# Supporting Information

## Shang et al. 10.1073/pnas.1801340115

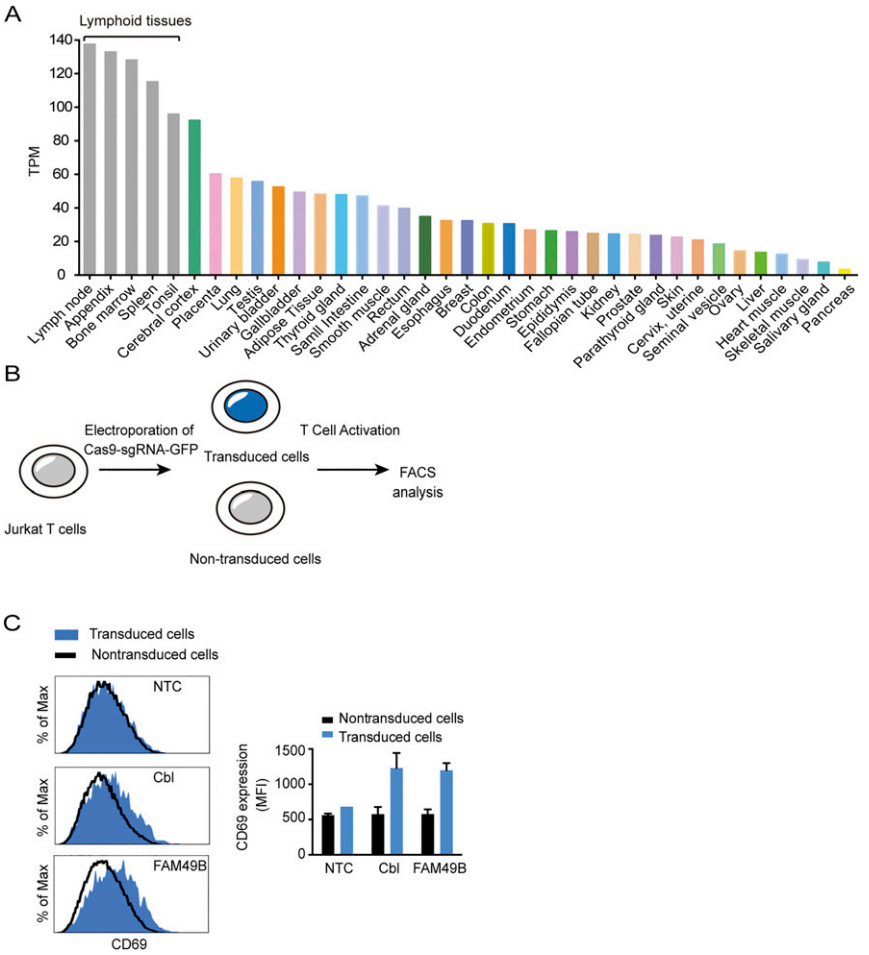


**Fig. S1.** Tissue expression profile of human FAM49B and hyperactivation of FAM49B KO T cells. (*A*) RNA-seq data of human FAM49B generated from the Human Protein Atlas (https://www.proteinatlas.org/ENSG00000153310-FAM49B/tissue). The lymphoid tissues are color-coded in gray. (*B*) Schematic of deleting targeting genes by electroporating cas9-sgRNA-GFP into Jurkat T cells. (*C*) FACS analysis of CD69 levels in Jurkat cells expressing sgRNAs targeting NTC, c-Cbl, or FAM49B upon overnight anti-TCR stimulation. Data are representative of three independent experiments.

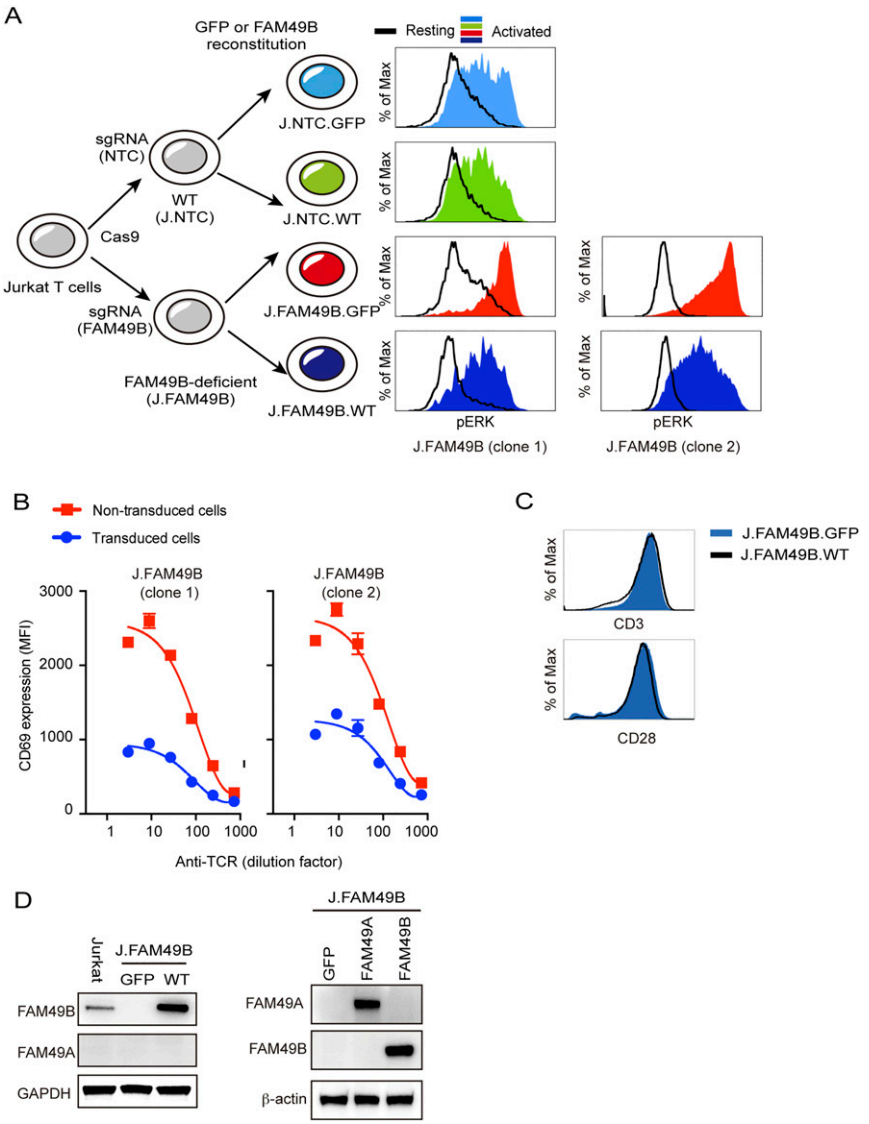


**Fig. S2.** Two different FAM49B KO clones have similar hyperactivation phenotypes. (*A*) FACS analysis of pERK phosphorylation in clone 1 and clone 2 described in Fig. 2*B*. (*B*) FACS analysis of CD69 levels in clone 1 and clone 2 upon different concentrations of anti-TCR C305 overnight activation. Data are representative of three independent experiments. (*C*) CD3 and CD28 surface expression levels of J.FAM49B.GFP and J.FAM49B.WT. (*D*) Western blot analysis of endogenous FAM49B and FAM49A expression level in Jurkat cells. GAPDH/β-actin were used as loading controls.

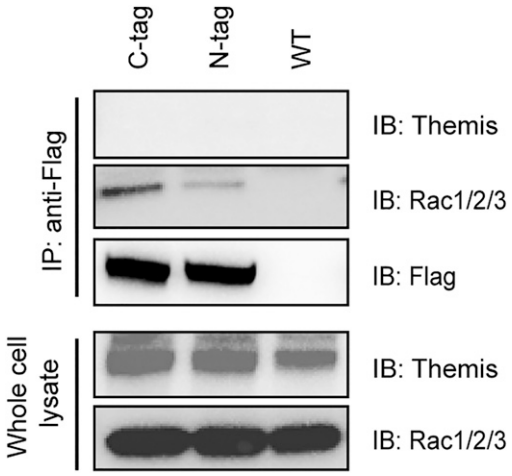


**Fig. S3.** Endogenous Rac, but not THEMIS, interacted with C-tagged FAM49B. The samples described in Fig. 3*C* were assessed by immunobloting (IB) using anti-Rac and anti-THEMIS antibodies.

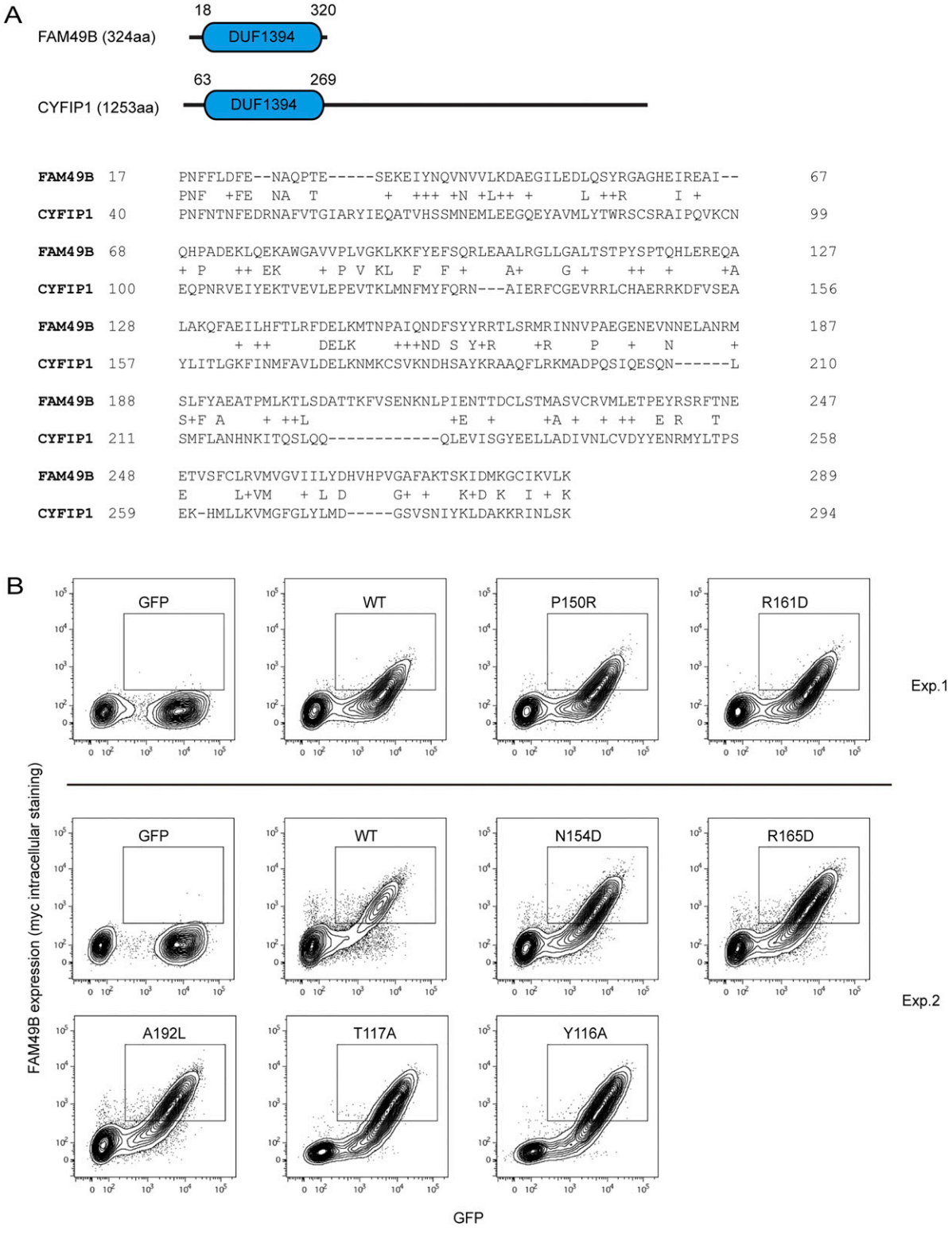


**Fig. S4.** FAM49B and CYFIP1 share similar DUF1394 domain. (*A*) Schematic of DUF1394 domain in FAM49B or CYFIP1 and protein sequence alignment of DUF1394 domain in FAM49B and CYFIP1. (*B*) FACS analysis of intracellular myc level and GFP expression level in the samples described in Fig. 4*B*.

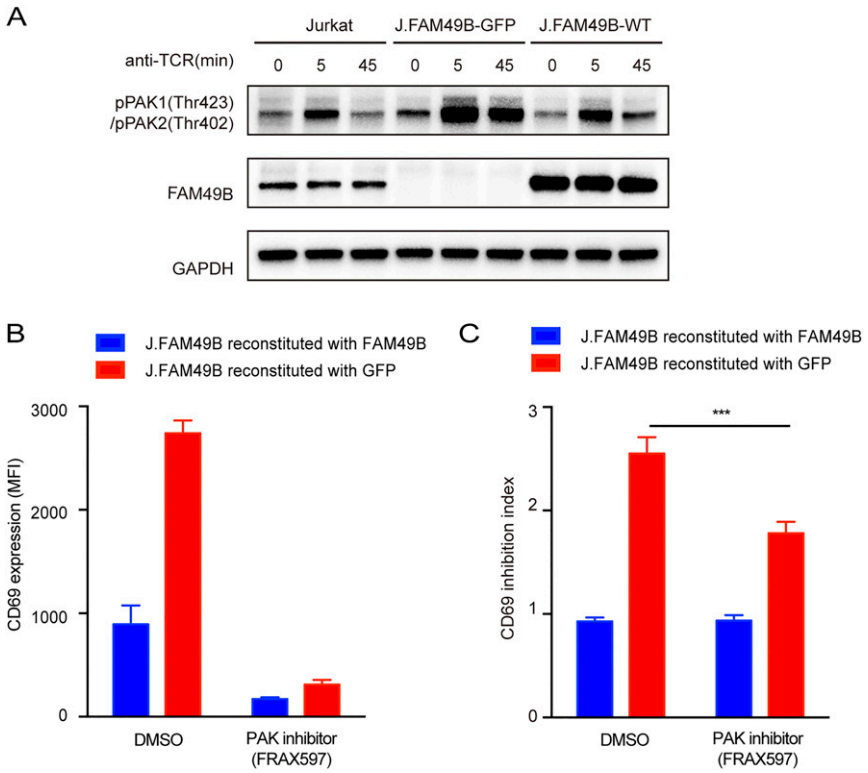


**Fig. S5.** J.FAM49B-WT and WT Jurkat cells had a similar PAK phosphorylation pattern and treatments of PAK inhibitor affected CD69 upregualtion. (*A*) The samples described in Fig. 5*B* and WT Jurkat cells were stimulated with anti-TCR for times indicated. Phosphorylation of PAK was assessed by immunoblotting. GAPDH was used as loading control. (*B*) FACS analysis of CD69 expression level of FAM49B deficient or sufficient cells. J.FAM49B cells were transduced with empty vector (GFP) or WT-FAM49B at an MOI of 0.5. These samples were activated upon anti-TCR stimulation in the presence of DMSO or PAK inhibitor (FRAX597, 1 μM). CD69 expression was measured by flow cytometry. (*C*) CD69 inhibition index was calculated by MFI of CD69 in $GFP^{-}$ population divided by that of $GFP^{+}$ population. $^{***}P < 0.001$.

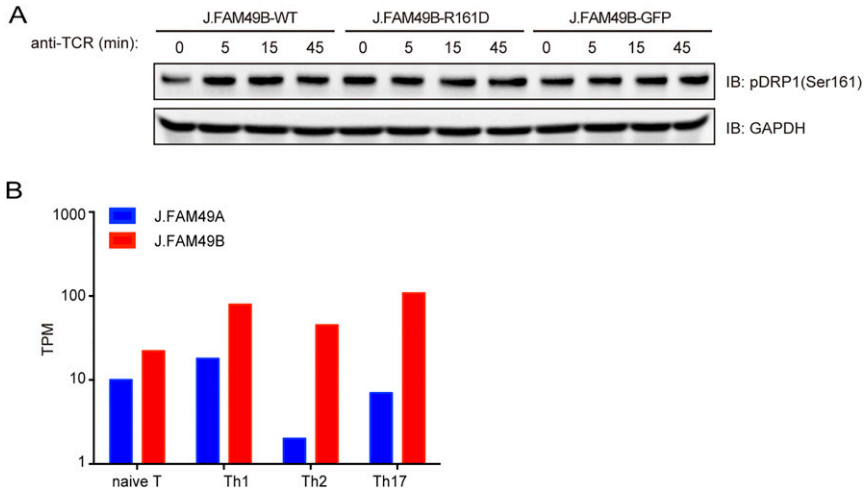


**Fig. S6.** DRP1 phosphorylation in FAM49B-deficient and FAM49B-reconstituted T cells and FAM49 expression in mouse T cells. (*A*) J.FAM49B cells reconstituted with WT-FAM49B, GFP or FAM49B (R161D) were stimulated with anti-TCR for indicated time. Phosphorylation of DRP1(Ser161) were assessed by immunoblotting. GAPDH was used as loading control. (*B*) Both FAM49A and FAM49B are expressed in mouse T cells (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-2582/).

## Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLSX)