Angulin-1 seals tricellular contacts independently of tricellulin and claudins

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Re: JCB manuscript #202005062

Dr. Mikio Furuse
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Dear Mikio,

Thank you for submitting your manuscript entitled "Angulin-1 seals tricellular contacts independently of tricellulin and claudins". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

As you will see, both reviewers agree that the role of angulin and the function of tricellular junctions in barrier function are important issues. However, as you will see, the two reviewer's differed in their opinions about the strength of the current dataset. The additional quantifications and clarifications requested by Reviewer 2 should be straightforward to carry out and should be completed. Reviewer 1 suggests a much more extensive set of additional experiments, which we recognize would require substantial expansion of the current work. Some of these should be straightforward to address with existing data or modest expansions of that data. Of the more substantive suggestions, the most central are the questions about potential mixing of adherens junction and tight junction proteins at tTJs, and additional experiments to clarify the relationship between ZO-1 and angulin (issues raised in that reviewer's points 5, 8, 9, 10, and 13-14)-these should be addressed experimentally. Other issues raised by this reviewer could be addressed by text clarifications. We realize this may require a significant extension of the current work.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
This manuscript by Sugawara et al aims to clarify the mechanism of tricellular tight junction (tTJ) formation and the role of angulin-1 in tricellular contacts and tight junction barrier function in MDCK cells. They describe the localization of tricellulin, angulin-2 and claudin-2 in sections of kidney, and then switch to MDCKII cells, where they carry out a characterization of the localization of occludin, tricellulin, claudin-2 and ZO-1, thus showing that tricellular central sealing elements contain all of these bicellular TJ proteins. They then carry out an analysis of angulin-1-KO cells, showing that the integrity of tricellular central sealing elements is disrupted, the accumulation of other TJ proteins...
and the deep tricellular contact sites detected by electron microscopy are undetectable upon KO of angulin-1. They correlate these morphological functions to an increase in paracellular permeability to fluorescein, whereas an increase permeability barrier to ions is seen only when both claudin-2 and angulin-1 are depleted. To understand the mechanisms involved in angulin-1 localization, they carry out GST pulldowns using different constructs, and identify and interaction between the C-terminal PDZ-binding motif with angulin-1 and the PDZ-2 domain of ZO-1. Through rescue with mutant and chimeric constructs, they implicate ZO-1 in anchoring. However, the localization of angulin-1 in cells lacking ZO proteins is not shown. Although the manuscript is clearly written and logically articulated, the data are sometimes minimalistic, leaving several questions open. Specifically, the mechanisms accounting for the phenotypes, namely what anchors angulin-1 to tTJs and how angulin-1 controls paracellular barrier to ions, are not sufficiently clarified. Furthermore, the functional redundancy between angulin-1 and JAM-A is not addressed.

MAJOR POINTS

1. A rod-like localization of angulins in tissues is shown for angulin-2 and tricellulin in kidney collecting ducts. However, in proximal tubules tricellulin is localized at dot-like structures and angulin-2 is not shown. This raises doubts about the actual significance of the rod-like structures. Are they specific of collecting ducts, are they associated with different membrane/cyttoplasmic/cytoskeletal structures? Do they exist in MDCKII cells, and are MDCKII cells (as opposed to MDCKI) an appropriate cellular model? Importantly, considering that MDCKII cells derive from the proximal tubule, and that studies on MDCKII cells focus on angulin-1 (and not angulin-2), it would be appropriate to show the localization of both angulins (1 and 2) in both proximal and distal (and collecting duct) tubules, and MDCKI and MDCKII cells. In fact, it would be useful to establish the occurrence of the rod-like structures not only in different parts of the nephron, but also in other epithelial tissues.

2. For completeness, the localization of ZO-2 and ZO-3 should be shown (in Supp. Figures), in addition to ZO-1 (Figure 2D), along central sealing elements.

3. The conclusion that bicellular TJ proteins such as claudin and ZO-1 are colocalized with tricellular TJ markers along the apicobasal axis at tTJ is well supported by the data. However, what is completely missing is the characterization of the localization of AJ components along the central sealing element. Are these components (nectin, cadherin, afadin, PLEKHA7, catenins, vinculin etc) displaced more basally along lateral sides, or are they intermingled with the tTJ components to form the central sealing elements? The EM analysis (Fig4) is suggest that the central column extends to the level of centrosomes. This makes it important to characterize to which extent molecular components of the AJ and the lateral contacts are co-localized with angulin-1.

4. How angulin-1-KO affects barrier to ions is not explained. This was assessed in cells that were also made KO for claudin-2, since the presence of claudin-2 makes TJ leaky to ions. Claudin-2-KO cells have a tighter barrier (higher TER >4000 ohms.cm²), and the KO of angulin-1 results in a drop in TER (to below 1000 ohms.cm²) in these cells. Because claudins control paracellular flux to ions, it is crucial to clarify why this drop in TER occurs. Does KO of angulin-1 affect the expression and/or localization of barrier-forming claudins?

5. The drop in TER (following KO of angulin-1 in the background of claudin-2-KO cells) is rescued in a similar manner by the WT angulin-1 and by a mutant lacking the PDZ-bonding motif (Figure S2F). This suggest that the role of angulin-1 in the control of TJ barrier to ions is independent of its interaction with ZO-1. If the expression and/or localization of claudins is disrupted upon angulin-1 KO, are they similarly rescued by WT and mutant? What does this say about ZO-1-independent mechanisms of tTJ assembly? Which transmembrane proteins whose function could be regulated by angulins are implicated?

6. Similarly, the barrier to larger molecules is rescued independently of the ZO-1-binding motif. This
raises the question of how the localization of JAM-A is affected by angulin-1. Is the relationship between JAM-A and angulin-1 similar to the relationship between occludin and tricellulin (Ikenouchi et al MBC 2008)? Is JAM-A upregulated in angulin-1-KO cells, as detected either by immunoblot or immunofluorescent localization? How does rescue of angulin-1-KO cells with either WT or mutant lacking the pbm affect any changes in JAM-A? Since angulins and JAM-A are the main Ig-like adhesion molecules of TJ it is important to establish their functional redundancy.

7. Page 6. The authors state "In angulin-1-KO cells, claudin-2 and ZO-1 were detected in the apical region containing TJs, but not in the lateral region at TCs (Fig.3C). I do not agree with this description, since claudin-2 labeling is detected in z-sections along all lateral contacts upon angulin-1 KO. This continuous lateral labeling, which is detected also in x-y images, is rescued by re-expression of angulin-1, resulting in clustered claudin-2 labeling at TCs. So, what the images say is that in WT cells claudin-2 is mostly clustered at TJ apically and at TC (by angulin-1), yet upon KO of angulin-1 it is "released" and diffuses all along lateral contacts basally. There is no comment and no explanation of this phenotype, which is relevant to the role of tTJ in controlling claudins.

8. The conclusion that ZO-1 anchors angulin-1 is supported by the pulldown data. However, the story is not as simple as the authors present it. ZO-1 is distributed circumferentially all along the apical TJ, whereas angulin-1 is clustered at tTJ. Where is angulin-1 localized in cells KO for ZO proteins? The authors acknowledge in the Discussion that "angulin-1 is responsible for the plasma membrane contact at TCs independently of tricellulin, claudins, JAM-A and binding to ZO-1". So, the mechanism for apical anchoring of angulin-1 is not clarified. Are apical polarity proteins are involved (Par3 for example)?

9. The conclusion (end of page 7) that "the angulin-1 pbm is responsible for the extended distribution of TJ constituents at TCs" is supported by the data showing that the mutant lacking the pbm fails to induce the redistribution of ZO-1 and claudin-2 along the basal central sealing elements. However, this also implies that the linkage to ZO-1 is irrelevant for the more basal-lateral localization of angulin-1, raising the question of its anchoring at these sites.

10. The same conclusion (end of page 7) also implies that the ability of the same mutant to rescue the barrier phenotypes to ions (figure S2F) is independent of ZO-1, and, as a possible consequence, of ZO-1-dependent recruitment of claudins to more basal locations. Thus, how does angulin-1 regulate barrier-forming claudins independently of its binding to ZO-1? (see also previous comment)

11. The localization of claudin-2 in angulin-1-KO cells rescued with the mutant lacking the pbm (Fig5B) appears apical, lateral and cytoplasmic. This is different from both the localization of claudin-2 in angulin-KO cells (where circumferential labeling is detected laterally in x-y images), and cells rescued with WT angulin-1 (where localization at TCs is detected). Can the authors explain this difference? Is claudin largely endocytosed here?

12. Although claudin-2 is detected laterally at TC in cells rescued with the chimeric construct (Fig 6 E-F-G), overall the rescue of the claudin-2 labeling with the chimeric construct is very, very different from the rescue with angulin-1 WT (Figure 2C). There is still a lot of cytoplasmic claudin-2 labeling (Fig.6G), suggesting that additional interactions of the pbm region of angulin-1 are required to re-establish the WT localization of claudin-2

13. The panels in Figure 6H-I actually belong to Figure 5, which contains data about the rescue with the mutant lacking the pbm. Here, the observation that the mutant rescues the barrier phenotypes suggests that some rearrangement of barrier-forming claudins occurs, independent of ZO-1. Yet, claudin-2 localization is clearly not rescued by the mutant (Figure 5B). This is confusing, and the mechanism for the rescue of the barrier to ions remains elusive, unless one assumes that angulin-1 itself can form a barrier to ions. Indeed, the conclusion (first paragraph, page 9) that "the angulin-1 pbm is not essential for the epithelial barrier function or plasma membrane contact at TC" leaves unresolved the issue of the mechanism of anchoring of angulins. If pbm-mediated interactions are not implicated in localizing angulin-1 at tTJ, what interactions recruit it there?
14. The conclusion that "the interaction between angulin-1 and ZO-1 supports the extended localization of claudin-2 along the apicobasal axis at TC" is based on evidence using the chimeric molecule. However, this experiment is complicated, because ZO-1 has many interactors, and the rescue could be due indirect interactions of ZO-1. To formally prove the implied assumption that claudin-2 interacts with the ZO-1 PDZ1 domain, it is necessary to compare the rescue with the constructs shown in Fig.6E with a similar construct lacking the PDZ1 domain. Otherwise, the precise mechanism remains fuzzy.

15. For completeness, it would be interesting to compare the binding of the cytoplasmic region of angulin-1 (Fig.6A) to the binding of the cytoplasmic region of JAM-A (with or without deletion of the pbm). And use afadin as a prey, in addition to ZO-1, to clarify the relevance of interaction with afadin (a ligand of JAM-A).

16. Figure 7 addresses the role of JAM-A, which reinforces the concept that the expression and localization of JAM-A in angulin-1-KO cells must be shown. The results shown in Figure 7A-B are puzzling. If angulin-1 is responsible for lateral-basal TC, why are these still detected at the level of desmosomes when few (or none) lateral central sealing elements are visible by immunofluorescence of angulin-1? Is angulin-2 upregulated under these conditions? Could angulin-2 be implicated in mediating the formation of TCs at the desmosome level?

17. Figure 7C-D. The authors claim that upon KO of 5 claudins plus KO of JAM-A "angulin-1 was localized at the only apical part of TCs". I do not agree. In x-y images, there is clear labeling for angulin-1 along the lateral borders, except that it is not clustered at TC but distributed uniformly along tricellular and bicellular contacts. Is this because angulin-1 takes the space left "available" by JAM-A? What is the relative localization of angulin-1 and JAM-A in WT cells, and in cells KO for one or the other?

18. EM shows that TCs are still present at the DS level in cells KO for 5 claudins and JAM-A (Figure 7D). This suggests, again, either that IF localization of angulin-1 at lateral TC is not significantly correlated with TCs detected by EM, or that, if angulin-1 is not involved, redundant molecules (angulin-2?) mediate the formation of TC in penta-claudin-JAM-KO cells. This cannot be tricellulin, since if angulin-1 recruits tricellulin (Figure 2b) and angulin-1 is not localized at lateral TC, as claimed by the authors, then tricellulin cannot be at lateral TC.

19. The EM phenotypes described in Figure 8E-F are largely anecdotal, and as noted by the authors, the conclusion that "tricellulin is required for the organization of tTJs by connecting short strands to the central sealing elements" is far from being justified in the absence of quantitative data and further investigations by super-resolution microscopy and freeze-fracture immunolabeling.

Additional issues

1. The labeling of immunofluorescent panels should be improved. For example, Figure 8C are tricellulin-KO cells, but it is not made explicit by the labeling. It's confusing.
2. The images in Figure 8F are insufficiently labelled and not at a sufficiently high magnification to make it clear what the authors mean by "caving-in" of TJ strands.
3. The results reported here are in disagreement with a previously reported role of tricellulin in controlling TER (Ikenouchi 2005), which requires clarification (beyond "different cell types").

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

Tricellular tight junctions (tTJs) seal the paracellular space at cell vertices in epithelia and are essential for epithelial barrier function. Tricellular junctions were first described in the 1970s, but their molecular organization and their functions in normal physiology and disease are only beginning to be understood and have recently attracted growing interest of cell biologists. In mammalian cells,
tTJs comprise the four-transmembrane domain protein tricellulin and immunoglobulin domain transmembrane proteins of the angulin family. Although previous work had shown that angulins recruit tricellulin to tTJs, the specific functions of angulins and of tricellulin in tTJ formation, and their respective contributions to tTJ structure have not been clear. Here Sugawara et al. describe the roles of angulin-1 and tricellulin in tTJ formation in MDCK cells using state-of-the-art genome editing, light and electron microscopy, biochemistry, and transepithelial electrical resistance (TER) measurements. They investigated the precise localization of angulin-1 and tricellulin and their interactions with claudins and with the TJ scaffold protein ZO-1. They show that TJ proteins (claudin-2, occludin, ZO-1) colocalize with tTJ proteins at basolateral extensions along vertices, and that the formation of these vertical extensions is dependent on angulin-1. Using both transmission and freeze-fracture electron microscopy the authors demonstrate that angulin-1 is required for the normal structure of the plasma membrane contacts and central sealing elements at cell vertices. Furthermore, they show that angulin-1 is required for normal transepithelial resistance and for limiting paracellular flux in claudin-2-deficient MDCK cells. They go on to show that angulin-1 binds to ZO-1 and that this interaction is required for the formation of basolateral tTJ extensions, but not for paracellular barrier function. Strikingly, the plasma membrane contact at vertices still forms in claudin-1, -2, -3, -4, -7 quintuple knockout cells lacking bicellular tight junction strands, as well as in cells additionally lacking the junctional adhesion molecular JAM-A, indicating that tTJs can assemble independently of bicellular TJs. Finally, they show that Tricellulin is required for the normal structure of tTJs with basolaterally extending TJ strands, but not for the formation of central sealing elements, and, most surprisingly, not for paracellular barrier function. This last finding is in discrepancy to earlier results from functional studies (e.g., Ikenouchi et al. 2005; Krug et al. 2009) that had suggested an essential role of tricellulin for barrier function. However, at the same time, the finding is consistent with, and may help explain, the subtle defects observed in Tricellulin knockout mice, as compared to the severe defects and embryonic lethality of angulin-1 knockout mice.

The study by Sugawara et al. provides important new insights into the molecular organization of tTJs and the interactions of tTJ proteins with bicellular TJ components, and identifies distinct roles of the two known tTJ components, angulin-1 and tricellulin, in tTJ assembly and barrier function. The results are convincing, of very high scientific quality and technical standard, and the text is very well written. However, the analysis of protein localization at TCs, as a central part of the paper, is entirely qualitative and needs to be quantified (see below).

Overall, this a very interesting study that clearly provides an important conceptual advance, with significant implications for epithelial biology in many different contexts and model systems. The work should therefore be of interest to the broad audience of readers of JCB.

The authors should address the following (minor) points before the work should be accepted for publication:

- The study would clearly benefit from a quantitative analysis of the immunofluorescence images. The length of the basolateral extensions of TJs along cell vertices is presented essentially as a binary trait ("extended" or "not extended"). Careful quantification of the length of the tTJ extensions, as well as of the enrichment of angulin-1 and tricellulin at apical and apico-lateral TCs would be informative.
- Besides the length of the basolateral extensions of tTJs, the distribution of angulin-1 or tricellulin between TCs and bicellular contacts appears to change in some of the genotypes shown. For instance, angulin-1 appears to spread into bicellular contacts in Claudin/JAM-A knockout cells (Fig. 7C; see also Fig. 7A, Fig. 8C). The authors should analyze the enrichment of angulin-1 at TCs compared to bicellular contacts, and discuss the results.
- Figure 1: the overall structure of the tissue, the shape of the cells and the organization of junctions around the TCs are difficult to apprehend. Several of the Tricellulin signals do not appear to be bordered by junctions from three cells. Highlighting the outlines of cells would be helpful here.
Overall, this figure does not seem to contribute much to the paper. Why did the authors choose to examine kidney tubules and collecting ducts, rather than planar epithelia (e.g. cochlea)?

- The authors should indicate the sample size (n) also for the electron microscopy data. Are apical (TJ level) and basolateral (DS level) sections for each genotype taken from the same cell or from different cells?
- p. 10: The statement that "ZO-1 was mostly colocalized with angulin-1 along the apicobasal axis at TCs, but was infrequently missing from some angulin rods" needs to be substantiated with numbers (sample size, penetrance of the phenotype).
- p. 10, Fig. 8E: "Horizontal ultrathin sections at the TJ level revealed that tricellulin KO cells contained either the plasma membrane contact or a gap at TCs (Fig. 8 E)." : indicate sample size and proportions of the two phenotypic classes ("contact" vs. "gap").
- On p. 14 the authors state that "The present observations indicate that the basic mechanisms for bicellular TJ formation and tTJ formation are independent of one another, but are coordinately organized in epithelial cells to form a functional paracellular barrier". They may consider discussing this interesting finding in the light of results from Drosophila, where bicellular and tricellular occluding junctions were also found to be assembled independently (Byri et al. Developmental Cell 2015).
- p. 9: check wording: "Angulin-1 was localized at the only apical part of TCs"
- p. 9: check wording: "which appeared to the apical cell-cell junction level"
- p. 14: missing word: "It will interesting to examine..."
Reviewer #1 (Comments to the Authors (Required)):

This manuscript by Sugawara et al aims to clarify the mechanism of tricellular tight junction (tTJ) formation and the role of angulin-1 in tricellular contacts and tight junction barrier function in MDCK cells. They describe the localization of tricellulin, angulin-2 and claudin-2 in sections of kidney, and then switch to MDCKII cells, where they carry out a characterization of the localization of occludin, tricellulin, claudin-2 and ZO-1, thus showing that tricellular central sealing elements contain all of these bicellular TJ proteins. They then carry out an analysis of angulin-1-KO cells, showing that the integrity of tricellular central sealing elements is disrupted, the accumulation of other TJ proteins and the deep tricellular contact sites detected by electron microscopy are undetectable upon KO of angulin-1. They correlate these morphological functions to an increase in paracellular permeability to fluorescein, whereas an increase permeability barrier to ions is seen only when both claudin-2 and angulin-1 are depleted. To understand the mechanisms involved in angulin-1 localization, they carry out GST pulldowns using different constructs, and identify an interaction between the C-terminal PDZ-binding motif with angulin-1 and the PDZ-2 domain of ZO-1. Through rescue with mutant and chimeric constructs, they implicate ZO-1 in anchoring. However, the localization of angulin-1 in cells lacking ZO proteins is not shown. Although the manuscript is clearly written and logically articulated, the data are sometimes minimalistic, leaving several questions open. Specifically, the mechanisms accounting for the phenotypes, namely what anchors angulin-1 to tTJs and how angulin-1 controls paracellular barrier to ions, are not sufficiently clarified. Furthermore, the functional redundancy between angulin-1 and JAM-A is not addressed.

We thank the reviewer for his/her critical reading of our manuscript and giving us comments for improvement of our study. The major criticism of the reviewer is the mechanisms on what anchors angulin-1 to the tTJs, and how angulin-1 controls the paracellular barrier. In the revised manuscript, we analyzed the localization of angulin-1 in ZO-1/ZO-2 double KO cells and ZO-1/ZO-2 double KO cells expressing a ZO-1 mutant lacking its PDZ2 domain, which is required for ZO-1 binding to angulin-1. The results suggest the possibility that angulin-1 autonomously assembles in tricellular contacts and recruits ZO-1 and claudins there. Furthermore, our additional data support the idea that angulin-1-mediated obliteration of tricellular contacts per se regulates the paracellular barrier to ions. These issues are extensively discussed in the revised manuscript. Please also see below for the detailed response.
MAJOR POINTS

1. A rod-like localization of angulins in tissues is shown for angulin-2 and tricellulin in kidney collecting ducts. However, in proximal tubules tricellulin is localized at dot-like structures and angulin-2 is not shown. This raises doubts about the actual significance of the rod-like structures. Are they specific of collecting ducts, are they associated with different membrane/cyttoplasmic/cytoskeletal structures? Do they exist in MDCKII cells, and are MDCKII cells (as opposed to MDCKI) an appropriate cellular model? Importantly, considering that MDCKII cells derive from the proximal tubule, and that studies on MDCKII cells focus on angulin-1 (and not angulin-2), it would be appropriate to show the localization of both angulins (1 and 2) in both proximal and distal (and collecting duct) tubules, and MDCKI and MDCKII cells. In fact, it would be useful to establish the occurrence of the rod-like structures not only in different parts of the nephron, but also in other epithelial tissues.

As we reported in the previous study, only angulin-1 was detected in proximal tubules, while only angulin-2 was detected in collecting duct among angulin family proteins in immunofluorescence (Higashi et al., JCS 2013). In the revised manuscript, we have presented angulin-1 staining in proximal tubules. Not only tricellulin but also angulin-1 are localized as dots, suggesting that the extension of tTJs to the basal direction depend on type of epithelial cells. Because of space limitation, the data of proximal tubules have been moved to Figure S1A and B.

In accordance with the reviewer’s suggestion, we have also shown rod-like distribution of tTJ and TJ proteins in the small intestine as another example in Figure 1. Accordingly, these results have been described in the text (line 115-117/Word of line 116-118/pdf). On the other hand, we do not agree on the reviewer’s view that MDCK II cells and MDCK I cells correspond to proximal and distal tubules, respectively. Although MDCK II cells were originated from proximal tubules, the expression pattern of claudins in MDCK II cells (claudin-1, -2, -3, -4 and -7) is much different from that of proximal tubules (claudin-2 and -10a), suggesting the loss of the proximal tubule property. We just use MDCK II cells as typical cultured cells with apicobasal extension of tTJs in this study regardless of its origin. Therefore, we have not analyzed MDCK I cells. In addition, “MDCKII renal epithelial cells” has been modified to “MDCK II cells” to avoid possible confusion (line 118/Word or line 119/pdf),
2. For completeness, the localization of ZO-2 and ZO-3 should be shown (in Supp. Figures), in addition to ZO-1 (Figure 2D), along central sealing elements.

We have shown the rod-like distribution of ZO-2 and ZO-3 at TCs in MDCK II cells in immunofluorescence in Figure S1 C and D and described it in the Results, line 128-129/Word or line 130-131/pdf.

3. The conclusion that bicellular TJ proteins such as claudin and ZO-1 are colocalized with tricellular TJ markers along the apicobasal axis at tTJ is well supported by the data. However, what is completely missing is the characterization of the localization of AJ components along the central sealing element. Are these components (nectin, cadherin, afadin, PLEKHA7, catenins, vinculin etc) displaced more basally along lateral sides, or are they intermingled with the tTJ components to form the central sealing elements? The EM analysis (Fig4) is suggest that the central column extends to the level of centrosomes. This makes it important to characterize to which extent molecular components of the AJ and the lateral contacts are co-localized with angulin-1.

As the reviewer pointed out, the distribution of AJ at TCs is of great interest. We examined the localization of AJ components and found that the alpha-catenin under tension and afadin showed extended localization along the apicobasal axis at TCs similar to angulin-1 and tricellulin in immunofluorescence. These data have been presented in Figure 3 and described in the Results, line 136-149/Word or line 137-150/pdf, and discussed in the Discussion, line 395-407/Word or line 404-416/pdf, in the revised manuscript. We also checked the localization of vinculin, but its staining at cell-cell contacts was very weak to evaluate the localization at tricellular contacts. Thus, we have not mentioned vinculin.

4. How angulin-1-KO affects barrier to ions is not explained. This was assessed in cells that were also made KO for claudin-2, since the presence of claudin-2 makes TJ leaky to ions. Claudin-2-KO cells have a tighter barrier (higher TER >4000 ohms.cm2), and the KO of angulin-1 results in a drop in TER (to below 1000 ohms.cm2) in these cells. Because claudins control paracellular flux to ions, it is crucial to clarify why this drop in TER occurs. Does KO of angulin-1 affect the expression and/or localization of barrier-forming claudins?

We have shown in the revised manuscript no reduction of claudin-4, a barrier-forming claudin, at tight junctions in claudin-2/angulin-1 KO cells in immunofluorescence (Figure
S3E, line 184-185/Word or line 185-186/pdf in the Results). In addition, we have added ultra-thin section EM images of paracellular gaps at tricellular contacts in claudin-2/angulin-1 KO cells (Figure S3I, line 200-201/Word or line 201-202/pdf in the Results). These observations suggest that TER drop in claudin-2/angulin-1 KO cells is not due to the loss of barrier-forming claudins in tight junctions, but due to tricellular gaps that permit the passage of ions.

5. The drop in TER (following KO of angulin-1 in the background of claudin-2-KO cells) is rescued in a similar manner by the WT angulin-1 and by a mutant lacking the PDZ-bonding motif (Figure S2F). This suggest that the role of angulin-1 in the control of TJ barrier to ions is independent of its interaction with ZO-1. If the expression and/or localization of claudins is disrupted upon angulin-1 KO, are they similarly rescued by WT and mutant? What does this say about ZO-1-independent mechanisms of tTJ assembly? Which transmembrane proteins whose function could be regulated by angulins are implicated?

As described in the previous response, the expression of barrier forming claudins is not disrupted in angulin-1/claudin-2 double KO cells. We showed in the original manuscript that angulin-1 Δpbm, which does not bind to ZO-1, can assemble in TCs and close the paracellular space there in angulin-1 KO cells. In addition, we have shown in the revised manuscript that 1) anguli-1 normally assembles in TCs in ZO-1/ZO-2 dKO cells expressing a ZO-1 mutant lacking PDZ2 domain, which is the binding domain to angulin-1 (see our response to the comment #8) and 2) the plasma membrane contact at TCs in claudin quinKO cells is mediated by angulin-1 (see our response to the comment #16). Taken together, these results are consistent with the idea that angulin-1 itself generates paracellular sealing at TCs.

6. Similarly, the barrier to larger molecules is rescued independently of the ZO-1-binding motif. This raises the question of how the localization of JAM-A is affected by angulin-1. Is the relationship between JAM-A and angulin-1 similar to the relationship between occludin and tricellulin (Ikenouchi et al MBC 2008)? Is JAM-A upregulated in angulin-1-KO cells, as detected either by immunoblot or immunofluorescent localization? How does rescue of angulin-1-KO cells with either WT or mutant lacking the pbm affect any changes in JAM-A? Since angulins and JAM-A are the main Ig-like adhesion molecules of TJ it is important to establish their functional redundancy.
We have compared the expression and localization of JAM-A in MDCK cells and angulin-1 KO cells. In MDCK II cells, JAM-A is localized along the lateral membrane domain as well as apical junctions and clear concentration of JAM-A along the apicobasal axis was not detected at TCs. This was shown in Fig S1E and described in the Results, line 129-132/Word or line 131-133/pdf. In angulin-1 KO cells, the expression and localization of JAM-A was not remarkably affected in immunofluorescence. We have shown these data in Figure S2F and in the Results (line 310-311/Word or line 317-319/pdf). Regarding possible functional redundancy between angulin-1 and JAM-A suggested by the reviewer, we hesitate to discuss it in the manuscript because angulins have a single Ig-like domain, whereas JAM family proteins have two Ig-like domains in the extracellular domain, indicating that they are not so close to each other. Indeed, JAM proteins are generally discriminated from nectins, which have three Ig-like domains in the extracellular domain.

7. Page 6. The authors state "In angulin-1-KO cells, claudin-2 and ZO-1 were detected in the apical region containing TJs, but not in the lateral region at TCs (Fig.3C). I do not agree with this description, since claudin-2 labeling is detected in z-sections along all lateral contacts upon angulin-1 KO. This continuous lateral labeling, which is detected also in x-y images, is rescued by re-expression of angulin-1, resulting in clustered claudin-2 labeling at TCs. So, what the images say is that in WT cells claudin-2 is mostly clustered at TJ apically and at TC (by angulin-1), yet upon KO of angulin-1 it is "released" and diffuses all along lateral contacts basally. There is no comment and no explanation of this phenotype, which is relevant to the role of tTJ in controlling claudins.

Consistent with the reviewer comment, we detected weak staining of claudin-2 along the lateral membrane in angulin-1KO cells. Therefore, our description, "In angulin-1-KO cells, claudin-2 and ZO-1 were detected in the apical region containing TJs, but not in the lateral region at TCs" was not correct. This sentence has been modified to " In angulin-1 KO cells, concentrated localization of claudin-2 and ZO-1 were detected in the apical region containing TJs, but not in the lateral region at TCs" in the Results, line 163-165/Word or line 165-166/pdf, in the revised manuscript.

Such a weak claudin-2 labeling along the lateral membrane was detected even in parent MDCK II cells and angulin-1 rescued cells, and we did not notice remarkable change in claudin-2 signals between these cells in our experiments. However, as the reviewer pointed out, the staining of the lateral claudin-2 in angulin-1 KO cells appeared more remarkable than that in angulin-1 rescued cells in the original manuscript. To address the reviewer's
comment, we repeated immunofluorescence staining of angulin-1 KO cells and the rescued cells with anti-claudin-2 antibody, but did not find remarkable change in diffuse claudin-2 staining along the lateral membrane in angulin-1 KO cells and the rescued cells. In the revised manuscript, we have replaced immunofluorescence images of claudin-2 in angulin-1 KO cells and the rescued cells with new data set in Figure 4C. Because we could not clearly detect the release of claudin-2 in angulin-1 KO cells, we do not mention this issue in the revised manuscript although the reviewer’s interpretation is reasonable.

8. The conclusion that ZO-1 anchors angulin-1 is supported by the pulldown data. However, the story is not as simple as the authors present it. ZO-1 is distributed circumferentially all along the apical TJ, whereas angulin-1 is clustered at tTJ. Where is angulin-1 localized in cells KO for ZO proteins? The authors acknowledge in the Discussion that "angulin-1 is responsible for the plasma membrane contact at TCs independently of tricellulin, claudins, JAM-A and binding to ZO-1". So, the mechanism for apical anchoring of angulin-1 is not clarified. Are apical polarity proteins are involved (Par3 for example)?

In accordance with the reviewer’s comment, we analyzed ZO-1/ZO-2 dKO MDCK II cells and found no angulin-1 assembly at tricellular contacts. We have added this data to the revised manuscript (Figure S4A and B). However, only with this result we cannot conclude that angulin-1 assembly at TCs depends on its interaction with ZO proteins because ZO-1/ZO-2 dKO cells show severe and complex phenotypes such as loss of apicobasal polarity, impaired TJ/AJ formation, and increased actomyosin-dependent tension (Otani et al., J. Cell Biol. 218:3372-3396, 2019). To avoid this complexity, we further analyzed the localization of angulin-1 at tricellular contacts in ZO-1/ZO-2 dKO cells expressing a ZO-1 mutant lacking PDZ2 domain, which is the binding domain to angulin-1. In these cells, angulin-1 went to TCs and its apicobasal extension at TCs was observed, suggesting that angulin-1 assembly is independent of ZO-1 binding. These results have been presented in Figure S4 C-E and described in the Results, line 250-264/Word or line 255-269/pdf. The role of the polarity complex proteins such as Par3 in the assembly of angulin-1 is of interest, but we would like to leave this issue for future studies because it is beyond the scope of the present study.

9. The conclusion (end of page 7) that "the angulin-1 pbm is responsible for the extended distribution of TJ constituents at TCs" is supported by the data showing that the mutant lacking the pbm fails to induce the redistribution of ZO-1 and claudin-2 along the basal
central sealing elements. However, this also implies that the linkage to ZO-1 is irrelevant for the more basal-lateral localization of angulin-1, raising the question of its anchoring at these sites.

Based on our observations, we should consider another possibility that angulin-1 autonomously assembles at tricellular contacts without the help of anchoring proteins, seals the extracellular space there, and recruits ZO-1. In the revised manuscript, we have discussed the mechanism of angulin-1 assembly in more detail by combining the results in this study and our recent publication (Oda et al., JBC 2020) in the Discussion, line 465-472/Word or line 475-483/pdf.

10. The same conclusion (end of page 7) also implies that the ability of the same mutant to rescue the barrier phenotypes to ions (figure S2F) is independent of ZO-1, and, as a possible consequence, of ZO-1-dependent recruitment of claudins to more basal locations. Thus, how does angulin-1 regulate barrier-forming claudins independently of its binding to ZO-1? (see also previous comment)

Our observations that angulin-1Δpbm rescued the barrier function in angulin-1 KO cells and angulin-1/claudin-2 double KO cells suggest that the apicobasal extension of claudins via ZO-1 may not be essential for the angulin-1-mediated paracellular barrier at TCs. We have mentioned this idea in the Discussion in the revised manuscript (line 457-461/Word or line 467-471/pdf).

11. The localization of claudin-2 in angulin-1-KO cells rescued with the mutant lacking the pbm (Fig5B) appears apical, lateral and cytoplasmic. This is different from both the localization of claudin-2 in angulin-KO cells (where circumferential labeling is detected laterally in x-y images), and cells rescued with WT angulin-1 (where localization at TCs is detected). Can the authors explain this difference? Is claudin largely endocytosed here?

12. Although claudin-2 is detected laterally at TC in cells rescued with the chimeric construct (Fig 6 E-F-G), overall the rescue of the claudin-2 labeling with the chimeric construct is very, very different from the rescue with angulin-1 WT (Figure 2C). There is still a lot of cytoplasmic claudin-2 labeling (Fig.6G), suggesting that additional interactions of the pbm region of angulin-1 are required to re-establish the WT localization of claudin-2

As the reviewer pointed out in his/her comments 11 and 12, the cytoplasmic staining of claudin-2 in part of images was remarkable in Fig. 6G, but this appeared due to technical
problem in our original experiments. We have redone the same experiments and obtained better images with less staining in the cytoplasm. As our conclusion, we cannot say significant difference in the cytoplasmic staining of claudin-2 between these cells. We have replaced these images in the revised manuscript in Figure 6B.

13. The panels in Figure 6H-I actually belong to Figure 5, which contains data about the rescue with the mutant lacking the pbm. Here, the observation that the mutant rescues the barrier phenotypes suggests that some rearrangement of barrier-forming claudins occurs, independent of ZO-1. Yet, claudin-2 localization is clearly not rescued by the mutant (Figure 5B). This is confusing, and the mechanism for the rescue of the barrier to ions remains elusive, unless one assumes that angulin-1 itself can form a barrier to ions. Indeed, the conclusion (first paragraph, page 9) that "the angulin-1 pbm is not essential for the epithelial barrier function or plasma membrane contact at TC" leaves unresolved the issue of the mechanism of anchoring of angulins. If pbm-mediated interactions are not implicated in localizing angulin-1 at tTJ, what interactions recruit it there?

As discussed in our previous responses, our observations prompted us to speculate that angulin-1 autonomously assembles at tricellular contacts and seals the extracellular space, resulting in paracellular barrier there. This means that angulin-1 itself can form a barrier to ions at tricellular contacts as discussed in the Discussion.

We agree with the reviewer about his/her comment "The panels in Figure 6H-I actually belong to Figure 5". Furthermore, additional data for revision prompted us to reorganize figures shown in the original manuscript. As a result, the barrier assay and EM images of angulin-1 KO cells expressing angulin-1 mutant lacking pbm in Figure 6H-I in the original manuscript have been moved and shown together with immunofluorescence staining as Figure 6D-E in the revised manuscript. Accordingly, the text has been moved to line 222-230/Word or line 225-234/pdf in the Results.

14. The conclusion that "the interaction between angulin-1 and ZO-1 supports the extended localization of claudin-2 along the apicobasal axis at TC" is based on evidence using the chimeric molecule. However, this experiment is complicated, because ZO-1 has many interactors, and the rescue could be due indirect interactions of ZO-1. To formally prove the implied assumption that claudin-2 interaction with the ZO-1 PDZ1 domain drives claudin-2 into lateral TCs it is necessary to compare the rescue with the chimeric constructs shown in Fig.6E with a similar construct lacking the PDZ1 domain. Otherwise, the precise mechanism remains fuzzy.
We agree with the reviewer. To confirm that the interaction between PDZ1 domain of ZO-1 and claudins supports ZO-1-mediated recruitment of claudin-2 along the apicobasal axis at TCs in a more sophisticated manner, we have established angulin-1 KO cells expressing a chimeric protein of angulin-1 mutant lacking the pbm domain but containing ZO-1 PDZ1 domain. In these cells, we found the extended distribution of claudin-2 along the apicobasal axis at TCs, supporting our conclusions. These data were added to Figure 7E-G and Figure S2D in the revised manuscript and described in the Results, lane 271-274/Word or line 276-279/pdf.

15. For completeness, it would be interesting to compare the binding of the cytoplasmic region of angulin-1 (Figure 6A) to the binding of the cytoplasmic region of JAM-A (with or without deletion of the pbm). And use afadin as a prey, in addition to ZO-1, to clarify the relevance of interaction with afadin (a ligand of JAM-A).

The reviewer’s suggestion is interesting, especially about analyses of possible interaction between angulin-1 and AJ-associated scaffold protein afadin because afadin shows extended localization along the apicobasal axis similar to angulin-1 as shown in Figure 3D. However, molecular interaction between angulin-1, JAM-A, ZO-1 and afadin appears complicated. ZO-1 binds to the C-terminus of JAM-A through its PDZ3 domain (Itoh et al., J Cell Biol 147:1351–1363, 1999) and to the C-terminus of angulin-1 through its PDZ2 domain. The C-terminus of JAM-A binds to the PDZ domain of afadin (Monteiro et al., Mol Biol Cell 24:2849-2860, 2013), and ZO-1 also binds to afadin (Ooshio et al., J Biol Chem 285:5003-5012, 2010). Because a lot of experiments will be needed to clarify the mechanism behind the TC localization of afadin, we would like to leave this issue for the next study, which is ongoing in our lab.

16. Figure 7 addresses the role of JAM-A, which reinforces the concept that the expression and localization of JAM-A in angulin-1-KO cells must be shown. The results shown in Figure 7A-B are puzzling. If angulin-1 is responsible for lateral-basal TC, why are these still detected at the level of desmosomes when few (or none) lateral central sealing elements are visible by immunofluorescence of angulin-1? Is angulin-2 upregulated under these conditions? Could angulin-2 be implicated in mediating the formation of TCs at the desmosome level?
We have shown the behavior of JAM-A in angulin-1 KO cells in immunofluorescence, in which we could not remarkable change (Figure S2E).

We have already discussed the discrepancy between lack of basal extension of angulin-1 in immunofluorescence and the plasma membrane contacts at TCs in Desmosome level in EM in the Discussion, line 446-452/Word or line 456-462/pdf. Due to technical problems in EM, we cannot evaluate the real depth of the Desmosome level, but we speculate that the plasma membrane contacts with desmosome in original Figure 7AB is just beneath of the apical region.

We do not think that angulin-2 is upregulated and is involved in tTJ formation only in claudin quinKO cells, but we cannot confirm that because of the lack of a characterized antibody that recognizes dog angulin-2. Instead, we knocked out angulin-1 in claudin quinKO cells and confirmed that the obtained cells have gaps at tricellular contacts and leakier barrier against the flux of FITC-dextran with 150 kDa. The data, which have been presented in Figure 8 C-E and described in the Results, line 294-300/Word or line 301-306/pdf, in the revised manuscript, suggest that angulin-1 is responsible for the plasma membrane contacts at TCs in claudin quinKO cells.

17. Figure 7C-D. The authors claim that upon KO of 5 claudins plus KO of JAM-A "angulin-1 was localized at the only apical part of TCs". I do not agree. In x-y images, there is clear labeling for angulin-1 along the lateral borders, except that it is not clustered at TC but distributed uniformly along tricellular and bicellular contacts. Is this because angulin-1 takes the space left "available" by JAM-A? What is the relative localization of angulin-1 and JAM-A in WT cells, and in cells KO for one or the other?

We thoroughly repeated immunofluorescence staining of claudin quinKO cells and claudin/JAM-A KO cells. As a result, angulin-1 signals along the lateral membrane in these cells were comparable with MDCK II cells. However, bicellular angulin-1 signals at the apical region in claudin quinKO cells and claudin/JAM-A KO cells were stronger than those in MDCK II cells. Moreover, this angulin-1 localization was more remarkable in claudin/JAM-A KO cells than claudin quinKO cells, supporting the possibility that angulin-1 and JAM-A compete the space for their localization as the reviewer pointed out. To address this possibility, we analyzed not only the localization of angulin-1 in MDCK cells and JAM-A KO cells but also the localization of JAM-A in MDCK cells and angulin-1 KO cells by immunofluorescence, but we could not find any evidence for significant interaction between angulin-1 and JAM-A. In accordance with these additional experiments, we have replaced Figure 7C-D in the original manuscript with Figure 8A and F in the revised manuscript.
Furthermore, we have presented the localization of angulin-1 in JAM-A KO cells and the localization of JAM-A in angulin-1 KO cells in Figure S2D-E. These results have been described in the Results, line 303-312/Word or line 309-319/pdf.

At this moment, we would like to avoid further discussion of the mechanism of bicellular localization of angulin-1 in claudin quinKO cells or claudin/JAM-A KO cells because we have no clear idea to explain this. Instead, we have discussed similarity of this phenomenon with the case in Drosophila, in which loss of septate junction integrity permits tricellular junction components to spread to bicellular contacts, in the Discussion, line 484-498/Word or line 495-509/pdf.

18. EM shows that TCs are still present at the DS level in cells KO for 5 claudins and JAM-A (Figure 7D). This suggests, again, either that IF localization of angulin-1 at lateral TC is not significantly correlated with TCs detected by EM, or that, if angulin-1 is not involved, redundant molecules (angulin-2?) mediate the formation of TC in penta-claudin-JAM-KO cells. This cannot be tricellulin, since if angulin-1 recruits tricellulin (Figure 2b) and angulin-1 is not localized at lateral TC, as claimed by the authors, then tricellulin cannot be at lateral TC.

Our response is same as that to the comment 16.

19. The EM phenotypes described in Figure 8E-F are largely anecdotal, and as noted by the authors, the conclusion that “tricellulin is required for the organization of tTJs by connecting short strands to the central sealing elements” is far from being justified in the absence of quantitative data and further investigations by super-resolution microscopy and freeze-fracture immunolabeling.

We performed quantification of the number of short TJ strands connected to a central sealing element in the whole freeze-fracture replica images which we obtained. We have added the data in Figure S5G in the revised manuscript.

Additional issues

1. The labeling of immunofluorescent panels should be improved. For example, Figure 8C are tricellulin-KO cells, but it is not made explicit by the labeling. It's confusing.
Thank you for pointing out our mistake. We have added the labeling of “Tricellulin KO cells” to the immunofluorescent panels in Figure 9C.

2. The images in Figure 8F are insufficiently labelled and not at a sufficiently high magnification to make it clear what the authors mean by "caving-in" of TJ strands.

We appreciate the author’s suggestion. We have added a schema that traces TJ strands and central sealing elements with "caving-in" in Figure 9H3 in the revised manuscript.

3. The results reported here are in disagreement with a previously reported role of tricellulin in controlling TER (Ikenouchi 2005), which requires clarification (beyond “different cell types”).

Although the reviewer’s comment is reasonable, so far we do not have any idea to mechanically explain the discrepancy in the impact of tricellulin in epithelial barrier function between tricellulin KO MDCK II cells in this study and tricellulin knockdown Eph4 cells in the previous study. On the other hand, we noticed a previous description that the impact of the loss of tricellulin in the tTJ structure in freeze-fracture replica differs between hair cells in utricular macula and hair cells in the organ of Corti in TricR497X/R497X mice (Nayak et al. 2013). This suggests that the role of tricellulin may differ depending epithelial cell types. In the revised manuscript, we have referred to this observation (line 535-538/Word or line 546-549/pdf).

In addition to the revision in accordance with the reviewer’s comments, we have further modified our manuscript regarding the following points.
1. During additional EM analyses of horizontal ultrathin sections of TCs, we obtained better images than the original ones. In Figure 5A in the revised manuscript, we would like to replace the original two images of the TJ level in angulin-1 KO cells with new ones containing clear lipid bilayers and narrower gaps, which are more consistent with the corresponding freeze-fracture replica images.
2. Although polyuria and polydipsia phenotype was reported in angulin-1-deficient mice, recent studies by other group could not reproduce the similar results in another strain of angulin-1-deficient mice. We have added this information to the Introduction. (line 81-82)
3. Very recently, the role of tricellulin and occludin in the complexity of TJ strands has been reported. We have referred to this publication. (line 512-514/Word or line 523-525/pdf)
4. Recently, mutations in angulin-1/LSR gene have been reported in patients of infantile intrahepatic cholestasis. We have referred to these reports. (line 552-554/Word or line 564-566/pdf)

5. There are many other small modifications for correctness or clarity of the manuscript without changing the meaning of the original manuscripts. These are shown in red letters in the revised manuscript.

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

Tricellular tight junctions (tTJs) seal the paracellular space at cell vertices in epithelia and are essential for epithelial barrier function. Tricellular junctions were first described in the 1970s, but their molecular organization and their functions in normal physiology and disease are only beginning to be understood and have recently attracted growing interest of cell biologists. In mammalian cells, tTJs comprise the four-transmembrane domain protein tricellulin and immunoglobulin domain transmembrane proteins of the angulin family. Although previous work had shown that angulins recruit tricellulin to tTJs, the specific functions of angulins and of tricellulin in tTJ formation, and their respective contributions to tTJ structure have not been clear.

Here Sugawara et al. describe the roles of angulin-1 and tricellulin in tTJ formation in MDCK cells using state-of-the-art genome editing, light and electron microscopy, biochemistry, and transepithelial electrical resistance (TER) measurements. They investigated the precise localization of angulin-1 and tricellulin and their interactions with claudins and with the TJ scaffold protein ZO-1. They show that TJ proteins (claudin-2, occludin, ZO-1) colocalize with tTJ proteins at basolateral extensions along vertices, and that the formation of these vertical extensions is dependent on angulin-1. Using both transmission and freeze-fracture electron microscopy the authors demonstrate that angulin-1 is required for the normal structure of the plasma membrane contacts and central sealing elements at cell vertices. Furthermore, they show that angulin-1 is required for normal transepithelial resistance and for limiting paracellular flux in claudin-2-deficient MDCK cells. They go on to show that angulin-1 binds to ZO-1 and that this interaction is required for the formation of basolateral tTJ extensions, but not for paracellular barrier function. Strikingly, the plasma membrane contact at vertices still forms in claudin-1, -2, -3, -4, -7 quintuple knockout cells lacking bicellular tight junction strands, as well as in cells additionally lacking the junctional adhesion molecular JAM-A, indicating that tTJs can assemble independently of bicellular TJs. Finally, they show that Tricellulin is required for the normal structure of tTJs with basolaterally extending TJ strands,
but not for the formation of central sealing elements, and, most surprisingly, not for paracellular barrier function. This last finding is in discrepancy to earlier results from functional studies (e.g., Ikenouchi et al. 2005; Krug et al. 2009) that had suggested an essential role of tricellulin for barrier function. However, at the same time, the finding is consistent with, and may help explain, the subtle defects observed in Tricellulin knockout mice, as compared to the severe defects and embryonic lethality of angulin-1 knockout mice.

The study by Sugawara et al. provides important new insights into the molecular organization of tTJs and the interactions of tTJ proteins with bicellular TJ components, and identifies distinct roles of the two known tTJ components, angulin-1 and tricellulin, in tTJ assembly and barrier function. The results are convincing, of very high scientific quality and technical standard, and the text is very well written. However, the analysis of protein localization at TCs, as a central part of the paper, is entirely qualitative and needs to be quantified (see below).

Overall, this a very interesting study that clearly provides an important conceptual advance, with significant implications for epithelial biology in many different contexts and model systems. The work should therefore be of interest to the broad audience of readers of JCB. The authors should address the following (minor) points before the work should be accepted for publication:

We are glad that the reviewer highly evaluates the significance of our study. The major criticism raised by the reviewer is the lack of quantification in morphological data. We will perform quantitative analyses and show the results in the revised manuscript.

- The study would clearly benefit from a quantitative analysis of the immunofluorescence images. The length of the basolateral extensions of TJs along cell vertices is presented essentially as a binary trait ("extended" or "not extended"). Careful quantification of the length of the tTJ extensions, as well as of the enrichment of angulin-1 and tricellulin at apical and apico-lateral TCs would be informative.

In accordance with the reviewer's comment, we performed the quantitative analysis in Fig. 4D, Fig. 6C, Fig. 7G, and Fig. 9D and E. For quantification of extended distribution of TJ or tTJ proteins along apicobasal axis at TCs, confocal sections along the Z-axis were obtained from each cell line. For each TC, the section of 1.2 µm or 1.7 µm basal from the section that contained the strongest signal of TJ markers was selected. Then, fluorescence signals of TJ
or tTJ proteins at the TC and those at a close bicellular junction were measured by line scan. When the maximum signal at the TC was 4-fold higher than that at the bicellular junction, we considered that the TJ or tTJ protein shows extended distribution along the apicobasal axis at the TC. By these measurements throughout a field of view, the ratio of the extended distribution was calculated. The data from three fields of view were analyzed.

• Besides the length of the basolateral extensions of tTJs, the distribution of angulin-1 or tricellulin between TCs and bicellular contacts appears to change in some of the genotypes shown. For instance, angulin-1 appears to spread into bicellular contacts in Claudin/JAM-A knockout cells (Fig. 7C; see also Fig. 7A, Fig. 8C). The authors should analyze the enrichment of angulin-1 at TCs compared to bicellular contacts, and discuss the results.

As the reviewer pointed out, angulin-1 spreads into bicellular contacts in Claudin quin KO cells and more remarkably in Claudin/JAM-A KO cells. Additional experiments provided some data related to this issue, but we still have no good idea to convincingly explain the mechanism of angulin-1 localization at bicellular contacts in these cells. We would like revised the manuscript as follows.

We have described the spread of angulin-1 into bicellular contacts in Claudin quinKO cells (line 287-289/Word or line 294-296/pdf) and in Claudin/JAM-A KO cells (line 303-305/Word or line 309-311/pdf) in the Results. Further western blotting analyses revealed that both claudin quinKO cells and claudin/JAM-A KO cells upregulate the angulin-1 level by unknown reasons, which might possibly lead to the leakage of angulin-1 from TCs to bicellular contacts. We have described these observations in Results (line 305-307/Word or line 312-314/pdf) and shown the data in Fig. S2G. On the other hand, loss of TJs in claudin may trigger the spread of angulin-1 into bicellular contacts by unknown reasons similar to the leakage of TCJ proteins into bicellular contacts in septate junction impaired fruitfly. We have discussed this issue in Discussion (line 484-498/Word or line 495-509/pdf). Stronger bicellular angulin-1 signals in claudin/JAM-A KO cells than in Claudin quinKO cells suggested a possibility that JAM-A and angulin-1 compete with each other in their bicellular localization at the apical region. However, this idea was rejected because we could not observe any bicellular spread of angulin-1 in JAM-A KO MDCK II cells. We have described these results in Results (line 307-312/Word or line 314-319/pdf) and shown the data in Fig. S2E-F.

• Figure 1: the overall structure of the tissue, the shape of the cells and the organization of junctions around the TCs are difficult to apprehend. Several of the Tricellulin signals do not
appear to be bordered by junctions from three cells. Highlighting the outlines of cells would be helpful here. Overall, this figure does not seem to contribute much to the paper. Why did the authors choose to examine kidney tubules and collecting ducts, rather than planar epithelia (e.g. cochlea)?

As the reviewer pointed out, in Figure 1A and B, the shape of cells is not clear because of the lack of background staining, while other images contain the overall structure of nephron segments by specific or background staining. Instead of highlighting the outline of cells, we have explained that the junctional staining are detected in the lumen side of epithelial tubes of the collecting ducts in the text in the Results (line 108-109/Word or line 109-110/pdf). Furthermore, in accordance of the reviewer’s suggestion, we have added the data of immunofluorescence staining of tTJ and TJ proteins in the small intestine as planar epithelia (Figure 1E-H). The reason why we chose kidney was that we can show the variation in the length of tricellular tight junctions in one organ. However, because of space limitation, we moved the IF images of the proximal tubules to Figure S1A and B. As the reviewer pointed out, part of tTJ marker signals do not appear to be bordered by junctions from three cells, but this is due to technical limitations. It is not easy to find tTJs that perfectly include three branches of bicellular TJs and tTJs within a 5 µm sections.

• The authors should indicate the sample size (n) also for the electron microscopy data. Are apical (TJ level) and basolateral (DS level) sections for each genotype taken from the same cell or from different cells?

We obtained dozens of EM images for horizontal ultrathin sections for each cell type and we are confident about our conclusions. However, a convincing quantitative analysis of the intercellular gap at TCs is not easy for EM images. We performed additional EM observations and selected the images containing TCs with three plasma membranes of clear lipid bilayers. From these images, we counted open or closed TCs at the TJ or AJC level based on our definition, which have been described in Materials and Methods, line 718-725/Word or line 733-740/pdf, and described the results in the legend for each figure. All the EM images are obtained from different cells. We described this in the figure legend in the revised manuscript.

• p. 10: The statement that "ZO-1 was mostly colocalized with angulin-1 along the
apicobasal axis at TCs, but was infrequently missing from some angulin rods" needs to be substantiated with numbers (sample size, penetrance of the phenotype).

In accordance with the reviewer's comment, we quantified colocalization of ZO-1 with angulin-1 rods in Tricellulin KO cells in Figure 9E in the revised manuscript.

• p. 10, Fig. 8E: "Horizontal ultrathin sections at the TJ level revealed that tricellulin KO cells contained either the plasma membrane contact or a gap at TCs (Fig. 8 E)." : indicate sample size and proportions of the two phenotypic classes („contact“ vs. „gap“).

Similar to our previous response, we have indicated the number of open and closed TCs at the TJ level of tricellulin KO cells we observed in the figure legend.

• On p. 14 the authors state that "The present observations indicate that the basic mechanisms for bicellular TJ formation and tTJ formation are independent of one another, but are coordinately organized in epithelial cells to form a functional paracellular barrier". They may consider discussing this interesting finding in the light of results from Drosophila, where bicellular and tricellular occluding junctions were also found to be assembled independently (Byri et al. Developmental Cell 2015).

We thank the reviewer to provide a chance to discuss this issue. Comparison between the vertebrate TJ-tTJ system and the invertebrate septate junctions-tricellular junctions system is of great interest. We have discussed this issue in the revised manuscript (line 488-498/Word or line 499-509/pdf).

• p. 9: check wording: "Angulin-1 was localized at the only apical part of TCs"

We have modified this sentence to "In claudin/JAM-A KO cells, angulin-1 signals were clearly detected at TCs as well as bicellular contacts at the level of apical junctions on immunofluorescence staining" in accordance with reviewer #1’s comment (line 303-304/Word or line 309-311/pdf).

• p. 9: check wording: "which appeared to the apical cell-cell junction level"

We have modified the original sentence "the plasma membrane contact at TCs was clearly observed in sections containing parallel plasma membranes with a narrow space, which
appeared to the apical cell-cell junction level” to "the plasma membrane contact at TCs was clearly observed in sections of the apical cell-cell junction level containing parallel plasma membranes with a narrow space” (line 313-315/Word or line 320-322/pdf).

• p. 14: missing word: “It will interesting to examine...”

"It will interesting to examine...“ have been corrected to ""It will BE interesting to examine...“.

In addition to the revision in accordance with the reviewer’s comments, we have further modified our manuscript regarding the following points.

1. During additional EM analyses of horizontal ultrathin sections of TCs, we obtained better images than the original ones. In Figure 5A in the revised manuscript, we would like to replace the original two images of the TJ level in angulin-1 KO cells with new ones containing clear lipid bilayers and narrower gaps, which are more consistent with the corresponding freeze-fracture replica images.

2. Although polyuria and polydipsia phenotype was reported in angulin-1-deficient mice, recent studies by other group could not reproduce the similar results in another strain of angulin-1-deficient mice. We have added this information to the Introduction. (line 81-82)

3. Very recently, the role of tricellulin and occludin in the complexity of TJ strands has been reported. We have referred to this publication. (line 512-514/Word or line 523-525/pdf)

4. Recently, mutations in angulin-1/LSR gene have been reported in patients of infantile intrahepatic cholestasis. We have referred to these reports. (line 552-554/Word or line 564-566/pdf)

5. There are many other small modifications for correctness or clarity of the manuscript without changing the meaning of the original manuscripts. These are shown in red letters in the revised manuscript.
May 25, 2021

RE: JCB Manuscript #202005062R

Dr. Mikio Furuse  
National Institute for Physiological Sciences  
Division of Cell Structure  
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Okazaki, Aichi 444-8787  
Japan

Dear Dr. Furuse:

Thank you for submitting your revised manuscript entitled "Angulin-1 seals tricellular contacts independently of tricellulin and claudins". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

The Reviewers have pointed out some typographical errors and places where Figure labeling should be added, and where clarification of Figure panels would be helpful. I do not think additional quantification is required, and do not think you need to shorten the Discussion. I would like you to briefly address in the text your reasoning for using a binary classification, as suggested by Reviewer 2. I do not think Fig 1A needs to be deleted, but perhaps a clearer explanation would address the issue raised by Reviewer 2. This should not require further external review.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure
legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

The following title is suggested:
Angulin-1 seals tricellular tight junctions independently of tricellulin and claudins

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
   a. Make and model of microscope
   b. Type, magnification, and numerical aperture of the objective lenses
   c. Temperature
   d. Imaging medium
   e. Fluorochromes
   f. Camera make and model
   g. Acquisition software
   h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstructions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements
regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

**It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.**

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

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

The authors have satisfactorily addressed most of my comments, and provided improved data, quantifications, and a substantial amount of new data. The Discussion should be shortened. Micrographs in Fig. 9G should be labelled. Supplementary data shown along Fig. 8 should be shown if possible in a Fig. S6 rather than Fig. S2, to avoid going back to an earlier Supl. Figure.

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

The authors have substantially revised the manuscript. They added new experimental data, including a detailed characterization of the interaction of Angulin-1 with ZO1. They also analyzed the localization of adherens junction and tight junction proteins at tricellular contacts, where components of the two types of junctions (surprisingly) appear to be intermingled. Overall, the new data and text revisions significantly strengthen the manuscript. I find the revised work suitable for publication in JCB, given that the authors address the remaining points listed below.

Most of my previous comments were addressed satisfactorily. The authors added quantitative data describing the basolateral extensions of TJs along cell vertices. However, surprisingly, this phenotype is still presented as a binary trait. Quantification of the length of the extensions would appear more appropriate here and should be technically feasible. However, if the authors cannot provide this kind of analysis, they need to explain the rationale for using a binary classification ("extended" or "not extended"), rather than analyzing junction length. Also, the description of this analysis in the Methods section is not clear:

Line 709, 714: "By these measurements throughout a field of view, the ratio of the extended distribution was calculated". It is not clear what the authors mean by "the ratio of the extended distribution". Please clarify.

As pointed out before, the images in Figure 1A (sections of renal collecting duct) are still confusing, as the overall structure and orientation of the tissue, the shape of the cells and the organization of bicellular junctions around tricellular junctions are not clear. Authors state in their rebuttal "The reason why we chose kidney was that we can show the variation in the length of tricellular tight junctions in one organ". I am not compelled by this argument, as the variation in the morphology and dimensions of junctions in these images is likely due to the irregular cell shapes and the plane of section. A 3-D reconstruction of the junctions encompassing an entire cell would be necessary to support this point. If more convincing images cannot be provided, Fig. 1A should be omitted from the paper. The images of small intestine (Fig. 1B) support the authors’ argument more convincingly. However, the orientation of cells and tissue (apical/basal; lumen) should be indicated in the images. Same for Fig. S1A.
Minor comments:
Figure 1A: The labels for anti-aquaporin-2 (AQP2) staining should read "AQP2", not "AOP2".
Line 1167 (legend to Figure 5): sample size is now stated, but please define what "n" is referring to: number of junctions, number of cells in the same specimen, or independent specimens?
Line 127: The statement "Angulin-1 was also diffusely distributed to the lateral membrane" is misleading. Signals at bicellular contacts are lower compared to TCs, but the term "diffusely" is suggestive of a mechanism that is not addressed here.
Line 131: "JAM-A were localized..." : was
Line 495: "Continuous bicellular TJs retained in angulin-1 KO cells, while angulin-1 sealed TCs...": "were" is missing
Fig. S2B: typo "Anguiln"