Online Methods

Reagents and antibodies.

S1P, SK1-I ((2R,3S,4E)-N-methyl-5-(4'-pentylphenyl)-2-aminopent-4-ene-1,3-diol), and mouse monoclonal polyubiquitin (K63-linkage specific) antibody were obtained from Enzo Life Sciences International. Other lipids and VPC23019 were from Avanti Polar Lipids. Wild type and Lys mutant ubiquitins were from Boston Biochem. Antibodies against the following were used for immunoblotting: p65, IκBα, IκBβ, IKKα and IKKβ, ubiquitin, TRAF3, TRAF5, and ERK2 (Santa Cruz Biotechnology); phospho-p65 (Ser536), phospho-IκBα (Ser32), phospho-p44/42 ERK1/2 (Thr202/Tyr204), phospho-IKKα/β (Ser176/180), phospho-JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), His-tag, tubulin, and TRAF2 (Cell Signaling); RIP1 (BD Transduction Laboratories). Rabbit polyclonal SphK1 and SphK2 antibodies were described previously [26] and rabbit polyclonal SphK1 (Ser225) phospho-specific antibody was from ECM Biosciences. Mouse anti-human CD40 antibody was kindly provided by Dr. D. Conrad (VCU). Expression plasmids for wild type TRAF2 and Flag-ΔRING-TRAF2 with 87 amino acids deleted from the N-terminal including the RING domain, and Flag-TRAF3 were kindly provided by Dr. B. Darnay (MD Anderson) and Dr. M. Karin (UCSD), respectively.

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Cell culture and transfections.

A7 melanoma cells, FM-516 normal immortal melanocytes, HeLa, HEK 293, and RPMI-8866 cells were cultured as described [27]. MEFs were isolated from E14 wild type or *sphk1*$^{-/-}$ embryos [28]. Cells were serum-starved overnight and then stimulated with TNF-α (10 ng/ml) for 10 min unless indicated otherwise. Cells were transfected with Lipofectamine Plus or GeneJuice for plasmids or Oligofectamine for siRNA. SphK1 and SphK2 were downregulated with sequence specific siRNA from Qiagen as previously described [26]. In some experiments, to confirm lack of off-target effects, cells were transfected with individual ON-TARGETplus SMARTpool siRNAs targeted to other SphK1 sequences (5'-GAAUCUCCUUCACGCUGA-3' and 5'-GGAAAGGUGUGUUUUGCAGU-3') and control siRNA (Dharmacon).

SphK1 activity.

SphK1 activity was determined exactly as described [27].

RIP1 immunoprecipitation.

Cell extracts were prepared in Hepes 20 mM pH 7.4 containing 150 mM NaCl, 10 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 10 mM NaF, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EGTA, 1 mM PMSF, 0.5% Triton X-100, 1:500 protease inhibitor cocktail (Sigma-Aldrich), and 1 mg/ml of N-ethylmaleimide. RIP1 was immunoprecipitated from 200 μg of cell lysate with 1 μg anti-RIP antibody overnight at 4°C. Immunoprecipitates were captured with protein A/G-plus agarose beads (Santa Cruz). After extensive washing, bound proteins were released by boiling in SDS-PAGE sample buffer and polyubiquitination of RIP1 determined by western blotting.

Electrophoretic mobility shift assays (EMSA).

EMSA were performed with 5 μg of nuclear protein and [α-^{32}P]dCTP-end-labelled double-strand oligonucleotides containing an NF-κB consensus binding site (10 fmol,10,000 cpm) exactly as described [29].

NF-κB reporter assays.

Cells were transiently transfected with NF-κB-luciferase and RSV-β-galactosidase or Renilla reporter plasmids using GeneJuice (EMD Biosciences). 48 hours later, cells were stimulated with TNF-α for 18 hours and combined measurements of luciferase and β-galactosidase activities determined with the Dual-Light chemiluminescent reporter gene assay (Applied Biosystems).

Reporter activity was expressed as relative luciferase units normalized to β-galactosidase or Renilla activity**.**

Immunofluorescence.

Cells grown on cover slips were fixed with 3% paraformaldehyde for 20 min, washed extensively, quenched by addition of 10 mM glycine in PBS, and permeabilized with 0.5% Triton X-100 for 3 min. After incubation with primary and appropriate fluorescent secondary antibodies (Molecular Probes), coverslips were mounted on slides with 10 mM n-propylgallate in 100% glycerol and visualized with a Zeiss LSM 510 laser confocal microscope. ImageJ version 1.6.0_12 image analysis software was used for computerized quantification of p65 nuclear fluorescence staining as described [30].

***In vitro* ubiquitination and protein purification.**

Ubiquitination assays were performed as described [22], with some modifications. Flag-tagged TRAF2 was purified from HEK 293 overexpressing cells with anti-Flag M2 affinity beads and eluted with Flag peptide (Sigma). His-tagged TRAF2 purified from Sf9 cells (Signal Chem) was used as the E3 ligase in some experiments. RIP1 was immunocaptured from A7 cells. Ubiquitination assays were carried out at 35°C for 2 hours in 50 mM Hepes, pH 7.8, containing 5 mM $MgCl_2$, 4 mM ATP, 50 nM E1, 10 μg ubiquitin (wild type, Lys 48 only or Lys 63 only), 150 nM UbcH5/Uev1a or UbcH13/Uev1a E2 complexes (Boston Biochem), and purified Flag-TRAF2 and RIP1 bound to agarose beads, in the absence or presence of various lipids. Reactions were stopped by boiling in SDS sample buffer, proteins resolved by SDS-PAGE, and immunoblotted**.** *In vitro* autoubiquitination reactions were carried out with purified recombinant His-TRAF2 captured on beads in the presence of ATP, ubiquitin, and UbcH13/Uev1a as described above.

Pulldowns with lipid affinity matrices.

Control, S1P-, sphingosine-, and LPA-coated agarose beads (Echelon Biosciences) equilibrated with binding buffer containing 10 mM Hepes (pH 7.8), 150 mM NaCl, 1:500 protease inhibitor cocktail, and 0.5% Igepal were mixed with cell extracts diluted in binding buffer and rocked for 2 hours at 4°C. Beads were washed 4 times with binding buffer and collected by centrifugation at 1000 rpm. After removing the supernatant, the beads were boiled in SDS-PAGE sample buffer and bound proteins analyzed by immunoblotting.

Quantitation of S1P by mass spectrometry.

Cell extracts (500 µg) were immunoprecipitated with anti-TRAF2 or anti-Flag antibodies, or control IgG. Lipids were extracted, and sphingolipids quantified by liquid chromatography, electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS, 4000 QTRAP, ABI) as described [26].

[^{32}P]S1P binding assays.

Cells overexpressing Flag-TRAFs or empty vector were lysed by freeze-thawing in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 1:500 protease inhibitor cocktail. Lysates (400 µg) were incubated with 150 µl anti-Flag antibody-conjugated agarose beads (Sigma-Aldrich) overnight at 4°C with agitation. The beads were then washed extensively and incubated without or with unlabeled lipids in the presence of [^{32}P]S1P (0.1 nM, 6.8 µCi/pmol) in 150 µl buffer containing 50 mM Tris (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 15 mM NaF, 0.5 mM NaV_3O_4 for 60 min at 4°C. Bound TRAFs were eluted with 40 µl Flag peptide (250 µg/ml). [^{32}P]S1P bound to the eluted proteins was quantified with a LS6500 scintillation counter (Beckman). S1P binding to His-TRAFs was measured as described [26].

Molecular docking, MD simulation and free energy calculation.

The molecular docking program AutoDock 4.0 was used for the automated molecular docking simulations [25]. Briefly, the PDBQT file was created and the AutoGrid algorithm and the Kollman all-atom charges were assigned for TRAF2 and Gasteiger-Marsili charges were assigned for the ligands. Complexes were selected according to the criteria of interacting energy combined with geometrical matching quality. These complexes were subjected to molecular dynamic simulations by AMBER9.0 software package solvated using a box of TIP3P water molecules extending at least 10 Å away from the boundary of any protein atoms. The MM_PBSA and Nmode module of AMBER program were used for the free energy calculation of the TRAF2-S1P complex.

Statistical analysis.

All experiments were repeated at least three times with consistent results. Data are means ± s.d. Statistical significance was assessed by two-tailed unpaired student's *t*-test. $P < 0.05$ was considered significant.