Peroxisomes exhibit compromised structure and matrix protein content in SARS-CoV-2-infected cells

Barbara Knoblach, Ray Ishida, Tom Hobman, and Richard Rachubinski

Corresponding author(s): Richard Rachubinski, University of Alberta

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(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.)

RE: Manuscript #E21-02-0074
TITLE: SARS-CoV-2 infection causes disintegration and collapse of the peroxisome compartment

Dear Dr. Rachubinski

Thank you for your patience. We have now received both reviews of your manuscript. While the first reviewer had overall a positive impression of the manuscript, the second reviewer, who is someone with expertise in the field of peroxisome biology, has raised several critical issues that will need to be addressed in order to move forward. Given the discrepancy in the reviewers comments, I read your manuscript to evaluate it. I agree with the second reviewer in particular regarding including ultrastructural morphology of the peroxisomes, which will strengthen your conclusions. Also I would add that additional time points to verify replication in Figure 1 is necessary as the amount of viral RNA detected could be due to just internalization of the virus (i.e. not replication).
I am sorry I cannot bring you more positive news right now but I hope you will be able to address this review and my comments and resubmit to Molecular Biology of the Cell.

Warm regards
Nihal Altan-Bonnet

Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Rachubinski,

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

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

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

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usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

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To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

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

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

Sincerely,

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

Reviewer #1 (Remarks to the Author):

This is a very well done study that illustrates the way that good cell biology can be leveraged to understand disease. The authors nicely demonstrate that SARS-CoV-2 infection leads to a breakdown of peroxisomes, with the most compelling data being presented on the defects in functional peroxisomes. The authors provide striking microscropic images of peroxisomes that lack PTS1 at late stages of infection. Future work will surely be focused on defining the mechanisms and consequences of these activities. My only concern with this study is the focus on a singele organelle. I would strongly encourage the authors to examine the morphology of other organelles within the cell. Lysosomes would be a useful comparison, but I leave it to the authors to decide how to address this point.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors observe that infection with the SARS-CoV-2 virus changes peroxisome-related immunostaining in two human cell lines. Figure 2 shows that two peroxisomal membrane proteins, PMP70 and PEX14, largely co-localize in puncta in uninfected cells but become more separated 72 hours following viral infection, with PMP70 losing its punctate pattern. Figure 3 shows in infected cells, PTS1-labeled puncta are replaced with diffuse PTS1 staining, suggesting that peroxisomes are lysed and/or lose matrix protein import ability in infected cells. Figure 4 probes the in vitro interaction between PEX14 and the SARS-CoV-2 ORF14 protein. Together, these data suggest that SARS-CoV-2 infection might impact peroxisome biology. Although interesting observations are reported, there are several limitations of the data that prevent a full appreciation of the significance of the findings.

1. The claim that PEX14 interacts with the viral ORF14 protein is weak.

1a. Figure 4 is confusing because MBP and MBP-ORF14 appear to be the same molecular mass. One would predict that the 73-amino acid ORF14 would increase the apparent molecular mass of MBP by ~8 kDa; such as shift is not apparent in the immunoblot of Figure 4.

1b. Figure 4 is mis-labeled, making it difficult to interpret. But assuming the "GST-ORF14" is a typo and should be "GST-PEX14", there is some indication of an interaction. However, it appears that the MBP-ORF14 protein is overloaded compared to the MBP protein, and a small amount of GST-PEX14(?) is present in the MBP control pulldown, calling into question the significance of the increased GST-PEX14(?) in the MBP-ORF14 pulldown. This putative PEX14-ORF14 interaction should be validated using an orthogonal approach. Such a validation is necessary because PEX14 was not among even the >22,000 proteins tentatively identified as interacting with SARS-CoV-2 proteins (Gordon et al., 2020).

2. The microscopy is not fully validated.

2a. Figure 3a-d shows that PMP70 and PTS1 antibodies label different structures. This result is surprising. The PMP70-reactive puncta are largely also recognized by the PEX14 antibody (Figure 2), suggesting that these are peroxisomes. However, the lack of PTS1 localization with PMP70 in these structures calls into question the identity of the puncta recognized by the PTS1 antibody. It would seem to be necessary to validate the PTS1 findings with another antibody, perhaps one that recognizes an individual peroxisomal matrix protein (e.g., catalase).

2b. To understand the importance of the findings of apparent peroxisome rearrangements during infection, it would be useful to have some context as to the timing of the observed changes relative to the course of infection. Does the virus kill the cells, and if so, when does this happen relative to the 72 hour time course of the observations? In other words, are these changes happening early or late in the infection process relative to other cellular changes? The authors seem to be proposing a direct impact of SARS-CoV-2 on peroxisomes (based on the interaction of ORF14 with PEX14), but because the supporting data are weak (see point 1), it would be useful to know more about the timeline of infection.

3. There are instances when the authors do not consistently and clearly distinguish between what they observe and what they conclude. For example:

3a. p. 6 - The authors note that "PMP70-labeled structures in infected cells...appeared to coalesce and sometimes fragment." This phrasing is unclear, as the authors are observing fixed cells and not live cell imaging. Perhaps "both larger and smaller PMP70-labeled structures were observed in infected cells, suggesting that peroxisome might be coalescing and fragmenting"?

3b. p. 9 - "Quantification of PTS1-labeled puncta showed that numbers of metabolically active peroxisomes...decreased..." This leap from observed "PTS1-labeled puncta" to "metabolically active peroxisomes" would be better linked with "suggested that" rather than "showed that".

3c. p. 9 - Similarly, the term "functional peroxisomes" would be more accurate as "PTS1-labeled peroxisomes."

4. There are additional instances in the text that could be clarified.

4a. p. 7 - The authors note that PMP70 staining "was more reticular than punctate" after noting in the previous sentence that PMP70 staining "exhibited characteristic punctate or elongated morphology." This is confusing.

4b. p. 9 - The statement that "SARS-CoV-2 has a greater negative effect on peroxisomes and their functionality than do other viruses" is an unnecessarily sweeping statement that might need to be toned down because no other viruses are evaluated in the present work, and because the cited papers may not have used comparable assays (cell lines, time points, antibodies, etc.) in their assessments.

4c. p. 9 - The statement that ORF14 was identified as "an interaction partner of various PEX proteins, including PEX3..., PEX11b..., and PMP70 (Gordon et al., 2020)" may be an overstatement. Although these proteins are among the over 22,000 proteins identified in the global experiment cited, these proteins are not among the 333 "high confidence interactions" reported in Gordon et al.

5. Some of the items specified in the MBoC data presentation guidelines appear to be missing or incomplete:

5a. Figure 3B: The bar graph shows the results of three independent experiments; these three mean values should be shown as points on the bar graphs (or as points without a bar graph as in Figure 1A).

5b. Figure 4: I did not find an indication in the methods or figure legend indicating how many times the results of Figure 4 were replicated.

5c. Methods: I did not find the method of cell line authentication or the frequency of testing for mycoplasm contamination in the methods.

April 29, 2021

Dr. Nihal Altan-Bonnet
Monitoring Editor
Molecular Biology of the Cell

RE: Manuscript #E21-02-0074

Dear Dr. Altan-Bonnet,

We hereby submit the revision of our manuscript entitled "SARS-CoV-2 infection causes disintegration and collapse of the peroxisome compartment". We have addressed the Reviewers' comments as follows:

Reviewer 1

In this manuscript, the authors observe that infection with the SARS-CoV-2 virus changes peroxisome-related immunostaining in two human cell lines. Figure 2 shows that two peroxisomal membrane proteins, PMP70 and PEX14, largely co-localize in puncta in uninfected cells but become more separated 72 hours following viral infection, with PMP70 losing its punctate pattern. Figure 3 shows in infected cells, PTS1-labeled puncta are replaced with diffuse PTS1 staining, suggesting that peroxisomes are lysed and/or lose matrix protein import ability in infected cells. Figure 4 probes the in vitro interaction between PEX14 and the SARS-CoV-2 ORF14 protein. Together, these data suggest that SARS-CoV-2 infection might impact peroxisome biology. Although interesting observations are reported, there are several limitations of the data that prevent a full appreciation of the significance of the findings.

1. The claim that PEX14 interacts with the viral ORF14 protein is weak.

1a. Figure 4 is confusing because MBP and MBP-ORF14 appear to be the same molecular mass. One would predict that the 73-amino acid ORF14 would increase the apparent molecular mass of MBP by ~8 kDa; such as shift is not apparent in the immunoblot of Figure 4.

We did additional experiments running either long SDS-PAGE gels or running Tris-acetate gels to better resolve a difference in migration between MBP alone and MBP-ORF14. We were unable to detect a notable difference in the migrations of these two proteins, suggesting that MBP-ORF14 exhibits an aberrant migration, the reason for which remains unknown. It must be emphasized that all recombinant DNA constructs used in this study were verified by sequencing and confirmed the correct fusion of SARS-CoV-2 ORF14 to MBP (see p. 14 of the revised manuscript).

1b. Figure 4 is mis-labeled, making it difficult to interpret. But assuming the "GST-ORF14" is a typo and should be "GST-PEX14", there is some indication of an interaction.

We thank Reviewer 1 for bringing this to our attention. The error has been corrected in the revised figure (now Figure 5).

However, it appears that the MBP-ORF14 protein is overloaded compared to the MBP protein, and a small amount of GST-PEX14(?) is present in the MBP control pulldown, calling into question the significance of the increased GST-PEX14(?) in the MBP-ORF14 pulldown. This putative PEX14-ORF14 interaction should be validated using an orthogonal approach. Such a validation is necessary because PEX14 was not among even the >22,000 proteins tentatively identified as interacting with SARS-CoV-2 proteins (Gordon et al., 2020).

The experiment was repeated and corrected for protein loading (see revised Figure 5). We wish to point out that this experiment has been performed multiple times and always showed a specific interaction between MBP-SARS-CoV-2-ORF14 and GST-PEX14. A reciprocal pull-down experiment in which GST alone or GST-PEX14 was immobilized to beads and incubated with either MBP alone or MBP-ORF14 also showed a specific interaction only between GST-PEX14 and MBP-ORF14. We opted for a pull-down assay as it demonstrates a direct interaction between binding partners. Demonstration of an interaction between ORF14 and PEX14 was done to suggest a mechanistic basis for the morphological results we observed. An additional orthogonal demonstration of the interaction between ORF14 and PEX14 is, we feel, beyond the scope of this current manuscript.

2. The microscopy is not fully validated.

2a. Figure 3a-d shows that PMP70 and PTS1 antibodies label different structures. This result is surprising. The PMP70-reactive puncta are largely also recognized by the PEX14 antibody (Figure 2), suggesting that these are peroxisomes. However, the lack of PTS1 localization with PMP70 in these structures calls into question the identity of the puncta recognized by the PTS1 antibody. It would seem to be necessary to validate the PTS1 findings with another antibody, perhaps one that recognizes an individual peroxisomal matrix protein (e.g., catalase).

We opted to use an antibody directed against thiolase (a PTS2-protein) to validate our PTS1 results (see new Figure 4). We chose to visualize a PTS2 protein (thiolase) over a PTS1 protein (catalase), as PTS1-targeted and PTS2-targeted proteins follow distinct import pathways into peroxisomes. We can now rule out that only PTS1-mediated peroxisomal matrix protein import is compromised in SARS-CoV-2-infected cells.

We also observed that the PMP70-signal and the thiolase-signal do not colocalize to a significant extent in mock-treated cells, thus supporting our previous observation that PTS1-containing and PMP70 localize to distinct regions of the peroxisomal compartment.

2b. To understand the importance of the findings of apparent peroxisome rearrangements during infection, it would be useful to have some context as to the timing of the observed changes relative to the course of infection. Does the virus kill the cells, and if so, when does this happen relative to the 72 hour time course of the observations? In other words, are these changes

happening early or late in the infection process relative to other cellular changes? The authors seem to be proposing a direct impact of SARS-CoV-2 on peroxisomes (based on the interaction of ORF14 with PEX14), but because the supporting data are weak (see point 1), it would be useful to know more about the timeline of infection.

Cell viability is now reported in a new Figure 1A. Because the viability of Huh-7 cells is not reduced at 24 and 48 hpi compared to mock-infected cells, and only moderately reduced at 72 hpi, we rule out that the morphological changes observed in peroxisomes in virus-infected Huh-7 cells are a consequence of cell death.

3. There are instances when the authors do not consistently and clearly distinguish between what they observe and what they conclude. For example:

3a. p. 6 - The authors note that "PMP70-labeled structures in infected cells...appeared to coalesce and sometimes fragment." This phrasing is unclear, as the authors are observing fixed cells and not live cell imaging. Perhaps "both larger and smaller PMP70-labeled structures were observed in infected cells, suggesting that peroxisome might be coalescing and fragmenting"?

We have revised the manuscript as suggested by Reviewer 1.

3b. p. 9 - "Quantification of PTS1-labeled puncta showed that numbers of metabolically active peroxisomes...decreased..." This leap from observed "PTS1-labeled puncta" to "metabolically active peroxisomes" would be better linked with "suggested that" rather than "showed that".

We have revised the manuscript as suggested by Reviewer 1.

3c. p. 9 - Similarly, the term "functional peroxisomes" would be more accurate as "PTS1-labeled peroxisomes."

We have revised the manuscript as suggested by Reviewer 1.

4. There are additional instances in the text that could be clarified.

4a. p. 7 - The authors note that PMP70 staining "was more reticular than punctate" after noting in the previous sentence that PMP70 staining "exhibited characteristic punctate or elongated morphology." This is confusing.

We have clarified this statement on p. 7 as follows: "In mock-infected cells, PMP70-labeled structures were seen to be dispersed throughout the cytosol and to exhibit both a punctate morphology characteristic of peroxisomes and an elongated morphology suggestive of localization to another compartment such as the ER (Figure 2; a, b and d)."

4b. p. 9 - The statement that "SARS-CoV-2 has a greater negative effect on peroxisomes and their functionality than do other viruses" is an unnecessarily sweeping statement that might need to be toned down because no other viruses are evaluated in the present work, and because the cited papers may not have used comparable assays (cell lines, time points, antibodies, etc.) in their assessments.

We have toned down this statement and now write on p.10 of the revised manuscript: "Reduced numbers of peroxisomes have been reported for cells infected by dengue, West Nile and Zika viruses (You *et al.*, 2015; Wong *et al.*, 2019); however, no disintegration of the peroxisome compartment during viral infection of cells like we have observed has been reported. Therefore, infection of cells by SARS-CoV-2 may more negatively affect the structure of peroxisomes and their functionality than do other viruses, although demonstration of this awaits future directly comparative studies."

4c. p. 9 - The statement that ORF14 was identified as "an interaction partner of various PEX proteins, including PEX3..., PEX11b..., and PMP70 (Gordon et al., 2020)" may be an overstatement. Although these proteins are among the over 22,000 proteins identified in the global experiment cited, these proteins are not among the 333 "high confidence interactions" reported in Gordon et al.

We have rephrased as "putative interaction partner" (p. 11 of the revised manuscript).

5. Some of the items specified in the MBoC data presentation guidelines appear to be missing or incomplete:

5a. Figure 3B: The bar graph shows the results of three independent experiments; these three mean values should be shown as points on the bar graphs (or as points without a bar graph as in Figure 1A).

The figure has been modified to show the individual data points, as requested by Reviewer 1.

5b. Figure 4: I did not find an indication in the methods or figure legend indicating how many times the results of Figure 4 were replicated.

This information is now included in the legend to Figure 5 (formerly Figure 4) on p. 23 of the revised manuscript.

5c. Methods: I did not find the method of cell line authentication or the frequency of testing for mycoplasm contamination in the methods.

The following information now appears on p. 13 of the revised manuscript:

Cell culture and virus infection

Vero E6 cells (American Type Culture Collection) used for the production of SARS-CoV-2 virus, Huh-7 cells (Thermo-Fisher) and SK-N-SH cells (Millipore-Sigma) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 100 U penicillin/mL, 100 U streptomycin/mL, 1 mM HEPES (Gibco), 2 mM glutamine (Gibco), 10% heat-inactivated fetal bovine serum (FBS) (Gibco) at 37°C in 5% CO_2. Cells were seeded the day before infection with SARS-CoV-2 and were infected at a MOI of 1.

Cell lines were limited to 20 passages, during which time they were monitored for aberrant morphology. After 20 passages, aliquots of new cells were revived. Cell lines upon receipt or when revived were tested for the presence of mycoplasma by PCR using primers specific for 16S rRNA gene of *Mycoplasma hyorhinis* (Eldering *et al.*, 2004). Cell cultures were tested for mycoplasma

every 6-8 weeks, and on the rare occasion that a positive test arose, cultures were discarded, and the cell line was revived.

Reviewer 2
This is a very well done study that illustrates the way that good cell biology can be leveraged to understand disease. The authors nicely demonstrate that SARS-CoV-2 infection leads to a breakdown of peroxisomes, with the most compelling data being presented on the defects in functional peroxisomes. The authors provide striking microscopic images of peroxisomes that lack PTS1 at late stages of infection. Future work will surely be focused on defining the mechanisms and consequences of these activities. My only concern with this study is the focus on a single organelle. I would strongly encourage the authors to examine the morphology of other organelles within the cell. Lysosomes would be a useful comparison, but I leave it to the authors to decide how to address this point.

We thank Reviewer 2 for the positive evaluation of our manuscript. We agree with Reviewer 2 that it would be interesting to consider the effects of SARS-CoV-2 infection on the integrity and morphologies of other organelles like lysosomes, but such investigations would require additional major studies that are beyond the scope of the present study on SARS-CoV-2 and peroxisomes.

We would like to thank the Reviewers for their insightful comments and you for the handling of our submission.

Sincerely,

Richard Rachubinski

RE: Manuscript #E21-02-0074R
TITLE: "Peroxisomes exhibit compromised structure and matrix protein content in SARS-CoV-2-infected cells"

Dear Dr. Rachubinski

Thank you for revising the manuscript and making changes to the title. We are now ready to move forward and accept your manuscript for publication in Molecular Biology of the Cell.

Thank you
Nihal Altan-Bonnet

Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Rachubinski:

Congratulations on the acceptance of your manuscript.

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

Within approximately four weeks you will receive a PDF page proof of your article.

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