EMBO reports

Homotypic SCOTIN assemblies form ER-endosome membrane contacts and regulate endosome dynamics

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Prof. Yoo

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting, but they also raise a number of concerns and have suggestions how to substantiate the conclusions that need to be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (April 9th). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

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We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends

in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

You can use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

Summary:
Key finding: this maunscript describes a previously unknown ability of the cytoplasmic domain of Scotin, an integral ER protein, to self-interact, and for the whole protein to also be in endosomes, which therefor become attached to ER by Scotin. Such a finding would be of significance and of genreal interest. At present it is not robustly documented.

This manuscript studies the type I ER membrane protein Scotin (SHSA5) in the context of endosomes. Scotin, both over-expressed and at more physiological levels (genomic insertion of GFP-11), localises fairly well to late endosomes marked by CD63. In cells lacking Scotin, these vesicles change their overall localization in cells (more centrifugal) and their relationship with the ER (lower PLA signal between late endosomal proteins and the ER). This is rescued by exogenous Scotin, but not when the entire 104 aa cytosolic domain is deleted, or when an internal group of 28 aa from near the start of this domain are missing. This entire domain is predicted to be disordered, and it is rich in prolines (~30%), with the 28 internal residues having a similar ratio of prolines to the rest of the domain. There is a striking finding that the entire cytoplasmic domain purified from bacteria forms large liquid droplets in vitro, while the peptide lacking the 28 internal residues does not. This leads the authors to conclude that Scotin is dually localized to ER and late endosomes, forming homotypic bridges to increase ER-endosome tethering.

While the overall idea is well supported by the in vitro finding of the liquid drop, there are several conclusions within the MS that need firming up and some minor issues over observations that do not fit the authors' descriptions.

Major issues:
Assessing ER-endosome contact: while PLA is a good way to probe proximity without having to (over-) express constructs, and can be used to see if knock-outs affect contact (e.g. PMID: 24105263) here the results are not useful enough. Firstly, they all rely on VAPA as the ER partner, so any change to the localization of VAPA to ER subcompartments will give a false signal. Secondly, the signals do not all make sense. Fig 3G shows that with Scotin KO and re-expressed Scotin held in the ER (RUSH system, pre biotin) there is virtually zero ER-endosome contact. If Scotin has such a dominant effect on endosome-ER proximity, then there should be data in other modalities, especially EM, to back this up. Otherwise, this suggests that the PLA signal can be heavily biased by unexplained factors. Finally, using MLN64 is potentially problematic as it sometimes partitions to endosome-mitochondrial contacts and this is not well understood.
Overall, transmission EM, which the MS already contains, is the gold-standard way of assessing contact, and I would expect that this should be used more, especially in key experiments. This can document the amount of each endosome that has ER on its surface, the length of each contact, and the distance across them. Endosomes can be marked using fluid-phase markers such as BSA-gold.

Where is Scotin?
Endogenous levels are only shown in Fig 2E, which shows partial colocalization with CD63. This should be put in context by repeating with other markers (LAMP, EEA1 and a negative control punctate marker) with some quantification.
However, there's now very little Scotin in the ER (Fig 2E, enlarged panels). One interpretation that fits the author's model is that all Scotin might be in junctional complexes in trans. This could be tested by co-expressing two different Scotin constructs on separate compartments to see if the ER localized protein largely enters into complexes: co-express GFP-Scotin in the ER (already made) with Cherry-Scotin-PRD targeted to another compartment (e.g. mitochondria, peroxisome) as a peripheral or integral membrane protein, such as the TMD of TOMM70 (PMIDs 9158224/ 19556461).

Compared to getting the localisation of protein expressed at endogenous levels,
other figures with over-expressed tagged constructs are not of great help as the constructs may be mislocalized by saturation of any targeting system that applies.

Minor

The claims implied by the wording of the title/abstract are too strong for the data presented. To support the current title, there would need to be comparison of multiple mutants of the cytosolic domain to show that the deleted region directly alters multimerization and nothing else (for example via an interaction partner) and/or reconstitution of the missing region using a

heterologous IDPR sequence that self assembles.

Page 9 (Line 246):
ER is not flat but likely tubular and much more curved than lyso/endosome
NB: interphase-> interface

Fig 5G: Str-Ii-SCOTIN Δ150-177 -SBP-EGFP is described as being in the ER but the pattern is more like a compartment adherent to intermediate filaments or similar, especially without biotin and also faintly with biotin (in the background).

Suppl Fig 5F: almost all the Str-Ii-SCOTIN Δ150-177 -SBP-EGFP construct runs 10 kD faster by SDS-PAGE when biotin is added. What does this indicate?

Very Minor
Fig 1A: needs key for blobs
Fig 5C: third blot down not aligned
Fig 5C: Text describing main result ("diminished", line 303) is too bland. Be more specific.

Referee #2:

In Yun et al., authors demonstrate that SCOTIN is a protein localizes to late endosomes and the homotypic assembly of SCOTIN controls membrane contact sites between the ER and endosomes. They further show that SCOTIN is needed for endosomal positioning due to its ability to bring endosomes and the ER together. I think the study is novel and contribute to the field of membrane contact sites, which is an emerging topic. However, I have one main concern regarding the ability of SCOTIN to localize at the ER and endosomes simultaneously to tether these compartments in cells (discussed below). Despite this, I find the study suitable for publication in EMBO Reports, given that the comments listed below are sufficiently addressed.

Major comments:
My main concern is the ability of SCOTIN to tether the ER and endosomes by localizing in both compartments simultaneously. The authors clearly show that SCOTIN can tether two membranes in vitro and its homotypic interaction is needed for the proximity of the ER and endosomes. This, however, does not conclude that the protein tethers these two organelles in a "MFN2-like interaction", i.e. a SCOTIN molecule in the ER interacting with another SCOTIN molecule on the endosome. A possibility is that the homotypic interaction of SCOTIN on the endosomal membrane, where it is mainly localized, would drive tethering via a different mechanism, e.g. interact with an ER protein. Along the same lines, if the homotypic interaction was responsible for tethering, the over-expression of SCOTIN should be sufficient to increase membrane contact sites; shown by many research groups that over-expression brings two membranes together.

Secondly, the ER localization of SCOTIN is not convincing and this supports my point. Many endosomal transmembrane proteins show ER localization, the site of their synthesis.

Using Str-Ii-SCOTIN-SBP-fluorescence protein to limit the SCOTIN localization to the ER is not ideal, as the ER looks unhealthy when this protein is over-expressed. To be able dissect the role of ER and lysosomal pools of SCOTIN to the membrane tethering, authors should use alternative methods. An option is attaching the cytosolic tail of SCOTIN to the well-established ER or lysosomal proteins, e.g. VAPA, SAC1, CD63, LAMP1.

Minor comments:
Line 122: It is not clear in the text why authors test Str-Ii-SCOTIN-SBP-DsRed "to verify the trafficking route" of Str-Ii-SCOTIN-SBP-EGFP. Please elaborate why this control was necessary.

Figure 3B and following similar images: The contrast of the PLA images is very low.

Line 179: "the outer membrane" should be "endosome delimiting membrane".

Line 185: "CELM" should be "CLEM".

Lines 201-208: seems repetitive.

Line 214: "SOCTIN" should be "SCOTIN"

Lines 308, 311, 313 "MLN63" should be "MLN64".

Referee #3:

In this paper, Authors examined subcellular localization of SCOTIN/SHISA5 and found that it mainly localizes on the late endosome (LE) membranes, in addition to the ER. By a combination of RUSH system and PLA analysis, they clarify that ER- as well as LE- localized SCOTIN are crucial for ER-LE contacts. Using an in vitro system, they show that the PRD domain of SCOTIN is required for in trans tethering of liposomes. The PRD domain deletion of SCOTIN elicits LE dispersion, suggesting that SCOTIN-mediated organelle tethering regulates LE perinuclear accumulation.
The RUSH and in vitro liposome analyses clearly show SCOTIN localization and tethering function. However, SCOTIN is also found in the LE lumen. This point shall be further analyzed to fully substantiate the conclusions of the paper

Major points
- SCOTIN localizes on endosomal membrane and lumen (Fig 2A & F). However, authors assumed that SCOTIN is an integral membrane protein, because of the predicted transmembrane domain. The authors should investigate whether SCOTIN is membrane integral, and whether its membrane integration is crucial for its organelle tethering function.

Minor points
- color differences in the time lapse images of Fig 6G & H are difficult to distinguish. Please change the color scale

Luca Scorrano

Dear Dr. Martina Rembold, the Editor of ***EMBO reports***,

Thank you for giving us the opportunity to submit a revised manuscript titled "**Self-assembly of SCOTIN on heterologous membranes controls endosome dynamics via ER membrane contact**" to *EMBO reports*. We appreciate the time and effort that you and the reviewers have dedicated to providing your valuable comments on our manuscript. We have been able to incorporate changes to reflect all the suggestions provided by the reviewers. We hope that our revised manuscript is satisfactory, as we have tried our best to address the issues raised by the reviewers.

The following are the highlights or our new data added to the revised manuscript:

- The artificial organelle membrane-targeted PRD chimera tether targeted the membrane.
- TEM analysis demonstrated that amino acids 150-177 of SCOTIN are necessary for ER and endosome membrane contact.
- Membrane integration of SCOTIN is crucial for membrane tethering.

Please see our point-by-point responses to the reviewers' comments below. Each answer is marked in **red** and indicated with an arrow before the next comment.

Answers to Reviewer 1.

Summary:

Key finding: this maunscript describes a previously unknown ability of the cytoplasmic domain of Scotin, an integral ER protein, to self-interact, and for the whole protein to also be in endosomes, which therefor become attached to ER by Scotin. Such a finding would be of significance and of general interest. At present it is not robustly documented.

This manuscript studies the type I ER membrane protein Scotin (SHSA5) in the context of

endosomes. Scotin, both overexpressed and at more physiological levels (genomic insertion of GFP-11), localizes fairly well to late endosomes marked by CD63. In cells lacking Scotin, these vesicles change their overall localization in cells (more centrifugal) and their relationship with the ER (lower PLA signal between late endosomal proteins and the ER). This is rescued by exogenous Scotin but not when the entire 104 aa cytosolic domain is deleted or when an internal group of 28 aa from near the start of this domain is missing. This entire domain is predicted to be disordered, and it is rich in prolines (~30%), with the 28 internal residues having a similar ratio of prolines to the rest of the domain. There is a striking finding that the entire cytoplasmic domain purified from bacteria forms large liquid droplets in vitro, while the peptide lacking the 28 internal residues does not. This leads the authors to conclude that Scotin is dually localized to ER and late endosomes, forming homotypic bridges to increase ER-endosome tethering.

While the overall idea is well supported by the in vitro finding of the liquid drop, there are several conclusions within the MS that need firming up and some minor issues over observations that do not fit the authors' descriptions.

Major issues:

Assessing ER-endosome contact: while PLA is a good way to probe proximity without having to (over) express constructs, and can be used to see if knock-outs affect contact (e.g. PMID: 24105263) here the results are not useful enough. First, they all rely on VAPA as the ER partner, so any change to the localization of VAPA to ER subcompartments will give a false signal.

→ To address the reviewer's concern, we performed two different kinds of experiments. First, to exclude the possibility of false PLA signals resulting from changes in the cellular localization of VAPA, we evaluated the endogenous VAPA distribution patterns in WT and KO cells with the general ER membrane marker Sec61β. The Airy-scan images show that the VAPA signals generally colocalize with that of Sec61β, similarly in KO and WT cells. We added superresolution images and plot profile graphs of VAPA in HeLa and KO cells.[Figures for referees not shown.]

→ Second, we performed a new PLA assay with another ER membrane protein, RTN4. PLA using RTN4 (the ER membrane partner) and Rab7 (the late endosome membrane partner) showed similar results: membrane contact between the ER and endosome was significantly reduced in SCOTIN-KO cells (Figure EV3A, B).

Second, the signals do not all make sense. Fig 3G shows that with Scotin KO and re-expressed Scotin held in the ER (RUSH system, pre biotin), there is virtually zero ER-endosome contact. If Scotin has such a dominant effect on endosome-ER proximity, then there should be data in other modalities, especially EM, to back this up. Otherwise, this suggests that the PLA signal can be heavily biased by unexplained factors.

→ First, we would like to thank the reviewer for pointing out this issue. It was a false-negative situation during the particle analysis macro in ImageJ. We were very cautious to avoid false positive detection of the PLA signal. For this reason, we usually set the threshold stringently. In this case, however, we did not realize that our threshold setting was too high to cause false negatives.

→ We requantified the PLA signals with lower threshold values. In this case, the overall numbers of PLA-positive spots increased, and PLA-positive spots in the cells of ER-retained SCOTIN (-Biotin) were clearly detected. In contrast to the negative control (with VAPA only staining), the number of PLA-positive spots was significantly different. For your reference, we presented old and new data below (side by side). In the revised manuscript, we replaced the figure with re-quantified data in Figure 5B, C. Additionally, in the Methods section, we explained in detail how we performed image analysis (particle analysis) using the ImageJ program.[Figures for referees not shown.]

Finally, using MLN64 is potentially problematic, as it sometimes partitions to endosome-mitochondrial contacts, and this is not well understood.

→ Thank you for the valuable information. To avoid any potential problem, we reperformed the PLA assay using VAPA-Rab7 (Figure 6D, E) and RTN4-Rab7 (Figure EV5F, G). In both cases, our finding that full-length SCOTIN increased ER-endosome membrane contact, but not the PRD or 150-177 deletion mutants, was reproduced.

Overall, transmission EM, which the MS already contains, is the gold-standard way of

assessing contact, and I would expect that this should be used more, especially in key experiments. This can document the amount of each endosome that has ER on its surface, the length of each contact, and the distance across them. Endosomes can be marked using fluid-phase markers such as BSA-gold.

→We completely agree with the reviewer's point that TEM is a better way to demonstrate contact. To meet the reviewer's critiques, we performed large sets of new experiments to visualize ER-endosome membrane contact in parental HeLa, SCOTIN-KO HeLa, SCOTIN-KO + SCOTIN-DsRed, and SCOTIN KO + SCOTINΔ150-177-DsRed HeLa cells. To assess ER-endosome membrane contact, we quantified the percentage of endosomes with ER on its surface, the length of each contact, and the distance between the ER and endosome membrane. Data presented in Figure 7.

→ Consistent with the PLA data, TEM analysis also confirmed that SCOTIN was proximate to the ER and endosome membranes. And the 150-177 region was required for this regulation.

Where is Scotin?
Endogenous levels are only shown in Fig 2E, which shows partial colocalization with CD63. This should be put in context by repeating with other markers (LAMP, EEA1 and a negative control punctate marker) with some quantification.

→ We presented superresolution images of endogenous SCOTIN (GFP11 knock-in cell) with marker proteins in Figure EV2D. Endogenous SCOTIN with Sec61β (an ER membrane marker), EEAl (early endosome marker), CD63 (late endosome marker), transferrin receptor (recycling endosome marker), and LAMP1 (lysosome marker) was tested. The plot profile macro in ImageJ was used to obtain line-scan graphs representing the fluorescence intensity of endogenous SCOTIN KI and the indicated marker proteins along the length of a line.

→ Additionally, we added new quantification data that measured the Pearson correlation coefficient between endogenous SCOTIN (GFP signal) and the indicated vesicle markers (Figure EV2E).

However, there is now very little Scotin in the ER (Fig 2E, enlarged panels).

→ SCOTIN was initially reported as a single-pass transmembrane protein localized in the ER and nucleus (PMID: 12135983). We also reported that overexpressed SCOTIN localizes within the subdomain of the ER, especially near the ERES (PMID: 34720018).

→ As we demonstrated in this manuscript, SCOTIN leaves the ER and is transported to other organelles, such as endosomes. Since most of the previous SCOTIN works have focused on the ER, we focused on emphasizing endosome membrane localization. To address the reviewer's question directly, we evaluated the localization of endogenous SCOTIN by costaining SCOTIN KI cells with RTN4, an ER marker, and CD63, an endosome marker, and observed Z stack projections (Figure 2E).

One interpretation that fits the author's model is that all Scotin might be in junctional complexes in trans. This could be tested by coexpressing two different Scotin constructs on separate compartments to see if the ER localized protein largely enters into complexes: coexpress GFP-Scotin in the ER (already made) with Cherry-Scotin-PRD targeted to another compartment (e.g. mitochondria, peroxisome) as a peripheral or integral membrane protein, such as the TMD of TOMM70 (PMIDs 9158224/19556461).

→ First, we would like to thank reviewer for this very interesting suggestion. We agree that it is worth trying whether forced expression of SCOTIN-PRD in trans functions to bring membranes (or organelles) closer.

→ As the reviewer suggested, we constructed MTS-PRD (mitochondria-targeted PRD) and CD63-PRD (endosome-targeted PRD) along with Sec61-PRD (ER-targeted PRD), and their colocalization was verified using superresolution image analysis (Figure 5D-G and Figure EV4D, E).

→ Based on this experiment, we observed that 1) forced expression of PRD on the membrane severely affects its morphology and 2) the distribution of the organelle membrane.

→ The endosome-targeted PRD chimera data show that SCOTIN (PRD) forms a junctional complex in transmembranes as well as membrane tethering (Figure 5D-G).

→ In contrast to MTS, which is distributed through the mitochondria, MTS-PRD accumulates at mitochondrial subdomains (Reviewer Figure 3)[Figures for referees not shown.]. In addition, cells transfected with MTS-PRD showed finely fragmented mitochondrial phenotypes.

Compared to getting the localization of protein expressed at endogenous levels, other figures with overexpressed tagged constructs are not of great help as the constructs may be mislocalized by saturation of any targeting system that applies.

→ As recommended, overexpressed SCOTIN data were minimized in revised manuscript in Figure EV1.

Minor

The claims implied by the wording of the title/abstract are too strong for the data presented. To support the current title, there would need to be comparison of multiple mutants of the cytosolic domain to show that the deleted region directly alters multimerization and nothing else (for example via an interaction partner) and/or reconstitution of the missing region using a heterologous IDPR sequence that self assembles.

→ To meet the reviewer's comments, we toned down the title, from the "Intrinsically

disordered region-mediated assembly of SCOTIN/SHISA5 on heterologous membranes controls endosome trafficking via ER contact regulation" to "**Self-assembly of SCOTIN on heterologous membranes controls endosome dynamics via ER membrane contact**". Additionally, the introduction, abstract and discussion sections were accordingly changed.

Page 9 (Line 246):
ER is not flat but likely tubular and much more curved than lyso/endosome

→ We changed the description.

NB: interphase-> interface

→ We apologize for these mistakes. Those typos are fixed.

Fig 5G: Str-Ii-SCOTIN Δ150-177 -SBP-EGFP is described as being in the ER but the pattern is more like a compartment adherent to intermediate filaments or similar, especially without biotin and faintly with biotin (in the background).

→ As the reviewer pointed out, we noticed that Str-Ii-SCOTIN(Δ150-177)-SBP-EGFP shows perinuclear condensed tubular structures. A recent study (preprint: Tom Cremer et al., 2022. bioRxiv doi: https://doi.org/10.1101/2022.03.24.485587) suggests that ER stress induces massive expansion of the perinuclear ER, congregating with Vimentin filaments.

→ Because Str-Ii-VSVG-SBP-EGFP also shows perinuclear expanded ER morphologies when it is entrapped in the ER, we speculate that the accumulation of cargo proteins in the ER (without biotin) might induce ER stress in the RUSH system.

Suppl Fig 5F: almost all the Str-Ii-SCOTIN Δ150-177 -SBP-EGFP construct runs 10 kD faster by SDS–PAGE when biotin is added. What does this indicate?

→ Yes. Not only Str-Ii-SCOTIN Δ150-177 -SBP-EGFP but also Str-Ii-SCOTIN-SBP-EGFP showed distinct bands by SDS–PAGE when biotin was added. We were aware of this phenomenon. We postulate that SCOTIN experiences cleavage during trafficking.

→ In fact, when we blot SCOTIN-Myc or SCOTIN Δ150-177-Myc, we also observed a fast

migrating band, indicating a cleavage event (see below)[Figures for referees not shown.] However, since it is beyond our current scope investigated in this manuscript, we did not incorporate these data in the revised manuscript.

Very Minor

Fig 1A: needs key for blobs

→ Yes. We changed the RUSH working model with a key for blobs.

Fig 5C: third blot down not aligned

→ Yes. We aligned the western blot image in the revised figure (Figure 6C).

Fig 5C: Text describing main result ("diminished", line 303) is too bland. Be more specific.

→ Yes. We changed the description of the GST pull-down results in the Results section.

Answers to Reviewer 2.

Yun et al. demonstrated that SCOTIN is a protein that localizes to late endosomes and that the homotypic assembly of SCOTIN controls membrane contact sites between the ER and endosomes. They further show that SCOTIN is needed for endosomal positioning due to its ability to bring endosomes and the ER together. I think the study is novel and contribute to the field of membrane contact sites, which is an emerging topic. However, I have one main concern regarding the ability of SCOTIN to localize at the ER and endosomes simultaneously to tether these compartments in cells (discussed below). Despite this, I find the study suitable for publication in EMBO Reports, given that the comments listed below are sufficiently addressed.

Major comments:

My main concern is the ability of SCOTIN to tether the ER and endosomes by localizing in both compartments simultaneously. The authors clearly show that SCOTIN can tether two membranes in vitro and its homotypic interaction is needed for the proximity of the ER and endosomes. This, however, does not conclude that the protein tethers these two organelles in a "MFN2-like interaction", i.e., a SCOTIN molecule in the ER interacting with another SCOTIN molecule on the endosome.

A possibility is that the homotypic interaction of SCOTIN on the endosomal membrane, where it is mainly localized, drives tethering via a different mechanism, e.g., interaction with an ER protein.

→ We agree that the possibility of homotypic interaction of SCOTIN on the endosomal membrane could drive tethering with another ER protein. To exclude this possibility, we performed PLA after reconstitution of the ER- or endosome-tethered PRD in SCOTIN-KO cells (Figure 5G). Although PRD on endosomes could slightly increase ER-endosome contact, the results show that when PRD attaches to both the ER and endosome membrane, it significantly tethers each membrane.

Along the same lines, if the homotypic interaction was responsible for tethering, the overexpression of SCOTIN should be sufficient to increase membrane contact sites, as shown by many research groups that overexpression brings two membranes together.

→ To address the reviewer's comments, we performed PLA with SCOTIN overexpression. For this, HeLa cells were transfected with SCOTIN-DsRed, and ER-endosome membrane proximity was assessed with PLA using VAPA-Rab7 (Figure 3B, C). We observed that SCOTIN overexpression itself increases the contact between the ER and endosome, as you mentioned.

Second, the ER localization of SCOTIN is not convincing and this supports my point. Many endosomal transmembrane proteins show ER localization, the site of their synthesis.

→ SCOTIN was initially reported as a single-pass transmembrane protein localized in the ER and nucleus (PMID: 12135983). We also reported that overexpressed SCOTIN localizes within the subdomain of the ER, especially near the ERES (PMID: 34720018).

→ As we demonstrated in this manuscript, some SCOTIN leaves the ER and is transported to other organelles, such as endosomes, while others remain in the ER (Figure 1F). Since most of the previous SCOTIN works have focused on the ER, we focused on emphasizing endosome membrane localization. To address the reviewer's question directly, we evaluated the localization of endogenous SCOTIN by costaining SCOTIN KI cells with RTN4 and CD63 and presented a Z stack projection (Figure 2E).

Using Str-Ii-SCOTIN-SBP-fluorescence protein to limit SCOTIN localization to the ER is not ideal, as the ER looks unhealthy when this protein is overexpressed.

→ We utilized Str-Ii-SCOTIN-SBP-EGFP to manipulate the cellular localization of SCOTIN to the ER or endosomes. As we mentioned above (line 240 in response letter), the accumulation of protein on the ER membrane induces perinuclear expansion of the ER membrane (preprint: Tom Cremer et al., 2022. bioRxiv doi: https://doi.org/10.1101/2022.03.24.485587).

→ We replaced the representative images of Str-Ii-SCOTIN-SBP-EGFP showing the ER tubule structure (Figure 5B).

To be able dissect the role of ER and lysosomal pools of SCOTIN to the membrane tethering,

authors should use alternative methods. One option is attaching the cytosolic tail of SCOTIN to well-established ER or lysosomal proteins, e.g., VAPA, SAC1, CD63, and LAMP1.

→ As the reviewer suggested, we constructed CD63-PRD (endosome-targeted PRD) along with Sec61-PRD (ER-targeted PRD). We found that endosome-targeted PRDs were closely localized with ER-targeted PRDs in the Airyscan superresolution image analysis (Figure 5D-F and Supplementary Figure 5D).

→ The membrane tethering between the ER and endosomes were evaluated with PLA in chimera expressed SCOTIN KO cells (Figure 5G).

Minor comments:

Line 122: It is not clear in the text why authors test Str-Ii-SCOTIN-SBP-DsRed "to verify the trafficking route" of Str-Ii-SCOTIN-SBP-EGFP. Please elaborate why this control was necessary.

→ Str-Ii-SCOTIN-SBP-DsRed is not the conrol for the Str-Ii-SCOTIN-SBP-EGFP. We used another construct for immunofluorescence imaging with endosome markers. We revised the manuscript to prevent this confusion in the Results section.

Figure 3B and following similar images: The contrast of the PLA images is very low.

→ We agree with the reviewer's comment that the representative PLA images are well distinguished. For better visualization, we edited PLA images with a lower threshold in Figure 3D, Figure 5B, and Figure 6G.

Line 179: "the outer membrane" should be "endosome delimiting membrane".

→ Yes. The sentence identified by the reviewer has been corrected in the revised manuscript.

Line 185: "CELM" should be "CLEM".

→ We apologize for these mistakes. Those typos are fixed.

Lines 201-208: seems repetitive.

→Yes. We changed the sentence to minimize repetition in the revised manuscript.

Line 214: "SOCTIN" should be "SCOTIN"

→ We apologize for these mistakes. Those typos are fixed.

Lines 308, 311, 313 "MLN63" should be "MLN64".

→ We apologize for these mistakes. Data using MLN64 were deleted in revised manuscript.

Answers to Reviewer 3.

In this paper, Authors examined subcellular localization of SCOTIN/SHISA5 and found that it mainly localizes on the late endosome (LE) membranes, in addition to the ER. By a combination of the RUSH system and PLA analysis, they clarified that ER- and LE-localized SCOTIN are crucial for ER-LE contacts. Using an in vitro system, they show that the PRD domain of SCOTIN is required for in trans tethering of liposomes. The PRD domain deletion of SCOTIN elicits LE dispersion, suggesting that SCOTIN-mediated organelle tethering regulates LE perinuclear accumulation.
The RUSH and in vitro liposome analyses clearly show SCOTIN localization and tethering function. However, SCOTIN is also found in the LE lumen. This point shall be further analyzed to fully substantiate the conclusions of the paper

Major points
- SCOTIN localizes on endosomal membrane and lumen (Fig 2A & F). However, the authors assumed that SCOTIN is an integral membrane protein because of the predicted transmembrane domain. The authors should investigate whether SCOTIN is membrane integral, and whether its membrane integration is crucial for its organelle tethering function.
→ We agree with the reviewer that we assumed that SCOTIN is an integral membrane protein. From the first report that SCOTIN is a single-pass transmembrane protein localized in the ER and nucleus (PMID: 12135983), all published studies designated SCOTIN as a transmembrane protein based on sequence analysis (PMID: 17889823, 15116103, 26868272, 34720018) without experimental evidence.

→ To address the reviewer's question directly, we performed a proteinase K protection assay after SCOTIN-Myc expression in SCOTIN KO cells. The data clearly show that SOTIN

localizes in the postnuclear supernatant fraction but not in the cytosolic fraction. Furthermore, proteinase K with or without Triton X-100 degraded SCOTIN-Myc, demonstrating that SCOTIN is not a luminal protein (Figure 2A).

→ To demonstrate whether membrane integration is crucial for its organelle tethering function, we quantified membrane proximity with PLA using SCOTIN truncation mutants. First, we transfected pcDNA, SCOTIN, TMPRD, or PRD-Myc into SCOTIN KO cells and performed superresolution fluorescence imaging (Figure EV3F). We quantified ER-endosome membrane contact using PLA. The data show that the membrane-anchored constructs (KO + SCOTIN or TMPRD) increase membrane contact, not in the cytosol domain only (KO + PRD), compared to the control (KO + pcDNA) (Figure 3G, H).

Minor points

- color differences in the time lapse images of Fig 6G & H are difficult to distinguish. Please change the color scale

→ As requested, we revised the figure showing the pseudocolored time lapse (Figure 8G, H).

Dear Prof. Yoo

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and recommend its publication.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

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Kind regards,

Martina Rembold, PhD
Senior Editor
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Referee #1:

I thank the authors for their extra work and constructive engagement with all the reviewers' comments - they have done a good job of addressing the outstanding issues

Referee #2:

The authors have addressed my points adequately.

Referee #3:

Authors addressed my concern. Congratulations on a beautiful story.

The authors have addressed all minor editorial requests.

Prof. Joo-Yeon Yoo
POSTECH
Life Science
San 31 Hyojadong
Pohang, Kyungbuk 37673
Korea, Republic of

Dear Prof. Yoo,

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