ORP3 Phosphorylation Regulates Phosphatidylinositol 4-phosphate and Ca^{2+} Dynamics at PM-ER Contact Sites

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MS TITLE: ORP3 Phosphorylation Regulates Phosphatidylinositol 4-phosphate and Ca^{2+} Dynamics at PM-ER Contact Sites

AUTHORS: Gergo Gulyas, Mira Sohn, Yeun Ju Kim, Peter Varnai and Tamas Balla
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

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

Reviewer 1

Advance summary and potential significance to field

In this paper, the authors investigate the less characterized ORP family member ORP3, as a potential regulator of lipid homeostasis at contact sites between the plasma membrane (PM) and the ER. They we show that ORP3 PM association is mainly determined by PI(4,5)P2 and to a lesser degree, by PI4P. In response to activation of cells, ORP3 robustly decreased PI4P and to a small extent phosphatidic acid (PA) and slightly increased cholesterol at the PM. Full activation of ORP3 was achieved when PKC activation was combined with Ca2+ increases, resulting in decreased PM PI4P levels and inhibition of Ca2+ entry via the store-operated Ca2+ entry pathway. In addition, they show that the C-terminal region adjacent to the strictly defined lipid transfer domain, is required for the proper localization and function of the ORP3 protein.

While at first glance, the paper appeared to be addressing an important area of interest, impacting plasma membrane lipid recycling as well as ER-PM junctions and SOCE, the paper suffers from sloppy editing and somewhat careless writing. In addition, the narrative is rather descriptive, confusing in some parts and does not lead to a strong conclusion. Last paragraph of discussion and last line of abstract do not really state what the main take home message is, rather two or three findings are listed. Then, there is the potentially interesting link between ORP3, PI4 depletion and SOCE, and also assembly of ORP3 in SOCE microdomains with STIM1. These interactions have not been examined in any depth and neither has the effect on SOCE/PI4P been elaborated. Nevertheless, the findings are of interest. I believe the authors need to carefully look at the results and manuscript and revise to improve the quality of this manuscript.

Comments for the author

Main concerns:

1. **Figure 1**:
C - confocal image quality is bad. It is difficult to differentiate between an ER network- like pattern and what is in the plasma membrane. Fluorescence images using an ER marker would have been good. Also, while the HH/AA mutation was reported in the manuscript to produce prominent ER-PM patches after PMA treatment, no clear difference was observed for the ORP3$^{HH/AA}$ images before and after PMA treatment. The authors need to explain why the ORP3$^{HH/AA}$ mutant behaves differently depending on whether it is tethered to VAPB or not.

More evidence of PM interaction is needed using TIRFM, which is better suit for the detection of ER-PM junctions at the cell periphery. In fact, I am not sure why these images were done using confocal while the rest are TIRFM images.

Quantitation is required for these images, since the authors are making a point in stating that mutating the PI4P binding site of ORP3 results in more pronounced ER-PM patches being formed.

2. **Fig S1**:
B, C - not clear why the control does not show an increase in PI4P after PMA. Are the cell lines in this and Fig. 1, same or different? If HEK293-AT1 cells are different from HEK- 293T, please explain why different cell lines were used in different experiments. Also, what else is different between these two cell types: Ca^{2+} influx, intracellular release etc.? Supplemental Fig 1C is not explained in the main text.

3. **Fig2:** TIRFM image with mutant alone (ORP3$^{HH/AA}$) should be provided.

4. **Figure S2**: A - In the rapamycin-treated PJ-dead cells, why is there no increase in PI4P? If this is again due to a different cell type being used, then this needs to be clearly stated from the beginning of the paper. Fig. 1 should contain both the patterns and characterization of the effects of different ORP3 constructs in the two cell types. Further, the rationale for selecting either cell type for different experiments should also be explained. As such, it is extremely confusing as to what has been done and why.

5. **Figure 4:**
- Comparison of responses with AngII and Tg must be given in the text. Why are they not the same (page 10)?
- Needs plasma membrane localization data for WT+BAPTA as well as HH/AA+STIM1/BAPTA. The authors needs to investigate if SOCE is required or whether STIM clustering is sufficient for ORP3 function.
- Does STIM1 cause ORP3 clustering directly or modulates ORP3 clustering via SOCE? No STIM1, STIM2, or Orai1 knockdown experiments have been done, so it is unclear what endogenous STIM1 is really doing. Is it specifically STIM1, or can either MAPPER, Ist2 or STIM2 also cluster with ORP3?
- The effects of PMA and Tg appear to be additive for HH/AA but is more than additive for HH/AA+STIM1. The authors should investigate this and provide and explanation for the observation.
- What is the effect of these different expressions and treatments on ER-Ca^{2+} release vs influx? Anything that affects ER-Ca^{2+} release will affect the influx. So, the authors needs to clarify whether the effects.

6. **Figure 6:**
- [Ca^{2+}]i measurements should utilize protocols to determine intracellular release vs influx.
- Data showing that AngII stimulates SOCE should be presented.
- For Ca^{2+} measurements, why did the authors used two different methods – BRET- based and Fura2? It is difficult to do a cross comparison of the Ca^{2+} responses for each condition tested. The authors should use just one Ca^{2+} measurement method for all experiments.
- Why does ORP3 cause decrease in stimulated Ca^{2+} increase with AngII but not ATP? I also do not understand why the authors chose to use ATP here instead of showing Ca^{2+} responses obtained with Tg and Tg+PMA. The authors should use the same conditions described in Fig. 4A-D, so that we can correlate the magnitude of Ca^{2+} responses obtained with the clustering of various ORP3 constructs.
- What is the mechanism underlying the decrease in SOCE? Is Orai1/STIM1 clustering altered or is the effect on the ER-PM junctions ?

7. **Figure S3** is not discussed in the paper.

8. **Figure 7:** Why does PI4P not increase after PMA in C? Is this again a cell line issue?.

Minor:
Figure 4: G and H are mislabeled (should be E and F). The description is also misstated in the text.

Reviewer 2

Advance summary and potential significance to field

The manuscript by G. Gulyás et al. provides important new advances on the function and regulation of the lipid transfer protein ORP3. This relatively poorly studied oxysterol binding protein related protein (ORP) is frequently overexpressed in cancer cells, and is associated with poor prognosis in numerous cancers including Burkitt's lymphoma, colorectal adenocarcinoma, B-cell-associated malignancies and testicular cancer. The functions of this lipid transfer protein in cells are not well understood, and the study by Gulyás et al. demonstrates regulation of membrane contact site localization of ORP3 by both SOCE and PKC activation, as well as by PM-localized PI(4,5)P2. These authors also provide evidence in cells that ORP3 recruitment to the PM causes a reduction in PI4P levels at the PM, supporting the idea that ORP3 functions in a manner similar to other ORPs in transferring PI4P out of the PM to the ER. Interestingly, the authors show that activation of ORP3 causes an inhibition of SOCE, thus demonstrating a novel feedback mechanism. The experimental data is very well done, and the conclusions novel and important. One major point that needs to be addressed is the nature of one of the constructs used for many experiments in this manuscript. The authors use a plasmid encoding ORP3, the viral T2A sequence as a linker, followed by VAPB (ORP3-T2A-VAPB). It is essential that the authors indicate whether their ORP3-T2A-VAPB construct is a fusion protein between ORP3 and VAPB, or whether the two proteins are simply co-expressed from

the same plasmid. In the former case, it is inappropriate to use the formuation “expressing ORP3 alone or together with VAPB”, as this phrase implies co-expression of two separate proteins. If ORP3-T2A-VAPB is a fusion protein, it should be described as “ORP3 fused to VAPB”, or “the ORP3-VAPB fusion protein”. If not, Figure 1B needs to be changed to show lack of continuity between the ORP3-mCherry protein and VAPB. The authors make extensive use of this protein, and see effects that are not seen (or only weakly) when expressing ORP3 alone (see points 1 and 3 below), so it is essential to clarify this point.

Comments for the author

1. Page 5, first paragraph. Why does the expression vector containing both the ORP3 and VAPB coding sequences (ORP3-T2A-VAPB) have a much more visible response to PMA than ORP3 alone (Figures 1 and 2)? The authors say that it is because this situation results in equivalent levels of expression of the two proteins. However, ORP3 binds to VAPA in addition to VAPB, and VAPA expression is significantly higher in cells than that of VAPB. It is not clear to this reviewer if ORP3 and VAPB are actually fused in this construct, or whether they are simply co-expressed from the same plasmid. These points should be clarified. Have the authors checked the levels of VAPA and VAPB expression in cells expressing the ORP3-T2A-VAPB construct compared to control cells, and if it is indeed a fusion between ORP3 and VAPB, compared these levels to that of the fusion protein?

2. Page 5, last paragraph. In Fig. 1C, the authors only show two of the four constructs described in Fig. 1B. This fact should be indicated, rather than stating “….localization of these constructs…”.

3. Page 6, first paragraph. In Fig. 1C, no difference is visible to this reviewer between untreated and PMA-treated cells expressing either ORP3 or ORP3-HHAA. Only the fusions of ORP3 with VAPB (WT ORP3 and the HH-AA mutant) show a detectable effect in the images shown. An explanation for this dramatic difference should be provided (see point 1 above), taking into account that whether ORP3 is actually fused to VAPB or is only co-expressed from the same plasmid makes a big difference in interpreting the results.

4. Page 6, second line. The authors refer to “quiescent cells”. Do they mean cells that are not dividing? If so, how are these Cos7 cells maintained in such a state? Does PMA cause increased proliferation?

5. Page 8. Suppl Fig. S2B does indeed show a dramatic decrease in PI(3,4)P2 and PI(3,4,5)P3 levels, but what is the justification that they were “…almost completely eliminated by Wm treatment”? The graph shows a reduction to 30-40%, not 0, so this claim needs to be explained more thoroughly. Also, a positive control in Fig. 2C using a PI(3,4)P2- and/or PI(3,4,5)P3-specific probe would be desirable, to show that the wortmannin treatment is effective in terms of protein recruitment. In Fig. 2C, the selective reduction of levels of the other PPIns were carried out by expression of phosphatases, not with drugs, so the situations are quite different.

6. - In Fig. 4B, it looks to me like the dark green and light green plots have been reversed, especially when compared to Fig. 4D. As it stands, Tg + BAPTA has a higher AUC than Tg + PMA + BAPTA in Fig. 4B, but is the opposite is shown in Fig. 4D.

- In Fig. 4D, what is meant by “open bars”, representing data from Figure 3/B?

-In the figure panel, “G” and “H” should be changed to “E” and “F”.

7. Page 12. Fig. 6 D and E, cited in the first paragraph, are not in Figure 6. Also, citation of Fig. 6F in the next paragraph seems to refer to Fig. 6D. Please check figure panels and their citations throughout the text.

8. Page 13, last paragraph. The speculation about interaction of the C-terminus of ORP3 with Sac1 or STIM1 is not supported by data shown, so should be moved (along with the last sentence) to the Discussion section.

9. Page 14, second paragraph. An additional explanation for previous results showing interaction of the ORP3 PH domain with 3-phosphorylated lipids may be that ORP3 localizes to other ER-organelle

contact sites. For example it has been shown that ORP3 functions in ER/NE-endosome interactions (Santos MF et al. 2018. J Biol Chem 293:13834-13848), as pointed out by the authors in the Introduction.

10. Overall the English usage in this manuscript is good, but there are some issues, so it should be carefully checked. For example, on Page 5, "…has been successfully used in our previous works to simultaneous expression of two proteins with fixed stoichiometry" should be changed to "…has been successfully used in our previous studies to simultaneously express two proteins with fixed stoichiometry."

Reviewer 3

Advance summary and potential significance to field

This manuscript entitled "ORP3 Phosphorylation Regulates Phosphatidylinositol 4-phosphate and Ca2+ Dynamics at PM-ER Contact Sites" by Gulyás et al. describes the mechanism of ORP3 association with the plasma membrane and how signaling affects this recruitment; moreover, this work shows the effect of ORP3 activation on PM PI(4)P and cholesterol, and Ca2+ signaling. The first part of the manuscript is really nicely performed. The second part is somewhat confusing and deserves more work.

Comments for the author

Major points:
- The title is somewhat confusing: the authors do not show that ORP3 is phosphorylated and that this phosphorylation is responsible for its activity.
The effect of PKC could be mediated by a mechanism not involving the phosphorylation of ORP3. This idea is present throughout the manuscript, but never demonstrated.
- Statistics are missing in some parts of manuscript. This challenges some conclusions. For instance Fig3, Fig6, the authors describe differences between samples, but they do not show that these differences are statistically significant. This leads to odd claims such as the following: P8: "low AngII concentrations that slightly elevated PI(4)P level in mock transfected control cells…" even if the difference in the graph is tiny, and no statistical analysis was performed. The authors should add statistical analyses if they want to conclude that there is a difference between samples.
- The paragraph "ORP3 responds to stimulation of Ca2+ mobilizing receptors" is unclear. The beginning of this part of the manuscript is devoid of references.
The justification of the experiments performed is hard to follow.
- Concerning the role of the carboxyl-terminal part of ORP3: how do the authors rule out that deleting the last 35 residues of ORP3 disrupts the folding of the protein? This would change the interpretation of the result. The authors should perform dynamic light scattering experiments on the ORD domain with or without the last 35 residues.
- Fig 2A: without PMA, there is an apparent increased recruitment at the PM of the HH/AA mutant compared to WT protein. Could the authors comment this?
- Page 10: what is the effect of STIM1 on ORP3 recruitment on the PM? In other words, is STIM1 required for ORP3 recruitment in ER-PM contacts?

Minor points:
- P3: "OSBP was the first lipid transfer proteins in which the paradigm of phosphatidylinositol 4-phosphate (PI4P)-gradient driven cholesterol/PI4P counter-transport between the ER and the Golgi complex has been described". OSBP was not the first LTP to be shown to exchange PI(4)P and cholesterol.
Osh4p/Kes1-mediated PI(4)P/sterol exchange was described in de Saint-Jean et al, JCB, 2011.
- P5: Rephrase: "This approach has been successfully used in our previous works to simultaneous expression of two proteins with fixed stoichiometry (Toth et al., 2012; Varnai et al., 2017)."
- P5:"The H631/632A (HH/AA) mutation has been reported to eliminate PI4P binding for the yeast OSH4 (Maeda et al., 2013)". The first paper to describe this mutation in Osh4p was de Saint-Jean et al, JCB, 2011.

- Some paragraphs in the Results section are devoid of concluding phrase (For instance “ORP3 responds to stimulation of Ca2+ mobilizing receptors”).
- The affiliation of one of the authors (Mira Sohn) is missing.

First revision

Author response to reviewers' comments

We would like to thank the Reviewers for their thorough reading of our manuscript, their critical comments and helpful suggestions. Our detailed responses to their comments are described below.

Reviewer 1

Comment 1:
Figure 1C - confocal image quality is bad. It is difficult to differentiate between an ER network-like pattern and what is in the plasma membrane. Fluorescence images using an ER marker would have been good. Also, while the HH/AA mutation was reported in the manuscript to produce prominent ER-PM patches after PMA treatment, no clear difference was observed for the ORP3HH/AA images before and after PMA treatment. The authors need to explain why the ORP3HH/AA mutant behaves differently depending on whether it is tethered to VAPB or not. More evidence of PM interaction is needed using TIRFM, which is better suit for the detection of ER-PM junctions at the cell periphery. In fact, I am not sure why these images were done using confocal while the rest are TIRFM images. Quantitation is required for these images, since the authors are making a point in stating that mutating the PI4P binding site of ORP3 results in more pronounced ER-PM patches being formed.
Answer 1:
Unfortunately, the submission site for JCS has put a limitation on the file size during the first submission stage, which required us to decrease the resolution of our Figures making their quality less than optimal. The original versions show these changes much better. Having said that, new images were obtained using the AT1-receptor expressing HEK cell line, with the different ORP3 constructs and mCherry-Sec61B as an ER marker. The old images have been replaced with new ones.
Confocal images were taken to also judge the ER association of the protein. Importantly, the VAP-B protein and the ORP3 proteins were expressed from a single plasmid but they were not fused. Separate expression was checked by WB analysis (now shown in Fig. 1C). The experiment was designed to test if there is enough endogenous VAP-B present to keep all the expressed ORP3 bound to the ER rather than just being in the cytosol. We tried to make this point clearer in the revised manuscript.
As for the suggested TIRF experiments and quantification, the Reviewer might have noticed that these were done in the subsequent parts of the study using the VAP co- expression condition and were quantified.
However, in response to the Reviewer’s comment, we did perform the TIRF experiments with or without VAP-B expression and looked at the PM interaction with quantification. Based on these TIRF analyses, the PM intensity increase was similar with or without the additional VAPB molecules, but the localization pattern was different. Namely, without concomitant VAPB expression, ORP3 (or its mutant form) showed a more uniform distribution on the PM with fewer recognizable patch indicative of the uniform translocation of the free cytosolic ORP3 not constrained by ER interaction. In contrast, punctate localization was more pronounced when VAPB was co-expressed as the larger portion of the ORP3 proteins were ER-membrane restricted. These are shown now in the paper in Fig. 2A and B.

Comment 2:
Fig S1B, C - not clear why the control does not show an increase in PI4P after PMA. Are the cell lines in this and Fig. 1, same or different? If HEK293-AT1 cells are different from HEK-293T, please explain why different cell lines were used in different experiments. Also, what else is different between these two cell types: Ca2+ influx, intracellular release etc.? Supplemental Fig 1C is not explained in the main text.

Answer 2:
The Reviewer is right when noticing this difference. Indeed, these graphs show results obtained in two different HEK293 cell lines. For several experiments (most not even shown in this manuscript) the initial characterization of the lipid transfer catalyzed by different ORP3 variants the HEK293T cells were used in the Varnai laboratory in Budapest. These cells express the simian virus 40 (SV40) large T antigen and achieve a larger level of expression of the BRET constructs. However, most subsequent experiments were performed at the NIH, using a HEK293 cell line that expresses the AT1 angiotensin receptor in order to see the effect of a strong Gq-receptor activation. Indeed, the PI4P increase after PMA stimulation is clearly more pronounced in the HEK293T cells, which may reflect a larger sensitivity of the response at higher expression levels of the BRET constructs or could be genuinely due to a different expression level of the component of the complicated PI4KA regulatory complex or PKCs. However, it is important to note that all of the changes that are exerted by ORP3 are the same in the two cell lines and across the Atlantic in two different laboratories. We more explicitly explain in the revised manuscript when the experiments were done in a different cell line. We corrected the missing reference to Supplemental Figure C in the text.

Comment 3:
Fig2: TIRFM image with mutant alone (ORP3$^{HH/AA}$) should be provided.

Answer 3:
The new Fig. 2 shows these images including the mutant ORP3 with parallel quantifications.

Comment 4:
Figure S2A - In the rapamycin-treated PJ-dead cells, why is there no increase in PI4P? If this is again due to a different cell type being used, then this needs to be clearly stated from the beginning of the paper. Fig. 1 should contain both the patterns and characterization of the effects of different ORP3 constructs in the two cell types.
Further, the rationale for selecting either cell type for different experiments should also be explained. As such, it is extremely confusing as to what has been done and why.
Answer 4:
See our response to question 2.

We do not believe that showing comparisons for all parameters with both cell lines would make our study stronger and would only make the paper unnecessarily complicated. We believe that as far as ORP3 is concerned, the effects are very clear regardless of the cells used and the only difference we noticed between the HEK293T and HEK293-AT1 cells was the larger PI4P increase to PMA in the former, which was small and often not apparent in the latter. For several parameters tested in COS-7 cells (mostly removed now for clarity) the ORP3 effects were the same suggesting that our conclusions are not cell-type dependent.

Comment 5:
Figure 4:
a) Comparison of responses with AngII and Tg must be given in the text. Why are they not the same (page 10)?
b) Needs plasma membrane localization data for WT+BAPTA as well as HH/AA+STIM1/BAPTA. The authors need to investigate if SOCE is required or whether STIM clustering is sufficient for ORP3 function.
c) Does STIM1 cause ORP3 clustering directly or modulates ORP3 clustering via SOCE? No STIM1, STIM2, or Orai1 knockdown experiments have been done, so it is unclear what endogenous STIM1 is really doing. Is it specifically STIM1, or can either MAPPER, Ist2 or STIM2 also cluster with ORP3?
d) The effects of PMA and Tg appear to be additive for HH/AA but is more than additive for HH/AA+STIM1. The authors should investigate this and provide and explanation for the observation.
e) What is the effect of these different expressions and treatments on ER-Ca2+ release vs influx? Anything that affects ER-Ca^{2+} release will affect the influx. So, the authors need to clarify whether the effects.

Answer 5:

a) We have inserted the missing comparison to the main text. The different responses to Tg or AngII are likely due to the larger and more rapid cytosolic Ca^{2+} increase generated by AngII which leads to a rapid increase in IP3 and IP3 receptor activation. Tg, in contrast, causes a slow Ca^{2+} leak from the ER and activates Ca^{2+} entry slowly. These different Ca^{2+} changes will activate PKCs to a different extent.

b) The plasma membrane localization data for HH/AA+STIM1/BAPTA have been now provided. We also provided additional data for the involvement of STIM1 itself by comparing the effects of STIM1 knock down or using Ca^{2+}-free medium to limit SOCE.

c) See answer above.

d) We are not certain that these differences warrant further analysis after seeing that STIM1 does not play a role beyond supporting Ca2+ influx and providing additional or stronger contacts. Overexpression of STIM1 clearly expands the ER-PM contacts which is probably why the response was disproportionally higher in the presence of STIM1, once TG was added.

e) Based on comparisons of the initial Ca2+ peaks after AngII addition, ORP3 expression does not appear to affect Ca2+ release.

Comment 6:

Figure 6:

a) [Ca2+]i measurements should utilize protocols to determine intracellular release vs influx.

b) Data showing that AngII stimulates SOCE should be presented.

c) For Ca2+ measurements, why did the authors used two different methods – BRET- based and Fura2? It is difficult to do a cross comparison of the Ca2+ responses for each condition tested. The authors should use just one Ca2+ measurement method for all experiments.

d) Why does ORP3 cause decrease in stimulated Ca2+ increase with AngII but not ATP? I also do not understand why the authors chose to use ATP here instead of showing Ca2+ responses obtained with Tg and Tg+PMA. The authors should use the same conditions described in Fig. 4A-D, so that we can correlate the magnitude of Ca2+ responses obtained with the clustering of various ORP3 constructs.

e) What is the mechanism underlying the decrease in SOCE? Is Orai1/STIM1 clustering altered or is the effect on the ER-PM junctions?

Answer 6:

a) We are not convinced that these protocols would add any new information beyond what is already shown by our results. The manuscript already has a lot of data and we performed a significant effort to make new experiments and focused our efforts on those that are more critical to improve the manuscript.

b) Although the effects of AngII stimulation of SOCE has been well-established in the literature (in fact we have not seen a Gq-coupled receptor that increases InsP3 that did not activate SOCE), we have added these experiments to the Results now shown in Fig. 5F.

c) The Fura-2 data in COS-7 cells was only meant to demonstrate that ORP3 has an effect on Ca2_ influx in different cell type using different methods for Ca2+- measurements. We think this has strengthened the case rather than weakened it. However, given the amount of new data added, we removed the Ca2+ data obtained in COS-7 cells to address this comment.

d) We are not sure what the Reviewer refers to here. Neither ATP in COS-7 cells, nor AngII in HEK293 cells affects Ca2+ release, only the influx phase. In each case the effect requires VAP-B co-expression (compare new Fig.7A-C. vs. Fig. S3A-C). However, as pointed out above, we have removed the COS-7 Ca^{2+} data.

e) We believe that the effects are related to ORP3 decreasing PI4P. However, we cannot rule out an effect on the ER side, such us direct interaction with Sac1 or STIM1. We have a whole new set of studies in progress to address this issue with alternative ways of testing this hypothesis including analysis of STIM1 clustering.

Comment 7:

Figure S3 is not discussed in the paper.

Answer 7:

Thank you for pointing this out. We have corrected this oversight.

Comment 8:
Figure 7: Why does PI4P not increase after PMA in C? Is this again a cell line issue?
Answer 8:
Yes, see answer under comment #1.

Comment 9:
Figure 4: G and H are mislabeled (should be E and F). The description is also misstated in the text.
Answer 9:
We thank the reviewer for spotting this mistake, which has been corrected.

Reviewer 2

Comment 1: Page 5, first paragraph. Why does the expression vector containing both the ORP3 and VAPB coding sequences (ORP3-T2A-VAPB) have a much more visible response to PMA than ORP3 alone (Figures 1 and 2)? The authors say that it is because this situation results in equivalent levels of expression of the two proteins.
However, ORP3 binds to VAPA in addition to VAPB, and VAPA expression is significantly higher in cells than that of VAPB. It is not clear to this reviewer if ORP3 and VAPB are actually fused in this construct, or whether they are simply co-expressed from the same plasmid. These points should be clarified. Have the authors checked the levels of VAPA and VAPB expression in cells expressing the ORP3-T2A-VAPB construct compared to control cells, and if it is indeed a fusion between ORP3 and VAPB, compared these levels to that of the fusion protein?
Answer 1:
ORP3 and VAP-B are expressed separately and not as a fusion from a single plasmid using the viral T2A sequence that allows stoichiometric expression of two separate polypeptides. We included Western-blot analysis to show that separate expression of the two proteins (new Fig. 1C). We are sorry if this was not all that clear in the original manuscript, which we corrected in the revised version. As for the expression of VAP-A and VAP-B, we could not determine their relative expression level in our cells as one would have to have antibodies that react with equal strength to the respective proteins. Our purpose of expressing VAP-B together with the ORP3 constructs was to make sure there was enough VAP-partner for their ER interaction in case the endogenous VAPs were not present in sufficient amounts.

Comment 2:.
Page 5, last paragraph. In Fig. 1C, the authors only show two of the four constructs described in Fig. 1B. This fact should be indicated, rather than stating "….localization of these constructs…".
Answer 2:
We appreciate the comment and corrected this sentence.

Comment 3:
Page 6, first paragraph. In Fig. 1C, no difference is visible to this reviewer between untreated and PMA-treated cells expressing either ORP3 or ORP3-HHAA. Only the fusions of ORP3 with VAPB (WT ORP3 and the HH-AA mutant) show a detectable effect in the images shown. An explanation for this dramatic difference should be provided (see point 1 above), taking into account that whether ORP3 is actually fused to VAPB or is only co-expressed from the same plasmid makes a big difference in interpreting the results.
Answer 3:
We have provided better images in the revised manuscript and clarified that the two constructs are not fused together.

Comment 4:
Page 6, second line. The authors refer to "quiescent cells". Do they mean cells that are not dividing? If so, how are these Cos7 cells maintained in such a state? Does PMA cause increased proliferation?
Answer 4:
We appreciate the comment, the "quiescent" was replaced with "unstimulated cells".

Comment 5: Page 8. Suppl Fig. S2B does indeed show a dramatic decrease in PI(3,4)P2 and PI(3,4,5)P3 levels, but what is the justification that they were "…almost completely eliminated

by Wm treatment"? The graph shows a reduction to 30-40%, not 0, so this claim needs to be explained more thoroughly. Also, a positive control in Fig. 2C using a PI(3,4)P2- and/or PI(3,4,5)P3-specific probe would be desirable, to show that the wortmannin treatment is effective in terms of protein recruitment. In Fig. 2C, the selective reduction of levels of the other PPIns were carried out by expression of phosphatases, not with drugs, so the situations are quite different.

Answer 5:

The BRET values never reach zero even when the lipid binding probes are fully disengaged from the membrane. Their luciferase component still excites to a small extent the membrane-bound Venus. This residual BRET is different for each of the probes we used and has been documented in previous publications describing these methods. Under these conditions PI(3,4)P2 and PI(3,4,5)P3-specific probes (the same probes are used in the BRET constructs) completely fall off the plasma membrane in confocal experiments as shown in previous studies (Toth et al, 2016, PMID: 26692031; Toth et al. 2019: PMID: 30790246).

Comment 6:

In Fig. 4B, it looks to me like the dark green and light green plots have been reversed, especially when compared to Fig. 4D. As it stands, Tg + BAPTA has a higher AUC than Tg + PMA + BAPTA in Fig. 4B, but is the opposite is shown in Fig. 4D.

Answer 6:

We thank the Reviewer for spotting the inconsistency. We have reviewed all of our Figures for proper labeling.

Comment 7:

In Fig. 4D, what is meant by "open bars", representing data from Figure 3/B?

Answer 7:

This was a mistake on our part as it referred to an earlier version of the Figure. We have corrected it in the revised manuscript.

Comment 8:

In the figure panel, "G" and "H" should be changed to "E" and "F".

Answer 8:

We thank the reviewer for noticing this discrepancy, and have corrected this mistake.

Comment 9:

Page 12. Fig. 6 D and E, cited in the first paragraph, are not in Figure 6. Also, citation of Fig. 6F in the next paragraph seems to refer to Fig. 6D. Please check figure panels and their citations throughout the text.

Answer 9:

We apologize for these mistakes and thank the reviewer for the comments. We made sure that Figure panels are correctly referenced in the revised manuscript.

Comment 10:

Page 13, last paragraph. The speculation about interaction of the C-terminus of ORP3 with Sac1 or STIM1 is not supported by data shown, so should be moved (along with the last sentence) to the Discussion section.

Answer 10:

We agree and have moved this speculation to the Discussion section.

Comment 11:

Page 14, second paragraph. An additional explanation for previous results showing interaction of the ORP3 PH domain with 3-phosphorylated lipids may be that ORP3 localizes to other ER-organelle contact sites. For example, it has been shown that ORP3 functions in ER/NE-endosome interactions (Santos MF et al. 2018. J Biol Chem 293:13834-13848), as pointed out by the authors in the Introduction.

Answer 11:

This is a very good and valid point and we have included this possibility both in the Results and the Discussion.

Comment 12:
Overall the English usage in this manuscript is good, but there are some issues, so it should be carefully checked. For example, on Page 5, "…has been successfully used in our previous works to simultaneous expression of two proteins with fixed stoichiometry" should be changed to "…has been successfully used in our previous studies to simultaneously express two proteins with fixed stoichiometry."
Answer 12:
Thank you for spotting this awkward sentence that has been overlooked during the many corrections that were made to the text before submission. We have changed the text as suggested.

Reviewer 3

Comment 1:
The title is somewhat confusing: the authors do not show that ORP3 is phosphorylated and that this phosphorylation is responsible for its activity. The effect of PKC could be mediated by a mechanism not involving the phosphorylation of ORP3. This idea is present throughout the manuscript, but never demonstrated.
Answer 1:
The phosphorylation of ORP3 by PKC has been elegantly demonstrated before in the Olkkonen laboratory (PMID: 25447204) and also have been confirmed in the present study (see additional Western Blot experiments that are now included in Fig. 1C). Although, we agree that the focus of the study was not on the phosphorylation process, we believe that the changes we have studied require the PKC-induced phosphorylation of ORP3 in agreement with the general consensus in the literature.

Comment 2:
Statistics are missing in some parts of manuscript. This challenges some conclusions. For instance, Fig3, Fig6, the authors describe differences between samples, but they do not show that these differences are statistically significant. This leads to odd claims such as the following: P8: "low AngII concentrations that slightly elevated PI(4)P level in mock transfected control cells…" even if the difference in the graph is tiny, and no statistical analysis was performed. The authors should add statistical analyses if they want to conclude that there is a difference between samples.
Answer 2:
We have carefully checked the manuscript and completed statistical analysis for effects that are relevant to the conclusions. We also changed the text not to claim differences when such analysis was not done because they were not relevant to the focus and conclusion of the study.

Comment 3:
The paragraph "ORP3 responds to stimulation of Ca2+ mobilizing receptors" is unclear. The beginning of this part of the manuscript is devoid of references. The justification of the experiments performed is hard to follow.
Answer 3:
We have added a reference to the Result section to support the statement that agonists induce PIP changes. Also, we sharpened the rational and justification of these experiments.

Comment 4:
Concerning the role of the carboxyl-terminal part of ORP3: how do the authors rule out that deleting the last 35 residues of ORP3 disrupts the folding of the protein? This would change the interpretation of the result. The authors should perform dynamic light scattering experiments on the ORD domain with or without the last 35 residues.
Answer 4:
We agree with the Reviewer on this point. To check the correct folding of the protein we would need to prepare pure recombinant proteins that requires a major effort as preparing these ORDs from mammalian cells is challenging even for a laboratory with expertise in producing recombinant proteins. Instead, we generated point mutations that predictably disrupt the association of the C-terminal domain with the core of the lipid binding domain. These mutants behaved the same way as the truncated protein. These new data have been added to the manuscript and our theory of how they might affect the transport and localization features have been discussed.

Comment 5:
Fig 2A: without PMA, there is an apparent increased recruitment at the PM of the HH/AA mutant compared to WT protein. Could the authors comment this?
Answer 5:
There is clearly a higher membrane binding affinity of this mutant, which causes some interaction with the membrane even before stimulation the extent of which varies from cell to cell. Based on the comments of other Reviewers, we have provided new pictures where such "basal" localization is not apparent (Fig. 1D and Fig. 2A).

Comment 6:
Page 10: what is the effect of STIM1 on ORP3 recruitment on the PM? In other words, is STIM1 required for ORP3 recruitment in ER-PM contacts?
Answer 6:
We have devoted a whole set of new experiments to address this question, which are included in the manuscript (new Fig.5E). Briefly, the conclusion of these studies is that STIM1 is needed because it supports store-operated Ca^{2+} entry but also may contribute to strengthening the ER-PM contact sites. Accordingly, overexpressed STIM1 increases the numbers and areas ER-PM contacts and hence increases the ORP3 signal found in these contact sites (shown in Fig. 5C and D).

Comment 7:
P3: "OSBP was the first lipid transfer proteins in which the paradigm of phosphatidylinositol 4-phosphate (PI4P)-gradient driven cholesterol/PI4*P* counter- transport between the ER and the Golgi complex has been described". OSBP was not the first LTP to be shown to exchange PI(4)P and cholesterol. Osh4p/Kes1-mediated PI(4)P/sterol exchange was described in de Saint-Jean et al, JCB, 2011.
Answer 7:
We partially agree. What we meant is that the whole detailed analysis of this process with the role of Sac1 and the rest has been described by the Antonny group in their Cell paper. Nevertheless, we have corrected this statement with more precise wording and included the truly important de Saint-Jean paper in the list of References.

Comment 8:
P5: Rephrase: "This approach has been successfully used in our previous works to simultaneous expression of two proteins with fixed stoichiometry (Toth et al., 2012; Varnai et al., 2017)."
Answer 8:
Thank you. All three Reviewers spotted this mistake. We have corrected this sentence.

Comment 9:
P5:"The H631/632A (HH/AA) mutation has been reported to eliminate PI4P binding for the yeast OSH4 (Maeda et al., 2013)". The first paper to describe this mutation in Osh4p was de Saint-Jean et al, JCB, 2011.
Answer 9:
Thank you for this correction that has now been addressed.

Comment 10:
Some paragraphs in the Results section are devoid of concluding phrase (For instance "ORP3 responds to stimulation of Ca2+ mobilizing receptors").
Answer 10:
We have attempted to correct these deficiencies.

Comment 11:
The affiliation of one of the authors (Mira Sohn) is missing.
Answer 11:
We have corrected this mistake.

Second decision letter

MS ID#: JOCES/2019/237388

MS TITLE: ORP3 Phosphorylation Regulates Phosphatidylinositol 4-phosphate and Ca2+ Dynamics at PM-ER Contact Sites

AUTHORS: Gergo Gulyas, Mira Sohn, Yeun Ju Kim, Peter Varnai, and Tamas Balla
ARTICLE TYPE: Research Article

All three reviewers now think your paper is publishable. However, reviewer #3 has several minor points of correction that could be incorporated into your paper. Please make these changes and I will accept your paper.

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

Reviewer 1

Advance summary and potential significance to field

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Comments for the author

I am satisfied with the revisions. I can now recommend publication of this paper.

Reviewer 2

Advance summary and potential significance to field

The manuscript by G. Gulyás et al. provides important new advances on the function and regulation of the lipid transfer protein ORP3. This relatively poorly studied oxysterol binding protein related protein (ORP) is frequently overexpressed in cancer cells, and is associated with poor prognosis in numerous cancers including BurkittÂ's lymphoma, colorectal adenocarcinoma, B-cell-associated malignancies and testicular cancer. The functions of this lipid transfer protein in cells are not well understood, and the study by Gulyás et al. demonstrates regulation of membrane contact site localization of ORP3 by both SOCE and PKC activation, as well as by PM-localized PI(4,5)P2. These authors also provide evidence in cells that ORP3 recruitment to the PM causes a reduction in PI4P levels at the PM, supporting the idea that ORP3 functions in a manner similar to other ORPs in transferring PI4P out of the PM to the ER. Interestingly, the authors show that activation of ORP3 causes an inhibition of SOCE, thus demonstrating a novel feedback mechanism. The experimental data is very well done, and the conclusions novel and important.

Comments for the author

The authors have added new experiments and made changes to the text, clarifying and addressing the issues raised. I have no further comments on this excellent study.

Reviewer 3

Advance summary and potential significance to field

I am satisfied with the response to my comments, and I think the manuscript has greatly improved, thus I recommend its publication.

Comments for the author

The manuscript would benefit from the following minor corrections:
- The conclusion of Fig2C is still unclear to this reviewer. During the ~7 first minutes following PMA treatment, PJ Sac1 and PJ -5 ptase induce the same effect on ORP3 recruitment to the PM. Then, the kinetics seem to be different (due to a secondary event?). However, the conclusion of the authors is that PI(4,5)P2 has the most important role in ORP3 recruitment. If that was the case, the quantification of the AUC should show it (and stats too), which is not the case.
The authors should be more cautious in their conclusion: PI4P and PI(4,5)P2 seem to be both important for ORP3 recruitment (as it is stated in the summary at the end of the discussion). They should correct the abstract and the text accordingly.
- Fig 1C: please provide a lower exposure of the ORP3-Venus western blot.
- FFAT stands for “two phenylalanines in an acidic tract”. Please correct.
- FFAT is a motif and not a domain. Please correct throughout the text.
- Fig. S2: keep the same color code for PIPs (panel A vs B)
- Define PJ (Pseudojanin) in the text (and not in the legend only).
- Please document the effect of siRNAs targeting STIM1 on STIM1 protein level
(Fig. 5)
- Fig 7A, B and C: please provide statistical analyses.

Second revision

Author response to reviewers' comments

We want to thank the Reviewer for his/her attention to the revised manuscript and for his/her helpful suggestions. In response to the comments we have made the following changes:

The conclusion of Fig2C is still unclear to this reviewer. During the ~7 first minutes following PMA treatment, PJ Sac1 and PJ -5 ptase induce the same effect on ORP3 recruitment to the PM. Then, the kinetics seem to be different (due to a secondary event?). However, the conclusion of the authors is that PI(4,5)P2 has the most important role in ORP3 recruitment. If that was the case, the quantification of the AUC should show it (and stats too), which is not the case.The authors should be more cautious in their conclusion: PI4P and PI(4,5)P2 seem to be both important for ORP3 recruitment (as it is stated in the summary at the end of the discussion).
They should correct the abstract and the text accordingly.

We have changed the Abstract to reflect the involvement of both PI4P and PI(4,5)P2.

- Fig 1C: please provide a lower exposure of the ORP3-Venus western blot.

We have replaced the pictures with a lower exposure one.

- FFAT stands for “two phenylalanines in an acidic tract”. Please correct.

Thank you, it was corrected

- FFAT is a motif and not a domain. Please correct throughout the text.

Indeed. We have changed it throughout.

- Fig. S2: keep the same color code for PIPs (panel A vs B)

Colors have been matched

- Define PJ (Pseudojanin) in the text (and not in the legend only).

It has been defined in the text now.

- Please document the effect of siRNAs targeting STIM1 on STIM1 protein level (Fig. 5)

Western blots have been added showing the effects of knock-down on STIM1 levels in Fig. 5.

- Fig 7A, B and C: please provide statistical analyses.

Areas under the curves (AUC) have been calculated and statistics performed. This is now shown in Fig. 7.

Third decision letter

MS ID#: JOCES/2019/237388

MS TITLE: ORP3 Phosphorylation Regulates Phosphatidylinositol 4-phosphate and Ca2+ Dynamics at PM-ER Contact Sites

AUTHORS: Gergo Gulyas, Mira Sohn, Yeun Ju Kim, Peter Varnai, and Tamas Balla
ARTICLE TYPE: Research Article

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