Nir1 constitutively localizes at ER-PM junctions and promotes Nir2 recruitment for PIP2 homeostasis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: Manuscript #E21-07-0356
TITLE: "Nir1 constitutively localizes at ER-PM junctions and promotes Nir2 recruitment for PIP2 homeostasis"

Dear Prof. Liou:

Thank you for submitting your manuscript "Nir1 constitutively localizes at ER-PM junctions and promotes Nir2 recruitment for PIP2 homeostasis" to Molecular Biology of the Cell. I have received reports from two experts in the field that have carefully read your manuscript.

As you will see from the attached reports, both reviewers indicate that the experiments are rigorous and convincing. While one reviewer considers the findings to advance the field and to be of broad interesting, the other reviewer feels that findings are a modest advance and that there is a lack of insight into the physiological relevance and regulation of the complex. Overall, I feel that carefully addressing the concerns that have been raised would be beneficial and improve the manuscript, and all comments should be addressed in the revision. In particular, the experiment suggested by Reviewer 1 to employ a PH domain sensor that will not respond to changes in IP3 seems reasonable and useful to support the conclusions of the manuscript.

Therefore, we would be happy to consider a revised manuscript that satisfies the joint concerns of the reviewers. We look forward to receiving your revised manuscript, together with a letter indicating the changes you have made and the responses to the reviewers’ comments.

Sincerely,

James Olzmann
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Liou,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.
In this manuscript, the role of Nir1 at ER-PM contact sites during the transfer of PI to the PM and subsequent PI(4,5)P2 replenishment is explored. This work demonstrates that Nir1 constitutively localizes to ER-PM contact sites in a way that is dependent on both the FFAT domain and the LNS2 domain, but is inhibited by the addition of a PITP domain. It was then shown that Nir1 recruits Nir2 to the contact sites though a direct interaction at a previously undefined region of the protein. Finally, it was determined that the presence of Nir1 at the contact sites and its interaction with Nir2 promotes PI(4,5)P2 replenishment downstream of PLC signaling. Overall, these findings advance the field by defining a previously unknown role of Nir1 and the PITP domain in Nir2 regulation and PI(4,5)P2 homeostasis.

For the most part, this manuscript presents compelling data for this new role of Nir1 in a clear and concise manner. The experiments are described in a logical manner that other labs will be able to reproduce. The authors grasp the significance of this work without overstating or understating the advances that these results bring to the field. However, some additional work can be done to provide more robust data.

Major weaknesses include:

1) A central finding is presented in figure 4E-F, that Nir1 is required for optimal PI(4,5)P2 resynthesis. These data rely on recovery profiles of a PI(4,5)P2 biosensor to estimate the impact on lipid synthesis. However, the PH domain used is well known to report a convolution of PI(4,5)P2 and IP3 dynamics (PMID: 12771127). Thus, another interpretation of the data is possible: that enhanced PLC activity occurs in the absence of Nir1, leading to sustained IP3 production and hence reduced recovery of the PH domain probe to the membrane. To be unambiguous, this experiment must be repeated in a manner that follows PI(4,5)P2 specifically, without confounds from IP3 levels. The simplest approach would perhaps be to repeat this experiment with sensors that do not respond to the large amounts of IP3 produced (PMID 18420701, 19769794).

2) The data in Fig 3 claims that the PITP domain of the Nir proteins is autoinhibitory. While the addition of the PITP domain clearly stops Nir1 localization and ER-PM contact sites, the data provided does not indicate that the mechanism of this inhibition is through autoinhibition of the Nir1 LNS2 domain by the PITP domain, although this is clearly a possibility. An alternative could perhaps be that clearance of PA from the plasma membrane by the PITP domain removes this ligand and stops binding by the LNS2 domain. This could easily be tested by using PA-deficient mutants of the PITP, which should also more easily localize to the contact sites like the deletions. Whilst this experiment would be intriguing, to be clear, we do not feel that this is a requisite for publication - just that it would be interesting to do! The alternative possibilities should be discussed, however.

Technical Points:

1) Number of cells used. Some of the experiments (Fig 1E, 4D) use low numbers of cells for quantification. Also, figure legends indicate x% of cells displaying certain patterns, but it is unclear how many cells were actually used. For example, does 80% of cells showing a certain pattern (n = 40 cells) mean that 40/50 cells showed that pattern or that 32/40 cells displayed that pattern (Fig 2B, 2C, 3B)? The same problem arises with statements like "colocalization observed in 30 cells," how many cells in total were imaged to produce the 30 cells that showed colocalization? (Fig 2F).

2) Methods of quantification. Line scans as those used in Fig 1D, 1F, 2F are somewhat arbitrary and thus don't show as meaningful co-localization as other methods used throughout the paper such as quantification of the percent of pixel overlap. Additionally, the methods section doesn't explicitly describe how certain localization patterns were defined, so it is unclear how percentage of cells displaying that localization pattern were calculated in Fig 2B, 2C, 3B, and 3F. Elsewhere, co-localization is not quantified by either method, such as in Fig 2D, 4A, 4B, 5A, and 5E. Therefore, some consistency in the analysis of the imaging data would be beneficial.

3) Representative images selected. The image in Fig 2B does not convincingly show Nir1-ΔLNS2 co-localization with the ER marker KDEL. This would be much more convincing if the magnified image showed the individual channels along with the merge. The same applies to figure 4A/B, and 5A, E.

4) Similarly, in Fig 3F, the Nir1 constructs with the added PITP domains are said to localize in the ER and the cytoplasm, but this is difficult to tell without other markers included in the cell. Thus markers should be included, or the interpretation changed to simply reflect the fact that contact site targeting is no longer present.

5) Controls shown. In Fig 4A, it would be useful to show the data from Nir2 expressed with the vector when H1R is overexpressed in order to clearly demonstrate that Nir1 lowers the threshold of Nir2 recruitment to ER-PM sites.

6) Description of statistical tests used. The figure legend of Fig 4 does not state how statistical significance was determined in Fig 4D, 4E, and 4F.

7) The paragraph in the introduction beginning with "While vesicular transport between membranes..." (p. 3) discusses the role of the PITP domain and its role in PIP2 replenishment, yet it is not explicitly stated that this domain has been proposed to facilitate direct PI/PA exchange at contact sites. This should be clearly articulated with credit to the appropriate citations (Kim et
Reviewer #2 (Remarks to the Author):

Nir2 and Nir3 are lipid transport proteins that support PIP2 production in the plasma membrane by exchanging phosphatidic acid (PA) and phosphatidylinositol (PI) between the ER and plasma membrane at contact sites between these organelles. Nir1 is a homologue of Nir2 and Nir3 but lacks the lipid transport domain found in the other Nir protein. The role of Nir1 in PIP metabolism has been unclear. This study shows that Nir1 is constitutively located at ER-plasma membrane contacts (unlike Nir2 and Nir3). Nir1 is found to interact with Nir2 and facilitate its recruitment to ER-plasma membrane contact sites. The study is very well done and convincing, though there a few minor issues listed below. However, it is only a modest advance in our understanding how PIP2 metabolism in the plasma membrane is regulated. It is certainly interesting that Nir1 promotes Nir2 recruitment to contact sites and this study identifies the portions of Nir1 that are required, but there is no insight into the physiological relevance of the recruitment, how recruitment is regulated, or whether Nir1 supports Nir2 function in any way beyond recruitment.

Minor issues:
1. In the Introduction, explain that the PITP domain is a lipid-binding domain that facilitates lipid transport.
2. Explain why histamine affects Nir2 and Nir3 localization.
3. Provide a citation to support the statement that histamine stimulation that increases PA in the plasma membrane.
4. In Fig 1F, what percent of Nir1 puncta do not co-localize with E-Syt2 and are they at other contact sites?
This document describes the changes we have made to the manuscript and our responses to reviewers’ comments.

Changes are highlighted in Yellow in the revised manuscript for easy identification.

From Monitoring Editor:

As you will see from the attached reports, both reviewers indicate that the experiments are rigorous and convincing. While one reviewer considers the findings to advance the field and to be of broad interesting, the other reviewer feels that findings are a modest advance and that there is a lack of insight into the physiological relevance and regulation of the complex. Overall, I feel that carefully addressing the concerns that have been raised would be beneficial and improve the manuscript, and all comments should be addressed in the revision. In particular, the experiment suggested by Reviewer 1 to employ a PH domain sensor that will not respond to changes in IP3 seems reasonable and useful to support the conclusions of the manuscript.

Therefore, we would be happy to consider a revised manuscript that satisfies the joint concerns of the reviewers. We look forward to receiving your revised manuscript, together with a letter indicating the changes you have made and the responses to the reviewers’ comments.

We thank the Monitoring Editor for the suggestions on improving our manuscript. We have carefully addressed all comments raised by the reviewers by performing additional experiments and revising the manuscript according to the suggestions. In particular, we have used a biosensor that does not respond to changes in IP3 as suggested by Reviewer 1 to repeat the PIP2 replenishment experiment. The results shown in the new Supplemental Figure 3 were consistent with our previous findings and support the conclusion that Nir1 regulates PIP2 resynthesis.

Reviewer #1 (Remarks to the Author):

Major comments:
1) A central finding is presented in figure 4E-F, that Nir1 is required for optimal PI(4,5)P2 resynthesis. These data rely on recovery profiles of a PI(4,5)P2 biosensor to estimate the impact on lipid synthesis. However, the PH domain used is well known to report a convolution of PI(4,5)P2 and IP3 dynamics (PMID: 12771127). Thus, another interpretation of the data is possible: that enhanced PLC activity occurs in the absence of Nir1, leading to sustained IP3 production and hence reduced recovery of the PH domain probe to the membrane. To be unambiguous, this experiment must be repeated in a manner that follows PI(4,5)P2 specifically, without confounds from IP3 levels. The simplest approach would perhaps be to repeat this
experiment with sensors that do not respond to the large amounts of IP3 produced (PMID 18420701, 19769794).

We thank the reviewer for the suggestions on improving our manuscript. To address this concern, we have repeated the PIP2 replenishment experiment using the GFP-Tubby PIP2 sensor that does not respond to IP3 as suggested (PMID 18420701, 19769794). As shown in the new Supplemental Figure 3 of the revised manuscript, the replenishment of plasma membrane PIP2 following receptor-induced hydrolysis monitored using GFP-Tubby was suppressed in siNir1-treated HeLa cells compared with siControl-treated cells. These results are consistent with our observations using the GFP-PLCδ-PH biosensor and support the conclusion that Nir1 is important for optimal PIP2 resynthesis in receptor-stimulated cells. In addition to the new figure, we have made the following change in the revised manuscript.

- (Page 9, after Figure 4E and F) "Similar results were observed using GFP-Tubby (Supplemental Figure 3), another biosensor that binds PIP2 selectively (Quinn et al., 2008)."

2) The data in Fig 3 claims that the PITP domain of the Nir proteins is autoinhibitory. While the addition of the PITP domain clearly stops Nir1 localization and ER-PM contact sites, the data provided does not indicate that the mechanism of this inhibition is through autoinhibition of the Nir1 LNS2 domain by the PITP domain, although this is clearly a possibility. An alternative could perhaps be that clearance of PA from the plasma membrane by the PITP domain removes this ligand and stops binding by the LNS2 domain. This could easily be tested by using PA-deficient mutants of the PITP, which should also more easily localize to the contact sites like the deletions. Whilst this experiment would be intriguing, to be clear, we do not feel that this is a requisite for publication - just that it would be interesting to do! The alternative possibilities should be discussed, however.

We thank the reviewer for suggesting an alternative explanation for results shown in Figure 3. We have made the following change in the revised manuscript to include this alternative possibility.

- (Page 8, after Figure 3G) “These results suggest that the PITP domain may provide an auto-inhibitory mechanism to limit Nir2 and Nir3 targeting to ER-PM junctions. Alternatively, the PITP domain may limit targeting of Nir proteins to ER-PM junctions by clearance of PA from the PM.”

Technical Points:
1) Number of cells used. Some of the experiments (Fig 1E, 4D) use low numbers of cells for quantification. Also, figure legends indicate x% of cells display certain patterns, but it is unclear how many cells were actually used. For example, does 80% of cells showing a certain pattern (n = 40 cells) mean that 40/50 cells showed that pattern or that 32/40 cells displayed that pattern (Fig 2B, 2C, 3B)? The same problem arises with statements like "colocalization observed in > 30
cells,” how many cells in total were imaged to produce the 30 cells that showed colocalization? (Fig 2F).

We have done additional experiments to increase the number of cells in Fig 1E (increased from 7-10 cells to 15-30 cells) and Fig 4D (increased from 11-15 cells to 18-23 cells). These two figures and the corresponding figure legends have been updated in the revised manuscript to include the additional cells. The conclusions of these figures remain the same as in the original manuscript.

In addition, we have modified the figure legends that indicated x% of cells display certain patterns to specify the number of cells that were actually used in Fig 2B, 2C, 3B.

- (Fig. 2B) “Nir1-ΔLNS2-mCh displayed cytosolic and ER localization in 100% of cells (n = 58, three experiments). Nir1-FFAA-mCh displayed PM localization in 86% of cells (49 out of 57, three experiments).”
- (Fig. 2C) “Nir1-594-974-mCh enrichment at the PM was observed in 56% of cells (18 out of 32, two experiments), while the other constructs displayed cytosolic localization in 100% of cells (n = 30, two experiments).”
- (Fig. 3B) “85% of cells (34 out of 40) expressing Nir2-ΔPITP-mCh displayed diffuse localization resembling WT Nir2, while 15% of cells (6 out of 40, two experiments) displayed enrichment in puncta.”

Moreover, we have modified the legend of Figure 2F to indicate that 100% of 30 cells imaged showed colocalization.

- (Fig. 2F) “Colocalization was observed in 100% of cells (n=30, two experiments)”

Overall, the figure legends in the revised manuscript have been modified to include the exact number of cells used to obtain the results. (Fig 1B, 1C, 2E, 2F, 3C, 3F, 3G, 4A, 4B, 5A, 5E).

2) Methods of quantification. Line scans as those used in Fig 1D, 1F, 2F are somewhat arbitrary and thus don't show as meaningful co-localization as other methods used throughout the paper such as quantification of the percent of pixel overlap. Additionally, the methods section doesn't explicitly describe how certain localization patterns were defined, so it is unclear how percentage of cells displaying that localization pattern were calculated in Fig 2B, 2C, 3B, and 3F. Elsewhere, co-localization is not quantified by either method, such as in Fig 2D, 4A, 4B, 5A, and 5E. Therefore, some consistency in the analysis of the imaging data would be beneficial.

We have addressed this concern by quantifying colocalization in Fig. 1D, 1F, 2F using Pearson’s correlation coefficient which is recommended for evaluating colocalization in biological microscopy (PMID: 21209361). New quantification results are shown next to the corresponding Line Scans in Fig 1D, 1F and 2F in the revised manuscript. We have also updated the “Colocalization Analysis” section in Materials and Method of the revised manuscript.
• Pearson’s correlation coefficients between two fluorescence images were calculated using the colocalization plugin JACoP (Just another colocalization plugin) of the ImageJ software (Bolte and Cordelières, 2006).

Additionally, we have explicitly described how certain localization patterns were defined to calculate percentage of cells displaying those patterns in Fig 2B, 2C, 3B and 3F by modifying the figure legends in the revised manuscript.

• (Fig. 2B, 2C, 3B): please see our response to Technical Point #1.
• (Fig. 3F) “100% of cells expressing N2-N1-mCh or N3-N1-mCh lost puncta localization (n = 35, two experiments).”

Moreover, we have performed quantification of colocalization using Pearson’s correlation coefficient in Fig 2D. A new graph showing the quantification has been added next to the original Fig 2D.

• (Fig. 2D) “Colocalization of GFP-2x-PASS with either Nir1-594-974-mCh (mean ± SEM; n = 23) or Nir1-FFAA-mCh (mean ± SEM; n = 27) was quantified using PCC.”

Lastly, the parameter of interest in Fig 4A, 4B, 5A, and 5E is translocation/puncta formation following receptor stimulation from 0 min to 2 min. Therefore, colocalization analyses are not applicable for these figures.

3) Representative images selected. The image in Fig 2B does not convincingly show Nir1-ΔLNS2 co-localization with the ER marker KDEL. This would be much more convincing if the magnified image showed the individual channels along with the merge. The same applies to figure 4A/B, and 5A, E.

We have followed the reviewer’s suggestion to make new magnified images show the individual channels along with the merge for Figure 2B, 4A, 4B, 5A and 5E in the revised manuscript.

4) Similarly, in Fig 3F, the Nir1 constructs with the added PITP domains are said to localize in the ER and the cytoplasm, but this is difficult to tell without other markers included in the cell. Thus markers should be included, or the interpretation changed to simply reflect the fact that contact site targeting is no longer present.

We thank the reviewer for the suggestion. We have removed the sentence that stated “cytosolic and ER localizations” in the revised manuscript to simply reflect the fact that targeting to ER-PM junctions is no longer present in Fig 3F.

5) Controls shown. In Fig 4A, it would be useful to show the data from Nir2 expressed with the vector when H1R is overexpressed in order to clearly demonstrate that Nir1 lowers the threshold of Nir2 recruitment to ER-PM sites.
Nir2 recruitment to ER-PM junctions when H1R is overexpressed has been published (Chang et al. 2013, Chang and Liou 2015), described in the text right before Figure 4A, and shown in Figure 4C. Since a proper control (co-expression with the vector vs. Nir1) has been included, we argue that it is redundant to add this data in Figure 4A.

6) Description of statistical tests used. The figure legend of Fig 4 does not state how statistical significance was determined in Fig 4D, 4E, and 4F.

We have modified the legend of Figure 4 to indicate how statistical significance was determined.

- (Fig 4D) “p < 0.05, two-way ANOVA”
- (Fig 4E) “p < 0.05, two-way ANOVA”
- (Fig 4F) “p < 0.05, t-test”

7) The paragraph in the introduction beginning with "While vesicular transport between membranes..." (p. 3) discusses the role of the PITP domain and its role in PIP2 replenishment, yet it is not explicitly stated that this domain has been proposed to facilitate direct PI/PA exchange at contact sites. This should be clearly articulated with credit to the appropriate citations (Kim et al and Yadav et al, 2015).

We have modified the introduction of the revised manuscript to articulate this point with credit to the appropriate citations.

- (Page 4) “The PITP domain of Nir2 and Nir3 has been proposed to facilitate direct PI/PA exchange at ER-PM junctions (Chang and Liou, 2015; Kim et al., 2015; Yadav et al., 2015).”

Reviewer #2 (Remarks to the Author):

Nir2 and Nir3 are lipid transport proteins that support PIP2 production in the plasma membrane by exchanging phosphatidic acid (PA) and phosphatidylinositol (PI) between the ER and plasma membrane at contact sites between these organelles. Nir1 is a homologue of Nir2 and Nir3 but lacks the lipid transport domain found in the other Nir protein. The role of Nir1 in PIP metabolism has been unclear. This study shows that Nir1 is constitutively located at ER-plasma membrane contacts (unlike Nir2 and Nir3). Nir1 is found to interact with Nir2 and facilitate its recruitment to ER-plasma membrane contact sites. The study is very well done and convincing, though there a few minor issues listed below. However, it is only a modest advance in our understanding how PIP2 metabolism in the plasma membrane is regulated. It is certainly interesting that Nir1 promotes Nir2 recruitment to contact sites and this study identifies the portions of Nir1 that are required, but there is no insight into the physiological relevance of the recruitment, how recruitment is regulated, or whether Nir1 supports Nir2 function in any way beyond recruitment.
We thank the reviewer for carefully reviewing the manuscript and for recognizing that this study is very well done and convincing. To our knowledge, this study is the first to demonstrate a positive role of Nir1 in regulating PIP2 homeostasis in mammalian cells. Our study also provides mechanistic insights into this positive regulation by demonstrating that Nir1 constitutively localizes at ER-PM junctions and enables Nir2 recruitment in stimulated cells without overexpressing the receptor. These findings suggest that co-expression with Nir1 is important for Nir2 recruitment to ER-PM junctions to mediate PIP2 replenishment in receptor-stimulated cells under physiological conditions. In our opinion, this study presents findings that stimulate progress in the field. Therefore, we submitted this study to be considered as a Brief Report of Molecular Biology of the Cell.

Minor issues:

1. In the Introduction, explain that the PITP domain is a lipid-binding domain that facilitates lipid transport.

To address this point, we have added an explanation and cited a reference in the Introduction of the revised manuscript.

- (Page 3) “A class of LTPs called PI transfer proteins (PITPs) are implicated in transfer of PI through a lipid-binding PITP domain that facilitates lipid transport (Cockcroft, 2012).”

2. Explain why histamine affects Nir2 and Nir3 localization.

We thank the reviewer for pointing out that we should explain why histamine affects Nir2 and Nir3 localization. We have added the explanation in the revised manuscript.

- (Page 5) “These puncta highly resembled Nir2 and Nir3 localizations at ER-PM junctions following PIP2 hydrolysis at the PM (Chang et al., 2013; Chang and Liou, 2015; Kim et al., 2015). To explore this possibility, we coexpressed Nir1-mCh with Nir2-YFP or Nir3-YFP and the histamine H1 receptor (H1R) in HeLa cells. H1R is a G-protein (Gq) coupled receptor that activates PLC to hydrolyze PM PIP2 following histamine stimulation (Panula et al., 2015). We have previously shown that histamine stimulation can trigger Nir2 and Nir3 translocation to ER-PM junctions via PLC and diacylglycerol kinases in HeLa cells overexpressing H1R (Chang and Liou 2015).”

3. Provide a citation to support the statement that histamine stimulation that increases PA in the plasma membrane.

We thank the reviewer for pointing out a mistake we made in a statement concerning Figure 3D. We have modified the statement in the revised manuscript.
(Page 8) “In line with previous findings (Kim et al., 2015), we observed robust Nir2-ΔPITP-mCh translocation to ER-PM junctions in response to receptor histamine stimulation that hydrolyzes PM PIP2 increases PA at the PM (Figure 3D).

4. In Fig 1F, what percent of Nir1 puncta do not co-localize with E-Syt2 and are they at other contact sites?

The experiments in Figure 1F were performed using TIRF microscopy which detects fluorescent signals within ~100 nm of the PM. This approach is suitable for monitoring ER-PM junctions but not other contacts. In these experiments, 100% of Nir1 puncta colocalize with E-Syt2 puncta, although the Nir1/E-Syt2 intensity ratio varies in individual puncta. These results support our conclusion that Nir1 constitutively localizes at ER-PM junctions.
Dear Prof. Liou:

Thank you for submitting your interesting discoveries to MBoC and for your careful responses to the reviewers' comments. The additional data and discussion have further strengthened your conclusions, and I am happy to accept your manuscript for publication in MBoC.

Sincerely,
James Olzmann
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Liou:

Congratulations on the acceptance of your manuscript. A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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