7 cells were pretreated with the indicated concentrations of SK1-I and stimulated with TNF-α (10 ng/ml) for 10 min (a) or pretreated without or with 10 μM SK1-I and then stimulated with TNF-α for the indicated times (b). Equal amounts of cell lysate proteins were analyzed by immunoblotting with specific antibodies. **c**, SphK1 activity in A7 cell lysates was measured in the presence of the indicated concentrations of SK1-I.



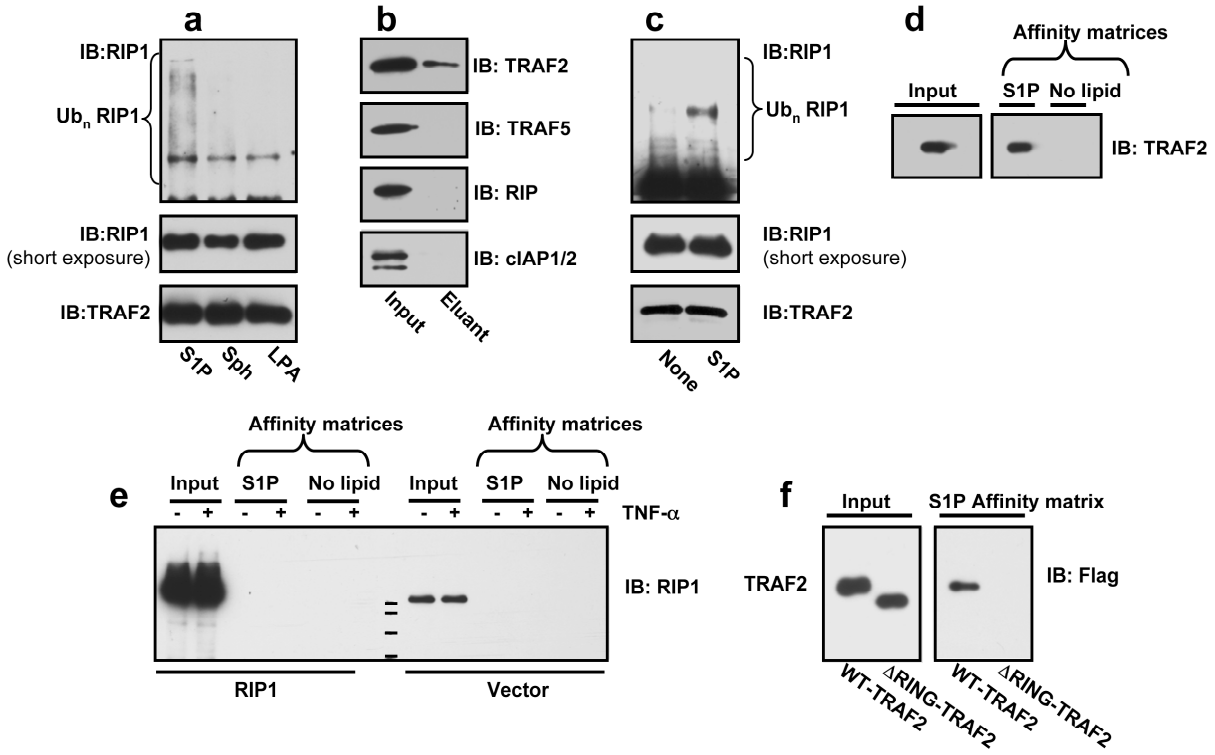
Supplementary Figure 4. SphK1 is critical for TNF-α-induced nuclear translocation of NF-κB. a, A7 cells grown on coverslips were treated without or with TNF-α for 30 min, fixed and stained with p65 antibody, and visualized by confocal microscopy. **b**, Cytosol and nuclear fractions were prepared from duplicate cultures and equal amounts of protein analyzed by immunoblotting with anti-p65 and anti-p50 antibodies. Antibodies against tubulin and lamin A/C were used as markers for cytosol and nuclear fractions, respectively, and to demonstrate equal loading and transfer.



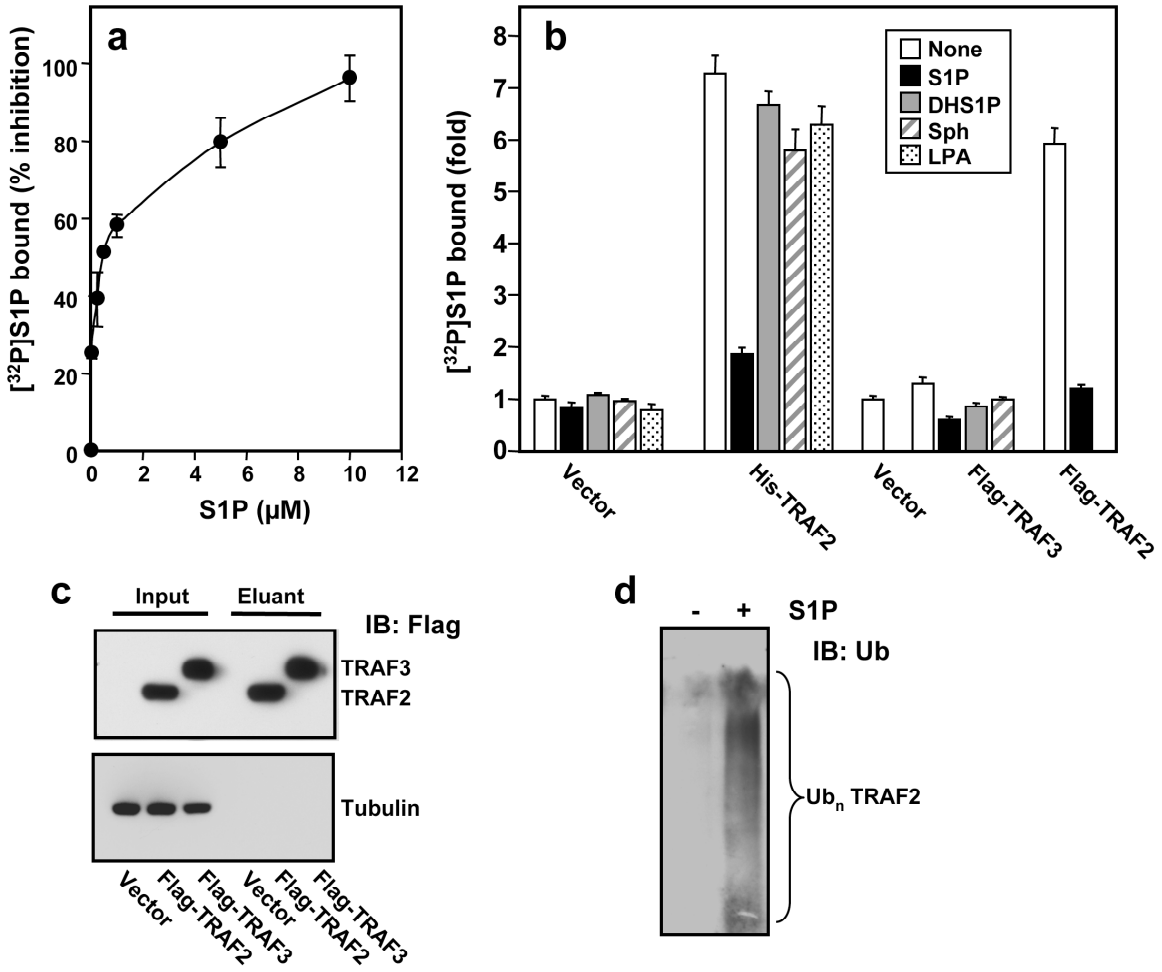
Supplementary Figure 5. Phosphorylation of IKKα/β and IκB stimulated by CD-40 and PMA/ionomycin is reduced by SphK1 inhibition or depletion. a,b, RPMI 8866 B-lymphoblastoid cells were pretreated without or with SK1-I (2.5 μM) for 30 min. Cells were stimulated with anti-CD40 antibody for the indicated times. (a) Equal amounts of cell lysate proteins were analyzed by immunoblotting with specific antibodies. (b) Levels of S1P in cells were measured by mass spectrometry. * $P < 0.01$. **c,** HEK 293 cells transfected with siControl or siSphK1 were treated without or with PMA (40 ng/ml) and ionomycin (1 μM) for 10 min and equal amounts of lysate proteins analyzed by immunoblotting with the indicated antibodies.



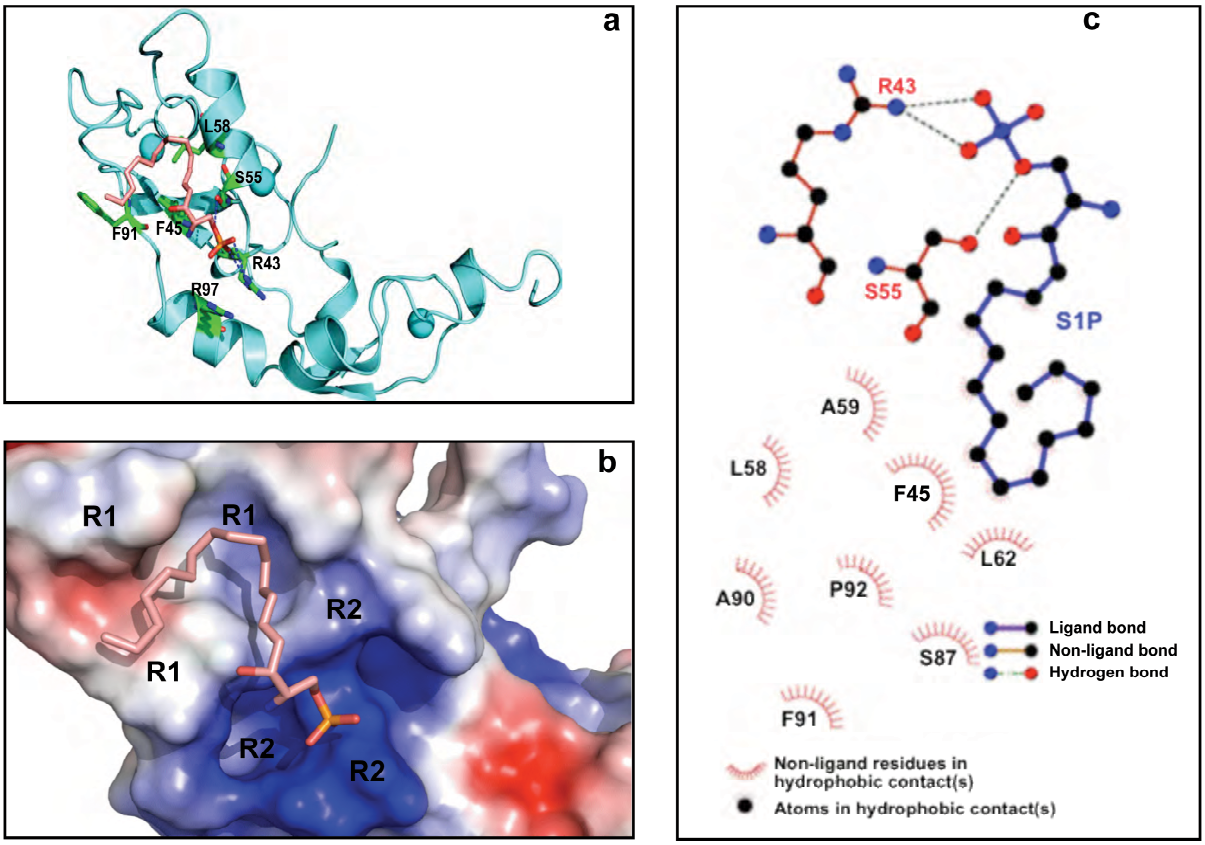
Supplementary Figure 6. TNF-α activates SphK1, yet inside-out signaling by S1P does not activate the canonical NF-κB pathway. a, A7 cells were treated with vehicle or TNF-α (10 ng/ml) for the indicated times and activation of SphK1 was determined by increased phosphorylation at Ser225. Equal amounts of cell lysates protein analyzed by immunoblotting with anti-phospho SphK1 (Ser225) antibody. The blot was stripped and re-probed with anti-p65 to demonstrate equal loading and transfer. Fold changes were determined by densitometry. **b**, HEK 293 cells were stimulated with TNF-α and SphK1 activity determined with an isoenzyme-specific enzymatic assay. **c,** HEK 293 cells (3.5 x 10^6) were stimulated with TNF-α for the indicated times and lipids were extracted from cells and media. S1P in cells or released into the media was determined by LC-ESI-MS/MS. * $p < 0.05$. **d,** A7 cells were stimulated without or with TNF-α (10 ng/ml) or S1P (100 nM) for the indicated times. **e,** A7 cells were pretreated with vehicle (DMSO) or VPC 23019 (10 μM) for 30 min and then stimulated without or with TNF-α (10 ng/ml) or S1P (100 nM) for 10 min. (d,e) Equal amounts of cell lysate proteins were immunoblotted with the indicated antibodies



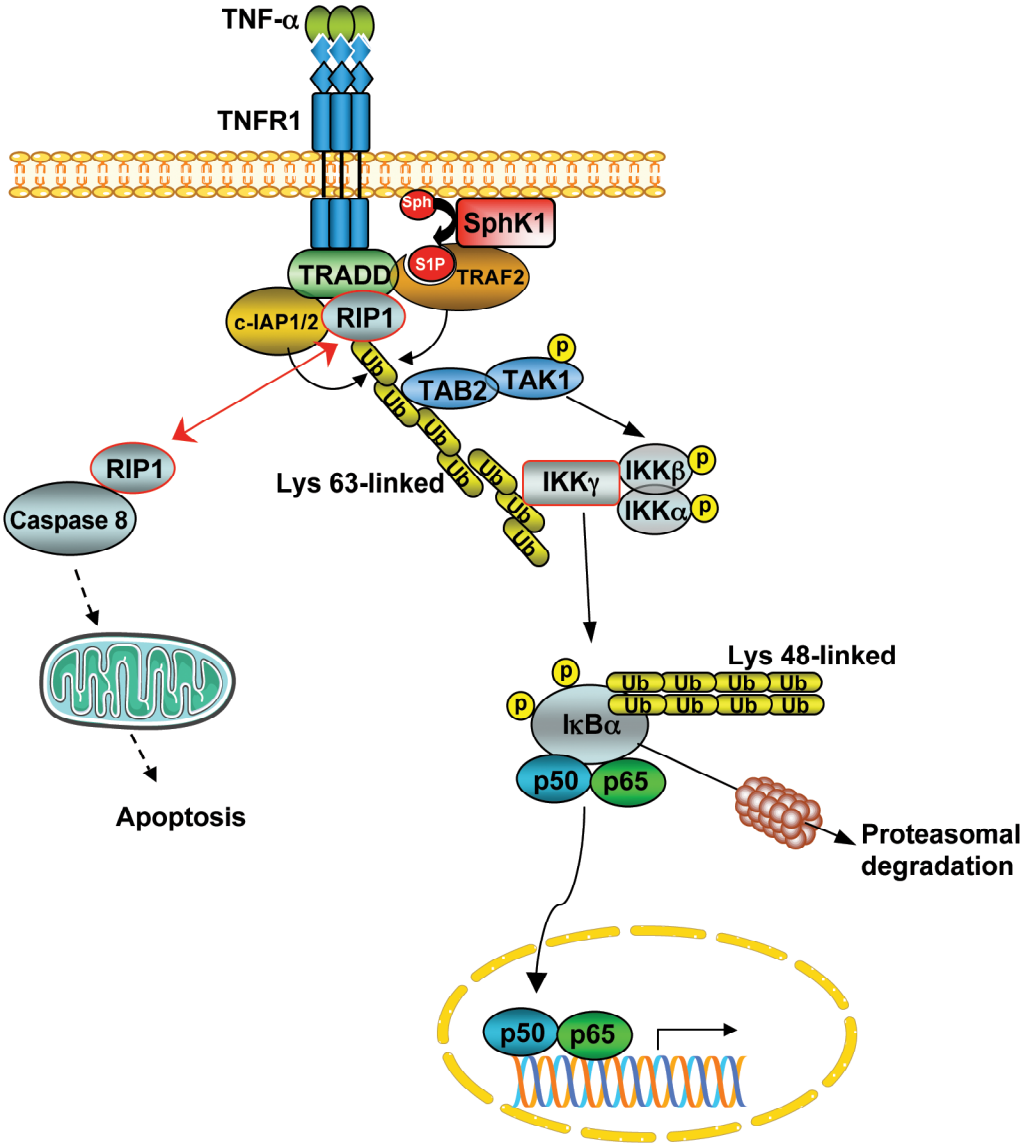
Supplementary Figure 7. S1P enhances the E3 ligase activity of purified TRAF2. a, *In vitro* ubiquitination of purified RIP1 was carried out with ATP, E1, Ubc13/Uev1a, ubiquitin, and Flag-TRAF2 immunopurified from HEK 293 cells, in the absence or presence of the indicated lipids (100 nM) and examined with anti-RIP1 antibody. **b,** Immunoblotting analysis of the immunopurified TRAF2 used for *in vitro* ubiquitination assays. **c.** Ubiquitination reactions were carried out with recombinant TRAF2 purified from Sf9 cells in the presence of ATP, E1, UbcH5a/Uev1a, and ubiquitin, without or with 100 nM S1P. RIP1 ubiquitination was examined with anti-RIP1 antibody and TRAF2 input with anti-TRAF2 antibody. **d,e,f Recombinant TRAF2 but not RIP1 or ΔRING-TRAF2 binds to S1P affinity beads. (**d**)** Recombinant TRAF2 purified from Sf9 cells was incubated with S1P beads, washed, and bound proteins analyzed by immunoblotting with anti-TRAF2 antibody. (e) HEK 293 cells transfected with vector or RIP1 were treated without or with TNF-α (10 ng/ml) for 10 min. Cell lysates were incubated with control (no lipid) or S1P affinity matrices, beads were washed and bound proteins analyzed by immunoblotting with anti-RIP1 antibody. (f) Cell lysates from HEK 293 cells transfected with Flag-TRAF2 or Flag-ΔRING-TRAF2 were incubated with S1P beads, washed, and bound proteins analyzed by immunoblotting with anti-Flag antibody. Inputs are shown in the left lanes.



Supplementary Figure S8. Specificity of binding of S1P to TRAFs. a, Immunocaptured Flag-TRAF2 was incubated with [^{32}P]S1P (0.1 nM) in the absence or presence of increasing concentrations of unlabeled S1P and [^{32}P]S1P bound to TRAF2 determined. Data are expressed as percent inhibition and are means ± s.d. **b,c,** Lysates from vector, Flag-TRAF2, and Flag-TRAF3 transfected HEK 293 cells and His-TRAF2 purified from Sf9 cells were incubated with anti-Flag agarose beads or with Ni-NTA-agarose beads. (b) Beads were washed and incubated with [^{32}P]S1P (0.1 nM) in the absence or presence of 1 μM unlabeled S1P, dihydro-S1P, sphingosine or LPA, then washed extensively and [^{32}P]S1P bound to TRAFs was eluted with Flag peptide or imidazole and radioactivity determined by scintillation counting. (c) Blots of inputs and eluted TRAFs were analyzed by immunoblotting with anti-Flag or anti-His antibodies, respectively. **d,** Autoubiquitination reactions were carried out with recombinant His-TRAF2 purified from Sf9 cells in the presence of ATP, UbcH13/Uev1a, and ubiquitin without or with S1P (100 nM) and ubiquitination was analyzed by immunoblotting with anti-ubiquitin antibody.



Supplementary Figure 9. Docking of S1P into the pocket of TRAF2. **a,** Docking of S1P (red) into the pocket of the RING domain of TRAF2, shown in cyan ribbon diagram, with Autodock 4.0. **b,** Surface contour of the binding site with S1P. R1 indicates the hydrophobic region; R2 indicates the positively charged region. The TRAF2 surface was colored by electrostatic potential. Figures were generated by Pymol. Estimated Ki values by AutoDock for S1P and dihydro-S1P are 8.74×10^{-7} and 5.37×10^{-4}, respectively (T=298.15 K). **c,** Schematic representation of the interaction of S1P with TRAF2 calculated by Ligplot. Thatched semi-circles indicate van der Waals contacts between hydrophobic protein residues and S1P. Hydrogen bonds are shown as green dashed lines. Note that the Arg43 and Ser55 residues in TRAF2 may stabilize the phosphate group of S1P.



Supplementary Figure 10. A model of the role of SphK1 and S1P in TRAF2 actions and regulation of NF-κB activation and the anti-apoptotic program initiated by TNF-α. Binding of TNF-α to the trimeric TNFR1 results in recruitment of TRADD, which recruits TRAF2, RIP1 and cIAP1/2. As SphK1 physically interacts with TRAF2, which enhances its activity, recruitment of TRAF2 to the TNFR1-associated complex also brings SphK1 to the plasma membrane where its substrate sphingosine resides, leading to formation of S1P. S1P in turn enables TRAF2 E3 ubiquitin ligase activity, suggesting a feed-forward type of interdependent regulation in which TRAF2 and SphK1 require and/or regulate the actions of the other. TRAF2-mediated RIP1 Lys 63-linked polyubiquitination acts as a scaffold to recruit and activate TAK1 and IKK complexes. IKK then phosphorylates IκBα leading to activation of the canonical NF-κB signaling pathway. Polyubiquitination of RIP1 also prevents its interaction with procaspase 8, limiting its processing to the effector form that initiates apoptosis.