Regulation of Hook1-mediated endosomal sorting of clathrin-independent cargo by γ-taxilin

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Original submission

First decision letter

MS ID#: JOCES/2021/258849

MS TITLE: Regulation of Hook1-mediated endosomal sorting of clathrin-independent cargo by γ-taxilin

AUTHORS: Satoru Higashi, Tomohiko Makiyama, Hiroshi Sakane, Satoru Nogami, and Hiromichi Shirataki
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers found the study interesting, well executed and organized and they also appreciated that experiments were well controlled and carefully quantified. From the reviewers' comments, you will see that there are few important points that require additional experiments. In particular, reviewers have pointed out lack of endogenous co-ip experiments and requirement for additional controls in these experiments. Further, more robust analysis is needed for quantification of tubular recycling endosomes. The specificity of the siRNA experiments should be addressed by addition of rescue experiment as pointed out by reviewer 2.

If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then

provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the current study, Higashi et al. identified Hook1, a microtubule- and cargo-tethering protein as a novel gamma-taxilin interactor. They then showed that depletion of gamma-taxilin in HeLa cells promoted formation of tubular structures that were positive for two Hook1-mediated cargo proteins, CD98 and CD147, recycling of CD98 to the plasma membrane, and CD147-mediated cell spreading. Conversely, overexpression of gamma-taxilin in HeLa cells suppressed CD98-positive tubular formation. Intriguingly, gamma-taxilin depletion had no effect on tubular formation of Hook1-independent cargos such as MHC-I and its recycling. Finally, they demonstrated that gamma-taxilin and CD98 competitively interacted with the C-terminal region of Hook1. On the basis of their findings, the authors suggested that gamma-taxilin negatively regulate the sorting of Hook1-mediated cargo proteins into tubular endosomes presumably by inhibiting the interaction between Hook1 and cargo proteins. Overall, the experimental approaches are well designed and the results obtained are largely convincing; however, there are a few issues that require additional clarification.

Comments for the author

Specific points:
1. Tubular structures of CD98 and CD147 were observed only in ?~30%? of the control HeLa cells (Fig. 2C and 2E). Does this mean that cells lacking these tubular structures (~70% of the control cells) contain the high expression level of gamma-taxilin? IF analysis of gamma-taxilin should be performed in Fig. 2. Also, co-localization data between gamma-taxilin and Hook1 would be informative for general readers.

2. The authors suggested in the last sentence of the abstract that gamma-taxilin inhibits the interaction between Hook1 and cargo proteins. Although gamma-taxilin has the ability to inhibit the Hook1-CD98 interaction (Fig. 7G), it is unclear whether it can also inhibit the Hook1-CD147 interaction, which is likely to promote cell spreading (Fig. 6). To complete this study, the authors should test this possibility.

3. According to the final model (Fig. 8), gamma-taxilin inhibits sorting of Hook1-mediated cargo proteins (CD98 and CD147) from sorting endosomes (SEs) to recycling endosomes (REs). Does this mean that both CD98 and CD147 are present on SEs rather than REs under gamma-taxilin-overexpressing conditions (Fig. 7A)? This should be experimentally tested by using appropriate endosomal markers. Moreover, this reviewer is also interested in the localization of Hook1 in gamma-taxilin-overexpressing cells. Is it localized in the cytosol or on REs?

4. The discussion section is too long and some parts are not so informative. It could be edited.

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors address the mechanisms by which clathrin-independent cargo are trafficked, and identify the protein gamma-taxilin as an important negative regulator of Hook1,

which links select clathrin-independent cargo to microtubules. The authors show that gamma-taxilin binds to Hook1 in the region of Hook1 that is involved in cargo selection and that interacts with receptors such as CD98 and CD147, thus preventing their selection into recycling tubules. Indeed, the authors demonstrated that gamma-taxilin KD leads to more CD98-containing tubular recycling endosomes and a faster rate of recycling, whereas overexpression of gamma-taxilin reportedly inhibits CD98-tubular endosome formation. Overall, this is an interesting manuscript that provides support for a model in which the gamma-taxilin binds to Hook1 to negatively regulate the formation of endosomal recycling tubules of clathrin-independent cargo that recycle directly from sorting endosomes.

Comments for the author

In Fig. 1, the authors show that Hook1 and Hook1 interact with gamma-taxilin, but not Hook3 by Y2H. Yet by IP, they only show Hook1 co-Ips and only do a bare minimum of controls. The authors need to show that in the co-IP Hook3 does not precipitate with gamma-taxilin. Additional controls, such as transfecting with HA-alpha or beta taxilin and attempting co-IP with Hook proteins should also be done.

The interactions shown by Y2H and co-IP appear generally robust, but this raises the important question as to whether co-Ips with endogenous proteins can be detected. This would be more convincing with regard to the physiological significance, and since antibodies are available, it is unclear why the authors neglected these experiments.
The authors maintain that the increase in TRE number is not an artifact of off-target effects because they used two different siRNA oligonucleotides. Can they formally prove this by rescue experiments where gamma-taxilin is reintroduced back into KD cells? Some form of rescue has become the standard in demonstrating that the KD of a specific protein is truly responsible for a given phenotype.
As a representative image, Fig. 7 does not really show that overexpressing GFP-gamma-taxilin leads to fewer TRE. This is not convincing.
The conclusion that gamma-taxilin and Hook1 compete for binding with CD98 should be fortified with competitive binding experiments.

While the manuscript does a reasonable job in asserting that gamma-taxilin might compete with CD98 for binding to Hook1, thus controlling sorting into TRE and recycling, some of the experiments done in this manuscript seem to be somewhat derivative in that they add some new information broadly, but refrain from addressing more central (and admittedly perhaps more challenging) questions. For example, the assessment that gamma-taxilin is perhaps involved in cell spreading and migration, is not unexpected and it is indeed rather complicated to unravel the role of recycling of integrins or these “green” TRE cargoes. On the other hand, if there is truly competition between gamma-taxilin and CD98 and other such cargo receptors for binding to Hook1, a very essential (and unaddressed) question is what regulates gamma-taxilin localization and binding to Hook1? To this reviewer, it seems as though this type of question is more likely to provide important information to the community.
Discussion is somewhat long and repetitive of information provided within the Results.

Reviewer 3

Advance summary and potential significance to field

The study of Higashi and colleagues reveals a role for γ-taxilin in endosomal recycling of selected cargoes (CD98 and CD147) internalized into the endocytic network through clathrin-independent endocytosis. The studies are generally well conducted and range from biochemical analysis of γ-taxilin, Hook1 and CD98 interactions through to functional analysis in the generation of tubular profiles and the process of cell spreading. There is good inclusion of positive and negative controls to reveal the selectivity and specificity of the observations and mechanisms. Overall, the study makes a significant contribution to the mechanistic understanding of endosomal sorting of cargo proteins that play important roles in a range of cellular processes.

Comments for the author

Overall, I recommend that the manuscript be accepted for publication subject to consideration of a few issues.

Main comments:
The various IP experiments rely on co-overexpression of tagged proteins. Can the interactions be observed with endogenous proteins? For example, does IP of HA-γ-taxilin bring down endogenous Hook1?
A positive tubulating cell is defined by having at least one tubule greater than 5 microns in length. What percentage of cells being analyzed are defined as being tubular because they have a single tubule? The analysis of the biogenesis of newly formed tubules is perhaps the weakest part of the manuscript. Were these experiments blinded? Was the frequency of tubule collapse altered by taxilin manipulation?
The discussion of ß2-adrenergic receptor must include reference to the SNX27-retromer-WASH pathway, which has been established by the van Zastrow lab to regulate the endosomal sorting of this cargo (e.g. Lauffer et al., 2010; Temkin et al., 2011). This pathway also serves to sort Basigin/CD147 and another CIE cargo GLUT1 (see Steinberg et al., 2013). The authors should discuss in more detail the link of their work with this and other endosomal retrieval and recycling pathways.

Minor points:
The lysate % needs to be stated in all relevant blots.

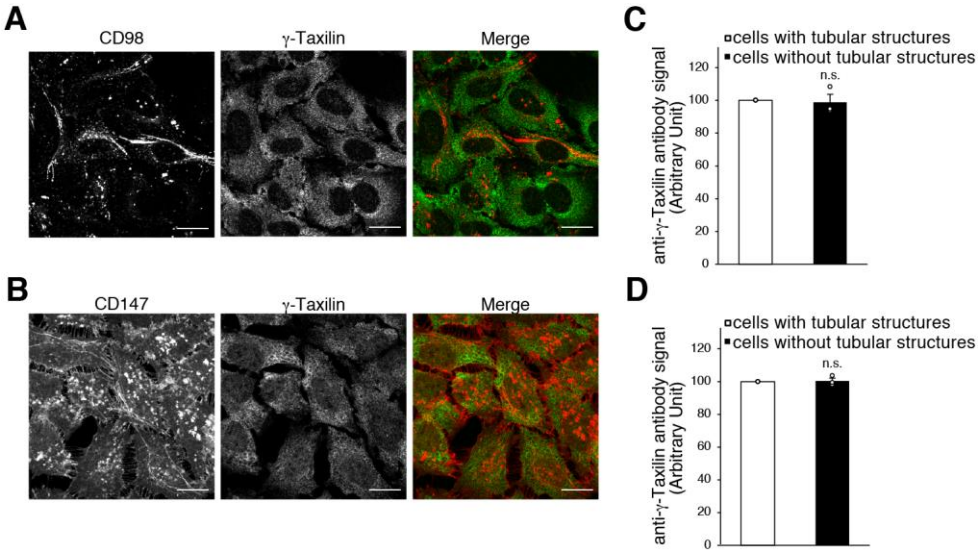
First revision

Author response to reviewers' comments

Our responses to Reviewer #1

1-1) Reviewer #1 asked whether cells lacking CD98- or CD147-positive tubular structures contain higher expression level of γ-taxilin than those forming these tubular structures.
According to the reviewer's comment, control HeLa cells in the antibody uptake assay were simultaneously immunostained with an anti-γ-taxilin antibody to measure the fluorescence intensity of γ-taxilin in cells with or without exhibiting CD98- or CD147-positive tubular structures. There was no significant difference in the fluorescence intensity of γ-taxilin between cells with or without these tubular structures as shown in the below figure. These results suggest that it is unlikely that cells lacking CD98- or CD147-positive tubular structures contain higher expression level of γ-taxilin than those forming these tubular structures. Although the reason why cells lacking CD98- or CD147-positive tubular structures were observed in about 70% of control cells is not known, the following possibility is thought. Since we observed in time-lapse imaging analysis that cells lacking these tubular structures at the start of observation newly formed these tubular structures during the period of observation and *vice versa*, it is thought that these tubular structures are highly dynamic and the antibody uptake assay shows only tubular structure status of cells just when cells are fixed. Our results are consistent with the report by another research group that CD98-positive tubular structures are observed in about 30% of control HeLa cells (see Kim Nguyen, N. T. *et al*. (2020). *Biochem Biophys Res Commun* 528, 220-226.). This is described in the revised manuscript (Page 11, lines 206-207). Although uninformative and repetitive description in the discussion section is deleted in the revised manuscript according to the reviewers' comment, the total word count of the revised manuscript is significantly increased due to the addition of other essential data required for the revision. Therefore, we are not able to show the below negative data in Figure 2 (word count: 65).

Additionally, since two other essential supplementary figures are added in the revised manuscript, the supplementary figure containing the below negative data is not able to be added due to limitations of the number of supplementary figure.



(**A** and **B**) Hela cells subjected to the antibody uptake assay were immunostained with an anti-γ-taxilin antibody. Scale bars: 20 μm.
(C and D) The fluorescence intensity of γ-taxilin in cells with or without exhibiting CD98- or CD147-positive tubular structures. Data are expressed as the mean ± s.e.m. (*n* = 3; >30 cells were analyzed in each experiment) n.s., not significant by two-tailed Student's *t*-test

1-2) Reviewer #1 asked whether γ-taxilin is co-localized with Hook1 in cells.
According to the reviewer's comment, we examined whether γ-taxilin is co-localized with Hook1 in HeLa cells. However, since both of available anti-γ-taxilin and anti-Hook1 antibodies were unfortunately produced in rabbit, only single staining patterns of each protein were able to be obtained. Then, we examined whether exogenously expressed GFP-γ-taxilin and myc-Hook1 are co-localized in cells. Immunocytochemical analysis showed that the staining patterns of GFP-γ-taxilin and myc-Hook1 were similar to those of the corresponding endogenous proteins and that GFP-γ-taxilin was co-localized with myc-Hook1. This is shown in Figure 1C,D and described in the revised manuscript (Page 9, line 180 - page 10, line 187).

Reviewer #1 asked whether γ-taxilin inhibits the Hook1-CD147 interaction.
According to the reviewer's comment, we examined whether γ-taxilin inhibits the Hook1-CD147 interaction. First, we performed a similar co-immunoprecipitation assay using pcDNA3-Flag-CD147 instead of pcDNA3-Flag-CD98 whether CD147 interacts with Hook1 and γ-taxilin. Flag-CD147 was co-immunoprecipitated with myc-Hook1 but not with γ-taxilin. The amount of myc-Hook1 co-immunoprecipitated with Flag-CD147 was significantly reduced in the cells expressing HA-γ-taxilin compared with control cells. This is shown in Figure S8 and described in the revised manuscript (Page 21, lines 464-466).

3-1) Reviewer #1 asked whether both CD98 and CD147 are present on SEs rather than REs under γ-taxilin-overexpressing conditions
According to the reviewer's comment, we examined whether overexpression of γ-taxilin affects the subcellular localization of CD98 and CD147. It has been reported that Hook1 depletion exhibiting the effect on the formation of CD98-positive tubular structures similar to that of γ-taxilin overexpression redirects CD98 to the EEA1-positive endosomes from the tubules associated with recycling. Then, we examined whether overexpression of γ-taxilin enhanced the co-localization of CD98 with EEA1-positive endosomes. When cells were simultaneously immunostained with an anti-EEA1 antibody, either CD98 or CD147 was hardly co-localized with EEA1-positive endosomes in control cells but both of CD98 and CD147 were significantly co-localized with EEA1-positive endosomes in cells overexpressing γ-taxilin. This is shown in Figure S7A-C and described in the revised manuscript (Page 20, lines 421-432).

3-2) Reviewer #1 asked whether Hook1 is localized in the cytosol or on REs under γ-taxilin - overexpressing conditions.
According to the reviewer's comment, we examined whether Hook1 is localized in the cytosol or on REs under γ-taxilin-overexpressing conditions. When cells were stained with an anti-Hook1 antibody, Hook1 was stained throughout the cytoplasm and often exhibited a tubule-vesicular pattern. On the basis of the observation, we thought that it is difficult to evaluate the effect of γ-taxilin overexpression on the subcellular localization of Hook1 by immunofluorescence analysis. Then, we performed subcellular fractionation to reveal whether γ-taxilin overexpression affects the subcellular localization of Hook1. Hook1 was almost present in the cytosolic fraction in control cells and overexpression of γ-taxilin did not affect the subcellular localization of Hook1. This is shown in Figure S7D and described in the revised manuscript (Page 20, lines 432-436).

4) Reviewer #1 pointed out that the discussion section is too long.
According to the reviewer's comment, uninformative and repetitive description was deleted in the discussion in the revised manuscript.

Our responses to Reviewer #2

1-1) Reviewer #2 suggested to show by IP that Hook3 is not precipitated with γ-taxilin.
According to the reviewer's comment, we examined using co-immunoprecipitation assay whether myc-Hook3 is precipitated with γ-taxilin or not. HA-γ-taxilin and myc-Hook3 were co-expressed in HeLa cells, and the cell lysates were immunoprecipitated with an anti-HA antibody. myc-Hook3 was not co-immunoprecipitated with HA-γ-taxilin, being consistent with the results obtained by Y2H. In addition, we performed a similar examination using pcDNA3-myc-Hook2 instead of pcDNA3-myc-Hook3. myc-Hook2 was co-immunoprecipitated with HA-γ-taxilin and *vice versa*, being consistent with the results obtained by Y2H. This is shown in Figure 1B and described in the revised manuscript (Page 9, lines 168-172).

1-2) Reviewer #2 suggested to show by IP that either α- or ß-taxilin is not precipitated with Hook proteins.
According to the reviewer's comment, we examined using co-immunoprecipitation assay whether myc-Hook1, -Hook2, and -Hook3 are precipitated with HA-α-taxilin or -ß-taxilin. HA-α-taxilin or -ß-taxilin and myc-Hook1, -Hook2, or -Hook3 were co-expressed in HeLa cells, and the cell lysates were immunoprecipitated with an anti-HA antibody. Either HA-α-taxilin or -ß-taxilin was not co-immunoprecipitated with each Hook protein, being consistent with the results obtained by Y2H. This is shown in Figure 1B and described in the revised manuscript (Page 9, lines 168-172).

2) Reviewer #2 suggested to examine by IP whether endogenous Hook1 is precipitated with endogenous γ-taxilin.
According to the reviewer's comment, we performed co-immunoprecipitation assay using anti-γ-taxilin and anti-Hook1 antibodies to examine whether endogenous Hook1 is interacted with endogenous γ-taxilin. However, we failed to detect the interaction between endogenous γ-taxilin and Hook1 by co-IP. Therefore, we examined whether overexpressed HA-γ-taxilin or myc-Hook1 is precipitated with endogenous Hook1 or γ-taxilin, respectively, but we also failed. The reason why we were not able to detect the interaction between γ-taxilin and Hook1 in above experiments is not known. However, since GFP-γ-taxilin and myc-Hook1, both of whose staining patterns were similar to those of the corresponding endogenous proteins, were co-localized as shown in Figure 1, it is possible that since the interaction between γ-taxilin and Hook1 might be very weak, a large part of the complex between γ-taxilin and Hook1 might be disrupted during the IP procedures, resulting that only a few part of the complex between γ-taxilin and Hook1 was able to be detected by IP. This is described in the revised manuscript (Page 9, line 176 - page 10, line 187).

3) Reviewer #2 suggested to perform rescue experiments where siRNA-resistant γ-taxilin is reintroduced back into KD cells to demonstrate that the KD of a specific protein is truly responsible for a given phenotype.
According to the reviewer's comment, we examined whether the effect of γ-taxilin depletion on the formation of CD98-positive tubular structures is attenuated by reintroducing plasmid containing

siRNA-resistant mutant of γ-taxilin into γ-taxilin-depleted cells. We constructed siRNA-resistant γ-taxilin expression plasmid (pEGFPC1-γ-taxilin-mt) containing five silent substitutions in the region complementary to γ-taxilin #1 siRNA and performed siRNA rescue experiments. The effect of γ-taxilin siRNA treatment on the formation of CD98-positive tubular structures was attenuated in pEGFPC1-γ-taxilin-mt-transfected cells but not pEGFPC1-γ-taxilin-transfected cells. GFP-γ-taxilin was expressed in pEGFPC1-γ-taxilin-mt-transfected cells but not pEGFPC1-γ-taxilin-transfected cells. The results support that the effect of γ-taxilin depletion on the formation of CD98-positive tubular structures is not due to an off-target effect of siRNA treatment. This is shown in Figure 2D-F. and described in the described manuscript (Page 11, lines 212-217; Page 30, lines 640-643; Page 31, lines 663-666).

4) Reviewer #2 pointed out that a representative image in Fig. 7 does not really show that overexpressing GFP-γ-taxilin leads to fewer TRE.
As the reviewer pointed out, the percentage of cells exhibiting CD98-positive tubular structures in representative images is not significantly different between GFP- and GFP-γ-taxilin overexpressing cells. Since in Figure 7A in the original manuscript, we intended to show that CD98-positive tubular structures were formed in GFP overexpressing cells but not GFP-γ-taxilin overexpressing, we presented misleading representative images. Then, to avoid confusion, exchanged representative images are shown in Figure 7A in the revised manuscript.

5) Reviewer #2 suggested to perform competitive binding experiments to fortify the conclusion that γ-taxilin and Hook1 compete for binding with CD98.
According to the reviewer's comment, we performed competitive binding experiments using various amounts of pcDNA3-HA-γ-taxilin. The expression levels of HA-γ-taxilin were increased in a dose-dependent manner but the expression levels of either Flag-CD98 or myc-Hook1 were not changed. The amounts of myc-Hook1 co-immunoprecipitated with Flag-CD98 were decreased in antiparallel with increasing expression levels of HA-γ-taxilin. This is shown in Figure 7H. and described in the revised manuscript (Page 21, line 466 - page 22, line 472).

6) Reviewer #2 suggested to describe about more central questions such as what regulates the localization of γ-taxilin and the binding of γ-taxilin to Hook1.
We agree to the reviewer's comment. It is important and essential issues to unravel a precise mechanism by which regulates the localization of γ-taxilin and the binding of γ-taxilin to Hook1. However, we have not yet obtained any data that resolve the issues. Then, taken together with the results obtained so far by other investigators, we describe our speculation concerning the issues in the revised manuscript. In the future, we hope to address the issues as another project. This is described in the revised manuscript (Page 28, lines 597-602).

7) Reviewer #2 pointed out that the discussion section is too long.

The same response to the comment 4) of the reviewer #1

Our responses to Reviewer #3

1) Reviewer #3 suggested to examine by IP whether exogenously expressed HA-γ-taxilin is precipitated with endogenous Hook1.

The same response to the comment 2) of the reviewer #2

2-1) Reviewer #3 asked what percentage of cells being analyzed are defined as being tubular because they have a single tubule?

We have already described in the original manuscript that about 35% of analyzed control cells contained at least one tubule greater than 5 microns. Our results are consistent with the previous report by Kim Nguyen, N. T. *et al* that CD98-positive tubular structures are observed in about 30% of control HeLa cells. This is described in the revised manuscript (Page 10, line 204 - page 11, line 206).

2-2) Reviewer #3 asked how the biogenesis of newly formed tubules was analyzed.
We agree to the reviewer's comment that counting or measuring newly formed tubules are affected by human bias. Here, we had counted the number of newly formed tubules and measured the length of newly formed tubules by three of the authors independently, and the results were matched between three of the authors. This is described in the revised manuscript (Page 34, lines 746-747).

2-3) Reviewer #3 asked the effect of γ-taxilin depletion on the frequency of tubule collapse
According to the reviewer's comments, we examined whether γ-taxilin depletion affects the frequency of tubule collapse in time-lapse imaging. There was no significant difference in the frequency of tubule collapse between control and γ-taxilin-depleted cell. This is described in the revised manuscript (Page 14, lines 294-297).

3) Reviewer #3 suggested to describe about other endosomal retrieval and recycling pathways such as the SNX27-retromer-WASH pathway in the discussion section.
We agree to the reviewer's comment and describe about other endosomal retrieval and recycling pathways such as the SNX27-retromer-WASH pathway in the revised manuscript (Page 23, line 495 - page 24, line 500).

4) Reviewer #3 pointed out that the lysate % needs to be stated in all relevant blots.

We agree to the reviewer's comment. The amount of cell lysate used for western blotting is expressed as lysate % in all relevant blots in the revised manuscript.

Second decision letter

MS ID#: JOCES/2021/258849

MS TITLE: Regulation of Hook1-mediated endosomal sorting of clathrin-independent cargo by γ-taxilin

AUTHORS: Satoru Higashi, Tomohiko Makiyama, Hiroshi Sakane, Satoru Nogami, and Hiromichi Shirataki
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but reviewer 2 has raised concerns with lack of evidence of endogenous gamma-taxilin and Hook1 binding. Reviewer 2 has also suggested additional experiments with overexpression of one binding partner. I hope that you will be able to carry these out because I would like to be able to accept your paper. When you submit your revised manuscript, please ensure that when plotting data to use a format that clearly shows the full distribution of the data such as a dot-plot or box-and-whisker-plot format.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the revised manuscript, the authors properly addressed this reviewer's concerns. I thus believe that the manuscript is now suitable for publication in Journal of Cell Science.

Comments for the author

I have no further comments to this manuscript.

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors address the mechanisms by which clathrin-independent cargo are trafficked, and identify the protein gamma-taxilin as an important negative regulator of Hook1, which links select clathrin-independent cargo to microtubules. The authors show that gamma-taxilin binds to Hook1 in the region of Hook1 that is involved in cargo selection and that interacts with receptors such as CD98 and CD147, thus preventing their selection into recycling tubules. Indeed, the authors demonstrated that gamma-taxilin KD leads to more CD98-containing tubular recycling endosomes and a faster rate of recycling, whereas overexpression of gamma-taxilin reportedly inhibits CD98-tubular endosome formation. Overall, this is an interesting manuscript that provides support for a model in which the gamma-taxilin binds to Hook1 to negatively regulate the formation of endosomal recycling tubules of clathrin-independent cargo that recycle directly from sorting endosomes.

Comments for the author

The authors have made improvements and addressed some, but not all of the issues raised by the reviewers in this revision. There remains some concern, despite the binding between over-expressed Hook1 and Hook2 with gamma-taxilin, that endogenous proteins cannot be detected interacting with one another in lysates from these cells--or even if one protein is endogenous. This reviewer would be more comfortable with additional controls to verify endogenous binding. If endogenous immune complexes are disrupted by co-ip, could the authors engineer CRISPR cells with endogenous levels of expression of Myc and HA-tagged proteins? Or could they show that antibodies to the endogenous proteins DO NOT precipitate the pair (i.e., only the Myc- or HA-antibodies), suggesting that this is really the case? It is somewhat concerning that with such excellent antibodies that co-ips cannot be done. Do the antibodies pull down Hook or taxilin but not binding partners? In addition, the more conceptual question raised (point #6) about the regulation of gamma-taxilin and its binding to Hook and localization was not addressed at all. A more minor issue is the improved Figure 7--insets are still needed to highlight the differences in observed tubules in these micrographs.

Reviewer 3

Advance summary and potential significance to field

Manuscript describes a link between gamma-taxilin and Hook1 in mediating endosomal cargo sorting. Their principle conclusion is that gamma-taxilin negatively regulates Hook1-mediated cargo proteins into recycling endosomes. Overall, the study adds additional insight into the molecular mechanisms of endosomal cargo sorting.

Comments for the author

The Authors have covered all of my concerns in their revisions and I recommend the manuscript for publication.

Second revision

Author response to reviewers' comments

Our responses to Reviewer #2

1) Reviewer #2 suggested to examine by IP whether endogenous Hook1 is precipitated with endogenous γ-taxilin.
According to the reviewer's comment, we performed co-immunoprecipitation assay using an anti-γ-taxilin antibody to examine whether endogenous Hook1 is interacted with endogenous γ-taxilin. However, we had failed to detect the interaction between endogenous γ-taxilin and Hook1 by co-IP in the first revised manuscript. We thought that the amount of co-immunoprecipitated Hook1 may be below detectable levels in the western blot detection system used in the first revised manuscript. Then, we attempted by use of a new higher-sensitive western blot detection system recently produced by Thermo Fisher scientific to detect co-immunoprecipitated Hook1 by western blotting. The sensitivity for detection of positive bands was dramatically raised beyond our expectations by use of the new higher-sensitive western blot detection system as shown in the below figure. In addition, we increased the amounts of IP samples loaded on SDS-PAGE compared to those used in the first revised manuscript. Under the conditions, we found that endogenous Hook1 was co-immunoprecipitated with endogenous γ-taxilin. This is described in the revised manuscript (Page 9, lines 176-180; Page 29, lines 612-613; Page 32 lines 696-697; Page33 lines 709-714; Page 44 lines 997-1002).

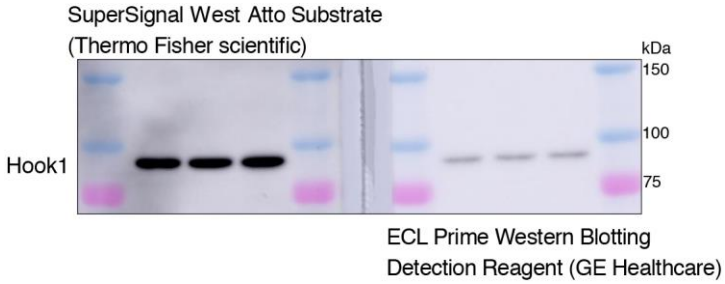


Figure 1. Detection of Hook1 in HeLa cell lysate (10 μg). Left and right show the western blots detected using SuperSignal West Atto Substrate (Thermo Fisher scientific, A38555) and ECL Prime Western Blotting Detection Reagent (GE Healthcare, RPN223), respectively. Both blots were simultaneously acquired using an Amersham Imager 600 (Amersham).

2) Reviewer #2 suggested to describe about more central questions such as what regulates the localization of γ-taxilin and the binding of γ-taxilin to Hook1.
We agree to the reviewer's comment. It is important and essential issues to unravel a precise mechanism by which regulates the localization of γ-taxilin and the binding of γ-taxilin to Hook1. Then, taken together with additional information, we extended the discussion concerning the issues in the revised manuscript. In the future, we wish to address the issues as another project and discuss concerning the issues in more detail. This is described in the revised manuscript (Page 28, lines 601-604).

3) Reviewer #2 suggested to add insets in Figure 7 in order to highlight the differences in observed tubules between GFP and GFP-γ-taxilin overexpressing cells.
According to the reviewer's comment, we added insets in Figure 7A to show that CD98-positive

tubular structures were prominently formed in GFP overexpressing cells rather than GFP-γ-taxilin overexpressing cells. This is shown in the insets of Figure 7 and described in the revised manuscript (Page 19, lines 419-421; Page 48 lines 1101-1102).

Third decision letter

MS ID#: JOCES/2021/258849

MS TITLE: Regulation of Hook1-mediated endosomal sorting of clathrin-independent cargo by γ-taxilin

AUTHORS: Satoru Higashi, Tomohiko Makiyama, Hiroshi Sakane, Satoru Nogami, and Hiromichi Shirataki
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.