

mTORC2 suppresses cell death induced by hypo-osmotic stress by promoting sphingomyelin transport

Yumiko Ono, Kenji Matsuzawa, and Junichi Ikenouchi

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Review Timeline:

Submission Date:	2021-06-25
Editorial Decision:	2021-07-28
Revision Received:	2021-12-28
Editorial Decision:	2022-01-19
Revision Received:	2022-01-20
Accepted:	2022-01-24

Monitoring Editor: Ian Macara

Scientific Editor: Andrea Marat

Transaction Report:

(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: <https://doi.org/10.1083/jcb.202106160>

July 28, 2021

Re: JCB manuscript #202106160

Prof. Junichi Ikenouchi
Kyushu University
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Fukuoka 819-0395
Japan

Dear Prof. Ikenouchi,

Thank you for submitting your manuscript entitled "mTORC2 suppresses cell death induced by hypo-osmotic stress by promoting sphingomyelin transport". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. I am pleased to let you know that all of the reviewers were quite positive, and found the work to be interesting, novel, and of high potential importance to the field. However, they each raise significant issues with several aspects of the study, which will require additional experimental work and revisions to the text. For these reasons we are unable to accept the manuscript for publication in its present form.

Reviewer #2 had mostly minor concerns, noting some controls that need to be added, or quantification of data.

Reviewer #1 was positive but notes that no direct evidence is provided that Rab35 is required for protection against hypo-osmotic shock or acts downstream of mTORC2 and is activated by osmotic shock (although there is published evidence that Rab35 GEFs are regulated by AKT). They suggest that in fact Rab35 might act in a parallel pathway, and propose that Rab35 depletion experiments will be necessary to resolve this issue. Several other issues are also raised, including whether Rab35GTP quickly translocates to the apical surface in response to osmotic shock and whether the PIP2 decrease shown in Fig 5 is dependent of mTORC2.

Reviewer #3 highlights the value of the new tool for studying sphingomyelin transport to the cell surface, but notes a lack of direct evidence that vesicle fusion to the PM is controlled by changes in the cortical actin cytoskeleton and PIP2. They suggest that this can be tested with pharmacological disruption of the actin cytoskeleton, and that statements regarding the mechanistic model should be toned down. This reviewer also commented on the need to increase the localization analysis of vesicles stained with the sphingomyelin probe, and suggests additional controls to further validate the probe. Additionally reviewer #3 suggests inhibition with Torin or the Rictor KO to demonstrate that AKT is activated through mTORC2.

We would be happy to consider a suitably revised version of your interesting manuscript. Please note that a full point-by-point response to each of the reviewer comments will be required with resubmission, and that the manuscript will be sent back to at least two of the external reviewers for their 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.

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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,

Ian Macara, Ph.D.
Editor

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

Journal of Cell Biology

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

Y. Ono et al. report here a role for mTORC2 in promoting expansion of the apical surface in response to hypo-osmotic shock in polarized epithelial cells. The authors showed that mTORC2 is required for the delivery of vesicles containing the apical marker PODXL and the apical lipid sphingomyelin, to make additional microvilli necessary for the apical surface expansion. They propose that the Rab35 GTPase acts downstream of mTORC2 in response to hypo-osmotic, and this pathway would favor vesicle fusion to the apical surface by remodeling the apical PI(4,5)P2 lipid domain and the apical F-actin cortex. Altogether, the mTORC2-Rab35 axis prevents membrane rupture and cell death after hypo-osmotic shock.

This manuscript contains several interesting and important observations, as well as the development of an original probe to follow the delivery of sphingomyelin by vesicular transport. While mTORC2 has already been involved in sphingomyelin biosynthesis in response to osmotic shock in cerevisiae and in actin remodeling, the proposed mechanism in mammalian cells appears quite different and involves PI(4,5)P2-actin remodeling downstream of the Rab35 GTPase. Overall, the experiments are convincing but more evidence showing a role of Rab35 downstream of mTORC2 are needed to back up some of the major conclusions. In my opinion, the manuscript would be an excellent fit to JCB when the authors have addressed the points below.

Major points

1. The conclusion that Rab35 acts downstream of mTORC2 and is activated by osmotic shock/mTORC2 is only based on epistasis/rescue experiments using the overexpression of Rab35 Q67L (active Rab35) after mTORC2 inactivation. However, Rab35 could act in a parallel, unrelated pathway and not in the proposed "mTORC2-Rab35 axis". In addition, there is no evidence that Rab35 is actually required for protecting cells against hypo-osmotic shock.

The authors should thus demonstrate that cells depleted of Rab35 display impaired localization of PODXL and sphingomyelin at the apical surface (Fig. 5A, 5D), accumulate sphingomyelin vesicles in the cytoplasm (Fig. 2D), do not expand their apical surface upon hypo-osmotic stress and eventually experience death in response to osmotic shock (Fig. 1A, 8D).

2. Along the same line, does Rab35 GTP quickly translocate at the apical surface upon shock, as expected if Rab35/OCRL was responsible for PI(4,5)P2 decrease observed in Fig. 8G and proposed in the model? In addition, does Rab35 GTP levels increase in a mTORC2-dependent manner after a hypo-osmotic shock (this could be measure by pull down assays as described in PMID 21737958)? Finally, the authors should demonstrate that the PI(4,5)P2 decrease shown in Fig. 5G is dependent on mTORC2.

3. SEM pictures of control cells, Rab35 depleted cells and rictor (or mTORC2) inhibited cells, both in iso-osmotic and in hypo-osmotic media, should be provided. According to the model, one expects less microvilli in Rab35- and rictor-depleted cells upon iso-osmotic conditions, and damage membranes upon hypo-osmotic shock.

Minor points

1. Fig. 2C: A western blot showing similar expression of the three GFP constructs should be shown to allow comparison.
2. Fig. 2F/4B, E: I don't understand the rationale of the Icyto index. Could the author justify this index? If the authors want to provide a measurement of Sphingomyelin in vesicle, the [total intensity of SS GFP Lys - the intensity of SS GFP Lys that is colocalized with Giantin]/Total GFP signal seems more appropriate.
3. Fig. 3D: The "total cell lysate" should be provided (as stated in the legend).
4. Fig. 4E: Raptor KO should be provided as a control.
5. Fig. 5B: x-axis, Time in hours, not minutes.
6. Fig. 6C: Is PODXL localization rescued as well?
7. Controls showing mScarlet and mScarlet-Rab35 Q67L in the absence of drug (Fig. 6C), and in cells not depleted of rictor (Fig. 7A and Fig. 7E) should be provided for comparison.
8. p.3 line 20: "50 mOsm/L" or "150 mOsm/L" (Fig. legend)
p.7 lines 9-10: There is something missing in this sentence.
9. The final model in Fig. 8J is very small and difficult to read. In addition, it does not show a reduction of the F-actin levels (but an increase of the mesh size of the actin cortex), which does not reflect the results. Furthermore, Rab35-GDP is supposed to be cytosolic and not attached to the PODXL tail (PMID 27040773).
10. The actin oxidase MICAL1 has been proposed to act as a Rab35 effector (PMID 28230050) and could be well involved in F-actin remodeling upon hypo-osmotic shock. This could be discussed as a potential mechanism, in parallel to OCRL1, to explain the observed results. In addition, Rab35 has been recently found as a negative regulator of mTORC1 (PMID 32503983). Could the authors discuss how this could fit with the observations reported here?

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

The mechanisms through which epithelial cells react to acute hypo-osmotic stress are poorly understood. Among several others, the role of ion channels, aquaporins, mechanoreceptors, lipid transporters and actin cytoskeleton has been suggested however the signaling cascades leading to the effective regulation of plasma membrane tension and cellular volume are not known. In the submitted manuscript, Ono et al. show the importance of sphingomyelin transport and its regulation by mTORC2 pathway and a regulator of cellular trafficking - Rab35. To do so, the authors use a set of molecular and genetic techniques allowing visualization of sphingomyelin in the context of various cellular compartments focusing on the Golgi apparatus and its vesicular transport to the plasma membrane. Overall, the data presented in the manuscript provide interesting insights into the response of epithelial cells to hyper-osmotic stress.

Minor comments:

- Fig. 1B and 1D are lacking legends on the graphs
- The authors should confirm that cell viability is not compromised by the osmotic shocks used (to eliminate other pathways such as apoptosis etc. as being involved).
- Fig S2B - the decrease in cav-1 signal should be quantified (eg apical-basal cav-1 intensity ratio)
- Fig2 - an impact of the stable expression of SS-GFP-Lys on SM levels should be assessed, namely the level of sphingomyelin in SS-GFP-Lys-expressing cells should be compared to the SM levels in parental cells
- p7 line 7-11 - this sentence needs fixing
- Fig 4B, E - show Pearson's coefficient aside or in supplementary material as it is a straight-forward measure of co-localization
- Have the authors looked at other sphingolipids? Is sphingomyelin really the critical feature here or vesicle transport? The authors may wish to reflect on the take home message that they would like to deliver with the title.
- Page 9 line 7, ref to Fig5D cites Rictor KD cells but figure is TORIN-treated WT cells.
- p10 and Fig6 It is not clear how the endothelial lumen formation is induced.
- page 13, line 9/10: no direct tension experiments were performed in this study, so formally, the statement "mTORC2-Rab35 activation [...] reduces plasma membrane tension by reducing actin cortex of apical membrane" is not justified - although of course the link between hypo-osmotic shock and membrane tension in combination with the cell rupture data hints into this direction
- Fig 6C is lacking a control where cells expressing Scarlett-Rab35 Q67L are mock-treated - does it change the localization of SS-GFP-Lys?
- fig. 7B: no legend is given

- Fig 7C-D - compare the increase of the fluorescence of the apical actin to the increase of fluorescence of the basal actin to support the claim about the apical actin accumulation upon Rictor KD

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

This interesting and novel manuscript describes a mechanism, the enhanced deliver of post-Golgi transport vesicles to the apical plasma membrane, that relieves hypo-osmotic stress in epithelial cells. This is in large part a well-supported model. In doing so the authors introduce and validate a useful tool for studying the transport of sphingomyelin to the cell surface. Although aspects of this study have been previously reported in other cell types, the importance of this compensatory response in epithelial cells has physiological significance, and the study is comprehensive. The conclusions of the authors are by and large well supported by the data presented with the exception of the assertion that vesicle fusion to the apical plasma membrane is controlled by changes in the cortical actin cytoskeleton. Specific points are as follows:

1. The assertion that changes in the actin cytoskeleton are the basis of the enhance transport of apically-destined vesicles is only circumstantially supported by evidence that changes in actin distribution correlate with this effect. Either a direct test of this assertion should be provided or language in the manuscript should be altered to present this as an as yet untested hypothesis. One approach would be to demonstrate that pharmacological disruption of the actin cytoskeleton, for example by Cytochalasin D, reverses the effect of mTORC2 inhibition. Similarly, the statement that alterations in PIP2 metabolism are the basis of the effect is not supported by direct functional experimentation.
2. Figure S3. The identity of the intracellular organelles that stain with the probe for sphingomyelin as post-Golgi transport carriers is an important aspect of this work. This is supported by the co-localization of the sphingomyelin probe with podocalyxin-like-1 but not E-cadherin (Figure 3) and the temperature based pulse/chase experiment depicted in Figure S5. However the co-localizations depicted in Figure S3 are not as convincing due to variations in the abundance and shape of the ssGFP-Lys staining. These localization studies should be repeated with the vesicles accumulated in the presence of Torin or the rictor knockout, in which the abundance of the vesicles should clarify co-localization or lack thereof.
3. Figure 1. The authors state that the expansion of the apical membrane does not result in dilution of the components, which is an important point. I believe the quantitation presented in Figure 1, Panels B and D represent total area stained. To support the authors' point this quantitation should be repeated as total intensity of staining.
4. Figure 2, Panel D. The ssGFP-lysenin probe is interesting and will be valuable to the research community. Validation is key. The results with the inhibitors in panel D are reasonable, but as the authors no doubt know, each inhibitor has drawbacks. The authors should also test inhibition of ceramide biosynthesis with Fumonisin B1 and the staining should be quantitated.
5. Figure 8, Panels A and B. AKT can be activated by a number of pathways, in addition to mTORC2. To demonstrate that the activation that is described in this figure is due to mTORC2 activation, the authors should demonstrate that it is blocked with Torin and/or in the rictor knockout.
6. Much of the data demonstrating a role of mTorc2 in the transport of sphingomyelin to the cell surface is derived from cells not subjected to hypo-osmotic stress. This indicates that this is a constitutive role as well as a role in response to osmotic stress. The authors should address this in the Discussion.



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Dec. 28, 2021

Prof. Ian Macara
Monitoring Editor
Journal of Cell Biology

Re: JCB manuscript #202106160

Dear Prof. Macara,

Please find attached the revised version of our manuscript, entitled “mTORC2 suppresses cell death induced by hypo-osmotic stress by promoting sphingomyelin transport”.

We are most grateful to you and the reviewers for the helpful comments regarding our previous manuscript. We have taken all these comments into consideration and have thoroughly revised the paper. Our point-by-point responses to the reviewer's comments and descriptions of the changes made to the manuscript are provided below.

We hope that the revised version of our paper is now suitable for publication in the *Journal of Cell Biology* as an Article and look forward to hearing from you at your earliest convenience.

Sincerely yours,

Junichi Ikenouchi M.D., Ph.D.
Professor

Monitoring Editor Comment

Reviewer #2 had mostly minor concerns, noting some controls that need to be added, or quantification of data.

Comment ME-1

Reviewer #1 was positive but notes that no direct evidence is provided that Rab35 is required for protection against hypo-osmotic shock or acts downstream of mTORC2 and is activated by osmotic shock (although there is published evidence that Rab35 GEFs are regulated by AKT). They suggest that in fact Rab35 might act in a parallel pathway, and propose that Rab35 depletion experiments will be necessary to resolve this issue. Several other issues are also raised, including whether Rab35GTP quickly translocates to the apical surface in response to osmotic shock and whether the PIP2 decrease shown in Fig 5 is dependent of mTORC2.

Response ME-1

By establishing and analyzing Rab35 knocked-out cell lines, we confirmed that Rab35 is essential for protection against hypo-osmotic stress (Fig. 7G). In addition, a pull-down assay of Rab35-GTP showed that activation of Rab35 by hypo-osmotic stress requires mTORC2 activity (Figs. 7K and 7L). Furthermore, Rab35 was activated at the apical membrane in a mTORC2 activity-dependent manner (Fig. 7M). With regard to PIP2, we demonstrated that its decrease in response to hypo-osmotic shock requires the activation of mTORC2 (Fig. 8D). Thus, we were able to answer the constructive comments raised by Reviewer 1 with new experimental results.

Comment ME-2

Reviewer #3 highlights the value of the new tool for studying sphingomyelin transport to the cell surface, but notes a lack of direct evidence that vesicle fusion to the PM is controlled by changes in the cortical actin cytoskeleton and PIP2. They suggest that this can be tested with pharmacological disruption of the actin cytoskeleton, and that statements regarding the mechanistic model should be toned down. This reviewer also commented on the need to increase the localization analysis of vesicles stained with the

sphingomyelin probe, and suggests additional controls to further validate the probe. Additionally reviewer #3 suggests inhibition with Torin or the Rictor KO to demonstrate that AKT is activated through mTORC2.

Response ME-2

We investigated whether fusion of vesicles containing sphingomyelin with the PM is promoted by destruction of actin cortex and reduction of PIP2. We found that reducing the level of PIP2 by forcibly localizing INPP5E, an enzyme that dephosphorylates the 5-phosphate of PIPs, to the PM promotes fusion of vesicles containing sphingomyelin (Fig. 8E). On the other hand, destruction of the actin cortex by cytochalasin treatment did not promote vesicle fusion. This is not surprising given that cytochalasin treatment disrupts not only actin cortex as a physical barrier to apical transport but also impairs apical membrane transport mediated myosin Vb, which is supported by the apical actin cortex (Kapitein et al. *Curr Biol* 2013). On the other hand, the decrease in PIP2 loosens the binding between the PM and the actin cytoskeleton, such as through the inactivation of Ezrin, but does not completely dissolve the actin cortex. Considering the yin and yang effects of the actin cortex on the delivery and fusion of the sphingomyelin-containing vesicles with the apical PM, we toned down the conclusion about the roles of actin cortex in our model. In addition, we further characterized the sphingomyelin probe we developed in this paper in detail, according to the comments of Reviewer 3 (Fig. S2). Lastly, we showed that the activation of AKT induced by hypo-osmotic stress is mTORC2 dependent (Figs. 7C and 7D).

Reviewer #1

Y. Ono et al. report here a role for mTORC2 in promoting expansion of the apical surface in response to hypo-osmotic shock in polarized epithelial cells. The authors showed that mTORC2 is required for the delivery of vesicles containing the apical marker PODXL and the apical lipid sphingomyelin, to make additional microvilli necessary for the apical surface expansion. They propose that the Rab35 GTPase acts downstream of mTORC2 in response to hypo-osmotic, and this pathway would favor

vesicle fusion to the apical surface by remodeling the apical PI(4,5)P₂ lipid domain and the apical F-actin cortex. Altogether, the mTORC2-Rab35 axis prevents membrane rupture and cell death after hypo-osmotic shock.

This manuscript contains several interesting and important observations, as well as the development of an original probe to follow the delivery of sphingomyelin by vesicular transport. While mTORC2 has already been involved in sphingomyelin biosynthesis in response to osmotic shock in cerevisiae and in actin remodeling, the proposed mechanism in mammalian cells appears quite different and involves PI(4,5)P₂-actin remodeling downstream of the Rab35 GTPase. Overall, the experiments are convincing but more evidence showing a role of Rab35 downstream of mTORC2 are needed to back up some of the major conclusions. In my opinion, the manuscript would be an excellent fit to JCB when the authors have addressed the points below.

Comment 1-1

1. The conclusion that Rab35 acts downstream of mTORC2 and is activated by osmotic shock/mTORC2 is only based on epistasis/rescue experiments using the overexpression of Rab35 Q67L (active Rab35) after mTORC2 inactivation. However, Rab35 could act in a parallel, unrelated pathway and not in the proposed "mTORC2-Rab35 axis". In addition, there is no evidence that Rab35 is actually required for protecting cells against hypo-osmotic shock.

The authors should thus demonstrate that cells depleted of Rab35 display impaired localization of PODXL and sphingomyelin at the apical surface (Fig. 5A, 5D), accumulate sphingomyelin vesicles in the cytoplasm (Fig. 2D), do not expand their apical surface upon hypo-osmotic stress and eventually experience death in response to osmotic shock (Fig. 1A, 8D).

Response 1-1

We established cell lines in which Rab35 was knocked out in order to definitively determine whether or not Rab35 was actually activated in protective response to hypo-osmotic stress (Figs. S5A and S5B). The transport of sphingomyelin to the apical membrane was severely impaired in Rab35 KO cells (Fig. S5C). In addition, in Rab35 KO cells, podocalyxin did not localize to the apical membrane and instead accumulated in intracellular vesicles (Fig. S5D). Rab 35 KO cells

rapidly ruptured upon exposure to hypo-osmotic medium, indicating that Rab35 is an essential component of the response mechanism to hypo-osmotic stress (Figs. 7G, 7H and 7I).

Comment 1-2

Along the same line, does Rab35 GTP quickly translocate at the apical surface upon shock, as expected if Rab35/OCRL was responsible for PI(4,5)P2 decrease observed in Fig. 8G and proposed in the model? In addition, does Rab35 GTP levels increase in a mTORC2-dependent manner after a hypo-osmotic shock (this could be measure by pull down assays as described in PMID 21737958)? Finally, the authors should demonstrate that the PI(4,5)P2 decrease shown in Fig. 5G is dependent on mTORC2.

Response 1-2

We probed the localization of Rab35-GTP by expressing the Rab35 binding domain (RBD) of the effector protein MICAL fused to GFP. GFP-MICAL-RBD was constitutively localized at the apical membrane regardless of whether the culture medium was hypo- or (Figs. 7M and 7N). Importantly, however, activation of Rab35 at the apical membrane was abolished by cell treatment with the mTOR inhibitor Torin-1 (Figs. 7M and 7N).

We confirmed that the amount of Rab35-GTP was significantly increased in cells subjected to hypo-osmotic stress, and that this activation was dependent on mTORC2 activity, by using a pull-down assay in combination with inhibitor treatments (Figs. 7K and 7L). Taken together, our results indicate that hypo-osmotic stress does not alter where Rab35 is activated but rather up-regulates the amount of activated Rab35. We also confirmed that the decrease of PIP2 caused by the hypo-osmotic stress was also suppressed by treatment with mTORC2 inhibitor (Figs. 8C and 8D).

Comment 1-3

SEM pictures of control cells, Rab35 depleted cells and rictor (or mTORC2) inhibited cells, both in iso-osmotic and in hypo-osmotic media, should be provided. According to

the model, one expects less microvilli in Rab35- and rictor-depleted cells upon iso-osmotic conditions, and damage membranes upon hypo-osmotic shock.

Response 1-3

According to the comments of Reviewer 1, we examined the morphology of the apical membranes of wild-type, Rab35 KO and Rictor KD cell cultured under both iso- and hypo-osmotic conditions (Figs. 5E and S5G). Microvilli formation was severely impaired in both Rictor KD and Rab35 KO cells. Furthermore, ruptured plasma membrane was a prominent feature of both Rab 35 KO and Rictor KD cells subjected to hypo-osmotic stress (Fig. 7I).

Comment 1-4

Fig. 2C: A westernblot showing similar expression of the three GFP constructs should be shown to allow comparison.

Response 1-4

According to the comment of Reviewer 1, we repeated the western blotting in question and replaced the image of Fig. 2C.

Comment 1-5

Fig. 2F/4B, E: I don't understand the rationale of the Icyto index. Could the author justify this index? If the authors want to provide a measurement of Sphingomyelin in vesicle, the [total intensity of SS GFP Lys - the intensity of SS GFP Lys that is colocalized with Giantin]/Total GFP signal seems more appropriate.

Response 1-5

I_{cytosol} was calculated as [total area of SS-GFP-Lys - the area of SS-GFP-Lys that is colocalized with Giantin] / Total area of GFP. The reason why I_{cytosol} was calculated using Area instead of Intensity of SS-GFP-Lys is as follows. As Lysenin preferentially binds to the sphingomyelin-containing membrane enriched with cholesterol (Ikenouchi et al. J Biol Chem 2012), the fluorescent intensity of SS-GFP-Lys does not necessarily reflect the absolute amount of sphingomyelin in the membrane. Therefore, in this study, we binarized the signal

of SS-GFP-Lys and measured the GFP-positive area instead of the fluorescent intensity of SS-GFP-Lys and used it as an index of the amount of vesicles containing sphingomyelin based.

The description of the quantification method has been revised to avoid misreading as follows.

Page 21 Line 23

I_{cytosol} was calculated as [total area of SS-GFP-Lys - the area of SS-GFP-Lys that is colocalized with Giantin] / Total area of GFP.

Comment 1-6

Fig. 3D: The "total cell lysate" should be provided (as stated in the legend).

Response 1-6

According to the comment of Reviewer 1, we added the data of the total cell lysate in Fig. 3D.

Comment 1-7

Fig. 4E: Raptor KO should be provided as a control.

Response 1-7

According to the comment of Reviewer 1, we added the data of Raptor KO cells in Figure 4E.

Comment 1-8

Fig. 5B: x-axis, Time in hours, not minutes.

Response 1-8

We corrected the unit of the x-axis in Fig. 5B.

Comment 1-9

Fig. 6C: Is PODXL localization rescued as well?

Response 1-9

When EpH4 cells expressing GFP-Podocalyxin-1 were treated with Torin-1, GFP-Podocalyxin-1 was distributed in intracellular vesicles and was not transported properly to the apical membrane, similar to SS-GFP-Lys under this condition (Fig. S4C). On the other hand, in cells expressing Scarlet-Rab35 Q67L, GFP-Podocalyxin-1 localized to the apical membrane even with Torin-1 treatment (Fig. S4C).

Comment 1-10

Controls showing mScarlet and mScarlet-Rab35 Q67L in the absence of drug (Fig. 6C), and in cells not depleted of rictor (Fig. 7A and Fig. 7E) should be provided for comparison.

Response 1-10

According to the comment of Reviewer 1, we added the images of SS-GFP-Lys-expressing cells together with mScarlet or mScarlet-Rab35 Q67L in the absence of mTOR inhibitors (Fig. 5F). We also added the images of wild-type cells expressing mScarlet or mScarlet-Rab35 Q67L in Fig. 6A.

Comment 1-11

p.3 line 20: "50 mOsm/L" or "150 mOsm/L" (Fig. legend)

Response 1-11

Thank you for pointing out the description error. We corrected the main text.

Comment 1-12

p.7 lines 9-10: There is something missing in this sentence.

Response 1-12

We corrected the sentence as follows.

Page 7 Line 24

To exclude the possibility that the effect observed by these mTOR inhibitors is due to the off-target effect and to narrow down which of the mTORC1 and mTORC2 pathways is responsible for the transport of sphingomyelin, we established EpH4 cells in which either Raptor or Rictor, essential scaffold proteins of mTORC1 or mTORC2 respectively, were knocked down (Liu and Sabatini, 2020) (Fig. 4D).

Comment 1-13

The final model in Fig. 8J is very small and difficult to read. In addition, it does not show a reduction of the F-actin levels (but an increase of the mesh size of the actin cortex), which does not reflect the results. Furthermore, Rab35-GDP is supposed to be cytosolic and not attached to the PODXL tail (PMID 27040773).

Response 1-13

We enlarged and revised the final model to reflect the reduction of F-actin levels under hypo-osmotic stress and the cytoplasmic localization of Rab35-GDP (Fig. 8H).

Comment 1-14

The actin oxidase MICAL1 has been proposed to act as a Rab35 effector (PMID 28230050) and could be well involved in F-actin remodeling upon hypo-osmotic shock. This could be discussed as a potential mechanism, in parallel to OCRL1, to explain the observed results. In addition, Rab35 has been recently found as a negative regulator of mTORC1 (PMID 32503983). Could the authors discuss how this could fit with the observations reported here?

Response 1-14

We appreciate thoughtful comment of reviewer 1. As reviewer 1 suggested, it is possible that rapid reduction of actin cortex under hypo-osmotic stress is mediated by two Rab35 effectors, by the redox enzyme MICAL1 through oxidation-mediated depolymerization of actin filaments and by OCRL-1 through the reduction of PIP2 level. We added the following sentences in the discussion.

Rab35 is also known to bind to and activate the redox enzyme MICAL family proteins, F-actin disassembly factors (Hung et al. 2011). Therefore, it is possible that the oxidation-mediated depolymerization of actin filaments by MICAL family proteins is involved in the rapid reduction of the actin cortex at apical membrane by hypo-osmotic stress. Thus, we propose that Rab35 initiates a two-pronged approach to reduce cortical actin at the apical membrane and thereby promote the transport of vesicles containing apical membrane components, one through OCRL-1 to decrease the amount of PI(4,5)P2 and destabilize the plasma membrane-actin tether and the other through MICAL family proteins to locally disassemble F-actin (Fig. 8H).

Since there was no difference in the apical transport of sphingomyelin-containing vesicle between wild-type cells and Raptor KD cells (Figs. 4C, 4D, 4E and 4F), we could not clarify the contribution of Rab35 as a negative regulator of mTORC1 in the adaptation mechanism to hypo-osmotic stress. We would like to make it clear in future research.

Reviewer #2

The mechanisms through which epithelial cells react to acute hypo-osmotic stress are poorly understood. Among several others, the role of ion channels, aquaporins, mechanoreceptors, lipid transporters and actin cytoskeleton has been suggested however the signaling cascades leading to the effective regulation of plasma membrane tension and cellular volume are not known. In the submitted manuscript, Ono et al. show the importance of sphingomyelin transport and its regulation by mTORC2 pathway and a regulator of cellular trafficking - Rab35. To do so, the authors use a set of molecular and genetic technics allowing visualization of sphingomyelin in the context of various cellular compartments focusing on the Golgi apparatus and its vesicular transport to the plasma membrane. Overall, the data presented in the manuscript provide interesting insights into the response of epithelial cells to hyper-osmotic stress.

Comment 2-1

Fig. 1B and 1D are lacking legends on the graphs.

Response 2-1

According to the comment of Reviewer2, we added the figure legends on the graphs of Fig. 1B.

Page 19 Line 18

Surface rendering of the plasma membrane area (S) by each antibody staining was performed using Imaris 9.6 software (Bitplane, Inc.).

Comment 2-2

The authors should confirm that cell viability is not compromised by the osmotic shocks used (to eliminate other pathways such as apoptosis etc. as being involved).

Response 2-2

We confirmed that wild-type cells did not undergo apoptosis when exposed to the hypo-osmotic condition used in this study using the FlipGFP-based caspase reporter (Zhang et al. *J. Am. Chem. Soc.* 2019). These images are presented in the revised manuscript as Figs. 7E and 7F.

Comment 2-3

Fig S2B - the decrease in cav-1 signal should be quantified (eg apical-basal cav-1 intensity ratio)

Response 2-3

According to the comment of Reviewer 2, we quantified the caveolin-1 signals of apical membrane and basolateral membrane to calculate the intensity ratio. These images and quantification are presented in the revised manuscript as Fig. S1G.

Comment 2-4

Fig2 - an impact of the stable expression of SS-GFP-Lys on SM levels should be assessed, namely the level of sphingomyelin in SS-GFP-Lys-expressing cells should be compared to the SM levels in parental cells.

Response 2-4

We confirmed that there is no change in the amount of sphingomyelin between parental cells and cells expressing SS-GFP-Lys. This quantification is presented in the revised manuscript as Fig. 2D.

Comment 2-5

p7 line 7-11 - this sentence needs fixing.

Response 2-5

This comment is same as Comment 1-12. We corrected this sentence as follows.

Page 7 Line 24

To exclude the possibility that the effect observed by these mTOR inhibitors is due to the off-target effect and to narrow down which of the mTORC1 and mTORC2 pathways is responsible for the transport of sphingomyelin, we established EpH4 cells in which either Raptor or Rictor, essential scaffold proteins of mTORC1 or mTORC2 respectively, were knocked down (Liu and Sabatini, 2020) (Fig. 4D)

Comment 2-6

Fig 4B, E - show Pearson's coefficient aside or in supplementary material as it is a straight-forward measure of co-localization

Response 2-6

We analyzed the Pearson's coefficient between the signals of SS-GFP-Lys and Giantin of Figure 4A and Figure 4D. In wild-type cells treated with DMSO, these two signals showed higher coefficient score, but the score decreased in cells treated with mTOR inhibitors and in Rictor KD cells (Figs. 4C and 4F).

Comment 2-7

Have the authors looked at other sphingolipids? Is sphingomyelin really the critical feature here or vesicle transport? The authors may wish to reflect on the take home message that they would like to deliver with the title.

Response 2-7

We appreciate thoughtful comment of Reviewer 2. We showed that under hypo-osmotic conditions, the apical membrane is selectively expanded and the transport of the apical membrane containing sphingomyelin visualized by SS-GFP-Lys is enhanced. Since inhibition of ceramide synthesis by HPA-12 (inhibitor of CERT) or Myriosin (inhibitor of serine palmitoyltransferase) impaired transport of podocalyxin-1 to the apical membrane, and sphingomyelin is highly accumulated at the apical membranes and is essential for microvilli formation in the EpH4 cells used in this study, we concluded that sphingomyelin is responsible for the apical transport. As Reviewer 2 suggested, sphingolipids other than sphingomyelin may also contribute to the formation of apical membranes as well as sphingomyelin. At present, we do not have the probes to visualize the intracellular transport of other sphingolipids such as glycolipids, but it should be clarified in future research whether other sphingolipids have similar functions in the apical transport.

Comment 2-8

Page 9 line 7, ref to Fig5D cites Rictor KD cells but figure is TORIN-treated WT cells.

Response 2-8

We corrected this sentence as follows.

Page 9 Line 23

As expected, podocalyxin failed to accumulate at the apical membrane in cells treated with Torin-1 (Fig. 5D).

Comment 2-9

p10 and Fig6 It is not clear how the endothelial lumen formation is induced.

Response 2-9

We added the experimental method about lumen formation of MDCK II cells in the Material and Methods section.

Page 35 Line 17**Lumen formation of MDCK II cells**

MDCK cysts were grown in 3D Matrigel cultures (BD), as described previously (Bryant et al., 2010). Drugs were added at the indicated final concentrations at the time of cell seeding. Cells were grown for 6–48 hours before fixation in 4% paraformaldehyde. The number of cysts with a single lumen or multiple lumens was measured and quantified as a percentage of the total number of measurements.

Comment 2-10

page 13, line 9/10: no direct tension experiments were performed in this study, so formally, the statement "mTORC2-Rab35 activation [...] reduces plasma membrane tension by reducing actin cortex of apical membrane" is not justified - although of course the link between hypo-osmotic shock and membrane tension in combination with the cell rupture data hints into this direction.

Response 2-10

As pointed out by Reviewer 2, we have not directly measured changes in plasma membrane tension, so this sentence is revised as follows:

Page 15 Line 16

Taken together, mTORC2-Rab35 activation by hypo-osmotic stress not only promotes apical transport of sphingomyelin to supply membrane, but also decreases the actin cortex underlining the apical membrane by reducing PI(4,5)P2 to facilitate the expansion of the apical membrane (Fig. 8H).

Comment 2-11

Fig 6C is lacking a control where cells expressing Scarlett-Rab35 Q67L are mock-treated - does it change the localization of SS-GFP-Lys?

Response 2-11

This comment is same as Comment 1-10. We added the images of SS-GFP-Lys expressing cells together with mScarlet or mScarlet-Rab35 Q67L in the absence of mTOR inhibitors in Fig. 5F.

Comment 2-12

fig. 7B: no legend is given.

Response 2-12

We added figure legend for Fig.6B in the revised manuscript.

Page 25 Line 6

(B) Time course change in the number of GFP-Lysenin-positive cells at the apical membrane was quantified. $N \geq 3$ independent experiments; error bar, s.d..

Comment 2-13

Fig 7C-D - compare the increase of the fluorescence of the apical actin to the increase of fluorescence of the basal actin to support the claim about the apical actin accumulation upon Rictor KD.

Response 2-13

We performed the quantification of signals of F-actin staining at apical membrane and basolateral membrane in wild-type cells and Rictor KD cells. The fluorescence intensity of F-actin is significantly up-regulated at the apical membrane as compared to the basolateral membrane in Rictor KD cells. These images and quantification are presented in the revised manuscript as Figs. 6C and 6D.

Reviewer #3

This interesting and novel manuscript describes a mechanism, the enhanced deliver of post-Golgi transport vesicles to the apical plasma membrane, that relieves hypo-osmotic stress in epithelial cells. This is in large part a well-supported model. In

doing so the authors introduce and validate a useful tool for studying the transport of sphingomyelin to the cell surface. Although aspects of this study have been previously reported in other cell types, the importance of this compensatory response in epithelial cells has physiological significance, and the study is comprehensive. The conclusions of the authors are by and large well supported by the data presented with the exception of the assertion that vesicle fusion to the apical plasma membrane is controlled by changes in the cortical actin cytoskeleton. Specific points are as follows:

Comment 3-1

The assertion that changes in the actin cytoskeleton are the basis of the enhance transport of apically-destined vesicles is only circumstantially supported by evidence that changes in actin distribution correlate with this effect. Either a direct test of this assertion should be provided or language in the manuscript should be altered to present this as an as yet untested hypothesis. One approach would be to demonstrate that pharmacological disruption of the actin cytoskeleton, for example by Cytochalasin D, reverses the effect of mTORC2 inhibition. Similarly, the statement that alterations in PIP2 metabolism are the basis of the effect is not supported by direct functional experimentation.

Response 3-1

We investigated whether fusion of vesicles containing sphingomyelin with the PM is promoted by destruction of actin cortex and reduction of PIP2. We found that reducing the level of PIP2 by forcibly localizing INPP5E, an enzyme that dephosphorylates the 5-phosphate of PIPs, to the PM promotes fusion of vesicles containing sphingomyelin (Fig. 8E). On the other hand, destruction of the actin cortex by cytochalasin treatment did not promote vesicle fusion (Fig. R1). This is not surprising given that cytochalasin treatment disrupts not only actin cortex as a physical barrier to apical transport but also impairs apical membrane transport mediated myosin Vb, which is supported by the apical actin cortex (Kapitein et al. *Curr Biol* 2013). On the other hand, the decrease in PIP2 loosens the binding between the PM and the actin cytoskeleton, such as through the inactivation of Ezrin, but does not completely dissolve the actin cortex (Fig. R2). Considering the yin and yang effects of the actin cortex on the delivery and

fusion of the sphingomyelin-containing vesicles with the apical PM, we toned down the conclusion about the roles of actin cortex in our model (Fig. 8H and related discussion).

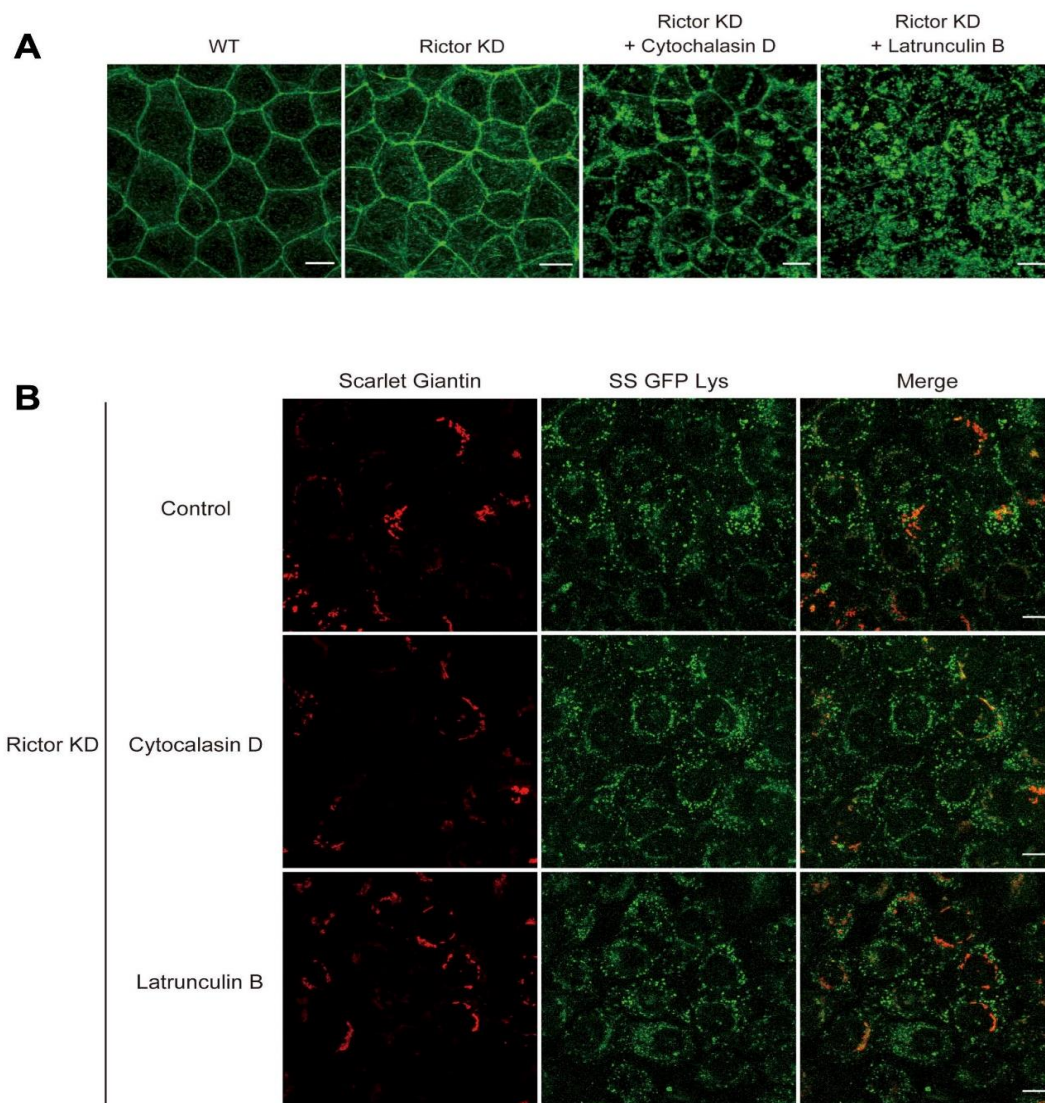


Figure R1

Disruption of actin cortex by treatment with cytochalasin D did not promote apical transport of sphingomyelin

(A) Wild-type cells, Rictor KD cells and Rictor KD cells treated with 2 $\mu\text{g/mL}$ cytochalasin D or 2 μM Latrunculin B were fixed and stained with phalloidin. Scale bar, 10 μm .

(B) Rictor KD cells expressing SS-GFP-Lys were treated with 2 $\mu\text{g/mL}$ cytochalasin D or 2 μM Latrunculin B for 3 hours. Scale bar, 10 μm .

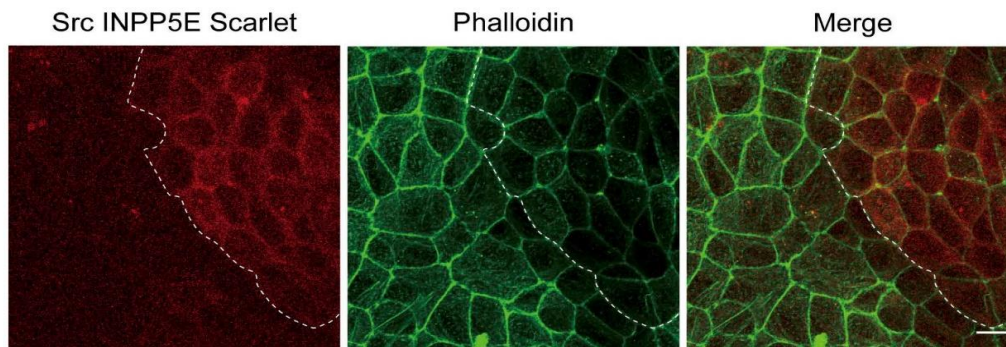


Figure R2

Expression of Src-INPP5E-Scarlet did not completely dissolve the actin cortex

EpH4 wild-type cells stably expressing Src-INPP5E-Scarlet were stained with phalloidin. Scale bar, 10 μm .

Comment 3-2

Figure S3. The identity of the intracellular organelles that stain with the probe for sphingomyelin as post-Golgi transport carriers is an important aspect of this work. This is supported by the co-localization of the sphingomyelin probe with podocalyzin-like-1 but not E-cadherin (Figure 3) and the temperature based pulse/chase experiment depicted in Figure S5. However the co-localizations depicted in Figure S3 are not as convincing due to variations in the abundance and shape of the ssGFP-Lys staining. These localization studies should be repeated with the vesicles accumulated in the

presence of Torin or the rictor knockout, in which the abundance of the vesicles should clarify co-localization or lack thereof.

Response 3-2

Following the instruction of Reviewer 3, we examined the co-localization of various organelle markers with vesicles containing SS-GFP-Lys accumulated in cells treated with Torin-1. SS-GFP-Lys containing vesicles and trans Golgi network marker (TGNP) were highly co-localized. These images are presented in the revised manuscript as Fig. S2.

Comment 3-3

Figure 1. The authors state that the expansion of the apical membrane does not result in dilution of the components, which is an important point. I believe the quantitation presented in Figure 1, Panels B and D represent total area stained. To support the authors' point this quantitation should be repeated as total intensity of staining.

Response 3-3

Following the comment from Reviewer 3, total intensity of staining was used to quantify the degree of dilution of each components during expansion of the apical membrane in Figs. 1C, 1D and 1G.

Comment 3-4

Figure 2, Panel D. The ssGFP-lysenin probe is interesting and will be valuable to the research community. Validation is key. The results with the inhibitors in panel D are reasonable, but as the authors no doubt know, each inhibitor has drawbacks. The authors should also test inhibition of ceramide biosynthesis with Fumonisin B1 and the staining should be quantitated.

Response 3-4

We examined the effects of Fumonisin B1 treatment on EpH4 cells according to the comment of Reviewer 3. However, massive cell death was induced after treatment with Fumonisin B1 (Figs. R3A and R3B). As already shown in previous studies, Fumonisin B1 treatment causes free sphinganine accumulation and

exhibit cytotoxicity in some epithelial cell lines such as LLC-PK1 (Yoo et al. *Toxicol. Appl. Pharmacol* 1992), HT-29(Schmelz et al. *Toxicol. Appl. Pharmacol.* 1998) and human keratinocytes(Tolleson et al. *Int. J. Oncol.* 1999). Treatment with Fumonisin B1 was performed at a low concentration (5 μ M) that did not induce cell death, but no decrease in the amount of sphingomyelin was observed in EpH4 cells and MDCK II cells used in this study (Figs. R3B, R3C and R3D). The effect of Fumonisin B1 may differ among cell types due to differences in the expressed enzyme sphinganine *N*-acyltransferase (ceramide synthase) isoforms. Considering our result and the toxicity to EpH4 cells, we thought that it is not appropriate to use Fumonisin B1 for the experiment of Fig. 2B and that the use of Myriocin and HPA-12, which do not induce free sphinganine accumulation, would be better.

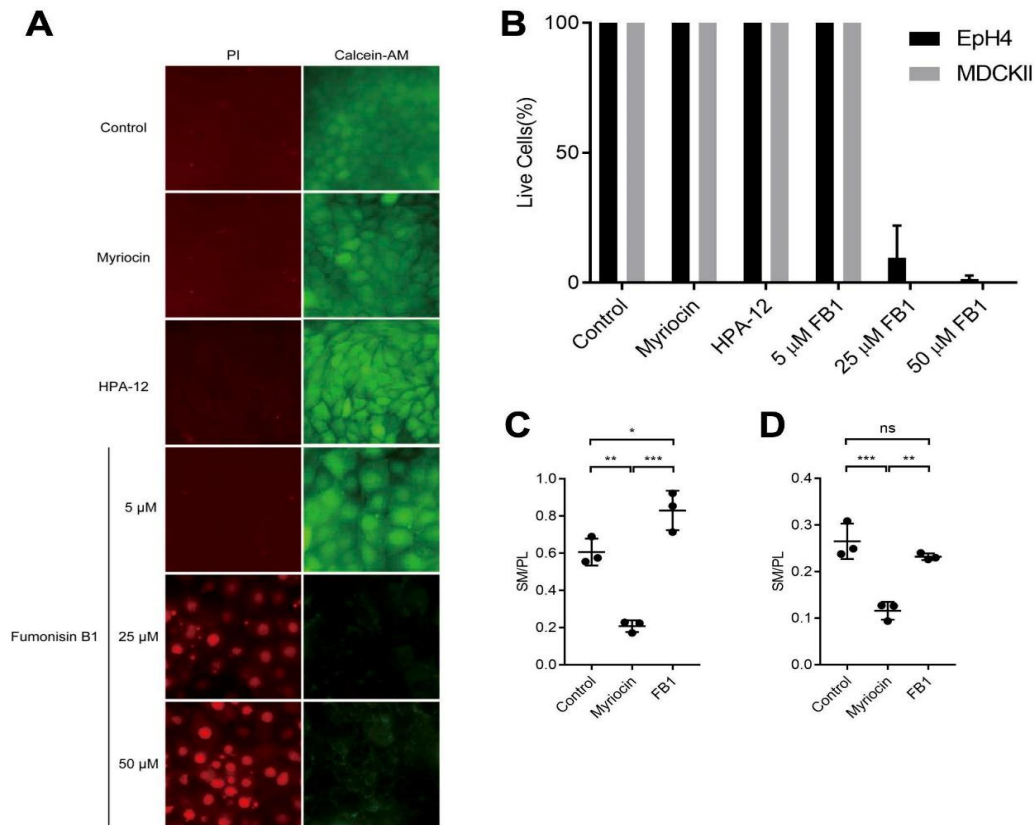


Figure R3

Fumonisin B1 treatment induces massive cell death in Eph4 cells and MDCK II cells

(A) Wild-type Eph4 cells treated with indicated inhibitors for 48 hours were stained with Calcein-AM (green; cytosol of live cells) and PI (red; nucleus of dead cells).

(B) Quantification of the ratio of live cells to total cells treated with the indicated inhibitors for 48 hours in Eph4 cells and MDCK II cells.

(C) (D) Total lipids were extracted from wild-type Eph4 cells (C) and MDCK II cells (D) treated with indicated inhibitors by Bligh and Dyer method. The ratio of the amount of sphingomyelin (mg/dL) to the total amount of phospholipids (mM) were quantified in both samples. $N = 3$ from independent experiments; error bar, s.d.; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.0002$ and ns, not significant by one-way ANOVA with Tukey's post-hoc test.

Comment 3-5

Figure 8, Panels A and B. AKT can be activated by a number of pathways, in addition to mTORC2. To demonstrate that the activation that is described in this figure is due to mTORC2 activation, the authors should demonstrate that it is blocked with Torin and/or in the rictor knockout.

Response 3-5

To examine whether the activation of AKT induced by hypo osmotic stress is dependent on the activation of mTORC2, we exposed cells to hypo-osmotic stress with or without Torin-1 treatment. Hypo-osmotic stress did not cause AKT activation when cells were pre-treated with Torin-1, indicating that the activation of AKT by hypo-osmotic stress is dependent on mTORC2 activation (Figs. 7C and 7D).

Comment 3-6

Much of the data demonstrating a role of mTorc2 in the transport of sphingomyelin to the cell surface is derived from cells not subjected to hypo-osmotic stress. This indicates that this is a constitutive role as well as a role in response to osmotic stress. The authors should address this in the Discussion.

Response 3-6

Following the comment of Reviewer 3, the following text is added to the Discussion.

Page 16 Line 24

We revealed that activation of the mTORC2-Rab35 pathway by hypo-osmotic stress promotes the apical transport of sphingomyelin and enables rapid expansion of the apical membrane. Notably, in mTORC2 (Rictor) KD or Rab35 knockout cell lines, the formation of apical membrane structures such as microvilli was impaired and the transport of sphingomyelin apical membrane was delayed, suggesting that sustained activity of the mTORC2-Rab35 pathway is required to maintain epithelial cell morphology and function even in steady-state epithelial cells. Furthermore, under iso-osmotic condition, RBD of MICAL-3, a reporter of activated Rab35, constitutively localizes to the apical membrane, and its localization is impaired by pharmacological

inhibition of the mTORC2 pathway, suggesting a constitutive role for the mTORC2-Rab35 axis at the apical membrane of polarized epithelial cells (Fig. 7M). Therefore, in addition to the molecular mechanism by which hypo-osmotic stress induces further activation of the mTORC2-Rab35 pathway, elucidation of the upstream signaling pathway responsible for the constitutive activity of mTORC2-Rab35 in polarized epithelial cells is a topic of immediate interest.

January 19, 2022

RE: JCB Manuscript #202106160R

Prof. Junichi Ikenouchi
Kyushu University
West 1st Building W1-D-809, 744 Moto-oka Nishi-ku Fukuoka
Fukuoka 819-0395
Japan

Dear Prof. Ikenouchi:

Thank you for submitting your revised manuscript entitled "mTORC2 suppresses cell death induced by hypo-osmotic stress by promoting sphingomyelin transport". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, please address reviewer #1's remaining minor comments.

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Sincerely,

Ian Macara, Ph.D.
Editor

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

Journal of Cell Biology

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

The authors convincingly addressed all my comments, and the revised manuscript provides clear conclusions supporting the proposed model. This is a very interesting piece of work connecting Rab35/actin/traffic of sphingomyelin/resistance to hypo-osmotic shock. I therefore fully support publication in JCB.

I have nevertheless two remaining remarks:

- Rab35 interacts with MICAL1 and MICAL3 via both high and low affinity binding sites (PMID 27552051 and PMID 28230050 studied this interaction in detail after the initial publication of Fukuda 2008). Importantly, at least 3 Rab GTPases interact with MICAL1 and MICAL3: Rab1, Rab8 and Rab35. Which MICAL3-RBD has been used in this study? To my knowledge and given the aforementioned crystallographic structures, there is no MICAL3-RBD that interacts only with Rab35. Therefore, GFP-MICAL3-RBD is unlikely to be a specific marker for Rab35-GTP. This should be at least discussed, and the associated data should be interpreted with caution.

- p. 15 line 8, the cited paper (Hung et al. 2011) has not addressed the Rab35/MICAL1 interaction nor its role in F-actin clearance. Please, correct the reference.

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

The authors have sufficiently addressed my previous critiques. A minor issue is that the panels in Figure S2 are not labeled. These labels should be added.