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Cooperation of membrane-translocated syntaxin4 and basement membrane for dynamic mammary epithelial morphogenesis

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Original submission

First decision letter

MS ID#: JOCES/2021/258905

MS TITLE: Cooperation of membrane-translocated syntaxin4 and basement membrane as a critical element for mammary epithelial morphogenesis

AUTHORS: Yuina Hirose and Yohei Hirai
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 find the topic important and the conclusions to be of potentially broad interest to the readership of JCS. However, they also raise a number of substantial criticisms that prevent me from accepting the paper at this stage. Reviewer 1 thoroughly identifies the current limitations of the study and suggests experimental approaches to address these issues. It is particularly important to have a fuller sense of how lumens form in this model spontaneously and the effect of loss of syntaxin4. I have some concern that the experiments suggested by the reviewers cannot be completed in a normal revision period. Accordingly, if you would like to request more time, please reach out to me. Alternatively, if you would like to propose a written revision plan for my review, you may do so. The reviewers do suggest that a suitably revised version might prove acceptable, if you can address their concerns. 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

Lumen formation by epithelial cells is one of the fundamental processes in organogensis. In mammary gland, dynamic lumen formation occurs by the stimulation with lactogenic hormones, where multi-layered epithelial cells are rearranged to developed lobular cysts containing a single polarized epithelium. Although it is thought that the epithelial lumen formation involves various elements, such as cell polarity, cell-cell adhesion, cell-substrate adhesion, the type and stiffness of extracellular matrix, hydrostatic pressure, etc., its whole picture has not been clarified yet. This study reports that EpH4 mouse mammary epithelial cells form cysts in Matrigel by prolactin stimulation. The authors focus on the role of extracellular syntaxin4, which is generally known a tSNARE exposed to the cytoplasm, and show that the artificial expression of syntaxin4 on the cell surface in EpH4 cells drastically accelerates lumen formation with cell migration to the basement membrane and the increase of E-cadherin degradation. The authors' in vivo findings potentially explain prolactin-induced mammary gland organogensis.

Comments for the author

The authors' finding that the extracellular syntaxin4 accelerates the cyst formation of EpH4 cells in 3D culture are potentially of great interest to understand a novel mechanism of epithelial lumen formation. The use of the Tet-off inducible expression system provided convincing results and each experiments are well done. However, the study still appears to be preliminary at least in two points. First, the authors' finding on lumen formation of EpH4 cells in Matrigel by prolactin treatment should be analyzed more intensively in comparison with syntaxin4-deficient EpH4 cells. Although the phenotype of Sig-T7-Stx4 EpH4 cells is dramatic, these cells are artificial and should be used to support and strengthen the data of parent EpH4 cells. To jump into Sig-T7-Stx EpH4 cells without extensive analyses of EpH4 cells makes this study somewhat premature. Second, the authors attribute the effects of Sig-T7-Stx4 expression on the behavior of EpH4 cells to accelerated degradation of E-cadherin, but the causal relationship is unclear at all.

Major points
1. Figure 1A is not sufficient for this study. Extracellular extrusion of syntaxin4 in mammary epithelial cells during lobular formation should be shown by immunostaining of the surface of isolated mammary epithelial cells without permeabilization.

2. Figure 4D. To strengthen the extrusion of syntaxin4, which is still under controversy, this should be also demonstrated in immunofluorescence staining by surface labeling without permeabilization.

3. Judging from the results shown in Figure 1E, this reviewer understands that parent EpH4 cells generate lumen structures in the presence of prolactin in syntaxin4-dependent manner. The authors should analyze this phonomenon in EpH4 cells more intensively by comparing syntaxin4 KO cells, including EM analyses, involvement of apoptosis, and cell migration in 2D and 3D cultures. Then, T7-Stx4 EpH4 should be presented as a syntaxin4 overexpressor, and Sig-T7-Stx4 EpH4 can be finally shown to strengthen the authors’ findings. In these experiments, the amount of the syntaxin4 extrusion need to be compared between these cell types to know the dose dependency of extracellular syntaxin4 on the cell behavior in lumen formation.

4. Do the author consider the extruded syntaxin4 to be exposed as a membrane bound form or excreted after digestion? Figure D needs molecular weight makers. Considering that the authors' group has extended the study of epimorphin, which is secreted by proteolytic cleavage, it is of much interest whether extruded syntaxin4 works as a membrane-bound form or a secreted form. Does the addition of the recombinant syntaxin4 lacking its transmembrane domain induce lumen formation of EpH4 cells?

5. The cleaved caspase 3 immunostaining in Figure 3D needs an appropriate positive control such as MDCK cysts, which undergo apoptosis according to the authors' description.

6. Figure 4A and movie1. It is hard to say from this movie that inner cells in the aggregates migrated outward and integrated into the outermost cell layer. To state that, the authors should label the all cells and track the movement of inner cells in higher resolution.

7. Figure 5B. Immunofluorescence staining of ZO-1 is not clear and the authors' statement is not convincing. The ZO-1 localization without DAPI should be shown.

8. Figure 5E. Quantification is needed. In this experiment, which cell type, Sig-T7 Stx4 EpH4 or parent EpH4, did the authors use for the cellular sheet?

9. Although the enhanced lysosomal degradation of E-cadherin by the expression of extracellular syntaxin4 is of some interest potentially, the data of degraded E-cadherin detected by CQ treatment in Figures 6 and 7 do not appear essential for this study. If the authors focus on E-cadherin, the point should be the amount of cell surface E-cadherin and E-cadherin-mediated cell-adhesive activity. The authors may follow the cell adhesion assay of E-cadherin-expressing L cells previously performed in Nagafuchi et al., J. Cell Biol. 127: 235–245 (1994).

Minor points
1. Figure 1B, right images lack a scale bar.

2. Line 150-157. The authors need to clearly describe which cell types provided these results. Moreover, cell surface biotinylation should be explained briefly as a method for detection of syntaxin4 extrusion in the text.

3. Line 179. The use of "stratified epithelia" for cell aggregates of EpH4 cells is confusing.

4. Line 306. It is still premature to say that the authors' observation in this study is EMT. A novel term to indicate the phenomenon is suggested.

Reviewer 2

Advance summary and potential significance to field

Comments for the author

The chief claim made by Hirose etal seems to be that prolactin causes secretion of STX4, and STX4-Ecad binding in the context of lrECM is required for cyst formation in EpH4 cells. The experiments embodied in this manuscript were thoughtful and creative, however, this manuscript is very difficult to follow and needs to be made more clear and more concise. Overall, I agree that the authors provided evidence commensurate with their chief claim.

1) In figure 6 authors claim co-assembly of E-cad and syntaxin4, but the representative images that were provided were not convincing. This claim would be better supported by proximity ligation assays and more quantitative co-localization analysis.

2) The authors referred multiple times to population or subpopulations of various proteins. This was a very confusing use of the population terms.

3) Syntaxin4 is probably more commonly discussed in the context of metabolism and diabetes. There are multiple known and putative receptors, and while the authors have shown previously that syntaxin4 binds E-cadherin in mammary epithelial cell lines many of those other receptors are expressed in mammary epithelia. Please add some discussion about the potential for alternative receptors in mammary epithelia.

First revision

Author response to reviewers' comments

Point-by-point answers

Reviewer1:
General comment
The findings in this study are potentially of great interest to understand a novel mechanism of epithelial lumen formation. However, behaviors between EpH4 cells and those deficient Stx4 should be compared more intensively before jumping to detailed analyses of Sig-T7-Stx4 cells · Also, authors should address the causal relationship between the effect of Sig-T7-Stx4 and accelerated degradation of E-cadherin.
Answer to general comment
We really appreciate this reviewer's careful reading, thoughtful suggestions and relatively positive comments. We have carried out additional experiments and tried to address reviewer's concerns, as mentioned below.

Major points (1)
Fig 1A: Extracellular extrusion of primary mammary epithelial cells should be shown by immuno-staining without permeabilization.
Answer (1)
We have isolated mammary epithelial cells from developing alveoli, cultured, and stained for Stx4 and cytoplasmic beta-actin, before and after permeabilization. The results were included in Fig 1A, which clearly demonstrate the extracellular extrusion of Stx4 in primary mammary epithelial cells.

Major point (2)
Fig 4D (we are sure that this comment is for Fig. 1D): Extracellular extrusion of Stx4 in EpH4 cells should also be shown by immuno-staining without permeabilization.
Answer (2)
We have done the experiment as suggested, which are shown in Supplementary Fig.S1.

Major point (3)
In order to strengthen author's findings, behaviors of parent EpH4 cells comparing Stx4-KO cells should be more intensively analyzed, then address the effect of Stx4-overexpression, before showing results from Sig-T7-Stx4 cells.
Answer (3)
We agree that the experiments using Sig-T7-EpH4 might sound arbitrary. We have done additional experiments and re-organized Fig 1(as well as supplementary figures). In Fig 1A, we added extracellular extrusion of Stx4 in primary mammary epithelial cells, as mentioned above. In Fig1B, the prolactin-provoked extracellular extrusion of Stx4 in EpH4 cells was shown with pull-down assay and quantification (also, immunostaining of surface Stx4 was shown in supplementary Fig 1S). In Fig 1C, the comparison of behaviors between parent EpH4 cells and EpH4-Stx4-KO cells in response to prolactin was shown. Additionally, by using the membrane-impermeable Stx4-antagonist (Hirose et al., J Cell Biochem 2018), we could additionally indicate that the effect of prolactin observed in parental EpH4 cells, but not in Stx4-KO cells, might be attributed to the extracellular exposure of Stx4. In Fig 1D, we show the generation of inducible T7-Stx4 over-expresser (T7-Stx4 EpH4), and compared 3D-cell behaviors of EpH4-Stx4-KO, EpH4 (T7-Stx4 EpH4 without induction of the transgene) and EpH4 overexpressing syntaxin4 (T7-Stx4 EpH4 with induction of the

transgene) in Matrigel. The membrane-impermeable Stx4-antagonist used in Fig 1C could not be tested for this experiment, because this antagonist is rapidly trapped by laminin in Matrigel and can not get accessible to the cells in the aggregates, however,the morphological changes looked dependent to the amount of extracellular Stx4. By this logical flow, the readers would consider “elucidation of 3D behaviors in cells with forcible expression of extracellular Stx4 (Sig-T7-Stx4)”is necessary as the next step. Accordingly, the original Fig 1F about Sig-T7-Stx4 cells was moved to Fig. 2A.

Major point (4)
Does extruded Stx4 act as membrane-bound form or secreted form, which has been shown for a cognate syntaxin, epimorphin?
Answer (4)
Previously, we have carefully analyzed the cleavage/secretion of epimorphin and successfully identified the cleavage site in this molecule: His residue in the membrane-proximal domain is necessary for the cleavage/secretion in epimorphin, and this His residue to Arg mutation dramatically reduced the cleavage efficiency (Hirai et al. JCS 2007). In Stx4, this position is Arg, implying that Stx4 is not efficiently cleaved/secreted off. Indeed, we have previously shown that the amount of the cleavage/secretion of Stx4 from cells was much lower than that of epimorphin (Kadono et al. BBRC 417, 2012). However, given the abundant expression of Stx4 in luminal epithelial cells, its functional exertion for epithelial morphogenesis might not require the effective cleavage/secretion. We added some sentences and a reference about these experiments in the Discussion (last paragraph).

Major point (5)
Fig. 3D: As a control, immnostaining of active caspase3 should be shown for MDCKII cells.
Answer (5)
We added immunostaining images for cleaved caspase3 in aggregates of MDCKII cells as well as Sig-T7-Stx4-EpH4 cells (ON and OFF) in Supplementary Fig. S2, which suggest apoptosis-independent lumen formation in Sig-T7-Stx4-EpH4 cells (ON).

Major points (6)
Fig 4A and movie1: The migration of the inner cells and their incorporation into the outermost cell layer are not clear. To state this, labeling of inner cells and tracking their movement in higher resolution might be necessary.
Answer (6)
The problem is that our time-lapse imaging machine is not a high-spec one and could not give us clearer images. We replaced this movie1 to an enlarged movie, so that readers might be able to trace the movement of the inner cells. We hope the quantification of the cellular incorporation into Matrigel-faced cell layers, which was added to Fig 5E, could support our statement.

Major points (7)
Figure 5B: Immunofluorescent staining of ZO-1 is not clear and the author's statement is not convincing. The ZO-1 localization without DAPI should be shown.
Answer (7)
We changed the original Z-stack images to that without DAPI-staining in Fig 5B, as suggested. In addition, to show firm cell-cell adhesion in dome-like structures instructed by extracellular syntaxin4 and Matrigel, we added an image of the upper plane stained with ZO-1, in Fig. 5B.

Major points (8)
Figure 5E: Quantification is needed. Author should show which cell types, Sig-T7-Stx4 EpH4 or parent EpH4, were used for the cellular sheet.
Answer (8)
We added quantification data in Fig 5E, as suggested. For the cellular sheet, Sig-T7-Stx4 EpH4 cells were used as indicated in the left schema.

Major point (9)
Lysosomal degradation could be of interest potentially, however, this might not be essential for this study. To show the importance of E-cadherin expression, the amount of cell surface E-cadherin and E-cadherin-mediated cell-adhesive activity should be analyzed. The authors may follow adhesion assay using E-cadherin expressing L (E-L)cells as had been conducted by Nagafuchi et al (JCB 127, 1994).
Answer (9)
Our data suggest that effects of extracellular Stx4 were not mediated by the changes in the amount of cell surface expression of full-length E-cadherin, but by E-cadherin-turnover and/or the dominant-negative effect by temporally generated tail-less E-cadherin. In Fig 7B and C, we have shown that E-L cells, which can adhere each other solely by E-cadherin, gradually weaken their adhesive property in response to extracellular Stx4 without alternation of the amount of cell surface full-length E-cadherin, but with the appearance of large amount of readily degrative tail-less E-cadherin. While rapid cell aggregation assay established by Nagafuchi did not show clear difference in EL cells w/wo Sig-T7-Stx4 and w/wo tail-less E-cadherin, functional ablogation of full-length E-cadherin might take more than a few days. In the revised manuscript, we added new data showing that the forced expression of the tail-less E-cadherin in E-L cells clearly weaken the E-cadherin-mediated cell adhesion without down-regulation of cell surface full-length E-cadherin, as well (new supplementary Fig S9). Also, we added several words/sentences for better understanding, hoping that the reviewer finds this revision could convince readers.

Minor points (1)
Figure 1B, right images lack a scale bar.
Answer (10)
We appreciate this comment, but we removed these images, as mentioned in Answer (3)

Minor points (2)
Line 150-157: The authors need to clearly describe which cell types provided these results. Also, cell surface biotinylation should be explained briefly as a method section.
Answer (11)
We added the cell type (as EpH4 cells) originally mentioned about in Line 150-157, as suggested. As for cell surface biotinylation, we have already mentioned in the Material and Method section in the original manuscript, as in “detection of proteins expressed at the cell surface”.

Minor points (3)
Line 179: The use of “stratified epithelia” for cell aggregates of EpH4 cells is confusing.
Answer (12)
We changed the word “stratified epithelia” to“cell aggregates”.

Minor points (4)
Line 306: It is still premature to say that the author's observation in this study is EMT. A novel term to indicate the phenomenon is suggested.
Answer (13)
We changed the words “leads to onset of EMT”(originally mentioned in Line 396) to “increases cellular motility”.

Reviewer2:
General comment
While the experiments embodied in this manuscript were thoughtful and creative, this manuscript is very difficult to follow and needs to be made clearer and more concise. Overall, this reviewer agree that the authors provided evidence commensurate with their chief claim
Answer to the general comment
We appreciate this reviewer's positive comment. We have tried to revise the text in more easily-understandable one.

Comment (1)
Fig 6: Images showing co-assembly of E-cad and syntaxin4 were not convincing. This claim would be better supported by proximity ligation assay and more quantitative co-localization analysis.
Answer (14)
We added line-scan analyses in confocal images (as Supplementary Fig S5), which support the co-localization of E-cadherin and Syntaxin4.

Comment (2)
The authors referred multiple times to population or subpopulations of various proteins. This was a very confusing use of the population terms
Answer (15)
We changed the usage of these words, to avoid possible confusions.
In the revised manuscript, “population” and “subpopulation” are used only for cells and for proteins, respectively.

Comment (3)
Syntaxin4 is more commonly discussed in the context of metabolism and diabetes and the counter molecules of syntaxin4, other than E-cadherin, are known to be expressed in mammary gland. Please add some discussion about the potential for alternative receptors in the mammary epithelia.
Answer (16)
Syntaxin4 and its counter molecules have been intensively investigated using, for example, pancreatic islet cells. However, most of these studies have focused on syntaxin4 as a t-SNARE protein and identified syntaxin4's counter molecules in the cytoplasm. In regard to cytoplasmic environment, mammary epithelial cells undoubtedly express these molecules and temporally interact with major subpopulation of cytoplasmic syntaxin4. However, these issues are obviously irrelevant to this study, since we analyzed only at the extracellular environment and identified E-cadherin as an extracellular target of syntaxin4. To avoid possible confusions, we referred possible cytoplasmic partners of syntaxin4, as well (in Discussion)

Second decision letter

MS ID#: JOCES/2021/258905

MS TITLE: Cooperation of membrane-translocated syntaxin4 and basement membrane for dynamic mammary epithelial morphogenesis

AUTHORS: Yuina Hirose and Yohei Hirai
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 see significant progress in revision but Reviewer 1 raises a number of substantial criticisms that prevent me from accepting the paper at this stage. Reviewer 2 also notes that it would be useful to edit the manuscript for clarity and style. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. 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.

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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

Only a part of the critiques by this reviewer have been addressed. The manuscript still needs revision in the following points.

Comments for the author

Unfortunately, only a part of the critiques by this reviewer have been addressed. The manuscript still needs revision in the following points.

Major 1. Figs 1 and 2. The amount of the syntaxin4 extrusion need to be compared between EpH4 with proalctin, T7-Stx4 EpH4 with prolactin, and Sig-T7-Stx4 EpH4 to know the dose dependency of extracellular syntaxin4 on the cell behavior in lumen formation.

2. If the EM analysis is not easy, the cysts of EpH4 with prolactin and T7-Stx4 EpH4 with prolactin needs to be analyzed via immunofluorescence staining with anti-ZO-1 and anti-E-cadherin as shown in Fig 2.

3. Fig 1C. The authors have added a new data of cell size of EpH4 cells and Stx4 KO EpH4 cells by prolactin treatment and the effect of r-F3. However, the authors have not mentioned the remarkable difference in cell size between EpH4 cells and Stx4 KO EpH4 cells. How do the authors explain the smaller cell size of Stx4 KO EpH4 in terms of the loss of Stx4? Does re-expression of Stx4 in Stx4 KO EpH4 cells recover cell size? This figure may be omitted.

4. Figs 7. Cell adhesion assay conducted in Nagafuchi et al (JCB 127, 1994) was based on cell dissociation experiments. The authors need to follow the same experiment. Even if the authors' cells do not show any remarkable difference of cell adhesive activity in such experiments, the negative data should be shown in supplemental figures for better understanding of the readers, and the author should discuss the results accordingly.

5. Line 270 and others. "rapid turnover of E-cadherin" is too speculative because there is no quantitative data. This term should be toned down.

6. Fig S1. The image of permeabilized EpH4 cells needs improvement. Alpha-tubulin staining is too poor to show permeabilization. Fluorescent phalloidin may be much better. In addition, a single channel image of Stx4 should be presented to show its subcellular localization. Whether prolactin-mediated Stx4 extrusion occurs in all the cells or a part of the cells should be shown and discussed.
Minor 1. Fig.1A. Extracellular extrusion of syntaxin4 in mammary epithelial cells during lobular formation have been shown in immunofluorescence staining. To show subcellular localization stx4, its staining also should be shown without merged with that of beta-catenin. Do only a part of cells show extracellular extrusion of stx4?

Reviewer 2

Advance summary and potential significance to field

This manuscript describes Syntaxin4 as a protein that extruded by mammary epithelial cells, binds E-cadherin, and participates in high-order mammary gland morphogenesis.

Comments for the author

The authors addressed my questions. The writing was improved upon revision, but still a little challenging to get through - I suggest a strong hand be taken by the copy editor.

Second revision

Author response to reviewers' comments

Point-by-point answers
Reviewer 1
(Comment 1). Figs 1 and 2. The amount of the syntaxin4 extrusion need to be compared between EpH4 with prolactin, T7-Stx4 EpH4 with prolactin, and Sig-T7-Stx4 EpH4 to know the dose dependency of extracellular syntaxin4 on the cell behavior in lumen formation.
<<Answer1>>: We added quantitative data showing the amount of the syntaxin4 extrusion in EpH4 with prolactin, T7-Stx4 EpH4 with prolactin, and Sig-T7-Stx4 EpH4 in Fig. S2, as suggested. Together with Fig 1C/2A/2B, this Fig S2 shows the effect of the expression mode (partial cells or total cells) and dose- dependency of extracellular Stx4 on luminal morphogenesis.

(Comment 2). If the EM analysis is not easy, the cysts of EpH4 with prolactin and T7-Stx4 EpH4 with prolactin needs to be analyzed via immunofluorescence staining with anti-ZO-1 and anti-E-cadherin as shown in Fig 2.
<<Answer 2>>: We added Immunofluorescence staining for ZO-1 and E-cadherin in the cysts of EpH4 with prolactin and T7-Stx4 EpH4 with prolactin in Fig. 1C, as suggested.

(Comment 3). Fig 1C. The authors have added a new data of cell size of EpH4 cells and Stx4 KO EpH4 cells by prolactin treatment and the effect of r-F3. However, the authors have not mentioned the remarkable difference in cell size between EpH4 cells and Stx4 KO EpH4 cells. How do the authors explain the smaller cell size of Stx4 KO EpH4 in terms of the loss of Stx4? Does re-expression of Stx4 in Stx4 KO EpH4 cells recover cell size? This figure may be omitted.
<<Answer 3>>: We agree that this figure possibly cause some confusion without data showing that re- expression of Stx4 in Stx4 KO EpH4 cells recover the cell size. We added data and explanation for re- expression of Stx4 in Stx4KO EpH4 cells in this figure, and moved to supplementary information (Fig. S1). We added sentences explaining the possible reason for the smaller size of Stx4-KO cells in the legend.

(Comment 4). Figs 7. Cell adhesion assay conducted in Nagafuchi et al (JCB 127, 1994) was based on cell dissociation experiments. The authors need to follow the same experiment. Even if the authors' cells do not show any remarkable difference of cell adhesive activity in such experiments, the negative data should be shown in supplemental figures for better understanding of the readers, and the author should discuss the results accordingly.
<<Answer 4>>: We performed the cell dissociation assay established by Nagafuchi and added the results in supplementary Fig. S7, as suggested. In this assay, EL-sigT7-Stx4 cells (ON and OFF) did not show remarkable differences in the instantaneous cell-cell adhesion. This negative result might be ascribed to the feature of parental L-cells, which endogenously possess or can actively form catenin-actin complex responsible for E-cadherin function. Actually, EL cells reportedly exhibit strong cell-cell adhesion without acquiring epithelial characteristics (Chen and Obrink, 1991, JCB), and we detected more obvious effects of inducible expression of “tailless E-cadherin” in EpH4 cells than EL cells. While nutrient-free

buffer should be used for this cell dissociation assay and we could not analyze the effect of E-cadherin turnover in a longer time window, EL cells cultured with normal medium gradually lost cell-cell adhesion in response to extracellular expression of syntaxin4 as shown in Fig 7C. In addition to showing the supplementary figure (Fig S7), we added several sentences for the explanation of the results in “Results” and “Discussion”, as suggested.

(Comment 5). Line 270 and others. "rapid turnover of E-cadherin" is too speculative because there is no quantitative >data. This term should be toned down.
<<Answer 5>>: We removed/changed the word “rapid” from "rapid turnover of E-cadherin" throughout the text, as suggested.

(Comment 6). Fig S1. The image of permeabilized EpH4 cells needs improvement. Alpha-tubulin staining is too poor to show permeabilization. Fluorescent phalloidin may be much better. In addition, a single channelimage of Stx4 should be presented to show its subcellular localization. Whether prolactin-mediated Stx4 extrusion occurs in all the cells or a part of the cells should be shown and discussed.
<<Answer 6>> We performed EpH4 staining again and added the results in Fig. 1B, as suggested. As the expressions of synatxin4 and b-actin were visualized with antibodies (Fig 1A), we used anti-b-actin antibodies, but not phalloidin. Also, we included a single channel image of Stx4, as suggested, which clearly shows the extracellular extrusion of Stx4 only in a part of the cells.

(Minor Comment 1). Fig.1A. Extracellular extrusion of syntaxin4 in mammary epithelial cells during lobular formation have been shown in immunofluorescence staining. To show subcellular localization stx4, its staining also should be shown without merged with that of beta-actin. Do only a part of cells show extracellular extrusion of stx4?
<<Answer>> In Fig 1A, the image for the staining of Stx4 without beta-actin was added, as suggested, which demonstrated that Stx4 was extruded in part of the cells.

Reviewer 2
(Comment) The authors addressed my questions. The writing was improved upon revision, but still a little challenging to get through - I suggest a strong hand be taken by the copy editor.
<<Answer>>: Before the submission of this revised manuscript, we have asked an English proofreading company to edit throughout the text, as suggested.

Others
In addition to the above-mentioned changes, we moved the original Fig S1 and S3 to Fig 1 and Fig 3, and, original Fig 1C to Fig S1, for better understanding. Accordingly, the number of original supplementary figures (S3~S7) was shifted one by one.

Third decision letter

MS ID#: JOCES/2021/258905

MS TITLE: Cooperation of membrane-translocated syntaxin4 and basement membrane for dynamic mammary epithelial morphogenesis

AUTHORS: Yuina Hirose and Yohei Hirai
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.

Reviewer 1

Advance summary and potential significance to field

The authors' observation that the extracellular syntaxin4 accelerates the cyst formation of EpH4 cells in 3D culture are potentially of great interest to understand a novel mechanism of epithelial lumen formation.

Comments for the author

The authors have addressed my critiques.

Reviewer 2

Advance summary and potential significance to field

STX4 is a T-snare protein that is typically associated with retrograde transport and with diseases like diabetes. Hirose and Hirai show that upon stimulation of mammary epithelial cells with the hormone prolactin STX4 is extruded outside the epithelial cells, binding to E-cadherin and initiating changes in self-organization within the glands such that cells were moving into contact with the basement membrane. This work shows an unexpected role for STX4 in mammary morphogenesis, and is possibly relevant to understanding the rearrangement of secretory luminal epithelial cells into a basement membrane experiencing position during lactation.

Comments for the author

The writing is much improved, thank you.