Fission yeast Pak1 phosphorylates anillin-like Mid1 for spatial control of cytokinesis

Joseph Magliozzi, Jack Sears, Lauren Cressey, Marielle Brady, Hannah Opalko, Arminja Kettenbach, and James Moseley

Corresponding Author(s): James Moseley, Dartmouth

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September 4, 2019

Re: JCB manuscript #201908017

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

Dear Dr. Moseley,

Thank you for submitting your manuscript entitled "Defining how Pak1 regulates cell polarity and cell division in fission yeast". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

Although all of the reviewers appreciate the breadth and quality of the work, they feel that it makes rather incremental advances in several directions (Pak1 regulation of cytokinesis, cell polarity, and cell separation) simultaneously, rather than making the kind of substantial advance in a focused area that would be required for JCB. For that reason, we cannot accept the paper for publication in JCB.

As indicated by reviewer #1 point #1, a version of the study that elucidated the mechanism whereby Pak1 regulates cytokinesis would be more appropriate. Similarly, versions that made a substantial mechanistic advance on the regulation of either polarity or cell separation could also become suitable for JCB. If you develop the story in one of these directions, we would be happy to consider it again.

Although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, I would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Daniel Lew, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

Polarized cell growth and contractile actomyosin ring (CAR) are known to coordinate in time and space during cytokinesis in fungal and animal cells. However, it remains poorly understood whether and how polarity pathway components directly regulate CAR and cell separation. In this study, the authors report the identification and validation of the substrates for the Cdc42-activated kinase, Pak1. The authors have demonstrated convincingly that the key polarity regulators (Scd1, Rga4), the cytokinetic components (Mid1, Cdc15, and Cyk3), and a stress-granule component (Sts5) are bona fide substrates of Pak1. In addition, the authors have convincingly shown that Pak1 plays a role in CAR assembly and cell separation. What is not clear is whether Pak1 regulates these processes through the newly identified substrates.

Major points:

1. To determine whether Pak1 controls CAR assembly through phosphorylation of Mid1, Cdc15, and Cyk3, the authors need to monitor CAR assembly in the triple mutant carrying either phosphomimetic or de-phosphomimetic mutations at the Pak1 phosphorylated residues of mid1, cdc15, and cyk3.

2. The evidence for Sts5 in mediating the role of Pak1 in cell separation is not strong. It is unclear how the stress granule-associated Sts5 could function in cell separation in any direct manner. In contrast, it is surprising why the authors did not explore whether Pak1 controls cell separation through its substrate Etd1, which has a well-characterized role in regulating septation through Spg1 and SIN (Garcia-Cortes and McCollum, JCB, 2009).

Minor points:

1. It is intriguing that "Pak1 strands" are connected to the "Rlc1 cables" (Figure 1B). Is it possible that Pak1 is associated with the actin filaments in the CAR? A LatA experiment would be informative.

2. Does Pak1 affect cell cycle progression, especially from late anaphase to the end of cell separation? If yes, could this contribute to its role in the regulation of cytokinesis?

3. The authors used rgd4 deletion cells as a control to rule out the cell-diameter impact of the pak1-as cells on cytokinesis. This argument can be strengthened by plotting the cell width measurements of the rgd4 deletion cells, together with those of pak1 mutant cells, in Figure S2A.

4. Organizational issues: A) the negative result on the function of Rlc1 phosphorylation by Pak1 can be described briefly in text, without actually showing the data (Figure 4). Alternatively, Figure 4 can

be moved to the supplemental materials; and B) this manuscript could focus on how Pak1 affect cytokinesis and cell separation. The part on the regulation of polarity proteins (Scd1 and Rgd4) by Pak1 seems to detract from the main message (Pak1 and cytokinesis).

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

The manuscript by Magliozzi et al. investigated how p21 kinase Pak1 regulates cell polarity and cytokinesis through phosphorylation its substrates. Pak1 family kinases are well studied for cell polarization, but their roles in cytokinesis are less understood. In this study, the authors found that Pak1 localizes to the division site during contractile ring formation and is involved in ring formation and septation. Genetic interaction with mid1 and rng2 mutants and septation index in pak1-as mutant are consistent with a role in Pak1 in cytokinesis. Then a large-scale phosphoproteomic screen was performed and several substrates in cell polarity and cytokinesis were discovered. Some of the substrates were confirmed by in vitro kinase assays. State-of-art approaches were used in this interesting study on the roles of polarity protein Pak1 in cytokinesis. Several concerns should be addressed in the revised version of the paper.

1. I did not find the section on Sts5 is a good fit with the manuscript. It is actually distracting since the roles of Sts5 on cell separation are not very dramatic from the results showed. Roles of Rng10 in cell separation are direct and better defined. I would like the authors to replace Sts5 with Rng10 in this part.
2. The Y-axes in figures should start with zero. Otherwise, the difference between wt and mutants are exaggerated.
3. In Figure 1B, the image was from airyscan microscope, what is the delay between the two channels? Is this possible that the structures observed had moved during the acquisition? Is Pak1 localization actin dependent?
4. It is better to show if CRIB and Pak1 colocalize or not in Fig. 1C.
5. Fig. 2D is confusing. No data for the double mutant at 25C? More spacing is needed between mid1 mutant and the double one.
6. In Fig. 5, Rng10 should be grouped in C since its main role is in septation.
7. Is it possible that Pak1 also regulates the tip localization of Scd1? The signals at cell tips are much brighter in pak1-as than wt. The intensity should be quantified.
8. In Fig. 7A, C2 domain of Mid1 should be illustrated. In 7C, the cells look weird, are the vacuole-like structures artifacts from deconvolution? Have you tried less iterations?
9. Any genetic interactions between pak1 and cdc15 mutants? How does Pak1 phosphorylation regulate Cdc15 in cytokinesis? How did you define the dynamics of Cdc15 punta? A FRAP assay might be more informative.
10. On page 15, last paragraph, not sure what yeast strains were broken by sonication. In the previous paragraph, the authors mentioned that yeast cells were lysed to 80% by coffee bean grinder, which is smart and economy.

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

This manuscript by Moseley and colleagues is in an interesting topic and has exceptionally high quality data, as is usual for work from this group. The manuscript describes work aimed at understanding how the fission yeast Pak1/Orb2 /Shk1 kinase regulates cell polarity and cytokinesis. There are two sides to the work. In the first part, they investigate Pak1 localization, its role in actin-myosin ring assembly, and also show some genetic interactions between Pak1 and dmf1/mid1 and

rng2. In this part, they also show that all of Pak-kinases effects are not via Rlc1. In the second part, they perform phospho-proteomics and uncover a number of substrates of Pak1. They show some experiments that reveal three elements downstream Pak1, with Cdc15 and Mid1 participating in cytokinesis, Scd1,Rga4, and Tea3 functioning in polarity and Sts5 in cell separation.

The work is very well done, but I have one major concern. I believe this paper straddles a large-scale proteomics effort story (a resource-type paper) and a mechanistic story and in the end does not do justice to either. I think the mechanistic analysis in figures 5-9 are fairly cursory, while parts of the front end of the paper are already described by other groups, although the current study admittedly adds a bit more to what is published. In light of this, I believe the paper does not merit publication in JCB, presently. I can see a number of ways the work can be expanded to provide detailed mechanisms, be it in actin-myosin ring function or in cell polarization or in cell separation.

Some additional comments

• The novelty is in the phospho-proteomics. It would be nice if this Figure had a heat map and clustering of the entire results instead just showing a table.
• Figure 6-8 should have Ala substitution experiments, which will further bolster conclusions from phenotypic studies.
• Regulation of Rga4 localization by Pak1 and Pom1is interesting (Fig 6). However, most of the genetics and cell biology studies in Fig 6-9 are too preliminary to conclude significance of the phospho-regulation. It would be better to have mechanistic insights about the phospho-regulation (e.g. protein-protein interaction and protein-lipid interaction).

Responses to Reviewer #1 (original comments in *italics*):

Polarized cell growth and contractile actomyosin ring (CAR) are known to coordinate in time and space during cytokinesis in fungal and animal cells. However, it remains poorly understood whether and how polarity pathway components directly regulate CAR and cell separation. In this study, the authors report the identification and validation of the substrates for the Cdc42-activated kinase, Pak1. The authors have demonstrated convincingly that the key polarity regulators (Scd1, Rga4), the cytokinetic components (Mid1, Cdc15, and Cyk3), and a stress-granule component (Sts5) are bona fide substrates of Pak1. In addition, the authors have convincingly shown that Pak1 plays a role in CAR assembly and cell separation. What is not clear is whether Pak1 regulates these processes through the newly identified substrates.

Major points:

1. To determine whether Pak1 controls CAR assembly through phosphorylation of Mid1, Cdc15, and Cyk3, the authors need to monitor CAR assembly in the triple mutant carrying either phosphomimetic or de-phosphomimetic mutations at the Pak1 phosphorylated residues of mid1, cdc15, and cyk3.

RESPONSE: We added the new Figure 5 based on this suggestion, with a focus on Mid1. We mapped Pak1-dependent phosphorylation sites on Mid1 both *in vitro* and *in vivo*, and then generated a Mid1(9A) mutant that largely abolishes phosphorylation by Pak1 *in vitro*. We integrated this mutant into the Mid1Nter construct. The non-phosphorylatable mutant showed nearly identical defects as *pak1-as* mutants (we also made the phosphomimetic version but it was non-informative loss of function, consistent with our experience that phosphomimetic mutants often disrupt protein function). These and other data indicated that Pak1 phosphorylates Mid1Nter to promote its interaction with Cdr2 nodes, suggesting that the defect might be rescued by synthetically retargeting the 9A mutant back to nodes. Indeed, using the GFP-GBP system we could restore node localization and suppress cytokinesis defects by tethering the 9A mutant back to nodes. These results mean that Pak1 promotes Mid1Nter localization to nodes but is not required for additional downstream activities of Mid1. We extended these analyses by showing that full-length Mid1(9A) exhibits synthetic defects when combined with *plo1-1*, leading to our final model that Pak1 and Plo1 act together to regulate Mid1 in time (Plo1) and space (Pak1).

2. The evidence for Sts5 in mediating the role of Pak1 in cell separation is not strong. It is unclear how the stress granule-associated Sts5 could function in cell separation in any direct manner. In contrast, it is surprising why the authors did not explore whether Pak1 controls cell separation through its substrate Etd1, which has a well-characterized role in regulating septation through Spg1 and SIN (Garcia-Cortes and McCollum, JCB, 2009).

RESPONSE: We appreciate the helpful criticism regarding a “weak point” in the paper. We thoroughly streamlined the revised manuscript by removing characterization of Pak1 substrates in cell polarity and septation, including Sts5. The revised manuscript now presents a focused and mechanism-oriented study on Pak1 regulating cytokinesis through Mid1. We agree with the

reviewer that Etd1 represents a likely candidate for Pak1 function in septation and will pursue this possibility in future work.

Minor points:

1. It is intriguing that "Pak1 strands" are connected to the "Rlc1 cables" (Figure 1B). Is it possible that Pak1 is associated with the actin filaments in the CAR? A LatA experiment would be informative.

RESPONSE: Thanks for the suggestion. We now present a latrunculin experiment along with a Lifeact colocalization experiment in the new Figures S1A-B.

2. Does Pak1 affect cell cycle progression, especially from late anaphase to the end of cell separation? If yes, could this contribute to its role in the regulation of cytokinesis?

RESPONSE: As shown in Figure S2E, the overall timing of cytokinesis is unaffected in *pak1* mutant cells because the extended CAR assembly phase is balanced by a shortened maturation phase. We have not observed any additional cell cycle defects in the *pak1* mutants.

3. The authors used rgd4 deletion cells as a control to rule out the cell-diameter impact of the pak1-as cells on cytokinesis. This argument can be strengthened by plotting the cell width measurements of the rgd4 deletion cells, together with those of pak1 mutant cells, in Figure S2A.

RESPONSE: We added these data in the revised Figure S1C.

4. Organizational issues: A) the negative result on the function of Rlc1 phosphorylation by Pak1 can be described briefly in text, without actually showing the data (Figure 4). Alternatively, Figure 4 can be moved to the supplemental materials;

RESPONSE: Thank you for this helpful suggestion. We shortened this text and moved the data to the supplemental Figure S2.

and B) this manuscript could focus on how Pak1 affect cytokinesis and cell separation. The part on the regulation of polarity proteins (Scd1 and Rgd4) by Pak1 seems to detract from the main message (Pak1 and cytokinesis).

RESPONSE: Again, thank you for this helpful suggestion, which was shared by the other reviewers. In revising the paper, we removed sections on cell polarity and cell separation substrates. We now present a more focused study of Pak1 and cytokinesis. We feel this

streamlined approach has greatly improved the impact of our work.

Responses to Reviewer #2 (original comments in *italics*):

The manuscript by Magliozzi et al. investigated how p21 kinase Pak1 regulates cell polarity and cytokinesis through phosphorylation its substrates. Pak1 family kinases are well studied for cell polarization, but their roles in cytokinesis are less understood. In this study, the authors found that Pak1 localizes to the division site during contractile ring formation and is involved in ring formation and septation. Genetic interaction with mid1 and rng2 mutants and septation index in pak1-as mutant are consistent with a role in Pak1 in cytokinesis. Then a large-scale phosphoproteomic screen was performed and several substrates in cell polarity and cytokinesis were discovered. Some of the substrates were confirmed by in vitro kinase assays. State-of-art approaches were used in this interesting study on the roles of polarity protein Pak1 in cytokinesis. Several concerns should be addressed in the revised version of the paper.

1. I did not find the section on Sts5 is a good fit with the manuscript. It is actually distracting since the roles of Sts5 on cell separation are not very dramatic from the results showed. Roles of Rng10 in cell separation are direct and better defined. I would like the authors to replace Sts5 with Rng10 in this part.

RESPONSE: We appreciate this helpful feedback. As discussed above, we have removed the sections on cell polarity and cell separation substrates. Our revised manuscript now focuses on Pak1 and cytokinesis, with new experiments on its regulation of Mid1. We hope to develop the Sts5 connection in future work. We agree that the role of Rng10 in cell separation has been defined well. In the revised manuscript, we have removed the list of putative substrates in cell separation. Therefore, we have kept Rng10 listed as a cytokinesis substrate so that it can still be highlighted as a potential Pak1 substrate, but we modified Figure 3B to clarify that Rng10 functions as a "septation protein."

2. The Y-axes in figures should start with zero. Otherwise, the difference between wt and mutants are exaggerated.

RESPONSE: Done.

3. In Figure 1B, the image was from airyscan microscope, what is the delay between the two channels? Is this possible that the structures observed had moved during the acquisition?

RESPONSE: The delay between the channels in this experiment was 4 seconds. These structures move on a much slower timescale, so this delay is unlikely to explain the localization difference. In addition, we have observed similar structures using an epifluorescence microscope that switches channels much faster (<1 sec). Thanks to the reviewer for raising this concern. We have added this information into the Methods section for other readers who might wonder about the role of channel switching.

Is Pak1 localization actin dependent?

RESPONSE: We performed a LatB experiment, which is presented in the new Figure S1A. At the stage when Pak1 normally localizes to the assembling ring, we found that LatB induced redistribution of Pak1 to puncta that partially colocalized with Rlc1.

4. It is better to show if CRIB and Pak1 colocalize or not in Fig. 1C.

RESPONSE: We attempted this experiment but did not obtain clear results due to extremely weak fluorescence of Pak1 fused to a range of red fluorophores. Although their distribution likely overlaps, our key result showing similar timing of CRIB and Pak1 recruitment to the division site is still supported by the data in Figure 1C.

5. Fig. 2D is confusing. No data for the double mutant at 25C? More spacing is needed between mid1 mutant and the double one.

RESPONSE: Thanks for the suggestions. We added more spacing and labeling of data.

6. In Fig. 5, Rng10 should be grouped in C since its main role is in septation.

RESPONSE: As discussed above, we kept Rng10 in the cytokinesis group but clarify that it functions as a "coiled-coil septation protein."

7. Is it possible that Pak1 also regulates the tip localization of Scd1? The signals at cell tips are much brighter in pak1-as than wt. The intensity should be quantified.

RESPONSE: Yes, Das et al. (*Science*, 2012) showed that Scd1-GFP intensity is confined to one cell tip in *pak1* mutant cells, and the signal is brighter than in wild type. For our revised manuscript, we removed the section on polarity substrates of Pak1, but we will be sure to state and reference this cell tip signal difference when reporting Pak1 effects on Scd1 in the future.

8. In Fig. 7A, C2 domain of Mid1 should be illustrated. In 7C, the cells look weird, are the vacuole-like structures artifacts from deconvolution? Have you tried less iterations?

RESPONSE: We have modified the Mid1 schematic (now Figure 5A) to include the C2 domain. We agree that the cells in Figure 4C (previously Figure 7C) have numerous vacuoles, which are often apparent when imaging a fluorescent protein in the cytoplasm (e.g. GFP-Mid1Nter). While the appearance of vacuoles may be more obvious after deconvolution, they are not artifacts of deconvolution. The vacuoles should be visible because GFP-Mid1Nter is cytoplasmic but

excluded from the vacuoles. Wild type fission yeast cells have roughly 80 vacuoles per cell (e.g. Bone et al., 1998, *Current Biology*), so it is not surprising to see them in these cells.

9. Any genetic interactions between pak1 and cdc15 mutants?

RESPONSE: Thanks for this helpful suggestion. We identified a synthetic growth defect for *orb2-34 cdc15-140* cells at 32˚C, the semi-permissive temperature for each single mutant. This growth defect correlated with gross defects in cell division. These new data are shown in the new Figures 4A-B.

How does Pak1 phosphorylation regulate Cdc15 in cytokinesis?

RESPONSE: We found that Pak1 is required for full recruitment of Cdc15 to the CAR, as *pak1-as* cells undergo cytokinesis with residual GFP-Cdc15 at cell tips. Cdc15 is a heavily phosphorylated protein that is targeted by at least 3 cell polarity kinases (Pak1, Pom1, and Kin1). Since the Gould lab has nicely described how phosphorylation directs Cdc15 localization and ligand-binding, we decided to focus our subsequent mechanistic studies on Mid1.

How did you define the dynamics of Cdc15 punta? A FRAP assay might be more informative.

RESPONSE: As defined in the Methods section, we defined Cdc15 puncta as dynamic if they persisted for less than 12 minutes in timelape imaging. We agree that FRAP experiments would provide additional information on these dynamics. However, given our attempts to streamline the paper with tighter focus on Mid1, we limited new experiments on Cdc15.

10. On page 15, last paragraph, not sure what yeast strains were broken by sonication. In the previous paragraph, the authors mentioned that yeast cells were lysed to 80% by coffee bean grinder, which is smart and economy.

RESPONSE: We routinely use sonication as an extra step in preparation of lysates for mass spectrometry experiments to ensure full disruption of cells.

Responses to Reviewer #3 (original comments in *italics*):

This manuscript by Moseley and colleagues is in an interesting topic and has exceptionally high quality data, as is usual for work from this group. The manuscript describes work aimed at understanding how the fission yeast Pak1/Orb2 /Shk1 kinase regulates cell polarity and cytokinesis. There are two sides to the work. In the first part, they investigate Pak1 localization, its role in actin-myosin ring assembly, and also show some genetic interactions between Pak1 and dmf1/mid1 and rng2. In this part, they also show that all of Pak-kinases effects are not via

Rlc1. In the second part, they perform phospho-proteomics and uncover a number of substrates of Pak1. They show some experiments that reveal three elements downstream Pak1, with Cdc15 and Mid1 participating in cytokinesis, Scd1,Rga4, and Tea3 functioning in polarity and Sts5 in cell separation.

The work is very well done, but I have one major concern. I believe this paper straddles a large-scale proteomics effort story (a resource-type paper) and a mechanistic story and in the end does not do justice to either. I think the mechanistic analysis in figures 5-9 are fairly cursory, while parts of the front end of the paper are already described by other groups, although the current study admittedly adds a bit more to what is published. In light of this, I believe the paper does not merit publication in JCB, presently. I can see a number of ways the work can be expanded to provide detailed mechanisms, be it in actin-myosin ring function or in cell polarization or in cell separation.

RESPONSE: We appreciate the reviewer's support for the topic and data, and we appreciate the recommendation for how to address the major concern. In response, we have removed the polarity and cell separation sections. In the revised manuscript, we have focused Pak1 regulation of cytokinesis through its new substrate Mid1.

Some additional comments

• The novelty is in the phospho-proteomics. It would be nice if this Figure had a heat map and clustering of the entire results instead just showing a table.

RESPONSE: Thanks for this suggestion. We have added a volcano plot as Figure 3A in the revised manuscript. We considered a number of options to visualize all of the results, and decided that the volcano plot best represents our data set and selection of putative Pak1 substrates.

• Figure 6-8 should have Ala substitution experiments, which will further bolster conclusions from phenotypic studies.
• Regulation of Rga4 localization by Pak1 and Pom1is interesting (Fig 6). However, most of the genetics and cell biology studies in Fig 6-9 are too preliminary to conclude significance of the phospho-regulation. It would be better to have mechanistic insights about the phospho-regulation (e.g. protein-protein interaction and protein-lipid interaction).

RESPONSE: Thanks for the constructive advice. Following this suggestion, we generated Ala substitutions at Mid1 phosphorylation sites, leading to our Mid1(9A) mutant. This mutant allowed us to conclude that Pak1 phosphorylates Mid1 to promote its association with nodes, based on a number of experiments in the new Figure 5. We believe that the new line of experiments provides mechanistic insights that improve the overall impact of our study.

April 15, 2020

RE: JCB Manuscript #201908017R-A

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

Dear Dr. Moseley:

Thank you for submitting your revised manuscript entitled "Fission yeast Pak1 phosphorylates anillin-like Mid1 for spatial control of cytokinesis". We would be happy to publish your paper in JCB provided the text is amended to cite the Saha papers, and perhaps discuss the oligomerization and other issues raised by the third reviewer where appropriate, and pending final revisions necessary to meet our formatting guidelines (see details below).

- Provide the main and supplementary texts as separate, editable .doc or .docx files
- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website paying particular attention to the guidelines for preparing images and blots at sufficient resolution for screening and production
- Provide tables as excel files
- Format references for JCB
- Add MW markers to blots in S2A
- Add or reposition scale bars in figures 1A,B 4G, S1E, (hard to see)
- Add paragraph after the Materials and Methods section briefly summarizing all "Online Supplementary Materials"

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

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

B. FINAL FILES:

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

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

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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 or call (212) 327-8588.

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

Sincerely,

Daniel Lew, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

The authors have addressed all of my comments thoughtfully. The revised manuscript focuses on how Pak1 phosphorylates Mid1 to control its association with cortical nodes for contractile ring assembly at the right place and right time. The story is much more mechanistic than before. I have no more concerns. Overall, this study addresses the important question of how polarity regulators control cytokinesis at the molecular and level, and it represents a significant contribution to the field of cytokinesis.

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

The authors have successfully addressed my concerns.

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

In this manuscript Magliozzi et al, have studied the role of Cdc42 activated PAK kinase in contractile actomyosin ring (CAR) assembly using S. pombe as a model system. The key findings in the paper are interesting and very well supported by the experiments and are in line with previously published work. Overall, this paper uses a range of techniques to identify and elegantly elucidate how Pak1 kinase regulates anilin-like protein Mid1 by phosphorylation during CAR assembly. It is an interesting paper, which is clearly written and partially convincing. Although this paper has been sent as a revision, it is really a new submission due to the recasting of the manuscript and its message. The authors should address the following key questions to improve the manuscript prior to acceptance. If JCB policy allows, I am supportive of a second round of revisions.

Comments after reading other referee comments: I see the other referees are satisfied with the revisions. I think the work is a nice addition, but minimally, it needs to placed in the context of the Pollard lab papers. The experiments will flesh out the story and make a significant contribution, but I leave it to the editor for a decision on whether they are needed.

Major points:

1. Saha et al., 2012b has shown that Mid1 (309-452; M1-3) domain is crucial for Mid1 oligomerization and cells lacking this particular region didn't show any phenotype, and it normally localizes to the nodes. Note: two of the papers from Pollard's lab relevant to this story has not been cited (Saha et al., 2012a; b MBoC. The authors should cite and credit their work. How does this particular cluster of phosphorylation by Pak1 kinase at Mid1-N-terminus contribute to the CAR assembly.
2. It has been shown previously that 309-452 is crucial for mid1 oligomerization and that Mid1:1-520 forms octamers. The authors should check the oligomerization status of Mid1-N terminus with phospho-deficient (A) and phospho-mimetic (D/E) mutants.
3. Does the Plo1 sites at mid1-N-terminus (Alamonacid et al., 2011) prime the Pak1 phosphorylation of Mid1 to anchor Mid1 to the nodes?
4. The authors should check Mid1 phosphorylation dependent interaction analysis for some of the key node proteins, which is known to interact with Mid1. Does this interaction help to anchor Mid1 to nodes in early cytokinesis?
5. The authors should explain why the Mid1 full length protein with "A" doesn't show any phenotype in comparison to Mid1-Nterminus (9A). Is there any parallel mechanism that suppresses Mid1-full length 9A function? N-ter (9A) mutant shows a very strong cytokinesis phenotype, which may be due to mid1-Nter(9A) acting as a hypomorphic allele.

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

In this manuscript Magliozzi et al, have studied the role of Cdc42 activated PAK kinase in contractile actomyosin ring (CAR) assembly using S. pombe as a model system. The key findings in the paper are interesting and very well supported by the experiments and are in line with previously published work. Overall, this paper uses a range of techniques to identify and elegantly elucidate how Pak1 kinase regulates anilin-like protein Mid1 by phosphorylation during CAR assembly. It is an interesting paper, which is clearly written and partially convincing. Although this paper has been sent as a revision, it is really a new submission due to the recasting of the manuscript and its message. The authors should address the following key questions to improve the manuscript prior to acceptance. If JCB policy allows, I am supportive of a second round of revisions.

Comments after reading other referee comments: I see the other referees are satisfied with the revisions. I think the work is a nice addition, but minimally, it needs to placed in the context of the Pollard lab papers. The experiments will flesh out the story and make a significant contribution, but I leave it to the editor for a decision on whether they are needed.

Major points:

1. Saha et al., 2012b has shown that Mid1 (309-452; M1-3) domain is crucial for Mid1 oligomerization and cells lacking this particular region didn't show any phenotype, and it normally localizes to the nodes. Note: two of the papers from Pollard's lab relevant to this story has not been cited (Saha et al., 2012a; b MBoC. The authors should cite and credit their work. How does this particular cluster of phosphorylation by Pak1 kinase at Mid1-N-terminus contribute to the CAR assembly.

RESPONSE: We have added references to both Saha papers, and we thank the reviewer for pointing out this omission. We wish to note that the data for Mid1 oligomerization by domain M3 in the Saha 2012b paper have recently been shown to be based on a contaminating protein, and not by Mid1 itself. The Pollard lab's most recent paper on Mid1 N-terminus (Chatterjee and Pollard, Biochemistry, 2019) states that the Saha 2012b oligomerization result "is explained by precipitation of our bacterial preparation of Mid1p-N452 when it was concentrated, leaving a bacterial contaminant in the supernatant, which we later identified as ArnA by mass spectrometry. The ArnA polypeptide has the same mobility as Mid1p-N452 as determined by SDS–PAGE, and the ArnA protein consists of eight subunits." The inability of Mid1 N-terminus to form oligomers or octamers is further supported by Sun et al. (*Developmental Cell*, 2015). Therefore, we agree that adding references to the Saha papers improves our manuscript, but we have not discussed oligomerization of Mid1 N-terminus because it appears doubtful.

2. It has been shown previously that 309-452 is crucial for mid1 oligomerization and that Mid1:1-520 forms octamers. The authors should check the oligomerization status of Mid1-N terminus with phospho-deficient (A) and phospho-mimetic (D/E) mutants.

RESPONSE: As discussed above, the Mid1 N-terminus does not appear to form octamers based on recent studies (Sun et al., *Dev Cell* 2015; Chatterjee and Pollard, *Biochemistry* 2019). However, in the conclusion of our paper, we state the possibility that phosphorylation regulates oligomerization by condensation of the Mid1 N-terminus based on the recent paper from the Pollard lab (Chatterjee and Pollard, *Biochemistry*, 2019).

3. Does the Plo1 sites at mid1-N-terminus (Alamonacid et al., 2011) prime the Pak1 phosphorylation of Mid1 to anchor Mid1 to the nodes?

REPONSE: At this point, we do not know the sequence of phosphorylation by Plo1 and Pak1, but we agree that this question represents an interesting future direction.

4. The authors should check Mid1 phosphorylation dependent interaction analysis for some of the key node proteins, which is known to interact with Mid1. Does this interaction help to anchor Mid1 to nodes in early cytokinesis?

RESPONSE: We hope to determine how each of these interactions is regulated by Pak1 phosphorylation in future studies.

5. The authors should explain why the Mid1 full length protein with "A" doesn't show any phenotype in comparison to Mid1-Nterminus (9A). Is there any parallel mechanism that suppresses Mid1-full length 9A function? N-ter (9A) mutant shows a very strong cytokinesis phenotype, which may be due to mid1-Nter(9A) acting as a hypomorphic allele.

RESPONSE: The Mid1 C-terminus contains domains that anchor Mid1 to the cortex by binding to lipids in the absence of Mid1 N-terminus interacting with Cdr2 and Gef2. Thus, the Mid1 N-terminus is entirely reliant upon interactions that are regulated by Pak1, while the full-length Mid1 protein is not.