JCB

TRPC3 channel gating by lipids requires localization at the ER-PM junctions defined by STIM1

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September 17, 2021

Re: JCB manuscript #202107120

Dr. Shmuel Muallem
National Institute of Health
NIDCR
Building 10, Room 5N-102
Bethesda, MD 20892

Dear Dr. Muallem,

Thank you for submitting your manuscript entitled "TRPC3 channel gating by lipids requires localization at the membrane contact site ER-PM junctions". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the reviewers find that your study addresses an important and interesting question of interest to the readership of JCB. However, while they appreciate the quality of the biophysical and biochemical aspects of your study, we agree with their assessment that significantly further cell biological evidence for your conclusion is necessary, in particular to address their concerns regarding the indirect nature of the ER-PM contact analysis. Therefore, for further consideration at JCB the reviewer concerns would need to be addressed in full, with extensive new experimental data where requested.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Andrea L. Marat, Ph.D.
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The work of Liu et al., uses electrophysiological and biochemical approaches to explore the role of ER-PM junctions and plasma membrane lipids in the regulation of TRPC3 function. The authors appear to have conducted their biophysical and biochemical characterizations to a high standard, although some comments on analysis are noted in the minor comments section. The authors major interpretation of their datasets is that TRPC3 is recruited to ER-PM junctions, in a VAPB-dependent manner, to control its receptor-mediated activation. While some of their datasets are suggestive of an important role for ER-PM junctions in regulating TRPC3 function, their results are very correlative, and lack rigor in terms of molecular analysis to appreciate if the roles of VAPB and E-Syt1 are direct as the authors suggest, or more complicated and mediated through indirect mechanisms. Below are some comments that will hopefully improve the work:
There are no line or page numbers, so I refer to the related figure(s) when offering comments.

Major comments:
1. General point: the work is not very accessible to a broader readership and is written for a very specific, TRP-minded subset of investigators. The accessibility issue is partly due to the polymodal nature of TRPC activation which requires many point mutations /complicated experimental designs, but the authors can help by more clearly and carefully detailing rationale for experiments and their interpretation of results. Further, it might be helpful to include a table (could be include in the supplement) of the different point mutations, their predicted role, and what new findings this study shows. Further, diagrams of what is explicitly being tested and/or a summary diagram detailing their model that can be referred to throughout the MS would be very helpful.

2. Data presentation and figure organization requires attention. The authors should consider the following suggestions to increase accessibility and enhance presentation of their data:
a. Be consistent with text sizes and the use of bold vs regular fonts
b. Align (when possible) graphs, lettering, axis etc.
c. The reviewer suggests to include SEM in both directions for transparency (as in Fig.3C), however if they you are to include only one error bar, they should have error bars going in the same direction within a figure panel. Figures Fig.1D, 1G, 1J, 2A, 3I, 3L, 3O, 4A etc. have upper error bars for one condition and lower error bars in a different direction.

3. Figure 1: Does the surface expression of M3 receptor change in response to altered expression of TRPC3 mutants, E-Syt1, VAPB, or carbachol? This control experiment would add addition rigor to the dataset and further support that the differences are not due to changes in receptor density.

4. Does knockout of STIM1 appreciably change ER-PM membrane contact sites? The authors continuously state that ER/PM junctions are disrupted, but has this been measured? Of course, it will alter STIM1-depednent signaling at ER-PM contacts and the ability of STIM1 to interact with TRPC, but will it change steady-state ER-PM junctions? Given the redundancy of ER-PM tethers in yeast and the numerous ER-PM junction-forming proteins in mammalian cells, it would be surprising if steady-state ER-PM junctions change much at all, however perhaps this has been measured using electron-microscopy? The reviewer is simply unaware of specific measurements. Understanding this issue may add clarity to the authors experiments.

5. Related to Figure 3. Unlike E-Sy2 and E-Sy3, E-Syt1 and ANO8 respond to changes in intracellular calcium to alter ER-PM junctions. This is interesting because neither protein would be expected to participate in steady-state ER-PM junction formation, but rather be recruited following depletion of ER Ca2+, or in the case of E-Syt1 a rise in intracellular Ca2+ that may come from voltage-gated Ca2+ channels. In the case of TRPC-E-Syt1, following receptor activation, do changes in current density with E-Syt1-/- or overexpression still occur in STIM1-/- cells? This would inform if STIM1 is required for these effects. This comment is trying to encourage the authors to more carefully consider how E-Syt1 is mediating changes in TRPC.

6. More broadly, can the authors visualize translocation of TRPC3 at ER-PM junctions and if they increase following Gq-receptor activation? The authors measure whole-cell currents, and make statements regarding ER-PM junctions based on changes in proteins expected to be present in these junctions. This is very reasonable, but if TRPC3 is translocated to ER-PM junctions through VAPB interactions, one may expect to be able to visualize this recruitment, like ORAI channels or KV2 channels, bona fide ER-PM localized ion channels.

7. Related to comment 6, do VAPB-TRPC3 interactions change under carbachol conditions? One hypothesis is that these interactions may be predicted to increase, similar to VAPB-NIR2 interactions, this would add further support to the authors hypothesis that VAPB helps facilitate translocation of TRPC3 to ER-PM junctions.

Minor Comments:

1. Introduction: the section on lipid regulation of TRPC channel function should be rewritten for clarity. The first half of the paragraph is fine, the second half needs to be focused better and simply refers to 'lipids' without detailing which specific lipid binds and/or activates the channel.

2. How are the authors quantifying their WB data. For example, Fig.1C in their control group all data points are at 1.0. Are these paired measurements? If so, are the other conditions paired and analyzed relative to this group? If not, the average should be at 1.0 with the other data points spread around this value.

3. No statistical information or N (number of days of experiments) vs n (number of cells/currents) is included in the manuscript.

Reviewer #2 (Comments to the Authors (Required)):

Transient Receptor Potential (TRP) channels of the canonical (TRPC) channels are cation channels and mediated receptor evoked signals. Although it is known that these channels are lipid regulated, the exact mechanism is unclear. Recent structural work published by others defined a number of sites within TRPC molecules which may be critical for this regulation and the manuscript by Liu et al is a tour-de-force to define the functional relevance of several of these sites and to address the question whether and how TRPC3 channels target and are regulated by these lipid site at ER-PM junctions. The data in the figures is densely packed and overloaded with many subpanels in an often rather chaotic order, making the manuscript not very readable. In the first half of the paper, the authors build on their own already published work (Lee et al 2014) to more closely define the interaction of TRPC3 with STIM1. This part could be shortened (see also concerns below) to put more emphasis on the second and more interesting part defining the functional relevance of the lipid regulatory sites.

Major concerns:

1. To define ER-PM junctions the authors rely on co-IP and biochemical experiments to show surface expressed protein. However, this is a very indirect method and does not demonstrate that TRPC3 moves or localizes preferentially to a specialized MCS. To show that TRPC3 is targeted to MCS, confocal images showing tagged TRPC3 moving to the same clusters as tagged STIM1 or ESyT molecules is required. As mentioned also below, FRET experiments showing interaction of TRPC3 and its key mutant with STIM1 need to be included. Because TRPC3 channels may not show an exclusive localization at the plasma membrane, using TRPC3 channels with an extracellular HA tag might uncover differences in clusters or surface localization using non-permeabilzed HEK wt or STIM1 deficient HEK cells.

2. Figure S1A: it appears that the input of TRPC3L241S is higher in the Co-IP with STIM1 than wt or I807S. To quantify differences in interaction, FRET or other more quantitative interaction experiments need to be included.

3. The reason for the discrepancy regarding C-terminal deletions with the study by Wedel et al 2003 should be addressed. Could this difference be due to the different tag that was included in the Wedel et al study? Is the difference due to the different experimental approaches (Ca imaging vs. patch-clamp)? The IV relationships shown in Fig. S1F show leak currents and not the expected TRPC3 IV. If the cells contain non-functional TRPC3 channels, the IV relationship should show almost flat lines. It is also dangerous to record currents at -100 mV when the ramp starts at -100 mV as capacitance artifacts may persist if the cells are not optimally compensated, therefore analysis at -80 or -90 mV should be performed to validate. Would the D793X deletion as in Wedel et al also not show Ca2+ entry when recorded in STIM1 deficient HEK cells?

4. Figure 1D,G: The sample sizes are very small. Are these from several independent transfections? The overall current density of TRPC3+STIM1 (D) is not smaller than L241S+STIM1 (I) despite the increased surface expression and increased interaction (2014 paper). Is there a difference in TRPC3 surface amounts or current density when TRPC3 is expressed in HEK wt cells versus HEK STIM1-/- cells? With co-overexpression it is difficult to control for similar amounts of both proteins. The lack of an effect with the I807S mutant is due to an increased current density of the vector control (~-23 pA/pF versus 15pA/pF for wt and ~8 pA/pF for the L241S controls). All vector controls should go into one bar graph. I would recommend recording all constructs in HEK wt and HEK STIM1-/- cells with an n of 12-15 for each condition. All conditions (Fig.1 F,I,L should be statistically analyzed together using one way Anova). The statistical tests that were used to indicate significance have not been described. This needs to be added for each figure legend.

4. In Fig. 1M the current densities (now represented as absolute values or as outward currents? - unclear from the Figure legend), now greatly exceed the values measured above for 1μM carbachol stimulation. The I807S mutant has not reached steady state, therefore, Vmax cannot be determined. In 1R the overall Vmax is smaller compared to 1N, which contradicts the

findings of F and I. Again, sample sizes are very small. The interesting differences in apparent Km values could be due to STIM1- Orai1 mediated currents contributing to the effects seen in Fig 1 M-0, as carbachol would also trigger ICRAC in these cells. Does treatment with BTP-2 also obliterate differences in apparent Km? Alternatively, does re-expression of STIM1 in the knock-out background rescue the differences in apparent Km? Figure 1P does not contain surface expression of the mutants in the STIM1 knock-out background.

5. Figure 2: Please include traces showing IV relationship after GSK treatment. It is not entirely clear if the GSK induced differences are different from receptor operated. At 1 µM Carbachol, the Vmax is also increased for L241S.

6. (see also 1) Figure 3: To quantify recruitment to ER-PM junctions, TIRF microscopy with tagged constructs in conjunction with MAPPER would be a more appropriate method compared to overall surface expression. The arrangements of the panels is chaotic in Figure 3 and very difficult to follow.
7. For better comparison, why were currents in Fig. 3I not measured with 1 µM carbachol as was done in Fig. 3C? Are the differences measured with L241S and siESYT1 significant at 10 mM Carbachol?

8. Currents measured in Figure 4H have dramatically shifted reversal potentials and do not display the shoulder seen in Figure 4B. This indicates that GSK either dramatically shifts the selectivity of TRPC3 or that other channels are recruited.

9. The second part of the paper investigates in detail the functional relevance of TRPC3 residues interacting with lipids as shown in structural work published by Fan et al. The results of Figure 5 are interesting, although the connection to the previous data is not so clear cut. How do the authors explain the fact that the W334 mutation again shows a clear shift in the reversal potential? This is an interesting finding but not fully investigated. A shift in reversal potential indicates altered selectivity of the pore. Does this mutant conduct more Na+ ions, thereby increasing current densities? This could explain the apparent discrepancy of increased CD despite reduced surface expression. Demonstrating that lipid binding alters pore selectivity would be important and needs to be further validated by ion replacement experiments.

9. In Figure 6, IV relationships are missing. Do the authors observe a similar shift in Erev in W334A mutants when stimulated with GSK?
To pinpoint the targeting site of TRPC3 to ER/PM junctions the authors mutate further residues. They claim that two phenylalanines in an acidic tract are important, however in TRPC3 the sequence is not FFAT but FYAY, the phrasing should be more careful.

10. Figure 8: many of the effects Ciii or Diii are very small and only borderline significant. Although fig. 8H shows that overall expression levels are not different when many cells are lysed, how do the authors control for equal expression in the cells that they recorded from?

11. Fig 8. Ii and Mi: what is the reason for the beginning inward currents before the application of carbachol? Is this ICRAC?

Results section contains some mislabels, i.e. (W33A instead of W334A) - first paragraph of "The lipid site 1 affects.." A few other typos and grammar mistakes are found.

Response to reviewers' comments

We would like to express our appreciation for the thoughtful and constructive comments by both reviewers that we hope help us to improve the manuscript and its accessibility. We also appreciate the positive evaluation of the manuscript and its potential significance. To address all reviewers' comments, we performed several additional experiments as listed below:

In response to Reviewer 1:

1) Measured the effect of the TRPC3(L241S) and TRPC3(I807S) mutants on the surface expression of the M3Rs.

2) Measured the effect of VAPB on the surface expression of the M3Rs.

3) Measured effect of carbachol stimulation on TRPC3+VAPB by Co-IP and by FRET.

4) Measured MAPPER puncta in wild-type and STIM1$^{-/-}$ cells by TIRF.

5) Measured effect of STIM1 on TRPC3 puncta in response to stimulation with 100 µM carbachol.

6) Measured effect of E-Syt1 expression on TRPC3 current expressed in STIM1-/- cells to determine if STIM1 is required for the E-Syt1 effects. These experiments resulted in important finding.

In response to Reviewer 2:

7) Measured effect of cell stimulation on STIM1-TRPC3 FRET and the effect of E-Syt1 of the FRET.

8) Measured FRET of STIM1+TRPC3, STIM1+TRPC3(L241S) and STIM1+TRPC3(I807S) in response to stimulation with 100 µM carbachol.

9) Measured effect of TRPC3 C terminal deletions on Ca^{2+} influx.

10) Compared TRPC3 current expressed in wild-type and STIM1$^{-/-}$ cells.

11) Measured rescue by STIM1 of Km for carbachol stimulation of TRPC3 current in STIM1$^{-/-}$ cells.

12) Provided traces showing I/V relationship for GSK stimulated TRPC3 current.

13) Showed the effect of PI(4,5)P_2 depletion on the reversal potential of TRPC3 stimulated with carbachol and with GSK. This analysis resulted in important new observation.

14) Provided I/Vs for TRPC3 and TRPC3(W334A) ± rapamycin treatment stimulated with carbachol and b) TRPC3 and TRPC3(W334A) ± rapamycin stimulated with GSK.

Response to specific comments:

Reviewer #1 (Comments to the Authors (Required)):

The work of Liu et al., uses electrophysiological and biochemical approaches to explore the role of ER-PM junctions and plasma membrane lipids in the regulation of TRPC3 function. *The authors appear to have conducted their biophysical and biochemical characterizations to a high standard, although some comments on analysis are noted in the minor comments section*. The authors major interpretation of their datasets is that TRPC3 is recruited to ER-PM junctions, in a VAPB-dependent manner, to control its receptor-mediated activation. While some of their datasets are suggestive of an important role for ER-PM junctions in regulating TRPC3 function, their results are very correlative, and lack rigor in terms of molecular analysis to appreciate if the roles of VAPB and E-Syt1 are direct as the authors suggest, or more complicated and mediated through indirect mechanisms.

Response: Thank you for the strong positive comments. We conducted additional experiments, including FRET and TIRF analysis as suggested by reviewer 2 (Figures 1C and 3H FRET and 3I-J TIRF) to strengthen the conclusion of the importance of localization at the ER/PM junctions for regulation by E-Syt1. We also show that E-Syt1 effects are strictly dependent on STIM1.

Below are some comments that will hopefully improve the work:
There are no line or page numbers, so I refer to the related figure(s) when offering comments.

Major comments:

1. General point: the work is not very accessible to a broader readership and is written for a very specific, TRP-minded subset of investigators. The accessibility issue is partly due to the polymodal nature of TRPC activation which requires many point mutations /complicated experimental designs, but the authors can help by more clearly and carefully detailing rationale for experiments and their interpretation of results. Further, it might be helpful to include a table (could be include in the supplement) of the different point mutations, their predicted role, and what new findings this study shows. Further, diagrams of what is explicitly being tested and/or a summary diagram detailing their model that can be referred to throughout the MS would be very helpful.

Response: We appreciate pointing out this issue and we have elaborated on the rationale for the experiments and their interpretation to the extent allowed by the length limitation imposed by the journal.
As suggested, we added a table to the supplement with all the mutations we tested, their predicted role, and what new findings this study shows. We also added a model summarizing the findings, which is now labelled Figure 9.

2. Data presentation and figure organization requires attention. The authors should consider the following suggestions to increase accessibility and enhance presentation of their data:
a. Be consistent with text sizes and the use of bold vs regular fonts
b. Align (when possible) graphs, lettering, axis etc.
c. The reviewer suggests to include SEM in both directions for transparency (as in Fig.3C), however if they you are to include only one error bar, they should have error bars going in the same direction within a figure panel. Figures Fig.1D, 1G, 1J, 2A, 3I, 3L, 3O, 4A etc. have upper error bars for one condition and lower error bars in a different direction.

Response: Thank you for the suggestions for edits. Figures A and B are adapted. Now we use single error bars in all Figures. The single error bars are used for clarity so that the average trace lines can be visible. For this reason, we used either the positive or negative bars to minimize the overlap and increase clarity.

3. Figure 1: Does the surface expression of M3 receptor change in response to altered expression of TRPC3 mutants, E-Syt1, VAPB, or carbachol? This control experiment would add

addition rigor to the dataset and further support that the differences are not due to changes in receptor density.

Response: Thank you for the suggestion. We now show in supplementary Figure 1J and Figure 8G that the TRPC3 mutants, receptor stimulation and VAPB had no effect on the surface expression of the M3 receptors.

4. Does knockout of STIM1 appreciably change ER-PM membrane contact sites? The authors continuously state that ER/PM junctions are disrupted, but has this been measured? Of course, it will alter STIM1-depednent signaling at ER-PM contacts and the ability of STIM1 to interact with TRPC, but will it change steady-state ER-PM junctions? Given the redundancy of ER-PM tethers in yeast and the numerous ER-PM junction-forming proteins in mammalian cells, it would be surprising if steady-state ER-PM junctions change much at all, however perhaps this has been measured using electron-microscopy? The reviewer is simply unaware of specific measurements. Understanding this issue may add clarity to the authors experiments.

Response: To specifically assay the ER/PM junctions associated with STIM1 we used the MAPPER construct since it is based on STIM1 transmembrane and polybasic domains to target it to STIM1 ER and PM localization sites. The results in the new supplementary Figure 2A show that deletion of STIM1 reduced the number of the junctions (reduced number of puncta) and likely their size (reduced fluorescence intensity). Moreover, supplementary Figure 3G shows that deletion of STIM1 eliminated the effect of the tether E-Syt1 on TRPC3 current. Previous work reported that knockdown of E-Syt1 reduced MAPPER fluorescence intensity (Chang et al., 2013).

5. Related to Figure 3. Unlike E-Sy2 and E-Sy3, E-Syt1 and ANO8 respond to changes in intracellular calcium to alter ER-PM junctions. This is interesting because neither protein would be expected to participate in steady-state ER-PM junction formation, but rather be recruited following depletion of ER Ca2+, or in the case of E-Syt1 a rise in intracellular Ca2+ that may come from voltage-gated Ca2+ channels. In the case of TRPC-E-Syt1, following receptor activation, do changes in current density with E-Syt1-/- or overexpression still occur in STIM1-/- cells? This would inform if STIM1 is required for these effects. This comment is trying to encourage the authors to more carefully consider how E-Syt1 is mediating changes in TRPC.

Response: Thank you for this very important question, which resulted in an important observation. We tested the effect of E-Syt1 in STIM1$^{-/-}$ cells and the results in supplementary Figure 3G show that deletion of STIM1 eliminated the effect of the tether E-Syt1 on TRPC3 current. This indicates that STIM1 dominates formation and stabilization of the junctions. The role of STIM1 as the dominant tether of the ER/PM junctions is now discussed.

6. More broadly, can the authors visualize translocation of TRPC3 at ER-PM junctions and if they increase following Gq-receptor activation? The authors measure whole-cell currents, and make statements regarding ER-PM junctions based on changes in proteins expected to be present in these junctions. This is very reasonable, but if TRPC3 is translocated to ER-PM junctions through VAPB interactions, one may expect to be able to visualize this recruitment, like ORAI channels or KV2 channels, bona fide ER-PM localized ion channels.

Response: Indeed, we do think that a signaling complex is formed in response to cell stimulation. A good way to show this is by FRET measurement. Therefore, we measured FRET between TRPC3 and STIM1. The new results in Figure 3H show that carbachol increased STIM1-TRPC3 FRET although carbachol had no effect on TRPC3 surface expression (Figure 3G). Moreover, E-Syt1 also increased the TRPC3-STIM1 FRET that was further increased by carbachol stimulation. These findings provide additional evidence for the role of the ER/PM junctions in the regulation of TRPC3 and assembly of the complexes in the junctions.

In additional experiments, we used TIRF to analyze TRPC3 and TRPC3 mutants at the ER/PM junction. Remarkably, Figures 3I and 3J show that TRPC3 puncta could be readily observed by TIRF microscopy and that STIM1 increased the intensity of the TIRF signal, although it was not increased further by cell stimulation. These findings provide further evidence for the localization of TRPC3 at the ER/PM junctions.

7. Related to comment 6, do VAPB-TRPC3 interactions change under carbachol conditions? One hypothesis is that these interactions may be predicted to increase, similar to VAPB-NIR2 interactions, this would add further support to the authors hypothesis that VAPB helps facilitate translocation of TRPC3 to ER-PM junctions.

Response: Measurement by Co-IP (Figure S5J) and independently by FRET (Figure S5K) show that the TRPC3-VAPB interaction is not affected further by cell stimulation. It is likely that the interaction is either constitutive or saturated by the required overexpression.

Minor Comments:

1. Introduction: the section on lipid regulation of TRPC channel function should be rewritten for clarity. The first half of the paragraph is fine, the second half needs to be focused better and simply refers to 'lipids' without detailing which specific lipid binds and/or activates the channel.

Response: Thank you. We revised the paragraph by splitting it to two paragraphs and revised some of the text, which should improve clarity.

2. How are the authors quantifying their WB data. For example, Fig.1C in their control group all data points are at 1.0. Are these paired measurements? If so, are the other conditions paired and analyzed relative to this group? If not, the average should be at 1.0 with the other data points spread around this value.

Response: Thank you. All blots were quantified by ImageJ, as in our previous publications. This is now indicated in the method section under total and surface expression (ImageJ was used to quantify bend intensity of all blots. All values are normalized to the surface/input or IP/input of TRPC3 in unstimulated cells.).

3. No statistical information or N (number of days of experiments) vs n (number of cells/currents) is included in the manuscript.

Response: Thank you. This information is now included in the method section.

Reviewer #2 (Comments to the Authors (Required)):

Transient Receptor Potential (TRP) channels of the canonical (TRPC) channels are cation channels and mediated receptor evoked signals. Although it is known that these channels are lipid regulated, the exact mechanism is unclear. Recent structural work published by others defined a number of sites within TRPC molecules which may be critical for *this regulation and the manuscript by Liu et al is a tour-de-force to define the functional relevance of several of these sites and to address the question whether and how TRPC3 channels target and are regulated by these lipid site at ER-PM junctions*. The data in the figures is densely packed and overloaded with many subpanels in an often rather chaotic order, making the manuscript not very readable. In the first half of the paper, the authors build on their own already published work (Lee et al 2014) to more closely define the interaction of TRPC3 with STIM1. This part could be shortened (see also concerns below) to put more emphasis on the second and more interesting part defining the functional relevance of the lipid regulatory sites.

Response: Thank you for finding the work interesting and thorough.

The first part of the manuscript examined the effect of localization of TRPC3 at the ER/PM junctions on channel function. The role of localization at the ER/PM junctions on channel function and activation by receptor stimulation was not examined for **any** TRP channel. Thus, the findings in the first part of the manuscript are completely new and critical for understanding regulation of the channels by lipids. The TRPC3(L241S) and TRPC3(I807S) mutants are used as tools to further demonstrate the importance of localization at the ER/PM junctions and do not reproduce in any way the findings of the previous publication.

Major concerns:

1. To define ER-PM junctions the authors rely on co-IP and biochemical experiments to show surface expressed protein. However, this is a very indirect method and does not demonstrate that TRPC3 moves or localizes preferentially to a specialized MCS. To show that TRPC3 is targeted to MCS, confocal images showing tagged TRPC3 moving to the same clusters as tagged STIM1 or ESyT molecules is required. As mentioned also below, FRET experiments showing interaction of TRPC3 and its key mutant with STIM1 need to be included. Because TRPC3 channels may not show an exclusive localization at the plasma membrane, using TRPC3 channels with an extracellular HA tag might uncover differences in clusters or surface localization using non-permeabilzed HEK wt or STIM1 deficient HEK cells.

Response: Thank you for this useful and informative suggestions. As suggested, we measured the effect of cell stimulation and E-Syt1 on STIM1-TRPC3 FRET and TRPC3 mutants and the results are shown in Figures 1C and 3H. Figure 1C shows that TRPC3(L241S) increases TRPC3-STIM1 FRET while TRPC3(I807S) reduces the FRET. In addition, cell stimulation increases the FRET between STIM1 and TRPC3 and TRPC3(L241S), but not with TRPC3(I807S). These mutants resulted with minimal effect of surface expression. Figure 3H shows that stimulation by carbachol increases the STIM1-TRPC3 FRET and E-Syt1 increased the FRET before and after cell stimulation. Importantly, these increases in FRET occurred with no change in surface expression of TRPC3, indicating that cell stimulation causes recruitment of TRPC3 to the STIM1 domain. Finally, in Figure 3I and 3J we used TIRF microscopy to show that STIM1 increases TRPC3 puncta at the ER/PM junctions. Together, the results provide multiple and independent evidence for recruitment and function of TRPC3 at the ER/PM junctions.

2. Figure S1A: it appears that the input of TRPC3L241S is higher in the Co-IP with STIM1 than wt or I807S. To quantify differences in interaction, FRET or other more quantitative interaction experiments need to be included.

Response: The slight increase in input in this experiment cannot explain the large increase in Co-IP that was examined in more details in (Lee et al., 2014). Measurement of FRET shown in the new Figure 1C confirms the increased interaction of TRPC3(L241S) with STIM1.

3. The reason for the discrepancy regarding C-terminal deletions with the study by Wedel et al 2003 should be addressed. Could this difference be due to the different tag that was included in the Wedel et al study? Is the difference due to the different experimental approaches (Ca imaging vs. patch-clamp)? The IV relationships shown in Fig. S1F show leak currents and not the expected TRPC3 IV. If the cells contain non-functional TRPC3 channels, the IV relationship should show almost flat lines. It is also dangerous to record currents at -100 mV when the ramp starts at -100 mV as capacitance artifacts may persist if the cells are not optimally compensated, therefore analysis at -80 or -90 mV should be performed to validate. Would the

D793X deletion as in Wedel et al also not show Ca2+ entry when recorded in STIM1 deficient HEK cells?

Response: We do not know how and why Wedel et al were able to measure retention of Ca^{2+} influx, especially with the available structures of TRPC3. The long pole helix controls the channel inner pore gate. Thus, it is not surprising that massive truncation of the pole helix, as was done by Wedel et al studies should inhibit the channel. Further, Wedel et al.'s truncation also deletes part of the all-important rib helix that is essential for forming the TRPC3 (and all other TRP channels) tetrameric assembly. Our truncation at 782 was selected to at least avoid truncation of the structurally essential rib helix. Moreover, deletion of only the last 8 residues resulted in inhibition of TRPC3 function. Finally, we now include Ca^{2+} influx measurements in Figures S1H and S1I, which show that the truncation mutants have no Ca^{2+} influx activity when expressed in wild-type or $STIM1^{-/-}$ HEK cells. $STIM1^{-/-}$ cells have low native Ca^{2+} influx and even this influx is inhibited by the TRPC3 mutants.

The I/V is indeed flat, and the leak current is very small. We changed the current scale to full scale to illustrate this point. We are very much aware of the capacitive and leak currents and make sure that they are not part of the data. As is customary, the -100 mV current is actually read at -99.9 mV to avoid any potential contribution of the capacitive current.

4. Figure 1D,G: The sample sizes are very small. Are these from several independent transfections? The overall current density of TRPC3+STIM1 (D) is not smaller than L241S+STIM1 (I) despite the increased surface expression and increased interaction (2014 paper). Is there a difference in TRPC3 surface amounts or current density when TRPC3 is expressed in HEK wt cells versus HEK STIM1-/- cells? With co-overexpression it is difficult to control for similar amounts of both proteins. The lack of an effect with the I807S mutant is due to an increased current density of the vector control (~-23 pA/pF versus 15pA/pF for wt and ~8 pA/pF for the L241S controls). All vector controls should go into one bar graph. I would recommend recording all constructs in HEK wt and HEK STIM1-/- cells with an n of 12-15 for each condition. All conditions (Fig.1 F,I,L should be statistically analyzed together using one way Anova). The statistical tests that were used to indicate significance have not been described. This needs to be added for each figure legend.

Response: With all respect, we do not agree that the sample size is small. Measuring current in 11-13 separate cells from at least 3 separate transfections is a large sample by current standard measurements that usually rely on 4-6 cells. We now indicate in the methods that current measurements are from at least three separate transfections and the number of cells shown as the dotes of individual measurements in the columns. The comparison of the currents at each carbachol concentration are given in Figure 1M.

To address the question of current in wild-type and $STIM1^{-/-}$ cells, we added new data in supplementary Figure S2C-E in which the current was measured at the same day in the two cell types stimulated at maximal agonist concentration. Maximal current density is the same in wild-type and $STIM1^{-/-}$ cells.

Indeed, control for expression level is needed when recording from multiple single cells in different experiments. To do so we use fluorescence and always measuring control and experimental conditions in parallel transfection and in the same experiments. TRPC3 and the mutants are tagged with YFP and cells are selected for recording based on fluorescent level to ensure comparable expression. This is reflected in the similar Vmax for TRPC3 and the mutants and in particular the similar currents at the various carbachol concentrations in $STIM1^{-/-}$ cells (Figure 1P).

Since the currents at Figures 1F, 1G and 1L were recorded at different days and different transfections, analyzing them together is not appropriate or informative. The same day analysis at a giver concentration were done for the results in Figures 1M and 1P.

4. In Fig. 1M the current densities (now represented as absolute values or as outward currents? - unclear from the Figure legend), now greatly exceed the values measured above for 1µM carbachol stimulation. The I807S mutant has not reached steady state, therefore, Vmax cannot be determined. In 1R the overall Vmax is smaller compared to 1N, which contradicts the findings of F and I. Again, sample sizes are very small. The interesting differences in apparent Km values could be due to STIM1- Orai1 mediated currents contributing to the effects seen in Fig 1 M-0, as carbachol would also trigger ICRAC in these cells. Does treatment with BTP-2 also obliterate differences in apparent Km? Alternatively, does re-expression of STIM1 in the knock-out background rescue the differences in apparent Km? Figure 1P does not contain surface expression of the mutants in the STIM1 knock-out background.

Response: It is now indicated that Figure 1M and 1Q show data from outward current. Vmax can be obtained from the fit and only requires current measurements with sufficient agonist concentration. Again, absolute current levels cannot be compared unless measured in the same day. When these are done, as in the new supplementary Figure S2C-E discussed above, there was no difference in current density measured at 100 µM carbachol in wild-type and STIM1$^{-/-}$ cells.

It is not clear to us how channel inhibitors (BTP-2 or others) will change the Km. Note that deletion of STIM1 affected the apparent affinity for carbachol with minimal effect on Vmax and channel inhibition is not equivalent to a change in the apparent affinity to agonist stimulation.

Thank you for the important question about the STIM1 rescue experiments. The new supplementary Figures S2F-J show that re-expression of STIM1 in STIM1$^{-/-}$ cells rescues the high affinity for receptor stimulation.

5. Figure 2: Please include traces showing IV relationship after GSK treatment. It is not entirely clear if the GSK induced differences are different from receptor operated. At 1 µM Carbachol, the Vmax is also increased for L241S.

Response: I/V for GSK stimulation are not included in Figure 2. The I/V are the same shape for GSK and receptor stimulation. The reversal potentials are discussed below in relation to the comments concerning Figure 4.

6. (see also 1) Figure 3: To quantify recruitment to ER-PM junctions, TIRF microscopy with tagged constructs in conjunction with MAPPER would be a more appropriate method compared to overall surface expression. The arrangements of the panels is chaotic in Figure 3 and very difficult to follow.

Response: Thank you for the suggestions. New Figures 1C and 3H show the FRET results and Figure 3I-J show the TIRF results. Cell stimulation and E-Syt1 increase TRPC3-STIM1 FRET.

The panels were rearranged for clarity.

7. For better comparison, why were currents in Fig. 3I not measured with 1 µM carbachol as was done in Fig. 3C? Are the differences measured with L241S and siESYT1 significant at 10 mM Carbachol?

Response: a) The results with 0.3 µM are used to show the maximal response; b) we measured the response to the super-maximal concentration of 1 mM carbachol, and the response is the same in control and cells treated with siE-Syt1.

8. Currents measured in Figure 4H have dramatically shifted reversal potentials and do not display the shoulder seen in Figure 4B. This indicates that GSK either dramatically shifts the selectivity of TRPC3 or that other channels are recruited.

Response: Thank you for pointing this important point that resulted in significant conclusions about regulation by $PI(4,5)P_2$. The I/V curves for activation by GSK were replaced with better examples. We have analyzed the effect of $PI(4,5)P_2$ depletion on TRPC3 reversal potential when stimulated with carbachol or activated by GSK. As can be seen in Figures 4D for stimulation with carbachol, the reversal potential for TRPC3 and TRPC3(L241S) are similar and are not statistically different, while the reversal potential of TRPC3(I807S) is different from that of TRPC3 and TRPC3(L241S) and has higher K^+/Na^+ selectivity. Notably, $PI(4,5)P_2$ altered the reversal potential for carbachol stimulation to equalize TRPC3 and TRPC3(L241S) K^+/Na^+ selectivity to that of TRPC3(I807S). The increased K^+ selectivity was specific for receptor stimulation, since the activation of all mutants by GSK had the same selectivity that was not affected by $PI(4,5)P_2$ depletion. This suggests that $PI(4,5)P_2$ modulates the TRPC3 pore selectivity and the effect of $PI(4,5)P_2$ is regulated by receptor stimulation.

9. The second part of the paper investigates in detail the functional relevance of TRPC3 residues interacting with lipids as shown in structural work published by Fan et al. The results of Figure 5 are interesting, although the connection to the previous data is not so clear cut. How do the authors explain the fact that the W334 mutation again shows a clear shift in the reversal potential? This is an interesting finding but not fully investigated. A shift in reversal potential indicates altered selectivity of the pore. Does this mutant conduct more Na+ ions, thereby increasing current densities? This could explain the apparent discrepancy of increased CD despite reduced surface expression. Demonstrating that lipid binding alters pore selectivity would be important and needs to be further validated by ion replacement experiments.

Response: Thank you again for bringing to our attention your point about lipids and reversal potential. The W334A increased both the inward and outward current and does not appreciably affect the reversal potential at the Na^+ and K^+ gradients used in the experiments in Figure 5. The increased current is likely due to increased open probability. However, this was not examined in this study, which is focused on the regulation by lipids.

9. In Figure 6, IV relationships are missing. Do the authors observe a similar shift in Erev in W334A mutants when stimulated with GSK?

To pinpoint the targeting site of TRPC3 to ER/PM junctions the authors mutate further residues. They claim that two phenylalanines in an acidic tract are important, however in TRPC3 the sequence is not FFAT but FYAY, the phrasing should be more careful.

Response: a) Example I/Vs are now included for stimulation by carbachol and GSK. Based on your comment 8 and the effects of $PI(4,5)P_2$ and receptor stimulation on the reversal potential are now shown and discussed in relation to Figure 4.

b) The FFAT motif was discovered with the two FF and named as such, but subsequent studies showed that Y can substitute one of the F. A scoring system was devised and can be found in (Murphy and Levine, 2016), but the name of FFAT motif was retained and is used as such in the literature.

10. Figure 8: many of the effects Ciii or Diii are very small and only borderline significant. Although fig. 8H shows that overall expression levels are not different when many cells are lysed, how do the authors control for equal expression in the cells that they recorded from?

Response: The responses are statistically significant. All TRPC3 and mutants used for current are tagged with YFP and the experimentalist selected cells that show clear plasma membrane expression and attempted to select cells with similar fluorescence in each set of experiments and within a recording day.

11. Fig 8. Ii and Mi: what is the reason for the beginning inward currents before the application of carbachol? Is this ICRAC?

Response: No, this is not CRAC current since the stores are not depleted. This is likely a small spontaneous TRPC3 current observed when the solution is changed to nominally Ca^{2+}-free solution to maximize the inward Na^{+} current.

12. Results section contains some mislabels, i.e. (W33A instead of W334A) - first paragraph of "The lipid site 1 affects.." A few other typos and grammar mistakes are found.

Response: Thank you. We attempted to correct these mistakes.

Chang, C.L., T.S. Hsieh, T.T. Yang, K.G. Rothberg, D.B. Azizoglu, E. Volk, J.C. Liao, and J. Liou. 2013. Feedback regulation of receptor-induced Ca2+ signaling mediated by E-Syt1 and Nir2 at endoplasmic reticulum-plasma membrane junctions. *Cell Rep*. 5:813-825.

Lee, K.P., S. Choi, J.H. Hong, M. Ahuja, S. Graham, R. Ma, I. So, D.M. Shin, S. Muallem, and J.P. Yuan. 2014. Molecular determinants mediating gating of Transient Receptor Potential Canonical (TRPC) channels by stromal interaction molecule 1 (STIM1). *J Biol Chem*. 289:6372-6382.

Murphy, S.E., and T.P. Levine. 2016. VAP, a Versatile Access Point for the Endoplasmic Reticulum: Review and analysis of FFAT-like motifs in the VAPome. *Biochim Biophys Acta*. 1861:952-961.

January 19, 2022

Re: JCB manuscript #202107120R

Dr. Shmuel Muallem
National Institute of Health
NIDCR
Building 10, Room 5N-102
Bethesda, MD 20892

Dear Dr. Muallem,

Thank you for submitting your revised manuscript entitled "TRPC3 channel gating by lipids requires localization at the ER-PM junctions defined by STIM1". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that both reviewers raise important issues regarding the presentation of the figures and the clarity of writing, which were points raised during the first round of review. Our general policy is that papers are considered through only one revision cycle; however, we are open to one additional round of revision that carefully and completely addresses the reviewers' constructive feedback on presentation. In addition, we agree with reviewer #2 that you should provide a colocalization analysis at the plasma membrane.

Please be advised that we will consult with reviewer #2 again, and that all issues must be completely addressed upon resubmission. Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Andrea L. Marat, Ph.D.
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The revised work of Liu et al., has conducted several new experimental series to strengthen their major conclusions. Overall, these new additions have strengthened several aspects of the work. While there are still many follow-up experiments required to extensively test their overriding hypothesis that lipid-dependent regultion of TRPC3 requires STIM1-centric ER-PM membrane contact sites, the volume of experiments suggests their results and conclusions will be of interest to the TRP field.

There are several outstanding comments that the authors believe should strongly considered to elevate the presentation of their work:

1. The figures still require much work. Practically all figures have serious alignment issues with many panels offset, displaying different sizes of fonts, line thicknesses etc.
2. Their model, although a nice addition, can be further refined to optimize its presentation. An obvious change would be to significantly decrease the thickness of the membranes.
3. The writing is still very dense and TRPC3-centric. Of course, this is to be expected given the authors interest in this important channel. However, to increase accessibility to other non-TRP specialists, and people from other fields, decreasing the density of information and clearly noting rationale for experiments (hypothesis) and interpretation of results is strongly suggested.

Reviewer #2 (Comments to the Authors (Required)):

In the revised version of the manuscript, the authors have conducted several important new experiments and analysis. Especially experiments conducted in cells lacking STIM1 with transient re-expression help to more clearly define STIM1 dependent effects, but now the manuscript should be rearranged to include some of the supplemental data in the main figures. However, several concerns persist and TRPC3 induced currents should be comparable on different days of transfection if DNA concentration and transfection methods remain identical (see previous comment #4). How else can the results be repeated/reproduced?

Both reviewers criticized the design and layout of the figures and readability of the manuscript for a general audience. Especially regarding the figures, little improvement is seen in the revised manuscript. Font sizes and styles still vary greatly, spelling mistakes are still present (i.e. Fig. 3I, Fig. S2a), letters and axis are not aligned and scaled to ease comparison and figures are still densely packed. The beginning the results section starts with a discussion of previous results and the results of supplementary Figure 1, when it should start with major results seen in Figure 1.

To further declutter the figures and to make the flow of the figures easier for the reader, in Figure 1 after panel B, the analysis of the current densities (defined as CD to shorten the axis labels) should be presented in the absence of STIM1 to show that there is no significant difference in CD between the mutant genotypes (suggestion of previous comment #4), supporting the claim (see reply of the authors) that there is no/little difference in surface expression. As HEK wt cells contain a significant amount of STIM1, this mutant comparison is best done in STIM1 deficient cells (as in fig S2, move to Fig.1). However, in the first version of the manuscript, Fig. 1C (old) showed a small but significant increase in surface expression TRPC3-L241 in the presence of carbachol ($p<0.016$) when compared to wt+Cch, which in the new version is not indicated anymore and could be due to endogenous STIM1.

I would recommend to then follow with the data from Fig. 1P-R, clearly indicating no difference between the mutants over a range of CCh concentrations in the absence of STIM1. Then move data from fig. S2 F-J (show error bars of traces in the same direction) to main Figure 1 to now very clearly show that the presence of STIM1 alters the concentration dependence of TRPC3 towards CCh. In the results section the authors state "a major role of STIM1 is controlling the affinity of the receptor for activation of TRPC3 and predict that the effect of the mutants should be altered in the absence..." Phrasing needs to be more careful, as not the affinity of the M3 receptor is changed, but rather the accessibility of TRPC3 towards DAG/lipids, as further investigated below.

Then continue with the mutant analysis in presence and absence of STIM1. Since Vmax and the apparent Km are calculated showing the outward currents, it is more consistent to show similar outward current traces for the mutants. The IV curves in Figure 1 could be shifted to the supplemental figures. The authors added new experiments in Fig. S2 C-E, but now 100 µM CCh induced CD of the same value as 1 µm CCh in the previous experiments (~ -23 pA/pF Fig. 1L vs S2C?)). What is the reason for this discrepancy? Figure 3 E,F also show very different current densities of WT (-40 pA/pF vs. -15 pA/pF in fig1) and L241S (-80 pA/pF vs -12 pA/pF in fig 1) with the scrambled control when compared to results of Figure 1. If such a variability is present, then differences with and without STIM1 as shown in Fig. 1D-L, are difficult. Vmax in Figure 3 is now affected by the mutants (in contrast to fig.1).

After establishing clear differences in the presence and absence of STIM1 and showing the rescue with transient expression of STIM1 within a main Figure, FRET data could follow, but needs to show the different genotypes without stimulation grouped and with stimulation grouped together. Differences in FRET ratios are quite small. Because STIM1 is an ER-localized protein, FRET between TRPC3 and STIM1 does not necessarily indicate that this interaction takes place at ER-PM junctions unless FRET is measured in the plane of the PM with TIRF (not clear from the legend). As the underlying data contains labeled proteins, the authors can add colocalization analysis at the plasma membrane to show that TRPC3-STIM1 colocalization is much larger at the puncta when compared to outside. This effect should not be present with the I807S mutant and could more clearly address the concern #1. In STIM1 deficient cells, no puncta of TRPC3 should appear.

The VAPB data is not included in the model.

Response to Reviewers comments-R2

Reviewer #1 (Comments to the Authors (Required)):

The revised work of Liu et al., has conducted several new experimental series to strengthen their major conclusions. Overall, these new additions have strengthened several aspects of the work. While there are still many follow-up experiments required to extensively test their overriding hypothesis that lipid-dependent regulation of TRPC3 requires STIM1-centric ER-PM membrane contact sites, the volume of experiments suggests their results and conclusions will be of interest to the TRP field.

Response: Thank you for appreciating the significance of the findings.

There are several outstanding comments that the authors believe should strongly considered to elevate the presentation of their work:

1. The figures still require much work. Practically all figures have serious alignment issues with many panels offset, displaying different sizes of fonts, line thicknesses etc.

Response: Some of the differences in appearance are because of the need to use different analysis and drawing programs (Origin, Prism etc.…) dictated by data equalization and analysis. We attempted to be more consistent and further revised drawing of the figures.

To increase clarity and following Reviewer 2's suggestions, we extensively revised the figures. First, we transferred almost all I/V curves, almost all surface and Co-IP blots, and several example current traces to the supplement. Per journal policy, the manuscript is restricted to 9 Figures and 5 supplementary Figures. Therefore, we included the model as a panel in Figure 9 to free one Figure. This noticeably reduces panel density in all Figures and allowed us to split the complicated old Figure 6 into two new Figures, now Figure 6 and Figure 7. With these changes, we believe the presentation of the results is significantly simplified and the clarity of the manuscript is markedly improved.

2. Their model, although a nice addition, can be further refined to optimize its presentation. An obvious change would be to significantly decrease the thickness of the membranes.

Response: The thickness of the membranes was changed and VAPB interaction with TRPC3 was added.

3. The writing is still very dense and TRPC3-centric. Of course, this is to be expected given the authors interest in this important channel. However, to increase accessibility to other non-TRP specialists, and people from other fields, decreasing the density of information and clearly noting rationale for experiments (hypothesis) and interpretation of results is strongly suggested.

Response: We do appreciate this issue raised by both reviewers and are struggling with it since the text is limited to 40,000 characters and the current text already exceeds this (by permission). Revising the Figures presentation as detailed above helped in simplifying the presentation. We revised the text to follow the revised Figures and while doing so, we simplified the text and increased the readability of the manuscript to the best of our ability.

Reviewer #2 (Comments to the Authors (Required)):

In the revised version of the manuscript, the authors have conducted several important new experiments and analysis. Especially experiments conducted in cells lacking STIM1 with

transient re-expression help to more clearly define STIM1 dependent effects, but now the manuscript should be rearranged to include some of the supplemental data in the main figures. However, several concerns persist and TRPC3 induced currents should be comparable on different days of transfection if DNA concentration and transfection methods remain identical (see previous comment #4). How else can the results be repeated/reproduced?

Response: With transient transfection, variations in current density are unavoidable when presenting actual current densities without normalization, as we did. Variations increase when using transfection of multiple plasmids, even when keeping the total cDNA amount the same. This is caused by different promotors and slight variabilities in the quality of cDNA preparations. In addition, transfection efficiency changes with cell passage despite using new batches after 10-15 passages. Finally, it is nearly impossible to maintain the same current density with studies that take close to 4 years to complete. To account for these, we always run a wild-type TRPC3 controls and compare Vmax, Km, reversal potentials and other kinetic parameters with sets of experiments performed together and at the same time. In addition, every recording day starts with current recording from 2-3 cells expressing wild-type TRPC3 to make sure the current density of a set of experiments to be compared are within the same range. Therefore, all comparisons are within the same set of experiments, but differences within sets are simply unavoidable and we do not do such comparisons in the current studies. It is not clear to us how it is possible to do this type of experiments in any other way.

Both reviewers criticized the design and layout of the figures and readability of the manuscript for a general audience. Especially regarding the figures, little improvement is seen in the revised manuscript. Font sizes and styles still vary greatly, spelling mistakes are still present (i.e., Fig. 3I, Fig. S2a), letters and axis are not aligned and scaled to ease comparison and figures are still densely packed. The beginning the results section starts with a discussion of previous results and the results of supplementary Figure 1, when it should start with major results seen in Figure 1.

Response: Some of the differences in appearance are because of the need to use different analysis and drawing programs (Origin, Prism etc.…) dictated by data equalization and analysis. We attempted to be more consistent and further revised drawing of the figures.

To increase clarity, we followed your suggestions and extensively revised the figures. First, we transferred almost all I/V curves, almost all surface and Co-IP blots and several example current traces to the supplement. Per journal policy, the manuscript is restricted to 9 Figures and 5 supplementary Figures, so we included the model as a panel in Figure 9 to free one Figure. This noticeably reduced panel density in all Figures and allowed us to split the complicated old Figure 6 into two new Figures, now Figure 6 and Figure 7. With these changes, we believe the presentation of the results is significantly simplified and the clarity of the manuscript is markedly improved.

To further declutter the figures and to make the flow of the figures easier for the reader, in Figure 1 after panel B, the analysis of the current densities (defined as CD to shorten the axis labels) should be presented in the absence of STIM1 to show that there is no significant difference in CD between the mutant genotypes (suggestion of previous comment #4), supporting the claim (see reply of the authors) that there is no/little difference in surface expression. As HEK wt cells contain a significant amount of STIM1, this mutant comparison is best done in STIM1 deficient cells (as in fig S2, move to Fig.1). However, in the first version of the manuscript, Fig. 1C (old) showed a small but significant increase in surface expression TRPC3-L241 in the presence of carbachol ($p<0.016$) when compared to wt+Cch, which in the new version is not indicated anymore and could be due to endogenous STIM1.

Response: We greatly appreciate the suggestions. As indicated above, we followed your suggestions and made all changes, including changing current density to CD in all Figures, transferring the STIM1 rescue experiments to Figure 1 and rearranging the results exactly as you described. As indicated above, we moved the example traces on the effect of STIM1 on the current activated by 1 µM carbachol to the supplement. These changes resulted in a much-simplified presentation of the key findings.

The effect of the TRPC3(L241S) mutants on surface expression was always included and was not removed. In the current version it is in Figure 1, panel M

I would recommend to then follow with the data from Fig. 1P-R, clearly indicating no difference between the mutants over a range of CCh concentrations in the absence of STIM1. Then move data from fig. S2 F-J (show error bars of traces in the same direction) to main Figure 1 to now very clearly show that the presence of STIM1 alters the concentration dependence of TRPC3 towards CCh. In the results section the authors state "a major role of STIM1 is controlling the affinity of the receptor for activation of TRPC3 and predict that the effect of the mutants should be altered in the absence..." Phrasing needs to be more careful, as not the affinity of the M3 receptor is changed, but rather the accessibility of TRPC3 towards DAG/lipids, as further investigated below.

Response: Please see response to the previous comment and the rearrangement of Figure 1. Moreover, we followed these suggestions to simplify presentation of the other Figures by reducing the number of panels in each Figure.

We slightly revised the indicated section. However, we kept the statement that STIM1 regulates the affinity for receptor activation of TRPC3 since STIM1 does affect the apparent Km for activation of TRPC3 by carbachol (Figure 1) but does not affect the apparent Km for GSK(DAG).

Then continue with the mutant analysis in presence and absence of STIM1. Since Vmax and the apparent Km are calculated showing the outward currents, it is more consistent to show similar outward current traces for the mutants. The IV curves in Figure 1 could be shifted to the supplemental figures. The authors added new experiments in Fig. S2 C-E, but now 100 µM CCh induced CD of the same value as 1 µm CCh in the previous experiments (~ -23 pA/pF Fig. 1L vs S2C?)). What is the reason for this discrepancy? Figure 3 E,F also show very different current densities of WT (-40 pA/pF vs. -15 pA/pF in fig1) and L241S (-80 pA/pF vs -12 pA/pF in fig 1) with the scrambled control when compared to results of Figure 1. If such a variability is present, then differences with and without STIM1 as shown in Fig. 1D-L, are difficult. Vmax in Figure 3 is now affected by the mutants (in contrast to fig.1).

Response: We focused mostly on the inward currents since they better represent physiological conditions, as physiological membrane potential is between the resting membrane potential of –60 mV and the depolarized membrane potential of -40 to -20 mV.
The traces, I/V curves, and time courses were shifted to the supplement as suggested.
As explained above, the CD can change between batches of cells, preparations of cDNA, and transfection conditions. This is the reason behind comparing effects within the same sets of experiments.

After establishing clear differences in the presence and absence of STIM1 and showing the rescue with transient expression of STIM1 within a main Figure, FRET data could follow, but needs to show the different genotypes without stimulation grouped and with stimulation grouped together. Differences in FRET ratios are quite small. Because STIM1 is an ER-localized protein, FRET between TRPC3 and STIM1 does not necessarily indicate that this interaction takes place at ER-PM junctions unless FRET is measured in the plane of the PM with TIRF (not clear from

the legend). As the underlying data contains labeled proteins, the authors can add colocalization analysis at the plasma membrane to show that TRPC3-STIM1 colocalization is much larger at the puncta when compared to outside. This effect should not be present with the I807S mutant and could more clearly address the concern #1. In STIM1 deficient cells, no puncta of TRPC3 should appear.

Response: The comment about FRET is not clear to us because this is exactly as the FRET data are shown, before and after stimulation. This presentation mode clearly shows the effect of the mutants independent of cell stimulation and the effect of receptor stimulation on the FRET.

Once unfolded STIM1 only communicates with the plasma membrane. Depletion of ER Ca^{2+} due to receptor stimulation causes STIM1 to unfold, allowing interaction with plasma membrane $PI(4,5)P_2$ at the ER/PM junctions. Therefore, FRET represents interaction between STIM1 and TRPC3 at the ER/PM junctions. This is now clarified in the text.

In these studies, we avoided the colocalization experiments because they are not informative in the context of what we are asked to show. Co-localization by confocal microscopy has a resolution at best of 200 nm. Super-resolution microscopy has a resolution of 50 nm, and both are inferior to the more quantitative and FRET that has a resolution of 2-8 nm. We now present example images in Figure S1Q-R that illustrate the limitation of confocal microscopy and discuss this in the text.
Please note that since we could not see increased surface expression by biotinylation, which is more stringent than supper-resolution microscopy as it is retained after detergent extraction, we needed to use FRET and TIRF to evaluate the effect of the mutations and of the ER/PM junctions on both STIM1-TRPC3 interaction and TRPC3 activation by receptor stimulation. The results with FRET and TIRF provide much stronger and more quantitative results than co-localization for the type of interaction examined in the present studies.

The VAPB data is not included in the model.

Response: Thank you. VAPB and its interaction with TRPC3 is now included in the model.

February 25, 2022

RE: JCB Manuscript #202107120RR

Dr. Shmuel Muallem
National Institute of Health
NIDCR
Building 10, Room 5N-102
Bethesda, MD 20892

Dear Dr. Muallem:

Thank you for submitting your revised manuscript entitled "TRPC3 channel gating by lipids requires localization at the ER-PM junctions defined by STIM1". As you will see, the reviewer is now supportive of publication once the remaining issues are completely addressed. Regarding your figures, while we appreciate the improvements made, we agree with the reviewer that the remaining presentation issues must be resolved to ensure your figures are legible, consistent, and mistake free. In redoing the figure layout, you should also consider using the entire space available on the page. The exclusive use of red/green in certain graphs to distinguish conditions also needs to be changed to make the figures legible to all readers. Please also note that the figure limit for a JCB Article is 10 main text figures, not 9, therefore your model must be moved to figure 10. While our limit for SI figures is typically 5, we can allow an extension as your 6 SI figures are very data rich. With your final submission, please provide a full point-by-point response outlining how you have addressed all remaining reviewer comments as appropriate.

Once these issues are completely resolved we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: * Scale bars must be present on all microscopy images, including inset magnifications (you may alternatively indicate the diameter of the inset)*. * Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. *

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) * Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies

(e.g. RFP antibody). Please also indicate the acquisition and quantification methods for immunoblotting/western blots. *

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures, however we can permit an extension given the amount of data in your supplementa figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Jodi Nunnari, Ph.D.
Editor-in-Chief

Andrea L. Marat, Ph.D.
Senior Scientific Editor

Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

This is the second revision of Liu et al. of their manuscript describing the regulation of TRPC3 channel gating by lipids in conjunction with their localization to ER-PM junctions. The authors have mostly addressed previous comments, rearranged figures and improved the flow of the paper to ease readability, but the manuscript and figures still remain dense.

With the exception of Figure 9, the figures are improved, however the authors response regarding the differences in appearance is correctable:
Authors response: Some of the differences in appearance are because of the need to use different analysis and drawing programs (Origin, Prism etc....) dictated by data equalization and analysis. We attempted to be more consistent and further revised drawing of the figures.

1. This is not a solid argument. Figures can be exported in a format that allows graphic programs (Adobe Illustrator, Corel draw, photoshop, inkscape, etc) to ungroup, change line thicknesses, font sizes etc. The figures are improved, but still lack consistency. Sometimes current density is abbreviated, other times not, font sizes still differ, Apparent Km abbreviated or not, typos STILL exist (i.e. Fig 3E), subpanels are partially cut (Fig. 5A, E)
Fig 5G and J cannot be separate panels as J is a magnification of G.
Both reviewers mentioned twice that one-sided error bars should be directed towards the same side, this is still not the case in many panels.

Perhaps the editors can decide on whether this is acceptable or not.

2. Author Response: We focused mostly on the inward currents since they better represent physiological conditions, as physiological membrane potential is between the resting membrane potential of -60 mV and the depolarized membrane potential of -40 to -20 mV..

reviewer: But then why are Figures 1(B-D), Figures 6, 7 and 8 based entirely on outward currents?

3. Minor point:
Reviewer comment was: However, in the first version of the manuscript, Fig. 1C (old) showed a small but significant increase in surface expression TRPC3-L241 in the presence of carbachol ($p<0.016$) when compared to wt+Cch, which in the new version is not indicated anymore and could be due to endogenous STIM1. (was not answered) See below for figure from 1. Submission to second submission.

Cannot paste figures in here, so please compare figure 1C from original submission (two significant comparisons) to current Figure 1M (one significant comparsion).

4. Reviewer comment: In the results section the authors state "a major role of STIM1 is controlling the affinity of the receptor for activation of TRPC3 and predict that the effect of the mutants should be altered in the absence..." Phrasing needs to be more careful, as not the affinity of the M3 receptor is changed, but rather the accessibility of TRPC3 towards DAG/lipids, as further investigated below.

Author reply:
We slightly revised the indicated section. However, we kept the statement that STIM1 regulates the affinity for receptor activation of TRPC3 since STIM1 does affect the apparent Km for activation of TRPC3 by carbachol (Figure 1) but does not affect the apparent Km for GSK(DAG).

Reviewer: But as GSK is added from the outside, any local effect that STIM1 or the ER/PM junctions would have regarding accessibility towards DAG/lipids would be overridden. Therefore, the argument in the discussion that the effects are not due to access and interaction of lipids is not necessarily correct and might only be clearly answered by measuring levels of PIP2 and DAG in the presence and absence of STIM1. It also does not become entirely clear if the lipid binding sites are selective in binding PIP2 and/or DAG. A very nice review by the lead author (doi: 10.15252/embr.201744331) summarizes how lipid regulation takes place at ER-PM contact sites, disturbing the junctional architecture therefore will have multiple effects.

5. Reviewer: ..... In STIM1 deficient cells, no puncta of TRPC3 should appear.
Could you comment on whether this is the case or not?

The revised model figure can still be improved and also contains some floating fragments of structures. Since this should be an easy to comprehend model, structural models for the proteins distract. The legend needs to explain the colors and symbols used in the model. I.e. difference between dark red and light red phospholipid head groups. The G proteins activated by M3 are missing. PMCA can be left out to simplify. Clustering of ORAI should be more prominent.

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"Several modes of regulation of the TRPC channels by PI(4,5)P2. PI(4,5)P2 (as) shown here (is) are providing the DAG substrate that activates the channel, (thereby its levels) controls access of DAG to its site of action in the channel pore, and PI(4,5)P2 regulates channel selectivity and localization of the channels at the ER/PM junctions" ??

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Response: The editor indicated that the Figures limit is 10 Figures and therefore we moved the model to Figure 10, which allowed us to revise the results in Figure 9 exactly as was done for the other Figures.

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Both reviewers mentioned twice that one-sided error bars should be directed towards the same side, this is still not the case in many panels.

Perhaps the editors can decide on whether this is acceptable or not.

Response: We tried before ungrouping and regrouping and found that this markedly reduced resolution. We now managed to redraw almost all figures with one graphic program and use similar size figures and insure uniformity.

We apologize for not noticing the errors in the Figures and the remaining use of different abbreviations. There were all corrected. We now use throughout CD for current density and Apparent Km µM.

The whole reason for using single error bars is that we can use selective, up or down bars to improve clarity of presentation and reduce overlap of bars. Therefore, we kept bars directions.

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reviewer: But then why are Figures 1(B-D), Figures 6, 7 and 8 based entirely on outward currents?

Response: Outward current was used only in Figure 1B. In Figures 6 and 7 both the inward and outward currents are shown. In Figure 6 this was necessary since the mutants differentially changed the two currents by affecting channel gating. To show this, it was necessary to show both the inward and outward currents. In Figure 7 the text indicates that the inward currents are shown in Figure S4A-J. This was simply because it was not possible to fit all the panels in one Figure and the more clean data with GSK are obtained with the outward current, since depletion of $PI(4,5)P_2$ resulted in small GSK-activated inward currents.

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Cannot paste figures in here, so please compare figure 1C from original submission (two significant comparisons) to current Figure 1M (one significant comparsion).

Response: We apologize for not understanding the comment. The second significance was given to show that the difference between the surface expression of the wild-type TRPC3 and TRPC3(L241S) is not significant. However, since all other difference are also not significant, we removed this to avoid confusion and kept only the significant difference.

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Response: First, thanks for the complement about the review! We agree that the selectivity of the pore lipid cannot be determined from our measurements. However, in this case we rely of the Cryo-EM structure in which DAG was observed in the pore of three TRPC channels, TRPC3, TRPC5 and TRPC6. We think that these observations make it clear that the pore lipid is DAG and our conclusion is justified. We attempted to clarify this point in the discussion.

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Response: Actually, as indicated in the manuscript, TRPC3 puncta are observed in unstimulated cells. It appears that TRPC3 tends to show puncta independent of STIM1. However, no change in puncta is observed in stimulated STIM1$^{-/-}$ cells.

Model: We do not see any floating structures. Please note that the E-Syts and ANO8 do not span the plasma membrane, only interacts with PI(4,5)P_2 at the inner leaflet and PLC and IP_3 are cytoplasmic. All colors and abbreviations are now defined in the legend. The G proteins activated by M3 were added. PMCA is a central component of the Ca^{2+} signal and is included in the model. The density of Orai1 was increased slightly. We were not able to increase if further since we use images of the structures and they cannot be overlapped by adding additional Orai1 images without masking other structures.

Discussion: Thank you. This was corrected.