SUPPLEMENTAL MATERIALS

Gab2 Plays a Crucial Role in inflammatory Signaling and Endothelial Dysfunction

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Major Resources Table

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent - Male	Mice	The Jackson Laboratory Bar Harbor ME	B6.129S4-*Gab2*^{tm1Hhg}/Mmjax MMRRC Stock No: 32101-JAX | Gab2$^{-/+}$	# MMRRC032101	https://www.jax.org/strain/012590
Parent - Female	Mice	The Jackson Laboratory Bar Harbor ME	B6.129S4-*Gab2*^{tm1Hhg}/Mmjax MMRRC Stock No: 32101-JAX | Gab2$^{-/+}$	# MMRRC032101	https://www.jax.org/strain/012590

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
anti-human VCAM1 antibody	Cell Signaling Technology	13662	5µg/ml		https://www.cellsignal.com/products/primary-antibodies/vcam-1-e1e8x-rabbit-mab/13662?Ntk=Products&Ntt=13662
Anti-ICAM1 antibody	Santa Cruz Biotechnology	Sc-8439	1ug/ml		https://www.scbt.com/p/icam-1-antibody-g-5
Gab1 antibody	Santa Cruz Biotechnology	Sc-133191	1ug/ml	J2516	https://www.scbt.com/p/gab-1-antibody-h-7
Gab2 antibody	Santa Cruz Biotechnology	sc-365590	2ug/ml	A3017	https://www.scbt.com/p/gab-2-antibody-h-6
Gab2 rabbit antibody	Sigma-Aldrich	HPA001368	2ug/ml		https://www.sigmaaldrich.com/catalog/product/sigma/hpa001368?lang=en&region=US&gclid=Cj0KCQjw28T8BRDbARIsAEOMBcwoiSm2Gvn--IPKMLQVaBUmZj3WsOL6KlYxmK61z8lYkPLrzwoZDd4aAshCEALw_wcB

p-NF-Kb antibody	Cell Signaling Technology	3033	Concentration not specified by CST. Used 1;1000 dilution	-	https://www.cellsignal.com/products/primary-antibodies/phospho-nf-kb-p65-ser536-93h1-rabbit-mab/3033?Ntk=Products&Ntt=3033
p-ERK1/2 antibody	Cell Signaling Technology	4370S	Concentration not specified by CST. Used 1;1000 dilution	Lot17 Ref 01/2018	https://media.cellsignal.com/pdf/4370.pdf
p-Akt (S473) antibody	Cell Signaling Technology	9271S	Concentration not specified by CST. Used 1;1000 dilution	-	https://www.cellsignal.com/products/primary-antibodies/phospho-akt-ser473-antibody/9271
ERK antibody	Cell Signaling Technology	9102S	Concentration not specified by CST. Used 1;1000 dilution	-	https://www.cellsignal.com/products/primary-antibodies/p44-42-mapk-erk1-2-antibody/9102
Akt antibody	Cell Signaling Technology	9272	Concentration not specified by CST. Used 1;1000 dilution	Lot22 Ref; 06/2010	https://www.cellsignal.com/products/primary-antibodies/akt-antibody/9272
p-SAPK/JNK (Thr183/Tyr185) antibody	Cell Signaling Technology	9251	Concentration not specified by CST. Used 1;1000 dilution		https://www.cellsignal.com/products/primary-antibodies/phospho-sapk-jnk-thr183-tyr185-antibody/9251?Ntk=Products&Ntt=9251

p-p38-MAPK (Thr180/Tyr182) antibody	Cell Signaling Technology	4631	Concentration not specified by CST. Used 1;1000 dilution		https://www.cellsignal.com/products/primary-antibodies/phospho-p38-mapk-thr180-tyr182-12f8-rabbit-mab/4631?Ntk=Products&Ntt=4631
GAPDH antibody	Santa cruz Biotechnology	sc-365062	5ug/ml	-	https://www.scbt.com/p/gapdh-antibody-g-9
Ly-6G antibody	BioLegend	127602	5ug/ml	-	https://www.biolegend.com/fr-fr/search-results/purified-anti-mouse-ly-6g-antibody-4767
MPO antibody	R&D systems	AF3667	5ug/ml	-	https://www.rndsystems.com/products/human-mouse-myeloperoxidase-mpo-antibody_af3667
Histone H3 (Cit-Arg17, Arg2-Arg8)antibody	Novus Biologicals	NB100057135	5ug/ml		https://www.novusbio.com/products/histone-h3-antibody_nb100-57135

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
pMSCV-GAB2-IRES-GFP		Benjamin Neel, NYU School of medicine	
pUMVC		AddGene, MA	
VSVG		AddGene, MA	

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
HUVECs	Lonza	Unknown -pooled	https://bioscience.lonza.com/lonza_bs/US/en/Primary-and-Stem-Cells/p/000000000000184943/HUVEC-%E2%80%93-Human-Umbilical-Vein-Endothelial-Cells%2C-Pooled%2C-

Data & Code Availability

Description	Source / Repository	Persistent ID / URL

Other

Description	Source / Repository	Persistent ID / URL
Gab1 siRNA (5′-AAUACUACGAGUGUGUUAACU -3′	Sigma	-
Gab2 siRNA (5′-UUUAAGGCACACAUUCAGGGC -3′)	Sigma	
c-Src: siRNA 5′-CGGCTCCAGATTGTCAACAACACAG-3′	Sigma	
Fyn: siRNA 5′-GAGCGACAGCTATTGTCCTTTGGAA-3′	Sigma	
Yes: siRNA 5′-CAGGTGGTGTTACTATATTTG-3′	Sigma	

Reagents

TNFα, IL-1β, and ELISA kit for human MCP-1 were purchased from R&D Systems (Minneapolis, MN). LPS (*E. Coli* O111:B4) and Gab2 polyclonal antibodies were from Sigma Aldrich (St. Louis, MO). Phospho-specific or total antibodies against ERK, JNK, TAK1, p38 MAPK, c-jun, p65, Akt, TRAF2, RIP1, and VCAM1 were obtained from Cell Signaling Technology (Danvers, MA). Monoclonal antibodies against Gab2, ubiquitin, phospho-tyrosine, SHP2, PLCγ2, P85α, ICAM1, Cav1, c-Src, Fyn, Yes, and TNFR1 were procured from Santa Cruz Biotechnology (Dallas, TX). ELISA kits for mouse TNFα, IL-1β, IL-6, MCP1, and human IL-8 were purchased from Invitrogen (Carlsbad, CA). Ly-6G antibody was from BioLegend (San Diego, CA). Fibrin antibody was purchased from EMD Millipore, MA, USA.

Silencing of Gab1, Gab2, c-Src, Fyn, or Yes

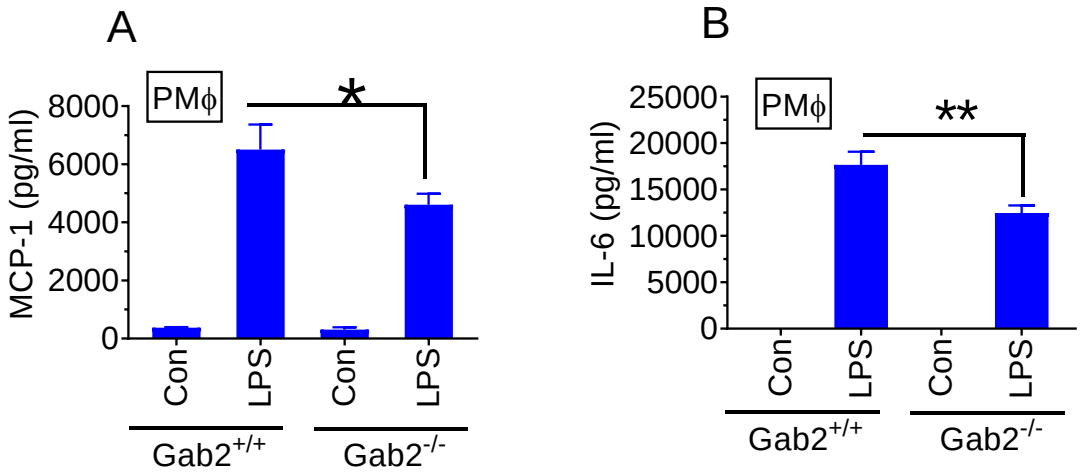
HUVEC were transfected with siRNA specific for Gab1, Gab2, c-Src, Fyn, or Yes using Lipofectamine RNAIMAX reagent (Invitrogen). The siRNA sequence for Gab1: AAUACUACGAGUGUGUUAACU, Gab2: UUUAAGGCACACAUUCAGGGC, Fyn: GAGCGACAGCTATTGTCCTTTGGAA, c-Src: CGGCTCCAGATTGTCAACAACACAG, Yes: CAGGTGGTGTTACTATATTTG. In controls, HUVEC were transfected with scrambled oligonucleotides of the above sequences. Briefly, when endothelial cell monolayers reached 70% confluency, they were washed once with serum-free medium, and then incubated with 200 nM of specific siRNA or a scrambled siRNA in Lipofectamine RNAIMAX reagent in serum-free medium. Eight hours post-transfection, the medium was changed to the growth medium (EBM-2 medium containing 2% FBS and growth supplements). The transfected cells were cultured for 48 h before they were used in experiments.

Plasmids, retrovirus, and RT-PCR

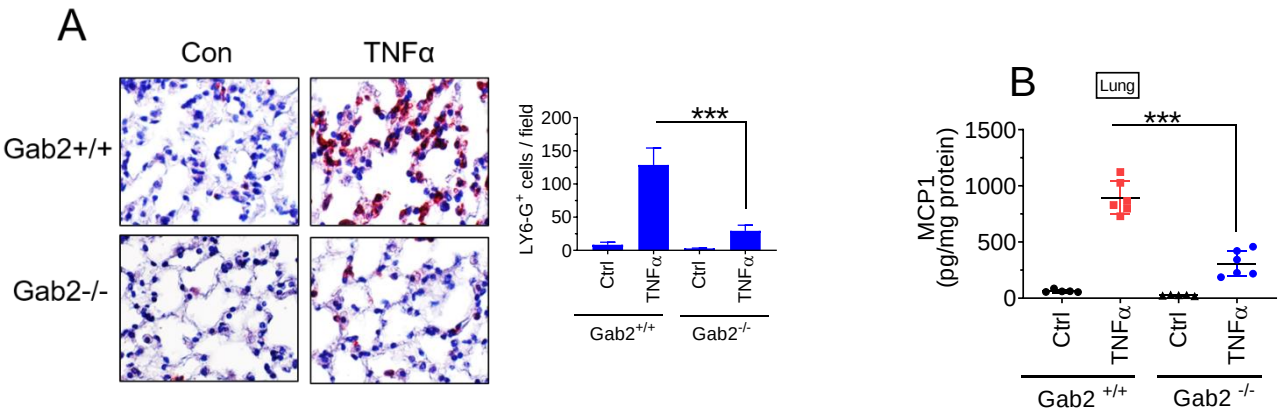
A retroviral construct expressing Gab2 (pMSCV-GAB2-IRES-GFP) was a gift of Benjamin Neel (NYU School of Medicine). To produce Gab2 retrovirus, 293 T17 cells (8×10^6) were co-transfected with the retroviral plasmid (4 μg) together with two packaging plasmids, pUMVC (3 μg), and VSVG (1 μg; Addgene, MA) using FuGENE HD (24 μl, Promega, WI). Control retrovirus (CV, pMSCV-IRES-GFP) was produced using the same procedure. Retrovirus harvested from cell supernatants 48 h post-transfection were filtered through 0.45 μm low protein-binding sterile filter.

HUVEC cells were infected with Gab2 or control retrovirus including polybrene (8 μg/ml; Sigma) during the infection. Experiments were conducted after 48 h of infection. In experiments involving the use of inhibitors of PI3K, SHP2, or TAK1, they were added to cells 1 h before stimulation of cells with agonists. At the end of the treatment period, the supernatant was removed, and the cells were solubilized in RNAzol. The total RNA was extracted, and cDNA was prepared by reverse transcription (iScript cDNA Synthesis Kit, Bio-Rad) and processed for qRT-PCR analysis (ABI PRISM 7000 Real-Time PCR System). The results were expressed as a relative expression or fold-increase over control.

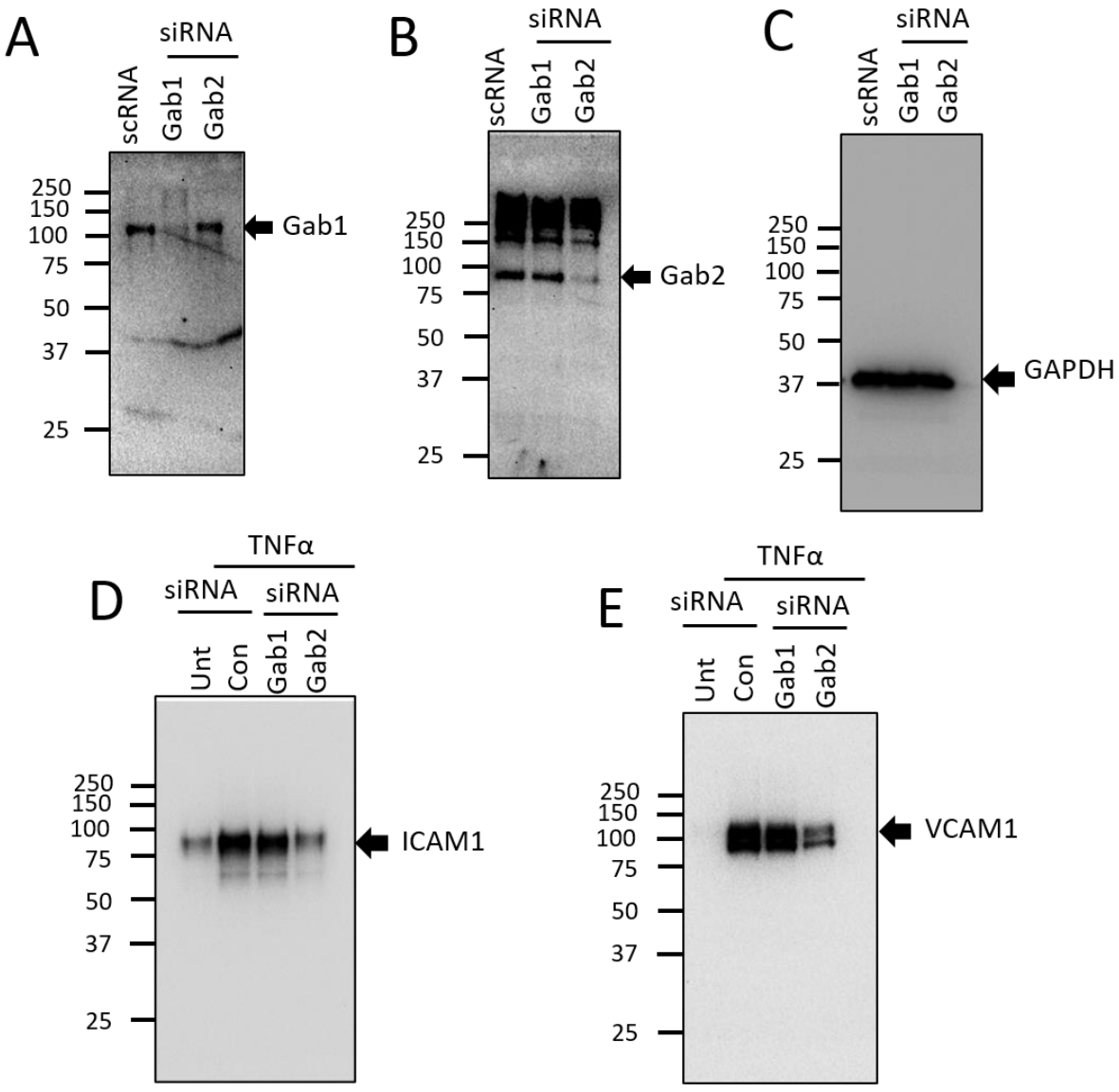
Supplementary figure I. Gab2 deficiency suppresses LPS-induced cytokine expression in peritoneal macrophages (PMϕ). Peritoneal macrophages were isolated from Gab2$^{-/-}$ and WT littermate control mice and treated with LPS (2 μg/ml) for 15 h. MCP-1 **(A)** and IL-6 **(B)** levels in the supernatants were measured in ELISA. *,$p < 0.05$; **, $p < 0.01$

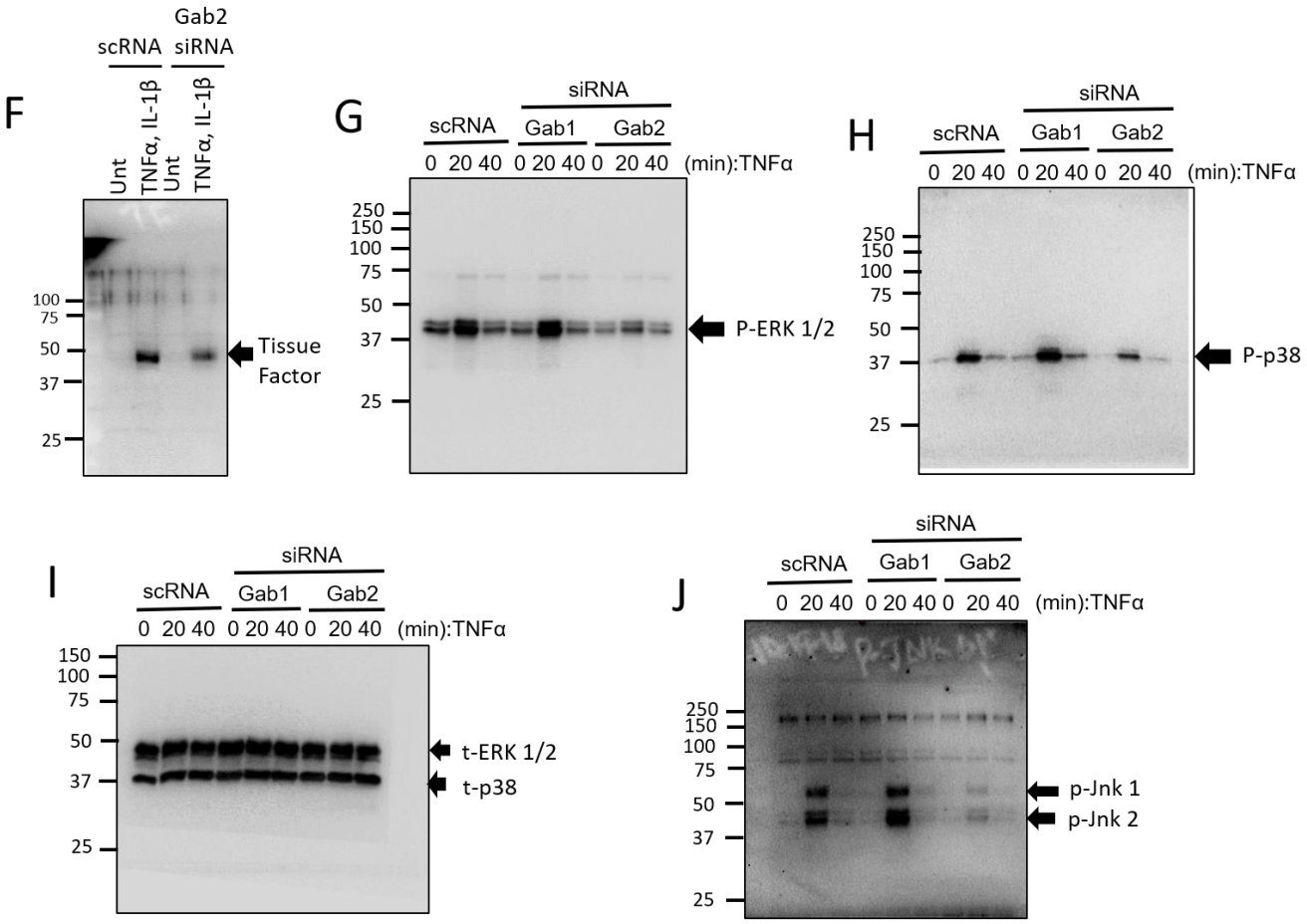


Supplementary Figure II. Gab2 deficiency attenuates TNFα-induced neutrophil infiltration and increased MCP-1 expression. Gab2$^{-/-}$ and WT littermate mice were injected with TNFα (50 µg/kg b.w) intravenously via the tail vein. Four h following TNFα injection, the mice were euthanized, the lung tissues were collected and processed for immunohistochemistry and measuring cytokines. **(A)** The lung tissue sections were stained with a Ly-6G antibody for neutrophil infiltration. **Left,** Representative images of tissue sections stained with LY-6G. **Right,** Count of LY-6G–stained cells/field. **(B)** MCP-1 levels in the lung tissue extracts.



Supplementary Figure III: Uncropped immunoblot images that correspond to cropped images shown in various figures as indicated in parenthesis) of the main manuscript. **(A)** Gab1, **(B)** Gab2, **(C)** GAPDH (Figure 1A); **(D)** ICAM1, **(E)** VCAM1 (Figure 1B); **(F)** TF (tissue factor) (Figure 1E); **(G)** p-ERK1/2, **(H)** p-p38, **(I)** t-ERK1/2 and t-p38, **(J)** p-JNK1/2 (Figure 3A); **(K)** p-p65 and p-c-jun, **(L)** p-Akt (Figure 3B); **(M)** Gab2 immunoprecipitation, **(N)** Gab2 tyrosine-phosphorylation (Figure 4A); **(O)** SHP2 (Figure 2A); **(P)** c-Src, **(Q)** Fyn, and **(R)** Yes (Figure 4D); **(S, T)** p-TAK1 (Figure 5A); **(U)** poly-Ub-TAK1 (Figure 5E).

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Gab2 siRNA
Unt
TNFα, IL-1β
Unt
TNFα, IL-1β
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75
50
37
25
Tissue Factor
G
scRNA
siRNA
Gab1
Gab2
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P-ERK 1/2
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siRNA
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Gab2
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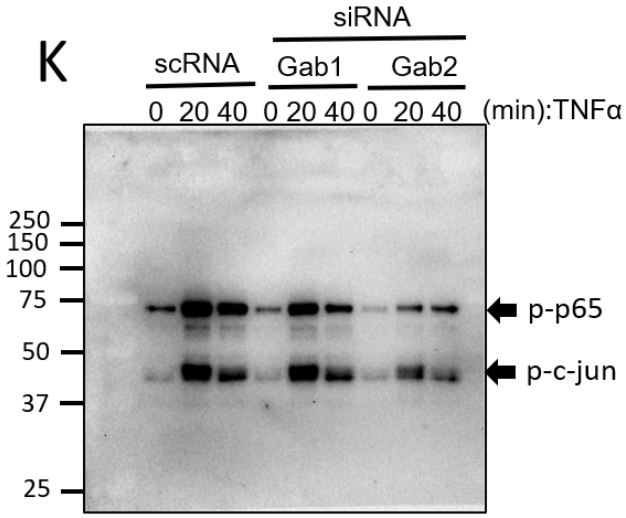
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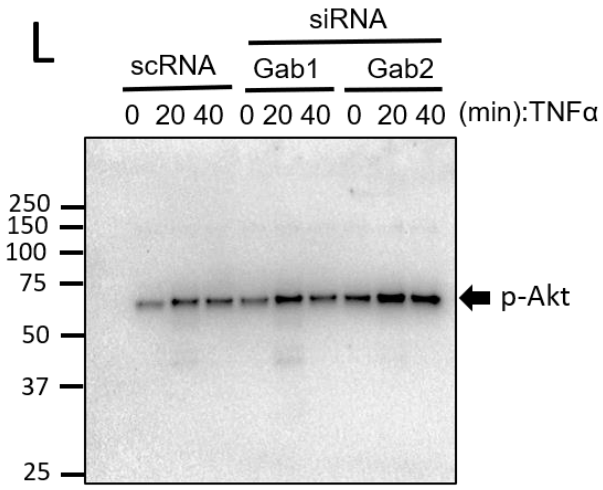
p-c-jun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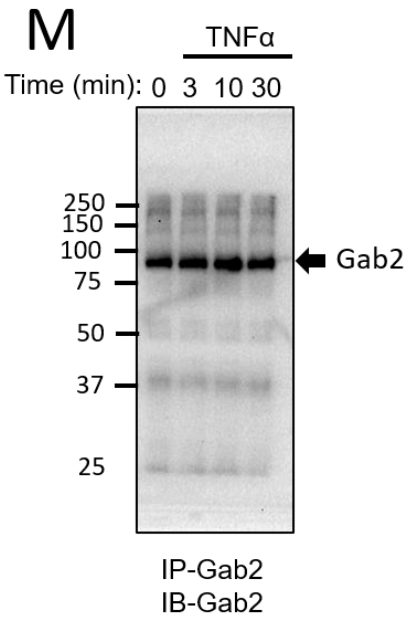
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100
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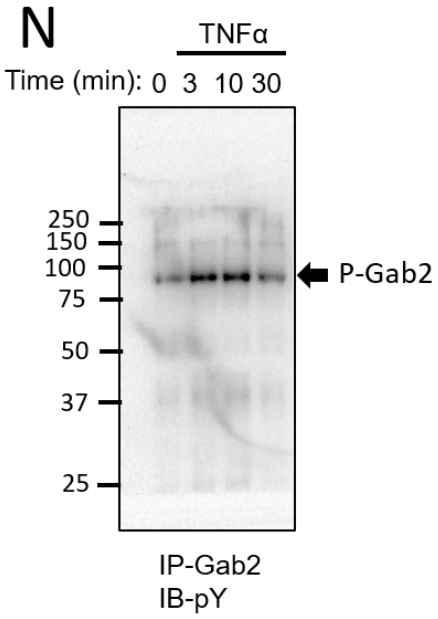
37
25
Gab2
IP-Gab2
IB-Gab2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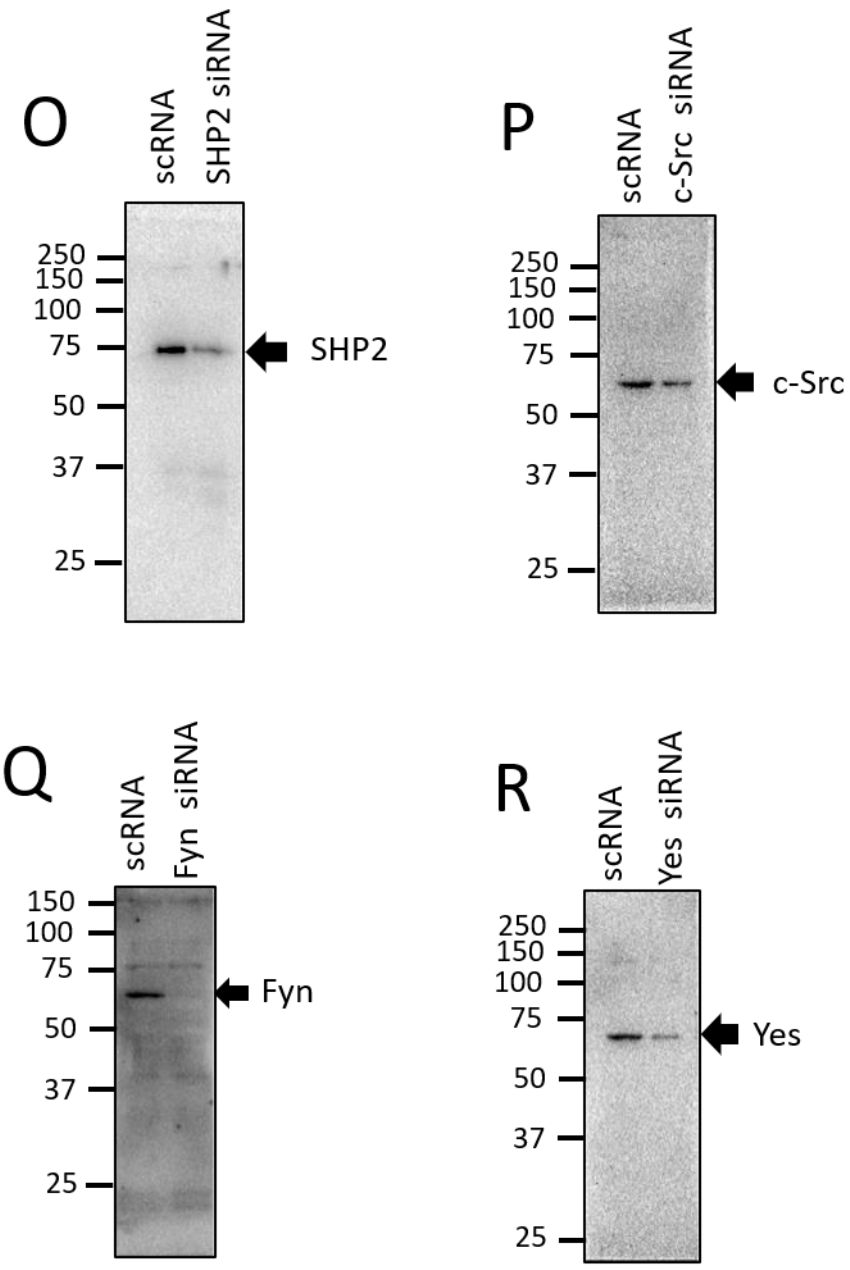
P-Gab2
IP-Gab2
IB-pY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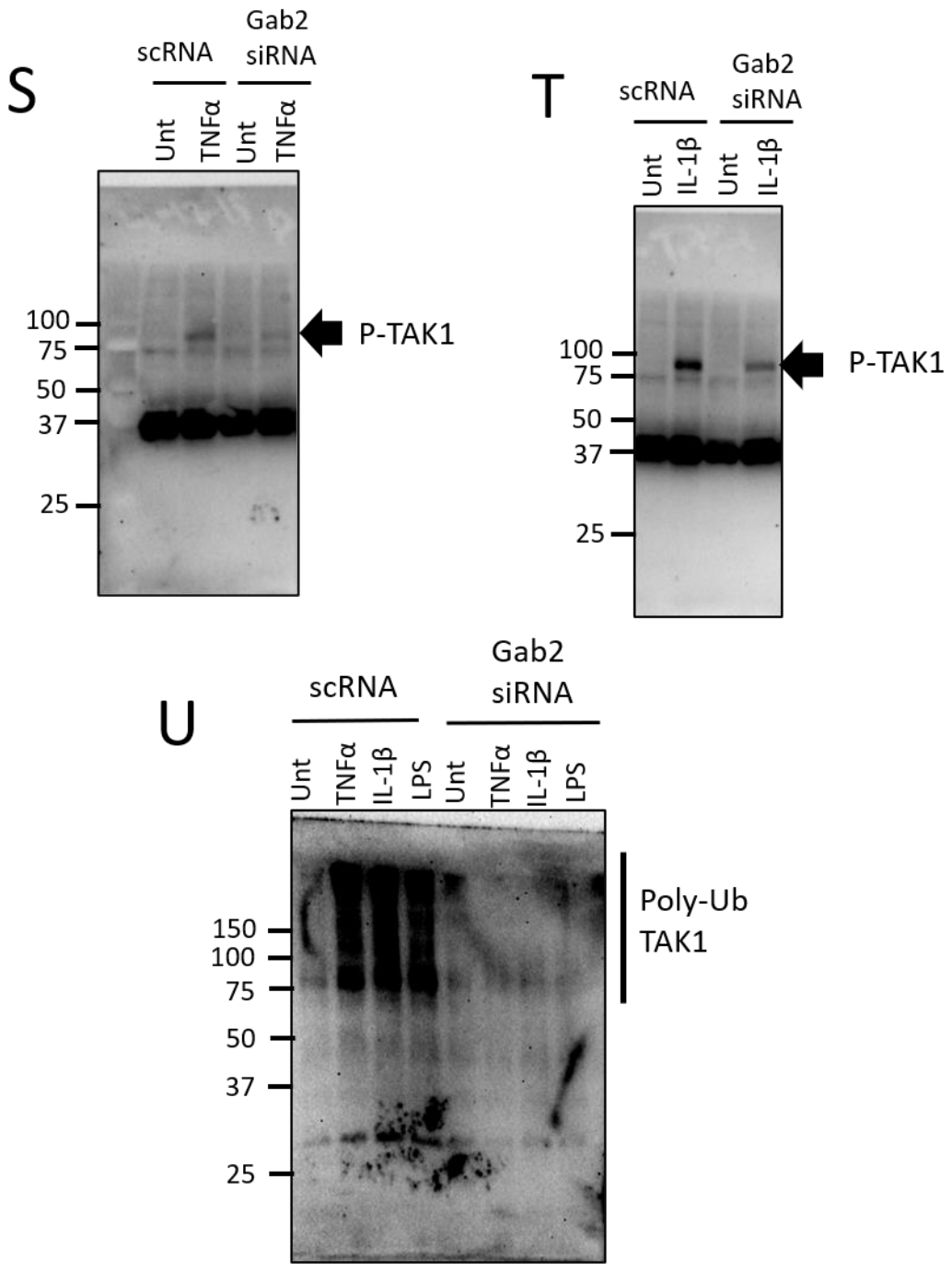
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