The surface of lipid droplets constitutes a barrier for endoplasmic reticulum-resident integral membrane proteins

Rasha Khaddaj, Muriel Mari, Stéphanie Cottier, Fulvio Reggiori and Roger Schneiter
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MS TITLE: The surface of lipid droplets constitutes a barrier for endoplasmic reticulum residential integral membrane spanning proteins

AUTHORS: Rasha Khaddaj, Muriel Mari, Stephanie Cottier, Fulvio Reggiori, and Roger Schneiter
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. In preparing your revision, please focus on all the comments raised by reviewer #2 and aim to provide a better physiological significance of your results to address the concerns of reviewer #1.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

The reagents may have use to probe inter-organellar contacts with LDs.

Comments for the author

Khaddaj et al. describe experiments in which the ability of ER transmembrane proteins to translocate to the lipid droplet (LD) surface is tested. Mammalian PLIN3, an LD-resident protein that is thought to normally target LDs from the cytosol, is fused to two ER resident proteins with a GFP in between. In these chimeras PLIN3 is facing out. The authors show conclusively that these probes cause a zippering of the ER surface with the LD surface. This is shown by normal fluorescence microscopy, EM immuno-gold, and BiFC. It is shown in yeast cells and in human cells. In contrast, if PLIN3 is on the ER-luminal surface, it fails to bind to the droplet surface. The interesting exception is that in this case it does bind to the LD surface in the absence of FIT proteins (in yeast). Along the same lines, if PLIN3 is tethered to mitochondria, there in a clear association of mitochondria and droplets.

The results are convincing and the paper is clearly written. But to me I don't see how the results could have been otherwise. ER membrane proteins are typically associated co-translationally into the ER. The two proteins used, Wbp1 and Sec61, are part of large complexes (the oligosaccharyl transferase and the Sec61 channel, respectively) in the membrane. Considering the hydrophobic nature of the noncytosolic side of LD membranes, I cannot imagine the energetics by which these proteins would be partitioned in the LD monolayer even if they were laterally mobile. However, the PLIN3 part of the fusion proteins would be attracted to the LD surface (as it is normally from the cytosol), and the authors showed that this was indeed true. So I don't know what new is learned. For the luminal probe, as very little droplet surface area would be available to these ER probes, it is not surprising that they fail to localize, especially if their lateral mobility is limited by their participation in large complexes. I'm not sure if the FIT2Δ result signifies that the droplet is inwardly invaginating on their own in the absence of FIT2 proteins or rather that the invagination is promoted by the PLIN3 fusions once the first molecules sense a small increase in the amount of TAG exposed to the luminal side of the ER membrane.

I agree with the authors that these probes could be used to force LD contacts to test other hypotheses regarding inter-organellar communication, but this alone is not sufficient reason to justify publication here, in my opinion. But the data are of high quality, and they warrant publication somewhere.

Reviewer 2

Advance summary and potential significance to field

Khaddaj et al study the interaction of resident integral ER membrane proteins with lipid droplets (LDs). Although LDs bud from the ER, they consist of a phospholipid monolayer coating a core of neutral lipids while the ER consists of a phospholipid bilayer, raising the question of how resident ER proteins behave at the interface of these organelles. By using a set of artificial reporters that contain targeting sequences to both domains (PLIN3 for LDs and two resident integral proteins - Wbp1 and Sec61 - for the ER), the authors present evidence that ER proteins can associate with LDs not directly (ie targeting the LD surface monolayer) but instead by rearranging the ER membrane around the LDs.

This is an interesting study that addresses the pathways of ER membrane protein interactions with LDs. The experiments appear well performed and described in a clear and concise manner. In my view, a more detailed EM analysis would have benefited the study and strengthen some of the

conclusions, however the imaging experiments are overall well controlled. I have few suggestions which I believe could improve the manuscript:

Comments for the author

1. How does an unrelated resident ER membrane protein distribute in the cells carrying the Wbp1/Sec61-PLIN3 reporters? Does the overexpression of these reporters affect only the LD-ER contact sites or they have a more general effect on the overall morphology of the ER? On a relevant note, do these cells grow like wild-types (no PLIN3 tag)?

2. The EM analysis documents a strong difference between the Wbp1- and the Sec61- PLIN3 chimeras in their capacity to induce "wrapping" of ER membranes (Fig. 2), however throughout the remaining results the two fusions appear to function in a similar manner with respect to the ER repositioning. It would be helpful if the authors could clarify this.

3. I feel that the physical properties of the LDs associated with the ER reporters need to be better defined. In Fig. 1, both PLIN3-ER chimeras clearly float at the LD fractions. It is not evident to me what - biochemically - represents this strong enrichment. Does this reflect ER membrane wrapped around the LD that can still float? An unmodified ER membrane protein control would be helpful in Fig. 1G - for example what is the behavior of the endogenous Wbp1/Sec61 proteins in the same assay and cells ? Along similar lines, if FIT proteins allow the accessibility of the luminal PLIN3 reporter, is that also reflected in the flotation properties of Pmt1-GFP-PLIN3, as one would expect?

4. Although the association of Wbp1/Sec61-PLIN3 to LDs is quantitated, the LD association of the luminal Pmt1-GFP-PLIN3 in the FIT mutants is not. This is an important experiment and it should be reported.

Additional minor comments:

With respect to the reporter distribution in the FIT mutant (page 9, Fig. 5A and B) "…following the same kinetics as observed in a wild-type background". Two hours post-induction and overnight do not really constitute "kinetic" analysis, that statement needs to be modified.

The results demonstrating the requirement of FIT proteins for the access of the ER luminal reporters are based on cells fed with oleate (Fig. 5). Do the authors observe the same effect in LDs induced in the absence of oleate (i.e. stationary phase)?

Page 6: "immune-electron microscopy" should read "immuno-electron microscopy"

First revision

Author response to reviewers' comments

>We would like to thank the two referees for their helpful comments on this manuscript. These have been addressed as detailed (>in blue) below.

Reviewer 1 Advance summary and potential significance to field
The reagents may have use to probe inter-organellar contacts with LDs.

Reviewer 1 Comments for the author
Khaddaj et al. describe experiments in which the ability of ER transmembrane proteins to translocate to the lipid droplet (LD) surface is tested. Mammalian PLIN3, an LD-resident protein that is thought to normally target LDs from the cytosol, is fused to two ER resident proteins with a GFP in between. In these chimeras PLIN3 is facing out. The authors show conclusively that these probes cause a zippering of the ER surface with the LD surface. This is shown by normal fluorescence microscopy, EM immuno-gold, and BiFC. It is shown in yeast cells and in human cells.

In contrast, if PLIN3 is on the ER-luminal surface, it fails to bind to the droplet surface. The interesting exception is that in this case it does bind to the LD surface in the absence of FIT proteins (in yeast). Along the same lines, if PLIN3 is tethered to mitochondria, there in a clear association of mitochondria and droplets.

The results are convincing and the paper is clearly written. But to me I don't see how the results could have been otherwise. ER membrane proteins are typically associated co-translationally into the ER. The two proteins used, Wbp1 and Sec61, are part of large complexes (the oligosaccharyl transferase and the Sec61 channel, respectively) in the membrane. Considering the hydrophobic nature of the noncytosolic side of LD membranes, I cannot imagine the energetics by which these proteins would be partitioned in the LD monolayer even if they were laterally mobile. However, the PLIN3 part of the fusion proteins would be attracted to the LD surface (as it is normally from the cytosol), and the authors showed that this was indeed true. So I don't know what new is learned. For the luminal probe, as very little droplet surface area would be available to these ER probes, it is not surprising that they fail to localize, especially if their lateral mobility is limited by their participation in large complexes. I'm not sure if the FIT2Δ result signifies that the droplet is inwardly invaginating on their own in the absence of FIT2 proteins or rather that the invagination is promoted by the PLIN3 fusions once the first molecules sense a small increase in the amount of TAG exposed to the luminal side of the ER membrane.

I agree with the authors that these probes could be used to force LD contacts to test other hypotheses regarding inter-organellar communication, but this alone is not sufficient reason to justify publication here, in my opinion. But the data are of high quality, and they warrant publication somewhere.
>We thank the referee for his critical comments. LDs have previously been shown to emerge towards the ER lumen in the absence of FIT proteins, i.e., even in the absence of a luminal PLIN3 {Choudhary et al., 2015, J Cell Biol, 211, 261-71}. Based on these observations, we think that the luminal PLIN3 detects and reports these luminal LDs, but does not induce luminal emergence of LDs as proposed by the reviewer.
>There are data sets, based on imaging or mass spectrometry of both yeast and mammalian cells reporting the presence of integral membrane proteins on LDs. Based on these data, we think that it is valid to ask whether integral membrane proteins could actually localize to LDs. In addition, we entertained the hypothesis that LDs could be positioned in the ER lumen, not only in the FIT mutant, but possibly also in wild-type cells, which is in agreement with our previous report that ER luminal probes can detect and decorate LDs in wild-type cells {Mishra et al., 2016, J Cell Sci, 129, 3803-3815}. The present study was one way to address this hypothesis further. If LDs were luminally oriented our membrane-anchored reporters would have been expected to localize to them by lateral relocalization within the plane of the ER membrane. They do not, and for us, this is an important step forward.

Reviewer 2 Advance summary and potential significance to field
Khaddaj et al study the interaction of resident integral ER membrane proteins with lipid droplets (LDs). Although LDs bud from the ER, they consist of a phospholipid monolayer coating a core of neutral lipids while the ER consists of a phospholipid bilayer, raising the question of how resident ER proteins behave at the interface of these organelles. By using a set of artificial reporters that contain targeting sequences to both domains (PLIN3 for LDs and two resident integral proteins - Wbp1 and Sec61 - for the ER), the authors present evidence that ER proteins can associate with LDs not directly (ie targeting the LD surface monolayer) but instead by rearranging the ER membrane around the LDs.

This is an interesting study that addresses the pathways of ER membrane protein interactions with LDs. The experiments appear well performed and described in a clear and concise manner. In my view, a more detailed EM analysis would have benefited the study and strengthen some of the conclusions, however the imaging experiments are overall well controlled. I have few suggestions which I believe could improve the manuscript:
>We would like to thank this reviewer for his appreciation of this manuscript and for his constructive comments.

Reviewer 2 Comments for the author
1. How does an unrelated resident ER membrane protein distribute in the cells carrying the Wbp1/Sec61-PLIN3 reporters? Does the overexpression of these reporters affect only the LD-ER contact sites or they have a more general effect on the overall morphology of the ER? On a relevant note, do these cells grow like wild-types (no PLIN3 tag)?
>We address this point in a new supplementary figure, Fig. S3, and describe these concerns on page 12. Fig. S3A shows the localization of the ER luminal marker mCherry-HDEL in cells expressing the soluble cytosolic GFP-PLIN3 or the membrane-anchored PLIN3 grown with oleic acid and Fig. S3B,C show the localization of Wbp1-mScarlet (B) and Sec61-mScarlet (C) in cells expressing the membrane-anchored PLIN3. In the absence of oleic acid, the nuclear ER in these cells appear to be normal. In oleic acid grown cell, however, the ER strongly proliferates and shows the circular colocalization with LDs. This is specific to the expression of the membrane-anchored PLIN3 as it is not observed in cells expressing GFP-PLIN3 alone (Fig. S3A). These results are thus consistent with the BiFC data, indicating the expression of these membrane anchored reporters induce wrapping of the ER around LDs.
>Cells expressing these membrane-anchored fusion proteins grow similar to wild-type cells. This is now shown in a new supplementary figure, Fig. S1, and described on page 7.

2. The EM analysis documents a strong difference between the Wbp1- and the Sec61- PLIN3 chimeras in their capacity to induce “wrapping” of ER membranes (Fig. 2), however throughout the remaining results the two fusions appear to function in a similar manner with respect to the ER repositioning. It would be helpful if the authors could clarify this.
>It is correct that the two membrane-anchored reporters show slight differences in their interaction with LDs. This point is now discussed on page 14.

3. I feel that the physical properties of the LDs associated with the ER reporters need to be better defined. In Fig. 1, both PLIN3-ER chimeras clearly float at the LD fractions. It is not evident to me what - biochemically - represents this strong enrichment. Does this reflect ER membrane wrapped around the LD that can still float? An unmodified ER membrane protein control would be helpful in Fig. 1G - for example what is the behavior of the endogenous Wbp1/Sec61 proteins in the same assay and cells ? Along similar lines, if FIT proteins allow the accessibility of the luminal PLIN3 reporter, is that also reflected in the flotation properties of Pmt1-GFP-PLIN3, as one would expect?
>Figure 1G now contains blots for the native versions of Wbp1 and Sec61 as well and marker enrichment between cells grown with and without oleic acid is now shown and described on page 6. These data show that the LD fraction is “contaminated” with ER markers but the PLIN3-containing fusion proteins appear to be enriched to a higher degree on the floating LD fraction compared to the ER native versions of Sec61 and Wbp1.
>Figure S2B now shows that Pmt1-GFP-PLIN3 is enriched on LDs isolated from scs3Δ yft2Δ double mutants, but not wild-type cells, which is consistent with the imaging data. This is now described on page 10 of the revised version of the manuscript.
>To more precisely decipher the nature of the association of these proteins with the floating LD fraction, these fractionations experiments would have to be performed on a more quantitative level and under conditions that would possibly allow disruption of the interaction of PLIN3 with the LD surface. While this is certainly a valid point, we think that it goes beyond the scope of the present study, in which we use fractionation as a more qualitative readout to confirm that the obtained biochemical data are in agreement with the imaging results.

4. Although the association of Wbp1/Sec61-PLIN3 to LDs is quantitated, the LD association of the luminal Pmt1-GFP-PLIN3 in the FIT mutants is not. This is an important experiment and it should be reported.
>Pmt1-GFP-PLIN3 colocalizes to 81% with Erg6-mCherry in the FIT mutant, but only to 5% in wild-type cells, these data are now shown in Fig. 5 and described on page 10.

Additional minor comments:

With respect to the reporter distribution in the FIT mutant (page 9, Fig. 5A and B) “…following the same kinetics as observed in a wild-type background”. Two hours post-induction and overnight do not really constitute “kinetic” analysis, that statement needs to be modified.
>”kinetics” was replaced by time-dependence.

The results demonstrating the requirement of FIT proteins for the access of the ER luminal reporters are based on cells fed with oleate (Fig. 5). Do the authors observe the same effect in LDs induced in the absence of oleate (i.e. stationary phase)?
>The new panel A in Figure S2 now shows that Pmt1-GFP-PLIN3 colocalizes to 80% with Erg6-mCherry after overnight induction in FIT mutants grown in the absence of oleic acid.

Page 6: “immune-electron microscopy” should read “immuno-electron microscopy”
>This has been corrected on pages 7, 21, and 23 of the revised manuscript.

Second decision letter

MS ID#: JOCES/2020/256206

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AUTHORS: Rasha Khaddaj, Muriel Mari, Stephanie Cottier, Fulvio Reggiori, and Roger Schneiter
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.

Reviewer 1

Advance summary and potential significance to field

While the data are lovely and consistent and of publication quality, I still don't see a conceptual advance. Yes I agree that many proteomic studies show transmembrane proteins localizing to LDs, but there has never been convincing evidence (of which I'm aware) that show hydrophilic domains of proteins directly interacting with the LD core. So it would have been novel if the ER portion of the chimeras actually integrated into the LD monolayer, as it would be so energetically unfavorable. I am also aware of the authors' previous study of binding of LD proteins that are targeted to the luminal surface, and, of course, the paper from the Prinz lab implicating FIT2 in vectorial budding outward. The present paper seems to confirm that result.

Comments for the author

See above.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed my concerns; minor issue: typo in "Doxycycline" label in Figure S1.

Comments for the author

N/A