A modified lysosomal organelle mediates non-lytic egress of reovirus

Isabel Fernandez de Castro, Raquel Tenorio, Paula Ortega, Jonathan Knowlton, Paula Zamora, Chris Lee, José J. Fernández, Terence Dermody, and Cristina Risco

Corresponding Author(s): Cristina Risco, National Center for Biotechnology

Review Timeline:

Submission Date:	2019-10-18
Editorial Decision:	2019-12-03
Revision Received:	2020-02-20
Editorial Decision:	2020-03-11
Revision Received:	2020-03-31

Monitoring Editor: Billy Tsai

Scientific Editor: Marie Anne O'Donnell

Transaction Report:

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

DOI: https://doi.org/10.1083/jcb.201910131

November 29, 2019

Re: JCB manuscript #201910131

Dr. Cristina Risco
National Center for Biotechnology
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Spain

Dear Dr. Risco,

Thank you for submitting your manuscript entitled "A modified lysosomal organelle mediates non-lytic egress of reovirus" to the Journal of Cell Biology. The manuscript has now been assessed by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that the reviewers note that the study is currently quite descriptive and, although the observations are novel and interesting, they suggest that some mechanistic insight into how lysosomes are co-opted by this virus would be necessary for JCB. Although your manuscript is intriguing, we feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, we would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Although we regret that we are not able to consider your manuscript further, we have discussed your manuscript with the editors of Life Science Alliance (http://www.life-science-alliance.org/) and they would like to offer publication of a slightly revised version of this manuscript in LSA. LSA would expect you to address the technical comments (points 2 and 3 of rev#2) as well as the comments regarding the terminology (both reviewers). A mechanistic extension is NOT required for LSA. LSA is our academic editor-led, open access journal launched as a collaboration between RUP, EMBO Press and Cold Spring Harbor Press. You can use the link below to initiate an immediate transfer of your manuscript files and reviewer comments to LSA.

Link Not Available

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for your interest in the Journal of Cell Biology.

Sincerely,

Billy Tsai, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

In this manuscript, the authors set out to investigate the cellular mechanisms exploited by non-membrane bound reoviruses for non-lytic virus exit. TEM and 3D-ET revealed that exiting reoviruses were clustered in membrane-bound structures which appeared to contact and fuse with the PM. Molecular characterization and live cell imaging indicated that the virus-carrying membrane compartments were derived from lysotracker, LAMP-1-positive structures that look like lysosomes. These lysosome-like structures are revealed to 'feed' the egress compartment with newly assembled virions through membrane connections.

Overall, while this is very interesting and well-done, the study remains largely descriptive (morphological).

As pointed out by the authors themselves in the discussion;

1- There is no real molecular understanding of how these organelles are:
-formed
-recruited
-used for virus transport
-used for virus egress
-how they fuse

Nor is the identification or role of the tubules within determined.

The role of pH within these organelles is discussed but not investigated or addressed.

There is no molecular basis established to distinguish between the so-called SOs and MCs.

I think that the new acronyms, membranous compartment (MC) and sorting organelles (SO) do the paper a disservice. These are virus-modified cellular organelles, would it not be best to define them and then call them as such.

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

Comments

In this study, the authors investigated late infection steps of reovirus including intracellular transport and non-lytic egress of the virus. By using confocal and super resolution microscopy, 2D and 3D TEM, and CLEM, they found that mature virions are collected by lysosome-like organelles that bud to form membrane carriers (MCs), which then release virions by fusing with plasma membranes. The finding that lysosome-related organelles are involved in selecting and delivering mature virion for non-lytic egress is interesting and novel. Unfortunately, the property of so called "modified lysosomes" is not clearly defined. Moreover, cellular mechanisms underlying the late infection steps (such as lysosome recruitment, SO and MC formation, MC fusion with PM) are not studied at all.

Specific comments:
1. The property of "modified lysosomes" that serves as sorting organelles for mature virions is not clearly defined. The authors showed that these lysosome-related organelles are lysotracker- and LAMP-positive. Do these structures contain cathepsins? Are they catalytic active?
2. The authors stated that virus infection causes changes in the size, number and distribution of lysosomes labeled by LysoTracker (Fig. 4B). These data should be quantified. Moreover, more lysosomal markers should be examined and quantified, such as LAMP1 and cathepsin.
3. NH_4Cl disrupts lysosome acidity and thus inhibits lysosome function. It is not clear from the data (Fig. 7A, C) whether LAMP1-positive structures are not recruited or that they cannot enclose mature virion.
4. In addition to property of SOs, the cellular mechanisms regarding lysosome recruitment and SO formation are not studied at all. For example, how are lysosomes recruited? Why lysosomes but not endosomes are recruited? Any specific labeling on lysosomes links them to VI or vice versa? Some surface labeling can be tested, such as phosphoinositides and PS.
In addition, lysosome transport machinery can be tested such as RAB7-dynein and Arl8-kinesin. What does "modified lysosome" mean? Are lysosomes modified to be recruited or that they are modified to select mature virions? How are they modified? This term is unclear and confusing.
5. From the EM images, neither VI nor MC seem to be enclosed by membranes.

February 20, 2020

Dear Drs. Tsai and O'Donnell,

Thank you for facilitating the review of our manuscript and providing the constructive and useful comments of the editors and reviewers. Our study is indeed the first to examine the non-lytic process used by reovirus to exit infected cells. Reovirus egress from transformed cells can occur during lysis, which likely does not occur in vivo, especially in key target tissues such as the CNS. We discovered a novel egress pathway for reovirus in which lysosomes are recruited and modified to build the egress machinery. Our manuscript is the result of a multiyear collaboration that will serve as the foundation for many future studies of this pathway in our laboratories as well as in others.

Following the requests of the editors and reviewers, we have extended our study to provide some mechanistic insights into how lysosomes are co-opted by reovirus. Our revised manuscript includes the following new findings:

- We provide additional details about the lysosomes co-opted by the virus (in response to requests of reviewers 1 and 2). We have assessed the pH of these organelles and observed that reovirus modifies the pH from ~ 4.5-5 to ~ 6.1 after recruitment to viral inclusions (VIs) and before incorporation of virions.

- We labeled endosomes with specific markers and confirmed that they are not recruited to viral inclusions (reviewer 2).

- We quantified the distribution, number, and size of individual lysosomes labelled with anti-LAMP-1 in mock-infected and reovirus-infected cells (reviewer 2). Reovirus infection is associated with an increase in the number and size of lysosomes. We conducted immunofluorescence assays with a new anti-σ1 antibody, which yielded improved images of the sorting organelles. We include these new images in Figure 7 of the revised manuscript (reviewer 2).

- We found that the dynein inhibitor ciliobrevin A does not inhibit reovirus egress. Consequently, it is unlikely that dynein motors are used to traffic lysosomes to VIs (reviewer 2).

Reviewer 1 mentions that there is no molecular basis established to distinguish between the so-called sorting organelles (SOs) and membranous carriers (MCs) and that there is no identification or role of the filaments within the organelles. We are working hard to answer those questions, but this work is part of a distinct project that will take us quite some time to complete.

Thank you again for your comments and the opportunity to resubmit a modified version of our paper. A response to all points raised by both reviewers is included at the end of this letter. We look forward to hearing from you to know whether a revised manuscript with our additional studies would be considered for publication.

With best wishes.

Sincerely,

Cristina Risco and Terence Dermody

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

In this manuscript, the authors set out to investigate the cellular mechanisms exploited by non-membrane bound reoviruses for non-lytic virus exit. TEM and 3D-ET revealed that exiting reoviruses were clustered in membrane-bound structures which appeared to contact and fuse with the PM. Molecular characterization and live cell imaging indicated that the virus-carrying membrane compartments were derived from lysotracker, LAMP-1-positive structures that look like lysosomes. These lysosome-like structures are revealed to 'feed' the egress compartment with newly assembled virions through membrane connections.

Overall, while this is very interesting and well-done, the study remains largely descriptive (morphological).

As pointed out by the authors themselves in the discussion;

1. There is no real molecular understanding of how these organelles are:
-formed
-recruited
-used for virus transport
-used for virus egress
-how they fuse

Nor is the identification or role of the tubules within determined.

Response: We have provided additional details about the lysosomes co-opted by reovirus. In new experiments, we found that reovirus induces an increase in the number and size of lysosomes (new Fig. S3). In addition, we studied the distribution of late and recycling endosomes and confirmed that endosomes are not recruited to VIs. Considering that lysosomes and endosomes normally use the same transport machinery, we hypothesize that specific components of lysosomes are detected for recruitment to VIs. The number of potential candidates is considerable, and their identification will require a new study.

2. The role of pH within these organelles is discussed but not investigated or addressed.

Response: We have directly determined the pH of these organelles (new Fig. S4). The reovirus-induced modification of lysosomes includes two main features. First, reovirus induces an increase in the number and size of lysosomes (Fig. S3). Lysosomes recruited to VIs have a variety of pH values, but the largest lysosomes, either with or without virions contained within, have a

pH of ~ 6.1, higher than the pH of perinuclear lysosomes in uninfected cells (Fig. S4). Second, the lysosome-derived SOs do not appear to contain lysosomal proteases. We conducted immunofluorescence assays using a mouse monoclonal anti-cathepsin B antibody (Calbiochem CA10) and found that the antibody labeled lysosomes but not SOs recruited to VIs (Figure 1). A negative immunolabeling result is not conclusive. However, if proteases are retained inside SOs containing virions, these enzymes likely would not be functional, as the luminal pH of SOs is 6.1, and the optimal pH of lysosomal proteases is 4.5-5.0.

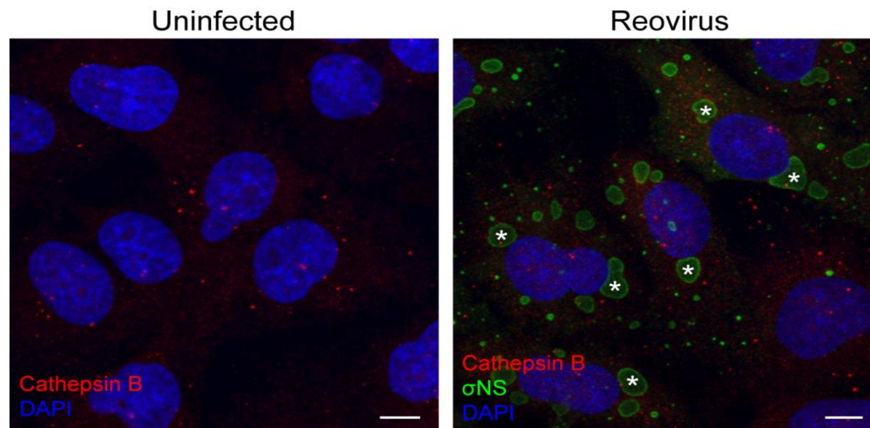

Figure 1. Lysosome-like organelles labeled with anti-cathepsin B are not recruited to viral inclusions (asterisks). Scale bar, 10 μm.

3. There is no molecular basis established to distinguish between the so-called SOs and MCs.

Response: MCs are formed from SOs as shown in 2D and 3D TEM (Figs. 3 and 9). Both organelles are labelled with anti-LAMP-1 antibodies and contain mature virions only. A more detailed characterization of their composition will require a new study.

4. I think that the new acronyms, membranous compartment (MC) and sorting organelles (SO) do the paper a disservice. These are virus-modified cellular organelles, would it not be best to define them and then call them as such.

Response: SOs are derived from lysosomes, and MCs are formed from SOs. Neither of these organelles is a cellular organelle, which is why we gave them a different name. This nomenclature is in keeping with that used for replication-associated organelles formed by many viruses. SOs and MCs stem from cellular organelles, but they are unique, hence they are called by a different name.

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

Comments:

In this study, the authors investigated late infection steps of reovirus including intracellular transport and non-lytic egress of the virus. By using confocal and super resolution microscopy, 2D and 3D TEM, and CLEM, they found that mature virions are collected by lysosome-like organelles that bud to form membrane carriers (MCs), which then release virions by fusing with plasma membranes. The finding that lysosome-related organelles are involved in selecting and delivering mature virion for non-lytic egress is interesting and novel. Unfortunately, the property of so called "modified lysosomes" is not clearly defined. Moreover, cellular mechanisms underlying the late infection steps (such as lysosome recruitment, SO and MC formation, MC fusion with PM) are not studied at all.

Specific comments:

1. The property of "modified lysosomes" that serves as sorting organelles for mature virions is not clearly defined. The authors showed that these lysosome-related organelles are lysotracker- and LAMP-positive. Do these structures contain cathepsins? Are they catalytic active?

Response: Please see our response to Comment 2 of Reviewer 1.

2. The authors stated that virus infection causes changes in the size, number and distribution of lysosomes labeled by LysoTracker (Fig. 4B). These data should be quantified. Moreover, more lysosomal markers should be examined and quantified, such as LAMP1 and cathepsin.

Response: We have quantified the size, number, and distribution of lysosomes labeled by anti-LAMP-1. These data are now shown in new Fig. S3. Please see our response to Comment 2 of Reviewer 1.

3. NH_4Cl disrupts lysosome acidity and thus inhibits lysosome function. It is not clear from the data (Fig. 7A, C) whether LAMP1-positive structures are not recruited or that they cannot enclose mature virion.

Response: To avoid inhibition of virus entry, cells were treated with NH_4Cl at 24 h post-infection. At that time, lysosomes are observed at the VI periphery. Using immunofluorescence and electron microscopy, we imaged lysosomes surrounding VIs in the presence and absence of NH_4Cl and observed that in the presence of NH_4Cl, lysosomes near VIs do not contain virions (Fig. 7). We improved the images shown in Fig. 7A by repeating the immunofluorescence experiments with a new anti-σ1 antibody that yielded clearer results.

4. In addition to property of SOs, the cellular mechanisms regarding lysosome recruitment and SO formation are not studied at all. For example, how are lysosomes recruited? Why lysosomes but not endosomes are recruited? Any

specific labeling on lysosomes links them to VI or vice versa? Some surface labeling can be tested, such as phosphoinositides and PS.

In addition, lysosome transport machinery can be tested such as RAB7-dynein and Arl8-kinesin.

Response: We studied the distribution of late and recycling endosomes and confirmed that endosomes are not recruited to VIs. Please see our response to Comment 1 of Reviewer 1.

We tested the effect of dynein inhibitor ciliobrevin A on reovirus release and found that ciliobrevin A does not inhibit viral egress (Figure 2). Therefore, it is unlikely that dynein motors are used to traffic lysosomes to VIs.

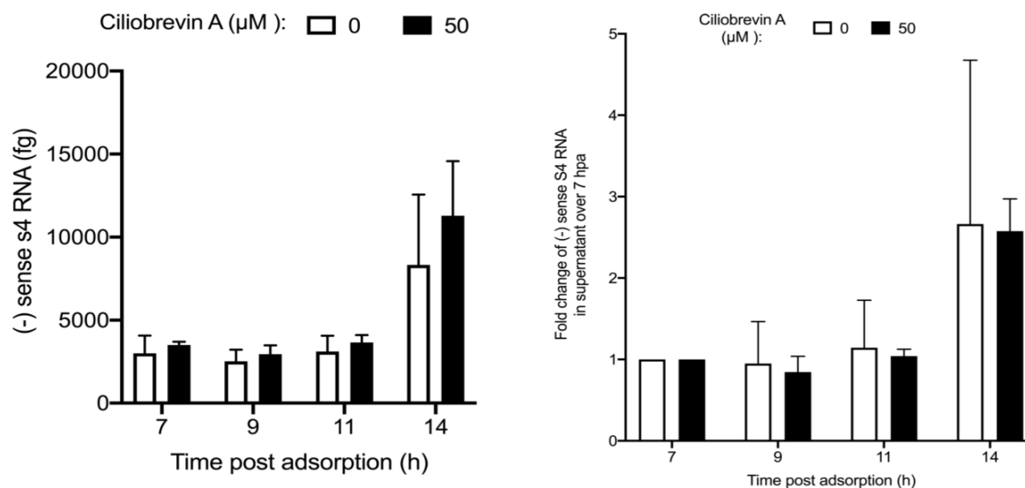

Figure 2. Effect of ciliobrevin A on reovirus egress. Hela cells were adsorbed with reovirus T3D at an MOI of 20 PFU/cell and treated with 50 μM ciliobrevin A at 7 h post adsorption. At the indicated intervals following drug treatment, the supernatant was collected, total RNA was purified, and reovirus genomic S4 RNA was quantified by RT-qPCR. S4 RNA levels were used as a surrogate for released virus to avoid the complication of transferring ciliobrevin A to indicator cells used in routine assays of viral infectivity.

What does "modified lysosome" mean? Are lysosomes modified to be recruited or that they are modified to select mature virions? How are they modified? This term is unclear and confusing.

Response: Variation in the colors of the fluorescent probes used to assess pH indicate that the lysosomes recruited to VIs have a variety of luminal pH values (Fig. S4). However, those containing virions are larger (as shown in the quantification in Fig. S3) and less acidic (as shown by their yellowish color, Fig. S4) than the characteristic perinuclear lysosomes in uninfected cells. Thus, the

pH of lysosomes appears to be modified after recruitment to the VI periphery possibly before the incorporation of mature virions.

5. From the EM images, neither VI nor MC seem to be enclosed by membranes.

Response: The reviewer is correct about VIs, as these structures are formed from collections of membranous tubules and vesicles derived from the ER and are not enclosed by membranes. These structures have been described in two previous publications from our group (Fernandez de Castro et al., mBio 2014; Tenorio et al., mBio 2018). However, MCs are indeed enclosed by membranes, as demonstrated by 2D TEM and 3D electron tomography (Figs. 2, 9, and S2).

March 11, 2020

RE: JCB Manuscript #201910131R-A

Dr. Cristina Risco
National Center for Biotechnology
Macromolecular Structures
Darwin 3
Campus UAM, Cantoblanco
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Spain

Dear Dr. Risco:

Thank you for submitting your revised manuscript entitled "A modified lysosomal organelle mediates non-lytic egress of reovirus". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

- Provide the main and supplementary texts as separate, editable .doc or .docx files
- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website *paying particular attention to the guidelines for preparing images and blots at sufficient resolution for screening and production*
- Articles can have up to five supplementary figures, there are currently eight - please combine where appropriate
- Add scale bars to figures 4C, inset S1A,

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, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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 video files: See our detailed guidelines for preparing your production-ready images, http://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.

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

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 Journal of Cell Biology.

Sincerely,

Billy Tsai Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

The authors have a done a nice job addressing some of my initial points. They provide additional understanding of the organelles themselves and from which cellular compartments they are formed, or not formed in this case.

As with the previous version the additional experiments are well controlled, and although the study remians largely descriptive the observation that reovirus hijacks two distinct cellular compartments to facilitate replication and egress is interesting and promises to open up additional lines of research.

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

The authors provided additional data in the revised manuscript to clarify some of the property of so called "modified lysosomes" (which may not contain cathepsin B (data now shown) and have a

higher pH) and to show that endosomes are not recruited. These new data are helpful, but they did not provide any mechanistic explanations on any of the key issues raised in the original comments (lysosome recruitment, SO and MC formation, MC fusion with PM).