Bro1 stimulates Vps4 to promote Intralumenal Vesicle Formation during Multivesicular Body biogenesis

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March 17, 2021

Re: JCB manuscript #202102070

Dr. David J Katzmann  
Mayo Clinic  
Biochemistry and Molecular Biology  
200 First St. SW  
Rochester, MN 55905

Dear Dr. Katzmann,

Thank you for submitting your manuscript entitled "Bro1 stimulates Vps4 activity to promote Intraluminal Vesicle Formation during MVB biogenesis". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. As you will see, your paper received very positive reviews from all three reviewers. Congratulations!

A few minor questions were raised by Reviewers #1 and #2 that you should address in a revised manuscript or in your response to the reviewers' comments. Reviewer #3 raised an interesting alternative hypothesis that should be relatively easy to address with one additional experiment - "a Western blot that compares the liberation of GFP from GFP-Cps1 with that from GFP-Ub-Cps1". There is no need to test a Ub-independent cargo such as what was also suggested by the reviewer.

Your revision will likely not need to be sent back to the reviewers for further review. Barring any unforeseen issue, we will be glad to assess your revised manuscript to expedite the handling of your paper.

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.

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

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

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

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,
Scott Emr, PhD  
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD  
Senior Scientific Editor, Journal of Cell Biology

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

This in an exciting study by David Katzmann and co-workers. In it, they address how the ESCRT machinery coordinates the formation of intraluminal vesicles (ILVs) during the biogenesis of multivesicular bodies with the sorting of ubiquitinated membrane proteins into the growing ILVs. They focus on Bro1 and their data suggest that Bro1 is a key player in this process with a dual function. On the one hand, Bro1 can stimulate ESCRT-III assembly (their earlier work: PMID: 21263029). On the other hand, they show that Bro1 through its V-domain directly stimulates the activity of the AAA - ATPase Vps4, in conjunction with ubiquitin. Thus, Bro1 probably regulates Vps4 mediated ESCRT-III remodelling at several steps and thereby helps to coordinate cargo sorting with membrane budding and scission. To arrive at these conclusions, they used budding yeast as a model system and combined beautiful EM tomography of MVBs, morphometric analysis, live cell imaging and in vitro assays.

Their work has important implications for our understanding of the ESCRT machinery in general and brings to attention new aspects of its complex regulation during ILV budding.
minor points:

1. Please present the WB data from Figure 1, full length and clipped GFP on a single blot. I find the results difficult to evaluate if the blots are cropped.
2. In Figure 6, it would be interesting to test if other Bro1 mutations (e.g. M1 or M2) that do not affect Vps4 ATPase activity, modulate MVB sorting of protein cargo.
3. The subcellular fraction in Figure 8 shows no differences in the steady state distribution of Snf7. To me all the factions (expect for Vps4 of course) of the different mutants (also bro1Δ) show approximately the same ratio of Snf7 in the soluble and in the membrane fractions. If anything, there is perhaps a subtle difference in in Bro1M4/M8, with relatively more of the mutant proteins in the pellet fraction.
4. Figure 4e: Please clarify: when using V domains of S. castellii Bro1 or human HD-PTP, was Vps4 from the corresponding species used?
5. In the discussion the authors speculate that Bro1 might detect free UB released by Doa4 - I really like the idea, but I think that the experiment in Supplementary Figure 2B and in Figure 3 argues against the idea since there is apparent MVB sorting (at least of NPC) in Doa4 mutants. It would be easy to test if in this mutant MVB cargo (UB-CPS vs CPS) would be sorted.

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

Tseng et al have investigated the role of the yeast Bro1 protein in MVB biogenesis and protein and lipid sorting into ILVs of the MVB. Bro1 contains three structural domains: N-terminal Bro1 domain, V domain, and PRR segment. The starting point for the study is the remarkable discovery that the V domain of Bro1 is sufficient to sustain ILV formation, but MVB cargo apparently fail to be sorted to the ILVs. Bro1 interacts with ESCRT-III subunits and Vps4 and the authors find that recombinant, purified Bro1 V domain stimulates Vps4 ATPase activity, likely by promoting Vps4 oligomerization. Ubiquitin is reported to enhance this activity of Bro1 V domain. The study makes a strong case for the conclusion that the Bro1 V domain stimulates Vps4 activity and that this couples Vps4- and ESCRT-dependent sorting of MVB cargo sorting.

In general, the study is distinguished by its rigor and comprehensive approach. The combination of tomography of MVBs and biochemical analyses of Vps4 ATPase activity are superb. I have no technical concerns regarding these aspects of the study. My only technical concern lies with the use of a fluorescence micrographs to measure efficiency of MVB sorting, expressed as penetrance of the phenotype (i.e., proportion of cells exhibiting a particular phenotype); it's unclear how to me how sorting was determined (plus or minus) from micrographs and how penetrance in the population relates to sorting mechanism. Wouldn't scoring GFP release from fusion proteins be a more accurate measure?

A minor note is that the manuscript is difficult to read because it is extremely dense with specific information (listing detailed aspects of cited information, qualifications in interpretations of data, and excessive citations. The authors may wish to consider edits to make it more readable.

Reviewer #3 (Comments to the Authors (Required)):
The authors analyzed the MVB sorting defects caused by the deletion of the BOD domain of Bro1. Surprisingly, loss of BOD caused only a partial reduction of ILVs present in MVBs and thus did not mimic the MVB morphology of a bro1 deletion. Consistent with this partial ILV formation phenotype, the lipid NBD-PC was trafficked to the lumen of the vacuole. However, protein cargoes of the MVB pathway such as CPS and Mup1 exhibited a strong trafficking defect, suggesting that MVB protein sorting was much more affected by the BOD deletion than ILV formation. In vitro biochemical studies identified a physical interaction between the V domain of Bro1 and the Vps4 MIT domain. This interaction increased the ATPase activity of Vps4, most likely by promoting Vps4 assembly. Furthermore, binding of ubiquitin to the V domain resulted in an additional stimulation of Vps4 ATPase activity. Together, these and additional data support a model in which Bro1 acts as a key MVB regulator by receiving input from ubiquitinated cargoes, the cargo sorting system and the ESCRT-III polymer and using this information to trigger Vps4 activity at proper time. The presented data are of high quality and support the conclusions of the manuscript. Because of the complexity of the system and general lack of understanding how ILVs are formed, it is not surprising that the manuscript is not able to outline the step-by-step events orchestrated by Bro1. However, the apparent difference in severity between the cargo-sorting and the ILV formation phenotypes observed in this study is intriguing and might give some important insight into Bro1 function. I can see two scenarios: either ILV formation can happen without protein cargo or the BOD deletion causes a loss of cargo selectivity. The latter case could be caused by premature de-ubiquitination of cargo, which might result in inefficient sorting of MVB cargo and entrapment of non-cargo. These models could be tested by western blots that compare the liberation of GFP from GFP-Cps1 with that from GFP-Ub-Cps1 and test if GFP-Dap2 might become a cargo (by western blot). Also the use of Ub-independent cargoes such as the MIT(L64D)-GFP-FYVE construct published by S.Mageswaran et al., 2013 could shed light on the cargo sorting problems of the BOD deleted strains. If the data suggest that ILVs indeed can form without or with less cargo, it would have profound consequences for the understanding of the ILV formation mechanism.
Dear Drs. Casadio and Emr,

We greatly appreciate your time and consideration in evaluating our manuscript as well as the positive reception by the reviewers. We have performed the experiments suggest by the reviewers and appended the results to this letter. We addressed the points reviewers raised here or within the text. In compliance with journal guidelines, we have added a summary for supplemental materials.

**Reviewer 1 comments:**

This in an exciting study by David Katzmann and co-workers. In it, they address how the ESCRT machinery coordinates the formation of intraluminal vesicles (ILVs) during the biogenesis of multivesicular bodies with the sorting of ubiquitinated membrane proteins into the growing ILVs. They focus on Bro1 and their data suggest that Bro1 is a key player in this process with a dual function. On the one hand, Bro1 can stimulate ESCRT-III assembly (their earlier work: PMID: 21263029). On the other hand, they show that Bro1 through its V-domain directly stimulates the activity of the AAA - ATPase Vps4, in conjunction with ubiquitin. Thus, Bro1 probably regulates Vps4 mediated ESCRT-III remodeling at several steps and thereby helps to coordinate cargo sorting with membrane budding and scission. To arrive at these conclusions, they used budding yeast as a model system and combined beautiful EM tomography of MVBs, morphometric analysis, live cell imaging and in vitro assays. Their work has important implications for our understanding of the ESCRT machinery in general and brings to attention new aspects of its complex regulation during ILV budding.

**minor points:**

1. *Please present the WB data from Figure 1, full length and clipped GFP on a single blot. I find the results difficult to evaluate if the blots are cropped.*

**Response:** We have replaced the cropped blots in Figure 1B & 1C with intact images displaying full-length GFP-fusions, Ub-modified species and clipped GFP. (The blots had been cropped to save space in the previous version of this figure.)

2. *In Figure 6, it would be interesting to test if other Bro1 mutations (e.g., M1 or M2) that do not affect Vps4 ATPase activity, modulate MVB sorting of protein cargo.*

**Response:** We thank the reviewer for suggesting this idea. In Response Figure 1, we have examined GFP-Cps1 sorting in the context of mutants M1 and M7, and we found that these mutants do not disrupt GFP-Cps1 cargo sorting. This result indicates that Bro1 with mutations that do not disrupt stimulation of Vps4 ATPase activity are not defective for cargo sorting. We include these findings in the manuscript in Figure S4C and main text line 237-238.

![Δbro1: Vector  BRO1  BRO1M1  BRO1M7](image)

**Response Figure 1. bro1M1 and bro1M7 do not disrupt GFP-Cps1 sorting.** Δbro1 cells were transformed with GFP-Cps1 and either vector, BRO1, BRO1M1, or BRO1M7 plasmids to assess the impact of these mutations on MVB sorting. Scale bar = 5 μm.

3. *The subcellular fraction in in Figure 8 shows no differences in the steady state distribution of Snf7. To me all the factions (expect for Vps4 of course) of the different mutants (also bro1Δ) show approximately...*
the same ratio of Snf7 in the soluble and in the membrane fractions. If anything, there is perhaps a subtle difference in in Bro1M4/M8, with relatively more of the mutant proteins in the pellet fraction.

Response: Quantitation of blots from multiple experiments support the conclusions that 1) bro1Δ is different from WT although we agree the difference is subtle, and 2) M4 and M8 are similar to WT in being different from bro1Δ (i.e. they complement the defect in Snf7 fractionation). This result contrasts with disruption of other Vps4 regulators that stabilize Snf7 membrane association. This point is the focus of our analysis. We appreciate the reviewer’s comment regarding a possible increased membrane association for M4 and M8, and will explore this issue in future studies.

4. Figure 4e: Please clarify: when using V domains of S. castellii Bro1 or human HD-PTP, was Vps4 from the corresponding species used?

Response: We thank the reviewer for raising this point and apologize for any confusion. V domains of S. castellii, human HD-PTP and S. cerevisiae were titrated against 0.5μM of S. cerevisiae Vps4. We have specified the species of Vps4 used in the manuscript to clarify this point (Line 203) and the figure legend (Line 736).

5. In the discussion the authors speculate that Bro1 might detect free UB released by Doa4 - I really like the idea, but I think that the experiment in Supplementary Figure 2B and in Figure 3 argues against the idea since there is apparent MVB sorting (at least of NPC) in Doa4 mutants. It would be easy to test if in this mutant MVB cargo (UB-CPS vs CPS) would be sorted.

Response: We thank the reviewer for proposing this interesting experiment. As indicated in Figure 2A, both Ub-GFP-Cps1 and GFP-Cps1 fail to appreciably sort into the vacuolar lumen in bro1Δ cells, as assessed by live cell microscopy. Similarly, we did not observe appreciable MVB sorting of Ub-GFP-Cps1 or GFP-Cps1 in bro1Δ doa4Δ cells. To address this issue further, we employed immunoblot analysis of GFP liberation, as in Figures 2B, 2C.

To validate this immunoblot assay for GFP liberation with Ub-Cps1, we first analyzed GFP-Cps1 and Ub-GFP-Cps1 in WT, vps4Δ, bro1Δ, snf7Δ, or pep4Δ strains (Response Figure 2A). To our surprise, the conversion of Ub-GFP-Cps1 to free GFP did not display dependence upon vacuolar protease activity (i.e., generation of free GFP in pep4Δ Ub-GFP-CPS); this result precluded use of this approach for analysis of Ub-Cps1 vs Cps1. However, liberation of free GFP from Mup1-GFP and Mup1-GFP-Ub is dependent upon vacuolar protease activity (Response Figure 2B) and were therefore suitable cargoes for the reviewer’s suggested comparison. GFP liberation from these cargoes in bro1Δ and bro1Δ doa4Δ cells revealed no differences (Response Figure 2C, 2D). We similarly did not observe any distinctions in Mup1-GFP and Mup1-GFP-Ub MVB sorting as assessed by live cell microscopy. We have added the GFP liberation assay presented here in Response Figure 2C as Figure S2A.

Based on both these data and the previously presented data using NBD-PC (Figure 3C, as alluded to by the reviewer) we conclude that Bro1 is not detecting free Ub generated by Doa4. We address this idea in the discussion proposing the possibility as well as clarifying that the results do not support this mechanism (Line 375-379).
Response Figure 2. Justification for use of Mup1-GFP and Mup1-GFP-Ub, instead of GFP-Cps1 and Ub-GFP-Cps1, in the GFP liberation assay. Lysates were generated from the indicated strains expressing the indicated GFP-tagged MVB cargoes - GFP-CPS and Ub-GFP-CPS (A), or Mup1-GFP and Mup1-GFP-Ub (B-D) - and subjected to western blotting with anti-Cps1 antibodies (A) or anti-GFP antibodies (A-D). Pgk1 western blotting serves as a loading control. * indicates statistically significant difference. # indicates statistically significant difference compared to WT. ns indicates statistically insignificant difference.

Reviewer 2 comments:
Tseng et al have investigated the role of the yeast Bro1 protein in MVB biogenesis and protein and lipid sorting into ILVs of the MVB. Bro1 contains three structural domains: N-terminal Bro1 domain, V domain, and PRR segment. The starting point for the study is the remarkable discovery that the V domain of Bro1 is sufficient to sustain ILV formation, but MVB cargo apparently fail to be sorted to the ILVs. Bro1 interacts with ESCRT-III subunits and Vps4 and the authors find that recombinant, purified Bro1 V domain stimulates Vps4 ATPase activity, likely by promoting Vps4 oligomerization. Ubiquitin is reported to enhance this activity of Bro1 V domain. The study makes a strong case for the conclusion that the Bro1
V domain stimulates Vps4 activity and that this couples Vps4- and ESCRT-dependent sorting of MVB cargo sorting.

In general, the study is distinguished by its rigor and comprehensive approach. The combination of tomography of MVBs and biochemical analyses of Vps4 ATPase activity are superb. I have no technical concerns regarding these aspects of the study. My only technical concern lies with the use of a fluorescence micrographs to measure efficiency of MVB sorting, expressed as penetrance of the phenotype (i.e., proportion of cells exhibiting a particular phenotype); it's unclear how to me how sorting was determined (plus or minus) from micrographs and how penetrance in the population relates to sorting mechanism. Wouldn't scoring GFP release from fusion proteins be a more accurate measure?

Response: We thank the reviewer for the overall positive assessment of our work. This final issue regarding the assessment of protein cargo sorting is complex, and we appreciate the reviewer’s request for clarification. In short, we feel that presenting the quantified qualitative assessment of GFP-cargo sorting along with the quantification of GFP liberation from certain cargoes provide the best assessment of function - both generally and for comparison to NBD-PC sorting for which only the 1st approach is applicable. To clarify the procedure for analyzing fluorescence images, cells with WT cargo sorting signal – as indicated by predominant lumenal fluorescence and an absence of appreciable limiting membrane accumulation (Figure S1B for NBD-PC example) - were scored manually; this has been clarified in the methods section (Line 543-545). The explicit relationship between penetrance in the population and the sorting mechanism is at one level unclear. However, we surmise that reduced function of the MVB sorting machinery leads to decreased penetrance of cargo sorting - both for protein cargo sorting and NBD-PC sorting. The final question/statement that GFP liberation is a more accurate measure of sorting is not a conclusion we support. While the aforementioned Ub-GFP-CPS analysis (with Pep4-independent GFP cleavage) provides a striking example of the limitations of GFP cleavage analysis, we also encountered complex proteolytic patterns and varied expression levels that complicated interpretation for some of these MVB cargoes. GFP liberation from Sna3-GFP and Mup1-GFP was not subject to such complications and was incorporated in the initial submission to support the microscopy approach. Additionally, we have examined GFP liberation from Cos5-GFP and Mup1-GFP-Ub, which are consistent with the results from Sna3-GFP and Mup1-GFP. These analyses have been added to Figure S2A and B. We believe the discrepancy between the degree of penetrance observed by microscopy (less sorting apparent) and the extent of GFP liberation (more liberation apparent) is a consequence of sensitivity: while western blotting allows visualization of low levels of free GFP, free GFP within the 3-dimensional vacuole lumen is more difficult to appreciate compared to the signal within the vacuolar membrane. We believe that documenting defective MVB cargo sorting by these two complementary methods adds value to the analysis and strengthens interpretation of the results.
Response Figure 3. *bro1*<sup>Δmut</sup> does not support efficient Cos5-GFP sorting. Lysates were generated from the indicated strains expressing the indicated GFP-tagged Cos5 and subjected to western blotting with anti-GFP or anti-Pgk1 antibodies.

A minor note is that the manuscript is difficult to read because it is extremely dense with specific information (listing detailed aspects of cited information, qualifications in interpretations of data, and excessive citations. The authors may wish to consider edits to make it more readable.

Response: We apologize for the density but felt that this was largely unavoidable due to the complex nature of the story being told and the desire to acknowledge previous contributions of others. We have attempted to further simplify the writing.

Reviewer 3 comments:

The authors analyzed the MVB sorting defects caused by the deletion of the BOD domain of Bro1. Surprisingly, loss of BOD caused only a partial reduction of ILVs present in MVBs and thus did not mimic the MVB morphology of a *bro1* deletion. Consistent with this partial ILV formation phenotype, the lipid NBD-PC was trafficked to the lumen of the vacuole. However, protein cargoes of the MVB pathway such as CPS and Mup1 exhibited a strong trafficking defect, suggesting that MVB protein sorting was much more affected by the BOD deletion than ILV formation. In vitro biochemical studies identified a physical interaction between the V domain of Bro1 and the Vps4 MIT domain. This interaction increased the ATPase activity of Vps4, most likely by promoting Vps4 assembly. Furthermore, binding of ubiquitin to the V domain resulted in an additional stimulation of Vps4 ATPase activity. Together, these and additional data support a model in which Bro1 acts as a key MVB regulator by receiving input from ubiquitinated cargoes, the cargo sorting system and the ESCRT-III polymer and using this information to trigger Vps4 activity at proper time. The presented data are of high quality and support the conclusions of the manuscript.

Because of the complexity of the system and general lack of understanding how ILVs are formed, it is not surprising that the manuscript is not able to outline the step-by-step events orchestrated by Bro1.

However, the apparent difference in severity between the cargo-sorting and the ILV formation phenotypes observed in this study is intriguing and might give some important insight into Bro1 function. I can see two scenarios: either ILV formation can happen without protein cargo or the BOD deletion causes a loss of cargo selectivity. The latter case could be caused by premature de-ubiquitination of cargo, which might result in inefficient sorting of MVB cargo and entrapment of non-cargo. These models could be
tested by western blots that compare the liberation of GFP from GFP-Cps1 with that from GFP-Ub-Cps1 and test if GFP-Dap2 might become a cargo (by western blot). Also the use of Ub-independent cargoes such as the MIT(L64D)-GFP-FYVE construct published by S. Mageswaran et al., 2013 could shed light on the cargo sorting problems of the BOD deleted strains. If the data suggest that ILVs indeed can form without or with less cargo, it would have profound consequences for the understanding of the ILV formation mechanism.

Response: We agree with the two hypotheses put forth by the reviewer. The data presented in Figure 2A and in Response Figure 4 does not support the conclusion that loss of cargo selectivity has occurred: Dap2-GFP (a vacuolar protein that is not an MVB cargo) does not enter the MVB pathway as assessed by microscopy or western blotting for free GFP. Additionally, we did not conclude that ILV formation can occur independently of cargo, but rather that the efficiency of cargo incorporation is dramatically perturbed in the context of bro1ΔBOD. ILV formation assessed by NBD-PC sorting was dependent upon early ESCRT function (supplemental Figure 3B). We interpret this result to indicate that early ESCRT recruitment (dependent upon Ub-cargoes) is required for recruitment/nucleation of ESCRT-III, subsequently “licensed” by Bro1ΔBOD. We have clarified this point to avoid confusion (line 158-159).

We thank the reviewer for this suggestion that premature de-ubiquitination may compromise MVB sorting in this context. However, immunoblotting presented in Figure 2B and 2C reveals no obvious alterations in Ub-modified forms of Sna3 or Mup1 at steady state. As indicated in our response to Reviewer 2, Ub-GFP-Cps1 exhibits unexpected vacuole-independent GFP liberation precluding its use in this analysis. However, GFP liberation from Mup1-GFP and Mup1-GFP-Ub were equivalent to each other both in bro1ΔBOD and bro1ΔBOD doa4Δ cells. These collective data do not support the conclusion that premature de-ubiquitination has compromised cargo selectivity. We have highlighted this conclusion in the manuscript.

We appreciate the suggestion to explore MIT(L64D)-GFP-FYVE sorting in bro1ΔBOD cells as V domain binds MIT(L64D). However, we feel this line of investigation is better left to future studies. We are unable to appreciate how the outcome of this analysis would impact our interpretation that there is a defect in coordination of cargo recognition and ILV formation in cells expressing Bro1ΔBOD as their sole form of Bro1, nor how it would enable the conclusion that ILVs are truly devoid of any protein cargo. As such we did not request this construct from the Babst group.

![Figure 4. bro1ΔBOD liberates similar amounts of GFP from GFP-Dap2 compared to bro1Δ and WT yeast. Lysates were generated from the indicated strains expressing GFP-Dap2 cargoes subjected to western blotting with anti-GFP or anti-Pgk1 antibodies.](image)
Dear Dr. Katzmann,

Thank you for submitting your revised manuscript entitled "Bro1 stimulates Vps4 to promote Intraluminal Vesicle Formation during Multivesicular Body biogenesis". In our view, you have addressed each of the reviewers' comments, most of which were fairly minor issues. Thank you for contributing this very interesting study and for your thorough responses to each of the reviewers' comments. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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   - Please revise the summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

2) 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. 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.

3) 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 in the text for readers who may not have access to referenced manuscripts.

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

Thank you for this interesting contribution, we look forward to publishing your paper in the Journal of Cell Biology.

Sincerely,

Scott Emr, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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