JCB

STED imaging of endogenously tagged ARF GTPases reveals their distinct nanoscale localizations

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July 14, 2022

Re: JCB manuscript #202205107

Prof. Francesca Bottanelli
Freie Universität Berlin
Thieallee 63
Berlin 14195
Germany

Dear Prof. Bottanelli,

Thank you for submitting your manuscript titled "Gene editing and super-resolution microscopy reveal multiple distinct roles for ARF GTPases in cellular membrane organization." The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We also asked reviewers to assess whether the manuscript would be a good fit for the Article format or more suitable in the Tool format. We invite you to submit a revision as a Tools paper if you can address the reviewers' key concerns, as outlined here.

You will see that reviewers feel the work is technically sound and that the cell lines with endogenously tagged ARFs could be important reagents for future studies dissecting the mechanisms of their functions. However, reviewers also express concerns regarding several conclusions that are not well supported by the data as well as inconsistencies in the appearance of ERGIC53-positive compartments in different figures. There are also requests to confirm that the tagged ARFs are functional and to validate the STED colocalization methodology. We feel these requests are fair and should be addressed in full. The remaining comments seem to be fairly minor requests for additional information and clarifications that we do not believe will require additional experiments.

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

GENERAL GUIDELINES:

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

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

Abstract and title: The summary should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

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Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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

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

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

Sincerely,

Elizabeth Miller, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

The manuscript by Wong-Dilworth et al from the Botanelli lab reports an approach for localizing endogenous ARF GTPases (ARF1,3,4&5) within the secretory pathway. Importantly, the entire work relies on endogenous tagging of ARF GTPases (often cell lines with two endogenously tagged markers were used). Also, the markers of different compartments such as beta-COP and ERGIC-53 were CRISPR engineered. The imaging was performed using STED microscopy and therefore shows the localization of all of these proteins below the diffraction limit. Overall, the data are of really high quality and the manuscript is well presented. My only concern is that the conceptual advance we gain for this work is limited. I am not sure whether this manuscript was submitted for publication as a regular article, or as a "Tool" article. I definitively recommend considering it as a Tool paper. For a regular original article, I think that the conceptual advance we gain from this work is rather limited and as such, I think it does not fit as a JCB paper. For instance, the work does not experimentally test conclusions based on their localization data. Many things remain unclear such as the role of ARF1 and ARF4 in bidirectional trafficking, the nature and biological role of Golgi-attached ERGIC elements. Are these anterograde carriers and are the ARFs that are found on them promoting the maturation of ERGIC elements towards the Golgi? Do these ERGIC elements contain SNARE molecules that support their role as anterograde carriers? These and other questions remain open. I am not saying that the authors need to test every possible conclusion, but at least some further exploration is definitively necessary. Therefore, I support the publication of this work as a Tool article, but not as a regular JCB paper. However, the decision whether to consider it a Tool article or not has to be made by the authors and the Editor. Apart from the issue of novelty, I have only few remarks on the text:

1- The authors write about Figure 2 that "COPI clusters are observed at the outermost rim of the cis-Golgi". I think that this needs to be reworded. Firstly, this is not evident at all from the image show. Secondly, it is not possible to make this statement based on a single confocal section. Rather, it requires 3D imaging of the Golgi and the COPI associated with it. I don't think that any further experiment is necessary, but only a re-wording. Why don't the authors write something like COPI appeared to form puncta (or clusters) around the Golgi ribbon. The same applies to text about Figure 5.

2- The statement that Golgin97 is further away from COPI clusters than the cis-Golgi should be supported by quantification.

3- In Figure 2E, I can see several places where ERGIC-53 positive elements perfectly colocalize with GM130. Is this ERGIC-53 present in the cis-Golgi?

4- This is purely linguistic, but the term "peripheral" ERGIC is more appropriate than "distal". In trafficking, "distal" usually refers to something that is downstream along the secretory pathway.

5- Figure 2I: again, the text describing the image does not fit with what I see. I don't see COPI and clathrin on the curved rim of the cisternae.

6- Normally, I leave it to the authors to choose the references they want to cite, unless I think that an important/relevant paper was missed. The paper from the Hauri lab, showing that depletion of ARF1+ARF4 results in tubulation of the ERGIC, is not cited. I think this is relevant for the current work and should be referred to.

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

In this study by Wong-Dilworth et al., the authors generated endogenously tagged ARF cell lines and then performed both fixed and live-cell STED microscopy using several Golgi markers.

They find that the different ARF genes occupy different localizations, with ARF1,3 enriched at trans compartments and ARF1,4,5 enriched at cis and ERGIC compartments. Furthermore, they find that even those ARFs that localize to the same compartment appear to occupy separate subdomains.

The results largely agree with previous reports but there are some interesting new details, such as the segregation of different ARFs into adjacent, but separate, subdomains. In principle the reagents reported here could provide a benefit to the field, to be used in future studies dissecting the mechanism and implications of the observed differential localizations. However I have several technical concerns that I think are important to address if these reagents will be for future mechanistic dissections.

Major points:
1. Previous studies have shown that it is quite difficult to tag ARF proteins without disrupting their function. For example, see Pubmed ID 20214751 (Jian, Kahn et al). The authors' own results with ARF3 highlight this possibility, as a longer tag was needed to see localization to the Golgi. The authors do try to test the function of their fusion proteins, and find that their tags do not interfere with cell growth, or COPI or clathrin recruitment (Fig. S2), but the authors do not report whether these cellular behaviors are perturbed by knockout of any individual ARF gene. Given the known redundancy in the function of the Golgi ARF genes, it is possible that single ARF gene deletions grow fine, and have normal COPI and clathrin recruitment. In which case, using these behaviors as phenotypes to test the function of their individually tagged Arf proteins is not sufficient to discern whether they are functional or not. And in that case, perhaps the authors could use the known phenotypes of double-knockouts to help them more rigorously validate their knock-in constructs.

2. It seems important for the authors to validate their STED colocalization approach by using two fluorophores to label the same protein and observe the amount of co-localization, with the expectation that it would be nearly complete. Otherwise it is hard to know if the observed subdomain localization of the closely related ARFs might be an artifact of the imaging approach.

3. The appearance of the ERGIC53-positive membrane compartments seems very different in Figure 4b compared to Figure 4a. Why is this?

4. Figure 5 is used to assert that ARF4 colocalizes better with COPI than does ARF1 but I do not find this convincing. In the zoomed-out portion of Figure 5a, I can see plenty of overlap between ARF1 and COPI, and in the zoomed-out portion of Figure 5b, I can see plenty of COPI structures that do not have ARF4. Furthermore, in 5c,d, distance measurements are presented but it seems to me that the 3D nature of the compartments prevents drawing conclusions based on small differences in 2D distance measurements, as these may not reflect the true distances in 3D. Is there a better method for quantifying the overlap? (like Manders?)

Minor points:
5. The authors state in the introduction: "The observation that ARF pairs needed to be depleted from cells to yield a trafficking defect (Volpicelli-Daley et al., 2005) lead to the hypothesis that ARFs may act as heterodimers, rather than acting redundantly." However, unless I am mistaken, in Volpicelli-Daley et al. they did not propose that Arfs act as heterodimers. Furthermore, if two genes are redundantly required, it is very unlikely that they would act as a heterodimer, as disrupting either single component would usually disrupt the function of a heterodimer.

6. The authors state that "ARF4 and ARF5 exclusively localize to segregated nanodomains on the ERGIC (Supplementary figure 5b)" - but there is significant overlap of ARF4 and ARF5 in the top part of the panel showing the imaging data, so therefore I think they are not *'exclusively'* localized to segregated nanodomains?

7. The authors state "ARF4 could contribute to anterograde flow or provide an early recycling platform from the ERGIC to the Golgi (Figure 4f)." But isn't it equally likely, or even more likely, that ARF4 on the ERGIC would be producing COPI vesicles for

returning cargos to the ER?

8. The different ARF genes are sometimes referred to as "isoforms" by the authors, but I think generally 'isoforms' refers to different splice-forms of the same gene, and 'paralogs' is better used to refer to similar genes?

9. I found it initially confusing that imaging data and quantification were sometimes presented adjacently but with the ARF genes in reverse order (i.e. Figure 5c, ARF1 is on top, but Figure 5d, ARF5 is on top).

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

ARF GTPases are master regulators of membrane traffic. Mammalian cells express multiple ARFs that share high sequence identity. ARF1, ARF3, ARF4, and ARF5 are involved in membrane traffic at the Golgi and ER-Golgi interface, but it is currently unclear whether their functions are overlapping or distinct, and what specific processes they may regulate. The manuscript by Wong-Dilworth et al. aims to clarify the physiological roles of these ARFs by investigating their endogenous localization, and by determining their spatial relationships with Golgi traffic components.

The main methodology is STED microscopy of HeLa cells edited via CRISPR-Cas9 to endogenously tag different ARFs with Halo, SNAP, or ALFA tags. ARFs were imaged individually or in pairs in fixed or live cells, and their relative positions to one another and other markers (ERGIC, Golgi, or TGN) were quantified. Endogenous labeling of these proteins is an important methodological advance that overcomes pervasive artifacts due to protein overexpression. Critically, the authors validated the functionality of the labeled ARFs in a haploid cell line, further demonstrating that their tagging strategy is robust and effective.

Overall, the data strongly support the conclusions regarding different localizations and roles in Golgi membrane traffic for the various ARFs tested. The figures are well constructed and clear, and the microscopy data are of very high quality. Graphical summaries present in many of the figures are appreciated. The authors analyzed a complex system about as well as could be expected with current methods. However, I have some technical comments.

1) My main concern stems from how images were analyzed and quantified. ARF-positive structures are pleomorphic and display varying degrees of co-staining, so it is unclear what criteria were used to select the group of structures that ultimately made it into quantifications¬¬. Are all the structures in a cell being quantified? Or only those that show some degree of overlap? There are staining heterogeneities throughout the Golgi (for example Fig. 3 C & D). If the entire Golgi isn't being quantified, how are specific areas selected in an unbiased manner? Is there a randomization step when selecting mini-stacks for quantification?

2) The experiment in Fig. 4 A & B is hard to interpret due to ¬the strikingly different staining quality of SNAP-ERGIC53 in panel A vs. panel B. There appears to be much higher levels of ERGIC53 in panel B, which could have artificially amplified the degree of association between ARF4 and ERGIC53. In fact, the SNAP-ERGIC53 stain looks radically different from previous images presented in Figs. 2 and 3. If endogenous ERGIC53 cannot be visualized in this experiment (via an ERGIC53-Fluorescent Protein KI in a triple KI line), then cells expressing similar levels of SNAP-ERGIC53 should be used to compare the association with ARF1 or ARF4. Moreover, the quantification of structures that co-label with ARF1 and ARF4 is somewhat misleading (Fig. 4 C & D). The authors refer to these structures as ERGIC elements that contain both markers, but in that experiment ERGIC53 wasn't visualized altogether with ARF1 and ARF4, so it is not certain that those structures are actually ERGIC.

3) I don't totally understand the conclusion from Fig.5 regarding the "enrichment" of ARF4/5 in COPI structures. In intact ribbons, the insets from Fig. 5 A & B do not look qualitatively different. It's unclear what the authors mean by "enrichment" in this context.

4) There is an error in the title preceding Fig. 6. "Type II ARFs ARF1 and ARF3 are the sole ARFs localizing to TGN membranes". Those ARFs are Type I.

5) COPI has previously been reported by EM to be present throughout the Golgi stack except on the trans-most cisterna. Can those results be reconciled with what is seen here? Is COPI simply much more concentrated on the cis-Golgi and Golgi-associated ERGIC than on distal Golgi compartments?

6) The preponderance of evidence now argues against GRASP65 and GM130 being stacking factors.

Beyond these technical issues, it must be acknowledged that although the current study represents a careful, thorough description of ARF localization, it does not directly test mechanism. This rigorous contribution will provide an important basis for further mechanistic studies, but the JCB editor will need to weigh whether it is the right fit.

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01.10.2023

Dear Dr. Miller and Dr. Simon,

We are resubmitting a revised version of our manuscript "**Gene editing and super-resolution microscopy reveal multiple distinct roles for ARF GTPases in cellular membrane organization**" for consideration as a Tool article at JCB.

We thank all the reviewers for their comments and for carefully reading our manuscript. We believe we have thoroughly addressed all the points raised by the reviewers. Their criticisms have led to a more solid version of the manuscript that addresses concerns about 1) the functionality of endogenously tagged ARF GTPases and 2) the limitations of super-resolution STED microscopy.

In particular, we have noticed that non-homogeneous labelling of elongated tubular structures is observed in a control sample where the same protein (ARF1-Halo) was labelled with both dyes used for multi-color live-cell STED (Fig. S6 E). After various troubleshooting sessions, we concluded that this is an artifact caused by a slight chromatic shift in the Z direction of the objective, which we cannot control or correct (see answer to reviewer 2 for a more detailed explanation). Unfortunately, we have missed this artifact because our earlier control experiments have been carried out with vesicular markers (COPI and clathrin), where the chromatic aberration is not apparent (Fig. S6 F), which is why we missed this information. We appreciate that reviewer 2 pushed us to perform even more control experiments, as this has had and will continue to have a positive impact on our downstream research. While we think that the segregation between ARF1 and ARF3 on tubules is more striking in the ARF1+ARF3 sample than in the control, we believe we cannot quantitatively support this observation. We have therefore removed the conclusion that ARF1 and ARF3 are segregated on post-Golgi tubules from the revised manuscript. We continue to pursue understanding the functional differentiation between ARF1 and ARF3 with complementary biochemical approaches which are better suited.

Below you will find a detailed answer to all the reviewers' comments.

All changes in the text are highlighted by track changes:
Changes in figures:
Figure 1: better labelling of the Y axes in panel
Figure 2: distal changed to peripheral in panel I
Figure 3: distal changed to peripheral in panel K
Figure 4: images in panel A and B changed as requested by the reviewers. Quantification in D repeated as requested by the reviewers.
Figure 5: order of the images in C inverted as suggested by the reviewers
Figure 7: order of images in E inverted as suggested by the reviewers
Figure 8: selected crops and graphical summary changed
Figure S1: order of images changed (ARF1 is now on top)
Figure S2: panels C,D,H and I added with more control experiments as suggested by the reviewers
Figure S3: quantification added in panel H and I as suggested by the reviewers
Figure S5: panel C shows new control experiments performed as suggested by the reviewers
Figure S6: Old supplementary figure 6 is now panel A in S6. Old supplementary figure 7a and b are now panel B and C in S6. S6D is the new source data for the quantification in figure 4D where ERGIC53 was expressed in the ARF1/ARF4 double KI cell line. Panel E and F are new control experiments suggested by the reviewers.
Looking forward to hearing back from you,

Sincerely,

Francesca Bottanelli

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

The manuscript by Wong-Dilworth et al from the Botanelli lab reports an approach for localizing endogenous ARF GTPases (ARF1,3,4&5) within the secretory pathway. Importantly, the entire work relies on endogenous tagging of ARF GTPases (often cell lines with two endogenously tagged markers were used). Also, the markers of different compartments such as beta-COP and ERGIC-53 were CRISPR engineered. The imaging was performed using STED microscopy and therefore shows the localization of all of these proteins below the diffraction limit. Overall, the data are of really high quality and the manuscript is well presented. My only concern is that the conceptual advance we gain for this work is limited. I am not sure whether this manuscript was submitted for publication as a regular article, or as a "Tool" article. I definitively recommend considering it as a Tool paper. For a regular original article, I think that the conceptual advance we gain from this work is rather limited and as such, I think it does not fit as a JCB paper. For instance, the work does not experimentally test conclusions based on their localization data. Many things remain unclear such as the role of ARF1 and ARF4 in bidirectional trafficking, the nature and biological role of Golgi-attached ERGIC elements. Are these anterograde carriers and are the ARFs that are found on them promoting the maturation of ERGIC elements towards the Golgi? Do these ERGIC elements contain SNARE molecules that support their role as anterograde carriers? These and other questions remain open. I am not saying that the authors need to test every possible conclusion, but at least some further exploration is definitively necessary. Therefore, I support the publication of this work

as a Tool article, but not as a regular JCB paper. However, the decision whether to consider it a Tool article or not has to be made by the authors and the Editor. Apart from the issue of novelty, I have only few remarks on the text:
We thank the reviewer for the constructive comments and understand their reasoning. We will be submitting the article as a tool article. We omitted going in depth into the ARF1 and ARF4/5 story, as we have a detailed manuscript in preparation for further research on the topic. We are very excited about multiple publications diving into the mechanisms of ARF function coming from this work. We would also like to clarify that, while all other markers were indeed gene-edited, that was not the case for the ERGIC53 marker, which was expressed transiently.

1- The authors write about Figure 2 that "COPI clusters are observed at the outermost rim of the cis-Golgi". I think that this needs to be reworded. Firstly, this is not evident at all from the image show. Secondly, it is not possible to make this statement based on a single confocal section. Rather, it requires 3D imaging of the Golgi and the COPI associated with it. I don't think that any further experiment is necessary, but only a re-wording. Why don't the authors write something like COPI appeared to form puncta (or clusters) around the Golgi ribbon. The same applies to text about Figure 5.
We have re-written the text as suggested (line 147 and 295-296)

2- The statement that Golgin97 is further away from COPI clusters than the cis-Golgi should be supported by quantification.
We have now quantified the distance between COPI and clathrin versus cis- and trans-Golgi markers (Fig. S3).

3- In Figure 2E, I can see several places where ERGIC-53 positive elements perfectly colocalize with GM130. Is this ERGIC-53 present in the cis-Golgi?
Indeed, we do observe some ERGIC53 co-localizing with GM130, which could be due to 1) ERGIC53 transiting through the cis-Golgi, particularly when ERGIC53 is overexpressed; and/or 2) the fact that the ribbon is highly tridimensional, making it hard to clearly separate the two structures due to poor Z resolution of 2D STED microscopy. To overcome the 2nd issue, all quantifications were done on favorably oriented side-view ministacks, which show a clear segregation between ERGIC53 and the cis-Golgi (Fig. S3 A). 3D STED on whole Golgi ribbons was also attempted, but the loss of resolution in the X-Y when performing 3D STED experiments was unfortunately deleterious to the overall quality of the images.

4- This is purely linguistic, but the term "peripheral" ERGIC is more appropriate than "distal". In trafficking, "distal" usually refers to something that is downstream along the secretory pathway.
We have changed the term “distal” to “peripheral”.

5- Figure 2I: again, the text describing the image does not fit with what I see. I don't see COPI and clathrin on the curved rim of the cisternae.
We have re-written the text (line 167). However, while the whole Golgi ribbon images are harder to interpret, the nocodazole data (Fig. S3 C) clearly shows vesicular structures in focus at the edges of the disk-shaped structures, which is the cis-Golgi defined by GM130. This observation strongly suggests a rim localization.

6- Normally, I leave it to the authors to choose the references they want to cite, unless I think that an important/relevant paper was missed. The paper from the Hauri lab, showing that depletion of ARF1+ARF4 results in tubulation of the ERGIC, is not cited. I think this is relevant for the current work and should be referred to.
We agree with the reviewer that the article by Ben-Tekaya et al., 2010 is a relevant reference and have added it to the discussion (lines 453-456). We have also added a sentence to integrate the reference in the discussion.

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

In this study by Wong-Dilworth et al., the authors generated endogenously tagged ARF cell lines and then performed both fixed and live-cell STED microscopy using several Golgi markers.

They find that the different ARF genes occupy different localizations, with ARF1,3 enriched at trans compartments and ARF1,4,5 enriched at cis and ERGIC compartments. Furthermore, they find that even

those ARFs that localize to the same compartment appear to occupy separate subdomains.

The results largely agree with previous reports but there are some interesting new details, such as the segregation of different ARFs into adjacent, but separate, subdomains. In principle the reagents reported here could provide a benefit to the field, to be used in future studies dissecting the mechanism and implications of the observed differential localizations. However I have several technical concerns that I think are important to address if these reagents will be for future mechanistic dissections.
We understand the technical concerns of the reviewer, and we thank them for the thoughtful review. We have included more controls to show that Halo-tagged ARFs do not show the same defect of the KO ARF cell lines (see answer to 1 below). We have now included imaging controls to show that co-localization is observed when using two secondary antibodies targeting the same primary antibody (Fig. S5 C) and two dyes labelling the same Halo target (Fig. S6, E and F). However, for the live-cell STED control, heterogeneities between the two labels were observed on elongated tubules, weakening one of the conclusions about the segregation of ARF1 and ARF3 on post-Golgi tubules (Fig. S6 E). We have performed multiple controls using fixed, live-cell samples and beads, and we have reached the conclusion that these heterogeneities are due to a small chromatic shift in the Z direction between the two channels that we cannot correct. This shift, unfortunately, affects elongated structures in STED mode; however, this effect appears negligible in confocal mode and on punctae-like structures (Fig. S6 F). Unfortunately, this was not caught because, thus far, the control experiments of the kind carried out in Figure S6 E had only been carried out on vesicular markers like COPI and clathrin—where the shift is negligible and overlap is always observed.

Major points:
1. Previous studies have shown that it is quite difficult to tag ARF proteins without disrupting their function. For example, see Pubmed ID 20214751 (Jian, Kahn et al). The authors' own results with ARF3 highlight this possibility, as a longer tag was needed to see localization to the Golgi. The authors do try to test the function of their fusion proteins, and find that their tags do not interfere with cell growth, or COPI or clathrin recruitment (Fig. S2), but the authors do not report whether these cellular behaviors are perturbed by knockout of any individual ARF gene. Given the known redundancy in the function of the Golgi ARF genes, it is possible that single ARF gene deletions grow fine, and have normal COPI and clathrin recruitment. In which case, using these behaviors as phenotypes to test the function of their individually tagged Arf proteins is not sufficient to discern whether they are functional or not. And in that case, perhaps the authors could use the known phenotypes of double-knockouts to help them more rigorously validate their knock-in constructs.

Pennauer et al., 2021(https://doi.org/10.1083/jcb.202106100) observed specific phenotypes in ARF KO cell lines:
- ARF1 KO = reduced recruitment of vesicle coats
- ARF4 KO = defect in KDEL dependent retrograde transport
- ARF1/4 and ARF4/5 double KO were not viable

Based on these observations, the following controls were already included (Figure S2 panel E-G) or added to Figure S2
- COPI and clathrin recruitment is not altered in ARF1 KI cells (panels E-G)
- KDEL receptor localization is not altered in ARF4 KI (panel C and D)
- ARF1/4 and ARF4/5 double KI are viable and do not show any discernable phenotypic or growth defect (panel H and I)

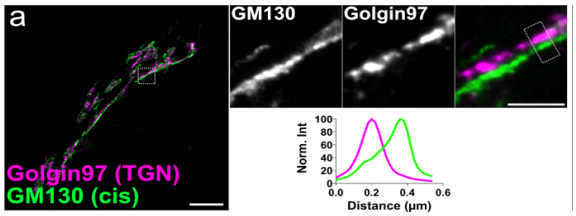
The text was changed accordingly to include the new data (120-127).

2. It seems important for the authors to validate their STED colocalization approach by using two fluorophores to label the same protein and observe the amount of co-localization, with the expectation that it would be nearly complete. Otherwise it is hard to know if the observed subdomain localization of the closely related ARFs might be an artifact of the imaging approach.
In Figure S5 we show co-localization of both STED channels (Alexa Fluor 594, Atto647N) in fixed cells when ARF1-2ALFA is labelled with a single anti-ALFA antibody and two primaries conjugated to Alexa Fluor 594 or Atto647N. Multiple boxes were drawn to obtain profiles to show the co-localization. The co-localization is stricking when compared with the double KI samples in panel A and B where segregating nano-domains are observed.
In Figure S6 E, we show co-localization of the two labels in images of ARF1-Halo simultaneously labelled with JFX650-Halo and JF571-Halo. The crops highlight heterogeneous labelling of elongated tubular

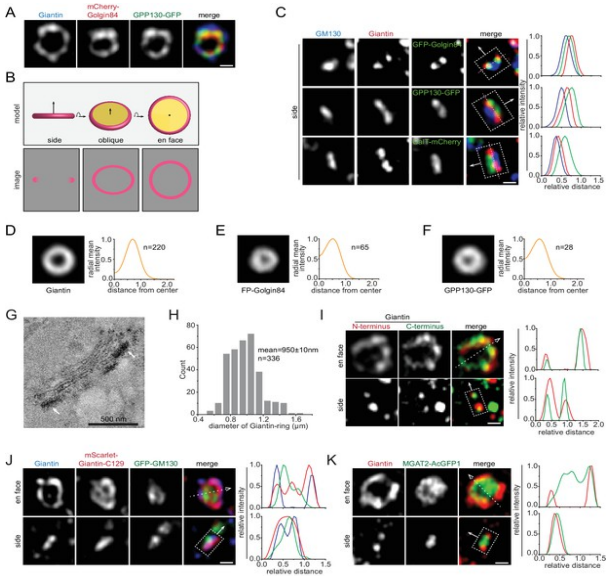
structures as explained above. On the other hand, the observed chromatic shift on peripheral vesicular structures was only ~ 30 nm.
The text has been re-written to include an explanation of the two control experiments (205-207 and 258-260). Additional quantification of the chromatic shift is shown in Figure S6 F and is negligible in comparison to the average distance between ARF1 and ARF4 nano-domains on peripheral ERGICs. Figure 8 was changed accordingly, and the results and discussion have been rephrased.

3. The appearance of the ERGIC53-positive membrane compartments seems very different in Figure 4b compared to Figure 4a. Why is this?
This was a concern that was also shared by reviewer 3. We believe that the discrepancies were due to different expression levels of the marker SNAP-ERGIC53. We have repeated the experiment and picked representative cells showing comparable levels of expression (see new Figure 4). However, we would like to point out that the final conclusion drawn from the data does not change. (ARF1 is excluded from Golgi-associated ERGICs and only defines a subset of peripheral ERGICs, while ARF4 has a greater overlap with the ERGIC marker.)

4. Figure 5 is used to assert that ARF4 colocalizes better with COPI than does ARF1 but I do not find this convincing. In the zoomed-out portion of Figure 5a, I can see plenty of overlap between ARF1 and COPI, and in the zoomed-out portion of Figure 5b, I can see plenty of COPI structures that do not have ARF4. Furthermore, in 5c,d, distance measurements are presented but it seems to me that the 3D nature of the compartments prevents drawing conclusions based on small differences in 2D distance measurements, as these may not reflect the true distances in 3D. Is there a better method for quantifying the overlap? (like Manders?)
We agree with the reviewer that quantification of a tridimensional structure as the Golgi is extremely complicated. Various quantification methods have been tested over the years, including the classical co-localization standards (Manders, Pearsons) and various custom-made programs such as a radial distribution program that would tell the user how much signal from A is present within a certain distance from B. Unfortunately, they all failed to yield meaningful measurements. We believe that Manders and Pearsons are not useful when applied to super-resolved structures where the overlap is minimal.
For the quantification, we decided to simplify the geometry of the Golgi by using nocodazole-induced ministacks as it has been done before (see below). The Golgi is a very asymmetrical and tridimensional structure, and any co-localization is hard to quantify. As for the selection of the line profiles shown in the crops, areas where the Golgi unwinds and cisternae are observed side-by-side were picked (as an example Figure 2 A is shown).



As for the selection of the ministacks to be quantified, we selected side-views and en-face ministacks, as has been done before in the Lu lab
Tie et al., 2018 https://elifesciences.org/articles/41301, Figure 1 presented below)
Tie et al., 2016 https://pubmed.ncbi.nlm.nih.gov/26764092/
Tie et al., 2022 https://rupress.org/jcb/article-abstract/221/6/e202109114/213180/Visualizing-intra-Golgi-localization-and-transport



(Figure 1 Tie et al., 2018): En-face ministacks (like in panel K) were picked for quantification of the distance of COPI and clathrin from the cisternae, while side-view ministacks (panel C) were picked to quantify the distance between cisternae. We have added this information in the materials and methods.

We agree that despite the fact that we were able to get a good idea of what to expect in terms of co-localization from whole Golgi images, they are very hard to quantify. Therefore, we have rephrased the statements about the co-localization in whole Golgi ribbon images (Line 303).

Minor points:

5. The authors state in the introduction: “The observation that ARF pairs needed to be depleted from cells to yield a trafficking defect (Volpicelli-Daley et al., 2005) lead to the hypothesis that ARFs may act as heterodimers, rather than acting redundantly.” However, unless I am mistaken, in Volpicelli-Daley et al. they did not propose that Arfs act as heterodimers. Furthermore, if two genes are redundantly required, it is very unlikely that they would act as a heterodimer, as disrupting either single component would usually disrupt the function of a heterodimer.

We thank the reviewer for the constructive comment. We agree that Volpicelli-Daley et al. talks about ARF pairs. We have re-written the sentence “The observation that ARF pairs needed to be depleted from cells to yield a trafficking defect (Volpicelli-Daley et al., 2005) lead to the hypothesis that ARFs may act in pairs”. And indeed, disruption of one component of a heterodimer would disrupt function, unless very closely related ARFs could somehow pick up the function of the missing ARF without causing any visible phenotype.

6. The authors state that “ARF4 and ARF5 exclusively localize to segregated nanodomains on the ERGIC (Supplementary figure 5b)” – but there is significant overlap of ARF4 and ARF5 in the top part of the panel showing the imaging data, so therefore I think they are not *’exclusively’* localized to segregated nanodomains?

We agree with the reviewer and have removed “exclusively” from the text (line 352).

7. The authors state “ARF4 could contribute to anterograde flow or provide an early recycling platform from the ERGIC to the Golgi (Figure 4f).” But isn’t it equally likely, or even more likely, that ARF4 on the ERGIC would be producing COPI vesicles for returning cargos to the ER?

In the text, we indeed suggest that ARF4 could either have a role in transport from the ERGIC to the cis-Golgi or from the ERGIC back to the ER. Further work from the lab (manuscript in preparation) shows that ARF4 (and not ARF1) segregated in carriers containing anterograde ERGIC-to-Golgi directed cargo.

8. The different ARF genes are sometimes referred to as “isoforms” by the authors, but I think generally ‘isoforms’ refers to different splice-forms of the same gene, and ‘paralogs’ is better used to refer to similar genes?

We have changed “isoforms” to “paralogs” throughout the text.

9. I found it initially confusing that imaging data and quantification were sometimes presented adjacently but with the ARF genes in reverse order (i.e. Figure 5c, ARF1 is on top, but Figure 5d, ARF5 is on top).
We have changed the order in Figure 5C and 7F

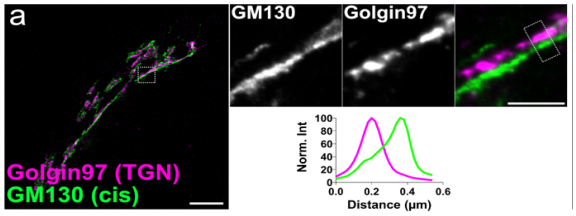
Reviewer #3 (Comments to the Authors (Required)):

ARF GTPases are master regulators of membrane traffic. Mammalian cells express multiple ARFs that share high sequence identity. ARF1, ARF3, ARF4, and ARF5 are involved in membrane traffic at the Golgi and ER-Golgi interface, but it is currently unclear whether their functions are overlapping or distinct, and what specific processes they may regulate. The manuscript by Wong-Dilworth et al. aims to clarify the physiological roles of these ARFs by investigating their endogenous localization, and by determining their spatial relationships with Golgi traffic components.

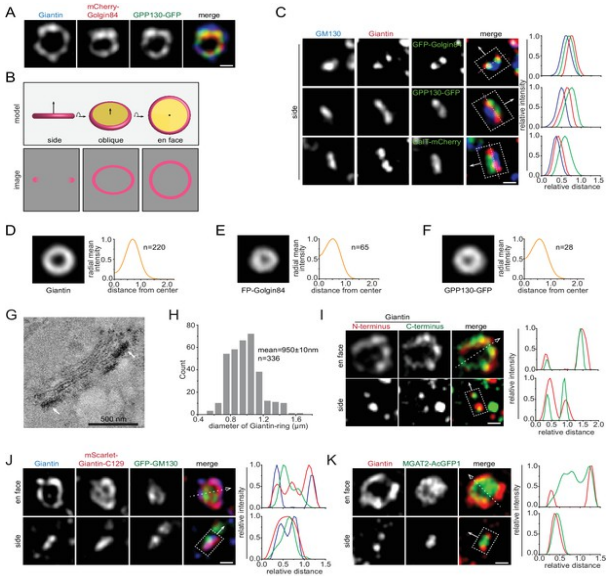
The main methodology is STED microscopy of HeLa cells edited via CRISPR-Cas9 to endogenously tag different ARFs with Halo, SNAP, or ALFA tags. ARFs were imaged individually or in pairs in fixed or live cells, and their relative positions to one another and other markers (ERGIC, Golgi, or TGN) were quantified. Endogenous labeling of these proteins is an important methodological advance that overcomes pervasive artifacts due to protein overexpression. Critically, the authors validated the functionality of the labeled ARFs in a haploid cell line, further demonstrating that their tagging strategy is robust and effective.

Overall, the data strongly support the conclusions regarding different localizations and roles in Golgi membrane traffic for the various ARFs tested. The figures are well constructed and clear, and the microscopy data are of very high quality. Graphical summaries present in many of the figures are appreciated. The authors analyzed a complex system about as well as could be expected with current methods. However, I have some technical comments.

1) My main concern stems from how images were analyzed and quantified. ARF-positive structures are pleomorphic and display varying degrees of co-staining, so it is unclear what criteria were used to select the group of structures that ultimately made it into quantifications¬¬. Are all the structures in a cell being quantified? Or only those that show some degree of overlap? There are staining heterogeneities throughout the Golgi (for example Fig. 3 C & D). If the entire Golgi isn't being quantified, how are specific areas selected in an unbiased manner? Is there a randomization step when selecting mini-stacks for quantification?
We understand the limitation and the concerns of the reviewer. Indeed, the Golgi is a very asymmetrical and tridimensional structure, and any co-localization is hard to quantify. As for the selection of the line profiles shown in the crops, areas where the Golgi unwinds and cisternae are observed side-by-side were picked (as an example Figure 2 A is shown).



As for the selection of the ministacks to be quantified, we selected side-views and en-face ministacks, as has been done before in the Lu lab
Tie et al., 2018 https://elifesciences.org/articles/41301, Figure 1 presented below)
Tie et al., 2016 https://pubmed.ncbi.nlm.nih.gov/26764092/
Tie et al., 2022 https://rupress.org/jcb/article-abstract/221/6/e202109114/213180/Visualizing-intra-Golgi-localization-and-transport



(Figure 1 Tie et al., 2018): En-face ministacks (like in panel K) were picked for quantification of the distance of COPI and clathrin from the cisternae, while side-view ministacks (panel C) were picked to quantify the distance between cisternae. We have added this information in the materials and methods.

We have tried to come up with a way to automatically select the structures to be analyzed, but we have not arrived at a solution due to the very heterogeneous nature of the structures under analysis. We believe that all of the previous publications that quantitatively mapped Golgi proteins faced the same limitation. As such, the structures included in the quantification were also hand-picked. We tried to remove some bias in the selection by having one person pick the ministacks and another person unbiasedly doing the quantification.

2) The experiment in Fig. 4 A & B is hard to interpret due to ¬the strikingly different staining quality of SNAP-ERGIC53 in panel A vs. panel B. There appears to be much higher levels of ERGIC53 in panel B, which could have artificially amplified the degree of association between ARF4 and ERGIC53. In fact, the SNAP-ERGIC53 stain looks radically different from previous images presented in Figs. 2 and 3. If endogenous ERGIC53 cannot be visualized in this experiment (via an ERGIC53-Fluorescent Protein KI in a triple KI line), then cells expressing similar levels of SNAP-ERGIC53 should be used to compare the association with ARF1 or ARF4. Moreover, the quantification of structures that co-label with ARF1 and ARF4 is somewhat misleading (Fig. 4 C & D). The authors refer to these structures as ERGIC elements that contain both markers, but in that experiment ERGIC53 wasn't visualized altogether with ARF1 and ARF4, so it is not certain that those structures are actually ERGIC.
This was a concern that was also shared by reviewer 2. We believe that the discrepancies were due to different expression levels of the marker SNAP-ERGIC53. Additionally, the differences in ERGIC morphology between Figure 2 and 3 are also due to the fact that the cells in Figure 2 and 3 are PFA-fixed cells, in which the tubular-vesicular network of the ERGIC may be disrupted. We have repeated the experiment and picked representative cells showing comparable levels of expression (see new Figure 4). However, we would like to point out that the final conclusion drawn from the data does not change. (ARF1 is excluded from Golgi-associated ERGICs and only defines a subset of peripheral ERGICs while ARF4 has a greater overlap with the ERGIC marker.)
As for the second point, we have now carried out the quantification on double KI ARF1 and ARF4 cells that also express a YFP-ERGIC53 marker, and the quantification has been implemented in Figure 4D.

3) I don't totally understand the conclusion from Fig.5 regarding the "enrichment" of ARF4/5 in COPI structures. In intact ribbons, the insets from Fig. 5 A & B do not look qualitatively different. It's unclear what the authors mean by "enrichment" in this context.
We agree that quantitative conclusions cannot be drawn from the intact ribbon images, and we have re-phrased the sentence stating the enrichment of ARF4 in COPI clusters (lines 303).

4) There is an error in the title preceding Fig. 6. "Type II ARFs ARF1 and ARF3 are the sole ARFs localizing to TGN membranes". Those ARFs are Type I.
Error was corrected.

5) COPI has previously been reported by EM to be present throughout the Golgi stack except on the trans-most cisterna. Can those results be reconciled with what is seen here? Is COPI simply much more concentrated on the cis-Golgi and Golgi-associated ERGIC than on distal Golgi compartments?
We agree with the reviewer that COPI is more concentrated on the cis-Golgi and ERGIC. We have now quantified the distance of COPI clusters from cisternae defined by GM130 and Golgin97 (Figure S3, H and I). While on average, COPI clusters are seen close to the cis-Golgi (panel H), many COPI clusters are also observed closer to the TGN marker Golgin97 (panel I).

6) The preponderance of evidence now argues against GRASP65 and GM130 being stacking factors.
We agree that evidence argues against GRASP65 and GM130 as stacking factors, and we have rephrased the text calling them cisternal markers.

Beyond these technical issues, it must be acknowledged that although the current study represents a careful, thorough description of ARF localization, it does not directly test mechanism. This rigorous contribution will provide an important basis for further mechanistic studies, but the JCB editor will need to weigh whether it is the right fit.

March 6, 2023

Re: JCB manuscript #202205107R

Prof. Francesca Bottanelli
Freie Universität Berlin
Thieallee 63
Berlin 14195
Germany

Dear Prof. Bottanelli,

Thank you for submitting your revised manuscript entitled "Gene editing and super-resolution microscopy reveal multiple distinct roles for ARF GTPases in cellular membrane organization." The manuscript has been seen by two of the original reviewers whose full comments are appended below.

You will see that Reviewer #3 remains supportive regarding the suitability of this study for JCB. However, Reviewer #2 again raises the question of whether the tagged ARFs in the knock-in cell lines are functional and asks for validation by direct comparison with ARF knockouts. We agree that the reviewer's concern here is reasonable because the HAP1 cell line used is different from the HeLa cells used by Pennauer et al. in their analysis of ARF knockouts. While we agree that direct comparison with ARF knockouts in HAP1 cells would conclusively demonstrate functionality and significantly strengthen the study, we don't feel that this is absolutely necessary. In our view, the viability of the double knock-in cells is reasonable evidence of functionality. However, if you are unable to add ARF knockout studies please revise the text to tone down claims of complete functionality. Please also address the comment about statistical analysis of Figure S2.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

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

Sincerely,

Elizabeth Miller, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

The authors claim to have addressed concerns regarding whether their new tagged-ARF tools are functional, but this reviewer disagrees.

The study from the Spiess lab (Pennauer et al.) cited in the rebuttal letter actually reported that beta-COP intensity and localization was unaffected by any ARF knockouts (Figs. 4A and 4C in Pennauer et al.). The Spiess lab paper also did not examine clathrin staining. Therefore the claim in the rebuttal letter that Fig. S2E-G in the authors manuscript, which examine beta-COP and clathrin localization, addresses whether the mutants are functional is misleading.

Even if the authors were examining the same phenotypes that were previously reported by the Spiess lab, the authors have failed to include key controls required to establish the functionality of the tagged ARF protein tools. In Figure S2, the authors never show images or quantification for ARF knockout cells. It is not sufficient to rely upon a previous publication's report of phenotypes. To validate these new reagents the authors need to directly compare the tagged cells to *both* wild-type *and* knockout cells in the same experiment. Function can only be demonstrated by showing that there is a phenotype in knockout

cells that is not displayed by the tagged ARF cell lines. Such an experiment is important in any case, but is especially important because the authors are using a different cell line (HAP1) than was used in the Spiess lab paper (HeLa) they cite in their rebuttal letter.

If the authors are having trouble seeing phenotypes in their own hands with ARF gene knockouts, GM130 intensity/localization (and ratio of GM130 to beta-COP, AP-1, and GGA2) appeared to have the most significant phenotype in the Spiess lab study for ARF gene knockouts (Figs. 2 and 4 in Pennauer et al.).

Furthermore, the data in Fig. S2 has not been statistically analyzed. Once the proper experiments are performed with needed controls, there should also be statistics performed to test significance.

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

The reviewers thoroughly addressed my comments and suggestions. I have no additional comments on the new version of the manuscript.

Freie Universität Berlin

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Institute of Chemistry and Biochemistry
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14195 Berlin

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E-Mail francesca.bottanelli@fu-berlin.de

16.03.2023

Dear Dr. Miller and Dr. Simon,

We are resubmitting a revised version of our manuscript "**Gene editing and super-resolution microscopy reveal multiple distinct roles for ARF GTPases in cellular membrane organization**" for consideration as a Tool article at JCB.

Below you will find a details response to the concerns raised by Reviewer 2.

Word files with track changes are provided.

Looking forward to hearing back from you,

Francesca Bottanelli

The authors claim to have addressed concerns regarding whether their new tagged-ARF tools are functional, but this reviewer disagrees.

We agree with the reviewer that data with KO cell lines would be beneficial. However, we believe that the data on the viability of the double KI cell lines sufficiently supports functionality of the tagged ARFs. We have toned down the conclusions about complete functionality of the tagged ARFs in the text and removed the comparison with Pennauer et al. (Lines 120-124).

The study from the Spiess lab (Pennauer et al.) cited in the rebuttal letter actually reported that beta-COP intensity and localization was unaffected by any ARF knockouts (Figs. 4A and 4C in Pennauer et al.). The Spiess lab paper also did not examine clathrin staining. Therefore the

claim in the rebuttal letter that Fig. S2E-G in the authors manuscript, which examine beta-COP and clathrin localization, addresses whether the mutants are functional is misleading.

Even if the authors were examining the same phenotypes that were previously reported by the Spiess lab, the authors have failed to include key controls required to establish the functionality of the tagged ARF protein tools. In Figure S2, the authors never show images or quantification for ARF knockout cells. It is not sufficient to rely upon a previous publication's report of phenotypes. To validate these new reagents the authors need to directly compare the tagged cells to *both* wild-type *and* knockout cells in the same experiment. Function can only be demonstrated by showing that there is a phenotype in knockout cells that is not displayed by the tagged ARF cell lines. Such an experiment is important in any case, but is especially important because the authors are using a different cell line (HAP1) than was used in the Spiess lab paper (HeLa) they cite in their rebuttal letter.

If the authors are having trouble seeing phenotypes in their own hands with ARF gene knockouts, GM130 intensity/localization (and ratio of GM130 to beta-COP, AP-1, and GGA2) appeared to have the most significant phenotype in the Spiess lab study for ARF gene knockouts (Figs. 2 and 4 in Pennauer et al.).

While we believe that the tools provided by Pennauer and colleagues will be beneficial to understand the function of ARFs in living cells, we think that subtle differences in Golgi shapes could also be due to clonal selection and the fact that only one KO clone was used for downstream phenotypic analysis. A less compact Golgi could give rise to a lower density of coat proteins and an unchanged total amount of coat protein as expressed by the lower coat/GM130 ratio (Figure 4). Additionally, the authors use GM130 staining to assess the volume of the Golgi (Figure 2) and observe an enlarged Golgi in various KO cell lines. However, later do not quantify that the volume is restored to WT levels in their rescue experiments (Figure 6). Therefore, we believe that total amount of recruited coat is an acceptable parameter to assess the functionality and ability of tagged proteins to recruit downstream effectors.

In connection to that, we are currently working with the cell lines from Pennauer et al. and preliminary data shows that gene edited AP-3 dissociates from the membranes in the ARF1 KO cells and expression of tagged ARF1 restores the WT phenotype.

Furthermore, the data in Fig. S2 has not been statistically analyzed. Once the proper experiments are performed with needed controls, there should also be statistics performed to test significance.

To carry out the statistical analysis on Fig. S2, we changed the visualization of the data to show the means of the various replicate experiments together with all the data points representing every single cells. Then, one-way ANOVA (Fig. S2, F and G) and *t* test (Fig. S2 D) were carried out. We have modified Fig. S2, figure legend and methods (Lines 670-672).

March 22, 2023

RE: JCB Manuscript #202205107RR

Prof. Francesca Bottanelli
Freie Universität Berlin
Thieallee 63
Berlin 14195
Germany

Dear Prof. Bottanelli,

Thank you for submitting your revised manuscript entitled "Gene editing and super-resolution microscopy reveal multiple distinct roles for ARF GTPases in cellular membrane organization." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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

2) Figure formatting: Tools may have up to 10 main text figures. Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add a scale bar for the magnifications in Fig. 3A.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments).
*** If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) The title should be less than 100 characters including spaces. Your current title exceeds this limit so we suggest the following title instead: "STED imaging of endogenously tagged ARF GTPases reveals their distinct nanoscale localizations."

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." JCB formatting does not allow for a supplementary methods section, please move all of this material to the main materials and methods section. You do not need to make any edits since the materials and methods section does not count towards the overall character limit.

6) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or

gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. Please add a reference citation for the anti-KDELR1 antibody.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

8) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed. JCB formatting does not allow for supplementary references, please remove this section and add any non-duplicate references to the main reference list.

9) Supplemental materials: Tools generally may have up to 5 supplemental figures and 10 videos. You currently exceed this but we will be able to give you the extra space. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

10) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

11) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

12) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped blot images, please be sure to provide one Source Data file for each figure that contains blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

15) Journal of Cell Biology also requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

16) Materials and data sharing: As a condition of publication, authors must make protocols and unique materials (including, but not limited to, cloned DNAs; antibodies; bacterial, animal, or plant cells; and viruses) described in our published articles freely available upon request by researchers, who may use them in their own laboratory only. All materials must be made available on

request and without undue delay. We strongly encourage to deposit all the cell lines/strains and reagents generated in this study in public repositories.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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