EMBO reports

A longer isoform of Stim1 is a negative SOCE regulator but increases cAMP modulated NFAT signaling

Mona Knapp, Dalia Alansary, Vanessa Poth, Kathrin Foerderer, Frederik Sommer, David Zimmer, Yvonne Schwarz, Nicolas Künzel, Achim Kless, Khaled Machaca, Volkhard Helms, Timo Mühlhaus, Michael Schroda, Annette Lis, and Barbara Niemeyer
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Dear Prof. Niemeyer

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be September 9th in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

***IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.***

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) The accession number for the mass spectrometric proteomic data and the database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Please remember to provide a reviewer password if the datasets are not yet public.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Regarding data quantification

The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

10) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

This important manuscript by Dr Niemeyer and colleagues describes the effects of a novel STIM1 splice variant, Stim1A, on SOCE and NFAT translocation. Stim1 A contains an extra 31 amino acids in the C-terminus, which results in it acting as a dominant negative regulator of Orai1, accelerating fast Ca-dependent inactivation. Despite reducing channel activity, the authors show that Stim1A increases Ca2+-dependent NFAT translocation into the nucleus. Careful mutagenesis identifies a major role for D503 in the regulation of Orai1 gating by Stim1A. Using differential proteomics, they find that the variant associates less than wt Stim1 with PDE8b, a phosphodiesterase that breaks down cAMP. Stim1A seems to alter the crosstalk between calcium entry with cAMP signalling, providing a new link between these signaling pathways. The experiments are nicely done and the findings are novel and important and will be of widespread interest. I very much enjoyed reading the manuscript and support publication in EMBO Reports.

The majority of the paper addresses the role of Stim1A in the gating of Orai1 and the underlying molecular mechanism. This section is novel and convincing. The second part focusses on the link between Stim1A, cAMP and NFAT. This is also intriguing but there are a few loose ends that should be addressed, largely in the Discussion.

The fast CDI experiments should ideally include hyperpolarizing steps to a range of negative potentials rather than to just one. In addition, the authors are probably correct in attributing the effect of Stim1A to CDI, but this would be strengthened by comparing EGTA with BAPTA in the patch pipette or using Ba2+ rather than Ca2+ as the charge carrier.

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The identification of the charged series of residues required for Stim1A gating of Orai1, and the MSD data are very elegant. The authors have nicely dissected out the mechanism and the results are convincing.

To link the first part of the paper with the second (cAMP), it would be interesting to check what forskolin or inhibition of PDE8B does on fast CDI. It has been suggested that PKA causes fast CDI of Orai1 by phosphorylation of Ser34. (Zhang et al., 2019).

The PDE8B/cAMP/NFAT translocation part is interesting but needs a little tightening up. This is more for the Discussion than a need for new experiments. i) The proteomics work was done in HEK cells but NFAT translocation was in SHSY-5Y cells. PDE8B has a relatively narrow tissue distribution, being found mainly in thyroid and testis and prostate gland. Is it expressed in SHSY-5Y cells? ii) The authors cite the Son et al. paper (PNAS 2020) but those authors did not show calcineurin and NFAT were associated with AKAP79 and Orai1. The relevant paper here is Kar et al. (Current Biology 2014). iii)Do the authors see similar effects in the HEK293DKO cells that they see in SHSY cells? If they express Stim1 or Stim1A, are the differences reduced when cAMP is increased? iv) in 7g, at time 0 (no stimulation), why is cAMP high? Presumably this is basal levels, so would one expect a slower decline in basal activity to have much effect on PKA activity?

Referee #2:

This s/m reports the identification of a new STIM1 variant termed STIM1A that is expressed in selective cell types with high level in testicular cells and Astrocytes. The authors continue to analyze the effect of STIM1A and conclude that STIM1A function as a partial dominant negative of STIM1 function. Of particular significance is the finding that STIM1A controls cAMP in addition to Ca2+ influx to specifically modulate the PKA regulation of calcineurin and NFAT regulation. The results presented support the main conclusions and the findings add significantly to understanding STIM1 function, Ca2+ signaling and report on a novel additional mode of regulation of NFAT and gene regulation. I have fairly minor comments relating to controls and few suggestion to extend and solidify the findings a bit.

Comments:

1. The introduction is very long and goes too far in describing all potential STIM1 variants and their effect and should be

condensed for clarity.

2. Ca2+-dependent inactivation: This require several additional controls. How STIM1A affect fast Ca2+-depend inactivation of STIM1+Orai1? Do mutations of the exon A residues that eliminate the effect of STIM1A on Ca2+ influx also eliminate the increased FCDI observed with STIM1A? Do mutations of the residues mediating FCDI in STIM1A and in STIM1 eliminate the effect of STIM1A on Ca2+ influx and the current measured with STIM1A and the effect of STIM1A on Ca2+ influx and current by STIM1+Orai1?

3. Additional controls: What is the effect of STIM1A on native SOCs? The finding in Figure 2c,d requires a control of the effect of STIM1 and STIM1A on the influx in the absence of Orai1. This will address the first part of the comment.

4. FRET studies: These can be extended by measuring the time course of STIM1-STIM1, STIM1A- STIM1A, STIM1-Orai1 and STIM1A-Orai1 FRET to determine whether STIM1A affect the rate of clustering and activation of Ca2+ influx.

5. One of the "Fig. 3a,b" in p6 should be 3a,c and all references to Fig. S3 in p7, Fig. S3 regarding the STIM1A mutants should be Fig. S4. The TG data in Fig. 4d appears to be switched where the blue is lower than orange, opposite from the traces. Please correct.

6. Do the mutants in exon A other than D503A that eliminate the effect of STIM1A on Ca2+ influx, in particular the AAAA mutant, also have no effect on cAMP, PKA and NFAT?

7. Many of STIM1 effects on NFAT requires localization at particular ER/PM junction domain. The authors should test if the same holds for STIM1A. Perhaps the simplest is to test whether STIM1A(ΔK) behaves like STIM1A with respect to activation of Orai1, inhibition of STIM1 function and activation of NFAT. Note that STIM1(ΔK) does not activate NFAT. This also offer a control for the pharmacological drug. Does PF rescue activation of NFAT by STIM1(ΔK)?

Referee #3:

This study focuses on a splice variant of STIM1, the Ca2+-sensing regulator of the store-operated calcium entry (SOCE) channels, referred as STIM1A. In depth analysis of the tissue distribution, the SOCE regulatory function, and the involvement in NFAT activation is described. The main conclusion of the Authors is that STIM1A is a dominant negative regulator of SOCE but it preferentially recruits cAMP signaling to converge with calcium on NFAT activation. Overall, this work can be a useful addition to the calcium signaling literature and most of the studies are thoughtfully designed and apparently well executed but there are some significant weaknesses of the present version:
-On the role of cAMP in the NFAT activation, the data with the PDE8 inhibitor are straightforward. But the cell permeable cAMP analogues have similar effects on both STIM1 and STIM1A rescues, and the cAMP records show only an early difference between them whereas the NFAT activation traces seem to progressively diverge. Some additional studies like testing if cell permeable analogues prevent the effect of the PDE8 inhibitor and also including in the cAMP pharmacology in Fig7 the STIM1A D503A mutant that lacks the dominant negative effect on SOCE, would be useful. The prediction for the mutant is that in the presence of cell permeable cAMP it will duplicate the effect of STIM1 on the NFAT activation.

-All the studies use only pharmacological ER Ca2+ depletion for testing the STIM1A engagements. The Authors might want to show if STIM1 vs STIM1A makes a difference during stimulation of the cells with a physiological calcium mobilizing agonist.

-Nanomolar calcium concentration is not in a linear relationship with the Fura Ratio and the Rmax & Rmin might be different in the different measurements. Therefore, showing nM Ca2+ concentration on the y axis would be appropriate in all the cytoplasmic calcium measurements. Furthermore, Fig2AB has to include the non-rescued cells or a reference in the text is needed to a publication where the Authors showed STIM1 rescue vs mock rescue.

-It would be useful to show in the various comparisons the expression levels of STIM1, STIM1A and STIM1A D503A because differences could contribute to some of the observed differences.

We thank all reviewers for their constructive criticism and the suggestions of additional experiments. With these additional experiments we were able to gain a deeper understanding of the cAMP-SOCE crosstalk and how this is affected by the splice variant STIM1A. A new figure (Figure 8) is now included and defines PIP Kinases as new targets for regulation by cAMP. In addition, new Figure EV5 provides additional data regarding FCDI and expression levels of PDE and ADCY in the investigated cell types. Figure EV2 is also appended. A new model figure summarizes our data in a comprehensive manner. The manuscript has been in part rewritten to include the new data and also the suggestions of the reviewers. Below, please find our detailed point-to-point reply, additional data for the reviewers and all changes that we made.

Referee #1:

This important manuscript by Dr Niemeyer and colleagues describes the effects of a novel STIM1 splice variant, Stim1A, on SOCE and NFAT translocation. Stim1 A contains an extra 31 amino acids in the C-terminus, which results in it acting as a dominant negative regulator of Orai1, accelerating fast Ca-dependent inactivation. Despite reducing channel activity, the authors show that Stim1A increases Ca2+-dependent NFAT translocation into the nucleus. Careful mutagenesis identifies a major role for D503 in the regulation of Orai1 gating by Stim1A. Using differential proteomics, they find that the variant associates less than wt Stim1 with PDE8b, a phosphodiesterase that breaks down cAMP. Stim1A seems to alter the crosstalk between calcium entry with cAMP signalling, providing a new link between these signaling pathways. The experiments are nicely done and the findings are novel and important and will be of widespread interest. I very much enjoyed reading the manuscript and support publication in EMBO Reports.

We thank the reviewer for the positive evaluation.

The majority of the paper addresses the role of Stim1A in the gating of Orai1 and the underlying molecular mechanism. This section is novel and convincing. The second part focusses on the link between Stim1A, cAMP and NFAT. This is also intriguing but there are a few loose ends that should be addressed, largely in the Discussion.

The fast CDI experiments should ideally include hyperpolarizing steps to a range of negative potentials rather than to just one. In addition, the authors are probably correct in attributing the effect of Stim1A to CDI, but this would be strengthened by comparing EGTA with BAPTA in the patch pipette or using Ba2+ rather than Ca2+ as the charge carrier.

We agree with the reviewer and conducted additional experiments using EGTA as an intracellular chelator. The protocol even with BAPTA had included several steps, but CDI was not prominent at the second step. We also added experiments to include the single point mutant STIM1A_D503A in the analysis of FCDI as also suggested by reviewer #2. This new data now is shown in new Figure EV5 (S5) and discussed in the results section (page 8). We conducted FCDI experiments with the STIM1AD503A mutant in both buffering conditions and also normalized FCDI to the initial peak current. While we find that STIM1A_D503A rescued FCDI in BAPTA conditions, it did not rescue currents or FCDI in EGTA conditions, uncovering an additional Ca2+ dependency. Please see revised results and discussion, reply to reviewer #2 and discussion regarding S34 below.

The authors argue that the effects in Fig 2a and b are not due to differences in Stim1 versus Stim1A expression, and cite the western blot in Fig 1g. However, the data in 2a and b were from MEFs whereas 1g is from HEK293 cells. The authors probably should use the same cell type to make the argument they do. They seem to have done this in Fig 4c; perhaps that should be referred to, not

1g.

The reviewer is correct. WB in Fig 1g was after expression from bi-cistronic constructs using IRES mcherry in HEK DKO cells, but the same constructs were used for experiments in Fig. 2a. We have used these murine constructs in Figure 2a in MEF cells but also in HEK cells as shown in Figure 2 c,d. However, to directly correlate expression levels with function, we indeed used tagged constructs in Figure 4 and measured the relative fluorescence intensity (RFI) to control for equal expression of STIM1, STIM1A and STIM1A_D503A as quantified in 4c. We have adjusted our text (page 5 and page 6) to account for this. We also conducted new imaging experiments with directly tagged constructs and calibrated the Fura2 signals to show absolute changes in Ca2+ (see revised Figure S2 (EV2 E,F)). We do not see any evidence that addition of 31 amino-acids negatively affected the amounts of translated protein of either tagged (Fig. 4c, Fig EV2) or of untagged constructs (Fig. 1G).

In Fig 2, Stim1A is co-expressed keeping Stim1 constant. This is an important experiment and the conclusion would be strengthened by some quantification of Stim1 levels being constant despite varying Stim1a levels. What happens if the Stim1A levels increase further? Is there a steady state reduction in SOCE which cannot be reduced further by Stim1A? And roughly how do these levels of Stim1 and Stim1A expression compare with those tissues where both proteins are found (heart, astrocytes, testis)?

We went back and investigated additional tissues that were tested for RNA expression in Fig. 1 and Fig. S1A and quantified the protein amounts of STIM1A vs. STIM1wt. In none of the investigated tissues (except the Sertoli cell line TM4) we find significantly higher amounts of STIM1A protein compared to STIM1wt protein levels (Fig. R1, below).
The 1:1 coexpression data (Fig. 2a,b) shows that we still see a phenotype on SOCE, but it also becomes apparent that it would be very difficult to see any significance with traces between STIM1:STIM1A ratio of 1:1 (blue trace in Figure 2a) and those of a 0:1 ratio (red trace). The finding that astrocytes from mice lacking splicing (showing ~ 32% of Stim1A) display an increased SOCE (Fig 2 e and i) argues for the in-vivo relevance of such a ratio. We also find that protein expression of both variants in heart appears to show a developmental profile. This is highly interesting and will be investigated in a follow-up study. In addition, our NFAT translocation data argues for effects of STIM1A that are independent of its decreased Ca2+ entry.

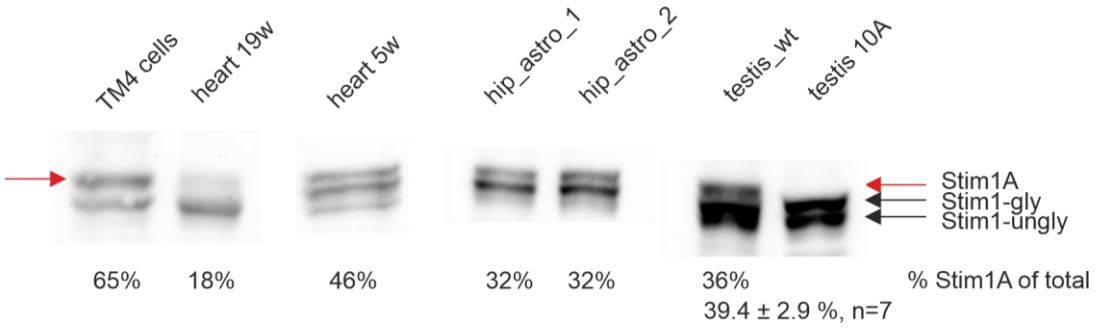


Fig. R1
Quantification of STIM1 protein amounts of selected tissues.

The identification of the charged series of residues required for Stim1A gating of Orai1, and the MSD data are very elegant. The authors have nicely dissected out the mechanism and the results are convincing.
We very much appreciate the recognition of these approaches.

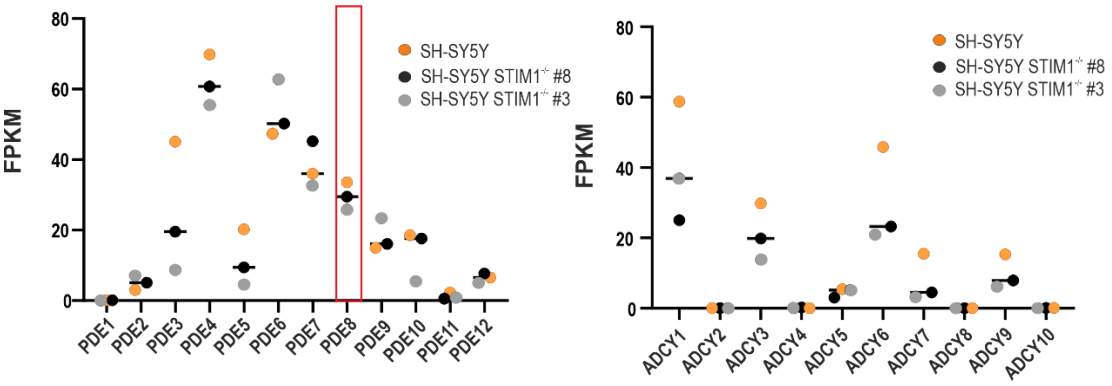
To link the first part of the paper with the second (cAMP), it would be interesting to check what forskolin or inhibition of PDE8B does on fast CDI. It has been suggested that PKA causes fast CDI of Orai1 by phosphorylation of Ser34. (Zhang et al., 2019).

This is an interesting suggestion, however, to explain the effects that STIM1A has on gating, we believe our data already harbor the answer to the reviewer's question. Fig. S2 shows that STIM1A reduces SOCE to a similar degree upon co-expression with different Orai homologs (Orai1, 2 or 3) in cells without expression of endogenous Orai channels (HEK TKO cells). As Ser34 is unique to the long form of Orai1 and not present in Orai2 or Orai3, altered phosphorylation of Ser34 cannot be the cause of the reduced current/SOCE. Our docking data and the investigation of the ETON region mutant of Orai1 instead point towards a destabilized interaction with the ETON region, which is identical in all of the Orai homologs. These arguments are now included in the discussion (Line 366)
Indeed, the Orai1R77E mutation masks an additional Domain A induced effect. We believe that the destabilized gating with Orai channels causes a change in FCDI. We did not observe any significant effect of incubation with the PDE8 inhibitor on the kinetics or the total amount of Ca2+ entry (revised Fig.7,DE), thus our data points to a role of PDE8 on NFAT translocation that requires the presence of STIM1 (SOCE), but is independent of the effect of SOCE reduction. New additional data (new Fig. 8) point towards a role of cAMP in the facilitated generation of PIP2. The results section and the discussion is amended to refer to the new data.

The PDE8B/cAMP/NFAT translocation part is interesting but needs a little tightening up. This is more for the Discussion than a need for new experiments. i) The proteomics work was done in HEK cells but NFAT translocation was in SHSY-5Y cells. **PDE8B has a relatively narrow tissue distribution, being found mainly in thyroid and testis and prostate gland. Is it expressed in SHSY-5Y cells? ii)** The authors cite the Son et al. paper (PNAS 2020) but those authors did not show calcineurin and NFAT were associated with AKAP79 and Orai1. The relevant paper here is Kar et al. (Current Biology 2014). iii**) Do the authors see similar effects in the HEK293DKO cells that they see in SHSY cells?** If they express Stim1 or Stim1A, are the differences reduced when cAMP is increased? iv) in 7g, at time 0 (no stimulation), why is cAMP high? Presumably this is basal levels, so would one expect a slower decline in basal activity to have much effect on PKA activity?

We thank the reviewer for the helpful input.

i) We addressed expression of all major PDE's and AC by performing RNA seq analysis of either the mock transfected parental SHSY-5Y cell line as well as on two independent CRISPR/Cas9 cells lines in which STIM1 was deleted. Clone #8 was used for the NFAT translocation experiments and Ca-imaging as shown in Fig 7d, new Fig. EV2 E,F. Fig. EV5D demonstrates that PDE8 indeed is expressed although at a slightly lower level compared to PDE3, 6 and 7, although the FPKM differences are rather small. We mentioned these results within the text on page 9, line 320. We also checked ADCY counts (Fig EV5E).



ii) The authors cite the Son et al. paper (PNAS 2020) but those authors did not show calcineurin and NFAT were associated with AKAP79 and Orai1. The relevant paper here is Kar et al. (Current Biology 2014).

We are sorry for this omission. We have now corrected the text on page 9, line 304 ff to cite Kar et al., 2014, Samanta et al 2015 and the new report showing the association of AKAP with Orai1 (Kar et al., PNAS 2021), see also revised discussion.

iii) Do the authors see similar effects in the HEK293DKO cells that they see in SHSY cells?

We indeed originally did first translocation experiments in the HEK293DKO cells as shown below, Fig R2 a,b and see a similar tendency. We also checked in SH-SY5YDKO cells and see a more dramatic difference on NFAT translocation (Fig R2 c,d, but low n). As we were concerned about a possible effect due to the lack of STIM2 (see Son et al., PNAS2020), and as a double knock-out background is less physiological, we decided to continue with the SH-SY5Y single STIM1-/- cells, which contain endogenous levels of STIM2 (STIM1 FPKM 50, STIM2 15 in CTR cells). However, this data indicates that NFAT translocation in these cells does not require STIM2 and we have included a sentence in the discussion.

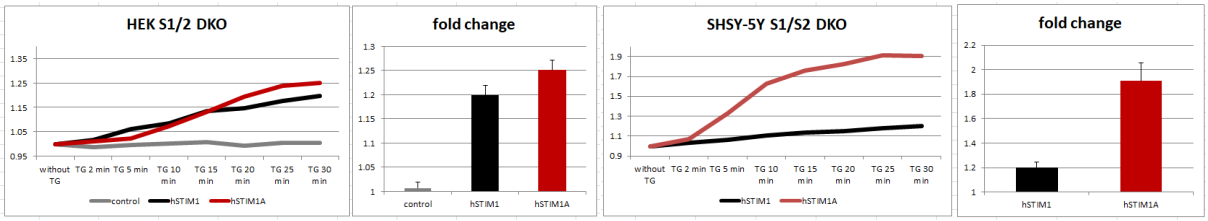


Fig. R2 NFAT translocation (N/C) after TG induction in HEK S1/2 DKO (2 biological replicates) and after expression in SHSY-5Y S1/2 DKO cells (1 biological replicate, 2 technical).

iv) in 7g, at time 0 (no stimulation), why is cAMP high? Presumably this is basal levels, so would one expect a slower decline in basal activity to have much effect on PKA activity?

We have done additional controls for bleaching and for the effects of control treatment without Tg. cAMP levels are normalized and not necessarily high at time point 0 (non-ratiometric indicator). With the new controls it becomes apparent that Tg (Ca2+) leads to a decline of basal levels of cAMP, likely due to Ca2+-CAM dependent PDE activity and STIM1 dependent recruitment of PDE8 to the signaling complex. This decline appears slowed in the case of STIM1A expression, which is in line with a reduced interaction of STIM1A with PDE8.

As shown with further controls, see response to reviewer 2 and 3 and also included as new Figure 8, the effects of STIM1 and STIM1A and the PDE8 inhibitor PF on NFAT translocation are absent with deletion of the STIM1 polybasic domain (STOP after 762), which showed some reduction of SOCE. This new data suggests that the effects may be mediated by PIP2 levels and we speculated that cAMP may cause an increased production of PIP2 by activating PIPkinase, thus facilitating recruitment of AKAP79 and STIM1 to the NFAT-SOCE signalosome. Indeed ISA2011-B, an inhibitor of PIPK abolished the cAMP effect. See new Figure 8, results and modified discussion. As both cAMP analogs have an effect on NFAT translocation, we believe this is not due to PKA activation but rather to an increased activity of PIP5K.

Referee #2:

This s/m reports the identification of a new STIM1 variant termed STIM1A that is expressed in selective cell types with high level in testicular cells and Astrocytes. The authors continue to analyze the effect of STIM1A and conclude that STIM1A function as a partial dominant negative of STIM1 function. Of particular significance is the finding that STIM1A controls cAMP in addition to Ca2+ influx to specifically modulate the PKA regulation of calcineurin and NFAT regulation. The results presented support the main conclusions and the findings add significantly to understanding STIM1 function, Ca2+ signaling and report on a novel additional mode of regulation of NFAT and gene regulation. **I have fairly minor comments relating to controls and few suggestion to extend and solidify the findings a bit.**

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We agree that the introduction was rather lengthy and condensed the text.

2**. Ca2+-dependent inactivation: This require several additional controls. How STIM1A affect fast Ca2+-depend inactivation of STIM1+Orai1? Do mutations of the exon A residues that eliminate the effect of STIM1A on Ca2+ influx also eliminate the increased FCDI observed with STIM1A? Do mutations of the residues mediating FCDI in STIM1A and in STIM1 eliminate the effect of STIM1A on Ca2+ influx and the current measured with STIM1A and the effect of STIM1A on Ca2+ influx and current by STIM1+Orai1?**

We would like to thank the reviewer for this interesting point. We conducted additional patch-clamp experiments with co-expression of STIM1, STIM1A or STIM1A_D503A with ORAI1 in STIM deficient HEK cells. We conducted these experiments both with BAPTA and with EGTA in the internal solution. We also introduced a new analysis of this data to account for potential effects of differential current densities on FCDI. This data is now included in Fig EV5 (see also below) and confirmed a strong dependency on intracellular Ca buffering on FCDI as expected. In conditions of stronger Ca-buffering, STIM1A_D503A rescued current densities and rescued normalized FCDI. Switching to EGTA containing internal solution, uncovered a Ca2+ dependency of the point mutant: Current densities remained reduced, and FCDI was not rescued. However, as detailed below and see also new experiments (Fig. 8, revised Figure S5), the effects of STIM1A or the STIM1A_D503A mutant on NFAT translocation appear mostly independent of the altered gating phenotypes. Revised Figure 7 now shows the long-term Calcium measurements including S1AD503A mutant with or without addition of PF.
Figure 5 also shows that mutation of ORAI1R77E, implicated to be involved in Ca2+ dependent inactivation (REF), masks the effect of STIM1A on SOCE or current densities.

To us it is not entirely clear whether the reviewer is referring to the mutations in STIM1A (D503A) or in Orai1 (R77) In case the reviewer refers to the effect of D503, please see above.

In case the reviewer refers to the effect of R77: *We analyzed FCDI data with R77 which masks the A effect. Interestingly, R77E leads to a strong increase in FCDI and also much reduced currents (Fig.5) with Wt STIM1, confirming a stabilizing role of R77 in gating, but the increased FCDI of STIM1A is lost with R77 (Fig R3). Because the effects on NFAT translocation appear independent of the FCDI effects, we did not include Fig R3.*

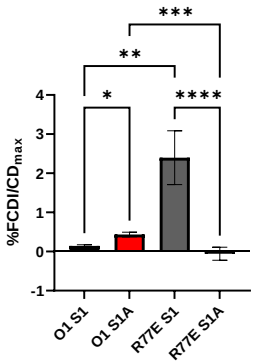


Fig. R3. Normalized FCDI of R77 in combination with STIM1 wt or STIM1A

3. Additional controls: What is the effect of STIM1A on native SOCs?
The finding in Figure 2c,d requires a control of the effect of STIM1 and STIM1A on the influx in the absence of Orai1. This will address the first part of the comment.

Because HEKwt cells (Fig 2 CD) still include endogenous Orai protein, we conducted experiments as shown in Figure S2 in HEK triple Orai knock-out cells lacking Orai1, Orai2 and Orai3 (described in Alansary et al, JCS 2020, Fig. S1, included below), the results (Fig. S2A-D) demonstrate that STIM1A scales SOCE in a similar manner with all Orai homologs and significantly decreases SOCE for each Orai homolog independently. This is important as it shows that only conserved regions of the proteins mediate this reduced activation (see text page 3). In these TKO cells we also observe that the D503A mutant fully rescues SOCE for ORAI2 (Fig. R4, below). Without overexpression of ORAI, no SOCE is seen in these cells (see below). This argues that at least in HEK cells, it is not likely that another SOC channel is significantly recruited by STIM1A. We cannot fully exclude that this may be the case for native SOCs in cells normally expressing STIM1A. Downregulation of Stim1A in astrocytes via siRNA or by recording from astrocytes unable to generate Stim1A, SOCE is increased (Fig 2), confirming the dominant-negative effect of domain A on native SOCE.
The revised Figure S2 (EV2, EF) now includes additional Fura2 experiments conducted in STIM1 deficient cells with native SOC channel expression with normalized data to show absolute Calcium values. These cells were also transfected with a vector control and show that endogenous SOCE (no overexpression of ORAI1) is absent without STIM or STIM1A. In addition, Figure 2A shows that in MEF cells, Stim1A elicits SOCE without overexpression of Orai1. The vector only control for corresponding experiments can be found (Ramesh et al., 2021), Supplementary Figure 2D.

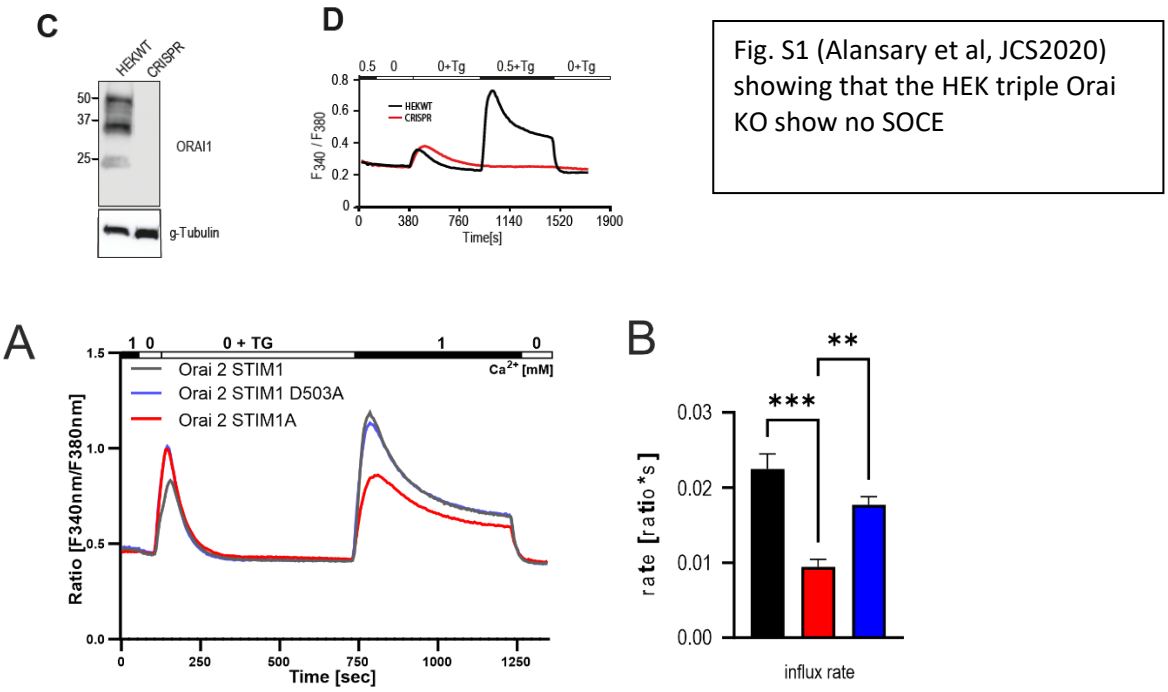
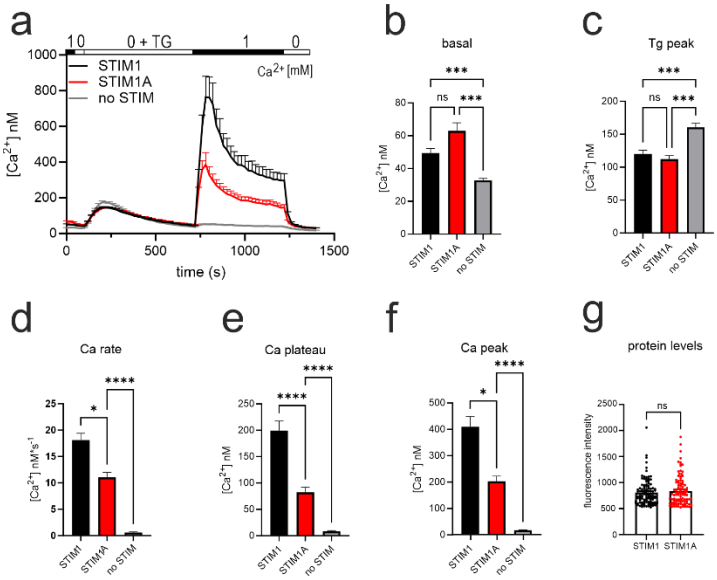


Fig. S1 (Alansary et al, JCS2020) showing that the HEK triple Orai KO show no SOCE

Fig. R4 STIM1A_D503A rescues reduced STIM1A-ORAI2 mediated SOCE in HEK triple Orai KO cells.

4. FRET studies: These can be extended by measuring the time course of STIM1-STIM1, STIM1A-STIM1A, STIM1-Orai1 and STIM1A-Orai1 FRET to determine whether STIM1A affect the rate of clustering and activation of Ca2+ influx.

We analyzed the rate of Ca2+ increase in all our imaging experiments and find a consistent decrease in the rate of Ca entry with expression of STIM1A. In contrast, downregulation of STIM1A increased influx rates (Fig. 2g, j) of Fura2 ratios. We conducted additional experiments to calibrate Ca influx rates below (d) for traces described in the revised Figure S2.



These results are consistent with the slower rate of current development, which could indeed be due to a slower rate of cluster formation. We carefully rechecked all out data and also found a small mistake with the significance bars in Fig. 4d. The corrected Fig. 4D shows that the single point mutant rescues the decreased SOCE rate for Orai1 and also for Orai2 (Fig. R4B), which indicates that the insertion of 31 amino acids does not per se slow cluster formation in a dramatic fashion. This is discussed in the results (page 7) and is the reason that we did not repeat FRET experiments. Please see also below (reply to point 8) for substantial new experiments that we found more relevant to understand the role of STIM1A.

5. **One of the "Fig. 3a,b" in p6 should be 3a,c and all references to Fig. S3 in p7, Fig. S3 regarding the STIM1A mutants should be Fig. S4. The TG data in Fig. 4d appears to be switched where the blue is lower than orange, opposite from the traces. Please correct**

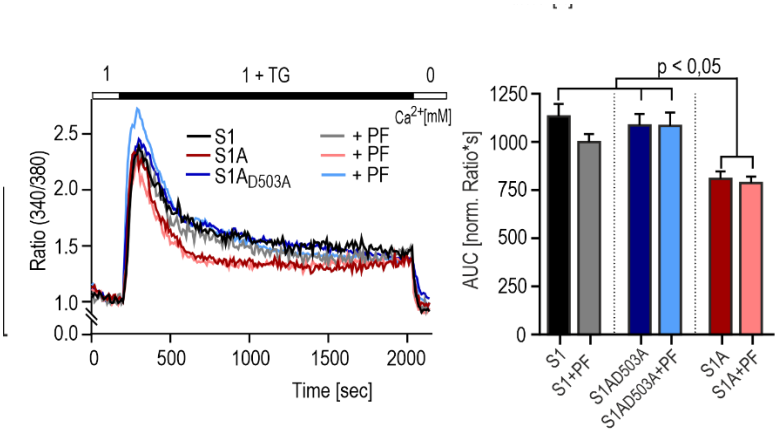
We thank the reviewer for pointing out our mislabeling regarding Figure S3 which should have read S4 and is now corrected. We rechecked analysis of the imaging data in Figure 4. Indeed, the TG peak (around 200sec in trace Fig 4b) for STIM1A D503A was a little lower than for wt or STIM1A. However, the SOCE trace (750 to 1250 sec in b) shows a complete rescue. A small mistake in the significance bars of the rates has been corrected (see above). In all SOCE parameters (lower bars in d: rate, peak and plateau), D503A rescued the decreased SOCE phenotype.

6. Do the mutants in exon A other than D503A that eliminate the effect of STIM1A on Ca2+ influx, in particular the AAAA mutant, also have no effect on cAMP, PKA and NFAT?

The STIM1 D503A mutant, which eliminates the effect of domain A on Ca2+ influx (however, see new FCDI data in EGTA conditions), still shows the increased translocation of NFAT (Fig. 6DE). We have added the Fura imaging trace of D503A over the entire time frame of a translocation experiment to Fig 7D. Together with new data and experiments suggested by the reviewer, see below and shown in new Figure 8, it becomes apparent that the effects of STIM1A on cAMP regulated enhancement of NFAT translocation are independent of differences in the absolute amount of Ca2+ entry, but as detailed below, are rather due to cAMP effects on PIP2 and not on PKA.
In our proteome pull-down approach, we had also included STIM1A_D503A to see if we can find mutant specific differences in protein interactions. However, for none of the hits described in Figure 6 and also not for PDE8B, we find that the differential interaction is altered for STIM1A_D503A versus STIM1A. Thus, we expect the mutant to behave like STIM1A in all experiments involving differential recruitment of PDE8. This information is now included in the results section page 9.

	mean LFQ S1A_D503A	mean LFQ STIM1A	mean LFQ STIM1	A vs D503 pvalue	STIM1 vs D503 pvalue	STIM1 vs A pvalue
KIAA2026	362	937	62328	0.9303	0.0001	0.0001
LOXHD1	391	451	5982	0.9355	0.0002	0.0002
CYB5R3	569	421	167	0.0526	0.0006	0.0061
FBLN1	1959	1392	19285	0.9049	0.0089	0.0077
PDE8B	6418	7064	37627	0.9464	0.0148	0.0162
SOD1	272	458	2047	0.7549	0.0204	0.0312
SYT2	4240	5028	18037	0.8732	0.0268	0.0333
EFEMP1	229	273	855	0.8536	0.0342	0.0443

We also conducted NFAT translocation experiments with the 4A mutant, which also showed less PF facilitated NFAT translocation when compared to STIM1. As insertion of domain A hinders the recruitment/activation of PDE by STIM1, it is not likely that the mutations would obliterate the steric hindrance.



updated Figure 6 D, E

7. Many of STIM1 effects on NFAT requires localization at particular ER/PM junction domain. The authors should test if the same holds for STIM1A.

The reviewer raises a very interesting point. In HEK293 cells we did not find a difference in the localization between STIM1 and STIM1A either alone (Fig.3, see Mander's coefficients mentioned within the text page 6, line 199) or in the presence of ORAI1 (Fig. 3d). However, immune-histological localization of both variants in testis indicates that, in vivo, both variants partially concentrate at different ER-PM junctional regions (see preprint: (Knapp et al., 2020). This data is not included in the current manuscript as we are currently creating testis-specific total STIM1 knock-out animals to unambiguously define the specificities of the applied antibodies. Future studies which go beyond the functional analysis presented in this manuscript will be conducted to address the in vivo role of STIM1A.

8. Perhaps the simplest is to test whether STIM1A(ΔK) behaves like STIM1A with respect to activation of Orai1, inhibition of STIM1 function and activation of NFAT.
Note that STIM1(ΔK) does not activate NFAT. This also offer a control for the pharmacological drug.

This is an excellent idea and we created both tagged variants with deletion of the C-terminal polybasic domain (translational STOP codon inserted after nucleotides encoding amino-acid 672). We then conducted both additional Calcium imaging experiments using the same global protocol as applied for the NFAT translocation experiments. New Fig 8 A and B show the expected decrease in total amount of Calcium entering the cell when comparing STIM1 to STIM1A. Deletion of the polybasic domain (PBD) reduced the AUC significantly for STIM1 but still showed Calcium entry which was higher than STIM1A induced entry. Deletion of the polybasic domain in STIM1A did not lead to a further reduction in Calcium entry. However, as indicated by the reviewer and demonstrated in Figure 8C, deletion of the PBD abolished NFAT translocation for both STIM1 and STIM1A and this effect was not rescued by PF. This new data confirms that NFAT translocation does not linearly scale with the amount of Calcium entry (as reported before, see references in discussion) and also that a local

signalosome integrating PIP2 rich domains and local Calcium entry is critical. Targeting of the STIM1-C-terminal domain to PIP2 rich domains is critical for facilitating anchoring of the AKAP-CAN-STIM1-ORAI signalosome (see (Son et al., 2020)) and also see (Kar et al., 2021a; Kar et al., 2021b)). This data prompted us to speculate if the effects of PF and cAMP analogs are due to an increase in PIP2 levels. Indeed, in S. cerevisiae, activity of the phosphatidylinositol-4-phosphate 5-kinase can be regulated by cAMP (Kato, H. et al., JBC 1989). Experiments using the new phosphatidylinositol-4-phosphate 5-kinase inhibitor ISA-2011B (Kunkl et al., 2017) indicate that indeed, inhibition of phosphatidylinositol-4-phosphate 5-kinase abolishes the cAMP dependent enhancement of NFAT translocation (Fig. 8D). This new data is included in the result and discussion section and provides an important link between the effects of PF and cAMP analogs and NFAT translocation. Results and discussion have been amended to include these new results.

Referee #3:

This study focuses on a splice variant of STIM1, the Ca2+-sensing regulator of the store-operated calcium entry (SOCE) channels, referred as STIM1A. In depth analysis of the tissue distribution, the SOCE regulatory function, and the involvement in NFAT activation is described. The main conclusion of the Authors is that STIM1A is a dominant negative regulator of SOCE but it preferentially recruits cAMP signaling to converge with calcium on NFAT activation. Overall, this work can be a useful addition to the calcium signaling literature and most of the studies are thoughtfully designed and apparently well executed but there are some significant weaknesses of the present version:

-On the role of cAMP in the NFAT activation, the data with the PDE8 inhibitor are straightforward. But the cell permeable cAMP analogues have similar effects on both STIM1 and STIM1A rescues, and the cAMP records show only an early difference between them whereas the NFAT activation traces seem to progressively diverge. Some additional studies like testing if cell permeable analogues prevent the effect of the PDE8 inhibitor and also including in the cAMP pharmacology in Fig7 the STIM1A D503A mutant that lacks the dominant negative effect on SOCE, would be useful. The prediction for the mutant is that in the presence of cell permeable cAMP it will duplicate the effect of STIM1 on the NFAT activation.

We thank the reviewer for the comments. Our initial findings triggered by the results of the proteomic data indicated that compared to STIM1, STIM1A interacts less with PDE8B, potentially leading to reduced degradation of cAMP, if the association of STIM1 with PDE8 facilitates its activation. Indeed, the PDE inhibitor (PF-also resulting in less cAMP degradation) is less potent with STIM1A compared to STIM1 (Fig. 7B) and RflincA measurements indicate a reduced initial cAMP degradation with STIM1A compared to STIM1 (Fig.7F). We expect the cell permeant cAMP analogs to mostly override differences that we see between STIM1 and STIM1A differential regulation of the cAMP hydrolyzing enzyme PDE8, as they would flood the cells with supramaximal cAMP concentrations. Indeed, although we see a tendency in the absolute amount of translocated NFAT to be slightly less with the analogs for STIM1A, this difference is not significant. What was surprising was that both analogs have similar effects, excluding a sole PKA dependent effect. As suggested by reviewer #2 we created several new constructs to test the influence of the polybasic domain on NFAT translocation. New Figure 8 shows that the PF effects are abolished upon deletion of the PBD from both STIM1 and STIM1A while for the full length constructs we reproduced the differential PF effect. We then conducted both additional Calcium imaging experiments using the same global protocol as applied for the NFAT translocation experiments. New Fig 8 A and B show the expected decrease in total amount of Calcium entering the cell when comparing STIM1 to STIM1A. Deletion of the polybasic domain (PBD) reduced the area under the curve (AUC) significantly for STIM1 but still showed Calcium entry which was higher than STIM1A induced entry. Deletion of the polybasic domain in STIM1A did

not lead to a further reduction in Calcium entry. However, as demonstrated in Figure 8C, deletion of the PBD abolished NFAT translocation for both STIM1 and STIM1A and this effect was not rescued by PF. This new data also confirms that NFAT translocation does not linearly scale with the amount of Calcium entry (as reported by others before) but that a local signalosome integrating PIP2 rich domains and local Calcium entry is critical.

These data indicate that the targeting of the STIM1-C-terminal domain to PIP2 rich domains is critical for facilitating anchoring/efficient functioning of the AKAP-CAN-STIM1-ORAI signalosome (see (Kar et al., 2021a; Kar et al., 2021b; Son et al., 2020)). This data prompted us to speculate if the effects of PF and cAMP analogs are due to an increase in PIP2 levels.
Indeed in S. cerevisiae activity of the phosphatidylinositol-4-phosphate 5-kinase can be regulated by cAMP (Kato et al., 1989). Experiments using the new phosphatidylinositol-4-phosphate 5-kinase inhibitor ISA-2011B (Kunkl et al., 2017) indicate that indeed, inhibition of phosphatidylinositol-4-phosphate 5-kinase abolishes the cAMP dependent enhancement of NFAT translocation. This new data is included in the result and discussion section.

All the studies use only pharmacological ER Ca2+ depletion for testing the STIM1A engagements. The Authors might want to show if STIM1 vs STIM1A makes a difference during stimulation of the cells with a physiological calcium mobilizing agonist.

We performed additional experiments using Carbachol as mobilizing agent and the same global protocol as for TG. In contrast to TG activation, 100 µM Carbachol resulted in a faster rise in intracellular Calcium and again STIM1A showed a reduction (Fig. R5). Carbachol was more efficient than TG in inducing NFAT translocation (Fig. R5C). Importantly, although Cch was more efficient activator of NFAT translocation, we saw no significant reduction of NFAT translocation with STIM1A despite lower global Ca2+ (Fig. R5B), however the relative difference Cch/TG was less. We speculate that differences that we see with cAMP modulated translocation at submaximal stimuli might be overridden by supramaximal carbachol stimulation, as Carbachol efficiently stimulates phosphoinositide turnover (Quist et al, 1987, Baron et al., 1989). We agree that more work is needed to unravel the in vivo role of STIM1A in the heart, testis and astrocytes with a combination of Gq and Gs stimulatory pathways and will follow up on this after this initial characterization. Because the data with Carbachol do not add sufficient mechanistic detail, we did not include this data in the manuscript.

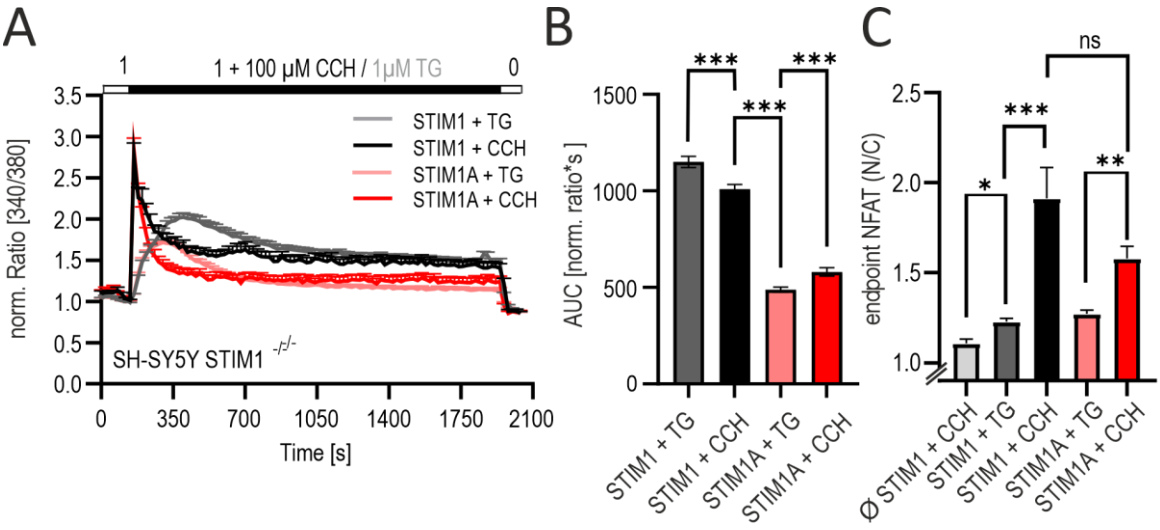


Figure R5: Comparison Tg versus Carbachol on Ca2+ and NFAT Translocation

A. Traces showing average changes (mean±s.e.m.) with normalized ratio over time in response to perfusion of different stimuli as indicated in the upper bar with mcherry-tagged constructs expressed in SH-SY5Y STIM1-/- cells (clone#8).
B. Quantification of the Area Under Curve (AUC) of ratios shown in (F). ***p<0.001, ordinary one-way Anova indicated only for selections (26< n <105) mean±s.e.m.
C. Normalized NFAT ratios (nuclear/cytoplasma) after 30 min with comparable stimulation as shown in (F). *p<0.05, **p<0.01 ***p<0.001, Kruskal-Wallis Anova (13 < n <103).

Nanomolar calcium concentration is not in a linear relationship with the Fura Ratio and the Rmax & Rmin might be different in the different measurements. Therefore, showing nM Ca2+ concentration on the y axis would be appropriate in all the cytoplasmic calcium measurements.

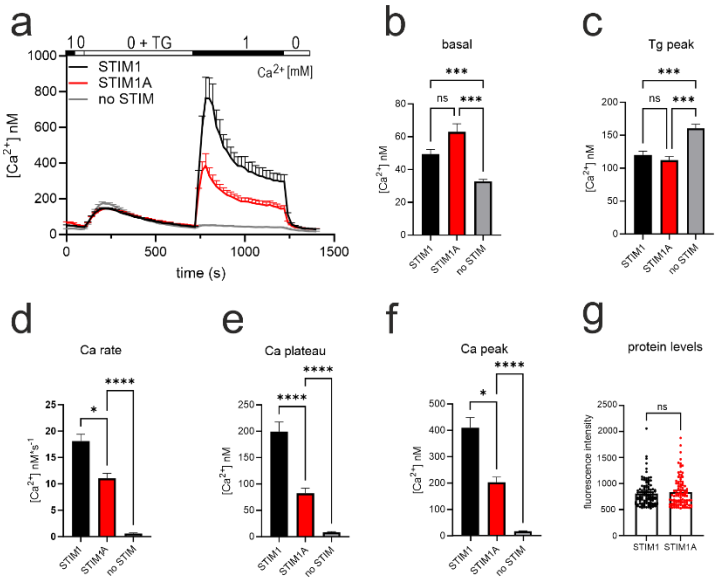


Fig. R5/EV2. Expression of control vector, STIM1 or STIM1A in SH-SH5A STIM1-/- cells. After Ratio measurement, cells were calibrated and ratios were converted into nM Calcium concentration a) time course of SOCE, b-g) quantification of parameters

The reviewer is correct that showing calibrated data is preferable. In our experience, the clear differences we see by showing ratios (we do not overload cells by prolonged incubation, use only low Fura concentrations and physiological external Ca concentrations) are recapitulated with calibrated data. For the large number of experiments shown in the manuscript, we cannot go back and calibrate as many of them were done on a set-up that now has a new light bulb, altering the parameters of the set-up. However, we performed a new set of imaging data with subsequent calibration and all reported differences (rate, peak and plateau of SOCE) remained significantly different. Traces and quantification of plateau and expression levels are included in the revised Figure EV2 EF (see above for all parameters) and mentioned in the results section.

Furthermore, Fig2AB has to include the non-rescued cells or a reference in the text is needed to a publication where the Authors showed STIM1 rescue vs mock rescue.

The reviewer is right and we included the reference to Ramesh et al. (MEF, Fig S2d) on page 5 line 161. Please also note vector control in new Fig. EV2 EF.

It would be useful to show in the various comparisons the expression levels of STIM1, STIM1A and STIM1A D503A because differences could contribute to some of the observed differences.

We were particularly concerned about this issue, although expression from the same strong promoter and inclusion of only 93 nucleotides would likely not affect translational efficiency. We therefore carefully quantitated protein levels for untagged constructs on WB (Fig 1G). To unambiguously

control for protein levels of constructs we also used for the NFAT translocation assays, we used m-cherry tagged constructs (see Figure 4c/new Fig EV2F) for quantification of directly tagged constructs, RFI values see 4C, EV2F). We do not see any differences in expression levels, Figure 4c includes expression of STIM1A_D503A, see also Fig. R3 for rescue of Orai2 in HEK TKO cells.

References

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Dear Prof. Niemeyer

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are positive about the study and request only minor changes to clarify text and figures. Please ensure to discuss the fact that NFAT translocation in HEK cells does not require STIM2 (referee 1). Referee 3 is concerned about the biological relevance of the findings. Upon discussing this point further with the referee, we suggest to include the carbachol stimulation data (Reviewer Figure 5) in the manuscript and to discuss the implications on the potential biological relevance of STIM1A.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Data availability section: Please remove the reviewer access information and add a link that resolves to the dataset in the respective database.

- Please check again whether all comments from our data editor in the figure legends have been addressed. I note that e.g., the comments for Fig. 1C (letters, arrows) and Fig. 1G (bars, error bars) have not been addressed and the comments in the EV figure legends also need your attention (see attached file).

- Please remove the Supplementary tables from the manuscript file and upload them individually as Table EV# files. Alternatively, you could combine all tables into one 'Reagents table', which is typeset into the methods section. See also the relevant information under "Structured methods" in
https://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- Citation of preprints: Please cite Knapp et al in the text as (preprint: Knapp et al, YEAR) and in the reference list as: Author names (YEAR) Title. bioRxiv doi: xyz [PREPRINT]

- Reference format: Names should not be in capital letters, the year needs to be in brackets and et al should be used if there are more than 10 authors. See also
https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Please move all information on funding to the Acknowledgement section.

- Figure callouts should be alphabetical, if possible, and all panels should be referenced. In this respect we note:
Fig 1C is called out before 1A.
Fig 4C is called out before 1H and 2E.
Fig EV4A callout is missing.
Fig EV5E+F callouts are missing.

- Methods: you mention that you calculate FRET efficiency according to van Rheenen et al, but do not provide the corresponding reference.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

The authors have carefully and thoroughly addressed the comments made and the revised paper has been strengthened by the addition of new data, particularly the inclusion of cAMP on PIP5K. Overall, this is an important paper that describes how a new longer isoform of STIM1 reduces calcium entry but enhances NFAT activation, through inhibition of PDE8.

The authors have nicely addressed my original review but I nevertheless have a few minor
comments.

Fig 2A, it would be helpful to show the absence of SOCE in the background recording.

Typos- p5; empty brackets after Ramesh et al., 2021; 6 lines ups from end of 2nd para: ...presence of absence...

Just a comment to the authors- most studies in HEK 293 cells have found PDE4 as the major isoform expressed (e.g. Shen and Cooper, 2013; Houslay papers). It is surprising that the only isoform in this study is PDE8. This could be a consequence of using STIM1-/2- cells, although one feels that is unlikely,

In my original review, one point I made was : The PDE8B/cAMP/NFAT part is interesting but needs tightening up.....
The authors reply with four sections to their answers: i)-iv). In iii), I had asked: Do the authors see similar effects in HEK293DKO cells that they see in SHSY cells? In their reply, the authors state that" However, these data indicates that NFAT translocation in these cells (HEK) does not require STIM2 and we have included a sentence in the discussion.

This is an important point but I was unable to see the sentence in the discussion. It should be included.

Referee #2:

Excellent and significant paper

Referee #3:

I appreciate the efforts of the Authors, and accept their responses. However, the Authors' decision to omit from the manuscript the phenotype they obtained for their novel splice variant in cells stimulated through a cell surface receptor (Figure R5) is unfortunate, and raises questions on the biological relevance of the findings. The results in the present version seem to be confined to testing the outcomes of pharmacological depletion of the ER Ca2+ store.

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November 23, 2021

To the editors of EMBO reports, dear Dr. Rembold,

We have edited our final manuscript according to the recommendations by the editor and reviewers. The requested additional data is now included in EV Fig 5 and is discussed in the results and discussion section. Reviewer 1 asked us to include a sentence regarding STIM2 in the discussion which we did, as we did not see any translocation upon deletion of the STIM1 PBD despite the presence of STIM2. (The HEK DKO data was only performed once and cannot be cited as data not shown). We carefully checked the EV figure legends and went through the other editorial comments.
References were reformatted.

Please let me know if any additional editorial changes are necessary.

With best regards,

Prof. Barbara Niemeyer
Saarland University
Molecular Biophysics
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Homburg 66421
Germany

Dear Barbara,

Thank you for implementing some final changes. I have uploaded the manuscript text you sent and am now very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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THINGS TO DO NOW:

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Barbara A. Niemeyer
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-53135V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	in General, at least three biological replicates with each three technical replicates were performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For Calcium imaging experiments, preactivated cells with initial Fura2 ratios > 0.5 were excluded as these indicate dying cells. These criteria are preestablished and applied to all experiments.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes, sometimes experiments were analyzed in a blinded fashion and key experiments were sometimes repeated by a different experimentator
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, samples were always analyzed for sample distriubtion (normalilty adn Lognormality test of raw or normalized data), depending on the outcome of this test different statistics were applied as specified in each figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	see above, Normality and Lognormality tests, followed by ordinary one way anova if normal distrubted or by Kruskal-Wallis Anova for multiple comparsions if non-normal distribution.
Is there an estimate of variation within each group of data?	no

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	in general we assume unequal variance to be on the critical side.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	yes, this information is included in the table, antibody verified by Antibodypedia
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	all cell lines are listed in the materials and methods and are routinely checked for mycoplasma contamination

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C56Bl6J, 10A Con in C56BlJ background, SPF housing in animal facility, source of mice are cited int the paper and only used for organ collection.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA, only organ collection was performed done by trained personell
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Yes
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO