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Lipid droplets form a network interconnected by the endoplasmic reticulum through which their proteins equilibrate

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MS TITLE: Lipid droplets form a network interconnected by the endoplasmic reticulum through which droplet-localized proteins equilibrate

AUTHORS: Stéphanie Cottier 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.)

Thank you for submitting this interesting manuscript. As you will see, both of the reviewers agree that the manuscript addresses an important question and provides interesting new insights into lipid droplet biology. The reviewers provide succinct comments, suggestions, and requests for clarifications. I feel that addressing these comments will further strengthen this manuscript. I would be pleased to see a revised manuscript.

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 experiments described in this manuscript directly address important questions in protein trafficking: to what extent and by what mechanism to proteins equilibrate between LDs? It also reinforces the notion that LDs communicate through their connections with the ER. An unknown issue has been the extent to which seipin, which lies in the ER underneath the droplet, allows entrance and exit of droplet proteins to and from an LD. One can conclude from this study that seipin does not prevent proteins from exiting the LD surface.
While the key findings of this paper may fit with best guesses of some in the field of how the system works, it is wonderful to see these ideas directly tested and confirmed. Using a 4Δ strain to show rapid movement of Erg6 between ER and droplet fit well with the proposed model. Beyond this, the authors have convincingly shown that seipin is immobile once it is a part of the ER/LD junction. This is an additional novel and important observation. Overall, this work should move the field forward. For example, the question could now be asked: how – not whether – seipin permits protein flow out from the droplet.

Comments for the author

Cottier and Schneiter report experiments to probe movement of lipid droplet (LD) proteins between droplets.
In yeast virtually all LDs reside on the ER. To assess trafficking the authors mate cells that each contains unique markers of proteins, specifically Dga1-3mCherry and Erg6-mCitrine. The chimeric proteins are expressed only before mating using the galactose promoter to avoid the complication of new protein synthesis after mating. Cell fusion during mating is monitored with soluble CFP within one of the pair, and an ER marker is also used in some experiments. Cells are monitored below an agar pad underneath a coverslip.

First, the authors find that Dga1 and Erg6 both are found on LDs in the other cell after 40 min of fusion. Cell fractionation does not show significant protein in a post-organelle supernatant, suggesting that do not traffic through the cytosol. Time-lapse experiments showed that both proteins reach a new steady state after about 20 min post-fusion. Next, the authors compared the migration of ER (monitored with Sec63-mCherry) vs. Erg6, and found, not surprisingly, that the ER protein migrated into the opposite fused cell faster than Erg6 offering strong evidence that the ER is the conduit of protein flow. That trafficking of Dga1 and Erg6 depends on ER fusion is shown by a blockade in this process, which results in much slower LD protein trafficking. In contrast to Erg6, mitochondria migrate much more slowly into the opposite cell. Using a 4KO strain that cannot synthesize neutral lipid, the authors next show Erg6, displaced to the ER in this case, was fully functional in migrating to LDs in the mated cell, and, in fact, migrated much faster by this route. Finally, the authors find that migration is considerably slower in seipin knock-out cells. Seipin itself, does not appreciably exchange between droplets in the two cells.

The experiments described in this manuscript directly address important questions in protein trafficking: to what extent and by what mechanism to proteins equilibrate between LDs? It also reinforces the notion that LDs communicate through their connections with the ER. An unknown issue has been the extent to which seipin, which lies in the ER underneath the droplet, allows entrance and exit of droplet proteins to and from an LD. One can conclude from this study that seipin does not prevent proteins from exiting the LD surface.

While the key findings of this paper may fit with best guesses of some in the field of how the system works, it is wonderful to see these ideas directly tested and confirmed. Using a 4Δ strain to show rapid movement of Erg6 between ER and droplet fit well with the proposed model. Beyond this, the authors have convincingly shown that seipin is immobile once it is a part of the ER/LD junction. This is an additional novel and important observation. Overall, this work should move the field forward. For example, the question could now be asked: how – not whether – seipin permits protein flow out from the droplet.

There are a few questions that should be addressed assuming the editor invites a revision:

(1) Much of Dga1 initially appears in the ER in most of the experiments (such as shown in Fig. 2). How do the authors account for this in their kinetics? It seems that the calculation of the rate of movement of Dga1 from cell to partner might greatly overestimate the actual trafficking from droplet to droplet of this protein. This is less of an issue with Erg6, which is more specifically localized to droplets at the start of the experiment.

(2) Overexpression of the two proteins could generate artifacts. As Erg6 is long-lived, a control should be added to follow endogenous (but fluorescently tagged) Erg6 in this type of experiment. Cycloheximide could be added to block new synthesis, although, considering its half-life, new synthesis should be minimal during the 20-minute period measured after fusion that these cells should be followed.

(3) A control should also be provided, such as BODIPY staining, showing minimal trafficking of intact LDs from cell to cell in the minutes after fusion, to rule out this alternative trivial model for transfer of LD proteins.

(4) I am struck by the lack of apparent mixing of the two proteins on the same droplet in several figures, such as Fig 2B. The merges show clear separation of the two colors in the ring of LDs in the bottom cell. So maybe the proteins are not as freely diffusible on the LD surface as between droplets? This seems worthy of a comment.

(5) I find the data in Fig. 1C confusing. Droplets should float in the protocol, yet the LD markers are in the pellet fraction. Also, assuming there is more total protein in the supernatant (cytosol and small membranes) than in the pellet (mainly mitochondria and ER), running out equal protein masses as the authors have done underestimates the percentage of signal in the supernatant. Finally, the panel should also include a cytosolic marker. A caveat may also be warranted to indicate that this experiment measures steady-state levels, not flux.

(6) In the seipin KO experiment, did the authors see evidence of two LD populations? As some droplets separate from the ER in seipin KO cells, I would imagine that a population of droplets would not yield any if its LD proteins (if dis-attached from the ER) while another population may traffic its proteins readily if remaining attached. The kinetics the authors report could represent an average between these populations.

Reviewer 2

Advance summary and potential significance to field

This paper by Cottier and Schneiter uses a yeast mating-based fluorescent screening system to study in cell dynamics and redistribution of LD proteins.
They find that protein targeting to LDs requires seipin and connection to the ER rather than LD-LD fusion. Using time-lapse imaging they were able to measure the kinetics of LD protein redistribution. The experiments are well-controlled, and the results are adequately interpreted. The manuscript well-written and easy to follow. Experimental rationale and conclusions are sufficiently explained. Overall, the results of this study are interesting and provide new insights on LD protein in cell targeting and the role of ER-LD bridges. The story will be of interest to the LD community and suitable for publication in the current version.

Comments for the author

The manuscript provides interesting new insights into the kinetics of protein targeting between LD and ER. Few questions remain unanswered. Incorporating answers and explanations in the text would enhance the clarity of the work.

1- The authors mentioned that both marker proteins displayed similar behavior suggesting that the transfer is not protein specific. Can the authors comment on what they think determines the directionality of LD protein retargeting? In non-mating yeast, what triggers the transfer of proteins from LD to ER and vice versa?

2- Can the authors comment on the mobility of LDs between mating yeast cells?
In some instances, it appears that LD remain in one location for 30-40 minutes
(e.g. Fig 2E). Are LDs inherited during mating?

3- The no LD quadruple knock-out experiment is particularly informative. The authors mention that neutral lipid enzymes transfer from WT to LD-null cells.
Can the authors comment on the transfer on neutral lipids through the ER of mating cell?

4- How do the kinetics of Erg6 ER->ER transfer compare to Dga1 and Sec63 in the 4-deltax4-delta mating experiment?

5- In the WTx4-delta mating group, do the kinetics of ER->LD relocation coincide with the formation on new LDs in the 4-delta strain and does that explain the slower kinetics?

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 (>) below.

Reviewer 1 Comments for the author
…There are a few questions that should be addressed assuming the editor invites a revision:
>we thank this reviewer for his appreciation of these results and for his constructive criticism.

(1) Much of Dga1 initially appears in the ER in most of the experiments (such as shown in Fig. 2). How do the authors account for this in their kinetics? It seems that the calculation of the rate of movement of Dga1 from cell to partner might greatly overestimate the actual trafficking from droplet to droplet of this protein. This is less of an issue with Erg6, which is more specifically localized to droplets at the start of the experiment.
>In our kinetics, we record the decrease in fluorescence in the donor cell and the simultaneous increase in fluorescence in the acceptor half of the zygote. Thus, in matings between wild-type cells shown in Figure 2, the steady-state levels of the protein within the ER is taken into account. It is correct that the levels of Dga1 in the ER appear to be higher than that of Erg6, which we now take into account in the text on page 6. However, given that both marker proteins, Erg6 and Dga1, relocalize with similar kinetics, Dga1 being only slightly faster, the elevated ER levels of Dga1 do not appear to strongly affect the readout. This is possibly due to the fact that LD->ER relocalization is rate limiting, not ER->LD localization (Figure 6).

(2) Overexpression of the two proteins could generate artifacts. As Erg6 is long-lived, a control should be added to follow endogenous (but fluorescently tagged) Erg6 in this type of experiment. Cycloheximide could be added to block new synthesis, although, considering its half-life, new synthesis should be minimal during the 20-minute period measured after fusion that these cells should be followed.
>Transfer rates of chromosomally tagged Erg6-mCitrine are now shown in a new Figure S1A, B. They follow transfer rates of Dga1-3mCherry, but do not level off at later time points, because the synthesis cannot be repressed. This is described in the text, page 6, and the Discussion, page 14. The experiment cannot be performed in presence of cycloheximide as it blocks the mating reaction.

(3) A control should also be provided, such as BODIPY staining, showing minimal trafficking of intact LDs from cell to cell in the minutes after fusion, to rule out this alternative trivial model for transfer of LD proteins.
>We have tested different staining methods to follow the movement of LDs upon zygote formation but they do not work because they either bleach too quickly or have a too high aqueous pool to immediately label all the LDs, i.e., even those in the non-labelled mating partner. We thus provide

labelling data using the yeast perilipin homolog Pet10-mScarlet, which is a soluble LD marker that transfers to potential acceptor LDs much slower that the membrane-anchored markers Erg6 and Dga1 used throughout the study. The new Figure S1C, D shows that such Pet10-mScarlet marked LDs do not rapidly move into the acceptor half of the zygote upon fusion of the gametes. Thus, movement of LDs into the acceptor half of the zygote does not account for the observed rapid transfer of the LD resident proteins (page 7).

(4) I am struck by the lack of apparent mixing of the two proteins on the same droplet in several figures, such as Fig 2B. The merges show clear separation of the two colors in the ring of LDs in the bottom cell. So maybe the proteins are not as freely diffusible on the LD surface as between droplets? This seems worthy of a comment.
>We and other have previously shown that LD proteins can rapidly move over the LD surface using FRAP. The slight shift in colocalization of the two color channels observed in some of the magnifications is due to mechanical vibration of the instrument upon motorized switching of filter cubes for CFP/YFP/mCherry imaging. We have added a corresponding explanation in the Materials and Methods section (page 18).

(5) I find the data in Fig. 1C confusing. Droplets should float in the protocol, yet the LD markers are in the pellet fraction. Also, assuming there is more total protein in the supernatant (cytosol and small membranes) than in the pellet (mainly mitochondria and ER), running out equal protein masses as the authors have done underestimates the percentage of signal in the supernatant. Finally, the panel should also include a cytosolic marker. A caveat may also be warranted to indicate that this experiment measures steady-state levels, not flux.
>Figure 1C shows the results of a differential fractionation experiment. This is not a flotation protocol that is typically used to isolate “the floating LD fraction” from the top of a sucrose gradient. A soluble control, GAPDH, and an LD marker, Ayr1, are now included. The aim here is to show that these LD marker proteins are membrane associated, not that they biochemically fractionate and enrich with LDs.

(6) In the seipin KO experiment, did the authors see evidence of two LD populations? As some droplets separate from the ER in seipin KO cells, I would imagine that a population of droplets would not yield any if its LD proteins (if dis-attached from the ER) while another population may traffic its proteins readily if remaining attached. The kinetics the authors report could represent an average between these populations.
>Yes, the acquisition of the LD markers in seipin mutant cells is heterogenous, resulting in some LDs that clearly show the marker while others do not. Thus, as the reviewer suggests, the kinetics represent an average between these populations. This is now specified in the text, page 10/11.

Reviewer 2 Comments for the author
The manuscript provides interesting new insights into the kinetics of protein targeting between LD and ER. Few questions remain unanswered. Incorporating answers and explanations in the text would enhance the clarity of the work.
>We thank the reviewer for this appreciation of the results and for these suggestions.

1- The authors mentioned that both marker proteins displayed similar behavior, suggesting that the transfer is not protein specific. Can the authors comment on what they think determines the directionality of LD protein retargeting? In non-mating yeast, what triggers the transfer of proteins from LD to ER and vice versa?
>This is of course a very important and pertinent question that we tried to address in the discussion section of the revised manuscript, page 14/15. In non-mating cells, it is likely the shrinkage of LDs upon lipolysis that induces LD->ER relocalization. Under the mating conditions analyzed here, the cues must be different as we observe not only LD->ER relocalization but also ER->LD targeting, see Discussion page 14/15.

2- Can the authors comment on the mobility of LDs between mating yeast cells? In some instances, it appears that LD remain in one location for 30-40 minutes (e.g. Fig 2E). Are LDs inherited during mating?
>A similar concern was raised by Reviewer 1, point 3. We now use LDs marked by Pet10-mScarlet to show that LDs themselves move into the acceptor part of the zygote only slowly, i.e., in a time frame where transfer of the membrane-anchored markers is already levelling off. These new data

are now shown in Figure S1C, D. Ultimately, however, the zygote contains the LDs of both of the parental gametes.

3- The no LD quadruple knock-out experiment is particularly informative. The authors mention that neutral lipid enzymes transfer from WT to LD-null cells. Can the authors comment on the transfer on neutral lipids through the ER of mating cell?
>It is possible that neutral lipids would actually also transfer from wild-type cells into the ER of the quadruple mutants and thereby promote LD formation, we now explicitly mention this on page 10/11 of the revised version. When we stain LDs with C12-Bodipy in one of the mating partners, this fluorescent lipid analogue moves to acceptor LDs with similar kinetics to that observed for the membrane anchored protein reporters, Erg6 and Dga1.

4- How do the kinetics of Erg6 ER->ER transfer compare to Dga1 and Sec63 in the 4-deltax4-delta mating experiment?
>The ER->ER transfer rate of Sec63 is slightly slower (8.8 +/- 2.4 min, N=6) than that of Erg6 (5.6 +/- 1.6 min; see Figure 6G). We cannot compare it to Dga1, as we would need to use a catalytically dead mutant version, which by itself might affect the transfer rate.

5- In the WTx4-delta mating group, do the kinetics of ER->LD relocation coincide with the formation on new LDs in the 4-delta strain and does that explain the slower kinetics?
>The rate of ER->LD transfer is faster than that of LD->ER transfer in matings with the quadruple mutant. LD formation in the ER of the quadruple mutant occurs after approximately 15+/- 5 min of cytoplasmic mixing, i.e., at a time point where the marker proteins have mostly exchanged in matings between wild-type cells. Thus, compared to these transfer rates, the appearance of new LDs from the ER of the quadruple mutant is slow.

Second decision letter

MS ID#: JOCES/2021/258819

MS TITLE: Lipid droplets form a network interconnected by the endoplasmic reticulum through which they equilibrate their proteins

AUTHORS: StÃ©phanie Cottier 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. Thank you for submitting this very interesting lipid droplet manuscript to JCS! I am looking forward to its publication in JCS and I am sure that the community will appreciate the interesting discoveries that you report.