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

Sergio E. Alvarez^{1,*,3}, Kuzhuvelil B. Harikumar^{1,*}, Nitai C. Hait¹, Jeremy Allegood¹, Graham M. Strub¹, Eugene Kim¹, Michael Maceyka¹, Hualiang Jiang², Cheng Luo², Tomasz Kordula¹, Sheldon Milstien¹, and Sarah Spiegel¹

¹Department of Biochemistry and Molecular Biology and the Massey Cancer Center, Virginia Commonwealth University School of Medicine, 1101 E. Marshall Street, Richmond, VA 23298, USA

²State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, P. R. China

Abstract

TNF receptor-associated factor 2 (TRAF2) is a key component in NF- κ B signaling triggered by TNF- α 1,2. Genetic evidence indicates that TRAF2 is necessary for polyubiquitination of receptor interacting protein 1 (RIP1) 3 that then serves as a platform for recruitment and stimulation of I κ B kinase (IKK) leading to activation of the transcription factor NF- κ B. Although TRAF2 is a RING domain ubiquitin ligase, direct evidence that TRAF2 catalyzes the ubiquitination of RIP1 is lacking. TRAF2 binds to sphingosine kinase 1 (SphK1) 4, one of the isoenzymes that generates the pro-survival lipid mediator sphingosine-1-phosphate (S1P) inside cells. Here we show that SphK1 and production of S1P is necessary for Lys 63-linked polyubiquitination of RIP1, phosphorylation of IKK and I κ B α , and I κ B α degradation, leading to NF- κ B activation. Surprisingly, these responses were mediated by intracellular S1P independently of its cell surface G protein-coupled receptors. S1P specifically binds to TRAF2 at the N-terminal RING domain and stimulates its E3 ligase activity. S1P, but not dihydro-S1P, dramatically increased recombinant TRAF2-catalyzed Lys 63- but not Lys 48-linked polyubiquitination of RIP1 *in vitro* in the presence of the ubiquitin conjugating enzymes (E2) UbcH13 or UbcH5a. Our data reveal that TRAF2 is a novel intracellular target of S1P, and that S1P is the missing co-factor for TRAF2 E3 ubiquitin ligase activity, suggesting a new paradigm for regulation of Lys 63-linked polyubiquitination. These results also highlight the key role of SphK1 and its product S1P in TNF-

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[†]Address correspondence to Sarah Spiegel, Dept. of Biochemistry and Molecular Biology, VCU School of Medicine, P.O. Box 980614, 1101 E. Marshall St., Richmond, VA 23298, Tel: (804) 828-9330, FAX: (804) 828-8999, sspiegel@vcu.edu.

^{*}These authors contributed equally to this work

³Present address: IMIBIO-SL CONICET, Ejército de los Andes 950, San Luis 5700, Argentina

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Author Contributions S.E.A. and K.B.H. planned and performed most experiments, with assistance from N.C.H., G.M.S., E.Y.K., J.A., and M.M. C.L. and H.J. performed molecular docking, T.K. contributed to the planning of the experiments, S.M. and S.S. conceived the study, contributed to planning of the experiments and wrote the manuscript.

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α signaling and the canonical NF- κ B activation pathway important in inflammatory, anti-apoptotic, and immune processes.

Engagement of the TNF receptor results in assembly of multi-component receptor-associated signaling complexes by adaptors including TNFR1-associated death domain (TRADD), the RING domain ubiquitin ligases, such as TRAF2, and RIP1, which activate the I κ B kinase (IKK) complex, composed of two highly homologous kinase subunits, IKK α /IKK1 and IKK β /IKK2, and a regulatory subunit NEMO/IKK γ . Phosphorylation of I κ B α by the IKK complex leads to its Lys 48-linked polyubiquitination and subsequent proteasomal degradation liberating the NF- κ B dimer, a transcription factor consisting of p65 and p50 subunits, which enters the nucleus and regulates transcription of target genes 1,2,5. It has previously been demonstrated that the interaction of SphK1 with TRAF2 and subsequent activation of SphK1 links TNF- α signals to activation of NF- κ B 4, yet the mechanism of the involvement of SphK1 in the canonical NF- κ B pathway has not been elucidated. To this end, expression of SphK1 was downregulated with small interfering RNA (siRNA), which reduced its levels by more than 70% without affecting SphK2 (Supplementary Fig. 1a). Depletion of SphK1 significantly decreased TNF- α -stimulated phosphorylation of IKK α , IKK β , $\alpha\delta$ I κ B α (Fig. 1a), and NF- κ B DNA binding and reporter activities (Supplementary Fig. 1b,c,d). In contrast, depletion of SphK2 had no significant effects (Supplementary Fig. 1a,d). To exclude off-target effects, SphK1 expression was also downregulated with siRNAs targeted to two other regions of the SphK1 sequence and both inhibited TNF- α -induced phosphorylation of I κ B α and IKK α / β (Supplementary Fig. 2a). Similar results were obtained in several other cell types (Supplementary Fig. 2b), suggesting that SphK1 has a general role in the canonical NF- κ B pathway.

To conclusively demonstrate the involvement of SphK1, we also used pharmacological and genetic approaches. The specific SphK1 inhibitor, SK1-I, which decreases intracellular S1P levels 6, reduced TNF- α -induced I κ B α degradation and phosphorylation of IKK α / β (Fig. 1b) and in a dose-dependent manner that correlated with inhibition of SphK1 activity (Supplementary Fig. 3a,c), yet did not have a significant effect on S1P-induced ERK1/2 phosphorylation (Fig. 1b), as expected for S1P triggered GPCR signaling pathways 7. Activation of c-Jun N-terminal kinase (JNK) by TNF- α was also diminished by depletion of SphK1 (Fig. 1a) or its inhibition (Supplementary Fig. 3b). Likewise, SK1-I reduced activation of p38 (Supplementary Fig. 3a). Finally, upon TNF- α treatment, phosphorylation of I κ B α and IKK α / β was absent in *sphk1*^{-/-} mouse embryonic fibroblasts (MEFs) compared with that in *sphk1*^{+/+} MEFs (Fig. 1c). Immunofluorescence and western blotting revealed that SphK1 depletion diminished TNF- α -induced translocation of p65 and p50 from the cytosol to the nucleus (Supplementary Fig. 4a,b). Similarly, *sphk1*^{-/-} MEFs were also impaired in TNF- α -induced translocation of p65 to the nucleus and transfection of SphK1 rescued this defect, whereas catalytically inactive SphK1 did not (Fig. 1d). Thus, SphK1 is required for TNF- α -induced activation of IKK α / β , I κ B α phosphorylation, and nuclear translocation of NF- κ B. Moreover, CD40-induced phosphorylation of IKK and I κ B α in a B lymphoid cell line was attenuated by SK1-I, which reduced intracellular levels of S1P (Supplementary Fig. 5a,b). Likewise, downregulation of SphK1 also reduced phosphorylation of IKK and I κ B α by phorbol 12-myristate 13-acetate (PMA) and

ionomycin stimulation (Supplementary Fig. 5c), supporting the general importance of SphK1 in NF- κ B activation.

Activation of SphK1 typically results in spatially restricted formation and secretion of S1P that acts in an autocrine or paracrine manner to activate its GPCRs on the cell surface 7. As S1P₁ and S1P₃ receptors may activate NF- κ B through G protein signaling 8,9, it was important to determine whether “inside-out signaling” by S1P could be responsible for SphK1-dependent activation of NF- κ B. In agreement with previous studies 4,10,11, TNF- α activated SphK1 (Supplementary Fig. 6a,b), increased intracellular mass levels of S1P and enhanced its transport out of cells (Supplementary Fig. 6c). Surprisingly however, exogenously added 100 nM S1P or dihydro-S1P, which only lacks the double bond in S1P and binds to and activates all of the S1P receptors 7, did not have any detectable effects on phosphorylation of I κ B α or its degradation, nor did they stimulate IKK phosphorylation in A7, HEK 293, or HeLa cells (Fig. 1b,f,g, Supplementary Fig. 6d,e). Yet, this concentration of S1P and dihydro-S1P, which is much greater than the K_d values for the S1P receptors, activated ERK1/2 in these cells (Fig. 1b,f,g, Supplementary Fig. 6d), indicating that the lack of effect on the NF- κ B pathway is not due to an inability of S1P to signal through its cell surface receptors. Moreover, the S1P₁/S1P₃ antagonist VPC23019 did not affect TNF- α -induced phosphorylation of IKK and I κ B α or its degradation in these cells (Supplementary Fig. 6e). Furthermore, in contrast to TNF- α , neither S1P nor dihydro-S1P stimulated NF- κ B reporter activity (Fig. 1e). To corroborate the notion that the actions of SphK1 were due to intracellular generated S1P, we took advantage of our previous observation that only high concentrations of exogenously added S1P significantly increase its intracellular levels 12. While S1P at 100 nM had no significant effects (Fig. 1e,f), treatment with 10 μ M S1P, which increased the total intracellular pools of S1P by 7-fold (Fig. 1h), enhanced phosphorylation of IKK α / β and I κ B α (Fig. 1f,g) and NF- κ B reporter activity (Fig. 1i). This high concentration of S1P also enhanced the response to a suboptimal dose of TNF- α (Fig. 1f,i). Importantly, however, a high concentration of dihydro-S1P, which has the same rate of uptake as S1P 13 and increases its intracellular level to a similar or even greater extent (Fig. 1h), did not mimic the effects of S1P on activation of the NF- κ B pathway (Fig. 1g). Thus, intracellular S1P generated by the activation of SphK1 specifically regulates TNF- α -induced NF- κ B in a S1P receptor-independent manner.

Unlike Lys 48-linked polyubiquitination, which targets proteins for proteasomal degradation, abundant evidence has demonstrated that Lys 63-linked or regulatory ubiquitination plays a critical role in signaling activation 5. One of the best characterized examples is the non-degradative Lys 63 polyubiquitination of RIP1 that serves as a scaffold to recruit proteins containing specific ubiquitin binding domains, resulting in recruitment and phosphorylation of the IKK complex leading to the activation of NF- κ B 5,14,15. Figure 2a shows that the high molecular weight species of RIP1 rapidly formed in response to TNF- α were greatly diminished by depletion of SphK1. Analysis of immunoprecipitated RIP1 by immunoblotting with an ubiquitin-specific antibody verified that knockdown of SphK1 reduced polyubiquitination of RIP1 (Fig. 2b). Furthermore, siSphK1 markedly reduced TNF- α -induced conjugation of ectopically expressed ubiquitin with RIP1 (Fig. 2c). An antibody that specifically recognizes Lys 63-linked ubiquitin chains 16 revealed that endogenous Lys 63-linked polyubiquitination of RIP1 was stimulated by TNF- α , consistent

with a previous study 17, which was greatly attenuated by SphK1 depletion (Fig. 2d). As was previously reported 18–20, following TNF- α stimulation, TRAF2 itself was polyubiquitinated and this was dramatically reduced in cells depleted of SphK1 (Fig. 2e). Taken together, these results suggest that SphK1 and intracellularly generated S1P are important for Lys 63-linked polyubiquitination of RIP1 leading to recruitment and activation of the IKK complex.

Although it has been shown that RIP1 ubiquitination is dependent on TRAF2 expression 3, evidence that TRAF2 directly catalyzes the ubiquitination of RIP1 is still lacking. Because intracellularly generated S1P was required for optimal TRAF2 and RIP1 polyubiquitination following stimulation by TNF- α , and SphK1 interacts with TRAF2 4, we examined whether S1P is required for direct RIP1 ubiquitination by TRAF2 *in vitro*. In agreement with previous studies 20,21, incubation of purified RIP1 with recombinant TRAF2, ubiquitin, the ubiquitin-activating enzyme E1, and Ubc13/Uev1a, an E2 that facilitates Lys 63 polyubiquitination, failed to produce ubiquitinated RIP1. Remarkably however, addition of S1P induced efficient TRAF2-mediated ubiquitination of RIP1 (Fig. 3a). In sharp contrast, dihydro-S1P did not mimic the effect of S1P (Fig. 3a). Neither sphingosine nor LPA, lipids structurally related to S1P, enhanced *in vitro* ubiquitination of RIP1 by TRAF2 (Supplementary Fig. S7a). Furthermore, TRAF2 with a deletion of the N-terminal 87 amino acids containing the RING domain (RING-TRAF2), which cripples its E3 ligase activity, failed to ubiquitinate RIP1 in the absence or presence of S1P (Fig. 3b,c), underscoring the importance of the E3 ligase activity of TRAF2. To determine if the ubiquitin conjugated to RIP1 *in vitro* was Lys 63 linked, we examined TRAF2-mediated polyubiquitination of RIP1 with wild type ubiquitin and its mutants containing only one lysine at either position 48 (Lys 48) or 63 (Lys 63). S1P enhanced incorporation of wild type and Lys 63 only ubiquitin into RIP1, whereas there was little or no incorporation of the Lys 48 only mutant in the presence of S1P (Fig. 3b,c). Even with the promiscuous E2 enzyme Ubc5a, S1P was still capable of stimulating TRAF2-mediated polyubiquitination of RIP1 with wild type and Lys 63 only ubiquitin and not with Lys 48 only ubiquitin. This effect was also dependent on the presence of the RING domain of TRAF2 (Fig. 3c).

Although TRAF2 can act as an adaptor for cIAP1 and cIAP2 that can themselves serve as E3 ligases for RIP1 21–23, neither cIAPs nor TRAF5 were associated with purified TRAF2 used for *in vitro* ubiquitination assays (Supplementary Fig. 7b). Moreover, S1P also enhanced the E3 ligase activity of recombinant TRAF2 purified from Sf9 insect cells (Supplementary Fig. 7c), further demonstrating that TRAF2 itself is the target of S1P.

Because our results show that S1P is required for the *in vitro* E3 ligase activity of TRAF2, it was important to confirm that TRAF2 is a direct target of intracellularly produced S1P. To this end, we first examined binding of TRAF2 to S1P immobilized on agarose beads. Both ectopically expressed Flag-TRAF2 (Fig. 4a) and endogenous TRAF2 (Fig. 4b) were pulled down by matrices carrying S1P but not by control, sphingosine, or LPA matrices. Pre-incubation with exogenous S1P abolished binding of endogenous TRAF2 to the S1P affinity beads (Fig. 4b). Moreover, recombinant TRAF2 purified from Sf9 insect cells also bound to S1P beads, whereas RIP1 and RING-TRAF2 did not (Supplementary Fig. 7d,e,f).

Next, direct binding of S1P to TRAF2 was evaluated. ^{32}P -labeled S1P specifically bound to Flag-tagged TRAF2 that was eluted from anti-Flag agarose beads with Flag peptide. Of note, binding to TRAF2 was abolished by addition of excess cold S1P but not by dihydro-S1P, LPA, or sphingosine (Fig. 4c), in agreement with the specific requirement for S1P to activate the E3 ligase activity of TRAF2 (Fig. 3a). Binding of ^{32}P -S1P to TRAF2 was reduced by 50% at a concentration of 0.5 μM unlabeled S1P (Supplementary Fig. 8a). Finally, we sought to examine whether endogenous S1P is bound to TRAF2 *in vivo* and whether this interaction is influenced by TNF- α , which activates SphK1. Cells were treated with TNF- α and the sphingolipids in TRAF2 immunoprecipitates were measured by mass spectrometry (LC-ESI-MS/MS). Of all the sphingolipids present in cells, only S1P was bound to TRAF2 (Fig. 4d, Supplementary Table 1), and this association was significantly increased by TNF- α (Fig. 4d). Moreover, as expected from a specific interaction, much more S1P was bound to ectopically expressed TRAF2 and this association was also further enhanced by treatment with TNF- α . However, there was no detectable S1P associated with RING-TRAF2 as measured by LC-ESI-MS/MS (Fig. 4e,f), which also did not bind to S1P affinity beads (Supplementary Fig. 7f), suggesting that the binding site for S1P is within the RING domain of TRAF2.

Molecular modeling of S1P into the RING domain of the crystal structure of TRAF2 24 revealed that it docked remarkably well in a 16 Å long binding cavity consisting of a hydrophobic region (F45, L58, A59, L62, A90, F91 and F92) and positively charged region (R43 and R97), which may stabilize the phosphate group of S1P (Fig. 4g, Supplementary Fig. 9). Further molecular dynamic simulation and free energy calculation indicates the binding for S1P is -8.07 kcal/mol while dihydro-S1P shows instability in binding with TRAF2 during the dynamic simulation process, consistent with the inability of dihydro-S1P to bind or activate TRAF2. Indeed, estimated K_i values by AutoDock 25 for S1P and dihydro-S1P are 8.74×10^{-7} and 5.37×10^{-4} , respectively ($T=298.15$ K). In contrast, no binding was detected to TRAF3 (Supplementary Fig. 8b,c). In agreement, sequence alignment and homology modeling did not identify an obvious binding pocket near residue Lys97 of TRAF3 (corresponding to Ser87 in TRAF2). S1P also stimulated *in vitro* autoubiquitination of recombinant TRAF2 (Supplementary Fig. 8d).

Hence, S1P is a missing cofactor for TRAF2, an E3 ligase that plays an important role in Lys 63-linked polyubiquitination of RIP1 and consequent regulation of NF- κB activation and the anti-apoptotic program initiated by TNF- α 1,2,5. Increased RIP1 Lys 63 ubiquitination prevents switching of RIP1 from a prosurvival to proapoptotic adaptor protein (Supplementary Fig. 10). These findings also resolve the puzzle associated with the cytoprotective effects of SphK1 and S1P 7. Although S1P and dihydro-S1P are equally potent ligands for the five S1P receptors, only S1P suppresses apoptosis as only S1P and not dihydro-S1P binds to and activates TRAF2. Our study also provides a mechanistic explanation for the numerous observations of the importance of SphK1 in inflammatory, anti-apoptotic, and immune processes.

METHODS SUMMARY

For details of expression plasmids, proteins, antibodies, cell lines, and methods for immunoprecipitations, immunofluorescence, pulldowns, S1P binding, and mass spectrometry, see the online-only Methods. *In vitro* ubiquitination assays were performed as described previously 22, with some modifications, as described in Online Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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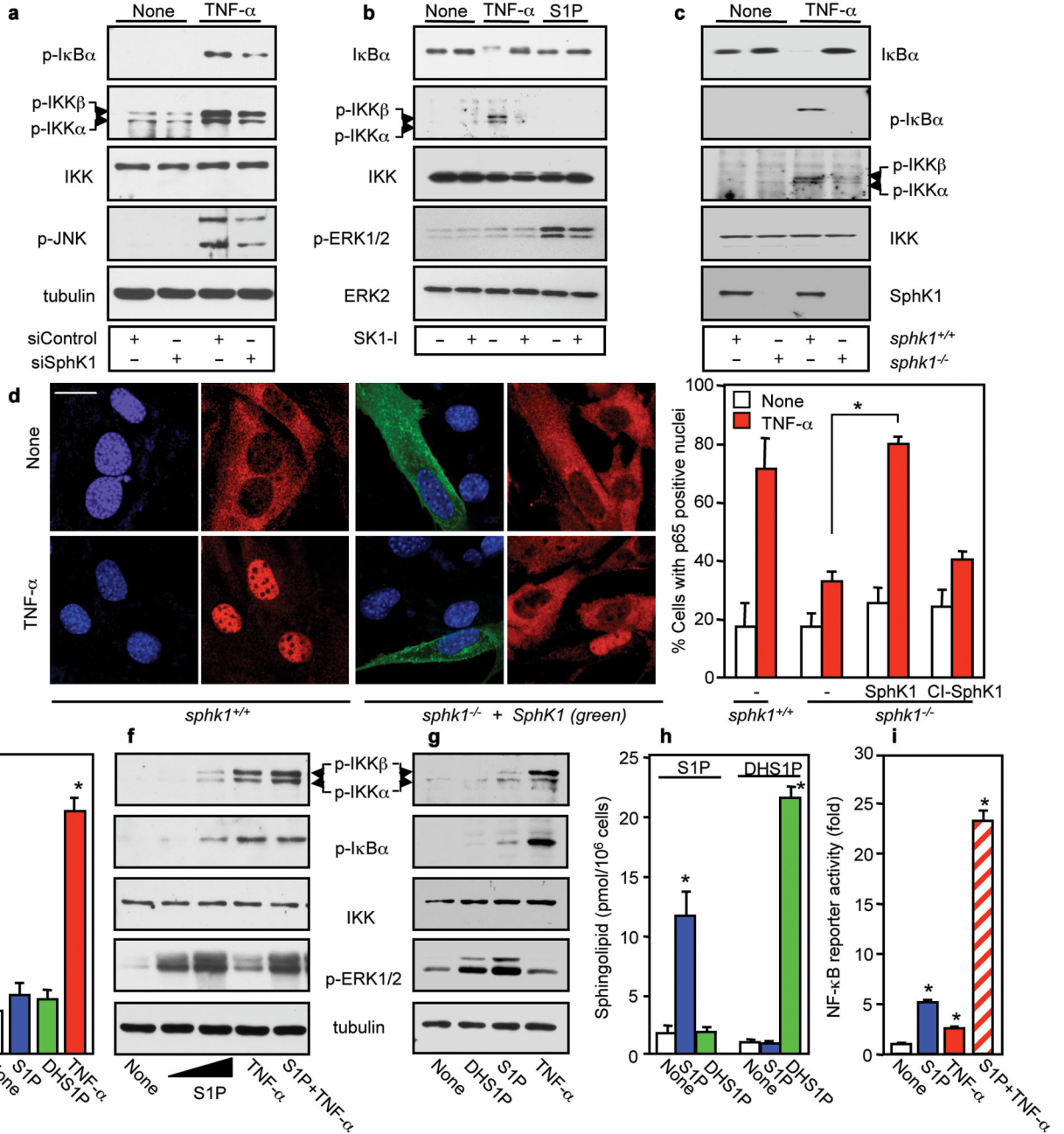


Figure 1. SphK1 and intracellular S1P are necessary for NF-κB activation by TNF-α independently of S1P receptors

a. HEK 293 cells transfected with siControl or siSphK1 were treated with TNF-α and analyzed by immunoblotting. **b.** A7 cells were pretreated with SK1-I (10 μM) and stimulated with TNF-α or S1P (100 nM). **c.** *sphk1*^{+/+} and *sphk1*^{-/-} MEFs were stimulated with TNF-α. **d.** *sphk1*^{+/+} or *sphk1*^{-/-} MEFs transfected with V5-SphK1 or catalytically-inactive SphK1^{G82D} (CI-SphK1) were treated with TNF-α, stained with Hoechst (blue), and p65 (red) and V5 (green) antibodies, and visualized by confocal microscopy. Bar, 20 μm.

Percentages of cells with p65 positive nuclei are shown. * $P < 0.01$. **e**, NF- κ B reporter activity was determined in A7 cells stimulated with TNF- α , 100 nM S1P or dihydro-S1P (DHS1P). **f**, A7 cells were stimulated with TNF- α (1 ng/ml) or S1P (100 nM or 10 μ M) for 10 min. **g**, HeLa cells were stimulated with TNF- α (1 ng/ml), 10 μ M S1P or DHS1P. **h**, Sphingolipids were analyzed by LC-ESI-MS/MS. **i**, NF- κ B reporter activity was determined in HeLa cells stimulated with TNF- α (1 ng/ml), S1P (10 μ M), or both. * $P < 0.01$ compared to None. n=3.

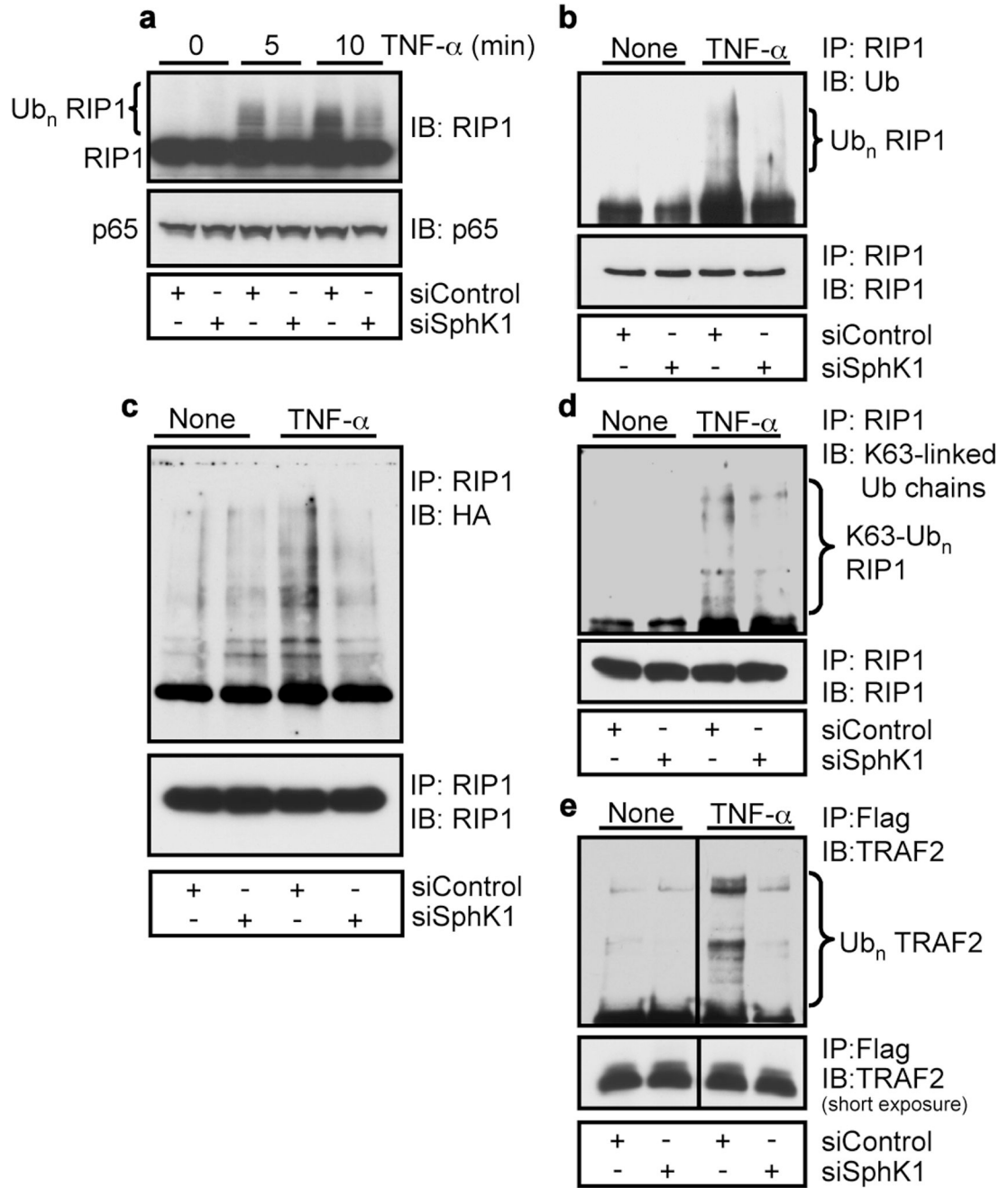


Figure 2. SphK1 is required for TNF- α -induced Lys 63-linked polyubiquitination of RIP1
a, A7 cells transfected with siControl or siSphK1 were stimulated with TNF- α (10 ng/ml). Proteins were immunoblotted with anti-RIP1 or anti-p65 antibodies. **b**, Lysates were immunoprecipitated with anti-RIP1 antibody and analyzed with anti-ubiquitin antibody. **c**, Lysates from cells transfected with HA-Ub and stimulated with TNF- α were immunoprecipitated with anti-RIP1 antibody and analyzed with HA antibody. **d**, Lysates were immunoprecipitated with anti-RIP1 antibody and proteins analyzed with Lys 63-specific polyubiquitin antibody. **e**, HEK 293 cells transfected with siControl or siSphK1

were transfected with Flag-TRAF2 and stimulated with TNF- α . Proteins were pulled down with anti-Flag beads and analyzed with anti-TRAF2 antibody.

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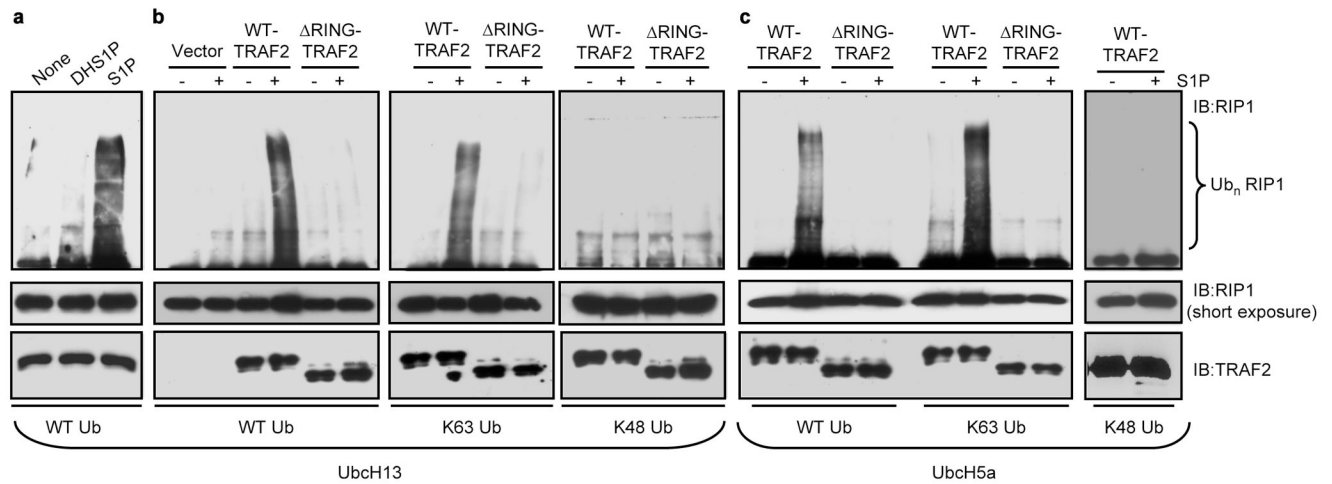


Figure 3. S1P is required for TRAF2-mediated Lys 63-linked polyubiquitination of RIP1 *in Vitro*

a, *In vitro* ubiquitination of purified RIP1 was carried out with ATP, E1, Ubc13/Uev1a, ubiquitin, and TRAF2 with the indicated lipids (100 nM) and examined with anti-RIP1 antibody. **b,c**, Ubiquitination reactions were carried out with purified WT-TRAF2 or RING-TRAF2 in the presence of UbcH13/Uev1a (**b**) or UbcH5a/Uev1a (**c**) as E2s and ubiquitin proteins (WT, Lys 63 only, or Lys 48 only), without or with 100 nM S1P. RIP1 ubiquitination was determined with anti-RIP1 antibody and TRAF2 input with anti-TRAF2 antibody.

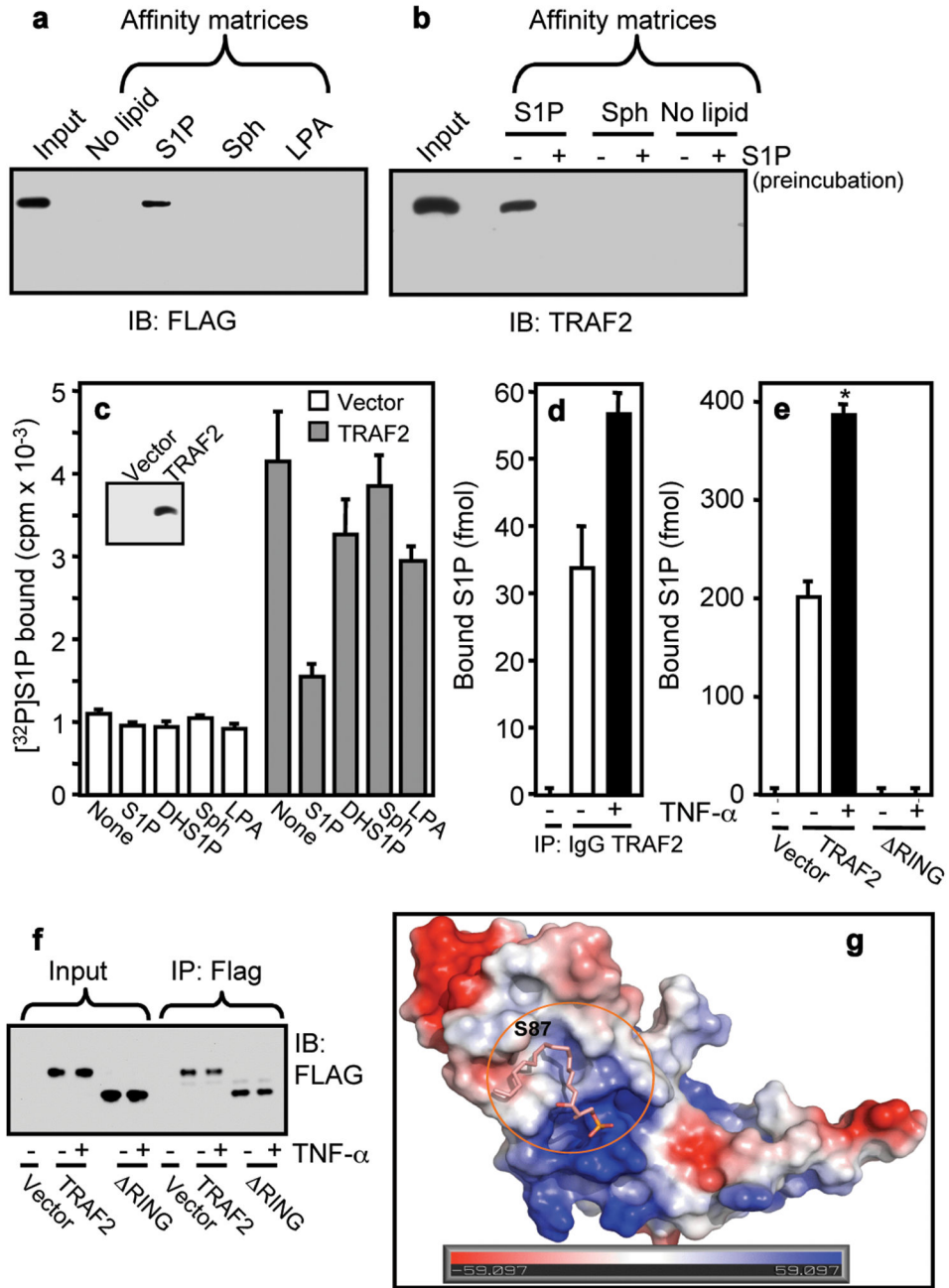


Figure 4. Specific binding of S1P to TRAF2

a, Lysates from HEK 293 cells transfected with Flag-TRAF2 were incubated with control (no lipid), S1P, LPA, or sphingosine affinity matrices. **b**, Lysates from naïve cells were pre-treated with 10 μM S1P, followed by pull-down with control (no lipid), S1P, or sphingosine affinity matrices and bound proteins analyzed by immunoblotting. **c**, Lysates from vector or Flag-TRAF2 transfected cells were incubated with anti-Flag agarose beads. Beads were washed and incubated with $[^{32}\text{P}]\text{S1P}$ (0.1 nM) in the absence or presence of 1 μM unlabeled S1P, dihydro-S1P, sphingosine or LPA, and $[^{32}\text{P}]\text{S1P}$ bound to TRAF2 was eluted with Flag

peptide and radioactivity determined. Insert, blot of eluted TRAF2. **d**, Naïve cells were stimulated with TNF- α . Lysates were immunoprecipitated with anti-TRAF2 antibody or control IgG and bound sphingolipids determined by LC-ESI-MS/MS. Of all of the sphingolipids present in these cells (Supplementary Table 1), only S1P was detected in the immunocomplexes. **e,f**, Cells transfected with vector, Flag-TRAF2, or Flag- RING-TRAF2 were stimulated with TNF- α . Lysates were immunoprecipitated with anti-Flag antibody. **(e)** Bound S1P determined by LC-ESI-MS/MS. **(f)** Immunoblot with anti-Flag antibody. **g**, Docking of S1P into the pocket of the RING domain of TRAF2. Surface contour of the binding site with S1P was colored by electrostatic potential.