Supplemental Figures

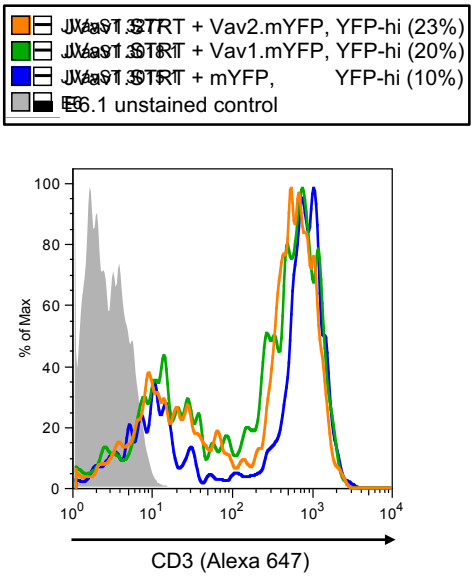

Figure S1. Vav1 and Vav2 expression do not alter surface TCR levels. Vav1-deficient cells expressing SLP-76 (J.Vav1.ST) were transiently transfected with the indicated mYFP-tagged Vav constructs, or an mYFP control, and stained for surface CD3. Populations expressing matched, high levels of YFP (blue), Vav1.YFP (green), and Vav2.YFP (orange) were gated and compared to unstained control cells (gray).

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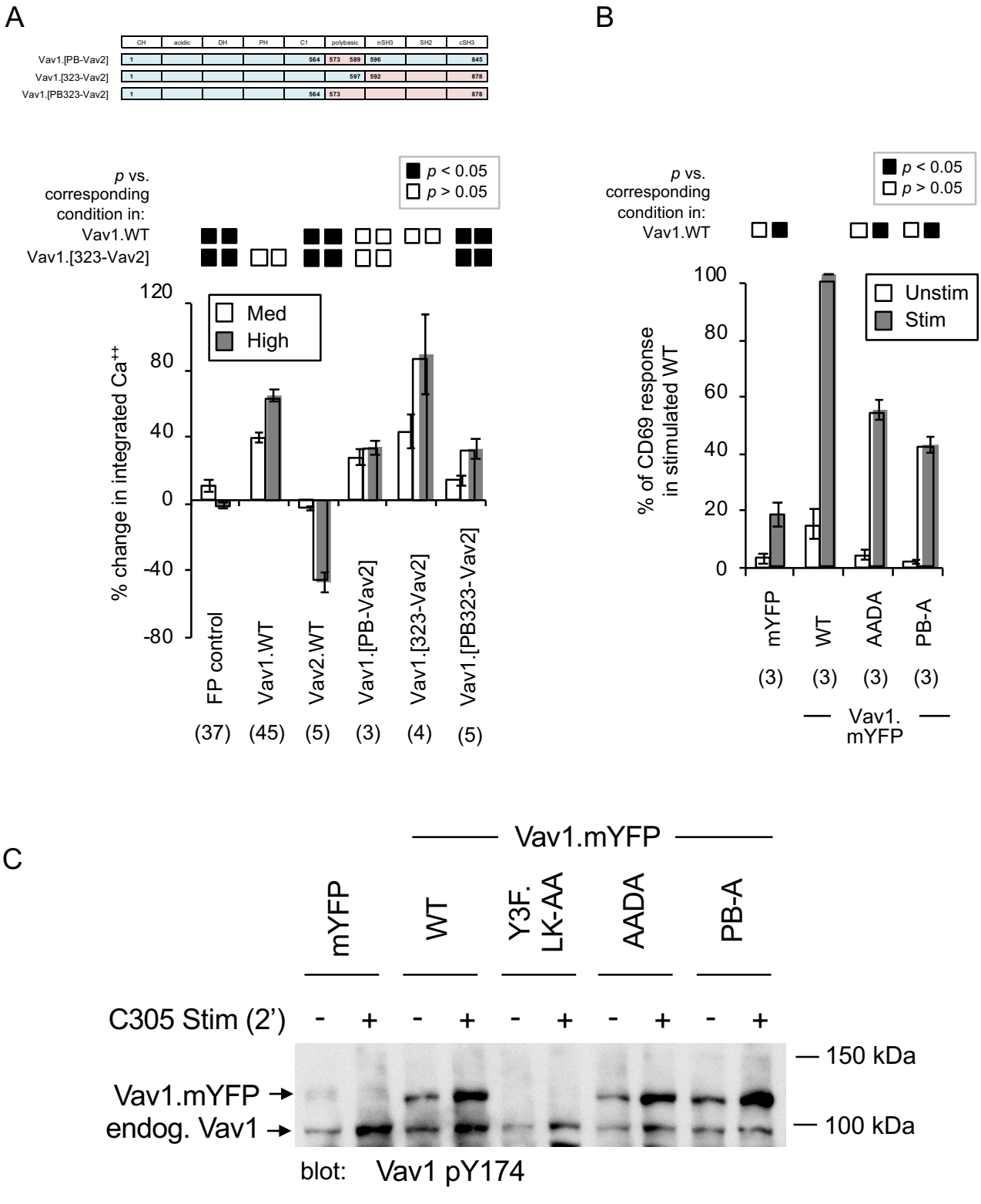

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Figure S2. The polybasic region of Vav1 supports TCR-dependent signaling, but does not impact the tyrosine phosphorylation of Vav1. A) The calcium responses of the indicated mYFP-tagged Vav1/Vav2 chimeras were monitored as in Fig. 1C and are presented as in Fig. 1D. **B)** CD69 levels were determined by flow cytometry for J.Vav1 cells transfected with the indicated Vav1.mYFP chimeras and either left unstimulated or stimulated with 10 ng/ml OKT3. All CD69 responses are normalized by expression as a percentage of the CD69 mean fluorescent intensity observed in stimulated J.Vav1 cells expressing Vav1.mYFP. Graphs depict the cumulative mean ± SEM for all salient experiments. Statistical information is presented as above. **C)** Vav1 chimeras were expressed in wild-type Jurkat E6.1 cells, which were either left unstimulated or stimulated for 2 minutes with C305. Total lysates were analyzed by western blotting for Vav1 pY174. The positions of endogenous Vav1 and the exogenous Vav1 chimeras are indicated. Representative of two independent experiments.

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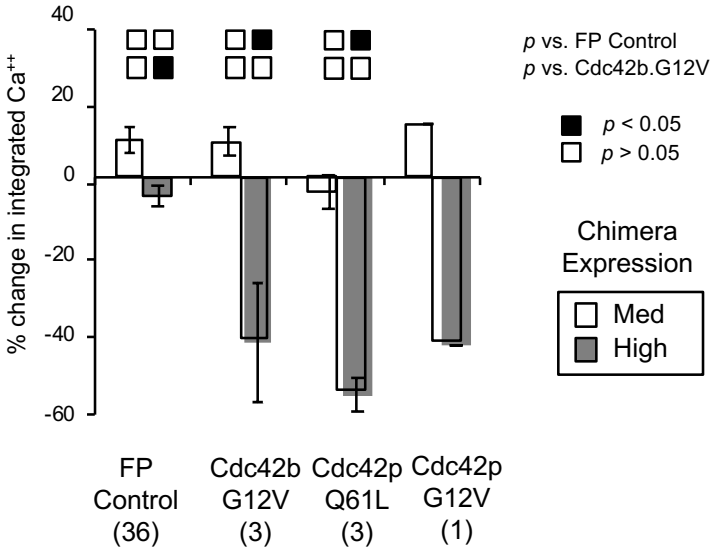
A



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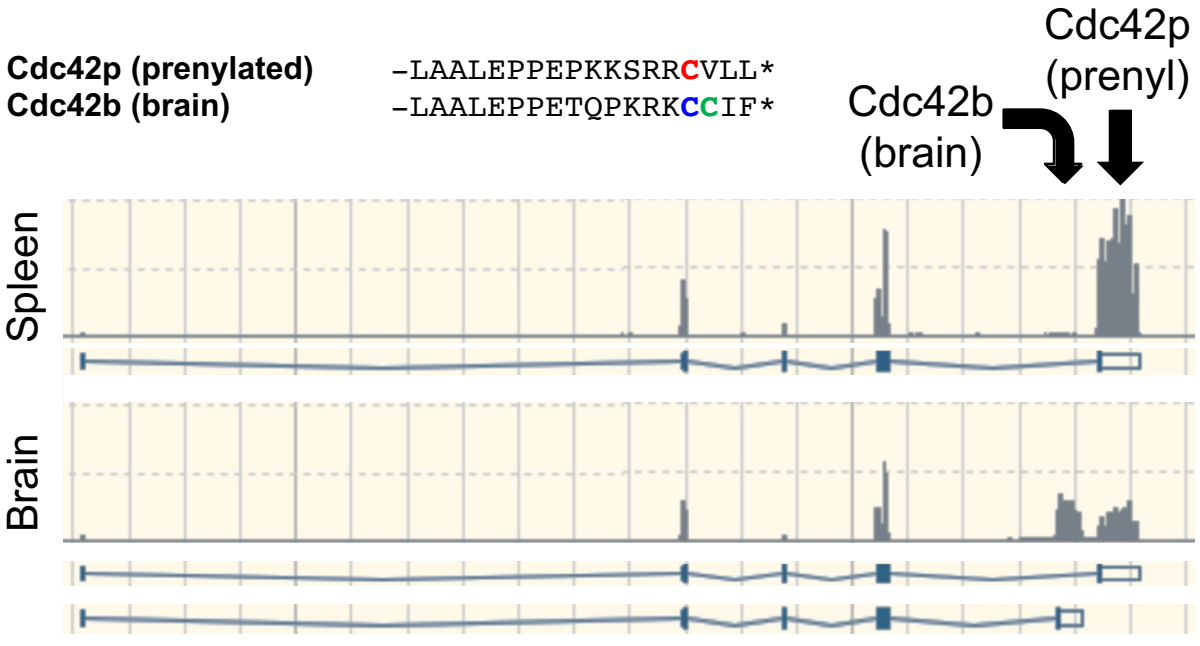

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Figure S3. Both Cdc42 isoforms and activating mutations suppress calcium signaling. **A)** The calcium responses of J.Vav1 cells transfected with either a fluorescent protein control or corresponding constitutively active Cdc42 constructs were assessed as in Fig. 3D. The Cdc42p (NP_001034891.1) splice variant is the ubiquitous prenylated form of Cdc42 that is found in hematopoietic cells. The Cdc42b (NP_426359.1) splice variant has been used widely but is primarily expressed in the CNS. Q61L and G12V are the GTPase-inactivating amino acid mutations most commonly used to maintain Rho GTPases in the GTP-bound, active state. **B)** Top: C-terminal amino acid sequence differences between the two major isoforms of Cdc42. Cdc42p is consistently prenylated at C188 (red). Cdc42b can be prenylated at C188 (blue) and atypically palmitoylated at C189 (green). Bottom: RNASeq expression profiles of the two isoforms in murine hippocampus (brain) and spleen. The spleen only expresses the prenylated isoform.

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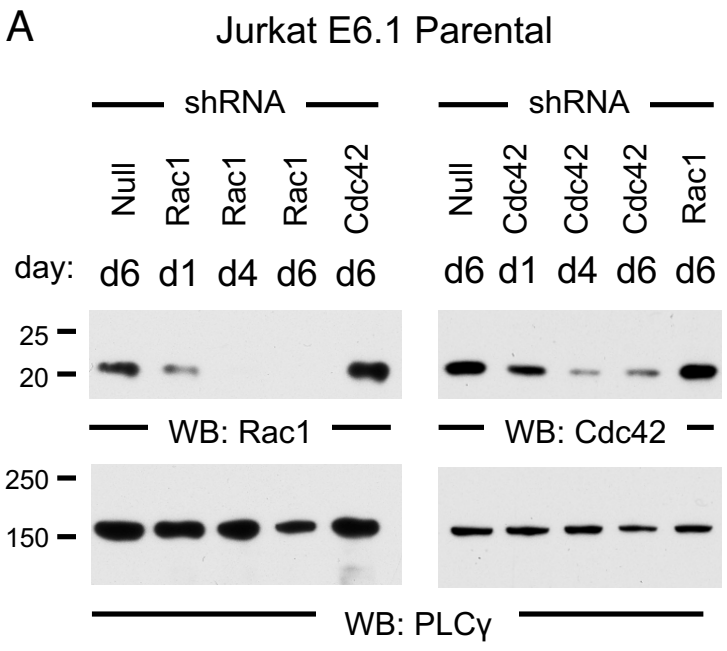

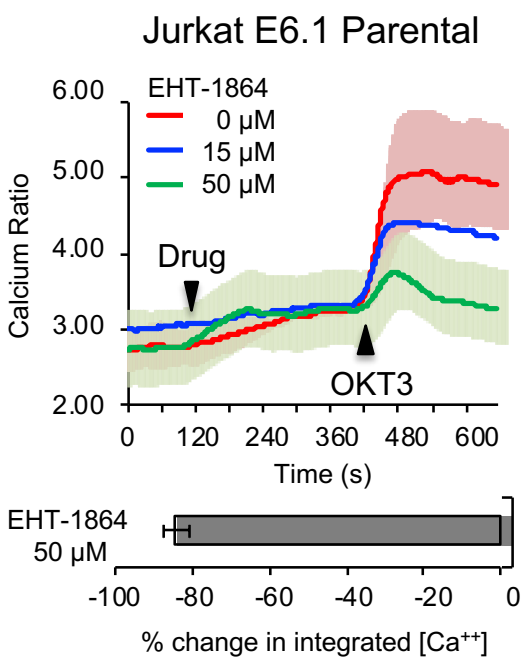

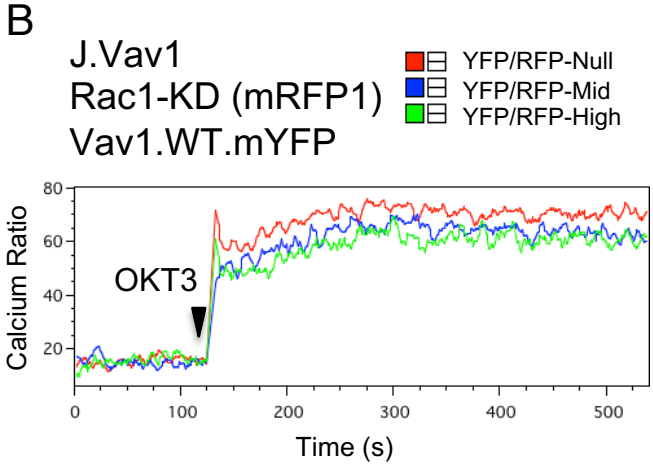

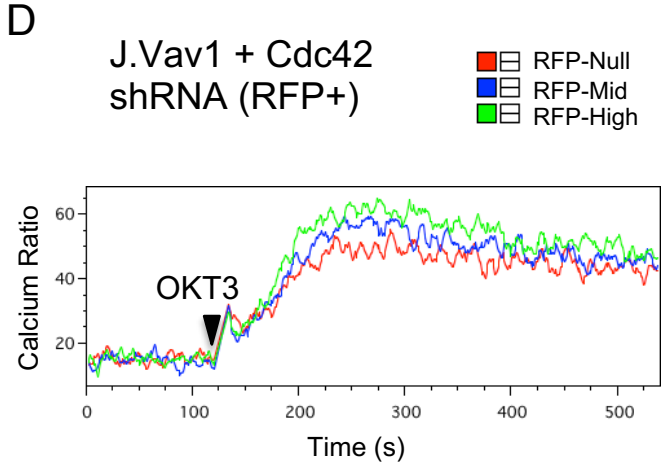

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Figure S4. Rac1 contributes to Vav1-dependent calcium responses while Cdc42 mediates the suppressive effects of Vav2 on TCR-mediated calcium entry. A) Jurkat E6.1 cells were transiently transfected with vectors that express Rac1- or Cdc42-targeting shRNAs and an mRFP1 reporter. Lysates were prepared at the indicated days post-transfection and analyzed by western blotting. **B)** J.Vav1 cells were transiently transfected with vectors encoding wild-type Vav1 and the Rac1-targeting vector described above. At day 4 post-transfection calcium responses were assessed by flow cytometry using ratiometric Indo-1 imaging following stimulation with 120 ng/ml OKT3. Responses are presented for populations expressing undetectable (YFP/RFP-Null), moderate (YFP/RFP-Mid), or high (YFP/RFP-High) levels of Vav1.mYFP and the mRFP1 reporter. **C)** The Rac inhibitor EHT-1864 impairs TCR-induced calcium entry. EHT-1864 fluorescence precludes the use of Indo-1 and with the calcium-bound FuraRed signal; therefore, Jurkat E6.1 cells were loaded with FuraRed and calcium levels were estimated by flow cytometry using the inverse of the calcium-free FuraRed signal (488 nm excitation, 695/40 bandpass emission filter). The upper plot displays the responses of cells treated with EHT-1864 as indicated and then stimulated with OKT3 (1 µg/ml). Two experiments were performed with 50 µM EHT-1864 and a DMSO control; one experiment also tested 15 µM EHT-1864. Mean responses are shown, with shaded regions indicating the range between replicates. The lower chart displays the percent change in the integrated calcium response observed with 50 µM EHT-1864 relative to the DMSO treated controls (mean ± SD, n=2). **D)** J.Vav1 cells were transiently transfected with the Cdc42-targeting vector described above. At day 4 post-transfection calcium responses were assessed by flow cytometry using ratiometric Indo-1 imaging following stimulation with 120 ng/ml OKT3. Responses are presented for populations expressing undetectable (RFP-Null), moderate (RFP-Mid), or high (RFP-High) levels of mRFP1.

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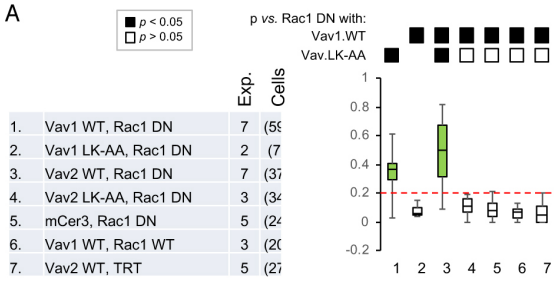
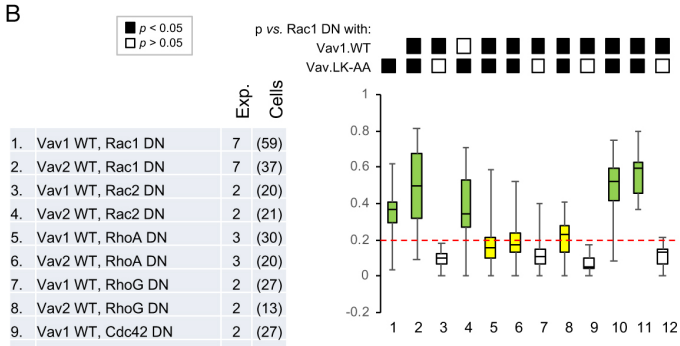
A



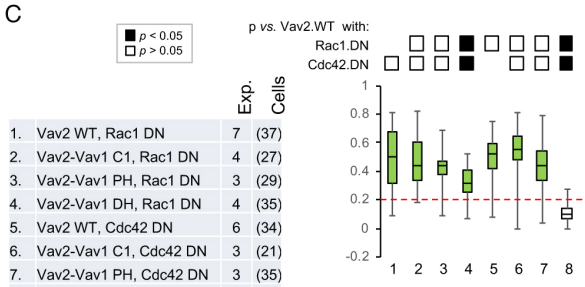
		Exp.	Cells
1.	Vav1 WT, Rac1 DN	7	(59
2.	Vav1 LK-AA, Rac1 DN	2	(7
3.	Vav2 WT, Rac1 DN	7	(37
4.	Vav2 LK-AA, Rac1 DN	3	(34
5.	mCer3, Rac1 DN	5	(24
6.	Vav1 WT, Rac1 WT	3	(20
7.	Vav2 WT, TRT	5	(27

B



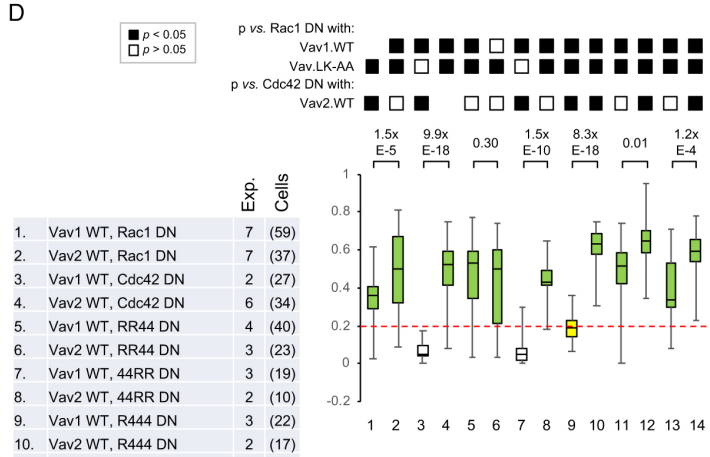
		Exp.	Cells
1.	Vav1 WT, Rac1 DN	7	(59)
2.	Vav2 WT, Rac1 DN	7	(37)
3.	Vav1 WT, Rac2 DN	2	(20)
4.	Vav2 WT, Rac2 DN	2	(21)
5.	Vav1 WT, RhoA DN	3	(30)
6.	Vav2 WT, RhoA DN	3	(20)
7.	Vav1 WT, RhoG DN	2	(27)
8.	Vav2 WT, RhoG DN	2	(13)
9.	Vav1 WT, Cdc42 DN	2	(27)
10.	Vav2 WT, Cdc42 DN	6	(34)
11.	Vav1.[GEF-V2], Cdc42 DN	2	(14)
12.	Vav2 LK-AA, Cdc42 DN	3	(21)

C



		Exp.	Cells
1.	Vav2 WT, Rac1 DN	7	(37)
2.	Vav2-Vav1 C1, Rac1 DN	4	(27)
3.	Vav2-Vav1 PH, Rac1 DN	3	(29)
4.	Vav2-Vav1 DH, Rac1 DN	4	(35)
5.	Vav2 WT, Cdc42 DN	6	(34)
6.	Vav2-Vav1 C1, Cdc42 DN	3	(21)
7.	Vav2-Vav1 PH, Cdc42 DN	3	(35)
8.	Vav2-Vav1 DH, Cdc42 DN	4	(39)

D



		Exp.	Cells
1.	Vav1 WT, Rac1 DN	7	(59)
2.	Vav2 WT, Rac1 DN	7	(37)
3.	Vav1 WT, Cdc42 DN	2	(27)
4.	Vav2 WT, Cdc42 DN	6	(34)
5.	Vav1 WT, RR44 DN	4	(40)
6.	Vav2 WT, RR44 DN	3	(23)
7.	Vav1 WT, 44RR DN	3	(19)
8.	Vav2 WT, 44RR DN	2	(10)
9.	Vav1 WT, R444 DN	3	(22)
10.	Vav2 WT, R444 DN	2	(17)
11.	Vav1 WT, 4R44 DN	3	(16)
12.	Vav2 WT, 4R44 DN	2	(14)
13.	Vav1 WT, Cdc42 F56W DN	3	(33)
14.	Vav2 WT, Cdc42 F56W DN	3	(29)

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Figure S5. Statistical analyses of colocalization in 'GEF-trap' assays. Co-clustering data shows an average of the fractional overlaps of the clustered areas identified using the dominant negative (DN) GTPase and SLP-76 channels, as a function of the DN GTPase and co-expressed Vav chimera, for all GEF-trap assays presented in the main text. Boxes enclose the second and third quartiles and whiskers indicate minimum and maximum values. Values below the dashed line correspond to no visible co-clustering. Small boxes above each chart denote statistical comparisons between the condition directly below and the condition indicated at left. The interquartile boxes are colored as follows: green for mean colocalization values statistically indistinguishable from or greater than the Vav1.WT/Rac1.DN pairing; yellow for mean colocalization values intermediate between and statistically distinguishable from both the Vav1.WT/Rac1.DN and Vav1.LK-AA/Rac1.DN pairings; white for mean colocalization values statistically indistinguishable from the Vav1.LK-AA/Rac1.DN pairing. Statistical comparisons were only performed for experiments conducted on the same instrument, using the same tagging schema, with the Vav chimera tagged with mCerulean3, the SLP-76 chimera tagged with mYFP, and the GTPase tagged with TRT. Alternative tagging schema yielded similar results. The total numbers experiments and cells complying with our inclusion criteria are indicated. Student's t-tests were performed considering each cell as a replicate. Similar significance values are obtained using experimental averages. **A)** Data supporting Fig. 4C; n≥3 experiments were performed for all constructs, with n≥2 experiments conforming to statistical inclusion criteria. No fewer than 3 cells were observed per condition per experiment. **B)** Data supporting Fig. 5A-B; n≥3 experiments were performed for all constructs, with n≥2 experiments conforming to statistical inclusion criteria. No fewer than 5 cells were observed per condition per experiment. **C)** Data supporting Fig. 6B; n≥3 experiments were performed for all constructs, with n≥3 experiments conforming to statistical inclusion criteria. No fewer than 4 cells were observed per condition per experiment. **D)** Data supporting Fig. 6D; n≥3 experiments were performed for Vav1 constructs and n≥2 experiments for Vav2 constructs, with n≥2 experiments conforming to statistical inclusion criteria. No fewer than 6 cells were observed per condition per experiment. Statistical comparisons are also included for pairwise comparisons of Vav1 and Vav2 with each chimeric GTPase.

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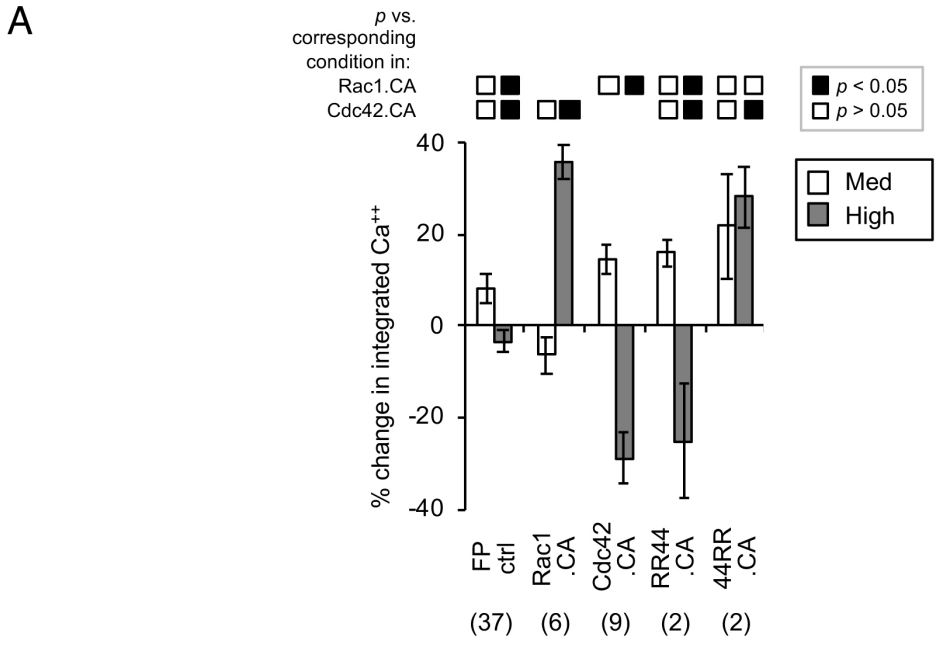

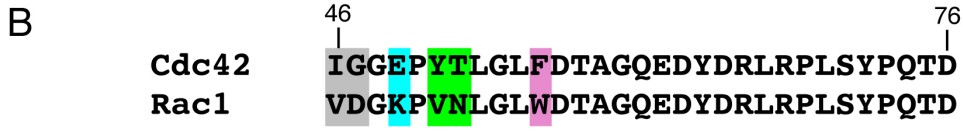

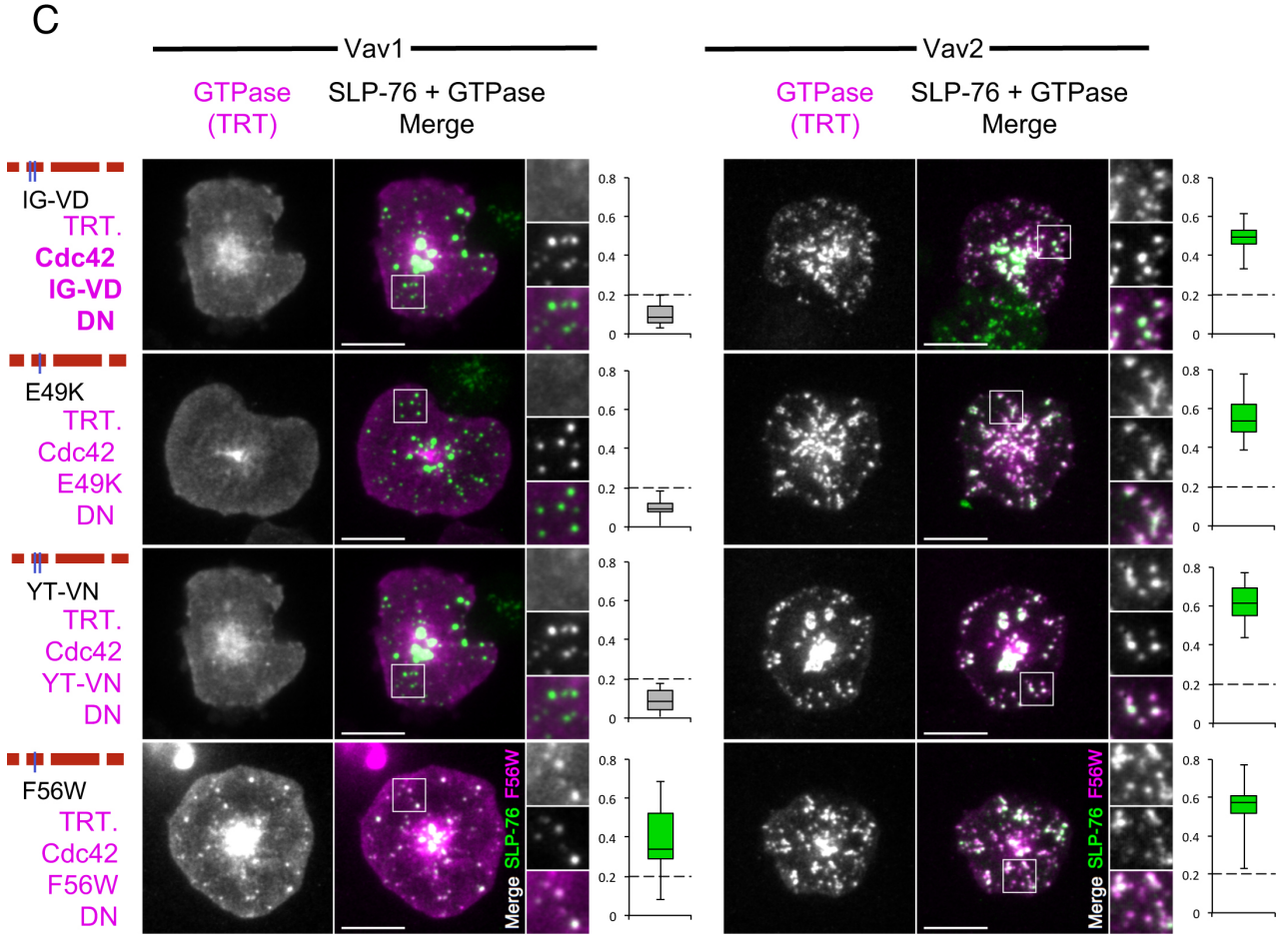

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Figure S6. Identification of the residues responsible for the exclusion of Cdc42 from Vav1 and the assessment of their impacts on calcium entry. A) Constitutively active hybrid GTPases derived from Rac1 and Cdc42 were tagged with 3xFlag.TRT and expressed in J.Vav1 cells. The TCR-induced calcium responses of cells expressing different levels of these chimeras were integrated over time and are expressed as the percent change in the integrated calcium response observed in TRT-negative cells in the same tube. Calcium responses and statistical calculations are presented as in Fig. 3D. **B)** The complete sequence of the GTPase segment that determines the differential binding of Cdc42 to Vav1 and Vav2. All amino acids differing between Rac1 and Cdc42 were tested for effects on GEF binding in the indicated groupings. **C)** J14.SY cells were co-transfected with vectors encoding a TRT-tagged dominant negative (DN) Rac1/Cdc42 chimera and either mCer3-tagged Vav1 (left) or Vav2 (right). Bars at the far left identify the relative positions of the substitutions from Rac1 into Cdc42. Images were acquired and presented as in Figure 4A (n≥3 experiments for all construct pairs). The panels for the Cdc42 F56W mutant are identical to those in Figure 6D.

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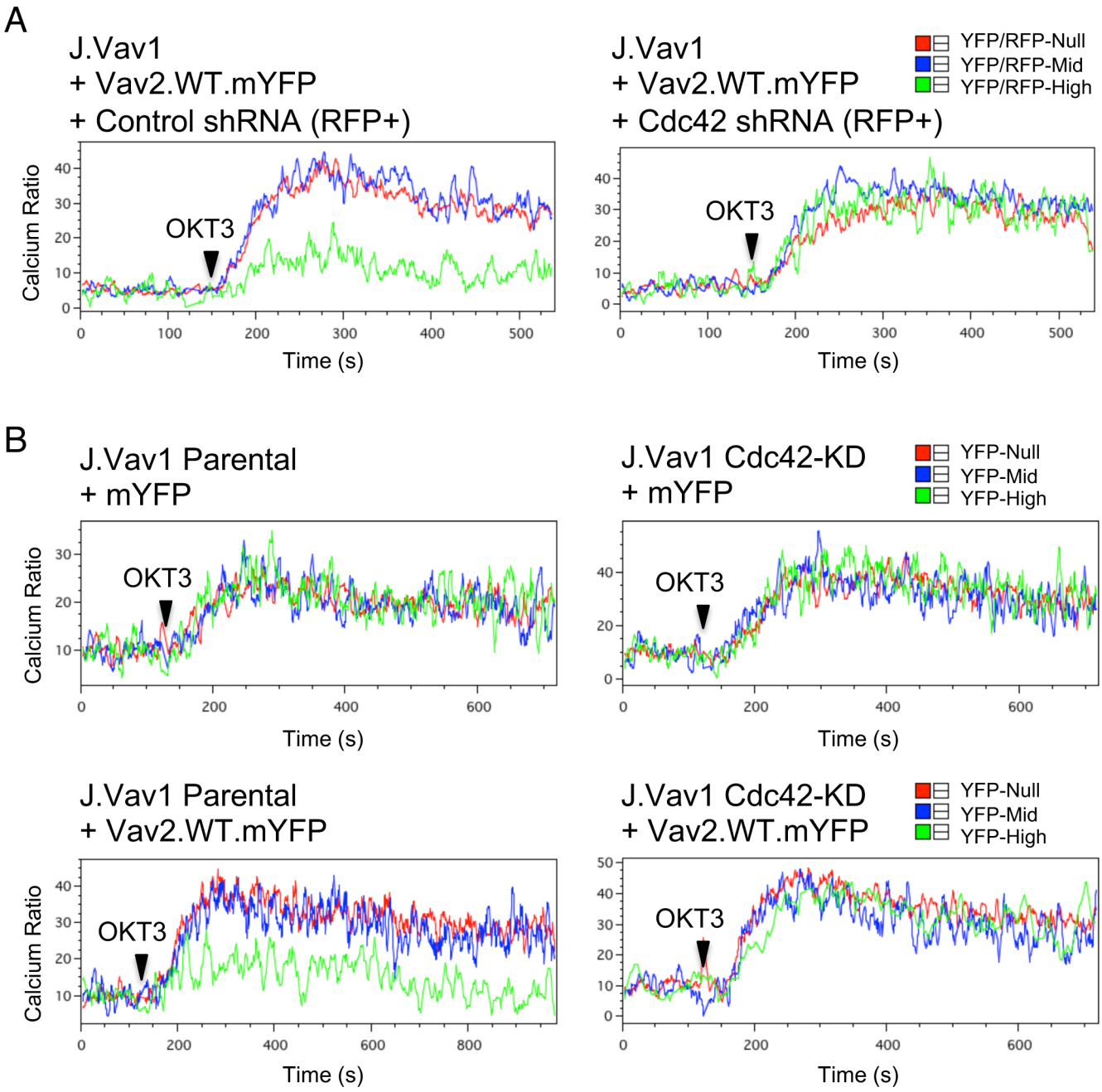

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Figure S7. Rac1 contributes to Vav1-dependent calcium responses while Cdc42 mediates the suppressive effects of Vav2 on TCR-mediated calcium entry. A) J.Vav1 cells were transiently transfected with a vector encoding wild-type Vav2.mYFP and either a control targeting vector or the Cdc42-targeting vector used in Fig. S4A-B. At day 4 post-transfection calcium responses were assessed by flow cytometry using ratiometric Indo-1 imaging following stimulation with 75 ng/ml OKT3. Responses are presented for populations expressing undetectable (YFP/RFP-Null), moderate (YFP/RFP-Mid), or high (YFP/RFP-High) levels of Vav2.mYFP and mRFP1. One of two similar experiments is shown. **E)** J.Vav1 cells were stably transduced with lentiviral vector encoding a Cdc42-specific shRNA. Parental and Cdc42-KD J.Vav1 cells were transiently transfected with vectors encoding either mYFP or wild-type Vav2.mYFP. Calcium responses were assessed by flow cytometry using ratiometric Indo-1 imaging following stimulation with 60 ng/ml OKT3. Responses are presented for populations expressing undetectable (YFP-Null), moderate (YFP-Mid) or high (YFP-High) levels of Vav2.mYFP.

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