JCB

Quantitative CLEM reveals the ultrastructural distribution of endogenous endosomal proteins

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July 27, 2021

Re: JCB manuscript #202106044

Prof. Judith Klumperman
UMC Utrecht
Department of Cell Biology
University Medical Center Utrecht
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Utrecht 3584 CX
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Dear Dr. Klumperman,

Thank you for submitting your manuscript entitled "Revealing the ultrastructural distribution of endogenous proteins using quantitative correlative microscopy; a closer look at endosomal regulators." The manuscript was assessed by expert reviewers, whose comments are appended to this letter.

Overall, the reviewers are enthusiastic about your study, noting that it is well done, provides an important advance in our understanding of the endocytic pathway, and appropriate for the JCB Tools format. Thus, we invite you to submit a revision if you can address their comments, as outlined here.

Most of the comments we feel can be easily addressed with text revisions. Reviewer #2 suggests adding a PI(3)P probe to confirm that the EEA1-positive late endosomes are also Pi(3)P-positive. We believe this would make an excellent addition to your study providing further support for EEA1 localization and also potentially new information regarding the localization of this lipid along the endolysosomal system. We strongly encourage you to address this comment with new data, using either a fixable probe that is expressed in the cells, or a probe that is applied to the sections. You will see that Reviewer #3 is somewhat concerned about lack of a significant conceptual advance, however this is not required for the Tools format.

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 Tools 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.

Figures: Tools 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.

Title: The title should be less than 100 characters including spaces and should be concise but accessible to a general readership. We do not feel that your current title will be accessible to a broader cell biology audience. Therefore we suggest the following title: "Quantitative correlative microscopy reveals ultrastructural distribution of endogenous endosomal proteins."

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.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Tools may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of measures to limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors if necessary to decide on 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,

Sean Munro, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

1. Summary of the paper

This paper describes a technique for performing CLEM on ultrathin cryosections where antibodies are used to identify the location of specific proteins and then the organelles corresponding to those fluorescent spots are identified in the transmission electron microscope. CLEM is not new and incredible results have previously been achieved, including by this group. Labelling ultrathin cryosections on grids, imaging the fluorescence and then correlating the fluorescent label with gold label in the EM has been described before (eg Oorschot et al. 2014 Methods in Cell Biology and Viccidomini et al., 2008 Traffic). However, unlike these previous studies, in this study they find the structures that stained positive by fluorescence in the EM without the use of gold label, enabling the ultrastructural characterisation of organelles that are positive for proteins where the antibodies do not work for immunoEM. It is not unusual for antibodies that give a good signal by immunofluorescence to give little or no signal by immunoEM. Thus, this technique is potentially very useful. Furthermore, this manuscript provides novel information on the localisation of key regulators in the endocytic pathway, revealing an unexpected localisation of EEA1, normally considered a marker of early endosomes, additionally on Rab5 negative late endosomes and lysosomes. I think that this paper may inspire others to have a go at this technique and therefore make it more accessible. Over all the data is clearly and beautifully presented and will definitely be of interest to the readers of JCB.

2. Main points of the paper

Figure 1 addresses overlap between endocytic regulators by conventional immunofluorescence and clearly shows that Rab 5 and Rab7 positive punctae are largely separate, as expected. The authors also state that: 'Rab5-positive spots largely overlapped with EEA1 and APPL1 staining (Fig. 1D, E, I)'. I think this is a bit misleading as there appears to be a considerable amount of Rab5 that overlaps with neither marker. That APPL1 and EEA1 are largely found in separate Rab5 positive endosomes is in line with published findings.

Figure 2 clearly shows that antibodies to Rab 5 and Rab 7 give a good immunofluorescent signal on ultrathin cryo sections whilst giving no signal using immunogold.

After optimising fixation conditions to preserve antigenicity whilst preserving ultrastructure as best as possible, on section CLEM is performed to demonstrate the ultrastructure of Rab 5 and Rab7 positive compartments. Organelles are classified into different subtypes based on previous observations and Rab5 is clearly demonstrated to be associated with vesicles and tubules, early endosomes and occasionally late endosomes whilst Rab7 is predominantly localised to late endosomes and lysosomes (Figure 3).

As expected APPL1 and EEA1 are found on distinct compartments with APPL1 found on tubules and vesicles whilst, more surprisingly, EEA1 is found on vacuolar structures that include Rab5 negative late endosomes and lysosomes (Figures 4/5). Interestingly these structures were also largely negative for Rab7 although were sometimes found adjacent to Rab7 positive tubules and vesicles. The presence of EEA1 on late endosomes/lysosomes was reproduced in different cell lines, albeit to different extents in the different lines.

3. Additional points:

The CLEM method of Viccidomini et al., (Traffic 2008) should be cited in the introduction.

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

This manuscript reports a high-quality CLEM study in which the authors correlate the localization, by immunofluorecence microscopy, of several endogenous endosomal regulator proteins with the underlying EM ultrastructure of Tokuyasu frozen thin sections. The proteins localized include Rab5, Rab7, EEA1 and APPL1, all of which have been widely used as markers of endosomal compartments. The novel element of the present study is the ability to perform 'on-section' immunofluorescence microscopy of antigens that cannot successfully be labelled using immunogold electron microscopy and precisely register the underlying ultrastructure. The limitations of axial resolution in the light microscope were circumvented using thin EM sections of 90 nm (compared to an approximately 700 nm Z section derived from a pinhole set to 1AU in the confocal microscope), resulting in confidence that the fluorescence was emitted from the correlated structures observed in the electron microscope. The new method expands on previously published high resolution CLEM techniques utilizing exogenously added fluorescent probes or EGFP-tagged proteins using Tokuyasu frozen thin sections correlated with confocal microscopy (Eline van Meel et. al. (2019) Traffic 20, 346-356) or super-resolution microscopy (Christian Franke et. al. (2019) Traffic 20, 601-617). The authors have verified their methodology by showing that Rab 5 and Rab 7 antibodies predominantly immunolabel compartments that, upon ultrastructural interrogation, were revealed to be early endocytic vesicles/early endosomes and late endosomes respectively and have gone on to demonstrate an unexpected association of EEA1 with late endocytic compartments.

The manuscript provides no new mechanistic insights into the endocytic pathway, However, it does contain several very important and valuable methodological insights and developments that deserve to be widely known and understood by the many members of the cell biology community who carry out immunolocalization studies on fixed samples. One linsight is that many attempted immunogold labelling attempts likely fail due to inadequate penetration of the gold-conjugated secondary reagents into Tokuyasu thin sections (since primary antibodies detected with secondary fluorochrome-conjugated antibodies yield successful IF localisations in the same sections). Another insight that deserves to be widely understood and followed is the need to have an optimal fixation protocol and in particular to consider the effects of longer incubation times with formaldehyde on reducing the fluorescence microscopy signal for many antibodies. In this regard, the thorough exploration of the effect of formaldehyde and glutaraldehyde fixation on antigenicity disclosed in Table 1 and supplementary figures S1 and S2 is to be commended.

Because of the lack of mechanistic insight this manuscript is best suited to be a Tool in the Journal of Cell Biology.

Minor points:

1) P2. The authors refer to late endosomes fusing with autophagosomes and lysosomes. These fusions result in organelles that have been termed autolysosomes and endolysosomes from which lysosomes are reformed. It would be useful to refer to a review of these processes eg Chonglin Wang & Xiaichen Wang (2021) J Cell Biol. 220, e202102001. When the authors define lysosomes morphologically on P14 it seems likely that the lysosomes to which they refer include endolysosomes and autolysosomes and organelles on all stages of the lysosome reformation pathway(s). They should certainly discuss this pint and may wish to make it clear that their lysosome compartment includes all such organelles.
2) P3 last paragraph. The authors state that Rab7 marks late endosomes and lysosomes displaying morphological elements indicative of degradation. However, they are also aware from previous work to which the senior author has contributed that some lysosomes are Rab7-negative but Arl8b-positive - Marlieke Jongsma et al.(2020) EMBO J 39, doi:10.15252/embj.2019102301, which they have cited on P2.. They should make it clear that not all lysosomes are Rab7-positive.
3) P4, Results section, end of 1st paragraph: A 4th explanation may be the limited penetration / accessibility of antibodies into organelles with densely packed lumens.
4) P4 second paragraph. If the Rab5-positive vesicles-tubules occasionally contained internalized BSA-gold and in 23% of cases displayed a clathrin coat, how can it be be said that they meet all the criteria of endocytic vesicles ? Some of them do and others do not.
5) P4 third paragraph, sixth line, 'plane' not 'plain'.
6) P6, last paragraph, 5th line: delete 'reversely' and add 'respectively' so that the sentence reads '.....Rab 5 and Rab 7 are distributed over early and late compartments respectively.'
7) P7, second paragraph, line 11 'confirming their endocytic nature' would be more appropriately written as 'consistent with their endocytic nature.'
8) P7, third paragraph. Have the authors tried localising a PI(3)P probe on their sections to confirm that their EEA1-positive late endosomes are also Pi(3)P-positive ? If that was the case it would strengthen the proposal that EEA1 is on late endosomes because it is binding to PI(3)P.
9) The use of endocytosed BSA-gold is limited in the context of these experiments since, when the gold flocculates following degradation of the BSA, it aggregates into a clump that does not fill the lumen of the compartment under investigation and, as the authors allude to, may well be present out of the plane of section. This would give a false negative for uptake of this endocytic tracer. The authors should mention/discuss the use of fluid phase tracers which fill the lumen of endocytic organelles

such as lysine-fixable dextran:fluorochrome-conjugated reagents (which may be compared with antibodies used in IF and in which the fluorochrome may also be immunolabelled with antibodies and gold conjugates, thus providing correlation at EM level) or HRP followed by the DAB reaction (although this will obscure underlying ultrastructure within the thin section).

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

This study analyzes the distribution of proteins implicated in the function of the endocytic pathway using an improved CLEM methodology. CLEM (correlative light/electron microscopy) techniques have been extensively used in cell biology to analyze by EM structures previously detected by immunofluorescence. Typically, these methods involve the analysis of cells by immunofluorescence followed by processing for EM, thin sectioning, EM analysis and superimposition of EM and fluorescence images. This manuscript describes a methodology for a direct correlation of immunofluorescence and EM images using sectioned non-plastic embedded material. The technique is based on the generation of cryosections of fixed cells which are then first processed for immunofluorescence, thus allowing to detect antigens at endogenous level, and subsequently examined by EM. In addition to making possible a more accurate correlative analysis, this methodology allows the examination of hundreds of organelles in tiled EM images. The authors employ this novel quantitative CLEM technique to systematically analyze organelles of the endocytic pathway (prelabeled by an endocytic pulse of BSA-gold) in HeLa cells. More specifically, they examine the localization of two Rabs, Rab5, Rab7, and of two Rab effectors, APPL1 and EEA1. In agreement with previous findings, APPL1 and EEA1 were found to localize to distinct organelles with little to no overlap. APPL1 positive structures were primarily small vesicles and tubules. A considerable percentage of EEA1 positive endosomes lacked Rab5 localization entirely, confirming that Rab5 is relegated to the earliest stages of endocytosis and EEA1 is found also on downstream stations. Few EEA1 positive structures were found to be positive for Rab7, suggesting the existence of a pool of EEA1-positive, Rab5/Rab7 negative endosomes that are transitioning into bona fide endolysosomes. The authors also carried out EEA1 labeling experiments in several other cell lines with similar results.

The strong point of this study is the development of a sensitive, quantitative CLEM protocol to examine ultrastructure of organelles positive for potentially low-abundance endogenous proteins. The experiments are of excellent technical quality. A weakness of this study is the lack of substantial conceptual advancement, as new information about the organization of the endocytic pathway is modest. For this reason and its focus on methodology, this work is better suited for a more specialized journal.

Specific comment

The authors conclude that APPL1- and EEA1-positive endosomes represent organelles of two distinct pathways. However, they only show that the two compartments are different, they do not provide dynamic information. There is evidence that at least a subpopulation of APPL-positive endocytic vesicles can convert to classical early endosomes and even that EEA1 positive endosome can be reverted back to APPL1 positive endosomes by manipulations that deplete PI3P on them (PMID: 19303853). Thus, the cartoon of Fig. 7 is an oversimplification.

Answer to the reviewers

L.S.,

We thank the reviewers for their thorough evaluation of our manuscript. We were very pleased to see that our work is well received! We agree with most of the comments and based on the reviewers' suggestions made textual changes, performed new experiments and incorporated new data in figures and supplementary figures. Besides, we extended some of our analyses with additional data points, none of which were cause to alter any conclusions drawn from the data before. All comments are addressed point-by-point below and significant adaptations are highlighted in yellow in the revised manuscript. In addition, we made small changes to text and legends to shorten the manuscript and increase readability, without affecting any of the data or conclusions of the paper.

We hope that this revised version of our paper is now found acceptable for publication in the Journal of Cell Biology.

Sincerely,

Judith Klumperman

Editor:

1. Reviewer #2 suggests adding a PI(3)P probe to confirm that the EEA1-positive late endosomes are also PI(3)P-positive. We believe this would make an excellent addition to your study providing further support for EEA1 localization and also potentially new information regarding the localization of this lipid along the endolysosomal system.
    - *We added an extra CLEM dataset based on the use of a 2xFYVE-GFP PI(3)P probe (New Figure 5 and new Figure S5). Our new data show the co-localization of EEA1 and PI(3)P on Rab5-negative late endosomes, indicating a role for PI(3)P in membrane association of EEA1 on late endosomes.*
2. The guideline for manuscript length in JCB is 40,000 characters (Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends).
    - *Our manuscript contains 43,608 characters, 36,320 in the main text, the rest in the figure legends. We hope this is acceptable.*
3. 10 main text figures (https://jcb.rupress.org/site/misc/ifora.xhtml)
    - *Our manuscript contains 7 main figures*
4. There is a limit of 5 supp. Figures
    - *We have combined supp. Figures 4, 5 and 6 to reduce the total to 5 supp. figures*
5. Title: The title should be less than 100 characters including spaces and should be concise but accessible to a general readership. We do not feel that your current title will be accessible to a broader cell biology audience. Therefore we suggest the following title: "Quantitative correlative microscopy reveals ultrastructural distribution of endogenous endosomal proteins."
    - *We changed the title as suggested*
6. 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.
    - *We have access to and have registered all used raw data*

7. 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 have highlighted significant changes, including those suggested by the reviewers, in yellow.*

Reviewer #1

1. The authors also state that: 'Rab5-positive spots largely overlapped with EEA1 and APPL1 staining (Fig. 1D, E, I)'. I think this is a bit misleading as there appears to be a considerable amount of Rab5 that overlaps with neither marker. That APPL1 and EEA1 are largely found in separate Rab5 positive endosomes is in line with published findings.
    - *We agree. The statement has been adapted to: 'Rab5-positive spots partially overlapped with EEA1 (26%) or APPL1 (45%) staining.' Page 3, par. 2.*
2. The CLEM method of Viccidomini et al., (Traffic 2008) should be cited in the introduction.
    - *We fully agree and added the reference to: Page 2 par. 3: '… labeling to ultrastructure (Mohammadian et al., 2019; Cortese et al., 2012; Vicidomini et al., 2008).'*

Reviewer #2

1. P2. The authors refer to late endosomes fusing with autophagosomes and lysosomes. These fusions result in organelles that have been termed autolysosomes and endolysosomes from which lysosomes are reformed. It would be useful to refer to a review of these processes eg Chonglin Wang & Xiaichen Wang (2021) J Cell Biol. 220, e202102001. When the authors define lysosomes morphologically on P14 it seems likely that the lysosomes to which they refer include endolysosomes and autolysosomes and organelles on all stages of the lysosome reformation pathway(s). They should certainly discuss this point and may wish to make it clear that their lysosome compartment includes all such organelles.
    - *This is a good point. We adapted the text at several sites to make this clearer: On page 1, par. 2, where we also added the suggested reference; in materials and methods, page 15, par. 'Definition of endo-lysosomes by EM morphology'.*
2. P3 last paragraph. The authors state that Rab7 marks late endosomes and lysosomes displaying morphological elements indicative of degradation. However, they are also aware from previous work to which the senior author has contributed that some lysosomes are Rab7-negative but Arl8b-positive - Marlieke Jongsma et al.(2020) EMBO J 39, doi:10.15252/embj.2019102301, which they have cited on P2. They should make it clear that not all lysosomes are Rab7-positive.
    - *This is a good point. We address this now in the discussion (Page 10, par. 1: 'Notably, all compartments studied in this paper were selected by IF labeling for CLEM analysis. Hence, non-labeled compartments were not taken into account, such as for example late endosomes/lysosomes that do not contain Rab7 but Arl8b or Rab9 (Jongsma et al., 2020). Future studies, with additional markers, may lead to the identification of additional endo-lysosomal compartments or sub-domains. ').*
3. P4, Results section, end of 1st paragraph: A 4th explanation may be the limited penetration/ accessibility of antibodies into organelles with densely packed lumens.
    - *Indeed, when performing immunostaining on permeable samples as cryosections the local environment can affect antigen availability and labeling efficiency. However, in case*

of on-section CLEM we use the same sections and primary antibodies for fluorescence imaging as for immuno-EM, so primary antibody penetration is similar for both labeling methods. Furthermore, all proteins under study adhere to the cytosolic site of membranes. Therefore, we do not think limited penetration is an applicable explanation in our case.

4. P4 second paragraph. If the Rab5-positive vesicles-tubules occasionally contained internalized BSA-gold and in 23% of cases displayed a clathrin coat, how can it be said that they meet all the criteria of endocytic vesicles? Some of them do and others do not.
    - *This is indeed confusing. We have re-formulated this sentence: Page 5, par. 2: 'In agreement with a role in endocytosis, they occasionally contained internalized BSA-gold 5nm and 23% of the correlated vesicles-tubules showed a distinctive clathrin coat.'*
5. P4 third paragraph, sixth line, 'plane' not 'plain'.
    - *We changed the word to 'plane'. (Page 6, par. 3)*
6. P6, last paragraph, 5th line: delete 'reversely' and add 'respectively' so that the sentence reads '.....Rab 5 and Rab 7 are distributed over early and late compartments respectively.'
    - *We changed the sentence; Page 6 par. 3, :'… Rab5 is generally distributed over vesicles, tubules and early endosomes, while Rab7 is found over late endosomes and lysosomes.'*
7. P7, second paragraph, line 11 'confirming their endocytic nature' would be more appropriately written as 'consistent with their endocytic nature.'
    - *We agree and adapted the sentence accordingly (Page 6, par. 1).*
8. P7, third paragraph. Have the authors tried localising a PI(3)P probe on their sections to confirm that their EEA1-positive late endosomes are also PI(3)P-positive ? If that was the case it would strengthen the proposal that EEA1 is on late endosomes because it is binding to PI(3)P.
    - *This is a great suggestion. We performed the requested experiment using a 2xFYVE-GFP PI(3)P probe, which turns out to work very well in our CLEM method and allows triple labeling with EEA1 and Rab5. The data obtained indeed show that Rab5-negative, EEA1-positive late endosomes contain PI(3)P, providing an explanation for the presence of EEA1 on late endosomes. These data and the quantitations are presented in a new version of Figure 5 and new Figure S5.*
9. The use of endocytosed BSA-gold is limited in the context of these experiments since, when the gold flocculates following degradation of the BSA, it aggregates into a clump that does not fill the lumen of the compartment under investigation and, as the authors allude to, may well be present out of the plane of section. This would give a false negative for uptake of this endocytic tracer. The authors should mention/discuss the use of fluid phase tracers which fill the lumen of endocytic organelles such as lysine-fixable dextran:fluorochrome-conjugated reagents (which may be compared with antibodies used in IF and in which the fluorochrome may also be immunolabelled with antibodies and gold conjugates, thus providing correlation at EM level) or HRP followed by the DAB reaction (although this will obscure underlying ultrastructure within the thin section).
    - *In our studies we have used BSA-gold as a general marker for endocytic compartments, but we are aware that this can give false negatives (as the reviewer rightfully points out). We therefore refrained from quantitative studies with this probe and only used it as a positive control for endocytosis. We have added a discussion point on page 5, par. 3: 'Of note, the presence of BSA5 cannot be used to measure the total number of organelles*

reached by endocytic marker, since some negative organelles may contain BSA5 outside the plane of sectioning especially when colloidal gold particles become clustered after degradation of BSA. In case quantitative studies on the entire endo-lysosomal system are required, the use of lysine-fixable fluorescent fluid phase markers is advised, which fill the entire lumen of endocytic organelles.'

- *Fluorescent fluid phase markers would indeed fill the entire lumen and probably increase the number of positive compartments. As illustrated by the new data using the 2xFYVE-GFP probe, fluorescence is preserved in cryosections, which obviates the need for antibody labeling to visualize fluorescent fluid phase markers. The HRP-DAB protocol is not compatible with cryosections, since cryosections are differentially contrasted than plastic EM and the DAB precipitates are poorly visible, if at all.*

Reviewer 3

1. The authors conclude that APPL1- and EEA1-positive endosomes represent organelles of two distinct pathways. However, they only show that the two compartments are different, they do not provide dynamic information. There is evidence that at least a subpopulation of APPL-positive endocytic vesicles can convert to classical early endosomes and even that EEA1 positive endosome can be reverted back to APPL1 positive endosomes by manipulations that deplete PI3P on them (PMID: 19303853). Thus, the cartoon of Fig. 7 is an oversimplification.
    - *We adapted the scheme in Fig. 7 by adding two arrows between EEA1 and APPL1 endosomes and added the following sentence to the text (Page 12, par. 2): 'Previous studies show that APPL1 and EEA1 endosomes dynamically interact and can interconvert (Kalaidzidis et al., 2015; Zoncu et al., 2009). Our studies indicate that these interactions are sparse or short-lived, since we find only a very small fraction (5%) of APPL1 co-localizing with EEA1 (Fig. 4D).')*

October 26, 2021

RE: JCB Manuscript #202106044R

Prof. Judith Klumperman
University Medical Center Utrecht
Department of Cell Biology University Medical Center Utrecht Heidelberglaan 100, AZU Rm G02.525
Utrecht 3584 CX
Netherlands

Dear Prof. Klumperman,

Thank you for submitting your revised manuscript entitled "Quantitative correlative microscopy reveals the ultrastructural distribution of endogenous endosomal proteins." 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) Figures limits: Tools may have up to 10 main text figures.

3) Figure formatting: 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 scale bars to Figures 3A/B, 4A, and 6A/B.

4) 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. 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."

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."

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. If antibodies are not commercial please add a reference citation if possible.

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.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Tools may have up to 5 supplemental figures and 10 videos. 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. JCB does not allow for supplementary methods descriptions so please move the 'Supplementary Note 1' text to the main materials and methods section.

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.

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.

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The authors have satisfactorily addressed all my comments on the previous version of the manuscript.