The ER cholesterol sensor SCAP promotes CARTS biogenesis at ER-Golgi membrane contact sites

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Re: JCB manuscript #202002150

Dr. Yuichi Wakana
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Dear Dr. Wakana,

Thank you for submitting your manuscript entitled "The ER cholesterol sensor SCAP promotes CARTS biogenesis at ER-Golgi 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 while the reviewers find the topic of your manuscript interesting, they have differing opinions on the suitability of your study currently for JCB. I agree that your finding that SCAP promotes CARTS biogenesis is of significant potential interest to the readership of JCB, and will open up future potential work into the molecular details. However, I agree that experimental revisions are necessary to support your key claims. In particular, your revision must address these points for reconsideration at JCB:

1. Strengthening the data that Sac1(K2A) mutant is indeed in the ER side of ER/TGN contacts and not on the Golgi side as shown by many studies (Rev2. Point 1). Your iFRAP experiments alone are not convincing enough to support this claim.
2. Testing if the defect is specific for CARTS (Rev 2, point 2)
3. Showing interaction between endogenous proteins (Rev 2. General comments and Rev3 point 2.)
4. Testing the mutant SCAP suggested by Rev3 point 5.
5. Testing if the transcriptional activity of SREBP1/2 is involved (Rev 2. point 6)

However, I do not expect you to expand the study with more cholesterol measurements (point 1 of Rev3), or testing the of target effects (point 7 of Rev3). Furthermore, while points 3 and 4 of Rev3 are valid, they do not need to be experimentally addressed in this study.

In addition, please be sure to address all minor comments.

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

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.
Reviewer #1 (Comments to the Authors (Required)):

This is a beautiful and extremely interesting paper showing a role for a key ER cholesterol sensor (SCAP) in the formation of a class of Golgi derived transport vesicles called CARTS. SAC1 (PtdIns4P phosphatase) is involved in the process by which VAP on the ER and OSBP on the Golgi (or VAP and CERT) transport cholesterol or ceramides, respectively, in exchange for PtdIns4P, between ER and late Golgi. Here the authors report that SCAP is found in association with SAC1 at these sites and its depletion increases PtdIns4P levels at the TGN. This is accompanied by a decrease in the formation of CARTS vesicles.
of CARTS. This is super cool and beautifully demonstrated here. My comments are minor and offered with the goal of improving clarity for the reader.

When ER cholesterol is low, SCAP escorts SREBPs into COP-II vesicles. When cholesterol is high, SCAP binds INSIG and blocks SREBP transport. Y234A locks SCAP to INSIG independent of cholesterol, and Y298C blocks INSIG binding, which should yield constitutive transport to Golgi (their ref 58). Yet the authors do not see this and surprisingly, their CARTS and PI4P phenotypes are the same despite two different types of mutation. I think it would be important to explain more clearly possible explanations for why the two distinct classes of mutants give the same phenotype--fewer CARTS and more PI4P. Is it just differences in expression level? Different cell types? Similar effects on INSIG localization? This is really important to address and clarify for the reader, together with a clearer explanation of the differences in the findings compared with Ref. 58.

Otherwise the work is important and should be published without delay in JCB.

Minor: Supp FIG1A,B seem more important than Figs. 1 & 3; Discussion line 4 replace Posterior with Subsequent

-Suzanne Pfeffer (Signed review)

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

This manuscript addresses regulation of secretion via Golgi-derived carriers referred to as 'CARTS'. In earlier studies, it was shown that depletion of factors required for VAP-A/B- and OSBP-mediated ER-TGN contacts sites cause a delay in secretion and misprocessing of glycans of CARTS cargo. The authors proposed that cholesterol and/or sphingolipid transport/biosynthesis contributes to regulated CARTS formation at the Golgi. The starting point for this study is the discovery that SCAP associates with components of the cholesterol/PI4P counter transport machinery that operates at sites of the ER-TGN contact. The authors report that SCAP associates with a VAP-A/OSBP/Sac1 complex that is proposed to be enriched at sites of ER-TGN contact. That SCAP influences OSBP-mediated cholesterol transport is suggested by indirect evidence showing that the OSBP cholesterol counter-ligand, PI4P, accumulates on Golgi membranes in SCAP depleted cells. In addition, OSBP is reported to accumulate at sites of ER-endosome contact in SCAP depleted cells. Assays of CARTS-mediated protein secretion show that CARTS formation at the Golgi and rate of cargo secretion are significantly reduced in SCAP depleted cells and that mutant SCAPs that are unable to sense cholesterol in the SREBP pathway, do not rescue CARTS secretion.

This is a high quality, well-controlled study that makes a generally compelling case that SCAP functions to promote secretion via CARTS formation at the TGN. It’s an interesting finding, in part because this appears to be an unexpected role for SCAP that is unrelated to its function in controlling SREBP activation, though at present it is unclear how SCAP might regulate CARTS formation. Despite the strengths of the study, I have three general concerns.

1. The conclusion that Sac1/K2A accumulates in ER membrane domains that are site of ER-TGN contact is at odds with published reports showing that its localization favors the Golgi, in comparison to the native sequence protein whose localization favors the ER. The co-localization analyses of antigens residing in the peri-nuclear region are ambiguous, as signals in this region of
cells are quite dense. The results of the photobleaching experiment shown in Figure 1C show that some amount of GFP-Sac1 accesses ER membrane, but most does not and this pool can reasonably be assumed to reside in the Golgi. Note that Dippold et al (Cell, 2012; not cited) used Sac1/K2A to deplete Golgi membrane of PI4P. More generally, the conclusion that native Sac1 is enriched in a specialized ER-Golgi contact site sub-domain is not rigorously demonstrated in this study or in the authors' previous publication (ref 34). Zewe et al 2018 (not cited) presented a rigorous analysis of Sac1 localization, concluding that Sac1 is not enriched in any particular aspect of the ER. It may be results obtained for tagged, ectopically over-expressed proteins used in this study are misleading. The small amount of SCAP that is observed to localize to the Golgi is also in line with the small amount that co-purifies with ER-Golgi tethering proteins.

2. The appearance of PAUF in figure 5B raises a concern that SCAP depletion causes a general deficiency of Golgi function. In the lysates, no modified/mature from of PAUF is observed in the SCAP KD lane, and the mobility of the secreted fraction also appears to be reduced, suggesting that Golgi-dependent glycosylation is compromised by SCAP depletion. Thus, Golgi function may be generally compromised and it is important to determine if this is the case as it relates to the issue of specificity of SCAP for the CARTS pathway. Is glycosylation of other secreted glycoproteins affected?

3. Does SCAP depletion affect secretion in general, or just CARTS, as the authors suggest? The conclusion would be more rigorously tested if the authors examined a non-CARTS cargo, obviously if it does not exhibit the same pattern. In addition, the conclusion that CARTS forms immediately adjacent to sites of ER-Golgi contact is not rigorously supported. Visible light microscopy does not provide sufficient resolution to support the conclusion, at least as implemented. In addition,

4. Minor comment: Many of the micrographs would be more easily inspected (by me, at least) if the grayscale were inverted.

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

This manuscript tried to establish that the Scap/SREBP complex is required to export cholesterol from the ER to Golgi by stimulating the PI4P phosphatase activity of Sac1 in the ER, and thus required to maintain cholesterol levels in Golgi to support secretion of proteins through CARTS. Unfortunately, the model shown in Fig. 9 is not supported by the data provided in the manuscript. The paper is further weakened by the lack of controls.

1. A key prediction made by the model shown in Fig. 9 is that deficiency in Scap should impair the ER to Golgi transport of cholesterol. However, there is no data to demonstrate that knocking down Scap increases amounts of cholesterol in the ER and decreases that in the Golgi. In Fig. 4A the authors argued that Filipin staining may not be sensitive enough for this purpose. This should not be used as an excuse, as other approaches such as measuring ACAT activity and determining cholesterol contents in purified subcellular organelles by MS can be used to determine the amounts of cholesterol in ER and Golgi.

2. The Scap/SREBP/Sac1/VAP/OSBP complex was demonstrated through overexpressed Scap and OSBP. The existence of the same complex should be demonstrated by endogenously expressed proteins.

3. Another key prediction made by the model shown in Fig. 9 is that the interaction between Scap and Sac1 is stimulated by cholesterol. This point should also be demonstrated by endogenously expressed proteins.
4. It is unclear why the authors did not consider the roles of Insig in their model. Is it possible that the interaction between Scap and the Sac1/VAP/OSBP complex is mediated by Insig? Addressing this question is particularly important to interpret the results of 25-HC, as the sterol binds both OSBP and Insig.

5. The data regarding Scap mutants are confusing. Why did overexpression of Scap(Y298C), the constitutively active mutant, fail to induce cleavage of SREBP2 in Fig. 8a? Actually, the best Scap mutant to demonstrate the model shown in Fig. 9 should be the DL mutant used in Fig. 2, which is capable of binding cholesterol but is retained in the ER regardless of cholesterol content in the ER. According to the interaction data shown in Fig. 2, expression of this mutant should support secretion through CARTS, and this activity should not be inhibited by cholesterol depletion.

6. How did the authors conclude that the effect of SREBP1/2 knockdown was owing to the function of the precursor but not the transcriptional activity of the cleaved nuclear form? In order to make this conclusion, the authors need to show that expression of SREBP2(R519A) that cannot be cleaved restores CARTS-mediated secretion in cells deficient in SREBP2.

7. The potential off target effects of all the siRNA and shRNA experiments were not controlled.
September 15, 2020
Dear Drs. Balla and Marat,

We very much appreciated the deadline extension you granted under the global situation caused by the COVID-19 pandemic. We also thank the reviewers for helping us to improve the quality of our paper. We have been able to address most of the reviewers’ concerns. Especially, our new data obtained with a Golgi transport-defective SCAP mutant strongly support our claim that ER-localized SCAP regulates PI4P turnover at ER-Golgi membrane contact sites (MCSs) and the biogenesis of CARTS at the TGN, thus showing a new role of SCAP in the ER membrane under cholesterol-fed conditions. We believe that our findings open new avenues for further study of MCSs and shed light on the roles of cholesterol in membrane trafficking. We hope with our new data you will find our paper suitable for publication in the Journal of Cell Biology.

We thank you for your support.
Please find below our responses to the reviewers’ concerns.

Sincerely yours,
Yuichi Wakana

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(Key concerns)

1. Strengthening the data that Sac1(K2A) mutant is indeed in the ER side of ER/TGN contacts and not on the Golgi side as shown by many studies (Rev2. Point 1). Your iFRAP experiments alone are not convincing enough to support this claim.

Previous papers (Liu et al., 2008; Cheong et al., 2010) claimed that GFP-Sac1 localizes to the cis-Golgi membranes. However, our previously published data (Appendix Fig. 1) and new unpublished data (Appendix Fig. 2) in HeLa cells stably expressing GFP-Sac1 showed almost perfect colocalization with VAP-A and VAP-B (markers of the ER side of the MCSs), but not with the control ER marker
SERCA2 or only partially with Golgi markers (cis-Golgi: GM130 and GPP130; medial-Golgi: mannosidase II; and TGN: TGN46). Our new data revealed that this is also the case for transiently expressed GFP-Sac1 WT and K2A upon co-staining with VAP-A and GM130 (Fig. S1 D, arrowheads). To distinguish between ER-Golgi MCSs and the Golgi membranes more clearly, we treated cells with the microtubule-depolymerizing agent nocodazole to induce the formation of dispersed Golgi ministacks in the cytoplasm (Cole et al., 1996), and investigated the distributions of GFP-Sac1 WT and K2A in relation to those of VAP-A, Bap31, GM130, and TGN46 (Fig. S1 A-C). Our results clearly show colocalization of GFP-Sac1-positive small punctate elements with VAP-A (Fig. S1 A, arrowheads), but not with the control ER marker Bap31 (Fig. S1 B), or only partially with GM130 or TGN46 (Fig. S1 C), further supporting our claim that both GFP-Sac1 WT and K2A are predominantly localized in the ER side of ER-Golgi MCSs, rather than in the Golgi membranes.

2. Testing if the defect is specific for CARTS (Rev 2, point 2)

We now examined the effect of SCAP knockdown on transport of VSV-G, which is exported from the TGN in carriers distinct from CARTS (Wakana et al., 2012, 2013). Our new data (Fig. S5 C) show that SCAP knockdown did not substantially affect VSV-G export from the TGN as compared to its inhibitory effect on CARTS-mediated GPI transport (Fig. 7 A). This result suggests the specificity of SCAP for the CARTS pathway.

3. Showing interaction between endogenous proteins (Rev 2. General comments and Rev3 point 2.)

By using new antibodies against Sac1 and SCAP together with magnetic beads, we have succeeded in detecting an interaction between endogenous Sac1 and SCAP, as shown in Fig. 1 F of our revised manuscript.

4. Testing the mutant SCAP suggested by Rev3 point 5.
We have established a stable cell line expressing SCAP D451A/L452A (Golgi transport defective mutant) and showed that this mutant, like the WT protein, can reverse the inhibitory effects of SCAP knockdown on the biogenesis of CARTS and PI4P turnover (Fig. 8, C and D). This result strongly supports our claim that SCAP regulates these functions in the ER membrane.

5. Testing if the transcriptional activity of SREBP1/2 is involved (Rev 2. point 6)

To test this, we would have needed to establish a stable cell line expressing SREBP2 (R519A), which cannot be cleaved by S1P, because overexpression of SREBP2 severely inhibits PAUF secretion. However, a previous paper (Shao and Espenshade, 2014) reported that the stably expressed R519A mutant reduces expression levels of SCAP. Our data also showed that treatment of HeLa cells with the S1P inhibitor PF-429242 in cholesterol-fed conditions decreased SCAP expression (Appendix Fig. 3). Because of such technical difficulties, we did not test this mutant. However, our new data on the rescue effects of the Golgi transport defective SCAP mutant (D451A/L452A) suggests that the transcriptional activity of SREBPs is not required. We assume that this data fully answers the reviewer’s concern.

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

When ER cholesterol is low, SCAP escorts SREBPs into COP–II vesicles. When cholesterol is high, SCAP binds INSIG and blocks SREBP transport. Y234A locks SCAP to INSIG independent of cholesterol, and Y298C blocks INSIG binding, which should yield constitutive transport to Golgi (their ref 58). Yet the authors do not see this and surprisingly, their CARTS and PI4P phenotypes are the same despite two different types of mutation. I think it would be important to explain more clearly possible explanations for why the two distinct classes of mutants give the same
We thank Dr. Pfeffer for her appreciation of our work and for her comments to improve the clarity of our manuscript. In the paper by Nohturfft et al. (1998), the authors did not include IF data, but inferred the localization of SCAP Y298C mutant based on its endo H sensitivity. Fig. 5B (lanes 15 and 16) in their paper shows that large amounts of the Y298C pool is endo H sensitive (ER form) both in the presence and absence of sterol, whereas a minor fraction of the Y298C pool is localized to the Golgi complex (where it becomes endo H resistant). These results are consistent with our IF data. Interestingly, a recent paper (Kuan et al., 2020) suggests that polyubiquitination of SCAP at K305 by RNF5 promotes transport of SCAP from the ER to the Golgi complex. Since Y298 is located in the putative RNF5-binding domain of SCAP, the Y298C mutation might inhibit RNF5 binding and polyubiquitination, hence causing most of the mutant to be retained in the ER at steady state.

Given the above situation, the Y298C mutant might not provide a clear answer. We therefore tested other constructs with mutations in the sterol-sensing domain of SCAP, L315F and D443N. These mutations like Y298 mutation cause resistance to the cholesterol-induced conformational change and abrogate Insig binding even in the presence of sterols. Although we could not test the effect of the D443N mutant because of its low expression, the L315F mutant with the expression level comparable to that of wild-type SCAP exhibited similar phenotypes to the Y298C mutant.

In the discussion section of the revised manuscript, a possible explanation of why the two distinct types of mutants give the same phenotypes is clearly mentioned (p. 16, lines 9-13).
Minor: Supp FIG1A,B seem more important than Figs. 1 & 3; Discussion line 4 replace Posterior with Subsequent

The pointed parts have been changed as recommended (Fig. 2, A and C; p. 15, line 5).

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

1. The conclusion that Sac1/K2A accumulates in ER membrane domains that are site of ER–TGN contact is at odds with published reports showing that its localization favors the Golgi, in comparison to the native sequence protein whose localization favors the ER. The co-localization analyses of antigens residing in the peri-nuclear region are ambiguous, as signals in this region of cells are quite dense. The results of the photobleaching experiment shown in Figure 1C show that some amount of GFP–Sac1 accesses ER membrane, but most does not and this pool can reasonably be assumed to reside in the Golgi. Note that Dippold et al (Cell, 2012; not cited) used Sac1/K2A to deplete Golgi membrane of PI4P. More generally, the conclusion that native Sac1 is enriched in a specialized ER–Golgi contact site sub-domain is not rigorously demonstrated in this study or in the authors’ previous publication (ref 34). Zewe et al 2018 (not cited) presented a rigorous analysis of Sac1 localization, concluding that Sac1 is not enriched in any particular aspect of the ER. It may be results obtained for tagged, ectopically over-expressed proteins used in this study are misleading. The small amount of SCAP that is observed to localize to the Golgi is also in line with the small amount that co-purifies with ER–Golgi tethering proteins.

Previous papers (Rohde et al., 2003; Liu et al., 2008; Cheong et al., 2010) report that endogenous Sac1 and transiently and stably expressed GFP-Sac1 WT localize not only to the peripheral ER but also to the perinuclear structures, consistent with our data (Fig. 1 A). The results in these papers do not exclude the possibility that a pool of Sac1 localizes to the perinuclear ER. Moreover, the presence of Sac1 K2A in the perinuclear region of the ER is possible because, as described in the
text, some ER integral membrane proteins use a di-lysine motif for recycling from the perinuclear area to the peripheral area in the ER (Wakana et al., 2008; Lavieu et al., 2010).

It should be noted that the iFRAP kinetics for Sac1 K2A is essentially the same as that for Sac1 WT (Fig. 1 C), indicating that the localization of Sac1 WT and K2A is quite similar. In addition, the time frame of the iFRAP experiments should also be recognized. Within 4 min, the time required for 50% reduction in the perinuclear fluorescence intensity after photobleaching, Sac1 transport from the Golgi complex to the ER could not occur. Therefore, it is reasonable to assume that this reduction is due to the redistribution of Sac1 within the same membrane from the perinuclear area to the peripheral area.

The data in the paper by Dippold et al. (Cell, 2009[2012]) do not necessarily exclude the possibility that Sac1 K2A localized to the ER side of the MCSs, but not to the Golgi membranes. It is possible that ER-localized Sac1 K2A depletes Golgi PI4P via the ER cholesterol/Golgi PI4P exchange at ER-Golgi MCSs. Also, a recent paper by Venditti et al. (2019) reported in-trans activity of Sac1 at ER-Golgi MCSs.

We would like to emphasize that Zewe et al. (2018) showed that Sac1 is not enriched specifically at “ER-plasma membrane (PM)” MCSs, but did not particularly study its “ER-Golgi” MCS localization. They did not look at ER-Golgi MCSs because they monitored ER-PM enrichment by comparing TIRF signal (only few nanometers above the glass, which corresponds mostly to PM signal) to the total signal to elaborate on ER-PM MCSs. The authors claimed in their paper that “Sac1 does not enrich at ER-PM MCS”. Moreover, Zewe et al. (2018) also used GFP-Sac1 and stated that “expressed Sac1 exhibits an identical localization to the endogenous protein” with citation to Rohde et al., 2003.

We do agree with the reviewer’s view that immunofluorescence signals in the perinuclear region of cells are quite dense. To distinguish between ER-Golgi MCSs
and Golgi membranes more clearly, we treated cells with nocodazole (Fig. S1 A-C). The results showed the colocalization of GFP-Sac1 K2A as well as WT with the ER-Golgi MCS marker VAP-A.

Our claim on this issue is described in the response to Key concern 1.

The papers by Dippold et al. (2009) and Zewe et al. (2018) are cited in the revised manuscript.

2. The appearance of PAUF in figure 5B raises a concern that SCAP depletion causes a general deficiency of Golgi function. In the lysates, no modified/mature form of PAUF is observed in the SCAP KD lane, and the mobility of the secreted fraction also appears to be reduced, suggesting that Golgi-dependent glycosylation is compromised by SCAP depletion. Thus, Golgi function may be generally compromised and it is important to determine if this is the case as it relates to the issue of specificity of SCAP for the CARTS pathway. Is glycosylation of other secreted glycoproteins affected?

Thank you for this valuable comment. As described in the response to Key concern 2, the revised manuscript contains a VSV-G transport assay to show the specificity of SCAP in the CARTS pathway. We assume that this data excludes the global Golgi dysfunction and fully answers the reviewer’s concern.

3. Does SCAP depletion affect secretion in general, or just CARTS, as the authors suggest? The conclusion would be more rigorously tested if the authors examined a non-CARTS cargo, obviously if it does not exhibit the same pattern.

As described above, the VSV-G transport assay has been performed.

In addition, the conclusion that CARTS forms immediately adjacent to sites of ER-Golgi contact is not rigorously supported. Visible light microscopy does not
Our BiFC approach has an advantage of detecting the specific class of ER-Golgi MCSs (VAP-A/OSBP complex-containing ones) even in the high density of the ER membrane in the perinuclear region. Our new data in which CARTS were imaged by STED super-resolution microscopy reveal that CARTS were located in the close vicinity of BiFC-positive perinuclear region (Fig. 6 A, arrowheads), supporting our conclusion.

4. Minor comment: Many of the micrographs would be more easily inspected (by me, at least) if the grayscale were inverted.

It is true that some people do prefer inverted micrographs, but others don’t. We prefer to keep these images as originally shown.

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

1. A key prediction made by the model shown in Fig. 9 is that deficiency in Scap should impair the ER to Golgi transport of cholesterol. However, there is no data to demonstrate that knocking down Scap increases amounts of cholesterol in the ER and decreases that in the Golgi. In Fig. 4A the authors argued that Filipin staining may not be sensitive enough for this purpose. This should not be used as an excuse, as other approaches such as measuring ACAT activity and determining cholesterol contents in purified subcellular organelles by MS can be used to determine the amounts of cholesterol in ER and Golgi.

After several attempts we found some technical difficulties in addressing this issue. Considering the suggestion made by the editor, we would like to solve this issue another time in the future, since this will require extensive additional technical effort.
2. The Scap/SREBP/Sca1/VAP/OSBP complex was demonstrated through overexpressed Scap and OSBP. The existence of the same complex should be demonstrated by endogenously expressed proteins.

As described in the response to Key concern 3, our new data (Fig. 1 F) showed an interaction between endogenous Sac1 and SCAP. ER-Golgi MCSs are highly dynamic in repeating association and dissociation of the two organelle membranes and probably because of this, no papers have shown an interaction between endogenous VAP-A and OSBP as far as we know. For the same reason, it seems quite difficult to show the existence of a complex of SCAP/SREBP/Sac1/VAP-A/OSBP only by endogenous proteins. We would like to address this issue in the near future.

3. Another key prediction made by the model shown in Fig. 9 is that the interaction between Scap and Sac1 is stimulated by cholesterol. This point should also be demonstrated by endogenously expressed proteins.

Considering the suggestion made by the editor, we did not experimentally address this issue for this paper. In addition to addressing this point, we would also like to test whether cholesterol-bound SCAP activates Sac1, sometime in the near future.

4. It is unclear why the authors did not consider the roles of Insig in their model. Is it possible that the interaction between Scap and the Sac1/VAP/OSBP complex is mediated by Insig? Addressing this question is particularly important to interpret the results of 25-HC, as the sterol binds both OSBP and Insig.

Considering the suggestion made by the editor, we did not address this issue for this paper. This is also an interesting question which we would like to address in the near future.
This relates to the comments of Reviewer #1. On the one hand, in the paper by Nohturfft et al. (1998), SCAP Y298C and SREBP2 were transiently co-overexpressed to induce cleavage of SREBP2. On the other hand, we used a stable cell line expressing only SCAP Y298C. This result fits with the localization of the mutant (that is, mostly ER distribution at steady state).

Actually, the best Scap mutant to demonstrate the model shown in Fig. 9 should be the DL mutant used in Fig. 2, which is capable of binding cholesterol but is retained in the ER regardless of cholesterol content in the ER. According to the interaction data shown in Fig. 2, expression of this mutant should support secretion through CARTS, and this activity should not be inhibited by cholesterol depletion.

Thank you for this valuable comment. As described in the response to Key concern 4, our new data with the suggested mutant (Fig. 8, C and D) support our model that ER-localized SCAP regulates PI4P turnover and CARTS biogenesis.

As described in the response to Key concern 5, we were not able to address this concern by testing SREBP2 (R519A), however, we consider that our new data with the Golgi transport defective SCAP mutant (Fig. 8, C and D) fully answers the reviewer’s concern.
7. The potential off target effects of all the siRNA and shRNA experiments were not controlled.

Considering the suggestion made by the editor, we did not fully address this issue. Rescue experiments were performed for SCAP knockdown (Fig. 8, C and D).

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October 1, 2020

RE: JCB Manuscript #202002150R

Dr. Yuichi Wakana
Tokyo University of Pharmacy and Life Sciences
1432-1 Horinouchi Hachioji
Tokyo 192-0392
Japan

Dear Dr. Wakana:

Thank you for submitting your revised manuscript entitled "The ER cholesterol sensor SCAP promotes CARTS biogenesis at ER-Golgi membrane contact sites". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

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Sincerely,

Tamas Balla, MD PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

My major concerns were with the assertion that Sac1 K2A is enriched in ER membrane at ER-TGN MCS. The authors have provided a considerable amount of new data which make a compelling case for their assertion. In addition, new data make a case that SCAP has a principal function in the CARTS pathway, rather than a general role in Golgi function. The only thing I find disappointing with the revised manuscript is the lack of SREBP answers on the potential roles of SREBP, but this is a substantial study as is.