CD2AP links actin to PI3 Kinase activity to extend epithelial cell height and constrain cell area

Youn Wang and William Brieher

Corresponding Author(s): William Brieher, University of Illinois, Urbana-Champaign

Review Timeline:

| Event                        | Date       |
|------------------------------|------------|
| Submission Date              | 2018-12-17 |
| Editorial Decision           | 2019-01-25 |
| Revision Received            | 2019-08-26 |
| Editorial Decision           | 2019-10-01 |
| Revision Received            | 2019-10-13 |

Monitoring Editor: Ian Macara

Scientific Editor: Tim Spencer

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/N/A
January 25, 2019

Re: JCB manuscript #201812087

Dr. William M Brieher
University of Illinois, Urbana-Champaign
601 S. Goodwin Avenue
Urbana, IL 61801

Dear William,

We have now received reports from three external reviewers on your manuscript "CD2AP links actin to PI3 Kinase activity to extend epithelial cell height and constrain cell area". As you will see from the appended comments, they all found the work to be important and interesting. However, there was a general feeling by all three reviewers that the manuscript lacks some essential controls, is somewhat correlative, and falls short of demonstrating a convincing link by CD2AP between actin and PI3K activity. Unfortunately, therefore, we are unable to accept the manuscript in its present form. However, we would be willing to consider a suitably revised version that addresses the issues raised by the reviewers. We note that any revised manuscript would additionally require a point-by-point response to each of these issues, and would likely be returned to the same reviewers for evaluation.

Reviewer #1 is fairly enthusiastic but feels that the issue of the functional relevance of the CD2AP dependent recruitment of PI3K is not sufficiently resolved, and notes that potentially an effect of PI3K activity on cell proliferation would impact cell height. They also ask if downstream signaling (through AKT) is regulated by CD2AP.

Reviewer #2 feels that the main conclusion is not sufficiently supported. They also ask if CD2AP depletion alters PI3K expression. Both reviewers point out that the 1-355aa fragment does not localize properly, so cannot be used to determine if p85 binding is sufficient for function.

Reviewer #3 is concerned about missing controls for multiple experiments, and is concerned by the in vitro data on actin polymerization.

Overall, we feel that these comments are appropriate, and would need significant experimental work to address them, but that this is necessary to support your main conclusions.

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,
http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. 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.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. 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.

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

Sincerely,

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

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology
ORCiD: 0000-0003-0716-9936

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

This paper addresses the interesting problem of how cell height is regulated. This is still a poorly-understood question, but one of obvious significance given that the heights of epithelial and endothelial cells differ between tissues, but are consistent within tissues: this implies that cell height must be physiologically regulated. Earlier studies reported roles for the actomyosin cytoskeleton and signalling (notably PI3-kinase), but these were essentially phenomenological and have yet to gel into a coherent mechanistic picture. Here the authors bring these elements together to show that CD2AP recruits PI3K to the cortex (especially at cell-cell junctions) to promote cell height. A simple model would have CD2AP act as a cortical recruiter of PI3K, whose downstream signalling then controls cell height. One interesting twist here is the authors’ evidence that some kind of feedback loop is involved, where PI3K can regulate F-actin which can, in turn,
influence both CD2AP and PI3K localization.

Overall, I think that the authors are on to something that may be very interesting. Although individual elements of the story have been reported before (e.g. a role for PI3K in controlling cell height, the association of p85 with CD2AP), a strength of the paper is how it can bring these disparate observations into a potentially novel, larger picture. However, to my mind that picture could be clarified if the authors were to address a few open questions.

1. A key question is how much of the functional impact of CD2AP in MDCK cells is mediated by its recruitment of PI3K. My impression (which might be wrong) is that much of the data would fit with a model where PI3K mediates the impact of CD2AP on cell height (e.g. the data in Fig 8) - i.e. CD2AP would recruit PI3K to regulate cell height. But the authors conclude that the interaction between the two proteins is not sufficient, because CD2AP 1-335, which can bind p85, doesn't rescue F-actin or cell area. However, the CD2AP 1-335 mutant doesn't appear to localize to junctions. If so, it remains possible that all CD2AP needs is to be both junctional and to recruit p85. Is it possible for the authors to target CD2AP 1-335 to junctions, e.g. by fusion with a cadherin or catenin?

2. The impact of actin on the CD2AP-PI3K pathway. In the model shown in Fig 5E the authors postulate that actin regulation downstream of PI3K can feed-back to influence CD2AP. However, this doesn't readily fit with their observation that LY29004 decreases junctional F-actin but not junctional CD2AP. Can they resolve this by providing further insight into the molecular nature of that feedback? In particular, in this, and earlier work, they show that CD2AP stabilizes F-actin. Might this be involved in the feedback: perhaps this serves, in turn, to stabilize CD2AP to effectively recruit PI3K?

3. Is there a relationship to cell population control? The authors show that CD2AP and PI3K inhibition causes both cell shortening and spreading. If the monolayers are confined to the same overall area, does this imply that cell number is reduced? If so, might control of cell number be the mechanism that allows CD2AP-PI3K to control cell height (fewer cells, less cell-cell compression, flatter cells?)? Or might CD2AP-PI3K coordinately regulate a pathway that can influence both proliferation and mechanics (e.g. Yap/Taz)?

Specific issues

a) Is downstream signalling from PIP3 modulated by CD2AP? The authors show that cortical/junctional levels of PIP3 require CD2AP. Does this translate to downstream signalling? For example, are phospho-Akt levels altered? (This could be easily tested by western analysis.)

Small (near-trivial) details

i) How do the authors distinguish between junctional and cortex (i.e. define the difference) for their quantitative analyses of fluorescence?

ii) The text refers to co-IP of p110 and CD2AP and references data in Fig 3F. But Fig 3F is the PIP3 biosensor. The p110 data seems to have gotten lost.

iii) Fig 4e. Could they authors label the X-axis to identify which are control or LY-treated?
Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Wang et al. investigated the mechanisms that determine the shape of epithelial cells. Previous work has demonstrated an important role for PIP3 in apical-basal polarity and promoting epithelial cell height (Gassama-Diagne et al., 2006; Roman-Fernandez et al., 2018). In this paper, the authors suggest that the actin-binding protein CD2AP promotes epithelial cell height by recruiting PI3-Kinase to lateral contacts between cells. Specifically, they show that knock-down of CD2AP reduces epithelial height. Next, they show that CD2AP co-localizes with a PI-3K and that knock-down of CD2AP reduces the level of PI-3K. In addition, disrupting actin decreases the intensity of both PIP3 and CD2AP.

The connection between actin and phosphoinositide synthesis is an important question, however, I felt like the authors fell short of clearly demonstrating that CD2AP links actin to PI3 Kinase activity, which may have been, in part, their presentation of the data. See my detailed criticisms below:

Main caveats:

1) I think the point that the authors can separate the actin-binding and PI3-Kinase function should be made more clear. I missed it the first time I read through the paper because key experiments are presented separately. Some suggestions are: 1) Characterize 1-475aa, 1-335aa, and 336-605aa fragments all in the same figure. Currently the quantitations are on different axes and you can't compare the relative contributions of each fragment. 2) Showing that 336-605aa binds actin is really important and I suggest putting it in the main figure.

2) The authors test whether p85α interaction is sufficient to rescue CD2AP depletion and suggest that that is not the case (1-335 a. a. expression does not rescue F-actin levels nor cell spreading area). However, this could be because the 1-355 a. a. protein fragment does not localize (or express) properly to the membrane (this should be shown), thus failing to properly localize PI3K at the lateral membranes. Could expressing of a myristoylated-form of PI3K rescue CD2AP depletion? This, if possible, would be the most convincing experiment to demonstrate that PI3K is downstream of CD2AP.

3) PIP3 exhibits apical-basal polarity (Gassama-Diagne et al., 2006; Roman-Fernandez et al., 2018) and yet it seems like these treatments are causing global decreases in F-actin. Why is it that when you have PIP3 enriched in a specific domain, the effect is not restricted to that domain? Are the cells losing polarity?

4) For the cross-section images of cells illustrating the height, one cannot see the cells. Need better images or labeling.

5) Does CD2AP affect localization or possibly protein levels? The authors need to check PI3-K protein levels by Western to show that CD2AP knock-down is not altering PI3-K expression. In addition, the expression of all CD2AP fragments should be tested to determine whether they are expressed at equivalent levels.

Minor points:
1) Check the manuscript carefully for grammar. In particular, the last few sentences of the "N-
terminal CD2AP inhibits barbed-end dynamics of actin filaments" section.

2) In the 3rd results section, there are references to figures that do not exist, such as p110y levels. The line scans showing a correlation of peak CD2AP signal and PH-GFP signal is Figure 3E but the authors list Figures 3G, H, and I (panels H and I are not present in the manuscript). Other parts in this section also incorrectly recall figure panels.

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

Wang and Brieher examine the role of CD2AP in epithelial polarization using shRNA of CD2AP in MDCK cells. The find that a modest knockdown of CD2AP expression results in a dramatic loss of actin polymerization and flattening of the cells. Because the phenotype is similar to cells that are treated with PI3 kinase inhibitors and because CD2AP is known to bind to CD2AP, they then focus on PI3 kinase localization and activation. They show that CD2AP colocalizes with the P85 subunit of PI3 kinase in punctate structures in the adherens junctions and that loss of CD2AP also results in lower P85 as well as diminished PIP3 in cellular junctions. This was also phenocopied when actin was inhibited with LatrunculinB. They were able to rescue the phenotype with overexpression of a construct expressing the N-terminal half of CD2AP consisting of the SH3 domains and the proline motifs. Surprisingly, the C-terminal coiled-coiled domain, and the capping protein binding motif were not required. To verify that this fragment of CD2AP has some actin regulatory capacity, the 1-475 fragment of CD2AP was used to interrogate its potential role in actin polymerization in vitro and it did slow actin polymerization.

The described phenotype is quite dramatic and implicates CD2AP as an important regulator of actin polymerization in polarized epithelium. Given how important this observation is, it is critical that controls are in place. However, important controls are lacking in many of the experiments. In addition, since the SH3 domains of CD2AP bind a wide variety of different binding partners including ASAP, ARAP, SH3BP1, RICH1, it is unclear whether the mechanism can be completely explained by the p85 interaction of CD2AP. Most of the data is correlative, and other binding partners were not ruled out.

1. In figure 1, actin staining of WT and an CD2AP ShRNA cell line are shown. The reduction in CD2AP expression is only about 50%, but the phenotype is dramatic. While the phenotype is certainly consistent with previous publications, it would be important to show that this is not a clonal phenotype. Is the nuclear staining background? Or does this represent the residual CD2AP expression as 50% reduction would not likely lead to the complete absence of signal as shown in 1A.

2. In figure 3, there is a correlation between decreased CD2AP expression and decreased p85 expression. A relocalization of p85 from membranes to the cytosol might be expected, but this appears to be a loss of expression. Immunoblotting for p85 might help interpret these images. The callout to Figure 3F in the text refers to a co-IP, but the figure 3F is quantitation of the image.

3. Figure 4 shows results of cells treated with PI3K inhibitor where CD2AP localization is unchanged, but there is a change in cell morphology and dramatic loss of p85. The low power images show that in some cells, there is a dramatic change in CD2AP localization, not see in the high power image and what appears to be increased CD2AP expression. Immunoblotting for p85 and CD2AP would be useful here to support the statement in the text that there is no change in CD2AP levels.

4. Figure 5 shows that latrunculin treatment results in decreased CD2AP and p85 localization at cell
junctions. A simple interpretation of this data could argue that impaired actin polymerization induced by CD2AP downregulation can also explain all of the data. The cell spreading and height is not shown for these experiments. The text states that CD2AP and p85 levels significantly drop, but this statement would require immunoblotting.

5. Figure 6 shows the rescue with various CD2AP mutants, but the FL CD2AP control is missing. It would also be important to know what level of overexpression is being generated here. An important caveat to these experiments is the presence of potentially about 50% of the WT FL CD2AP which could be complementing some of the function of the mutants. The simplest explanation is that one of the other proline motifs is important or that multiple proline motifs are important. Some more mechanistic experiments might be useful here.

6. Figure 7 shows the effect of the 1-475 CD2AP mutant on in vitro actin polymerization. The data is somewhat surprising and concludes unsatisfyingly that there must be some type of biochemical activity in the segment of CD2AP that extends beyond the SH3 domains. Again, positive and negative controls are missing here like the full-length protein as well as the 1-335 mutant.
We sincerely thank all three reviewers for taking the time to review our manuscript to make it better. We appreciate how much time and effort it takes to review a long paper. We have responded to most of your questions and suggestions with either an experiment or by changing the text. We also thank all three reviewers for excellent suggestions worthy of future follow-up studies. The main changes to the manuscript are a test to see if changes in cell height are due to cell density/packing (figure 2), whether targeting the SH3 domains or PI3K to the membrane rescues CD2AP knockdown phenotypes (figure 8), and whether full length CD2AP or the SH3 domains alone inhibit actin polymerization (figure 9). Other control experiments and changes in the text were also done. Thank you again for your critiques. Detailed responses are below.

**Reviewer #1**

1. A key question is how much of the functional impact of CD2AP in MDCK cells is mediated by its recruitment of PI3K. My impression (which might be wrong) is that much of the data would fit with a model where PI3K mediates the impact of CD2AP on cell height (e.g. the data in Fig 8) - i.e. CD2AP would recruit PI3K to regulate cell height. But the authors conclude that the interaction between the two proteins is not sufficient, because CD2AP 1-335, which can bind p85, doesn’t rescue F-actin or cell area. However, the CD2AP 1-335 mutant doesn’t appear to localize to junctions. If so, it remains possible that all CD2AP needs is to be both junctional and to recruit p85. Is it possible for the authors to target CD2AP 1-335 to junctions, e.g. by fusion with a cadherin or catenin?

We thank the reviewer for this excellent idea! We fused EGFP-CAAX (Zhang et al., 2014) at the C-terminus to CD2AP 1-329, and expressed it in CD2AP knockdown MDCK cells. We show that CD2AP-1-329-EGFP-CAAX localized to the plasma membrane (Figure S3D), and quantification of immunofluorescence images show that CD2AP-1-329-EGFP-CAAX restored F-actin and PI3K p110γ along cell borders and constrained cell area back to wild-type cell levels (Figure 8, A-E). CD2AP-1-329-EGFP-CAAX is a tad toxic in epithelial cells, and we had a hard time getting stable monolayers with these cells. Nevertheless, we could score the rescue in subconfluent cells and obtain enough data to conclude that one major function of CD2AP in epithelial cell morphology is to recruit PI3K to the membrane.

2. The impact of actin on the CD2AP-PI3K pathway. In the model shown in Fig 5E the authors postulate that actin regulation downstream of PI3K can feed-back to influence CD2AP. However, this doesn't readily fit with their observation that LY29004 decreases junctional F-actin but not junctional CD2AP. Can they resolve this by providing further insight into the molecular nature of that feedback? In particular, in this, and earlier work, they show that CD2AP stabilizes F-actin. Might this be involved in the feedback: perhaps this serves, in turn, to stabilize CD2AP to effectively recruit PI3K?

Drawing the feedback loop to CD2AP is speculative. We talk about the model in greater detail now in the discussion section. The general idea is that CD2AP binds to PI3K, and it binds to F-actin. So, it could be just polymer mass. The more actin polymer generated
near cell-cell contacts, the more CD2AP binding sites, and therefore more PI3K binding sites near the membrane. Again, it's speculative, and we state that it is speculative in the discussion. The molecular nature of the feedback loop from actin to PI3K is certainly a topic of interest for the future. We think that could get complicated, and it will require a number of experiments to sort out.

3. *Is there a relationship to cell population control?* The authors show that CD2AP and PI3K inhibition causes both cell shortening and spreading. If the monolayers are confined to the same overall area, does this imply that cell number is reduced? If so, might control of cell number be the mechanism that allows CD2AP-PI3K to control cell height (fewer cells, less cell-cell compression, flatter cells?)? Or might CD2AP-PI3K coordinately regulate a pathway that can influence both proliferation and mechanics (e.g. Yap/Taz)?

Another excellent point. It is established that PI3K-Akt signaling regulates cell cycles, so changes in membrane areas might be a by-product of cell packing. We compared cell height and cell area in sparse cultures. Under these conditions, we still see cell area and cell height phenotypes resulting from CD2AP depletion. Therefore, changes in spread area and height are not due to cell packing. This data is presented in figure 2 in the revised manuscript. Of course, there still could be/probably is a proliferation phenotype as the reviewer suggests, but cell crowding is not necessary for the changes in membrane proportions. Examination of CD2AP effects on cell population control are underway.

*Specific issues*

a) *Is downstream signalling from PIP3 modulated by CD2AP?* The authors show that cortical/junctional levels of PIP3 require CD2AP. Does this translate to downstream signalling? For example, are phospho-Akt levels altered? (This could be easily tested by western analysis.)

We agree that the downstream signaling pathway from PIP3 is major question. What we would like to do is start from scratch in a new study to see if CD2AP is signaling through Akt or some other pathway to the actin cytoskeleton. Some papers out there argue PIP3 regulates actin through Akt. Others show Akt-independent signaling. It's likely, then, that the signaling pathway will depend on cell type and context, which is why we would like to start this line of investigation from ground zero.

i) *How do the authors distinguish between junctional and cortex (i.e. define the difference) for their quantitative analyses of fluorescence?*

Previous results from our lab (Tang and Brieher, 2013, JCB) had a detailed analysis of CD2AP dependent actin organization in these cells. Wording from that paper crept into this manuscript, but we are not looking at the actin at the level of detail, here. We replaced “junctional” with “lateral”. By “cortex” we are referring to the “apical actin cortex” and have adjusted the manuscript accordingly.
ii) The text refers to co-IP of p110 and CD2AP and references data in Fig 3F. But Fig 3F is the PIP3 biosensor. The p110 data seems to have gotten lost.

We really apologize for that. We accidentally uploaded an old version of the Figure. P110γ data is now provided in Figure 4, D-F.

iii) Fig 4e. Could they authors label the X-axis to identify which are control or LY-treated?

We labeled the figures as per the reviewer’s request. It can be found in Figure 5E.

Reviewer #2

Main caveats:
1) I think the point that the authors can separate the actin-binding and PI3-Kinase function should be made more clear. I missed it the first time I read through the paper because key experiments are presented separately. Some suggestions are: 1) Characterize 1-475aa, 1-335aa, and 336-605aa fragments all in the same figure. Currently the quantitations are on different axes and you can’t compare the relative contributions of each fragment. 2) Showing that 336-605aa binds actin is really important and I suggest putting it in the main figure.

We thank the reviewer for this suggestion, which is a better way to present the data. We combined characterizations of the fragments in figure 7. We moved the actin binding data 336-605 to the main figures (figure 9M).

2) The authors test whether p85α interaction is sufficient to rescue CD2AP depletion and suggest that that is not the case (1-335 a.a. expression does not rescue F-actin levels nor cell spreading area). However, this could be because the 1-355 a.a. protein fragment does not localize (or express) properly to the membrane (this should be shown), thus failing to properly localize PI3K at the lateral membranes. Could expressing of a myristoylated-form of PI3K rescue CD2AP depletion? This, if possible, would be the most convincing experiment to demonstrate that PI3K is downstream of CD2AP.

We really appreciate this thoughtful and constructive comment. We performed the experiment, and it worked! The result is shown in figure 8. Reviewer 1 had a similar idea, and a detailed answer can be found above in our response to reviewer 1.

3) PIP3 exhibits apical-basal polarity (Gassama-Diagne et al., 2006; Roman-Fernandez et al., 2018) and yet it seems like these treatments are causing global decreases in F-actin. Why is it that when you have PIP3 enriched in a specific domain, the effect is not restricted to that domain? Are the cells losing polarity?

Another good question. The cells are losing a lot of actin in the “apical” membrane when PI3K
is inhibited. Yet, the kinase isn’t in that domain. We don’t have explanation for this. Loss of polarity is a good lead hypothesis. We should look at cell polarity as a function of PI3K and actin and CD2AP, but the paper is getting kind of big now, so in order to do a good job on polarity, we should probably reserve that for another study.

4) For the cross-section images of cells illustrating the height, one cannot see the cells. Need better images or labeling.

We have how provided better orthogonal view images illustrating cell height in Figure 1E and Figure 5B.

5) Does CD2AP affect localization or possibly protein levels? The authors need to check PI3-K protein levels by Western to show that CD2AP knock-down is not altering PI3-K expression. In addition, the expression of all CD2AP fragments should be tested to determine whether they are expressed at equivalent levels.

Following the reviewer’s suggestion, we have now included western blot quantification data in Figure S1, showing that CD2AP knock-down does not alter PI3-K expression. In addition, we provide plots of rescued actin intensity versus expression levels of all CD2AP fragments by immunofluorescence staining (Figure 7, C-H). The expression levels of all CD2AP constructs in shCD2AP-MDCK cells and endogenous CD2AP in wild-type MDCK cells are readily comparable. Since the transfection rates and the amount of cDNA expression in individual cells varied, and western blot only gives total protein expression, would not properly reflect the heterogeneity, we did not use it to evaluate the efficiency of different CD2AP fragments.

Minor points:
1) Check the manuscript carefully for grammar. In particular, the last few sentences of the "N-terminal CD2AP inhibits barbed-end dynamics of actin filaments" section.

We thank the reviewer for pointing this out, we have rewrote that paragraph, please see the content under subtitle: “The N-terminal and C-terminal segments of CD2AP bind to F-actin”.

2) In the 3rd results section, there are references to figures that do not exist, such as p110γ levels. The line scans showing a correlation of peak CD2AP signal and PH-GFP signal is Figure 3E but the authors list Figures 3G, H, and I (panels H and I are not present in the manuscript). Other parts in this section also incorrectly recall figure panels.

We really apologize for that. We accidentally uploaded an old version of the Figure. P110γ data is now provided in Figure 4, D-F.
Reviewer #3

1. In figure 1, actin staining of WT and an CD2AP ShRNA cell line are shown. The reduction in CD2AP expression is only about 50%, but the phenotype is dramatic. While the phenotype is certainly consistent with previous publications, it would be important to show that this is not a clonal phenotype. Is the nuclear staining background? Or does this represent the residual CD2AP expression as 50% reduction would not likely lead to the complete absence of signal as shown in 1A.

We understand the concern. The phenotype is not clonal. We didn’t pick a single colony and propagate it for use throughout the study. The data for the study involved dozens of knockdowns and rescues. We saw dramatic effects on actin with 50% knockdown in our previous paper, and you can see evidence that the depleted cells were occupying more space in XY in that paper. That paper also required dozens of knockdowns.

The nuclear staining is mysterious. We see it in the knockdown cells. Don’t see it very much in the wildtype cells in this manuscript or in our previous paper on CD2AP (Tang and Brieher, JCB, 2013). So, it could be the relocalization of the residual CD2AP, or the appearance of a non-specific epitope in the knockdown cells. In the future, we should move to knocking CD2AP out with genome editing to clarify the nuclear signal.

2. In figure 3, there is a correlation between decreased CD2AP expression and decreased p85 expression. A relocalization of p85 from membranes to the cytosol might be expected, but this appears to be a loss of expression. Immunoblotting for p85 might help interpret these images. The callout to Figure 3F in the text refers to a co-IP, but the figure 3F is quantitation of the image.

As suggested by the reviewer, we now provide western blots of PI3-K p85α and p110γ in Figure S1, showing that CD2AP knock-down does not alter PI3-K expression. We think that the significant p85α signal drop may be due to dilution effects from cell expansion. We mistakenly uploaded the wrong version of Figure 3 last December, really apologize for that, the complete data is now provide in Figure 4.

3. Figure 4 shows results of cells treated with PI3K inhibitor where CD2AP localization is unchanged, but there is a change in cell morphology and dramatic loss of p85. The low power images show that in some cells, there is a dramatic change in CD2AP localization, not see in the high power image and what appears to be increased CD2AP expression. Immunoblotting for p85 and CD2AP would be useful here to support the statement in the text that there is no change in CD2AP levels.

We have removed the line stating that there is no change in CD2AP levels. If CD2AP levels did respond to PI3Kinase, that would be interesting, but it doesn’t add to this story. The “dramatic change in cell localization” seen in some cells in the low magnification image is
actually disruption of the cell monolayer because some of the cells are starting to round up. That’s why the high magnification image comes from a region that doesn’t contain rounded up cells.

4. Figure 5 shows that latrunculin treatment results in decreased CD2AP and p85 localization at cell junctions. A simple interpretation of this data could argue that impaired actin polymerization induced by CD2AP downregulation can also explain all of the data. The cell spreading and height is not shown for these experiments. The text states that CD2AP and p85 levels significantly drop, but this statement would require immunoblotting.

We are not sure if we understand the question. While CD2AP inhibits actin assembly in vitro, it promotes actin assembly in cells. Cell spreading data is provided. We didn’t measure cell height at this point because our previous work showed that disrupting actin assembly via knockdown of CRMP1, EVL, WAVE2, and Arp2/3 results in thin, highly spread cells (Yu-Kemp, Kemp, and Brieher, JCB, 2017). We altered the text to state “Quantification showed that actin, CD2AP, and PI3K levels at cell-cell boundaries significantly dropped upon LatB treatment (Fig. 6, B and C)…” which is supported by the data.

5. Figure 6 shows the rescue with various CD2AP mutants, but the FL CD2AP control is missing. It would also be important to know what level of overexpression is being generated here. An important caveat to these experiments is the presence of potentially about 50% of the WT FL CD2AP which could be complementing some of the function of the mutants. The simplest explanation is that one of the other proline motifs is important or that multiple proline motifs are important. Some more mechanistic experiments might be useful here.

We added the FL CD2AP rescue experiment to Figure 7. By quantifying fluorescence, most of the constructs are over-expressed by about 20-50% over the endogenous levels present in wildtype cells (figures 7C – 7H). Linear regression analysis shows that for most of the constructs that rescue actin, the correlations between CD2AP expression and actin intensity are matched, suggesting the rescuing construct is using a related/similar mechanism for actin assembly as the endogenous. The correlations do not hold for the construct lacking one of the proline rich sequences showing that this construct is less efficient at rescuing actin.

Regarding mechanism, we targeted the SH3 domains alone and PI3 Kinase alone to the plasma membrane and scored for actin rescue and restoration of cell proportions. Those results are presented in figure 8 in the revised manuscript.

6. Figure 7 shows the effect of the 1-475 CD2AP mutant on in vitro actin polymerization. The data is somewhat surprising and concludes unsatisfyingly that there must be some type of biochemical activity in the segment of CD2AP that extends beyond the SH3 domains. Again, positive and negative controls are missing here like the full-length protein as well as the 1-335 mutant.
We corrected our original hypothesis thanks to the reviewer’s suggestion. We quantified actin elongation rates in the presence or absence of FL CD2AP or 1-335 mutant (Figure 9, A-D), to our surprise, 1-335 retards F-actin growth. 1-335 does not rescue F-actin and cell shape because it’s not on the plasma membrane. From these new results, we now conclude that CD2AP’s binding to F-actin and suppression of (+) end actin assembly is not sufficient to account for its function in cells.
October 1, 2019

RE: JCB Manuscript #201812087R

Dr. William M Brieher
University of Illinois, Urbana-Champaign
601 S. Goodwin Avenue
Urbana, IL 61801

Dear Dr. Brieher:

Thank you for submitting your revised manuscript entitled "CD2AP links actin to PI3 Kinase activity to extend epithelial cell height and constrain cell area". The paper has no been seen again by the original reviewers and, although reviewers #1 and 2 have some minor remaining points, all three now recommend acceptance. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

Please respond to and/or address the remaining reviewer issues in the final revised manuscript. Also please be sure to provide a point-by-point rebuttal to each of these remaining reviewer comments.

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 and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) 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.

3) 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 (both in the figure legend itself and 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, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must
state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) 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 (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) 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.

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

7) 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.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools 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.

9) 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."

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

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

The authors have reasonably addressed all the questions that I raised in my earlier review. This is an informative paper and I think it makes a valuable contribution to a still-neglected area of cell biology.

There are only two tiny issues that I noticed in Fig 8 (which could be handled with a very minor revision).

1) I can see that p110 staining is restored at cell-cell contacts in 8B, but it looks like staining for p85 not restored (8a). Why would this be? Perhaps this is just a consequence of the limits of the
immunofluorescence reagents.
2) What does "MII" refer to in 8C-E?

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

The authors have very much improved the organization and logic of this paper. There were a few points that were confusing and could use clarification in the writing, and there were some minor typos, but otherwise I found the paper to be straightforward.

Conceptual points:

1) Figure 9: The idea that CD2AP links F-actin to PI3K is well supported by most of the data, but in the last figure they show that CD2AP 1-335 inhibits actin assembly, suggesting a linkage. If CD2AP 1-335 can bind actin and PI3K, why does it not rescue the knockdown?

Minor typos:
1) "CD2AP colocalizes with and binds to PI3K p85a at cell-cell boundaries." First sentence: ‘The flatted cells . . .’ should be flattened cells?

2) "PI3K and PIP3 membrane recruitment is diminished in CD2AP knockdown MDCK cells": Should be PIP3 synthesis and PI3K recruitment to be precise.

3) Figure 4F and other images of gels: I think it is journal policy to have size markers on all gel images.

4) "Effective cell area constriction requires SH3 domains of CD2AP and recruiting PI3K" section: ‘Our data further support the idea that maintaining proper epithelial cells architecture . . .’ cells should be cell.

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

I'm satisfied with the revisions.
We thank the reviewers for their most recent comments on our manuscript. Thank you all for your efforts. Specific responses are below.

**Reviewer #1**

The authors have reasonably addressed all the questions that I raised in my earlier review. This is an informative paper and I think it makes a valuable contribution to a still-neglected area of cell biology. There are only two tiny issues that I noticed in Fig 8 (which could be handled with a very minor revision).

1) I can see that p110 staining is restored at cell-cell contacts in 8B, but it looks like staining for p85 not restored (8a). Why would this be? Perhaps this is just a consequence of the limits of the immunofluorescence reagents.

Good point. Looks like we picked a bad image that doesn’t reflect the overall result. Perhaps we were too enamored with the morphology of those cells, that we didn’t pay close attention to the p85α staining. Other images show p85α accumulating at the junction. To make sure, we repeated the experiment and found many cells with p85α targeted to the membrane. We updated figure 8A, 8B to show the results that are more representative of the actual result.

2) What does "MII" refer to in 8C-E?

Sorry. Lab jargon that spilled into the figures. MII is our code for MDCK II cells. We changed the figure such that it now reads “WT-MDCK”.

**Reviewer #2**

The authors have very much improved the organization and logic of this paper. There were a few points that were confusing and could use clarification in the writing, and there were some minor typos, but otherwise I found the paper to be straightforward.

Conceptual points:

1) Figure 9: The idea that CD2AP links F-actin to PI3K is well supported by most of the data, but in the last figure they show that CD2AP 1-335 inhibits actin assembly, suggesting a linkage. If CD2AP 1-335 can bind actin and PI3K, why does it not rescue the knockdown?

Good question. Evidently binding to PI3K and to actin is not sufficient to rescue the knockdown. Reviewer #1 suggested an experiment where we targeted the SH3 domains of CD2AP to the membrane. This construct rescues. Therefore, it appears that CD2AP SH3
domains lack a membrane targeting region that is necessary for its biological activity in those processes that we are measuring. We added a comment about this to the discussion section that other unknown CD2AP interacting proteins are necessary for its recruitment to the membrane and for its ability to promote actin assembly, PI3K signaling, and lateral membrane extension. Identifying the putative CD2AP receptor on lateral membranes is high priority project for future research.

Minor typos:
1) "CD2AP colocalizes with and binds to PI3K p85a at cell-cell boundaries." First sentence: 'The flatted cells . . .' should be flattened cells?

2) "PI3K and PIP3 membrane recruitment is diminished in CD2AP knockdown MDCK cells": Should be PIP3 synthesis and PI3K recruitment to be precise.

3) Figure 4F and other images of gels: I think it is journal policy to have size markers on all gel images.

4) "Effective cell area constriction requires SH3 domains of CD2AP and recruiting PI3K" section: 'Our data further support the idea that maintaining proper epithelial cells architecture . . .' cells should be cell.

Thank you for pointing these out. We have corrected the typos, and size markers are on all the gels.

Reviewer #3

I'm satisfied with the revisions.

Thank you.