Rab14/MACF2 Complex Regulates Endosomal Targeting During Cytokinesis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Rytis,

I have received 3 reviews of your manuscript. Overall, the reviewers were positive regarding the novelty, but major concerns of the rigor of the work and statistical analysis came up in all 3 reviews, that I think you can work to revise and address the reviewers concerns (both major and minor).

One reviewer stated to me that "the improvements needed are do-able. Of course if data are analyzed more carefully, it might completely change their conclusions." "It’s impossible to know because of their lack of description of stat tests and n’s. The lack of explanation of statistical tests needs to be addressed."

Given the statistical analysis concerns, the resubmission is recommended to go out for re-review, if you choose to resubmit. Please let me know.

Sincerely,

Ahna Skop
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Prekeris,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor’s decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor’s and reviewers’ comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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To submit the rebuttal letter, revised manuscript, and figures, use this link: 

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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Reviewer #1 (Remarks to the Author):

The manuscript by Gibieza and colleagues, "Rab14/MACF2 complex regulates endosomal targeting to the abscission site during cytokinesis" is an attempt to examine the role of Rab14 in cytokinesis. The authors present a combination of biochemical and cell biological data in HeLa cells, to manipulate Rab14 and MACF2 to elucidate their relationship and roles in endosomes during cytokinesis.

This is a novel question, since previously Rab14 has only obliquely been implicated in cytokinesis. It was previously shown to play a role in apical membrane targeting and specification in polarised cells (Kitt et al 2008 and Lu/Wilson 2016). Regarding cytokinesis, Kelly et al 2009 had shown that Rab14 localizes to the cleavage furrow and midbody, and Lu/Wilson 2016 showed that Rab14 knockdown altered cdc42 activity and localization, increased mitotic spindle orientation and midbody displacement relative to the apical membrane. However, neither of these previous papers examined how Rab14 localized to furrow or midbody, nor pinpointed its role in cytokinesis (either furrowing or abscission). Furthermore, the authors as well as other groups previously showed involvement of other Rabs and endosomes in cytokinetic abscission.

The manuscript represents a lot of work, and shows some very interesting findings. The localization of Rab14 around the minus ends of microtubules at the outer ends of the intercellular bridge (ICB) is intriguing, because not much is known about the events at that location during abscission. They also report a previously unknown interaction of Rab14 with MACF2 and complex with CAMSAP3/Patronin. Thus Gibieza et al are addressing important questions and show some intriguing data. However, there are some major concerns about the rigour of the analyses, including statistical methods and controls. In some cases it is not stated or clear what was quantified and how, and in other cases quantification was not done. Finally some terminology is mis-used, including the use of "abscission site" in the title of the manuscript. Overall many of the conclusions are not
well supported, and for others it is difficult to judge, since the statistical tests used are not stated, and the nature of the control cells was not described. Some of the data point to a role for Rab14 in furrowing or early midbody formation, rather than in abscission itself.

Major Concerns:
1. The title of the paper and multiple figure titles and section headings refer to roles in abscission, but the analyses, at least as presented, do not examine abscission per se. In the live imaging, it is not clear how midbody formation or completion of abscission were ascertained/timed. In fixed images, the control is often shown at an early midbody stage, while the knockdown cell is shown at a later midbody stage. Indeed, some data suggest a role in earlier steps of cytokinesis such as cleavage furrow ingression or completion, or compaction of microtubules at the outer ends of the ICB.

2. The word "abscission site" is used improperly. The field has converged on using that term to mean where scission (the "final cuts") will occur, near the centre of the midbody. The outer ends of the ICB, where Rab14 and Fip1 appear to be enriched, are not the abscission sites. It is repeatedly stated that something localizes "to the ICB" or "to abscission site", when the localization would be more precisely described as to the outer ends of the ICB, not actually inside the ICB, and microns away from the actual abscission sites.

3. The lack of details on statistical methods make it impossible for the reader to judge whether appropriate tests were used, and whether the purported significant differences are actually significant. In all figure panels where p-values are shown, it is not stated what statistical test was used (nor in the Methods). Were data tested for normality? Was appropriate test used when more than two conditions are compared? For some "rescued" conditions, no p-values are stated. Also, what sample size n's were used for the statistical formula? All the legends contain this statement: "data shown are means and standard deviation from 3 independent experiments", but the number of dots in graphs vary widely.

4. Another major concern is the use of stable knockdown or knockout cell lines, and unclear nature of the control cells. For genes involved in cell division, how are these stable lines maintained? If 15-20% of cells fail to divide as stated, then with every passage of the cell line, more and more cells will become tetraploid, aneuploid, senescent, etc. What are the passage numbers? Furthermore, the nature of the control cells is not explained (was a non-targeting shRNA lentivirus used? Number of passages same as experimental lines?). It may be best to compare at least initially with transient knockdowns, so we would know what happens in the first cell division after the protein is lost. Are we looking at cytokinesis failure as a primary defect or a secondary effect?

5. For the live time-lapse imaging in Figures 1E, 2A, and 6A, there are several points of confusion. With the data presented, it is not clear which phase(s) of mitosis or cytokinesis were affected. Metaphase, anaphase, furrowing, or abscission were not distinguished from the data shown. First, the time units are not stated. Second, it is not clear what is meant to be observed in the time-lapse panels; labels and arrows are needed. (In 6A, does the cell take 370 minutes? Is this cell delayed in abscission completion or did it fail? It appears to still be connected at the last frame.) Third, it is unclear how "division time" was measured exactly and what was delayed. How was abscission completion ascertained? It is stated that the starting time zero is "starting from metaphase". However, metaphase can take many minutes, longer than anaphase.

6. Fig 2F-G: How was the actin intensity in the furrow versus poles measured? A point, box or linescan? The images appear to be some kind of projection of multiple image planes? From the
data is it not clear: does Rab14 have a role in F-actin organization in the furrow, at the outer ends of the ICB, or at the abscission sites in the midbody? The text refers to actin at abscission sites, but that is not clearly shown in the images, and the y axis on F says "furrow". For example images and analyses of F-actin at abscission sites (cut sites), see Wang/Bi 2019, or Dema/Gai 2018, or Dambournet/Echard 2011.

7. Localization of Rab14: First, Since there are many Rab's, the specificity of the antibody for subcellular localization is important. The appearance of Rab staining in the late midbody in 3B is different than that shown for Rab14 in a late midbody by Kelly EE et al, 2009. The authors should confirm the localization by showing the signal is lost in the Rab14 KD or KO, and/or by using an independent antibody. Second, The Rab14 dots appear to be less concentrated at the minus ends of midbody microtubules in later stage midbodies than early stage. Can this be quantified to compare Rab14 localization at different stages of the abscission process?

8. Figure 3D and Suppl Fig 2A: the co-localization of GFP-rab14 with EEA1: it appears that they are adjacent rather than coincident. Especially in Sup2A we would need to see the green channel alone and see more zoomed examples or single planes.

9. In Sup 2D,E, when does the cell actually complete division (scission of the ICB)? Is the Fip3 around the ends of the ICB through the entire process of midbody maturation and abscission? The conclusion of this section "affecting delivery of rab11/fip3 to the abscission site" is not supported. The abscission sites are very close to the center of the midbody; the fip3 dots are microns away.

10. Suppl Fig 3B,C: Halo-MACF2 looks non-specific. Another reagent is needed to show MACF2 localization.

11. Fig 5 EF: the conclusion that KD of MACF2 does not affect CAMSAP localization into midbody is not well supported by these two pictures. It would need to be quantified, using control and experimental cells at similar stages of cytokinesis/midbody stage. Also Suppl Fig4 shows only 50% knockdown of MACF by mRNA levels. Is the protein gone?

12. Fig 6 E-F: again the KD cell is at a much later stage (late midbody with abscission sites/constriction sites forming) than the control cell (very late central spindle/very early midbody). And there is no quantification here. This should be quantified, with matching stage midbodies, or removed.

Minor points:

1. In figure 2B, the authors say that a higher proportion of knockdown cells are "in telophase". What is actually being quantified? Legend says "with anti-tubulin antibodies". Telophase refers to chromatin state, the phase of mitosis when chromatin decondenses and nuclear envelope re-forms. It overlaps with cytokinesis, but final scission takes place after telophase is over, in the G1 of the following cell cycle (Gershony et al 2014). For that matter, what was the percentage of cells in other phases of mitosis (prophase, metaphase, anaphase)? Is there an effect on mitosis as well as cytokinesis?.

2. The term "central spindle" is used improperly by the authors, to refer to cell images that show early midbody/ICB stage. Central spindle is used in the literature to refer to the wider array of microtubules at the cell equator during furrow ingestion, as in in the middle image in 3C, where Rab14 is said not to be localized.
3. Figure 1E: Text states 17% of rab14-ko cells failed cytokinesis by regressing cleavage furrow. What percentage of control cells failed and regressed the furrow? What were n's of cells for each? It doesn't say in legend either.

4. Figure 2A: when did this cell complete abscission? Did this cell successfully complete cytokinesis or did it become binucleate?

5. Fig 2C-D. It would be better to plot individual cell division times, (as was done in 6B?), because averaging them together before plotting hides the variability in individual times.

6. The text for Fig 2D says "rab14-KO could also be rescued by over-expression of GFP-Rab14 (Figure 2D)". But, there is no statistical p value showing the rescue experiment is significantly different from KO.

7. Figure 3E text: "what appears to be a Fig1-endosome budding from a Rab14 EE..." is not convincing.

8. Figure 4 title and section heading: The wording Rab11 is used, even though Rab11 is shown nowhere. It may well be that Fip1 is a well known Rab11 effector, but this still seems a stretch for the title.

9. Fig 4A-B. The representative image for the rab14 ko shows a later stage midbody and later telophase than the control image (midbody is thinner, and chromatin is less compact). Where is the centrosome in the Rab14 KO cell? Where is line drawn in the KO cell? In the graphs, are the values of traces in KO cells significantly different from control cells? Same issues for Fig 6C-D. It seems odd that there is not a drop in green signal if the line goes through the nucleus.

10. For Fig 6B, how many cells were analyzed? The legend is again unclear saying the data are means from 3 experiments. Some dots are too close together to distinguish.

11. In the colour panels it is helpful to have the text label for each marker match the colour used, rather than just being white text.

12. Page numbers and line numbers would be helpful for the reviewers.

Reviewer #2 (Remarks to the Author):

This study investigates the role of the Rab14 GTPase in the final phase of cell division, cytokinesis. The authors decided to focus on this specific Rab because it was identified as a component of the midbody proteome in their previous work. They report that both overexpression of dominant-negative Rab14 and its knock-down/knock-out caused cytokinesis failure, which by time-lapse imaging was shown to occur late, just before abscission. Furthermore, they show that Rab14 vesicles accumulate at the minus ends of the central spindle microtubules during telophase and investigate a potential relationship between Rab14 and Rab11 GTPases. Finally, by using immunoprecipitation coupled with mass spectrometry they identify the microtubule-associated MACF2/CAMSAP3 complex as an interactor of Rab14 in telophase and report that MACF2 is required for proper Rab14 localization to the central spindle.
Our understanding of the mechanisms and pathways that control membrane trafficking in cytokinesis is still limited and therefore in my opinion the novel findings presented in this manuscript should attract the interest of MBoC readers. However, as detailed in my specific comments below, some parts of the paper, such as the analysis of the relationship between the two Rab GTPases, are not fully convincing and should be either strengthened or eliminated before I can support publication of this manuscript.

Specific comments:

1. The authors' statement that: "Rab14 and Rab11 may function in the same regulatory pathway" is based on an incorrect interpretation of their results, which point to a synergistic rather than an epistatic relationship between the two Rab GTPases. Indeed, one would not expect an increase in a specific phenotype after double knockdown or a rescue of Rab14 defects by Rab11 overexpression if the two GTPases were acting in the same pathway. These results instead indicate that the two proteins work in parallel pathways that converge on the same process, i.e. the delivery of membrane vesicles to the cleavage site.

2. It is also unclear why the authors decided to focus only on a potential relationship between Rab14 and Rab11, and not Rab35 for example. I presume that this is because of the previous interest of the Prekeris lab in Rab11, but a potential relationship between Rab14 and Rab35 cannot be excluded a priori.

3. I find the results involving Fip1 not very convincing. The two cells in Fig. 4A are clearly at two different stages as the DNA is still very condensed and the central spindle is much ticker in the control cell. Moreover, in both Fig 4B and 6D the graphs seem only to indicate that Fip1 vesicles are more abundant in the nucleus, but do not show any particular effect on their localization and/or recruitment to the central spindle and/or cleavage site. By contrast, the lack of recruitment of Fip3 vesicles to the central spindle in Rab14 knock-out cells is, in my opinion, clear and convincing. On the light of this and my previous comments, I would like to suggest to eliminate the Fip1 experiments and re-interpret the result by arguing that, although the experiments indicate that a potential relationship between Rab14 and Rab11 exists, this appears to be not simply linear but synergistic and complex.

4. The experiment in Fig. 2G is not very convincing because the Rab14 knock-out cell seems to be at an earlier stage than the control and this could of course explain the different levels of F-actin accumulation. I recommend to repeat this experiment by more carefully staging the cells.

5. The authors should clarify more precisely how they measured cell division time in their time-lapse experiments. They only indicated that they started from metaphase, which is not a precisely defined time-point during mitosis. I suggest to re-calculate these values by using anaphase onset and abscission as starting and end time-points, respectively. I don't think that this will lead to major changes to their results, but the data will be more convincing and accurate. It is also not specified what the numbers in the still images indicate and the scale bars are too small.

6. All the graphs are difficult to analyze because the median and SD lines are covered by the dots, especially in the case of time-lapse data. In addition, always in the time-lapse experiments, the number of dots does not much the number of cells filmed (see Figs. 2C and 2D), which is odd and should be corrected. Perhaps using a smaller point size and/or colors could solve these problems.
7. The authors must indicate which statistical test they used in every graph.

8. The data regarding the localization of Rab14 after MACF2 knock-down should be strengthened. The fluorescence levels of Rab14 in control vs MACF2 knock-down cells should be quantified and movies of control cells should be shown.

Minor

1. Although the paper is generally clearly written, it is evident that it was written by multiple hands because in some parts the English is not very good and contains a mix of British and American spelled words. Some typos are also present. More carefully proof-reading is necessary.

2. The figure and movie captions are too concise and often do not provide sufficient information to the reader to understand the data.

3. Some panels lack scale bars (e.g., 3E, 4D, 5C-F, 6C), in one the bar is clearly incorrect (left panel in 6C), and in many others the scale bars are too thin and the letters too small.

4. The titles of two subheadings in the results section "Rab14 regulates abscission" and "Rab14 and Rab11 are part of the same abscission regulatory pathway" are overstatements that are not supported by the results; see also comments above.

5. Figure 1E should be merged with Fig 2A.

6. MS data should be included and possibly deposited in a public repository.

Reviewer #3 (Remarks to the Author):

This study by Gibieza and colleagues identifies novel roles for Rab14 in cytokinesis and abscission. It begins from an unbiased approach, which is great. It demonstrates clear phenotypes for Rab14 KO and KD and identifies MACF2 as an interaction partner and potential effector. Overall, I think this study identifies a new player in a clearly important process, but I have both major and minor concerns that would need to be addressed prior to supporting its publication.

Major concerns/comments
I love a good schematic model, which I think would significantly help the readers in this study. I'm struggling to connect the Rab14 and Rab11 data here with what is known about Rab11/Rab35 from the literature. The header "Rab14 and Rab11 are part of the same abscission regulatory pathway" is confusing based on the data. Rab14 and Rab11 independently give a similar phenotype, and the phenotype is additive when both are knocked down. In my eyes, and please correct me if I'm wrong, this suggests they are in independent pathways. If they were in the same pathway, then the effect of knocking down each should be the same as knocking down both (breaking a chain in two spots is the same as breaking it in one). Obviously, this is contingent on KD efficiency, which is not shown for Rab11. Conversely, Rab11 OE rescues or bypasses Rab14 KD (it's not clear if the authors think this is a rescue or bypass), suggesting they might be in the same pathway.

Similar to above, the connection to actin at the bridge is ill-defined, and hinges on a single immunostaining experiment and latrunculin multinucleation experiment that could have alternative
interpretations. As the authors note, known effectors of Rab11/35 that might regulate actin disassembly have been identified. I would encourage a bit more examination in this area to see if they are mislocalized in Rab14KD/KO, if latrunculin rescues the division time and not just multinucleation, if latrunculin rescues actin over-assembly during Rab14-KD, etc. Is it possible the prolonged latrunculin treatment is slowing cell cycle instead of rescuing abscission defects, leading to reduced multinucleation?

More details are needed throughout regarding methods. I've pointed out many examples below. Importantly, validation of expression levels, KD, and CRISPR knock-in is missing. I do not see any validation of the CRISPR knock-ins. It is not clear from the methods how these cells were isolated. Did the authors sort a GFP+ polyclonal line? Did they sort clones? Can they show western blots of a GFP-Rab14 expression? Did they genomically validate KI? Does the GFP signal co-localize with Rab14 immunostaining or RFP-Rab14 expression? Additionally, more quantitation of imaging some experiments would further support the observations.

Minor concerns/comments:
Fig 1A: What are spectral points? Are those peptide hits? I understand it was in a previous paper, but I think it would help the reader to define what you mean here in the legend or methods.
Fig 1B: Are Rab29-DN (and maybe Rab21) not significantly different from control? It would be helpful to either state the remainder are NS or state their p values and say you moved forward with the most apparent one.
Fig 1B: I do not see a description of the DN plasmids in the text. How was this assay done? Are these GFP-tagged and only GFP cells counted? Were all DN constructs expressed at similar levels?
Fig. 1C & 1D: I hate western blots, but I would love to see some western blots. The rescue is incomplete in 1C, which is fine, but why? What is the KD efficiency? What is the expression of GFP-Rab14 relative to endogenous?
Fig 2: How do the authors define "Division Time"? "Starting at metaphase" means starting as soon as the metaphase plate is apparent? More importantly, how is dissolution of the bridge defined? This is such a small narrow structure that at lower resolution in between two cells is difficult to identify in every frame. A bit more about the criteria and methods here would be helpful.
Fig 2: Does LatA rescue the division time? Does LatA rescue the actin levels at the furrow?
Fig 2: Do the number of aborted cytokinesis events in their live-cell imaging support the data from the multinucleation assay? Does failed abscission fully account for Rab14-KO/KD-mediated multinucleation?

Coloring: What is MBoC policy on green/red combos? I would argue that any single channel imaging be in grayscale instead of green on black (and I fully advocate for inverting the grayscale if the authors agree).
Fig 3A: please label the insets.
What is the n value on the IP/MS that identified MACF2? Was it done just once? Are these spectral counts high enough to trust?
Fig 6: is Fip1 going into the nucleus in MACF1 KD? In the quantitative data, why is the nuclear signal similar to that in the cytoplasmic?
Fig S3: The yes/no quantitation of FIP3 localization upon MACF2 KD is unsatisfying. Is it possible to put some more informative numbers to this?

Discussion: "Rab14 is the only MB-associated Rab that is required for abscission"; I think this is perhaps a stretch considering the authors state they did not feel comfortable knocking down Rab5 and 7, which were also in the MB pool.

Why were some experiments done on collagen and some on fibronectin?
Reviewer #1:

Major Concerns:
1. The title of the paper and multiple figure titles and section headings refer to roles in abscission, but the analyses, at least as presented, do not examine abscission per se. In the live imaging, it is not clear how midbody formation or completion of abscission were ascertained/timed. In fixed images, the control is often shown at an early midbody stage, while the knockdown cell is shown at a later midbody stage. Indeed, some data suggest a role in earlier steps of cytokinesis such as cleavage furrow ingression or completion, or compaction of microtubules at the outer ends of the ICB.

While most of our data does suggest that Rab14 affects abscission, reviewer is correct that we cannot fully rule out the possibility that Rab14 may also affect the earlies steps of cytokinesis. Thus, we edited manuscript title, as well as titles of several figures and section headings to take that into account. We also removed references to abscission where our data does not directly test that.

As suggested by the reviewer we also added more detailed explanation how the timing of cytokinesis was measured (see Materials and Methods). Finally, we checked all the images (and replaced them where needed) to make sure that all images are stage-matched.

2. The word "abscission site" is used improperly. The field has converged on using that term to mean where scission (the "final cuts") will occur, near the center of the midbody. The outer ends of the ICB, where Rab14 and Fip1 appear to be enriched, are not the abscission sites. It is repeatedly stated that something localizes "to the ICB" or "to abscission site", when the localization would be more precisely described as to the outer ends of the ICB, not actually inside the ICB, and microns away from the actual abscission sites.

Our apologies if we were not clear in the text about our use of the term “abscission”. Reviewer is absolutely correct that term “abscission site” is meant to define the site of final scission. Obviously, Rab14 and FIP1 do not accumulate at the abscission site but instead Rab14 and FIP1 accumulate at the mins ends of central spindle microtubules. We never meant to state otherwise, thus, we edited the manuscript to make sure that this is very clear. The only proteins analyzed in this manuscript that do accumulate at the abscission site are FIP3 and Rab11. That has now been shown in numerous studies by several labs and we also show that in Figure 4C, last image at the right. Again, we edited the text to make that clear.

3. The lack of details on statistical methods make it impossible for the reader to judge whether appropriate tests were used, and whether the purported significant differences are actually significant. In all figure panels where p-values are shown, it is not stated what statistical test was used (nor in the Methods). Were data tested for normality? Was appropriate test used when more than two conditions are compared? For some "rescued" conditions, no p-values are stated Also, what sample size n's were used for
the statistical formula? All the legends contain this statement: "data shown are means and standard deviation from 3 independent experiments", but the number of dots in graphs vary widely.

We expanded Method section to provide all the details about the statistical tests used. We also made sure that all analyses (including rescues) have either p values or n.s. label (if not significant, p value larger than 0.05). Our apologies for misstatements “data shown are means and standard deviation from 3 independent experiments”. We also edited figure legends and Method section to correct that number of dots means the number of experimental replicates and n means number of cells analyzed.

4. Another major concern is the use of stable knockdown or knockout cell lines, and unclear nature of the control cells. For genes involved in cell division, how are these stable lines maintained? If 15-20% of cells fail to divide as stated, then with every passage of the cell line, more and more cells will become tetraploid, aneuploid, senescent, etc. What are the passage numbers? Furthermore, the nature of the control cells is not explained (was a non-targeting shRNA lentivirus used? Number of passages same as experimental lines?). It may be best to compare at least initially with transient knockdowns, so we would know what happens in the first cell division after the protein is lost. Are we looking at cytokinesis failure as a primary defect or a secondary effect?

We fully agree with the reviewer that knock-down or knock-out of house-keeping genes (especially involved in regulating mitosis) may have large effects on several cell functions, as well as lead to accumulation of aneuploidy/polyploidy. Indeed, Rab14-KO cells substantially slow down after passage 14-16 and usually die off by passage 20. Thus, we do not use KD or KO cells past 10th passage to avoid any cumulative defects. Additionally, for many knock-down experiments we made sure that defects can be rescued, suggesting that shown phenotypes are not due to indirect accumulation of genetic defects. Finally, for KD cells we used empty scrambled shRNA-vector as a negative control. We expanded Method Section to include all that information.

5. For the live time-lapse imaging in Figures 1E, 2A, and 6A, there are several points of confusion. With the data presented, it is not clear which phase(s) of mitosis or cytokinesis were affected. Metaphase, anaphase, furrowing, or abscission were not distinguished from the data shown. First, the time units are not stated. Second, it is not clear what is meant to be observed in the time-lapse panels; labels and arrows are needed. (In 6A, does the cell take 370 minutes?? Is this cell delayed in abscission completion or did it fail? It appears to still be connected at the last frame.) Third, it is unclear how "division time" was measured exactly and what was delayed. How was abscission completion ascertained? It is stated that the starting time zero is "starting from metaphase". However, metaphase can take many minutes, longer than anaphase.

In our time-lapse analyses we did look at times needed to go from metaphase-to-telophase and from telophase-to-abscission. Rab14-KOs clearly affected both, thus, we agree with the reviewer that in addition to abscission, Rab14 may also play a role in furrowing. We added this new data to Supplemental Figure 1D. We also edited the text
to incorporate this data and added (as suggested) labels and arrows to make that clear. We also added the time units to the figure legends.

Yes, in 6A it did take 370 minutes. Unfortunately, by minute 920 the time-lapse was completed, thus, we do not know if that particular cell has completed division.

We expanded our Method section to add information how division time was measured. Briefly, we identified different mitotic stages using following criteria: a) cells start metaphase when they round up and chromosomes are aligned in metaphase plate (as determined by DIC); b) cells enter anaphase when cells start elongating due to movement of opposing poles; 3) telophase starts when cells initiate cytokinetic cleavage; 4) cells complete cytokinesis when we cannot observe clearly identifiable midbody and the ICB. That allowed us to measure the time that took cell to pass through all these different stages, although in final figure we just report a total time for cells to go from metaphase (stage 1) to completion of cytokinesis (stage 4). The timing of cytokinesis completion is a bit tricky, since if cells are in very close proximity it is sometimes difficult to determine whether ICB has been severed. To minimize that we have plated cells in low density, however, it is likely that we may have overestimated time in some cells. Nevertheless, since the same criteria has been applied to all conditions, we have analyzed large number of cells for each condition, and the difference in division time between control and KO or KD cells is quite large, we are confident in this type of analysis.

6. Fig 2F-G: How was the actin intensity in the furrow versus poles measured? A point, box or linescan? The images appear to be some kind of projection of multiple image planes? From the data is it not clear: does Rab14 have a role in F-actin organization in the furrow, at the outer ends of the ICB, or at the abscission sites in the midbody? The text refers to actin at abscission sites, but that is not clearly shown in the images, and the y axis on F says "furrow". For example images and analyses of F-actin at abscission sites (cut sites), see Wang/Bi 2019, or Dema/Gai 2018, or Dambournet/Echard 2011.

To measure actin fluorescence in the furrow we selected region of interest (ROI) at the furrow as well as opposing poles of the cell. Fluorescence was them measured (as sum fluorescence) and normalized to the size of ROI. Finally, the ration of fluorescence in the furrow and poles were calculated to measure the enrichment of F-actin at the furrow. We expanded the Method section to provide more information about how analysis was done. Of note, the images are not projections but a single plane from the z-stack taken of the entire cell using 200 nm size z-step.

Since we have analyzed the actin in the entire ICB, as mentioned by reviewer, we cannot conclude that Rab14 regulates actin at the abscission. Thus, we re-worded the conclusion to say that Rab14 regulates actin at the ICB.

7. Localization of Rab14: First, since there are many Rab's, the specificity of the antibody for subcellular localization is important. The appearance of Rab staining in the late midbody in 3B is different than that shown for Rab14 in a late midbody by Kelly EE
et al, 2009. The authors should confirm the localization by showing the signal is lost in the Rab14 KD or KO, and/or by using an independent antibody. Second, The Rab14 dots appear to be less concentrated at the minus ends of midbody microtubules in later stage midbodies than early stage. Can this be quantified to compare Rab14 localization at different stages of the abscission process?

As suggested, we added immunofluorescence analysis of anti-Rab14 antibody in Rab14-KO cells (Figure 3C). It clearly shows that the signal is missing, demonstrating that staining shown in Figure 3A and B does represent Rab14 distribution during cell division.

We agree with the reviewer that Rab14 organelles do appear to be less concentrated as cell progresses from early to late telophase. That would be in agreement with our model that Rab14-endosomes dock at minus ends of microtubules early in telophase to allow more efficient budding and transport of the endosomes into the ICB. However, due to localization variability and issues of determining exact telophase stage (early versus mid or late) in fixed cells we could not generate a quantification that I could fully trust. Thus, we decided not to include that in the manuscript.

8. Figure 3D and Suppl Fig 2A: the co-localization of GFP-rab14 with EEA1: it appears that they are adjacent rather than coincident. Especially in Sup2A we would need to see the green channel alone and see more zoomed examples or single planes.

GFP-Rab14 and EEA1 are definitely present in the same organelles. To make that point better we added additional zoom-in insets in Figure 3D. As suggested, we also added zoom-in insets in Sup2A.

9. In Sup 2D,E, when does the cell actually complete division (scission of the ICB)? Is the Fip3 around the ends of the ICB through the entire process of midbody maturation and abscission? The conclusion of this section "affecting delivery of rab11/fip3 to the abscission site" is not supported. The abscission sites are very close to the center of the midbody; the fip3 dots are microns away.

Yes, in both cases cell appear to compete division since in the last time frame one cannot observe an ICB and the midbody. It was previously reported by our and other laboratories that during division Fip3 first go to the centrosomes then translocate to the MB and abscission site at late telophase. That can also be observed in Sup 2D, two dots at the beginning of the division are the centrosomal pools of Fip3. At the later telophase one can see the movement of Fip3 from centrosomes to the MB (marked by asterisk). In Rab14-KO cells (Sup2E) one can clearly see centrosomal pools (two dots). However, Fip3 does not move from centrosomes to MB (marked by asterisk). These time-lapse images, as well as immune-fluorescence data shown in Fig 4C-D support our conclusion that Rab14 directly or indirectly affects Fip3 targeting to the MB during division. We have edited the text in the manuscript to make sure that our rationale for this conclusion is described better.
10. Suppl Fig 3B,C: Halo-MACF2 looks non-specific. Another reagent is needed to show MACF2 localization.

The signal of Halo-MACF2 is indeed very dim. We do believe that the signal is real, since similar signal was not observed in control cells that were transfected with empty Halo vector. Generally, HeLa cells appear not tolerate even moderate MACF2 overexpression. We re-cloned MSACF2 in GFP-vector but got very similar results, very few transfected cells expressing very low levels of GFP-MACF2. We also tried to create stable cells lines using Halo-MACF2 as well as GFP-MACF2, with little success. Consequently, we decided to remove MACF2 imaging data, since we agree with the reviewer that the quality of images is not great.

11. Fig 5 EF: the conclusion that KD of MACF2 affects CAMSAP localization into midbody is not well supported by these two pictures. It would need to be quantified, using control and experimental cells at similar stages of cytokinesis/midbody stage.

As suggested, we replaced CAMSAP-GFP image in MACF2-KD cells with the one that is better stage-matched with control. We also added quantification of enrichment of CAMSAP3-GFP in the ICB in control and MSCFD2-KD cells (see Figure 5F).

12. Fig 6 E-F: again the KD cell is at a much later stage (late midbody with abscission sites/constriction sites forming) than the control cell (very late central spindle/very early midbody). And there is no quantification here. This should be quantified, with matching stage midbodies, or removed.

We have replaced the image with the one that better matches the mitotic stage of control cell. As suggested, we also added quantification of Rab14 accumulation at the minus ends of ICB microtubules in control and MACF2-KD cells (Figure 6G). We also added description of this analysis in the Method section.

Minor points:

1. In figure 2B, the authors say that a higher proportion of knockdown cells are "in telophase". What is actually being quantified? Legend says "with anti-tubulin antibodies". Telophase refers to chromatin state, the phase of mitosis when chromatin decondenses and nuclear envelope re-forms. It overlaps with cytokinesis, but final scission takes place after telophase is over, in the G1 of the following cell cycle (Gershony et al 2014). For that matter, what was the percentage of cells in other phases of mitosis (prophase, metaphase, anaphase)? Is there an effect on mitosis as well as cytokinesis?

We have used a definition of telophase as a stage between anaphase and interphase that ends at the abscission. By this definition, telophase starts with initiation of cytokinesis, specifically start of furrow ingress. Thus, staining cells with anti-tubulin antibodies and DAPI allowed us to visualize mitotic and central spindle, as well as chromatin decondensation, to categorize cells. We added couple sentences to the text...
to make that clear. We also added additional description of this analysis to the Method Section of the manuscript.

During this analysis we also counted cells in metaphase and found no difference between control and Rab14-KD cells. As suggested, we have added this data to the text of the manuscript. We did not count cells in anaphase, since it is a very transient phase and not enough anaphase cells could be found to generate reliable numbers.

2. The term "central spindle" is used improperly by the authors, to refer to cell images that show early midbody/ICB stage. Central spindle is used in the literature to refer to the wider array of microtubules at the cell equator during furrow ingression, as in in the middle image in 3C, where Rab14 is said not to be localized.

I appreciate reviewer comment regarding using “central spindle” term. Indeed, central spindle in metaphase and anaphase does refer to the array of microtubules at the cell equator. We also used this term for telophase cells since it is currently believed that the midbody and ICB microtubules are derived from central spindle microtubules compacted and crosslinked during cytokinesis. However, to eliminate this confusion we have changed the term “central spindle microtubules” to “ICB microtubules”.

3. Figure 1E: Text states 17% of rab14-ko cells failed cytokinesis by regressing cleavage furrow. What percentage of control cells failed and regressed the furrow? What were n's of cells for each? It doesn't say in legend either.

We did not observe any control cells to regress furrow. Obviously, that does not mean that this never happens, since our analyzed cell numbers (n=75) may just be too small to detect it. As suggested, we also added (to the manuscript text) the total numbers of cells analyzed.

4. Figure 2A: when did this cell complete abscission? Did this cell successfully complete cytokinesis or did it become binucleate?

Yes, this cell did complete cytokinesis. We added post-abscission time frame to the figure.

5. The text for Fig 2D says "rab14-KO could also be rescued by over-expression of GFP-Rab14 (Figure 2D)". But, there is no statistical p value showing the rescue experiment is significantly different from KO.

We compared division times between rescues and control and found no statistical difference. The “n.s” to mark that has been added to the figure.

6. Figure 3E text: "what appears to be a Fip1-endosome budding from a Rab14 EE..." is not convincing.
We realize that the resolution does not allow us to definitely state that Fip1 endosome can bud from Rab14-EE. We felt that image is interesting enough to add it, but in new version of the manuscript we made sure that our conclusion is marked as speculation.

7. Figure 4 title and section heading: The wording Rab11 is used, even though Rab11 is shown nowhere. It may well be that Fip1 is a well-known Rab11 effector, but this still seems a stretch for the title.

Title has been changed.

8. Fig 4A-B. Where is the centrosome in the Rab14 KO cell? Where is line drawn in the KO cell? In the graphs, are the values of traces in KO cells significantly different from control cells?
Same issues for Fig 6C-D. It seems odd that there is not a drop in green signal if the line goes through the nucleus.

We did draw line between the ICB and the presumptive centrosomal (spot on the outside of nucleus) pool of Fip1 endosomes to capture the Fip1 distribution. However, since we did not co-stain with centrosomal marker we cannot state that that Fip1 accumulation on the outside of the nucleus is actually centrosomes. We also did our best to stage-match the cells and the line analysis is actually average of four cells. We added better explanation about analysis to the Method section.

The reason for lack of drop in green signal at nucleus in KO and KD cells is that the line-scan analysis is an average of signal from 4 different cells. Each cell is a bit different and have different distance between ICB and nucleus. In control cells Fip1-endosomes are very tightly clustered and still show two clear peaks. In KO and KD cells Fip1-endosomes are widely scattered and that after averaging leads to lack in green signal drop.

9. For Fig 6B, how many cells were analyzed? The legend is again unclear saying the data are means from 3 experiments. Some dots are too close together to distinguish.

The experiment was repeated three times and multiple cells were analyzed I each biological replicate. Total, for each experimental condition we analyzed 21 cells. As suggested, we added information of cell number to the figure.

10. In the color panels it is helpful to have the text label for each marker match the color used, rather than just being white text.

As suggested we changed labels to match the color used in the images.

11. Page numbers and line numbers would be helpful for the reviewers.

Page and line numbers added.
Reviewer #2:

Specific comments:

1. The authors' statement that: "Rab14 and Rab11 may function in the same regulatory pathway" is based on an incorrect interpretation of their results, which point to a synergistic rather than an epistatic relationship between the two Rab GTPases. Indeed, one would not expect an increase in a specific phenotype after double knockdown or a rescue of Rab14 defects by Rab11 over-expression if the two GTPases were acting in the same pathway. These results instead indicate that the two proteins work in parallel pathways that converge on the same process, i.e. the delivery of membrane vesicles to the cleavage site.

**We fully agree with the reviewer that we cannot determine whether Rab11 and Rab14 work in the same or parallel pathway (or possibly combination of both). On one hand reviewer is totally correct that increase in severity of cytokinetic phenotype in double KO/KD would suggest that two proteins work in parallel. On the other hand, we do see the effect of Rab14-KO on Fip3 recruitment to the ICB, suggesting that they may, at least partially, work in the same pathway. We edited the text to make that very clear. We also expanded Discussion section to point out these possibilities.**

2. It is also unclear why the authors decided to focus only on a potential relationship between Rab14 and Rab11, and not Rab35 for example. I presume that this is because of the previous interest of the Prekeris lab in Rab11, but a potential relationship between Rab14 and Rab35 cannot be excluded a priori.

**We did look at Rab35 as well. As previously reported, during division Rab35 appear to be localized at plasma membrane of the ICB with little presence at the recycling and early endosomes. Our preliminary analysis also showed little effect of Rab14-KO on Rab35. Consequently, we decided to focus on possible connection between Rab11 and Rab14. We totally agree that Rab35 is also an important player in regulating ICB actin and abscission. Thus, we expanded Discussion section to discuss potential interplay between Rab14, Rab11 and Rab35.**

3. I find the results involving Fip1 not very convincing. The two cells in Fig. 4A are clearly at two different stages as the DNA is still very condensed and the central spindle is much ticker in the control cell. Moreover, in both Fig 4B and 6D the graphs seem only to indicate that Fip1 vesicles are more abundant in the nucleus, but do not show any particular effect on their localization and/or recruitment to the central spindle and/or cleavage site. By contrast, the lack of recruitment of Fip3 vesicles to the central spindle in Rab14 knock-out cells is, in my opinion, clear and convincing. On the light of this and my previous comments, I would like to suggest to eliminate the Fip1 experiments and re-interpret the result by arguing that, although the experiments indicate that a potential
relationship between Rab14 and Rab11 exists, this appears to be not simply linear but synergistic and complex.

We do agree with the reviewer that Fip3 data is much stronger than Fip1. We could eliminate Fip1 data, but I feel that is important to have a second Rab11 proxy (Fip1) analyzed, especially since our Fip3 analysis is based on overexpression of exogenous Fip3-GFP. Thus, we would like to keep Fip1 data in (unless reviewer feels very strongly about it). As suggested, we did re-write the parts of manuscript to focus more on Fip3 and slightly reinterpret the data.

4. The experiment in Fig. 2G is not very convincing because the Rab14 knock-out cell seems to be at an earlier stage than the control and this could of course explain the different levels of F-actin accumulation. I recommend to repeat this experiment by more carefully staging the cells.

We respectfully disagree with the reviewer. The cells shown at the Fig. 2G are about the same stage, or at least as much as can be determined in fixed images. We have added to the Method Section a better description of how cells were stage-matched. That hopefully will clarify how we stage-matched cells for this type of the analysis. Finally, the quantification shown in Figure 2F includes quantification from 12 cells, thus, presumably averaging out any potential slight mismatches in stage.

5. The authors should clarify more precisely how they measured cell division time in their time-lapse experiments. They only indicated that they started from metaphase, which is not a precisely defined time-point during mitosis. I suggest to re-calculate these values by using anaphase onset and abscission as starting and end time-points, respectively. I don't think that this will lead to major changes to their results, but the data will be more convincing and accurate. It is also not specified what the numbers in the still images indicate and the scale bars are too small.

As suggested, we have expanded the Method section to clarify our approach in measuring the division time. We do feel that we can accurately measure the division time, since our time resolution only 10 minutes (based on our time-lapse time) and we define entering metaphase when cells round up and that usually occurs within one time-lapse.

We also added to figure legend the explanation of the numbers of still images (they represent elapsed time). Unfortunately, we cannot change the scale bar since it is embedded into images by software and we cannot change them.

6. All the graphs are difficult to analyze because the median and SD lines are covered by the dots, especially in the case of time-lapse data. In addition, always in the time-lapse experiments, the number of dots does not much the number of cells filmed (see Figs. 2C and 2D), which is odd and should be corrected. Perhaps using a smaller point size and/or colors could solve these problems.
We decreased the size of the dots to make sure that SD lines are more visible. We also made sure that information about meaning and number of dots is consistent with the experimental design. Finally, we expanded the figure legends and Materials and Methods section to ensure that experiments are better described.

7. The authors must indicate which statistical test they used in every graph.

*We expanded Method section to better describe statistical methods, as well as approaches to perform various image analyses.*

8. The data regarding the localization of Rab14 after MACF2 knock-down should be strengthened. The fluorescence levels of Rab14 in control vs MACF2 knock-down cells should be quantified.

*As suggested, we added quantifications of Rab14 localization in control and MACF2-KD cells (Figure 6G).*

**Minor**

1. Although the paper is generally clearly written, it is evident that it was written by multiple hands because in some parts the English is not very good and contains a mix of British and American spelled words. Some typos are also present. More carefully proof-reading is necessary.

*Our apologies for that. Indeed, manuscript was written by two people, one from US and one from Europe. We have extensively edited the manuscript to fix this.*

2. The figure and movie captions are too concise and often do not provide sufficient information to the reader to understand the data.

*As suggested, we have expended Figure legends and Method section to provide more details.*

3. Some panels lack scale bars (e.g., 3E, 4D, 5C-F, 6C), in one the bar is clearly incorrect (left panel in 6C), and in many others the scale bars are too thin and the letters too small.

*We increased the size of scale bars, as well as added them where they are missing. Unfortunately, often the numbers for scale bars are imprinted in the image during its acquisition and we cannot change it. Thus, we also made sure that the sizes of scale bars are listed in corresponding figure legends.*

4. The titles of two subheadings in the results section "Rab14 regulates abscission" and "Rab14 and Rab11 are part of the same abscission regulatory pathway" are overstatements that are not supported by the results; see also comments above.
Subheadings have been changed.

5. Figure 1E should be merged with Fig 2A.

As suggested, we merged Fig 1E and Fig 2A (now Fig 1E).

Reviewer #3:

Major concerns/comments
I love a good schematic model, which I think would significantly help the readers in this study.

We a bit reluctant to make a model (unless reviewer insists on it) since there are still too many questions to be answered. For example, while we know that Rab14 is important for abscission and regulates actin depolymerization in ICB, we still do not know whether it works as a parallel pathway to Rab11 or whether Rab14 and Rab11 are parallel pathways. That prevents us from making a clear model figure. We have expanded the discussion section to clarify what we propose regarding Rab14 role during mitosis.

I'm struggling to connect the Rab14 and Rab11 data here with what is known about Rab11/Rab35 from the literature. The header "Rab14 and Rab11 are part of the same abscission regulatory pathway" is confusing based on the data. Rab14 and Rab11 independently give a similar phenotype, and the phenotype is additive when both are knocked down. In my eyes, and please correct me if I'm wrong, this suggests they are in independent pathways. If they were in the same pathway, then the effect of knocking down each should be the same as knocking down both (breaking a chain in two spots is the same as breaking it in one). Obviously, this is contingent on KD efficiency, which is not shown for Rab11. Conversely, Rab11 OE rescues or bypasses Rab14 KD (it's not clear if the authors think this is a rescue or bypass), suggesting they might be in the same pathway.

Reviewer is spot on about the difficulties to understand the interplay between Rab14 and Rab11. The connection with Rab35 is reasonably clear since we have not found any evidence that Rab14 affects Rab35 function, thus, Rab35 is clearly a separate parallel pathway. Connection to Rab11 is a bit more enigmatic and interesting. As reviewer pointed out there are some evidence that Rab11 and Rab14 work in parallel pathways, as well as that they may be part of the same pathway. Furthermore, these two options are not necessarily mutually exclusive. Understanding that is outside the scope of this manuscript but is something we are very interested in exploring in the future. This is also the reason that we decided not to make a model figure. We did expand the Discussion section to discuss the connections between Rab14, Rab11 and Rab35.

Similar to above, the connection to actin at the bridge is ill-defined, and hinges on a single immunostaining experiment and latrunculin multinucleation experiment that could
have alternative interpretations. As the authors note, known effectors of Rab11/35 that might regulate actin disassembly have been identified. I would encourage a bit more examination in this area to see if they are mislocalized in Rab14KD/KO, if latrunculin rescues the division time and not just multinucleation, if latrunculin rescues actin over-assembly during Rab14-KD, etc.

We agree with the reviewer that much more needs to be understood about the role of Rab14 in regulating actin at the ICB. However, this is not really the focus of this manuscript. What we want to focus on is demonstrating that Rab14 is needed for cytokinesis (never been shown before) and what are Rab14 effectors that contributes to Rab14 role during division, specifically MACF2/CAMSAP3. We have added more discussion about Rab14 and actin and are planning to explore that in our future studies. We also wanted to point out that the effect of latrunculin on actin in ICB has been already carefully analyzed in three really nice papers from Arnaud Echard’s laboratory, so it is becoming an established assay to test the role of actin depolymerization during cytokinesis.

More details are needed throughout regarding methods. I've pointed out many examples below. Importantly, validation of expression levels, KD, and CRISPR knock-in is missing. I do not see any validation of the CRISPR knock-ins. It is not clear from the methods how these cells were isolated. Did the authors sort a GFP+ polyclonal line? Did they sort clones? Can they show western blots of a GFP-Rab14 expression? Did they genomically validate KI? Does the GFP signal co-localize with Rab14 immunostaining or RFP-Rab14 expression? Additionally, more quantitation of imaging some experiments would further support the observations.

We have substantially expanded Method Section to include much more details about methods. Specifically:

a) expanded section describing various imaging analysis approaches
b) expanded section describing statistical analyses
c) expanded section describing KD, including adding qPCR data (Supplemental Figure 3A) confirming MACF2 KD as well as western blot confirming Rab14 KD (Supplemental Figure 1A).
d) expanded section describing CRISPR knock-in. That includes the explanation that the GFP-Rab14-KI line was genotyped. We also added western blot showing that GFP-Rab14-KI line expresses endogenous Rab14 tagged with GFP (Supplemental Figure 2C).

Minor concerns/comments:
Fig 1A: What are spectral points? Are those peptide hits? I understand it was in a previous paper, but I think it would help the reader to define what you mean here in the legend or methods.

It should say “spectral count”. Our apologies for the mistake. It has been corrected.

Fig. 1B: Are Rab29-DN (and maybe Rab21) not significantly different from control? It
would be helpful to either state the remainder are NS or state their p values and say you moved forward with the most apparent one.

As suggested, we added “ns” to figures to mark the treatments that are not statistically significant from control.

Fig 1B: I do not see a description of the DN plasmids in the text. How was this assay done? Are these GFP-tagged and only GFP cells counted? Were all DN constructs expressed at similar levels?

Yes, we transiently expressed GFP-tagged DN plasmids and then counted and analyzed only GFP-positive cells. In all cases we only analyzed cells expressing low to moderate levels of GFP to avoid defects caused by very high GFP-DN over-expression. We added explanation to Method section.

Fig. 1C & 1D: I hate western blots, but I would love to see some western blots. The rescue is incomplete in 1C, which is fine, but why? What is the KD efficiency? What is the expression of GFP-Rab14 relative to endogenous?

We have added a western blot showing the efficiency of Rab14 KD (Supplemental Figure 1A). It is a bit more complicated to measure levels of GFP-Rab14 relative to endogenous Rab14 since for this assay we used transient expression. Since we get about 35% transfection efficiency and only analyze cells that express low-to-moderate levels of GFP-Rab14 simply doing a western blot on the entire population would not be that informative. Considering that endogenous Rab14 appears to be expressed at moderate levels it is safe to say that GFP-Rab14 is likely expressing several fold over endogenous Rab14 levels.

Fig 2: How do the authors define "Division Time"? "Starting at metaphase" means starting as soon as the metaphase plate is apparent? More importantly, how is dissolution of the bridge defined? This is such a small narrow structure that at lower resolution in between two cells is difficult to identify in every frame. A bit more about the criteria and methods here would be helpful.

We expanded methods section to include a detailed explanation of how this analysis was done.

Fig 2: Does LatA rescue the division time? Does LatA rescue the actin levels at the furrow?

We did not test whether LatA rescue division time. The problem with this experiment is that LatA may also affect the time of cytokinetic actomyosin contractile ring formation and contraction. Thus, any changes in division time would be very hard to interpret.

As far the levels of f-actin in the ICB, it has now been shown by several other studies (especially from Dr Echard’s lab) that LatA treatment does decrease f-actin levels at the
ICB, thus, rescuing the defects caused by Rab11 and Rab35 KDs and KOs, since both of these Rabs are also known to regulate f-actin disassembly in the ICB. We have cited those papers in the manuscript.

Fig 2: Do the number of aborted cytokinesis events in their live-cell imaging support the data from the multinucleation assay? Does failed abscission fully account for Rab14-KO/KD-mediated multinucleation?

Yes, the percentage of divisions that lead to regression of the furrow (about 17%, stated in the text) matches multinucleation (~20% in cells overexpressing DN mutants, ~15% in rab14-KD cells and ~18% in rab14-KO cells; see Figures 1 and 2).

Coloring: What is MBoC policy on green/red combos? I would argue that any single channel imaging be in grayscale instead of green on black (and I fully advocate for inverting the grayscale if the authors agree).

As suggested, we switched to grayscale whenever single channel is shown.

Fig 3A: please label the insets.

Labels have been added.

What is the n value on the IP/MS that identified MACF2? Was it done just once? Are these spectral counts high enough to trust?

Yes, the MS has been done once. The spectral count of 2 is not very high but is not uncommon in immunoprecipitation/proteomics experiments. To make sure that the binding is real we have glutathione bead pull-down and immunoprecipitation experiments to confirm the binding (see Figure 5B). Importantly, binding is enhanced in GTP-bound Rab14, as would be expected if MACF2 is Rab14 effector proteins. This GTP-dependency further confirms that binding is real.

Fig 6: is Fip1 going into the nucleus in MACF1 KD? In the quantitative data, why is the nuclear signal similar to that in the cytoplasmic?

No, Fip1 does not go to nucleus as clear from images in Figure 5C. The quantification is derived from line scan analysis of four different cells. Each cell does have a slightly different nucleus positioning, thus, when Fip1 signal is scattered, as it is in MACF2-KDs, that averages out to lack of dip in position where nucleus would be.

Fig S3: The yes/no quantitation of FIP3 localization upon MACF2 KD is unsatisfying. Is it possible to put some more informative numbers to this?

FIP3 localization at the ICB is very hard to quantify since many labs (including ours) have shown that Fip3 is very dynamic. For example, during most of mitosis it associates with centrosomes and only moves to the ICB at late telophase and this ICB
accumulation contributes to triggering abscission. Consequently, accumulation of FIP3 at the ICB is a transient event that is best analyzed by time-lapse microscopy. Unfortunately, it is hard to quantify the amount of FIP3 at the ICB during this transient translocation. As the result, we simply report in how many cells we observe this FIP3 translocation to the ICB.

Discussion: "Rab14 is the only MB-associated Rab that is required for abscission"; I think this is perhaps a stretch considering the authors state they did not feel comfortable knocking down Rab5 and 7, which were also in the MB pool.

As suggested, we removed this statement.

Why were some experiments done on collagen and some on fibronectin?

All of the experiments were done in two different labs, one at University of Colorado and another at Lithuanian University of Health Sciences. Thus, each lab used different coverslip coating approaches. We did not realize about this difference until starting to put manuscript together. Our apologies for that. Importantly, we have no reason to believe that coating slides with collagen or fibronectin led to any differences in data.
RE: Manuscript #E20-09-0607R  
TITLE: “Rab14/MACF2 Complex Regulates Endosomal Targeting During Cytokinesis”

Monitoring Editor (Remarks to Author):

Dear Rytis,

I have received some comments from the previous reviewers after your revisions and I also agree that the graphs need attention and standard deviations needs addressing before publication.

Once I receive these back from you we can move forward. Please revise your manuscript, figure, methods/data analysis and upload a revised manuscript once you are finished.

I appreciate your patience and I look forward to your revised manuscript, especially the figures and data analysis.

Sincerely,
Ahna Skop
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Prekeris,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.
Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Revised manuscript "Rab14/MACF2 complex regulates endosomal targeting during cytokinesis" by Gibieza et al.

Most of my concerns have been addressed. However there are still 3 points which need correcting before it would be acceptable for publication.

1. Different statistical tests need to be used in some graphs, where more than two conditions are compared. It is possible that in some cases this might change the conclusion from the graph. Previously, p values were stated but the tests used to calculate those p values were not stated anywhere. Now the authors did add a statement to methods, saying that a t-test was performed on all normally distributed datasets, and M-W U test on non-normally distributed data. However, a t-test can compare one continuous variable between two groups. If three or more groups are compared, ANOVA should be used, because of the risk of Type I errors when multiple t-tests are used. For non-Gaussian distributions, Use the Mann-Whitney test only to compare two groups. To compare three or more groups, use the Kruskal-Wallis test followed by post tests. It is not appropriate to perform several Mann-Whitney (or t) tests, comparing two groups at a time. Reference: GraphPad website, for example: https://www.graphpad.com/guides/prism/latest/statistics/stat_checklist_unpairedttest.htm

2. In Figure 5F, the new graph added has a y axis that says "actin enrichment", when it is supposed to be showing campsap3 quantification. Not sure if this is a data error or a labeling error.
3. In images in Fig 1 and Fig 6 there are some frames with an arrow labeled "furrow" when what is pointed to is actually a midbody, after furrow ingression has already been completed.

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my comments, but unfortunately there are still some issues with their graphs that need to be corrected before publication.

In the graphs in Fig. 2 B and 2C, the individual data points do not match the number of cells. For example, in the control in 2B the authors indicated that they filmed 75 cells, but only 6 data points are shown on the graph. As each cell is an independent experiment, the data points should match the number of cells in the graphs in 2B and 2C.

Standard deviations are not shown in the graphs in Figs 1C, 5F and 6B. In 6B the mean lines are not visible for the control and KD2. As I suggested, this can be easily fixed by using different colors and/or shapes for the data points.
Reviewer #1:

1) Different statistical tests need to be used in some graphs, where more than two conditions are compared. It is possible that in some cases this might change the conclusion from the graph. Previously, p values were stated but the tests used to calculate those p values were not stated anywhere. Now the authors did add a statement to methods, saying that a t-test was performed on all normally distributed datasets, and M-W U test on non-normally distributed data. However, a t-test can compare one continuous variable between two groups. If three or more groups are compared, ANOVA should be used, because of the risk of Type I errors when multiple t-tests are used. For non-Gaussian distributions, Use the Mann-Whitney test only to compare two groups. To compare three or more groups, use the Kruskal-Wallis test followed by post tests. It is not appropriate to perform several Mann-Whitney (or t) tests, comparing two groups at a time. Reference: GraphPad website, for example: https://www.graphpad.com/guides/prism/latest/statistics/stat_checklist_unpairedttest.htm

We fully agree with the reviewer. Whenever more than two groups are compared we re-run the test using ANOVA. The new analysis did not change any conclusions, since all significant differences were re-confirmed. We added explanation about usage of ANOVA in Method section.

2) In Figure 5F, the new graph added has a y axis that says "actin enrichment", when it is supposed to be showing campsap3 quantification. Not sure if this is a data error or a labeling error.

Fixed. Our apologies for mistake.

3) In images in Fig 1 and Fig 6 there are some frames with an arrow labeled "furrow" when what is pointed to is actually a midbody, after furrow ingression has already been completed.

In Figures 1 and 6 Term “furrow” was replaced with “midbody”.

Reviewer #2:

1) In the graphs in Fig. 2 B and 2C, the individual data points do not match the number of cells. For example, in the control in 2B the authors indicated that they filmed 75 cells, but only 6 data points are shown on the graph.

Sorry for the confusion but the dots represent means from each independent experimental repeat (that included multiple cells). n – represents total number of cell analyzed. We re-wrote figure legend to make that clear.

2) Standard deviations are not shown in the graphs in Figs 1C, 5F and 6B. In 6B the
mean lines are not visible for the control and KD2. I suggested, this can be easily fixed by using different colors and/or shapes for the data points.

*Fixed.*
RE: Manuscript #E20-09-0607RR  
TITLE: "Rab14/MACF2 Complex Regulates Endosomal Targeting During Cytokinesis"

Dear Rytis,  
The revisions of the figures and manuscript has been sufficiently been made. Thank you for making your figures and data analysis more clear to the readers. We are honored to published your work in MBoC.

Sincerely,
Ahna Skop  
Monitoring Editor  
Molecular Biology of the Cell

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Dear Dr. Prekeris:  

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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