JCB

Rac1 promotes kidney collecting duct integrity by limiting actomyosin activity

Fabian Bock, Bertha Elias, Xinyu Dong, Diptiben Parekh, Glenda Mernaugh, Olga Viquez, Anjana Hassan, Venkateswara Amara, Jiageng Liu, Kyle Brown, Andrew Terker, Manuel Chiusa, Leslie Gewin, Agnes Fogo, Cord Brakebusch, Ambra Pozzi, and Roy Zent

Corresponding Author(s): Roy Zent, Vanderbilt University Medical Center

Review Timeline:

Submission Date:	2021-04-02
Editorial Decision:	2021-04-27
Revision Received:	2021-07-27
Editorial Decision:	2021-08-24
Revision Received:	2021-08-29

Monitoring Editor: Kenneth Yamada

Scientific Editor: Lucia Morgado-Palacin

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202103080

April 27, 2021

Re: JCB manuscript #202103080

Prof. Roy Zent
Vanderbilt University Medical Center
Medicine
Room C3210 MCN
Nashville, TN 37232

Dear Roy,

Thank you for submitting your manuscript entitled "Rac1 promotes kidney collecting duct integrity by limiting actomyosin activity" to the Journal of Cell Biology. The manuscript has now been assessed by three expert reviewers, whose reports are appended below. As you can see from the reviews provided by three leaders in the various overlapping research areas spanning the elements of this paper, there was potential interest in the conclusions, although the level of enthusiasm was mixed because one reviewer questioned its level of novelty. After a careful assessment of the reviewer feedback, we invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Two reviewers asked for in vivo data concerning myosin and p-MLC, and the concern about the immunostaining for Rac would need to be resolved. There were suggestions for further in vivo descriptive characterizations that would raise the level of interest in this in vivo evaluation of Rac roles, e.g., effects on laminin and cell shape. These reviewers provide various other suggestions and questions that we ask you to consider carefully. Overall, we encourage you to provide as much additional characterization as is practical for you within a reasonable revision period. The resubmitted manuscript will be returned to the reviewers for re-evaluation as they have requested, though the final decision about acceptability for publication will be made at the senior editor level if there are remaining reservations.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article 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.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for your interest in the Journal of Cell Biology and look forward to receiving a revised manuscript.

With kind regards,

Ken

Kenneth M. Yamada, MD, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

In the manuscript by Bock et al, "Rac1 promotes kidney collecting duct integrity by limiting actomyosin activity," the authors report that Rac1 does not play a major role in early branching morphogenesis of kidney collecting ducts, which the authors attribute to lack of Rac expression in ureteric buds during early kidney development. They show that Rac1 is required to maintain epithelial integrity, polarity, and physiologic function (i.e. urinary concentrating ability) in adult kidneys. Overall the manuscript was well written, experiments were well-conducted, appropriate

controls were performed, and the statistical analysis was acceptable. These results advance the field because they examine the function of Rac in tissue in vivo and in primary cells, which is in contrast to many studies that have used cell lines. In the Rac knockout animals, they show a dramatic decrease in E-cadherin and other junctional components and change in the distribution of proteins that are normally enriched in the apical compartment. (e.g. ezrin). They also demonstrate that this occurs via Wave2 and Arp2/3, which causes increase actomyosin contractility, thereby providing mechanistic insight into the signaling pathways controlled by Rac in these cells. The discussion section clearly delineates which of their findings are novel and which have already been observed in established cell lines.

A few comments to improve the manuscript:

1) Immunostaining for Rac was performed with a mouse antibody on mouse tissue. Single cell RNAseq datasets demonstrate it is expressed by ureteric bud during development (and has similar levels of expression in various tubule subtypes), yet the immunofluorescent staining does not indicate this. Please provide immunofluorescent experiments in the control and Rac f/f; HoxB7-cre knockout tissue (neonatal and adult) to validate the antibody and Rac1 localization.
2) Please address the fact that mutant kidneys are smaller, yet the authors report no change in morphogenesis and branching. What do they think the reason is?
3) Can they visualize changes in myosin or p-MLC in vivo in tissue sections in knockout animals compared to controls? What about phospho-Pak and phospho-cofilin? Do the results parallel the biochemical data from cultured collecting duct cells?

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

JCB 202103080 Bock....Zent
The authors selectively knocked out Rac1 from the ureteric bud (UB) starting at E10.5, when the UB starts to form. There was a mild defect in branching during development of the UB, which becomes the collecting duct (CD) of the mouse kidney. However, in older adult mice (6 months), they found progressive degeneration of the CD epithelium, with flattened cells, multilayering, and a urine concentration defect. (It is not clear if these defects resemble any known human disease.) To investigate the underlying mechanism, the authors turned to culture of the Rac1 knocked-out CD cells. By 6 months, there was a loss of E-cadherin at the junctions, a major epithelial polarity defect, and loss of tubulogenesis in 3D Matrigel culture. The cultured CD cells failed to form focal adhesions or adhere and spread normally. In culture there was again a loss of polarization and disorganization of the cytoskeleton. This was apparently independent of Pak1, which is a major downstream effector of Rac1. Instead, there was a defect in the WAVE2-Arp2/3 machinery for nucleating actin polymerization and lamellipodia formation. Myosin was overactivated and inhibition of myosin II by ML-7 or blebbistatin could partially rescue the defects caused by Rac1 KO.

Overall, this paper is interesting, well-presented and appropriate for JCB, and the data are largely convincing. There are a few points that I suggest should be addressed:

1. Loss of Rac1 function in polarized epithelial cells has previously been shown to affect laminin organization. See cited papers by O'Brien and Yu. Can they see if laminin organization is affected?

2. Figure 2 panel B. Why are there two images? What is the difference? Please rewrite to clarify.

3. Figure 3 panel D. In the Hoxb7, the cilia seem to be very short, but not absent.

4. Figure 6. Formally, this figure seems to show that global stimulation of Pak1 is not sufficient to rescue. This is not equivalent to showing that Pak1 is not involved. Please rewrite to clarify.

5. Figure 6. FTY720 is described as a Pak1 inducer. This is not correct. The drug activates a sphingosine receptor, which probably does many things, of which activation of Pak1 is only one. Please rewrite to clarify.

6. Figure 7 panel G. Can they obtain statistics on the difference between the two conditions?

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

The authors used targeted gene deletion in mice to investigate the role of Rac1 in the formation, maintenance, and function of the kidney collecting duct. Mutant mice have fewer, more dilated collecting ducts with impaired physiology along with eventual loss of cell polarity, loss of E-cadherin, and fibrosis. Primary cultures of collecting duct cells lacking Rac1 show defects in actin assembly, especially of those actin networks derived through Arp2/3. This results in an increase in myosin contractility in cells in culture, and the authors present a model in which unchecked myosin II contractility is largely responsible for the various phenotypes seen in the Rac1 knockout.

The issue is novelty. For example, showing that Rac1 is important for actin assembly and lamellipodia formation is consistent with the established view. The antagonistic relationship between Rac and myosin II contractility is also known from previous work. Therefore, the impact of the current study is limited to kidney physiology but does not extend to fundamental cell biology. A deeper, more extensive characterization of Rac1 knockout phenotypes in vivo could lead to interesting conclusions or hypotheses, but that might be a long ways away. The results as of now are somewhat descriptive and confirmatory of what is already known using other systems.

Specific comments:

The authors demonstrate an important role for Rac1 in actin assembly in cultured cells (figure 8). Is the same true in vivo? Quantitative imaging should be done to assess the effect of Rac1 knockout on actin polymer mass in apical vs lateral vs basal membranes in the collecting duct.

The authors also show an increase in myosin II activity in Rac1 knockout cells in culture. Is the same true in vivo? Quantitative imaging in the collecting duct should be used to compare the ratio of myosin II to actin (e.g., phalloidin stain) in the collecting duct. This experiment might work because much of the soluble myosin is extracted from cells following permeabilization. In addition, the authors should perform quantitative imaging to assess the levels of phosphorylated myosin in the collecting duct as a function of Rac1.

Recent work has implicated actin polymerization as necessary for maintaining cell-cell adhesion and stabilization of lateral membranes (PMID: 28630144, PMID: 29507127, PMID: 31871203). Is the same true in vivo? Quantitative imaging of membrane markers might reveal if the lateral membranes remain in contact or if they detach.

Extensive electron microscopy over the decades revealed numerous protrusions/invaginations

along lateral membranes. See, for example, figure 5 in PMID:3084501, figure 15 in PMID: 9502381, figures 11 and 13 in PMCID: PMC1712231. More recent work also showed such protrusions in cultured cells (PMID: 31871203). Are such protrusions evident in the collecting duct, and if so, are they Rac1 dependent as might be predicted?

Does Rac1 affect cell dimensions? Looking at the E-cadherin staining in figure 3A, I count roughly 20 cells surrounding the lumen in the WT conditions but ~30 cells in the Rac1 KO. Does this hold up over more extensive analysis? It would be interesting to count the number of cells per lumen and also cell number normalized to lumen size.

Also regarding figure 3A, is there a difference in cell height? WT cells appear to be more columnar than the Rac1 knockout cells. Does that hold up over more extensive quantification? If there are Rac-1 dependent differences in cell height, it would be interesting to relate these observations, experimentally, to current ideas on molecular mechanisms controlling the relative proportions of lateral membranes to apical and basal (e.g., PMID: 26585313, PMID: 28357057, PMID: 31723006).

Reviewer 1:

Comment 1: Immunostaining for Rac1 was performed with a mouse antibody on mouse tissue. Single cell RNAseq datasets demonstrate it is expressed by ureteric bud during development (and has similar levels of expression in various tubule subtypes), yet the immunofluorescent staining does not indicate this. Please provide immunofluorescent experiments in the control and Rac f/f; HoxB7-cre knockout tissue (neonatal and adult) to validate the antibody and Rac1 localization.

Response: This is a valid point. To further test the antibody, we have performed a full histological spatiotemporal assessment of Rac1 using control and mutant mouse tissue of different embryonic and adult stages. These were immuno-stained for Rac1 combined with the ureteric bud/collecting duct marker DBA and analyzed by confocal microscopy. On a protein level we again find that Rac1 is not expressed until the newborn stage explaining the absence of a severe branching morphogenesis defect in conditional knockout mice (Hoxb7:Rac1f/f). Rac1 staining in DBA-positive ducts of the knockout mice was absent at all stages. This is the new supplementary figure 1. Interestingly, these studies revealed that the intracellular location of Rac1 changes over time from predominantly basal in newborns to more apical in aged adults. Rac1 is known to be differentially located and activated along the apicobasal axis which allows dynamic age-dependent functional specialization of actin cytoskeletal protrusions along the lateral membrane and maintenance of epithelial cell shape to prevent mesenchymalization (Couto et al., 2017; Yagi et al., 2012). Consequently, we believe that in the renal epithelium these distinct Rac1 patterns are critically important to dynamically control the integrity of the lateral membrane and thus cell shape and function as outlined in our experimental work addressing the comments by reviewer 3 and incorporated into new Figure 9.

Additionally, we are aware that available transcript-level data (such as RNAseq) suggest that Rac1 is expressed in the UB. However, mRNA can be a poor proxy for final protein abundance (e.g. due to cell cycle variations or asynchronous protein-RNA relationships) which was extensively reviewed elsewhere recently (Edfors et al., 2016; Liu et al., 2016). Specifically, highly dynamic non-steady state biological processes such as vertebrate embryonic development produce poor correlations between mRNA and protein levels unless extensive mathematical corrections are employed (Peshkin et al., 2015). Moreover, even in the aging kidney transcriptomic and proteomic profiling has revealed that functionally important biological changes on the protein level occur in the absence of corresponding mRNA changes (Takemon et al., 2021). For these reasons we decided to focus on protein levels instead of transcripts when assessing Rac1.

Comment 2: Please address the fact that mutant kidneys are smaller, yet the authors report no change in morphogenesis and branching. What do they think the reason is?

Response: This is an important point. Based on previous literature regarding the role of Cdc42 (the other major small Rho GTPase controlling epithelial branching) in kidney branching morphogenesis as well as the known role of Rac1 in epithelial branching we hypothesized that Rac1 would be essential for branching and that the Hoxb7:Rac1$^{f/f}$ mice would develop a catastrophic or lethal developmental phenotype. Hence, we were surprised that kidneys formed and matured well into adulthood. However, a mild branching morphogenesis defect exists. We delineate our reasoning and interpretation of the data in the result section for Figure 2:

"*The relatively mild branching morphogenesis phenotype of the Hoxb7:Rac1$^{f/f}$ was surprising as Rac1 has been shown to play a critical role in epithelial cell polarization (Lof-Ohlin et al., 2017; O'Brien et al., 2001; Yu et al., 2005). We therefore investigated when the phenotype first became evident and the mechanism underlying it. Kidneys from E13 and E15.5 Hoxb7:Rac1$^{f/f}$ mice had no obvious developmental abnormalities, when compared to Rac1$^{f/f}$ controls (Fig. 2A). However, a subtle branching defect was seen at E18.5 (fewer and more dilated papillary ducts) that worsened over time and became clearly visible in newborn mice (fewer and more dilated CDs) (Fig. 2A). This late mild branching phenotype suggested that Rac1 plays a role in regulating advanced stages of UB development. To determine whether this was due to temporal Rac1 expression, we investigated its expression levels during UB development. Rac1 is undetectable in the E15.5 UB of wild type mice (Fig. 2B) but readily detectable in newborns and enriched in adult mouse and human CDs (Fig. 2, C-E). Thus, Rac1 expression only occurs in late branching morphogenesis, which likely accounts for its relatively minor role in UB development.*''

The ultimate determinants of kidney size are complex and we do not know precisely how this is controlled by Rac1 in the collecting system. During murine kidney development by E16.5 nearly 85% of branching is complete but the number of nephrons that each UB tip induces increases dramatically during late development (from E.16.5 to birth) (Cebrian et al., 2004). Thus, it is conceivable that a relatively minor contribution of Rac1 to the final stages of branching morphogenesis explains a noticeable difference in adult kidney size between WT and mutant. However, we believe that other mechanisms are at play (or may even dominate). In our manuscript we show that Rac1 maintains adult epithelial integrity. Loss of epithelial integrity in mutant mice leads to fibrosis (see Fig. 1) which eventually contributes to a decrease in functional renal tissue and organ atrophy. Additionally, we find marked abnormalities in epithelial cell shape (Fig. 9) with decreased cell height which likely contributes to smaller renal papillae in the mutants and thus a decrease in overall kidney size.

Comment 3: Can they visualize changes in myosin or p-MLC in vivo in tissue sections in knockout animals compared to controls? What about phospho-Pak and phospho-cofilin? Do the results parallel the biochemical data from cultured collecting duct cells?

Response: This is an excellent suggestion. We have dissected renal papilla (developmentally derived from the UB) of adult control (Rac1$^{f/f}$) and Hoxb7:Rac1$^{f/f}$ mice and performed immunoblotting for p-MLC and MLC. Indeed, the p-MLC/MLC ratio is increased in papillary lysates of mutant vs control mice (Fig. 8 A). Furthermore, utilizing fresh frozen renal papilla of aged (10 months) mice we were able to visualize phosphorylated myosin (p-MLC) in Rac1 deficient CD epithelium *in vivo*. Consistent with our *in vitro* findings there was increased p-MLC in Rac1 deficient epithelial cells. Compared to Rac1$^{f/f}$ mice where p-MLC was apical, p-MLC was mostly basolateral in mutant mice indicating increased actomyosin contractility along the vertical axis (Fig. 9, G and H). In agreement with the conceptual framework highlighted by reviewer 3 regarding molecular mechanisms controlling epithelial cell height this suggests that Rac1 is critical to restrict actomyosin activity along the lateral membrane. These findings likely explain the differences in *in-vivo* epithelial morphology (see experimental work addressing reviewer 3).

Similarly, we have performed immunoblotting for phospho-Pak1 and t-Pak1 of control and mutant renal papilla *in vivo* confirming the *in vitro* findings. This is now included in Figure 6 (D and E) alongside the *in vitro* data.

Reviewer 2:

Comment 1: Loss of Rac1 function in polarized epithelial cells has previously been shown to affect laminin organization. See cited papers by O'Brien and Yu. Can they see if laminin organization is affected?

Response: We agree that this is a relevant point and that the correct orientation of laminin is an important outcome of Rac1 signalling in epithelial polarization during development. To that end, we first assessed the basement membrane of adult (10 months) papillary inner medullary CDs of control and Hoxb7:Rac1$^{f/f}$ mice by performing transmission electron microscopy (TEM) (Fig. 3 H). This revealed that mutant basement membranes were severely abnormal with significant thinning indicating abnormal epithelial polarity (Fig. 3, H and I). However, when we performed confocal microscopy of fresh isolated renal papillae of aged mice that were immunostained for laminin (α1 subunit) we did not find obvious differences in basal localization between controls and mutants (Fig. 3 J). This indicates that in CDs Rac1-dependent epithelial polarity is likely not controlled by autocrine basal laminin assembly contrasting our data in mature epithelial cells with those obtained from developmental models of epithelial morphogenesis (e.g. O'Brien and Yu). Prior reports that studied the effect of Rac1 on laminin assembly utilized developmental models of epithelial morphogenesis to study the process of polarization (e.g MDCK cysts formation). We on the other hand find that Rac1 plays a minor role in renal epithelial morphogenesis in the CD during development but is a key regulator in the maintenance of adult epithelial organization and polarity. Consequently, we find a different effect of Rac1 on the ECM which underscores a novel role of Rac1 in maintaining polarity and basement membrane integrity in mature epithelial cells. This data is now included in the main manuscript in the result section and in new Fig. 3, H-J.

> *"Polarized deposition of a basement membrane is required for epithelial polarity (Morrissey and Sherwood, 2015). We next assessed the basement membrane of adult papillary inner medullary CDs of control and Hoxb7:Rac1$^{f/f}$ mice by performing transmission electron microscopy (TEM) (Fig. 3 H). This revealed that mutant basement membranes were severely abnormal with significant thinning (Fig. 3, H and I). It has been shown that during development Rac1 orients epithelial polarity via an autocrine pathway leading to basal laminin assembly (O'Brien et al., 2001; Yu et al., 2005). To test whether similar mechanism apply to the CD of adult mice we assessed laminin localization in inner medullary CDs of control and Hoxb7:Rac1$^{f/f}$ mice. There were no obvious differences in basal laminin (α1 subunit) localization between controls and mutants (Fig. 3 J) indicating that in CDs Rac1-dependent epithelial polarity is likely not controlled by autocrine basal laminin assembly"*

Comment 2: Figure 2 panel B. Why are there two images? What is the difference? Please rewrite to clarify.

Response: We have rewritten Figure 2 legend and adjusted the figure to indicate that panel B shows Rac1 (as single channel, red) on the left and the merged image (with E-cadherin, green) on the right with a focused area (indicated by the white dashed box) below.

Comment 3: Figure 3 panel D. In the Hoxb7, the cilia seem to be very short, but not absent.

Response: We have rephrased the corresponding result section to indicate that there is a ciliary defect but that they are not absent.

Comment 4: Figure 6. Formally, this figure seems to show that global stimulation of Pak1 is not sufficient to rescue. This is not equivalent to showing that Pak1 is not involved. Please rewrite to clarify.

Response: Yes, we agree. To prevent over-interpretation of the data we have re-phrased the corresponding results section as follows:

> *"These results show that global stimulation of Pak1 is unable to rescue the epithelial integrity defects of Rac1$^{-/-}$ CD epithelial cells. Thus, although Rac1 activates Pak1 in CD cells, it is unlikely to be the key Rac1 dependent regulator of CD cell integrity."*

Comment 5: Figure 6. FTY720 is described as a Pak1 inducer. This is not correct. The drug activates a sphingosine receptor, which probably does many things, of which activation of Pak1 is only one. Please rewrite to clarify.

Response: This is a valid point. FTY720 is structurally similar to S1P and we have now added the literature outlining the GTPase independent mechanisms of sphingosine-induced activating autophosphorylation of Pak1 (Bokoch et al., 1998). Furthermore, we have rephrased the corresponding result section accordingly:

> *"To define whether the abnormal phenotypes in the Rac1$^{-/-}$ CD cells were due to the inability to activate Pak1, we treated them with FTY720, a synthetic sphingosine-like analog with the ability to, among other things, directly activate Pak1 via a Rho GTPase independent mechanism (Bokoch et al., 1998; Ke et al., 2013; Liu et al., 2011)."*

Comment 6: Figure 7 panel G. Can they obtain statistics on the difference between the two conditions?

Response: Yes, we have now measured Arp2 intensity within a region of interest around the junctional area (new panel H in Fig. 7) to obtain statistics corresponding to Fig. 7 F and G.

Reviewer 3:

General Comment: The issue is novelty. For example, showing that Rac1 is important for actin assembly and lamellipodia formation is consistent with the established view. The antagonistic relationship between Rac and myosin II contractility is also known from previous work.

Response: We agree that the antagonistic relationship between Rac1 and myosin II contractility is known and there are models that show that Rac1 controls myosin activity largely via a mutual antagonism with RhoA as the major small Rho GTPase controlling myosin contractility. These mechanisms have been shown to also occur in models of developmental epithelial morphogenesis (Chauhan et al., 2011; Martin et al., 2016). However, we believe that this is not the major novelty of our study. Firstly, the fact that Rac1 is not essential for ureteric bud branching morphogenesis is surprising and shows that there is important interplay between the different Rho GTPAses during ureteric bud branching morphogenesis. Secondly, we show that Rac1 plays a critical role in the homeostasis of mature epithelial cells such as maintaining normal polarization, morphology and the consequent normal function and localization of critical transporters. This is mediated by Rac1 dependent restriction of actomyosin via Arp2/3-dependent actin cytoskeletal branching. This significantly extends prior studies that largely utilized models of developmental epithelial morphogenesis (e.g. C. elegans embryos, Drosophila or the murine lens)(Chauhan et al., 2011; Georgiou and Baum, 2010; Martin et al., 2016). We thus highlight a novel function for Rac1 in the mature kidney epithelium for which we provide a mechanistic framework that likely applies to other mature mammalian epithelial systems.

General Comment: A deeper, more extensive characterization of Rac1 knockout phenotypes in vivo could lead to interesting conclusions or hypotheses.

Response: We thank the reviewer for making us aware of certain literature that we were unaware of. We have performed extensive experimental work to show that Rac1-dependent actin dynamics at the lateral membrane control lateral membrane integrity and thus epithelial cell shape. This adds another layer of novelty (new Figure 9) and we thank this reviewer for the insights.

Comment 1: The authors demonstrate an important role for Rac1 in actin assembly in cultured cells (figure 8). Is the same true in vivo? Quantitative imaging should be done to assess the effect of Rac1 knockout on actin polymer mass in apical vs lateral vs basal membranes in the collecting duct.

Response: Assessing the effect of Rac1 on the actin cytoskeleton *in vivo* is a critical point. In addressing this we faced a few technical challenges. (1) Actin fibers are delicate and decay rapidly upon tissue harvesting thus commonly processed tissue does not readily stain positive for phalloidin. (2) Many *in vivo* fixation or tissue processing techniques, especially those using methanol or acetone, do not preserve the actin cytoskeleton well. (3) In order to visualize the cellular network and its cytoskeleton so that we could perform high resolution 3D imaging *in vivo* required us to develop methodologies that resulted in optical clearing so that deep tissue imaging was possible. (4) Many optical clearing techniques, especially those using organic

solvents, dehydrate the tissue and thus distort tissue morphology which was critical to be maximally preserved in our assessment. We circumvented these problems by performing transcardial perfusion to achieve rapid systemic delivery of a mixture of 2% glutaraldehyde (rapid fixation) and 2% paraformaldehyde (rapid penetration). We then dissected renal papilla (derived from the ureteric bud) of control ($Rac1^{f/f}$) and mutant ($Hoxb7{:}Rac1^{f/f}$) mice, performed thick cryosectioning (50µm) and phalloidin labelling followed by simple immersion aqueous-based optical clearing (refractory index matching, RIMS) and super-resolution confocal microscopy (Airy Scan) of the renal papilla. Consistent with our *in vitro* findings mutant CDs in the papilla have a severe defect in apical and lateral F-actin (Fig. 9, A-C). Additionally, we find striking abnormalities in epithelial morphology. Compared to the typical inner medullary columnar to cuboidal epithelium (as seen in control mice), mutant collecting ducts display decreased cell height and deep apical membrane invaginations reminiscent of p120-catenin deficient epithelial cells that have excess myosin contractility along the lateral membrane (see response to comments below).

Comment 2: The authors also show an increase in myosin II activity in Rac1 knockout cells in culture. Is the same true in vivo? Quantitative imaging in the collecting duct should be used to compare the ratio of myosin II to actin (e.g., phalloidin stain) in the collecting duct. This experiment might work because much of the soluble myosin is extracted from cells following permeabilization. In addition, the authors should perform quantitative imaging to assess the levels of phosphorylated myosin in the collecting duct as a function of Rac1.

Response: We have quantified the p-MLC/MLC by western blotting isolated renal papilla (inner medulla, derived from the ureteric bud). Mutant ($Hoxb7{:}Rac1^{f/f}$) have a significantly increase p-MLC/MLC ratio indicating increased actomyosin activation (Fig. 8 A). Next, utilizing fresh frozen tissue we visualized phosphorylated myosin (p-MLC) in Rac1 deficient inner medullary papillary CDs *in vivo* by immunostaining and confocal microscopy. Consistent with the immunoblot and our *in vitro* findings there was increased p-MLC in Rac1 deficient epithelial cells (Fig. 9, G and H). Compared to $Rac1^{f/f}$ control mice where p-MLC was apical, p-MLC was mostly basolateral in mutant mice indicating increased actomyosin contractility along the vertical axis (Fig. 9, G and H). In agreement with the conceptual framework highlighted by this reviewer regarding molecular mechanisms controlling epithelial cell height, this suggests that Rac1 is critical to restrict actomyosin activity along the lateral membrane likely explaining the abnormal epithelial morphology (decreased height, lateral membrane defects, apical invaginations) seen in the mutants. We further studied lateral membrane integrity as outlined below.

Comment 3: Recent work has implicated actin polymerization as necessary for maintaining cell-cell adhesion and stabilization of lateral membranes (PMID: 28630144, PMID: 29507127, PMID: 31871203). Is the same true in vivo? Quantitative imaging of membrane markers might reveal if the lateral membranes remain in contact or if they detach.

Response: To address this we performed transmission electron microscopy (TEM) of papillary inner medullary CDs of perfusion-fixed fresh kidney papillae of control ($Rac1^{f/f}$) and mutant ($Hoxb7{:}Rac1^{f/f}$) mice. As shown in new Figure 9 D and E we again note the decrease in epithelial cell height with apical invaginations. Furthermore, mutant lateral cell-cell membranes show protrusions that do not interdigitate and fail to attach (Fig. 9 F) which is consistent with defective lateral actin cytoskeletal dynamics and increased myosin contractility.

Comment 4: Extensive electron microscopy over the decades revealed numerous protrusions/invaginations along lateral membranes. See, for example, figure 5 in PMID:3084501, figure 15 in PMID: 9502381, figures 11 and 13 in PMCID: PMC1712231. More recent work also showed such protrusions in cultured cells (PMID: 31871203). Are such protrusions evident in the collecting duct, and if so, are they Rac1 dependent as might be predicted?

Response: We performed TEM *in vivo* to address this. Please see our response to the previous comment.

Comment 5: Does Rac1 affect cell dimensions? Looking at the E-cadherin staining in figure 3A, I count roughly 20 cells surrounding the lumen in the WT conditions but ~30 cells in the Rac1 KO. Does this hold up over more extensive analysis? It would be interesting to count the number of cells per lumen and also cell number normalized to lumen size.

Response: The representative images in Fig. 3A were randomly chosen ureteric bud/collecting duct cross-sections of somewhat comparable real size to allow full visualization of E-cadherin. We did count and compared additional cross-sections but did not find any difference. In fact, when we quantified the cell number normalized to lumen size in controls vs mutant papillary adult CDs *in vivo* we find the ratio to be significantly decreased due to a decrease in cell number and increase in lumen size. This is a starting point for a follow-up study which is currently ongoing. We found a striking proliferation defect upon injury in Rac1 deficient CD epithelium. Furthermore, *in vitro* and *in vivo* injury-models revealed that Rac1-dependent actin cytoskeletal dynamics are required for cell cycle regulation during CD epithelial repair. A manuscript addressing the molecular mechanism whereby the seemingly dichotomous Rac1 functions of cell cycle control and actin cytoskeletal regulation are unified in non-transformed epithelial cells to restore cell number during repair. This is currently in preparation and we believe that this is a separate aspect beyond the scope of the current study. However, we further addressed cell dimension regulation by Rac1 in experiments outlined below.

Comment 6: Also regarding figure 3A, is there a difference in cell height? WT cells appear to be more columnar than the Rac1 knockout cells. Does that hold up over more extensive quantification? If there are Rac-1 dependent differences in cell height, it would be interesting to relate these observations, experimentally, to current ideas on molecular mechanisms controlling the relative proportions of lateral membranes to apical and basal (e.g., PMID: 26585313, PMID: 28357057, PMID: 31723006).

Response: We were intrigued by the cited literature on how lateral membrane integrity directs overall epithelial morphology. We have outlined Rac1-dependent lateral membrane differences and differences in epithelial morphology above. To experimentally address whether lateral Rac1-dependent actin dynamics are involved we have performed fluorescence recovery after photobleaching (FRAP) experiments of the lateral membrane of isolated $Rac1^{f/f}$ and $Rac1^{-/-}$ CD cells live stained for F-actin (SiR actin) (Fig. 9, I and J). SiR actin has recently successfully been used in FRAP experiments by Ghokin et al. elegantly demonstrating that the actin filaments of Red Blood Cells are indeed dynamic (Gokhin et al., 2015). Given their apicobasal polarization defect $Rac1^{-/-}$ CD cells do not readily form a well polarized monolayer under standardized

conditions of a polarization assay. To circumvent this problem we plated control and mutant CD cells at high density on transwell inserts, incubated with 100nm SiR actin for 6h (a concentration known not affect actin dynamics)(Lukinavicius et al., 2014), and then imaged patches of epithelial cells that have started to form a layer and performed FRAP at the sites of initial lateral cell-cell contact formation. Our FRAP images and recovery curves demonstrate that actin dynamics at the lateral membrane are significantly impaired with severely reduced molecular diffusion kinetics in $Rac1^{-/-}$ compared to control CDs (Fig. 9, I and J). Inspired by reference PMID 28357057 by Vivian Tang we next assessed the functional relevance of impaired lateral F-actin dynamics in $Rac1^{-/-}$ CD cells for restoration of morphology by examining the rise and height of F-actin at lateral membranes in regenerating CD epithelial layers *in vitro*. For this we performed a scratch assay of confluent CD cells and imaged areas adjacent to the scratch as soon as they were covered with cells of comparable density between $Rac1^{f/f}$ controls and $Rac1^{-/-}$ mutants. This took significantly longer for mutant cells given the known migration defect (Fig. S5 D and E). The cells were then fixed and labeled with phalloidin. Confocal microscopy with z-stacking of these regenerating CD epithelial layers indicates that while control epithelial cells were able to regenerate F-actin along lateral cell-cell borders reaching full epithelial cell height, the regeneration in $Rac1^{-/-}$ CDs was incomplete with short lateral membranes, apical invaginations and cell-cell junction defects (Fig. 9, K and L). The defects were inducible with Arp2/3 inhibition in control cells and reversible with actomyosin inhibition in mutant cells indicating that Arp2/3 dependent actin branching and restriction of actomyosin are likely causally involved in this Rac1 dependent phenotype.

In Summary, this work indicates that Rac1 is a critical regulator of the lateral epithelial membrane. Rac1 maintains and restores lateral membrane integrity and lateral actin dynamics resulting in restriction of actomyosin contractility - likely via Arp2/3-branched networks - thereby promoting normal epithelial morphology. We feel that the new insights gained from these experiments add novelty to the work. These data are described in the result section, further commented on in the discussion, and the methods are outlined in greater detail in the methods section.

Results:

Rac1 controls epithelial morphology by maintaining lateral F-actin height and lateral membrane stability

Branched Arp2/3 dependent actin networks at lateral cell-cell borders are critical for normal actomyosin activity, lateral cell-cell junction stability and normal epithelial morphology (Efimova and Svitkina, 2018; Li et al., 2020). Given that Rac1 in CD cells promotes Arp2/3 branched actin networks and restricts actomyosin we examined whether Rac1 is required for normal epithelial morphology and lateral membrane integrity. To characterize epithelial morphology in vivo in greater detail we isolated fresh frozen papilla, which contains inner medullary CDs of columnar to cuboidal morphology (Qiao et al., 1999). We performed thick cryo-sectioning combined with optical clearing for full confocal 3D visualization of phalloidin-labeled collecting ducts. Longitudinal as well as transverse cross-sections revealed that mutant (Hoxb7:$Rac1^{f/f}$) epithelial cells have decreased apical F-actin density and decreased lateral F-actin height (Fig. 9, A-C), causing the epithelial cells to be shorter, more rounded and have apical invaginations at cell-cell junctions which was confirmed with electron microscopy (TEM) (Fig. 9, D and E). Furthermore, TEM of papillary Hoxb7:$Rac1^{f/f}$ epithelium revealed detaching lateral cell-

cell contacts in Hoxb7:Rac1$^{f/f}$ CDs indicating compromised lateral membrane integrity (Fig. 9 F). This correlated with increased actomyosin activity (pMLC) along the apicobasal axis with more activated myosin II basolaterally in mutant Rac1-deficient epithelium compared to a more apical localization in controls (Fig. 9, G and H). These data suggest that Rac1 dependent restriction of actomyosin along lateral membranes is required to maintain normal epithelial cell height, lateral membrane integrity and overall morphology in vivo.

Rac1 promotes lateral actin dynamics and restores lateral F-actin and normal morphology
Since Rac1 is critical to maintain normal lateral F-actin and CD epithelial morphology we next asked whether Rac1 also actively promotes lateral actin dynamics and restoration of epithelial morphology. To assess whether Rac1 controls lateral actin dynamics at cell-cell contacts in live CD cells we performed FRAP of live SiR-actin labeled Rac1$^{f/f}$ and Rac1$^{-/-}$ cells. We bleached F-actin at lateral sites of initial cell-cell contact formation in newly forming monolayers (Fig. 9 I). Lateral F-actin of Rac1$^{-/-}$ CD cell layers recovers significantly slower than controls indicating impaired actin dynamics at lateral membranes (Fig. 9, I and J). Next, to test whether the lateral F-actin defects are functionally relevant for morphological restoration we assessed lateral F-actin height in regenerating CD cell layers in vitro. For this we performed a scratch assay of confluent CD cells and imaged areas adjacent to the scratch as soon as they were covered with cells of comparable density (Fig. S5, D and E). This took significantly longer for mutant cells given the known migration defect (Fig. S5, D and E). F-actin of regenerating control CD layers was restored to a tight network across cell-cell junctions (apical cross-sections) and lateral F-actin reached full epithelial cell height leaving no apical invaginations (in xz dimension) (Fig. 9 K). Rac1$^{-/-}$ CD epithelial cell layers however demonstrated incomplete regeneration with defective F-actin at cell-cell junctions and shorter lateral F-actin with apical invaginations at cell-cell junctions (Fig. 9, K and L). This was inducible in control cells with Arp2/3 inhibition (CK666) and reversible in Rac1$^{-/-}$ CD cells with actomyosin inhibition (blebbistatin) suggesting that Arp2/3 branched actin networks and restriction of actomyosin are likely causally involved in this Rac1 dependent restoration of lateral F-actin and epithelial morphology (Fig. 9, K and L). In summary, these data indicate that Rac1 by restricting actomyosin not only maintains and but actively promotes lateral actin dynamics and restores epithelial morphology.

Discussion:

We demonstrate that Rac1 in mature epithelial cells in vivo controls lateral membrane F-actin and actomyosin activity thereby maintaining lateral membrane integrity and overall epithelial morphology. This extends previous in vitro data showing that dynamic Arp2/3-dependent actin protrusions at lateral cell membranes are critical to prevent myosin-dependent contractility from constantly unzipping cell-cell adhesions (Li et al., 2020). Our data suggest that Rac1 is an upstream regulator of this critical process required to maintain tissue integrity. In the developing Drosophila pupal notum it has long been established that Rac1 activity along an apicobasal gradient creates a polarized actin cytoskeleton and determines epithelial cell shape (Couto et al., 2017; Georgiou and Baum, 2010). Our data extends this function of Rac1 to a mature mammalian epithelial system in vivo and provides a mechanistic framework of how Rac1 maintains mature mammalian epithelial morphology.
Our data contrast studies that show Rac1-dependent epithelial morphology is largely determined by a Rac1-RhoA antagonism (Chauhan et al., 2011; Martin et al., 2016).

These studies however used developmental systems of epithelial morphogenesis (e.g., the embryonic lens or C. elegans embryos) likely suggesting that Rac1 dependent mechanisms of maintaining mature mammalian epithelial cell shape differ. It is possible that epithelia at different ages use distinct molecular mechanisms to not only maintain but also restore morphology (e.g., after mechanical injury). In fact, in an in vitro injury model we find that Rac1 promotes the restoration of lateral F-actin at cell-cell contacts to thereby promote the re-establishment of a normal morphology in a regenerating epithelial cell layer. Mechanical considerations of epithelial repair imply that the molecular mechanisms that shape the lateral membrane are likely fundamental in determining the regeneration of a differentiated epithelium (Tang, 2017). Future studies could elucidate whether Rac1 is a key player in modulating and restoring cell-cell interfaces during homeostasis or post-injury repair. in other epithelial systems.

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August 24, 2021

RE: JCB Manuscript #202103080R

Prof. Roy Zent
Vanderbilt University Medical Center
Medicine
Room C3210 MCN
Nashville, TN 37232

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

The authors have appropriately addressed my questions and concerns from the first review, and the paper is improved.

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

The revised paper is now acceptable for publication.

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

This is a quality revision that has addressed all of my comments. My overall assessment of the first submission was that it lacked it novelty. I was of the opinion that the first version simply showed a role for Rac in cell shape and tissue organization, which is to be expected. The authors responded with several new experiments and a clearly written rebuttal, which among other things, pointed out the importance of the work for kidney development even if fresh insight into basic cell biology might have been lacking. Now, my overall assessment is that it is important work showing the role of Rac in controlling epithelial cell height and actin organization/dynamics in the kidney. The authors added several new experiments to show the effect of Rac on epithelial cell height and on actin organization and dynamics. These are quality experiments with clear results. I appreciate and respect that many of these experiments were performed in vivo, which is more challenging than the in vitro cell culture systems that I work with.

I found the changes in cell height especially interesting and novel because this important part of epithelial biology receives comparatively little experimental attention. I thought imaging the kinetics at which epithelial cells grow taller after wound healing was a clever idea that could yield more information in the future. I also think it will be interesting to continue to look in vivo for the relationships between cell height versus apical/basal area and cell number in the collecting duct. Our research using cultured cells shows that it takes fewer cells to build a monolayer when cells get thin because apical and basal surfaces expand at the expense of lateral membrane - presumably because cells defend cell volume. It would be more interesting to see how those parameters play out in vivo. By the sound of it, the authors are going to do such work in the future.

Again, I think this a significant manuscript, and I thank the authors for responding to my comments with experiments and changes to the text and new sections in the discussion.