SI Materials and Methods

Reagents and antibodies

Sphingosine-1-phosphate (S1P), sphingosine, and lysophosphatidic acid (LPA) were obtained from Echelon Biosciences. Thapsigargin (Tg) and tunicamycin (Tm) were from Sigma-Aldrich. TNFα was from Shenandoah Biotechnology and geldanamycin (GA) was from Cayman. TNFα inhibitor was from Calbiochem and TNFα neutralizing antibody was from Cell Signaling. Full length human recombinant HSP90α and GRP94 were from ProSpec. The following antibodies were used for western blot, immunoprecipitation, or immunofluorescence assay: rabbit monoclonal HSP90α, rabbit polyclonal HSP90β, rabbit polyclonal GRP94, rabbit monoclonal IRE1α, rabbit polyclonal TRAF2, rabbit monoclonal RIP1, rabbit monoclonal STUB1, rabbit monoclonal phospho-JNK (Thr183/Tyr185), rabbit monoclonal phospho-IKK (Ser176/180), and rabbit polyclonal Histone H3 antibodies (Cell Signaling); mouse monoclonal p50, mouse monoclonal p65, rabbit polyclonal IκBβ, rabbit polyclonal phospho-IκBα, mouse monoclonal IKK, and rabbit polyclonal ubiquitin antibodies (Santa Cruz Biotechnology); mouse monoclonal HSP90α, rabbit polyclonal GRP94, rabbit polyclonal CAMP antibodies (LifeSpan Biosciences); mouse monoclonal RIP1 antibody (BD Bioscience); mouse monoclonal polyubiquitin (K63-linkage specific) antibody (Enzo Life Sciences); mouse monoclonal GFP antibody (Origene Technologies); monoclonal β-actin-peroxidase conjugated antibody (Sigma-Aldrich); rabbit polyclonal sphingosine kinase 1, mouse monoclonal sphingosine kinase 2 (ECM Biosciences); and mouse monoclonal HRP-conjugated His6 antibody (Medical & Biological Laboratories Co Ltd.).

Cell Culture

Because mammalian epidermis is continuously threatened by perturbants from the external environment, we employed cultured human epidermal keratinocytes (KC) as our primarily model for most of these studies. Normal human KC isolated from neonatal foreskins were grown in serum-free KC growth medium containing 0.07 mM calcium chloride and growth supplements (Invitrogen), as described previously (1) under the UCSF Institutional Review Board approval protocol. Human lung carcinoma A549 and macrophage cell line RAW264.7 were maintained in DMEM containing 10% FBS, respectively, as described previously (2). Culture medium was switched to KC growth medium containing reduced growth factor (to 50%) for KC and DMEM containing 1% FBS for other cells one day prior to treatment.

Animal and *Ex vivo* experiments

S1P1, S1P2, S1P3, or S1P1/3 receptors- and SPHK1-*null* mice were kindly provided by Drs. Timothy Hla (Weill Cornell Medical College), Richard L. Proia (National Institute of Health) and Julie Saba (Children's Hospital Oakland Research Institute). Mice are generated and genotyped as described previously (3). *Ex vivo* (organ culture) skin studies were conducted, as described previously (1). Briefly, full thickness pieces of murine skin harvested from each S1P receptors- or SPHK1-*null* mice or appropriate littermate controls (wild type) were placed on filter papers, dermis side down, and cultured at the air-medium interface in KC growth medium. Tg (100 nM) or vehicle (DMSO) was epicutaneously applied (20 μl/cm^2) followed by incubation for 24 hrs. All experiments and procedures were performed according to protocols approved by the SFVAMC

Institutional Animal Care and Use Committee.

Expression of plasmid for TRAF2

The pCMV6-AC-TRAF2-GFP vector purchased from OriGene was used for overexpression of human TRAF2 gene. The plasmid was transformed into *Escherichia coli* DH5α and purified by Qiagen plasmid Midi prep system using standard methods (4).

Expression and Purification of Recombinant HSP90 proteins

The pCMV6-XL4-HSP90AA1 (Origene) was used as a template for PCR amplification of three DNA fragments coding the full length (Full) (Met1-Asp732), the N-terminal (N) domain (Met1-Glu236) or the middle and C-terminal (MC) domain (Lys293-Gly697) of HSP90 isoform 1. The PCR primers are listed below. Each PCR product of HSP90 was cloned into pET28b (Novagen) with the In-Fusion Advantage PCR cloning kit (Clontech). All plasmids were designed to express the recombinant protein with the His6-tags at both N- and C-termini, and the entire coding regions were checked by DNA sequencing. The resulting expression plasmids were transformed into *Escherichia coli* BL21 (DE3) strain and expressed for 4 hrs at 37 °C after induction with 1 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested at 7,000 × g for 5 mins and resuspended in Buffer A (20 mM sodium phosphate pH 7.4, 0.5 M NaCl). Cells were disrupted by sonication, and cell debris was removed by centrifugation (15,000 × g, 30 mins, 4 °C). The cleared supernatants were loaded on a nickel-nitrilotriacetic acid-agarose column (HisTrap FF-1mL, GE Healthcare) operated on an $ÄKTA_{FPLC}$ liquid chromatography system (GE Healthcare) pre-equilibrated with Buffer A, and His_6-tagged proteins were

eluted using a linear 60-1000 mM imidazole gradient in Buffer A. Peak fractions were pooled, concentrated (10-kDa MWCO; Vivaspin® 20, Sartorius), and further purified on a HiTrap DEAE HP-1mL anion exchange chromatography column (GE Healthcare) pre-equilibrated with Buffer B (20 mM sodium phosphate, pH 7.4). Proteins were eluted by using a linear 0-500 mM NaCl gradient in Buffer B. The fractions were pooled and dialyzed against Buffer C (20 mM Tris-HCl, pH 7.4). The concentration of each HSP90 protein was determined by using the following extinction coefficient: ϵ_{280} = 59,850 M^{-1} cm^{-1} for the full length, ϵ_{280} = 16,065 M^{-1} cm^{-1} the N-domain, ϵ_{280} = 42,315 M^{-1} cm^{-1} for the MC-domain of HSP90.

List of primers for plasmid construction

HSP90α f1 (PCR for InFusion-cloning of HSP90α Full [1-732], CCGCGCGGCAGCCATATGCCTGAGGAAACC; HSP90α f2 (PCR for InFusioncloning of HSP90α N-domain [1-236]), CCGCGCGGCAGCCATATGGACCAACCGATG; HSP90α f2 (PCR for InFusioncloning of HSP90α MC-domain [293-697]), CCGCGCGGCAGCCATATGAAGCCCATCTGG; HSP90α r1 (PCR for InFusioncloning of HSP90α Full [1-732]), CTTGTCGACGGAGCTGTCTACTTCTTCCAT; HSP90α r2 (PCR for InFusion-cloning of HSP90α N-domain [1-236], CTTGTCGACGGAGCTTTCAGCCTCATCATC; HSP90α r3 (PCR for InFusion-cloning of HSP90α MC-domain [293-697]), CTTGTCGACGGAGCTACCCAGACCAAGTTT; pET28α f (PCR of pET28 for InFusion-cloning), AGCTCCGTCGACAAGCTTGCGGCCGCACTC; pET28α r (PCR

of pET28 for InFusion-cloning), ATGGCTGCCGCGCGGCACCAGGCCGCTGCT; HSP90_200 (DNA sequence primer, GCATCTGTTGGTGTCTGGAT; HSP90α 800 (DNA sequence primer), GAAAGTAACTGTGATCAC; HSP90α 1400 (DNA sequence primer), TGCTCCTTTTGATCTGTTTG.

Quantitative RT-PCR

Relative mRNA expression was assessed by quantitative RT-PCR (*q*RT-PCR) using SensiMix^{TM} SYBR PCR Master Mix (Bioline) as described previously (1). Briefly, Total RNA was isolated from cell lysates using RNeasy mini kit (Qiagen), followed by preparation of cDNA using SensiFAST^{TM} cDNA synthesis kit (Bioline). The used primer sets for PCR are listed below and in our prior study (1). The thermal cycling conditions were 95˚C for 10 mins, 95˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 15 sec, repeated 40 times on ABI Prism 7900HT (Applied Biosystems). mRNA expression was normalized to levels of GAPDH. Values shown represent mean (± SD) for three independent assays. The following primer sets were used: SPHK1, 5-CATCCAGAAGCCCCTGTGTAG-3' and 5'-CTGCTCATAGCCAGCATAATGG-3'; SPHK2, 5-TGCTCCATGAGGTGCTGAAC-3' and 5'-GCCCACAGGCATCTTCACA-3'; S1P4, 5-GTCTTTGGCTCCAACCTCTG-3' and 5'-CTGCTGCGGAAGGAGTAGAT-3'; S1P5, 5-CGACAGAGATGGTGATGGTG-3' and 5'-CTTTTGGTCTTCCCAGGACA-3'; ICAM-1, 5-CGTGGGGAGAAGGAGCTGAA-3' and 5'-CAGTGCGGCACGAGAAATTG-3'; IL-8, 5-TCTGGCAACCCTAGTCTGCT-3' and 5'-AAACCAAGGCACAGTGGAAC-3'; MMP-9, 5-TTGACAGCGACAAGAAGTGG-3' and 5'-GCCATTCACGTCGTCCTTAT-3'.

Western blot analysis

Western blot analysis was performed as described previously (1). Cell lysates, prepared in radioimmunoprecipitation assay buffer, were resolved by electrophoresis on 4-12% Bis-Tris protein Gel (Invitrogen). Nuclear and cytoplasmic fractions were prepared using extraction reagents (Thermo Scientific), following the manufacturer's instructions. Resultant bands were blotted onto polyvinylidene difluoride membranes, probed with appropriate antibodies, and detected using enhanced chemiluminescence (Thermo Scientific). The intensity of bands was measured with a LAS-3000 (Fuji Film).

ELISA for CAMP Quantification

CAMP content in cell lysates or conditioned medium was quantitated using an LL-37 human enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotech) in accordance with the manufacturer's instructions.

Immunofluorescence

KC were pretreated with GA for 30 mins or were transfected with IRE1α, HSP90α, or GRP94 *si*RNA (20 nM) prior to incubation with Tg or Tm for the appropriate time period. CAMP or NF-κB distribution was assessed using anti-CAMP (LifeSpan BioSciences) or anti-NF-κB p65 (Santa Cruz Biotechnology), respectively. The secondary antibodies were Alexa Fluor® 488 goat anti-rabbit IgG, Alexa Fluor® 488 goat anti-mouse IgG, or Alexa Fluor® 568 donkey anti-rabbit IgG antibodies (Invitrogen). Cells were counterstained with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI)

(Vector Laboratories) for nuclear visualization. Slides were examined with a Carl Zeiss Axio fluorescence microscope.

Transfections of *si*RNAs or pTRAF2-GFP

KC were transfected with 20 nM *si*RNAs for IRE1α, SPHK1, SPHK2 (two sources *si*RNA [Santa Cruz Biotechnology and Invitrogen] were used in different set of studies), HSP90α, GRP94 (Invitrogen), TRAF2, STUB1 (Santa Cruz Biotechnology), or scrambled control (Invitrogen) using siLentFect lipid reagent (Bio-Rad), as previously described (1). In some experiments, cells were transfected with different sequences of *si*RNAs to exclude off-target effects. KC were transfected with pTRAF2-GFP using DNA transfection reagent, HilyMax (Dojindo).

Immunoprecipitation

Cell lysates were prepared in IP lysis/wash buffer (Thermo Scientific) containing protease inhibitor cocktail (Roche) and 1mg/ml N-ethylmaleimide in accordance with the manufacturer's instructions. RIP1, GFP, HSP90α, or GRP94 were immunoprecipitated from 200 μg of cell lysates with 1 μg anti-RIP, anti-GFP, anti-HSP90α, or anti-GRP94 antibodies overnight at 4°C. Immunoprecipitates were captured with protein A/G plus agarose beads (Thermo Scientific). After extensive washing, bound proteins were released by boiling in SDS-PAGE sample buffer and performed western blot analysis.

Isolation of S1P binding proteins

Control-, S1P-, and sphingosine-coated beads (Echelon Biosciences) equilibrated with the binding/washing buffer (10 mM Hepes buffer pH 7.4, 150 mM NaCl, protease inhibitor cocktail, and 0.5% (*v/v*) Igepal were mixed cell lysates or recombinant HSP90s (full-length [full], Middle + C-terminal [MC] or N-terminal [N] domain) and incubated for 2 hrs with continuous motion at 4°C. The beads were washed 5 times with binding/washing buffer. Proteins are eluted from beads by the binding/washing buffer containing 0.1 mg/ml of S1P or by boiling the beads with the SDS-PAGE sample buffer. The eluted proteins were analyzed by SDS-PAGE and western blotting.

Protein identification by Mass Spectrometry

Proteins were separated on a 4-12% gradient Bis-Tris protein gel (Invitrogen), bands were visualized by coomassie brilliant blue staining and then excised, followed by processing Mass Spectrometry at the Stanford University Mass Spectrometry facility. Briefly, gel was digested as previously described (5) using trypsin (Promega) for overnight at 37°C. Peptides were extracted from the gel bands subjected to LC-MS where the liquid chromatography was an Eksigent nano2D operated at 600 nL/min equipped with a Bruker Michrom Captive Spray source. The Mass Spectrometer was a LTQ Orbitrap Velos operated in data dependent acquisition (DDA) mode in which the most intense multiply charged precursor ions were selected and fragmented in the ion-trap. The data was searched using SEQUEST software (Thermo Fisher Scientific) on a Sorcerer platform, exported and visualized by Scaffold.

Bio-layer Interferometry Assay

S1P, sphingosine, or LPA bindings to the full length (Full), the N-terminal (N) domain, or the domain consisting of both middle and C-terminal (MC) domain of purified HSP90α were measured by bio-layer interferometry on an Octet RED 384 system (ForteBio) at the Biosensor Core Facility, UCSF Center for Advanced Technology. Full, N, or MC domains of HSP90α equilibrated with PBS buffer were dispensed into 384-well plates at a volume of 100 µl per well. Operating temperature was maintained at 27°C with 1000 rpm rotary agitation. Ni-NTA biosensor tips (ForteBio) were pre-wet for 10 mins with buffer to establish a baseline before protein immobilization. First, Full of His_6-tagged HSP90α proteins were capture-immobilized onto the biosensors for 2 mins at a concentration of 50 µg/ml. The immobilization level attained was 1.5 nm. Binding association of serial dilutions of each lipid with the Full of HSP90α-loaded biosensor tips was monitored for 30 sec, and subsequent disassociation in PBS buffer was monitored for 30 sec. Subsequently, N- or MC-domains of His_6-tagged HSP90 proteins were immobilized onto the biosensor tips, which were regenerated by rinsing 40 sec in 0.3M imidazole, 0.05% SDS, followed by the determination of binding with each lipid as described above. The apparent affinities of HSP90 proteins with each lipid were calculated from steady-state response measurements (Octet User Software version 8.0).

S1P quantification

KC transfected with pTRAF2-GFP using DNA transfection reagent, HilyMax (Dojindo) for 24 hrs were incubated with Tg or TNFα for 10 mins. Cells were lysed in IP lysis/wash buffer (Thermo Scientific) containing protease inhibitor cocktail (Roche). HSP90α or TRAF2 were immunoprecipitated from 500 µg of cell lysates with 2.5 µg anti-HSP90α or

anti-TRAF2 antibodies overnight at 4°C. Immunoprecipitates were captured with protein A/G plus agarose beads (Thermo Scientific) and washed 4 times with IP lysis/wash buffer. To recover/extract sphingolipids bound HSP90α or TRAF2, 500 μl of 1% formic acid (*v*/*v*) in acetonitrile containing C_{17}-S1P (20 pmol) as an internal standard was added and incubated at 60°C for 20 mins. The sphingolipid extraction was repeated by the addition of another 500 μl of 1% formic acid (*v*/*v*) in acetonitrile containing C_{17}-S1P (20 pmol). The extracted lipids (total 1ml) dried using a vacuum system (Vision, Seoul, Korea) were re-dissolved in methanol and analyzed by LC-ESI-MS/MS (API 4000 QTRAP mass, AB/SCIEX) by selective ion monitoring mode (The precursor ions of S1P [m/z 380.3] and dihydro-S1P [m/z 382.3] were cleaved into fragment ions of m/z 264.2 and m/z 284.2, respectively) as described previously (6, 7). Data were analyzed using Analyst 1.4.2 software (Life Technologies).

Enzyme activity assays for SPHK1 and SPHK2

KC pretreated with a specific SPHK1 inhibitor, PF-543 (1 μM or 2.5 μM) for 30 mins were incubated with Tg (50 nM) for 24 hrs. SPHK1 and SPHK2 activities were determined as described previously (8, 9). Briefly, cell lysates in 20 mM Tris (pH7.4) containg 5 mM EDTA, 5 mM EGTA, 3 mM β-mercaptoethanol, 5% glycerol, protease inhibitors (Sigma-Aldrich) and phosphatase inhibitors (Roche) were incubated with 10 μL of 200 μM C_{17}-Sphingosine as a SPHK substrate. To assay each isoform of SPHK activity, 0.5% Triton X-100 or 1 M KCl (for SPHK 1 and SPHK2, respectively) were added into assay buffer and then incubated at 37°C for 30 mins. Enzyme reactions were terminated by the addition of $CHCl_3$: MeOH: HCl (8:4:3, *v*/*v*/*v*). C_{17}-sphinganine-1-

phosphate (100 pmol) was added as an internal standard. The organic phage separated by addition of $CHCl_3$ was dried using a vacuum system (Vision). The dried residue was re-dissolved in MeOH and then analyzed by LC-ESI-MS/MS (API 3200 Triple quadruple mass, AB/SCIEX) as above and the activities of SPHK1 and SPHK2 are expressed as C_{17}-S1P pmol per mg protein per min.

Statistical analyses

Experiments were repeated at least three times. For each experiment, results from triplicate samples were expressed as the mean ± standard deviation (SD). Significance between groups was determined with unpaired Student *t* test. The P values were set at <0.01.

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SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. ER stress-mediated S1P production by SPHK1 increases CAMP expression in KC. KC transfected with SPHK1 or scrambled control *si*RNA were incubated with Tg. CAMP protein levels were assessed by immunofluorescence staining (*A*). Phosphorylated IκBα, IκBβ and subunit of NF-κB, p65, were assessed by Western blot analysis using indicated antibodies (*B*). Nuclear translocation of NF-κB was also assessed by immunofluorescence staining (*C*). Green and blue staining corresponds to CAMP (*A*), NF-κB (*B*) and nuclear staining, respectively.

Fig. S2. Exogenous S1P stimulates CAMP production in SPHK1 silenced cells. KC were incubated with exogenous S1P (at indicated concentrations) or Tg (50 nM) for 24 hrs (*A*). KC transfected with SPHK1 or scrambled control *si*RNA were incubated with exogenous S1P (1 μM) or Tg (50 nM) for 24 hrs (*B*, *C*). CAMP (*A*, *C*), SPHK1 and SPHK2 (B) mRNA levels were assessed by *q*RT-PCR.

Fig. S3. S1P1, S1P2, S1P3, S1P4 or S1P5 knockdown does not influence ER stress-mediated increases in CAMP expression. S1P1, S1P2, and S1P3 *null*, as well as S1P1 and S1P3 double *null* mouse skin were obtained 24 hrs following topical application (20 μl/cm^2) of Tg (100 nM) or vehicle (DMSO) on dorsal skin. Murine CAMP (mCAMP) mRNA (*A-C*) and protein (MW 18kDa) (*D-F*) levels assessed by *q*RT-PCR or Western blot analysis, respectively. KC transfected with S1P4, S1P5 or scrambled control *si*RNA were incubated with Tg (20 nM) for 24 hrs. S1P4, S1P5 (*G*), and CAMP (*H*) mRNA levels were assessed by *q*RT-PCR.

Fig. S4. Morphological changes are not evident in S1P1, S1P2, S1P3, and S1P1 and S1P3 double *null* mouse skin under ER stress. S1P1, S1P2, and S1P3 *null*, as well as S1P1 and S1P3 double *null* mouse, and wild type (WT) skin were obtained 24 hrs following topical application (20 μl/cm^2) of Tg (100 nM) or vehicle (DMSO) on dorsal skin. Skin morphology was assessed using hematoxylin and eosin–stained sections.

Fig. S5. IRE1α is required for NF-κB activation in response to ER stress. KC transfected with IRE1α or scrambled control *si*RNA were incubated with a sub-toxic concentration of another ER stressor tunicamycin (Tm, 0.12 μM). CAMP mRNA levels were assessed by *q*RT-PCR (*A*). Phosphorylated IκBα, IκBβ and subunit of NF-κB, p50 and p65, were assessed by Western blot analysis (*B*). Nuclear translocations of NF-κB

were also assessed by immunofluorescence staining. Green and blue staining corresponds to NF-κB and nuclear staining, respectively.

Fig. S6. TRAF2 and STUB1 are necessary for ER stress-induced CAMP stimulation. TRAF 2 and STUB1, which have E3 ubiquitin ligase activity, are involved in ER stress-induced CAMP production. pTRAF2-GFP overexpressed KC were transfected with TRAF2, STUB1 or scrambled control *si*RNA and then exposed to Tg (20 nM) for 24 hrs. TRAF2, STUB1 (*A*), and CAMP protein (*B*) levels were assessed by Western blot analysis. *si*RNA specifically suppressed target protein expression.

Fig. S7. IRE1α or HSP90α silencing does not change S1P production in response to ER stress. KC transfected with IRE1α, HSP90α or scrambled *si*RNA were exposed Tg (20 nM) for 24 hrs. S1P and dihydro-S1P levels were assessed by LC-ESI-MS/MS.

Fig. S8. Interaction of proteins in a signaling complex of S1P-HSP90s-IRE1α-TRAF2-RIP1. KC overexpressed with pTRAF2-GFP were transfected with SPHK1 or scrambled *si*RNA followed by incubating with Tg (20 nM) for 10 mins. Cell Lysates were immunoprecipitated with anti-GFP antibody and immunoblotted with SPHK1 antibody (*A*) or RIP1 antibody (*D*). SPHK1 (*B*) and TRAF2 (*C*) protein levels were assessed by Western blot analysis. Lysates from cells transfected with SPHK1 or scrambled control *si*RNA and treated with Tg were immunoprecipitated with anti-GRP94 antibody (*E*) or anti-HSP90α antibody (*F*) and immunoblotted with indicated antibodies.

Fig. S9. HSP90α and TRAF2 bind to S1P, but not dihydro S1P in response to ER stress and TNFα receptor activation, respectively. Lysates from pTRAF2-GFP-overexpressed KC incubated with Tg (20 nM) or TNFα (10 ng/ml) were immunoprecipitated with anti-HSP90α (*A-C*, *G-I*) or anti-TRAF2 (*D-F*, *J-L*) antibodies,

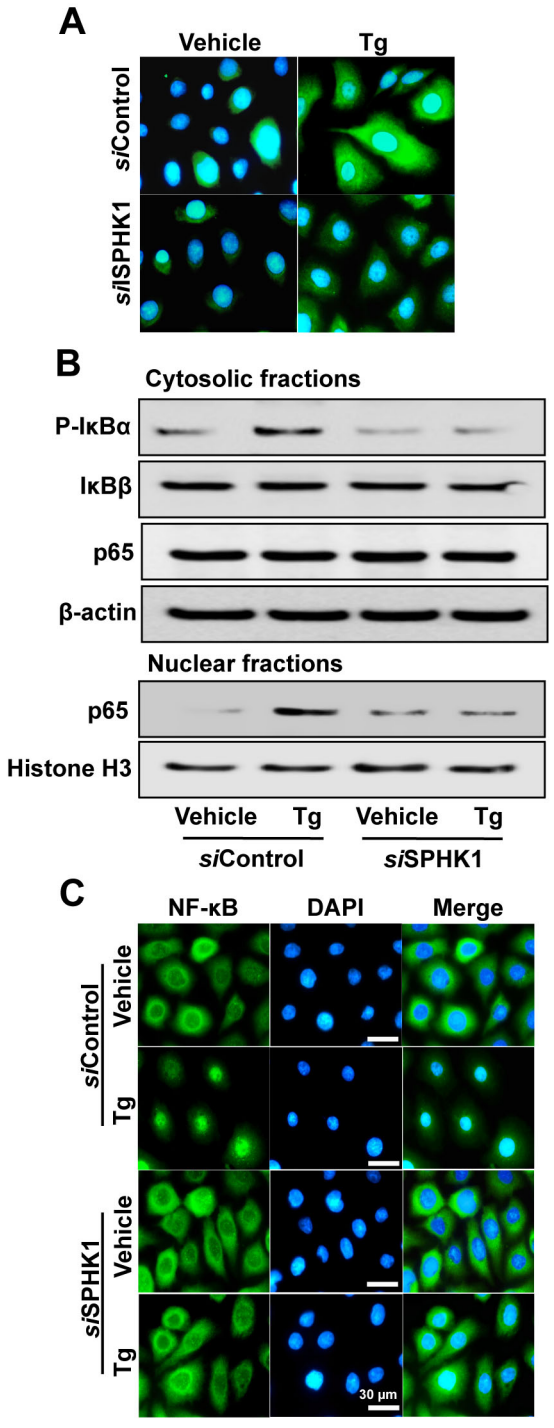
and bound S1P (*A-F*) and dihydro-S1P (*G-L*) were determined by LC-ESI-MS/MS. The precursor ions of S1P (m/z 380.3) and dihydro-S1P (m/z 382.3) were cleaved into fragment ions of m/z 264.2 and m/z 284.2, respectively.

Fig. S10. Both HSP90α and GRP94 are required for increased CAMP production initiated by ER stress. KC transfected with HSP90α, GRP94 or scrambled control *si*RNA were incubated with Tg (20 nM). CAMP mRNA (*A*) and protein (*B, C*) levels were assessed by *q*RT-PCR and Western blot analysis, respectively. Nuclear translocations of NF-κB were examined by immunofluorescence (*D*). Arrows indicate NF-κB positive nuclei.

Fig. S11. Neither sphingosine nor LPA binds to HSP90α. Bindings of full length, N-terminus, or MC-terminus constructs of HSP90α to either sphingosine (*A*), lysophosphatidic acid (LPA) (*B*) or S1P (*C*) were assessed by Bio-Layer Interferometry (Octet®).

Fig. S12. ER stress stimulates CAMP mRNA expression in both alveolar epithelial cell (A549) and macrophage (RAW264.7) through S1P–HSP90 signaling pathway. Cells pre-incubated with GA (0.5 μM) or PF-543 (0.5 – 1 μM) were treated with Tg (100 nM) for 24 hrs. Spliced XBP1, an indicator of ER stress, and CAMP mRNA were assessed by RT-PCR (*A, C*) or *q*RT-PCR (*B, D*), respectively.

Fig. S1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Vehicle
Tg
Vehicle
Tg
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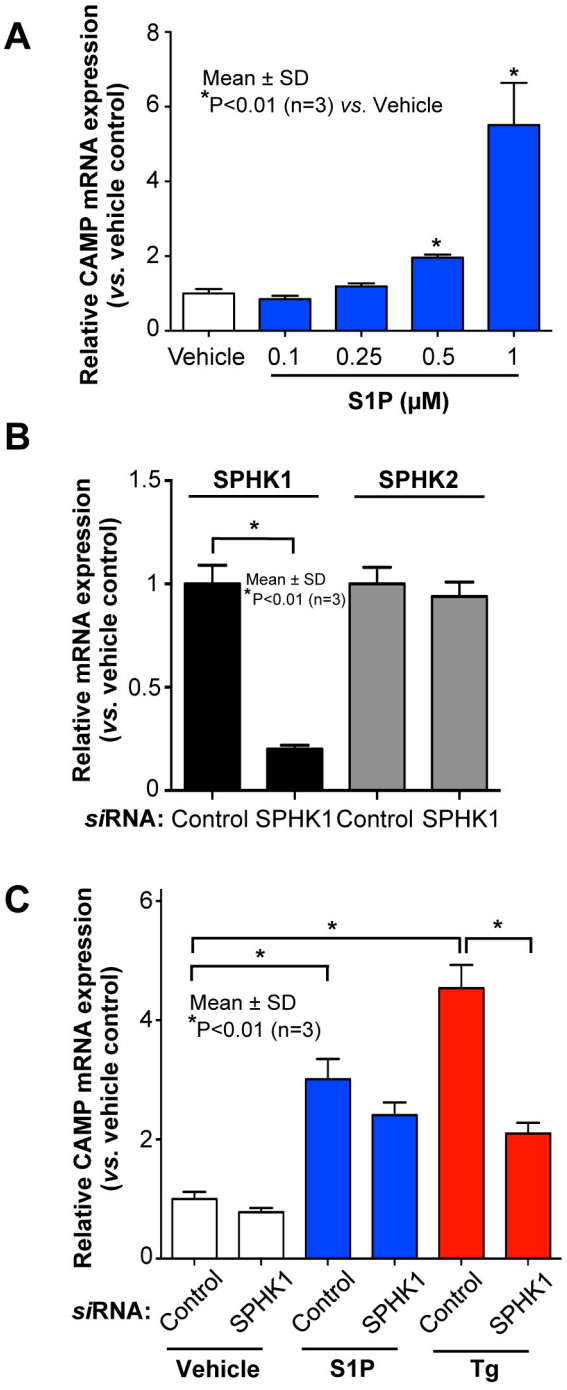
Fig. S2


A
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Mean ± SD
*P<0.01 (n=3) vs. Vehicle
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0.1
0.25
0.5
1
S1P (μM)
B
Relative mRNA expression (vs. vehicle control)
SPHK1
SPHK2
Mean ± SD
*P<0.01 (n=3)
siRNA: Control SPHK1 Control SPHK1
C
Relative CAMP mRNA expression (vs. vehicle control)
Mean ± SD
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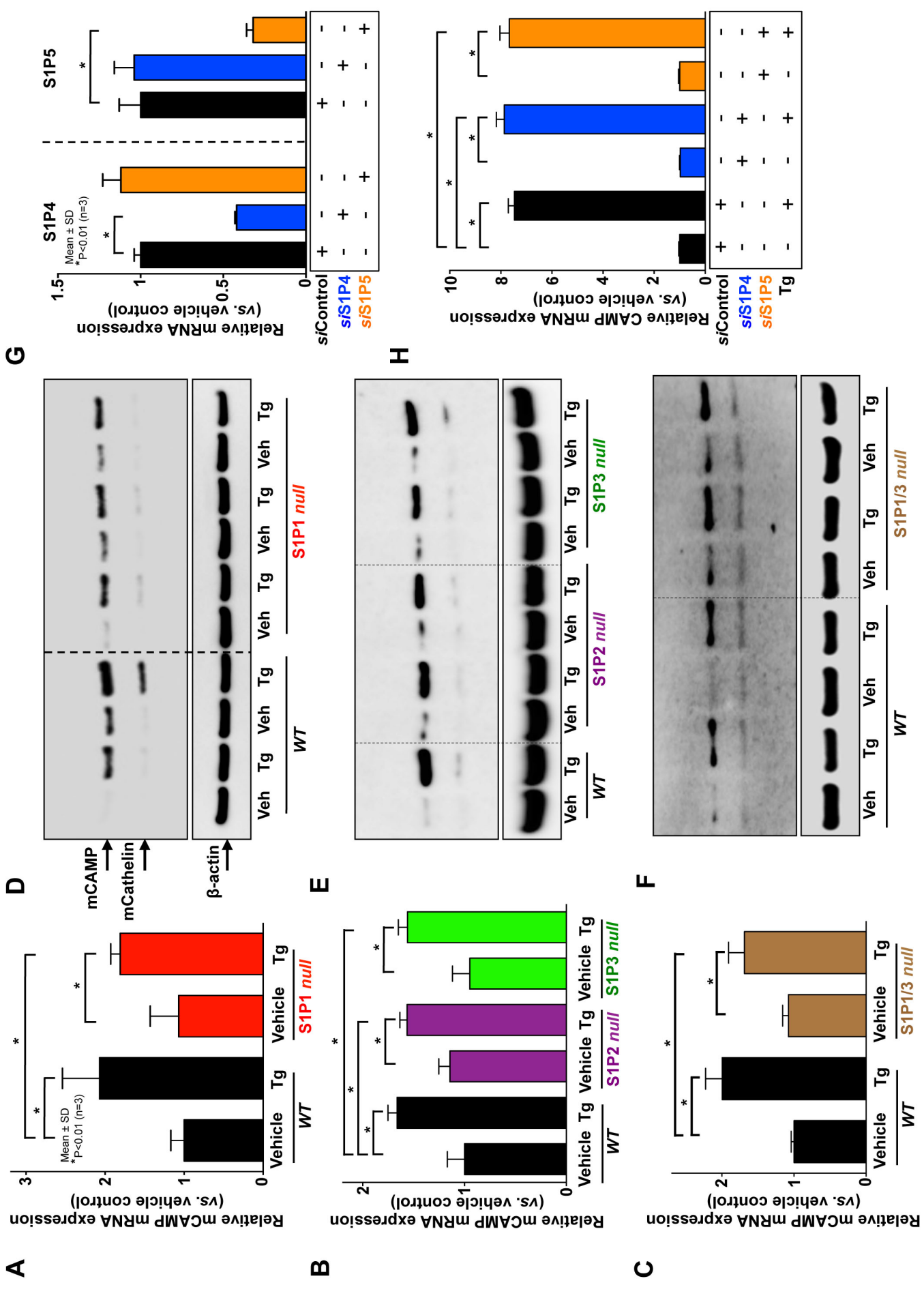
Vehicle
S1P
Tg

Fig. S3


A
Relative mCAMP mRNA expression (vs. vehicle control)
Mean ± SD
*P<0.01 (n=3)
Vehicle Tg Vehicle Tg
WT
S1P1 null
B
Relative mCAMP mRNA expression (vs. vehicle control)
Vehicle Tg Vehicle Tg Vehicle Tg
WT
S1P2 null
S1P3 null
C
Relative mCAMP mRNA expression (vs. vehicle control)
Vehicle Tg Vehicle Tg
WT
S1P1/3 null
D
mCAMP
mCathelin
β-actin
Veh Tg Veh Tg Veh Tg Veh Tg
WT
S1P1 null
E
Veh Tg Veh Tg Veh Tg Veh Tg Veh Tg Veh Tg
WT
S1P2 null
S1P3 null
F
Veh Tg Veh Tg Veh Tg Veh Tg
WT
S1P1/3 null
G
Relative mRNA expression (vs. vehicle control)
S1P4
S1P5
Mean ± SD
*P<0.01 (n=3)
siControl
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Relative cAMP mRNA expression (vs. vehicle control)
siControl
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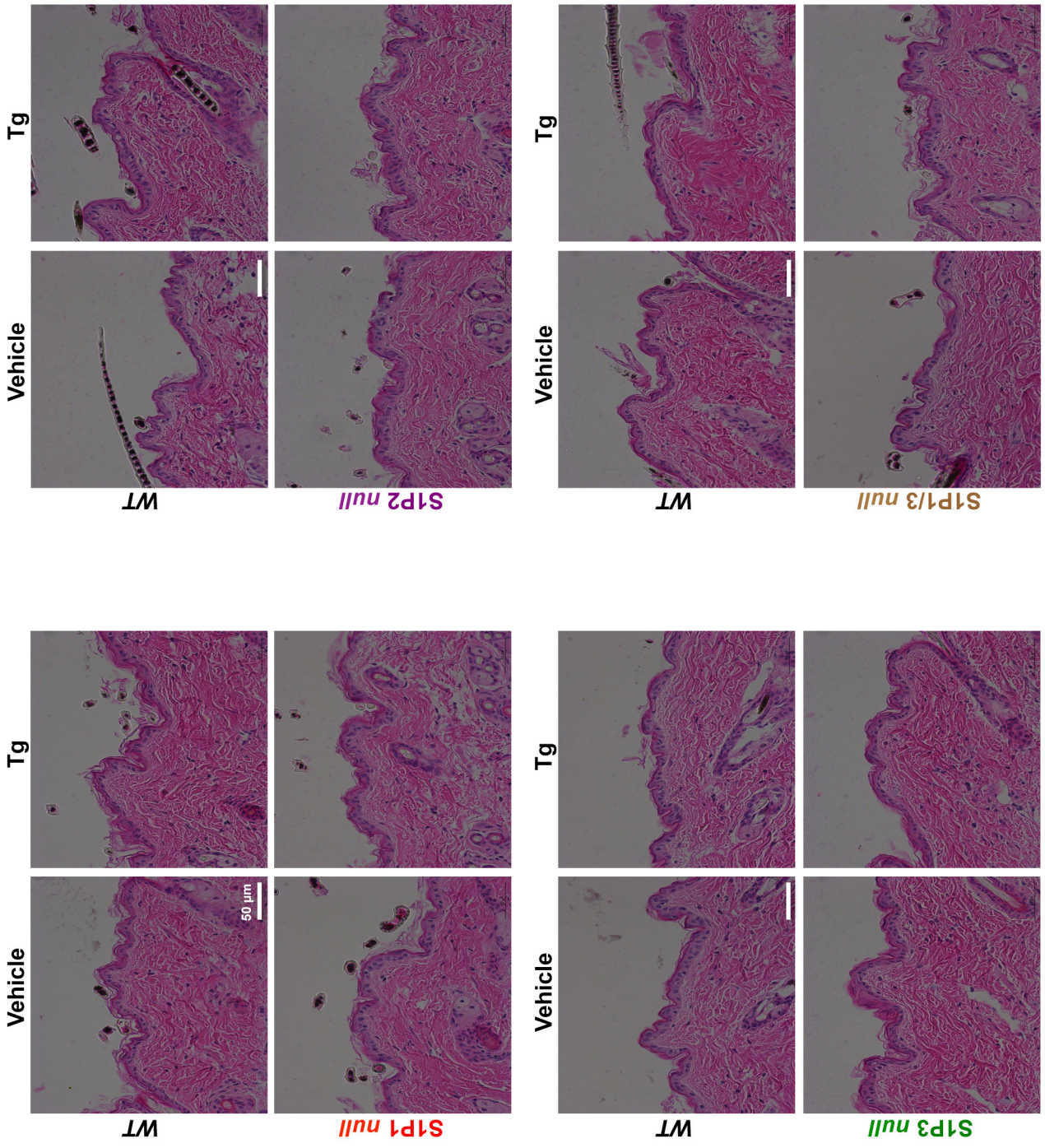
siS1P5
Tg

Fig. S4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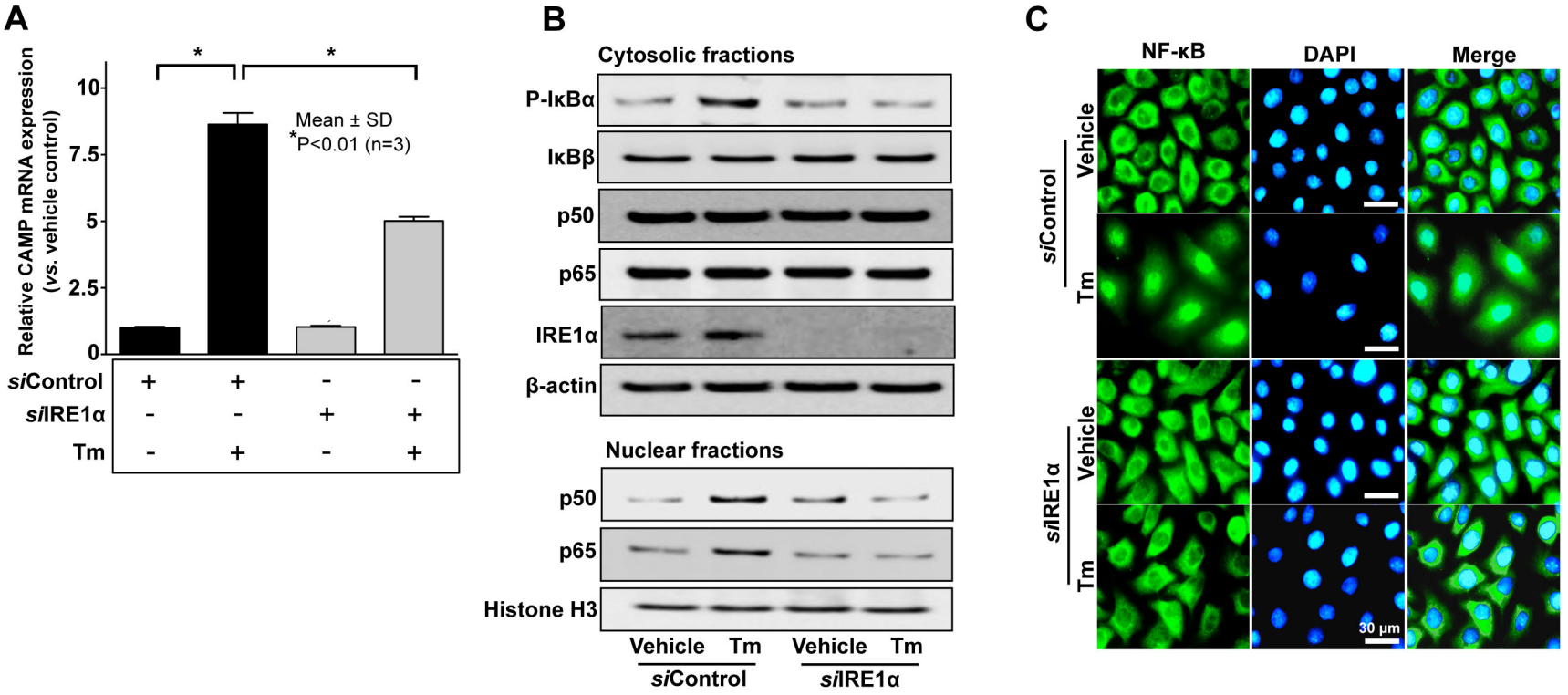

A
Relative CAMP mRNA expression (vs. vehicle control)
10
7.5
5
2.5
0
*
*
Mean ± SD
*P<0.01 (n=3)
siControl + + - -
siIRE1α - - + +
Tm - + - +
B
Cytosolic fractions
P-IκBα
IκBβ
p50
p65
IRE1α
β-actin
Nuclear fractions
p50
p65
Histone H3
Vehicle Tm Vehicle Tm
siControl
siIRE1α
C
NF-κB
DAPI
Merge
siControl
Vehicle
Tm
siIRE1α
Vehicle
Tm
30 μm

Fig. S6

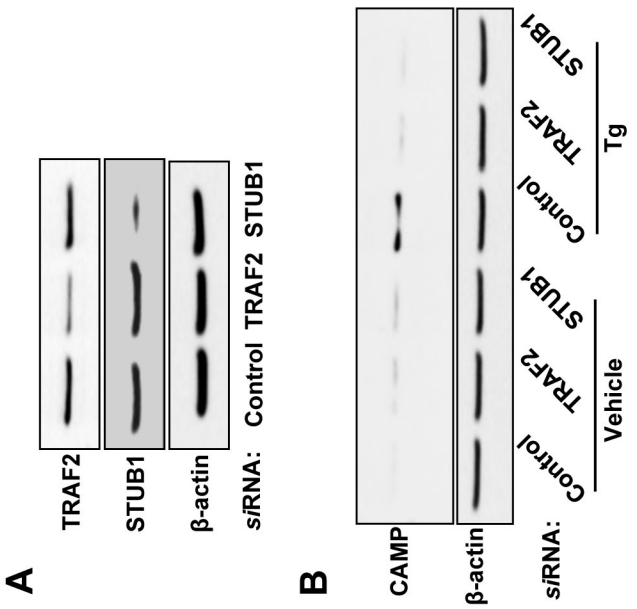

A
TRAF2
STUB1
β-actin
siRNA: Control TRAF2 STUB1
B
CAMP
β-actin
siRNA: Control TRAF2 STUB1 Control TRAF2 STUB1
Vehicle
Tg

Fig. S7

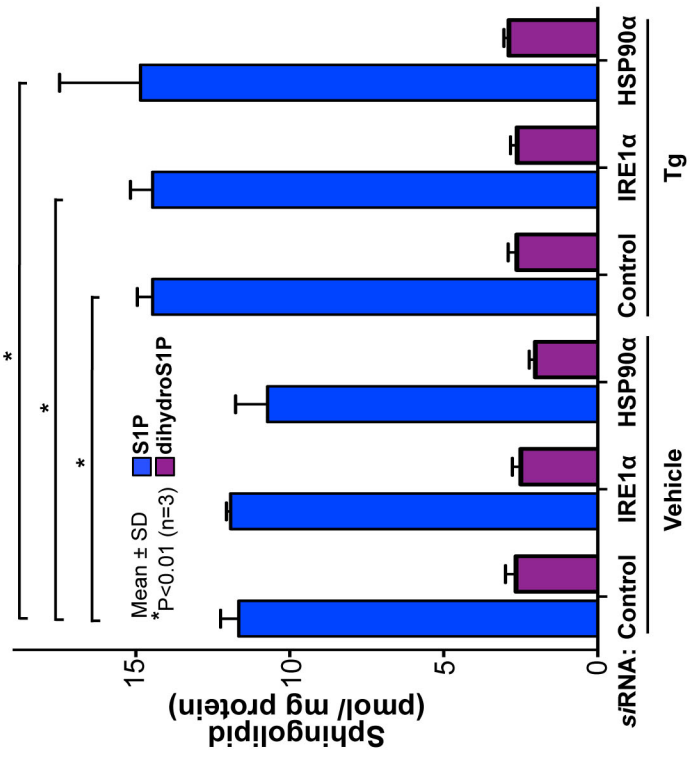

Sphingolipid
(pmol/ mg protein)
0
5
10
15
Mean ± SD
*P<0.01 (n=3)
S1P
dihydroS1P
*
*
*
siRNA: Control
IRE1α
HSP90α
Control
IRE1α
HSP90α
Vehicle
Tg

Fig. S8

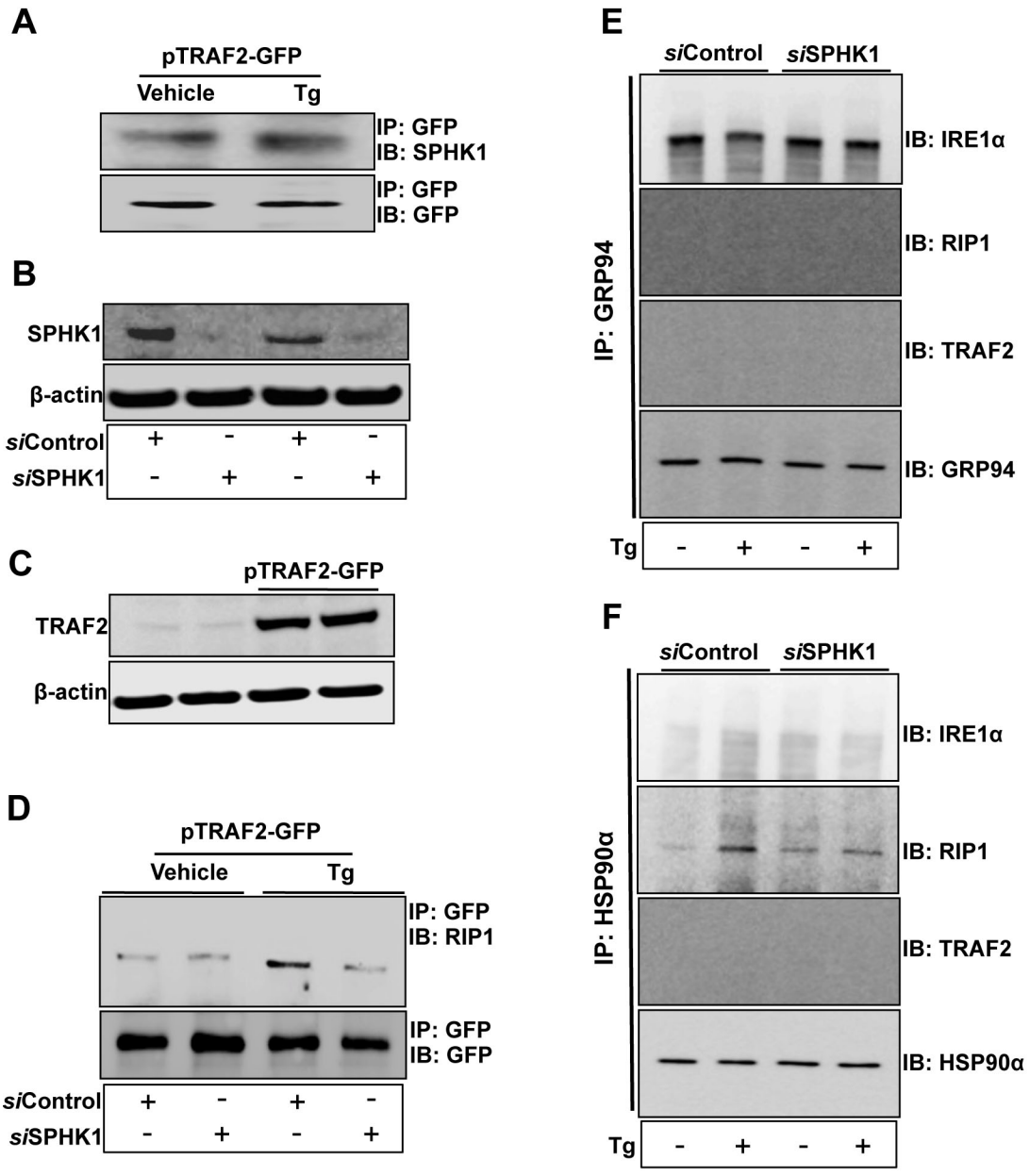
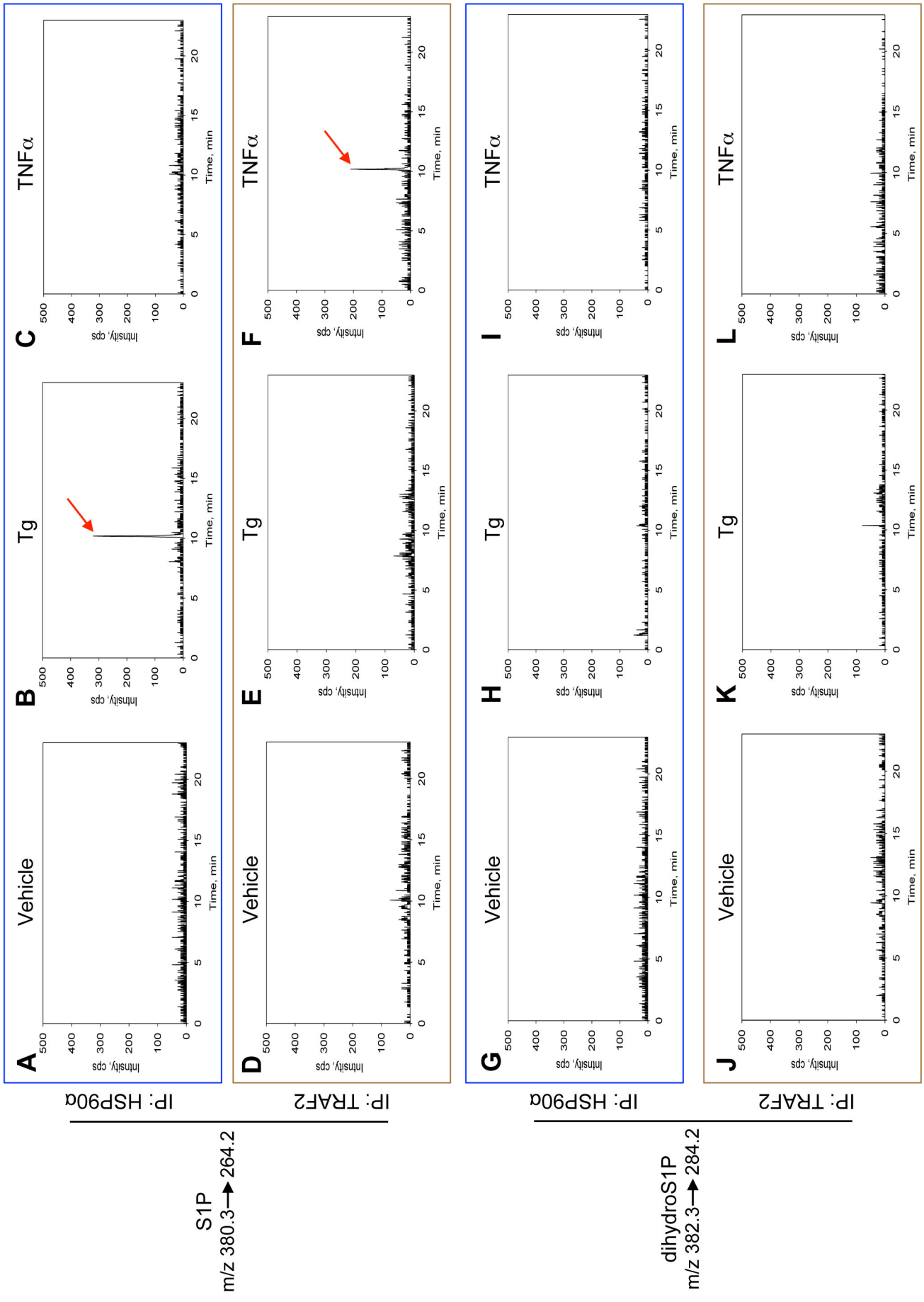
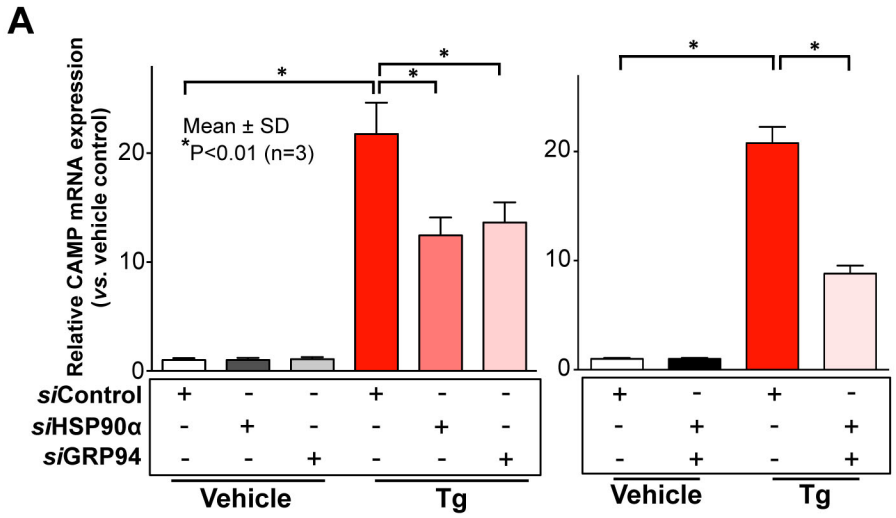
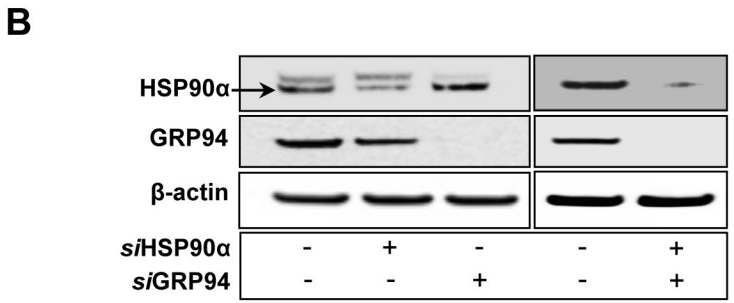
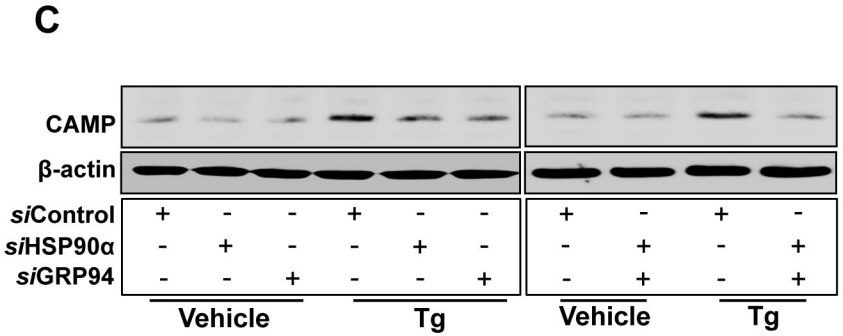

A
pTRAF2-GFP
Vehicle
Tg
IP: GFP
IB: SPHK1
IP: GFP
IB: GFP
B
SPHK1
β-actin
siControl + - + -
siSPHK1 - + - +
C
pTRAF2-GFP
TRAF2
β-actin
D
pTRAF2-GFP
Vehicle
Tg
IP: GFP
IB: RIP1
IP: GFP
IB: GFP
siControl + - + -
siSPHK1 - + - +
E
siControl
siSPHK1
IP: GRP94
IB: IRE1α
IB: RIP1
IB: TRAF2
IB: GRP94
Tg - + - +
F
siControl
siSPHK1
IP: HSP90α
IB: IRE1α
IB: RIP1
IB: TRAF2
IB: HSP90α
Tg - + - +

Fig. S9


A
Vehicle
B
Tg
C
TNFα
D
Vehicle
E
Tg
F
TNFα
G
Vehicle
H
Tg
I
TNFα
J
Vehicle
K
Tg
L
TNFα
Intnsity, cps
Time, min
IP: HSP90α
IP: TRAF2
IP: HSP90α
IP: TRAF2
S1P
m/z 380.3→264.2
dihydroS1P
m/z 382.3→284.2


A
Relative CAMP mRNA expression
(vs. vehicle control)
Mean ± SD
*P<0.01 (n=3)
siControl
siHSP90α
siGRP94
Vehicle
Tg
Vehicle
Tg


B
HSP90α
GRP94
β-actin
siHSP90α
siGRP94


C
CAMP
β-actin
siControl
siHSP90α
siGRP94
Vehicle
Tg
Vehicle
Tg

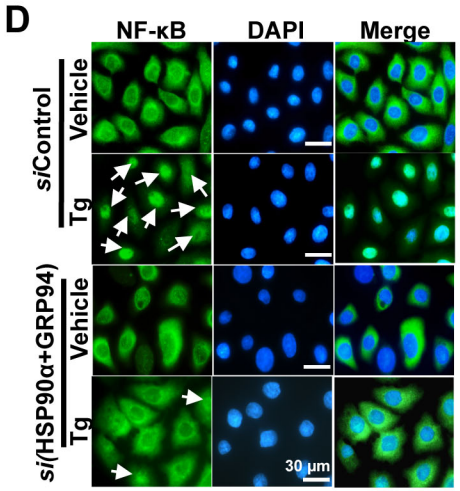
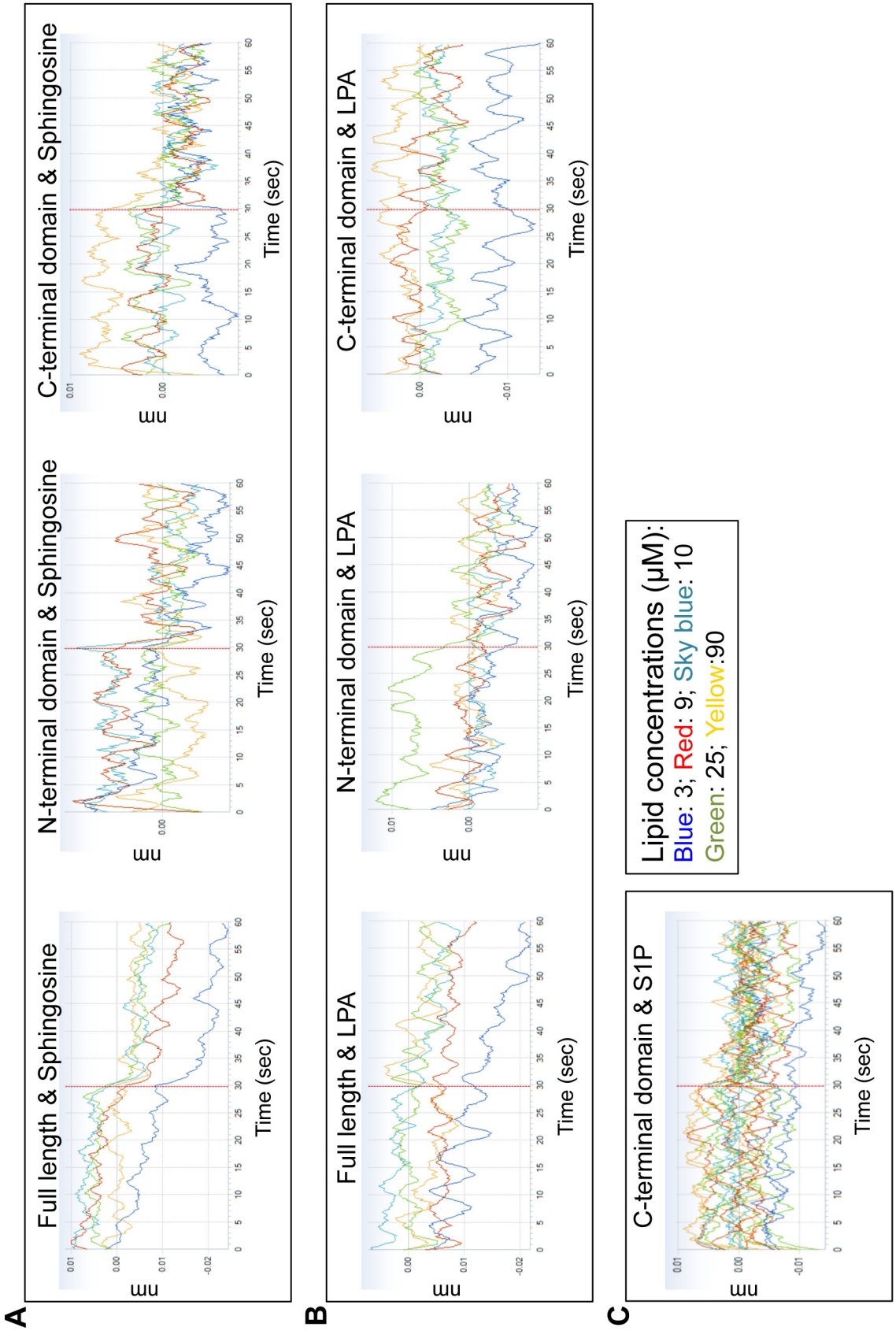

D
NF-κB
DAPI
Merge
siControl
Vehicle
Tg
si(HSP90α+GRP94)
Vehicle
Tg
30 μm

Fig. S11


A
Full length & Sphingosine
N-terminal domain & Sphingosine
C-terminal domain & Sphingosine
B
Full length & LPA
N-terminal domain & LPA
C-terminal domain & LPA
C
C-terminal domain & S1P
nm
Time (sec)
Lipid concentrations (μM):
Blue: 3; Red: 9; Sky blue: 10
Green: 25; Yellow:90

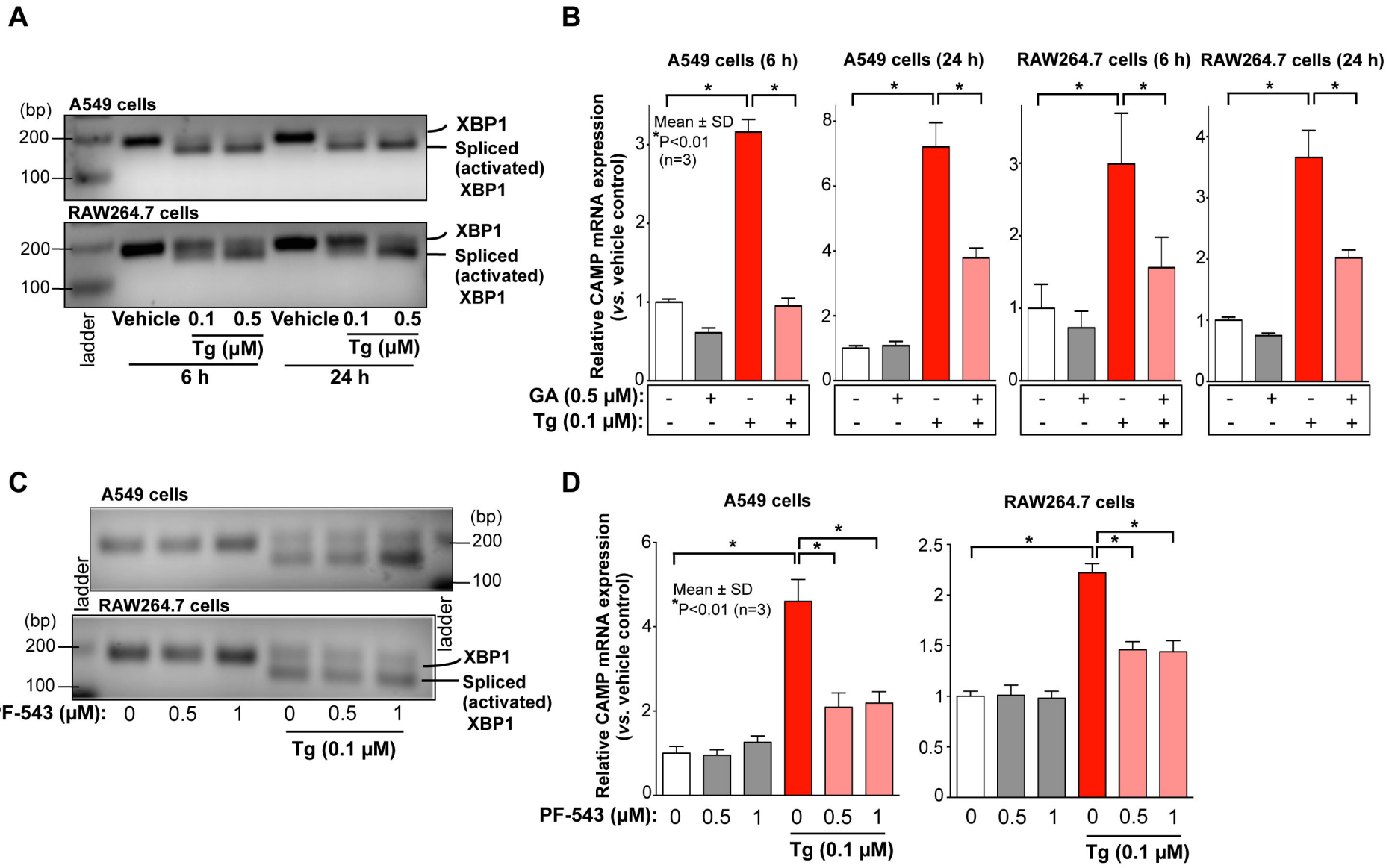

A
(bp)
A549 cells
200
100
XBP1
Spliced (activated) XBP1
RAW264.7 cells
200
100
XBP1
Spliced (activated) XBP1
ladder
Vehicle 0.1 0.5 Vehicle 0.1 0.5
Tg (μM)
Tg (μM)
6 h
24 h
B
A549 cells (6 h)
A549 cells (24 h)
RAW264.7 cells (6 h)
RAW264.7 cells (24 h)
Relative CAMP mRNA expression (vs. vehicle control)
Mean ± SD
*P<0.01
(n=3)
GA (0.5 μM):
Tg (0.1 μM):
C
A549 cells
(bp)
200
100
ladder
RAW264.7 cells
(bp)
200
100
ladder
XBP1
Spliced (activated) XBP1
PF-543 (μM): 0 0.5 1 0 0.5 1
Tg (0.1 μM)
D
A549 cells
RAW264.7 cells
Relative CAMP mRNA expression (vs. vehicle control)
Mean ± SD
*P<0.01 (n=3)
PF-543 (μM): 0 0.5 1 0 0.5 1
Tg (0.1 μM)

Table S1. TNFα-independent pathway accounts for regulation of CAMP expression

Treatment	Relative mRNA expression (*vs*. vehicle control)			
	MMP-9	**ICAM-1**	**IL-8**	**CAMP**
Vehicle	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.12
TNFα inhibitor[1] (0.5 μM)	0.59 ± 0.03	0.77 ± 0.05	0.74 ± 0.05	nd[2]
TNFα (10 ng/ml)	8.06 ± 0.40*	3.49 ± 0.18*	3.19 ± 0.17*	0.99 ± 0.32
TNFα + TNFα inhibitor	2.01 ± 0.14**	2.48 ± 0.12**	1.40 ± 0.07**	nd[2]
TNFα antibody (1 μg/ml)	0.92 ± 0.08	0.80 ± 0.06	1.05 ± 0.09	nd[2]
TNFα + TNFα antibody	1.25 ± 0.07**	1.03 ± 0.12**	1.11 ± 0.11**	nd[2]
Vehicle	nd[2]	nd[2]	nd[2]	1.00 ± 0.12
TNFα inhibitor	nd[2]	nd[2]	nd[2]	1.40 ± 0.17
Tg (0.02 μM)	nd[2]	nd[2]	nd[2]	7.45 ± 0.71*
Tm (0.12 μM)	nd[2]	nd[2]	nd[2]	6.24 ± 0.54*
Tg+TNFα inhibitor	nd[2]	nd[2]	nd[2]	7.06 ± 0.90*
Tm+TNFα inhibitor	nd[2]	nd[2]	nd[2]	6.48 ± 0.63*
Vehicle	nd[2]	nd[2]	nd[2]	1.00 ± 0.22
TNFα antibody	nd[2]	nd[2]	nd[2]	1.04 ± 0.21
Tg	nd[2]	nd[2]	nd[2]	7.80 ± 1.22*
Tm	nd[2]	nd[2]	nd[2]	11.75 ± 1.78*
Tg+TNFα antibody	nd[2]	nd[2]	nd[2]	7.68 ± 1.17*
Tm+TNFα antibody	nd[2]	nd[2]	nd[2]	11.45 ± 1.87*

Mean ± SD, $p<0.01$ (n=3), **vs*. Vehicle control, ***vs*. TNFα; [1]TNFα inhibitor, 6,7-Dimethyl-3-((methyl-(2-(methyl-(1-(3-trifluoromethyl-phenyl)-1H-indol-3-ylmethyl)-amino)-ethyl)-amino)-methyl)-chromen-4-one, diHCl; [2]nd, not determined