Supporting Information

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SI Materials and Methods

Cells and Constructs. Jurkat-KCa3.1 T cells were generated as previously described (1) and cultured in RPMI + 10% FBS. GFP-tagged class II PI3K C2α (PI3KC2α) and PI3KC2β were kindly provided by J. Domin, Imperial College, London, United Kingdom. The PI3KC2β kinase dead mutant was generated by substituting lysine 850 to arginine and tripartite motif containing protein 27 (TRIM27) Really Interesting New Gene (RING) mutant was generated by substituting C3/H1/C4/C5 in the cysteine-rich zinc binding domain to S3/Q1/S4/S5 (2).

All T-cell transfection were performed using AMAXA reagents according to the manufacturer's protocols (Amaxa Biosystems). Human CD4 T cells were isolated from peripheral adult blood buffy coats (New York Blood Center) using the CD4 isolation kit from Miltenyi Biotec according to manufacturer's protocol. We routinely obtained >95% CD4 T cells as assessed by FACS.

Antibodies. Anti-TRIM27 antibodies were purchased from IBL America and anti-PI3KC2β were purchased from BD Transduction Laboratories. Anti-PI3KC2β antibody M02 (clone 3E5) (Novus Biologicals) was used to both immunoblot and immunoprecipitate mouse PI3KC2β.

In Vivo Ubiquitination Assay. GFP-tagged PI3KC2α and PI3KC2β were expressed with or without FLAG-TRIM27 together with His_6-ubiquitin in HEK 293 cells. Transfected cells were then lysed in 6 M guanidinium and ubiquitinated proteins were purified using Nickel (Ni)-NTA beads followed by Western blotting with anti-GFP or anti-FLAG antibodies, as previously described (3).

To assess whether TRIM27 stimulates K48, K63, poly-, or monoubiquitination of PI3KC2β, HEK 293 cells were transfected with GFP-PI3KC2β and FLAG-TRIM27. Twenty-four hours later, cells were lysed in 1% SDS lysis buffer and, after diluting to 0.1% SDS, PI3KC2β was immunoprecipitated with anti-GFP antibodies and then Western blotted with the Lys48-specific antibody (Millipore; clone Apu2), the polyubiquitin-specific antibody (Millipore; clone FK1), of the Lys63-specific antibody (Millipore; clone Apu3), as previously described (4, 5).

Whole-Cell Patch Clamp. ***CD4 T cells.*** Whole-cell patch clamping was performed on activated CD4 T cells 48 h after stimulation with anti-CD3 and antiCD28 antibodies, as previously described (6), with some modification (7).

Jurkat-KCa3.1 T cells. Whole-cell patch clamping on Jurkat-KCa3.1 T cells was performed as previously described (1).

To verify that TRIM27 modulated KCa3.1 channel activity via PI3KC2β, which resulted in changes in levels PI3P, PI3P (100 nM) was added into the pipette solution during patch clamping in Jurkat-KCa3.1 cells overexpressing GFP-TRIM27 (8). PI(3)P diC16 [$C_{41}H_{45}Na_3O_{16}P_2$ (C_6)] as well as other PIs were purchased from Echelon Biosciences and used according to specifications at a concentration of 100 nM in the pipette solution.

siRNA Transfections and Lentiviral Infection with GFP-TRIM27(WT) or TRIM27(RING MT). For siRNA transfection, unstimulated human CD4 T cells or Jurkat-KCa3.1 cells were electroporated using AMAXA reagents (Amaxa Biosystems) according to the manufacturer's protocol. For human CD4 T cells, cells were rested overnight after transfection, and subjected to whole-cell patch clamp following stimulation for 2 d with anti-CD3 and anti-CD28 antibodies. Jurkat-Kca3.1 underwent whole-cell patch clamp 48 h after transfection (1). The following TRIM27 siRNAs (Qiagen) were used: siRNA 1, sense strand: 5′-GAGUUACUCGGGAGGGAAATT-3′; antisense strand: 5′-UUUCCCUCCCGAGUAACUCAG-3′; siRNA 2, sense strand: 5′-CACCUCUGUUAUGAGAUAATT-3′; antisense strand: 5′-UUAUCUCAUAACAGAGGUGGG-3′.

For rescue of siRNA-transfected cells, T cells were infected with a vesicular stomatitis virus-G pseudotyped HIV derived lentivirus expressing either FLAG-TRIM27(WT) or FLAG-TRIM27(RING MT) togther with internal ribosome entry site-GFP, as previously described (7, 9). GFP postive cells were then subjected to whole cell patch clamp.

Immunoprecipitation and Western Blots. Immunoprecipitation and Western blots were performed as previously described (8, 10).

Intracellular Ca^{2+} Activity. Cells were loaded at 1×10^6 cells/mL with 5 μM Fura-2:00 AM ester (Molecular Probes) in RPMI medium for 30 min at room temperature, washed, and then resuspended in RPMI. Cells were attached to poly(L)lysine-coated coverslips for 20 min in a RC-20 bath flow chamber (Warner Instrument) and fura-2 fluorescence was recorded (Delta Ram; PTI Inc.) at excitation wavelengths of 340 and 380 nm. Background fluorescence was obtained by treating the cells with 100 mM $MnCl_2$ in the end of the experiment. Data are represented as the ratio 340/380 after background subtraction. Cells were perfused with the bath solution (composition described before) in the presence or absence of extracellular Ca^{2+} and stimulated with 5 μg/mL of anti-CD3 cross linked with 5 μg/mL of rat anti-mouse IgG.

PI3K Assay. HEK 293 cells were transfected with GFP-PI3KC2β WT or kinase dead mutant alone or together with TRIM27 (WT) or TRIM27 (RING MT). Cells were lysed in the presence of proteinase inhibitors, phosphatase inhibitors, and 5 μM ubiquitin-aldehyde (Boston Biochem), and PI3K assay was performed on anti-GFP immunoprecipitates, as previously described (1). Lymphocytes from *TRIM27*$^{-/-}$ and *TRIM27*$^{+/+}$ mice were lysed as above and PI3K assay was performed on anti-PI3KC2β (Novus Biologicals) immunoprecipitates, also as previously described (1).

Generation of *TRIM27*$^{-/-}$ Mice. ES cell line (clone ID 345D11, strain 129/ola) that contained an exon-trapping plasmid pUPA integrated between exon 1 and exon 2 of the *TRIM27* gene was purchased from The Center for Modeling Human Disease at the University of Toronto. (http://www.cmhd.ca/genetrap/index.html). ES cells (strain 129/ola) were injected into C57BL/6 blastocyst by the transgenic facility at the New York University Langone Medical Center (New York) and chimeric mice were obtained. *TRIM27*$^{+/-}$ mice were backcrossed five generations with C57BL/6 and then used to generate *TRIM27*$^{-/-}$ C57BL/6 mice in these studies.

Primers used to genotype mice: TRIM27 F WT (mouse chromosome 13 genomic contig, GenBank Accession Number NT_039578.7, 10099908–10099931) CATTTAGCCACTCTCAGACTCGGG; TRIM27 R (mouse chromosome 13 genomic contig, GenBank Accession Number NT_039578.7, 10100140–10100166). AGCGAGTGAAATGATACAGGTGACAGC; pUPA F KO GATAAGTTGCTGGCCAGCTTACCTCCC. The WT PCR product (primers TRIM27 F WT and TRIM27 R) is 259 bp and the knockout PCR product (primers pUPA F KO and TRIM27 R) is 600 bp.

Purification and Differentiation TRIM27$^{-/-}$ and TRIM27$^{+/+}$ CD4 Th Subsets. $CD4^+$ T cells were purified on MACS beads (Miltenyi Biotech) from WT or TRIM27$^{-/-}$ spleens as previously described (11). Various CD4 T-cell subsets were generated by

culturing CD4 cells purified from spleen cells under Th1 polarizing conditions (100 U/mL IL-12 and anti–IL-4) or Th2 polarizing conditions (100 U/mL IL-4, anti–IFN-γ) for 4–6 d.

Quantitative Real-Time PCR. Total RNA was isolated using TRIzol reagent and then reverse transcribed using random hexamer primers. Quantitative PCR was then assessed using SYBR Green 1 by iCycler iQ (BioRad) using gene specific primers purchased from Qiagen.

Cytokine Assays. For cytokine assays, TRIM27$^{-/-}$ and TRIM27$^{+/+}$ CD4 T cells were stimulated for 48 h with anti-CD3/CD28 antibodies and, after resting overnight, were restimulated for 3 d together with splenocytes in U-bottom 96-well plates at a ratio of 1:8 (T cells:splenocytes) in the presence of various concentrations of staphylococcal enterotoxin E (*SEE*). Cytokines were assessed in supernatants using the BD Cytometric Bead Array Cytokine Kit.

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3. Campanero MR, Flemington EK (1997) Regulation of E2F through ubiquitin-proteasome-dependent degradation: Stabilization by the pRB tumor suppressor protein. *Proc Natl Acad Sci USA* 94:2221–2226.
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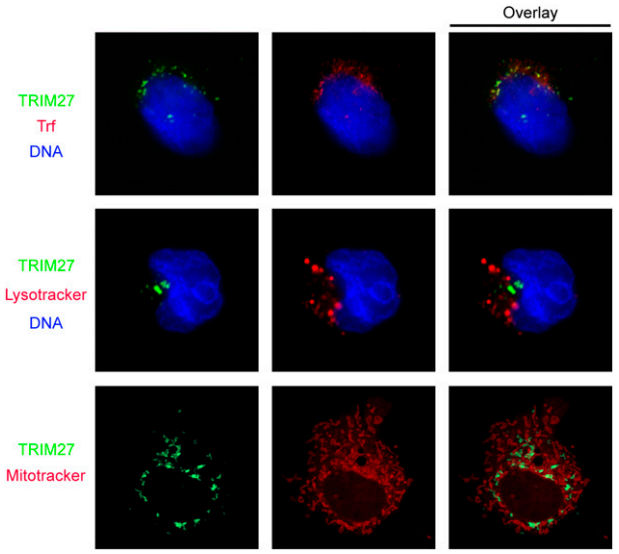

Fig. S1. TRIM27 partially colocalizes with the transferrin receptor. COS cells expressing GFP-TRIM27 were incubated with rhodamine-transferrin (trf), lysotracker, and mitotracker (Molecular Probes), and colocalization was assessed by fluorescence. Magnification, 60×.

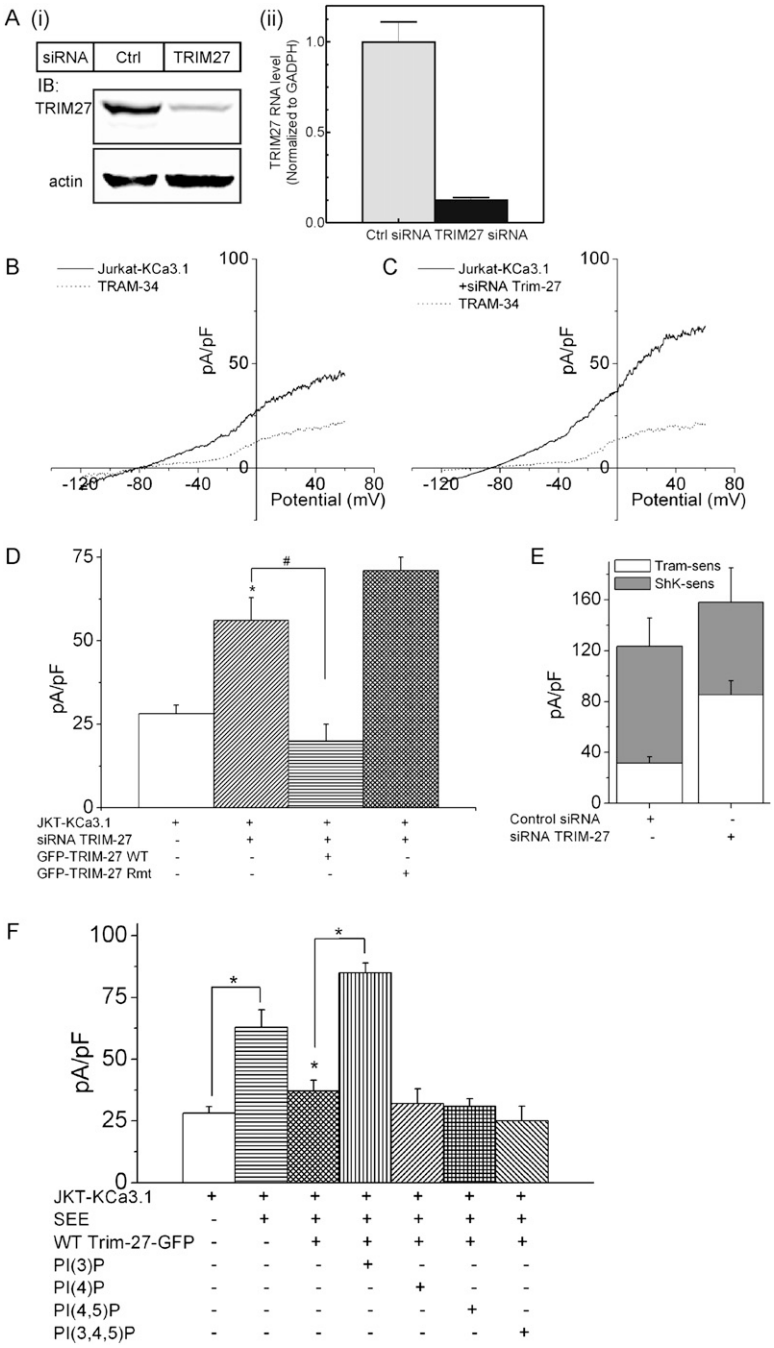

Fig. S2. TRIM27 negatively regulates KCa3.1 channel activity in Jurkat T cells overexpressing KCa3.1 (Jurkat-KCa3.1) and human CD4 T cells. siRNA knockdown of TRIM27 in Jurkat-KCa3.1 cells was assessed by (*Ai*) Western blot and (*ii*) real-time PCR. The Western blot in *Ai* was reprobed with β-actin to demonstrate equal loading between lanes, and the results in *Aii* show the relative amount of TRIM27 expression standardized to a GAPDH control ($n = 4$). Whole-cell patch clamp of (*B*) control or (*C*) TRIM27 siRNA-transfected cells. (*D*) Bar graph summary of TRAM34 inhibited currents plotted at +60 mV from Jurkat-KCa3.1 cells transfected with ctrl or TRIM27 siRNA shown in *B* and *C*. Shown are the average from 10 cells per condition. $*P < 0.001$. TRIM27 siRNA knockdown cells rescued with either FLAG-TRIM27(WT) and FLAG-TRIM27(RING MT). (*E*) Purified human CD4 cells were transfected with an siRNA to TRIM27 and then stimulated with anti-CD3 and CD28 antibodies for 48 h. Shown are the bar graph summary of TRAM34 (KCa3.1) and Shk (Kv1.3) inhibited currents plotted at +60 mV. (*F*) PI3P (100 nM) was added into the pipette solution during patch clamping in Jurkat-KCa3.1 cells overexpressing GFP-TRIM27. Plotted are means ± SE from at least three independent experiments. Also shown in *F* is the failure of PI4P, PI(4,5)P_2, or PI(3,4,5)P_3 to rescue. $*P < 0.05$.

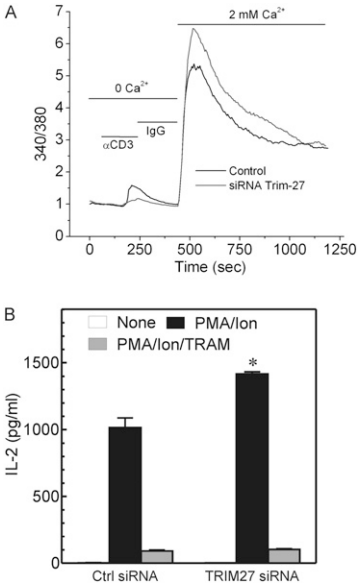

Fig. S3. Increased Ca^{2+} influx and IL-2 production following siRNA knockdown of TRIM27 in Jurkat-KCa3.1 cells. (*A*) Cells in Fig. S2 were loaded with Fura-2:00 AM (5 mM) and Ca^{2+} flux was determined after cross-linking with anti-CD3 antibodies. (*B*) Control or TRIM27 siRNA-transfected cells were cultured with phorbol myristate acetate/Ionomycin, and IL-2 secretion in the supernatant was determined 24 h after stimulation in the absence or presence of TRAM34, as described. $*P < 0.05$.

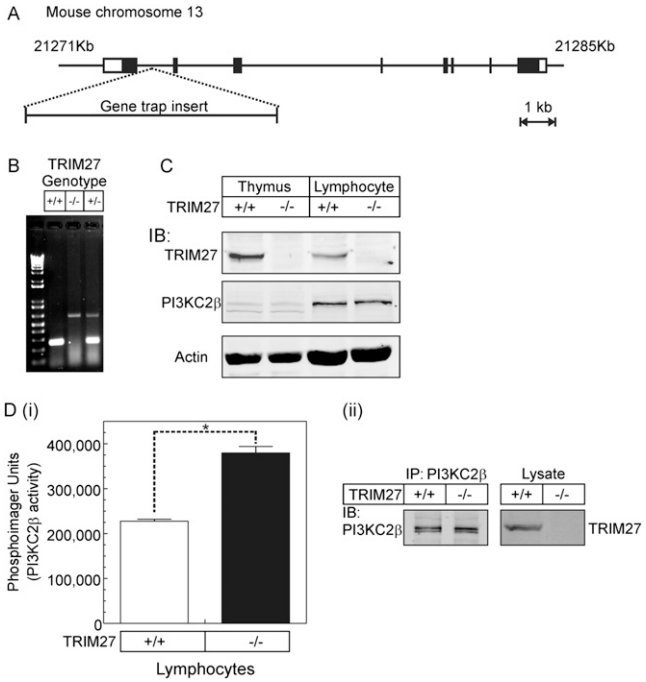

Fig. S4. Generation of *TRIM27*$^{-/-}$ mice. (*A*) Schematic representation of the *TRIM27* gene of clone 345D11 showing the insertion of pUPA in the intron between exons 1 and 2 of the *TRIM27* gene. (*B*) To amplify the WT *TRIM27* allele, oligoneucleotides were synthesized that were complementary to genomic DNA on either side of the insertion site of pUPA and used to amplify the WT locus by PCR (bp 259). The mutant TRIM27 locus was amplified by PCR using the same 3′ oligo used to amplify the WT locus and an oligo close to the 3′ end pUPA (bp 600). (*C*) Total lysates were isolated from spleen and thymus of *TRIM27*$^{-/-}$ and *TRIM27*$^{+/+}$ mice and Western blotted with antibodies to TRIM27 and PI3KC2β. The same blot was then reprobed with antibodies to β-actin to demonstrate equal loading of protein in both lanes. (*Di*) Lymphocytes from *TRIM27*$^{-/-}$ and *TRIM27*$^{+/+}$ mice were lysed and PI3K assay was performed on anti-PI3KC2β immunoprecipitates. $*P < 0.05$. (*ii*) Fifty percent of the immunoprecipitate in *Di* was immunoblotted with anti-PI3KC2β antibodies to demonstrate equal amounts of PI3KC2β protein in the two samples. Lysates were also immunoblotted with anti-TRIM27 antibodies.

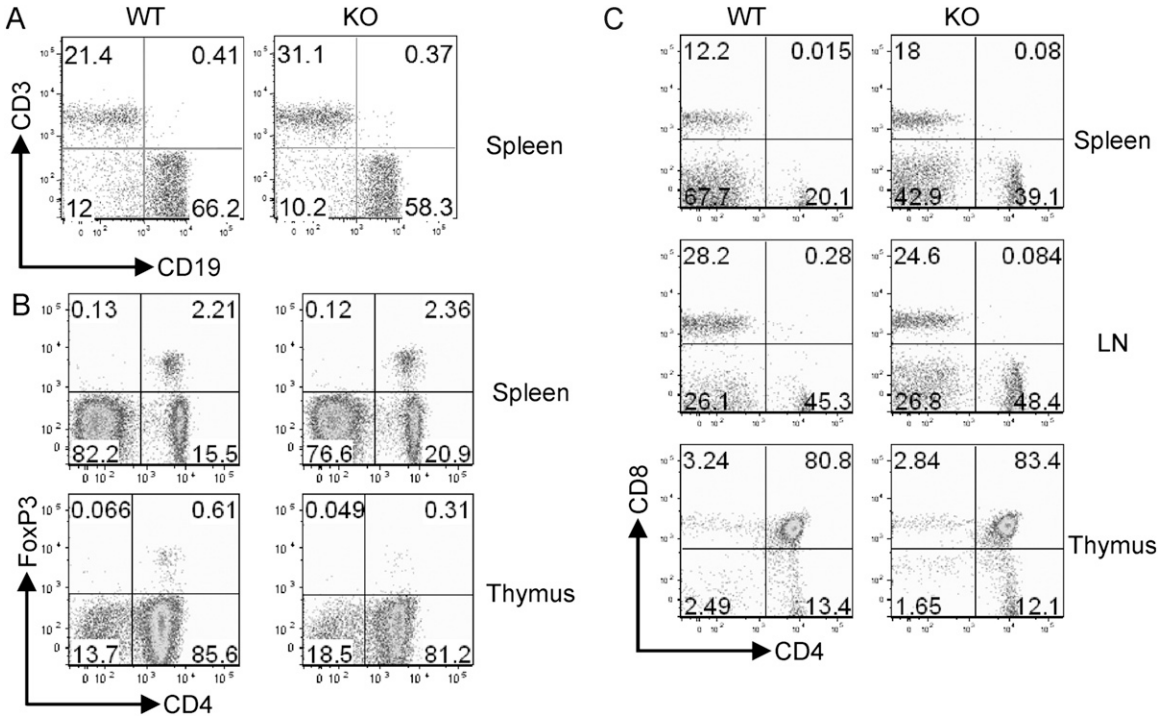

Fig. S5. T- and B-cell development is normal in *TRIM27*$^{-/-}$ mice. Cells were isolated from spleen, thymus, and lymph node (LN) from WT and *TRIM27*$^{-/-}$ mice and stained with antibodies to (*A*) CD3 and CD19, (*B*) CD4 and FoxP3, or (*C*) CD4 and CD8 followed by FACS analysis. All experiments shown are representative of at least four experiments performed on cells isolated from at least four pairs of independent mice.

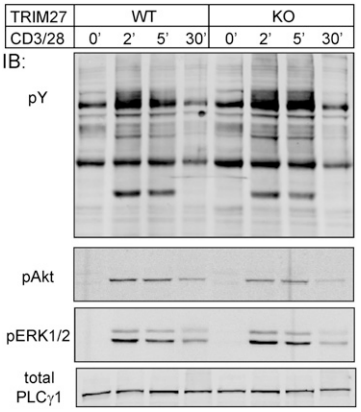

Fig. S6. Activation of T-cell receptor (TCR) stimulation of proximal signaling pathways is similar between TRIM27$^{-/-}$ and WT Th0 cells. TRIM27$^{-/-}$ and WT Th0 cells were stimulated with anti-CD3/CD28 antibodies for various times and Western blotted with antibodies to phosphotyrosine (pY), phospho-AKT (pAKT), or phospho-ERK (pERK1/2).

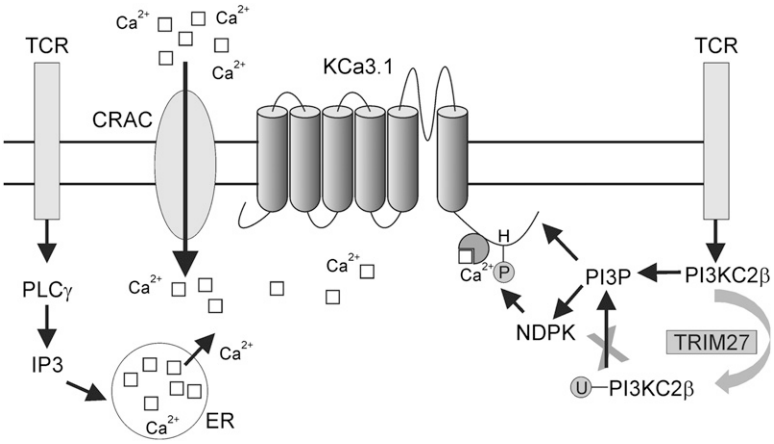

Fig. S7. Model for TCR-stimulated activation of KCa3.1 mechanism for regulation by TRIM27. Activation of two signaling pathways is required for TCR-stimulated activation of KCa3.1 and T-cell activation. Signal 1: TCR activation of PLCγ results in the generation of IP_3, stimulating release of Ca^{2+} from the endoplasmic reticulum, opening of calcium release-activated Ca^{2+} channels (CRAC), and the influx of Ca^{2+}; signal 2: TCR stimulation also activates PI3KC2β leading to the generation of PI(3)P, which is required for nucleoside diphosphate kinase B (NDPK-B) to phosphorylate histidine 358 in the carboxy-terminus (CT) of KCa3.1. Both binding of Ca^{2+} to the calmodulin bound to the CT of KCa3.1 and phosphorylation of H358 in CT of KCa3.1 by NPDK-B is required for KCa3.1 activation. TRIM27 inhibits signal 2 by ubiquitinating and inhibiting PI3KC2β's activity.