Vav2 Lacks Ca^{2+} Entry-Promoting Scaffolding Functions Unique to Vav1 and Inhibits T Cell Activation via Cdc42

Michael A. Fray, John C. Charpentier, Nicholas R. Sylvain, Maria-Cristina Seminario and Stephen C. Bunnell
DOI: 10.1242/jcs.238337

Editor: Ana-Maria Lennon-Duménil

Review timeline
Original submission: 20 August 2019
Editorial decision: 24 September 2019
First revision received: 23 December 2019
Accepted: 6 January 2020

Original submission

First decision letter

MS ID#: JOCES/2019/238337

MS TITLE: Vav2 Lacks Calcium Entry-Promoting Scaffolding Functions Unique to Vav1 and Actively Inhibits T Cell Activation by Targeting Cdc42

AUTHORS: Michael A. Fray, John C. Charpentier, Nicholas R. Sylvain, Maria-Cristina Seminario, and Stephen C. Bunnell
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The article by Fray et al. entitled "Vav2 lacks calcium entry-promoting scaffolding functions unique to Vav1 and actively inhibits T cell activation by targeting Cdc42" studies the effects of Vav isoforms on calcium responses triggered in Jurkat T cells stimulated by anti-CD3 antibodies. Interestingly, the authors show that Vav1 and Vav3 increase the TCR-induced calcium signal whereas Vav2 inhibits it.

Comments for the author

In a first part, Fray et al. have designed multiple Vav1-Vav2 chimeras to try to pinpoint the structural differences that could account for the opposite responses observed for the two isoforms. It is difficult from the results to have a clear picture of the Vav1 and Vav2 domains responsible for the observed functional divergence, probably because several non-linear portions of Vav1 or Vav2 are likely to be involved. For example, the GEF domain of Vav2 seems to be key to induce calcium response inhibition (Fig. 3B). Nevertheless, the contribution of the neighboring polybasic has been a bit overlooked in the studies presented. The amino acid sequences alignment (Fig 2C) of the so-called polybasic region actually shows strong differences that correlate with the opposite observed functional effects: Vav2 lacks all the basic amino acids there that are found in Vav1 and Vav3 and actually contains rather acidic residues. The authors have to design and test chimeric proteins containing this region of Vav2 swapped with the polybasic region of Vav1, and vice versa. This will allow to get more definitive insight into an eventual role of this region in Vav1 and Vav2 functions.
The second part uses a nice 'GEF trap' strategy to study Vav-Rho interaction in live cells.
In addition to the evidence presented in this manuscript regarding a role for Cdc42 in inhibiting calcium responses induced by Vav2 using expression of a CA form of Cdc42 and a pharmacological inhibitor of Cdc42, I think more definitive experiments have to be carried out to prove the main point of this article. In addition to evidences based on the reported imaging data suggesting an exclusive interaction between Vav2 and Cdc42, the effects of Vav1, Vav2 and Vav3 on Cdc42 activation are presently lacking in this manuscript. Measurements of Cdc42-GTP levels using, for example, biochemical pull-down experiments need to be performed to really show a unique and catalytically-dependent effect of Vav2 on Cdc42 that would not be shared by Vav1 and Vav3. Moreover, using an RNAi approach, the authors should test whether the inhibition of calcium responses induced by Vav2 is lost in Cdc42-knocked-down cells.

Other points:
Fig. 1E: The degree of co-clustering between Vav1, Vav2 or mYFP, and SLP-76 should be quantified and shown as depicted in Fig. 4C and other imaging figures.
Sup Fig 2B: The article by Wirth et al. shows that Cys188 from the brain isoform of Cdc42 can actually be also prenylated and not necessarily palmitoylated. This needs to be corrected.
Contrary to Arrieumerlou et al. (JI, 2000) who showed that active Rac inhibits calcium responses in Jurkat cells, Fray et al. show here an opposite effect of active Rac1 and Rac2 (Fig. 3D). The reasons for this discrepancy have to be addressed. Experiments using Rac1 DN should affect actin polymerization and consequently, the ability of cells to spread on coverslips. It does not look to be the case from the images presented in Fig 4C. This point should be discussed.
I do not understand the black and white squares used for statistical analyses.
Fig. 6B is mentioned in the text when Fig. 6D is actually described.
Fig 2 has been duplicated in the manuscript.
Please add page numbers in the manuscript.

Reviewer 2

Advance summary and potential significance to field

This is a well written and comprehensive paper examining a longstanding conundrum in T cell signaling and Vav biology. It has been known for some time that Vav1 and Vav3, but not Vav2 can support intracellular calcium release following engagement of the T cell receptor, but the mechanism by which this occurred was unknown. In this study from Dr. Bunnell and colleagues,

they have identified a conserved polybasic motif within Vav1 that is not found in Vav2 that contributes to this difference in function. Moreover, they show quite elegantly that unlike Vav1, which has specificity for Rac1, Vav2 has specificity for Cdc42, which also suppresses intracellular calcium release following TCR engagement. Altogether, this study provides important insight into Vav biology that will be appreciated by immunologist and cell biologists alike.

Comments for the author

This is a well written and comprehensive paper examining a longstanding conundrum in T cell signaling and Vav biology. It has been known for some time that Vav1 and Vav3, but not Vav2 can support intracellular calcium release following engagement of the T cell receptor, but the mechanism by which this occurred was unknown. In this study from Dr. Bunnell and colleagues, they have identified a conserved polybasic motif within Vav1 that is not found in Vav2 that contributes to this difference in function. Moreover, they show quite elegantly that unlike Vav1, which has specificity for Rac1, Vav2 has specificity for Cdc42, which also suppresses intracellular calcium release following TCR engagement. Altogether, this study provides important insight into Vav biology that will be appreciated by immunologist and cell biologists alike. However, I have a few minor concerns that should be addressed prior to acceptance at the JCS:

The data in the manuscript point to the activation of Cdc42 by Vav2 as being a critical reason as to why Vav2 can inhibit calcium mobilization. Since Cdc42 is activated downstream of TCR engagement, it suggests that the spatial and temporal activation of Cdc42 GEFs is regulated to ensure that active Cdc42 does not interfere with calcium mobilization. The authors define a mutation F56W in Cdc42, which allows the interaction with Vav1. It would be very interesting determine the effect on calcium mobilization when this Cdc42 mutant is expressed in JVav1 cell reconstituted with Vav1 or Vav2.

1. Page 9 line 10 - refer to Figure 2B at the end of the sentence : ....mutation nearly eliminating the response (Fig. 2B)

2. Page 13, lines 7, 9, and 12. Wrongly refer to Fig. 6B. Should be 6D.

First revision

Author response to reviewers' comments

Fray *et al.*, JOCES/2019/238337

Point by point responses follow below. Some comments reference data that will not appear in the digital response to reviewers. A formatted copy of this letter, including figures, can be found among the supplemental data files.

Reviewer 1

Advance Summary and Potential Significance to Field:

The article by Fray et al. entitled "Vav2 lacks calcium entry-promoting scaffolding functions unique to Vav1 and actively inhibits T cell activation by targeting Cdc42" studies the effects of Vav isoforms on calcium responses triggered in Jurkat T cells stimulated by anti-CD3 antibodies. Interestingly, the authors show that Vav1 and Vav3 increase the TCR-induced calcium signal whereas Vav2 inhibits it.

Comments for the Author:

In a first part, Fray et al. have designed multiple Vav1-Vav2 chimeras to try to pinpoint the

structural differences that could account for the opposite responses observed for the two isoforms. It is difficult from the results to have a clear picture of the Vav1 and Vav2 domains responsible for the observed functional divergence, probably because several non-linear portions of Vav1 or Vav2 are likely to be involved. For example, the GEF domain of Vav2 seems to be key to induce calcium response inhibition (Fig. 3B). Nevertheless, the contribution of the neighboring polybasic has been a bit overlooked in the studies presented. The amino acid sequences alignment (Fig 2C) of the so- called polybasic region actually shows strong differences that correlate with the opposite observed functional effects: Vav2 lacks all the basic amino acids there that are found in Vav1 and Vav3 and actually contains rather acidic residues. **(1)** The authors have to design and test chimeric proteins containing this region of Vav2 swapped with the polybasic region of Vav1, and vice versa. This will allow to get more definitive insight into an eventual role of this region in Vav1 and Vav2 functions.

The second part uses a nice 'GEF trap' strategy to study Vav-Rho interaction in live cells.
In addition to the evidence presented in this manuscript regarding a role for Cdc42 in inhibiting calcium responses induced by Vav2 using expression of a CA form of Cdc42 and a pharmacological inhibitor of Cdc42, I think more definitive experiments have to be carried out to prove the main point of this article. In addition to evidences based on the reported imaging data suggesting an exclusive interaction between Vav2 and Cdc42, the effects of Vav1, Vav2 and Vav3 on Cdc42 activation are presently lacking in this manuscript. **(2)** Measurements of Cdc42-GTP levels using, for example, biochemical pull-down experiments need to be performed to really show a unique and catalytically- dependent effect of Vav2 on Cdc42 that would not be shared by Vav1 and Vav3. **(3)** Moreover, using an RNAi approach, the authors should test whether the inhibition of calcium responses induced by Vav2 is lost in Cdc42-knocked-down cells.

Main point **R1.1**: *"The authors have to design and test chimeric proteins containing this region of Vav2 swapped with the polybasic region of Vav1, and vice versa."*

As per the reviewer's suggestion, we transposed the 'polybasic' region of Vav2 into Vav1. As anticipated, this attenuated the TCR-induced calcium response. However, the observed effect narrowly missed the significance threshold ($p = 0.054$ for Vav1.[PB-Vav2] *vs.* Vav1.WT within high- expressing populations, Fig. S2A). Because the inhibitory effect was consistent, we anticipate that further repeats would reveal a significant difference between the populations. We hypothesized that the effect of this swap could be counterbalanced, to a degree, by the destabilization of the closed state of Vav1. This could occur as a result of shortening of the linker normally present in Vav1 from 31 to 17 amino acids, which could impact the ability of the C-terminal SH3 domain to fold back upon the catalytic core of Vav1 in the proper orientation. A similar 'opening' effect is observed with the Y3F mutation, the LK-AA mutation, and may account for the enhanced responses of the Vav1.[323-Vav2] chimera. Therefore, we also examined the impact of the 'polybasic' swap in the context of the Vav1.[323-Vav2] chimera. In this context, the incorporation of the Vav2 PB region significantly reduced calcium entry ($p = 0.036$ for Vav1.[PB323-Vav2] *vs.* Vav1.[323-Vav2] within high-expressing populations, Fig. S2A). As a further test of the importance of the PB region in an 'open' variant of Vav1, we also evaluated the effect of the AADA mutation on calcium entry on the Vav1.Y3F.LK-AA mutant, in which the closed state has been doubly disrupted. In this context, the mutation of the PB region significantly attenuated TCR-induced calcium entry ($p = 0.038$ for Vav1.Y3F.LK-AA.AADA *vs.* Vav1.Y3F.LK-AA within high-expressing populations, Fig. 2D). Together, these data demonstrate that the polybasic region of Vav1 contributes significantly to TCR-mediated calcium entry, even if it is not strictly required for this effect. Finally, we now show that the AADA and PB-AA mutations impair TCR- induced CD69 upregulation as well as calcium entry, but do not do so by hindering the tyrosine phosphorylation of Vav1 (Fig. S2B-C).

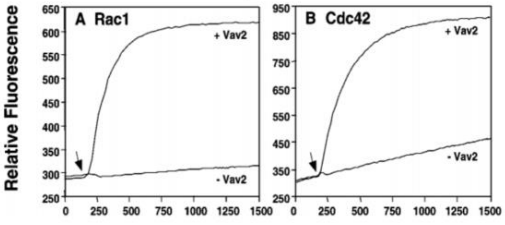
The reviewer also inquired about the converse experiment, in which the PB region of Vav1 is transposed into Vav2. However, we already know that the substitution of the Vav2 catalytic core into Vav1 abolishes the ability of Vav1 to support calcium entry, even though this chimera retains the polybasic linker from Vav1 (Fig. 3B). Further, we know that the Vav1.[GEF-Vav2] chimera retains a limited dominant negative function in the context of the LK-AA mutation. These findings already demonstrate that the Vav1 PB region is unable to convey a positive scaffolding function upon chimeras that contain the catalytic core of Vav2, regardless of their

catalytic activity. This also indicates that the DH, PH, and/or C1 domains that comprise the catalytic core of Vav1 contribute to calcium entry in a non-catalytic manner. This is now discussed in the text.

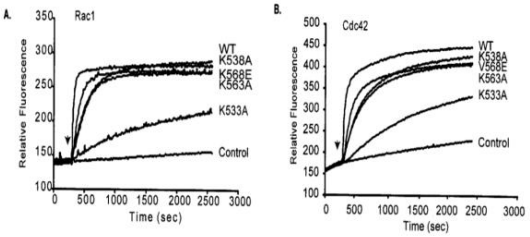
Main point **R1.2**: *"Measurements of Cdc42-GTP levels using, for example, biochemical pull-down experiments need to be performed to really show a unique and catalytically-dependent effect of Vav2 on Cdc42 that would not be shared by Vav1 and Vav3."*

As requested, we attempted to perform kinetic assays of Vav1 and Vav2 GEF function using a commercial kit based on the incorporation of mant-GTP into substrate Rho GTPases. Since such assays have already been performed using bacterially-produced recombinant fragments encompassing the catalytic cores (the DH-PH-C1 module) of Vav1 and Vav2 (see below), we attempted to extend these findings by using full-length Vav1 and Vav2 proteins. To this end, we subcloned wild-type Vav1.mYFP and activated (Y3F) versions of our Vav1.mYFP and Vav2.mYFP chimeras into vectors that appended a tandem affinity purification tag C-terminal to mYFP. The tag employed consists of a proximal 6xHis tag and a distal streptavidin binding peptide (SBP) tag. Previous work in our lab established that similarly tagged forms of mYFP can be highly enriched using streptavidin agarose and are efficiently released under mild-conditions using biotin. We know that this tagging strategy does not perturb the recruitment of Vav1 or of other signaling molecules into signaling microclusters. All three constructs were stably transduced into HEK 293T cells and were abundantly expressed in these cells, although the Y3F variants accumulated to lower levels than wild- type Vav1. The Y3F variants of Vav1 and Vav2, but not wild-type Vav1, dramatically altered the morphology of the 293T cells in a manner consistent with cellular transformation, indicating that the Y3F mutant GEFs were active in this context. We then purified the Vav chimeras on streptavidin agarose and attempted to elute them using biotin. Whereas full-length wild-type Vav1 was eluted efficiently, the Y3F variants showed increased degradation and were retained on the beads under comparable conditions. We suspect the both phenomena are the result of the constitutive opening of the Vav proteins, which may lead to increased protease access and to the aggregation of their more disordered structures. Although we were able to demonstrate that the streptavidin agarose beads themselves do not interfere with the commercial exchange assay, we were unable to detect any activity from the bead-bound Y3F mutant GEFs. Given more time, we expect that we would be able to purify the wild-type variants of Vav1 and Vav2, and to activate them *in vitro* using recombinant Lck, or to identify conditions more suitable to the recovery of the mutationally activated GEFs.

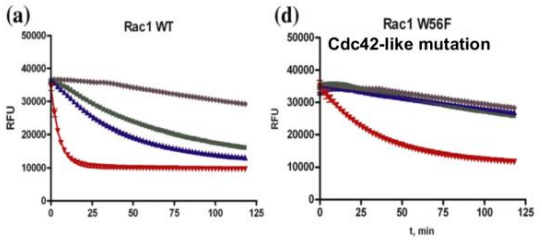
Abe 2000 (mant-GTP binding)
50nM Vav2 GEF *vs.* 2μM GTPase



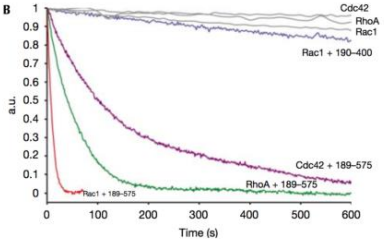
Booden 2002 (mant-GTP binding)
50nM Vav2 GEF *vs.* 2μM GTPase



Chrencik 2008 (Bodipy-GDP release)
125 nM Vav1 GEF *vs.* 1 μM GTPase



Rapley 2008 (mant-GDP release)
1.6 μM Vav1 GEF *vs.* 0.4 μM GTPase



At this point, we feel that our *in vitro* GEF trap assay, coupled with our new data implicating Cdc42 in the suppressive functions of Cdc42, is sufficient to reinterpret the existing kinetic data (assuming one discards those datasets that appear to suffer from technical defects, as noted in our discussion). The kinetic data that meets our criteria exists in pairs that are remarkably consistent with one another (*i.e.* Abe & Booden for Vav2 and Chrencik and Rapley for Vav1, see above). In this regard, the strength of our study is not that it identifies, for the first time, a difference in the catalytic specificities of Vav1 and Vav2, but rather that this difference is reflected in the behaviors of Vav1 and Vav2 *in vivo*, in their full- length forms, when acting against appropriately lipidated versions of Rho GTPases, and that this difference explains, at least in part, the distinctive behaviors of Vav1 and Vav2 in T cells. Further, the Chrencik and Rapley studies highlight the importance of GTPase residue 56 in governing the catalytic efficicacy of Vav1 towards the GTPase, just as our data supports a role for this residue in the selective trapping of Rac1 but not Cdc42 by Vav1.

Main point **R1.3**: *"Moreover, using an RNAi approach, the authors should test whether the inhibition of calcium responses induced by Vav2 is lost in Cdc42-knocked-down cells."*

We now include evidence that the suppression of TCR-mediated calcium responses by Vav2 requires the expression of Cdc42 (Fig. S7A-B). In the first approach we transiently co-transfected J.Vav1 cells with a vector expressing a Vav2 chimera and vectors encoding either a non-targeting shRNA or a Cdc42-targeting shRNA in conjunction with an mRFP1 reporter. However, this approach was challenging because Vav2 expression typically waned before appropriate levels of Cdc42 suppression were achieved. In a second approach, we stably transduced J.Vav1 cells with a puromycin-selectable lentiviral vector encoding a distinct Cdc42-targeting shRNA. When Vav2 was first overexpressed in this Cdc42-deficient line, the Vav2-dependent suppression of TCR-mediated calcium entry was largely abolished. Unfortunately, this line was difficult to maintain, most likely because Cdc42 plays an important role in cell division. Nevertheless, these data indicate that the suppression of Cdc42 attenuates the suppressive effect of Vav2, whether the suppression is transient or stable, and that this attenuation is observed with two distinct Cdc42-targeting shRNAs, but not with a control non-targeting shRNA.

In addition, we now include data indicating that the residual TCR-induced calcium responses of J.Vav1 cells are enhanced, to a degree, by the suppression of endogenous Cdc42 (Fig. S4B). This is consistent with the observation that Cdc42, in contrast to Rac1, is activated normally in J.Vav1 cells, most likely via alternative GEFs belonging to the PIX and/or DOCK families [Phee 2005; PMID: 15864311].

Other points:

- Fig. 1E: The degree of co-clustering between Vav1, Vav2 or mYFP, and SLP-76 should be quantified and shown as depicted in Fig. 4C and other imaging figures.

In contrast to the GEF trap experiments, which were performed using fixed cells, the relevant studies were performed in live cells to visualize the persistence and movement of SLP-76 microclusters over time. The spinning-disc confocal imaging system used for these studies cannot acquire live images in an interleaved fashion. Consequently, any pair of Vav (mYFP) and SLP-76 (TRT) images is not truly simultaneous, and is separated by the exposure time required for each channel. Since microclusters move during this period of time, these datasets are not suitable for the co-localization analyses performed in the GEF trap studies. We elected not to pursue these studies any further because we also demonstrated that scaffolding domains that we previously identified as impacting cluster persistence, *i.e.* the CH domain and the C-terminal adaptor module, proved functionally interchangeable in this study [Sylvain 2011].

- Sup Fig 2B: The article by Wirth et al. shows that Cys188 from the brain isoform of Cdc42 can actually be also prenylated and not necessarily palmitoylated. This needs to be corrected.

We thank the reviewer for bringing this to our attention and have revised the text and

corresponding supplemental figure accordingly. We added an additional reference that further addresses this issue.

- Contrary to Arrieumerlou et al. (JI, 2000) who showed that active Rac inhibits calcium responses in Jurkat cells, Fray et al. show here an opposite effect of active Rac1 and Rac2 (Fig. 3D). The reasons for this discrepancy have to be addressed.

The reviewer is correct in pointing out that the Arrieumerlou study demonstrated that CA Rac has a dominant negative effect on TCR-mediated calcium entry. However, this study also demonstrated that DN Rac has a dominant negative effect on TCR-mediated calcium entry. This presents something of an internal contradiction, and raises some concern.

It is also notable that the catalytically inactive Vav1.LK-AA knock-in mouse causes significant defects in T cell development and activation, yet produces no defect in TCR-mediated calcium entry, even though this mutation abolishes the activation of Rac1 by the TCR [Saveliev 2009; PMID 20009105]. This suggests that Rac1 is not normally a major player in TCR-mediated calcium entry. In contrast, a 2001 study demonstrated that Rac2 does play a major role in TCR-mediated calcium entry [Yu 2001; PMID 11581314]. Rac2 has been similarly implicated in antigen-induced calcium responses in both B cells and mast cells [Croker 2002; PMID 11907095][Baier 2014, PMID 24399839].

We now present new data indicating that the effect of the Rac inhibitor EHT-1864 on wild-type Jurkat E6.1 cells (Fig. S4C) is very large relative to the effect of Rac1-KD in J.Vav1 cells reconstituted with Vav1.WT (Fig. S4B). This indicates that other Rac isoforms, such as Rac2, may be the primary targets of EHT-1864 in Jurkat T cells. This is consistent with the known ability of EHT to target Rac2 in addition to Rac1 [Shutes 2007; PMID 17932039]. Our own data indicates that CA Rac2 is a more potent enhancer of TCR-induced calcium responses than CA Rac1, as it, unlike Rac1, operates at moderate levels of expression (Fig. 3D). Finally, our data also suggests that Vav1 is poor activator of Rac2, as it is virtually incapable of 'GEF-trapping' DN Rac2 (Fig. 5A).

We have added text describing the new data in the results section pertaining to Fig. S4.

- Experiments using Rac1 DN should affect actin polymerization and consequently, the ability of cells to spread on coverslips. It does not look to be the case from the images presented in Fig 4C. This point should be discussed.

We understand this expectation, as we had this concern ourselves. In retrospect, we note that the phenomena associated with the CA GTPases manifested most profoundly in the higher expressing subset of cells (Fig 3D). In contrast, most of the 'GEF trap' imaging (Figs. 4-6) was done with cells expressing moderate levels of the exogenous DN GTPases, and have added text emphasizing this fact. This level of expression permits the visualization of the labeled GTPase but may be insufficient to prevent the activation of endogenous GTPases. However, we did find that the DN form of RhoG, rather than Rac1, inhibited contact formation, as is noted in the legend for Fig. 5.

- I do not understand the black and white squares used for statistical analyses.

We regret that our presentation of statistical data was confusing. We used this presentation in order to present the data *in toto*, rather than cherry-picking comparisons that achieved significance. We have revised our figure legends to state that "*Small boxes above each chart depict p values for statistical comparisons between cells expressing matched levels of the chimeras indicated directly below and at left*." We hope that this is clearer. For example, in Figure 1D there are two rows of boxes. The boxes in these rows represent comparisons to either the fluorescent protein control (upper row) or the Vav1.WT chimera (lower row), as is indicated at left. Within each row, these boxes occur in pairs that correspond to the expression levels the chimeras that are being compared. Thus, the left-hand boxes above the Vav3 column indicate that the calcium responses observed in cells expressing moderate levels of the Vav3 chimera cannot be distinguished from the responses of cells expressing moderate levels of either the fluorescent protein control (upper) or the Vav1.WT chimera (lower). Similarly, the right-hand boxes above the

Vav3 column indicate that the calcium responses observed in cells expressing high levels of the Vav3 chimera differ significantly from those of cells expressing high levels of the fluorescent protein control (upper), but do not differ significantly from the responses of cells expressing high levels of the Vav1.WT chimera (lower).

- Fig. 6B is mentioned in the text when Fig. 6D is actually described.

We thank the reviewer for catching the error. This has been corrected in the text.

- Fig 2 has been duplicated in the manuscript.

We inadvertently duplicated the figure when uploading a second, high-resolution version. This has been corrected.

- Please add page numbers in the manuscript.

We have added page numbers to the manuscript.

Reviewer 2

Advance Summary and Potential Significance to Field:

This is a well written and comprehensive paper examining a longstanding conundrum in T cell signaling and Vav biology. It has been known for some time that Vav1 and Vav3, but not Vav2 can support intracellular calcium release following engagement of the T cell receptor, but the mechanism by which this occurred was unknown. In this study from Dr. Bunnell and colleagues, they have identified a conserved polybasic motif within Vav1 that is not found in Vav2 that contributes to this difference in function. Moreover, they show quite elegantly that unlike Vav1, which has specificity for Rac1, Vav2 has specificity for Cdc42, which also suppresses intracellular calcium release following TCR engagement. Altogether, this study provides important insight into Vav biology that will be appreciated by immunologist and cell biologists alike.

Comments for the Author:

This is a well written and comprehensive paper examining a longstanding conundrum in T cell signaling and Vav biology. It has been known for some time that Vav1 and Vav3, but not Vav2 can support intracellular calcium release following engagement of the T cell receptor, but the mechanism by which this occurred was unknown. In this study from Dr. Bunnell and colleagues, they have identified a conserved polybasic motif within Vav1 that is not found in Vav2 that contributes to this difference in function. Moreover, they show quite elegantly that unlike Vav1, which has specificity for Rac1, Vav2 has specificity for Cdc42, which also suppresses intracellular calcium release following TCR engagement. Altogether, this study provides important insight into Vav biology that will be appreciated by immunologist and cell biologists alike. However, I have a few minor concerns that should be addressed prior to acceptance at the JCS:

The data in the manuscript point to the activation of Cdc42 by Vav2 as being a critical reason as to why Vav2 can inhibit calcium mobilization. Since Cdc42 is activated downstream of TCR engagement, it suggests that the spatial and temporal activation of Cdc42 GEFs is regulated to ensure that active Cdc42 does not interfere with calcium mobilization. **(1)** The authors define a mutation F56W in Cdc42, which allows the interaction with Vav1. It would be very interesting determine the effect on calcium mobilization when this Cdc42 mutant is expressed in JVav1 cell reconstituted with Vav1 or Vav2.

Main point R2.1: *"...determine the effect on calcium mobilization when this Cdc42 mutant [F56W] is expressed in JVav1 cell reconstituted with Vav1 or Vav2."*

If we are interpreting the reviewer's comment correctly, the goal of these experiments would be

to determine *1)* whether the Cdc42.F56W mutant, which allows Cdc42 to interact with Vav1, would cause Vav1 to suppress TCR-mediated calcium entry via the catalytic activation of a Cdc42-driven inhibitory effect, or *2)* whether the F56W mutation would alter the effector function of Cdc42 so as to enhance TCR-mediated calcium entry, in which case the Cdc42.F56W mutant might confer upon Vav2 a calcium-promoting activity. We agree that these are interesting issues. However, the interpretation of these experiments would be challenging. For example, Vav1 contributes to TCR- mediated calcium entry via non-catalytic mechanisms. This effect would be expected to counteract, to a degree, any antagonism mediated by the action of Vav1 on the Cdc42.F56W mutant, complicating the analysis of the Vav1/Cdc42.F56W pair. Further, even though Vav2 interacts with Rac1 and Rac2, and is known to activate Rac1, the Cdc42-dependent suppression of TCR-mediated calcium signals is dominant. Thus, it is unlikely that the co-expression of the Cdc42.F56W mutant would alter the

behavior of Vav2 unless endogenous Cdc42 was also eliminated. Our experience suggests that it would be very difficult to obtain enough triply-transfected cells to obtain useful calcium data.

To address the functions of our chimeric GTPases in a more direct manner, we now report the effects of the CA RR44 and CA 44RR chimeras on TCR-induced calcium entry. Although these chimeric GTPases bind to Vav1 and Vav2 in a manner determined by their N-termini (Figs. 6D and S5D), we now show that their impacts on calcium entry are determined by their C-termini (Fig. S6A). Since the C-terminus of Cdc42 is sufficient to antagonize calcium entry, it is highly likely that the F56W mutation, which is within the N-terminal fragment, would not interfere with the antagonistic effect of Cdc42 on calcium entry. While it would be interesting to determine whether the RR44 chimera and the Cdc42.F56W mutant could overcome the non-catalytic calcium-promoting functions of Vav1, we feel that this is beyond the scope of the current manuscript.

Other points:

- Page 9 line 10 - refer to Figure 2B at the end of the sentence : ....mutation nearly eliminating the response (Fig. 2B)

This has been added to the text.

- Page 13, lines 7, 9, and 12. Wrongly refer to Fig. 6B. Should be 6D.

We thank the reviewer for catching the error. This has been corrected in the text.

Second decision letter

MS ID#: JOCES/2019/238337

MS TITLE: Vav2 Lacks Calcium Entry-Promoting Scaffolding Functions Unique to Vav1 and Inhibits T Cell Activation via Cdc42

AUTHORS: Michael A. Fray, John C. Charpentier, Nicholas R. Sylvain, Maria-Cristina Seminario, and Stephen C. Bunnell
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

-

Comments for the author

I have carefully reviewed the revised version of the manuscript by Fray et al. Although not all the additional experiments I had asked for seemed to have worked out, the authors have made a big effort to address all my comments. They have included new data showing that RNAi-mediated depletion of Cdc42 supresses the inhibitory effect of Vav2 on calcium responses. This is an important result that reinforces the main point of the article. Therefore, I believe that the paper can now be accepted for publication.

Reviewer 2

Advance summary and potential significance to field

I am satisfied with the revision and the response to my prior comments.

Comments for the author

This is a well designed and executed study dissecting the different roles of Vav proteins in T cell signaling.

I am satisfied with the revision and the response to my prior comments.