Cdc42 prevents precocious Rho1 activation during cytokinesis in a Pak1-dependent manner

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Submission to Review Commons: 17 June 2022
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Reviewer 1

Evidence, reproducibility and clarity

This manuscript focuses on the role of Cdc42 in Rho1 activation during fission yeast cytokinesis. The primary finding is that active Cdc42 and its downstream effector Pak1 prevents accumulation of active Rho1 and the synthesis of cell wall material, at early stages of cytokinesis and despite the local recruitment of the Rho1 GEF Rgf1. The data supporting these conclusions are reasonably sound.

Additional experiments are presented to suggest that Cdc42 and Pak1 negatively regulate Rgf1, this conclusion is not as strongly supported (though it may be true)

These study relies on a newly described probe for active Rho1. However this probe is not sufficiently well validated.

Overall the manuscript was not assembled with sufficient care and rigor, these deficits could be readily corrected.

The major point of the paper is that Cdc42 and Pak1 negatively regulate Rho1 activation. However, during late cytokinesis, active Cdc42 and active Rho1 co-exist at the division site. Thus, Cdc42 activation induces a delay in Rho1 activation, but how this delay is overcome is not investigated or even discussed. Indeed, while the delay is shown the transience of this inhibition is not explicitly mentioned. At a minimum, the authors should highlight this point for the readers.

Specific points

1. - RBD probe
This probe is central to this manuscript. However, there is insufficient validation of its target. Figure 1 shows the localization and its independence of Rho2. The authors should provide direct evidence that it recognizes Rho1 (for example using a repressible promotor or an anchor away approach).

At various places in the manuscript the authors refer to this probe as "Rho-probe", RBD-probe, RBD, RBD-(mNG or tdTomato). On page 11 the authors state, "As per our observations, we refer to the Rho-probe signal at the division site as active Rho1 from here onwards." Yet, in the very next paragraph they refer to the localization of the "Rho probe".
This is also an issue with the figures. For example, in figures 4B,C ; 5B,C; 6B; 7B,C the

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figures are titled either "Rho1 activation at division site", "Rho1-probe at division site"; "Rho1-probe appearance at division site" ; "Rho1-probe in non-constricting rings".
In fig 3, RBD-nNG is quantified in a graph entitled "localizaton [sic] of Rho1-GEFs at division site"

In all figures but two, 5c and 7c, the authors quantify Rho1 activation by the presence or absence of the probe, rather than a quantitative measure or the extent of recruitment of the probe. This could be analyzed my quantitatively.

2. - Regulation of Rgf1 by Cdc42 and Pak1.
The results shown in figure 8 show that "early Rho1 activation in gef1 mutants is not Rgf3-dependent". Figure 9 establishes "loss of rgf1 prevents premature Rho1 activation in gef1? cells restoring it to normal in late anaphase (Fig.9A, B)." This finding indicates that Rgf1, but not Rgf3, is required for Rho1 premature activation. This finding doesn't rule out the possibility that Cdc42 and Pak1 might be required to turn off RhoGAPs to allow active Rho1 to accumulate. This analysis concludes with this unclear and ungrammatical sentence, "While we were unable to assess the Rho-probe in the rgf1? rgf2? double mutants due to its lethality [sic; is the Rho probe lethal?], our observations suggest that apart from Rgf1 early Rho1 activation in gef1? cells is either due to activation of Rgf2 or due to inhibition of Rga5." The conclusion that this regulation is due to control of Rgf1 should be toned down. E.g. from the abstract: "We provide functional and genetic evidence which indicates that Pak1 regulates Rho1 activation likely via the regulation of its GEF Rgf1."

Referees cross-commenting

I think reviews are appropriate and speak for themselves.

Significance

This manuscript ties together several recent papers from the author's lab on the control of Cdc42 activation during cytokinesis and older papers on the role of Rho1 in Bgs1 activation. It provides missing information into the temporal regulation of septum assembly.

The authors make a point of the similarities of fission yeast cytokinesis to animal cell cytokinesis. Indeed the second sentence reads, "The fission yeast model system divides via an actomyosin-based contractile ring, which is assembled in the medial region of the cell, as in animal cells (Balasubramanian et al., 2004; Pollard, 2010).". However, the authors fail to point out the many differences between yeast and animal cell cytokinesis until the last paragraph of the discussion. If the authors want to include the similarities in the introduction, they should also include the differences. For example, ring assembly is independent of Rho1 activation in fission yeast, but dependent on RhoA activation in animal cells.

This work will be of interest to biologists working on yeast cell division. To a lesser extent it will be of interest to biologists interested in cytokinesis and coordination of distinct GTPase pathways.

Additional points

1. - The text is overly wordy and needs extensive revision. Many of the experiments could be explained more clearly and with somewhat less genetic jargon. The introduction has quite a bit of extraneous information and lacks relevant facts, such as the function of Bgs1, which is central to the results.

2. - page 4 "GEFs promote GTP binding, thus keeping the GTPase active while the GAPs increase GTP hydrolysis, thus promoting GTPase inactivation." GEFs promote GTP binding, but they do not keep the GTPase active (an inhibitor of a RhoGAP would do that), they activate the GTPases.

3. - The current literature on animal cell cytokinesis indicates little direct role in cytokinesis, rather than the author's statement, "In larger eukaryotes, the role of Cdc42 activation has been reported mostly in meiotic division events such as polar body extrusion in oocytes, but not much is known about its role in cytokinesis in somatic cell division (Drechsel et al., 1997; Na and Zernicka-Goetz, 2006)." See for example, PMID 10898977, 10871280 which indicate Cdc42 does not play a major role during cytokinesis in at least a few systems where it has been analyzed.

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Reviewer 2

Evidence, reproducibility and clarity

In many fungal cells, including fission yeast, the deposition of a new cell wall (a septum) between daughter cells is essential for cytokinesis. Cell wall synthases are trafficked to and activated at the division site, and dysregulated trafficking and/or synthase activation can lead to cytokinetic defects.

In this study, the authors use fluorescent probes for Cdc42 and Rho1 activity and live-cell imaging to investigate the timing and regulation of Rho1 activity in fission yeast, and specifically, the role of Cdc42 in regulating Rho1. Summary of the proposed model: Gef1 -> active Cdc42 -> Pak1 --| Rgf1 -> active Rho1 -> septum formation

Major comments

1. As far as I can gather from the authors' description in the manuscript and quick literature search, this will be the first publication in S. pombe utilizing the HR1-C2 domain of Pkc2p as fluorescent probe for active Rho1 (RBD-mNG). While a comparable domain of S. cerevisiae Pkc1p (not "pck2" as referenced by the authors in Page 25) has been used for similar purposes, given the importance of this probe and the precedent it sets in the S. pombe literature, it is imperative that proper tests are performed to validate that its localization reflects activity of Rho1 and nothing else (such as membrane binding of the C2 domain or transcriptional regulation of the pkc2 promoter). Such tests should also be independent of the hypotheses central to the current study (i.e., effects of Gef1, Pak1, Rgf1/2 on the timing of RBD-mNG localization). Can the authors provide data to address this point? Examples include, but not limited to, rho1 mutants, expression of constitutively active Rho1, or temporary expression of dominant-negative Rho1.

2. Related, M&M does not provide sufficient details about the amino-acid positions corresponding to the "RBD" domain of Pkc2, thus precluding readers from reproducing the experiments. This needs to be clarified.

3. In Figure 1B, RBD1-mNG localizes clearly to the medial region of rho2? cells when the Rlc1-tdTomato ring has not formed. Does this mean that Rho2 has a major role in forming the contractile ring that is independent of Rho1 activation? On this other hand, however, data in Fig. S2BC suggest that RBD-mNG does not localize to the medial region in rho2? cells until Rlc1-tdTomato ring forms (the timing of which seems normal). This discrepancy needs to be addressed.

4. Given the nature of RBD-mNG localization, it seems unavoidable to have some level of arbitrariness in measuring the onset of its localization at the division site. It would be advisable for the authors to be specific in M&M about how they defined the onset of localization, i.e., whether it was based on universal threshold in signal intensity, ratio, etc. or on manual curation (ideally double-blind).

Minor comments

1. Throughout the manuscript, there are quite a few places where inconsistencies in genetic nomenclature can cause confusion to readers. Below are some examples. Figs. 6B, 7B, 10B: pak1(-ts), shk1, and orb2-34 (including faint labels under category marks in 6B). Fig. 9B (gef1+ rgf1?) vs 9C (rgf1?). Wild-type alleles are implicit in some figures, while explicit in others.

2. The first hypothesis (Fig. 1C) is that the AMR might regulate Rho1 activation. The ring is disrupted with LatA, but Rho remains active. They cite this as evidence that the AMR does not activate Rho1, but were the cells treated before or after the rings formed? If before, then the experiment demonstrates what the authors claim, but if after, it only shows that the AMR is not essential to maintain Rho activity.

3. Page 8: "Time-lapse imaging of cells simultaneously expressing CRIB-3xGFP and RBD- tdTomato [...] while Rho1 is activated ~20 minutes after SPB duplication (Fig. 2B)." This appears to refer to Fig. 2C.

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4. Page 9: "[...] Rgf1 and Rgf3 localize as early as the time of ring assembly at an average SPB distance of 4-5 μm (Fig. 3D)." This sentence is confusing. How was the average calculated over the earliest ring assembly in non-time-lapse data? Fig. 3DE show distances between SPBs as short as 2.5 μm, not 4-5 μm, and average of ~8 μm for all cells at different stages of mitosis. This confusion needs to be clarified.

5. Fig. 5. Both the intensity and onset of RBD-mNG localization were affected by cdc42g12v expression. These two may form a causative relationship: reduced overall RBD signal may cause failed detection of early RBD localization. Can the authors compare cells with similar mean RBD-mNG signal intensities (Fig. 5B) and confirm that the timing of appearance at the division site is still delayed in gef1+ cdc42g12v relative to gef1+ empty?

Referees cross-commenting

I find all reviewer comments fair and have nothing specific to add.

Significance

The mechanisms governing septum formation during cytokinesis represent a key regulatory step in cytokinesis. Prior work showed that the Rho GTPases Cdc42 and Rho1 together control the timing of septum formation in S. pombe, and in S. cerevisiae, similar antagonistic regulation between Cdc42 and Rho1 has been reported previously (Atkins et al., J. Cell Biol. 2013 202: 231-240; Onishi et al., J Cell Biol. 2013 202: 311-329), but the precise molecular mechanisms remained unclear.

This is an important study that addresses the interplay between two major Rho-type small GTPases involved in cell division of many eukaryotic cells, and highlights their roles outside of the regulation of contractile ring. However, there are some issues that need to be addressed prior to publication, as listed above.

Keywords for the reviewer's field of expertise: S. pombe, S. cerevisiae, cytokinesis, Rho1, Cdc42, septum, MEN/SIN, genetics, cell biology, biochemistry.

Reviewer 3

Evidence, reproducibility and clarity

Onwubiko et al., present a clear and well written manuscript detailing the mechanistic understanding of how Rho1 is activated in a timely manner to ensure cytokinesis occurs in a scheduled manner at the end of telophase. Using fission yeast as a model system, and with the development of a novel Rho1 biosensor, they implicate a series of GTPases, exchange factors, GTPase activating proteins and kinases acting downstream of Cdc42 in the timely activation of Rho1. Specifically, they find that Cdc42 prevents premature Rho1 activation in early anaphase in a manner requiring the kinase Pak1. They observe that the Rho1 activators Rgf1 and Rgf3 localise to the division site in early anaphase, but Rho1 doesn't get activated until late anaphase, suggesting that control mechanisms ensure that these GEFs are held inactive, or that RhoGAP activity is able to balance this activation in early anaphase. This suppression of Rho1 activity in early anaphase requires Cdc42 and Pak1 and implicate (by omission) Rgf1, rather than Rgf3, is the relevant GEF.

I liked this manuscript, it was clearly written the experimental progression was logical and the data were easy to interpret from the figures. The conclusions were precise, believable and not overstated. The manuscript provides novel observations and through good use of a series of rescues/mutants, illuminates a pathway that is held in check by Cdc42 to ensure timely Rho1 activation. The novel Rho1 probe is exciting and shows well differently regulated pools of active Rho1 at the division site and the growing tips. I thought the co- imaging/measurement of ring placement and SBP duplication allowed a really clear understanding of the kinetics during this rapid phase of the cell cycle. A critique of the study is that the the mechanism by which Cdc42 controls Pak1, and by which Pak1 controls Rgf1/Rgf2 is left unclear. I guess there could always be a molecular expansion of these points (e.g., how does Cdc42 control Pak1; how does Pak1 control Rgf1; how is Rgf activity restricted when localised), but I think that would only enhance, rather than change, the level of detail of the paper's message. I think the paper's current conclusions

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stand on their own, the data is clear and believable, the experiments are well performed. There are a number of observations in the paper that are left open for future studies, and I think this is a positive (e.g., any separable role of Rgf1/Rgf2 and how Rga5 integrates into this pathway. As such, I am tempted to recommend accept with only minor amendments as outlined below.

1. P8 P15: should the call out be to Fig 2C, rather than 2B.
2. P14 L17: should it be 'gef1+ rgf3-', not 'gef1+, rgf3+'
3. Structure wise, I thought the section on Rga5 didn't really fit well on P16; it seemed sandwiched between two sections on GEFs. Is there a more appropriate place to place these data - perhaps between the paragraph breaks on P17? Related to this data, the conclusion on P16 suggests 'other' regulators of RhoGAP activity act to repress Rho1 function. Would 'additional' regulators of RhoGAP activity be more appropriate as there is some function contributed by Rga5?
4. In Fig 10b, you haven't defined orb2-34. Is it the rgf1-delete?
5. I find the sentence at the top of P18: 'Rho1 activation in pak1+ rgf1+....at 25oC and 35.5oc occurred at longer and similar SBP distances' quite hard to interpret. Could you perhaps expand it to make your message clearer?

Significance

I think the advance here is a genetic understanding of control mechanisms that order the exit from mitosis. The interplay between numerous GTPases and kinases must ensure a timely and ordered progression through M-exit, but it is often unclear how these activities are coordinated. A strength of the yeast system is that dependencies can be clearly visualised and the authors do a good job here to order the enzymatic activities needed to activate Rho1 in a timely manner for cytokinesis at the end of telophase.

I think this manuscript will be of interest to those interested in the cell biology of mitotic exit, the interplay between kinases and GTPases and those interested in the systems/netwoek biology of these processes. The description of a new Rho1 biosensor is an excellent tool for the community.

I am a cell biologist (mammalian) with interests in M-exit programmes that ensure a timely and ordered reestablishment of interphase architecture.

Author response to reviewers' comments

We thank the reviewer for their comments. We are encouraged that the reviewers found our research “important study that addresses the interplay between two major Rho-type small GTPases involved in cell division” and “of interest to those interested in the cell biology of mitotic exit”. We agree with the comments raised by the reviewers and have provided new data as per their recommendation. We have also made changes to the text and format of the paper. We feel that with these changes the manuscript is stronger and we thank the reviewers for their suggestions. Below we provide a detailed response to the reviewers’ comments.

Reviewer #1

This manuscript focuses on the role of Cdc42 in Rho1 activation during fission yeast cytokinesis. The primary finding is that active Cdc42 and its downstream effector Pak1 prevents accumulation of active Rho1 and the synthesis of cell wall material, at early stages of cytokinesis and despite the local recruitment of the Rho1 GEF Rgf1. The data supporting these conclusions are reasonably sound.

Additional experiments are presented to suggest that Cdc42 and Pak1 negatively regulate Rgf1, this conclusion is not as strongly supported (though it may be true)

These study relies on a newly described probe for active Rho1. However this probe is not sufficiently well validated.

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Overall the manuscript was not assembled with sufficient care and rigor, these deficits could be readily corrected.

The major point of the paper is that Cdc42 and Pak1 negatively regulate Rho1 activation. However, during late cytokinesis, active Cdc42 and active Rho1 co-exist at the division site. Thus, Cdc42 activation induces a delay in Rho1 activation, but how this delay is overcome is not investigated or even discussed. Indeed, while the delay is shown the transience of this inhibition is not explicitly mentioned. At a minimum, the authors should highlight this point for the readers.

We are encouraged by the fact that the reviewer found our data “reasonably sound”. We agree that this manuscript does not provide the molecular details of how Cdc42 inhibits Rho1 activation. Our genetic data suggests that this is likely mediated by multiple pathways possibly involving the regulation of the Rho regulators Rgf1, Rgf2 and Rga5. We are currently investigating the molecular details of this regulation and hope to report it in another manuscript.

Our data shows that while Cdc42 inhibits Rho1, the SIN pathway is essential for Rho1 activation regardless of the presence of Cdc42. While Cdc42 is activated at the division site as the ring completes assembly, the SIN pathway is activated immediately prior to ring constriction similar to that of Rho1 activation. It is possible that once the SIN is activated at the division site, it overcomes Cdc42-mediated Rho1 inhibition. We have highlighted this in the discussion section of this manuscript and are currently investigating the molecular details of this regulation.

Specific points
1 - RBD probe
This probe is central to this manuscript. However, there is insufficient validation of its target. Figure 1 shows the localization and its independence of Rho2. The authors should provide direct evidence that it recognizes Rho1 (for example using a repressible promotor or an anchor away approach).

We thank the reviewers for their comments on the RBD probe. We have now provided validation for the RBD-probe. We have used *rho1* temperature-sensitive and switch-off mutants to show loss of RBD-probe localization in these mutants. This data is provided I the revised manuscript in Fig1 and Supplementary fig. S1.

At various places in the manuscript the authors refer to this probe as "Rho-probe", RBD-probe, RBD, RBD-(mNG or tdTomato). On page 11 the authors state, "As per our observations, we refer to the Rho-probe signal at the division site as active Rho1 from here onwards." Yet, in the very next paragraph they refer to the localization of the "Rho probe".
This is also an issue with the figures. For example, in figures 4B,C ; 5B,C; 6B; 7B,C the figures are titled either "Rho1 activation at division site", "Rho1-probe at division site"; "Rho1-probe appearance at division site" ; "Rho1-probe in non-constricting rings".

We agree that these multiple terms to describe the probe is confusing. We have restricted the terms to either “RBD-mNG” or “RBD-tdTomato” when reporting the data and use “Rho-probe” for descriptive purposes.

In fig 3, RBD-nNG is quantified in a graph entitled "localizaton [sic] of Rho1-GEFs at division site"

We thank our reviewers for identifying this error in our labeling of the graph in Fig. 3E. This figure now reads “Localization of Rgf1, Rgf3, active Rho1 at the division site”

In all figures but two, 5c and 7c, the authors quantify Rho1 activation by the presence or absence of the probe, rather than a quantitative measure or the extent of recruitment of the probe. This could be analyzed my quantitatively.

We appreciate this comment and provide this response in order to clarify our reasoning for presenting this data. We quantified the intensities of RBD-mNG or RBD-tdTomato where ever relevant to the question we are addressing for each experiment performed. Where we look at Rho1

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activation at the division site with respect to SPB distances, we are reporting the differences in the timing of Rho activation with respect to mitotic progression. However, in Figures 5c and 7c, and now also Fig 1 of the revised manuscript, we quantified the intensities of the probe as this indicated the changes in overall active Rho1 levels under our experimental conditions. We have added in the text for earlier experiments where we do not report intensity measurements for the active Rho probe that we do not observe any differences in the intensity levels.
2 - Regulation of Rgf1 by Cdc42 and Pak1.
The results shown in figure 8 show that "early Rho1 activation in gef1 mutants is not Rgf3-dependent". Figure 9 establishes "loss of rgf1 prevents premature Rho1 activation in gef1Δ cells restoring it to normal in late anaphase (Fig.9A, B)." This finding indicates that Rgf1, but not Rgf3, is required for Rho1 premature activation. This finding doesn't rule out the possibility that Cdc42 and Pak1 might be required to turn off RhoGAPs to allow active Rho1 to accumulate.
This analysis concludes with this unclear and ungrammatical sentence, "While we were unable to assess the Rho-probe in the rgf1Δ rgf2Δ double mutants due to its lethality [sic; is the Rho probe lethal?], our observations suggest that apart from Rgf1 early Rho1 activation in gef1Δ cells is either due to activation of Rgf2 or due to inhibition of Rga5."

We thank you for your insight and agree with these remarks. We could not investigate Rho1 activation in *rgf1Δ rgf2Δ* double mutants since the double mutants are inviable. We have re-worded the sentence to reflect our findings appropriately.

The conclusion that this regulation is due to control of Rgf1 should be toned down. E.g. from the abstract: "We provide functional and genetic evidence which indicates that Pak1 regulates Rho1 activation likely via the regulation of its GEF Rgf1."

We have now removed this statement from the abstract. We have also clarified in the discussion that the molecular details of how Cdc42 inhibits Rho1 is not known and needs to be investigated. While our data suggests that the regulator Rgf1 and Rga5 may be involved in the process the details are unclear and we are currently investigating this regulation.

SECTION B - Significance

This manuscript ties together several recent papers from the author's lab on the control of Cdc42 activation during cytokinesis and older papers on the role of Rho1 in Bgs1 activation. It provides missing information into the temporal regulation of septum assembly.

The authors make a point of the similarities of fission yeast cytokinesis to animal cell cytokinesis. Indeed the second sentence reads, "The fission yeast model system divides via an actomyosin-based contractile ring, which is assembled in the medial region of the cell, as in animal cells (Balasubramanian et al., 2004; Pollard, 2010).". However, the authors fail to point out the many differences between yeast and animal cell cytokinesis until the last paragraph of the discussion. If the authors want to include the similarities in the introduction, they should also include the differences. For example, ring assembly is independent of Rho1 activation in fission yeast, but dependent on RhoA activation in animal cells.

We thank the reviewer for pointing out this deficiency in our writing. We have now amended the introduction to highlight the differences between Rho1 activity in fission yeast and animal cells during cytokinesis. We have added the following text to the Introduction section.
"The animal Rho1 homolog RhoA is required for ring formation and is essential for cytokinesis (Basant and Glotzer, 2018). While in yeast, Rho1 is essential for septum formation, the current literature suggests that it is dispensable for ring formation (Onishi et al., 2013; Yoshida, 2009). In fission yeast where both the actomyosin ring and the septum have important roles in the proper coordination of cytokinesis, Rho1 has no reported roles in ring formation but is essential for septation (Balasubramanian et al., 2004)."

This work will be of interest to biologists working on yeast cell division. To a lesser extent it will be of interest to biologists interested in cytokinesis and coordination of distinct GTPase pathways.

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Additional points

1 - The text is overly wordy and needs extensive revision. Many of the experiments could be explained more clearly and with somewhat less genetic jargon. The introduction has quite a bit of extraneous information and lacks relevant facts, such as the function of Bgs1, which is central to the results.

We have now modified the text to remove unnecessary genetic jargon. We have also provided additional text to describe the role of Bgs1 in the Introduction.

2 - page 4 "GEFs promote GTP binding, thus keeping the GTPase active while the GAPs increase GTP hydrolysis, thus promoting GTPase inactivation." GEFs promote GTP binding, but they do not keep the GTPase active (an inhibitor of a RhoGAP would do that), they activate the GTPases.

We thank the reviewers for highlighting this error. We have corrected this sentence, which now reads “GEFs promote GTP exchange to activate the GTPase, while the GAPs increase GTP hydrolysis to promote GTPase inactivation.”

3 - The current literature on animal cell cytokinesis indicates little direct role in cytokinesis, rather than the author's statement, "In larger eukaryotes, the role of Cdc42 activation has been reported mostly in meiotic division events such as polar body extrusion in oocytes, but not much is known about its role in cytokinesis in somatic cell division (Drechsel et al., 1997; Na and Zernicka-Goetz, 2006)."
See for example, PMID 10898977, 10871280 which indicate Cdc42 does not play a major role during cytokinesis in at least a few systems where it has been analyzed.

We thank our reviewer for this observation and agree that this statement can be expanded to further explain the role of Cdc42 in animal cytokinesis. The paragraph has been re-written as follows-Pg5 - “In animal cells, the direct role of Cdc42 in cytokinesis remains indefinite. In Xenopus embryos and mouse fibroblasts for example, constitutively active Cdc42 impairs cytokinesis completion (Drechsel et al., 1997). However, in other cases such as in mouse embryonic stem cells, Cdc42 was only critical for development but not cytokinesis (Chen et al., 2000). RNA interference in animal cells demonstrate that that while RhoA is required for cytokinesis, Cdc42 is not required for this process (Jantsch- Plunger et al., 2000). Cdc42 also promotes spindle positioning and polar body extrusion in mouse oocytes, but it is not known whether its localization at these spindles affects RhoA (Na and Zernicka-Goetz, 2006). Thus, the role of Cdc42 in the cytokinetic process may be cell-type specific, and these data highlight the importance for more investigation to elucidate Cdc42 regulation in dividing cells (Jordan and Canman, 2012).”

Reviewer #2

In many fungal cells, including fission yeast, the deposition of a new cell wall (a septum) between daughter cells is essential for cytokinesis. Cell wall synthases are trafficked to and activated at the division site, and dysregulated trafficking and/or synthase activation can lead to cytokinetic defects.
In this study, the authors use fluorescent probes for Cdc42 and Rho1 activity and live-cell imaging to investigate the timing and regulation of Rho1 activity in fission yeast, and specifically, the role of Cdc42 in regulating Rho1. Summary of the proposed model: Gef1 -> active Cdc42 -> Pak1 --| Rgf1 -> active Rho1 -> septum formation

Major comments

(1) As far as I can gather from the authors' description in the manuscript and quick literature search, this will be the first publication in S. pombe utilizing the HR1-C2 domain of Pkc2p as fluorescent probe for active Rho1 (RBD-mNG). While a comparable domain of S. cerevisiae Pkc1p (not "pck2" as referenced by the authors in Page 25) has been used for similar purposes, given the importance of this probe and the precedent it sets in the S. pombe literature, it is imperative that proper tests are performed to validate that its localization reflects activity of Rho1 and

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nothing else (such as membrane binding of the C2 domain or transcriptional regulation of the pkc2 promoter). Such tests should also be independent of the hypotheses central to the current study (i.e., effects of Gef1, Pak1, Rgf1/2 on the timing of RBD-mNG localization). Can the authors provide data to address this point? Examples include, but not limited to, rho1 mutants, expression of constitutively active Rho1, or temporary expression of dominant-negative Rho1.

We agree with the reviewer and now provide data to show loss of the localization of the Rho-probe RBD-mNG in rho1 mutants. Using temperature-sensitive and switch-off mutants we show that under mutant conditions the RBD-mNG localization is lost at the division site and also from the cell ends. This provides strong evidence that the probe detects active Rho1 in the cells.

(2) Related, M&M does not provide sufficient details about the amino-acid positions corresponding to the "RBD" domain of Pkc2, thus precluding readers from reproducing the experiments. This needs to be clarified.

We now provide in the materials and methods the details of how this probe was generated including the base pairs of the budding yeast *PKC1* and the fission yeast *pck2* promoter.

(3) In Figure 1B, RBD1-mNG localizes clearly to the medial region of rho2Δ cells when the Rlc1-tdTomato ring has not formed. Does this mean that Rho2 has a major role in forming the contractile ring that is independent of Rho1 activation? On this other hand, however, data in Fig. S2BC suggest that RBD-mNG does not localize to the medial region in rho2Δ cells until Rlc1-tdTomato ring forms (the timing of which seems normal). This discrepancy needs to be addressed.

In response to the issue raised here, we do not see active Rho1 at the division site of cells without rings. However, after cytokinesis, while cells are in septation, although the ring has disappeared, active Rho1 lingers at the division site. The cell shown in the panel is a septated cell after ring constriction completes. We have included DIC panels of these cells to show that active Rho1 lingers in septating cells.

(4) Given the nature of RBD-mNG localization, it seems unavoidable to have some level of arbitrariness in measuring the onset of its localization at the division site. It would be advisable for the authors to be specific in M&M about how they defined the onset of localization, i.e., whether it was based on universal threshold in signal intensity, ratio, etc. or on manual curation (ideally double-blind).

We have updated the methods to describe that “onset of localization” was performed via double-blind visual observations.

Minor comments

(1) Throughout the manuscript, there are quite a few places where inconsistencies in genetic nomenclature can cause confusion to readers. Below are some examples.
Figs. 6B, 7B, 10B: pak1(-ts), shk1, and orb2-34 (including faint labels under category marks in 6B).
Fig. 9B (gef1+ rgf1Δ) vs 9C (rgf1Δ). Wild-type alleles are implicit in some figures, while explicit in others.

We have corrected these inconsistencies.

(2) The first hypothesis (Fig. 1C) is that the AMR might regulate Rho1 activation. The ring is disrupted with LatA, but Rho remains active. They cite this as evidence that the AMR does not activate Rho1, but were the cells treated before or after the rings formed? If before, then the experiment demonstrates what the authors claim, but if after, it only shows that the AMR is not essential to maintain Rho activity.

We agree with the reviewer that this is an important distinction. We have modified this statement to “These results indicate that while at the division site the actin cytoskeleton is not required for maintaining Rho1 activation, it is necessary at the growth sites of interphase cells.”

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(3) Page 8: "Time-lapse imaging of cells simultaneously expressing CRIB-3xGFP and RBD-tdTomato [...] while Rho1 is activated ~20 minutes after SPB duplication (Fig. 2B)." This appears to refer to Fig. 2C.

We thank the reviewers for catching this error in the text. We have now corrected it, showing timelapse as Fig. 2C, and an Image of cells simultaneously expressing CRIB and RBD as Fig. 2B.

(4) Page 9: "[...] Rgf1 and Rgf3 localize as early as the time of ring assembly at an average SPB distance of 4-5 μm (Fig. 3D)." This sentence is confusing. How was the average calculated over the earliest ring assembly in non-time-lapse data? Fig. 3DE show distances between SPBs as short as 2.5 μm, not 4-5 μm, and average of ~8 μm for all cells at different stages of mitosis. This confusion needs to be clarified.

We thank the reviewer for observing this mistake in our writing and interpretation. We agree that the text does not reflect the accurate interpretations of the data collected and have now fixed these errors. The current sentence reading "In an asynchronous population of cells, we find that Rgf1 and Rgf3 localize as early as the time of ring assembly at an average SPB distance of 4-5μm." has now been replaced with the description shown below-"Using the distance between SPBs of anaphase cells as a proxy for timing of cytokinesis, we find that in most anaphase cells, Rgf1-GFP and Rgf3-eGFP was localized at the division site at very early stages in anaphase (Fig. 3D, E). This can be observed by the short distance between the SPBs of ~2μm (Fig. 3D). We also measured the distance for which active Rho1 appeared at the division site, and find that at the distance between SPBs of ~10μm, active Rho1 was present at the division site in ~50% of the population of control cells (Fig. 3E)."

(5) Fig. 5. Both the intensity and onset of RBD-mNG localization were affected by cdc42g12v expression. These two may form a causative relationship: reduced overall RBD signal may cause failed detection of early RBD localization. Can the authors compare cells with similar mean RBD-mNG signal intensities (Fig. 5B) and confirm that the timing of appearance at the division site is still delayed in gef1+ cdc42g12v relative to gef1+ empty?

We thank the reviewers for pointing this out and appreciate the opportunity to further clarify our observations. While there is clear decrease in Rho-probe intensity at the division site of on cells expressing *cdc42G12V*, we did see some variation in the extent of the decrease likely due to the variation in the expression levels of *cdc42g12V*. To provide a more accurate analysis of our observation we have shown the changes in the timing and intensities of Rho-probe localization. However, due to the noisy nature of the data we cannot compare the intensities in individual cells at specific spindle pole body distance between cells. As observed cdc42G12V significantly reduces Rho1 activity globally, not just at the division site. To cherry-pick cdc42G12V cells with similar active rho1 intensity to assess time of Rho1 activation may lead to subconscious data manipulation and will not address how early Rho1 activation is regulated.

Reviewer #3

Onwubiko et al., present a clear and well written manuscript detailing the mechanistic understanding of how Rho1 is activated in a timely manner to ensure cytokinesis occurs in a scheduled manner at the end of telophase. Using fission yeast as a model system, and with the development of a novel Rho1 biosensor, they implicate a series of GTPases, exchange factors, GTPase activating proteins and kinases acting downstream of Cdc42 in the timely activation of Rho1. Specifically, they find that Cdc42 prevents premature Rho1 activation in early anaphase in a manner requiring the kinase Pak1. They observe that the Rho1 activators Rgf1 and Rgf3 localise to the division site in early anaphase, but Rho1 doesn't get activated until late anaphase, suggesting that control mechanisms ensure that these GEFs are held inactive, or that RhoGAP activity is able to balance this activation in early anaphase. This suppression of Rho1 activity in early anaphase requires Cdc42 and Pak1 and implicate (by omission) Rgf1, rather than Rgf3, is the relevant GEF.

I liked this manuscript, it was clearly written the experimental progression was logical and the data were easy to interpret from the figures. The conclusions were precise, believable and not overstated. The manuscript provides novel observations and through good use of a series of

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rescues/mutants, illuminates a pathway that is held in check by Cdc42 to ensure timely Rho1 activation. The novel Rho1 probe is exciting and shows well differently regulated pools of active Rho1 at the division site and the growing tips. I thought the co-imaging/measurement of ring placement and SBP duplication allowed a really clear understanding of the kinetics during this rapid phase of the cell cycle. A critique of the study is that the the mechanism by which Cdc42 controls Pak1, and by which Pak1 controls Rgf1/Rgf2 is left unclear. I guess there could always be a molecular expansion of these points (e.g., how does Cdc42 control Pak1; how does Pak1 control Rgf1; how is Rgf activity restricted when localised), but I think that would only enhance, rather than change, the level of detail of the paper's message. I think the paper's current conclusions stand on their own, the data is clear and believable, the experiments are well performed. There are a number of observations in the paper that are left open for future studies, and I think this is a positive (e.g., any separable role of Rgf1/Rgf2 and how Rga5 integrates into this pathway. As such, I am tempted to recommend accept with only minor amendments as outlined below.

1. P8 P15: should the call out be to Fig 2C, rather than 2B.

We thank reviewer for their highlighting this error in our text. We have now fixed it.

2. P14 L17: should it be 'gef1+ rgf3-', not 'gef1+, rgf3+'

We have fixed this error and further clarified the terms for easy understanding.

3. Structure wise, I thoughst the section on Rga5 didn't really fit well on P16; it seemed sandwiched between two sections on GEFs. Is there a more appropriate place to place these data - perhaps between the paragraph breaks on P17? Related to this data, the conclusion on P16 suggests 'other' regulators of RhoGAP activity act to repress Rho1 function. Would 'additional' regulators of RhoGAP activity be more appropriate as there is some function contributed by Rga5?

We have now moved this section to the end of the section on Rho1 regulators after we discuss the Rho1 GEFs. We have also modified the text to clarify that multiple regulators are likely involved in the regulation of Cdc42-mediated Rho1 inhibition.

4. In Fig 10b, you haven't defined orb2-34. Is it the rgf1-delete?

The mutant orb2-34 is a temperature sensitive allele of the *pak1* kinase. To avoid confusion, we have replaced the allele name with *pak1-ts* in figure 10 and in the text.

5. I find the sentence at the top of P18: 'Rho1 activation in pak1+ rgf1+....at 25oC and 35.5oc occurred at longer and similar SBP distances' quite hard to interpret. Could you perhaps expand it to make your message clearer?

We thank the reviewer for pointing this out. These statements have now been re-written for clarity. 'Rho1 activation in pak1+ rgf1+....at 25ºC and 35.5ºC” has been changed, and now reads as follows:
“The timing of RBD-mNG localization at the division site occurs late in cytokinesis during late anaphase as depicted by longer SPB distances in pak1+ rgf1+, pak1-ts rgf1+, and pak1+ rgf1Δ cells at 25ºC (Fig.10B). As previously shown, RBD-mNG localizes to the division site in early anaphase in pak1-ts rgf1+ cells at the restrictive temperature (35.5ºC, Fig. 7A, B). In agreement with our reasoning, early RBD-mNG localization in pak1-ts mutants at 35.5ºC was rescued in the absence of rgf1 (Fig. 10A, B).”

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Original submission

First decision letter

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MS TITLE: Cdc42 prevents precocious Rho1 activation during cytokinesis in a Pak1-dependent manner

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Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

I am happy to tell you that based on your revisions to address the reviewers concerns on your review commons submission that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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