Supplemental material 1

Tabel S1 shows all primer used in this study. Fig. S1 shows the quantification of the localization of GFP and GFP-tagged PH domains at the plasma membrane. Fig. S2 shows the interaction of the purified isolated PH of SKAP55 with F- or G-Actin *in vitro.* Fig. S3 shows expression levels of the TCR and CD18 upon knockdown of SKAP55 and re-expression of wild type or various SKAP55 mutants. Fig. S4 shows a structural comparison of the isolated PH domain and the DM-PH tandem of SKAP55. Fig. S5 shows a model of the recruitment of the ADAP/SKAP55-module and LFA-1 activation in T cells. Fig. S6 shows that the deletion of the PH domain within full length SKAP55 did not attenuate TCR-mediated adhesion.

Table S1

Name of the primer	Sequence of the primer
K116M SKAP55-for	5'-ATCAAGCAAGGATACTTGGAGAtGAAAAGCAAAGATCATAGTTTC-3'*
K116M SKAP55-rev	5'-GAAACTATGATCTTTGCTTTTCaTCTCCAAGTATCCTTGCTTGAT-3'*
R131M SKAP55-for	5'-TGGATCGGAGTGGCAGAAGatgTGGTGTGTTGTCAGCAGAGG-3'*
R131M SKAP55-rev	5'-CCTCTGCTGACAACACACCAcatCTTCTGCCACTCCGATCCA-3'*
K152E SKAP55-for	5'-GAGAAGAGCAAGCAGCCCgAgGGGACCTTCCTCATTAAG-3'*
K152E SKAP55-rev	5'-CTTAATGAGGAAGGTCCCcTcGGGCTGCTTGCTCTTCTC-3'*
K152M SKAP55-for	5'-GAGAAGAGCAAGCAGCCCAtgGGGACCTTCCTCATTAAG-3'*
K152M SKAP55-rev	5'-CTTAATGAGGAAGGTCCCcaTGGGCTGCTTGCTCTTCTC-3'*
D120K SKAP55-for	5'-TACTTGGAGAAGAAAAGCAAAaAaCATAGTTTCTTTGGATCGGAGTGG-3'*
D120K SKAP55-rev	5'-CCACTCCGATCCAAAGAAACTATGtTtTTTGCTTTTCTTCTCCAAGTA-3'*
D120A SKAP55-for	5'-TACTTGGAGAAGAAAAGCAAAGcTCATAGTTTCTTTGGATCGGAGTGG-3'*
D120A SKAP55-rev	5'-CCACTCCGATCCAAAGAAACTATGAgCTTTGCTTTTCTTCTCCAAGTA-3'*
SK55-shRNA-for	5´-GATCCCCGAAAGAATCCTGCTTTGAATTCAAGAGATTCAAAGCAGGATTCTTTCTTTTTGGAAA-3´
SK55-shRNA-rev	5´-AGCTTTTCCAAAAAGAAAGAATCCTGCTTTGAATCTCTTGAATTCAAAGCAGGATTCTTTCGGG-3´
Mlu1SKAP55-for	5'-CCCACGCGTATGCAGGCCGCCGCCCTC-3'#
Not1 SKAP55-rev	5'-CCCGCGGCCGCTCATCTTTCTTCCACTTC-3'#
SKAP55 sh-res-for	5'CGAAGAGATTCCAAGAAgGAgTCgTGtTTcGAgCTGAACTCCCAGGATAGG-3'
SKAP55 sh-res-rev	5'-CCTATCCTGGGAGGTCAGcTCgAAaCAcGAcTCcTTCTTGGAATCTCTTCG-3'

***** Lowercase letters indicate changed nucleotide

Underlined sequences indicate restriction sites

Figure S1. Localization and quantification of GFP and GFP-tagged fusion proteins at the PM

(A) Jurkat T cells were transfected with constructs encoding GFP the GFP-tagged PH domains of AKT, PLCδ or SKAP55. 24h after transfection, cells were fixed, permeablized, stained with TRITC-phalloidin to visualize F-Actin, and then imaged by confocal laser scanning microscopy. The fluorescence intensities of GFP or the GFP-tagged proteins (green curves) together with the F-Actin (red curves) were measured at 9 o'clock in fluorescence profiles perpendicularly to the cell membrane (indicated by white bars) a selfmade MATLAB (Mathworks Inc.) tool. (B) The obtained profiles were analyzed by determining the middle of the F-Actin curve (red) and dropping a perpendicular that divides the green curve (GFP-tagged proteins) in halves (areas A and B). (C) Using Adobe Photoshop CS3, the area A (close to the membrane) and B (cytoplasm) were determined to calculate the ratio of fluorescence intensity at the plasma membrane of individual cells as described in the Material and Method section. Bar at the lower right corner: 5µm

Figure S2. *In vitro* interaction studies of the isolated PH domain of SKAP55 with F- or G-Actin.

20 μ g of the purified His-tagged wild-type PH domain of SKAP55 (WT) or of the purified His-tagged K152E mutant of SKAP55 (K152E) were incubated in the absence (-) or presence of F-Actin (+) for 30min at room temperature. Proteins were used in a co-sedimentation assay by high-speed ultracentrifugation (150.000g). Pellet (P) and supernatant (S) were analyzed by SDS-PAGE and proteins were visualized by Coomassie blue staining (left panel). The F-Actin-binding protein α-Actinin served as positive control (right panel) (n=2). (B) 20 μ g of the purified wild type His-tagged PH domain of SKAP55 (WT) or of the His-tagged K152E mutant of SKAP55 (K152E) were incubated in the absence (-) or presence of G-Actin (+) for 30min at room temperature. Upon initiation of F-Actin polymerization for 30 min at 24° C

proteins were used in a co-sedimentation assay by high-speed ultracentrifugation (150.000 g). Pellet (P) and supernatant (S) were analyzed by SDS-PAGE and proteins were visualized by Coomassie blue staining (left panel). The F-Actin-binding protein α-Actinin served as positive control (right panel) (n=2). (C) F- or G-Actin was incubated with 20 μ g of the purified His-tagged PH domain of SKAP55 (WT) for 1h at 4° C. His-tagged PH domain of SKAP55 was immunoprecipitated using anti-His mAbs coupled to Protein G agarose. Precipitates of anti-His mAbs conjugated to Protein G agarose only in the presence of F- or G-Actin served as controls to exclude unspecific binding of antibodies and Protein G agarose to Actin Precipitates were analyzed by SDS-PAGE and proteins were visualized by Coomassie blue staining (n=2). Positions of Actin, α-Actinin and the PH domain of SKAP55 are indicated.

Figure S3. Expression levels of the TCR and CD18 upon knock-down of SKAP55 and re-expression of wild type or various SKAP55 mutants.

(A) Jurkat T cells were transfected with suppression/re-expression constructs that do not suppress endogenous SKAP55 (shC), or reduces the protein level of SKAP55 (shSKAP55) and re-express a FLAG-tagged shRNA-resistant wild type form of SKAP55 (WT) or SKAP55 mutants (K116M, R131M, and K152E). Transfectants were used for flow cytometric analysis of the TCR and CD18 surface expression within the GFP gate. (n=2) (B) Jurkat T cells were transfected with suppression/re-expression constructs that do not suppress endogenous SKAP55 (shC), or reduces the protein level of SKAP55 (shSKAP55) and re-express a FLAG-tagged shRNA-resistant wild type form of SKAP55 (WT) or SKAP55 mutants (D120K, K152E, or D120K/K152E). Transfectants were used for flow cytometric analysis of the TCR and CD18 surface expression within the GFP gate. (n=2)

Figure S4. Structural comparison of the PH domain and the PH-DM tandem of SKAP55

(A) Aspartatic acid D129 or lysine K161 in SKAP-HOM corresponds with D120 or K152 in SKAP55 (in bold). (B) Shown is the overlay of ^{1}H-^{15}N HSQC spectra of 270µM isolated PH domain of SKAP55 (blue) with 135µM SKAP55 of the DM-PH tandem (cyan). Some significantly shifting residues are indicated for illustration. (C) Residues in the SKAP-HOM PH domain homologous to the residues shifting significantly in the SKAP55 PH domain in the presence of the DM domain are coloured cyan in the structure of SKAP-HOM DM-PH (PDB code: 2otx). The β1-β2-loop residues that are not assigned in NMR spectra (including residue D120) are colored orange. The structure is overlayed with IP_4 co-crystallized with the PH domain of AKT (1unq) in order to indicate its likely position. Residues K116, R131, D120 and K152 are shown as sticks.

Figure S5. Model of the ADAP/SKAP55-module recruitment and LFA-1 activation in T cells

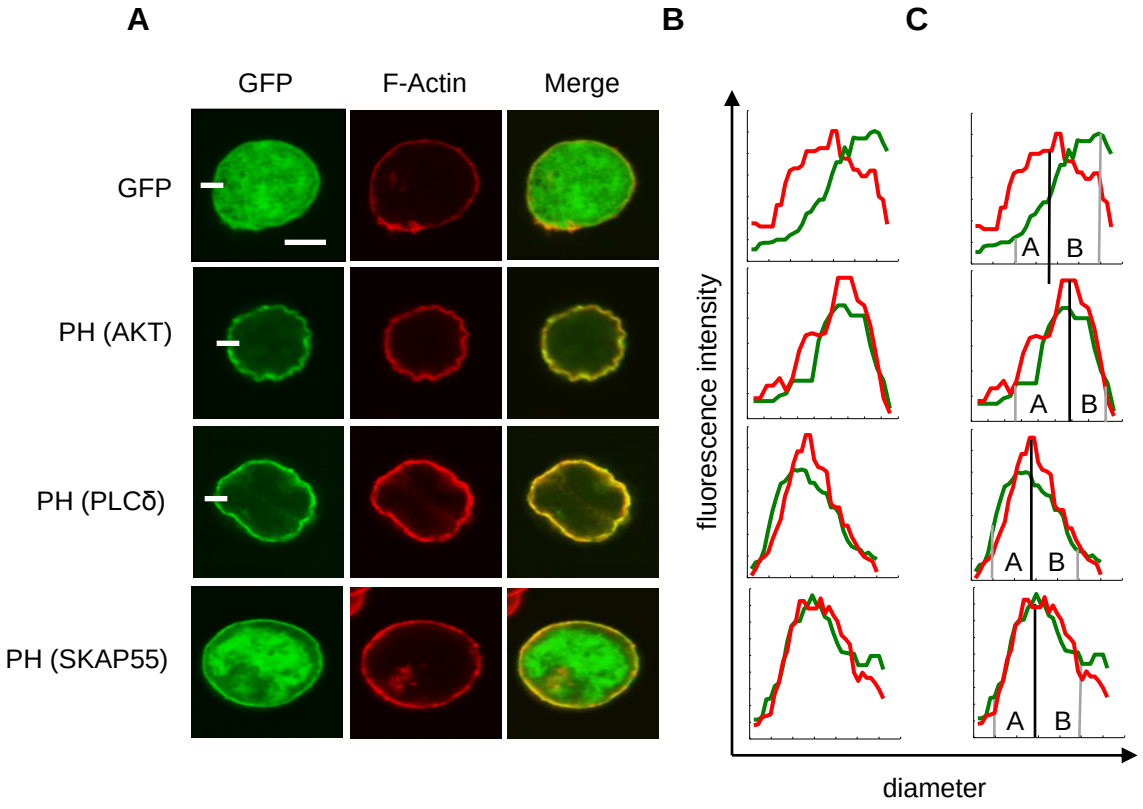
We have recently identified two pools of the ADAP/SKAP55-signaling module that are either associated with RIAM or RAPL in resting T cells (A). (B) Upon TCR-triggering SKAP55 can use two independent (or complementary) routes for plasma membrane targeting. One route would encompass an inducible association of the ADAP/SKAP55-module with SLP-76 which itself binds via Gads to phosphorylated LAT, whereas the second route would involve Actin binding via the released PH domain of SKAP55 to provide a link between LFA-1 and Talin.

Figure S6. Deletion of the PH domain within full-length SKAP55 did not attenuate TCR-mediated adhesion.

(A) Jurkat T cells were transfected with suppression/re-expression constructs which do not suppress endogenous SKAP55 (shC), or reduce the protein level of SKAP55 (shSKAP55) and re-express a FLAG-tagged shRNA-resistant wild type form of SKAP55 (WT) or the deletion of the PH domain within full length SKAP55 (ΔPH). 48h after transfection, lysates were

analyzed by Western blotting with the indicated antibodies. (B) Jurkat T cells transfected as described in A were analyzed for their ability to adhere to ICAM-1-coated wells in a resting state or upon stimulation with two different concentrations of CD3 antibodies (1 μ g/ml and 5 μ g/ml), respectively. Adherent cells were counted and calculated as percentage of input (n=3). (mean $\pm$ SD; *p$\leq$0.05; **p$\leq$0.01) (C) Jurkat T cells transfected as described in A were left untreated or stimulated with CD3 antibodies (CD3). Lysates were used for immunoprecipitation of LFA-1 using anti-CD11a antibodies. Precipitates were analyzed by Western blotting using the indicated antibodies (n=2).

Figure S1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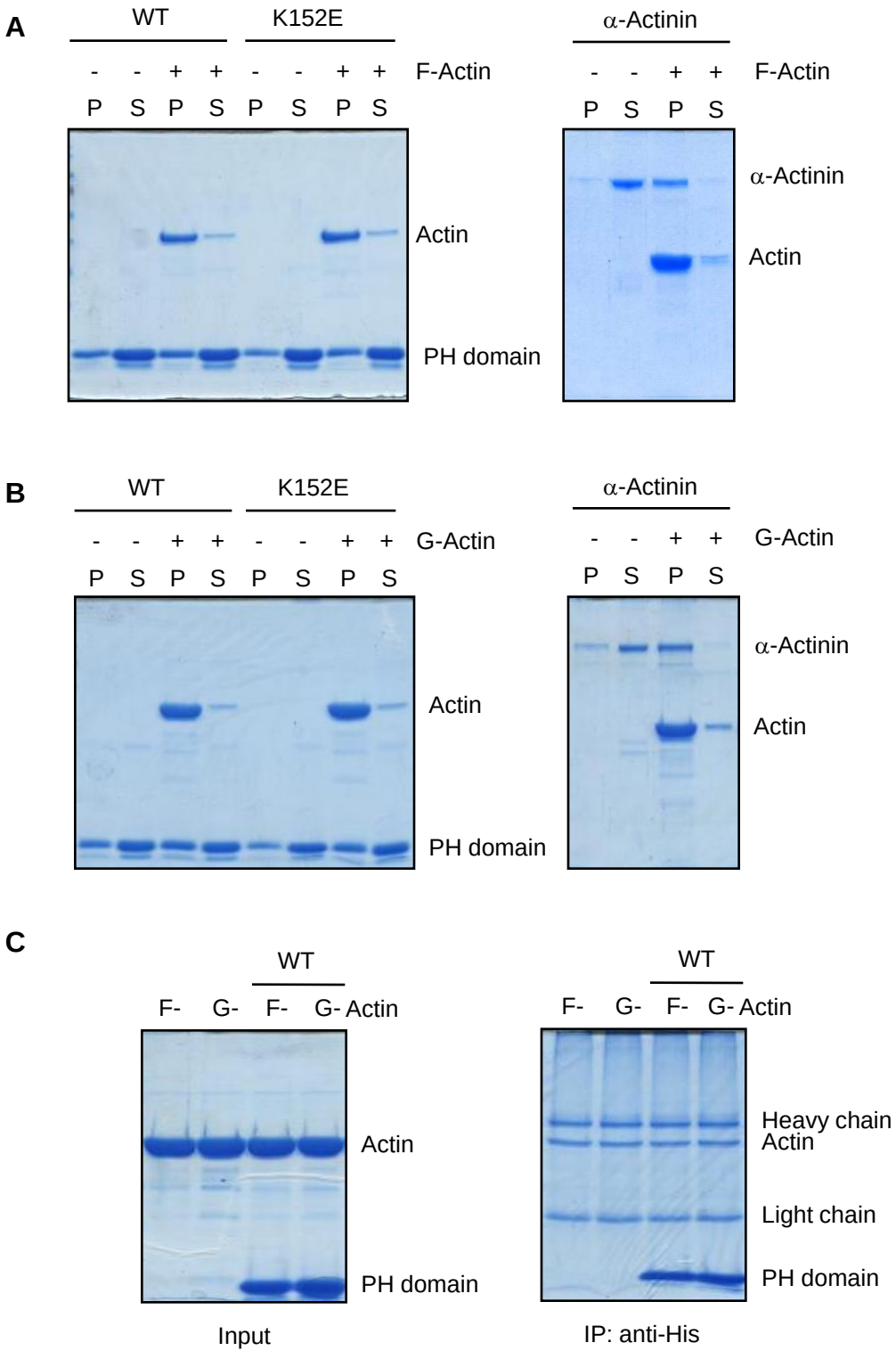
Merge
GFP
PH (AKT)
PH (PLCδ)
PH (SKAP55)
fluorescence intensity
diameter
A
B

Figure S2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B
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C
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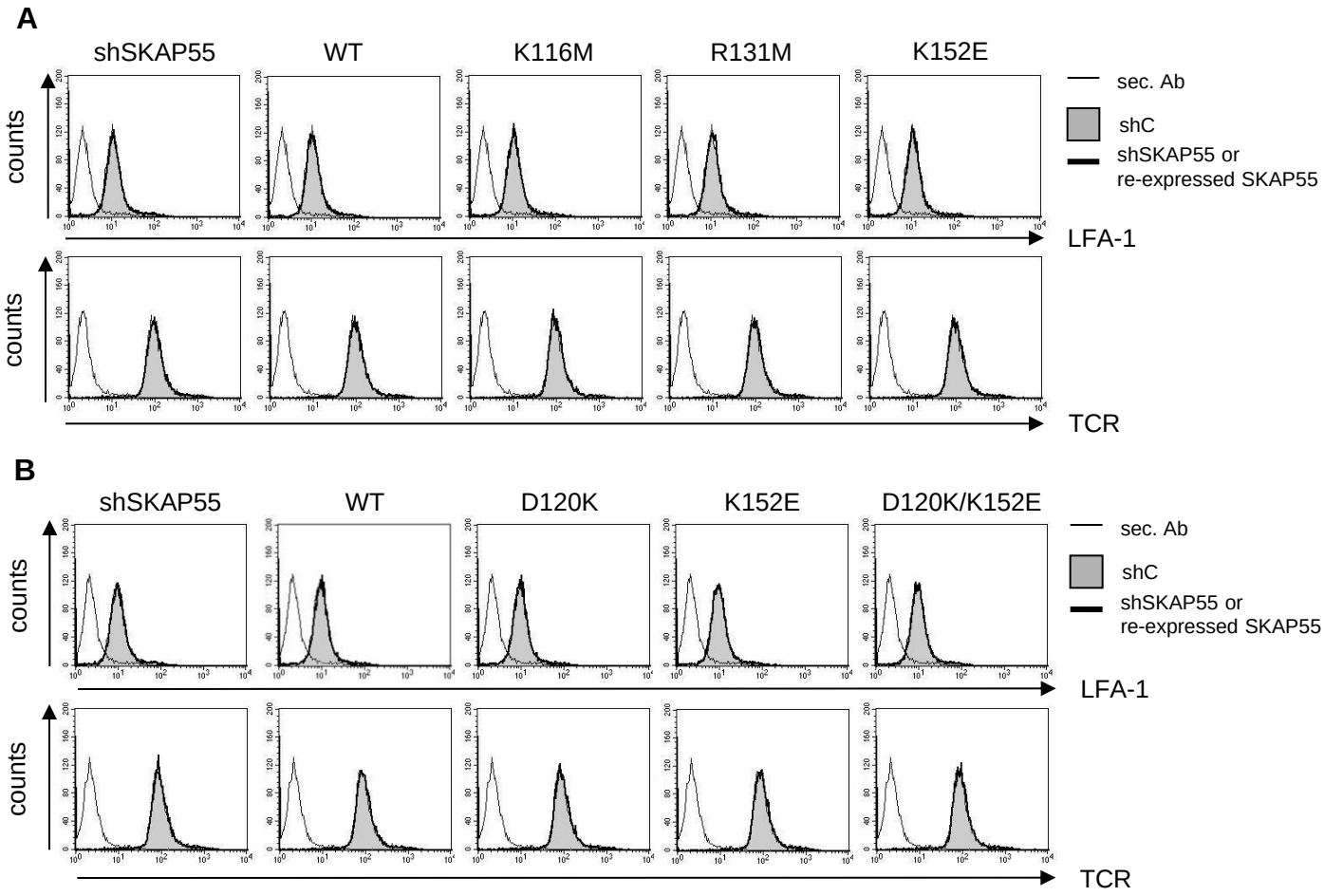
PH domain
IP: anti-His

Figure S3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WT
K116M
R131M
K152E
counts
LFA-1
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shC
shSKAP55 or
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B
shSKAP55
WT
D120K
K152E
D120K/K152E
counts
LFA-1
TCR
sec. Ab
shC
shSKAP55 or
re-expressed SKAP55

Figure S4

A

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SKAP-HOM
VLKAGYLEKRRKDHSFLGFEWQKRWCALSKTVFYYYGSDKDKQQKGEFAIDGYSVRMNNTLRKDGKKDCCFEISAPDKRIYQFTAASPKDAEEWVQQLKFVLQ
SKAP55
                  120                                                 152
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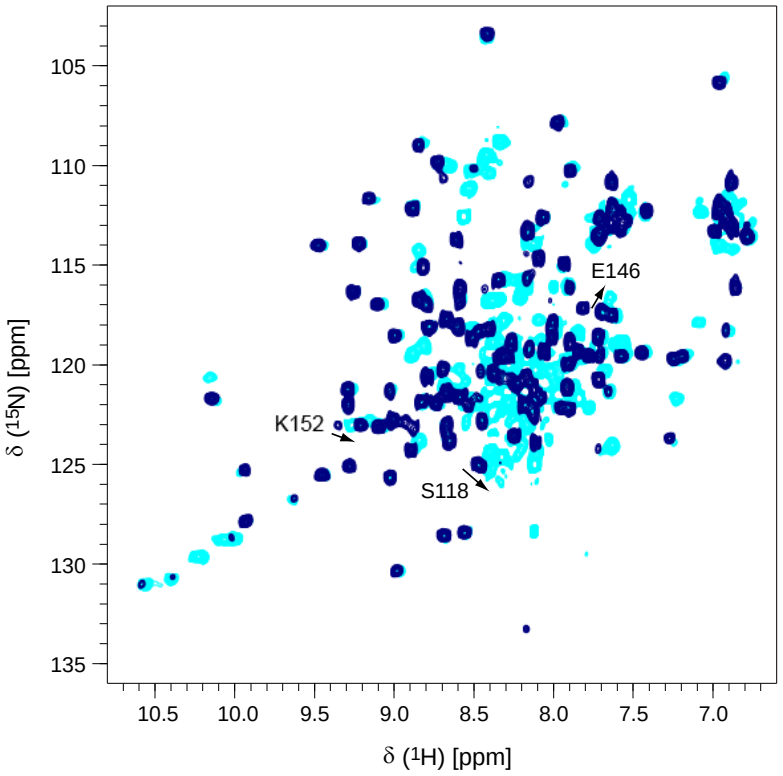
B



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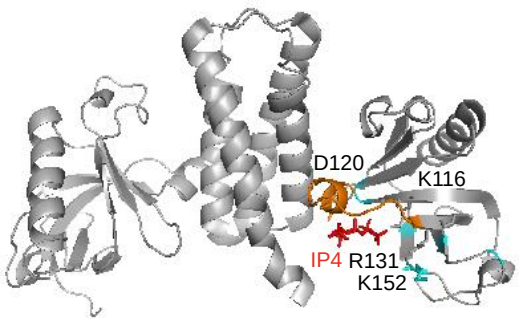

Figure S5

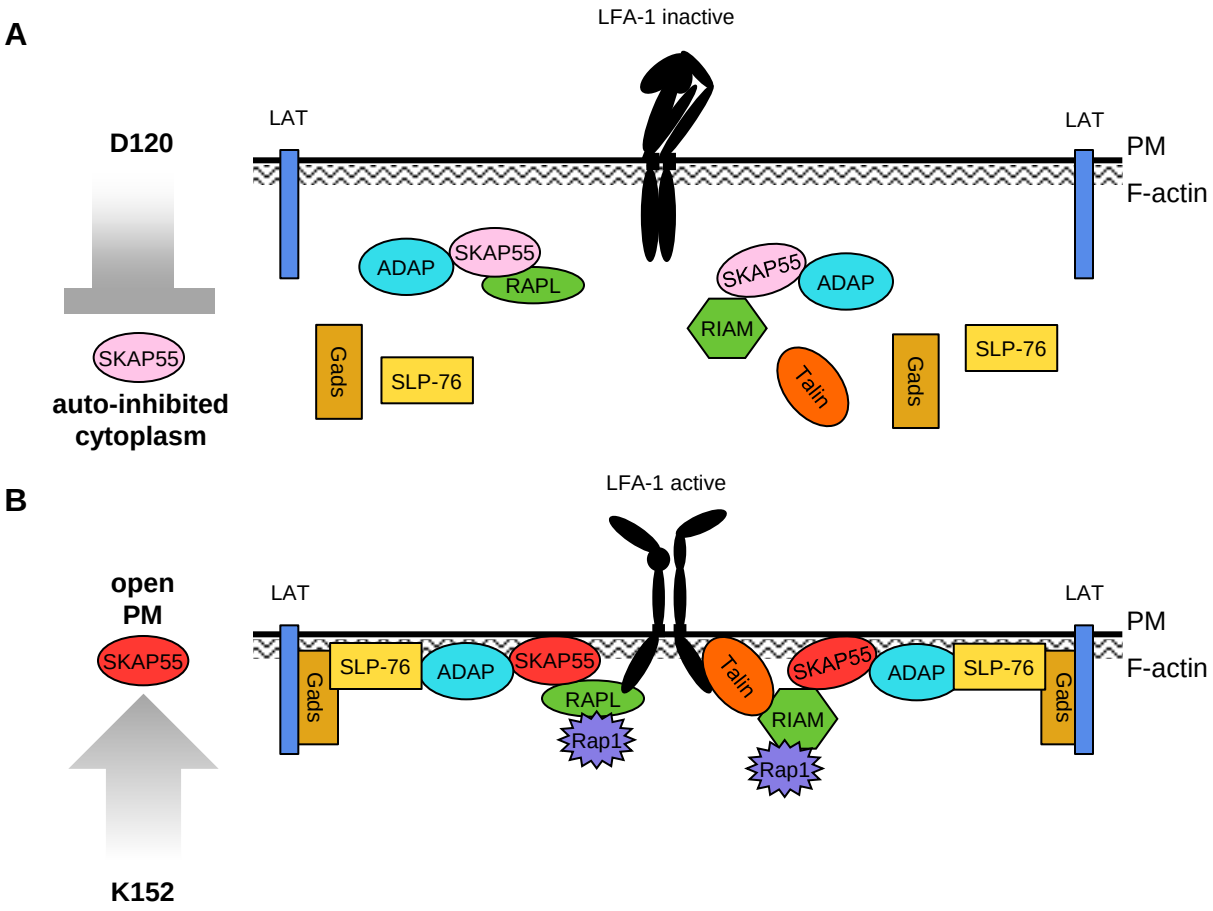
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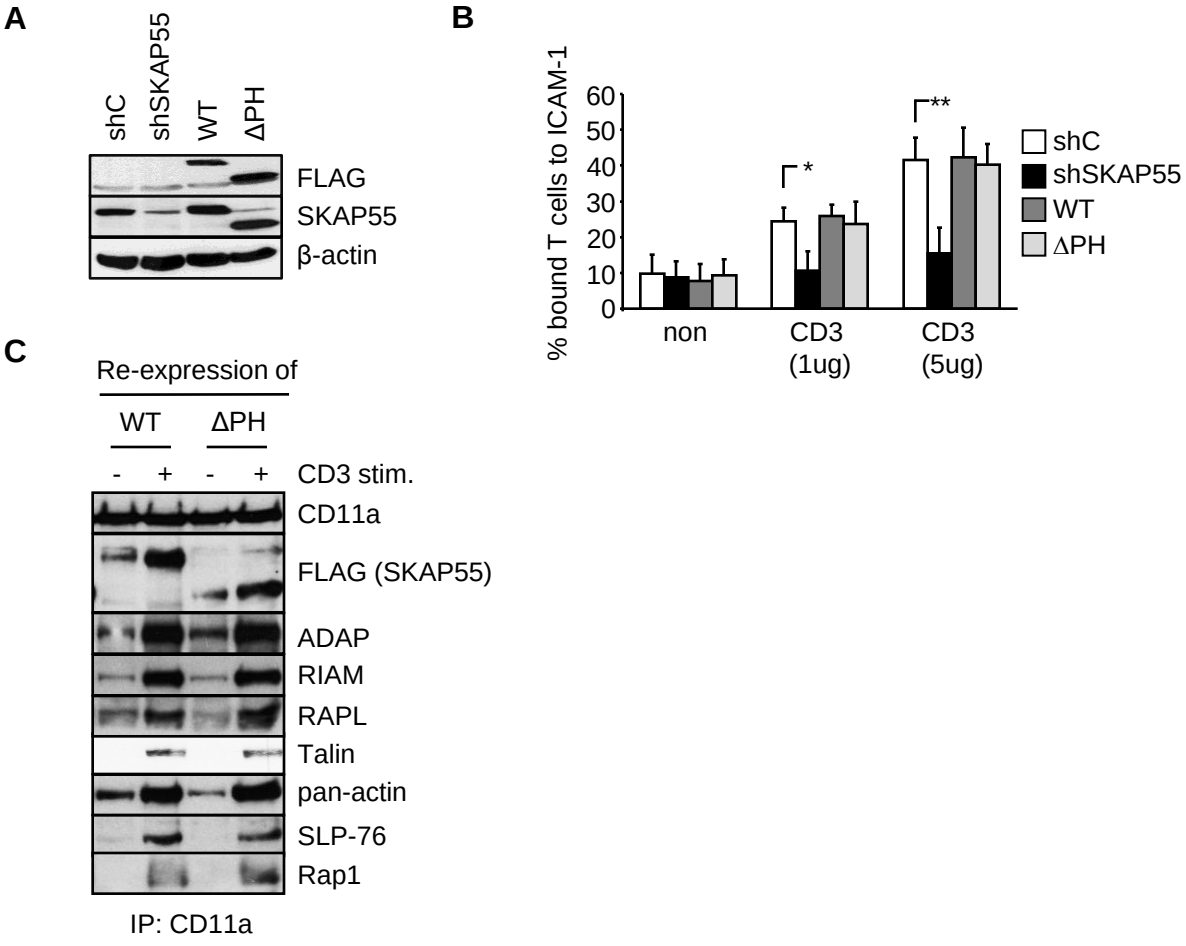
Figure S6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50
40
30
20
10
0
*
**
non
CD3 (1ug)
CD3 (5ug)
shC
shSKAP55
WT
ΔPH
C
Re-expression of
WT
ΔPH
- + - +
CD3 stim.
CD11a
FLAG (SKAP55)
ADAP
RIAM
RAPL
Talin
pan-actin
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Rap1
IP: CD11a