# Arf6 anchors Cdr2 nodes at the cell cortex to control cell size at division

Hannah Opalko, Kristi Miller, Hyun-Soo Kim, Cesar Vargas-Garcia, Abhyudai Singh, Michael-Christopher Keogh, and James Moseley

*Corresponding Author(s): James Moseley, Dartmouth College*



| **Review Timeline:** | | |
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| | Submission Date: | 2021-09-29 |
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## 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.)

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# <u>Revision 0</u>

# Review #1

## 1. How much time do you estimate the authors will need to complete the suggested revisions:

### Estimated time to Complete Revisions (Required)

### (Decision Recommendation)

Less than 1 month

## 2. Evidence, reproducibility and clarity:

### Evidence, reproducibility and clarity (Required)

**Summary:**

In this paper, a new component in cell cycle/cell size signaling is described in the fission yeast, Schizosaccharomyces pombe. The GTPase Arf6 was identified using a synthetic genetic array approach with both an allele of the cell cycle regulator cdc25 and the deletion of cdr2, a protein kinase that influences cell size at division. The loss of Arf6 was found to affect the size of cdc25-22 cells at permissive temperature in a manner dependent on Cdr2. Using further genetic strategies and cell imaging, it was discovered that Arf6 is involved in organizing Cdr2-Cdr1-Wee1 cortical nodes in fission yeast, which in turn affect cell size control, mitotic progression, and cytokinesis. Arf6 was found to co-localize to these nodes in a Cdr2-dependent and GEF Syt22-dependent manner. Reciprocally, in arf6Δ cells, it was reported that Cdr1 does not localize to cortical nodes, Wee1 inhibitory phosphorylation is attenuated, and Cdr2 nodes are somewhat disorganized and less stably associated with the plasma membrane. Structure-function analysis was performed on Arf6 validating the hypothesis that membrane binding is required for Arf6 influence on cortical nodes. The data and overall conclusion that Arf6 is involved in the organization and function of Cdr2-based nodes are convincing. The methods and analysis are satisfactory.

**Major comments:**

1. It is stated about the data in Fig2: "These data show that GTPase cycling is required for Arf6 localization to nodes" . . . I missed the evidence that GTPase cycling is necessary. I only found the evidence that GTP binding is required.
2. "Arf6 localization to nodes depends on the state of its bound nucleotide under control of the GEF Syt22 and the GAP Ucp3." The role of the GAP Ucp3 was not tested so please rephrase this

conclusion.
3. The images in Figure 5 should be accompanied by quantification of the phenotypes described. Generally, quantification of images should be included throughout to bolster descriptive conclusions.
4. Two mutants of Mid1 were included to emphasize the role of Arf6 in organizing the activities of medial cortical nodes but because there is no understanding of why these alleles would have synthetic interactions with arf6Δ or that arf6Δ would affect so drastically the localization of Cdr1 or Cdr2 organization, these data don't further advance our understanding of what Arf6 is doing at nodes.

**Minor comments:**

1. Please explain what the cdc25-dD mutant is and provide citation.
2. It is stated: "Thus, we were interested to find that two of the mutants SS/SL with cdc25-22 (arf6Δ and mpn1Δ) showed increased cell length at division (Fig S1A)." The data show that there is no statistically significant difference between wildtype and arf6Δ cell lengths but there is with cdc25-22 so I found the wording of this sentence confusing. I recommend describing cell lengths of both single and double mutants separately.
3. "Specific functions for yeast Arf6 have been poorly understood", meaning have not been identified?

## 3. Significance:

### Significance (Required)

**Significance:**

The discovery of Arf6's roles advances our understanding of the players involved in organizing Cdr2-based cortical molecular assemblies, cell size control, and cell cycle control. It also opens up new lines of research in terms of learning how Arf6 actually does this and characterizing the regulation of the GEF that in turn regulates Arf6. Although not discussed, investigating why Cdr1 localization cannot occur even though Cdr2 nodes persist in arf6Δ cells could also be a fruitful line of future inquiry. This paper should appeal to cell cycle control and cell size control enthusiasts of which I am one.

# Review #2

## 1. How much time do you estimate the authors will need to complete the suggested revisions:

### Estimated time to Complete Revisions (Required)

**(Decision Recommendation)**

Between 1 and 3 months

# 2. Evidence, reproducibility and clarity:

### Evidence, reproducibility and clarity (Required)

**Summary:**

In their manuscript titled "Arf6 anchors Cdr2 nodes at the cell cortex to control cell size at division", Opalko et al. identify the Arf6 GTPase as an important component of the Cdr2-Wee1 pathway in fission yeast. They employed light microscopy to visualise and track the colocalisation of Cdr2 and Arf6, and the localisation of Cdr2 in the absence of Arf6. They conclude that Arf6 in its GTP-bound form is required to anchor Cdr2 at the middle of the cell by probing Arf6 localisation in deletions of the Arf6 GEF Syt22, GTP-locked and GDP-locked Arf6 mutants. Finally, they combined Arf6 deletion with other node mutants to demonstrate that Arf6 participates in cytokinesis.

**Major comments:**

While this work combines multiple techniques to establish Arf6 as a Cdr2 anchor, the primary concern is the lack of quantification of several data. Specifically:

1. Colocalisation of Arf6 with Cdr2 needs to be quantified. In nodes where Arf6 is absent, does Cdr2 remain associated with the membrane on its own?
2. Quantification of the time that each node spends on the membrane (from formation to disappearance) in Fig. 2B and 3C needs to be performed. This is not apparent based on the images.
3. The intensity of the Cdr2 cluster i.e. number of molecules in Cdr2 clusters also seems to change in arf6Del cells. This change needs to be quantified since if the number of molecules per cluster is reduced in arf6D cells, this could explain the shorter duration the cluster spends on a whole on the membrane.
4. Quantification of the number of Cdr2 clusters at middle of the cell vs. away in WT and arf6Del cells needs to be performed.
5. Quantification of more than 1 cell in Fig. S3D is required. In the example shown, the Cdr1-3XGFP signal in the arf6Del cell does not appear very different from WT.
6. Representative image of the FRAP (before, during and after) is required in Fig 3C.
7. Quantification of data in 3D is required.
8. In Fig. 4D, what is the localisation of Cdr2 with respect to Syb1 in WT cells?
9. Quantification is required in Fig. 5A and B.
10. Proper description of the statistical tests performed and the rationale behind the choice of test need to be included.

# 3. Significance:

### Significance (Required)

This work identifies Arf6 as a new player in the Cdr2-Wee1 pathway, which determines the timing of mitotic entry in fission yeast based on a threshold surface area.

This finding adds significantly to existing knowledge in the field regarding the control systems that dictate fission yeast mitotic entry.

This work will be of general interest to people working in the field of cell growth and size.

My expertise is in fission yeast cell biology.

**Referee Cross-commenting**

I agree with the comments from the other reviewers. Quantification of the data will greatly improve the manuscript.

# Review #3

## 1. How much time do you estimate the authors will need to complete the suggested revisions:

### Estimated time to Complete Revisions (Required)

### (Decision Recommendation)

Less than 1 month

## 2. Evidence, reproducibility and clarity:

### Evidence, reproducibility and clarity (Required)

**Summary:**

In this manuscript, the authors showed that fission yeast GTPase Arf6 is a novel component of the cell size controlling pathway. This protein ensures the localisation of the Cdr2 kinase, a critical protein kinase in this regulatory system, to nodes at the medial cell cortex, thereby ensuring selective, local activation of this kinase at nodes. Methodologies used include yeast genetics and cell biology.

**Major comments:**

- Are the key conclusions convincing?
Yes.
- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
No.
- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.
No. The exact mechanism by which Arf6 localises Cdr2 to nodes remains elusive. However, this issue, I believe, is beyond the scope of this work.
- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.
NA.
- Are the data and the methods presented in such a way that they can be reproduced?
Yes.
- Are the experiments adequately replicated and statistical analysis adequate?
Yes.

**Minor comments:**

- Specific experimental issues that are easily addressable.
No.
- Are prior studies referenced appropriately?
Yes.
- Are the text and figures clear and accurate?
Yes.
- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?
Yes (see specific points below).
- Specific points:
Arf6
Please describe previously known facts about Arf6; I guess that this protein was first identified as a protein required for bipolar growth (Fujita, 2008). In addition, the authors need to discuss their finding shown in this study in relation to this previous knowledge.

page 3, arf6Δ and mpn1Δ
Please give a brief explanation for Mpn1. Otherwise, mpn1Δ could be deleted.

page 4, Sad1 and Rcl1
Please explain what these proteins are, followed by appropriate references.

Figure S2G
If the authors have immunoblotting data to show the amount of various Arf6 mutant proteins, please present them. If not, that is fine; this is not a request.

page 5, Figure S3F

S3F appears in the text prior to S3A-3E. For the sake of proper ordering of figures, it would be better to change S3F to S3A and rearrange figure images accordingly.

page 5, These data show that Cdr2 becomes enriched
show-suggest

page 8, EMM-Uri
EMM-Ura

# 3. Significance:

## Significance (Required)

**- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.**

This study has identified a new, rather unexpected, molecule that regulates cell size in fission yeast. This is Arf6, a conserved GTPase that has been linked to the formation of multivalent protein assemblies at the plasma membrane in animal cells. It is known that in fission yeast the Cdr2 kinase (belonging to the conserved SAD-family) is localised to the medical cell cortex as nodes, by which it inhibits the Wee1 kinase at this site, leading to maintaining cell size (cell surface area) when cells divide. It has been proposed that at the late G2 phase when the number of Cdr2 molecules reaches beyond a certain level, Wee1 is inactivated, leading to activation of Cdc2/Cdk1 and mitotic entry.

**- Place the work in the context of the existing literature (provide references, where appropriate).**

Several proteins localising to nodes have been identified and Arf6 is a new regulator of these node components. Importantly, it regulates Cdr2 localisation. Thus, Arf6 is a novel cell sizer that works closely with Cdr2.

**- State what audience might be interested in and influenced by the reported findings.**

A broad range of researchers who are interested in cell cycle control, cell morphogenesis and GTPase-regulated signal transduction pathways.

**- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.**

My research fields include cell cycle control, genome stability and chromosome segregation, and I use mainly fission yeast as a system. I used to work on cell polarity control in fission yeast and therefore, I am still interested in the topic that this manuscript deals with.

**Referee Cross-commenting**

I agree with comments raised by the other two referees. As they pointed out, quantification of the data and some qualification of the text are necessary.

Takashi Toda

# Revision Plan

Review COMMONS

**Manuscript number:** RC- 2021-01014
**Corresponding author(s):** James Moseley

## 1. General Statements

*Our study identifies a new and unexpected role for the conserved GTPase Arf6 in regulating cell size at division through a signaling pathway that regulates Wee1. We are pleased that all 3 reviewers were enthusiastic about the significance and technical quality of the work. All 3 reviewers commented that the work will appeal broadly to researchers interested in cell growth, cell morphogenesis, and general cell biology. For revisions, the reviewers requested additional quantification for several of the mutant phenotypes described in the paper. We agree that this quantification will strengthen our conclusions, and we do not anticipate any difficulties in completing the requested experiments within 1-2 months. Below, we provide a point-by-point response to the reviewer comments, which are broken into planned revisions (i.e. experimental) and completed revisions (i.e. text-based).*

## 2. Description of the planned revisions

*Reviewer #1*
***Major comments:***

*3. The images in Figure 5 should be accompanied by quantification of the phenotypes described. Generally, quantification of images should be included throughout to bolster descriptive conclusions.*

AUTHOR RESPONSE: We agree and will quantify defects in single mutants versus double mutants from biological triplicate experiments for Figure 5. From our initial analysis, this quantification is likely to reveal very strong synthetic defects for the double mutants, as shown in the representative images.

*Reviewer #2*
***Major comments:***

*While this work combines multiple techniques to establish Arf6 as a Cdr2 anchor, the primary concern is the lack of quantification of several data. Specifically:*

*1. Colocalisation of Arf6 with Cdr2 needs to be quantified. In nodes where Arf6 is absent, does Cdr2 remain associated with the membrane on its own?*

AUTHOR RESPONSE: We will quantify the percentage of Cdr2 nodes that contain Arf6 through colocalization experiments. In addition, we will perform timelapse colocalization experiments to test how colocalization with Arf6 affects the dynamics of individual Cdr2 nodes.

*2. Quantification of the time that each node spends on the membrane (from formation to disappearance) in Fig. 2B and 3C needs to be performed. This is not apparent based on the images.*

AUTHOR RESPONSE: We will measure this timing from our movies and will add the resulting data into the full revision of our manuscript.

*3. The intensity of the Cdr2 cluster i.e. number of molecules in Cdr2 clusters also seems to change in arf6Del cells. This change needs to be quantified since if the number of molecules per cluster is reduced in arf6D cells, this could explain the shorter duration the cluster spends on a whole on the membrane.*

AUTHOR RESPONSE: Thank you for this helpful idea. We will measure the intensity of Cdr2 nodes in wild type versus *arf6Δ* cells. These data will be added into the full revision of our manuscript.

*4. Quantification of the number of Cdr2 clusters at middle of the cell vs. away in WT and arf6Del cells needs to be performed.*

AUTHOR RESPONSE: We will quantify these numbers as suggested. The data will be added into the full revision of our manuscript.

*5. Quantification of more than 1 cell in Fig. S3D is required. In the example shown, the Cdr1-3XGFP signal in the arf6Del cell does not appear very different from WT.*

AUTHOR RESPONSE: We will add line scans for additional cells as suggested. In addition, we will mark overlapping peaks (colocalization) versus non-overlapping peaks (no colocalization) in these graphs. Thus far, our images show that Cdr1 still forms clusters on the cell cortex in *arf6Δ* but these clusters do not colocalize with Cdr2 nodes. This conclusion will be strengthened by following the reviewer's helpful suggestion.

*7. Quantification of data in 3D is required.*

AUTHOR RESPONSE: We agree and will quantify the percentage of cells with Wee1-mNG at nodes for wild type versus *arf6*Δ strains.

*8. In Fig. 4D, what is the localisation of Cdr2 with respect to Syb1 in WT cells?*

AUTHOR RESPONSE: In our experience, Cdr2 does not localize to cytoplasmic clusters in wild type cells, so we predict that Cdr2 and Syb1 will not colocalize. To test this prediction, we will perform this experiment and add the results into the revised manuscript.

*9. Quantification is required in Fig. 5A and B.*

AUTHOR RESPONSE: We agree that this quantification will strengthen our conclusions. We will quantify these phenotypes and add the results in the full revision of our manuscript.

3. Description of the revisions that have already been incorporated in the transferred manuscript

*<u>Reviewer #1</u>*
***Major comments:***
*1. It is stated about the data in Fig2: "These data show that GTPase cycling is required for Arf6 localization to nodes" . . . I missed the evidence that GTPase cycling is necessary. I only found the evidence that GTP binding is required.*

AUTHOR RESPONSE: Thanks for this helpful comment. We revised the text to state that "GTP binding is required…" as suggested.

*2. "Arf6 localization to nodes depends on the state of its bound nucleotide under control of the GEF Syt22 and the GAP Ucp3." The role of the GAP Ucp3 was not tested so please rephrase this conclusion.*

AUTHOR RESPONSE: Thanks for this helpful comment. We removed Ucp3 from this sentence as suggested.

*4. Two mutants of Mid1 were included to emphasize the role of Arf6 in organizing the activities of medial cortical nodes but because there is no understanding of why these alleles would have synthetic interactions with arf6Δ or that arf6Δ would affect so drastically the localization of Cdr1 or Cdr2 organization, these data don't further advance our understanding of what Arf6 is doing at nodes.*

AUTHOR RESPONSE: We apologize for any confusion stemming from our description of these two mutants, which have different effects on the functional and physical interactions between Cdr2 and Mid1. We have clarified their differences in the revised test. Specifically, Mid1-Nter interacts with Cdr2 and requires functional Cdr2 nodes for proper cytokinesis. This mutant provides a genetic background to test the functionality of Cdr2 nodes in cytokinesis. In contrast, Mid1(Δ400-450) does ***not*** interact with Cdr2 and provides a sensitized system to study the contribution of other proteins (e.g. Arf6) in anchoring Cdr2 nodes without reinforcement from Mid1.

In the revised text, we state: "The Mid1 N-terminus (Mid1-Nter) is necessary and sufficient for its cytokinesis function, and the localization and function of Mid1-Nter requires Cdr2 (Celton-Morizur et al., 2006; Almonacid et al., 2009). Unlike the Mid1(Δ400-450) mutant that cannot interact with Cdr2, the Mid1-Nter mutant interacts with Cdr2 and depends on Cdr2 nodes for cytokinesis, meaning that Mid1-Nter provides a system to examine the function of Cdr2 nodes in cytokinesis."

***Minor comments:***

*1. Please explain what the cdc25-dD mutant is and provide citation.*

AUTHOR RESPONSE: We added this information in the revised text. The Materials and Methods section now states: "The *cdc25-dD* mutant (*dD* abbreviates *degron-DAmP*) reduces Cdc25 levels by addition of a degradation tag (degron) in combination with truncation of the 3'UTR (Breslow et al., 2008; Deng et al., 2014)."

*2. It is stated: "Thus, we were interested to find that two of the mutants SS/SL with cdc25-22 (arf6Δ and mpn1Δ) showed increased cell length at division (Fig S1A)." The data show that there is no statistically significant difference between wildtype and arf6Δ cell lengths but there is with cdc25-22 so I found the wording of this sentence confusing. I recommend describing cell lengths of both single and double mutants separately.*

AUTHOR RESPONSE: For clarity, we revised the text to focus on the cell length of double mutants. The text now states: "Thus, we were interested to find that the mutant *arf6Δ* was SS/SL with *cdc25-22* and increased cell length at division when combined with *cdc25-22* (Fig S1A)."

*3. "Specific functions for yeast Arf6 have been poorly understood", meaning have not been identified?*

AUTHOR RESPONSE: Thanks for this comment. We deleted the vague statement, and the sentence now reads: “We have demonstrated a clear role for Arf6 in organizing nodes for cell size control.”

Reviewer #2

***Major comments:***

*6. Representative image of the FRAP (before, during and after) is required in Fig 3C.*

AUTHOR RESPONSE: A full montage for representative cells (both wild type and *arf6*Δ) are provided in Figure S3C.

*10. Proper description of the statistical tests performed and the rationale behind the choice of test need to be included.*

AUTHOR RESPONSE: We revised the text to address this point. In the Methods section, we now state: “One-way ANOVA followed by Tukey’s multiple comparison test was used to assess differences for Figures 1F-G, S1C, S2H, and S3A (left panel). This test was selected to compare every mean within an experiment to each other. Unpaired t-tests with Welch’s correction were performed for Figure S1E and for each time point in Figures 3C and S3D. This test was selected to compare two datasets that are not assumed to have equal variance. All statistics and graphs were made using Prism8 (GraphPad Software).”

Reviewer #3

**Minor comments:**

*Arf6*

*Please describe previously known facts about Arf6; I guess that this protein was first identified as a protein required for bipolar growth (Fujita, 2008). In addition, the authors need to discuss their finding shown in this study in relation to this previous knowledge.*

AUTHOR RESPONSE: Thank you for this comment. We added the following sentences at the bottom of page 3: “Previous studies identified a role for fission yeast Arf6 and its regulatory GEF (Syt22) and GAP (Ucp3) in bipolar cell growth (Fujita, 2008; Fujita and Misumi, 2009, 2011), but a role in cell cycle progression has not been reported. Our data suggest a novel function for active Arf6 in the Cdr2 cell cycle pathway.”

*page 3, arf6Δ and mpn1Δ*
*Please give a brief explanation for Mpn1. Otherwise, mpn1Δ could be deleted.*

AUTHOR RESPONSE: We agree that Mpn1 distracts from the main conclusions. We have removed this reference to Mpn1 from the main text.

*page 4, Sad1 and Rcl1*
*Please explain what these proteins are, followed by appropriate references.*

AUTHOR RESPONSE: Done. We revised the text to state: “To test the timing of Arf6 node localization, we tracked mitosis with the spindle pole body marker Sad1 (Hagan and Yanagida, 1995), and we tracked cytokinesis with the myosin-II regulatory light chain Rlc1 (Naqvi et al., 2000; Le Goff et al., 2000).”

*Figure S2G*
*If the authors have immunoblotting data to show the amount of various Arf6 mutant proteins, please present them. If not, that is fine; this is not a request.*

AUTHOR RESPONSE: Unfortunately, we do not have these immunoblots. Our Arf6 mutant proteins were tagged with mNeonGreen, and our lab do not currently have an anti-mNG antibody that is sufficient to detect these proteins in fission yeast cell lysates. These *arf6-mNG* strains all exhibit similar levels of whole-cell fluorescence, so we believe that these mutant proteins are expressed at similar levels.

*page 5, Figure S3F*
*S3F appears in the text prior to S3A-3E. For the sake of proper ordering of figures, it would be better to change S3F to S3A and rearrange figure images accordingly.*

AUTHOR RESPONSE: Done.

*page 5, These data show that Cdr2 becomes enriched*
*show-suggest*

AUTHOR RESPONSE: Done.

*page 8, EMM-Uri*
*EMM-Ura*

AUTHOR RESPONSE: We supplement our media with uridine to select for *ura4* auxotrophic mutations, so the media is EMM-Uri as described.

## 4. Description of analyses that authors prefer not to carry out

*Not applicable (we will perform all of the requested analyses).*

October 15, 2021

Re: JCB manuscript #202109152T

Dr. James Moseley
Dartmouth College
412 Remsen Department of Biochemistry
Hanover, NH 03755

Dear Dr. Moseley,

Thank you for submitting your manuscript entitled "Arf6 anchors Cdr2 nodes at the cell cortex to control cell size at division". We apologize for the delay in communicating our decision to you. We have assessed your manuscript, the reviewer reports, and your revision plan. We feel that the broader question of how growth is controlling cell-cycle transitions is important and this adds a new element in a well-studied model system. Therefore we would like to invite you to submit a revision if you can address all the reviewers' concerns, as outlined in your revision plan.

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.

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 a Transfer is < 20,000, 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: Transfers may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

*** 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.

*** 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 made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the 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

Supplemental information: There are strict limits on the allowable amount of supplemental data. Transfers may have up to 3 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.

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.

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,

Arshad Desai
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

## Response to Reviewers (original comments in *italics*)

*Reviewer #1 (Evidence, reproducibility and clarity (Required)):*

***Summary:***

*In this paper, a new component in cell cycle/cell size signaling is described in the fission yeast, Schizosaccharomyces pombe. The GTPase Arf6 was identified using a synthetic genetic array approach with both an allele of the cell cycle regulator cdc25 and the deletion of cdr2, a protein kinase that influences cell size at division. The loss of Arf6 was found to affect the size of cdc25-22 cells at permissive temperature in a manner dependent on Cdr2. Using further genetic strategies and cell imaging, it was discovered that Arf6 is involved in organizing Cdr2-Cdr1-Wee1 cortical nodes in fission yeast, which in turn affect cell size control, mitotic progression, and cytokinesis. Arf6 was found to co-localize to these nodes in a Cdr2-dependent and GEF Syt22-dependent manner. Reciprocally, in arf6Δ cells, it was reported that Cdr1 does not localize to cortical nodes, Wee1 inhibitory phosphorylation is attenuated, and Cdr2 nodes are somewhat disorganized and less stably associated with the plasma membrane. Structure-function analysis was performed on Arf6 validating the hypothesis that membrane binding is required for Arf6 influence on cortical nodes. The data and overall conclusion that Arf6 is involved in the organization and function of Cdr2-based nodes are convincing. The methods and analysis are satisfactory.*

***Major comments:***

*1. It is stated about the data in Fig2: "These data show that GTPase cycling is required for Arf6 localization to nodes" . . . I missed the evidence that GTPase cycling is necessary. I only found the evidence that GTP binding is required.*

AUTHOR RESPONSE: Thanks for this helpful comment. We revised the text to state that “GTP binding is required…” as suggested.

*2. "Arf6 localization to nodes depends on the state of its bound nucleotide under control of the GEF Syt22 and the GAP Ucp3." The role of the GAP Ucp3 was not tested so please rephrase this conclusion.*

AUTHOR RESPONSE: Thanks for this helpful comment. We removed Ucp3 from this sentence as suggested.

*3. The images in Figure 5 should be accompanied by quantification of the phenotypes described. Generally, quantification of images should be included throughout to bolster descriptive conclusions.*

AUTHOR RESPONSE: We agree and have quantified these phenotypes. The new Figure 5E quantifies the phenotypes shown in panel 5D, and the new Figures S3I-K quantify the localization and morphology defects shown in panels 5A-B.

*4. Two mutants of Mid1 were included to emphasize the role of Arf6 in organizing the activities of medial cortical nodes but because there is no understanding of why these alleles would have synthetic interactions with arf6Δ or that arf6Δ would affect so drastically the localization of Cdr1 or Cdr2 organization, these data don't further advance our understanding of what Arf6 is doing at nodes.*

AUTHOR RESPONSE: We apologize for any confusion regarding the description of these two mutants, which have different effects on the functional and physical interactions between Cdr2 and Mid1. We have clarified their differences in the revised test. Specifically, Mid1-Nter interacts with Cdr2 and requires functional Cdr2 nodes for proper cytokinesis. This mutant provides a genetic background to test the functionality of Cdr2 nodes in cytokinesis. In contrast, Mid1(Δ400-450) does ***not*** interact with Cdr2 and provides a sensitized system to study the contribution of other proteins (e.g. Arf6) in anchoring Cdr2 nodes without reinforcement from Mid1.

In the revised text, we state: "The Mid1 N-terminus (Mid1-Nter) is necessary and sufficient for its cytokinesis function, and the localization and function of Mid1-Nter requires Cdr2 (Celton-Morizur et al., 2006; Almonacid et al., 2009). Unlike the Mid1(Δ400-450) mutant that cannot interact with Cdr2, the Mid1-Nter mutant interacts with Cdr2 and depends on Cdr2 nodes for cytokinesis, meaning that Mid1-Nter provides a system to examine the function of Cdr2 nodes in cytokinesis."

***Minor comments:***

*1. Please explain what the cdc25-dD mutant is and provide citation.*

AUTHOR RESPONSE: We added this information in the revised text. The methods section now states: "The *cdc25-dD* mutant reduces Cdc25 levels by addition of a degradation tag (degron) in combination with truncation of the 3'UTR (Breslow et al., 2008; Deng et al., 2014)."

*2. It is stated: "Thus, we were interested to find that two of the mutants SS/SL with cdc25-22 (arf6Δ and mpn1Δ) showed increased cell length at division (Fig S1A)." The data show that there is no statistically significant difference between wildtype and arf6Δ cell lengths but there is with cdc25-22 so I found the wording of this sentence confusing. I recommend describing cell lengths of both single and double mutants separately.*

AUTHOR RESPONSE: For clarity, we revised the text to focus on the cell length of double mutants. The text now states: "Thus, we were interested to find that *arf6Δ* was SS/SL with *cdc25-22* and increased cell length at division when combined with *cdc25-22* (Fig S1A)."

*3. "Specific functions for yeast Arf6 have been poorly understood", meaning have not been identified?*

AUTHOR RESPONSE: Thanks for this comment. We deleted the vague statement, and the sentence now reads: “We demonstrated a clear role for Arf6 in organizing nodes for cell size control.”

*Reviewer #1 (Significance (Required)):*

***Significance:***

*The discovery of Arf6's roles advances our understanding of the players involved in organizing Cdr2-based cortical molecular assemblies, cell size control, and cell cycle control. It also opens up new lines of research in terms of learning how Arf6 actually does this and characterizing the regulation of the GEF that in turn regulates Arf6. Although not discussed, investigating why Cdr1 localization cannot occur even though Cdr2 nodes persist in arf6Δ cells could also be a fruitful line of future inquiry. This paper should appeal to cell cycle control and cell size control enthusiasts of which I am one.*

*Reviewer #2 (Evidence, reproducibility and clarity (Required)):*

***Summary:***

*In their manuscript titled "Arf6 anchors Cdr2 nodes at the cell cortex to control cell size at division", Opalko et al. identify the Arf6 GTPase as an important component of the Cdr2-Wee1 pathway in fission yeast. They employed light microscopy to visualise and track the colocalisation of Cdr2 and Arf6, and the localisation of Cdr2 in the absence of Arf6. They conclude that Arf6 in its GTP-bound form is required to anchor Cdr2 at the middle of the cell by probing Arf6 localisation in deletions of the Arf6 GEF Syt22, GTP-locked and GDP-locked Arf6 mutants. Finally, they combined Arf6 deletion with other node mutants to demonstrate that Arf6 participates in cytokinesis.*

***Major comments:***

*While this work combines multiple techniques to establish Arf6 as a Cdr2 anchor, the primary concern is the lack of quantification of several data. Specifically:*

*1. Colocalisation of Arf6 with Cdr2 needs to be quantified. In nodes where Arf6 is absent, does Cdr2 remain associated with the membrane on its own?*

AUTHOR RESPONSE: We quantified the percentage of Cdr2 nodes that also contain Arf6, the result is shown in the new Figure S2A. We found that most but not all Cdr2 nodes contain Arf6.

*2. Quantification of the time that each node spends on the membrane (from formation to disappearance) in Fig. 2B and 3C needs to be performed. This is not apparent based on the images.*

AUTHOR RESPONSE: We added a new figure quantifying Cdr2 node dynamics in wild type versus *arf6Δ* cells. In the new Figure S3A, we compared Cdr2-mEGFP localization in a single cell at timepoint 0 to each subsequent timepoint using a Pearson correlation coefficient (PCC). If nodes are stable, then the PCC remains high throughout the time course, while a decrease in PCC over time indicates dynamic changes. The new Figure S3A shows that PCC decreases more rapidly in *arf6Δ* versus wild type. We also performed time-lape imaging of *arf6-mNG cdr2-mCherry* cells with the goal of documenting Cdr2 and Arf6 lifetimes at individual nodes. However, we encountered significant photobleaching (particularly in the red channel) that precluded meaningful interpretation even after optimizing with different fluorophores and imaging conditions. We will continue working on this line of experiments as a goal for future studies.

*3. The intensity of the Cdr2 cluster i.e. number of molecules in Cdr2 clusters also seems to change in arf6Del cells. This change needs to be quantified since if the number of molecules per cluster is reduced in arf6D cells, this could explain the shorter duration the cluster spends on a whole on the membrane.*

AUTHOR RESPONSE: Thank you for this helpful idea. We performed this analysis in the new Figure S2M, which shows that node intensity is reduced in *arf6Δ* cells.

*4. Quantification of the number of Cdr2 clusters at middle of the cell vs. away in WT and arf6Del cells needs to be performed.*

AUTHOR RESPONSE: We agree and performed this analysis, which is presented in the new Figure S2L. We found that *arf6Δ* cells have increased number of Cdr2 clusters at cell tips and decreased number of Cdr2 clusters at cell sides when compared to wild type cells.

*5. Quantification of more than 1 cell in Fig. S3D is required. In the example shown, the Cdr1-3XGFP signal in the arf6Del cell does not appear very different from WT.*

AUTHOR RESPONSE: Thanks for the feedback. We added line scans for additional cells as suggested. In addition, we marked the peaks of Cdr2-RFP and Cdr1-3xGFP in these graphs to demonstrate where they overlap (see revised Figure S3F). Our images and analysis show that Cdr1 still forms clusters on the cell cortex in *arf6Δ*, but these clusters do not colocalize with Cdr2 nodes.

*6. Representative image of the FRAP (before, during and after) is required in Fig 3C.*

AUTHOR RESPONSE: A full montage for representative cells (both wild type and *arf6Δ*) are provided in Figure S3B.

*7. Quantification of data in 3D is required.*

AUTHOR RESPONSE: We agree and quantified the percentage of cells with Wee1-mNG at nodes for wild type versus *arf6Δ* strains. These data are shown in the new Figure S3D.

*8. In Fig. 4D, what is the localisation of Cdr2 with respect to Syb1 in WT cells?*

AUTHOR RESPONSE: We performed this experiment and show the result in the new Figure S3H. We found that Cdr2 does not colocalize with Syb1 in wild type cells.

*9. Quantification is required in Fig. 5A and B.*

AUTHOR RESPONSE: We agree and quantified these results as suggested. The new Figures S3I-K quantify the localization and morphology defects of cells from Figures 5A-B. In addition, the new Figure 5E shows quantification of the defects in Figure 5D.

*10. Proper description of the statistical tests performed and the rationale behind the choice of test need to be included.*

AUTHOR RESPONSE: Thanks for this recommendation. We added a new section in the Methods under the heading “Statistical Analysis.” The revised text now states: “One-way ANOVA followed by Tukey’s multiple comparison test was used to assess differences for Figures 1F-G, S1C, S2I, and S2J (left panel). This test was selected to compare every mean within an experiment to each other. Unpaired t-tests with Welch’s correction were performed for Figures S1E, S2L-M, S3D, S3I-K, and for each time point in Figures 3C, S3A, and S3C. This test was selected to compare two datasets that are not assumed to have equal variance. All statistics and graphs were made using Prism8 (GraphPad Software).”

*Reviewer #2 (Significance (Required)):*

*This work identifies Arf6 as a new player in the Cdr2-Wee1 pathway, which determines the timing of mitotic entry in fission yeast based on a threshold surface area.*

*This finding adds significantly to existing knowledge in the field regarding the control systems that dictate fission yeast mitotic entry.*

*This work will be of general interest to people working in the field of cell growth and size.*

My expertise is in fission yeast cell biology.

**Referee Cross-commenting**

I agree with the comments from the other reviewers. Quantification of the data will greatly improve the manuscript.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

**Summary:**

In this manuscript, the authors showed that fission yeast GTPase Arf6 is a novel component of the cell size controlling pathway. This protein ensures the localisation of the Cdr2 kinase, a critical protein kinase in this regulatory system, to nodes at the medial cell cortex, thereby ensuring selective, local activation of this kinase at nodes. Methodologies used include yeast genetics and cell biology.

**Major comments:**

- Are the key conclusions convincing?
Yes.
- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
No.
- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.
No. The exact mechanism by which Arf6 localises Cdr2 to nodes remains elusive. However, this issue, I believe, is beyond the scope of this work.
- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.
NA.
- Are the data and the methods presented in such a way that they can be reproduced?
Yes.
- Are the experiments adequately replicated and statistical analysis adequate?
Yes.

**Minor comments:**

- Specific experimental issues that are easily addressable.
No.
- Are prior studies referenced appropriately?
Yes.

*- Are the text and figures clear and accurate?*
*Yes.*
*- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?*
*Yes (see specific points below).*
*- Specific points:*
*Arf6*
*Please describe previously known facts about Arf6; I guess that this protein was first identified as a protein required for bipolar growth (Fujita, 2008). In addition, the authors need to discuss their finding shown in this study in relation to this previous knowledge.*

AUTHOR RESPONSE: Thank you for this comment. We added the following sentences at the bottom of page 3: "Previous studies identified a role for fission yeast Arf6 and its regulatory GEF (Syt22) and GAP (Ucp3) in bipolar cell growth (Fujita, 2008; Fujita and Misumi, 2009, 2011), but a role in cell cycle progression has not been reported. Our data suggest a novel function for active Arf6 in the Cdr2 cell cycle pathway."

*page 3, arf6Δ and mpn1Δ*
*Please give a brief explanation for Mpn1. Otherwise, mpn1Δ could be deleted.*

AUTHOR RESPONSE: We agree that Mpn1 distracts from the main conclusions. We have removed this reference to Mpn1 in the main text.

*page 4, Sad1 and Rcl1*
*Please explain what these proteins are, followed by appropriate references.*

AUTHOR RESPONSE: Done. We revised the text to state: "To test the timing of Arf6 node localization, we tracked mitosis with the spindle pole body marker Sad1 (Hagan and Yanagida, 1995), and we tracked cytokinesis with the myosin-II regulatory light chain Rlc1 (Naqvi et al., 2000; Le Goff et al., 2000)."

*Figure S2G*
*If the authors have immunoblotting data to show the amount of various Arf6 mutant proteins, please present them. If not, that is fine; this is not a request.*

AUTHOR RESPONSE: Unfortunately, we do not have these immunoblots. Our Arf6 mutant proteins were tagged with mNeonGreen, and our lab do not currently have an anti-mNG antibody that is sufficient to detect these proteins in fission yeast cell lysates. These arf6-mNG strains all exhibit similar levels of whole-cell fluorescence, so we believe that these mutant proteins are present at similar levels.

*page 5, Figure S3F*
*S3F appears in the text prior to S3A-3E. For the sake of proper ordering of figures, it would be better to change S3F to S3A and rearrange figure images accordingly.*

AUTHOR RESPONSE: Done. Note that this figure panel has been moved and now appears as Figure S2J.

*page 5, These data show that Cdr2 becomes enriched*
*show-suggest*

AUTHOR RESPONSE: Done.

*page 8, EMM-Uri*
*EMM-Ura*

AUTHOR RESPONSE: We supplement our media with uridine to select for ura4 auxotrophic mutations, so the media is EMM-Uri as described.

*Reviewer #3 (Significance (Required)):*

***- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.***

*This study has identified a new, rather unexpected, molecule that regulates cell size in fission yeast. This is Arf6, a conserved GTPase that has been linked to the formation of multivalent protein assemblies at the plasma membrane in animal cells. It is known that in fission yeast the Cdr2 kinase (belonging to the conserved SAD-family) is localised to the medical cell cortex as nodes, by which it inhibits the Wee1 kinase at this site, leading to maintaining cell size (cell surface area) when cells divide. It has been proposed that at the late G2 phase when the number of Cdr2 molecules reaches beyond a certain level, Wee1 is inactivated, leading to activation of Cdc2/Cdk1 and mitotic entry.*

***- Place the work in the context of the existing literature (provide references, where appropriate).***

*Several proteins localising to nodes have been identified and Arf6 is a new regulator of these node components. Importantly, it regulates Cdr2 localisation. Thus, Arf6 is a novel cell sizer that works closely with Cdr2.*

***- State what audience might be interested in and influenced by the reported findings.***

*A broad range of researchers who are interested in cell cycle control, cell morphogenesis and GTPase-regulated signal transduction pathways.*

***- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.***

*My research fields include cell cycle control, genome stability and chromosome segregation, and I use mainly fission yeast as a system. I used to work on cell polarity control in fission yeast and therefore, I am still interested in the topic that this manuscript deals with.*

***Referee Cross-commenting***

*I agree with comments raised by the other two referees. As they pointed out, quantification of the data and some qualification of the text are necessary.*

*Takashi Toda*

November 22, 2021

RE: JCB Manuscript #202109152R

Dr. James Moseley
Dartmouth College
412 Remsen Department of Biochemistry
Hanover, NH 03755

Dear Dr. Moseley:

Thank you for submitting your revised manuscript entitled "Arf6 anchors Cdr2 nodes at the cell cortex to control cell size at division". We have now assessed your revised manuscript and 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 ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Reports is normally < 20,000, 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. Please note that in the JCB Report format, the Results and Discussion sections should be merged into a single "Results and Discussion" module.

2) Figures limits: Reports may have up to 5 main text figures.

3) Figure formatting:
*** Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please, include MWM in main Fig 1H.

*** Scale bars must be present on all microscopy images, including inset magnifications. Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. Please, include scale bars in main Fig 2B (inset), 2D, 3B (inset), 3D (inset), 4D, 5A (inset) and supplementary Fig 2B, 2D (inset), 2F (inset), 3B, 3G (inset), 3H (inset).

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. Please, add the number of independent data points to the legend of supplementary Fig 1E (same as in C-D?), 2L-M . Also, could you please indicate how many cells were analyzed per experiment in main Fig 5E and supplementary Fig 3F?

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 in your study (i.e. t-tests), 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." We realize that statistical tests are included in the material and methods, but could you please also include them in the corresponding figure legend?

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."

Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

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. Species seem to be missing for anti-wee1 and anti-cdc2 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 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. Reports may normally have up to 3 supplemental figures. There is no limit for supplemental tables. However, please 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 (please see any recent JCB paper for an example of this summary).

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."

13) *** A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

14) 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.

15) Materials and data sharing: All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

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

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

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

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

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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 made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the 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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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu.

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

Sincerely,

Arshad Desai
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

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A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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AUTHOR RESPONSE: Done.

2) Figures limits: Reports may have up to 5 main text figures.

AUTHOR RESPONSE: Done – our paper has 5 main text figures.

3) Figure formatting:
*** Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please, include MWM in main Fig 1H.

AUTHOR RESPONSE: Done.

*** Scale bars must be present on all microscopy images, including inset magnifications. Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. Please, include scale bars in main Fig 2B (inset), 2D, 3B (inset), 3D (inset), 4D, 5A (inset) and supplementary Fig 2B, 2D (inset), 2F (inset), 3B, 3G (inset), 3H (inset).

AUTHOR RESPONSE: Done.

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. Please, add the number of independent data points to the legend of supplementary Fig 1E (same as in C-D?), 2L-M . Also, could you please indicate how many cells were analyzed per experiment in main Fig 5E and supplementary Fig 3F?

AUTHOR RESPONSE: Done.

Statistical methods should be explained in full in the materials and methods.

AUTHOR RESPONSE: Done.

*** For figures presenting pooled data the statistical measure should be defined in the figure legends.

AUTHOR RESPONSE: Done.

*** 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 in your study (i.e. t-tests), 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." We realize that statistical tests are included in the material and methods, but could you please also include them in the corresponding figure legend?

AUTHOR RESPONSE: Done.

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.

AUTHOR RESPONSE: Done.

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."

Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

AUTHOR RESPONSE: Done.

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. Species seem to be missing for anti-wee1 and anti-cdc2 antibodies.

AUTHOR RESPONSE: Done.

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.).

AUTHOR RESPONSE: Done.

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.

AUTHOR RESPONSE: Done.

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A summary of all supplemental material should appear at the end of the Materials and methods section (please see any recent JCB paper for an example of this summary).

AUTHOR RESPONSE: Done.

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.

AUTHOR RESPONSE: Done.

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."

AUTHOR RESPONSE: Done.

13) *** A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

AUTHOR RESPONSE: Done.

14) 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.

AUTHOR RESPONSE: OK.

15) Materials and data sharing: All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

AUTHOR RESPONSE: OK.