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Review COMMONS

Cdc42 reactivation at growth sites is regulated by local cell-cycle-dependent loss of its GTPase-activating protein Rga4 in fission yeast

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Review timeline

Submission to Review Commons:	18 August 2020
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Reviewer 1

Evidence, reproducibility and clarity

This manuscript from Das and colleagues reports a new phenotype in fission yeast cells upon transient cell division arrest, where cells re-initiate growth earlier than unchallenged cells and without completion of cell separation. The phenotype, which the author dub as PrESS (Polar Elongation Sans Separation), indicates that the growth restart is regulated independently of cell separation. The authors propose the interesting idea that cell growth is resumed after a given time, independently of cell separation, and competes with cell separation. However, they do not test this directly. From a candidate screen for regulators of the process, they focus on a Cdc42 GAP, Rga4, in the absence of which 3% more cells exhibit the PrESS phenotype and suggest Rga4 re-localization to cell poles during mitosis helps arrest cell growth during cell separation. Unfortunately, while the PrESS phenotype is intriguing, most experiments are not performed in a quantitative manner, which prevents any conclusive interpretation, at least at this stage. The text jumps to conclusion without solid experimental support. The aspects that would need to be addressed to support the claims made are listed below.

Major comments:

1. The method to block cytokinesis need to be better characterized. The authors use 10µM Latrunculin A to disrupt the actin cytoskeleton. This is a very low concentration, as previously used concentrations to disrupt actin structures are between 50µM and 200µM. Disruption of actin structures, especially the actin ring, needs to be directly monitored in these conditions.

2. With a few exceptions, experiments are performed on fixed cells rather than with timelapse experiments, without specific fluorescent markers to stage the cell cycle, which removes precision in assessing the phenotype. Specifically:

-How is septum closure monitored? This is not indicated in the methods. Advancement of septation is very difficult to evaluate from DIC images, removing confidence in measurements of timing between septation completion and growth initiation.

-The PrESS phenotype is mostly quantified from the length of septated cells. It would be more convincing to show timelapse imaging of septated cells growing at their old ends. For instance, Fig 2B is not convincing of the PrESS phenotype: this should be shown live, ideally with fluorescent markers of cell growth.

-Scd1, Scd2 and Myo52 localization would be best investigated through time-lapse imaging to look at the transition of localization from the septum to the old cell poles. As presented, the "gradual" localization of Myo52 claimed is not supported. None of these data are quantified.

-For figures 5D-E and 5G, how are early anaphase and late anaphase cells attributed to these classes? The WT cells appear to have SPB marker, but not the cdc2-as cells.

3. Quantification of the PrESS phenotype is missing in several experiments:

-In Fig 1D, it needs to be quantified in the rng2-D5 mutant, as well as WT cells treated the same way.

-In Fig 2A, the % of mitotic cells showing the PrESS phenotype needs to be quantified.

-What is the basis for the claim that scd1? PrESS cells are more polarized than scd1? cells? Of course, two cells linked by a septum will have a larger aspect ratio than a single cell. Perhaps individual cells are also more polarized, but this is not evident from the image shown. Cell dimensions (of one cell compartment) should be measured to substantiate the claim.

-PrESS phenotype quantification is missing in Fig 6A.

4. Quantification of fluorescence signals is lacking, specifically:

-The idea that poles compete with cell division for polarity factors draws from the proposal that Cdc42 activity fades from the septum faster in PrESS cells than wildtype cells. This is not clear from the time-lapse shown in Figure 3B and needs to be quantified. Similarly, the early activation at cell poles should be quantified. This is essential to support the model of competition of the pole to the septum.

-The idea that Rga4 at the poles of mitotic cells helps prevent growth is in part based on the claim that there is more Rga4 at cell poles in mitotic cells. From the images in figure 5A-B, I am not convinced that there is a clear change in Rga4 distribution at the cell periphery during the cell cycle. In the projection images of figure 5B, Rga4 can be seen to localize at all cell poles, irrespective of cell cycle stage. What seems to vary more is the amount of cytosolic Rga4, which appears higher in mitosis and G1/S than in G2. Quantitative description of these distributions (for instance tip to side or cortex to cytosol fluorescence levels) would be required to make any firm claim on localization changes. These measurements should replace those currently presented in Fig 5D, where Rga4 localization at cell poles is quantified in a binary manner (present or absent), which is not representative of the images shown in Fig 5B. Cell tip amounts likely vary along a continuum, not in a step manner.

5. The idea of an independent timer for growth re-initiation is interesting but is not tested. One simple way to test it would be to perform shorter or longer LatA treatments and assess the septation to growth interval. If there is an independent timer to re-initiate growth independently of cell separation, then the time between end growth and septum closure should be inversely linked with the length of the LatA treatment.

6. The reason for the higher proportion of rga4? (and gef1?) PrESS cells is not clear. Is there a higher fraction of mitotic cells showing the PrESS phenotype, or are there more mitotic cells in these cultures? To convince of a phenotype, it will be necessary to quantify ONLY cells that are in mitosis during the LatA treatment, or show that the PrESS phenotype also happens in other cell cycle stages. This is absolutely essential, as the current work is built on a barely statistically significant 3% difference from an unsynchronized culture. In the rga4? panel of Fig 6A, the only septated cell looks very large, similar to the PrESS reported.

However, these cells are not treated with LatA. This suggests that rga4? may have delays in cell division, independently of additional treatments, which further complicates the interpretation of a small % difference. Thus, I am not convinced of the role of Rga4 in preventing PrESS.

7. The data displayed in figure 6 is not complete enough to allow for easy interpretation. The authors indicate in the text that lysis is suppressed by rga4?, but lysis is not quantified. It is not clear whether lysis is restricted to cell division or also happens during cell growth (the top-right panel shows a single lysed cell). The growth curve data (which lacks standard deviations) indicates a genetic interaction between the Nak1-Mor2 fusion protein and rga4?, but 1) may not directly report on lysis, and 2) does not show whether in the double mutant growth at cell poles prevents separation-driven lysis, or lack of Rga4 function at the division site is the cause of the lysis suppression. The claimed PrESS phenotype is also not quantified.

Minor comments:

1. Please include page numbers

2. The authors indicate in the introduction that "Rga6 and Rga3 appear to play minor roles in polarity and sporulation". I am not aware of any Cdc42 GAP with a role in sporulation.

3. In Fig 1D, what the time refers to relative to temperature shift is not clear.

Significance

It is difficult to comment on the significance of this study at this stage. In its current form, this study is not robust enough to convince of the claims made (re-initiation of growth after timer period; role of Rga4 GAP relocalization to cell poles during mitosis to block growth). If these claims can be substantiated by careful, systematic quantitative analysis, the timer model will be very interesting, suggesting a possible regulation of polarized cell growth by the cell cycle.

Referees Cross-Commenting

Having read the other reviewers' comments, I can see that all reviewers concur in thinking that the topic is interesting, but that the data do not support the proposed conclusions.

Reviewer 2

Evidence, reproducibility and clarity

In this manuscript, the authors propose a novel Rga4-dependent inactivation of Cdc42 in cell tips during a cell division. At first, they found temporary abrogation of an actin ring produced end growing cells with a septum (PrESS), suggesting that a restart of cell elongation after cytokinesis is regulated independently of cell separation. After several genetic and cell biological analyses, the authors found Rga4 was involved in this growth pattern regulation by localizing to the old cell end during mitosis.

Major comments:

1) First of all, I cannot find any novelty of a phenotype of PrESS. To date, many mutant strains showing an elongated cell body with multi-septa have been identified in S. pombe (i.e. sec8, end4, ppb1 KO, etc.). What is a difference in the phenotype of PrESS with them?

2) Previously I found a low amount of Lat-A produced a large population of an elongated cell with the septum. The authors have shown no clear evidence that the actin cytoskeletal organization is fully recovered after a temporary treatment with Lat-A. I think the authors cannot ignore the possibility that a low amount of Lat-A after washouts produces the PrESS phenotype.

3) In figure 4D, a septation index in Lat-A untreated cells should be shown. In addition, a frequency of the PrESS phenotype elevated only 3% in rga4 KO although the authors insisted the difference was statistically significant. It seems that there is not a big difference between gef1 and rga4. Is the role of Rga4 minor in the control of regrowth in cell tips after mitosis?

4) In this manuscript, the authors just observed the Rga4 localization pattern in the cell cortex during the restart of cell growth. They should consider testing what happens in the restart of cell growth if a force-localizing Rga4 in the old cell end.

Minor comments:

1) No evidence (or citation) is shown in the following sentence; "We have previously shown that in gef1 cells, the old end competes with the new end for Cdc42 more effectively than wild type."

2) Quantitative data is required in figure 6A.

3) In figure 6B, does the pale blue curve indicate a growth of rga4+ Nak-Mor2+ cells?

Significance

The focus of this study is interesting, but the data lacks sufficient persuasiveness. In particular, the importance of rga4 in controlling the restart of cell elongation after mitosis needs to be carefully examined. Otherwise, it would be judged to be no significant progress.

Referees cross-commenting

I believe that reviewer 1 asked for a redo of most of the experiments and validation of the mechanism proposed by the authors, so this reviewer decided that the paper needed more than six months to be revised.

I think that Reviewer 3's opinion is essentially the same as Reviewer 1's, but I believe it was suggested that the time frame for revising was unknown because the paper itself needed to be fundamentally revised (This may be a near euphemistic rejection, probably).

I decided that a month would be enough time for the authors to address the technical problem. After conducting those experiments, the authors should judge for themselves the validity of the model proposed in their manuscript, and if they thought it was scientifically sound, they could submit a revised manuscript.

While there are differences as to the need for revisions and the length of time it would take to do so, I feel that each reviewer's suggestions are reasonably. Therefore, in accordance with the journal policy, editor should decide. I will respect the judgement of editor.

Reviewer 3

Evidence, reproducibility and clarity

This paper addresses an interesting question on the timing of cell polarization during the cell cycle in fission yeast. In that system, polar growth stops during mitosis and resumes following cell separation. Polarity regulators are repurposed and relocalized to the division site during cytokinesis. What is the trigger for repolarization after cytokinesis? Here the authors use transient actin depolymerization during mitosis to perturb the system (delaying actin-dependent cytokinesis) and observe the effects. From this analysis they conclude that resumption of polarity is not dependent on completion of cytokinesis or cell separation, but instead that some (uncharacterized) cell cycle cue at the end of mitosis triggers polarization by removing the Cdc42-directed GAP, Rga4, from the cell poles. This is an intriguing and plausible hypothesis. However, I did not think that the conclusions were adequately supported by the data presented.

Specific comments:

1. The conclusion drawn from Fig. 1 and highlighted in the first paragraph of the Discussion is that old end polar growth is not dependent on cell separation: wasn't this already known from the fact that (e.g.) septin mutants resume growth without separating?

2. The paper is organized around observation of the "PrESS" phenotype, which is described differently in different parts of the paper. In the Discussion, it is described as "polarized cell growth [initiated] during cytokinesis" but the acronym and initial description refer to "separation" and most cells appear to complete cytokinesis. This highlights what I found to be a major ambiguity in trying to interpret the results: the timing of initiation of polar growth is never clearly measured, and many conclusions are drawn based on a phenotype that relies on other factors like the efficiency/rate of cell separation. The only data that seemed to address timing directly were in Fig. 1C, which has several issues (below). The remainder of the paper views the presence of growing cells that failed to separate as an indication of "premature" polar growth, but it seems that could equally reflect poor cell separation (as in septin mutants). As there is no obligate link between cell separation and polar growth, it seems to me that PrESS per se need not imply anything about the timing of polar growth relative to cytokinesis.

3. Fig. 1C scores the time from "septum ingression" to "old end growth onset" but no description was provided of how these times were determined. The text refers to the time of "septum closure", but again with no indication of how this was determined. Is there a difference between septum ingression and closure? How were these times scored?

4. Also on the issue of timing, in Fig. 3A: at what time were these pictures taken relative to LAT washout or initiation of ring constriction? In Fig. 3B, Cdc42 activation is shown to occur before cell separation, but on what basis do the authors conclude that Cdc42 activation occurs "earlier" at the ends? Here time "0" is not defined: how does it relate to the time of LAT washout or the time of initiation of ring constriction? Is "0" for the untreated and treated samples comparable in some way or are the times arbitrary? Fig. 3C: how does 2 hours of recovery relate to the time of septation? Might the Scd1/2/Myo52 be present at the center earlier on?

5. The questions above (2-4) highlight a major difficulty with the issue of how to tell whether the timing of polar growth is "normal" or "premature" in a given condition. One would ideally like to know when polar growth initiates relative to the time when MOR signaling turns on (or is there some other event the authors would pick?). But as the perturbations employed here (LAT treatment, or mutant strains) would presumably affect the timing of MOR onset, and no measure of that timing was provided, I did not understand how the authors inferred the relative timing of polar growth.

6. A central finding of the paper is that Rga4 localizes to cell poles during mitosis (Fig. 5). I found it hard to perceive the Rga4 at the poles that the authors highlight-I assume the scoring must count instances of very faint puncta, and I am uncertain as to the significance of these. I appreciate the time averaging strategy used in Fig. 5B, but I had a hard time seeing the pole enrichment in late anaphase (the cytoplasm as a whole seems darker in this cell-I would suggest this be shown for several cells), and the pole signal highlighted by the arrows seemed very faint in Fig. 5C. Thus, it seemed a gross exaggeration to draw Rga4 distribution in anaphase as being uniform at poles and sides, as done in the cartoon in Fig. 7 and hinted at in some of the text. The less punctate Rga4 distribution at cell sides during mitosis was more convincing than the presence of Rga4 at poles, but the significance of this less punctate pattern was unclear.

7. Fig. 6 shows that the lytic phenotype resulting from constitutive MOR pathway activity is rescued by deleting Rga4. The authors conclude that Rga4 specifically prevents old end growth during cell separation, and resumption of growth occurs upon Rga4 removal. But it is far from clear that the phenotype is rga4-specific: would other mutants from Table S2 also rescue? If so, that might argue against a specific role for Rga4 in timing the resumption of polar growth.

8. The authors conclude that "after division, Cdc42 activation at the cell ends requires both removal of Rga4 from the cell ends and MOR activation". But to establish a requirement for "removal of Rga4 from the cell ends" would require an experiment that specifically prevents Rga4 removal from the ends, and no such experiment is presented.

9. It seems to me that there are three scenarios that could explain loss of Rga4 from the poles. First, as the authors assume, some cell cycle signal may remove Rga4 specifically from poles, making the poles permissive for Cdc42 activation. Alternatively, some other regulatory event may activate growth at the poles despite the low level presence of Rga4, and such growth may then

promote Rga4 removal, as suggested by earlier findings that LAT treatment of interphase cells allowed some Rga4 to be seen at poles (Das et al. 2007). Finally, one could imagine a feedback loop whereby Cdc42 activation leads to Rga4 exclusion, which facilitates further Cdc42 activation. Distinguishing between these options would require an analysis of the relative timing of Cdc42 activation and Rga4 removal from the poles. As detection of Rga4 at cell ends was difficult in any case, determining the timing of its loss may be problematic. But without such an analysis, the authors' assumption that Rga4 removal is the primary event seems premature.

Other issues:

- The conclusion that Cdc42 at the old end "siphons Cdc42 away from the division site" seems reasonable but I did not see any experimental test that established this to be true.

- The authors show that Sty1 is not required for the resumption of growth before cell separation, but the more interesting question is what happens to Cdc42 in the sty1 mutant: does Cdc42 return to the poles earlier (after the LAT perturbation) than in the wildtype?

- The MOR pathway is discussed as though its effects are exclusively mediated by Sts5, but isn't Gef1 another target of the MOR that directly affects Cdc42 and hence polar growth?

- Just out of curiosity: do cells that resume old-end growth but fail to separate subsequently do NETO?

Significance

This paper addresses an interesting question on the timing of cell polarization during the cell cycle in fission yeast. In that system, polar growth stops during mitosis and resumes following cell separation. Polarity regulators are repurposed and relocalized to the division site during cytokinesis. What is the trigger for repolarization after cytokinesis? Here the authors use transient actin depolymerization during mitosis to perturb the system (delaying actin-dependent cytokinesis) and observe the effects. From this analysis they conclude that resumption of polarity is not dependent on completion of cytokinesis or cell separation, but instead that some (uncharacterized) cell cycle cue at the end of mitosis triggers polarization by removing the Cdc42-directed GAP, Rga4, from the cell poles. This is an intriguing and plausible hypothesis. However, I did not think that the conclusions were adequately supported by the data presented.

Referees cross-commenting

You are correct that my review might be viewed as a rejection in the sense that I did not see what technically feasible experiments could be done to really support their model. However, it may be that a re-worked paper would be more persuasive because of factors that I did not appreciate. So I support the idea of letting the authors address the comments on whatever timescale they may need.

Author response to reviewers' comments

We would like to thank the reviewers for their time and thoughtful comments. We are encouraged that the reviewers consider our findings intriguing and appreciate the novelty of this phenotype. We agree with the reviewers' comments, and have provided additional quantitative data to support our findings wherever requested. In addition to our responses listed below, we would like to emphasize how the phenotype reported in the paper is distinct from cell separation mutants that have been reported in fission yeast in the past. Mutants that fail cell separation eventually grow from the ends, resulting in a multi-septated phenotype. Here however, using chemical perturbation we have uncoupled growth and cytokinesis such that these cells initiate growth while the septum is still forming or maturing. This uncoupling leads to sequestration of the polarity machinery away from the division site and towards the growing end, thus, resulting in a failure to separate. Thus while

these cells physically resemble cell separation mutants, they are distinct in that they initiate end growth while the septation process is ongoing. To the best of our knowledge, such a phenotype has not been reported in the literature. To clarify this point, we have modified the name of the phenotype as follows, **p**ola**r** **e**longation **s**imultaneous with **s**eptation (PrESS).

Below we address each comment with additional data and/or detailed explanation as needed.

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

This manuscript from Das and colleagues reports a new phenotype in fission yeast cells upon transient cell division arrest, where cells re-initiate growth earlier than unchallenged cells and without completion of cell separation. The phenotype, which the author dub as PrESS (Polar Elongation Sans Separation), indicates that the growth restart is regulated independently of cell separation. The authors propose the interesting idea that cell growth is resumed after a given time, independently of cell separation, and competes with cell separation. However, they do not test this directly. From a candidate screen for regulators of the process, they focus on a Cdc42 GAP, Rga4, in the absence of which 3% more cells exhibit the PrESS phenotype and suggest Rga4 re-localization to cell poles during mitosis helps arrest cell growth during cell separation. Unfortunately, while the PrESS phenotype is intriguing, most experiments are not performed in a quantitative manner, which prevents any conclusive interpretation, at least at this stage. The text jumps to conclusion without solid experimental support. The aspects that would need to be addressed to support the claims made are listed below.

Major comments:

1. The method to block cytokinesis need to be better characterized. The authors use 10µM Latrunculin A to disrupt the actin cytoskeleton. This is a very low concentration, as previously used concentrations to disrupt actin structures are between 50µM and 200µM. Disruption of actin structures, especially the actin ring, needs to be directly monitored in these conditions.

We find that with a 10µM LatA treatment, the actomyosin ring is disrupted, as observed with Phalloidin staining (Supplemental Figure 1B). Similarly, upon LatA treatment, Rlc1-tdTomato, an actomyosin ring marker, appears disintegrated and no longer appears as a ring. Upon recovery, we observe remnants of the original ring as marked with Rlc1-tdTomato, however a second ring is formed which then undergoes constriction, as shown in Supplemental Figure 1A. Thus, a mild 30-minute treatment with 10µM LatA treatment delays cytokinesis since the cell has to reassemble the ring in order for cytokinesis to proceed. Similar results were also observed in (Swulius et al, 2018, PNAS) where 10µM LatA treatment disrupts the actomyosin ring.

2. With a few exceptions, experiments are performed on fixed cells rather than with timelapse experiments, without specific fluorescent markers to stage the cell cycle, which removes precision in assessing the phenotype. Specifically:

-How is septum closure monitored? This is not indicated in the methods. Advancement of septation is very difficult to evaluate from DIC images, removing confidence in measurements of timing between septation completion and growth initiation.

We agree that brightfield imaging is not the most reliable way to monitor septum closure. For these experiments, cells have to be imaged for hours with a time resolution of one minute to accurately capture septum closure and onset of growth. Using fluorescently labeled ring or septum markers is complicated as they often bleach before the cells even recover or lead to toxicity. Thus, we used brightfield imaging to detect septum closure and record corresponding onset of growth. However, to confirm our data, we also monitored ring closure with Rlc1- tdTomato and analyzed growth via time-lapse imaging. In these experiments we used a longer time interval to prevent bleaching or toxicity. Regardless, our data show that, in PrESS cells, growth initiates on average about 7.5 minutes after ring closure compared to 40 minutes in non- PrESS cells (Supplemental Figure 1D). For clarity, we have also provided a description of how brightfield imaging was used to detect septum closure (Supplemental Figure 1E). These data indicate that growth at the ends of PrESS cells initiates around the same time and sometimes even before the ring/septum closes.

-The PrESS phenotype is mostly quantified from the length of septated cells. It would be more convincing to show timelapse imaging of septated cells growing at their old ends. For instance, Fig 2B is not convincing of the PrESS phenotype: this should be shown live, ideally with fluorescent markers of cell growth.

We have provided a time-lapse movie of end growth with respect to septum closure in Figure 1 and in Supplemental Movie S1. We also show time-lapse image of end growth in PrESS cells in figure 2Aii and Supplemental Movie S2. In addition, as suggested by the reviewer, we now show a growth marker, active Cdc42, as determined by CRIB-3xGFP localization, in Figure 2B. In support of Figure 2B in the previous version of the manuscript, we find that only synchronized cells which were treated during mitosis show CRIB-3xGFP at the ends while they fail cell separation, displaying the PrESS phenotype.

-Scd1, Scd2 and Myo52 localization would be best investigated through time-lapse imaging to look at the transition of localization from the septum to the old cell poles. As presented, the "gradual" localization of Myo52 claimed is not supported. None of these data are quantified.

We now provide quantification of Scd2-GFP and Myo52-tdTomato intensities at the cell ends over time. We show that, in PrESS cells, Scd2-GFP and Myo52-tdTomato transition to the cell ends and away from the cell middle (Figure 4B). Scd2-GFP and Myo52-tdTomato intensities were measured from when they first appear at the division site and compared to their intensities at the cell ends. In untreated cells, we did not see any increase in the intensities at the ends compared to the division site. In PrESS cells, the intensities at the ends gradually increased over time as compared to the division site, eventually reaching a ratio of over 1. This agrees with a similar pattern observed for active Cdc42, as determined by CRIB-3xGFP intensity (Figure 3C). Together, these analyses provide an explanation for how Cdc42 activity and consequent growth initiate at PrESS cell ends while the division site fails separation. Scd1- 3xGFP shows lower signal and bleaches rapidly, thus we were unable to show a similar pattern via time-lapse imaging. However, since Scd2 localizes Scd1, time-lapse imaging of the former is a good indicator of the localization of the whole Cdc42 regulatory complex.

-For figures 5D-E and 5G, how are early anaphase and late anaphase cells attributed to these classes? The WT cells appear to have SPB marker, but not the cdc2-as cells.

Since Rga4-GFP is present in the cytoplasm and excluded from the nucleus, the nucleus is easily distinguishable in these cells. Additionally, Rga4-GFP localizes to the constricting ring, with constriction itself starting near the end of anaphase B (Das et al, 2007; Cortes et al, 2018, PLOS Genet). Thus, we determined the cell cycle stage of *rga4-GFP*-expressing cells based on the shape of the nucleus and the localization of Rga4-GFP at the division site, as shown in Supplemental Figure S5D.

3. Quantification of the PrESS phenotype is missing in several experiments:

-In Fig 1D, it needs to be quantified in the rng2-D5 mutant, as well as WT cells treated the same way.

We have removed the *rng2-D5* mutant data from the revised manuscript. Given the difficulty of maintaining an appropriate temperature for this temperature-sensitive mutant, as well as the presence of pleiotropic effects, we were not able to satisfactorily determine the timing of growth resumption or the PrESS frequency in the *rng2-D5* mutant compared to septation. We had used *rng2-D5* mutants to show that the PrESS phenotype was not simply due to a pleiotropic effect of LatA treatment, but rather was due to delaying cytokinesis in dividing cells. As an alternate evidence to show that the PrESS phenotype is not due to pleotropic effects of LatA treatment we now show that the PrESS frequency scales with the duration of the LatA treatment (Figure 3E). Cells with a shorter duration of LatA treatment show a smaller PrESS frequency, indicating that it is not just the LatA treatment, but rather the duration of the treatment and the resulting cytokinetic delay that causes this phenotype.

-In Fig 2A, the % of mitotic cells showing the PrESS phenotype needs to be quantified.

We now provide this data in Supplemental Figure S2A. As is evident from the data provided, PrESS cells were only observed in cells undergoing mitosis at the time of LatA treatment.

-What is the basis for the claim that scd1Δ PrESS cells are more polarized than scd1Δ cells? Of course, two cells linked by a septum will have a larger aspect ratio than a single cell. Perhaps individual cells are also more polarized, but this is not evident from the image shown. Cell dimensions (of one cell compartment) should be measured to substantiate the claim.

After careful analysis of the cell dimensions of *scd1Δ* PrESS cells, we did not see a significant change in their aspect ratio compared to untreated cells. We have removed this statement from the manuscript. This does not in any way change the conclusions of this study.

-PrESS phenotype quantification is missing in Fig 6A.

These data are now provided in the revised Figure 7D. While we did not see any PrESS-like phenotype in *rga4+* cells constitutively activating the MOR pathway, we did see PrESS-like cells in *rga4Δ* mutants under similar conditions. Interestingly, even with low levels of active MOR (+thiamine conditions), we observed a significant number of PrESS-like cells. Furthermore, we observed lysed cells in *rga4+* cells constitutively activating the MOR pathway under both low and high expression conditions. The number of lysed cells was significantly reduced in *rga4Δ* cells under similar conditions. Together, this indicates that in the absence of *rga4*, lysis is rescued in cells constitutively activating the MOR pathway, and these cells instead show growth at the cell ends.

4. Quantification of fluorescence signals is lacking, specifically:

-The idea that poles compete with cell division for polarity factors draws from the proposal that Cdc42 activity fades from the septum faster in PrESS cells than wildtype cells. This is not clear from the time-lapse shown in Figure 3B and needs to be quantified. Similarly, the early activation at cell poles should be quantified. This is essential to support the model of competition of the pole to the septum.

We now provide quantification of the transition of CRIB-3xGFP signal from the cell middle to the ends in PrESS cells (Figure 3C). In PrESS cells, CRIB-3xGFP intensity at the cells ends increases over time with respect to the intensity at the division site. In contrast, in non-PrESS cells, the ends do not show CRIB-3xGFP, and the intensity at the division site does not change throughout cytokinesis until the cells separate.

In addition, we also monitored when Cdc42 is activated at the cell ends with respect to completion of anaphase B. We find that Cdc42 activation at the cell ends occurs at a fixed time after anaphase B in PrESS and non-PrESS cells. Thus, while cytokinesis in PrESS cells is prolonged due to reassembly of the actomyosin ring, Cdc42 activation at the ends resumes at a fixed time with no delay. Together, these findings indicate that Cdc42 activation and consequent growth at the cells ends occurs at a fixed time in the cell cycle.

-The idea that Rga4 at the poles of mitotic cells helps prevent growth is in part based on the claim that there is more Rga4 at cell poles in mitotic cells. From the images in figure 5A-B, I am not convinced that there is a clear change in Rga4 distribution at the cell periphery during the cell cycle. In the projection images of figure 5B, Rga4 can be seen to localize at all cell poles, irrespective of cell cycle stage. What seems to vary more is the amount of cytosolic Rga4, which appears higher in mitosis and G1/S than in G2. Quantitative description of these distributions (for instance tip to side or cortex to cytosol fluorescence levels) would be required to make any firm claim on localization changes. These measurements should replace those currently presented in Fig 5D, where Rga4 localization at cell poles is quantified in a binary manner (present or absent), which is not representative of the images shown in Fig 5B. Cell tip amounts likely vary along a continuum, not in a step manner.

We now provide quantification of Rga4-GFP intensity at the cell ends during different cell cycle stages. We agree that regardless of the cell cycle stage, the signal of Rga4-GFP at the ends is weak. Thus, to capture sufficient signal to quantitatively distinguish between cells in G2 and mitosis, we

used projections of short time-lapse movies (5mins) as described in the Materials and Methods. With this approach, we show increased intensity of Rga4-GFP at the cell ends in mitotic cells as compared to G2 or G1/S cells (Figure 6C). We also quantified the ratio of Rga4- GFP intensity at the cortex with respect to the cytoplasm throughout the cell cycle (Supplemental Figure S6B). We show that the ratio of Rga4-GFP at the cortex to the cytoplasm in G2 is increased compared to late mitosis. While our findings indicate that Rga4 localization is regulated in a cell-cycle-dependent manner, it is unclear how these changes affect its ability to regulate Cdc42.

5. The idea of an independent timer for growth re-initiation is interesting but is not tested. One simple way to test it would be to perform shorter or longer LatA treatments and assess the septation to growth interval. If there is an independent timer to re-initiate growth independently of cell separation, then the time between end growth and septum closure should be inversely linked with the length of the LatA treatment.

We thank the reviewer for this suggestion. Indeed, we find that shorter treatments with LatA decrease the PrESS frequency, as shown in Figure 3E. If growth initiation is triggered by a timer, then increasing the cytokinetic delay should give the cell ends a head start over completion of cytokinesis, resulting in a high PrESS frequency, while decreasing the delay should have the opposite effect. In addition, we also show that Cdc42 activation at the ends in PrESS cells occurs at a fixed time after completion of anaphase B, just as in non-PrESS cells (Figure 3D). Together these findings support the idea that growth at the ends initiates at a fixed time.

6. The reason for the higher proportion of rga4Δ (and gef1Δ) PrESS cells is not clear. Is there a higher fraction of mitotic cells showing the PrESS phenotype, or are there more mitotic cells in these cultures? To convince of a phenotype, it will be necessary to quantify ONLY cells that are in mitosis during the LatA treatment, or show that the PrESS phenotype also happens in other cell cycle stages. This is absolutely essential, as the current work is built on a barely statistically significant 3% difference from an unsynchronized culture. In the rga4Δ panel of Fig 6A, the only septated cell looks very large, similar to the PrESS reported. However, these cells are not treated with LatA. This suggests that rga4Δ may have delays in cell division, independently of additional treatments, which further complicates the interpretation of a small % difference. Thus, I am not convinced of the role of Rga4 in preventing PrESS.

We now provide data to show that mitosis is not delayed in *rga4Δ* mutants suggesting that the fraction of mitotic cells is similar in asynchronous populations of *rga4*□ wild type cells (Supplemental Figure S5C). We do not see any differences in the duration of different cytokinetic events in *rga4Δ* mutants compared to wild type cells. Therefore, the increase in the PrESS frequency observed in the *rga4Δ* cells is attributable to a role for Rga4 in the regulation of Cdc42 activity at the cells ends during mitosis. We agree that a 3% increase in the PrESS frequency in *rga4*□ mutants, while statistically significant, is only a mild increase. However, our data strongly suggest that PrESS cells arise only in mitotic cells that have been treated with LatA. This means that there is an upper limit to the number of cells that are able to show the PrESS phenotype which is dependent on the fraction of mitotic cells in the population at the time of treatment. Since mitosis is not prolonged in these cells, the upper limit for potential PrESS cells in *rga4Δ* mutants is similar to that of wild type cells. The increase in PrESS frequency observed in these mutants is thus likely due to an enhanced advantage of the ends over the division site in *rga4* mutants.
The reviewer raises a good point that a 3% increase in PrESS frequency observed in the *rga4Δ* mutant could be an artifact. By itself, we agree that this result alone is insufficient to make a case for Rga4 in regulating the timing of cell growth after division. However, we also provide additional data that Rga4 localization changes in a cell-cycle-dependent manner (Figure 6), with Rga4 levels at cell ends increasing during mitosis and decreasing during interphase. We also show that *rga4*□ cells constitutively activating the MOR pathway show Cdc42 activation and end growth in septated cells, resulting in a PrESS-like phenotype (Figure 7). While our data also show a small increase in the PrESS frequency in *gef1*□ mutants, *gef1*□ mutants constitutively activating the MOR pathway do not show a PrESS-like phenotype. Furthermore, deleting another Cdc42 GAP, *rga6*, in cells constitutively activating the MOR pathway also does not result in a PrESS-like phenotype. Together, these data suggest that loss of *rga4* in particular is necessary for end growth activation and make a much stronger case for the role of Rga4 in regulating the initiation of Cdc42 activation after division.

7. The data displayed in figure 6 is not complete enough to allow for easy interpretation. The authors indicate in the text that lysis is suppressed by rga4Δ, but lysis is not quantified. It is not clear whether lysis is restricted to cell division or also happens during cell growth (the top-right panel shows a single lysed cell). The growth curve data (which lacks standard deviations) indicates a genetic interaction between the Nak1-Mor2 fusion protein and rga4Δ, but 1) may not directly report on lysis, and 2) does not show whether in the double mutant growth at cell poles prevents separation-driven lysis, or lack of Rga4 function at the division site is the cause of the lysis suppression. The claimed PrESS phenotype is also not quantified.

We now provide quantification of cell lysis in cells expressing *nak1-mor2* (Figure 7C). As has been reported earlier (Gupta et al, 2014), *nak1-mor2*-expressing cells lyse during cytokinesis at the division site. Similarly, we observed cell lysis at the division site. Our quantification shows that cell lysis is rescued in *rga4Δ* mutants expressing *nak1-mor2*. We now also provide the quantification of the PrESS-like phenotype observed in these cells. The statistics for the growth curve are also provided.

In order to determine whether the PrESS-like phenotype and the rescue of cell lysis in *rga4Δ* mutants are due to activation of Cdc42 at the ends, we monitored CRIB-3xGFP at the cell ends of septated cells in *nak1-mor2*-expressing *rga4+* and *rga4Δ* cells. We find that while CRIB- 3xGFP is present at the ends in only in a small fraction of septated *rga4+* cells, it is present at the ends in a large majority of septated *rga4Δ* cells. This provides further evidence that loss of *rga4* in cells with a constitutively active MOR pathway results in a PrESS-like phenotype.

Minor comments:

1. Please include page numbers

Page numbers are now provided.

2. The authors indicate in the introduction that "Rga6 and Rga3 appear to play minor roles in polarity and sporulation". I am not aware of any Cdc42 GAP with a role in sporulation.

We have corrected this statement.

3. In Fig 1D, what the time refers to relative to temperature shift is not clear.

We have removed this data from the revised manuscript.

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

In this manuscript, the authors propose a novel Rga4-dependent inactivation of Cdc42 in cell tips during a cell division. At first, they found temporary abrogation of an actin ring produced end growing cells with a septum (PrESS), suggesting that a restart of cell elongation after cytokinesis is regulated independently of cell separation. After several genetic and cell biological analyses, the authors found Rga4 was involved in this growth pattern regulation by localizing to the old cell end during mitosis.

Major comments:

1) First of all, I cannot find any novelty of a phenotype of PrESS. To date, many mutant strains showing an elongated cell body with multi-septa have been identified in S. pombe (i.e. sec8, end4, ppb1 KO, etc.). What is a difference in the phenotype of PrESS with them?

We agree that PrESS cells resemble the cell separation failure phenotype reported in different cytokinetic mutants. The fundamental difference between cell separation failure in cytokinetic mutants and lack of cell separation in the PrESS phenotype is the timing of end growth. In cells in which separation fails due a defect either in membrane trafficking or glucanase delivery, cells initiate growth only after the septum forms; they have an opportunity to separate, but fail to do so.

In PrESS cells, cytokinesis is delayed, and the ends initiate growth even before the septum has had a chance to undergo separation. This is shown in our analysis of the timing of end growth initiation in a well-known cell separation mutant. While this phenotype has been reported in several mutants, we decided to use a septin mutant since these cells are otherwise healthy with no observable pleiotropic effects. While we observe cell separation failure in *spn1Δ* mutants, growth at the ends of these cells initiates well after septum formation, unlike in PrESS cells, in which growth initiates during septation (Figure 1C and D). This further suggests that the PrESS phenotype arises due to growth activation at the ends even as cytokinesis is delayed.

2) Previously I found a low amount of Lat-A produced a large population of an elongated cell with the septum. The authors have shown no clear evidence that the actin cytoskeletal organization is fully recovered after a temporary treatment with Lat-A. I think the authors cannot ignore the possibility that a low amount of Lat-A after washouts produces the PrESS phenotype.

We now provide data to show that the actomyosin ring reassembles in cells that recover from the 30-minute LatA treatment and that cytokinesis proceeds (Supplemental Figure S1B). In PrESS cells, the reassembled actomyosin ring undergoes constriction, the primary-septum- synthesizing enzyme Bgs1 is delivered to the division site, and the septum appears normal. Moreover, we find that only mitotic cells that have undergone LatA treatment show a PrESS phenotype (Figure 2). These observations indicate that the PrESS phenotype only occurs in a certain population of cells in a certain cell cycle stage and these cells indeed delay cytokinesis as the ring has to be reassembled.

To the point that the PrESS phenotype may be an artefact of residual LatA remaining in the samples after the wash, one would expect to see the same frequency of PrESS cells regardless of duration of treatment. Instead we find that the duration of treatment corresponds to the PrESS frequency observed (Fig. 3E). We observed a progressive drop in PrESS frequency with decreasing treatment durations. Finally, after washout, we observe the PrESS phenotype only in the first cell cycle, and in subsequent cell cycles the cells are able to divide and grow normally (Figure 1A and Supplemental Figure S3C). These results indicate that the PrESS phenotype is not due to some trace level of LatA that may remain in the media after washout.

3) In figure 4D, a septation index in Lat-A untreated cells should be shown. In addition, a frequency of the PrESS phenotype elevated only 3% in rga4 KO although the authors insisted the difference was statistically significant. It seems that there is not a big difference between gef1 and rga4. Is the role of Rga4 minor in the control of regrowth in cell tips after mitosis?

We now provide the septation indices of the untreated cells in this figure (now Figure 5) in Supplemental Figure S5A and B. We did not see any increase in the septation index of *rga4Δ* mutants compared to wild type cells. This suggests that the increase in PrESS frequency is not simply due to a septation defect in these cells. Moreover, we also provide data to show that *rga4Δ* mutants do not show any cell cycle delays. Therefore, the increase in the PrESS frequency observed in *rga4Δ* cells indicates a role for Rga4 in the regulation of Cdc42 activity at the cells ends during mitosis. We would like to highlight the fact that, since PrESS cells are only observed in cells that are in mitosis at the time of LatA treatment, there is an upper limit to the possible fraction of PrESS cells in an asynchronous population, which is dependent on the fraction of mitotic cells in the population. Since *rga4Δ* cells do not show a cell cycle delay, they do not have a larger fraction of mitotic cells compared to wild type cells. Thus, the higher PrESS frequency observed in these cells is due to an enhanced ability to activate Cdc42 at their cell ends over their division site.

A role for Rga4 in regulating Cdc42 during cell division is further supported by the fact that the localization pattern of Rga4 is cell-cycle-dependent, given that Rga4 localizes to the ends during mitosis (Figure 6), and loss of *rga4* in cells constitutively activating the MOR pathway yields a PrESS-like phenotype (Figure 7).

4) In this manuscript, the authors just observed the Rga4 localization pattern in the cell cortex during the restart of cell growth. They should consider testing what happens in the restart of cell growth if a force-localizing Rga4 in the old cell end.

This is an interesting idea. A recent report has shown that artificially localizing Cdc42 GAPs to the cortex blocks Cdc42 activity and leads to cell death (Gerganova et al, 2020, BioRxiv). In that same

report, oligomerizing the GAP at the cortex enabled displacement of the GAP from the ends and allowed Cdc42 activation at these sites. This indicates that the GAP's ability to inactivate Cdc42 is influenced by its protein properties and localization pattern. In agreement with this, we find that localization pattern of Rga4 changes throughout the cell cycle. In interphase, Rga4 appears more punctate along the cell sides, and in mitosis it appears diffuse at the cortex all the way to the cell ends. Our results suggest a role for Rga4 in inhibiting Cdc42 activity at the cell ends, and this inhibition is lifted in *rga4Δ* mutants. If force-localizing Rga4 blocks Cdc42 activity at the ends, this will be indeed a good confirmation of our findings.
However, if the ends remove the force-localized Rga4 and then activate Cdc42, this does not disprove our hypothesis. This is because the cell-cycle-dependent changes in the behavior of Rga4 most likely influence its ability to localize and thus inactivate Cdc42. Thus, to understand how Rga4 regulates Cdc42 in a cell-cycle-dependent manner at the ends, we will first need to investigate the molecular mechanism of how the cell cycle alters Rga4. This is currently under investigation in the lab, and the findings will be reported in our next manuscript on this project.

Minor comments:

1) No evidence (or citation) is shown in the following sentence; "We have previously shown that in gef1 cells, the old end competes with the new end for Cdc42 more effectively than wild type."

The reference for this statement is now provided.

2) Quantitative data is required in figure 6A.

We have now provided a quantification of PrESS and lysed cells in this experiment.

3) In figure 6B, does the pale blue curve indicate a growth of rga4+ Nak-Mor2+ cells?

Yes. These cells show a significant decrease in growth rate due to cell lysis, as has been reported by Gupta et al, 2014.

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

This paper addresses an interesting question on the timing of cell polarization during the cell cycle in fission yeast. In that system, polar growth stops during mitosis and resumes following cell separation. Polarity regulators are repurposed and relocalized to the division site during cytokinesis. What is the trigger for repolarization after cytokinesis? Here the authors use transient actin depolymerization during mitosis to perturb the system (delaying actin-dependent cytokinesis) and observe the effects. From this analysis they conclude that resumption of polarity is not dependent on completion of cytokinesis or cell separation, but instead that some (uncharacterized) cell cycle cue at the end of mitosis triggers polarization by removing the Cdc42-directed GAP, Rga4, from the cell poles. This is an intriguing and plausible hypothesis. However, I did not think that the conclusions were adequately supported by the data presented.

Specific comments:

1. The conclusion drawn from Fig. 1 and highlighted in the first paragraph of the Discussion is that old end polar growth is not dependent on cell separation: wasn't this already known from the fact that (e.g.) septin mutants resume growth without separating?

We agree that cells which fail to separate are able to grow at their ends. Given that growth resumption occurs simultaneous to cell separation in wild type cells, here we asked if the timing of growth resumption would be delayed in cell separation mutants. Our findings indicate that the timing of growth resumption is not dependent on cytokinetic events or cell separation, and instead depends on a cell cycle cue. We have rephrased this in the text for clarification.

2. The paper is organized around observation of the "PrESS" phenotype, which is described differently in different parts of the paper. In the Discussion, it is described as "polarized cell growth [initiated] during cytokinesis" but the acronym and initial description refer to "separation"

and most cells appear to complete cytokinesis. This highlights what I found to be a major ambiguity in trying to interpret the results: the timing of initiation of polar growth is never clearly measured, and many conclusions are drawn based on a phenotype that relies on other factors like the efficiency/rate of cell separation. The only data that seemed to address timing directly were in Fig. 1C, which has several issues (below). The remainder of the paper views the presence of growing cells that failed to separate as an indication of "premature" polar growth, but it seems that could equally reflect poor cell separation (as in septin mutants). As there is no obligate link between cell separation and polar growth, it seems to me that PrESS per se need not imply anything about the timing of polar growth relative to cytokinesis.

We have rephrased this statement to highlight that PrESS cells initiate polarized cell growth during septation. We also provide data that show that Cdc42 is activated at the cell ends at a fixed time after anaphase B in both non-PrESS and PrESS cells (Figure 3D). Furthermore, we find that the frequency of the PrESS phenotype correlates with the duration of the LatA treatment (the extent of cytokinesis delay) (Figure 3E). Together, these results indicate that Cdc42 activation and growth resumption at the cell ends are not dependent on cytokinesis or cell separation, but are rather on a cell-cycle-dependent timer.

3. Fig. 1C scores the time from "septum ingression" to "old end growth onset" but no description was provided of how these times were determined. The text refers to the time of "septum closure", but again with no indication of how this was determined. Is there a difference between septum ingression and closure? How were these times scored?

We monitored septum ingression in control and PrESS cells using brightfield microscopy. The time point at which the gap between the two ingressing sides of the septum closes is considered the end of septum ingression and the time of septum closure. We now provide an example of this analysis in Supplemental Figure S1C. In addition, we also provide data to show the timing of growth resumption at the ends with respect to ring closure, which was detected by the ring marker Rlc1-tdTomato (Supplemental Figure S1D). The timing of end growth with respect to ring closure is consistent with the timing observed with respect to septum closure.

4. Also on the issue of timing, in Fig. 3A: at what time were these pictures taken relative to LAT washout or initiation of ring constriction? In Fig. 3B, Cdc42 activation is shown to occur before cell separation, but on what basis do the authors conclude that Cdc42 activation occurs "earlier" at the ends? Here time "0" is not defined: how does it relate to the time of LAT washout or the time of initiation of ring constriction? Is "0" for the untreated and treated samples comparable in some way or are the times arbitrary? Fig. 3C: how does 2 hours of recovery relate to the time of septation? Might the Scd1/2/Myo52 be present at the center earlier on?

Figure 3A (now Figure SD3 in the revised manuscript) shows cells 3 hours after LatA wash out. This is specified in the legend. We now provide a quantitative analysis of Cdc42 activity at the cell ends with respect to the cell middle (Figure 3C). In both control and PrESS cells, we measured levels of active Cdc42 at cell ends, normalized to the cell middle, beginning with when Cdc42 became active at the cell middle. In non-PrESS cells, active Cdc42 levels at the ends remains low or non-existent until the cell separates. In PrESS cells, active Cdc42 levels at the ends increase gradually as compared to the cell middle. In Figure 3A and B, we now use ring closure, as determined with the marker Rlc1-tdTomato, as time zero. These results in Figure 3A-D together indicate that Cdc42 activity returns to the ends of PrESS cells at a fixed time after mitosis, independent of cytokinetic events.

In Figure 4 we now provide a quantitative analysis of Scd2 and Myo52 levels at cell ends, normalized to the cell middle. We show that, similar to active Cdc42, Scd2 and Myo52 levels at the ends of PrESS cells increase gradually as compared to the cell middle. The Scd2 and Myo52 levels were measured from the onset of signal at the division site. We were unable to perform time-lapse imaging of Scd1-3xGFP in a similar manner due to rapid photo-bleaching of this fluorophore. However, since Scd1 localization depends on Scd2, Scd2 is a good marker for Scd1 localization. Our results indeed indicate that, during cytokinesis, Scd1, Scd2, and Myo52 localize to the cell middle as expected. However, in PrESS cells, the levels at the middle gradually diminish while those at the ends increase over time. Thus, our results in Figures 3 and 4 collectively show that, in PrESS cells, active Cdc42, its regulators, and its downstream effector appear at the cell ends at a fixed time

after mitosis, independent of cytokinetic events.

5. The questions above (2-4) highlight a major difficulty with the issue of how to tell whether the timing of polar growth is "normal" or "premature" in a given condition. One would ideally like to know when polar growth initiates relative to the time when MOR signaling turns on (or is there some other event the authors would pick?). But as the perturbations employed here (LAT treatment, or mutant strains) would presumably affect the timing of MOR onset, and no measure of that timing was provided, I did not understand how the authors inferred the relative timing of polar growth.

We thank the reviewer for this suggestion. One of the best cellular timers in fission yeast during division is mitotic progression as determined by the spindle pole body distance. Taking advantage of this timer, we now show that Cdc42 activation at the cell ends in non-PrESS and PrESS cells occurs at a fixed time after anaphase B (Figure 3D). This indicates that a cell-cycle- dependent cue determines when the ends activate Cdc42 and resume growth. In these experiments, anaphase B was determined based on the distance between the spindle pole bodies, marked by Sad1-mCherry.

6. A central finding of the paper is that Rga4 localizes to cell poles during mitosis (Fig. 5). I found it hard to perceive the Rga4 at the poles that the authors highlight-I assume the scoring must count instances of very faint puncta, and I am uncertain as to the significance of these. I appreciate the time averaging strategy used in Fig. 5B, but I had a hard time seeing the pole enrichment in late anaphase (the cytoplasm as a whole seems darker in this cell-I would suggest this be shown for several cells), and the pole signal highlighted by the arrows seemed very faint in Fig. 5C. Thus, it seemed a gross exaggeration to draw Rga4 distribution in anaphase as being uniform at poles and sides, as done in the cartoon in Fig. 7 and hinted at in some of the text. The less punctate Rga4 distribution at cell sides during mitosis was more convincing than the presence of Rga4 at poles, but the significance of this less punctate pattern was unclear.

We now provide a quantitative analysis of Rga4-GFP levels at the cells ends in G2, mitotic, and G1/S cells using time projections of 5-minute movies imaged every 10 seconds (Figure 6C). We find that Rga4-GFP levels at the ends increase in mitosis as compared to G2 and G1/S. We also find that Rga4-GFP signal at the cortex increases during mitosis compared to the cytoplasm (Supplemental Figure S6B). We have now modified the model in Figure 8 to accurately reflect the Rga4 signal we observe at the cell ends.

7. Fig. 6 shows that the lytic phenotype resulting from constitutive MOR pathway activity is rescued by deleting Rga4. The authors conclude that Rga4 specifically prevents old end growth during cell separation, and resumption of growth occurs upon Rga4 removal. But it is far from clear that the phenotype is rga4-specific: would other mutants from Table S2 also rescue? If so, that might argue against a specific role for Rga4 in timing the resumption of polar growth.

We thank the reviewers for this suggestion. Our observation with *rga4Δ* mutants constitutively activating the MOR pathway indicates that, in untreated wild type cells, Rga4 prevents end growth before cell separation, and its removal allows growth resumption at the ends. To confirm if this is indeed specific to Rga4, we performed similar experiments with *rga6Δ* and *gef1Δ* mutants. We selected the *rga6Δ* mutant as it is another Cdc42 GAP, but does not show an increase in PrESS frequency compared to wild type cells. We do not see any rescue of cell lysis or the PrESS-like phenotype in these mutants when constitutively activating the MOR pathway. We also used *gef1Δ* mutants, as these cells show a higher PrESS frequency than wild type cells. In these mutants we also did not observe a rescue of cell lysis or any PrESS-like cells upon constitutive MOR pathway activation. Together, these results indicate that, in the context of constitutive MOR pathway activation, rescue of cell lysis and the presence of PrESS-like cells are specific to the absence of *rga4*, thus demonstrating a role for Rga4 in preventing end growth before cell separation.

8. The authors conclude that "after division, Cdc42 activation at the cell ends requires both removal of Rga4 from the cell ends and MOR activation". But to establish a requirement for "removal of Rga4 from the cell ends" would require an experiment that specifically prevents Rga4 removal from the ends, and no such experiment is presented.

We agree that the use of the term “removal” may not accurately describe how Rga4 levels at the cell ends decrease during G1/S and G2 since the mechanism of this event is unclear. A more accurate term would be “loss” of Rga4 from the cell ends. Our lab is currently investigating the molecular mechanism of cell-cycle-dependent regulation of Rga4 localization. As we gain further insight into this mechanism, we will be able to design experiments to increase or decrease Rga4 localization at the cell ends and monitor growth resumption at those ends.
Regardless, it has been shown in a recent study that artificially localizing the GAP domain of Rga4 to the cortex prevents Cdc42 activation, leading to cell death. In these cells, artificially oligomerizing the GAP enables Cdc42 activation at the ends. This observation is reminiscent of our findings where Rga4 localization undergoes cell-cycle-dependent changes, with the GAP localizing to the ends during mitosis when Cdc42 activation is inhibited, and mainly to the sides during interphase when end growth resumes.

9. It seems to me that there are three scenarios that could explain loss of Rga4 from the poles. First, as the authors assume, some cell cycle signal may remove Rga4 specifically from poles, making the poles permissive for Cdc42 activation. Alternatively, some other regulatory event may activate growth at the poles despite the low level presence of Rga4, and such growth may then promote Rga4 removal, as suggested by earlier findings that LAT treatment of interphase cells allowed some Rga4 to be seen at poles (Das et al. 2007). Finally, one could imagine a feedback loop whereby Cdc42 activation leads to Rga4 exclusion, which facilitates further Cdc42 activation. Distinguishing between these options would require an analysis of the relative timing of Cdc42 activation and Rga4 removal from the poles. As detection of Rga4 at cell ends was difficult in any case, determining the timing of its loss may be problematic. But without such an analysis, the authors' assumption that Rga4 removal is the primary event seems premature.

The three different scenarios presented by the reviewer, while quite plausible, are not mutually exclusive. It is not well understood what leads to initiation of Cdc42 activation at the ends after division. However, since polarized growth requires positive feedback activation of Cdc42, one can assume that a certain threshold level of active Cdc42 is required to establish a growing end. It is possible that presence of Rga4 at the cell ends may impede meeting this threshold, and thus prevent growth at that end. While it is possible that increased active Cdc42 may lead to loss of Rga4 from the ends, this may only be possible once Cdc42 activity levels reach this threshold. This is also supported by the fact that artificially localizing constitutively active Cdc42 to the cell sides does not establish a robust local site of Cdc42 activation and growth due to the presence of Rga4 at these sites (Lamas et al, 2020, Cells). A more detailed understanding of the mechanism of Rga4 regulation will provide a deeper insight into this process.

To provide additional evidence for the role of loss of Rga4 from the ends in growth initiation, we analyzed *rga4Δ* mutants constitutively activating the MOR pathway. Our findings show that, in these mutants, cell lysis is rescued and Cdc42 is activated at the cell ends, leading to a PrESS- like phenotype. Moreover, this observation is specific to Rga4. Our data thus show that growth initiates at the ends after division only once the MOR pathway is activated and Rga4 is lost from the cell ends.

Other issues:

•The conclusion that Cdc42 at the old end "siphons Cdc42 away from the division site" seems reasonable but I did not see any experimental test that established this to be true.

We have now provided a quantification of active Cdc42 localization in non-PrESS and PrESS cells (Figure 3C). In PrESS cells, we see that after the cell middle activates Cdc42 at the beginning of cytokinesis, Cdc42 is then also activated at the cell ends. The signal at the ends increases over time, while the signal at the cell middle decreases. We have changed the wording to avoid saying that Cdc42 activity is “siphoned” away from the cell middle, since we have not shown this.

•The authors show that Sty1 is not required for the resumption of growth before cell separation, but the more interesting question is what happens to Cdc42 in the sty1 mutant: does Cdc42 return to the poles earlier (after the LAT perturbation) than in the wildtype?

This is an interesting point and one that requires further investigation. While it is known that in *sty1* mutants treated with LatA, Cdc42 activity is restored at the cell end, the mechanism for how this is brought about is unclear. Future experiments will explore Cdc42 activation in the *sty1* mutant in PrESS cells.

•The MOR pathway is discussed as though its effects are exclusively mediated by Sts5, but isn't Gef1 another target of the MOR that directly affects Cdc42 and hence polar growth?

The MOR pathway terminal kinase Orb6 promotes growth and polarity. Polarized growth is maintained by the Orb6 kinase by preventing depolarized localization of Gef1 and by limiting polarized growth to the cell ends. As a result, loss of *gef1* rescues the *orb6* mutant's polarity defect, but not its growth defect or viability. On the other hand, Orb6 prevents growth via inhibiting the RNA-binding protein Sts5 and promoting protein synthesis. Loss of *sts5* in *orb6* mutants promotes growth and restores viability. Thus, here we focus more on the growth-promoting process of the MOR pathway. Indeed, even in cells constitutively activating the MOR pathway, Cdc42 activation does not polarize to the cell ends and remains at the division site. This results in cell lysis as the prematurely synthesized glucanases (as a result of MOR activation) are delivered to the division site before the septum forms. However, in *rga4Δ* mutants constitutively activating the MOR pathway, we see increased polarized Cdc42 activation at the cell ends and consequent growth at these ends, as well as a rescue of the cell lysis phenotype. Moreover, we did not see any lack of the PrESS phenotype in LatA-recovering *gef1Δ* mutants, nor did we see PrESS-like cells in *gef1Δ* mutants constitutively activating the MOR pathway. Thus, in this manuscript we have focused on the role of the MOR pathway in promoting growth via protein synthesis.

•Just out of curiosity: do cells that resume old-end growth but fail to separate subsequently do NETO?

We do indeed see some branching from the unseparated division site in a small fraction of PrESS cells. Thus, NETO is possible though rare in these cells. One potential explanation for this low number could be steric hindrance due to the presence of the septum.

Original submission

First decision letter

MS ID#: JOCES/2021/259291

MS TITLE: Cdc42 reactivation at growth sites is regulated by local cell-cycle-dependent loss of its GAP Rga4

AUTHORS: Julie Rich-Robinson, Afton Russell, Eleanor Mancini, and Maitreyi Das
ARTICLE TYPE: Research Article

Dear Dr. Das,

Thank you for submitting your manuscript together with the associated reports from Review Commons. I have gone through your responses to the reviewers comments and the revised manuscript. I think you have addressed all the reviewers concerns and questions with your thorough responses. Moreover, I do not feel there is a need to go back to the reviewers, so I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.