

# Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function.

Akira Saito, Tomohito Higashi, Yugo Fukazawa, Tetsuhisa Otani, Masashi Tauchi, Atsuko Higashi, Mikio Furuse, and Hideki Chiba

*Corresponding author(s): Tomohito Higashi, Fukushima Medical University*

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## Review Timeline:

Submission Date:	2020-07-17
Editorial Decision:	2020-08-19
Revision Received:	2020-11-27
Editorial Decision:	2021-01-18
Revision Received:	2021-01-22
Accepted:	2021-02-01

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*Editor-in-Chief: Matthew Welch*

## 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.)

RE: Manuscript #E20-07-0464

TITLE: Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function.

Dear Dr. Higashi,

I hope you are well during this challenging time for all of us.

Two reviewers have now reviewed your manuscript. While both find the basic story developed in this paper to be interesting and highly appropriate for *Molecular Biology of the Cell*, they have similar concerns that you will need to address in a revised version. The revision will require re-review.

(1) Controls: A key approach in your manuscript is the use of a Claudin2 KO line. However, this raises an issue addressed by both reviewers: what is the appropriate control for your experiments? Is it an MDCK II line with claudin 2 intact? Do you need to at least cite work involving more-familiar MDCK I lines? Please clarify why you used particular cell lines as negative controls, and why you chose not to include analysis of other lines that seem relevant. This is important for other figures in the paper (e.g., current Fig. 5).

(2) Mechanism: Both reviewers found the manuscript to be somewhat light on mechanism. The reviewers suggest different experiments that you could consider (overexpression, live imaging, more detailed analysis of TJ strand breaks, etc.) that could yield further mechanistic insight.

(3) Figure 4: Neither reviewer found Fig. 4 particularly compelling. Consider how you might improve this data, or consider focusing on the freeze fracture data from Fig. 5 instead.

(4) Consolidating data: Reviewer 1 suggests several places where you could consolidate your presentation to focus on the truly novel features of the analysis. It may be worthwhile to consider Reviewer 1's suggestions if they help to clarify and accentuate the novel findings in your work.

(5) Other Reviewer 2 comments: Reviewer 2 raises issues about Claudin3 expression, angulin localization, and has questions about the TER data in Fig. 6 that you should address.

In your revision you should also address the minor comments of the reviewers, and list your specific changes to address these comments.

Thanks again for submitting your work to MBoC. We look forward to receiving your revisions soon.

Best regards,  
Jeff Hardin  
Monitoring Editor  
Molecular Biology of the Cell

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Dear Dr. Higashi,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office ([mboc@ascb.org](mailto:mboc@ascb.org)).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at [mboc@ascb.org](mailto:mboc@ascb.org).

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Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors ([www.molbiolcell.org/info-for-authors](http://www.molbiolcell.org/info-for-authors)). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: [Link Not Available](#)

Please contact us with any questions at [mboc@ascb.org](mailto:mboc@ascb.org).

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
[mbc@ascb.org](mailto:mbc@ascb.org)

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Reviewer #1 (Remarks to the Author):

Saito and collaborators report that occludin and tricellulin jointly strength the epithelial barrier function by promoting the formation of anastomosing Tight Junction (TJ) strand network. While the observation that occludin and tricellulin contribute to the formation of anastomosing TJ strands and permeability properties of epithelial cells, the underlying mechanism of this function is not explored in these studies.

Some data is novel, while other observations have been previously published. Thus, it would be useful to consolidate the key novel findings into a single brief report that demonstrates contribution of occludin and tricellulin in the formation of anastomosing tight-junction strand network together with the in-silico model.

Concerns:

- 1.- Figure 1 characterizes MDCK-II Occludin/Tricellulin dKO cells. It would be useful to consolidate figures 1 and 2 as one figure.
- 2.- The re-distribution of tricellulin from tricellular to bicellular TJs in the absence of occludin has been already reported by Ikenouchi et al. 2008 (Figure 3). Thus, this figure can be presented as supplemental material.
- 3.- Since figures 4 and 5 describe the TJ structure by TEM and freeze-fracture electron microscopy, respectively, they should be consolidated as a single figure. Additionally, improved resolution of TEM images would be useful in order to visualize the electrodense zone, which is altered in the absence of occludin/tricellulin. It would also be useful to analyze the length and intra-membrane paracellular space of TJs to complement the barrier function results.
- 4.- One of the most interesting effects of occludin/tricellulin dKO is the reduction of branching points in TJ strands (Figure 5). Therefore, it would be interesting to analyze these strand branch points in occludin/tricellulin over-expressing cells. On the converse, the tricellulin KO cells exhibit a pearled strand meshwork that might suggest differential incorporation of claudins in TJs. The authors should address this point by investigating the expression/localization of other claudin members.
- 5.- What is the rationale for using MDCK-II claudin-2 KO cells as a "control" rather than MDCK-I cells, which lack Claudin-2.

Reviewer #2 (Remarks to the Author):

The manuscript "Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function" investigates the role of a tricellular TJ protein and TJ protein in single and double knock MDCKII cell lines. It remains unclear whether occludin and tricellulin modulate the complexity of the TJ strand network in epithelial cells and this is the central question of this manuscript. The role of Tricellulin in barrier formation is also unclear given the conflicting literature for and against Tricellulin in cell culture experiments. The manuscript utilizes a CRISPR mediated approach to remove the tTJ protein Tricellulin (TRIC) and the TJ protein Occludin (OCLN) paired with standard immunofluorescence and electron microscopy to assess the consequences on TJ formation and structure. The permeability of the TJ was assessed and a mathematical model is presented that is predictive of the changes to the TJ ultrastructure. Loss of either gene had little effect on TJ structure and function, loss of both proteins affected the permeability of the TJ and changed the ultrastructure of the TJ, specifically the

cross-links in the TJ strands (less anastomosed). This leads to a model whereby TRIC and OCLN play a role in the formation of the anastomosing meshwork of TJ strands, and contribute to the maintenance of epithelial barrier function.

Overall the paper was very straightforward and simple approach using CRISPR mediated knockdown to create different loss of function alleles. The analyses were classic approaches and for the most part generated solid and convincing data. The key result is the reduction in the anastomosing bridges in the double KO and this section was well done and supported by strong data and excellent freeze fraction EM images. The mathematical model was nicely supportive. The paper was very light on mechanism as to what the roles of TRIC and OCLN were in the formation of the bridges. A more sophisticated approach including live imaging of the TJ strands or the relationship of TRIC or OCLN to TJ breaks or PLA analysis of the tagged rescue constructs and which regions of the TJ they are functioning would have increased the impact especially if these proteins can be found at the branch points in a specific complex. A greater emphasis on experiments that test the model would have really made the manuscript compelling. For instance does overexpression of the rescue constructs stabilize the TJ leading to more bridges in MDCKII cells? That being said this is a straightforward and solid paper with clearly presented results that lead to a better understanding of how TRIC and OCLN may function

A key and clever approach, central to this paper, was the use of a Claudin2 KO line as the back ground to remove the highly permeable claudin-2 from the MDCK II cell line. Indeed the supplementary data showed that the transepithelial electric resistance measurement in this cell line had significantly increased. These cells were used as the "parental" line for the rest of the manipulations...however this line can't be called "wild type" by any means and should be called Cldn2-KO or parental line throughout. Wild-type means something very different. This is important in that the Cldn2-KO clearly changes either the morphology or the function of the TJ and it is not correct to use the words wildtype. A consistent comparison of the double KO to the original MDCK II cells (i.e. Cldn-2 gene intact) would be very worthwhile in particular in the context of Figure 5. Does the Cldn-2KO lead to more anastomosing bridges and thus the double KO in this background is simply a reversion of this to the level of the original MDCK II line?

The authors used a nice approach to compare control (Cldn2-KO) cells to double or single KO cells by co-seeding control cells (Cldn2-KO) cells tagged with NLS-GFP with the single or double knock outlines. None of the TJ or tTJ proteins were mislocalized in the absence of either Ocln or Tric or the dKO however the Claudin-3 immunolabeling was clearly reduced. The authors state "no drastic changes in the amount of junctional proteins, including claudin-3". A quantification of the protein levels would have helped here as it does appear that there was quite a reduction in the claudin-3 immunolabeling. This in parallel with a better quantification of the Western in Figure S3 would help the reader know if this was a general protein reduction or a loss from the TJ. Quantifying protein levels in the original MDCKII cell line compared to the Cldn2-KO (Figure S1) would help assure the reader that there hasn't been changes in the expression levels. For instance in Figure S1 it looks like TRIC has increased in the Cldn2-KO, while claudin-4 is decreased.

Tricellulin is still found at tTJs in Ocln-KO cells but overall the intensity of tricellulin at bicellular TJs is increased in Ocln-KO cells compared with the Cldn2-KO control and this was very nicely quantified and presented in a good scatter plot analysis. This does lead to the question, what happens to angulin-1/LSR does it also spread in the bicellular TJ? However the supplemental data (Figure S3) for the angulin-1/LSR was confounded by the fact that the angulin-1 immunolabeling was in the same channel as the NLS-GFP making it very difficult to assess any changes to angulin-1. Please repeat this analysis using Angulin in the red channel so that the control cells are more easily distinguished as it looked like in the Ocln-KO and in the double KO the angulin-1 had spread in the bicellular TJ compared to the GFP cells. A similar quantification of the angulin-1 distribution in the tTJ versus the bTJ similar to that of Tricellulin would strengthen this data greatly.

As mentioned above for Figure S3 the western analysis of the different proteins levels of the TJ and tTJ proteins should be quantified compared to the actin control. As it stands it looks like the angulin-1 levels have increased greatly in the Ocln-KO and perhaps the Claudin-4 levels as well.

Figure 4 was not particularly effective in the presentation of the TJ. The focus or the resolution was not sufficient to really determine if the TJ were intact or if there were any changes to the ultrastructure. Either remove this figure (given that Figure 5 is far more convincing) or replace the panels with more convincing data.

In contrast Figure 5 was very convincing and provided a very nice analysis of the TJ strand network. The graph should be a scatter plot and for this graph the error bars are the 95% confidence interval rather than the S.D. displayed in all the other figures (the reader is left wondering why the difference)? This figure needs the P values indicated above each genotype compared to control. There is a spelling mistake in Figure 5 legend.

The conclusion that the TJ strands were less branched and the network less complex was a key finding from this paper. However given that the double KO is in the context of the claudin-2 KO it would be nice to have the analysis done on the original MDCK II line as it is possible that the claudin-2 KO increases the number of bridges which would fit with the model very well.

The conclusion from the first part of Figure 6 wasn't consistent with the data present. All three lines Tric-KO #2, Ocln-KO #1 and Ocln-KO #2 had a statistically significant decreases in TER compared to control and the mean TER from the graph seemed to be equivalent. This suggests that both TRIC and OCLN may play a role in the TER and that there might be variations in the

responses of the two different cell lines? This fits also better with the conclusion stated in the following section based on the double KO. "indicating that tricellulin and occludin are required for the establishment of permeability barrier for ions"  
The use of the rescue constructs was a nice approach and used very effectively and these constructs rescued the reduction in TER. For the dextran permeation experiments the concluding statement was a bit confusing as it appeared that the permeability barrier for macromolecules was restored in the dKO#1+Flag-TRIC cells and dKO#1+Flag-OCN cells. Therefore it seems more likely that either Tric or Ocln can rescue and are not "jointly required for the establishment of a tight permeability barrier against ions and macromolecules".

For Figure 6, Supplemental Table 1 is key but reading supplemental table 1 was quite confusing. To make this figure and data easier to parse...rather than a bar graph, a scatter plot needs to be used to observe the spread in the TER and P[app] values. This figure would be much easier to digest if the P value or \*, \*\* or NS were placed above each sample (that way the reader doesn't have to flip back and forward between the supplemental and the data figure).  
Finally for Figure 6 could this be more explicit stated on the graph what the #1 and #2 indicate as well in the legend.

Figure 7 and the modeling that support this figure I do not have the necessary expertise to comment on. However the implications of the model fit very well with the literature and with the discussion.

Figure 8 was an interesting model and nicely summarized the finding. The data lacking in support of this model is of course the live imaging of the dynamics of TJ formation and breakage. It is surprising that the authors didn't test their lines with a GFP tagged Claudin or another TJ component to visualize the live dynamics of TJ reformation. However that is likely beyond the scope of this manuscript.

Other comments:

It is presumed a one-way anova was used for Figure 6 but please specify this.

The purpose of Figure S6 was very unclear.

The authors make use of some less rigorous language that should be corrected:

- Page 6, line 10: "Transepithelial electric resistance (TER) measurement showed that Cldn2-KO MDCK II cells exhibited drastically reduced permeability". A simpler statement would be increased resistance by XX fold.
- Page 8, line 11: "Also, there was no huge difference in the average number of horizontal TJ strands among these cell lines". Please be specific was there a statistically significant difference or not? If not then simply state this.
- Figure 7 legend: "The break in the TJ strand drastically reduce[sic] the electric resistance".

There are numerous grammatical and spelling errors.



RE: Manuscript #E20-07-0464

TITLE: Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function.

Dear Dr. Higashi,

I hope you are well during this challenging time for all of us.

Two reviewers have now reviewed your manuscript. While both find the basic story developed in this paper to be interesting and highly appropriate for *Molecular Biology the Cell*, they have similar concerns that you will need to address in a revised version. The revision will require re-review.

We deeply appreciate the editor and reviewers for reviewing our manuscript in such a challenging time and providing insightful feedback which substantially strengthened our paper. We have performed additional experiments and statistical analyses according to the reviewers' suggestions, and incorporated changes into the revised manuscript and figures. We hope that our revision satisfactorily addresses all the concerns the editor and reviewers have noted.

(1) Controls: A key approach in your manuscript is the use of a Claudin2 KO line. However, this raises an issue addressed by both reviewers: what is the appropriate control for your experiments? Is it an MDCK II line with claudin 2 intact? Do you need to at least cite work involving more-familiar MDCK I lines? Please clarify why you used particular cell lines as negative controls, and why you chose not to include analysis of other lines that seem relevant. This is important for other figures in the paper (e.g., current Fig. 5).

Thank you very much for your comment.

Regarding your inquiry, we used a *Cldn2*-KO MDCK II cells for several reasons, described below.

(i) To the best of our knowledge, MDCK II cells are one of the most commonly-used cells among the researchers of cell-cell junctions.

(ii) MDCK I cells are relatively unstable (Dukes JD et al., *BMC Cell Biology*, 2011) compared with MDCK II cells and tend to change their morphology and function during the process of repeated cell cloning.

(iii) Knockout of *Cldn2* in MDCK II cells does not alter the morphology or stability, and improves their barriers against ions (Tokuda S et al., *PLoS One*, 2014).

(iv) Claudin-2-bearing TJ strands appear to be discontinuous in the glutaraldehyde-fixed freeze-fracture samples because some of particles are attached with the ectoplasmic (E)-face (Furuse M et al., *JCB*, 2001). In contrast, claudin-2-free TJ strands are mostly continuous and suitable for quantitative analysis.

Thus, we decided to use the *Cldn2*-KO MDCK II cells as a starting material and isolated all of the KO cells from them. We believe that the *Cldn2*-KO MDCK II cells are most suitable for negative controls.

However, we agree with the editor that it would be helpful to include analyses of *Cldn2*-KO MDCK II cells in comparison to parental MDCK II cells, in which claudin-2 is intact. Thus, we added freeze-fracture electron microscopy analysis of parental MDCK II cells and compared them with *Cldn2*-KO cells. New data are presented as the revised Figures S2.

(2) Mechanism: Both reviewers found the manuscript to be somewhat light on mechanism. The reviewers suggest different experiments that you could consider (overexpression, live imaging, more detailed analysis of TJ strand breaks, etc.) that could yield further mechanistic insight.

We established cell lines stably overexpressing occludin or tricellulin, and analyzed them using freeze-fracture electron microscopy. The TJ strand complexity was increased, especially in the occludin-overexpressing cells, and the permeability for ions and 10-kD macromolecules was decreased compared with that in the control cells. We also conducted a simulation using a mathematical model with parameters based on the observation of overexpressed cells and the simulation results were in good agreement with the experimental data. These results further support our working hypothesis that tricellulin/occludin regulates epithelial barrier through TJ strand network complexity, and we believe these results provide new insights into the mechanism of TJ strand network formation. New data are presented in the revised Figure 6.

(3) Figure 4: Neither reviewer found Fig. 4 particularly compelling. Consider how you might improve this data, or consider focusing on the freeze fracture data from Fig. 5 instead.

We modified the sample preparation method for TEM (see the reply for the Reviewer #1) and obtained improved images for each cell clone. New images are included in the revised Figure 3.

(4) Consolidating data: Reviewer 1 suggests several places where you could consolidate your presentation to focus

on the truly novel features of the analysis. It may be worthwhile to consider Reviewer 1's suggestions if they help to clarify and accentuate the novel findings in your work.

We agree with Reviewer 1's comment about the original Figure 3, and we have moved it to the supplemental information (revised Figure S5). For Figures 1 and 2, we decided to keep them as they are, because consolidating them would make them too small to clearly see details in the immunostaining images. The TEM images and freeze-fracture replica images are consolidated as a single figure (revised Figure 3).

(5) Other Reviewer 2 comments: Reviewer 2 raises issues about Claudin3 expression, angulin localization, and has questions about the TER data in Fig. 6 that you should address.

We analyzed the band intensities of western blots. We also quantified the fluorescence intensities of Cldn3 at cell-cell junction and angulin-1 at tricellular junctions. We added immunoblot quantification for parental MDCK II cells in the revised Figure S1, immunoblot quantification for KO cells in the revised Figure S4, immunostaining quantification in the revised Figure 2, and the tTJ intensity data of angulin-1 in the revised Figure S5.

For TER data, we presented the values in the scatter plot and added statistical significance data. The data are shown in the revised Figure 4.

In your revision you should also address the minor comments of the reviewers, and list your specific changes to address these comments.

Thanks again for submitting your work to MBoC. We look forward to receiving your revisions soon.

Best regards,  
Jeff Hardin  
Monitoring Editor  
Molecular Biology of the Cell

-----  
Dear Dr. Higashi,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors ([www.molbiolcell.org/info-for-authors](http://www.molbiolcell.org/info-for-authors)). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: <https://www.mbcpapers.org/cgi-bin/main.plex?el=A6o1Ktw2A7rAb1I4A9ftdT8CZDjXEAY4jkDz7cVYhwZ>

Please contact us with any questions at [mboc@ascb.org](mailto:mboc@ascb.org).

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.



Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
[mbc@ascb.org](mailto:mbc@ascb.org)

We thank the reviewers for their helpful comments, which will strengthen this article. We respond to the comments on a point-by-point basis below.

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Reviewer #1 (Remarks to the Author):

Saito and collaborators report that occludin and tricellulin jointly strength the epithelial barrier function by promoting the formation of anastomosing Tight Junction (TJ) strand network. While the observation that occludin and tricellulin contribute to the formation of anastomosing TJ strands and permeability properties of epithelial cells, the underlying mechanism of this function is not explored in these studies.

Some data is novel, while other observations have been previously published. Thus, it would be useful to consolidate the key novel findings into a single brief report that demonstrates contribution of occludin and tricellulin in the formation of anastomosing tight-junction strand network together with the in-silico model.

We appreciate the Reviewer for reviewing our manuscript and providing us with helpful comments and suggestions.

Concerns:

1.- Figure 1 characterizes MDCK-II Occludin/Tricellulin dKO cells. It would be useful to consolidate figures 1 and 2 as one figure.

We appreciate the Reviewer for the suggestion. However, we would like to keep Figures 1 and 2 as they are, because downsizing of them would make it difficult to recognize confined signals especially for tTJ proteins.

2.- The re-distribution of tricellulin from tricellular to bicellular TJs in the absence of occludin has been already reported by Ikenouchi et al. 2008 (Figure 3). Thus, this figure can be presented as supplemental material.

We agree with the Reviewer that the re-distribution of tricellulin in OCLN-deficient cells has already been reported by Ikenouchi et al. Thus, we have moved these data; they are now included as the revised Figure S5.

3.- Since figures 4 and 5 describe the TJ structure by TEM and freeze-fracture electron microscopy, respectively, they should be consolidated as a single figure. Additionally, improved resolution of TEM images would be useful in order to visualize the electrodense zone, which is altered in the absence of occludin/tricellulin. It would also be useful to analyze the length and intra-membrane paracellular space of TJs to complement the barrier function results.

We also agree with the Reviewer on this point. For the original manuscript, we used polyethylene terephthalate (PET) filters to culture cells for TEM analysis. Since the stiffness of PET is different from the resin-embedded cells, it was difficult to make good ultrathin sections. We repeated the TEM analysis using polycarbonate filters, and obtained new images with improved resolution and quality. We included the new data in the revised Figure 3 together with the freeze-fracture data.

4.- One of the most interesting effects of occludin/tricellulin dKO is the reduction of branching points in TJ strands (Figure 5). Therefore, it would be interesting to analyze these strand branch points in occludin/tricellulin over-expressing cells. On the converse, the tricellulin KO cells exhibit a pearled strand meshwork that might suggest differential incorporation of claudins in TJs. The authors should address this point by investigating the expression/localization of other claudin members.

We examined freeze-fracture replicas of *Tric/Ocln*-dKO cells stably overexpressing Flag-OCLN or Flag-TRIC, and analyzed the branching points of TJ strands. Although TJs in the Flag-TRIC-overexpressing cells appears to be similar to those of the control cells, the TJ strands in the Flag-OCLN-overexpressing cells are more branched and the barrier function of these cells are improved compared with the control cells, which are consistent with our working hypothesis. In addition, we noticed that the TJ strands in the OCLN-overexpressing cells (and part of those in the TRIC-overexpressing cells) are thicker than usual. We have added these data as the revised Figure 6. We compared the morphology of the *Tric*-KO cells with that of the control cells, and did not find that strands in the *Tric*-KO cells were especially "pearled" (see the revised Figures. 3 and S6). To show the expression levels of claudins, we added quantification data of immunoblotting in the new Figure S4. We also quantified the immunofluorescence signal intensity of claudin-3 at TJs and added the data in the revised Figure 2. As the junction localization of claudin-

3 is similarly reduced in the *Tric*-KO, *Ocln*-KO and *Tric/Ocln*-dKO cells and the decrease of TJ complexity and barrier function are evident only in the *Tric/Ocln*-dKO cells, it may not be likely that change in claudin-3 localization is directly associated with TJ strand branching or barrier.

5.- What is the rationale for using MDCK-II claudin-2 KO cells as a "control" rather than MDCK-I cells, which lack Claudin-2.

We used MDCK II cells because this cell line is robust and stable in its epithelial phenotype. Although MDCK I and MDCK II cells are derived from the same stock, MDCK I cells are relatively unstable and may change their morphological and physiological properties in the processes of repeated transfection and cell cloning. One paper describing the differences of MDCK subclones comments "MDCK II are the most commonly used strain and we would recommend them to researchers new to using MDCK cells, unless they have a specific reason to use one of the other strains" (Dukes JD et al., *BMC Cell Biology*, 2011). The only problem with the MDCK II cell line is its leaky barrier, which we overcame by knocking out the *Cldn2* gene.

Reviewer #2 (Remarks to the Author):

The manuscript "Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function" investigates the role of a tricellular TJ protein and TJ protein in single and double knock MDCKII cell lines. It remains unclear whether occludin and tricellulin modulate the complexity of the TJ strand network in epithelial cells and this is the central question of this manuscript. The role of Tricellulin in barrier formation is also unclear given the conflicting literature for and against Tricellulin in cell culture experiments. The manuscript utilizes a CRISPR mediated approach to remove the tTJ protein Tricellulin (TRIC) and the TJ protein Occludin (OCLN) paired with standard immunofluorescence and electron microscopy to assess the consequences on TJ formation and structure. The permeability of the TJ was assessed and a mathematical model is presented that is predictive of the changes to the TJ ultrastructure. Loss of either gene had little effect on TJ structure and function, loss of both proteins affected the permeability of the TJ and changed the ultrastructure of the TJ, specifically the cross-links in the TJ strands (less anastomosed). This leads to a model whereby TRIC and OCLN play a role in the formation of the anastomosing meshwork of TJ strands, and contribute to the maintenance of epithelial barrier function.

Overall the paper was very straightforward and simple approach using CRISPR mediated knockdown to create different loss of function alleles. The analyses were classic approaches and for the most part generated solid and convincing data. The key result is the reduction in the anastomosing bridges in the double KO and this section was well done and supported by strong data and excellent freeze fraction EM images. The mathematical model was nicely supportive. The paper was very light on mechanism as to what the roles of TRIC and OCLN were in the formation of the bridges. A more sophisticated approach including live imaging of the TJ strands or the relationship of TRIC or OCLN to TJ breaks or PLA analysis of the tagged rescue constructs and which regions of the TJ they are functioning would have increased the impact especially if these proteins can be found at the branch points in a specific complex. A greater emphasis on experiments that test the model would have really made the manuscript compelling. For instance does overexpression of the rescue constructs stabilize the TJ leading to more bridges in MDCKII cells? That being said this is a straightforward and solid paper with clearly presented results that lead to a better understanding of how TRIC and OCLN may function

We appreciate the Reviewer for the encouraging comments and useful suggestions. To obtain insights into the mechanisms, we analyzed the freeze-fracture replicas of Flag-OCLN- or Flag-TRIC-overexpressing cells. Both cell types had an increased complexity of TJ strand network and improved barrier (although some of them were not statistically significant), which suggests that occludin and tricellulin stabilize the TJ strand branches. We also conducted a computer simulation based on the branching frequency measured in overexpressed cells and compared the simulated permeability with the experimental data. They corresponded well, further supporting our hypothesis. We show these data as the revised Figure 6.

We could not undertake a live-imaging approach because TJ strands in the epithelial cells cannot be visualized even using super-resolution microscopes. Previous studies which visualized TJ strands with live imaging used fibroblasts (L cells, Sasaki et al., *PNAS*, 2003 and Rat-1 cells, Van Itallie CM et al., *MBoC*, 2017).

A key and clever approach, central to this paper, was the use of a Claudin2 KO line as the back ground to remove the highly permeable claudin-2 from the MDCK II cell line. Indeed the supplementary data showed that the transepithelial electric resistance measurement in this cell line had significantly increased. These cells were used as the "parental" line for the rest of the manipulations...however this line can't be called "wild type" by any means and should be called been called *Cldn2*-KO or parental line throughout. Wild-type means something very different. This is important in that the *Cldn2*-KO clearly changes either the morphology or the function of the TJ and it is not correct to use the words wildtype. A consistent comparison of the double KO to the original MDCK II cells (i.e. *Cldn2* gene intact) would be very worthwhile in particular in the context of Figure 5. Does the *Cldn2*-KO lead to more anastomosing bridges and thus the double KO in this background is simply a reversion of this to the level of the original MDCK II line?

We thank the Reviewer for considering our strategy to use *Cldn2*-KO MDCK II cells to be a clever approach. We agree that calling the parental *Cldn2*-KO cells "wild type" is confusing. Thus, we decided to use the name "control (Ctrl)" in the revised manuscript and replaced all "wild type" labels with "Ctrl" in the revised figures. Also, we compared the morphology of TJ strands in (*Cldn2*-intact) WT cells with that of *Cldn2*-KO cells and found that the knockout of *Cldn-2* gene leads to less anastomosing bridges. These data are shown in the new Figure S2.

The authors used a nice approach to compare control (*Cldn2*-KO) cells to double or single KO cells by co-seeding control cells (*Cldn2*-KO) cells tagged with NLS-GFP with the single or double knock outlines. None of the TJ or tTJ proteins were mislocalized in the absence of either *Ocln* or *Tric* or the dKO however the Claudin-3 immunolabeling was clearly reduced. The authors state "no drastic changes in the amount of junctional proteins, including claudin-3". A quantification of the protein levels would have helped here as it does appear that there was quite a reduction in the claudin-3 immunolabeling. This in parallel with a better quantification of the Western in Figure S3 would help the reader know if this was a general protein reduction or a loss from the TJ. Quantifying protein levels in the original MDCKII cell line compared to the *Cldn2*-KO (Figure S1) would help assure the reader that there hasn't been changes in the expression levels. For instance in Figure S1 it looks like TRIC has increased in the *Cldn2*-KO, while claudin-4 is decreased.

Since the extent of claudin-3 localization at TJs vary from experiment to experiment, we quantified multiple independent preparations of immunostaining data and realized that a significantly smaller amount of claudin-3 was localized at TJs in the *Tric*-KO, *Ocln*-KO and *Tric/Ocln*-dKO cells compared with the Ctrl cells (revised Figure 2). We replaced the figures for *Tric*-KO and *Ocln*-KO cells in the revised Figure 2 to show representative images. We quantified the expression levels of TJ and AJ proteins using multiple preparations of samples in immunoblot analysis, and found that there were no significant differences between the Ctrl cells and KO cells. These data are presented in the revised Figure S4. We also did the same quantification analysis using the parental MDCK II cells and *Cldn2*-KO cells. Although the expression levels of tricellulin and claudin-4 varied, there was no significance in the averaged expression levels from multiple preparations. We have included these data in the revised Figure S1.

Tricellulin is still found at tTJs in *Ocln*-KO cells but overall the intensity of tricellulin at bicellular TJs is increased in *Ocln*-KO cells compared with the *Cldn2*-KO control and this was very nicely quantified and presented in a good scatter plot analysis. This does lead to the question, what happens to angulin-1/LSR does it also spread in the bicellular TJ? However the supplemental data (Figure S3) for the angulin-1/LSR was confounded by the fact that the angulin-1 immunolabeling was in the same channel as the NLS-GFP making it very difficult to assess any changes to angulin-1. Please repeat this analysis using Angulin in the red channel so that the control cells are more easily distinguished as it looked like in the *Ocln*-KO and in the double KO the angulin-1 had spread in the bicellular TJ compared to the GFP cells. A similar quantification of the angulin-1 distribution in the tTJ versus the bTJ similar to that of Tricellulin would strengthen this data greatly.

We again appreciate the reviewer's kind words. We agree that the question posed by the reviewer is interesting. To answer the question, we quantified the distribution of angulin-1 similarly to tricellulin. To tease apart the nls-GFP and angulin-1 signals, we mix-cultured each KO cell line and control cells expressing nls-GFP, stained angulin-1 using a red fluorescent dye, Cy3, and quantified the distribution of angulin-1. No significant change in angulin-1 localization was observed. The results suggest that occludin-mediated exclusion of tricellulin from the bicellular TJs is independent of angulin-1. Alternatively, since angulin-1 is abundantly localized at lateral membranes in addition to tTJs (we don't know why angulin-1 at the lateral membrane does not recruit tricellulin), the subtle increase in fluorescence at bicellular TJs in the *Ocln*-KO cells might have been buried and would therefore have not been detected. We presented the quantification data of angulin-1 in the revised Figure S5.

As mentioned above for Figure S3 the western analysis of the different proteins levels of the TJ and tTJ proteins should be quantified compared to the actin control. As it stands it looks like the angulin-1 levels have increased greatly in the *Ocln*-KO and perhaps the Claudin-4 levels as well.

We quantified the band intensity of immunoblot samples from four independent preparations and evaluated them. Although the expression levels of some proteins including angulin-1 and claudin-4 varied to some extent from experiment to experiment, there was no statistically significant difference. We show one representative image for each blot together with quantification analysis results in the new Figure S4B.

Figure 4 was not particularly effective in the presentation of the TJ. The focus or the resolution was not sufficient to really determine if the TJ were intact or if there were any changes to the ultrastructure. Either remove this figure (given that Figure 5 is far more convincing) or replace the panels with more convincing data.

This is also pointed out by the Reviewer 1 and we agree. We obtained new TEM images, in which kissing points of TJs are clearly visualized. The new images are included in the revised Figure 3.

In contrast Figure 5 was very convincing and provided a very nice analysis of the TJ strand network. The graph should be a scatter plot and for this graph the error bars are the 95% confidence interval rather than the S.D. displayed in all the other figures (the reader is left wondering why the difference)? This figure needs the P values

indicated above each genotype compared to control. There is a spelling mistake in Figure 5 legend.

The conclusion that the TJ strands were less branched and the network less complex was a key finding from this paper. However given that the double KO is in the context of the claudin-2 KO it would be nice to have the analysis done on the original MDCK II line as it is possible that the claudin-2 KO increases the number of bridges which would fit with the model very well.

To quantify the frequency of branching points, we considered two quantification methods, described below.

1) Measuring the length between branching point to branching point

This method is relatively simple and s.d. and p-values can be calculated, but it may not evaluate the average branching frequency correctly. The sizes of membrane surface harboring TJ strands visible in the freeze-fracture replicas are varied. At the edges of the TJ-bearing membrane surface (at which the fracture plane goes into cytosol, extracellular fluid, or at the edge of the replica), we need to measure "the length between the branching point to the edge" or in some cases (especially in dKO cells, which have seldom branching points), edge-to-edge length, which does not reflect the exact branching frequency. If we do not include such edge-containing strands from the analysis, we need to remove a large part of relatively long strands, which are especially evident in dKO cells.

2) Calculating the average branch frequency (by dividing branching point counts by total strand length)

We counted branching points (three way: 1, four way: 2) and measured the TJ strand length for each replica. Since the sizes of TJ-containing membranes vary from replica to replica, it was not appropriate to average the values of the replicas (by doing this, we would be able to calculate s.d. and p-values). Instead, we just summed up the number of branching points from all replicas and divided it by total TJ strand length. This calculation gives only one value and we cannot provide s.d.. Thus, we calculated upper and lower 95% confidence intervals for branching probability using the Pearson distribution. Using the branching frequency of the control cells as the hypothesized ratio, we calculated the p-values using the Poisson's exact rate test, and presented them in the revised Figure 3, 6, and S2. We also appreciate the Reviewer for pointing out the typo in the legend of the original Figure 5 (revised Figure 3). As mentioned above, we also analyzed the complexity of the TJ strands in the original MDCK II cells and presented the data in the revised Figure S2.

The conclusion from the first part of Figure 6 wasn't consistent with the data present. All three lines Tric-KO #2, Ocln-KO #1 and Ocln-KO #2 had a statistically significant decreases in TER compared to control and the mean TER from the graph seemed to be equivalent. This suggests that both TRIC and OCLN may play a role in the TER and that there might be variations in the responses of the two different cell lines? This fits also better with the conclusion stated in the following section based on the double KO. "indicating that tricellulin and occludin are required for the establishment of permeability barrier for ions"

The use of the rescue constructs was a nice approach and used very effectively and these constructs rescued the reduction in TER. For the dextran permeation experiments the concluding statement was a bit confusing as it appeared that the permeability barrier for macromolecules was restored in the dKO#1+Flag-TRIC cells and dKO#1+Flag-OCLN cells. Therefore it seems more likely that either Tric or Ocln can rescue and are not "jointly required for the establishment of a tight permeability barrier against ions and macromolecules".

We have removed the statement "indicating that tricellulin is dispensable for establishing tight barrier" from the revised manuscript, and rephrased the paragraph to more precisely state the conclusion of this section: "at least either of tricellulin or occludin is required for the establishment of a tight permeability barrier against ions and macromolecules"

For Figure 6, Supplemental Table 1 is key but reading supplemental table 1 was quite confusing. To make this figure and data easier to parse....rather than a bar graph, a scatter plot needs to be used to observe the spread in the TER and P[app] values. This figure would be much easier to digest if the P value or \*, \*\* or NS were placed above each sample (that way the reader doesn't have to flip back and forward between the supplemental and the data figure). Finally for Figure 6 could this be more explicit stated on the graph what the #1 and #2 indicate as well in the legend. We presented the data with scatter plots, including bars of 25, 50 and 75% percentiles. We also added asterisks and n.s. labels to indicate statistical significances. Finally, we also added descriptions for the meaning of #1 and #2 in the legend of the revised Figure 4 (original Figure 6).

Figure 7 and the modeling that support this figure I do not have the necessary expertise to comment on. However the implications of the model fit very well with the literature and with the discussion.

We appreciate the reviewer's comment.

Figure 8 was an interesting model and nicely summarized the finding. The data lacking in support of this model is of course the live imaging of the dynamics of TJ formation and breakage. It is surprising that the authors didn't test their lines with a GFP tagged Claudin or another TJ component to visualize the live dynamics of TJ reformation. However that is likely beyond the scope of this manuscript.

We strongly would like to perform live imaging of TJ strands in epithelial cells, which would reveal the dynamics of claudins and TAMPs. We hope that technical innovations will make this possible in the future.

Other comments:

It is presumed a one-way anova was used for Figure 6 but please specify this.

One-way ANOVA is required to use Fisher's LSD or Scheffe's test, which use F statistic. We used Tukey-Kramer's test, which does not require one-way ANOVA.

The purpose of Figure S6 was very unclear.

Since we used a newly established anti-tricellulin monoclonal antibody in our manuscript, we included information on the immunogen sequence, specific immunostaining data and sequence homology among human (immunogen), dog (MDCK II cells) and mice (frozen sections) tricellulin.

The authors make use of some less rigorous language that should be corrected:

- Page 6, line 10: "Transepithelial electric resistance (TER) measurement showed that Cldn2-KO MDCK II cells exhibited drastically reduced permeability". A simpler statement would be increased resistance by XX fold.
- Page 8, line 11: "Also, there was no huge difference in the average number of horizontal TJ strands among these cell lines". Please be specific was there a statistically significant difference or not? If not then simply state this.
- Figure 7 legend: "The break in the TJ strand drastically reduce[sic] the electric resistance".

We appreciate the reviewer for pointing these out. We have now corrected these descriptions.

There are numerous grammatical and spelling errors.

For the revised manuscript, we have had our manuscript proofread by native-English speaking scientific editors from the Editing Department of our university.

RE: Manuscript #E20-07-0464R

TITLE: "Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function."

Dear Dr. Higashi,

Many thanks for your revised manuscript. I am happy to say that the reviewers and I find the revision much improved. It will be ready for publication after you address the extremely minor data analysis questions that Reviewer 2 asks about.

I know that these times are very challenging, and I very much appreciate our team's work on this interesting story. Thanks again for choosing MBoC!

Best regards,

Jeff Hardin  
Monitoring Editor  
Molecular Biology of the Cell

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Dear Dr. Higashi,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office ([mboc@ascb.org](mailto:mboc@ascb.org)).

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): [Link Not Available](#)

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
[mbc@ascb.org](mailto:mbc@ascb.org)

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Reviewer #1 (Remarks to the Author):

The authors have addressed all key concerns.

Reviewer #2 (Remarks to the Author):

Overall the authors have done a very good job of responding to the prior comments. The inclusion of new data has greatly strengthened the manuscript and the conclusions. Overall the revised manuscript and new data provide strong support for the authors conclusions that tricellulin and occludin regulate epithelial barrier through TJ strand network complexity. There are only a few minor points that could be made to help with the reader's ability to interpret the figures.

Specifically the text is much better and the designation of the Cldn2-KO MDCKII cells as control helped with the interpretation throughout. The comparison of the original MDCKII cells and the Cldn2 KO in Supplemental Figure 2 was for instance a helpful addition to the paper.

The original comment with regards to a lack of overall mechanism was partially addressed by the inclusion of a new Figure 6. Overall the new Figure 6 did very much strengthen the conclusions with additional data on the overexpression of the different proteins in the dKO lines. Only one small issue arises in that the authors reverted to the bar graph for Figure 6C and D. It would be beneficial to keep to the scatter plots that most of the data is now presented as. This allows the reader to visualize the spread and number of individual data points. A violin plot would be especially useful given the large numbers in D. Could it be made more clear in the as to what the dashed lines represent?

For the dextran experiments in Figure 6F was there sufficient power for these experiments as only 4 to 5 trace flux experiments were carried out.

The manuscript was nicely strengthen through the inclusion of quantification of both Westerns and immunolabeling and this much helped ensure that the Cldn2-KO cells and the KO cells were not grossly different. Of interest was that the angulin-1/LSR was not different and thus the tricellulin increase in intensity in the bicellular TJ is independent. The inclusion of the scatter plots in this Figure S5 was a great improvement.

The new TEM images in Figure 3 were much more effective. It would have been nice to include scatter or violin plots for the quantitation in C and D in Figure 3.





RE: Manuscript #E20-07-0464R

TITLE: "Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function."

Dear Dr. Higashi,

Many thanks for your revised manuscript. I am happy to say that the reviewers and I find the revision much improved. It will be ready for publication after you address the extremely minor data analysis questions that Reviewer 2 asks about.

I know that these times are very challenging, and I very much appreciate our team's work on this interesting story. Thanks again for choosing MBoC!

We appreciate you for reviewing our manuscript in the challenging time. According to the reviewer's comment, we made several graphs and incorporated them to the revised manuscript. We hope that we have satisfactorily addressed all the concerns in the new revised version.

Best regards,

Jeff Hardin  
Monitoring Editor  
Molecular Biology of the Cell

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Reviewer #1 (Remarks to the Author):

The authors have addressed all key concerns.

We are grateful for all your help in our manuscript.

Reviewer #2 (Remarks to the Author):

Overall the authors have done a very good job of responding to the prior comments. The inclusion of new data has greatly strengthened the manuscript and the conclusions. Overall the revised manuscript and new data provide strong support for the authors' conclusions that tricellulin and occludin regulate epithelial barrier through TJ strand network complexity. There are only a few minor points that could be made to help with the reader's ability to interpret the figures.

Thank you for kind and constructive comments.

Specifically the text is much better and the designation of the Cldn2-KO MDCKII cells as control helped with the interpretation throughout. The comparison of the original MDCKII cells and the Cldn2 KO in Supplemental Figure 2 was for instance a helpful addition to the paper.

We agree with the reviewer. This characterization also provides a fundamental platform for future studies using Cldn2-KO MDCK II cells.

The original comment with regards to a lack of overall mechanism was partially addressed by the inclusion of a new Figure 6. Overall the new Figure 6 did very much strengthen the conclusions with additional data on the overexpression of the different proteins in the dKO lines. Only one small issue arises in that the authors reversed to the bar graph for Figure 6C and D. It would be beneficial to keep to the scatter plots that most of the data is now presented as. This allows the reader to visualize the spread and number of individual data points. A violin plot would be especially useful given the large numbers in D. Could it be made more clear in the as to what the dashed lines represent?

Thank you for your comment. We agree with the review that visualization of all data points would be beneficial for the readers to grasp the spread and number of data points. Thus, we created scattered plots on the TJ strand segment

length in the revised Figures S7A. In this figure, we can easily see that there are more longer segments in the *Tric/Ocln*-dKO cells compared with other clones.

As we discussed in the prior rebuttal letter, observed TJ strand segments are categorized into three groups: closed segment (both ends of the strand are connected to other strands), semi-open strand (one end is connected and other end is free or missing) and open segment (both ends are missing). Although we differentially plotted them in the scatter graph, it is difficult to do statistics on this data because *Tric/Ocln*-dKO cells have longer segments and many of them are semi-open and open segments. Since the lengths of semi-open and open segments do not reflect the actual length of the segment, they cannot be compared directly with the lengths of closed segments. Thus, we decided to keep the bar graphs in the Figures 3C, 6C and S2D for statistics and also include scatter plots in Figure S2E and S7A for visualization of data point distribution.

For visualization of horizontal strand number data points, we used histograms instead of scatter or violin plots because the values are discrete. We incorporated them in the revised S2G and S7B.

We added descriptions about the dashed lines in the legends of Figure 6C and 6D.

For the dextran experiments in Figure 6F was there sufficient power for these experiments as only 4 to 5 trace flux experiments were carried out.

From the previous experiments shown in Figure 4, we assumed that the s.d. of Papp values for 4-kD dextran is  $3.6 \times 10^{-9}$  [cm/sec]. Simulation analysis predicted that OCLN-OE cells exhibit reduced permeability by  $10 \times 10^{-9}$  [cm/sec]. If we use  $n=5$  and  $n=4$  data, the power will be 0.969 and 0.902, respectively (calculated by R software). If the change in the permeability is too subtle, this test cannot detect the difference (e.g. difference =  $3 \times 10^{-9}$  [cm/sec] results in power = 0.213)

The manuscript was nicely strengthened through the inclusion of quantification of both Westerns and immunolabeling and this much helped ensure that the *Cldn2*-KO cells and the KO cells were not grossly different. Of interest was that the angulin-1/LSR was not different and thus the tricellullin increase in intensity in the bicellular TJ is independent. The inclusion of the scatter plots in this Figure S5 was a great improvement.

The new TEM images in Figure 3 were much more effective. It would have been nice to include scatter or violin plots for the quantitation in C and D in Figure 3.

Thank you again for kind comments. As shown above, we included scatter plots and histograms for Figure 3, C and D.

RE: Manuscript #E20-07-0464RR

TITLE: "Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function."

Dear Dr. Higashi,

Many thanks for making the additional minor revisions to your manuscript. I am pleased to say that your work is now ready for publication in *Molecular Biology of the Cell*.

Many thanks for sending your very interesting work to MBoC.

Best regards,  
Jeff Hardin  
Monitoring Editor

*Molecular Biology of the Cell*

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Dear Dr. Higashi:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at [www.molbiolcell.org/toc/mboc/0/0](http://www.molbiolcell.org/toc/mboc/0/0) is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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