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Tex2 is required for lysosomal functions at TMEM55-dependent ER membrane contact sites

Yuanjiao Du, Weiping Chang, Lei Gao, Lin Deng, and Wei-Ke Ji

Corresponding Author(s): Wei-Ke Ji, Huazhong University of Science and Technology

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July 15, 2022

Re: JCB manuscript #202205133

Dr. Wei-Ke Ji
Geisel School of Medicine at Dartmouth

Dear Dr. Ji,

Thank you for submitting your manuscript entitled "Tex2 is required for lysosomal functions at TMEM55-dependent ER membrane contact sites". 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 both reviewers are enthusiastic about your study, an opinion we share. However, they also have several concerns. All of them should be addressed but two stand out as particularly important. Both reviewers question the claim that Tex2 transports lipids (Rev 1, pt 7 and Rev 2, pt 4). If stronger evidence cannot be provided, the claim should be removed. Rev 2 also raises significant concerns about the interpretation of the results presented in Figs. 9 and 10 (pts 5 and 6). It is important to address these concerns. All the other issues raised by the reviewers should also be addressed. The reviewers' comments are constructive and it should be possible to address many of them without substantial experimentation.

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, and acknowledgments. Count does not include materials and methods, figure legends, 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.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find 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

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

In this manuscript by Du et al., the authors investigate the localization and function of Tex2, a SMP domain-containing ER membrane protein. They found that Tex2 preferentially localizes at ER tubules and becomes enriched at contact sites between the ER and late endosome/lysosomes (LE/lys) when co-expressed with TMEM55, a LE/Lys membrane protein. TMEM55-dependent enrichment of Tex2 at ER-LE/lys contacts is regulated by PI4P produced by type II PI4Ks. In addition, they showed that TEX2 KO cells exhibit defective lys functions.

This study is of great interest to cell biologists with many new findings and nice results. Nevertheless, the first half and the second half of the study are not well connected.

Specific comments:
1. The first half of the study demonstrates that the N-terminus of Tex2 is important for mediating its interaction with TMEM55 and localization to ER-LE/Lys MCS. Are the interaction with TMEM55 and localization to ER-LE/Lys MCS important for the lysosomal functions of Tex2? This can be tested by rescuing the Tex2 KO using the N-terminal deletion mutant and the N-terminal only mutant of Tex2.
2. Is the localization of TMEM55 and PI4KIIa/b altered in Tex2 KO cells?
3. The authors also demonstrate the involvement of phosphoinositide in Tex2 interaction with TMEM55. Can the PH domain deletion mutant of Tex2, which can still localize to ER-LE/Lys MCS and interact with TMEM55 without being affected by PI4KII, rescue the lysosomal defects in Tex2 KO cells?
4. Does the increase in PI3P at LE/Lys account for the lysosomal defects? Can the defects be rescued by a PI3K inhibitor such as VPS34in?
5. A recent study (PMID: 34663803) showed that Tex2 deficiency contributes to accumulation of PI4,5P2 at endosomes. The authors should re-analyze the data shown in Figure S9B to see if there is an increase in PI4,5P2 at endosomes by comparing with PI4,5P2 at plasma membrane, where PI4,5P2 is enriched.
6. Figure S9. It is better to use P4M, a PI4P specific biosensor to measure PI4P. The measurements should be performed to test if there is an increase in PI4P distribution at LE/lys vs Golgi and plasma membrane
7. (Line 393-394) The authors claimed that the lipid transfer activity of Tex2 is important for the lysosomal defects in Tex2 KO cells. They showed that the SMP domain Tex2 can bind various lipids but did not show that it confers lipid transfer activity. It should be noted that SMP domain can function as a dimerization domain (PMID: 26686281). The authors should either demonstrate the lipid transfer activity of the SMP domain of Tex2 between 2 membranes or remove/modify this claim.

Minor points
1. Figure 3A. Explain the experiment and the control. What are the two conditions for the volcano plot? TMEM55B is among a low hit. Why is TMEM55B selected? What are the other hits that ranked higher than TMEM55B? Why use a rat database (line 589) if the experiment was done using 293 cells
2. Figure 5. Use a phosphatase dead (point mutation) to show that the phosphatase activity is important for the interaction
3. Line 116-125. It should be refereeing to Figure 2, not figure 1, in this paragraph.
4. Line 65-66. This paper did not show Tex2 localization at ER-LE/Lys MCS
5. Figure 3E. The yellow arrow on the middle panel of LAMP1-mCh is not at the correct position.
6. Line 191. Typo "associated"

7. Figure label. Figure 9H should be Figure 9G and vice versa

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

In this manuscript Du and co-workers identify a new type of ER-lysosome contact site, which depends on the interaction between the ER localized SMP protein Tex2 and the lysosomal weak PI phosphatase TMEM55B, previously implicated in retrograde lysosome transport. They show that this contact site is important for endosomal lipid homeostasis and the maintenance of lysosomal pH and thus the activity of autolysosomes. The contact site formation is regulated by the activity of lysosomal PI4KII and the PH-domain of Tex2, whereas its function requires the lipid binding SMP domain of Tex2, suggesting that lipid transfer is involved.
Tex2 homologues have been implicated in lipid transfer and ER-organelle contact sites in yeast and worms although incompletely understood, and Tex2 is poorly described in mammals. Hence, this study is timely and important as it identifies the role of Tex2 in a new type of ER-lysosome contact sites, and elucidates the mechanistic and functional aspects of this contact site in mammalian cells. The identification of TMEM55 as the lysosomal binding partner of Tex2 is interesting and opens up for further studies regarding the role of ER in control of lysosome positioning and function. The work is overall well conducted and presented in a logical way. The studies have been carried out in a rigorous manner and the images are clear and convincing. Before I can recommend publication, the following issues need to be clarified and addressed:

Specific points:
Fig 1A. It would be informative to comment on the localization of the the ER-luminal marker, which gives a similar localization pattern as Halo-E-Syt1 in Fig. S1B, in order to explain better the sheet like localization. An image showing the colocalization between Halo-E-Syt1 and ER-tagged RFP could further strengthen this point.

Fig. 3A. Please describe better in the legend what is shown and compared in the volcano plot.
The recruitment of GFP-Tex2 positive ER to TMEM55B overexpressing perinuclear lysosomes is convincing. To strengthen the argument that these are indeed ER-lysosome contact sites, it would be nice to include ultrastructural analysis, such as correlative light and electron microscopy.

Fig. 4H Please show the expression of purified GST-control protein compared to TMEM55B.

Fig. 6. I find it difficult to understand how higher level of PtdIns4P species on lysosomes prevent contact site formation if the PH domain of Tex2 interacts with these species? Should this not rather stabilize the MCS?
Fig. 7-8. The authors should be careful to claim that Tex2 is a lipid transfer protein unless they show lipid transfer activity by in vitro analysis.
Fig. 8E. Please describe in the methods how the analysis was performed (which software, manual or automatic segmentation of lysosomes, fluorescence intensity, etc?).

Fig. 9. Please explain how the LC3-dots were quantified and how the results were normalized. I assume that they were normalized to RFP-LC3 (LC3deltaG?).
KO of Tex2 presumably leads to an increased pH in lysosomes as shown by the visualization of a pHluorine-tagged LAMP1 construct in Fig. 8. However, in Figure 9F, GFP-LC3 accumulates in lysotracker positve autolysosomes in Tex2 KO cells. Since lysotracker labels acidic compartments, how can the authors explain this paradox? To clarify this discrepancy, it would be informative to measure the pH in endosomes in control and Tex2 KO cells. How do the authors envision how the Tex2-TMEM55 mediated ER-lysosome contact sites regulate lysosomal pH? Direct mechanism, or indirect by regulating lysosome positioning of lipid homeostasis?

Fig. 10. As a PtdIns3P reporter, p40PX-GFP is mainly found on OFP-EEA1 positive compartments as expected (Fig S9E, F). In Tex2 KO cells, however, p40PX-GFP is found on LAMP1-mCh compartments. The authors suggest that this is due to an enrichment of PtdIns3P in LE/Lys, but they neither precede to investigate this further, nor do they provide an explanation why Tex2 depletion could lead to an upregulation of PtdIns3P on lysosomes. I think that an alternative explanation could be that the maturation of endosomes is impaired (impaired phosphoinositide and RAB-GTPase switch) by the loss of ER-endosome contact sites, leading to a hybrid compartment positive for EEA1, LAMP1 and PtdIns3P (and thus not an increase in the PtdIns3P levels as such). It will be important to clarify this question by additional experiments using tagged probes and markers, but also by the labelling of endogenous EEA1 and LAMP1 by immunofluorescence imaging, where antibodies are commercially available. The highly specific PtdIns3P probe 2xFYVE should be used in addition to verify the results obtained by using p40PX-GFP.

Fig. S3. The authors need to verify the level of KD for RTN4, REEP5, ARL6P1, RTN1 and RTNs by WB or qPCR to be able to draw the conclusion that the tubular ER localization of Tex2 is independent of these proteins.

Fig. S4. From the representative images shown, it is plausible that GFP-Tex2 (similar to RTN4-GFP) counteracts the increased sheet formation caused by overexpression of Climp63-Halo, thus contributing to tubular ER shaping as the authors suggest. A quantification of sheets/tubules in the different conditions is required to support this conclusion.

FigS5A, B. Please explain in the legend and/or methods how this analysis was carried out and how R/r was defined (which software, manual or automatic segmentation of lysosomes, fluorescence intensity or area, etc?).
The axis is a bit misleading, claiming to show percentage, but rather shows the fraction of cells with dispersed or perinuclear lysosomes, counting up to a total of approximately 200 cells per condition, if I understand it correctly. The data could rather be represented as % of cells with perinuclear lysosomes, of the total cell population set to 100.

TMEM55B is involved in the transport of lysosoms to the cell centre important for lysosomal function (PMID: 29146937). In Fig. S5B, Tex2 counteracts the perinuclear clustering of TMEM55B positive lysosomes, whereas the deltaNT mutant (likely cytosolic) does not. Is it possible that the connection of TMEM55B positive lysosomes to the ER by Tex2 precludes the retrograde transport of lysosomes? Have the authors observed any change in lysosome positioning (perinuclear clustering) in Tex2KO cells? The overexpression of TMEM55B likely increases the perinuclear clustering of lysosomes, so this question is best answered by using the labelling of another lysosomal protein, like LAMP1.
Fig. S5C. I assume that the numbers indicate the time point (seconds?) of selected frames from a movie. Please indicate the frame rate of the movie and explain better in the legend. In my experience, a frame rate of 2-5Hz is suitable for tracking of individual vesicles, which might otherwise be lost. Please upload the corresponding movie. How many movies/endosomes is this representative of?

Fig. S5D. Please upload the corresponding movie.

Fig. S6B. How can you exclude that the perinuclear cloud observed upon co-expression of GFP-Tex2-KI and Halo-TMEM55B is not associated with the Golgi in TG treated cells? It would be informative to investigate this using a Golgi marker and high-resolution imaging.

Fig S6D. Is this quantified after 6 or 12 hours of TG treatment?. How many experiments were performed for this analysis?

Missing in the Methods:

Please describe the generation of the GFP-Tex2-KI and Tex2 KO in the methods.

From which supplier was the siRNA oligos purchased?

For the rescue experiments, please describe how the cells were reconstituted with Tex2. Transient or stable expression of HALO-Tex2?

Describe the reagents used, eg. thapsigargin, lysotracker, bafilomycin, rapamycin.

Typos and comments:
Although clearly understandable, the language of the manuscript needs improvement.
Manuscript line 178 refers to Fig. 3I, should be 3J.

Manuscript line 179: ------"co-localization analysis based on x-y andy-z projections of 3D rendering". I cannot find the data for this co-localization analysis
Manuscript line 291, Fig. 68G should be 6G.

1st Revision - Authors' Response to Reviewers: October 17, 2022

Dear Dr. Ji,

Thank you for submitting your manuscript entitled "Tex2 is required for lysosomal functions at TMEM55-dependent ER membrane contact sites". 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.

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The revised manuscript, including title page, abstract, introduction, results, discussion, and acknowledgements, has ~35000 characters, not including spaces.

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.

The revised manuscript has 10 main text figures, and figures are prepared according to the policies.

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.

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The revised manuscript has 5 supplemental figures and 2 videos, with a summary of all supplemental material at the end of the Materials and methods section.

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here: https://rupress.org/jcb/pages/submission-guidelines#revised

The Source Data containing fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures is submited along with the revised manuscript and point by point rebuttal letter.

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find 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.

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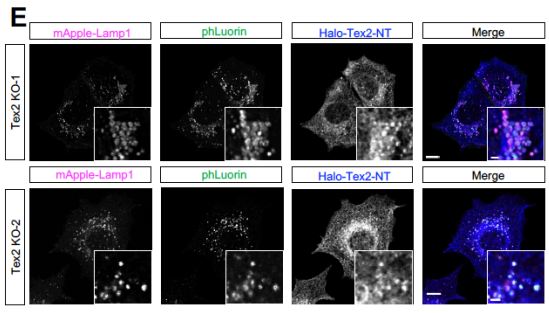
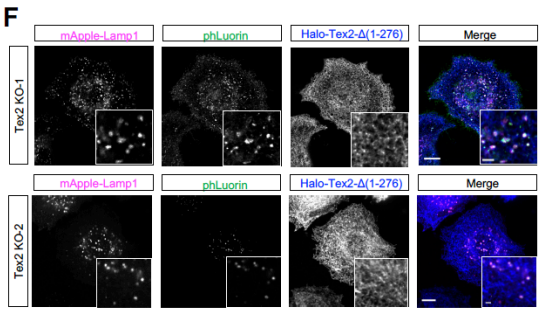
Reviewer #1 (Comments to the Authors (Required)):

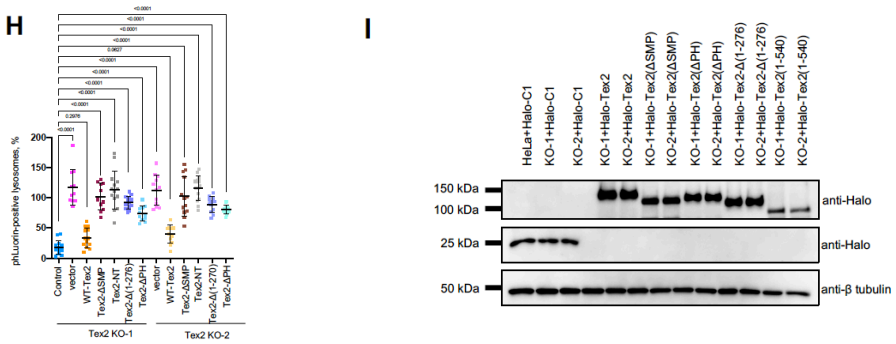
In this manuscript by Du et al., the authors investigate the localization and function of Tex2, a SMP domain-containing ER membrane protein. They found that Tex2 preferentially localizes at ER tubules and becomes enriched at contact sites between the ER and late endosome/lysosomes (LE/lys) when co-expressed with TMEM55, a LE/Lys membrane protein. TMEM55-dependent enrichment of Tex2 at ER-LE/lys contacts is regulated by PI4P produced by type II PI4Ks. In addition, they showed that TEX2 KO cells exhibit defective lys functions.

This study is of great interest to cell biologists with many new findings and nice results. Nevertheless, the first half and the second half of the study are not well connected.
We are truly grateful for your constructive comments and insightful suggestions. To strenghen the logical flow of this manuscript, we have made three modifications in the revised manuscript. First, we added new data showing that the Tex2-TMEM55B interaction was required for lysosomal function. Second, we changed the positions of two figures. Specifically, the original Fig. 7I (Tex2 binds glycerophospholipids and ceramides) is moved to the Fig. 9 of the revised manuscript. Moreover, the original Fig. S5. (A potential role of Tex2-TMEM55B interaction in retrograde transport of LE/lys.) is moved to the Fig. 6 in the main text of the revised manuscript. In addition, we have rephrased some sentences to make this manuscript more logical and easier to read.

Specific comments:
1. The first half of the study demonstrates that the N-terminus of Tex2 is important for mediating its interaction with TMEM55 and localization to ER-LE/Lys MCS. Are the interaction with TMEM55 and localization to ER-LE/Lys MCS important for the lysosomal functions of Tex2? This can be tested by rescuing the Tex2 KO using the N-terminal deletion mutant and the N-terminal only mutant of Tex2.
We thank reviewer for this insightful suggestion. As suggested, we tested whether Halo-Tex2-Δ(1-276), the TMEM55B-binding defective mutant, or the Tex2-NT could resuce the lysosome defect in Tex2 KO. Our results showed that Tex2-NT could not significantly rescue the phenotype in these two Tex2 KO clones (Fig. 7, E and H), indicating that Tex2-NT was not sufficient for restoring the lysosomal defects. Interestingly, the TMEM55B-binding defective mutant, Tex2-Δ(1-276), could restore the lysosomal defects but to a very limited extent (Fig. 7, F and H). Collectively, these results suggested that Tex2-TMEM55B interaction is required but not sufficient in rescuing the phenotype.
The new results were shown below and incorporated in the present manuscript as Fig. 7, E, F, H and I.

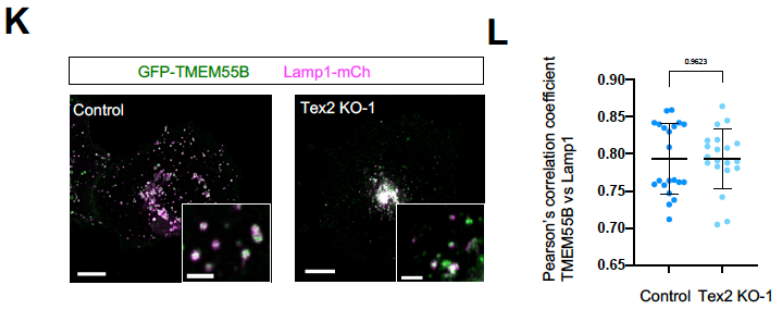




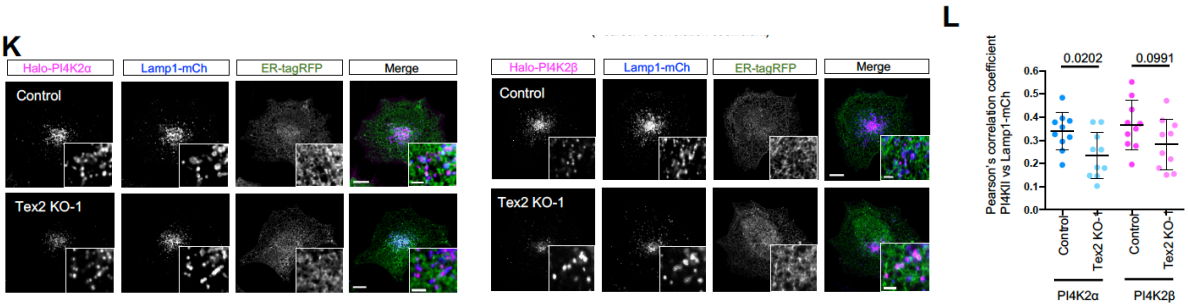


2. Is the localization of TMEM55 and PI4KIIa/b altered in Tex2 KO cells?

Thanks for the comment. We examined the localization of TMEM55 and PI4KIIa/b in control or Tex2 KO HeLa cells. In case of TMEM55, our results showed that Tex2 KO did not alter the localization of Halo-TMEM55B relative to LE/lys, though we observed that more LE/lys were at perinuclear region, suggesting a role of Tex2 in LE/lys trafficking. These results were shown below and incorporated in the revised manuscript as Fig. 4, K and L.

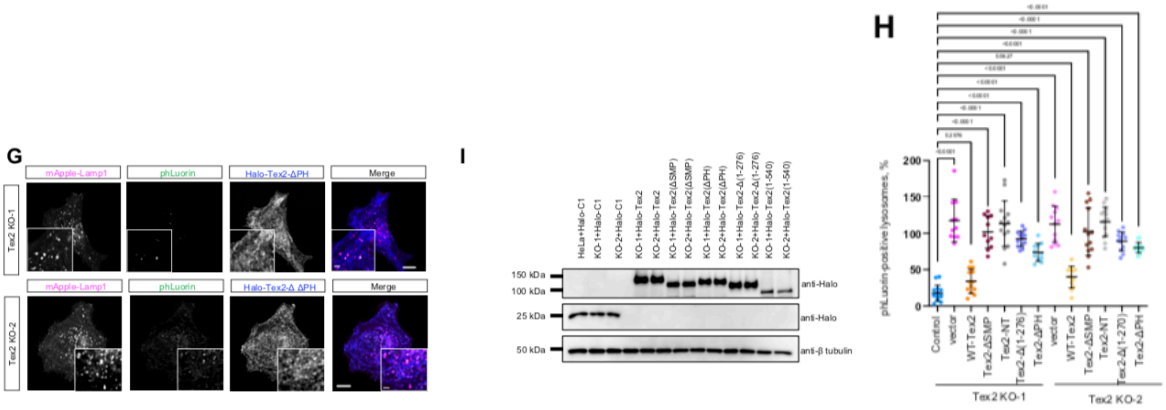


In case of PI4KIIa/b, we found that Tex2 KO did not substantially affect the localization of Halo-PI4KIIa/b relative to LE/lys, and these results were shown below and incorporated in the revised manuscript as Fig. 5, K and L.



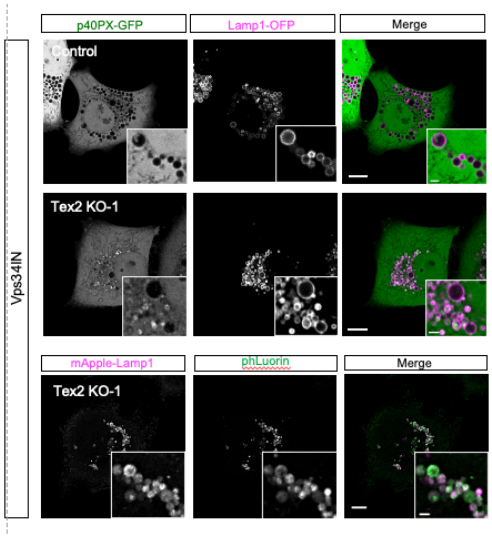
3. The authors also demonstrate the involvement of phosphoinositide in Tex2 interaction with TMEM55. Can the PH domain deletion mutant of Tex2, which can still localize to ER-LE/Lys MCS and interact with TMEM55 without being affected by PI4KII, rescue the lysosomal defects in Tex2 KO cells?

Thanks for the comment. As suggested, we tested whether Halo-Tex2-ΔPH could resuce the lysosome defect in Tex2 KO. Interestingly, our results showed that Tex2-ΔPH could partially rescue the phenotype (Fig. 7, G and H), indicating that the PH domain of Tex2 was not strictly required but could somehow promote the lysosomal function. These new results were shown below and incorporated in the present manuscript as Fig. 7, G and I.



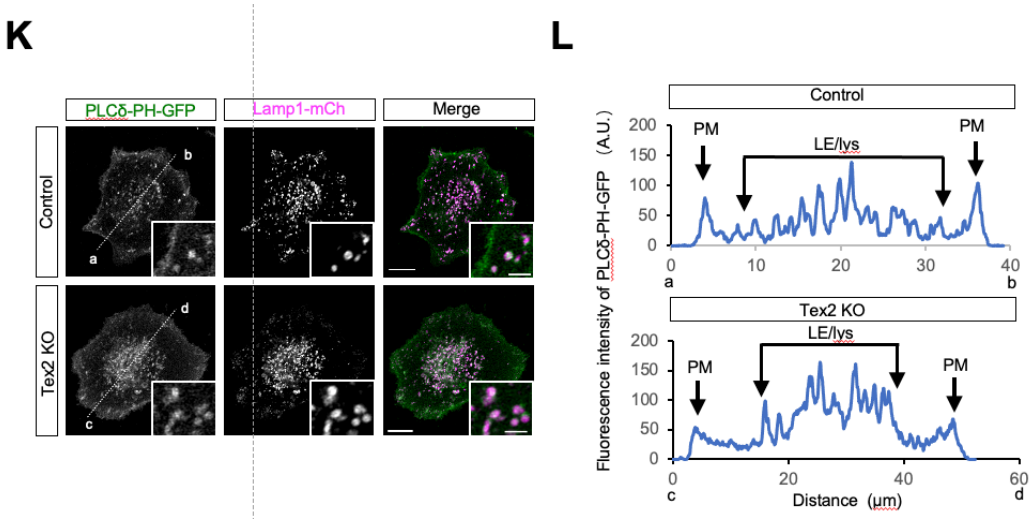
4. Does the increase in PI3P at LE/Lys account for the lysosomal defects? Can the defects be rescued by a PI3K inhibitor such as VPS34in?

We thank reviewer for this insightful suggestion. As suggested, we tested whether the lysosomal defect can be rescued by the PI3K inhibitor VPS34in. Our results showed that the defect could not be substantially rescued by VPS34in, indicating that PI3P accumulation did not account for the lysosomal defect. The effeciency of VPS34in-mediated PI3K inhibition was verified by the cytosolic distribution of PI3P probe p40PX-GFP.



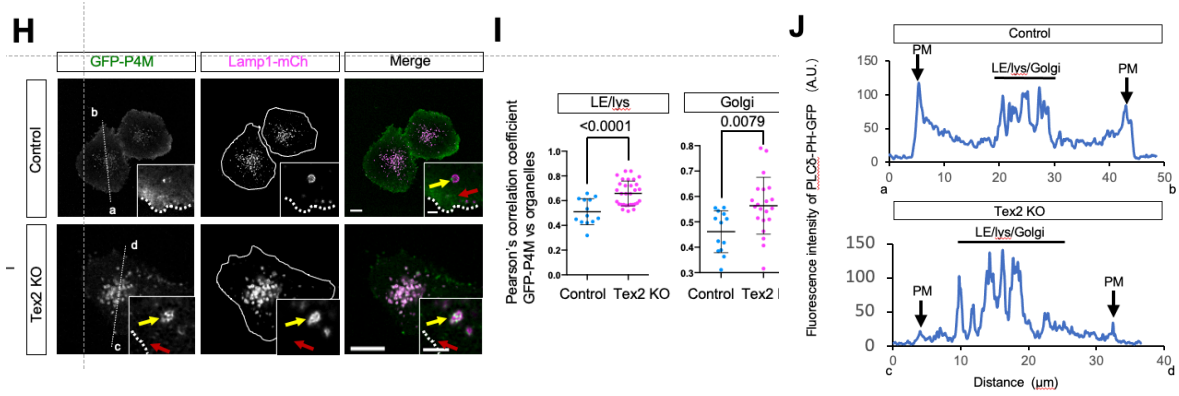
5. A recent study (PMID: 34663803) showed that Tex2 deficiency contributes to accumulation of PI4,5P2 at endosomes. The authors should re-analyze the data shown in Figure S9B to see if there is an increase in PI4,5P2 at endosomes by comparing with PI4,5P2 at plasma membrane, where PI4,5P2 is enriched.

We thank reviewer again for such insightful comment. In the original manuscript, we analyzed the PI4,5P2 at LE/lys by Pearson's correlation coefficient, and found no substantial changes. As suggested, we re-analyzed the PI4,5P2 at LE/lys relative to the PI4,5P2 on the PM by linescan, and found that the ratio of PI4,5P2 at LE/lys to the PM was higher in Tex2 KO HeLa cells compared to control cells, consistent with the recent study. These new results were shown below and incorporated in the present manuscript as Fig. S5, K and L.



6. Figure S9. It is better to use P4M, a PI4P specific biosensor to measure PI4P. The measurements should be performed to test if there is an increase in PI4P distribution at LE/lys vs Golgi and plasma membrane

We thank reviewer again for such insightful comment. As suggested, we re-analyzed the PI4P at LE/lys relative to the Golgi or the PM, and found that PI4P at LE/lys or at the Golgi was slightly higher in Tex2 KO compared with control cell. In addition, we analyzed the PI4P at LE/lys or Golgi relative to the the PM by linescan, and found that the ratio of PI4P at LE/lys/Golgi to the PM was higher in Tex2 KO HeLa cells compared to control cells. These results were shown below and incorporated in the present manuscript as Fig. S5, H, I and J.



7. (Line 393-394) The authors claimed that the lipid transfer activity of Tex2 is important for the lysosomal defects in Tex2 KO cells. They showed that the SMP domain Tex2 can bind various lipids but did not show that it confers lipid transfer activity. It should be noted that SMP domain can function as a dimerization domain (PMID: 26686281). The authors should either demonstrate the lipid transfer activity of the SMP domain of Tex2 between 2 membranes or remove/modify this claim.

We appreciate reviewer for this great point. We agreed with reviewer. Since SMP domains could mediate protein dimmerization in addition to lipid transfer, our results could not distinguish which function of the SMP domain of Tex2 was responsible for the lysosomal function, and thus our results only suggested that the lipid transfer activity of Tex2 was essential for the lysosomal function. Therefore, we deleted the claim throughout the revised manuscript.

Minor points

1. Figure 3A. Explain the experiment and the control. What are the two conditions for the volcano plot? TMEM55B is among a low hit. Why is TMEM55B selected? What are the other hits that ranked higher than TMEM55B? Why use a rat database (line 589) if the experiment was done using 293 cells

We thank reviewer for the comments. These comments can be further divided into three sub-questions, which were addressed separately.

1) Figure 3A. Explain the experiment and the control. What are the two conditions for the volcano plot?

In the experiment, protein candidates coIPed with GFP-Tex2 were identified by mass spectrometry; while in the control, protein candidates coIPed with GFP-C1 empy vector were identified. By comparing the experiment and control group, candidates that were considered significant [-log (P value)>1.3; $p< 0.05$] were labeled in red [Log2 (fold change) >0; increased in abundance] or blue (Log_2 (fold change) <0; decreased in abundance).

2) TMEM55B is among a low hit. Why is TMEM55B selected? What are the other hits that ranked higher than TMEM55B?

As we mentioned in the manuscript, TMEM55B was among a low hit. To identify the 'true' adaptor for recruiting Tex2, we cloned all the genes coding the red protein candidates [Log2 (fold change) >0], and screened them all one by one by co-transfecting each protein candidate with GFP-Tex2 in COS7 cells. In the screening, only TMEM55B showed a strinkingly recruitment of Tex2 to LE/lys membranes. We were also suprised to see a low hit as TMEM55B that actually worked, and one possible explanation may be that Tex2-TMEM55B interaction was not always active, but could be turned on through certain mechanism (for example, regulation by PI4P species on LE/lys).

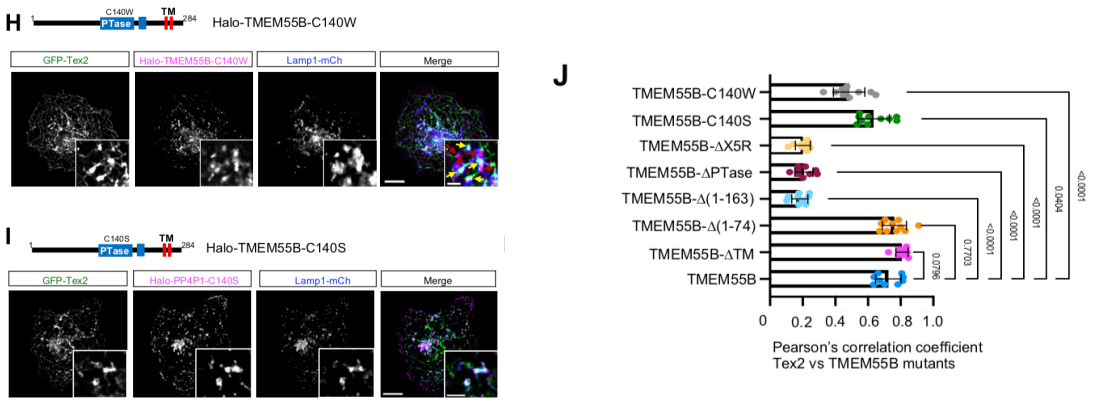
3) Why use a rat database (line 589) if the experiment was done using 293 cells

We thank reviewer for the comment. It was a mistake, and we actually used a human database [the UniProtKB human database (36,080 total entries, downloaded 2019.06.25)]. We have corrected this mistake in the revised manuscript.

2. Figure 5. Use a phosphatase dead (point mutation) to show that the phosphatase activity is important for the interaction

Thanks again for this comment. We made two TMEM55B phosphatase dead mutatants C140W and C140S (C133 in isoform 1 => C140 in isoform 2), and found that these two mutants were still able to recruit GFP-Tex2, but to a less extent compared to WT TMEM55B (Fig. 4, H, I and J), suggesting that the Tex2-TMEM55B interaction was not strictly dependent on phosphatase activity of TMEM55B, which was consistent with our results showing that Tex2 directly bound TMEM55B in in vitro pull-down assays. Yellow arrows indicated TMEM55B-positive LE/lys with GFP-Tex2 enrichments; while

red arrows denoted TMEM55B-positive LE/lys without GFP-Tex2 enrichments. Therefore, our results suggested that the phosphatase activity of TMEM55B is not essential for the interaction. Instead, the CX_5R motif was essential for binding Tex2.



3. Line 116-125. It should be refereeing to Figure 2, not figure 1, in this paragraph.
Thanks for catching this typo, and we have corrected this mistake in the revised manuscript.

4. Line 65-66. This paper did not show Tex2 localization at ER-LE/Lys MCS
Thanks for catching this mistake, and we have rephrased the sentences describing the NC paper in the revised manuscript, shown below.

'Tex2 is recently reported to cooperate with another SMP-containing protein PDZD8 and the PI(4,5)P2 phosphatases, OCRL-1 and UNC-26/synaptojanin to regulate endosomal $PI(4,5)P_2$ homeostasis in worms. However, whether and how Tex2 localized to MCSs remain elusive.'

5. Figure 3E. The yellow arrow on the middle panel of LAMP1-mCh is not at the correct position.
Thanks for catching this typo, and we have corrected this mistake in the revised manuscript.

6. Line 191. Typo "associated"
Thanks for catching this typo, and we have corrected this mistake in the revised manuscript.

7. Figure label. Figure 9H should be Figure 9G and vice versa
Thanks for catching this mislabeling, and we have corrected this mistake in the revised manuscript.

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

In this manuscript Du and co-workers identify a new type of ER-lysosome contact site, which depends on the interaction between the ER localized SMP protein Tex2 and the lysosomal weak PI phosphatase TMEM55B, previously implicated in retrograde lysosome transport. They show that this contact site is important for endosomal lipid homeostasis and the maintenance of lysosomal pH and thus the activity of autolysosomes. The contact site formation is regulated by the activity of lysosomal PI4KII and the PH-domain of Tex2, whereas its function requires the lipid binding SMP domain of Tex2, suggesting that lipid transfer is involved.
Tex2 homologues have been implicated in lipid transfer and ER-organelle contact sites in yeast and worms although incompletely understood, and Tex2 is poorly described in mammals. Hence, this study is timely and important as it identifies the role of Tex2 in a new type of ER-lysosome contact sites, and elucidates the mechanistic and functional aspects of this contact site in mammalian cells. The identification of TMEM55 as the lysosomal binding partner of Tex2 is interesting and opens up for further studies regarding the role of ER in control of lysosome positioning and function. The work is overall well conducted and presented in a logical way. The studies have been carried out in a rigorous manner and the images are clear and convincing.
Before I can recommend publication, the following issues need to be clarified and addressed:

We are truly grateful for your constructive comments and insightful suggestions.

Specific points:
Fig 1A. It would be informative to comment on the localization of the the ER-luminal marker, which gives a similar localization pattern as Halo-E-Syt1 in Fig. S1B, in order to explain better the sheet like localization. An image showing the colocalization between Halo-E-Syt1 and ER-tagged RFP could further strengthen this point.

Thanks for this comment. We co-transfected Halo-E-Syt1 and ER-tagRFP (ER luminal marker) in COS7 cells, and found that, in contrary to GFP-Tex2, Halo-E-syt1 did not show specific localization on the ER, relative to the general ER luminal marker. These results were shown below and incorporated in the present manuscript as Fig. S1,C and D.

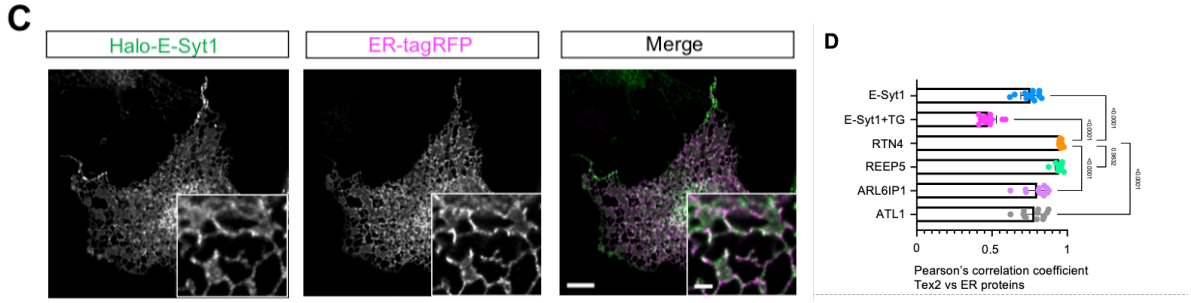


Fig. 3A. Please describe better in the legend what is shown and compared in the volcano plot.
Thanks for the comment. We have described the volcano plot in details in the revised manuscript, shown below.

'Volcano plot of protein candidates coIPed with Tex2 in HEK293 cells, compared to protein candidates coIPed with GFP tag only. Candidates that were considered significant [-log (p

value)>1.3; p< 0.05] were labeled in red [Log2 (fold change) >0; increased in abundance] or blue (Log_2 (fold change) <0; decreased in abundance).'

The recruitment of GFP-Tex2 positive ER to TMEM55B overexpressing perinuclear lysosomes is convincing. To strengthen the argument that these are indeed ER-lysosome contact sites, it would be nice to include ultrastructural analysis, such as correlative light and electron microscopy.

We thank reviewer for this comment. As suggested, we directly observed the recruitment of GFP-Tex2-labeled ER membranes to Halo-TMEM55B-positive lysosomes in COS7 cells using correlative light and electron microscopy (CLEM). Consistent with high-resolution confocal microscopy results, GFP-Tex were strongly recruited to Halo-TMEM55B-positive perinuclear lysosomes, and transmission electron microscopy images showed that at the perinuclear region, where GFP-Tex2 and Halo-TMEM55B were highly co-enriched, LE/lys were tightly associated with ER membranes. We had tried our best in the CLEM assay but we undersood that the quality of CLEM image may not be optimal, because upon overexpression of TMEM55B, most LE/lys were tightly clustering at perinuclear region along with Tex2-labeled ER membranes, making the resolution of lysosomes and ER membranes techinically challenging even under TEM. In additon, there was no core facilities capable of doing CLEM in our city, and we had to send our samples to our collaborator in another city, rendering doing CLEM more difficult. These results were shown below and incorporated in the present manuscript as Fig. 2 K.

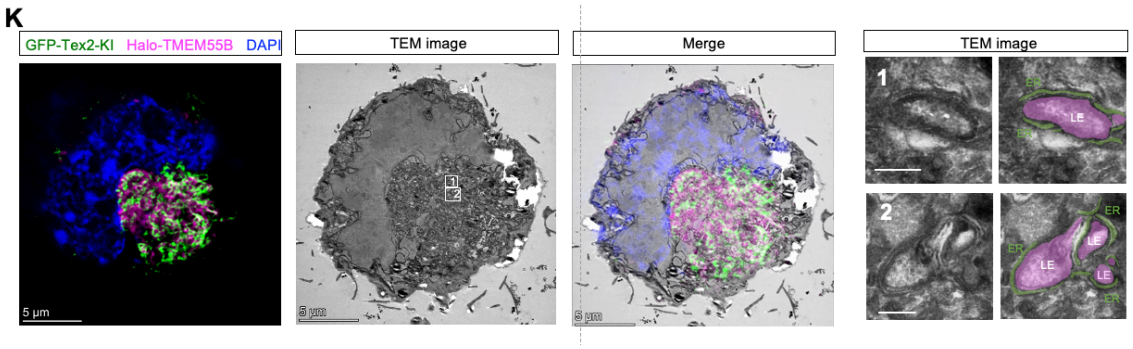


Fig. 4H Please show the expression of purified GST-control protein compared to TMEM55B.

Thanks for the comment. We have provided the commassie blue staining of purified GST-control protein in the in vitro pulldown assay. These results were shown below and incorporated in the present manuscript as Fig. 3 H.

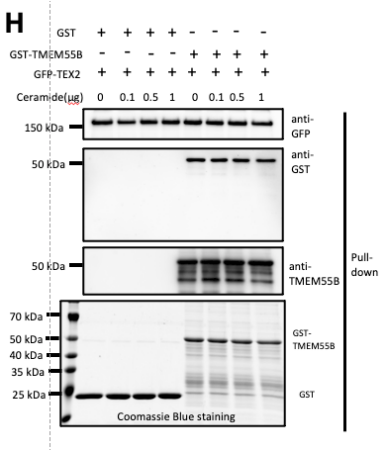


Fig. 6. I find it difficult to understand how higher level of PtdIns4P species on lysosomes prevent contact site formation if the PH domain of Tex2 interacts with these species? Should this not rather stabilize the MCS?

We thank reviewer for this great comment. These results were also puzzling us. One potential explanation was that the interaction between PH domain of Tex2 and PtdIns4P species on lysosomes interfered with the binding of Tex2-NT to TMEM55B (intramolecular competition model).

In particular, purified Tex2-PH bound PI4P, PI(3,4)P2, and PI(4,5)P2 in vitro (Fig. S4 C), but this interaction was not strong enough to mediate the ER-LE/lys MCSs (Fig. S4, B, D and H, and our preliminary results not shown in this manuscript). Therefore, we speculated that the transient binding of these PIPs to the PH domain of Tex2 may hamper the 'productive' interaction between Tex2-NT and TMEM55B, thus negatively regulating the recruitment of Tex2 by TMEM55B.

In addition, to get a better understanding of the regulation of Tex2-TMEM55 by PI4KII activity, we depleted PI4KIIs by siRNAs to test if Tex2 recruitment to LE/lys was enhanced. Indeed, depletion of PI4KIIα or PI4KIIβ significantly promoted the recruitment of GFP-Tex2 to LE/lys in absence of exogenous TMEM55B, as revealed by the higher extent of colocalization between GFP-Tex2 and Lamp1-mCh in PI4KII-depleted cells. These results were shown below and incorporated in the present manuscript as Fig. 5, H, I and J.

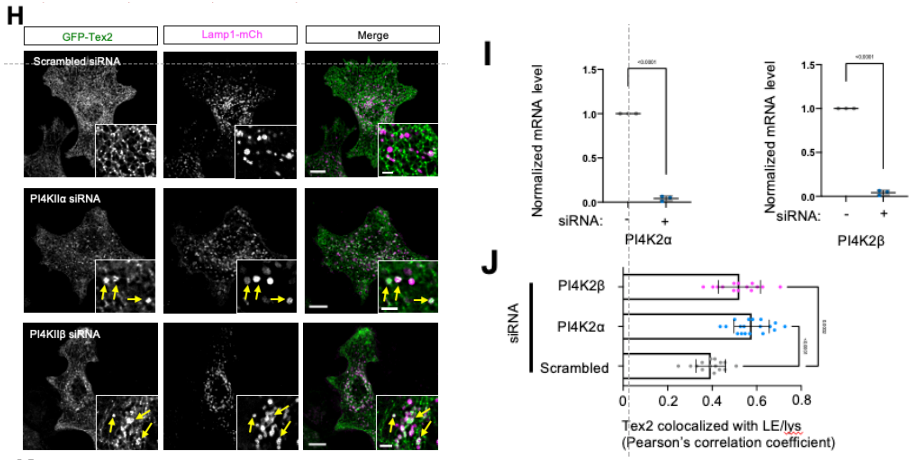


Fig. 7-8. The authors should be careful to claim that Tex2 is a lipid transfer protein unless they show lipid transfer activity by in vitro analysis.

We thank reviewer for this great suggestion. As we mentioned above, we agreed with reviewer. Since SMP domains could mediate protein dimmerization in addition to lipid transfer, our results could not distinguish which function of the SMP domain of Tex2 was responsible for the lysosomal function, and our results only suggested that the lipid transfer activity of Tex2 may be essential for the lysosomal function. Therefore, we deleted the claim throughout the revised manuscript.

Fig. 8E. Please describe in the methods how the analysis was performed (which software, manual or automatic segmentation of lysosomes, fluorescence intensity, etc?).

Thanks for this comment. We described the quantification method in the materials and methods section of the revised manuscript, as shown below.

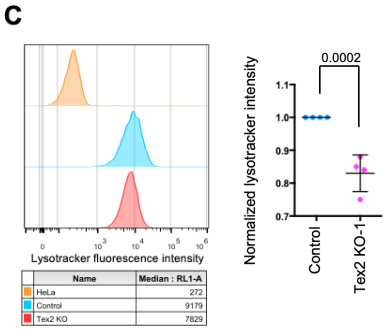
'**Measurement of the percentage of PHluorin-positive lysosomes in cells.** PHluorin-positive lysosomes were manually counted with assistance of Cell Counter, a plugin of ImageJ (2.1.0/1.53c; NIH), and lysosomes were manually counted in the same way based on Lamp1-mApple

fluorescence. The percentage of PHluorin-positive lysosomes in a cell was quantified by the number of PHluorin-positive lysosomes divided by Lamp1-mApple positive lysosomes.'

Fig. 9. Please explain how the LC3-dots were quantified and how the results were normalized. I assume that they were normalized to RFP-LC3 (LC3deltaG?).
Thanks for the comment. Yes, we counted GFP-LC3 dots by hands using ImageJ plugin Cell Counter, followed by the normalization of the number of GFP-LC3 dots to the fluorescence intensity of RFP-LC3deltaG. The description was added to the legend of Fig. 8, E and H in the revised manuscript.

KO of Tex2 presumably leads to an increased pH in lysosomes as shown by the visualization of a pHluorine-tagged LAMP1 construct in Fig. 8. However, in Figure 9F, GFP-LC3 accumulates in lysotracker positve autolysosomes in Tex2 KO cells. Since lysotracker labels acidic compartments, how can the authors explain this paradox? To clarify this discrepancy, it would be informative to measure the pH in endosomes in control and Tex2 KO cells. How do the authors envision how the Tex2-TMEM55 mediated ER-lysosome contact sites regulate lysosomal pH? Direct mechanism, or indirect by regulating lysosome positioning of lipid homeostasis?
We thank reviewer for these insightful comments. These comments can be further divided into two sub-questions, which were addressed separately.

1) KO of Tex2 presumably leads to an increased pH in lysosomes as shown by the visualization of a pHluorine-tagged LAMP1 construct in Fig. 8. However, in Figure 9F, GFP-LC3 accumulates in lysotracker positve autolysosomes in Tex2 KO cells. Since lysotracker labels acidic compartments, how can the authors explain this paradox? To clarify this discrepancy, it would be informative to measure the pH in endosomes in control and Tex2 KO cells.
As suggested, we carefully examined the pH in LE/lys in control and Tex2 KO cells using lysotracker by a quantitative and high-throughout method flow cytometry. In this assay, unstained WT HeLa cells were used as a negative control. Notably, we found that Tex2 KO only resulted in a moderate effect (~15% reduction compared to control) on the LE/lys pH, but the effect was not strong. This line of evidence may explain why lysotracker could still label lysosomes in Tex2 KO cells. The result shown below was a representative of 4 independent assays, and was incorporated as Fig. S5 C in the revised manuscript.

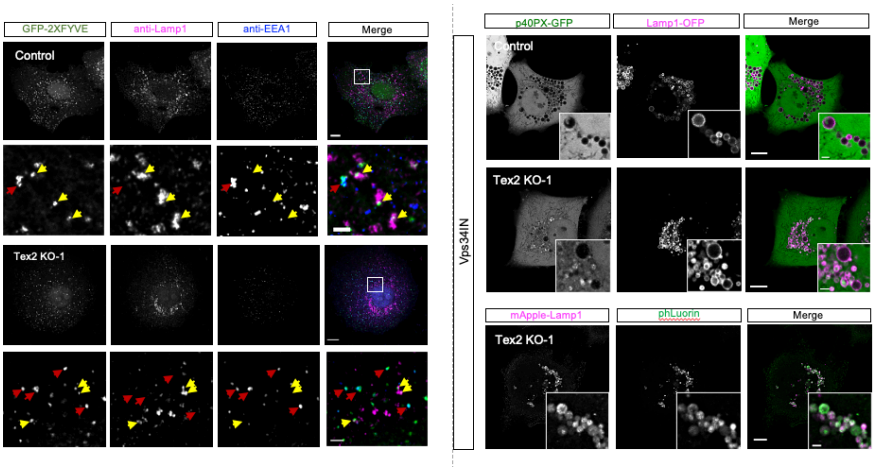


2) How do the authors envision how the Tex2-TMEM55 mediated ER-lysosome contact sites regulate lysosomal pH? Direct mechanism, or indirect by regulating lysosome positioning of lipid homeostasis?
We thank reviewer for this insightful suggestion. We speculated that the Tex2-TMEM55 mediated ER-lysosome contact sites indirectly regulate lysosomal pH possibly through regulating lipid composition of lysosomal membranes due to the following reasons. First, Tex2 KO did not strongly affect the LE/lys pH, suggesting a indirect role of Tex2 for lysosomal pH maintanence. Second,

Tex2-NT, a Tex2 truncation mutant capable of interacting with TMEM55B and could regulate lysosomal positioning, did not rescue the lysosomal defect in Tex2 KO. Third, as a potential lipid transporter at MCSs, we envisioned that the primary role of Tex2 was to mediate lipid exchange to regulate the lipid composition of the ER and LE/lys membranes.

Fig. 10. As a PtdIns3P reporter, p40PX-GFP is mainly found on OFP-EEA1 positive compartments as expected (Fig S9E, F). In Tex2 KO cells, however, p40PX-GFP is found on LAMP1-mCh compartments. The authors suggest that this is due to an enrichment of PtdIns3P in LE/Lys, but they neither precede to investigate this further, nor do they provide an explanation why Tex2 depletion could lead to an upregulation of PtdIns3P on lysosomes. I think that an alternative explanation could be that the maturation of endosomes is impaired (impaired phosphoinositide and RAB-GTPase switch) by the loss of ER-endosome contact sites, leading to a hybrid compartment positive for EEA1, LAMP1 and PtdIns3P (and thus not an increase in the PtdIns3P levels as such). It will be important to clarify this question by additional experiments using tagged probes and markers, but also by the labelling of endogenous EEA1 and LAMP1 by immunofluorescence imaging, where antibodies are commercially available. The highly specific PtdIns3P probe 2xFYVE should be used in addition to verify the results obtained by using p40PX-GFP.

We appreciate reviewer for these insightful suggestions. As suggested, we examined the PI3P level at EE or LE/lys in control or Tex2 KO cells using GFP-2xFYVE probing PI3P, anti-Lamp1 antibody labeling LE/lys, and anti-EEA1 antibody marking EEs in immunofluorescence. In contrary to our results using p40PX-GFP, we did not observe a substantial alteration in PI3P level between control and Tex2 KO cells. In the figure shown below, red arrows indicated PI3P at EEs, while yellow arrows denoted PI3P at LE/lys. The inconsistency made us question whether Tex2 KO affected the distribution of PI3P. Supporting this notion, inhibition of PI3P production on endosomes by PI3K inhibtor Vps34in did not substantially rescue the lysosomal defect resulting from Tex2 KO. The inhibition efficiency of Vps34IN-mediated PI3K inhibition was verified by the cytosolic distribution of PI3P probe p40PX-GFP. Therefore, these PI3P results (Fig. 10 in the original manuscript) were removed from the revised manuscript.



To get a better understanding how Tex2 KO affected lysosomal functions, we isolated the ER membranes and LE/lys membranes and analyzed the lipid compositon of these two membranes by non-targeted lipidomics using LC-MS/MS. The purity of LE/lys and ER membrane fractions were verified by western blots (Fig. 9, F and G). Lipidomics results showed that the levels of PG, Cer, DG of the ER or LE/lys fractions was not substantially affected (Fig. 9H). In contrast, the levels of PC and SPH of the ER fraction were significantly increased (Fig. 9H). Importantly, the level of SPH of

LE/lys was siginificantly increased in Tex2 KO HeLa cells compared to that of control cells, whereas the level of ceramide was not substantially changed upon Tex2 KO. This line of evidence suggest that the aberrant cuumulation of SPH might account for the lysosomal dysfunction in Tex2 KO cells. Indeed, accumulation of SPH, which were observed in lysosomes of Niemann-Pick disease type C patient cells (te Vruchte et al., 2004), were reported to disturb lysosomal calcium homeostasis (Hoglinger et al., 2015), a prerequisite for lysosomal functions and autophagy (Tedeschi et al., 2019). Moreover, the levels of PC and PE were also significantly changed in LE/lys fractions of Tex2 KO cells. In addition, our microscopy results using lipid probes showed that levels of PI(4,5)P2 and PI4P at LE/lys were higher in Tex2 KO than those in control cells. Collectively, these lines of evidence showed that Tex2 depletion resulted in a defect in lipid composition of LE/lys membranes, which might accounted for the lysosomal defect in Tex2 KO cells.

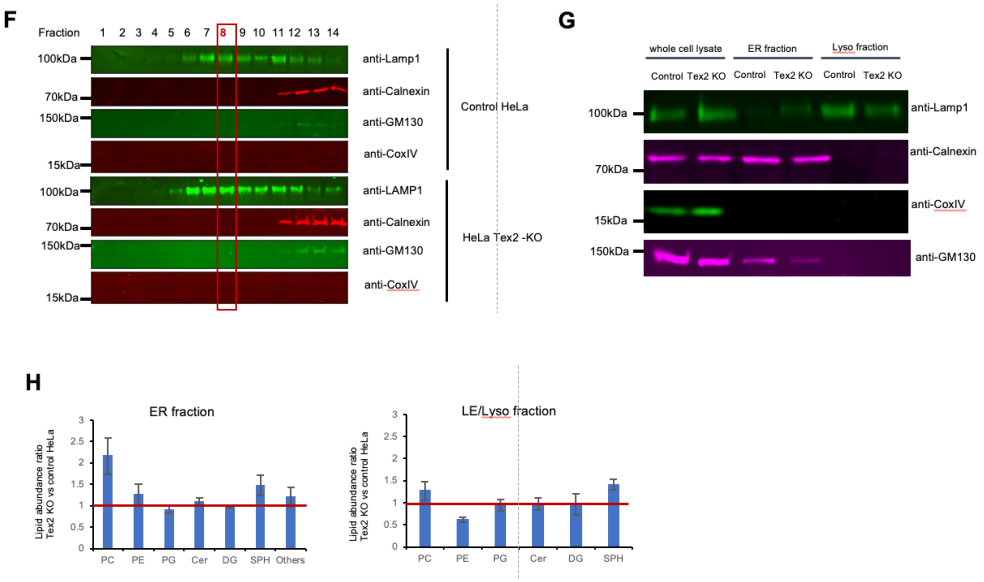


Fig. S3. The authors need to verify the level of KD for RTN4, REEP5, ARL6P1, RTN1 and RTNs by WB or qPCR to be able to draw the conclusion that the tubular ER localization of Tex2 is independent of these proteins.

Thanks for the comment. We examined mRNA levels of these ER shaping proteins in siRNA-treated cells by qPCR. These results were shown below, and were incorporated in the revised manuscript as Fig. S2 J.

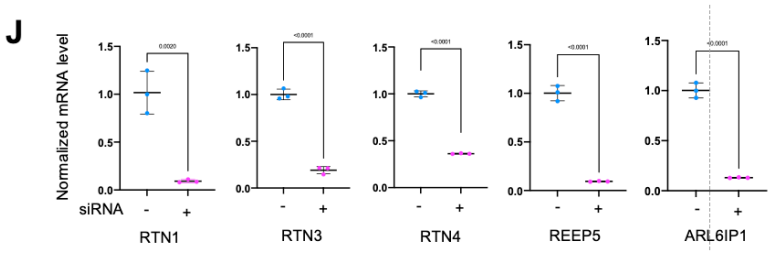
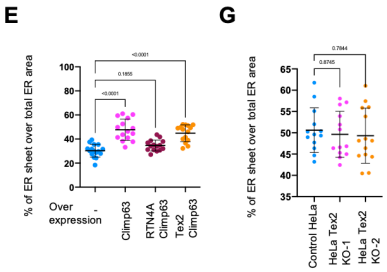


Fig. S4. From the representative images shown, it is plausible that GFP-Tex2 (similar to RTN4-GFP) counteracts the increased sheet formation caused by overexpression of Climp63-Halo, thus contributing to tubular ER shaping as the authors suggest. A quantification of sheets/tubules in the different conditions is required to support this conclusion.

Thanks for the comment. We quantified % of ER sheet over total ER area. The quantification method was described in the materials and methods section, shown below. Notably, our results showed that

Tex2 KO appeared not to substantially affect the tubular ER network at periphery, though Tex2 overexpression could counteract Climp63 to some extent, suggesting a redundant role of these tubular ER-resident proteins in the formation and/or maintenance of the tubular ER network. These quantification results were shown below, and were incorporated in the revised manuscript as Fig. S3, E and G.

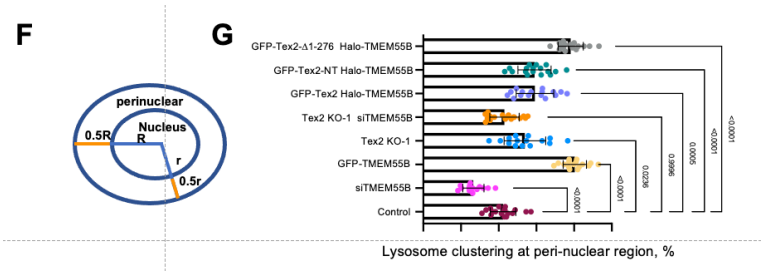
‘**Measurement of ER sheet abundance in cells.** Following methods described previously (Shibata et al., 2010) with modifications, ER sheet abundance was measured by calculating the percentage of the areas of ER sheet over the total ER area. In this quantification, the areas of ER sheets and tubules were determined from the fluorescence of Climp63 and RTN4, respectively, after subtraction of background. ‘



FigS5A, B. Please explain in the legend and/or methods how this analysis was carried out and how R/r was defined (which software, manual or automatic segmentation of lysosomes, fluorescence intensity or area, etc?).
Thanks for the comment. We explained how the analysis of LE/lys positioning in the materials and methods section shown below.

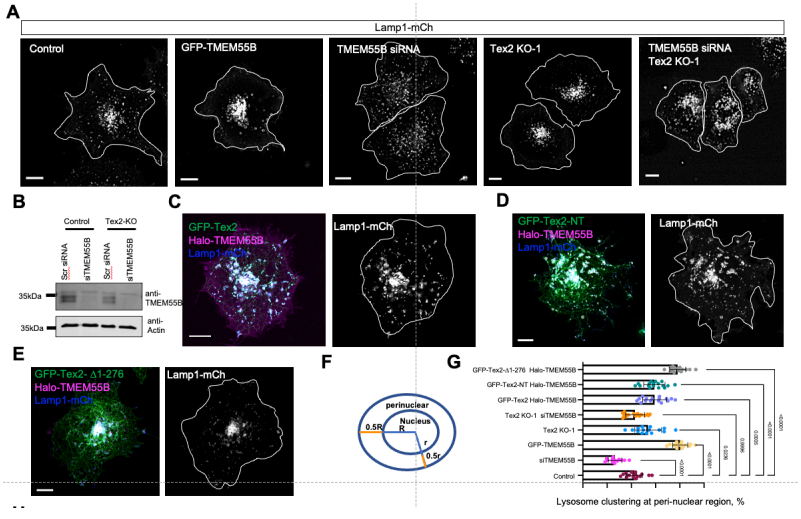
Quantification of the LE/lys positioning. Following methods described previously (Gao et al., 2022), the number of LE/lys was counted manually with assistance of ImageJ plugin Cell counter. For quantification of LE/lys positioning, perinuclear regions of cells were defined as shown in Fig. 7 F. Briefly, R was defined by the longer radius of the oval-shaped nucleus, while r was defined by the shorter radius of the oval-shaped nucleus. The length of R or r was measured by ImageJ. The perinuclear region was defined by a region within a distance of 0.5R/r to nucleus rim. To calculate the percentage of perinuclearly LE/lys, we manually counted the number of total LE/lys and perinuclearly LE/lys with the assistance of an ImageJ plugin Cell counter.

The axis is a bit misleading, claiming to show percentage, but rather shows the fraction of cells with dispersed or perinuclear lysosomes, counting up to a total of approximately 200 cells per condition, if I understand it correctly. The data could rather be represented as % of cells with perinuclear lysosomes, of the total cell population set to 100.
Thanks for the comment. We re-analyzed this dataset using the method shown above, and removed the Fig. S5 B of the original manuscript. These quantification results were shown below, and were incorporated in the revised manuscript as Fig. 6, F and G.



TMEM55B is involved in the transport of lysosoms to the cell centre important for lysosomal function (PMID: 29146937). In Fig. S5B, Tex2 counteracts the perinuclear clustering of TMEM55B positive lysosomes, whereas the deltaNT mutant (likely cytosolic) does not. Is it possible that the connection of TMEM55B positive lysosomes to the ER by Tex2 precludes the retrograde transport of lysosomes? Have the authors observed any change in lysosome positioning (perinuclear clustering) in Tex2KO cells? The overexpression of TMEM55B likely increases the perinuclear clustering of lysosomes, so this question is best answered by using the labelling of another lysosomal protein, like LAMP1.
Fig. S5C. I assume that the numbers indicate the time point (seconds?) of selected frames from a movie. Please indicate the frame rate of the movie and explain better in the legend. In my experience, a frame rate of 2-5Hz is suitable for tracking of individual vesicles, which might otherwise be lost. Please upload the corresponding movie. How many movies/endosomes is this representative of?
We thank reviewer for these insightful comments. These comments can be further divided into two sub-questions, which were addressed separately.

1) TMEM55B is involved in the transport of lysosoms to the cell centre important for lysosomal function (PMID: 29146937). In Fig. S5B, Tex2 counteracts the perinuclear clustering of TMEM55B positive lysosomes, whereas the deltaNT mutant (likely cytosolic) does not. Is it possible that the connection of TMEM55B positive lysosomes to the ER by Tex2 precludes the retrograde transport of lysosomes? Have the authors observed any change in lysosome positioning (perinuclear clustering) in Tex2KO cells? The overexpression of TMEM55B likely increases the perinuclear clustering of lysosomes, so this question is best answered by using the labelling of another lysosomal protein, like LAMP1.
As suggested, we examined a role of Tex2-TMEM55B interaction in regualting LE/lys positioning. We observed that LE/lys were substantially confined to perinuclear regions upon TMEM55B overexpression (Fig. 6, A, F and G), in accord with a reported role of TMEM55B in promoting the retrograde trafficking of LE/lys (Willett et al., 2017). Consistently, siRNA-mediated TMEM55B depletion resulted in a much more dispersed distribution of LE/lys compared to control HeLa cells (Fig. 6, A and G). Interestingly, we found that LE/lys were more clustered at perinuclear region in Tex2 KO cells comapred to the control cells, similar to the phenotype resulting from the suppression of another SMP-containing lipid transporter PDZD8 (Gao et al., 2022). Importantly, Tex2 KO could significantly rescue the dispersed distribution of LE/lys resulted from TMEM55B depletion (Fig. 6A, B, and F), suggesting that Tex2 may antagonize the effects of TMEM55B in the retograde tafficking. In addition, we observed that co-expression of GFP-Tex2 or GFP-Tex2-NT along with Halo-TMEM55B could partially allievated the clustering of LE/lys at perinuclear regions caused by Halo-TMEM55B overexpression (Fig. 6, C, D, F and G). However, co-expression of Tex2 without the NT (Tex2-Δ1-276) with TMEM55B had no effect (Fig. 6, E and G), suggesting a role of Tex2-NT in the regulation of TMEM55B-mediated LE/lys trafficking.



2) Fig. S5C. I assume that the numbers indicate the time point (seconds?) of selected frames from a movie. Please indicate the frame rate of the movie and explain better in the legend. In my experience, a frame rate of 2-5Hz is suitable for tracking of individual vesicles, which might otherwise be lost. Please upload the corresponding movie. How many movies/endosomes is this representative of?
We described the videos in details in the legend of Fig. 6, H and I, and also added the legends of video1/2 at the bottom of figure legend section in the revised manuscript, shown below. We also uploaded the two corresponding movies along with revised manuscript, representative of 12 COS7 cells transfected with ER-GFP and Lamp1-mCh, and 9 COS7 cells transfected with GFP-Tex2, Halo-TMEM55B, and Lamp1-mCh from 3 independent time-lapse imaging assays. The speed of the two videos could not reach 2-5Hz, due to the limitation of the microscope we used (LSM900) in 2-color or 3-color imaging.

Video 1. Representative time-lapse imaging of a COS7 cell expressing Lamp1-mCh (magenta) and ER-GFP (green) with a lysosome undergoing retrograde transport. Time interval 1.63 sec. Scale bar, 2μm. **Video 2**. Representative time-lapse imaging of a COS7 cell expressing GFP-Tex2 (green), Halo-TMEM55B (magenta), and Lamp1-mCh (blue) with a Halo-TMEM55B-positive lysosome tightly associating with GFP-Tex2-labeled ER membranes during intracellular transport. Time interval 2.53 sec. Scale bar, 2μm.

Fig. S5D. Please upload the corresponding movie.

Thanks for the comment. The corresponding movie was uploaded along with the revised manuscript.

Fig. S6B. How can you exclude that the perinuclear cloud observed upon co-expression of GFP-Tex2-KI and Halo-TMEM55B is not associated with the Golgi in TG treated cells? It would be informative to investigate this using a Golgi marker and high-resolution imaging.

Thanks for the comment. We examined the colocalization between GFP-Tex2-KI and MGAT2-Halo (a Golgi marker) upon DMSO or TG (6h), and we found that TG treatment did not substantially enhance the recruitment of Tex2 to the Golgi region, though the quantification showed a moderate increase level in colocalization (Fig. S3, I and J). In addition, endogenous GFP-Tex2 was still substantially recruited to Halo-TMEM55B-positive LE/lys, but not the Golgi, upon ER stress (Fig. S3, M and N).

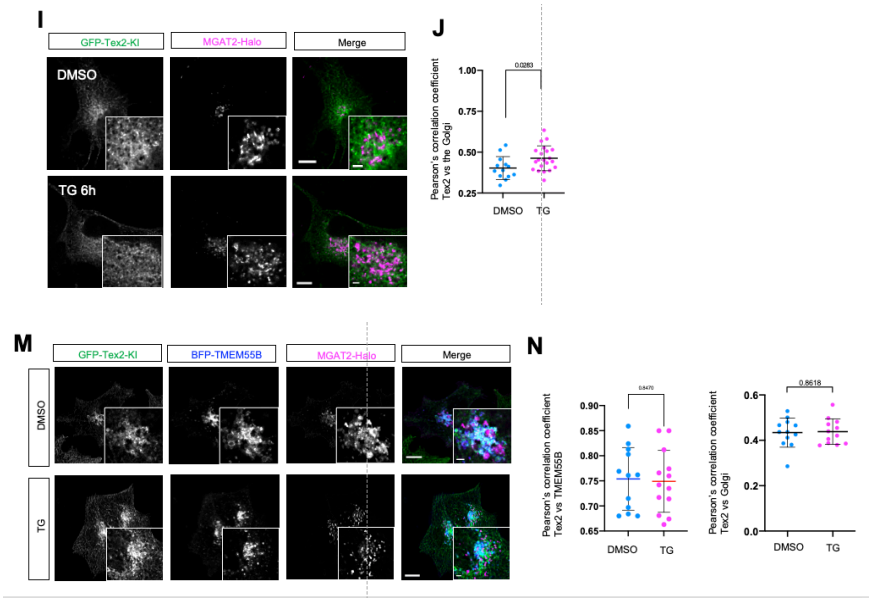


Fig S6D. Is this quantified after 6 or 12 hours of TG treatment?. How many experiments were performed for this analysis?

Thanks for the comment. This quantification was after 12 hours of TG treatment from more than 3 independent assays.

Missing in the Methods:

Please describe the generation of the GFP-Tex2-KI and Tex2 KO in the methods.

Thanks for the comment. We described the generation of the GFP-Tex2-KI and Tex2 KO in the methods, shown below.

CRISPR-Cas9-mediated gene editing. To make Tex2 KO HeLa cell lines, two gRNAs (5'-CCTCTGCACGTGCACTTTAG-3' and 5'-CAAGTTGGCCATGACCCCGC-3') were used to delete ~190 bp from exon 1 of Tex2 gene (Fig. S1 G). Complementary gRNAs were annealed and subcloned into the pSpCas9(BB)-2A-GFP (pX-458) vector (Addgene #48138) between BbsI endonuclease restriction sites. Upon transfection, HeLa cells were grown in antibiotic-free medium for 48 hours, followed by single cell sorting by flow cytometry. Two independent clones were verified by imaging and western blots (Fig. S1 H). To make GFP-Tex2-KI HeLa cell line, a single gRNA (5'-CCGGCAATGACAAGTCTGTA-3') was used to target the N-terminus of the Tex2 gene. HeLa cells were transfected with plasmids encoding the gRNA and a donor construct containing superfolder GFP (sfGFP) and two homologous arms using Lipofectamine 2000 (Fig. S1 E). 48 hours after transfection, single clones were sorted based on GFP fluorescence by flow cytometry. A positive clone was verified by imaging and western blots (Fig. S2 F).

From which supplier was the siRNA oligos purchased?

All the siRNA oligos used in this study were purchased from Ribobio (Guangzhou, China).

For the rescue experiments, please describe how the cells were reconstituted with Tex2. Transient or stable expression of HALO-Tex2?

Thanks for the comment. Two Tex2 KO clones were reconstituted with transient expression of Halo-Tex2 or related mutants. This information was added in the legends of corresponding figures in the revised manuscript.

Describe the reagents used, eg. thapsigargin, lysotracker, bafilomycin, rapamycin.

Thanks for the comment. All these reagents were described in the materials and methods section of the revised manuscript.

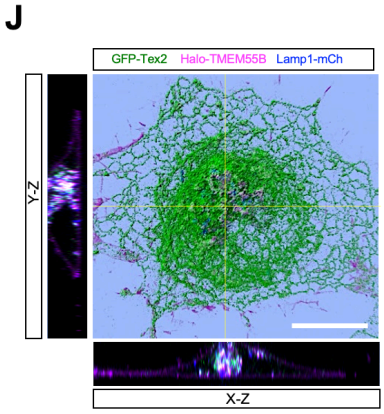
Typos and comments:
Although clearly understandable, the language of the manuscript needs improvement.

Thanks for this comment. As suggested, we have tried our best to polish the language of the manuscript and made it easier to read.
Manuscript line 178 refers to Fig. 3I, should be 3J.

Thanks for this catching the mistake. This msitake was corrected in the revised manuscript.

Manuscript line 179: ------"co-localization analysis based on x-y andy-z projections of 3D rendering". I cannot find the data for this co-localization analysis

Thanks for the comment. The x-y and y-z projections were shown in Fig. 2 J.



Manuscript line 291, Fig. 68G should be 6G.

Thanks for this catching the mistake, which was corrected in the revised manuscript.

December 5, 2022

RE: JCB Manuscript #202205133R

Dr. Wei-Ke Ji
Geisel School of Medicine at Dartmouth

Dear Dr. Ji:

Thank you for submitting your revised manuscript entitled "Tex2 is required for lysosomal functions at TMEM55-dependent ER membrane contact sites". The paper has now been seen again by the original reviewers, both of whom recommend acceptance. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

As you will see, reviewer #2 has raised a few very minor points that will need to be addressed in the final revision. The issues related to figure presentation should be easy to fix but please do provide a full response to this reviewer's final point regarding choice of statistical test.

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 and Tools is < 40,000, not including spaces. Count includes the abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, materials and methods, figure legends, references, tables, or supplemental legends. You are currently below this limit but please bear it in mind when revising.

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

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please provide the weight marker values for the blots in figure 9B and Supplementary figure 4C.

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 (both in the figure legend itself and 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, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) 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 (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

6) 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.

7) 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.).

8) 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.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. At the moment, you meet this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material (in addition to the supplementary figure legends) should appear at the end of the Materials and methods section.

10) 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. It should begin with "First author name(s) et al..." to match our preferred style.

11) 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."

12) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

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) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts.
While we see that you have provided these source data blots, they should be labeled as they appear in the figures (i.e. the conditions of each lane should be indicated at the top of each blot). In addition, the anti-TMEM55B blot from figure 3H appears to be missing from the source data file; please provide it with the final revised version.

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).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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.

Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit https://rupress.org/jcb/pages/submission-guidelines#videoSummaries.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7-14 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can

work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu.

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

Sincerely,

William Prinz, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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

In this revised manuscript, the authors have addressed all the concerned I raised with new experimental results and modified certain claims. It does not require revision.

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

In this revised manuscript from Du et al., the authors have addressed all my concerns, and I recommend the manuscript for publication in JCB. By performing important additional experiments and controls, they have strengthened the data regarding the role of Tex2 mediated ER-endosome contacts in lysosome function and positioning, related to the interaction with TMEM55 and a role for Tex2 in lipid transfer.
Thank you for providing a better description of the conditions for the videos in FigS6H,I, including frame rates. I apologize that I accidently wrote 2-5Hz, instead of 0.2-0.5 Hz. Your frame rates are within those values, and thus very suitable for your analysis.
I spotted a few minor errors that the authors might want to change before publishing:
The y-axes of some of the graphs do not start at "0". It would be good to indicate a broken axis in these cases. (FigS3 G, J N, FigS5 C,I, Fig1I, Fig4L)
Fig6G, X-axis numbering missing (%)
Line 248: Fig4H I, should be Fig3H, I (GST pulldown blots)
FigS5C. One sample t-test for normalized data might be a better test than the unpaired students t-test.

2nd Revision - Authors' Response to Reviewers: December 12, 2022

December 5, 2022

RE: JCB Manuscript #202205133R

Dr. Wei-Ke Ji
Geisel School of Medicine at Dartmouth

Dear Dr. Ji:

Thank you for submitting your revised manuscript entitled "Tex2 is required for lysosomal functions at TMEM55-dependent ER membrane contact sites". The paper has now been seen again by the original reviewers, both of whom recommend acceptance. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

As you will see, reviewer #2 has raised a few very minor points that will need to be addressed in the final revision. The issues related to figure presentation should be easy to fix but please do provide a full response to this reviewer's final point regarding choice of statistical test.

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Checked.

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

Checked.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please provide the weight marker values for the blots in figure 9B and Supplementary figure 4C.

We thank editor for this point. As suggested, the weight marker values for Supplementary figure 4C were added in the current revised manuscript.
Figure 9B showed blots of a native gel of purified Tex2-SMP proteins, and we did not run a native protein ladder for this assay. Therefore, we can not add weight marker values for this panel.

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 (both in the figure legend itself and 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, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

As suggested, we made a stament in the statistical analysis section of the revised manuscript by adding the sentence "Data distribution was assumed to be normal but this was not formally tested."

5) 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 (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

Checked.

6) 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.

Checked.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
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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.).

Checked.

8) 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.

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Checked.

10) 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. It should begin with "First author name(s) et al..." to match our preferred style.

Du. et al shows that Tex2, a potential lipid transporter on tubular endoplasmic reticulum, is recruited to late endosomes/lysosomes by endosome-resident phosphatidylinositol 4-phosphatases TMEM55 to regulate the lipid compositions, trafficking and functions of lysosomes.

11) 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."

Checked.

12) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

As suggested, we reformated the names of authors in the author contribution section of revised manuscript.

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.

We provided ORCID ID for each author.

14) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts.
While we see that you have provided these source data blots, they should be labeled as they appear in the figures (i.e. the conditions of each lane should be indicated at the top of each blot). In addition, the anti-TMEM55B blot from figure 3H appears to be missing from the source data file; please provide it with the final revised version.

We thank editor for this suggestion. As suggested, we re-labled all of source data blots as they appeared in the figures. In addition, we also added the souce data blot for anti-TMEM55B in figure 3H.

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).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

Checked.

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

Checked.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

We uploaded an image of a fixed GFP-Tex2 (green) knock-in HeLa cell with Halo-TMEM55B (magenta) and DAPI staining (blue) , showing a substantial recruitment of endogenous GFP-Tex2 to Halo-TMEM55B-positive late endosomes/lysosomes (LE/Lys) as well as a strinking peri-nuclear clustering of LE/lys.

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.

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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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Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit https://rupress.org/jcb/pages/submission-guidelines#videoSummaries.

Checked.

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Please contact the journal office with any questions, cellbio@rockefeller.edu.

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

Sincerely,

William Prinz, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

We are truly grateful to both reviewers for their constructive comments and insightful suggestions, which significantly improve the quality of this manuscript.

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

In this revised manuscript, the authors have addressed all the concerned I raised with new experimental results and modified certain claims. It does not require revision.

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

In this revised manuscript from Du et al., the authors have addressed all my concerns, and I recommend the manuscript for publication in JCB. By performing important additional experiments and controls, they have strengthened the data regarding the role of Tex2 mediated ER-endosome contacts in lysosome function and positioning, related to the interaction with TMEM55 and a role for Tex2 in lipid transfer.
Thank you for providing a better description of the conditions for the videos in FigS6H,I, including frame rates. I apologize that I accidently wrote 2-5Hz, instead of 0.2-0.5 Hz. Your frame rates are within those values, and thus very suitable for your analysis.
I spotted a few minor errors that the authors might want to change before publishing:

The y-axes of some of the graphs do not start at "0". It would be good to indicate a broken axis in these cases. (FigS3 G, J N, FigS5 C,I, , Fig4L)

We thank reviewer for this point. As suggested, we re-plot these graphs with y-axes starting at "0".

Fig6G, X-axis numbering missing (%)

We thank reviewer for pointing out this mistake. We have added the labels of X-axis of fig.6G in the revised manuscript.

Line 248: Fig4H I, should be Fig3H, I (GST pulldown blots)

Thanks for pointing out this mistake. We have corrected the typo in the revised manuscript.

FigS5C. One sample t-test for normalized data might be a better test than the unpaired students t-test.

Thanks for this suggestion. As suggested, we re-analyzed the normalized data using one sampe t-test.