JCB

Notch1 cortical signaling regulates epithelial architecture and cell-cell adhesion

Matthew White, Kyle Jacobs, Tania Singh, Lakyn Mayo, Annie Lin, Christopher Chen, Young-wook Jun, and Matthew Kutys

Corresponding Author(s): Matthew Kutys, University of California, San Francisco

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April 13, 2023

Re: JCB manuscript #202303013

Dr. Matthew L. Kutys
University of California, San Francisco
Department of Cell and Tissue Biology
513 Parnassus Avenue, HSW-613
San Francisco, CA 94143

Dear Matthew,

We have now received comments on your manuscript "Notch1 cortical signaling regulates epithelial architecture and cell-cell adhesion" from three external reviewers with expertise in this area. While I am pleased to be able to report that the reviewers found the study to be interesting and well-written, they each have significant issues with the data and conclusions that will need substantial revision, including further experiments, before the manuscript could be considered further by JCB. If you choose to work towards a revised version of the study, we will require a preliminary description from you of the changes you would plan to include, with outlines of the additional experimental approaches. Please note that any revised version of the manuscript will be re-reviewed by the original referees, and that only one major round of revision is allowed by the journal.

One key issue raised by all the referees concerns the role of the ICD and the conclusion that Notch1 controls epithelial architecture and proliferation independently of transcription. Reviewer #3 notes a lack of evidence proving the molecular mechanism for transcription independent Notch signaling, and suggests that the ICD-KO mutant acts as a dominant negative. Two of the reviewers also raise the concern that only MCF10A cells are used, which are not a good model of mammary ductal epithelium (they do not have tight junctions, and express the basal marker Krt14, for example) and the 3D model is not actually "organotypic" and is not "ductal tissue". Moreover, Reviewer #3 wonders about the relevance of using the 3D model if the same phenotypes are observed with cells grown on 2D hydrogels. We do not think the suggestion of reviewer #3 to use a mix of MCF7 and MCF10A cells would work, or be a solution to the issue, but it is possible to make organoids from primary mammary tissue that can be genetically modified with lentivirus. An alternative would be to focus on the Notch1 signaling and tone down all references to mammary glands.

Additionally, reviewer #1 notes that biological replicates are essential, and technical replicates (multiple measurements from one cell culture) are insufficient. JCB generally requires at least triplicate biological replicates, with all data points being displayed. Finally, there are several comments about the novel binding partner FAM83H concerning localization and if over-expression can rescue Notch KO.

Although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that a complete point-by-point response to each of the reviewer comments will be needed with any revised manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Ian Macara, Ph.D.
Editor
The Journal of Cell Biology

Tim Fessenden, Ph.D.
Scientific Editor
Journal of Cell Biology

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

In this manuscript, White and co-workers report a transcription-independent role of Notch1 in regulating the actin cortex, cell-cell contact stability and EGRFR signaling to regulate mammary epithelial integrity. They show that deletion of Notch1, but not just the Notch intracellular domain, leads to a morphogenetic defect in an in vitro mammary gland tube model as a result of hyperproliferation. This phenotype can be rescued by inhibition of EGFR that is not maintained inactive in the KO due to altered cortical organization and junction stability. Finally, they identify FAM83H as a new Notch interactor and effector of the cortical/junction phenotype through unknown mechanisms.

Overall this is an interesting and well-written manuscript that nicely extend the previous work by the authors and others and will be interesting to the cell biology community. The data is of high quality and convincing. I have only a few suggestions to strengthen some of the conclusions:

The role of the ICD is somewhat confusing. On the other hand the deletion of the ICD is not sufficient to trigger the actin/morphogenesis phenotype indicating that the ICD is not required for the effect of Notch (Fig1), but then later it is shown that prevening the cleavage of the ICD by DAPT triggers adhesion destabilization. Given the apparent involvement of ICD, how do the authors explain the lack of phenotype in the ICD KO?

- Could the authors express the ICD alone in the Notch1-KO cells to see if this can rescue the effects? Does the ICD in this case localize to the nucleus?

- A control of DAPT -treatment in the ICD-KO would be helpful to exclude off-target effects of this drug that is known to have other targets.

- Where is the FAM83H localized in the notch-KO vs ICD-KO cells?

Other points:

Fig 2 I: Would be helpful to have a adherens junction marker here to distinguish junctional vs for example subcortical actin localization of EGFR

Supplementary Fig. 4C: IP would need a IgG control to confirm specificity of the interactions

Statistics should not be done from single cell measurements within a cell culture as these do not represent independent observations and artificially inflate the n numbers and decrease p-values. Displaying the single data points is useful but the statistics should be done comparing means from independent experiments.

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

In this manuscript, White et al. combine 2D monolayer culture and a sophisticated 3D organotypic model of MCF10A cells to address the role of the "two arms" of Notch1 activity, transcriptional signaling and cell-cell adhesion, in ductal morphogenesis. The function of Notch1 was either fully ablated (Notch1-KO), or alternatively only the transcriptional signaling arm was ablated by deleting the intracellular domain of Notch1 (ICD-KO). In the 3D model, Notch1-KO, but not ICD-KO, cells failed to maintain proper epithelial cell architecture, and instead, the lumen was filled with mutant cells. The authors move on to show that this transcription-independent Notch1 cortical signaling mechanism stabilizes adherens junctions and cortical actin, and restrains EGFR signaling activity.
The authors move on with a really cool approach where they unbiasedly profiled differential Notch1 interacting proteins leading to the identification FAM83H as a Notch1 binding partner. Finally, they show that deletion of FAM83H largely recapitulates the Notch1-KO phenotype in the 3D organotypic model. The experimental approaches are sound, and the data presented convincing and of high quality. This is an elegant, coherent manuscript that was a pleasure to read.

I have one major question/concern about the conclusions:

Based on the Notch1-KO phenotype, and lack of similar phenotype in ICD-KO epithelium, the authors conclude that Notch1 controls epithelial cell architecture and proliferation in a manner independent of transcription.
I wonder how the authors can exclude the possibility that the observed Notch1-KO phenotype is not the result of co-operation of "cortical signaling" and transcriptional signaling. In other words, it is assumed that a Notch1 variant that would maintain the transcriptional signaling but be deficient in "cortical signaling", would recapitulate the Notch1-KO 3D phenotype. But does it?

If it is not feasible to generate such a Notch1 construct, then what about rescueing the Notch1-KO phenotype? If the authors are

correct, expression of ICD should not rescue the Notch1-KO phenotype. Is this the case?

Other comments:

1. It is also concluded that cell proliferation increase occurs non-cell autonomously in Notch1-KO, through enhanced sensitivity to EGF and internalization of EGFR. Perhaps I missed something, but I quite did not understand which experiment shows that this is a non-cell autonomous phenomenon? A mosaic deletion of Notch1 and/or mixing of fluorescently labeled control and Notch1-KO cells would perhpas answer this question.

2. For datasets containing three or more samples, one-way ANOVA with a post-hoc test was used. It remained unclear if all significant differences were indicated in Figures. For example, In Fig. 1F, did ICD-KO differ from SCR?

3. I would have appreciated that the video (and Fig. 2A) includes also the control sample. Based on the videos and SFig. 2A, it is concluded that spindle orientation is different in Notch1-KO. Quantification would be streghten this statement.

4. The authors report that dnMAML expression decreases transcript levels of Notch transcriptional target genes HES1 and HEY1 (SFig. 1D). However, I failed to observe any difference in Hes1 expression compared to SCR. Overall, as the sample size in SFig.1D was low, I wonder if the statistical test used is appropriate (i.e. should a non-parametric test be used instead).

5. The authors write that "Notch1 junctional accumulation is coincident with a six-fold increase in γ-secretase-mediated cleavage of ICD (cleavage-specific Notch1 V1754 antibody) with only a marginal increase in total Notch1 protein levels (Figure 4D)." To me a 2-fold increase (with *** stats) it is a bit more than marginal.

6. In several places the authors refer to their model as organotypic human mammary ductal epithelium. I would appreciate if the authorw paid a bit more attention to the description of the model, as a fast reader might think that this indeed is a model of primary human ductal cells, not a cell line. In addition to the main text, e.g. M&M reads "Duct tissues were fixed", etc.

7. The previous point brings me to my last point. If I understood the 3D system correctly, MCF10A cells form a single-cell layered epithelium in the 3D microfluidic set-up used in the study. This differs greatly from the in vivo bilayered organization of the mammary epithelium consisting of outer basal and inner luminal cells. Furthermore, in vivo, Notch1(-3) is active in luminal cells with a key role in luminal cell fate determination. Do the authors think that Notch1/Fam83h system functions the same way in the bilayered mammary epithelium in vivo? Some discussion on this matter would be most welcome.

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

In this manuscript, White and colleagues analyse the potentially differential effects of the complete loss of the Notch1 receptor (NOTCH1-KO) as compared to the deletion of the intracellular domain of Notch1 (ICD-KO) in affecting epithelial proliferation and adhesion. The rational is that ICD-KO construct is transcriptionally dead but retains the adhesion properties of the full-length receptor. They perform this study in a microfluidic organotypic system that they have They perform this study in a microfluidic organotypic system that they have previously characterised and published (Polacheck et al., 2017).

In my view, this study suffers from two major flaws, as I elaborate below: a lack of physiological relevance of the cell line chosen for these studies (when mammary cells do not exist as a monolayer in vivo) and a lack of evidence proving the molecular mechanism that would mediate the so-called "transcription independent Notch1 signalling" that in my view has not been demonstrated in sufficient depth to be convincing.

1- Related to this latter point, the authors found a different phenotype for Notch1-KO and ICD-KO mutant cells, whereas the ICD-KO mutation has similar effects as a dnMAML mutant. However, in theory, dnMAML should block downstream transcriptional signalling from all Notch receptors, unlike the Notch1-specific ICD-KO. To evaluate which receptors are expressed, the authors should check the levels of Notch 1-4 in the cell line MCF10A.

2- I do not think the authors gathered enough evidence to say that the phenotype of dnMAML is transcription independent. There is a formal possibility that the ICD-KO mutant also acts as a dominant negative, for example by sequestering ligands (and in this case it would affect all Notch paralogues, thus explaining the different phenotypes between Notch1-KO and ICD-KO). Actually, this possibility fits with the fact that dnMAML and ICD-KO mutations do phenocopy each other.
To investigate this, the levels of the targets HES1 and HEY1 should also be checked in ICD-KO cells and not only in dnMAML mutants.

3- In addition, I do not understand why dnMAML mutant cells are only examined in Fig. 1 and not throughout the study, to evaluate consistency with the ICD-KO phenotypes?

4- If exactly the same phenotype is observed in cells grown on 2D compliant hydrogels and in the microfluidic device, why using the 3D organotypic model? The authors are invited to elaborate on the way the 3D organotypic ducts help to answer the relevant questions? Switching from one to another system without rationale is not acceptable.

5- In vivo, basal mammary cells express Notch ligands and luminal mammary cells express the receptors. MCF10A is a cell line that presents basal features (i.e. it expresses the basal cytokeratin KRT14 and not luminal KRT19, see Figure 3 in https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6125218/); what happens in a luminal line like MCF7? Introducing the Notch1-KO and ICD-KO mutations in MCF7 cells may allow to estimate the magnitude of transcription and signalling-independent effects versus the phenotypes that depend on ligand-receptor interaction signalling.

6- In the same line of thought, is it possible to co-culture a basal and luminal cell line, such as MCF10A and MCF7, to recapitulate a more physiological setting with the microfluidic 3D organotypic cultures used here?

7- Relative to Fig. 2G: is there a difference in EGFR phosphorylation in the absence of recombinant EGF? Is there a phenotype when cells are incubated without EGF?

8- Relative to Fig. 2K: what is the effect of Erlotinib on SCR cells? Potentially, any kind of anti-proliferative pathway would lead to the same phenotype. Incidentally, as a control for Erlotinib treatment, the WB in Suppl Fig. 4C should also be performed in the presence of Erlotinib (condition shown in Suppl Fig. 4D).

9- Relative to Fig. 3: in terms of adherens junctions, NOTCH1-KO cells have the same phenotype as DAPT-treated cultures. How do the authors explain this? What is the proposed mechanism? How can they conclude on a transcription-independent effect of DAPT? What do ICD-KO mutant cells do? The analyses of adherens junctions' distribution and cortical actin organization on the ICD-KO and dnMAML mutant cells must be included.

10- I disagree with the sentence: "visualizing live actin dynamics following treatment with DAPT revealed dissolution of cortical actin fibers within 30 minutes (Supplementary Fig. 5E), a rapid response that further supports a transcription independent Notch1 function." 30 minutes is long enough to be linked to early transcriptional responses.

11- Relative to Fig. 4. As also listed above as one of my main concerns, I think that studying the subcellular localization of Notch in a monolayer system while normal mammary cells compose a pseudo-stratified bi-layered epithelium is not physiologically relevant.

12- I do not think the authors should refer to "Notch1-KO monolayers" if this mutation induces hyperproliferative multilayered outgrowths and lumen cell filling. For example, at page 4: "NOTCH1-KO cells similarly adhere to the channel architecture and progressively form a monolayer". At page 5: "we observed no difference in nuclear YAP localization between SCR and NOTCH1-KO monolayers". Page 5 again: "NOTCH1-KO monolayers have diminished EGFR..." ...and in several other instances. It is not always clear when the authors use 2D or 3D cultures.

13- Related to the sentence: "During this transition, immunofluorescence staining revealed Notch1 progressively accumulates at cell-cell interfaces (Figure 4A, B). High magnification confocal micrographs further showed that E-cadherin most strongly localizes to apical domains in the polarized state, while Notch1 and cortical actin intensity is highest at lateral cell membranes (Figure 4C)."
Notch1 is shown to accumulate with higher confluency and in polarized conditions in the WB in Fig. 4D; however, it is not clear if the accumulation at cell-cell interfaces is specific or simply a consequence of more abundant expression.

14- In Fig. 4F, it is not shown that recombinant soluble DLL4 works in activating Notch signalling. To prove this, the expression of the endogenous Hes1 and Hey targets has to be assessed, because the GFP reporter might simply not be sensitive enough. Also, in Fig. 4E, it is unclear why a second band appear in the nuclear extract; is this form recognized by the cleaved Notch antibody (val1744)?

15- In Fig. 5B the increase in Notch1 expression in lysates from Polarised cells (P) is not evident, actually it seems like Notch1 levels are reduced, generating confusion and inconsistency with the results presented in Fig. 4D.

16- The fact that FAM83H-KO partially phenocopies the NOTCH1-KO phenotype does not mean that it mediates this effect. Indeed in Fig. 5G, the statement: "FAM83H co-immunoprecipitates with E-cadherin, but this interaction is lost in NOTCH1-KO" is incorrect because the interaction is reduced but clearly not lost. Further experimental evidence must be provided to conclude that 'Notch1 cortical signalling functions through FAM83H". For example, can overexpression of FAM83H rescue the NOTCH1-KO phenotype?

Minor comments:
• Figure 1D, the X-axis title should be changed from "Duct diameter variance (μm)" to "Duct diameter (μm)". Also, specify the statistical test used to define p-values throughout all Figures.

• Mention in the legend of Figure 2G that cells were treated with a high EGF concentration (20 ng/ml).
• Mention in in the legend of Figure 2H that it represents a comparison of 2 different concentrations of EGF (2 ng/ml vs 20 ng/ml).
• More generally, the use of bar charts should be restricted to the representation of counts or proportions. If the goal of the figures is to compare data distribution, please prefer boxplots or violin plots representations. Please change in every Figure where relevant.

Response to Reviewers

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

In this manuscript, White and co-workers report a transcription-independent role of Notch1 in regulating the actin cortex, cell-cell contact stability and EGRFR signaling to regulate mammary epithelial integrity. They show that deletion of Notch1, but not just the Notch intracellular domain, leads to a morphogenetic defect in an in vitro mammary gland tube model as a result of hyperproliferation. This phenotype can be rescued by inhibition of EGFR that is not maintained inactive in the KO due to altered cortical organization and junction stability. Finally, they identify FAM83H as a new Notch interactor and effector of the cortical/junction phenotype through unknown mechanisms.

Overall this is an interesting and well-written manuscript that nicely extend the previous work by the authors and others and will be interesting to the cell biology community. The data is of high quality and convincing. I have only a few suggestions to strengthen some of the conclusions:

We appreciate the reviewer's positive assessment.

The role of the ICD is somewhat confusing. On the other hand the deletion of the ICD is not sufficient to trigger the actin/morphogenesis phenotype indicating that the ICD is not required for the effect of Notch (Fig1), but then later it is shown that prevening the cleavage of the ICD by DAPT triggers adhesion destabilization. Given the apparent involvement of ICD, how do the authors explain the lack of phenotype in the ICD KO?

We also appreciate the opportunity to clarify this point through text changes and additional experimentation. We previously identified that the Notch1 transmembrane domain (TMD) is the essential domain of Notch1 for regulating endothelial adherens junctions, and the cortical signaling activity of the TMD requires removal of the ICD from the TMD (Polacheck et al., 2017). To examine the hypothesis that proteolytic activation of Notch1 and subsequent removal of the ICD from the TMD is a necessary step for epithelial Notch1 cortical signaling, we demonstrate: 1) acute DAPT treatment phenocopies *NOTCH1^{KO}* adhesion phenotypes (Fig S4D-H), 2) *ICD^{KO}* cells are insensitive to DAPT treatment (Fig S4I), and 3) expression of the TMD, but not ICD, is sufficient to rescue *NOTCH1^{KO}* adherens junction and actin defects (Fig 3G-I). Thus, to the reviewer's point, our data support a model in which DAPT blocks a critical step of ICD cleavage from TMD, which is 'bypassed' in *ICD^{KO}* cells. Why ICD removal is required for TMD cortical signaling is still unclear, and is the focus of an independent ongoing study. We now clarify this point on pages 6-7 and in the discussion.

- Could the authors express the ICD alone in the Notch1-KO cells to see if this can rescue the effects? Does the ICD in this case localize to the nucleus?

In Fig 3G and Fig S5A-D, we express the ICD as well as the TMD in *NOTCH1^{KO}* cells. Expressed ICD localizes to the nucleus and increases *HEY1* mRNA transcript expression, but fails to rescue *NOTCH1^{KO}* defects. Expression of the TMD in *NOTCH1^{KO}* cells, in contrast, prevents focal adherens junctions and normalizes cortical actin organization.

- A control of DAPT -treatment in the ICD-KO would be helpful to exclude off-target effects of this drug that is known to have other targets.

In Fig S4I, we quantify the effects on DAPT on *ICD^{KO}* cells compared to SCR. We show *ICD^{KO}* cells maintain stable adherens junctions irrespective of whether they were treated with DAPT.

- Where is the FAM83H localized in the notch-KO vs ICD-KO cells?

Our efforts to localize endogenous FAM83H via immunostaining using paraformaldehyde fixation were unsuccessful. In this revision we report that, with methanol fixation, endogenous FAM83H localizes at and proximal to lateral cell-cell interfaces in SCR and *ICD^{KO}*, but this localization is lost in *NOTCH1^{KO}* cells (Fig 5J) consistent with E-cadherin co-immunoprecipitation experiments (Figure 5I). Additionally, we now include biochemical evidence that FAM83H complexes with TMD, and that TMD increases FAM83H-E-cadherin co-immunoprecipitation in *NOTCH1^{KO}* cells (Fig 5I, K-L).

Other points:

Fig 2 I: Would be helpful to have a adherens junction marker here to distinguish junctional vs for example subcortical actin localization of EGFR

> We now include an actin overlay in Fig 2K, as well as an E-cadherin overlay with the TR-EGF internalization experiments in Fig S2H.

Supplementary Fig. 4C: IP would need a IgG control to confirm specificity of the interactions

> FRET and co-immunoprecipitation experiments have established EGFR and E-cadherin complex at the plasma membrane and perturbing homophilic E-cadherin adhesions is sufficient to disrupt the complex (Sullivan et al. 2022), (Proux-Gillardeaux et al. 2021), (Curto et al. 2007). Consistent with these previous reports, EGFR-E-cadherin co-immunoprecipitation is reduced in *NOTCH1*^{KO} cells coincident with defects in adherens junctions, which is supported by immunofluorescence staining (Fig 2K). Altogether, we are confident in the specificity of the EGFR-E-cadherin interaction now in Fig S2E.

Statistics should not be done from single cell measurements within a cell culture as these do not represent independent observations and artificially inflate the n numbers and decrease p-values. Displaying the single data points is useful but the statistics should be done comparing means from independent experiments.

> We thank the reviewer for this important recommendation. We have updated all graphs, statistics, and associated figure legends to ensure that statistics are performed on independent biological observations and that all statistical comparisons are displayed on graphs.

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

In this manuscript, White et al. combine 2D monolayer culture and a sophisticated 3D organotypic model of MCF10A cells to address the role of the "two arms" of Notch1 activity, transcriptional signaling and cell-cell adhesion, in ductal morphogenesis. The function of Notch1 was either fully ablated (Notch1-KO), or alternatively only the transcriptional signaling arm was ablated by deleting the intracellular domain of Notch1 (ICD-KO). In the 3D model, Notch1-KO, but not ICD-KO, cells failed to maintain proper epithelial cell architecture, and instead, the lumen was filled with mutant cells. The authors move on to show that this transcription-independent Notch1 cortical signaling mechanism stabilizes adherens junctions and cortical actin, and restrains EGFR signaling activity. The authors move on with a really cool approach where they unbiasedly profiled differential Notch1 interacting proteins leading to the identification FAM83H as a Notch1 binding partner. Finally, they show that deletion of FAM83H largely recapitulates the Notch1-KO phenotype in the 3D organotypic model. The experimental approaches are sound, and the data presented convincing and of high quality. This is an elegant, coherent manuscript that was a pleasure to read.

> We thank the reviewer for their very positive assessment.

I have one major question/concern about the conclusions:

Based on the Notch1-KO phenotype, and lack of similar phenotype in ICD-KO epithelium, the authors conclude that Notch1 controls epithelial cell architecture and proliferation in a manner independent of transcription.
I wonder how the authors can exclude the possibility that the observed Notch1-KO phenotype is not the result of co-operation of "cortical signaling" and transcriptional signaling. In other words, it is assumed that a Notch1 variant that would maintain the transcriptional signaling but be deficient in "cortical signaling", would recapitulate the Notch1-KO 3D phenotype. But does it?

> This is an excellent suggestion. An active goal of our laboratory is to engineer a Notch1 construct that preserves transcriptional, but not cortical signaling. To date, this has proven difficult as the putative domains necessary for cortical signaling (TMD) are also intimately tied to Notch1 receptor activation, as we recently contributed to the description of in (Kwak et al. 2022).
>
> Included in this revision and elaborated on below, we now express the ICD or TMD in *NOTCH1*^{KO} cells and demonstrate domain-specific phenotypic rescue with TMD. ICD expression is sufficient for transcriptional signaling but does not rescue the *NOTCH1*^{KO} phenotype. In addition, qPCR and biochemical analyses across the Notch1 mutant cell lines (Fig S1B) and during low confluence to polarized transitions (Fig S5E) do not support a role for Notch1 transcriptional signaling in this specific morphodynamic process. However, in other morphogenic contexts we agree that transcription and

cortical signaling may be coordinated, and at the reviewer's suggestion we now discuss how cooperativity between Notch1 transcriptional and cortical signaling may operate *in vivo*.

If it is not feasible to generate such a Notch1 construct, then what about rescueing the Notch1-KO phenotype? If the authors are correct, expression of ICD should not rescue the Notch1-KO phenotype. Is this the case?

In Fig 3G-I and Fig S5, we express the ICD or TMD in *NOTCH1^{KO}* cells. ICD localizes to the nucleus and increases the Notch transcript *HEY1*, decreases E-cadherin expression, but does not rescue *NOTCH1^{KO}* cortical actin and cell multilayering. In contrast, expression of Notch1 TMD normalizes the adherens junction and cortical actin phenotypes.

Other comments:

1. It is also concluded that cell proliferation increase occurs non-cell autonomously in Notch1-KO, through enhanced sensitivity to EGF and internalization of EGFR. Perhaps I missed something, but I quite did not understand which experiment shows that this is a non-cell autonomous phenomenon? A mosaic deletion of Notch1 and/or mixing of fluorescently labeled control and Notch1-KO cells would perhpas answer this question.

We apologize for this confusing language. Our conclusion is that the proliferative increases are inherent to *NOTCH1^{KO}* cells, but dependent on enhanced sensitivity to EGF and associated EGFR mitogenic signaling. In this revision, we illustrate this point further by quantifying internalized EGF and the *NOTCH1^{KO}* proliferation dependence on EGF (Fig S2H-I). We have removed non-cell autonomous and clarified the text describing this finding.

2. For datasets containing three or more samples, one-way ANOVA with a post-hoc test was used. It remained unclear if all significant differences were indicated in Figures. For example, In Fig. 1F, did ICD-KO differ from SCR?

We regret this oversight and thank the reviewer for the suggestion. We now include all statistical comparisons on graphs.

3. I would have appreciated that the video (and Fig. 2A) includes also the control sample. Based on the videos and SFig. 2A, it is concluded that spindle orientation is different in Notch1-KO. Quantification would be streghten this statement.

We now include the control timelapse in Fig 2A. Additionally, we also have quantified spindle orientation in Fig 2B,D.

4. The authors report that dnMAML expression decreases transcript levels of Notch transcriptional target genes HES1 and HEY1 (SFig. 1D). However, I failed to observe any difference in Hes1 expression compared to SCR. Overall, as the sample size in SFig.1D was low, I wonder if the statistical test used is appropriate (i.e. should a non-parametric test be used instead).

In addressing this point, we identified that the *HES1* and *HEY1* x-axis labels were mistakenly swapped in the original Fig S1D. In the new Figure S1B, we have expanded our qPCR sample size and analysis to include SCR, *NOTCH1^{KO}*, *ICD^{KO}*, and dnMAML cell lines. Consistent with the previous observation, we report a marked decrease in *HES1* mRNA transcripts upon dnMAML expression, but the reviewer correctly identified no significant difference in *HEY1*. Interestingly, overexpression of ICD triggers increased *HEY1*, but not *HES1*, transcript expression in MCF10A (Fig S5D). Notably, there are no significant *HES1* or *HEY1* transcript differences across the other three control and Notch1 mutant lines.

5. The authors write that "Notch1 junctional accumulation is coincident with a six-fold increase in γ-secretase-mediated cleavage of ICD (cleavage-specific Notch1 V1754 antibody) with only a marginal increase in total Notch1 protein levels (Figure 4D)." To me a 2-fold increase (with *** stats) it is a bit more than marginal.

We thank the reviewer for this critical point. Regrettably, during figure preparation the asterisk label was inadvertently shifted slightly giving the appearance of *** stats for total ICD (which was intended for cleaved Notch1). There is no statistical difference between low confluence and polarized total ICD in Fig 4D. We clarify this in the text, as well as now include all statistical comparisons on graphs.

6. In several places the authors refer to their model as organotypic human mammary ductal epithelium. I would appreciate if the authorw paid a bit more attention to the description of the model, as a fast reader might think that this indeed is a model of primary human ductal cells, not a cell line. In addition to the main text, e.g. M&M reads "Duct tissues were fixed", etc.

> We appreciate the opportunity to describe our model more accurately, including its limitations in modeling mammary physiology. We have extensively edited the text to ensure that we accurately describe our 3D engineered in vitro system throughout the results, discussion, and methods.

7. The previous point brings me to my last point. If I understood the 3D system correctly, MCF10A cells form a single-cell layered epithelium in the 3D microfluidic set-up used in the study. This differs greatly from the in vivo bilayered organization of the mammary epithelium consisting of outer basal and inner luminal cells. Furthermore, in vivo, Notch1(-3) is active in luminal cells with a key role in luminal cell fate determination. Do the authors think that Notch1/Fam83h system functions the same way in the bilayered mammary epithelium in vivo? Some discussion on this matter would be most welcome.

> This is an excellent suggestion. In the revised text, we carefully describe our experimental model and highlight its advantages and limitations, including the differences between MCF10A and basal and luminal mammary epithelia. We also now discuss how Notch1 cortical signaling may coordinate with transcriptional signaling during mammary developmental morphogenesis *in vivo*.

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

In this manuscript, White and colleagues analyse the potentially differential effects of the complete loss of the Notch1 receptor (NOTCH1-KO) as compared to the deletion of the intracellular domain of Notch1 (ICD-KO) in affecting epithelial proliferation and adhesion. The rational is that ICD-KO construct is transcriptionally dead but retains the adhesion properties of the full-length receptor. They perform this study in a microfluidic organotypic system that they have They perform this study in a microfluidic organotypic system that they have previously characterised and published (Polacheck et al., 2017).

In my view, this study suffers from two major flaws, as I elaborate below: a lack of physiological relevance of the cell line chosen for these studies (when mammary cells do not exist as a monolayer in vivo) and a lack of evidence proving the molecular mechanism that would mediate the so-called "transcription independent Notch1 signalling" that in my view has not been demonstrated in sufficient depth to be convincing.

> We appreciate the opportunity to strengthen our manuscript by addressing the reviewer's specific comments below. To summarize our response to these two main points, in this revision we:
>
> 1) Provide additional experimentation in support of the transcription-independent Notch1 cortical signaling mechanism.
>   a. We illustrate that ICD expression in *NOTCH1^{KO}* cells localizes to the nucleus and increases *HEY1* transcript levels, but does not rescue *NOTCH1^{KO}* cell-cell adhesion defects.
>   b. We show expression of the Notch1 transmembrane domain (TMD) in *NOTCH1^{KO}* cells is sufficient to restore adherens junction and cortical actin organization.
>   c. We expand our qPCR analyses and show that Notch1 transcriptional targets *HES1* and *HEY1* levels do not increase when Notch1 is activated in polarized cells.
>   d. We demonstrate that there are no differences in Notch2-4 protein expression or in *HES1* and *HEY1* transcript levels in the mutant Notch1 cell lines.
>   e. We expand our examination of dnMAML cells and in all cases dnMAML phenotypes are consistent with *ICD^{KO}*.
>
> 2) Downplay references to mammary gland physiology.
>
> We firmly believe that this work stands out by establishing a new mechanism for Notch1 signaling in epithelia that directly regulates tissue morphogenesis, cell architecture, and cell-cell adhesion. Nonetheless, we agree that our model and MCF10A do not fully represent a mammary ductal epithelium. We therefore have focused our narrative on Notch1 cortical signaling in epithelia and deemphasized references to mammary glands through the following changes:
>   a. Clearly describe the architecture of our model platform, discussing its experimental advantages and limitations relative to *in vivo* architecture. We also discuss limitations of MCF10A as a model cell line.

b. At the request of the reviewer, expand our previous investigations of Notch1 cortical signaling in other epithelial cells to include MCF7. MCF7 Notch1 mutant cell lines phenocopy key observations in MCF10A.
c. Clarify the distinction of this cortical signaling mechanism from classic Notch sender-receiver models of lateral induction or inhibition, and discuss how Notch1 transcriptional and cortical signaling may function cooperatively *in vivo*.

1- Related to this latter point, the authors found a different phenotype for Notch1-KO and ICD-KO mutant cells, whereas the ICD-KO mutation has similar effects as a dnMAML mutant. However, in theory, dnMAML should block downstream transcriptional signalling from all Notch receptors, unlike the Notch1-specific ICD-KO. To evaluate which receptors are expressed, the authors should check the levels of Notch 1-4 in the cell line MCF10A.

In Fig S1A we now include a western blot of all Notch receptors in the Notch1 mutant cell lines. We observe no significant changes in Notch2-4 protein levels in $NOTCH1^{KO}$ or ICD^{KO} MCF10A. Additionally, in Fig S1B, we have expanded our qPCR sample size and analysis to include SCR, $NOTCH1^{KO}$, ICD^{KO}, and dnMAML cell lines. We demonstrate that *HES1* transcripts are markedly decreased in dnMAML cells. Interestingly, overexpression of Notch1 ICD increases expression of *HEY1*, but not *HES1* (Fig S5D) in MCF10A. There are no significant differences in *HES1* or *HEY1* transcripts in the other cell lines.

2- I do not think the authors gathered enough evidence to say that the phenotype of dnMAML is transcription independent.

dnMAML is an established tool to globally suppress Notch transcription (Maillard et al. 2004). Our data demonstrates that dnMAML expression suppresses transcript expression of the Notch target gene *HES1* in MCF10A (Fig S1B).

There is a formal possibility that the ICD-KO mutant also acts as a dominant negative, for example by sequestering ligands (and in this case it would affect all Notch paralogues, thus explaining the different phenotypes between Notch1-KO and ICD-KO). Actually, this possibility fits with the fact that dnMAML and ICD-KO mutations do phenocopy each other. To investigate this, the levels of the targets HES1 and HEY1 should also be checked in ICD-KO cells and not only in dnMAML mutants.

As requested, in contrast to dnMAML cells, ICD^{KO} cells have no significant differences in *HES1* or *HEY1* transcript levels relative to SCR or $NOTCH1^{KO}$ (Fig S1B). SCR cells phenocopy ICD^{KO} and dnMAML cells across several key assays, and ICD^{KO} cells express lower levels of Notch1 ECD than SCR (Fig 1A), which altogether does not support a ICD^{KO} dominant negative effect on other Notch receptors from ligand sequestration.

3- In addition, I do not understand why dnMAML mutant cells are only examined in Fig. 1 and not throughout the study, to evaluate consistency with the ICD-KO phenotypes?

Our original submission included examination of dnMAML 3D duct morphology (in Figure 1) and cell proliferation (in Supplementary Figure S2) as secondary confirmation of the absence of phenotype in ICD^{KO} cells. At the request of the reviewer, we now include examine EGFR internalization (Fig 2L, Fig S2G) and cell architecture, adherens junctions, and cortical actin (Fig 3B,D, Fig S4C) in dnMAML cells. In all cases, dnMAML phenotypes are consistent with ICD^{KO}.

4- If exactly the same phenotype is observed in cells grown on 2D compliant hydrogels and in the microfluidic device, why using the 3D organotypic model? The authors are invited to elaborate on the way the 3D organotypic ducts help to answer the relevant questions? Switching from one to another system without rationale is not acceptable.

The tissue morphogenic phenotype (which cannot be fully capture by 2D models) associated with loss of Notch1 cortical signaling was revealed by the 3D model, and facilitated identifying the driving cell behaviors. Transitioning to tailored 2D hydrogels that captured key aspects of this phenotype permitted high resolution analysis of cell architecture and adhesion, mechanical behavior, and biochemical signaling that was not possible in the 3D system. We now include explanation of this experimental approach on page 4.

5- In vivo, basal mammary cells express Notch ligands and luminal mammary cells express the receptors. MCF10A is a cell line that presents basal features (i.e. it expresses the basal cytokeratin KRT14 and not luminal KRT19, see Figure 3 in https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6125218/); what happens in a luminal line like MCF7? Introducing the Notch1-KO and ICD-KO mutations in MCF7 cells may allow to estimate the magnitude of transcription and signalling-independent effects versus the phenotypes that depend on ligand-receptor interaction signalling.

Luminal mammary cells also express Notch ligands (Bouras et al. Cell Stem Cell. 2008), (Nguyen et al. 2018). MCF10A show both basal and luminal expression profiles in 3D culture, including luminal KRT8/18 (Qu et al. 2015). However, as requested, in Fig S3A-D we examine *NOTCH1*^{KO} and *ICD*^{KO} MCF7 cells. Notch1 accumulates at lateral cell-cell interfaces in SCR MCF7. Loss of Notch1 leads to increases in MCF7 focal adherens junctions, cortical actin disorganization, cell multilayering, and proliferation. These phenotypes are not observed in *ICD*^{KO} MCF7 cells, consistent with observations in MCF10A.

Nonetheless, we agree that MCF10A, and our 3D model, are not true physiologic representations of human mammary epithelia. The primary focus of this manuscript is Notch1 cortical signaling and the connection to epithelial morphogenesis, cell architecture, and adhesion. In this revision, we therefore deemphasize the description of our 3D system as an organotypic mammary duct, include limitations of MCF10A as a model cell line, and discuss of how Notch1 cortical and transcriptional signaling likely cooperate *in vivo*.

6- In the same line of thought, is it possible to co-culture a basal and luminal cell line, such as MCF10A and MCF7, to recapitulate a more physiological setting with the microfluidic 3D organotypic cultures used here?

We appreciate the reviewer's suggestion. However, while of luminal origin, MCF7 are a transformed mammary cell line and an artificial MCF10A-MCF7 bilayer system would present several limitations in modeling the *in vivo* mammary bilayer interface. We are actively investigating the interplay of Notch1 cortical and transcriptional signaling during mammary morphogenesis *in vivo*, but this effort is outside the scope of this work.

7- Relative to Fig. 2G: is there a difference in EGFR phosphorylation in the absence of recombinant EGF? Is there a phenotype when cells are incubated without EGF?

NOTCH1^{KO} increases in cell proliferation are lost when cells are incubated without EGF (Fig S2J). Further, using labelled EGF ligand (TR-EGF) we demonstrate increased ligand internalization in *NOTCH1*^{KO} cells (Fig S2H).

8- Relative to Fig. 2K: what is the effect of Erlotinib on SCR cells? Potentially, any kind of anti-proliferative pathway would lead to the same phenotype. Incidentally, as a control for Erlotinib treatment, the WB in Suppl Fig. 4C should also be performed in the presence of Erlotinib (condition shown in Suppl Fig. 4D).

Erlotinib slows the proliferation of MCF10A (Glaysher et al. 2014). The mechanisms of the E-cadherin-EGFR interaction have been studied extensively (Sullivan et al. 2022), (Rea et al. 2018), (Proux-Gillardeaux et al. 2021), (Moreno et al. 2022), and is not a focus of this study. In the revised manuscript, we demonstrate that, relative to SCR or *ICD*^{KO} cells, *NOTCH1*^{KO} cells have increased EGFR internalization (Fig 2K-L), TR-EGF internalization (Fig S2H-I), and phosphorylation of EGFR (Fig 2I,J), and that *NOTCH1*^{KO} increases in cell proliferation are lost when cell are incubated without EGF (Fig S2J) or with Erlotinib (Fig 2M-O).

9- Relative to Fig. 3: in terms of adherens junctions, NOTCH1-KO cells have the same phenotype as DAPT-treated cultures. How do the authors explain this? What is the proposed mechanism? How can they conclude on a transcription-independent effect of DAPT?

We appreciate the opportunity to clarify this point through text changes and additional experimentation. We previously identified that the Notch1 transmembrane domain (TMD) is the essential domain of Notch1 for regulating endothelial adherens junctions, and the cortical signaling activity of the TMD requires removal of the ICD from the TMD (Polacheck et al., 2017). To examine the hypothesis that proteolytic activation of Notch1 and subsequent removal of the ICD was a necessary step for epithelial Notch1 cortical signaling, we show: 1) acute DAPT treatment phenocopies *NOTCH1*^{KO} adhesion

phenotypes (Fig S4D-H), 2) *ICD^{KO}* cells are insensitive to DAPT treatment (Fig S4I), and 3) expression of the TMD, but not ICD, is sufficient to rescue *NOTCH1^{KO}* adherens junction and actin defects (Fig 3G-I). Thus, to address the reviewer's point, DAPT blocks a critical step of ICD removal from TMD, which is 'bypassed' in *ICD^{KO}* cells. Why ICD removal is required for TMD cortical signaling is still unclear, and is the focus of an independent ongoing study. We clarify this point on pages 6-7 and in the discussion.

What do ICD-KO mutant cells do? The analyses of adherens junctions' distribution and cortical actin organization on the ICD-KO and dnMAML mutant cells must be included.

ICD^{KO} cells were extensively characterized in the original manuscript, including adherens junction and cortical actin organization. In this revision, we further demonstrate that *ICD^{KO}* cells maintain stable adherens junctions irrespective of whether they were treated with DAPT (Fig S4I). Quantification of adherens junctions and cortical actin organization in dnMAML cells are now in Fig 3B,D.

10- I disagree with the sentence: "visualizing live actin dynamics following treatment with DAPT revealed dissolution of cortical actin fibers within 30 minutes (Supplementary Fig. 5E), a rapid response that further supports a transcription independent Notch1 function." 30 minutes is long enough to be linked to early transcriptional responses.

We have removed this specific sentence from the text.

11- Relative to Fig. 4. As also listed above as one of my main concerns, I think that studying the subcellular localization of Notch in a monolayer system while normal mammary cells compose a pseudo-stratified bi-layered epithelium is not physiologically relevant.

We have refocused the narrative of our manuscript to emphasize the novel connection between Notch1 cortical signaling and epithelial cell architecture and cell-cell adhesion. The subcellular localization of Notch1 is an important and relevant aspect of Notch1 cortical signaling, and is consistent across four human epithelial cell lines examined here (MCF10A, MCF7, Caco-2, 16HBE14o-). However, we acknowledge the current limited relevance to mammary physiology. We now address the limitations of our model and of MCF10A cells, and include discussion on how Notch1 cortical and transcriptional signaling may operate in a bilayer epithelium.

12- I do not think the authors should refer to "Notch1-KO monolayers" if this mutation induces hyperproliferative multilayered outgrowths and lumen cell filling. For example, at page 4: "NOTCH1-KO cells similarly adhere to the channel architecture and progressively form a monolayer". At page 5: "we observed no difference in nuclear YAP localization between SCR and NOTCH1-KO monolayers". Page 5 again: "NOTCH1-KO monolayers have diminished EGFR..." ...and in several other instances. It is not always clear when the authors use 2D or 3D cultures.

We no longer use monolayer in reference to *NOTCH1^{KO}* cells. We have also ensured in the text and figure legends that the experimental setups are clearly described.

13- Related to the sentence: "During this transition, immunofluorescence staining revealed Notch1 progressively accumulates at cell-cell interfaces (Figure 4A, B). High magnification confocal micrographs further showed that E-cadherin most strongly localizes to apical domains in the polarized state, while Notch1 and cortical actin intensity is highest at lateral cell membranes (Figure 4C)."
Notch1 is shown to accumulate with higher confluency and in polarized conditions in the WB in Fig. 4D; however, it is not clear if the accumulation at cell-cell interfaces is specific or simply a consequence of more abundant expression.

We thank the reviewer for the opportunity to clarify this point. In the original submission, the asterisk label was inadvertently shifted during figure preparation, giving the appearance of *** stats for total ICD (which was intended for cleaved Notch1 V1754). There is no statistical difference in Notch1 expression between low confluence and polarized conditions (Fig 4D). Therefore, the accumulation at cell-cell interfaces is specific and not due to expression changes.

14- In Fig. 4F, it is not shown that recombinant soluble DLL4 works in activating Notch signalling. To prove this, the expression of the endogenous Hes1 and Hey targets has to be assessed, because the GFP reporter might simply not be sensitive enough. Also, in Fig. 4E, it is unclear why a second band appear in the nuclear extract; is this form recognized by the cleaved Notch antibody (val1744)?

Recombinant (rDll4) was immobilized on the substrate to activated Notch1, not soluble (Varnum-Finney et al. 2000). To the reviewer's point, rDll4 led to an expected increase in proteolytic Notch1 activation (visualized by increased V1754 western blot), but no reporter expression (Fig 4F). We now assess *HES1* and *HEY1* transcripts under these conditions and report no significant differences (Fig S5D), consistent with the GFP reporter. We agree that the appearance of this second band is interesting, and we speculate on why this might be in the discussion. We now include an immunoblot in Fig 4E using the cleaved Notch1 V1754 antibody and observe a second band in the nuclear extract.

15- In Fig. 5B the increase in Notch1 expression in lysates from Polarised cells (P) is not evident, actually it seems like Notch1 levels are reduced, generating confusion and inconsistency with the results presented in Fig. 4D.

We apologize for this confusion. As mentioned previously in point #13, this issue stems from the asterisk label in the original Figure 4D being inadvertently shifted to give the appearance of *** stats for total ICD (which was intended for cleaved Notch1 V1754). There is no statistical difference between low confluence and polarized total ICD (Fig 4D). We will clarify this in the text, as well as include all statistical comparisons on the graph. Therefore, the observation in Fig 5B is consistent.

16- The fact that FAM83H-KO partially phenocopies the NOTCH1-KO phenotype does not mean that it mediates this effect. Indeed in Fig. 5G, the statement: "FAM83H co-immunoprecipitates with E-cadherin, but this interaction is lost in NOTCH1-KO" is incorrect because the interaction is reduced but clearly not lost.

FAM83H^{KO} phenocopies the tissue and cell-cell adhesion defects observed in *NOTCH1*^{KO} cells. We have changed the above referenced sentence to: "FAM83H-E-cadherin co-immunoprecipitation is significantly reduced in *NOTCH1*^{KO} cells relative to SCR or *ICD*^{KO} cells (Fig 5I)".

Further experimental evidence must be provided to conclude that 'Notch1 cortical signalling functions through FAM83H". For example, can overexpression of FAM83H rescue the NOTCH1-KO phenotype?

Overexpression of FAM83H does not rescue the *NOTCH1*^{KO} cell-cell adhesion phenotype. Based on the data in this revision, this is likely due to an inability to localize to cell-cell interfaces without Notch1 cortical signaling. To conclude Notch1 cortical signaling functions through FAM83H, we show:

1) *FAM83H*^{KO} phenocopies the tissue and cell-cell adhesion defects observed in *NOTCH1*^{KO} cells (original submission, Fig 5H).
2) *NOTCH1*^{KO} cells have significantly reduced FAM83H-E-cadherin co-immunoprecipitation relative to SCR or *ICD*^{KO} cells (original submission, Fig 5I).
3) With methanol fixation, endogenous FAM83H localizes at and proximal to cell-cell interfaces in SCR and *ICD*^{KO} cells, but not *NOTCH1*^{KO} cells (Resubmission, Fig 5J).
4) A SNAP-tagged TMD (SNAP-TMD) complexes with E-cadherin and FAM83H when expressed in *NOTCH1*^{KO} cells (Resubmission, Fig 5K).
5) Expression of SNAP-TMD in *NOTCH1*^{KO} cells increases FAM83H-E-cadherin co-immunoprecipitation (Resubmission, Fig 5L).

Minor comments:

• Figure 1D, the X-axis title should be changed from "Duct diameter variance (μm)" to "Duct diameter (μm)". Also, specify the statistical test used to define p-values throughout all Figures.

In Fig 1D, we are reporting frequency of variance in duct diameter to quantitate duct tortuosity, so "Duct diameter variance" is the correct label. Figure legends include description of all statistical tests.

• Mention in the legend of Figure 2G that cells were treated with a high EGF concentration (20 ng/ml).

This has been added to the legend.

• Mention in in the legend of Figure 2H that it represents a comparison of 2 different concentrations of EGF (2 ng/ml vs 20 ng/ml).

This has been added to the legend.

• More generally, the use of bar charts should be restricted to the representation of counts or proportions. If the goal of the figures is to compare data distribution, please prefer boxplots or violin plots representations. Please change in every Figure where relevant.

We have updated all figures to include all statistical comparisons and changed graphs to violin plots where appropriate.

August 25, 2023

RE: JCB Manuscript #202303013R-A

Dr. Matthew L. Kutys
University of California, San Francisco
Department of Cell and Tissue Biology
513 Parnassus Avenue, HSW-613
San Francisco, CA 94143

Dear Dr. Kutys:

Thank you for submitting your revised manuscript entitled "Notch1 cortical signaling regulates epithelial architecture and cell-cell adhesion". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). Please also attend to remaining concerns (points 2 and 3) from Reviewer 3 by revising the text accordingly.

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, http://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 abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, figure legends, materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main figures and 5 supplemental figures/tables.
** Please reduce supplemental figures to 5.

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. Please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.
** Please include molecular weight markers on all protein gels.

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.

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. We also provide a report from SciScore and an associate score, which we encourage you to use as a means of evaluating and improving the methods section.

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 reconstitutions, 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 figures. 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 an ORCID ID for all authors.

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.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

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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).

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-- 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,

Ian Macara, Ph.D.
Editor
The Journal of Cell Biology

Tim Fessenden
Scientific Editor
Journal of Cell Biology

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

The authors have made substantial efforts to address all comments of this reviewer and the manuscript is now in great shape for publication

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

The authors have adequately addressed all the points that I raised.

I still have one minor comment:
The new data on presented in Fig. 2D on quantification of spindle angles is not presented in a reader-friendly format and is in fact quite difficult to grasp. I guess one challenge is the low n (13)? Perhaps Rose plots?

It also remained unclear what the degrees stand for (i.e. what is 0 degrees and what is 90 degrees) - one can kind of deduce that but a description in the Figure legend would be appropriate.
I also failed to find information on how the statistics in Fig. 2D was performed.

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

The revised manuscript has been improved by addressing important concerns raised by the 3 referees. I am fairly satisfied with the additional experiments and changes included in the revised manuscript. However, there are still 4 points which, in my view, have not been clarified:
1- The new Figure panels 3G-I provide important data showing that indeed only the TMD domain can rescue the Notch1-KO

phenotype, whereas ICD O/E fails to rescue. However, the cortical actin/AJ phenotypes of ICD O/E in the context of Notch1-KO look very different to me compared to the ones produced by Notch1-KO+GFP (in Fig. 3G). Why?
2- I still do not understand why deletion of ICD (in ICD-KO cells) does not elicit changes in expression of the direct Notch target genes HES1 and HEY1 (in Fig. S1B). Also, why cell polarization leads to a decrease in HES/HEY expression (in Fig. S5E)? Why DAPT increases ICD cleavage but no target transcription? and why no target upregulation is observed upon recombinant Dll4 activation of the pathway and receptor cleavage (in Fig. S5D)? In the Discussion (page 10), the authors propose that unclear intricate mechanisms, potentially linked to unverified nuclear mechanotransduction, may gate the transcriptional activity of cleaved ICD. I thus reiterate my previous point and still do not understand how the authors can discriminate between transcription-independent cortical signalling and ICD-dependent transcriptional signalling if the expected changes in direct transcriptional Notch targets are not observed in their experimental setting.
3- Regarding the co-IP between E-cadherin and FAM83H, in Fig. 5I the interaction between E-cad and FAM83H is decreased also in ICD-KO conditions compared to the SCR control. To clarify this point, the new experiments presented in Fig. 5K-L should be performed also in Notch1-KO+ICD O/E conditions, as in Fig. 3G. This would address the question if also ICD can interact with FAM83H and E-cadherin, although possibly with a lower affinity than TMD. In addition, these experiments could clarify if ICD O/E in a Notch1-KO context can cooperate with TMD to enhance the co-IP between E-cadherin and FAM83H, as shown for TMD alone in Fig. 5L.
4- Finally, the immunofluorescence for Notch1 in Fig. S4I shows an increase of Notch1 at the membrane only in some cells in the SCR+DAPT panel, where every cell should be equally exposed to DAPT, why? And in ICD-KO+DAPT, the Notch1 signal is still present with a non-homogeneous distribution in different cells, differently from the ICD-KO panel in Fig. S3A, where the signal is almost undetectable. These inconsistencies need to be addressed. What domain is recognized by the anti-Notch1 antibody used in these experiments? And how confident are the authors on the reliability of this antibody, which is also used in many other experiments?

- One last minor comment is that in response to my previous point #12: I do not think the authors should refer to "Notch1-KO monolayers" if this mutation induces hyperproliferative multilayered outgrowths and lumen cell filling; the authors say in the rebuttal: "We no longer use monolayer in reference to NOTCH1-KO cells". However, in the revised Results at page 4, I read: "SCR and NOTCH1-KO cells similarly adhere to the channel architecture and progressively form a monolayer" and in other sentences throughout the text the mutant multi-layered ducts are still named monolayers.