Multivalent interactions make adherens junction-cytoskeletal linkage robust during morphogenesis

Kia Perez-Vale, Kristi Yow, Ruth Johnson, Amy Byrnes, Tara Finegan, Kevin Slep, and Mark Peifer

Corresponding Author(s): Mark Peifer, UNC-Chapel Hill

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June 1, 2021

Re: JCB manuscript #202104087

Dr. Mark Peifer
UNC-Chapel Hill
Department of Biology University of North Carolina at Chapel Hill CB#3280; Coker Hall
Chapel Hill, NC 27599-3280

Dear Dr. Peifer,

We have now received feedback on your interesting manuscript "Multivalent interactions make adherens junction-cytoskeletal linkage robust during morphogenesis" from three external referees with expertise in the field. As you will see from the appended comments, all the referees voiced considerable enthusiasm for the study, but a few issues were raised that will need to be addressed before we can move forward with publication.

In particular, reviewer #1 raised a number of concerns, including the need for independent validation of delta-FAB mutants (point 2). With respect to reviewer #1's point 1, we agree with the reviewer that experimental examination of the role of the Rap1-binding RA domains in mechanical signal transduction would significantly increase the impact of the study, and we would encourage you to consider including the suggested experiments. However, we acknowledge that this is not strictly needed to support the main conclusions of the paper so we will not require such experiments for resubmission. We hope that you will be able to address each of the reviewers' other points, though, most of which are minor and will involve only textual modifications.

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.

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***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.

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

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

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu.

Sincerely,

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

Lucia Morgado Palacin, PhD
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Reviewer #1 (Comments to the Authors (Required)):

This paper investigates three well-characterized protein interaction domains of the adherens junction regulator Canoe/Afadin, asking whether they are required for Canoe localization and function in the Drosophila embryo. The focus is on detailed phenotypic analyses of domain deletion alleles generated at the canoe locus to understand how individual domain deletions impact endogenously expressed Canoe. A crystal structure of the PDZ domain is reported and used to design the PDZ domain deletion. A previously characterized null canoe allele was used to judge the severity of each domain deletion allele. Surprisingly, deletion of the PDZ domain, which can bind AJ receptors E-cadherin and Echinoid, had minimal effect on Canoe localization, embryo ectoderm morphogenesis, or development into viable and fertile adult flies. Although slightly more impactful, deletion of the FAB domain, known to mediate actin interactions, also had relatively mild effects, and permitted development of adult flies. Deletion of the Rap1-binding RA domains had the strongest effects: the expressed protein failed to localize to high-tension AJs, where Canoe normally does; tissue markers were disrupted almost as much as in null canoe mutants; and full embryonic lethality occurred. The paper makes/consolidates several important points about
Canoe/Afadin: (1) domains shown to mediate interactions with key components of AJs are dispensable; (2) a phosphorylation site recently reported to be required for mechanical signal transduction is dispensable when removed from the endogenous locus rather than from over-expressed protein; and (3) the Rap1-binding RA domains seem essential for mechanical signal transduction. Drawing these conclusions from detailed in vivo phenotypic analyses of endogenously expressed domain deletion alleles is a strength of the paper, but issues should be addressed.

1. For publication in JCB, I would expect further characterization of the role of the Rap1-binding RA domains in mechanical signal transduction. Compared to WT Canoe, how is Canoe-deltaRA affected by experimentally decreasing and increasing junctional actomyosin activity? Also, the Peifer lab recently showed that the Rap1-GEF Dizzy is required for Canoe localization to tri-cellular junctions, sites of high junctional tension, and that Dizzy has some enrichment at these sites-how is Dizzy affected by decreasing and increasing junctional actomyosin activity? Experiments like these would be informative, whether results are positive or negative. Since a number of the paper's key results confirm past results involving over-expressed proteins in Drosophila or mammalian cell culture, greater characterization of the role of the Rap1-binding RA domains would increase the paper's contributions to the field.

2. The main strategy to delete the PDZ domain and the FAB domain resulted in domain-deleted proteins being co-expressed with a small of amount of truncated protein containing the domains of interest. The viability of delta-FAB mutants was confirmed with a distinct domain deletion approach, but this was not done for the delta-PDZ mutants. Thus, it remains possible that the mild phenotype of the delta-PDZ mutant is the result of inter-allelic complementation, possibly through the formation of functional complexes containing complementary forms of abnormal Canoe. This is a missing piece of an otherwise thorough genetic analysis.

Minor concerns

1. The authors say "cno null mutants are zygotically lethal, and reintroducing CnoWT restored zygotic and M/Z viability and fertility (Fig 5A), verifying our "knock-in" rescue strategy." It seems the comparison should be with the cno-deltaRA mutants, since CnoWT was knocked-in to the cno-deltaRA locus.

2. It should be clarified in the text whether the cno-deltaFAB mutants described in Fig S5 (generated by the distinct domain deletion approach) were only used in this figure, or also in other figures.

3. In the third line of the Fig 2 legend, "Dorsal closure" should be "Ventral closure".

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The manuscript from Perez-Vale et al describes what constitutes a detailed "structure-function" study of Canoe, the Drosophila homolog of the conserved family of Afadin scaffold proteins, best known perhaps for their role as linkers between epithelial cell adherens junctions and cortical microfilaments. In their analysis, the authors focus on three major domains of the Canoe protein: the (pair of) N-terminal Rap GTPase binding domains RA1 and RA2; the central PDZ domain, through which Canoe binds cell-surface adhesion proteins; and the C-terminal actin-binding domain (FAB). The authors separately delete each of these three domains, in the context of the endogenous canoe gene locus, and assess the phenotypic consequences on several key processes of early Drosophila embryogenesis, an established setting for elucidating the cell-biological basis of developmental events, as well as on the localization pattern of the mutant Canoe proteins at this developmental stage. In addition, the authors report on the crystal structure of the Canoe PDZ domain, when complexed with the C-terminal region of the adhesion protein Echinoid.

Major observations made in the study include:

- Strong requirements for intact RA domains, underscoring the significance of association with Rap-GTPase for Canoe function and mechanosensitive localization;
- A surprisingly weak requirement for an intact PDZ domain for both Canoe localization and function;
- Presence of an intact FAB domain, considered to be responsible for interfacing with the cytoskeleton, is shown to be functionally important, although considerably less critical than what might be expected.

I found this to be a thorough, well-designed and well-executed study. My criticisms and suggestions for improvement have to do primarily with (non-trivial) matters of data presentation and manuscript organization (see below). A separate issue to consider relates to the significance of the reported results and the progress they represent. There is certainly much to learn here for researchers specifically interested in Canoe (and by inference- Afadin family protein) structure and function. The more general take-home message proposed by the authors, favoring a view of adherens junctions as complex structures, whose functional output relies on multiple, partially overlapping/compensatory interactions between the relevant molecular elements- rather than on a simple signal-transduction pathway- is a reasonable, albeit not-unexpected conclusion of this study, but which is bound to motivate future study of these issues.

Manuscript improvement issues:

1. The Results section and Figure 1 should begin with a clear description and visual presentation of the experimental framework. The schematics of Cno proteins used in Figure 4A (to which the CnoΔRA variant should be added) are, for example, appropriate for achieving this purpose. Furthermore, the detailed description of the CRISPR-based gene editing scheme used to (serendipitously) generate CnoΔRA- which should be reported, of course- is distracting and not of general interest, and much of it can justifiably appear as supplementary data.

2. The organization of the main text (and in particular, the portion describing the data appearing in Figures 3 and 4) is confusing at times, and requires considerable back-and-forth from the reader. I would recommend that, at the very least, most of the text referring to Figures 4 and 5 precede that associated with Figure 3, and that the section on the PDZ domain crystal structure appear together with additional results related to PDZ domain function. Furthermore- the authors should consider a more extensive re-organization, in which, for the most part, the manuscript proceeds by describing the results obtained for the different deletions, domain-by-domain. If this approach is adopted, the order of CnoΔRA, CnoΔPDZ and finally CnoΔFAB makes sense for a variety of reasons.
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In this work Perey-Vake et al studied the domain-function of the adherens junction adapter protein Canoe (afadin) in Drosophila morphogenesis. The main findings are that PDZ and FAB domains are not required for viability and AJ localization. However, the N-terminal RA domains are important for Canoe localization and function.

The conclusions are based on genetic engineering of the endogenous Canoe locus and subsequent analysis of epithelial morphogenesis phenotypes via fluorescence imaging. I find the experiments to be of high quality and supporting the main claims of the manuscript. While this work does not directly reveal a new function of any of the Canoe domains, the overall conclusion on the role of multivalent interaction networks of multidomain adapter proteins underlying structural/functional robustness is important and timely.

I find the amount of data and quantification is sufficient for publication. Even though I would be curious to see if deletions of the disordered linker regions especially the c-terminus would have an effect on Canoe function. One change I suggest is to move the domain structure of Canoe and its known interactions to Figure 1. This would help the reader to navigate through the deletion mutants more easily and setup the scope of the work at the beginning.
Response to Reviewers: JCB 202104087.

We are very grateful to the reviewers for their generally supportive response to our initial manuscript and their very helpful feedback. The three reviewers had a diverse set of suggestions for strengthening the manuscript. Prompted by their suggestions, over the past several months we carried out multiple new experiments, including most of those suggested by the Editor and Reviewers to strengthen the mechanistic insights. The new experiments included the experiment you as Editors particularly noted: generating versions of CnoWT and CnoΔPDZ in which the rest of the cno coding sequence is deleted and verifying that these lines are still viable and fertile, thus eliminating concern about potential interallelic complementation. We addressed other issues raised by the Reviewers about the relationship between mechanosensing and function, exploring the effects of reducing junction tension, using a ROCK inhibitor, on recruitment of wild-type Canoe and CnoΔRA—this revealed interesting effects on junctional stability. We also found that Rap1 is essential for tricellular junction recruitment of Cno, that CnoΔRA localization to junctions under tension can be rescued by co-expression with wild-type Cno. Finally, we explored the roles of the PDZ and FAB domains in another issue, the developing pupal eye, revealing that some domains are more important in one tissue than in another. In addition, we substantially revised the text and Figures to address concerns of all three Reviewers. These data and presentation changes are included in new Figure 4, new Suppl. Figure 6, and revised Figures 1, 3, 4, and 9. We have also included in the Supplemental material a “Marked-Up” copy of the text as a PDF with significant text additions/ revisions marked in red.

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interactions with key components of AJs are dispensable; (2) a phosphorylation site recently reported to be required for mechanical signal transduction is dispensable when removed from the endogenous locus rather than from over-expressed protein; and (3) the Rap1-binding RA domains seem essential for mechanical signal transduction. Drawing these conclusions from detailed in vivo phenotypic analyses of endogenously expressed domain deletion alleles is a strength of the paper, but issues should be addressed.

We appreciate this accurate summary.

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We agree that further exploring the role of the RA domains in mechanosensing is an important issue. The reviewer suggested exploring how Cno\(\Delta\)RA is affected by increasing or decreasing tension. This is a technically challenging experiment, as the same machinery that regulates myosin activity during germband extension is also essential for cellularization, so it needs to be manipulated in an acute fashion. While optogenetic tools are being developed to allow this, constructing stocks to allow us to do this in the \(cno\Delta\)RA maternal/zygotic mutant background would be very challenging. However, the use of ROCK inhibitors to reduce tension was something we could do acutely, and we carried out that experiment (New Figure 4). The result was very interesting and surprising.

While we might have naively expected that reducing tension would reduce the issues caused by reduction in Cno function, we found the opposite. As was previously observed by Simoes et al. 2010, inhibiting ROCK altered planar polarization of junctional proteins, enhancing Baz planar polarity to DV borders—similarly we noted that it reduced wild-type Cno accumulation on AP borders. Even more striking, ROCK inhibition in wild-type embryos led to cell junction separation at some AP borders, thus mimicking Cno loss! In \(cno\Delta\)RA maternal/zygotic mutants this effect was even more striking, with further enhancement of the junction separation phenotype, conversion of some cells to a hexagonal shape, and cell separation at many or all tricellular junctions (TCJs)! These data were very exciting and prompt a new hypothesis—junctional tension is required to recruit stabilizing proteins like Cno (and speculatively, also proteins like Vinculin and Ajuba) to those junctions. With tension reduced, this reinforcement does not occur, and junctions separate.

In parallel we carried out two additional experiments to further explore the role of the RA domain in mechanosensing. Our previous work during cellularization revealed that Rap1 acts on Canoe localization in both RA-dependent and RA-independent ways, but we had
not examined any later stages. We thus examined Canoe TCJ enrichment analysis in a Rap1 RNAi mutant background. This revealed that wild-type Cno TCJ enrichment is strongly reduced in the Rap1 mutants, and planar polarity of Cno is reversed, consistent with what we observed for the cnoΔRA mutant, thus suggesting that Cno enrichment to junctions under tension requires Rap1. This is part of Revised Figure 3. Second, we extended an earlier observation we made during cellularization — while cnoΔRA-GFP has defects in localization during cellularization when it is the only Canoe protein present, localization of the mutant protein is rescued if expressed in a wild-type background, suggesting that endogenous Canoe can associate directly or indirectly with the mutant protein. Here we examined whether presence of the endogenous wild-type Canoe protein can rescue CnoΔRA localization to TCJs during germband extension, sites of elevated tension. Intriguingly, this restored TCJ enrichment of CnoΔRA. These data are also included in Revised Figure 3.

You also suggested examining the localization of the GEF Dizzy, and whether it is recruited to sites of high tension. We do not have an antibody to Dizzy that works in situ, and the signal from the endogenously-tagged Dizzy-GFP is quite weak and thus there is strong background noise. We have included a Reviewer Figure at the end of this response illustrating this localization, and similar localization of a GAL4/UAS driven Dizzy-GFP, during the stages in question (stages 7 and early stage 8). At these stages there is not a strong TCJ localization, but the signal is sufficiently weak that we are reluctant to draw a stronger conclusion. We’d be happy to add one of these images to the manuscript, if the reviewer desired this, and have added to the Discussion the importance of exploring the role of Dizzy localization and function in the future.

2. The main strategy to delete the PDZ domain and the FAB domain resulted in domain-deleted proteins being co-expressed with a small of amount of truncated protein containing the domains of interest. The viability of delta-FAB mutants was confirmed with a distinct domain deletion approach, but this was not done for the delta-PDZ mutants. Thus, it remains possible that the mild phenotype of the delta-PDZ mutant is the result of inter-allelic complementation, possibly through the formation of functional complexes containing complementary forms of abnormal Canoe. This is a missing piece of an otherwise thorough genetic analysis.

You and the Editor both highlighted this important issue, and we addressed it as requested. The complexity of the canoe gene structure required a somewhat different strategy, which we had already embarked on before submission. In this strategy, we have deleted most of the remaining canoe coding sequence from both our CnoWT and our CnoΔPDZ lines, thus preventing production of CnoΔRA (New Suppl. Figure 6). We verified these new deletions by both PCR and Western blotting. We next verified that both CnoWT and CnoΔPDZ rescue viability and fertility in this now canoe null background. This rules out the possibility that the surprising phenotype of CnoΔFAB and CnoΔPDZ are due to interallelic complementation.

This critique also prompted us to further explore the roles of the FAB and PDZ domains by collaborating to examine their roles in a different tissue and time: the developing pupal eye (Revised Figure 9). The results were quite intriguing — in this tissue we confirmed that
both domains play supporting roles, and, that in the pupal eye the PDZ domain is more important than the FAB domain. This suggests particular protein interactions are more or less important in one tissue than in another, perhaps due to differences in how tension is applied to junctions in space and time. It will be exciting to examine this more broadly.

Minor concerns

1. The authors say "cno null mutants are zygotically lethal, and reintroducing CnoWT restored zygotic and M/Z viability and fertility (Fig 5A), verifying our "knock-in" rescue strategy." It seems the comparison should be with the cno-deltaRA mutants, since CnoWT was knocked-in to the cno-deltaRA locus.

Good point. We have rephrased that as follows (page 8): “Both cno null mutants and the cno∆RA mutant are zygotically and maternal/zygotically lethal, and reintroducing CnoWT restored zygotic and M/Z viability and fertility (Fig 5A), verifying our “knock-in” rescue strategy.”

2. It should be clarified in the text whether the cno-deltaFAB mutants described in Fig S5 (generated by the distinct domain deletion approach) were only used in this figure, or also in other figures.

We thank the reviewer for the suggestion. The text has been updated (see page 8)

3. In the third line of the Fig 2 legend, "Dorsal closure" should be "Ventral closure".

We thank the reviewer for pointing out that mistake. The legend has been updated accordingly.

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We’re grateful for the positive comments.

Manuscript improvement issues:
1. The Results section and Figure 1 should begin with a clear description and visual presentation of the experimental framework. The schematics of Cno proteins used in Figure 4A (to which the CnoΔRA variant should be added) are, for example, appropriate for achieving this purpose. Furthermore, the detailed description of the CRISPR-based gene editing scheme used to (serendipitously) generate CnoΔRA - which should be reported, of course - is distracting and not of general interest, and much of it can justifiably appear as supplementary data. The suggestion for moving the schematics of the Cno protein to the beginning of Figure 1, allowing us to use them in the introduction and making it clearer where the paper is heading, was an excellent one. We incorporated that. We were reluctant to further shorten the in-text description of the CRISPR knock-in, which is only four sentences. We also left in the diagram as we think it is important to both explain the nature of cnoΔRA and to avoid the appearance we are hiding the serendipity involved. However, with the protein diagrams now leading that Figure, as you suggested, we think it’s much clearer.

2. The organization of the main text (and in particular, the portion describing the data appearing in Figures 3 and 4) is confusing at times, and requires considerable back-and-forth from the reader. I would recommend that, at the very least, most of the text referring to Figures 4 and 5 precede that associated with Figure 3, and that the section on the PDZ domain crystal structure appear together with additional results related to PDZ domain function. Furthermore - the authors should consider a more extensive re-organization, in which, for the most part, the manuscript proceeds by describing the results obtained for the different deletions, domain-by-domain. If this approach is adopted, the order of CnoΔRA, CnoΔPDZ and finally CnoΔFAB makes sense for a variety of reasons.

We have taken these suggestions to heart. As you suggested, we changed the order in which the mutant phenotypes are described, now using the order CnoΔRA, CnoΔPDZ and finally
CnoΔFAB. We agree this makes the manuscript more sensible and clearer. We also moved the images of junctional localization of CnoWT, CnoΔPDZ, and CnoΔFAB to Figure 4, to match the place in the text where they are first referenced. We chose to retain the images of TCJ enrichment and its quantification and that of planar polarity in Figure 3, as we first reference those properties in our description of CnoΔRA. We acknowledge that this order requires the reader to return to Figure 3 later in the paper but think this is balanced by the ability to directly compare the TCJ enrichment of all of the mutants.

3. Figure legend comments:
• Figure 1D and 1E: why was Armadillo included in the immunoblot? What was the purpose of comparing protein levels between the 1-4 hour and 12-15 hour stages?

We thank the reviewer for the question. We have updated the Figure legend to explain the reasoning for the inclusion of the two time points and the blot for Arm “cnoΔRA maternal/zygotic mutants exhibit only the truncated protein at early time points (1-4 hr), but because 50% of the embryos receive a paternal wild-type copy of cno, wild-type Cno protein is seen at the 12-15 hr time point. Levels of the AJ protein Arm are not altered in cnoΔRA maternal/zygotic mutants.”. We also added a note later in the main text (page 5), when discussing Arm localization: “While Arm and Pyd localized to AJs in cnoΔRA mutants (Fig 2A vs B; H vs I; Fig S1C) and levels of Arm were not substantially altered (Fig 1D)…”

• Figure 2A,B- the term "dorsal closure" seems in error, to be replaced by "mesoderm invagination".

We thank the reviewer for pointing that out. The text has been updated.

• In general, the figure legends tend to be terse, and where possible, should be expanded to more fully describe what is shown in the figure itself.

We agree, but with the additional text added in Review we’re now over the “text limit”

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

In this work Perez-Vale et al studied the domain-function of the adherens junction adapter protein Canoe (afadin) in Drosophila morphogenesis. The main findings are that PDZ and FAB domains are not required for viability and AJ localization. However, the N-terminal RA domains are important for Canoe localization and function.

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I find the amount of data and quantification is sufficient for publication.
We’re grateful for this assessment.

Even though I would be curious to see if deletions of the disordered linker regions especially the c-terminus would have an effect on Canoe function.

We agree that mutations affecting the IDR are a very important next step, and our own lab’s work on Abl kinase has emphasized that for us. We have strengthened that suggestion in the discussion. See page 11.

One change I suggest is to move the domain structure of Canoe and its known interactions to Figure 1. This would help the reader to navigate through the deletion mutants more easily and setup the scope of the work at the beginning.

This was an excellent suggestion, shared by two reviewers. This allowed us to use this in the introduction and is a very nice change.
Dear Dr. Peifer:

Thank you for submitting your revised manuscript entitled "Multivalent interactions make adherens junction-cytoskeletal linkage robust during morphogenesis". We have now assessed your revised manuscript and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

A. MANUSCRIPT FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/site/misc/ifora.xhtml. Submission of a paper that exceeds these limits without prior discussion with the journal office will delay scheduling of your manuscript for publication.

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

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

3) Figure formatting:
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Scale bars must be present on all microscopy images, including inset magnifications. *** Please, add scale bars to main figure 2U, 5I-J, 9O-R.

*** Also, please avoid pairing red and green for those images in which separate channels or quantification graphs are not shown to ensure legibility for color-blind readers. Please change the color scheme of main figures 2J, 3A-B, 3M-N, 7C, 7F, 7I, L, 8E', 8F', 8F', 9C. We suggest changing the color scheme throughout the figure so that there is consistency between the panels (i.e. in main figure 2, if you leave panel E as red/green but switch J to, say, magenta/green, the lack of consistency may seem a bit strange). However, if you really want to just change the conflictive panels and leave the others, that is fine with us.

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. We are aware that, in most of the cases, n is indicated in the figure, but there are some panels (i.e. main figure 5G) in which this information is missing.

*** 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.). 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 (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."

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.

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

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

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   *** There are strict limits on the allowable amount of supplemental data. Articles/Tools may have
up to 5 supplemental figures.*** At the moment, you currently have 6 supplemental figures but one of them (S2) is a table - we allow an unlimited number of tables and supplemental tables -. We suggest that you label S2 as supplemental table. Please be sure to correct the callouts in the text to reflect this change.

*** 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. It should begin with "First author name(s) et al..." to match our preferred style.

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."

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B. FINAL FILES:

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3) It is JCB policy that if requested, original data images must be made available to the editors. Please ensure that you have access to all original data images prior to final submission.

4) Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the cover or table of contents. Images should be uploaded as .tif or .eps files and must be at least 300 dpi resolution.

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