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Cooperative interactions at the SLP-76 complex are critical for actin polymerization

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 February 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. I am sorry for the delay in getting back to you with a decision, owed largely to the high number of submissions and the editorial office holiday around the turn of the years. We have just now eventually received the last of four referee reports, all of which you will find attached below. I am pleased to inform you that in general, all of these expert reviewers consider your findings an important advance for understanding SLP-76-dependent T cell receptor signaling pathways. They nevertheless raise a varying number of concerns, regarding technical issue as well as presentation and interpretation, that would need to be adequately addressed before publication will be warranted. I am thus inviting you to prepare a revised version of the manuscript along the lines suggested by all four referees. In this respect, I am aware that especially the list of issues raised by referee 4 appears quite extensive, however it is clear that many of this referee's points concern really the presentation and discussion of the findings rather than experimental criticisms - and I agree that the presentational issues could probably be improved by careful revisions. In this regard, I should point out that we will have at this stage some more flexibility with the length limitations of the final version of the manuscript, so that you should be able to provide the requested careful descriptions and arrangements of main and supplementary figures. If necessary, we can of course discuss this further before resubmission.

In conclusion, should you be able to adequately address the various points raised, we would be happy to consider a revised manuscript for publication. Please be reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important that you diligently answer to all the various experimental and editorial points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website:

http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this paper Baard-Saad et al explore the interaction between Nck, vav, and SLP-76 using a range of techniques. These include biophysical analysis of purified proteins using AUC and ITC, and analysis of interactions in cells using FRET and immunoprecipitation (IP)/western blotting.
On the basis of their findings they propose a model whereby vav, nck and slp-76 form a 2:2:1 complex with vav binding directly to nck which in turn binds to SLP-76.
While much of their data is consistent with this model there are some important questions that need addressing.
1. The key gap in their study is that they have not convincingly demonstrated any direct interaction between vav and Nck using purified proteins. The techniques that they rely as evidence for a direct interaction such as FRET and IP with or without mutations only demonstrate that they exist in the same molecular complex in cells, NOT that they bind directly. The authors should adjust their interpretation of these experiments to reflect this fact. They infer a direct interaction when they demonstrate by AUC that a mixture of vav and nck form a 3 or 4:1 stoichiometry with the triply phosphorylated slp-76 peptide (Fig 1C). However this is not justified without further experiments. For example they could show that point mutations in vav or nck that do not affect direct binding to slp-76 disrupt formation of the larger 4:1 complex. Similarly they could show that mutations in vav which disrupt binding to slp-76 still allow formation of the 4:1 complex in AUC.

2. The infer from experiments in cells lacking nck or vav that nck binds directly to slp-76 and that vav binds to slp-76 via nck. This seems to contradict the experiments with purified proteins which show that vav binds directly to slp-76 peptides with a 10 fold higher affinity than nck binding to slp-76 peptides (Supp Table 1). How do the authors reconcile this major discrepancy?

3. In the ITC experiment with Nck shown in Sup Fig 1C it is difficulty to see how they established the stoichiometry as being 1:2 for pY113,128,145 and 1:1 for all the others, as reported in sup table 1. Instead it looks as though these stoichiometry were fixed for these fits. Either they should allow the stoichiometry to vary and show that the fits produce approximately 1:1 or 1:2 stoichiometry or they should do fits with stoichiometry fixed at 1:1 or 1:2 and show that one fits significantly better than the other.

Referee #2 (Remarks to the Author):

In this study the Barda-Saad and colleagues attempt to better define the composition and stoichiometry of interactions between SLP-76 with Nck and Vav1. Determining the way these proteins work together will provides a better understanding of the process of actin polimerization in response to TCR stimulation. They use a combination of techniques including ITC, FRET, and IP to examine the interactions between these proteins in both resting and TCR stimulated Jurkat and primary human T cells. The authors offer convincing evidence that Vav1 and Nck are able to interact in the absence of SLP-76 and that this interaction is enhanced by the presence of SLP-76 and TCR signals. They also provide a nice model outlining how all of these proteins could interact to promote actin polymerization.

In general, the findings are supported by the data and are of interest to the lymphocyte and signaling communities. The finding that Nck and Vav1 interact independent of TCR signals and SLP-76 is a novel finding and in combination with the proposed model involving the stoichiometric findings has the potential to alter the current thinking in the field. Enthusiasm would be increased if the following major issues were addressed:

1. Using ITC and ultracentrifugation, the authors show that Vav-1 can bind to both pY113/145 and pY113/128/145 peptides of SLP-76 (in isolation from Nck). In addition, the authors also show that Nck is necessary for Vav-1 to interact with SLP-76 in Jurkat T cells. As each of these findings is critical for the proposed model, further clarification is needed as to why what accounts for this disconnect.

2. The data presented strongly suggests that Nck can interact with Vav1 in a SLP-76 independent manner. Figure 2A clearly shows decreased FRET, but I question whether the expression of SLP-76 can improve the interaction given the slight, and nearly statistically significant trend ($p>0.053$). It may be a difference only in the cell lines used and J14 reconstituted with a wild-type SLP-76 would offer a better control. It may also strengthen the data by showing the earlier and later timepoints (1 and 5 minutes) as seen in Figure 4.

3. The authors argue that the SH3 domain of Nck is necessary for interacting with Vav1. The while FRET using the Vav1 W637 mutant is nearly zero (Figure 5), Vav1 and the Nck(SH3 mutant) is approximately 10%(Figure 4A). The immunoprecipitation data is also inconclusive and should be improved. The authors should comment upon the sufficiency of the Nck SH3 domain to mediate this interaction.

Minor issues:
1: The authors use a FRET Negative control, but fail to explain how this control was done in the text, legend or methods.

2: The authors claim that increased interaction between Nck and Vav1 occurs in stimulated cells because of increases in SLP-76 phosphorylation, but there is no 76kD band that in Figure 3E. To make this point, they should clearly point out where SLP-76 occurs in the blot in Figure 3E, repeat the experiment using a P-SLP-76 specific antibody, or show a SLP-76IP with phosphotyrosine western blot.

3: Figure 3D: Can SLP-76 be detected in the Vav1 IP?

4: The quality of the IP data in Figure 4C should be improved. The methods or legend should mention that the anti-GFP antibody cross-reacts to detect YFP.

5: The labels in Supplementary Figures 4 and 5 should be clarified. It is confusing to have the histograms labeled on the x-axis only as fluorescence intensity. The labels should be changed to reflect the molecule depicted (i.e. GFP or TCR).

6: References to Supplementary Figures 4C and 4D are switched within the text.

7: Figure S5A+B: Vav1 W637A-YFP levels are higher than the WT both in the western blot and by FACS. While this difference does not affect interpretation, it should be mentioned in the text.

8: The authors include a Supplementary Figure 6 with the paper, but fail to mention it in the text.

9: A previously published study showed that two molecules of SLP-76 can functionally interact (Jordan et al, 2008) and is referenced by the authors. However, the proposed model in Figure 7 does not address this possibility.

Referee #3 (Remarks to the Author):

Manuscript Number: EMBOJ-2009-73404

Barda-Saad et al "Cooperative interactions at the SLP-76 complex are critical for actin polymerization"

This manuscript describes a multidisciplinary approach that yields a great deal of molecular level information on how the SLP-76 complex assembles. The stoichiometry and nature of the contacts between SLP-76, Nck and Vav that are described in this paper considerably extend our knowledge of this signaling complex. The conclusions are well supported by the data and the breadth of the techniques used in this study provides a great deal of redundant information.

Of particular importance is the finding that the signaling complex consists of more than one copy of each participating protein (1:2:2, SLP-76:Nck:Vav). The signaling field is often summarized with cartoon depictions of signaling complexes with each protein indicated once - a clear over simplification. That said, stoichiometry is often difficult to establish and is therefore not well characterized for many systems. Thus, the findings presented here are highly significant and provide insight into one signaling complex that has to date been lacking.

In addition to the importance of these studies to the T cell signaling field, the work presented here should be of interest to a broader community as many of the molecular features of SLP-76, Vav and Nck are shared among many signaling proteins. My enthusiasm for this work is very high.

Referee #4 (Remarks to the Author):

This manuscript by Barda-Saad et al provides an in depth investigation of the mechanism by which a hematopoietic adaptor protein, known as SLP-76, recruits other signaling molecules required for TCR-induced actin polymerization. As explained below, the manuscript provides evidence for a novel view of the mechanism by which SLP-76 functions, which is significantly different from the currently accepted model that has appeared, virtually unaltered, in all reviews on this protein for the past 10 years. The authors provide multiple, independent lines of evidence for their model, which together are very convincing. However, the manuscript is overly long, and contains too much supplementary material, much of which cannot be understood by their target audience due to technical details that are not adequately explained. The manuscript therefore must be edited for conciseness and clarity.

Novelty and importance: SLP-76 is a hematopoietic adaptor protein that is absolutely required for antigen-receptor induced T cell responses, including TCR-induced actin polymerization. TCR-induced actin polymerization is important, because it helps facilitate a stable interaction of the T cell with antigen presenting cells. The mechanisms by which SLP-76 functions have been extensively studied, and the adaptor is known to possess 3 N-terminal tyrosine phosphorylation sites, which are known to bind to 3 signaling molecules: Vav, Nck and Itk. Based on extremely limited published evidence, it has been assumed that each of the above signaling molecules binds independently to one of the 3 phosphotyrosines found on SLP-76. However, previous attempts to sort out the role of each individual tyrosine phosphorylation site did not provide convincing evidence for this simple model, nor did they support any particular alternative mechanistic model for the function of SLP-76.
The way in which SLP-76 nucleates a signaling complex capable of inducing actin polymerization has thus remained an unsolved mystery.

This paper by Barda-Saad removes much of the mystery around this issue by providing biophysical, imaging, and genetic evidence for an alternative model for SLP-76 signaling. Their model proposes that SLP-76 binds directly to two molecules of Nck, which in turn recruit two molecules of Vav. They show that a direct interaction between Nck and Vav exists in the absence of SLP-76, and is required for SLP-76-mediated signaling events leading to actin polymerization. This model alters the presumed stoichiometry of the complex nucleated by SLP-76 and also alters the proposed topological arrangement of molecules within the complex. It therefore has important implications for all those who are interested in the study of antigen-receptor-induced signaling events in particular, and adaptor protein function in general.

Major Comments:
1. The supplementary material is much too long. Supplementary material should contain only data sets that are too large to be easily presented in printed form, or details that are of interest only to

aficionados.
I can offer the following suggestions for reducing the size of the supplementary data section:

-Data and methods that are essential to the central thesis of the paper (for example the biophysical data and methods, and the method of labeling the Vav protein, the methods for conducting FRET experiments) should be presented in a concise and easily understood format in the main manuscript.

-Essential controls that are integral parts of the main experiments should be presented as part of the experiment, in the main manuscript. For example, loading controls for IP assays should be presented as a separate panel in the IP assay figure, and not as supplementary data. Similarly, data establishing the effectiveness of siRNA reagents in reducing protein expression can be presented as an added panel in the main manuscript figures. To make room for these added panels, many of the panels in the main figures can be dramatically reduced in size - for example the histograms representing FRET efficiency can be dramatically reduced in size, or presented as tables.

Trivial controls, such as equivalent TCR expression in the various cell lines, should be mentioned as data not shown. Supplemental figure S3 can also be removed and mentioned as data not shown.

2. The biophysical data should be presented in a manner that can be followed by non-biophysicists, who comprise the majority of the readership of EMBO J. I thank the authors for citing appropriate references where readers can find more information on these techniques, but this is not sufficient. They should also provide the readers with a few sentences long explanation of how the data are interpreted, just as would be done if presenting this data in a seminar setting.

3. The manner of presentation of the ITC data is particularly unsatisfying for a number of reasons:

A. p6 of the manuscript states that the ITC experiments were performed in order to determine the binding affinities of Vav and Nck to SLP-76 phosphopeptides. The binding affinities calculated from these experiments should be presented in the main manuscript, so the reader can easily see this information without having to access the supplementary data.

B. The method by which the above-mentioned binding parameters were calculated is not at all clear. The supplementary material provides a list of the ITC experiments that were performed and the conclusions that were reached, without providing any insight (for the non-biophysicist) into how the ITC curves were interpreted to reach these conclusions. What do the X and Y axes of sup figs 1B and 1C indicate? How can one determine from these curves what is the affinity of binding and what is the number of binding sites? Why were these parameters not determined using more familiar and established methodologies (scatchard plots?).

C . In addition, the top of page 6 states that the ITC experiments demonstrate that at least 2 molecules of Vav1 or Nck associate to one molecule of SLP-76, and imply that Vav and Nck can bind directly (directly to each other? or directly to SLP-76?). I cannot figure out which particular pieces of data were interpreted to support these conclusions. It appears that none of the ITC data presented included experiments where both Vav and Nck were present, so how can the data support an interaction between Nck and Vav? If the ITC data provide critical support for this conclusion of the paper, they should be presented and interpreted in the main manuscript. If the data are "soft" and subject to multiple interpretations, then perhaps they should not be presented or discussed at all.

D. I am left wondering why the authors did not use more conventional methods to measure binding constants such as fluorescence polarization-based binding assays (which could readily measure binding of the labeled SLP-76 peptides to the much larger Nck and Vav proteins), or biacore (surface plasmon resonance)-based assays. I would like the authors to clarify what is the specific advantage of using ITC as compared to other types of binding assays, and what particular information and insights were derived by virtue of choosing this technique.
The impression given by the paper is that ITC data are open to multiple interpretations, since the binding constants are inferred from thermodynamic changes, but not directly measured.

I therefore strongly suggest that the ITC data should be replaced or supplemented by more conventional binding assays, which could be performed using the reagents already prepared for the ITC experiments. In addition to directly measuring the binding affinities of the two proteins for

SLP-76 phosphorylation sites, surface plasmon resonance (biacore) could be used to directly test their model whereby Nck cooperatively augments binding of Vav to SLP-76. This model could be tested by measuring the effect of preincubation with NCK on subsequent binding affinity of Vav. If the two proteins compete for the same sites, preincubation with Nck will increase the apparent Km for Vav binding (competitive inhibitor). On the other hand, if Nck facilitates Vav binding or indirectly recruits Vav, then preincubation with Nck should decrease the Km for Vav and/or increase the available number of Vav binding sites. In addition to providing more information, a more straightforward measurement would be easier to present in a concise format.

4. The AUC data convincingly show cooperative binding of Vav and Nck to phosphorylated SLP-76 peptides, to form larger complexes together than are formed by either binding partner alone.
The authors should explain whether they can exclude multimerization of the SLP-76 peptides as the basis of the unexpected stoichiometry. Presumably a multimerized peptide would not produce a substantial change in the sedimentation coefficient of the peptide alone, but would result in the appearance of larger than expected complexes, if all of the sites on the multimerized peptide bound to Vav or Nck.

5. I would like the authors to discuss whether the biophysical data are completely consistent with the imaging data. The biophysical data presented in supplementary table I suggest that Nck binds to the SLP-76 peptides with a slightly lower affinity (as compared to the binding of Vav to these same peptides). Yet, the FRET data presented in Fig 2 were interpreted as evidence that Vav cannot bind to SLP-76 in the absence of Nck. The authors therefore suggest that Nck bridges the interaction between SLP-76 and Vav. How can it be that Vav binds to the SLP peptides as well or better than Nck in vitro, but depends on Nck for binding to SLP-76 in cells?
An alternative explanation could be that binding of Nck to SLP-76 induces a conformational change that brings the SLP-76- and Vav-bound fluorophores in sufficient proximity to exhibit FRET. In other words, Nck does not necessarily influence the binding of SLP-76 to Vav, but only influences the manifestation of this binding as FRET. Can the authors exclude this explanation? If not, they should discuss both possible interpretations of their data.
To distinguish between these two interpretations, the authors should perform an immunoprecipitation assay, to test whether knock-down of Nck in fact disrupts the ability of Vav to bind to SLP-76 within TCR-stimulated cells.
In addition, the authors should demonstrate that the Nck siRNA treatment does not interfere with TCR-induced tyrosine phosphorylation of SLP-76.

6. The manuscript should cite the work of Wu et al Immunity 4:593-602 (1996), which first described the physical and functional interaction between Vav and SLP-76. Wu et al showed that mutation of the SH2 domain of Vav abolished its interaction with SLP-76. The authors of the current manuscript should discuss their results in light of this finding - if Vav is indirectly recruited to SLP-76 via an SH3 domain-mediated interaction with Nck, why is the SH2 domain of Vav required for binding to SLP-76?

7. What does the lane labeled "no ab" in fig3B, 3C and similar figures indicate? This is not mentioned in the figure legend or in the methods section.
As a control for the specificity of the co-IP reaction between Nck and Vav, the authors should perform the following control experiment:
Perform an anti-Vav IP from WT (E6.1) and from J.Vav cells, followed by blotting for Nck. If Nck is present in the WT IP but absent in the anti-Vav IP from J.Vav cells, this would constitute proof that Nck is truly co-precipitating specifically with Vav, and not non-specifically binding to the anti-Vav beads.

8. The experiment shown in fig 4C is not convincing.
To me it appears that the C-terminally mutated Nck construct exhibits a basal interaction with Vav. Perhaps a better quality experiment could be substituted for this one (if the co-IP signal were higher, it would be easier to distinguish true co-IP from background noise). In the revised figure 4C, the expression level of FP-tagged Nck constructs should be shown as an additional panel within the main figure, and not as supplementary data. The panel showing co-IP of Nck should be clearly labeled as such and not labeled as GFP-blot (all proteins in the experiment are FP-tagged).

9. Figures 6 B-D are intended to prove that the W637A mutation of Vav does not impair TCR

signaling in general, but only specifically impairs actin polymerization.
The thesis of the manuscript does not really require that the effects of the W637A mutation be restricted to actin polymerization. Since Vav is thought to cooperate with SLP-76 to mediate activation of PLC and NFAT (see Wu et al), it is entirely possible that a mutation that disrupts the recruitment of Vav to SLP-76 would disrupt these downstream signaling events.
However, the authors never explicitly show that the W671A mutation of Vav disrupts its recruitment to SLP-76. This experiment should be performed and added to the manuscript.

As they stand, the experiments shown in Figs 6 B-D have some defects that should be improved, if the experiments are not removed from the paper. The particular defects are listed below:

-The citation of Reynolds et al on p19 is the wrong reference. The Reynolds paper investigated the phenotype of Vav1-deficient mice, not J.Vav cells. The J.Vav cells were described in Cao et al, which for some reason is not cited in this paper. (the Yablonski paper describing the J14 cell line, which is also extensively used in this manuscript was also not cited for some reason). The Cao paper shows that (contrary to the observations seen in Vav1-deficient mice) J.Vav cells do not exhibit reduced PLC-gamma phosphorylation or reduced calcium flux. Figs 6B and C are therefore irrelevant and should be removed. The reduced calcium flux in J.Vav observed by the Barda-Saad group does not fit with the previous description of this cell line, and may reflect some experimental error.

-The error bars in Fig 6D are quite large. Is the response seen in WT or 637AVav-reconstituted J.Vav cells significantly different from the response seen in J.Vav cells alone?
This figure should be modified to show NFAT luciferase activity both in the presence and absence of stimulation. Then it would be easier to determine whether the W637A mutant can reconstitute TCR-induced NFAT activation.

Minor Comments:
1. Abbreviations should be defined the 1st time they are used. The abbreviation ITC appears on p6 of the manuscript but is defined only in the supplementary material. The abbreviation AUC is not defined, but it should be defined on p6, where the term analytical ultracentrifugation 1st appears. The abbreviation Sv is never defined.

2. The description of the ITC experiments on p6 of the results section should refer to binding of Nck and Vav to short SLP-76-derived phospho-peptides, and not to SLP-76.

3. The figure legends for all of the FRET histograms should indicate how many cells from how many experiments were analyzed to determine the average FRET efficiency and what the error bars represent.

4. Parts of the results section should be shortened, to focus more on the experiments and their interpretation, while reserving detailed discussion of the literature for the introduction and discussion sections. For example, the middle of p13 contains a long discussion of previously published work which does not contribute to a smooth presentation of the results. The following sentences should be removed: "Studies in the literature suggest.....prompted us to revisit the necessity of SLP-76 for Nck biding to Vav" .

5. What do the second and 3rd rows in Fig 3A represent? Do they show the same cell seen in the first row, but at different time points? Or different cells at the 3 min time point? This question relates also to Figures 4A and 6A (bottom of page).

6. Why is the W637A mutation referred to as a mutation in a proline-rich region of Vav on p17? Isn't this just a standard SH3 domain-inactivating mutation?

7. The expression "necessary and required" at the top of p18 is redundant. One of these words is sufficient.
8 . The results section beginning on the 3rd line of p18 lacks a subtitle. Also, the 1st 7 lines of this section (lines 3-9 on p 18) really belong in the discussion and should be deleted here.

9. The experiment performed in Figure 6A should be more clearly explained - do the results indicate

basal levels of actin, or TCR-stimulated actin polymerization? Also, what does the term "integrated intensity" indicate?

1st Revision - authors' response 30 April 2010

Reviewer #1

We appreciate the reviewer's comments and specific suggestions aimed at improving the paper. All the points suggested by the reviewer were addressed as detailed below:

(1) Based on AUC experiments, it seems that the binding affinity between Nck to VAV1 is weak as stated in the text (page 23, second paragraph). In a control experiment with VAV1-FAM and Nck at 4 M in the absence of SLP-76, we only observed a slight increase (~ 0.15 S) of the sedimentation coefficients of the mixture over the individual free components, indicating weak and rapidly reversible binding of the purified molecules in solution (data not shown). Given the weak affinity of these proteins in dilute solution in vitro, there seems to be little point to perform the suggested experiments in-vitro.
Instead we believe that the AUC experiment results strongly suggest a VAV-Nck interaction in the context of cooperative binding to phosphorylated SLP-76. It is difficult to interpret the AUC data without that conclusion. On the basis of these initial biophysical studies we turned our focus to an intensive analysis of the interaction in cells. Influenced by previous structural studies on the interaction of Grb2 and VAV1, we performed mapping experiments that support our model. The use of point mutations in Nck and VAV1 specific binding sites, both of which disrupt the interaction with functional consequences, seems to us to be the best evidence of the interaction in vivo.

(2) We would like to emphasize that the experiments demonstrating that Nck recruits VAV to SLP-76 were done in-vivo while the experiments showing that VAV binds directly to SLP-76 were done in-vitro. There is a potentially important difference in the form of the VAV1 protein used in these experiments. For technical reasons we generated and purified a fragment of VAV1 containing only the SH2 and SH3 binding domains. Our cellular experiments made use of full-length VAV1, which in vivo might be subject to additional cooperative interactions. Moreover as mentioned in the discussion section "This difference and the possibility of multiple, cooperative binding events in vivo in the setting of numerous other binding events may account for this difference in the in vitro and in vivo results" (page 24, second paragraph). It is known that relative affinities can be modulated by orders of magnitude when going from a diluted solution to the intracellular environment, because of the high volume exclusion of all intracellular proteins leading to strongly favor any reactions that produce more compact conformations of the complex (lower excluded volume), relative to the separate components. What do not change, however, are the (oligomeric) states of the proteins and their complexes, as well as the modes of interaction.
Again the in vitro experiments led us to more extensive in vivo studies and considering the reviewer's input and in order to strengthen the in-vivo data we added a set of experiments (Fig. 5D) in which mutant form of VAV1 W637A (which abrogates the direct interaction with Nck) and VAV1 wt were introduced into JVAV lacking the endogenous form of VAV1. Immunoprecipitation of SLP-76 and immunoblotting with anti VAV1 and Nck antibodies were performed. Co-precipitation of SLP-76 and VAV1 was dramatically reduced in the VAV1 W637A mutant cell lysates in contrast to an inducible interaction detected between SLP-76 and VAV wt proteins. This biochemistry data confirm the FRET data and indicate that Nck is required for the recruitment of VAV1 to SLP-76.

(3) We agree with the reviewer that visually it is indiscernible that while the inflection point for the triple phosphorylated peptide is at a molar ratio << 1, the binding to the other peptides is much weaker. Thus, we have to rely on the statistical results of the data analysis. Here, as the reviewer correctly guesses, the stoichiometry was fixed to either 1:1 or 1:2 and the quality of fit was compared. This is described in the supplemental methods: "Departing from the custom of traditional ITC analyses, SEDPHAT does not allow for unphysical non-integral values of binding

sites ('n-values')", and in the footnote to the Supplementary Table S1: "A model for two independent sites exhibiting the same binding parameters as the independent, non-cooperative binding to the SLPpY113,145 site and SLPpY128,145 site, respectively. For each site, the binding parameters were fixed to those observed independently". Furthermore, as described in the methods, the analysis was performed by globally fitting to two or more titrations. For the data questioned by the reviewer (Nck binding to triple phosphorylated peptide), the best single site fit results in a chi-square 2.6-fold larger than that for the non-cooperative two-site model (with parameters fixed to the separately measured individual sites). Moreover, the residuals are strongly systematic with the best-fit single site model, whereas they are almost randomly distributed for the two-site model. In order to address the reviewer's concern, we have added in the footnote of the table in the supplemental data: "The single-site model was rejected in the global fit due to highly systematic residuals and 2.6 fold larger chi-square of the fit than the two-site model that achieved nearly randomly distributed residuals".

Reviewer#2
We very much appreciate the reviewer's suggestions. We note that reviewer#1 had some similar issues, which we addressed above.

(1) Please see our response to reviewer#1, point #2. We agree with the reviewer that additional clarification should be provided, and have therefore added a new in-vivo experiment (Figure 5D) which confirms by biochemical approach the FRET data illustrated at Figure 2. This experiment is described in detailed in the results section (page 18, second paragraph). Furthermore, we added a paragraph to the discussion (page 24, second paragraph) highlighting the differences between the in-vivo and the in-vitro system. This is followed by a clarification of our data consistency suggesting that the first molecular model presented in Figure 7A is more likely than the one presented in Figure 7B (page 25, first paragraph).

(2) We apologize for not including the requested control sample and have now added it to the manuscript. In addition we have repeated this experiment >5 times. The data presented in Figure 3A-C strongly suggest that Nck binds to VAV1 independently of SLP-76. Two types of cells were compared- E6.1 (expressing endogenous SLP-76) and J14 (SLP-76 deficient T cells) ($p<0.07$). In order to determine whether reconstitution of SLP-76 deficient T cells (J14) with SLP-76 wt presents better Nck-VAV interaction than J14 cells, we performed two independent experiments in which we introduced VAV-YFP and CFP-Nck into J14 cells reconstituted with SLP-76 wt. We found no significant differences in the FRET efficiency between Nck and VAV expressed in these cells and those expressed in J14 cells ($p<0.412$). Please see page 14, first paragraph. Based on this data, we think that SLP-76 does not significantly affect the direct binding affinity between Nck and VAV. Since the interaction between Nck and VAV is independent of TCR stimulation, we do not see any significant differences in the FRET efficiency of these proteins in the presence or absence of SLP-76 and at different time point activation.

(3) We agree with the reviewer comment regarding the biochemistry data previously presented in Fig. 4C. We improved the immunoprecipitation data (Figure 4C). The presented data clearly demonstrate that point mutation in Nck c-terminal SH3 domain dramatically reduces the interaction of Nck with VAV1.

Minor issues:
(1) Due to text limitation the paragraph describing the FRET negative controls appeared at the supplementary text, however due to the reviewer's comment we moved this text to the main manuscript (page 29, third paragraph).

(2) We agree with the reviewer that SLP-76 band was difficult to identify in our previous Figure 3E, thus we added an arrow indicating where this protein precipitated (upper band close to 75kDa marker). In addition we added the blot of the membrane with anti SLP-76 antibody which confirms the location and the identity of the band pointed out in the anti phosphotyrosine blot as SLP-76, please see new Figure 3E.

(3) Figure 3D: We added as requested a new panel showing that SLP-76 can be detected in

stimulated cell lysates immunoprecipitated with anti-VAV antibody and not in unstimulated cell lysates, as expected.

(4) As correctly requested by the reviewer, the immunoprecipitation presented in Figure 4C was improved, please see new Figure 4C. In addition, we added the clarifying sentence that anti-GFP antibody cross reacts to detect YFP and CFP (page 28, first paragraph).

(5) The x-axis of Supplementary Figure S4 & S5 now termed as Supplementary Figure S4A, S4B, S4D and S4F was changed to "CFP or YFP fluorescence intensity" as requested.

(6) References to Supplementary Figure 4C and Supplementary Figure 4D were corrected.

(7) We agree with the reviewer's comment that although the difference in the expression level between VAV1 W637A to wt form does not affect result's interpretation, it should be mentioned in the text. We have added this in page 18, first paragraph.

(8) Supplementary Figure 6 now termed as Supplementary Figure 5 is described in details in Supplementary data, page 9 and the labeling technology is mentioned in the main manuscript, page 6, third paragraph.

(9) We agree with the reviewer that the question of whether multiple SLP-76 molecules interact in a complex has been raised previously, however our AUC data are not consistent with a complex comprised of multiple SLP-76 molecules associated with Nck and VAV1 at the measured ratio, while our cellular data do not address this point. For example a dimer of SLP-76 with Nck and VAV1 (with a ratio of 4:4:2) would have an apparent molecular weight of 350kDa and would migrate at greater than 10S far exceeding the observed 6 S. Thus, the measured composition in concert with the measured s-value makes it impossible to consider the idea of an oligomerized SLP-76 in the complex.

Reviewer#3
We appreciate the positive criticism of the reviewer who found our research novel and highly significant.

Reviewer#4
We appreciate the reviewer's comments and suggestions aimed at improving the paper. All the issues raised by the reviewers were addressed by providing new experimental data or textual clarifications as listed below:
(1) The manuscript was edited for conciseness and clarity as requested. Data presenting the effectiveness of siRNA reagent (old Supplementary Figure S2B) or protein expression (old Supplementary Figure S5A) were combined with the main data presented in Figure 2B and 5B, respectively. Data or methods describing the biophysical aspect of the paper were substantially elaborated, however due to space limitations the paragraph describing the labeling of VAV1 by Sortase or the ITC remained in the supplementary data. Supplementary Figure 4 panels which show the expression levels of the proteins and serve as controls for FRET analysis are in the supplementary data for space reasons and since they are of particular interest only for FRET specialists. Controls as TCR expression level were removed as requested by the reviewer.

(2) As mentioned above, we have rewritten the biophysical data and specifically the ITC section with much more details, to be understandable by non-biophysicists as requested. Please see supplementary data first section. Unfortunately we can not include this section in the main manuscript due to length limitations.

(3) For the ITC data: (A) The calculated binding affinities could not be moved to the main manuscript due to length limitations, however further detailed explanation was added. (B) We added explanations as to how the experiments were performed as well as how the data was interpreted

(Supplementary data first section). Axes are labeled. (C) We stated that two molecules of Nck and two molecules of VAV1 can bind to SLP-76. The molecular interaction is indicated in Figure 7A in which Nck bind to SLP-76 and recruits two molecules of VAV1. The data where both Nck and VAV1 are present in addition to SLP-76 are from sedimentation velocity analytical ultracentrifugation and not ITC. Please see Figure 1. (D) In fact, ITC technology is very well established in the biophysical field, and is even considered one of the GOLD STANDARDS for macromolecular interactions. Fluorescence polarization assays, would not have given meaningful insights, although they allow quantifying binding, since it is generally impossible from the measured change in anisotropy to distinguish the number of molecules binding to the labeled species. Surface plasmon resonance, as well, is a method that makes it very difficult to quantify multiple binding events, and is very much limited by complex surface effects. Incidentally, we are well versed in SPR (see our reviews in Ann Rev Biophys Biomol Struct 26;541, 1997, and Curr Opin Biotech 8;498, 1997), and have both fluorescence polarization and surface plasmon resonance well established in our laboratory, to be used for studies when appropriate. The present system is far exceeding in complexity the abilities of these methods.

(4) AUC data: The multi-signal sedimentation velocity analysis with the labeled SLP-76 peptides allows us to quantify the relative composition of the complexes. This suggests the 2:2:1 VAV:Nck:SLP-76 composition. If SLP-76 peptides were to dimerize, then a complex with the measured 2:2:1 composition would have to exhibit a stoichiometry of 4:4:2. Such a complex would have a molecular weight of ~ 350 kDa, and would be expected to sediment at >> 10 S, far exceeding the observed 6 S. Thus, the measured composition in concert with the measured s-value makes it impossible to consider the idea of an oligomerized SLP-76 in the complex. This clarification was added to the manuscript, please see page 10, first paragraph and page 25, first paragraph.

(5) The biophysical data are consistent with the imaging data. Some discussion of this point was made above in response to a similar question from reviewer#1 point#2. In order to address the reviewer#4's comments, statistical analysis was added to Supplementary Figure S2B which presents the FRET efficiency between SLP-76 and VAV1 in J-VAV cells. The interaction between these proteins decreases dramatically when either the SLP-76Y113F or SLP-76Y128F mutant is used. When we mutated both sites (Y113 & Y128), the FRET efficiency between SLP-76 to VAV1 was dramatically reduced but was not zero, most probably due to a weak interaction of Nck with Y145 site. Since in vivo VAV1 interactions require Nck and we see that two Nck molecules can bind SLP-76 our in vivo results as well as the in vitro data support the same potential stoichiometery of 2:2:1 (VAV1:Nck:SLP-76). We also added a textual clarification, (page 24, third paragraph). In addition, new data was added to the paper (Fig. 5D) confirming the observation that Nck recruits VAV1 to SLP-76. In these experiments, mutant VAV1 W637A (which its direct interaction with Nck was disrupted) and VAV1 wt were introduced into J-VAV lacking the endogenous form of VAV1. Immunoprecipitation of SLP-76 and immunoblotting with anti-VAV1 and anti-Nck antibodies were performed. Co-precipitation of SLP-76 and VAV1 was dramatically reduced in the VAV1 W637A mutant cell lysates in contrast to a constitutive interaction detected between SLP-76 and endogenous Nck. This biochemistry data confirm the FRET results and indicate that Nck is required for the recruitment of VAV1 to SLP-76. These experiments exclude the option that Nck does not influence the binding of SLP-76 to VAV1 but rather influences the manifestation of this binding detected by FRET. This experiment is described in detailed in the results section (page 18, second paragraph). Furthermore, we added a paragraph to the discussion (page 24, second paragraph) to further clarify this issue.

(6) As requested by the reviewer we cited the work done by Wu et al Immunity 4:593-602 (1996). Please see page 25, first paragraph. We believe that our work does not contradict Wu's study but rather adds another novel aspect to the triple molecular complex formation consisting of SLP-76, Nck and VAV1. We show for the first time that Nck and VAV1 directly interact both in unstimulated as well as in stimulated conditions. We show that the interaction between SLP-76 and VAV1 is inducible upon TCR interaction, although most of this is due to Nck (Figures 2B-C and Figure 5D), while we still do not preclude the possibility that minor fraction of VAV1 can interact with SLP-76 following TCR stimulation. In fact we propose such a model in Figure 7B, in which VAV SH2 domain binds SLP-76 directly, although we showed that this is not prominent interaction. Furthermore, it is possible that following TCR activation, interactions mediated by the VAV SH2

domain contributes to the overall cooperativity in the formation of the signaling complex. In that case if this domain is mutated the complex would not be as stable.

(7) We agree that the manuscript should explain this term. The lanes "No Ab" define the negative control samples. The samples represent cell lysates which underwent the same procedure as the experimental samples in the absence of a specific antibody, to eliminate the possibility of non specific binding between the cell lysates and the beads. We added this description in the text (page 30, second paragraph). In addition we performed the experiment suggested by the reviewer. Please see Supplementary Figure 2C. As shown, no precipitation between VAV1 and Nck was detected in J-VAV cells in contrast to E6.1. We added the description of this experiment in the text (page 14, second paragraph).

(8) We agree with the reviewer's comment and we have repeated this experiment, resulting higher quality data. Please see new Figure 4C. Since the membrane was reacted with anti-GFP antibody that cross reacts with YFP and CFP, we did not change the label but rather added a clarification to the Materials and Methods section regarding this cross reactivity. Please see page 28, first paragraph.

(9) As mentioned above, we added the requested experiment, please see Figure 5D and related text (page 18, second paragraph). Experiments presented in old Figure 6D were reproduced, please see new Figure 6B, in additional T-test data were added to the figure legend, please see page 37, first paragraph. As suggested by the reviewer, old Figures 6B-C were removed, the text was not changed and these experiments are reported. Cao et al and Yablonski et al papers were added, please see page 11, first paragraph.

Minor comments:
(1) The abbreviations ITC, AUC and Sv are defined. Please see, page 6 second and third paragraphs.

(2) The ITC experiments were done with short or long synthesized SLP-76 phosphopeptides. We added a clarification in the text, please see page 6, first paragraph.

(3) The number of experiments performed for the FRET data analysis is mentioned in the legend to figure. It is not practical to mention the cell number of each and every experiment since in some cases an image presents multiple cells.

(4) As requested, the sentence was removed.

(5) The rows at figures 3A, 4A and 6A represent different cell panels at a specific activation time point. Thus, we added a textual clarification in the legend to these figures.

(6) Mutation W637A is an SH3 domain (595-660) mutation. The VAV1-Nck binding sites, as mentioned in the discussion, resemble VAV1-Grb2 interaction sites (Nishida M. EMBO J. 20:2995, 2001). VAV1-Grb2 interface has been analyzed by crystallography and found to consist a few discontinuous sites: 600-623, 630-635 and 642-646 aa. The tetraproline region of VAV1 is bound to Grb2 in a manner similar to that of the proline-rich peptide bound to the SH3 domain. The tetraproline region of VAV1 forms a hydrophobic pocket in which Trp637 is the major component at the bottom of the Grb2-binding valley, substitution of Trp637 caused a large decrease (~40-fold) in the VAV1-Grb2 binding affinity. Based on this observation we introduced a point mutation in the N-terminal SH3 domain of VAV1, W637A, and indeed this mutation also abolished VAV1-Nck direct interaction.

(7) The textual correction has been made.

(8) The section was removed.

(9) The results in Figure 6A represent TCR-induced actin polymerization. As mentioned in Material & Methods section, the coverslips were coated with anti-CD3 antibody. The term "integrated intensity" means the integration of fluorescence intensity of cells labeled with phalloidin. This was clarified at the legend to Figure 6A (page 36 second, paragraph).

We hope that the additional experiments we have performed and added as well as the extensive clarifying textual changes we have done are satisfactory. Thanks to all reviewers for their thoughtful comments.

Acceptance letter 25 May 2010

Thank you for submitting your revised manuscript for our consideration. It has now been reviewed once more, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal

