Supplemental Information for

PHOSPHATIDYLINOSITOL MONOPHOSPHATES REGULATE OPTIMAL Vav1 SIGNALING OUTPUT

by

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This PDF file includes:

(1) Supplemental Figures S1 to S6 and legends (pages 2 to 10)

(2) Supplemental Tables S1 and S2 (pages 11 to 13)
FIGURE S1. Evolution of the KR in Vav family proteins

Amino acid sequence and charge features of the Vav1 KR region were analyzed in the indicated species. Positively charged residues are shown in red.
FIGURE S2. The Vav1 KR regulatory mechanism is specific for lymphocytes

(A) Activation of NFAT triggered by indicated Vav1 proteins in nonstimulated and BCR–stimulated DT40 cells. Data represent the mean ± SEM. Statistical values were obtained using the Mann–Whitney U test. Blue and salmon asterisks indicate the significance level compared with nonstimulated and BCR–stimulated Vav1WT–expressing cells, respectively. Black asterisks refer to the P values obtained between the indicated experimental pairs (in brackets). n = 3 independent experiments.
(B) Representative example of the abundance of the indicated Vav1 proteins and tubulin α (loading control) in the assays performed in A.

(C) Activation of SRF by the indicated Vav1 constructs in COS1 cells. Data represent the mean ± SEM. Statistical values were obtained using the Mann–Whitney U test. All the comparisons are referred to Vav1<sup>WT</sup>. 

\( n = 3 \) independent experiments, each performed in triplicate.

(D) Representative example of the abundance of the indicated Vav1 proteins and tubulin α (loading control) in the assays performed in C.

(E) Effect of the indicated proteins (top) in the F–actin cytoskeleton of COS1 cells. EGFPs and F–actin are shown in green and red, respectively. Areas of colocalization of Vav1 proteins and F–actin are shown in yellow (bottom panels). Scale bar, 20 \( \mu \)m. 

\( n = 3 \) independent experiments.

(F and H) Activation of SRF by the indicated Vav1 proteins in COS1 cells. Data represent the mean ± SEM. Statistical values were generated applying the Mann–Whitney U test using as comparative control the values obtained in Vav1<sup>D835–845</sup>–expressing (F) and Vav1<sup>D1–186</sup>–expressing (H) cells. 

\( n = 3 \) independent experiments, each performed in triplicate.

(G and I) Representative example of the abundance of the indicated Vav1 proteins and tubulin α (loading control) in the assays performed in F (G) and H (I).

(J) Activation of NFAT triggered by Vav1<sup>WT</sup> and indicated polyhistidine–tagged Vav2 (His–Vav2) proteins in nonstimulated and TCR–stimulated Jurkat cells. Data represent the mean ± SEM. Statistical values were obtained using the Mann–Whitney U test. Blue and salmon asterisks indicate the significance level compared with nonstimulated and TCR–stimulated Vav1<sup>WT</sup>–expressing cells, respectively. Black asterisks refer to the \( P \) values obtained between the indicated experimental pairs (in brackets). 

\( n = 3 \) independent experiments, each performed in duplicate.

(K) Representative example of the abundance of the indicated Vav proteins and endogenous tubulin α (loading control) in the assays performed in J. Vav2 was detected using an antibody to the polyhistidine tag. The asterisk pinpoints the residual signal from the previous blotting carried out with the antibody to polyhistidine residues.
FIGURE S3. The KR region is important for the localization of Vav1 in lymphocytes

(A) Immunoblot of cytoplasmic and plasma membrane fractions of nonstimulated and CD3–stimulated Jurkat cells showing the localization of the indicated EGFP–tagged Vav1 versions (top), a membrane–localized (TP1/36), and a cytosolic (tubulin α) protein. Please, note that the tubulin panel was generated using aliquots from the same experiment in an independent filter.
(B) Quantification of the distribution of the indicated Vav1 proteins in the membrane and cytosolic fractions of nonstimulated and stimulated Jurkat cells from the experiments shown in A. Data are shown as mean ± SEM. Statistical values were calculated using the Student’s t test relative to the data obtained in Vav1WT-expressing cells. n = 3 independent experiments.

(C) Example of the tyrosine phosphorylation of the indicated ectopically-expressed (GFP-pVav1) and endogenous Vav1 (pVav1) proteins in nonstimulated and TCR-stimulated Jurkat cells (top panel). As control, we include the Western blot of immunoprecipitated Vav1 with antibodies to the Vav1 DH domain (bottom panel).

(D) Quantification of the tyrosine phosphorylation levels of the indicated ectopically-expressed Vav1 proteins obtained in the experiments shown in C. Data are shown as mean ± SEM. Statistical values were calculated using the Student’s t test relative to the data obtained in Vav1WT-expressing cells. n = 6 independent experiments.

(E) Example (top panels) and quantification (bottom) of the effect of EGFP and indicated EGFP–tagged Vav1 versions (top) in the polymerization of actin inside the contact area and outside or at the peripheral area of the immune synapse. This data is part of the same experiment performed in Figure 3G. As in that case, values were obtained comparing the F–actin signal in these areas to the signal in other regions of both the T and B cell as detailed in the methods. Histograms represent the mean ± SEM and statistics were performed using two–way ANOVA and Dunnett’s multiple comparison tests using as reference control the detection of F–actin inside (blue) and outside (salmon) the contact area in Jurkat cells expressing the indicated EGFP–protein. n = 3 independent experiments.
FIGURE S4. The entire Vav1 C1–KR mediates phosphatidylinositol monophosphate binding

(A,B) Stained SDS–PAGE gels showing the purified MBPs used in Figure 5B,C. The migration of the molecular weight markers is shown on the right of each panel. KDa, kilodalton.

(C) Representative experiment showing the association of the specified MBP proteins (top) with the liposomes of the indicated composition (left). S, soluble (unbound) fraction; P, pelleted (bound) fraction. PC, 1–palmitoyl–2–oleoyl–sn–glycero–3–phosphocholine; PS, 1–palmitoyl–2–oleoyl–sn–glycero–3–phospho–L–serine. Proteins were stained with Coomassie.

(D) Quantification of the experiments shown in C. Data represent the mean ± SEM. Statistical values were obtained using two–way ANOVA followed by Dunnett’s test for multiple comparisons. n = 5 independent experiments.
(E) Stained SDS–PAGE gel showing the purified Vav1\textsuperscript{WT} protein used in Figure 5E. The migration of the molecular weight markers is shown on the right.

(F) Immunoprecipitation of indicated Vav1 proteins (top) with antibodies to epitopes located in the Vav1 DH domain (top panel on the left) and KR (top panel on the right). As control, we show the expression of each of the protein versions used aliquots of the total cellular lysates used in the immunoprecipitation experiments (bottom panel).

(G) Stained SDS–PAGE gels showing the purified MBPs used in Figure 6. The migration of the molecular weight markers is shown on the right of each panel.
FIGURE S5. Vav1 does not influence the localization of phosphatidylinositol monophosphates at the immune synapse.

WT and VAV1 knockdown (shVav1) Jurkat cells were transfected with EGFP-tagged domains that specifically recognize PI3P (NAPD oxidase PX domain), PI4P (four phosphate adaptor protein 1 PH domain) and PI5P (Ing2 PHD domain). Upon synapse formation, cells were fixed, stained with phalloidin, and subjected to confocal microscopy. The bioreporters and F-actin are seen as green and red signals. Areas of colocalization between the bioreporters and F-actin are seen in yellow.
The residues involved in the coordination of Zn$^{2+}$ are shown in green and shaded in dark gray. Basic residues present in the KR regions of the indicated proteins are shown in red. The basic residues present in the Ing2 PHD that contribute to binding to phosphatidylinositol monophosphates are also indicated in red.

**FIGURE S6. Comparison of the Vav1 C1–KR with the PHD–KR cassette of nuclear proteins**

The residues involved in the coordination of Zn$^{2+}$ are shown in green and shaded in dark gray. Basic residues present in the KR regions of the indicated proteins are shown in red. The basic residues present in the Ing2 PHD that contribute to binding to phosphatidylinositol monophosphates are also indicated in red.
TABLE S1. Sequence of oligonucleotides used in this study

| Mutant | DNA sequence of primer |
|--------|------------------------|
| **KR1**<sub>Mut</sub> | |
| F<sub>1</sub>* | 5’– CAAGATTTCGCAGGAACCAT(G)GAAGAAGGACAAGCTCCATC –3’ |
| R<sub>1</sub> | 5’– GATGGAGCTTGTCCTTTCCTCC(C)ATGGTCCCTGCGAAATCTTG –3’ |
| F<sub>2</sub> | 5’– GGACAAGCTCCATCG–AGGGCCCAGGACAAG –3’ |
| R<sub>2</sub> | 5’– CTTGCCCTGCGAATC –3’ |
| **KR2**<sub>Mut</sub> | |
| F<sub>1</sub> | 5’– CATCGAAGGGCCCAGGAC–AGAAAAGGAATGATTGG –3’ |
| R<sub>1</sub> | 5’– CCCAATTCATTCCTTTTCTGAATTCG –3’ |
| F<sub>2</sub> | 5’– TCTGCTAAGATGGAAGTGTTT(T)CAGGAATACTATG –3’ |
| R<sub>2</sub> | 5’– GAGGGATCCCATAGTATTCCTG –3’ |
| **CAAX** | |
| F<sub>1</sub> | 5’– GCGTGTTGCCTGGCATGACAGGAC –3’ |
| R<sub>1</sub> | 5’– [p]– GCTCTAGATCAGGAGAGGCAGCAATATTCGGAATAGTCTTCC –3’ |
| F<sub>2</sub> | 5’– GGAAGACTATTCCGAATATTGC–TGCGTCCTCTC –3’ |
| R<sub>2</sub> | 5’– GCTCTAGATCAGGAGAGGCAGCAATATTCGGA –3’ |
| **Δ835–845** | |
| F<sub>1</sub> | 5’– CCCCCTAACACTAAGTGGAGGAGAC –3’ |
| R<sub>1</sub> | 5’– GTCTCCACTCCACTGGAAAGG –3’ |
| **D578K** | |
| F<sub>1</sub> | 5’– CGCAGGAACCATGAAAGAAAGAAAGCTCCATCGAAGGCCC –3’ |
| R<sub>1</sub> | 5’– GGGGCTTGCGATGAGCTTCTCTC –3’ |
| **E591K** | |
| F<sub>1</sub> | 5’– CCCAGGACAAGAAAAGGAAATTAATTGGGTCTGCCTAAGATG –3’ |
| R<sub>1</sub> | 5’– CATCTTGACGACCAATTTATCCCTTCTCTTGTCG –3’ |
| **E598K** | |
| F<sub>1</sub> | 5’– GGGTCTGCCTAAGATGAAGGTTCTCGG –3’ |
| R<sub>1</sub> | 5’– GTATCCCTGAAACACTTTAGTATCCCTC –3’ |
| **G691V** | |
| F<sub>1</sub> | 5’– CCAACCGTTCTGATGACCTATCTGTTGCG –3’ |
| R<sub>1</sub> | 5’– CGCACCAGATAGTCAATCGAAGGTTTGGG –3’ |
| **MBP–C1** | |
| F<sub>1</sub> | 5’– GCAGGAACCATGTAAGGACAAGC –3’ |
| R<sub>1</sub> | 5’– GCTTTGTCCCTCTCATGTTGTCG –3’ |
| **MBP–KR** | |
| F<sub>1</sub> | 5’– CCTATAGATCGTGCCCATGGGAAAGATTTCGC –3’ |
| R<sub>1</sub> | 5’– GGAGGATGATCCCATAGTATCC –3’ |

*F, forward primer (in the case of a two–step mutagenesis protocol, the primers are referred to as F<sub>1</sub> and F<sub>2</sub> for the first and second step, respectively); R, reverse primer (in the case of a two–step mutagenesis protocol, the primers are referred to as R<sub>1</sub> and R<sub>2</sub> for the first and second step, respectively); [p]–, phosphorylated primer. Nucleotides used for the generation of the indicated mutations are shown in red. Nucleotides that have been inserted or deleted in the WT sequence are indicated in red parenthesis and with a red line, respectively.
### TABLE S2. Alignment of the amino acid sequences present at the C–terminus of C1 domains (continues in next page)

| Protein     | Region C–terminal to C1 domain* |
|-------------|---------------------------------|
| Vav1        | GHQDFFGTM3NDKLRSAQDKKKNELGLPMEVFQEQYGLPPPAIG |
| DGKα        | PTPFEGGSFVPENFVHLMVHRKQEGAKQCGGPOQFSPFSEIV |
| DGKβ        | PTPFEVGSNVTPEFVHLMVHRKQEGAKQCGGPOQFSPFSEIV |
| DGKδ        | SDCTQGQDQGQDITDDKHLEGNPSGACVCTCGSSDVLACVC |
| RasGRP2     | N³AQSVSLSAPSFSNLSMMMAFSSLFPESCQGS |
| PKCα        | PGADGPDTDDFSKHPHELQYSGTFCCGSLYYLIGQGMCDTCD |
| PKCβ (isoform 2) | GSSGPPDTDDFSKHPHELQYSGTFCCGSLYYLIGQGMCDTCD |
| PKCγ        | PGAGGPDTDDFSKHPHELQYSGTFCCGSLYYLIGQGMCDTCD |
| DGKγ        | CVTYSGASQSGEPKKELSTLCDDGELIDILLPSTCPCtvYSGS |
| DGKβ        | CTVYSGASQSGEPKKELSTLCDDGELIDILLPSTCPCtvYSGS |
| DGKα        | EVSTYASNDIGVQSVWVSGCESGCDCCQEISLSTLCDDGELIDILLPSTCPCtvYSGS |
| Vav3        | CVYNGEQGTLLEPNTNGLTPQVPQDPGLPMQVNYNSGTPPPAPE |
| RasGRP3     | RFCAPLSLSSGISPLPSLPPAQDVEFPVGTACHDLDLASITLV |
| RasGRP1 (isoform 2) | KNEVNPATENNTSVGPVSNLCSLGAIDRPEEGPTTFPHEA |
| RasGRP4     | RFDCPAGDAGPPAPVSTPAPASCGSEENSTYLLSTEPTGCQVLKAAA |
| PKD3        | SGVRKLSNVLGSPLCGLSLVTPLEGYPALPSESVENGPSIPWS |
| PKD1        | SGVRKLSNVLGSPLCGLSLVTPLEGYPALPSESVENGPSIPWS |
| PKD2 (fragment) | DGKTRNCAKTVPNCLIAGELAINGVMEEATEDFSEADSAIHMLO |
| PKCσ        | GMHDSVMPSQEPVDDNEDADLPSEETDG1AYISSRGGDSIQDSEED |
| DGKδ        | TTTLASGDIIDEDGAMPPQWLEGNPLVQATCVDTCGSSVLQD |
| DGKδ (isoform 2) | TTTLASGDIIDEDGAMPPQWLEGNPLVQATCVDTCGSSVLQD |
| DGKη        | TTTLASGDIIDEDGAMPPQWLEGNPLVQATCVDTCGSSVLQD |
| DGKε        | VMNTLSITDDDPLLPADEVMNPQWVEGNNPVSQOCAVCBGSGYQYSEQ |
| DGKι        | VSIMLNTDVLDAMPIVQNVPLSCYMVCQCQCGQPLCDYCMW |
| DEF8        | VSSVYSAEYLNICPETGLDSQDYCAECAPSLAVGPSEAECDDT |
| PLEKHM3     | SVTVKQGAEFLYVEEPIDLQDENAMLHAEPLAALVACCILS |
| B–RAF       | VNYQDLQDLPVSFTFIEPIFEASLAETLSTGSSPSASASISGQPI |
| TENC1       | QALPQVELRTNPQHERELLGSTMNLSKSNSTPFSFSLDMEE |
| PNS3        | GQVQLQEPAGSSSLSSLCDLPPVILSTMEGCGLLTYESTII |
| RhoGEF28    | MPPTPTVNNNTQPIGNSSTFIDOPGQLSTISSPVVPVLGTSTPV |
| ADCP5       | SQOEGLSDPSEPSTLTVSFSQNPCVEETOFTPLQEE1CDTSYNT |
| RacGAP1     | IPTLIGTVP1GEOMLADVQTSIPMSVVPVNEIEGLGTELGLT |
| AKAP13      | ATDDDQPSQLQAPITLSTLPVINMPISQPEPRPASVLLHDEATTAP |
| RAF         | VDDSN1QOLLFFPSTGSDGGVPALPSLTLGRSMESVSMPPSOSMST |
| RAF (isoform X5) | VDDSN1QOLLFFPSTGSDGGVPALPSLTLGRSMESVSMPPSOSMST |
| PKCι        | GHSLPQEPVMMDOSSSDAQTVPINVNSSLEDQVQEEEAMMTS |
| KMT2C       | GSHLDPSAPAGPSVVEASSNLQSNIPPIDFPPCTDPPPVPPGCLPN |
| ARAF        | VDMSTNQPPFPYVQDLQGGSGQLEAPSNPLNELLPTQPPFCTD |
| TNS1        | VPPSNVEFVPITTENAPNVQ%cEAGASGNTSLNEDGSDTVTSPVQ |
| ADCP1       | CGPDGWEAPVEDTFNDEFVETFEDLSQAREIEEQHJEYNAQINSNL |
| PDZD8       | GATRQIDTTNLLEQGQTTLGLPPVDAEASVNVTTTGFINT |
| Vav2        | ETSPADLDASAGPPGMVAMQNYKGNPPAPVVLFTQTGDVLELLG |

*Basic amino acid residues are shaded in red.
| Protein      | Region C-terminal to C1 domain                        |
|--------------|------------------------------------------------------|
| UNC13B       | LQAAE SCGAED TQNIMAH GWNL II BPEIFEVIVDVTVPN        |
| UNC13A       | LQAAE SSGAED TQNIIMVL GWNL II BPEIFELQEIFAVT        |
| UNC13C       | LQAAE SSGAED QTIIITAM EWLNL II PEHEVQEMQGIS         |
| STAC         | MLQLP GFRHHYSSPLLIE EQFGCI EVMTAAGNV DPPVYETL FGTS  |
| STAC2        | PGTSTSFHSFSSPLLV EPVPCATS EPSPTDGSG VDPYETLFGY      |
| STAC3        | FQPPGFGHSYSSPLSNQYACY DLSAAN DNPVYETL TGVIMAN       |
| KSR2         | LLIIQGDPAGLV TESVPCINP HDPPYSSL ISQTLF TNINQD       |
| KSR1         | ISFLPLTL RATESVPSDIINPVLEAAEP FGTLP ALI KPAPAMNL    |
| PKCδ         | TGTANS ETDIFO EFNIDMP HIF VNYMSTFCD GSLLWGLV XOG   |
| PKCζ         | TGSAINS ETMFHE FIDMP HIF VNYMSTFCD GSLLWGLA XOG     |
| PKCη         | AGLGQETPQVDSIS FSVNMP HFIYNY VPTFCD GSLLWGLL XOG    |
| PKCα         | TCQNNIN VDSIAEC FGINIP FSIYNY VPTFCD GSLLWGLIM XOG  |
| PKCβ (isooform X1) | PIPPEQS PGLVQVQGI TAYK GVVPTGVGWQ AAYVCCDK          |
| MRCKα        | PVFPDEQT GGLGIDPQ EGTAYEC VIF PAGVXQGQ ALAI VCDF  |
| MRCKβ (isoform X1) | PIPPEQS PGLVQVQGI TAYK GVVPTGVGWQ AAYVCCDK          |
| ROCK1        | SIEGMWLSVPV GNM STRG QYVVVS TILFYENQD EQOSNPMSVL   |
| CIT          | GLPAEYAT PTEAFC DMPGQLGT FPPSIS LECWMPF NNNSOQ      |
| PLEKHM1      | ESVGPASSDG FELVFSQGL ALASSQDEAEWDL WVEALQ VTPQOE   |
| CHN1         | QPDLSLVE HYSSCDLTLV IAHTKQ PMVMDIC EIES GLNSEGELY V  |
| CHN2         | QPDLSLVE HYSSCDLTLV IAHTOQ PMVMDIC EIEAK GLNSEGELY V |
| HMHA1        | KGCILQG LQFDFPSF PAASADPGFVIV YVCCQF EIAL QTV       |
| RhoGAP29     | GQLO LPGHI LFLQAEFTQVAK EPDGIPFII ICASEIEN ALCLO QGY |
| GMI          | GRIALPA TPLFGVDFQLQP DPEEPVPHYT KFPARIT GADQVQY      |
| MYO9A        | STYDPHELSS QGVELS LTSED TVPVLWVE LINYEM GLYTEGIV      |
| MYO9B        | STYDGK EPGVEPG FCVCVDSLTS DIASVPIVL ELLE VEM GLYTE   |
| BRD1         | LQSTAPA                                              |