JCB

CRISPR screens for lipid regulators reveal a role for ER-bound SNX13 in lysosomal cholesterol export

Albert Lu, Frank Hsieh, Bikal Sharma, Sydney Vaughn, Carlos Enrich, and Suzanne Pfeffer

Corresponding Author(s): Suzanne Pfeffer, Stanford University School of Medicine and Albert Lu, University of Barcelona

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June 15, 2021

Re: JCB manuscript #202105060

Dr. Suzanne R. Pfeffer
Stanford University School of Medicine
Department of Biochemistry
279 Campus Drive
Stanford, CA 94305-5307

Dear Dr. Pfeffer,

Thank you for submitting your manuscript entitled "CRISPR screens for lipid regulators reveal a role for ER-bound SNX13 in lysosomal cholesterol export". 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.

We find that a complete mechanistic investigation into the role of SNX13 is outside of the scope of the current study. However, we agree that a deeper analysis of SNX13 loss of function phenotypes as suggested by reviewer 2 needs to be performed. In particular, expanding your proposed model that SNX13 may act via enhanced formation of membrane contact sites would provide an interesting foundation for future in depth studies. In addition, we expect that you will be able to address all of the technical questions including those regarding measurement of cholesterol in your revised manuscript.

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 an Article is < 40,000, 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: Articles may have up to 10 main text figures. 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 to publication.

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. Articles may have up to 5 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,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Andrea L. Marat, Ph.D.
Senior Scientific Editor

Journal of Cell Biology

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

This paper describes a broad CRISPR knockout study of genes that are associated with changes in cholesterol distribution in cells and with the abundance of a late endosomal lipid, BMP. The results provide an abundance of targets for future investigation, and as discussed in the paper, they provide complementary information to other related screens. In general the studies were well-executed and analyzed appropriately, but there were some methodological issues that will be discussed in detail below. There was an examination of one hit from the screen, SNX13, and the effects of changes in expression of this protein were analyzed in some detail. In the end, though, even for this protein its actual role in transport of cholesterol or in the abundance of BMP is not made clear. An overall issue is that the screen identified a large number of genes, including transcriptional regulators, RNA-editing proteins, etc. While this is a valuable contribution that can serve as a resource for future investigations, it is hard to know which of these are primary effectors of cholesterol transport as compared to effects on general cell physiology.

Specific comments.

1. The measurements of stored cholesterol depend on binding of labeled PFO* to cholesterol in cells that have been permeabilized with a cholesterol-binding detergent, and then the labeled cells are stored at low temperature to 16 hours. There should be some validation of this method - either by more direct cholesterol measurements or by reference to previous validation studies.

2. In Figure 6, the measurements of area and number of fluorescent vesicles does not seem like an ideal measurement. Why not just measure the total fluorescence per cell, perhaps after thresholding to identify vesicles? The size of dots can be very sensitive to the threshold value used.

3. In Figure 8, they could stain lipid droplets without permeabilization that was required for immunofluorescence.

4. I don't understand the discussion on page 13. In U18-treated cells in Figure 2, knockout of SNX13 decreased both BMP and cholesterol. Nevertheless, the text states that increased BMP may facilitate cholesterol efflux in NPC1-deficient cells. It seems possible, for example, that as with other lipids cholesterol and BMP increase or decrease together, and reducing one reduces the other.

5. There is no firm conclusion about what role SNX13 plays - either directly or indirectly - on the distribution of cholesterol in cells.

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

This study uses several CRISPR-based screening approaches to reveal genes that positively and negatively regulate cholesterol and BMP efflux from lysosomes in the presence and absence of NPC1 function. They focus on one hit in particular, SNX13, a poorly characterized ER-localized protein, and show that SNX13 loss partially rescues the cholesterol accumulation in lysosomes when NPC1 activity is blocked.

The study utilizes an elegant screening approach, and identifies numerous unexpected and potentially exciting new genes associated with cholesterol and BMP intracellular trafficking. The BMP associated hits are particularly interesting since very little is known about this lipid and its trafficking regulation. The study appears rigorous and well conducted, and the experiments are conclusive and well quantified.

However, whereas the first half of the study focusing on the screen is thorough, the last section dealing with investigating the nature of the hits is less well developed. SNX13-KO/KD is shown to reduce cholesterol accumulation in U18666A treated cells. It is also shown that these cells accumulate cholesterol at their plasma membrane. Lipid droplets are also shown to accumulate in SNX13-KD +U18666A cells. Finally, SNX13 KD cells also have increased BMP pools at their lysosomes. As presented, these are mostly descriptive observations at present that lack an explanation. Experiments that further dissect the role of SNX13 in cholesterol/BMP efflux, or at least explain how SNX13 loss may promote a re-wiring of cholesterol sub-cellular distribution, will

significantly enhance the study. Specific suggestions below:

1) SNX13 associates with lipid droplets with OA, and SNX13 siRNA is correlated with increased TAG and fatty acids (Fig 8). Is this TAG increase due to increased DGAT1/2 enzyme abundance or activity? Alternatively is there more lipid uptake or de novo lipid synthesis in SNX13 siRNA cells?

2) It is notable that free cholesterol accumulates at the surface of SNX13 siRNA cells treated with U18666A. Do these cells have increased de novo cholesterol synthesis? Alternatively, is trafficking of cholesterol to the PM increased or altered in some way? ER-PM contact sites are major sites of cholesterol transport. Are these sites altered in SNX13 siRNA?

3) SNX14 and SNX13 are paralogs, and both are detected in the screening as decreasing lysosomal cholesterol in U18666A treated cells. It is unclear what their relationship is to one another. Does over-expression of one paralog rescue the loss of the other?

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

This study uses CRISPR screens to identify genes that alter levels of cholesterol and bis(monoacylglycero)phosphate (BMP) in lysosomes. The screens were also performed after cells were treated with the NPC1-inhibitor U18666A. Genes that participate in many cellular processes were found. The second half of the study focuses on SNX13, one of the genes that was found to decrease both cholesterol and BMP in lysosomes after U18666A treatment. Depletion of SNX13 after U18666A treatment is shown to increase cholesterol in the plasma membrane and increase levels of TAG and, as a result, the number of lipid droplets. While these are interesting findings, their significance is considerably oversold. Here are my major concerns.

1. The finding that knockdown of SNX13 significantly reduces lysosomal cholesterol accumulation following NCP1 inhibition is an important finding. However, there is no mechanistic insight into how occurs. A complete understanding is beyond the scope of this study, but some insight seems necessary for this to be appropriate for JCB.

2. Claims about cholesterol distribution in cells are based only on PFO*. Some of the most important findings should be verified with other methods and, if possible, by direct measurement of cholesterol levels in cellular compartments.

3. The CRISPER screens are well designed, but the results are rather disappointing and oversold. The authors may want to consider a more balanced discussion of the significance of their findings and, in general, the usefulness of this type of screen for investigating lipid trafficking and metabolism. Aside from the SNX13 findings, the results of the screens are only a modest advance in our understanding of intracellular cholesterol trafficking and BMP metabolism. What do the screens tell us about these processes that we did not know already? The results of the screen are also not presented in a way that is terribly useful. Figs. 4 and 5 are overwhelming and difficult to make sense of.

Thank you for submitting your manuscript entitled "CRISPR screens for lipid regulators reveal a role for ER-bound SNX13 in lysosomal cholesterol export". 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.

We find that a complete mechanistic investigation into the role of SNX13 is outside of the scope of the current study. However, we agree that a deeper analysis of SNX13 loss of function phenotypes as suggested by reviewer 2 needs to be performed. In particular, expanding your proposed model that SNX13 may act via enhanced formation of membrane contact sites would provide an interesting foundation for future in depth studies. In addition, we expect that you will be able to address all of the technical questions including those regarding measurement of cholesterol in your revised manuscript.

Thank you for the opportunity to revise this manuscript. As requested we have now used live cell microscopy to visualize and quantify the ability of SNX13 to maintain contact with lipid droplets (Supp. Movie #1) and to stabilize ER-lipid endolysosome membrane contacts (new Figure 9 and Supp. Movies #2 and #3). We have also improved the manuscript with additional data including direct cholesterol determination as requested and discussed below.

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

This paper describes a broad CRISPR knockout study of genes that are associated with changes in cholesterol distribution in cells and with the abundance of a late endosomal lipid, BMP. The results provide an abundance of targets for future investigation, and as discussed in the paper, they provide complementary information to other related screens. In general the studies were well-executed and analyzed appropriately, but there were some methodological issues that will be discussed in detail below. There was an examination of one hit from the screen, SNX13, and the effects of changes in expression of this protein were analyzed in some detail. In the end, though, even for this protein its actual role in transport of cholesterol or in the abundance of BMP is not made clear. An overall issue is that the screen identified a large number of genes, including transcriptional regulators, RNA-editing proteins, etc. While this is a valuable contribution that can serve as a resource for future investigations, it is hard to know which of these are primary effectors of cholesterol transport as compared to effects on general cell physiology.

Thank you for the positive comments regarding the execution and analysis of our study. We also appreciate the reviewer's general concerns and add new data here showing the SNX13 is a membrane contact site protein that has unexpected effects on accessible cholesterol distribution in cells lacking NPC1 function.

We have performed new experiments (new Fig. 9) to gain insight into a possible role for SNX13 in the regulation of intracellular cholesterol trafficking by stabilizing membrane contact sites. We have also extended our discussion focusing on screen hits that may play a direct role in cholesterol transport. We agree that hits derived from any genetic screening approach can always be indirectly related to the cellular process being studied, but screens can provide important new leads for future discovery.

Specific comments.

1. The measurements of stored cholesterol depend on binding of labeled PFO* to cholesterol in cells that have been permeabilized with a cholesterol-binding detergent, and then the labeled cells are stored at low temperature to 16 hours. There should be some validation of this method - either by more direct cholesterol measurements or by reference to previous validation studies.

We agree with the reviewer that PFO* does not measure total cholesterol, but rather detects accessible cholesterol. As requested, we have now carried out direct biochemical determination of total cellular cholesterol and find that SNX13 knockdown with U18666A treatment does not change the total cholesterol, rather it's distribution in cells--i.e. we see increases in accessible cholesterol at the plasma membrane and decreases in accessible cholesterol in lysosomes. We have included a new panel (Fig. 8D) and edited our manuscript accordingly. This was an important control and clarifying the text throughout to refer to accessible cholesterol was also important.

As for the PFO* staining protocol used in our "cholesterol" screens, the staining of two cultures (Control and U18666A-treated), each consisting of 600 million cells, required a full day of work. The next day cells were sorted. For that reason, we kept labeled cells in the cold for 16h prior sorting. It is important to keep in mind that sorted labeled cells were previously fixed in paraformaldehyde. For our microscopy studies we did not keep cells overnight: Control or U18666A-treated cells were fixed, permeabilized (or not; for PM cholesterol analysis), stained and mounted before microscopy inspection. We have clarified the methods section accordingly.

2. In Figure 6, the measurements of area and number of fluorescent vesicles does not seem like an ideal measurement. Why not just measure the total fluorescence per cell, perhaps after thresholding to identify vesicles? The size of dots can be very sensitive to the threshold value used.
Thank you for this suggestion--we agree--and now include total fluorescence over vesicles, the compartment in question. In permeabilized cells (Fig. 5), our PFO-fluorescence measurements on vesicles show a decreased PFO*-cholesterol accumulation in endolysosomes (Fig. 5F, K, Fig. S4C); in non-permeabilized cells (Fig. 7, Fig. S4D-F) we show increased PFO* fluorescence intensity at the plasma membrane.

3. In Figure 8, they could stain lipid droplets without permeabilization that was required for immunofluorescence.

The reason we permeabilized cells in Figure 7A was not only to show that LDs increase in SNX13-depleted cells, but also to further confirm that these cells do not accumulate cholesterol in endolysosomes (stained with PFO). We agree that we could stain lipid droplets in non-permeabilized cells, but we would not expect major changes between these versus permeabilized cells.

4. I don't understand the discussion on page 13. In U18-treated cells in Figure 2, knockout of SNX13 decreased both BMP and cholesterol. Nevertheless, the text states that increased BMP may facilitate cholesterol efflux in NPC1-deficient cells. It seems possible, for example, that as

with other lipids cholesterol and BMP increase or decrease together, and reducing one reduces the other.

We agree with the reviewer, and validations sometimes give unexpected results. CRISPR and siRNAs do not always give the same phenotypes, especially between two cell types. We have tried to make the text clearer throughout.

5. There is no firm conclusion about what role SNX13 plays - either directly or indirectly - on the distribution of cholesterol in cells.

We show that SNX13 increases membrane contact sites (new Figure 9). Further mechanistic analysis is beyond the scope of this study.

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

This study uses several CRISPR-based screening approaches to reveal genes that positively and negatively regulate cholesterol and BMP efflux from lysosomes in the presence and absence of NPC1 function. They focus on one hit in particular, SNX13, a poorly characterized ER-localized protein, and show that SNX13 loss partially rescues the cholesterol accumulation in lysosomes when NPC1 activity is blocked.

The study utilizes an elegant screening approach, and identifies numerous unexpected and potentially exciting new genes associated with cholesterol and BMP intracellular trafficking. The BMP associated hits are particularly interesting since very little is known about this lipid and its trafficking regulation. The study appears rigorous and well conducted, and the experiments are conclusive and well quantified.

However, whereas the first half of the study focusing on the screen is thorough, the last section dealing with investigating the nature of the hits is less well developed. SNX13-KO/KD is shown to reduce cholesterol accumulation in U18666A treated cells. It is also shown that these cells accumulate cholesterol at their plasma membrane. Lipid droplets are also shown to accumulate in SNX13-KD +U18666A cells. Finally, SNX13 KD cells also have increased BMP pools at their lysosomes. As presented, these are mostly descriptive observations at present that lack an explanation. Experiments that further dissect the role of SNX13 in cholesterol/BMP efflux, or at least explain how SNX13 loss may promote a re-wiring of cholesterol sub-cellular distribution, will significantly enhance the study. Specific suggestions below:

1) SNX13 associates with lipid droplets with OA, and SNX13 siRNA is correlated with increased TAG and fatty acids (Fig 8). Is this TAG increase due to increased DGAT1/2 enzyme abundance or activity? Alternatively is there more lipid uptake or de novo lipid synthesis in SNX13 siRNA cells?

SNX13-depleted cells exhibit increased numbers of LDs, even in cells where cholesterol esterification was inhibited by the ACAT1 inhibitor SANDOZ. How this happens will be an exciting avenue for future studies, but is beyond the scope of the present study.

2) It is notable that free cholesterol accumulates at the surface of SNX13 siRNA cells treated with U18666A. Do these cells have increased de novo cholesterol synthesis? Alternatively, is

trafficking of cholesterol to the PM increased or altered in some way? ER-PM contact sites are major sites of cholesterol transport. Are these sites altered in SNX13 siRNA?

We have performed orthogonal validation of our immunofluorescence data using a biochemical fluorometric assay to measure cholesterol in cell extracts (new Fig. 8D). Total cell cholesterol levels do not significantly change between cells treated with SNX13 siRNA. Thus, these data support the conclusion that SNX13 depletion triggers cholesterol redistribution from endolysosomes to the PM.

We now show that mammalian SNX13 is an inter-organelle tether, using live cell video microscopy experiments and a new method that uses hypotonic conditions to better visualize inter-organelle contacts (King et al., 2020; new Fig. 9 and Supplemental movies 2 and 3). Additionally, live cell video microscopy also revealed stable and prolonged association of SNX13-ER domains with lipid droplets (Supplemental movie 1), further suggesting tight SNX13-mediated ER-LD tethering activity. Finally, we have modified the manuscript discussing possible models for how SNX13 may regulate cholesterol transport in cells.

3) SNX14 and SNX13 are paralogs, and both are detected in the screening as decreasing lysosomal cholesterol in U18666A treated cells. It is unclear what their relationship is to one another. Does over-expression of one paralog rescue the loss of the other?

SNX14 and SNX13 knock out decreased accessible cholesterol in our screens. In our validation immunofluorescence experiments we also observed a significant overall decrease of vesicular PFO-fluorescence in both SNX13- and SNX14-depleted cells after U18666A treatment, at least in U2OS cells (new panels in Figure 5 (F, K)). While SNX13-depletion decreased the number of PFO-positive vesicles, SNX14 knockdown did not. Finally, under these conditions, the PM cholesterol redistribution observed in SNX13-depleted cells was not observed in cells lacking SNX14, indicating a more prominent role for SNX13 in this context.

Recently, a paper by Bonifacino and colleagues (Saric et al., 2021) described a role for another SNX13/SNX14 paralog, namely SNX19, in mediating ER-Endolysosome (EL) contacts. As in our present study, they also observed that SNX13-ER domains, but not SNX14-ER, co-localized with ELs. This is in agreement with the fact that the PX domains of SNX13 and SNX19, but not that from SNX14, bind endosomal phosphoinositides (Zheng et a., 2001; Mas et al., 2014; Saric et al., 2019). While we had previously observed close apposition between SNX13-positive ER structures and BMP-positive ELs (Fig. 4E, G, H), our new live cell microscopy data shown in Figure 9 further confirms that SNX13 plays a role in establishing ER-EL contacts, suggesting a role for SNX13-mediated interorganelle contacts in the regulation of cholesterol transport.

We do not discard the possibility that these two SNX paralogs regulate cholesterol transport in cells in a coordinated manner. Indeed, a recent proteome-wide study reporting protein-protein interactions (Huttlin et al., 2021) revealed that both SNX13 and SNX14 interact with one another, further supporting such a model.

We have also added siRNA-rescue experiments (Fig. S4A-C) by ectopically expressing a siRNA-resistant WT-SNX13. As previously reported by others (Mas et al., 2014), we experienced great difficulty at overexpressing SNX13, especially after siRNA transfections. Despite this limitation, we were able to image cells treated with SNX13 siRNA + U18666A and

quantify PFO-positive vesicles and PFO intensity of these vesicles in both rescued and non-rescued cells (Fig. S4A-C), as in the experiments shown in Figure 5E, F. Unfortunately, when attempting to perform PM-staining in rescue experiments, SNX13-overexpressing cells detached very easily, and could not be monitored.

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

This study uses CRISPR screens to identify genes that alter levels of cholesterol and bis(monoacylglycero)phosphate (BMP) in lysosomes. The screens were also performed after cells were treated with the NPC1-inhibitor U18666A. Genes that participate in many cellular processes were found. The second half of the study focuses on SNX13, one of the genes that was found to decrease both cholesterol and BMP in lysosomes after U18666A treatment. Depletion of SNX13 after U18666A treatment is shown to increase cholesterol in the plasma membrane and increase levels of TAG and, as a result, the number of lipid droplets. While these are interesting findings, their significance is considerably oversold. Here are my major concerns.

1. The finding that knockdown of SNX13 significantly reduces lysosomal cholesterol accumulation following NCP1 inhibition is an important finding. However, there is no mechanistic insight into how occurs. A complete understanding is beyond the scope of this study, but some insight seems necessary for this to be appropriate for JCB.

We agree with the reviewer that “a complete understanding of SNX13 function is beyond the scope of this study”, nevertheless we appreciate the reviewer’s comment asking for additional insight into the function of this protein. As discussed above, we performed live microscopy experiments to monitor increased membrane contact sites due to SNX13 protein.

2. Claims about cholesterol distribution in cells are based only on PFO*. Some of the most important findings should be verified with other methods and, if possible, by direct measurement of cholesterol levels in cellular compartments.

We have performed orthogonal validation using a biochemical assay to measure cholesterol in cell-free extracts. Using this method, we found that total cell cholesterol levels do not significantly change between cells treated with either Control siRNA or SNX13 siRNA. Thus, these data further support the notion that upon SNX13-depletion, cholesterol is redistributed from endolysosomes to the PM. We have included a new panel (Fig. 8D) and edited the text.

3. The CRISPER screens are well designed, but the results are rather disappointing and oversold. The authors may want to consider a more balanced discussion of the significance of their findings and, in general, the usefulness of this type of screen for investigating lipid trafficking and metabolism. Aside from the SNX13 findings, the results of the screens are only a modest advance in our understanding of intracellular cholesterol trafficking and BMP metabolism. What do the screens tell us about these processes that we did not know already? The results of the screen are also not presented in a way that is terribly useful. Figs. 4 and 5 are overwhelming and difficult to make sense of.

Sorry that the reviewer finds our results disappointing and oversold. This was a huge effort that complements work from several other labs and shows coordinate regulation of BMP and

cholesterol and a completely surprising link between NPC1 and SNX13. The study also indicates that we are reaching saturation in terms of our knowledge of cholesterol regulators.

We agree that Fig. 4 and 5 were overwhelming and apologize. We have now deleted Fig. 5 and Supplemental Fig. 1 and moved the previous Figs. 3 and 4 to the Supplement. Our previous Supplemental Figures 3 and 4 are now the new main Figures 3 and 4.

Additional FIGURE CHANGES MADE:

- New Panel C, D in Figure 8 showing nmol cholesterol/mg protein; E showing BMP +U18 by mass spec
- New Figure 9 demonstrating membrane contact sites plus 3 videos
- Former Fig.3 is now moved to Suppl Fig. 1.
- Former Fig.4 is now moved to Suppl Fig. 3.
- Former Suppl Fig. 5 is now Suppl Fig. 4.
- New Panels A, B and C in Supp Fig. 4. showing rescue of siRNA phenotype
- New Fig. 5F and K showing vesicular PFO intensity as requested

In summary, we believe that the manuscript has been made much more easily digestible and the conclusions have been bolstered significantly. We thank the reviewers for their time and helpful suggestions.

November 24, 2021

RE: JCB Manuscript #202105060R

Dr. Suzanne R. Pfeffer
Stanford University School of Medicine
Department of Biochemistry
279 Campus Drive
Stanford, CA 94305-5307

Dear Dr. Pfeffer:

Thank you for submitting your revised manuscript entitled "CRISPR screens for lipid regulators reveal a role for ER-bound SNX13 in lysosomal cholesterol export". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final manuscript you must also address reviewer #3's comments regarding the quantification of cholesterol. An analysis with SNX14 is however not required.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, 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.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, * including all inset magnifications*. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

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."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model

g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Andrea L. Marat, Ph.D.
Senior Scientific Editor

Journal of Cell Biology

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

The authors have successfully addressed the concerns in my original review.
The paper provides a wealth of new data on genes that would modify NPC1 mutant cells.
It also describes novel findings about how SNX13 may influence cholesterol transport through membrane contact sites.

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

The revised study addresses the majority of major concerns. The new experiments examine total cellular cholesterol versus cholesterol spatial distribution. The revision also now shows that SNX13 can influence ER membrane contact sites, a key observation for the current model. The differential contributions of SNX13 and SNX14 to the phenotypes examined here have also been further examined. SNX13 appears to play the primary role in the distribution of cholesterol versus SNX14. The text has also been updated in several sections to enhance clarity as requested.

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

My concerns have mostly been addressed, but one issues remains.

Figure 8D shows the direct quantification of cholesterol levels in cells treated with U18 and siRNA against SNX 13, an important control. It is not clear whether free or total cholesterol is being shown. In either, case both free cholesterol and cholesteryl ester levels should be given. If the total of free plus esterified cholesterol changes following SNX13 knockdown, then SNX13 could be regulating more than cholesterol distribution. This should be should be discussed. It would also be good if the same analysis was performed with SNX14.