Cholesterol transfer via endoplasmic reticulum contacts mediates lysosome damage repair

Maja Radulovic, Eva Wenzel, Sania Gilani, Lya Katrine Holland, Alf Håkon Lystad, Santosh Phuyal, Vesa Olkkonen, Andreas Brech, Marja Jäättelä, Kenji Maeda, Camilla Raiborg, and Harald Stenmark

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Yours sincerely,

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Referee #1:

Radulovic et al studied a new mechanism by which lysosomal membrane damage is repaired in ESCRT-independent manner by the formation of contacts between the ER and the lysosome that deliver cholesterol. According to the suggested model, the VAPA/B, an ER protein previously implicated in plasma membrane ER contacts, is needed for the formation of ER-lysosome contacts formed in response to lysosomal membrane damage. ORP1L, a cholesterol-binding protein is recruited to the membrane damage site by interacting with VAPA/B followed by accumulation of cholesterol in the lysosomal membrane. The authors also found that the PtdIns 4-kinase (PI4K2A) rapidly produce PtdIns4P on the damaged lysosomal membrane, serving to recruit ORP1L and cholesterol. The authors report that OSBP, a cholesterol-PtdIns4 transporter is also recruited to the damaged lysosomal membrane and in its absence the membrane repair is inhibited leading to cell death.

Overall, this is an interesting study that provide mechanistic details to the new lysosomal membrane repair process described last month by Tan and Finkel. The authors show a detailed lipid analysis of isolated control and damaged lysosomes. They show that ORP1L-mediated cholesterol transfer is essential to the repair process and showed that its recruitment to the damaged lysosomal membrane is regulated by PI4K2A and the accumulation of PtdIns4P on these membranes. In addition, the authors present data supporting the hypothesis that similar to its role in ER-Golgi contacts, OSBP may act to transfer cholesterol from the ER to the damaged lysosomal membrane in exchange to PtdIns4P.

There are few minor issues that require the authors' attention:
1. The data presented in Fig.2, showing the contacts between the ER and the damaged lysosomes is very exciting. The authors also tested for the effect of VAP knockout and claim that it prevents such contacts (Fig. EV4). It is important that this data should be quantified (similarly to that shown in Fig. 2B) and presented as part of Fig. 2. It would also be important to verify the presence of VAP proteins in the contacts shown in figure 2.
2. The authors should challenge their model by looking for VAP proteins in their purified lysosomes obtained from cells lacking (knockdown or knockout) either PI4K2A, ORP1L and or OSBP. Alternatively, biochemical approaches should be utilized to show interaction between VAPA/B and ORP1L or OSBP.
3. In the survival assay shown in Fig. 8C the authors describe LLOMe treatments of 0-180 min. It would be more suitable to test longer treatment periods.
4. Finally, and most importantly, the authors should better describe in the Discussion section the similarities and the differences between their and Tan and Finkel reports.
EMBOJ-2022-112677, response to reviewer’s comments

Reviewer comments in black font, response in blue.

Radulovic et al studied a new mechanism by which lysosomal membrane damage is repaired in ESCRT-independent manner by the formation of contacts between the ER and the lysosome that deliver cholesterol. According to the suggested model, the VAPA/B, an ER protein previously implicated in plasma membrane ER contacts, is needed for the formation of ER-lysosome contacts formed in response to lysosomal membrane damage. ORP1L, a cholesterol-binding protein is recruited to the membrane damage site by interacting with VAPA/B followed by accumulation of cholesterol in the lysosomal membrane. The authors also found that the PtdIns 4-kinase (PI4K2A) rapidly produce PtdIns4P on the damaged lysosomal membrane, serving to recruit ORP1L and cholesterol. The authors report that OSBP, a cholesterol-PtdIns4 transporter is also recruited to the damaged lysosomal membrane and in its absence the membrane repair is inhibited leading to cell death.

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We thank the reviewer for the excellent comments, and we have now quantified the EM data for the VAP double knockout cells as suggested. Indeed, the quantifications confirmed that there is no increase in ER-lysosome contact sites in VAP double knockout cells. Because of the importance of this result, we moved the complete VAP data set to a revised Figure 2C.

Regarding the presence of VAP proteins at the ER-Lysosome contacts, we performed immunofluorescence microscopy and detected co-occurrence between LAMP1 and VAPA positive lysosomes after 10 min of LLOMe treatment:

Ideally, we would have liked to confirm this result by immunoelectron microscopy, but the lack of antibodies suited for immunoelectron microscopy precluded this.

2. The authors should challenge their model by looking for VAP proteins in their purified lysosomes obtained from cells lacking (knockdown or knockout) either PI4K2A, ORP1L and or OSBP. Alternatively, biochemical approaches should be utilized to show interaction between VAPA/B and ORP1L or OSBP.

We tested the purity of immunoprecipitated lysosomes by excluding contamination from the ER membranes (as probed by Calnexin shown in Fig EV1). Since VAP proteins are ER-resident, we did not look for them in the purified lysosomal fractions. In our model, we assume that lysosomes are in contact with the ER membrane and that this contact is mediated by VAP proteins among others. However, we have performed the suggested co-IP experiment with GFP-ORP1L and GFP-OSBP (see figure for reviewer, below). In agreement with previous publications, we detected interactions of VAPB with both ORP1L and OSBP. Because interactions between VAP proteins and ORP1L and OSBP have been demonstrated in multiple papers previously, we suggest not to include this figure in our manuscript.
3. In the survival assay shown in Fig. 8C the authors describe LLOMe treatments of 0-180 min. It would be more suitable to test longer treatment periods.

We thank the reviewer for this suggestion, but we refrained from performing longer time points since cell viability was already quite impaired in OSBP depleted cells after 3 h of LLOMe treatment. However, we did strengthen our current 3h dataset by performing more experiments to reach higher cell numbers for the quantification.

4. Finally, and most importantly, the authors should better describe in the Discussion section the similarities and the differences between their and Tan and Finkel reports.

As suggested, in the revised manuscript we have better described similarities and differences between our and Tan & Finkel’s studies (second paragraph of the Discussion). Overall, the two papers complement each other well and jointly show the importance of PI4K2A and ER-lysosome contacts in lysosome repair. Differences partially exist in use of methodology (proximity biotinylation and in vitro assays in the Finkel paper, vs lipidomics and electron microscopy in our manuscript), and also in the fact that the Finkel paper focuses on phospholipid transfer whereas our paper focuses on cholesterol transfer. Finally, the Finkel paper identified a potential role for Ca²⁺ in PI4K2A recruitment whereas our manuscript shows a role for OSBP in removal of PtdIns4P on lysosomes and demonstrates its importance for viability.
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Thank you for submitting a revised version of your manuscript. There are now only a couple of minor revisions to be made. Would you please:

- Format the manuscript as a .doc file with no figures and no track changes
- Change the Conflict of interest section title to "DISCLOSURE AND COMPETING INTERESTS STATEMENT"
- Remove the author contributions section from the manuscript and add names on the AC/CRediT section of our submission website.
- Consider adding Source Data files containing the original photographs of the few western blots shown in the manuscript, and
- Add a synopsis image and text. The image could be take from the manuscript, and the text need only be two sentences and three or four bullet points.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Use the link below to submit your revision:

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All editorial and formatting issues were resolved by the authors.
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**Corresponding Author Name:** Harald Stenmark

**Manuscript Number:** EMBOJ-2022-112677

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     - the assay(s) and method(s) used to carry out the reported observations and measurements.
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     - an explicit mention of the biological and chemical entity(ies) that are being measured.
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