The Cdc42 GAP Rga6 promotes monopolar outgrowth of spores

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Re: JCB manuscript #202202064

Prof. Chuanhai Fu
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Dear Prof. Fu,

Thank you for submitting your manuscript entitled "The Cdc42 GAP Rga6 is required for promoting monopolar growth during spore germination." Your manuscript has been reviewed by three experts whose comments are appended below. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you will see, they all considered the observation of bipolar outgrowth from spores of rga6 delete mutants to be interesting. Your hypothesis that what gives Rga6 the ability to suppress bipolar growth is its localization to the membrane by PIP2 is interesting, but as you will see the reviewers had a number of technical issues and suggested experiments that would make that much more convincing. While it would also be interesting to elucidate the basis for the PIP2 distribution, that issue seems beyond the scope of this paper.

A potential alternative quantitative explanation for the special role of Rga6 on suppressing bipolar growth could be that it is simply a more highly expressed GAP in germinating spores. Have you compared the expression level of Rga6 in outgrowth-stage spores with that in vegetative cells? And would overexpression of other GAPs like Rga3 or Rga4 (e.g. from the ase1 promoter as you did with Rga6) suppress the bipolarity phenotype of rga6 mutant spores? These simple experiments could perhaps distinguish a quantitative model from your localization-based qualitative model to explain the different roles of the GAPs.

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, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

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

Thank you for submitting this interesting work to JCB. We look forward to seeing a revised version that addresses the reviewers' comments.

Sincerely,

Daniel Lew, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

Regulation of singularity in polarized cell growth is not well understood in any system. The authors attempted to address this problem using the monopolar growth displayed by the germinating spores of the fission yeast S. pombe as a model system. Through a series of well-designed and well-executed experiments, the authors have demonstrated convincingly that the Cdc42-GAP Rga6, but not other Cdc42-GAPs (Rga3 and Rga4), is required for promoting the monopolar growth, and this function requires both the GAP and PBR domains of Rga6. In addition, the authors have demonstrated clearly that the PBR domain is responsible for the localization of Rga6 to the non-growing region of the PM and this domain can direct the GAP domain of Rga3 or Rga4 to perform the Rga6 function. Furthermore, the authors presented suggestive evidence that Rga6 localizes to the non-growing region by binding to PIP2 via its PBR domain. Overall, this study is rigorous, and the major conclusion is well supported by the data. Some straightforward experiments to increase the scope and mechanistic depth of the study as well as some minor weaknesses are outlined below.

Major points:

1. Figure 4A: based on the localization patterns of Rga3, Rga4, and Rga6, it appears that Rga6 acts to prevent Cdc42 activation at the non-growing end, as the authors have convincingly demonstrated. In addition, Rga4 and Rga6 may share a role in preventing Cdc42 activation at the lateral sides of the cell. If so, the rga4 rga6 spores may germinate at two or more random locations. This proposed test could strengthen the story by demonstrating how distinct GAPs act alone or in concert to promote or ensure monopolar growth of the germinating spores at multiple levels.
2. Regarding the role of PIP2 in Rga6 localization (Figure 5): while the decreased localization of Rga6 caused by overexpression of the PIP2 biosensor Opy1 does suggest that Rga6 localizes to the non-growing regions by binding to PIP2, additional experimental test can further solidify the conclusion. If the authors' hypothesis is correct, conditional depletion of PIP2 at the PM should decrease the cortical localization of Rga6 and promote non-monopolar growth of the germinating spores.

Minor points:

1. In several places, the authors describe the role of Rga6 in "maintaining" monopolar growth. However, all the data suggest that Rga6 plays an essential role in "establishing" the monopolar growth by preventing Cdc42 activation at the non-growing regions.

2. Figure 2D, 2nd row (rga3 cells), 1st DIC image: this image has a much darker background than the rest of the images in the montage. Please clarify.
3. Figures 2D-2G: please indicate in the figure legend how the monopolar and bipolar growth patterns were determined unambiguously based on DIC imaging only (these spores were not stained by Calcofluor).
4. Figure 3C: please indicate what percentage of cells (n = ?) displayed oscillation of CRIB-GFP between the fast- and slow-growing ends for each strain.
5. Figure 3E, 2nd row (rga3 cells): adjust the brightness and contrast to ensure consistency between panels or choose a different cell.

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

This manuscript by Wei and colleagues examines the monopolar outgrowth of fission yeast spores and describes a role for the Cdc42 GTPase activating protein Rga6 in maintaining this mode of growth. The main findings of the paper are that rga6Δ spores often germinate in a bipolar manner and that this requires Rga6 GAP activity and localization to the cell cortex. Localization of Rga6 may involve binding to PI(4,5)P2, though this is indirectly shown. The conclusions of the manuscript are that "intrinsic properties of the spore plasma membrane may create spatial cues to specify the characteristic cortical localization of Rga6 and promote monopolar growth".

How monopolar vs. bipolar growth is regulated is a very interesting question. The data presented is generally clean and convincing (with a few comments below), but remains preliminary, leading to rather vague conclusions that "intrinsic properties of the spore plasma membrane may create spatial cues to specify the characteristic cortical localization of Rga6.". The true questions are whether there is anything special ("intrinsic properties") about the spore plasma membrane relative to vegetative cells, and what these "intrinsic properties" may be. The role of the main difference between spores and vegetative cells, i-e the presence of a rigid spore wall, is also completely unexplored.

My comments below first relate to specific findings of the paper, and then to their placement in the context of previous work.

In what way is spore outgrowth different relative to vegetative growth?
As suggested by the authors, a couple of observations suggest that spore outgrowth and vegetative growth are not regulated exactly in the same way. First, spore outgrowth is normally monopolar, whereas vegetative growth is bipolar. This is likely linked to the necessity to break the specific, rigid outer spore wall to initiate outgrowth (see Bonazzi et al, Dev Cell 2014; see below). Second, it is interesting that the one growing end of the germinating spore appears to grow faster than even the combined growth of both cell ends during mitotic growth. To my knowledge, this second observation is not directly linked to the monopolar mode of growth, as monopolar mutants do not generally show overall faster growth. The overall growth rate of germinating spores (adding the average values for the slow and fast-growing ends shown in Figure 3D) also seems rather constant between genotypes, with rga6Δ overall growth similar to that of WT or rga3Δ or rga4Δ spores, but with more equal growth between cell ends. This raises the question of why spore outgrowth should be faster than vegetative growth. Is this dependent on the size of the spore, the length of time since sporulation, or perhaps other characteristics?

Is the function of Rga6 specific to spore outgrowth?
The considerations above also raise the question of whether the bipolar phenotype of rga6Δ is specific to spore outgrowth, or whether it is a more general phenotype. Do rga6Δ cells generally shift growth towards bipolarity more easily? This can be tested for instance by measuring the proportion of bipolar rga6Δ (vegetative) cells, and by measuring the relative growth of the fast and slow-growing ends. It

Role of PI(4,5)P2:
The possible role of PI(4,5)P2 is poorly defined. The observation that Opy1 overexpression leads to reduced Rga6 cortical localization may suggest that Rga6 binds PI(4,5)P2, but the competition may also be through other indirect ways. To support this point, in vitro work would be required. If Rga6 localization is indeed dictated by that of PI(4,5)P2, this then raises the question of how PI(4,5)P2 is distributed to cell sides, which is not addressed.

Placement in context of previous work:
Prior work from the Minc lab showed that spore outgrowth required breakage of the rigid spore wall (Bonazzi, Dev Cell 2014). Thus, one possible (likely) reason for the monopolar mode of spore outgrowth is that spore wall breakage is difficult. Since polarity site stabilization relies on positive feedback with growth upon spore wall breakage, it is easy to imagine that this first site is preferred and a second one less likely to initiate. Thus, it is very interesting that bipolar outgrowth happens more frequently in rga6Δ cells and begs the question of whether spore wall breakage occurs more easily in this background or mechanical feedback between the cell wall and polarity is altered. This is not considered at all in the manuscript.

There is a rich prior literature, which has examined the role of protein (Cdc42 GTPase and regulators) dynamics using either S. pombe or S. cerevisiae yeast models in regulating monopolar vs bipolar mode of growth (Lew, Bi, Goryachev, Martin, to cite just a few labs), but this literature is unfortunately not cited here.

Recent publication from the Martin lab (Gerganova, Science Advances 2021) showed that Rga4 side localization is due to bulk flows of membrane from the secretion site organized by Cdc42 activity. Rga6 is not very different from Rga4: both localize to cell sides and the localization of both proteins depends on a poly-basic stretch of amino-acids. It would be interesting and important to consider Rga6 localization in this context.

Minor comments:

p. 3: the reference to GEFs activating Cdc42 locally is perhaps not so clear for Gef1, which was proposed to act as a "global" GEF (see Tay et al, JCS 2018).

p. 5: What is lipid YE? Is this meant to be liquid YE?

The methods do not explain clearly how measurements were made. For example, in Figure 1C, the very peaked distribution is probably the result of aligning curves to the max of each profile rather than the geometric end of the cell (which the scheme suggests).

Do the measurements of growth rate here correspond to previous measurements? Please cite the appropriate references for comparison.

Figure 2B: The calcofluor staining experiment is not all that clear. I do not understand how you still get staining of the growing cell ends and very prominent staining of the septa if calcofluor was not present in the media for 6h. This suggests that there is substantial unbound calcofluor in the media. Doing the same sort of analysis with labelled lectin may be a good alternative. Furthermore, in the enlarged examples shown, it looks like calcofluor is still excluded from both WT and rga4Δ "non-growing" end. While the rga6Δ phenotype is clear, it is not all that clear what is counted as monopolar or bipolar in the other backgrounds.

Figure 2D-2F: What the yellow arrowheads mark is not very clear. It is not the initiation of outgrowth, which started in earlier timepoints (as judged from drawing a line from the edges of the initial spore).

In Figure 3B (and generally Figure 3), it may be best to measure the actual CRIB intensity at each cell pole rather than provide a binary measure of presence/absence. Again, the rga6Δ phenotype is clear, but the statement that neither rga3Δ nor rga4Δ show phenotype may be overstated. They may show a weak intermediate phenotype.

Figure 4A: The Rga4-2mN localization appears to be in a spore that grows in a bipolar manner. Is that usual? In a WT background, this should be a rare instance. Perhaps a more representative example could be chosen.

Figures S3B: lanes are unlabelled.

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

Wei et al. report on the role of the Cdc42 GAP Rga6 in promoting monopolar growth in germinating/outgrowing spores of fission yeast. The study combines standard approaches for yeast cell biology including microscopy, quantitative analysis and genetics. The authors show that Rga6D cells exhibit premature bipolar growth in germinating spores, with 2 tips emerging from the spore body in place of only 1 in WT. Localization of Active Cdc42 (at the growing tip) and rga6 (at the non-growing part of the cell) confirms that Rga6 may act by restricting Cdc42 activity to one growing tip in WT cells. By generating truncation alleles the authors finally suggest that this effect requires the proper cortical localization of Rga6 and the GAP domain of the protein. Overall, the MS is of correct quality (some aspects need improvements, see below), and the phenotype interesting given that there are few studies of spore germination/outgrowth in Fungi. I list below some comments/critics for the authors to improve some aspects of the work:

General point: The microscopy images presented throughout the MS are somewhat below standard for Pombe, and in many the contrast is boosted making it hard to fully capture the claimed phenotypes. I think the authors should definitely improve many of these. This concerns especially CRIB images in Fig 1, Rga6/Crib images in Fig 4A and C, 5A and C.

1- Fig 1: The much faster growth of outgrowing tips in spores together with the more pronounced Cdc42 activity is interesting. Is this effect specific to outgrowing spores or is it caused by some competition for polarity/growth material between the two tips, as documented in previous work (PMID : 33257499)? For example do vegetative monopolar cells (e.g. in tea1D mutants) grow as fast as outgrowing spores, do they also have brighter CRIB signals as well?

A related point: In the ref PMID: 26960792 Rga6D vegetative cells are not reported to feature defects in polarity, but OE of Rga6 has a strong monopolar phenotype. This should be mentioned, as it kind of support the general claim of the paper. Also the OE

of Rga6 should also be tested in spores (they may not even polarize).

2- Fig 2 and Fig 6: While the calcofluor assay makes a convincing case for the bipolar phenotype of Rga6D the panels D and F are not so convincing, as cells do not appear to really grow at their rounded end. This raises a concern on the use of Caclofluor, (This is a standard to affect CWs and here cells are treated for 6h!) which could affect spore CW properties and account for the pronounced role of Rga6 activity in promoting monopolar growth, given results from Ref PMID: 24636258. Since this is the central claim of the paper, I would be strict with a much better analysis of growth patterns in outgrowing spores of Rga6D without Calcofluor. I suggest either to (i) use lectins, which have less damaging effects on CWs, (ii) track fiducial marks precisely to compute growth at both ends, and/or (iii) quantify the size ratio btw daughter cells after division which should somehow reflect how much both tips have grown.

3- Figure 4B and 5B: It would be much clearer to trace directly the intensity of the signal around the full cell contour as done in Fig 4D and 6E.

4- Fig 5. The data for a role for PiP2 and Opy1 are not very convincing. First, the authors should provide much better co-localization images and quantifications btw the two signals. Second the localization of Rga6 in an Opy1D should be documented to test if PiP2 levels are really linked to Rga6 localization, and third the OE data need better images and quantification. Finally, the phenotype in term of monopolar vs bipolar growth for both OE of Opy1 and Opy1D should be provided.

Minor:
- The word germination is used throughout, even in the title, but the authors are looking at outgrowth. Germination is normally used for the onset of exit from dormancy (~6-7h before most images shown in the paper).
- CRIB domains have been shown to rapidly wander around during the first phase of isotropic growth in Pombe spores (PMID: 26441355). How does Rga6 localize during this first phase ? Is it stable at a given location, or does it oscillate in front of active Cdc42? This would be important to document, to test if Rga6 localization at the back of the cell may for instance come from landmarks inherited during sporulation, or if the system of polarity spontaneously auto-assemble.
- The last sentence of the introduction comes out of nowhere, this little part introducing the key results could be improved.
- Some typos:
P5: Lipid YE => liquid YE.
P9: promoted => prompted

Responses to editor and reviewers' comments

We would like to thank the editor and all reviewers for the effort extended to review our manuscript and for the suggestions for our improvements. We appreciate their great enthusiasm extended to our work and have taken their insightful suggestions to prepare a careful revision that addresses all concerns raised in the last review. Specifically, we have added 26 new figures and 22 new supplementary figures in the revised manuscript. Details are shown below:

1) Revised Figure 1 (Original Figure 1). New strains, including *tea1Δ*, carrying CRIB-3GFP were created, imaged, and analyzed. All data are newly generated during the revision.

2) Revised Figure 2 (Original Figure 2). Revised Figure 2, B-D were newly generated; Figure 2E was generated using original microscopic data; Figure 2F were created based on original and new imaging data.

3) Revised Figure 3 (new). Revised Figure 3A is the original supplemental Figure S2; Revised Figure 3, B-E are newly generated data based on the editor and reviewers' comments.

4) Revised Figure 4 (Original Figure 3). Only C was changed based on suggestions.

5) Revised Figure 5 (Original Figure 4). All data were newly generated by using optimized microscopic settings, and a new strain expressing CRIB-3mCherry, instead of CRIB-mCherry was used.

6) Revised Figure 6 (Original Figure 5). All data were newly generated, based on reviewers' comments.

7) Revised Figure 7 (Original Figure 6). Only E and F were analyzed, based on suggestions.

8) Revised supplemental Figure S1. All, except E and F (Original Figures 1D and 1E), are new data.

9) Revised supplemental Figure S2. All are new data.

10) Revised supplemental Figure S3. All are new data.

11) Revised supplemental Figure S4. A-D are original supplemental Figure S3, A-D; E is new.

12) Revised supplemental Figure S5. All are new data.

Overall, our new data are consistent with our original conclusions. The point-by-point responses to comments are shown below by highlighted text in blue. The revised text in the manuscript is also highlighted in blue. We hope that the revised manuscript would be acceptable for publication.

Editor's comments

We are grateful for the editor's positive comments.

A potential alternative quantitative explanation for the special role of Rga6 on suppressing bipolar growth could be that it is simply a more highly expressed GAP in germinating spores. Have you compared the expression level of Rga6 in outgrowth-stage spores with that in vegetative cells? And would overexpression of other GAPs like Rga3 or Rga4 (e.g. from the ase1 promoter as you did with Rga6) suppress the bipolarity phenotype of rga6 mutant spores? These simple experiments could perhaps distinguish a quantitative model from your localization-based qualitative model to explain the different roles of the GAPs.

We thank the editor for giving the opportunity for revision.

In the revised manuscript, we have conducted the experiments, as suggested by the editor, to address the two questions stated above. To address the two questions, a reliable method should be employed to determine the growth polarity during spore germination.

The Calcofluor staining method used in our method was established previously in determining the growth stages of spores (Plante and Labbe, 2019; Plante et al., 2017). In this present work, we employed this established Calcofluor-staining method to study spore outgrowth. Nonetheless, two reviewers were concerned and asked whether the method affected the growth of spores. To address this concern, we determined the growth stages of spores, which had been stained with or without Calcofluor in parallel in triplicates, after the spores were cultured in rich medium (Calcofluor-free). Quantification results revealed that the established Calcofluor staining procedure did not appear to affect the growth of spores because the percentage of spores stained with or without Calcofluor at the indicated growth stages was comparable (see new Figure 2B and new supplemental Figures S1C and S1D). It is possible that the special properties of the outer spore wall (Tahara et al., 2020) protect spores against the potential detrimental effects of Calcofluor. Therefore, the Calcofluor staining method, established previously (Plante and Labbe, 2019; Plante et al., 2017) and used in the present study, can be used to reliably study spore germination and outgrowth. To complement the Calcofluor-staining method, we also employed long time-lapse light microscopy to determine the growth polarity of germinating spores that were not stained with Calcofluor (see responses to reviewers' comments below).

1) Question 1: *"have you compared the expression level of Rga6 in outgrowth-stage spores with that in vegetative cells"*.

As shown in new Figure 2B and new supplemental Figures S1C-D, ~60% and >80% of spores underwent outgrowth at 6 and 8 hours, respectively, after the spores were cultured in rich medium. Therefore, to address the question, we collected germinating spores at 6 and 8 hours after the spores were cultured in rich medium and compared the expression level of Rga6 in the germinating spores and in vegetative cells (See new Figure 3B). The result showed that the expression level of Rga6 was generally higher in vegetative cells than in germinating spores. Hence, this result suggests that the role of Rga6 in suppressing bipolar growth of germinating spores is not due to the increased expression of Rga6 during spore germination.

We further tested the expression level of the three Cdc42 GAPs (i.e., Rga3, Rga4, and Rga6) in germinating spores at 8 hours after the spores were cultured in rich medium (See new Figure 3C). The results showed that the expression level of Rga4, but not Rga3 and Rga6, was the highest during the spore outgrowth stage.

2）Question 2: "*would overexpression of other GAPs like Rga3 or Rga4 (e.g. from the ase1 promoter as you did with Rga6) suppress the bipolarity phenotype of rga6 mutant spores*".

To address this question, we created *rga6Δ* strains expressing Rga3-GFP or Rga4-GFP from the *ase1* promoter (i.e., overexpression) and tested whether overexpression of Rga3-GFP or Rga4-GFP could suppress the bipolar growth of spores caused by the absence of Rga6. First, we confirmed the expression of Rga3-GFP and Rga4-GFP, respectively, by western blotting analysis (see new supplementary Figure S5A). We then employed the Calcofluor staining method to assess the growth polarity of spores. As shown in new supplementary Figures S5B and S5C, the percentage of spores displaying bipolar growth was comparable in the three types of spores (i.e., *rga6Δ*, *rga6Δ*+Rga3-GFP, and *rga6Δ*+Rga4-GFP). In addition, we determined spore growth polarity by long time-lapse light microscopy (spores without Calcofluor staining). Similar results were obtained (See new supplemental Figure S5D). These results suggest that the bipolarity phenotype of spore growth is caused specially by the absence of Rga6.

Reviewer #1

Regulation of singularity in polarized cell growth is not well understood in any system. The authors attempted to address this problem using the monopolar growth displayed by the germinating spores of the fission yeast S. pombe as a model system. Through a series of well-designed and well-executed experiments, the authors have demonstrated convincingly that the Cdc42-GAP Rga6, but not other Cdc42-GAPs (Rga3 and Rga4), is required for promoting the monopolar growth, and this function requires both the GAP and PBR domains of Rga6. In addition, the authors have demonstrated clearly that the PBR domain is responsible for the localization of Rga6 to the non-growing region of the PM and this domain can direct the GAP domain of Rga3 or Rga4 to perform the Rga6 function. Furthermore, the authors presented suggestive evidence that Rga6 localizes to the non-growing region by binding to PIP2 via its PBR domain. Overall, this study is rigorous, and the major conclusion is well supported by the data. Some straightforward experiments to increase the scope and mechanistic depth of the study as well as some minor weaknesses are outlined below.

We thank the reviewer for the positive comments.

Major points

1. Figure 4A: based on the localization patterns of Rga3, Rga4, and Rga6, it appears that Rga6 acts to prevent Cdc42 activation at the non-growing end, as the authors have convincingly demonstrated. In addition, Rga4 and Rga6 may share a role in preventing Cdc42 activation at the lateral sides of the cell. If so, the rga4Δ rga6Δ spores may germinate at two or more random locations. This proposed test could strengthen the story by demonstrating how distinct GAPs act alone or in concert to promote or ensure monopolar growth of the germinating spores at multiple levels.

We followed the reviewer's suggestion to analyze the growth pattern of the double-deletion mutant *rga4Δrga6Δ*. The result showed that the growth pattern of *rga4Δrga6Δ* and *rga6Δ* spores was comparable, i.e., ~60% of *rga4Δrga6Δ* and *rga6Δ* spores grew in a bipolar manner (see new supplemental Figure S5, E-G). We did not see growth taking place from three or more random locations in *rga4Δrga6Δ* cells. This result suggests that Rga6 plays a dominant role in regulating the monopolar growth of spores.

2. Regarding the role of PIP2 in Rga6 localization (Figure 5): while the decreased localization of Rga6 caused by overexpression of the PIP2 biosensor Opy1 does suggest that Rga6 localizes to the non-growing regions by binding to PIP2, additional experimental test can further solidify the

conclusion. If the authors' hypothesis is correct, conditional depletion of PIP2 at the PM should decrease the cortical localization of Rga6 and promote non-monopolar growth of the germinating spores.

We thank the reviewer for the helpful suggestion.

The 1-phosphatidylinositol-4-phosphate 5-kinase *its3* is an essential gene, and functions to generate PI(4,5)P2 (Zhang et al., 2000). A temperature sensitive mutant, *its3-1*, was reported previously (Zhang et al., 2000). Therefore, we created a mutant strain expressing *its3-1* and used the *its3-1* strain to test whether conditional depletion of PIP2 at the plasma membrane could decrease the cortical localization of Rga6. Consistently, at restrictive temperature, i.e., 37℃, the staining of Opy1-2mNeonGreen and Rga6-tdTomato on the plasma membrane was much weaker in *its3-1* spores than wild-type spores (new supplemental Figures S3D and S3E). This result further supports our claim that Rga6 localizes to the plasma membrane in a PIP2-dependent manner. Additionally, We tested the effect of PI(4,5)P2 on the growth of germinating spores. As shown in supplemental Figure S3D, *its3-1*spores did not undergo outgrowth at all at the restrictive temperature, i.e., 37℃. By contrast, wild-type spores still underwent outgrowth at 37℃. This result suggests that PIP2 is essential for spore outgrowth. Hence, in addition to promoting the cortical localization of Rga6, PIP2 plays an uncharacterized essential role in regulating spore outgrowth. Therefore, it was impossible to assess the role of Rga6 in outgrowth specifically by conditional depletion of PIP2.

To strengthen the claim that Rga6 has an affinity for PIP2, we carried out liposome reconstitution assays using lipids PI(4,5)P2 and PC and recombinant proteins His-GFP and His-GFP-Rga6. As shown in new Figures 6C-6E, His-GFP-Rga6, but not His-GFP, efficiently decorated the microspheres coated with lipids containing PI(4,5)P2, and the decoration was enhanced by increasing the percentage of PI(4,5)P2 in the lipid mixture. Therefore, it is highly likely that Rga6 localizes to the plasma membrane in a PI(4,5)P2-dependent manner.

We further quantified the growth patterns of Opy1-GFP overexpressing and *opy1Δ* spores. As shown in new Figure 6I and new supplemental Figures S3A and S3B, more spores grew in a bipolar fashion when Opy1-GFP was overexpressed. Since overexpression of Opy1 decreased the localization of Rga6 on the plasma membrane, we considered this as a piece of evidence supporting the claim that Rga6 functions to ensure the monopolar growth of outgrowing spores.

Minor points:
>
> 1. In several places, the authors describe the role of Rga6 in "maintaining" monopolar growth. However, all the data suggest that Rga6 plays an essential role in "establishing" the monopolar growth by preventing Cdc42 activation at the non-growing regions.

We have corrected the indicated statements.

2. Figure 2D, 2nd row (rga3Δ cells), 1st DIC image: this image has a much darker background than the rest of the images in the montage. Please clarify.

The darker background of the first image in the row of the *rga3Δ* panel was caused by the automated image acquisition (less light exposure at only this timepoint by accident) during the live-cell imaging. To ensure consistent background, we have adjusted the contrast of the indicated image (see supplemental Figure S1E in the revised manuscript). The original place in Figure 2 was replaced with representative kymograph graphs of 10.5-hour live-cell imaging of *rga6Δ* spores (two types of growth patterns: monopolar outgrowth and bipolar outgrowth) (new Figure 2E), followed by a plot graph showing the extension speed of the slow-growing end of the indicated spores (new Figure 2F)

3. Figures 2D-2G: please indicate in the figure legend how the monopolar and bipolar growth patterns were determined unambiguously based on DIC imaging only (these spores were not stained by Calcofluor).

Since the extension speed of the slow-growing end of germinating spores is quite low (see new Figure 2E), it was challenging to determine the pattern of growth polarity unless long time-lapse light microscopy was carried out (as shown in new Figure 2E). To present the data precisely, we directly measured the extension speed of the slow-growing end based on the kymograph graphs and plotted each data point using dot plots (as shown in new Figure 2F). The method has been stated in the corresponding figure legend and in the revised text. As shown in new Figure 2F, the extension speed of the slow-growing end of *rga6Δ* was noticeable whereas the extension speed of the slow-growing end of WT, *rga3Δ*, and *rga4Δ* was quite unnoticeable or zero. This result is consistent with the data obtained by Calcofluor-white staining (new Figure 2D), supporting the claim that the absence of Rga6 caused bipolar growth of germinating spores.

Regarding original Figure 2G (new Figure 2H), protrusion from the two distinct ends of the mother cell at the beginning of the second division was quite apparent. Therefore, based on the emergence of the protrusion, we determined the pattern of growth polarity for the second division.

4. Figure 3C: please indicate what percentage of cells (n = ?) displayed oscillation of CRIB-GFP between the fast- and slow-growing ends for each strain.

In the revised manuscript, the indicated information has been added. Since the representative kymograph graphs of WT, *rga3Δ*, and *rga4Δ* indicate monopolar staining of CRIB-GFP, the percentage for these cells indicates monopolar localization of CRIB-GFP. Note that the kymograph graph of *rga6Δ* represents bipolar staining of CRIB-GFP. Therefore, the percentage for *rga6Δ* indicates bipolar oscillation of CRIB-GFP. The note has been clearly stated in the revised figure legend.

5. Figure 3E, 2nd row (rga3Δ cells): adjust the brightness and contrast to ensure consistency between panels or choose a different cell.

Brightness and contrast have been adjusted.

Reviewer #2

This manuscript by Wei and colleagues examines the monopolar outgrowth of fission yeast spores and describes a role for the Cdc42 GTPase activating protein Rga6 in maintaining this mode of growth. The main findings of the paper are that rga6Δ spores often germinate in a bipolar manner and that this requires Rga6 GAP activity and localization to the cell cortex. Localization of Rga6 may involve binding to PI(4,5)P2, though this is indirectly shown. The conclusions of the manuscript are that "intrinsic properties of the spore plasma membrane may create spatial cues to specify the characteristic cortical localization of Rga6 and promote monopolar growth".

How monopolar vs. bipolar growth is regulated is a very interesting question. The data presented is generally clean and convincing (with a few comments below), but remains preliminary, leading to rather vague conclusions that "intrinsic properties of the spore plasma membrane may create spatial cues to specify the characteristic cortical localization of Rga6.". The true questions are whether there is anything special ("intrinsic properties") about the spore plasma membrane relative to vegetative

cells, and what these "intrinsic properties" may be. The role of the main difference between spores and vegetative cells, i-e the presence of a rigid spore wall, is also completely unexplored.

My comments below first relate to specific findings of the paper, and then to their placement in the context of previous work.

We are grateful for the positive comments. We have followed the suggestions to perform new experiments and analyses to strengthen our conclusions.

As suggested by the authors, a couple of observations suggest that spore outgrowth and vegetative growth are not regulated exactly in the same way. First, spore outgrowth is normally monopolar, whereas vegetative growth is bipolar. This is likely linked to the necessity to break the specific, rigid outer spore wall to initiate outgrowth (see Bonazzi et al, Dev Cell 2014; see below). Second, it is interesting that the one growing end of the germinating spore appears to grow faster than even the combined growth of both cell ends during mitotic growth. To my knowledge, this second observation is not directly linked to the monopolar mode of growth, as monopolar mutants do not generally show overall faster growth. The overall growth rate of germinating spores (adding the average values for the slow and fast-growing ends shown in Figure 3D) also seems rather constant between genotypes, with rga6Δ overall growth similar to that of WT or rga3Δ or rga4Δ spores, but with more equal growth between cell ends. This raises the question of why spore outgrowth should be faster than vegetative growth. Is this dependent on the size of the spore, the length of time since sporulation, or perhaps other characteristics?

We thank the reviewer for pointing out our interesting finding that spore outgrowth and vegetative growth are not regulated exactly.

1) We agree with the reviewer that breaking the rigid outer spore wall plays a crucial role in directing the monopolar growth of spores, as shown in Bonazzi et al, Dev Cell 2014. Findings in Bonazzi et al, Dev Cell 2014 have laid a solid foundation for further studying the growth of spores. We have emphasized these crucial findings in multiple places in the present work. For example, the Introduction includes the statement:

"It has been shown that the mechanical properties of the outer spore wall play a crucial role in dictating the spore outgrowth that also requires a polar cap composed of polarity factors, including Cdc42, Bud6, and Bgs4 (Bonazzi et al., 2014)."

It is possible that in addition to the rigid spore wall, other factors, as shown in the present work, may contribute to the establishment of monopolar outgrowth of spores.

2) The fast growth of outgrowing spores may not be directly linked to the monopolar mode of growth. Nonetheless, we found that the vegetative growth of the fast-growing end of *tea1Δ* cells, which generally grow in a monopolar manner (Glynn et al., 2001; Taheraly et al., 2020), was also faster than that of wild-type cells (new Figure 1D). This is consistent with the finding reported previously (Taheraly et al., 2020). Intriguingly, CRIB-3GFP staining at the cell end was stronger in vegetative *tea1Δ* cells than in wild-type cells, and CRIB-3GFP staining at the cell end was the strongest in wild-type outgrowing spores (see new Figure 1C). Therefore, it appears that the growth speed is correlated with the strength of CRIB-3GFP staining. This is consistent the finding that the growth rate of cell surface plays a crucial role in controlling the stability of Cdc42-GTP domains (Haupt et al., 2018). These results also support the model proposed previously that growing ends may compete for a pool of factors regulating cell growth (Taheraly et al., 2020). Consistently, as pointed out by the reviewer, although the combined growth rates of the two ends of outgrowing WT, *rga3Δ*, *rga4Δ*, and *rga6Δ* spores were quite similar, the speed of the fast-growing end of outgrowing *rga6Δ* spores was slightly slower than that of WT, *rga3Δ*, *rga4Δ*, spores. Note that *rga6Δ* spores underwent abnormal bipolar outgrowth (see new Figure 2).

Despite the differential regulation of the growth of germinating and vegetative cells, we agree that we still did not fully understand why spore outgrowth should be faster than vegetative growth.

Therefore, we further tested the question raised by the reviewer, i.e., *"Is this dependent on the size of the spore, the length of time since sporulation, or perhaps other characteristics?"*

To test the above question, we plotted the growth speed of the fast-growing end of outgrowing spores against the size and the outgrowth time since the start of germination, respectively. Pearson correlation analysis gave coefficient of 0.068 and 0.252, respectively, suggestive of almost no correlation (see Figure R1 below). This result suggests that the speed of spore outgrowth does not depend on the size and the outgrowth time since the start of germination. Since this result did not appear to help address the question *"why spore outgrowth should be faster than vegetative growth"*, *we did not add it to the revised manuscript.*

Taken together, we favour the model that the monopolar growth mode of spores may concentrate Cdc42-GTP at the growing end to promote efficient outgrowth.
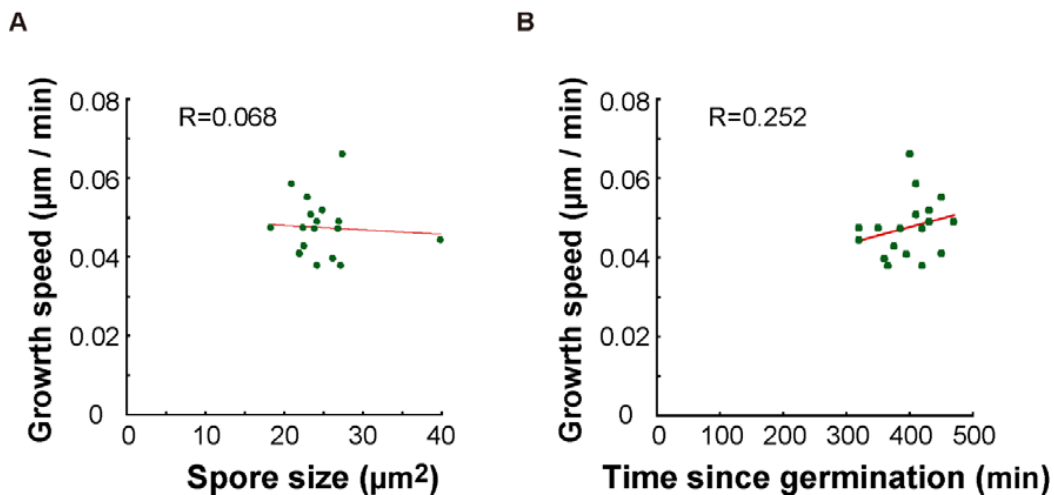

Figure R1: Plots of spore growth speed against spore size (A) and the time since germination (B). Pearson correlation analysis was carried out, and *R* indicates Pearson correlation coefficient.

Is the function of Rga6 specific to spore outgrowth?

The considerations above also raise the question of whether the bipolar phenotype of rga6Δ is specific to spore outgrowth, or whether it is a more general phenotype. Do rga6Δ cells generally shift growth towards bipolarity more easily? This can be tested for instance by measuring the proportion of bipolar rga6Δ (vegetative) cells, and by measuring the relative growth of the fast and slow-growing ends.

We have conducted the suggested experiments. Specifically, 1) quantify the vegetative cells displaying bipolar staining of CRIB-GFP and 2) measure the growth speed of the fast- and slow-growing ends of vegetative cells.

As shown in (supplemental Figure S2A), the percentage of wild-type and *rga6Δ* vegetative cells displaying bipolar CRIB-GFP was comparable. This result is consistent with the finding, reported previously (Revilla-Guarinos et al., 2016), that the absence of Rga6 only mildly affects the growth polarity of vegetative cells. In addition, the extension speed of the fast- and slow-growing ends of

7

wild-type and *rga6Δ* vegetative cells was comparable (supplemental Figure S2B). Therefore, the absence of Rga6 appear to play a minor role in regulating the growth polarity of vegetative cells. This result, together with the ones indicated below, support the conclusion that Rga6 plays a specific role in spore outgrowth.

1) Overexpression of Rga3 or Rga4 in *rga6Δ* spores did not rescue the phenotype of abnormal bipolar outgrowth (see new supplemental Figures S5A and S5B).

2) Depletion of *rga4* in *rga6Δ* spores did not appear to enhance the phenotype of abnormal bipolar outgrowth (see new supplemental Figures S5E-S5G).

Role of PI(4,5)P2:
> The possible role of PI(4,5)P2 is poorly defined. The observation that Opy1 overexpression leads to reduced Rga6 cortical localization may suggest that Rga6 binds PI(4,5)P2, but the competition may also be through other indirect ways. To support this point, in vitro work would be required. If Rga6 localization is indeed dictated by that of PI(4,5)P2, this then raises the question of how PI(4,5)P2 is distributed to cell sides, which is not addressed.

We agree with the reviewer that *in vitro* work is required to support the point that Rga6 binds PI(4,5)P2. Therefore, we first coated beads with PC and PI(4,5)P2, at different ratio, and then incubated the lipid-coated beads with recombinant proteins His-GFP-tagged Rga6 and His-GFP. We then assessed the localization of the recombinant proteins to the lipid-coated beads by confocal microscopy. The result clearly showed that His-GFP-Rga6, but not His-GFP, localized to lipid-coated beads carrying PI(4,5)P2 in a PI(4,5)P2-concentration dependent manner, suggesting that Rga6 binds PI(4,5)P2.

How PI(4,5)P2 is distributed to cell sides is an interesting question to pursue. Since the focus of this present work is to study the role of Cdc42 GAPs, particularly Rga6, in regulating spore outgrowth, we would pursue the question in a follow-up study.

Prior work from the Minc lab showed that spore outgrowth required breakage of the rigid spore wall (Bonazzi, Dev Cell 2014). Thus, one possible (likely) reason for the monopolar mode of spore outgrowth is that spore wall breakage is difficult. Since polarity site stabilization relies on positive feedback with growth upon spore wall breakage, it is easy to imagine that this first site is preferred and a second one less likely to initiate. Thus, it is very interesting that bipolar outgrowth happens more frequently in rga6Δ cells and begs the question of whether spore wall breakage occurs more easily in this background or mechanical feedback between the cell wall and polarity is altered. This is not considered at all in the manuscript.

We agree with the reviewer that the rigid spore wall plays a crucial role in dictating the monopolar outgrowth. Bonazzi, Dev Cell 2014 has presented solid data demonstrating the important function of the outer spore wall in regulating outgrowth and has laid a solid foundation for further studying spore growth.

As suggested by the reviewer, we examined the wall of wild-type and *rga6Δ* spores carefully by transmission electron microscopy (new Figures 3D and 3E). No noticeable difference of the spore wall between wild-type and *rga6Δ* spores was found. In addition, the average wall width of wild-type and *rga6Δ* spores was comparable (Figure 3E). Therefore, it is unlikely that the absence of Rga6 causes bipolar spore outgrowth through impairing the spore wall.

There is a rich prior literature, which has examined the role of protein (Cdc42 GTPase and regulators) dynamics using either S. pombe or S. cerevisiae yeast models in regulating monopolar vs bipolar

8

mode of growth (Lew, Bi, Goryachev, Martin, to cite just a few labs), but this literature is unfortunately not cited here.

We would like to thank the reviewer for pointing out this issue. In the revised manuscript, key research/review articles from the indicated groups have been added (8 from the Lew lab, 2 from the Bi lab, 3 from the Goryachev lab, 7 from the Martin lab, and 4 from the Minc lab).

Recent publication from the Martin lab (Gerganova, Science Advances 2021) showed that Rga4 side localization is due to bulk flows of membrane from the secretion site organized by Cdc42 activity. Rga6 is not very different from Rga4: both localize to cell sides and the localization of both proteins depends on a poly-basic stretch of amino-acids. It would be interesting and important to consider Rga6 localization in this context.

We agree with the reviewer that membrane flow is one of the main contributors functions to pattern the lateral localization of membrane-bound proteins, as reported by Professor Sophie Martin (Gerganova et al., 2021).

Despite similarity, the localization of Rga6 and Rga4 is not exactly the same. Although both Rga6 and Rga4 localizes to the lateral sides of the vegetative cell, the clusters formed by Rga4 and Rga6 on the lateral sides were found to rarely colocalize, as reported previously (Revilla-Guarinos et al., 2016). Therefore, the authors concluded that Rga4 and Rga6 may form different complexes on the lateral plasma membrane.

In the revised manuscript, we performed spinning disk live-cell microscopy again using optimum imaging conditions to examine the localization of Rga3, Rga4, and Rga6 in outgrowing spores. As illustrated in new Figure 5A in the diagrams, Rga3 localizes to the growing end of outgrowing spores, Rga4 localizes to only the two lateral sides of outgrowing spores (note that two large gaps were present on the cell cortex, at the two cell ends), and Rga6 decorates almost the entire cortical region except the extreme growing end of outgrowing spores (also see new supplemental Figure S2D). Hence, the distribution pattern of Rga4 and Rga6 is clearly different on the cortex of outgrowing spores.

Rga6 binds PI(4,5)P2 *in vitro* (new Figures 6D and 6E) and colocalizes with PI(4,5)P2, which is marked by Opy1-2mNeonGreen, on the cell cortex (new Figure 6A). In addition, overexpression of Opy1-2mNeonGreen delocalized Rga6 from the cell cortex (new Figures 6F and 6G). These results point towards the model that Rga6 localizes to the cell cortex in a PI(4,5)P2-dependent manner. Nonetheless, our data do not exclude the possibility of the contribution of membrane bulk flows to the characteristic localization of Rga6. We have cited the indicated work from Professor Sophie Martin and discuss the point brought up by the reviewer in the Discussion section.

Minor comments:
>
> p. 3: the reference to GEFs activating Cdc42 locally is perhaps not so clear for Gef1, which was proposed to act as a "global" GEF (see Tay et al, JCS 2018).

We thank the reviewer for pointing out the point. In the revised manuscript, we have cited the indicated work and changed the original text to ""Gef1 and Scd1 function as global and local Cdc42 GEFs, respectively".

sp. 5: What is lipid YE? Is this meant to be liquid YE?

9

The methods do not explain clearly how measurements were made. For example, in Figure 1C, the very peaked distribution is probably the result of aligning curves to the max of each profile rather than the geometric end of the cell (which the scheme suggests).

We have clearly stated how the measurements were done in the corresponding figure legends in the revised manuscript. The indicated data were measured and plotted by aligning curves to the geometric center of cell ends, as illustrated in the revised figures.

Figure 2B: The Calcofluor staining experiment is not all that clear. I do not understand how you still get staining of the growing cell ends and very prominent staining of the septa if Calcofluor was not present in the media for 6h. This suggests that there is substantial unbound Calcofluor in the media. Doing the same sort of analysis with labelled lectin may be a good alternative. Furthermore, in the enlarged examples shown, it looks like Calcofluor is still excluded from both WT and rga4Δ "non-growing" end. While the rga6Δ phenotype is clear, it is not all that clear what is counted as monopolar or bipolar in the other backgrounds.

We thank the reviewer for pointing out the issue. To ensure the reliability of the Calcofluor-white staining method, we carefully washed the dormant spores that had been stained with Calcofluor-white for 30 minutes and then cultured the stained spores in Calcofluor-free rich medium (YE) for the indicated time and collected the spores for confocal microscopic analysis. In the revised manuscript, we carried out rigorous experiments to test the reliability of using the Calcofluor-white staining to study spore germination/outgrowth (See new Figures S1C and S1D and Figure 2B). All Calcofluor-white staining experiments were then performed by following a consistent procedure.

1) We demonstrated, in the revised manuscript, that the Calcofluor-white staining method did not affect spore growth by performing microscopic analysis with stained and unstained spores in parallel (See new Figures S1C and S1D and Figure 2B). In fact, similar approach has been successfully used to monitor spore growth by other groups (Plante and Labbe, 2019; Plante et al., 2017).

2) By optimizing the staining procedure, we now showed clear and consistent staining. All Calcofluor-white staining was done at least three times for each type of spores. As shown in new Figures 2C and 2D, the data are consistent with the ones shown previously that Calcofluor-white marked one end of most of the WT, *rga3Δ*, and *rga4Δ* spores, but no end of most of the *rga6Δ* spores. These results unambiguously showed that WT, *rga3Δ*, and *rga4Δ* spores preferred to grow in a monopolar manner whereas *rga6Δ* spores preferred to grow in a bipolar manner.

In addition, to complement the Calcofluor-white staining method, we performed long time-lapse light microscopy (>10 hours) to directly observe spore growth and measured the average speed of the slow-growing of outgrowing spores. As shown in new Figures 2E and 2F, most of the WT, *rga3Δ*, and *rga4Δ* spores displayed quite slow growth or no growth of the slow-growing end. By contrast, *rga6Δ* spores displayed relatively fast growth of the slow-growing end. These results further support the conclusion that WT, *rga3Δ*, and *rga4Δ* spores grow in a monopolar fashion while *rga6Δ* spores grow in a bipolar fashion.

Figure 2D-2F: What the yellow arrowheads mark is not very clear. It is not the initiation of outgrowth, which started in earlier timepoints (as judged from drawing a line from the edges of the initial spore).

We thank the reviewer for pointing out the issue. We meant to mark the initiation of outgrowth. However, the arrowheads were added on the cells that had initiated outgrowth. We have corrected this in the revised manuscript and used the arrowheads to indicate the initiation of outgrowth. Note that the original Figures 2D and 2E become supplemental Figures 1E and 1F and that the original Figures 2F and 2G are now Figures 2G and 2H.

In Figure 3B (and generally Figure 3), it may be best to measure the actual CRIB intensity at each cell pole rather than provide a binary measure of presence/absence. Again, the rga6Δ phenotype is clear, but the statement that neither rga3Δ nor rga4Δ show phenotype may be overstated. They may show a weak intermediate phenotype.

Due to the dynamic nature of CRIB-GFP, the intensity of CRIB-GFP at cell ends was quite variable even within one population of cells. Nonetheless, it is straightforward to determine whether cell ends were marked by visible CRIB-GFP or not. Therefore, we feel that the binary measurements were helpful in determining the growth polarity and kept the figure in the revised manuscript.

Our data suggest that Rga3 and Rga4 play a very minor role in regulating spore outgrowth (see Figures 2C-2H). Nonetheless, to avoid overstatement, we have followed the suggestion to make statements, throughout the text, to avoid giving impression that Rga3 and Rga4 do not play a role in regulating spore outgrowth at all.

Figure 4A: The Rga4-2mN localization appears to be in a spore that grows in a bipolar manner. Is that usual? In a WT background, this should be a rare instance. Perhaps a more representative example could be chosen.

Few WT spores grew in a bipolar fashion (see Figures 2C and 2D). Since all cells showed in Figure 4A were snapshot images (acquisition at only one timepoint), it seems impossible to determine the growth polarity of the indicated spore.

As requested by reviewer 3, we performed imaging experiments again to improve image quality. The new data as shown are consistent with the ones shown previously. To illustrate clearly the localization of Rga3, Rga4 and Rga6, diagrams of the localization of the proteins were included at the bottom panel of the new Figure 5A.

Figures S3B: lanes are unlabelled.

Labels have been added. Note that the original Figure S3B is now new supplemental Figure S4A.

Reviewer #3

Wei et al. report on the role of the Cdc42 GAP Rga6 in promoting monopolar growth in germinating/outgrowing spores of fission yeast. The study combines standard approaches for yeast cell biology including microscopy, quantitative analysis and genetics. The authors show that Rga6D cells exhibit premature bipolar growth in germinating spores, with 2 tips emerging from the spore body in place of only 1 in WT. Localization of Active Cdc42 (at the growing tip) and rga6 (at the non-growing part of the cell) confirms that Rga6 may act by restricting Cdc42 activity to one growing tip in WT cells. By generating truncation alleles the authors finally suggest that this effect requires the proper cortical localization of Rga6 and the GAP domain of the protein. Overall, the MS is of correct quality (some aspects need improvements, see below), and the phenotype interesting given that there

are few studies of spore germination/outgrowth in Fungi. I list below some comments/critics for the authors to improve some aspects of the work:

We would like to thank the reviewer for the support and for the helpful suggestions.

General point: The microscopy images presented throughout the MS are somewhat below standard for Pombe, and in many the contrast is boosted making it hard to fully capture the claimed phenotypes. I think the authors should definitely improve many of these. This concerns especially CRIB images in Fig 1, Rga6/Crib images in Fig 4A and C, 5A and C.

Our lab is equipped with a PerkinElmer Ultraview spinning-disk confocal microscope carrying a Nikon Apochromat TIRF 100 x 1.49NA objective and a Hamamatsu C9100-23B EMCCD camera. The performance of the microscopic system has been consistently outstanding when we studied mitochondria (see (Rasul et al., 2021)) and microtubules (see (Shen et al., 2019; Zheng et al., 2020)). All microscopic images in the present work were acquired using the same microscope.

Most of the proteins presented in this study were expressed endogenously. Particularly, the expression level of Rga6 appeared to be quite low and the localization of Opy1 was both on the plasma membrane and the cytoplasm. CRIB was tagged with 1xGFP or 1xmCherry in the original figures, giving weak staining of cells. Given the nature of these proteins, we agree with the reviewer that microscopic observation was challenging. To improve imaging quality, we have optimized microscopic settings to acquire images and used CRIB-3xGFP or CRIB-3xmCherry, which were developed by Professor Sophie Marin (Vjestica et al., 2020), during the revision of the present work. We hope the images presented in the revised figures would be satisfactory.

All indicated figures have been improved and were replaced by new data in the revised manuscript. The conclusions from the new data are consistent. Specifically, new Figure 1 presents the data obtained by using CRIB-3GFP, instead of the original CRIB-GFP; new Figures 4A, 5A and 5C present the data acquired by using a higher laser power and a longer exposure time; new Figure 4C presents the data obtained by using CRIB-3mCherry, instead of the original CRIB-mCherry.

Fig 1: The much faster growth of outgrowing tips in spores together with the more pronounced Cdc42 activity is interesting. Is this effect specific to outgrowing spores or is it caused by some competition for polarity/growth material between the two tips, as documented in previous work (PMID : 33257499)? For example do vegetative monopolar cells (e.g. in tea1D mutants) grow as fast as outgrowing spores, do they also have brighter CRIB signals as well?

We would like to thank the reviewer for bring up this interesting point. We followed the suggestion to examine the vegetative growth of *tea1Δ* cells (see new data in Figure 1, A-D).

1) We confirmed that most of the *tea1Δ* cells grew in a monopolar fashion (new Figures 1A and 1B).
2) The speed of the fast-growing end of vegetative *tea1Δ* cells was significantly faster than that of vegetative WT cells but significantly slower than that of WT outgrowing spores (new Figure 1D).
3) The CRIB-3GFP intensity at the cell end was the highest in WT outgrowing spores, then in vegetative *tea1Δ* cells, and the lowest in vegetative WT cells (new Figure 1C).

Therefore, the data support the competition model as proposed previously (Taheraly et al., 2020).

A related point: In the ref PMID: 26960792 Rga6D vegetative cells are not reported to feature defects in polarity, but OE of Rga6 has a strong monopolar phenotype. This should be mentioned, as it kind

of support the general claim of the paper. Also the OE of Rga6 should also be tested in spores (they may not even polarize).

We would like to thank the reviewer for pointing out this important point.

1) We have cited the finding as the statement below in the revised manuscript.

"it has been reported that overexpression of Rga6 causes pronounced monopolar growth of vegetative cells (Revilla-Guarinos et al., 2016). This finding is consistent with the role of Rga6 in promoting monopolar outgrowth during germination."

2) We have also followed the suggestion to test the effect of Rga6 overexpression on spore outgrowth. As shown in new supplemental Figure S2, C-E, spores with Rga6 overexpressed were still able to undergo outgrowth. This is consistent with the localization of Rga6 that was over-expressed from the *cam1* promoter, i.e., cortical localization except the growing tip (new supplemental Figure S2D and S2E).

Fig 2 and Fig 6: While the Calcofluor assay makes a convincing case for the bipolar phenotype of Rga6D the panels D and F are not so convincing, as cells do not appear to really grow at their rounded end. This raises a concern on the use of Caclofluor, (This is a standard to affect CWs and here cells are treated for 6h!) which could affect spore CW properties and account for the pronounced role of Rga6 activity in promoting monopolar growth, given results from Ref PMID: 24636258. Since this is the central claim of the paper, I would be strict with a much better analysis of growth patterns in outgrowing spores of Rga6D without Calcofluor. I suggest either to (i) use lectins, which have less damaging effects on CWs, (ii) track fiducial marks precisely to compute growth at both ends, and/or (iii) quantify the size ratio btw daughter cells after division which should somehow reflect how much both tips have grown.

1) The reason of the no-growth impression of *rga6Δ* spores at the round end is due to the very slow speed of the round end (See new Figures 2E and 2F). In the revised manuscript, we moved the original Figures 2D and 2E to the supplemental Figures S1E and S1F and replaced with kymograph graphs of *rga6Δ* spores showing typical monopolar and bipolar outgrowth, respectively. Note that these unstained spores were observed for > 10 hours. Based on the trajectory/fiducial-mark of the round/slow-growing end, the round end grew in a quite slow but noticeable manner (the average speed of outgrowth, ~0.01 μm/min, for *rga6Δ* spores, i.e., 0.6 μm per hour). The average speed of the round end of WT, *rga3Δ*, and *rga4Δ* spores was unnoticeable or zero.

2) Calcofluor-white staining. We agreed with the reviewer that this is an important piece of data to support our claim. Therefore, we followed the suggestion to seek alternative methods. Unfortunately, as stated in the Result section (also see supplemental Figures S1A and S1B), Lectin staining is impossible for studying spore outgrowth. In general, it takes about 6 hours for the stained dormant spores to enter the stage of outgrowth but Lectin staining was stable less than 2 hours (supplemental Figures S1A and S1B).

Calcofluor-white staining has been used successfully to determine the growth stage of spores (Plante and Labbe, 2019; Plante et al., 2017). In the revised manuscript, we further carefully tested the effect of Calcofluor-white staining on spore growth. In fact, we did not culture spores in rich medium containing Calcofluor-white. Instead, we first stained dormant spores with Calcofluor-white staining for 30 minutes, and after washing, the stained spores were then cultured in Calcofluor-free rich medium for the indicated time (Figure 2A). "6 hours" indicated in the original Figure 2A and in the text was incorrect and should be "8 hours", in Calcofluor-free rich medium. We have clearly stated the method in the revised text.

13

We observed spore growth of Calcofluor-stained and unstained spores, which were cultured in Calcofluor-free rich medium, in parallel. As shown in new supplemental Figures S1C and S1D and Figures 2B, the quantification result of spore growth was comparable between the two populations, suggesting that pre-treatment by Calcofluor staining did not appear to affect spore growth. Note that both populations underwent outgrowth at 6 hours and with a long germ tube at 8 hours (new supplemental Figures S1C), which is consistent with the findings shown previously (Bonazzi et al., 2014; Plante et al., 2017). It could be that the special properties of the outer spore wall protect spores against the potential detrimental effects of Calcofluor-white.

Although the tailored procedure of Calcofluor-white staining has proven to be reliable for studying spore growth, we agreed with the reviewer that a complement method should also be considered. Therefore, we complemented all Calcofluor-white staining experiments by direct observation of spore growth by long time-lapse light microscopy (> 10 hours) (Figures 2E, 2F, 6I, S4E, S5D, and S5G) and measured the average growth speed of the round/slow-growing end to determine growth polarity (the fast-growing end is pronounced and measurements are not necessary in determining growth polarity). Consistently, only the absence of Rga6 caused pronounced growth of the round/slow-growing end (Figure 2F).

We feel that the two types of data unambiguously support our original claim that Rga6 plays a crucial role in dictating monopolar outgrowth of spores.

Figure 4B and 5B: It would be much clearer to trace directly the intensity of the signal around the full cell contour as done in Fig 4D and 6E.

We have followed that suggestion to perform the measurements, and the new results were consistent with the original ones.

Fig 5. The data for a role for PiP2 and Opy1 are not very convincing. First, the authors should provide much better co-localization images and quantifications btw the two signals. Second the localization of Rga6 in an Opy1D should be documented to test if PiP2 levels are really linked to Rga6 localization, and third the OE data need better images and quantification. Finally, the phenotype in term of monopolar vs bipolar growth for both OE of Opy1 and Opy1D should be provided.

1) As stated in the responses to point 1 above, the nature of Rga6 expression and Opy1 localization made it challenging to obtain crystal clear imaging data. Nonetheless, we improved the image quality by optimizing microscopic settings and hope the new data are acceptable (new Figures 6A and 6B). In addition, we performed reconstitution assays to test the affinity of recombinant proteins His-GFP-Rga6 and His-GFP (control) for lipid-coated microspheres (new Figures 6C-6E). This new data clearly showed that His-GFP-Rga6, but not His-GFP, bound PIP2-coated microspheres. We further reduced the synthesis of PI(4,5)P2 by compromising the function of Its3, which has been shown to be required for the synthesis of PI(4,5)P2, in the temperature sensitive strain *its3-1* at the restrictive temperature and tested the localization of Opy1 and Rga6. The data consistently showed that inhibiting synthesis of PI(4,5)P2 impaired the cortical localization of both Opy1 and Rga6 and also impaired outgrowth of spores (see supplemental Figure S3D).

2) We followed the suggestion to also test how the absence of Opy1 affects the cortical localization of Rga6 (see new Figures 6F and 6G). Interestingly, the absence of Opy1 did not appear to affect the cortical localization of Rga6 but it was consistent with the result shown previously that overexpression of Opy1 significantly impaired the localization of Rga6 to the cell cortex. Given the nature of the low endogenous expression of Rga6, it could be possible

14

that excessive PIP2 docking sites could be available for Rga6 regardless of the presence of Opy1. We would pursue this point in a follow-up study.

3) We have performed imaging again by using optimized microscopic settings. Measurements of the cortical localization of Rga6 clearly showed that overexpression of Opy1, but not the absence of Opy1, impaired the cortical localization of Rga6 (Figure 6G).

4) The growth polarity of the three types of spores were also determined, as suggested, by Calcofluor-staining and long time-lapse light microscopy. Consistent with the effect on the cortical localization of Rga6, overexpression of Opy1, but not the absence of the Opy1, caused a slightly but significantly increased percentage of spores displaying bipolar outgrowth, as shown by the Calcofluor-white staining data (supplemental Figures S3A and S3B; also compare with the *rga6Δ* data shown in new Figure 2D). Measurements using the long time-lapse microscopic movies showed that of the average speed of round/slow-growing ends of Opy1-overexpressing spores, but not the spores lacking Opy1, were also increased slightly but significantly (new Figure 6I).

<u>Minor:</u>
The word germination is used throughout, even in the title, but the authors are looking at outgrowth. Germination is normally used for the onset of exit from dormancy (~6-7h before most images shown in the paper).

We agree with the reviewer that "*germination*" is generally used for describing the onset of spores exiting from dormancy. However, "*germination*" was also used, in some studies (Plante and Labbe, 2019; Plante et al., 2017), to describe the whole growth of spores, including exiting dormancy, isotropic swelling, outgrowth, extension of the germ tube, and separation of the daughter cell from the mother spore. Despite this, we agree with the reviewer that we should be more careful in using the term *germination*. Therefore, in the revised manuscript, we used *outgrowth/outgrowing* in the statements where outgrowth was specifically referred and used *germination* in the statements where the whole growth of spores was referred.

CRIB domains have been shown to rapidly wander around during the first phase of isotropic growth in Pombe spores (PMID: 26441355). How does Rga6 localize during this first phase ? Is it stable at a given location, or does it oscillate in front of active Cdc42? This would be important to document, to test if Rga6 localization at the back of the cell may for instance come from landmarks inherited during sporulation, or if the system of polarity spontaneously auto-assemble.

We have followed the suggestion to observe the localization of Rga6-2mNeonGreen and CRIB-3mCherry (See the figure below). Note that the expression level of Rga6 was very low at 6 hours after dormant spores were cultured in rich medium and increased afterwards (i.e., most of the spores are at the isotropic growth stage or at the early stage of outgrowth) (see supplemental Figures 2C and 2D and Figure 3B). Since observation of Rga6-2mNeonGreen in spores undergoing outgrowth was already technically challenging (Figure 5A), it was expected to be more challenging to observe the localization of Rga6 in spores undergoing isotropic growth. For this, we refrained from adding the data below to the present work.
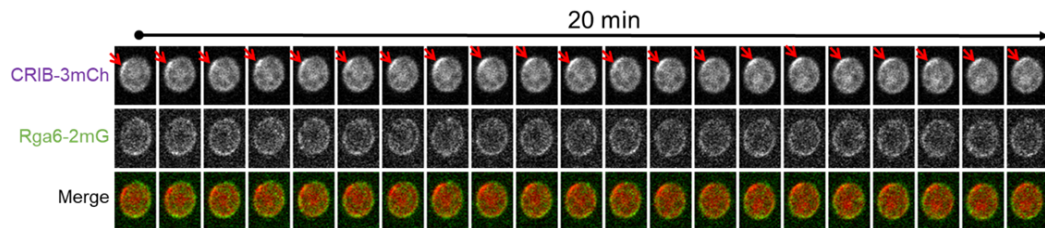
15

Figure R2: Z-slice images of a spore expressing Rga6-2mNeonGreen and CRIB-3mCherry. Red arrows mark the concentrated CRIB-3mCherry. Scale bar, 5 μm.

The last sentence of the introduction comes out of nowhere, this little part introducing the key results could be improved.

We have removed the last sentence in the original introduction and added statements to cover the key findings of this present work.

Some typos:
> P5: Lipid YE => liquid YE.
> P9: promoted => prompted

All have been corrected.

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October 6, 2022

RE: JCB Manuscript #202202064R

Prof. Chuanhai Fu
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Hefei, Anhui 230027
China

Dear Prof. Fu,

Thank you for submitting your revised manuscript entitled "The Cdc42 GAP Rga6 promotes monopolar outgrowth of spores." Your study has now been re-assessed by two of the original reviewers and both feel that their major concerns have been addressed. They do have a few remaining requests which we feel can be addressed through text revisions. But we would of course welcome any new data you can provide to address their comments if this can be done in a reasonable timeframe, particularly additional assays of the its3-1 mutant and the quantification of spore wall thickness. We would be happy to publish your paper in JCB pending these final revisions as well as any other changes necessary to meet our formatting guidelines (see details below).

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4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Please also indicate the acquisition and quantification methods for immunoblotting/western blots and type of membrane used.

5) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial please add a reference citation if possible.

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c. Temperature

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e. Fluorochromes

f. Camera make and model

g. Acquisition software

h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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Sincerely,

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Dan Simon, PhD
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Reviewer #2 (Comments to the Authors (Required)):

The authors have performed substantial work to address the comments from the reviewers and the paper is now much clearer. In particular, the interaction between Rga6 and PIP2 has been well strengthened and the assays used to measure mono-/bipolarity well explained and improved. I also appreciate the efforts made to investigate possible changes in the spore wall in rga6Δ by EM and agree with the authors that there does not appear to be any overt structural change prior to spore wall breakage. However, it is difficult to imagine how outgrowth happens without breakage of the spore wall. The spore wall must break twice in rga6Δ, for both poles to grow. It would have been interesting to document this by obtaining EM images of rga6Δ spores after outgrowth. To me how bipolarity of the spore outgrowth arises remains unclear, with open questions about whether rga6Δ affects mechanical feedback between cell (spore) wall and cell polarity.

On the question of whether the role of Rga6 is specific to spore outgrowth, the authors showed that vegetative bipolar cells are not more bipolar when rga6Δ. Because most cells are anyway bipolar, otherwise WT cells are not a very sensitive background to test this. It may have been interesting to test whether rga6Δ cells blocked in G1 or S phase, or combined with other weak monopolar mutants, shift more easily to bipolarity. I think it is still conceivable that rga6Δ vegetative cells are more prone to bipolar growth. Perhaps this can be mentioned.

The addition of the its3-1 mutant is interesting, but the 7h incubation at restrictive temperature is very long and can lead to many indirect effects. It would have been nice to use shorter times are restrictive temperature and also show the localization of Opy1 and Rga6 in its3-1 at permissive temperature. In Fig S3D, Opy1 localization looks already affected at time 0. Do these spores grow at permissive temperature?

Minor comments :

Fig 2D, 2H and others: typo in bipolar (bipoar)

Top of p. 9: please revise writing of "we created a temperature-sensitive mutant of its3" to "we used" or "we took opportunity of an existing temperature-sensitive mutant", or "we introduced a previously described temperature-sensitive mutant".

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

The authors have performed a significant number of revisions, which include novel experiments , analyses and text edits. These address many of my initial critics.

I just have one minor point regarding the new EM pictures presented in the novel Fig 3D. From the images presented ; it is hard to judge if spore walls (which are the black thine lines surrounding the main cell walls) are intact or not. The authors should consider mentioning this in the text, and maybe provide quantification of the thickness of the spore walls per se (and not of the whole cell walls).

We thank the editor and all reviewers for the kind support. We have followed the suggestions, provided during the first revision, to edit text and revise the supplementary Figure S3, D and E (i.e., the *its3-1* data). The point-by-point responses to the comments are shown below in blue.

Reviewer #2

The authors have performed substantial work to address the comments from the reviewers and the paper is now much clearer. In particular, the interaction between Rga6 and PIP2 has been well strengthened and the assays used to measure mono-/bipolarity well explained and improved. I also appreciate the efforts made to investigate possible changes in the spore wall in rga6Δ by EM and agree with the authors that there does not appear to be any overt structural change prior to spore wall breakage. However, it is difficult to imagine how outgrowth happens without breakage of the spore wall. The spore wall must break twice in rga6Δ, for both poles to grow. It would have been interesting to document this by obtaining EM images of rga6Δ spores after outgrowth. To me how bipolarity of the spore outgrowth arises remains unclear, with open questions about whether rga6Δ affects mechanical feedback between cell (spore) wall and cell polarity.

We agree with the reviewer that how the bipolar outgrowth of *rga6Δ* spores arises is an interesting question to be further studied. We will collaborate with biophysicists to employ a combination of cell biological and computational approaches to address the question. The statements below have been added to the Discussion section.

"Despite the specific function of Rga6 in promoting monopolar spore outgrowth, how the bipolar outgrowth arises in *rga6Δ* spores is unclear. Whether the absence of Rga6 affects the mechanical feedback between the spore wall and cell polarity remains to be further tested."

On the question of whether the role of Rga6 is specific to spore outgrowth, the authors showed that vegetative bipolar cells are not more bipolar when rga6Δ. Because most cells are anyway bipolar, otherwise WT cells are not a very sensitive background to test this. It may have been interesting to test whether rga6Δ cells blocked in G1 or S phase, or combined with other weak monopolar mutants, shift more easily to bipolarity. I think it is still conceivable that rga6Δ vegetative cells are more prone to bipolar growth. Perhaps this can be mentioned.

We thank the reviewer for the suggestions. Since the focus of the present work is the role of Rga6 in spore outgrowth, we will further test whether the absence of Rga6 affects the cell cycle of vegetative cells in follow-up studies using the suggested approaches. Note that the absence of Rga6, Rga4, or Rga3 did not appear to affect the growth of spores (Fig. S1, E and F). Therefore, it is unlikely that *rga6Δ* cells were blocked at G1 or S phases.

Whether the absence of Rga6 affects the cell polarity of vegetative cells has been carefully tested in the previous work (Revilla-Guarinos et al., 2016), revealing that the absence of Rga6 only mildly affects the growth polarity of vegetative cells. We also confirmed this finding (Fig. S2, A and B). Hence, the statements below were made in the Result section.

"No significant difference was found between wild-type and *rga6Δ* vegetative cells (Fig. S2, A and B), suggesting that Rga6 may play a very minor role in regulating the polarized growth of vegetative cells. Consistently, it has been reported that the absence of Rga6 only mildly affects the growth polarity of vegetative cells (Revilla-Guarinos et al., 2016)."

The addition of the its3-1 mutant is interesting, but the 7h incubation at restrictive temperature is very long and can lead to many indirect effects. It would have been nice to use shorter times are restrictive temperature and also show the localization of Opy1 and Rga6 in its3-1 at permissive temperature. In Fig S3D, Opy1 localization looks already affected at time 0. Do these spores grow at permissive temperature?

1) We have revised the original Fig. S3 E by performing the suggested experiments (see new Fig. S3, D and E). The new data were consistent, showing that the malfunction of Its3 impaired the localization of Opy1 and Rga6 to the spore plasma membrane.

2) As shown in new supplementary Figure S3, E, the *its3-1* spores were able to germinate at permissive temperature.

Minor comments :

Fig 2D, 2H and others: typo in bipolar (bipoar)

Corrected.

Top of p. 9: please revise writing of "we created a temperature-sensitive mutant of its3" to "we used" or "we took opportunity of an existing temperature-sensitive mutant", or "we introduced a previously described temperature-sensitive mutant".

We have rephased the statement as below.

"Therefore, we took opportunity of an existing temperature-sensitive mutant of its3"

Reviewer #3

The authors have performed a significant number of revisions, which include novel experiments, analyses and text edits. These address many of my initial critics.

I just have one minor point regarding the new EM pictures presented in the novel Fig 3D. From the images presented ; it is hard to judge if spore walls (which are the black thine lines surrounding the main cell walls) are intact or not. The authors should consider mentioning this in the text, and maybe provide quantification of the thickness of the spore walls per se (and not of the whole cell walls).

We thank the reviewer for pointing out the issue. We agree that it was challenging to analyze the outer spore wall because the electron-dense structures at the outmost layer of the spore wall were amorphous. Therefore, we refrained from measuring the thickness of the outer spore wall. Instead, the whole spore wall was analyzed. We have followed the suggestion to add the statements below in the Result section.

"We noticed that thin electron-dense structures, presumably the outer spore wall, were present at the outmost layer of the WT and *rga6Δ* spores (Fig. 3D). However, the thin electron-dense structures were amorphous, making it challenging to quantify the thickness of the outer spore wall. Therefore, we sought to examine the spore wall carefully."