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Molecular organization of cytokinesis node predicts the constriction rate of the contractile ring

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September 23, 2020

Re: JCB manuscript #202008032

Prof. Caroline Laplante
North Carolina State University
Molecular Biomedical Sciences
1051 William Moore Drive
Raleigh, North Carolina 27606

Dear Dr. Laplante,

Thank you for submitting your manuscript entitled "Molecular organization of cytokinesis node predicts the constriction rate of the contractile ring." The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Overall, all three reviewers were enthusiastic about the study but each also raised several concerns which will need to be addressed before this paper would be ready for publication in JCB. In particular the comment by Reviewer #2 that, as the nodes that form in mid1-deleted cells are likely different in composition and function, they should therefore be given a different name is an important one. Also important is Reviewer #2's request that some of the key experiments be repeated with with a mid1 temperature-sensitive mutant to alleviate concerns that some of the described phenotypic variation is due to the acquisition of suppressor mutations or are an indirect consequence of aneuploidy. Reviewer #3 requests additional details on the SMLM data processing method and imaging of control structures thought to be continuous (microtubules might be good for this) to rule out the possibility that the processing method itself introduces a node-like appearance to the images. Reviewers 1 and 2 also have questions regarding the relationship between the nascent and enduring strands in Δmid1, the composition of strands in Δnaa25 cells, and whether the Δnaa25 strands are really similar to the enduring strands in Δmid1.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

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

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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

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

Sincerely,

Karen Oegema, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

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

This is a very interesting paper where the authors have shown that cytokinetic nodes exist even in the absence of the anillin Mid1. The authors show that the nodes in mid1Δ mutants form via actin interaction. Loss of mid1 results in different properties of the contractile ring and this is likely due to the organization of the Type 2 Myosin Myo2 and the nature of the actin filaments involved in the ring assembly. The authors have taken advantage of super resolution microscopy in live cells and combined it with yeast genetics to create different models of node assembly and correlate it with the corresponding behavior of the contractile ring. This research while addressing some important questions in the field of actomyosin ring assembly and constriction, also raises some new question

about how actin filaments influence ring behavior. This is a well written manuscript with strong experimental data and I mostly concur with the conclusions. However, there are a few issues that can be addressed to further improve the manuscript

Major issues:
In Fig 1E the mid1Δ myp2Δ cells although showing a much slower constriction rate, appear to still display two modes of constriction. The authors attribute this to the fact that myp2 contributes to about 50% of the constriction rate in WT. Given that constriction in mid1Δ is bimodal, could it be possible that in mid1Δ myp2Δ double mutant, the usually fast constricting, nascent rings nearly fail to constrict. Indeed, several rings have near 0 constriction rates in these cells. This would suggest that Myp2 is needed for nascent ring constriction but not for enduring ring constriction.

Fig. 3C, and S2 shows that Myo2p heads spread into a zone with a smaller radius than WT cells (i.e. 7nm wider), yet they constrict significantly faster than WT cells (Fig. 1E). This somehow opposes the central idea of the paper, which is that a more compact radius of Myo2p heads would constrict slower, given that nascent strands give rise to faster ring constriction rates (Fig. 1E). A similar conundrum appears when their model shows that enduring strands show increased interaction between Myo2 and actin, even though these rings constrictions slowly. We do not feel this is counterintuitive as mentioned in the paper. It is possible that nascent strands show faster constriction due to increased myo2-actin interaction. On the other hand, in enduring strands the interaction is excessive and likely exceeds a threshold such that the spatial organization needed to effectively constrict the ring is lost. This would slow down ring constriction.

Fig. 3D and E show a wider spread of mEGFP-Myo2p after LatA treatment in mid1Δ. This is missing a control showing what the node diameter is in LatA treated WT cells. This will indicate if even low actin levels in the nodes would influence its assembly in WT cells.

The authors show data that disassembled actin rings due to latA treatment show consistent nodes. However, these nodes appear to diffuse or move away from the cell medial in mid1Δ cells while they stay at the cell medial region in WT. Are these mid1Δ nodes that diffuse or move away still cortical? The authors also show a few node-like structures in latA treated mid1Δ cells suggesting that actin is not necessary for node formation in mid1Δ cells. However, in fig 5 they show that nodes form at non-medial actin patches. How do they reconcile these contradicting observations?

The authors show that the acetyltransferase function of Naa25p is responsible for the spread of Myo2p heads on actin filaments, as bias exists for filaments decorated with acetylated tropomyosin vs unacetylated tropomyosin. Authors assume that in naa25Δ, actin filaments are decorated with unacetylated tropomyosin, thus leading a tightly linked association with Myo2p heads, and thus slower constriction rates as seen with enduring strands. I wonder if the slow constriction in these enduring strands is due to lack of acetylated actin filaments that depend on cdc12 or is due to a decrease in the level of cdc12 or some other node component. Have the authors looked at node composition in naa25 mutants?

Minor issues:
• Fig. 5B- Are these 3D-reconstructions of the division site? It is not clear in the text, or figure legends as the authors call these projections which they are likely not.
• The last sentence of the abstract is confusing. Replacing "... function of the contractile ring" with "...behavior of the contractile ring" would be more apt.
• Please check for extremely long sentences, which could be easily broken for clarity. Also

proofreading the manuscript will help with readability.

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

The manuscript by Kimberly Bellingham-Johnstun et al. described the architecture of cytokinesis nodes in anillin mid1 deletion mutant. In mid1 deletion cells, the precursor nodes of the contractile ring cannot form. However, many cells can assemble a misplaced ring after a delay. Using confocal microscopy and super-resolution SMLM, the authors found mid1 deletion cells assembled the ring through two pathways: nascent strands and enduring strands. The rings from the two populations constricted at different speeds. They think that acetylation of tropomyosin caused the compaction of Myo2 heads, which affect the rate of ring constriction. The authors concluded that their study establishes a relationship between the molecular organization of cytokinesis nodes and the mechanical function of the contractile ring. This is an interesting study on contractile ring architecture in a powerful model system. However, I think the manuscript has to be improved before drawing such a conclusion.

Major concerns:
1. A better definition of nodes is needed. I think the claim that "nodes assemble in mid1 deletion cells" in the abstract is misleading. Even with Dr. Laplante's groundbreaking work with Pollard (PNAS) on node architectures using FPLAM and this work, no conclusive evidence to show that the precursor nodes for ring assembly in wild type cells and the nodes in the assembled ring are the same structure. It's very likely the composition and stoichiometry are different besides Mid1. Without new firm evidence, the distinction needs to be made between the two structures. It is fair to say that no ring precursor nodes are in mid1 mutant cells because the so called nodes in mid1D cells are actin dependent.

2. It is tricky to work with mid1D cells and some of the key experiments should be repeated and conclusions confirmed using one of the available mid1 temperature-sensitive mutants (mid1-6, mid1-18, or mid1-366, etc). The authors most likely have noticed that healthier mid1D cells can easily take over the liquid culture. Depending on how long the cells are grown on plates and liquid culture before imaging, healthy mid1D cells can very easily dominate the whole culture at later stages. It's likely those cells pick up suppressors or become aneuploidy. Thus, the cells with nascent and enduring strands may not have the same genetic background. The cells with nascent strand may have suppressors or are aneuploidy. So it is too simplified to conclude the difference between the cells with nascent and enduring strands are due to myosin head compactness. Thus, it is essential to confirm the results by growing mid1 temperature-sensitive mutants at restrictive temperature for 2 or 4 hrs, which is much less likely to pick up suppressors. The cells can be synchronized using hydroxylurea (HU) instead of cdc25-22 to maintain the ts phenotype if necessary.

3. The study used both mid1D::ura4+ ura4-D18 and mid1D::natMX6 ura4-D18 cells. The authors must have noticed that ura4+ cells grow at least 50% faster than ura4-D18 cells. Thus, it is very important to specify which strain is used for each figure in the strain table, otherwise, it's hard to evaluate the time courses in various experiments.

4. Problems in Figures and Figure legends:
a. It's not necessary to explain various symbols in figure legends if they are clear in the figures.
b. No panel C in Fig. 4 legend.

c. How cell edges are determined in figures (with SMLM images) need to be explained.
d. Fig. S2 legend, what does "tip of the Myo2p" mean?
e. No outliers for box blots?
f. Figure 3 and several other figures, n, SD, and statistical test are not shown. To draw conclusion on whether the RDD are different, it is impossible to evaluate with a single number. No scale bars in figure 3 A, B and some other figures.
g. Before showing super-resolution images, it's better to show a bigger region of the division site so we know what part of the cells we are looking at, especially for the contractile ring.

Minor concern:
1. It's not necessary to mention DNA similarity between Mid1 and anillin on page 2.

2. The first sentence on page 3 is misleading and not logical. Mid1 is essential for the initial assembly of precursor node, but it does not mean that the node proteins will disperse evenly after it's departure prior to ring constriction. The roles of Mid1 are to bring the proteins together to the division site. The multivalent interactions among the proteins and plasma membrane can be maintained without Mid1 once assembled.

3. The cells with enduring strands have two SPBs close to each other. Have they finished mitosis with two separated nuclei? The cells with nascent and enduring strands may be at different cell-cycle stages. Thus, the proteins in the ring may have different phosphorylation status and thus contribute to the difference in ring constriction. This point should be discussed.

4. Actin orientation and stability in the nascent and enduring strands might be different, random or bipolar in nascent or monopolar in enduring strands, which could affect ring constriction and myosin head compactness. This point should also be discussed.

5. SPB separation marks the onset of mitosis, not anaphase A in S. pombe (page 4).

6. The constriction rates of contractile rings in naa25D cells are similar to those rings made from enduring strands in mid1D cells, which is used to support that the roles of Cdc8 acetylation in myosin distribution. But the ring formation looks quite different. Please discuss the caveat.

7. Page 15, is it true that only 10 ul was used to make gelatin pad? It might be too thin and dry out quickly.

8. Page 15, to count proteins, how images are projected? Max or sum intensity?

9. Page 15, last sentence mentioned the number of molecules per contractile ring are in Table S1, but S1 is the strain table.

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

This manuscript details the molecular organization of cytokinetic node proteins in mid1Δ fission yeast cells. In fission yeast, the cytokinetic ring assembles from precursor nodes that are positioned in the cell middle. Mid1 is known to provide the spatial signals for cytokinetic node positioning the right place. Past work has shown that nodes assemble in mid1 mutants, but they are displaced and may have altered activity (Huang 2008; Saha 2012). In the current study, Laplante and colleagues

employ single molecule localization microscopy (SMLM) to obtain nanometer resolution images of nodes in mid1 mutants. They also perform time lapse imaging of nodes becoming cytokinetic rings in mid1 mutants. The authors classify the cytokinetic rings in mid1Δ cells as nascent or enduring, and they characterize the constriction and molecular composition of nodes in these two strands. Their data suggest that some cytokinetic structures in mid1 mutant cells are protein aggregates that associate with actin structures. Some interesting differences are observed in the orientation of myosin motors with nodes from different mid1 mutant rings. Other interesting observations include potential suppression of mid1 mutant phenotypes by deletion of the formin for3, and an interesting role for tropomyosin acetylation in the organization of myosin motors in nodes.

The study presents a large amount of data that will be interesting for researchers studying different aspects of cytokinesis. The use of SMLM has the potential to reveal an unprecedented view of protein organization in nodes, although it comes with the difficulty of conflicting studies in the field (see below). Given the large amount of data, the paper does not always present a clear central message or conclusion. The title suggests a broad assessment of differently organized nodes, whereas it really compares two different classes of rings in mid1 mutants. In general, the paper has a number of strengths, and its overall significance and impact could be strengthened by streamlining the central model and by addressing the following concerns:

Main comments

1. What is the relationship between nascent strands and enduring strands? It seems that enduring strands are just nascent strands that do not become orthogonal prior to SIN activation. It would be helpful for the authors to describe the connection between these classes in more detail, and to consider them in the context of Huang et al.'s data regarding the ability of mid1 mutant rings to become orthogonal if SIN activation is delayed.

2. There is currently some controversy as to whether the constricting cytokinetic ring is comprised of nodes or of a homogenized mix of node proteins. On the one hand, Laplante et al. (PNAS 2016) provided super-resolution microscopy suggesting the presence of nodes in the ring. On the other hand, McDonald et al. (eLife, 2017) provided super-resolution microscopy suggesting the opposite: that the ring is not comprised of discreet nodes, but rather a more continuous array of myosin motors and other proteins. This second conclusion has been supported by cryoEM images (Swulius et al., PNAS 2018) and by modeling approaches (Nguyen et al., MBoC 2018), but it is clear that more work is needed to resolve this controversy. Data in the present study are consistent with the previous work by Laplante, but I remain a bit confused about whether it is influenced by data processing. For example, the text states: "Nodes in constricting contractile rings can be too densely packed to be clearly resolved even in SMLM images. We overcame this caveat by reconstructing SMLM datasets using subsets of camera frames." An alternative interpretation of nodes being too densely packed to resolve by SMLM would be that they are not nodes, but rather a more continuous structure. How were subsets of camera frames selected, and does this selection process impact whether nodes are seen or not? This concern becomes amplified by statements in the methods section that "clusters of localized emitters associated with cytokinesis structures were manually selected from the reconstructed SMLM images" and that "fitting results that did not converge properly and resulted in center positions outside the ring boundary or extremely large or small sigma values were also discarded from the final results." These statements imply that the authors have discarded data that do not fit in their model, as opposed to unbiased analysis of all data. The main conclusions in the paper rely heavily on the notion that rings are comprised of discreet nodes, so it seems imperative to remove any doubt about the presence of nodes in constricting rings. I do not mean to suggest that the authors are wrong, and to their credit they

address the discrepancy with the McDonald study, but the lingering controversy makes it hard to judge the impact of their subsequent data regarding nodes in rings.

3. Related to the previous comment, do contiguous non-nodal structures such as actin or septins appear punctate in similar SMLM experiments with similar data selection and processing? It would help for the authors to show that non-node structures (perhaps GFP-CHD in the ring? of septin-GFP?) are not punctate or node-like by their approaches. Perhaps such a control has already been performed, and the authors can point to such references.

4. If mid1Δ mutants have tighter Myo2 radius and more Myo2 associated, then why do they exhibit increased CAR constriction rate? In the discussion, it is proposed that more Myo2 leads to slower CAR constriction. For the readers understanding, more clarity possibly in the form of a cartoon model about how node radius and Myo2 levels compute to CAR constriction would be helpful.

Minor comments

5. On page 6, the authors use LatA treatment to conclude that nodes are "bound to actin filaments." I would suggest changing the wording because the data show that nodes are "dependent" on actin, not necessarily "bound."

6. In Figure 1E, a myp2Δ should be included in the constriction rate quantification.

7. In Figure 3E, it would be helpful to include the RDD of Myo2 in wildtype cells treated with LatA.

8. In Figure 5A, how is the timing of SPB splitting known since there is not an SPB marker in the top two strains? It would be helpful to provide more information about how time was determined in this experiment.

9. The result of for3Δ suppressing the phenotypes of a mid1Δ mutant is very interesting. Some images of these cells as well as quantification would be helpful for the reader as well as a bit more discussion/speculation in the text.

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

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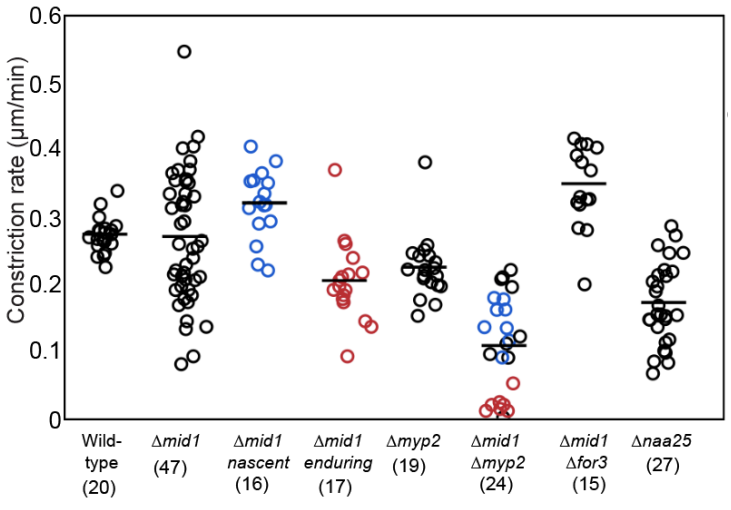
We thank the reviewer for the thorough revision of our work and for the thoughtful comments. Addressing each of these comments strengthen our work and improved the text. For easy navigation, we label each answer with **[A]**.

Major issues:
1- In Fig 1E the mid1Δ myp2Δ cells although showing a much slower constriction rate, appear to still display two modes of constriction. The authors attribute this to the fact that myp2 contributes to about 50% of the constriction rate in WT. Given that constriction in mid1Δ is bimodal, could it be possible that in mid1Δ myp2Δ double mutant, the usually fast constricting, nascent rings nearly fail to constrict. Indeed, several rings have near 0 constriction rates in these cells. This would suggest that Myp2 is needed for nascent ring constriction but not for enduring ring constriction.

[A]: To address this comment, we re-analyzed our data, acquired new data and identified the type of strand that made the contractile rings in the same manner we did for Δ*mid1* cells. We found that the enduring strands made slow constricting contractile rings (red) while the nascent strands made the fast constricting contractile rings (blue). We updated Figure 1E.

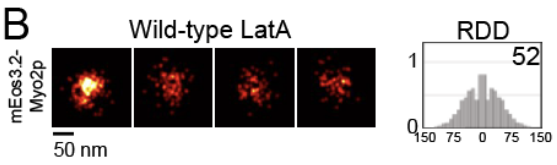


The movies did not always contain enough time points before ring assembly to determine which type of strand generated the contractile rings.

2- Fig. 3C, and S2 shows that Myo2p heads spread into a zone with a smaller radius than WT cells (i.e. 7nm wider), yet they constrict significantly faster than WT cells (Fig. 1E). This somehow opposes the central idea of the paper, which is that a more compact radius of Myo2p heads would constrict slower, given that nascent strands give rise to faster ring constriction rates (Fig. 1E). A similar conundrum appears when their model shows that enduring strands show increased interaction between Myo2 and actin, even though these rings constrictions slowly. We do not feel this is counterintuitive as mentioned in the paper. It is possible that nascent strands show faster constriction due to increased myo2-actin interaction. On the other hand, in enduring strands the interaction is excessive and likely exceeds a threshold such that the spatial organization needed to effectively constrict the ring is lost. This would slow down ring constriction.

[A]: This is an interesting interpretation. We added the following to the discussion to add it: “An optimal ratio of Myo2p heads bound to actin filaments may exist to achieve optimal constriction rate. A ratio of bound heads that exceed the optimal range, such as in enduring strands, would be counterproductive and impedes constriction. Why nascent strands produce rings that constrict faster than wild-type rings is unclear and further work will be necessary to identify other changes to the molecular organization of contractile rings made from nascent strands. Those changes may include but are not limited to increased contractile force by Myp2p and/or Myo51p, reduced drag forces or increased rate of septum deposition.”

3- Fig. 3D and E show a wider spread of mEGFP-Myo2p after LatA treatment in mid1Δ. This is missing a control showing what the node diameter is in LatA treated WT cells. This will indicate if even low actin levels in the nodes would influence its assembly in WT cells.



[A]: The Myo2p heads in ring nodes of wild-type cells treated with LatA show a radius of 52 nm comparable to broad band nodes (50 nm) prior to actin polymerization at the division plane. New Figure S2B shows the data.

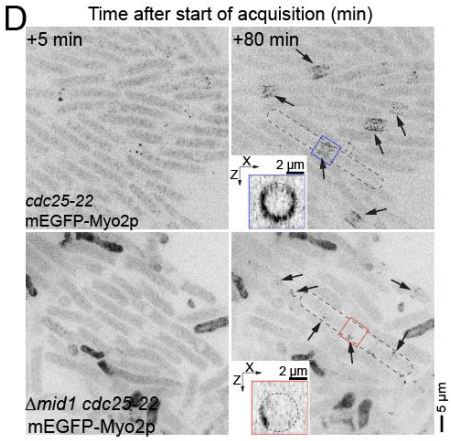
This information was also added to Result section 3. The previous sentence "The Myo2p heads distribute in a more relax zone of 49 nm of radius in arrested Δ*mid1 cdc25-22* cells released in LatA, comparable to the size of the zone occupied by Myo2p heads in the band nodes of wild-type cells (Figure 3E and S2D). " now reads " In these cells, the Myo2p heads distribute in a relax zone of 49 nm of radius, comparable to the size of the zone occupied by Myo2p heads in the band of P-nodes of wild-type cells and LatA treated wild-type cells (Figure 3E and S2B, E)."

4a- The authors show data that disassembled actin rings due to latA treatment show consistent nodes. However, these nodes appear to diffuse or move away from the cell

medial in mid1Δ cells while they stay at the cell medial region in WT. Are these mid1Δ nodes that diffuse or move away still cortical?

[A]: Yes, the nodes are cortical as seen in these XZ projections.

We added that nodes are cortical to text in Results section 3. Sentence now reads, “In contrast, P-nodes appeared randomly across the cortex in ∆mid1 cells as cortical complexes with no bias for the cell equator or other presumptive division plane.”



We also updated Figure 3D to include XZ projections across the cells showing the cortical localization of nodes in both wild-type and ∆*mid1* cells treated with LatA.

4b- The authors also show a few node-like structures in latA treated mid1Δ cells suggesting that actin is not necessary for node formation in mid1Δ cells. However, in fig 5 they show that nodes form at non-medial actin patches. How do they reconcile these contradicting observations?

[A]: Our data shows that, like in wild-type cells, actin is not essential for node assembly in ∆*mid1* cells (LatA treated ∆*mid1* cells Figure 3D and E). However, unlike wild-type cells, at the time nodes proteins appear in ∆*mid1* cells (cells not treated with LatA, Figure 5A) actin is already present in the cells.

The confusion may arise from the manner and timing of node protein recruitment in ∆*mid1* cells. Node protein recruitment in ∆*mid1* cells occurs in a visible stepwise manner. Step 1: Cytokinesis proteins concentrate in cytoplasmic aggregates near the cell tips at t = ~5-10 min. Step 2: These aggregates move from the cell tips to the presumptive division plane over a short period of ~5 min. Step 3: The cytokinesis proteins become cortical nodes assembled onto actin strands, the nascent strands at t = ~15-20 min.

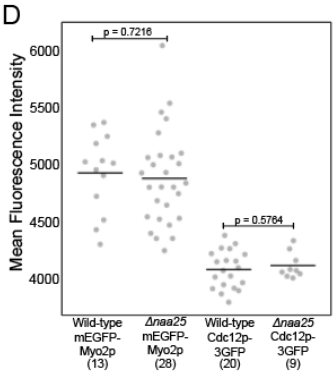
As nodes are cortical protein complexes (Wu et al. JCB. 2006) and the aggregates mentioned in step 1 are cytoplasmic, we do not refer to them as nodes. We also never observed organized protein clusters in those aggregates by SMLM. At this moment, we do not know the specific molecular organization of the proteins in those cytoplasmic aggregates. What we call “nodes” are the organized protein clusters at the cortex mentioned in step 3 (figure 5, t=6 min).

We identified confusing text and clarified this point by adding the following sentence to Result section 3: “Precursor nodes appeared ~50 min after shifting the cell cultures to room temperature in both genotypes, confirming that cytokinesis nodes assemble in the absence of actin filaments in either wild-type or ∆*mid1 cdc25-22* cells.”

We also added the following sentence to Result section 5: “Because nodes are defined as cortical protein complexes, we only refer to them as P-nodes once they contact the cortex, not when they are cytoplasmic aggregates with no obvious contact with the cortex.”

5- The authors show that the acetyltransferase function of Naa25p is responsible for the spread of Myo2p heads on actin filaments, as bias exists for filaments decorated with acetylated tropomyosin vs unacetylated tropomyosin. Authors assume that in naa25Δ, actin filaments are decorated with unacetylated tropomyosin, thus leading a tightly linked association with Myo2p heads, and thus slower constriction rates as seen with enduring strands. I wonder if the slow constriction in these enduring strands is due to lack of acetylated actin filaments that depend on cdc12 or is due to a decrease in the level of cdc12 or some other node component. Have the authors looked at node composition in naa25 mutants?

[A]: To address this comment, we compared the amount of Cdc12p and Myo2p between wild-type and Δ*naa25* cells by quantitative confocal microscopy. We measured the total amount of fluorescence of Cdc12p-3GFP in the contractile rings of Δ*naa25* cells and compared the values to wild-type controls. We found no significant difference between the total amount of fluorescence between the two strains suggesting that the levels of both Cdc12p and Myo2p are not decreased in Δ*naa25*.



We added the following to the Result section 6: “We measured no significant differences in the fluorescence intensity of mEGFP-Myo2p and Cdc12p-3GFP in constricting contractile rings in Δnaa25 and wild-type cells suggesting that the overall composition of these contractile rings is comparable (Figure S3D)”. We also added figure panel S3D.

Minor issues:

6- Fig. 5B- Are these 3D-reconstructions of the division site? It is not clear in the text, or figure legends as the authors call these projections which they are likely not.

[A]: The images show projections of the color-coded boxed areas in Figure 5A in the XZ plane. We added this information in the figure legend as well as a label in the figure panel. Figure legend for Fig. 5B now reads: “Orthogonal views of boxed regions in A *(*Δ*mid1* cell; 7 optical planes).”

7- The last sentence of the abstract is confusing. Replacing "... function of the contractile ring" with "...behavior of the contractile ring" would be more apt.

[A]: We have replaced the final sentence with “Our work establishes a predictive correlation between the molecular organization of nodes and the behavior of the

contractile ring.” For consistency, we also replaced the term “mechanical function” with the term “behavior” in the last sentence of the introduction.

8- Please check for extremely long sentences, which could be easily broken for clarity. Also proofreading the manuscript will help with readability.

[A]: We thank the reviewer for this comment. We identified multiple long sentences throughout the text and simplified them. We also proofread the manuscript and made changes that we thought would improve readability.

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

The manuscript by Kimberly Bellingham-Johnstun et al. described the architecture of cytokinesis nodes in anillin mid1 deletion mutant. In mid1 deletion cells, the precursor nodes of the contractile ring cannot form. However, many cells can assemble a misplaced ring after a delay. Using confocal microscopy and super-resolution SMLM, the authors found mid1 deletion cells assembled the ring through two pathways: nascent strands and enduring strands. The rings from the two populations constricted at different speeds. They think that acetylation of tropomyosin caused the compaction of Myo2 heads, which affect the rate of ring constriction. The authors concluded that their study establishes a relationship between the molecular organization of cytokinesis nodes and the mechanical function of the contractile ring. This is an interesting study on contractile ring architecture in a powerful model system. However, I think the manuscript has to be improved before drawing such a conclusion.

We thank the reviewer for the thorough evaluation of our work. We carefully addressed each concern with new experiments, new analyses or clarifying changes to the text. Please find our answers to each concern below labeled with **[A]**.

Major concerns:
1. A better definition of nodes is needed. I think the claim that "nodes assemble in mid1 deletion cells" in the abstract is misleading. Even with Dr. Laplante's groundbreaking work with Pollard (PNAS) on node architectures using FPLAM and this work, no conclusive evidence to show that the precursor nodes for ring assembly in wild type cells and the nodes in the assembled ring are the same structure. It's very likely the composition and stoichiometry are different besides Mid1. Without new firm evidence, the distinction needs to be made between the two structures. It is fair to say that no ring precursor nodes are in mid1 mutant cells because the so called nodes in mid1D cells are actin dependent.

[A]: We agree that the term “node” is too vague. We now clearly define nodes in the text by adding the sentence (Result section 5) "nodes are defined as cortical protein complexes", which refers to the original work where the term “node” was first coined (Wu et al. JCB. 2006). It is already known that nodes vary in protein composition throughout the cell cycle with three types of interphase nodes and cytokinesis nodes (Akamatsu et al., 2014; Deng and Moseley, 2013; Wu et al., 2003; Wu et al., 2006).

We also agree that future work will likely identify more characteristics that will further distinguish nodes into sub-categories. We therefore added a qualifier to the term node to clearly distinguish the different types of nodes in this work. We call “precursor nodes” the nodes of the broad band in wild-type cells and strand nodes of Δ*mid1* cells. We call “ring nodes” nodes found in the contractile ring.

To further address this concern, we added the following sentence to Results Section 2: “To differentiate between the types of nodes, we use the term “precursor nodes” (P-

nodes) to refer to nodes found in the broad band and strands. We use the term “ring nodes” (R-nodes) to refer to nodes of the contractile ring.”

2. It is tricky to work with mid1D cells and some of the key experiments should be repeated and conclusions confirmed using one of the available mid1 temperature-sensitive mutants (mid1-6, mid1-18, or mid1-366, etc). The authors most likely have noticed that healthier mid1D cells can easily take over the liquid culture. Depending on how long the cells are grown on plates and liquid culture before imaging, healthy mid1D cells can very easily dominate the whole culture at later stages. It's likely those cells pick up suppressors or become aneuploidy. Thus, the cells with nascent and enduring strands may not have the same genetic background. The cells with nascent strand may have suppressors or are aneuploidy. So it is too simplified to conclude the difference between the cells with nascent and enduring strands are due to myosin head compactness. Thus, it is essential to confirm the results by growing mid1 temperature-sensitive mutants at restrictive temperature for 2 or 4 hrs, which is much less likely to pick up suppressors. The cells can be synchronized using hydroxylurea (HU) instead of cdc25-22 to maintain the ts phenotype if necessary.

[A]: We are aware that Δ*mid1* cells can accumulate suppressor mutations over time. We took the following two precautions to ensure our phenotypes, specifically the presence of either nascent or enduring strands, are not caused by a random change in the genetic background of the cells.

Precaution 1: We imaged *mid1-366* expressing Rlc1p-tdTomato as nodes and contractile ring marker and Sad1-mEGFP as SPB marker to determine whether nascent strands are present only in cells that have acquired suppressor mutations. We incubated *mid1-366* Rlc1p-tdTomato Sad1-mEGFP cells at non-permissive temperatures and imaged at room temperature (we do not currently have a heated chamber on our microscope). In these cells, we observed contractile ring assembly from both nascent and enduring strands. The nascent strands make rings that constrict at 0.35 µm/min (n=11 cells) whereas enduring strands make rings that constrict at 0.22 µm/min (n=8 cells). Those rates are comparable to the Δ*mid1* constriction rates we measured for both nascent (p=0.5686) and enduring strands (p=0.0935). We also imaged *mid1-366* Rlc1p-tdTomato Sad1-mEGFP grown at room temperature and found that contractile ring assembly occurs by the coalescence of a band of equatorial nodes in ~80% of the cells or from the looping of a nascent strand in the remaining ~20% of the cells (n = 46 cells). The nascent strands appear at the presumptive division plane at t = 7.4 min, comparable to the time of appearance of nascent strands in Δ*mid1* cells. Therefore, nascent strands are not the result of a different genetic background.

We added the following passage to the Result section 1: “We considered that the two types of strands could result from differences in genetic backgrounds due to the accumulation of random suppressor mutations in Δ*mid1* cells. We imaged *mid1-366* Rlc1p-tdTomato Sad1p-mEGFP cells pre-incubated at non-permissive temperature and observed contractile ring assembly from both nascent and enduring strands. Contractile

rings made by nascent strands constricted at 0.35 µm/min whereas those made by enduring strands constricted at 0.22 µm/min (Figure S1A). Interestingly, *mid1-366* Rlc1p-tdTomato Sad1p-mEGFP cells grown at room temperature assemble contractile rings either by the coalescence of a band of equatorial nodes (~80% of the cells) or from the looping of a nascent strand (~20% of the cells, n = 46 cells). Therefore, nascent and enduring strands are the results of defective Mid1p function."

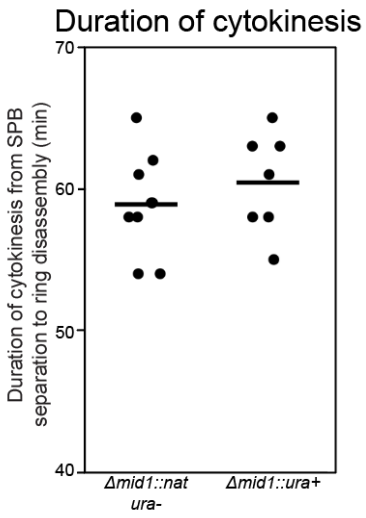
Precaution 2: The Δ*mid1::ura+* strain was generated in the Pollard lab multiple years ago and all the frozen strains were tested for the phenotypes described previously (Paoletti and Chang, 2000; Saha and Pollard, 2012a; Sohrmann et al., 1996). We generated all the Δ*mid1::natR* strains in our own lab. The phenotypes of the different strains were carefully compared, and all phenotypes measured were the identical across all deletion strains. In addition, we thawed fresh cultures from the -80˚C stocks to perform the experiments to be done the following week. The cultures were then discarded to prevent the accumulation of suppressor mutations over time.

Relevant to this concern is the short genomic distance between the *myo2* and *mid1* loci. The *myo2* and *mid1* loci are only 83 Kb apart from each other preventing us from recombining our mEos3.2-Myo2p, Myo2p-mEos3.2 and mEGFP-Myo2p constructs into the Δ*mid1* background. We had to delete the *mid1* gene from the Myo2-tagged strains for every new strain generating "new" Δ*mid1* strains every time and for each deletion. Deleting *mid1* was more efficient than N-terminal tagging Myo2p. Integrating at the N-terminus of the *myo2* gene has always been very inefficient in our hands throughout our experience working with Myo2p (Laplante et al. Curr Biol. 2015, Laplante et al. PNAS. 2016 and this work). All strains obtained were carefully compared for consistency of phenotypes.

In conclusion, given the multiple new *mid1* deletion strains we generated for this work, the careful comparison we performed for each strain and the results obtained with the temperature sensitive allele *mid1-366*, it is highly unlikely that the same phenotypes observed in all the strains are caused by random variations in the genetic background.

We entered the following information in the Methods:
Δ*mid1* cells can accumulate suppressor mutations overtime (Coffman et al., 2009; Lee and Wu, 2012; Tao et al., 2014). To avoid complications from different genetic backgrounds, all Δ*mid1* cells used in this study were carefully compared to ensure that all phenotypes measured were the identical across all deletion strains. In addition, we freshly thawed yeast cultures from the -80˚C stocks, grew them on YE5S plates for 2-3 days at 25˚C and then used them to prepare liquid cultures that were grown for 36 hours prior to imaging. Plates and liquid cultures were discarded and replaced with fresh cultures on a weekly basis to prevent the accumulation of suppressor mutations.

3. The study used both mid1D::ura4+ ura4-D18 and mid1D::natMX6 ura4-D18 cells. The authors must have noticed that ura4+ cells grow at least 50% faster than ura4-D18 cells. Thus, it is very important to specify which strain is used for each figure in the strain table, otherwise, it's hard to evaluate the time courses in various experiments.



[A]: We did not notice obvious differences in the growth of our different Δ*mid1* strains (ura+ versus ura-) during overnight cultures at 25˚C. To address this concern, we measured the total duration of mitosis between the two strains from spindle pole body separation until the disassembly of the contractile ring. We focused on mitosis as this is the phase of the cell cycle we compare between our different strains. We found no significant differences (student t-test p=0.4042) between the two different types of strains within that period of the cell cycle.

We added the following sentence to the Methods to reflect this: “Both Δ*mid1::natMX6* and Δ*mid1::ura4+* genetic backgrounds were utilized in this study, as the duration of cytokinesis from SPB separation to contractile ring disassembly between Δ*mid1::natMX6* cells (n=8) and Δ*mid1::ura4+* (n=7) was not significantly different (Student’s t-test, p=0.4042).”

4. Problems in Figures and Figure legends:

a. It's not necessary to explain various symbols in figure legends if they are clear in the figures.

[A]: We agree and deleted the markers from the figure legends.

b. No panel C in Fig. 4 legend.

[A]: We added the missing “C” label.

c. How cell edges are determined in figures (with SMLM images) need to be explained.

[A]: The cell edges were identified as described in Laplante et al. PNAS. 2016 Figure S2C. The following description was added to the methods. “The edge of the cell was identified by increasing the brightness of SMLM images to enhance the cytoplasmic background. The edge of the cell was located where the cytoplasmic background drops off at the interface with the space outside the cells (Laplante et al., 2016b).”

d. Fig. S2 legend, what does "tip of the Myo2p" mean?

[A]: This meant “tip of the Myo2p tails”. The legend has been corrected and the rest of the text searched for this mistake.

e. No outliers for box blots?

[A]: We changed the box plots to new versions that display the outliers.

f. Figure 3 and several other figures, n, SD, and statistical test are not shown. To draw conclusion on whether the RDD are different, it is impossible to evaluate with a single number.

[A]: All of the statistical results for the SMLM data is listed in Figure S4A and B. Since most of the comparisons between the distribution of localizations was significant between the markers using a KS test, we listed which comparisons were not significant at p <0.05. The number of nodes for each marker in each dataset is also listed. The KS test compares the maximum distance between CDF curves (also shown in Figure S4A) and a calculated critical for the desired significance level. It does not rely on SD for its calculation. The RDD output by our analysis pipeline is the average distribution of localizations within a certain node marker and does not have a SD associated with it, and therefore a SD value is not listed for RDD data. To make this information easier to find, we introduced this sentence into the Methods, “The results of the KS test comparisons and sample size of the super-resolution datasets can be found in Figure S4A and B.”

For confocal data, all relevant statistical tests used are listed in the Methods, and any significant results and associated sample sizes are indicated in the figure panel where the data is displayed.

g. No scale bars in figure 3 A, B and some other figures.

[A]: We added the missing scale bars.

h. Before showing super-resolution images, it's better to show a bigger region of the division site so we know what part of the cells we are looking at, especially for the contractile ring.

[A]: We clarified the use of Figure 2C in the legend by adding the following sentence: “Cropped images of contractile rings in wild-type and Δ*mid1* cells are shown as both 40 s and sequential 10 s reconstructions to demonstrate the ring node density within these structures.”

We also added the following to Result section 2: “Nodes in constricting contractile rings are densely packed but are clearly distinguished in images reconstructed using fewer camera frames (Figure 2D and E).”

Minor concern:

1. It's not necessary to mention DNA similarity between Mid1 and anillin on page 2.

[A]: The term “DNA” was removed from the sentence on page 2.

2. The first sentence on page 3 is misleading and not logical. Mid1 is essential for the initial assembly of precursor node, but it does not mean that the node proteins will disperse evenly after it's departure prior to ring constriction. The roles of Mid1 are to bring the proteins together to the division site. The multivalent interactions among the proteins and plasma membrane can be maintained without Mid1 once assembled.

[A]: We deleted the second part of the sentence. The sentence now reads: “Together, those observations supported the interpretation that Mid1p is responsible for the organization of cytokinesis proteins into nodes.”

Comments 3 and 4 were addressed together.

3. The cells with enduring strands have two SPBs close to each other. Have they finished mitosis with two separated nuclei? The cells with nascent and enduring strands may be at different cell-cycle stages. Thus, the proteins in the ring may have different phosphorylation status and thus contribute to the difference in ring constriction. This point should be discussed.

4. Actin orientation and stability in the nascent and enduring strands might be different, random or bipolar in nascent or monopolar in enduring strands, which could affect ring constriction and myosin head compactness. This point should also be discussed.

[A to 3 and 4]: To address a few different comments including these two comments, we added a new section to the discussion. “Enduring strands assemble from nascent strands that fail to loop into contractile rings or from contractile ring that do not disassemble at the end of cytokinesis. The making of an enduring strand from one of those preexisting cytokinesis structures requires For3p. For3p may be required to polymerize actin for the enduring strand either de novo or in the form of actin cables that bundle with the nascent strand or disassembling contractile ring. Enduring strands are persistent structures and cells that make a contractile ring from these strands are likely at a different phase of the cell cycle than cells that make their contractile ring for a nascent strand of from the coalescence of a band of P-nodes. As a result, the activity of important signaling pathways including the septation initiation network (SIN) may be different across these cellular contexts. Specifically, the SIN pathway regulates constriction, contractile ring integrity and septation in wild-type cells and also the appearance of contractile rings in Δ*mid1* cells (Huang et al., 2008; Schmidt et al., 1997; Wu et al., 2003).” These different contexts likely result in additional factors that impact contractile ring behavior like differential posttranslational modification of cytokinesis

proteins that may alter the actin cytoskeleton."

5. SPB separation marks the onset of mitosis, not anaphase A in S. pombe (page 4).

[A]: We removed that statement. The sentence "We used the separation of the SPBs, the onset of anaphase A, to time the assembly of a contractile ring from nascent strands in ∆mid1 cells (Wu et al., 2003)." now reads "We used the separation of the SPBs to time the assembly of a contractile ring from nascent strands in ∆mid1 cells (Wu et al., 2003).". To be consistent, we modified the sentence (introduction) "Under wild-type conditions, Mid1p localizes to Type 1 interphase nodes early in G2, transfers to Type 2 interphase nodes ~30 min prior to anaphase A and is subsequently joined by other cytokinesis proteins to form the cytokinesis nodes (Akamatsu et al., 2014; Guzman-Vendrell et al., 2013).", which now reads " Under wild-type conditions, Mid1p localizes to Type 1 interphase nodes early in G2, transfers to Type 2 interphase nodes ~30 min prior to spindle pole body (SPB) separation and is subsequently joined by other cytokinesis proteins to form the cytokinesis nodes (Akamatsu et al., 2014; Guzman-Vendrell et al., 2013)."

6. The constriction rates of contractile rings in naa25D cells are similar to those rings made from enduring strands in mid1D cells, which is used to support that the roles of Cdc8 acetylation in myosin distribution. But the ring formation looks quite different. Please discuss the caveat.

[A]: To discuss this difference between ∆*naa25* and ∆*mid1* cells, we added a sentence to the Result section 6: "Although contractile rings in ∆*naa25* cells assemble from the coalescence of a band of P-nodes, their constriction rate is comparable to that of contractile rings made from enduring strands in ∆*mid1* cells. The common factor between these two different contexts is possibly the unacetylated state of Cdc8p."

7. Page 15, is it true that only 10 ul was used to make gelatin pad? It might be too thin and dry out quickly.

[A]: We tested different volumes of gelatin for our gelatin pads and found that 10 µL was most suitable for SMLM. The gelatin pads are freshly made before our experiments are stable for that day when kept in a "humid box" and wrapped in a sealable bag.

8. Page 15, to count proteins, how images are projected? Max or sum intensity?

[A]: The images were sum projected as reported in the reference Wu and Pollard, Science, 2005. This information was added to the sentence in the Methods and now reads "To count proteins in contractile rings, we created sum projection images of fields of cells from stacks of 19 optical images separated by 0.36 µm (Wu et al., 2008; Wu and Pollard, 2005).".

9. Page 15, last sentence mentioned the number of molecules per contractile ring are in Table S1, but S1 is the strain table.

[A]: We fixed this mistake. The sentence now refers to Figure 1G.

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

This manuscript details the molecular organization of cytokinetic node proteins in mid1Δ fission yeast cells. In fission yeast, the cytokinetic ring assembles from precursor nodes that are positioned in the cell middle. Mid1 is known to provide the spatial signals for cytokinetic node positioning the right place. Past work has shown that nodes assemble in mid1 mutants, but they are displaced and may have altered activity (Huang 2008; Saha 2012). In the current study, Laplante and colleagues employ single molecule localization microscopy (SMLM) to obtain nanometer resolution images of nodes in mid1 mutants. They also perform time lapse imaging of nodes becoming cytokinetic rings in mid1 mutants. The authors classify the cytokinetic rings in mid1Δ cells as nascent or enduring, and they characterize the constriction and molecular composition of nodes in these two strands. Their data suggest that some cytokinetic structures in mid1 mutant cells are protein aggregates that associate with actin structures. Some interesting differences are observed in the orientation of myosin motors with nodes from different mid1 mutant rings. Other interesting observations include potential suppression of mid1 mutant phenotypes by deletion of the formin for3, and an interesting role for tropomyosin acetylation in the organization of myosin motors in nodes.

The study presents a large amount of data that will be interesting for researchers studying different aspects of cytokinesis. The use of SMLM has the potential to reveal an unprecedented view of protein organization in nodes, although it comes with the difficulty of conflicting studies in the field (see below). Given the large amount of data, the paper does not always present a clear central message or conclusion. The title suggests a broad assessment of differently organized nodes, whereas it really compares two different classes of rings in mid1 mutants. In general, the paper has a number of strengths, and its overall significance and impact could be strengthened by streamlining the central model and by addressing the following concerns:

We thank the reviewer for the positive comments and thoughtful criticisms of our work. We addressed each concern with new experiments, new analyses or updated text. Please find our answers, labeled with **[A]**, to each concern below.

Main comments

1. What is the relationship between nascent strands and enduring strands? It seems that enduring strands are just nascent strands that do not become orthogonal prior to SIN activation. It would be helpful for the authors to describe the connection between these classes in more detail, and to consider them in the context of Huang et al.'s data regarding the ability of mid1 mutant rings to become orthogonal if SIN activation is delayed.

[A]: To address a few different comments including this one, we added a new section to the discussion. “Enduring strands assemble from nascent strands that fail to loop into contractile rings or from contractile ring that do not disassemble at the end of cytokinesis. The making of an enduring strand from one of those preexisting cytokinesis

structures requires For3p. For3p may be required to polymerize actin for the enduring strand either de novo or in the form of actin cables that bundle with the nascent strand or disassembling contractile ring. Enduring strands are persistent structures and cells that make a contractile ring from these strands are likely at a different phase of the cell cycle than cells that make their contractile ring for a nascent strand of from the coalescence of a band of P-nodes. As a result, the activity of important signaling pathways including the septation initiation network (SIN) may be different across these cellular contexts. Specifically, the SIN pathway regulates constriction, contractile ring integrity and septation in wild-type cells and also the appearance of contractile rings in Δ*mid1* cells (Huang et al., 2008; Schmidt et al., 1997; Wu et al., 2003).” These different contexts likely result in additional factors that impact contractile ring behavior like differential posttranslational modification of cytokinesis proteins that may alter the actin cytoskeleton.”

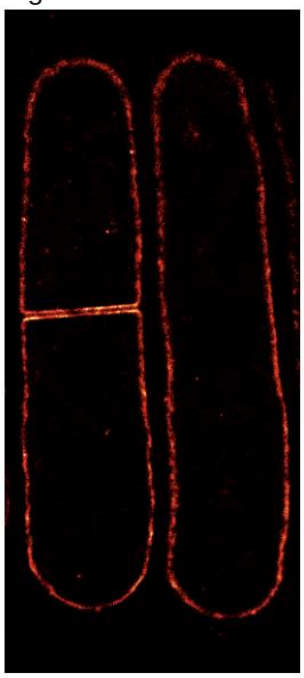
2. There is currently some controversy as to whether the constricting cytokinetic ring is comprised of nodes or of a homogenized mix of node proteins. On the one hand, Laplante et al. (PNAS 2016) provided super-resolution microscopy suggesting the presence of nodes in the ring. On the other hand, McDonald et al. (eLife, 2017) provided super-resolution microscopy suggesting the opposite: that the ring is not comprised of discreet nodes, but rather a more continuous array of myosin motors and other proteins. This second conclusion has been supported by cryoEM images (Swulius et al., PNAS 2018) and by modeling approaches (Nguyen et al., MBoC 2018), but it is clear that more work is needed to resolve this controversy. Data in the present study are consistent with the previous work by Laplante, but I remain a bit confused about whether it is influenced by data processing. For example, the text states: "Nodes in constricting contractile rings can be too densely packed to be clearly resolved even in SMLM images. We overcame this caveat by reconstructing SMLM datasets using subsets of camera frames." An alternative interpretation of nodes being too densely packed to resolve by SMLM would be that they are not nodes, but rather a more continuous structure. How were subsets of camera frames selected, and does this selection process impact whether nodes are seen or not? This concern becomes amplified by statements in the methods section that "clusters of localized emitters associated with cytokinesis structures were manually selected from the reconstructed SMLM images" and that "fitting results that did not converge properly and resulted in center positions outside the ring boundary or extremely large or small sigma values were also discarded from the final results." These statements imply that the authors have discarded data that do not fit in their model, as opposed to unbiased analysis of all data. The main conclusions in the paper rely heavily on the notion that rings are comprised of discreet nodes, so it seems imperative to remove any doubt about the presence of nodes in constricting rings. I do not mean to suggest that the authors are wrong, and to their credit they address the discrepancy with the McDonald study, but the lingering controversy makes it hard to judge the impact of their subsequent data regarding nodes in rings.

[A]: We understand the need for clear explanations of our methods as SMLM in live cells is still a new technique and is not commonly used. Nodes are manually selected by the user using a “cropping box” of 309 nm x 309 nm. The information within the box is then extracted and stored within a matrix to isolate the node from the rest of the image. The isolated nodes then have their center fit using a least squares calculation to map the calculated center to the region of greatest density of localized emitters. Since our isolation method allows for multiple objects to be selected within the same, we then push the nodes with fit centers through a rejection step to eliminate instances where multiple objects were included in the same 309 nm x 309 nm box. To do this, we compare the distribution of localizations from the fit center in the selected nodes and compare it to a gaussian distribution of set dimensions. If the distribution of localizations in the selected region is not gaussian, the node is rejected. Nodes are not rejected manually.

To better describe the reasoning behind the implementation of the rejection criteria we added the following to the methods section, “This step eliminates instances where two objects were included in the same 309 x 309 nm selection box and include only selections containing a single cluster.”

We also changed one sentence in the Methods from “For comparison purposes, all nodes were cropped from images reconstructed from 1,000 frames (5 s) to minimize blurring due to crowding and movement” to “For comparison purposes, all nodes were cropped from images reconstructed from 1,000 frames (5 s) to minimize blurring due to crowding and movement and allow for the selection of individual P-nodes and R-nodes” to clarify why we selected subsets of the full dataset.

We also added the following to the discussion to describe the findings in Swulius et al. 2018: “Electron cryotomography showed the organization of actin filaments within the contractile ring (Swulius et al., 2018). Although nodes could not be resolved with this technique, a ~60 nm gap between the actin filaments and the plasma a membrane is consistent with the size of the core of cytokinesis nodes.”



3. Related to the previous comment, do contiguous non-nodal structures such as actin or septins appear punctate in similar SMLM experiments with similar data selection and processing? It would help for the authors to show that non-node structures (perhaps GFP-CHD in the ring? of septin-GFP?) are not punctate or node-like by their approaches. Perhaps such a control has already been performed, and the authors can point to such references.

[A]: It expected that many protein structures that appear continuous by confocal microscopy will have a punctate appearance at a ~20-nm resolution. To determine whether our method of analysis was contributing to the punctate nature of

cytokinesis structures described in this work, we imaged cells expressing Pnmt41-mEos3.2-Rho2CAAX construct, which marks the plasma membrane, using the same acquisition settings used for the cytokinesis structures (see image on the right). The Pnmt41-mEos3.2-Rho2CAAX construct highlights the plasma membrane. The signal is not punctate even though some gaps are observed along the cell outlines, suggesting that our analysis does not artificially induce punctate organization in non-punctate structures.

4. If mid1Δ mutants have tighter Myo2 radius and more Myo2 associated, then why do they exhibit increased CAR constriction rate? In the discussion, it is proposed that more Myo2 leads to slower CAR constriction. For the readers understanding, more clarity possibly in the form of a cartoon model about how node radius and Myo2 levels compute to CAR constriction would be helpful.

[A]: We clarified the discussion by adding the following: “An optimal ratio of Myo2p heads bound to actin filaments may exist to achieve optimal constriction rate. A ratio of bound heads that exceed the optimal range, such as in enduring strands, would be counterproductive and impedes constriction. Why nascent strands produce rings that constrict faster than wild-type rings is unclear and further work will be necessary to identify other changes to the molecular organization of contractile rings made from nascent strands. Those changes may include but are not limited to increased contractile force by Myp2p and/or Myo51p, reduced drag forces or increased rate of septum deposition.”

We also modified the Figure 4C models to include expected outcomes of constriction rate.

Minor comments

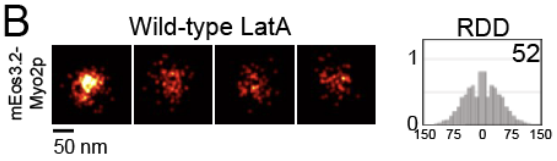
5. On page 6, the authors use LatA treatment to conclude that nodes are "bound to actin filaments." I would suggest changing the wording because the data show that nodes are "dependent" on actin, not necessarily "bound."

[A]: We found that nodes are not dependent on actin as nodes assemble in the absence of actin (LatA treated cells) in both Δ*mid1* and wild-type cells.

We have clarified this information in Result section 2. The passage now reads: “We determined whether P-nodes align onto strands in Δ*mid1* because they are bound to actin filaments. We treated wild-type and Δ*mid1* cells with 5 µM LatA to depolymerize the actin filament network, quickly mounted them and started time lapse confocal imaging within 2-3 min of addition of the drug. P-nodes in strands disperse along the cell cortex within 10 min of drug addition (Figure S1C). We observed the same outcome with rings nodes in both wild-type and Δ*mid1* cells. We obtained comparable results with 100 µM LatA except with faster node dispersal after the addition of the drug. Therefore,

depolymerizing the actin network releases P-nodes and R-nodes from strands and contractile rings."

6. In Figure 1E, a myp2Δ should be included in the constriction rate quantification.



[A]: Figure 1E was updated to add a column with the Δ*myp2* alone control. For consistency, Figure 1F was updated to show a kymograph of Δ*myp2* constricting contractile ring.

7. In Figure 3E, it would be helpful to include the RDD of Myo2 in wildtype cells treated with LatA.

[A]: We added RDD of Myo2 in wild-type cells treated with LatA to Figure S2 (Figure S2B).

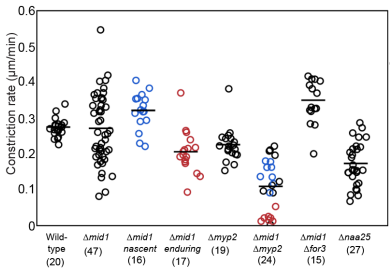
8. In Figure 5A, how is the timing of SPB splitting known since there is not an SPB marker in the top two strains? It would be helpful to provide more information about how time was determined in this experiment.

[A]: Cells are aligned at the time of ring assembled. To eliminate the confusion, we now set time = 0 min at the moment that the contractile ring is assembled. The figure label was updated to reflect this change and now says "Time from contractile ring assembled (min)".

9. The result of for3Δ suppressing the phenotypes of a mid1Δ mutant is very interesting. Some images of these cells as well as quantification would be helpful for the reader as well as a bit more discussion/speculation in the text.

[A]: We agree that further quantification of Δ*for3* suppressing the phenotypes of Δ*mid1* is of interest. We therefore quantified the penetrance of the branched phenotype in Δ*mid1* and Δ*mid1* Δ*for3* cells. We found a great reduction in the proportion of branched cells in a Δ*mid1* Δ*for3* background and added this sentence to the results to reflect this: "The morphology of Δ*mid1* Δ*for3* cells was more similar to that of wild-type cells with fewer branched cells (1%, n=67 Δ*mid1* Δ*for3* cells compared to 24%, n=94 Δ*mid1* cells)."

We also speculated as to biological significance of this in the discussion by adding the following: "Deleting For3p from Δ*mid1* cells partially rescues the branched morphology of Δ*mid1* cells (Figure 5D). For3p polymerizes the actin cables that are important for the



transport of cell polarity proteins necessary for tip growth (Feierbach and Chang, 2001; Martin and Chang, 2006). For3p localizes to cell tips by the function of the polarity protein Tea1p (Feierbach et al., 2004). Tea1p localizes to ectopic cortical sites in Δ*mid1* cells and may contribute to their branched morphology by promoting the growth of ectopic cell tips by recruiting For3p (Saha and Pollard, 2012a). Deleting For3p may thus prevent ectopic tip growth due to the lack of actin cables."

December 16, 2020

RE: JCB Manuscript #202008032R

Prof. Caroline Laplante
North Carolina State University
Molecular Biomedical Sciences
1051 William Moore Drive
Raleigh, North Carolina 27606

Dear Dr. Laplante,

Your revised manuscript has now been read by the three original reviewers. In light of their generally positive comments we would be happy to publish your paper in the JCB pending final revisions. Although it is not necessary to repeat the experiment suggested by Reviewer 2 of imaging the mid1 ts mutant at the restrictive temperature, we would ask that you address the remaining comments of this reviewer with textual changes while preparing your final submission.

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

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6) 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.

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c. Temperature
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Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

10) 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.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Karen Oegema, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

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

In this revised version of the manuscript, the authors have addressed the specific comments satisfactorily and provide additional data to address the questions raised. The combination of Super-resolution microscopy and yeast genetics is powerful and with this approach, the authors provide evidence that the nature of actin filaments can influence ring constriction. This manuscript is very well written and I strongly recommend this manuscript for publication in JCB. Congratulations to the authors for a job well done!

While I agree with reviewer 2 that mid1 ts mutant ideally should be analyzed at the restrictive temperature and that a room heater should be able to do the job, I am not convinced that the authors need to repeat this experiment. Under their imaging conditions, the authors are able to show that mid1 deletion and mid1 ts have similar phenotypes, indicating that this is unlikely due to a random suppressor mutant. Moreover, even at permissive temperatures, 20% mid1 ts shows looping of nascent strands further indicating that this is a mid1 specific phenotype. Thus, in my opinion, repeating the experiments at the restrictive temperature will not make any critical contribution to the story anymore.

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

I appreciate that the authors have addressed most of my concerns under the challenging conditions. But I still have some concerns to be dealt with.

Major concern:
Imaging mid1 temperature sensitive mutant at room temperature is not acceptable. The ts mutant can recover quickly after shifting to non-restrictive temperature. So no useful conclusions on ring formation and constriction can be drawn from the presented experiments. Inexpensive objective heater will be enough for this purpose.

Minor concerns:
1. The figures are not numbered.

2. Introduction, 3rd paragraph, the statement Mid1 transfers from type 1 node to type 2 node is misleading. How are type 2 nodes started? Do they colocalize with type 1 nodes? Is it more likely that type 1 nodes become type 2 nodes when more proteins are recruited?

3. Introduction, 4th paragraph, I won't say that "mid1D cells assemble functional constricting contractile ring". The sentence need to be modified because most of these cells have misplaced and disorganized septa. So the ring is not fully functional.

4. Figure 1A, no explanation of the red and blue symbols.

5. Does For3 have a role in the assembly of the nascent strands?

6. Fluorescence intensity, not fluorescent intensity.

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

The authors have done a nice job addressing reviewer comments with additional text and data. The issue regarding the presence of 'nodes' within intact rings remains an open question for me, but this paper provides a large amount of excellent data in other areas. I commend the authors on a very thorough and interesting piece of work.

1. The figures are not numbered.

[A] We did not add numbers to the figures as these are final submission figures and won't be used for reviews again.

2. Introduction, 3rd paragraph, the statement Mid1 transfers from type 1 node to type 2 node is misleading. How are type 2 nodes started? Do they colocalize with type 1 nodes? Is it more likely that type 1 nodes become type 2 nodes when more proteins are recruited?

[A] We carefully re-read the relevant section and modified it to clarify any confusion. The section now reads: "Mid1p localizes to Type 1 interphase nodes early in G2 and joins the Type 2 interphase nodes ~30 min prior to spindle pole body (SPB) separation (Akamatsu et al., 2014; Guzman-Vendrell et al., 2013). During mitosis, Mid1p localizes to cytokinesis nodes Mid1p leaves the contractile ring prior to the onset of constriction (Wu et al., 2006)."

We nonetheless answer the reviewer's questions to provide important information to the editors. The formation of Type I, Type II and cytokinesis nodes and their changes during the cell cycle has been thoroughly characterized in Akamatsu et al. 2014.

How are type 2 nodes started? Type 2 nodes form from the remnants of the disassembly of the contractile ring at the end of cytokinesis.
Do they colocalize with type 1 nodes? Type I and Type 2 nodes colocalize during interphase but not during mitosis. Quantitative measurements and computer simulations showed that these two types of nodes come together by a diffuse-and-capture mechanism: type 2 nodes diffuse to the equator and are captured by stationary type 1 nodes.
Is it more likely that type 1 nodes become type 2 nodes when more proteins are recruited? Type I and Type II nodes are separate protein complexes that come into contact by a "diffuse-and-capture" mechanism. Therefore, proteins of the Type II node proteins are not "recruited" to Type I nodes.

3. Introduction, 4th paragraph, I won't say that "mid1D cells assemble functional constricting contractile ring". The sentence need to be modified because most of these cells have misplaced and disorganized septa. So the ring is not fully functional.

[A] We rephrased the sentence to: "Despite their multiple cytokinesis defects, Δ*mid1* cells assemble contractile rings competent for constriction, raising an important question: What is the molecular organization of cytokinesis proteins in the absence of Mid1p?"

4. Figure 1A, no explanation of the red and blue symbols.

[A] Figure 1A does not have red and blue symbols that are not explained. However, figures S1A has red and blue symbols that we had not defined. We added a definition for those symbols in the legend of Figure S1A.

5. Does For3 have a role in the assembly of the nascent strands?

[A] For3p does not have a role in the assembly of nascent strands. We added the following sentence to clarify this point: "The formation of nascent strands and their proportion in the cell population were not changed in Δ*mid1* Δ*for3* cells, suggesting that For3p is dispensable for nascent strands."

6. Fluorescence intensity, not fluorescent intensity.

[A] We found two instances of "fluorescent intensity" in the methods section and changed those to "fluorescence intensity".