TMEM41B and VMP1 are scramblases and regulate the distribution of cholesterol and phosphatidylserine

Yang Li, Yichang Wang, Ximing Du, Tizhong Zhang, Hoi Yin Mak, Sarah Hancock, Holly McEwen, Elvis Pandzic, Renee Whan, Yvette Aw, Ivan E. Lukmantara, Yiqiong Yuan, Xiuju Dong, Anthony Don, Nigel Turner, Shiqian Qi, and Hongyuan Yang

Corresponding Author(s): Hongyuan Yang, UNSW Sydney and Shiqian Qi, Sichuan University

Review Timeline:

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Dear Hongyuan,

Thank you for submitting your manuscript entitled "TMEM41B and VMP1 are scramblases and regulate the distribution of cholesterol and phosphatidylserine". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that all three reviewers found your study to be important and timely. They also raise several concerns that can be addressed without additional experimentation. Most focus on the evidence that cholesterol and PS have an altered intracellular distribution after knockdown of VMP1 or TMEM4. The most significant concerns are about use of PS sensors (Rev 1, pts 3-4, Rev 2 pts 1-3, and Rev 3 pt 1). Addressing these concerns will take a considerable additional work but, as Rev 3 states, "What either the cholesterol or PS phenotypes have to do with loss of a scramblase activity at the ER is unclear." This reviewer suggests completely removing the PS data, which is worth considering. Alternatively, the claims about PS distribution can be considerably toned down and the concerns of the reviewers acknowledged and discussed.

Rev 2 suggests additional lipidomic characterization of cells lacking VMP1 or TMEM4 and an investigation of acyl chain composition affects scramblase activity (pts 5,6). Both are good ideas but are beyond the scope of this study.

The additional evidence requested by Rev 3 to support the claims of the activities of VMP1 and TMEM4 (pt 2) would strengthen the study. If evidence is available, it would be welcomed but, if not, the issues raised should be discussed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Reports may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior
***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

William Prinz, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

This study assigns biochemical function to two proteins, Vmp1 and TMEM41B, that are involved in several critical cellular processes (i.e. LD, autophagosome, and LDL particle biogenesis). The authors use state of the art fluorescence probes to investigate lipid distribution in cells and clever in-vitro assays to derive the conclusion that Vmp1/TMEM41B are lipid scramblases. The authors also demonstrate clearly that loss of Vmp1/TMEM41B impact LD morphology in a manner independent of the protein seipin, highlighting the importance of these proteins in organelle biogenesis.
Undoubtedly, this story would be of interest to the readers of JCB; however, the manuscript contains a few major flaws that need to be expanded on or corrected prior to acceptance:

**Major points:**

1) The authors clearly demonstrate that loss of Vmp1/TMEM41B leads to accumulation of DH4 on the plasma membrane. However, this tool may not be optimal to derive the conclusion that loss of Vmp1/TMEM41B causes re-distribution of cholesterol in cells. Increase in DH4 at the PM in Vmp1/TMEM41B KD cells also seems to be accompanied by a decrease in intracellular signal. It is not clear whether lysosomes decrease their cholesterol content in the absence of Vmp1/TMEM41B or if cholesterol is moderately re-distributed to the PM. A second method that corroborates these results (i.e. organelle fractionation followed by lipid analysis) would greatly increase the strength of the paper. Also along these lines, all quantifications of intensities are plotted as intensity at the PM/intensity in the cytoplasm. The authors should also include quantifications of PM intensity and cytoplasmic intensity alone.

2) Detection of PS by the GST-2xPH biosensor is a cornerstone of this study; however, results derived from this sensor seem to directly conflict with two other biosensors used for PS (GFP-evt2PH, Lact-C2-P4Mx2) shown in figure S2A. This certainly requires elaboration from the authors on why they chose to use this probe as opposed to the previous probes, or why the results for this probe can be interpreted differently.

3) Along similar lines, there are minor discrepancies between PS readout from the GST-2xPH probe and the lipidomics data published by the authors. Figure 3F-G shows no difference between WT and siTMEM41B cells for PS readout by the GST-2xPH biosensor when cells are permeabilized with Triton X-100, but lipidomics data shows a clear decrease in total PS for siTMEM41B cells. These discrepancies need to be discussed.

4) The authors did not describe how fluorescence intensities were quantified. This is a major portion of the paper, and should be discussed in depth in the M&M section.

**Minor points:**

1) Authors report no association between seipin and TMEM41B/Vmp1, but this could be re-worded to undetectable association. It is possible that weak interactions amongst the proteins provide for negative IP results.

2) While it is clear that both loss of Vmp1/TMEM41B or overexpression of these proteins do not rescue the loss of seipin, it is not demonstrated that they function completely independent of seipin. An alternative possibility is that seipin is simply epistatic to Vmp1/TMEM41B.

3) In the images shown in Supplemental figure 1A for the Vmp1siRNA cells, there are clearly fewer PLIN3 puncta prior to oleate addition; however, this trend is not mirrored in the quantification. Is this a representative image of siVmp1? Additionally, the authors conclude TMEM41B must function separate from seipin, but they do not perform time-lapse imaging for siTMEM41B as they did for siVmp1. This data should be shown, if the authors have performed the experiments

4) It may be interesting to identify a lipid species that Vmp1/TMEM41B does not act upon. That is,
perform the in-vitro assays with NBD-sterol or NBD-FFA. This would strengthen the conclusions of the paper.

Reviewer #2 (Comments to the Authors (Required)):

In this study, Li et al. examine the roles of the related ER-localized VMP1 and TMEM41B proteins in membrane lipid distribution. Previous studies have indicated that VMP1 and TMEM41B regulate lipid droplet homeostasis and autophagy. The current study confirms their roles in lipid droplet size control (but does not examine autophagy) and further elucidates their potential molecular function as lipid scramblases. The purified proteins appear to confer robust lipid scramblase activity when reconstituted into liposomes. Lipid scramblase activities (in addition to TMEM16 family members) in the ER have been long sought. Recently, TMEM41B has been implicated in coronavirus and flavivirus egress; this may be of interest but whether TMEM41B lipid scramblase activity is involved (or not) has not been addressed in the study.

Overall, the data support a role of the TMEM41B and VMP1 proteins in the control of membrane lipid distribution. The in vitro reconstitution assays may provide a major advance in revealing their primary function as lipid scramblases. However, a few experiments are needed to provide support for the authors' conclusions. In particular, methods to monitor phosphatidylserine could be improved.

Specific comments:

1. The use of detergents and the exogenous GST-2xPH(evt2) protein to monitor phosphatidylserine may be problematic. A potential concern is that the detergents could affect membrane lipid (e.g. cholesterol) organization resulting in increased phosphatidylserine exposure (or increased transbilayer movement in intracellular organelles or even loss of integrity of these compartments). It would be better to examine the localization of a GFP-2xPH(evt) fusion expressed in intact VMP41B and VMP1 knockdown cells. This should only detect phosphatidylserine in the cytoplasmic leaflet and this is an important consideration given that the confocal microscopy approaches in the study do not discern between membrane leaflets. Ideally, one would want to examine phosphatidylserine and cholesterol bilayer distribution using freeze-fracture cryo-electron microscopy (although these are challenging experiments that require experts in this technique).

2. Since GFP-evtPH and LactC2-GFP distribution were not altered in the single KD or KO cells, it may be useful to test for additive effects in double KD cells depleted of both VMP1 and TMEM41B.

3. As an alternative control, it may be useful to examine localization of the phosphatidylserine probes (GFP-evtPH, LactC2-GFP and GFP-tagged 2xPHevt) in cells transiently expressing the mutant PTDSS1 that does not undergo product feedback inhibition.

4. Phosphatidylserine levels are measured using lipidomics. It may be informative to measure other phospholipids as well, at both the class and species levels (providing acyl chain information). First of all, this may indicate whether the VMP1 and TMEM41B proteins display acyl chain specificity. In addition, a previous study by the Zhang lab suggested that VMP1 controls (promotes) SERCA activity in the ER. Work by the Vance lab and another study by Fu et al. 2013 (as well as other studies) have shown that SERCA activity is modulated by ER membrane lipid composition (increases in the PC/PE ratio reduce SERCA activity). Having a broader profile of lipidomic data may indicate whether ER lipid composition is altered in the mutant cells.
5. Regarding acyl chain specificity, it would be informative to use liposomes with different acyl chain compositions (in either the NBD-labelled lipids or the non-labelled lipids) in the in vitro assays. The scramblase assays used POPC and POPS, but do the VMP1 and TMEM41B proteins show preference for phospholipid species that are transported to the PM or those that are retained in the ER?

6. In the Abstract, the authors state that VMP1/TMEM41B function in lipid scrambling and distribution may be required for coronavirus infection. However, it is too preliminary to conclude this. For example, the NPC1 protein is required for ebola virus entry but NPC1 cholesterol transport activity is not required. Ultimately, a mutant form of the VMP1 and/or TMEM41B impaired in scramblase activity is required to test roles in lipid droplet biogenesis, autophagy, and viral infection.

Reviewer #3 (Comments to the Authors (Required)):

The authors probe the function of two transporter-like proteins of the ER, VMP1 and TMEM41B, that have been implicated in lipid droplet biogenesis and SARS-CoV-2 infection. Through purification and biochemical reconstitution, the authors provide compelling evidence that these proteins can catalyze phospholipid scramblase activity. This part of the study was done very well and provides an important contribution to the field as the identity of ER scramblases has been long sought. Knockdown of VMP1 and TMEM41b individually alters the accessibility of genetically-encoded cholesterol probes and increases their distribution at the plasma membrane. The authors nicely show lipid droplet morphology and sterol distribution phenotypes are independent of Seipin and that VMP1/TMEM41b are acting redundantly. The authors also report an alteration in PS accessibility to a probe although I found those data much less compelling. What either the cholesterol or PS phenotypes have to do with loss of a scramblase activity at the ER is unclear. The study also lacks evidence that VMP1 and TMEM41b catalyze scramblase activity within the native membrane environment, which can be quite challenging to do if other scramblases exist in the ER.

Primary concerns.

1. Figure 3 shows that fixed and digitonin or saponin permeabilized knockdown cells display increased staining of GST-2X-PH (PS sensor) relative to control cells. However, PS levels are unchanged and this difference goes away when fixed cells are treated with Triton X-100. The authors suggest the Triton is exposing a luminal pool (undefined organelle) of PS that is unavailable in the digitonin/saponin-treated cells. However, Figure 3A and B is showing probe staining of the plasma membrane and if the cells have been permeabilized, the probe should have access to both leaflets in all three detergents. Figure 2 shows an alteration of cholesterol availability at the plasma membrane, which should impact the ability of digitonin and saponin to permeabilize the plasma membrane (and possibly other membranes). Thus, the basis of the experiment is flawed as the action of the detergent on control and siRNA-treated cells may not be the same. Why the digitonin-treated cells look so different from the saponin-treated cells is also unclear. Even if these phenotypes are biologically meaningful, they don't provide a clear link to ER scramblase activity. My suggestion is that the authors should remove figure 3 and any mention of alterations of PS distribution from the other sections of the manuscript.

2. The authors have outstanding biochemical data supporting the scramblase activity of VMP1 and TMEM41B. Ideally, one would like to see some support for this activity in the native membrane
environment. The authors have knocked out VMP1 in HeLa cells - would it be possible to knockdown TMEM41B in this background and image the ER? One might expect ER morphological changes if lipid synthesis continued on the cytosolic face of the ER but failed to cross the bilayer efficiently to populate the luminal leaflet. A more direct assay would be to measure rates of flip-flop in ER microsomes from WT and double knockout/down cells.
Point-by-point Answers

Reviewer #1 (Comments to the Authors (Required):

This study assigns biochemical function to two proteins, Vmp1 and TMEM41B, that are involved in several critical cellular processes (i.e. LD, autophagosome, and LDL particle biogenesis). The authors use state of the art fluorescence probes to investigate lipid distribution in cells and clever in-vitro assays to derive the conclusion that Vmp1/TMEM41B are lipid scramblases. The authors also demonstrate clearly that loss of Vmp1/TMEM41B impact LD morphology in a manner independent of the protein seipin, highlighting the importance of these proteins in organelle biogenesis.

Undoubtedly, this story would be of interest to the readers of JCB; however, the manuscript contains a few major flaws that need to be expanded on or corrected prior to acceptance:

Answer: Many thanks for recognizing the importance of this work.

Major points:

1) The authors clearly demonstrate that loss of Vmp1/TMEM41B leads to accumulation of DH4 on the plasma membrane. However, this tool may not be optimal to derive the conclusion that loss of Vmp1/TMEM41B causes re-distribution of cholesterol in cells. Increase in DH4 at the PM in Vmp1/TMEM41B KD cells also seems to be accompanied by a decrease in intracellular signal. It is not clear whether lysosomes decrease their cholesterol content in the absence of Vmp1/TMEM41B or if cholesterol is moderately re-distributed to the PM. A second method that corroborates these results (i.e. organelle fractionation followed by lipid analysis) would greatly increase the strength of the paper. Also along these lines, all quantifications of intensities are plotted as intensity at the PM/intensity in the cytoplasm. The authors should also include quantifications of PM intensity and cytoplasmic intensity alone.

Answer: Thanks for the suggestion. We used two sensors (D4H and GRAM-G187L) to show there are more accessible cholesterol on the plasma membrane (PM) of the knockdown /knockout cells. Please note these live sensors are NOT quantitative, and the increased signal of the sensors does not necessarily mean there is an increase in the absolute amount of cholesterol in the PM. Rather, the results suggest that there could be altered distribution of cholesterol within the PM, e.g., the pool of cholesterol sequestered by sphingomyelin may redistribute to the pool accessible to D4H/PFO in TMEM41B/VMP1-deficient cells (please see Das et al. eLife 2014; 3: e02882). This redistribution of cholesterol may well result from the changes in the phospholipid content due to the loss of VMP1/TMEM41B. Likewise, the reduction of sensors in the lysosomes does not indicate a decrease of lysosomal cholesterol. Rather, there could be more cholesterol in the PM that is accessible to the sensors than that in the lysosomes of VMP1/TMEM41B deficient cells. In our manuscript, we did not claim there is more PM cholesterol or less lysosomal cholesterol in the TMEM41B/VMP1-deficient cells. This point has now been made clearer in the discussion. Future work will determine whether there are changes in the absolute amount of organelar cholesterol upon depleting TMEM41B/VMP1.

2) Detection of PS by the GST-2xPH biosensor is a cornerstone of this study; however,
results derived from this sensor seem to directly conflict with two other biosensors used for PS (GFP-evt2PH, Lact-C2-P4Mx2) shown in figure S2A. This certainly requires elaboration from the authors on why they chose to use this probe as opposed to the previous probes, or why the results for this probe can be interpreted differently.

Answer: Our data (Fig. S2G-I) and those from Tsuji et al (PNAS, 2019) have proven that GST-2xPH is a highly specific sensor for PS. It should be noted that GST-2xPH detect PS in fixed and permeabilized cells, whereas GFP evt2PH and LactC2 detect PS in live cells. We had hoped that the results from all three PS sensors would be consistent, but we must report what we observed. At this time, we cannot explain the differences between purified and live PS sensors. There could be many compounding factors in live cells that affect the sensitivity and specificity of the live PS sensors (see Tsuji et al, PNAS, 2019). We are, however, very confident with the data using GST-2xPH. We also believe that reporting these differences would stimulate further research on PS detection. We have included some explanations and pointed out the deficiencies of our current work in the discussion.

3) Along similar lines, there are minor discrepancies between PS readout from the GST-2xPH probe and the lipidomics data published by the authors. Figure 3F-G shows no difference between WT and siTMEM41B cells for PS readout by the GST-2xPH biosensor when cells are permeabilized with Triton X-100, but lipidomics data shows a clear decrease in total PS for siTMEM41B cells. These discrepancies need to be discussed.

Answer: The decrease in PS by lipidomic analyses is only borderline significant, and only in TMEM41B-deficient cells. The key point here is that there is no increase in total PS, which suggests a possible distribution issue.

4) The authors did not describe how fluorescence intensities were quantified. This is a major portion of the paper and should be discussed in depth in the M&M section.

Answer: Thanks for the suggestion. We have now included the method for quantitation in materials and methods.

Minor points:

1) Authors report no association between seipin and TMEM41B/Vmp1, but this could be reworded to undetectable association. It is possible that weak interactions amongst the proteins provide for negative IP results.

Answer: Done. Thanks.

2) While it is clear that both loss of Vmp1/TMEM41B or overexpression of these proteins do not rescue the loss of seipin, it is not demonstrated that they function completely independent of seipin. An alternative possibility is that seipin is simply epistatic to Vmp1/TMEM41B.

Answer: Thanks. We have modified relevant text.

3) In the images shown in Supplemental figure 1A for the Vmp1siRNA cells, there are clearly fewer PLIN3 puncta prior to olate addition; however, this trend is not mirrored in the quantification. Is this a representative image of siVmp1? Additionally, the authors conclude
TMEM41B must function separate from seipin, but they do not perform time-lapse imaging for siTMEM41B as they did for siVmp1. This data should be shown, if the authors have performed the experiments.

**Answer:** There appears to be some confusion here. The weak puncta in siCtrl cells are likely to be background noise, but not real PLIN3 puncta. The number of bright PLIN3 puncta is very similar between control and siVMP1 cells. We have not performed this experiment for siTMEM41B, although we expect similar results, given the similar impacts on mature LDs, cholesterol and PS distribution.

4) It may be interesting to identify a lipid species that Vmp1/TMEM41B does not act upon. That is, perform the in-vitro assays with NBD-sterol or NBD-FFA. This would strengthen the conclusions of the paper.

**Answer:** Thanks. There are many more nice controls we can perform, but we think our current data on scramblase activity are solid, as noted by all reviewers.

Reviewer #2 (Comments to the Authors (Required)):

In this study, Li et al. examine the roles of the related ER-localized VMP1 and TMEM41B proteins in membrane lipid distribution. Previous studies have indicated that VMP1 and TMEM41B regulate lipid droplet homeostasis and autophagy. The current study confirms their roles in lipid droplet size control (but does not examine autophagy) and further elucidates their potential molecular function as lipid scramblases. The purified proteins appear to confer robust lipid scramblase activity when reconstituted into liposomes. Lipid scramblase activities (in addition to TMEM16 family members) in the ER have been long sought. Recently, TMEM41B has been implicated in coronavirus and flavivirus egress; this may be of interest but whether TMEM41B lipid scramblase activity is involved (or not) has not been addressed in the study.

Overall, the data support a role of the TMEM41B and VMP1 proteins in the control of membrane lipid distribution. The in vitro reconstitution assays may provide a major advance in revealing their primary function as lipid scramblases. However, a few experiments are needed to provide support for the authors’ conclusions. In particular, methods to monitor phosphatidylserine could be improved.

**Answer:** Thanks for recognizing the importance of this work.

Specific comments:

1. The use of detergents and the exogenous GST-2xPH(evt2) protein to monitor phosphatidylserine may be problematic. A potential concern is that the detergents could affect membrane lipid (e.g. cholesterol) organization resulting in increased phosphatidylserine exposure (or increased transbilayer movement in intracellular organelles or even loss of integrity of these compartments). It would be better to examine the localization of a GFP-2xPH(evt) fusion expressed in intact VMP41B and VMP1 knockdown cells. This should only detect phosphatidylserine in the cytoplasmic leaflet and this is an important consideration given that the confocal microscopy approaches in the study do not discern between membrane leaflets. Ideally, one would want to examine phosphatidylserine and cholesterol bilayer distribution using freeze-fracture cryo-electron microscopy (although
these are challenging experiments that require experts in this technique).

**Answer:** This is debatable. In fact, it could be argued that using purified probes on fixed/permeabilized cells is a better approach. This is because many factors in live cells may affect the sensitivity and specificity of the live PS sensors (see Tsuji et al, PNAS, 2019). Moreover, for detecting cholesterol, purified probes (ALOD4/PLO) on fixed/permeabilized cells are considered more quantitative than live sensors (see Trinh et al, PNAS, 2020; Das et al. *eLife* 2014; 3: e02882; both cited in our manuscript). We had hoped that the results from all three PS sensors would be consistent, but we must report what we observed. At this time, we cannot explain the different results between purified and live PS sensors. We are, however, very confident with the sensitivity and specificity of GST-2xPH: see Fig. S2G-I and Tsuji et al, PNAS, 2019. We also believe that reporting these differences would stimulate further research on PS detection. We have included some explanations in the discussion.

2. Since GFP-evtPH and LactC2-GFP distribution were not altered in the single KD or KO cells, it may be useful to test for additive effects in double KD cells depleted of both VMP1 and TMEM41B.

**Answer:** Thanks. We did the experiments but there is still no change in GFP-evtPH or LactC2-GFP distribution in the double knockdown cells.

3. As an alternative control, it may be useful to examine localization of the phosphatidylserine probes (GFP-evtPH, LactC2-GFP and GFP-tagged 2xPHevt) in cells transiently expressing the mutant PTDSS1 that does not undergo product feedback inhibition.

**Answer:** Thanks. This is a nice control only for the specificity of the live probes. It will not help this study.

4. Phosphatidylerine levels are measured using lipidomics. It may be informative to measure other phospholipids as well, at both the class and species levels (providing acyl chain information). First of all, this may indicate whether the VMP1 and TMEM41B proteins display acyl chain specificity. In addition, a previous study by the Zhang lab suggested that VMP1 controls (promotes) SERCA activity in the ER. Work by the Vance lab and another study by Fu et al. 2013 (as well as other studies) have shown that SERCA activity is modulated by ER membrane lipid composition (increases in the PC/PE ratio reduce SERCA activity). Having a broader profile of lipidomic data may indicate whether ER lipid composition is altered in the mutant cells.

**Answer:** Thanks for the suggestion. This will be investigated in future work as indicated by the editors.

5. Regarding acyl chain specificity, it would be informative to use liposomes with different acyl chain compositions (in either the NBD-labelled lipids or the non-labelled lipids) in the in vitro assays. The scramblase assays used POPC and POPS, but do the VMP1 and TMEM41B proteins show preference for phospholipid species that are transported to the PM or those that are retained in the ER?
Answer: Thanks for the suggestion. This will be investigated in future work as indicated by the editors.

6. In the Abstract, the authors state that VMP1/TMEM41B function in lipid scrambling and distribution may be required for coronavirus infection. However, it is too preliminary to conclude this. For example, the NPC1 protein is required for ebola virus entry but NPC1 cholesterol transport activity is not required. Ultimately, a mutant form of the VMP1 and/or TMEM41B impaired in scramblase activity is required to test roles in lipid droplet biogenesis, autophagy, and viral infection.

Answer: Agreed, and that is why we used the term “may be”.

Reviewer #3 (Comments to the Authors (Required)):

The authors probe the function of two transporter-like proteins of the ER, VMP1 and TMEM41B, that have been implicated in lipid droplet biogenesis and SARS-CoV-2 infection. Through purification and biochemical reconstitution, the authors provide compelling evidence that these proteins can catalyze phospholipid scramblase activity. This part of the study was done very well and provides an important contribution to the field as the identity of ER scramblases has been long sought. Knockdown of VMP1 and TMEM41b individually alters the accessibility of genetically-encoded cholesterol probes and increases their distribution at the plasma membrane. The authors nicely show lipid droplet morphology and sterol distribution phenotypes are independent of Seipin and that VMP1/TMEM41b are acting redundantly. The authors also report an alteration in PS accessibility to a probe although I found those data much less compelling. What either the cholesterol or PS phenotypes have to do with loss of a scramblase activity at the ER is unclear. The study also lacks evidence that VMP1 and TMEM41b catalyze scramblase activity within the native membrane environment, which can be quite challenging to do if other scramblases exist in the ER.

Answer: Many thanks for recognizing the importance of the work.

Primary concerns.

1. Figure 3 shows that fixed and digitonin or saponin permeabilized knockdown cells display increased staining of GST-2X-PH (PS sensor) relative to control cells. However, PS levels are unchanged and this difference goes away when fixed cells are treated with Triton X-100. The authors suggest the Triton is exposing a luminal pool (undefined organelle) of PS that is unavailable in the digitonin/saponin-treated cells. However, Figure 3A and B is showing probe staining of the plasma membrane and if the cells have been permeabilized, the probe should have access to both leaflets in all three detergents. Figure 2 shows an alteration of cholesterol availability at the plasma membrane, which should impact the ability of digitonin and saponin to permeabilize the plasma membrane (and possibly other membranes). Thus, the basis of the experiment is flawed as the action of the detergent on control and siRNA-treated cells may not be the same. Why the digitonin-treated cells look so different from the saponin-treated cells is also unclear. Even if these phenotypes are biologically meaningful, they don't provide a clear link to ER scramblase activity. My suggestion is that the authors should remove figure 3 and any mention of alterations of PS distribution from the other sections of the manuscript.
**Answer:** The likelihood that cholesterol may somewhat impact the effect of the detergents in knockdown cells is very small. It should be noted that the increased signal of cholesterol sensors at the plasma membrane (PM) in the KD/KO cells does not necessarily mean there is more cholesterol on the PM, since these live sensors are not quantitative (see our answer to Point 1 by reviewer 1). It is highly likely that there is no change in total PM cholesterol in the KD/KO cells, but rather some redistribution of cholesterol between the three pools of PM cholesterol.

As for detergents, it is well known that low level of digitonin or saponin only disrupts the integrity of the plasma membrane but leaving other internal organelles intact. Please see the classic JCB paper by Bill Balch and colleagues (Plutner et al, JCB, 1992) in which the authors demonstrated that digitonin only selectively disrupted the plasma membrane and VSVG protein can still be transported from the ER to the Golgi in those cells. Please also see Ohsaki et al, Histochem Cell Biol, 2005. Therefore, under the conditions we used, GST-2xPH cannot access the luminal side of the ER, Golgi etc. The argument that “if the cells have been permeabilized, the probe should have access to both leaflets in all three detergents” is incorrect.

Overall, based on our own observation and those of others (Tsuji et al, PNAS, 2019), we are confident with the GST 2xPH data. Moreover, we disagree with this reviewer’s comment “Even if these phenotypes are biologically meaningful, they don't provide a clear link to ER scramblase activity”. PS is made in the ER but the PS on the cytoplasmic side of the ER is actively transported to other organelles by lipid transfer proteins such as ORP5. Moreover, vesicular trafficking from the ER will impact the distribution of PS in other organelles. Therefore, the scramblase activity at the ER will impact the overall PS distribution in all organelles. In fact, the change in cholesterol distribution most likely is a secondary effect of altered PS distribution (see discussion). We have therefore decided to retain the PS data.

Nevertheless, we have added a paragraph in the discussion to point out the weakness/deficiencies of our data and future directions.

2. The authors have outstanding biochemical data supporting the scramblase activity of VMP1 and TMEM41B. Ideally, one would like to see some support for this activity in the native membrane environment. The authors have knocked out VMP1 in HeLa cells - would it be possible to knockdown TMEM41B in this background and image the ER? One might expect ER morphological changes if lipid synthesis continued on the cytosolic face of the ER but failed to cross the bilayer efficiently to populate the luminal leaflet. A more direct assay would be to measure rates of flip-flop in ER microsomes from WT and double knockout/down cells.

**Answer:** These are nice suggestions for future investigations. Measuring the rates of lipid flip-flop in purified microsomes can be very challenging.
April 13, 2021

RE: JCB Manuscript #202103105R

Prof. Hongyuan Yang
UNSW Sydney
School of Biotechnology and Biomolecular Sciences, University of New South Wales
Sydney 2052
Australia

Dear Prof. Yang:

Thank you for submitting your revised manuscript entitled "TMEM41B and VMP1 are scramblases and regulate the distribution of cholesterol and phosphatidylserine". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."
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   b. Type, magnification, and numerical aperture of the objective lenses
   c. Temperature
   d. Imaging medium
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