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Coronaviral ORF6 protein mediates inter-organelle contacts and modulates host cell lipid flux for virus production

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1st Editorial Decision **24th Oct 2022**

Dear Binbin,

Thank you for submitting your manuscript to The EMBO Journal. We have now received comments from four reviewers with expertise in viral replication (reviewers #1 and #3) and lipid droplet-organelle interactions (reviewers #2 and #4), which are included below for your information.

As you will see from the reports, the reviewers find the study interesting, but they also raise multiple substantial concerns with the study, in particular regarding the data presentation, conclusiveness and quantification. The reviewers find that insufficient support is provided for the role of SARS-CoV-2 ORF6 function in lipid biogenesis, inter-organelle contact site re-organisation, and the relevance of these contact sites for viral replication.

Based on the overall interest expressed in the referee reports and your willingness to engage in a major revision as expressed in the preliminary revision plan provided during the pre-decision consultation, I would like to invite you to address the comments of all reviewers in a revised version of the manuscript. From the editorial side, providing further insight into ORF6 homodimerisation is not required, and I agree that a detailed further investigation in this ORF6 property is not needed. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

Due to the extensive nature of the revision, I have extended the revision time to four months. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this four-month deadline, please let us know in advance to arrange an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication, and I look forward to receiving the revised manuscript.

Best regards,

Ieva

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Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and

conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days;

Referee #1:

Yue et al investigated the role of the SARS-CoV-2 ORF6 protein on host lipid droplets. The authors demonstrate that the predicted amphipathic helices of ORF6 are required for homodimerization and lipid droplet interaction. Their data suggests that ORF6 expression increases lipid droplet numbers, and using chemical alterations (ATGLi, DGAT1/2i, CQ), suggest that ORF6 specifically induces lipid droplet biogenesis. Next they investigated ORF6 localization to the ER, suggesting that the viral protein tethers lipid droplets to the ER. Finally, they look at ORF6 localization to mitochondria, again suggesting that it acts as an anchor between lipid droplets and mitochondria. This paper uses a combination of generally well-performed immunoprecipitations, confocal and electron microscopy experiments to support their hypotheses, but some clarification/added experiments are needed to help readers navigate this very dense paper.

Major comments:

1. Authors identify many membrane contact sites induced by ORF6 expression and chemical inhibitions, but the only assays used to confirm the phenotype in SARS-CoV-2 infections are western blots and plaque assays. Can an increase in inter-organelle contact sites be confirmed in infected cells by EM for conclusive evidence that replication organelles are at these contact sites.
2. Figure 3F needs the comparative non untreated control
3. ATGLi treatment usually results in the accumulation of larger lipid droplets, the chosen images do not show this. Does ORF6 expression change the size of the lipid droplets with or without ATGLi treatment?
4. Does orf6 dimerization matter for the LD/mito or LD/ER contact sites? Can the authors determine what part of the protein is interacting with DGAT1/2 and if dimerization is required?
5. While the manuscript has compelling data, it is very dense and could use some streamlining by possibly moving some panels to the supplemental figures (like Fig 5J-N) or text based changes.
Minor comments:
1. What about seipin localization? If the authors argue that ORF6 is involved in LD biogenesis, then is it near seipin a major biogenesis protein?
2. Figure 3I and 5B have strikingly different LD/cell numbers despite being the same conditions (3X inhibitors +/- ORF6)?
3. How were lipid droplets/cell or Mito/LD contact sites quantified? The methods section did not explain.
4. Figures 1 and 4 are difficult to navigate as panels are non-intuitively laid out.
5. Figure 6, please add quantification of the images from S5C (the -OA condition) to compare next to these +OA quants?
6. Please quantify the differences between the +/- ORF6 of Fig 6G and +/- virus in Fig 6J since text states significantly higher/more.

Referee #2:

This manuscript entitled "Viral Protein Links Lipid Droplets, the ER, and Mitochondria, and Modulates Lipid Flux for Virus Production" by M. Yue and collaborators describes the role of the ORF6 protein of SARS-CoV-2 in mediating endoplasmic reticulum (ER)-lipid droplet (LD) and LD-mitochondria contacts.
The authors first showed that amongst the proteins encoded by the genome of SARS-CoV-2, ORF6 is localized to LDs. Then, they characterized the molecular mechanism of the interaction of ORF6 with LDs and showed that ORF6 promotes the biogenesis of LDs by binding with DGAT1/2. Moreover, they showed that ORF6 promotes the formation of ER-LD contacts, promotes lipolysis by binding to ATGL, and link mitochondria to LDs, and finally peroxisome to LDs.
Although many of these results are interesting, the experiments described are not strong enough to support the authors' claims. Controls are missing and some data are over-interpreted. The authors should really add complementary experiments to make their point.

Below are some of the main issues with the manuscript:

Major points:
1. There are many experiments which are not associated with quantifications (for instance, Fig. 1G, 2G, 4E...); Co-localizations should be quantified (Pearson correlation for instance). Moreover, some quantifications are not properly done: statistics should be performed between independent samples, while here statistics (Fig. 3B, 3E, 3I etc...) are done considering individual cells as independent samples. The experiments should be repeated multiple times and statistics performed between experiments (using the mean of several cells quantified per experiment).
2. Figure 1A, C: ORF6 is bound to LDs but some of the staining is not associated with LDs: what is the other localization of ORF6?
3. Figure 1G: the localization of the ORF6-4Q mutant should be determined: is it localized in the cytosol or is it associated with the endoplasmic reticulum or another organelle?
4. The role of the amphipathic helices AH1 and AH2 should be further investigated:
a. Are AH1 and AH2 alone able to recruit a protein at the surface of LDs? (could be answered using AH1- and AH2-GFP fusion proteins)
b. Are AH1 and AH2 sufficient to associate with the surface of LDs? This should be answered in an in vitro simplified system using synthetic peptides and artificial LDs. This kind of experiment could help understand the function of AH1 vs AH2.
c. The role of the dimerization of ORF6 remains unclear:
i. Figure 2: the data shown in this figure are over-interpreted: for instance, the authors claim that the AH1 helix alone is responsible for dimerization even if the level of dimerization of the deltaAH2 mutant is reduced by about 10-fold. The relative contribution of AH1 and AH2 really needs to be clarified.
ii. the deltaAH2 mutant does not really associate with LDs. This is not analyzed in this way in the manuscript: only the role of AH1 is discussed.
iii. What is the effect of the mutations described in Figure 1 (LL-QQ and LI-QQ) on dimerization? Why did the authors only use the 4Q mutation in their analysis?
iv. The authors propose 2 models for the role of AH1 and AH2: in figure 1, AH1 and AH2 are both involved in binding lipid droplets, and in figure 2, AH1 is involved in ORF6 dimerization while AH2 is involved in binding to LDs. Are these models mutually exclusive and in that case which model is correct? If I understood correctly, the authors favor the second model. In that case, they should prove that this model is correct. Can AH1 be replaced by a dimerization domain? How does AH1 dimerize? The identification of point mutations disrupting the dimerization could be a valuable information. Can AH2 be replaced by another amphipathic helix known to interact with LDs?
v. Figure 2E: the amount of ORF6-6His protein pulled-down by ORF6-GST is really higher than that of the mutants ORF6-GST. Could the authors comment this point.
5. Figure 3: the authors claim that the ORF6 4Q mutant does not promote LD growth but there is no quantification shown. The same experiment as in Fig. 3A-B should be performed with this mutant.
6. Figure 3F: why did the authors test the interaction of ORF6 with DGAT1/2? What is the mechanism of interaction between ORF6 and DGAT1/2? Could the authors identify point mutations disrupting the interaction? It would be useful to understand the role of the interaction in the activity of DGAT enzymes. Does ORF6 expression increase the level of DGAT1 and DGAT2? Is the effect transcriptional/post-traductional?
7. Figure 4A: markers of other organelles (lipid droplets, mitochondria, endosomes...) should be added to control the purity of the fractions.
8. Figure 1 and Figure 4: is ORF6 present at the surface of LDs or in ER-LD contacts?
9. Figure 4: is ORF4 promoting the formation of ER-LD contacts? ER-LD contacts should be quantified in control and ORF4 expressing cells.
10. Figure 4C: are DGAT proteins localized in the ER? Could the interaction with DGAT be involved in the formation of ER-LD contacts?
11. Figure 4: the analysis of the ORF4DNTD and ORF6DH1 mutant is not sufficient to conclude "that the interactions between ORF6-BAP31 and/or ORF6-USE1 were important for the formation of direct ER-LD contacts". The authors should silence BAP31 and USE1 and quantify ER-LD contacts induced by ORF6.
12. Figure 4E: the colocalization should be quantified and/or EM experiments performed.
13. Are BAP31 and USE1 more localized in ER-LD contacts when ORF6 is over-expressed?
14. Figure S4C: the control images are missing. The modification of UBXD8 localization should be quantified.
15. Page 14 line 292: "Furthermore, compared to non-infection, SARS-CoV-2 infection enhanced the interaction of ATGL with Plin2 and UBXD8". Is the interpretation of this experiment correct?
16. Figure 6A: markers of other organelles (lipid droplets, ER, endosomes...) should be added to control the purity of the fractions
17. Figure 6: does ORF6 promote the association of LDs with mitochondria?
18. There are many different localizations of ORF6 that are described in this paper; what is the percentage of the protein present in each of these localizations?
19. Figure 6C-D: it would be more appropriate to perform the quantification in cells not treated with OA: in cells treated with OA, LDs are huge and the interpretation of the date becomes difficult.
20. Figure 6E and F: this experiment is biased by the effect of ORF6 on the number of LDs. At the resolution of fluorescence microscopy, the increased number of LDs will indeed increase the apparent number of LD-mito contacts even if the number of contacts is unchanged.
21. Figure 6G: some staining on these images are over-saturated and make the interpretation of the data difficult.
22. The co-localizations shown Figure S6A are not convincing: this should be quantified.

23. The interpretation of the experiment shown Fig. S6C is odd. The mutant lacking both AH1 and AH2 is missing.
24. Figure 7F: is there an effect of silencing MTX1, MTX2, and/or SAMM50 on the number of LD and the number of mitochondria? If so, this could bias the interpretation of this experiment.
25. Figure S7: again the same controls are missing and no quantification is shown; is there an effect of ORF6 on the number of peroxisome? And the effect of ORF6 on the number of LDs affects the interpretation of the result.

Minor points:
1. The introduction could be shortened.
2. Figure 2G: show the bodipy alone panels.
3. Figure 3 A: what is the meaning of the white and yellow ROIs? It is not described in the legend.
4. Figure S2: there are 2 B panels.
5. Figure S3C is never called. The KD of BAP31 and USE1 seems to affect the ER morphology. Could the authors comment.
6. Figure J: change the LUTs.
7. The materials and methods does not describe the methodologies used to quantify images (IF and EM)

Referee #3:

In this manuscript, Yue and colleagues report the role of SARS-CoV-2 accessory gene ORF6 as an important factor to modulate LD biogenesis during viral infection. They identified two conserved amphipathic helices in ORF6 that are required for the association with the LD membrane. They also demonstrate through crosslinking and pull-downs that the AH1 is required for ORF6 dimerization, and that dimerization is essential for ORF6 recruitment to the LD. Furthermore, they show how ORF6 is involved in the connection between LD and both the ER and mitochondria through the interaction with BAP31 and USE1 for the ER-LDs side and the mitochondrial SAMM complex to mediate the interaction with the mitochondria. Mechanistically, the authors show that ORF6 expression is able to activate lipogenesis through interaction with DGAT1 and DGAT2 and activate lipolysis of triacylglycerol and transfer of fatty acids from LD to mitochondria through activation of ATGL.
Overall, the data are well presented and appear for most aspect solid. Their mechanistic characterization of the role of overexpressed ORF6 is well performed, looking at many aspects performing interaction studies, mutational analysis, and imaging.
My main critique is that there are few experiments performed to validate their results in an infectious system, a limit that the authors themselves highlight in the manuscript discussion. Among the experiments conducted in the infection system, the authors show how viral infection alters the interactors of ATGL, mirroring their observations in transfected cells. In addition, they analyzed the viral titers upon knock-down or inhibition of the factors they identified as interactors (DGAT1 and DGAT2 and several others).
However, additional validation can be performed in the infectious system to prove that the observations made in the context of ORF6 overexpression can be translated in an infectious system. For instance, the authors never showed the localization of ORF6 in infected cells and considering that this is the main point of the manuscript, they should prove that during infection indeed ORF6 localizes clearly on LD and that mitochondria relocalize in their proximity (do ER/LD and LD/mitochondria contact sites increase also during infection?). Moreover, all the experiments performed in an infectious system require the analysis of the cell viability to prove that the perturbations applied (knock-down, inhibitors or chemicals such as OA) do not alter cell viability in absence of viral infection.
Another important misconception that the author perpetrate throughout the manuscript is to refer to "viral replication" while measuring secreted infectivity (virus titers). This can be misleading since viral replication refers to a specific step (viral genome replication) of the virus infectious cycle.
The experiment they perform (virus titers) measures infectivity secreted from the cells and thus cannot rule out that the effect they observe is due to alteration in either entry, genome replication, virus assembly and/or virus release.
It is important that this point is clear, and the author have to either tune down their conclusion by stating that the exact step is still unknown and might not be "replication" or they need to perform additional experiments (such as entry assays with pseudoviruses to exclude entry and intracellular RNA quantification during viral infection to prove the reduction of viral replication) that unequivocally points toward an effect on viral replication.

Point-by-point comments
Viability assays: Figure 5A and 5B: experiments with inhibitors require the analysis of cells viability and WB that shows that in all conditions the levels of ORF6 are equal.
All perturbations performed on infected cells require a viability control to prove that alterations of viral infectivity/replication are not due to cells death. Please include viability test for all treatments (inhibitors, chemicals or siRNA).

>Figure 6D-F: The authors observe more mito-LD contact per cell upon ORF6 overexpression. However, they also see double the amount of LD in this condition. Using this kind of quantification, they cannot exclude that contact sites simply increase because more LD are presents. Number of LD/mito contact should be calculated on the single LD level (number of contacts for

each LD), ideally including ORF6 positive and ORF6 negative droplets. Does the triple KD affect the number of LD? This could help solve this problem, since if KD does not affect LD number, the reduction in the number of LD-mito contact sites is indeed specific. In addition, there is no statement on how contact sites are calculated in the material and methods section. Please add a section explaining how this quantification has been performed.

>Quantification of the IF is performed on 25 cells. However, the authors do not state how many biological replicates they used. Please ensure that at least two independent biological replicates are analyzed

>I am not satisfied with the experiments showing the tethering of the ER to the LD. While the biochemical studies seem solid, the imaging approaches are not. ER membranes in the immunogold preparations are poorly visible and while extensive gold label is present, a single field of view without quantification is not enough to prove that ER is wrapping LD. The authors should provide high resolution TEM images of transfected and mock cells and quantify the number of LD wrapped or not by ER membranes. Similarly for the confocal studies, the authors should provide quantification of the colocalization observed in the context of the ORF6 and ORF6 mutants with the ER and LD.

Minor points
>Induction of LD has been previously reported by https://www.nature.com/articles/s41467-022-31097-7 - Farley and co-workers showed that ORF6 induces LD and DGAT1 inhibitors are able to reduce viral titers in infected cells. Please cite this recent paper and discuss their results in light of your findings.

>Figure 6G: please include quantification (colocalization at the different time points)
Figure 6J: please include quantification. Also there seems to be bleed through between the two channels in the infected cells (BODIPY signal looks filamentous in the zoom panel of the infected cell).

>Sup Fig1E: Rig-1 should read Rig-I

Referee #4:

In the manuscript by Yue et al., the authors explore the functional role of the SARS-CoV-2 protein ORF6, which they find targets to the surface of lipid droplets through two putative amphipathic helices. The authors introduce several glutamine mutations into hydrophobic residues of the helices, causing a loss of lipid droplet targeting. The authors find overexpression of ORF6 in mammalian cells promotes larger lipid droplets and increased TAG storage, which they propose occurs via an interaction detected between ORF6 and DGAT1/2. Somewhat paradoxically, however, the expression of ORF6 also leads to increased TAG lipolysis, which they propose is mediated by interactions between ORF6 and ATGL. The authors also provide evidence that inhibiting lipid droplet formation or inhibiting lipolysis decreases viral titers. In addition to these data, the authors explore interacting partners of ORF6 based on published IP/MS datasets, identifying several ER and mitochondrial proteins that interact with ORF6 in their assays, including the mitochondrial SAM complex. The authors demonstrate that depleting a number of these interacting partners in host cells reduces viral infectivity.

While there are a number of interesting observations in the manuscript, the argument that ORF6 simultaneously promotes lipid droplet biogenesis and lipolysis is confusing and must be explored in greater detail. Further the conclusions of the manuscript would be greatly strengthened if effects on viral infectivity were tested by mutating ORF6 (i.e using the 4Q mutation) rather than knocking down host proteins, which in each case are known to have severe pleiotropic effects.

Major points:
1. All experiments were done in the paper with either gain of function ORF6 expression or correlative experiments measuring viral titers in host cells with various treatments. However, loss of function experiments are necessary to strongly conclude that ORF6 and its targeting to lipid droplets is important for viral replication.
2. The ability of ORF6 to promote both biogenesis of lipid droplets and their lipolysis is confusing. ORF6 was shown throughout the manuscript to bind to DGATs, ATGL, BAP31, USE1, SAMM50, MTX1, and MTX2 in a manner dependent on a 7 amino acid stretch of protein. While the N-terminal deletion construct can be expressed in cells, is it possible this construct is improperly folded, leading to the loss of interactions?
3. Likewise, these interactions may not be indicative of functional roles. Do DGATs or ATGL become more targeted to LDs when ORF6 is overexpressed and does this depend on the N-terminus of ORF6?
4. The viral titer decrease in KD of BAP31/USE1 and in SAMM50/MTX1/MTX2 may be due to effects on ER or mitochondrial function, respectively, rather than loss of binding to ORF6. The SAM complex is required for mitochondrial morphology, cristae architecture, and proper respiration, for example. These conclusions would be stronger if the ORF6 interaction with each protein were specifically interrupted.
5. The conclusion that ORF6 promotes lipid droplet-mitochondrial contact may be due to increased lipid droplet number in overexpression cells or increased TAG content, but not necessarily due to an interaction with the SAM complex. Does DGAT overexpression cause similar increases in contact between the organelles in their assay?

Dear Ieva and Reviewers,

We are deeply grateful for your great efforts and constructive comments towards our manuscript (EMBOJ-2022-112542) titled "Viral Protein Links Lipid Droplets, the ER, and Mitochondria, and Modulates Lipid Flux for Virus Production". The detailed responses follow here. Please note that the response text is highlighted in blue. Many thanks for your attentions.

Point-by-point response:

Referee #1 (Report for Author)

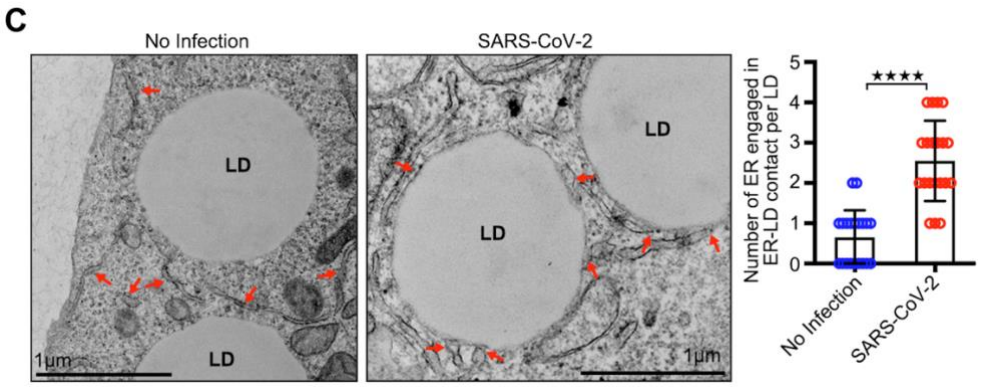
Yue et al investigated the role of the SARS-CoV-2 ORF6 protein on host lipid droplets. The authors demonstrate that the predicted amphipathic helices of ORF6 are required for homodimerization and lipid droplet interaction. Their data suggests that ORF6 expression increases lipid droplet numbers, and using chemical alterations (ATGLi, DGAT1/2i, CQ), suggest that ORF6 specifically induces lipid droplet biogenesis. Next they investigated ORF6 localization to the ER, suggesting that the viral protein tethers lipid droplets to the ER. Finally, they look at ORF6 localization to mitochondria, again suggesting that it acts as an anchor between lipid droplets and mitochondria. This paper uses a combination of generally well-performed immunoprecipitations, confocal and electron microscopy experiments to support their hypotheses, but some clarification/added experiments are needed to help readers navigate this very dense paper.

Response: Many thanks for your valuable and positive comments and conclusion of our work.

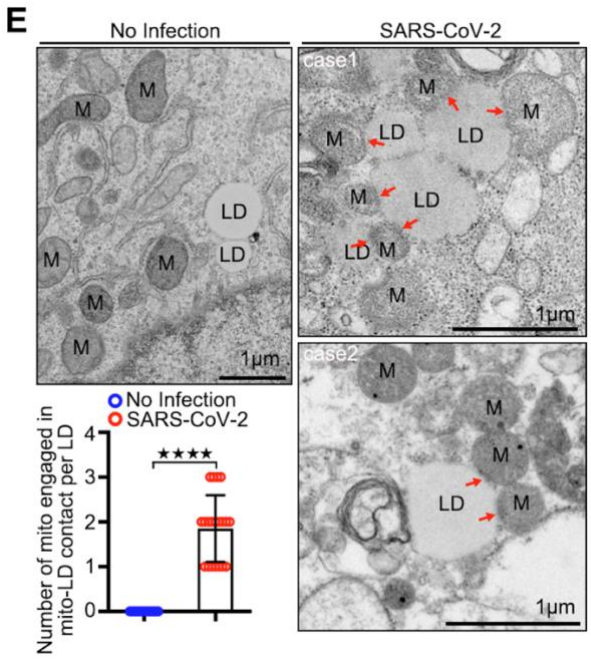
Major comments:
1. Authors identify many membrane contact sites induced by ORF6 expression and chemical inhibitions, but the only assays used to confirm the phenotype in SARS-CoV-2 infections are western blots and plaque assays. Can an increase in inter-organelle contact sites be confirmed in infected cells by EM for conclusive evidence that replication organelles are at these contact sites.

Response: Thank you for your instructive suggestions. Beside western blot and plaque assays, by using confocal, we have shown that LDs contact with mitochondria in SARS-CoV-2 infected cells (**Original Manuscript Fig 6J**).

As your suggested, we have confirmed the inter-organelle contacts in SARS-CoV-2-infected cells by TEM (**Revised Manuscript Fig 4C and 6E**).



Revised Manuscript Fig 4C. Representative transmission electron micrograph of non-infected or SARS-CoV-2 infected Vero-E6 cells. Red arrows mark the ER. LD, lipid droplets. Scar bar represents 1 μm. Quantification of number of ER engaged in ER-LD contact per LD. 20 LDs of two independent experiments were counted. Two-tailed Unpaired Student's t-test, ****p < 0.0001.



Revised Manuscript Fig 6E. Representative transmission electron micrograph of non-infected or SARS-CoV-2 infected Vero-E6 cells. Red arrows mark the mitochondria-LD contact. LD, lipid droplets. M, mitochondria. Scar bar represents 1 μm. Quantification of number of mitochondria engaged in mitochondria-LD contact per LD. 20 LDs of two independent experiments were counted. Two-tailed Unpaired Student's t-test, ****p < 0.0001.

2. Figure 3F needs the comparative non untreated control

Response: 0 h means non-untreated control, we have corrected it.

3. ATGLi treatment usually results in the accumulation of larger lipid droplets, the chosen images do not show this. Does ORF6 expression change the size of the lipid droplets with or without ATGLi treatment?

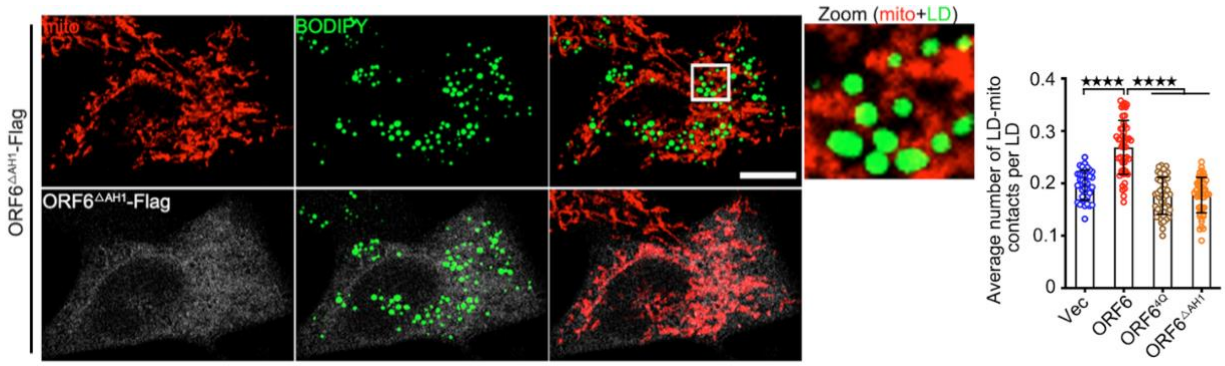
Response: We are grateful for the Reviewer's critical comments. We used HeLa cells and Vero-E6 cells under 200 μM OA (12 h) treatment for confocal microscopy, larger LDs were rarely observed in these two cells. In order to accumulate larger LDs, we treated HeLa cells with 500 μM OA for 16 h. We found that under mock treatment, ORF6 expression increased the number of small LDs (1μm<), may due to lipogenesis, and slightly decreased the number of big LDs (>2μm), may due to lipolysis; while under ATGLi treatment, ORF6 expression increased the number of small LDs (1μm<), but has no obvious effect on the number of big LDs (>2μm) (**Response Letter Fig 1**).

Figures for referees not shown.

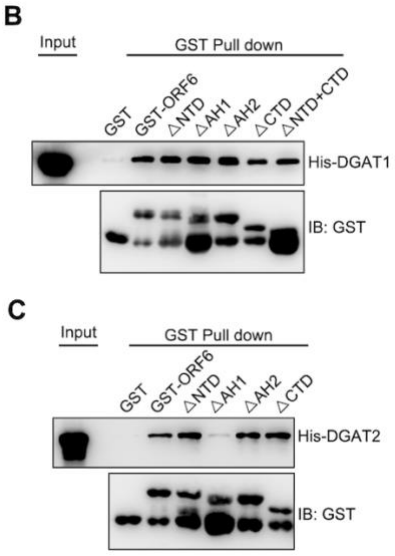
4. Does orf6 dimerization matter for the LD/mito or LD/ER contact sites? Can the authors determine what part of the protein is interacting with DGAT1/2 and if dimerization is required?

Response: Thank you for your insightful comments. We found that AH1 and AH2 domains on ORF6 are responsible for its dimerization, and the dimerization is required but not sufficient for LD localization (**Revised Manuscript Fig 2D-G**), ORF6△AH1 failed to link LDs to the ER. (**Revised Manuscript Fig 4E**). As your suggested, we have examined the mitochondrial-LDs contact in ORF6△AH1 expressed cells, we found that ORF6△AH1 failed to mediate LD/mito contact (**Revised Manuscript Fig EV5D**). These data suggest that ORF6 dimerization matter for the LD/mito or LD/ER contact sites.

By *in vitro* GST pull-down, we found that ORF6△AH1 still binds DGAT1, but not DGAT2 (**Revised Manuscript Fig EV2B-C**). Unfortunately, we failed to obtain the truncation ORF6△AH1+AH2 due to too few amino acids. Based on our GST pull-down result, we proposed that ORF6 binds DGAT1 via AH1+AH2 domains because ORF6△AH1, ORF6△AH2, and ORF6△NTD+CTD all interact with DGAT1, and ORF6 binds DGAT2 via AH1 domain. Considering that ORF6△AH2 failed to dimerize but still bind DGAT1/2, indicating that dimerization of ORF6 is not critical for its interaction with DGAT1/2.



Revised Manuscript Fig EV5D. HeLa cells expressing ORF6△AH1-Flag were treated with 200 μM OA for 12 h, and then were fixed and stained with anti-Flag (white) and anti-Tom20 (red). Tom20 represents mitochondria marker. LDs were labeled with BODIPY-493/503 (green). Cells were imaged by confocal microscopy. Scar bar represents 10 μm. Quantification of average number of LD-mito contacts per LD (contact number divided by LD number). 37 cells (Vector), 50 cells (ORF6), 50 cells (ORF6^{4Q}), and 50 cells (ORF6△AH1) from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ****$p < 0.0001$.



Revised Manuscript Fig EV2B and EV2C. The interactions of GST tagged ORF6 or the mutants with the His tagged DGAT1 and His tagged DGAT2 were analyzed by GST pull-down assay.

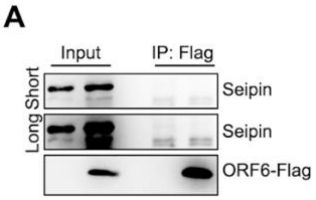
5. While the manuscript has compelling data, it is very dense and could use some streamlining by possibly moving some panels to the supplemental figures (like Fig 5J-N) or text based changes.

Response: Thank you for your insightful comments and helpful suggestions. We have moved Fig 5J-N to supplemental figures in the revised manuscript.

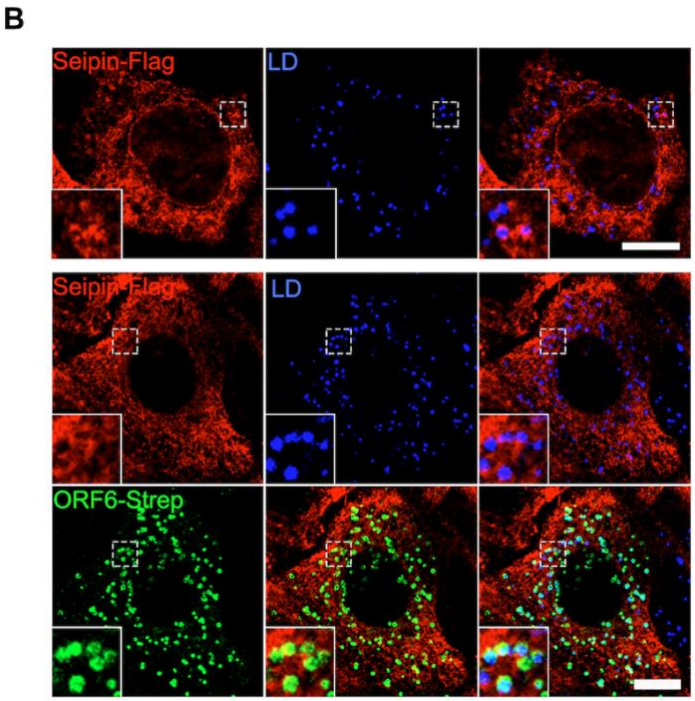
Minor comments:

1. What about seipin localization? If the authors argue that ORF6 is involved in LD biogenesis, then is it near seipin a major biogenesis protein?

Response: Thank you for your instructive suggestions. As discussed in our manuscript (lines 455-456), we have examined the interaction between ORF6 and Seipin, and their interaction is negative (**Appendix Figure S4A**). Furthermore, we observed weak colocalization between ORF6 and Seipin by confocal microscopy (**Appendix Figure S4B**).



Appendix Figure S4A. HEK293T cells were transfected with ORF6-Flag. Protein interactions were detected by immunoprecipitation with anti-Flag beads and immunoblotting analysis with Seipin antibody.



Appendix Figure S4B. HeLa cells were transfected with ORF6-Strep and Seipin-Flag for 22 h and treated with 200 μM OA for 2 h, then fixed and stained with anti-Flag (red) and anti-Strep (green). LDs were labeled with LipidTOX Deep Red (blue). Cells were imaged by confocal microscopy. Scar bar represents 10 μm.

2. Figure 3I and 5B have strikingly different LD/cell numbers despite being the same conditions (3X inhibitors +/- ORF6)?

Response: We are grateful for the Reviewer's comments. As we have mentioned in our figure legends, in Fig 3I, HeLa cells stable expressing vector or ORF6-Flag were treated or untreated

with 50 μM ATGL inhibitor, along with DMSO or 1 μM DGAT1 inhibitor (DGAT1i) or/and 2 μM DGAT2 inhibitor (DGAT2i) for 24 h **without OA treatment**. In this experiment, we examined the effect of ORF6 expression on LD biogenesis under steady-state conditions.

In Fig 5B, HeLa cells were transfected with ORF6-Flag for 12 h and treated **with 200 μM OA** for another 12 h, the medium was removed and replaced with fresh complete culture containing 1 μM DGAT1 inhibitor and 2 μM DGAT2 inhibitor for 24 h. Meanwhile, 50 μM ATGL inhibitor or 100 μM Chloroquine (CQ) was added and allowed to incubate for 24 h or 4 h to block lipolysis or lipophagy, respectively. In this experiment, we examined the effect of ORF6 expression on lipolysis and lipophagy under OA stimulation.

3. How were lipid droplets/cell or Mito/LD contact sites quantified? The methods section did not explain.

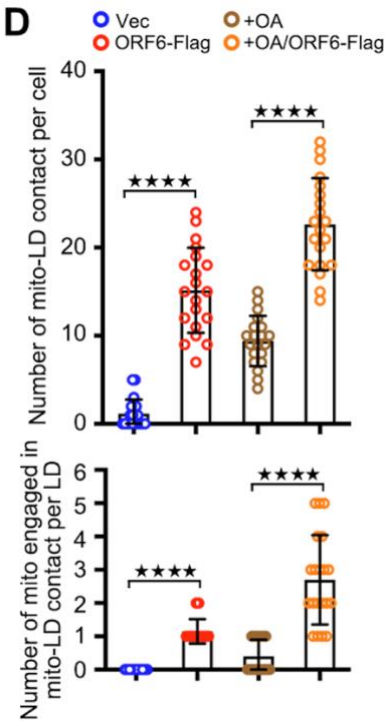
Response: We are grateful for the Reviewer's critical comments. Colocalization-based analysis of the contact sites of mitochondria-LDs and ER-LDs were performed using a plugin named colocalization in ImageJ. Contacts automatically identified by colocalization plugin with white pixels representing potential contact sites and were counted manually. We have added the colocalization analysis in Methods.

4. Figures 1 and 4 are difficult to navigate as panels are non-intuitively laid out.

Response: We apologize for non-intuitively laid out. We have re-arranged these two figures to make them easy to follow.

5. Figure 6, please add quantification of the images from S5C (the -OA condition) to compare next to these +OA quants?

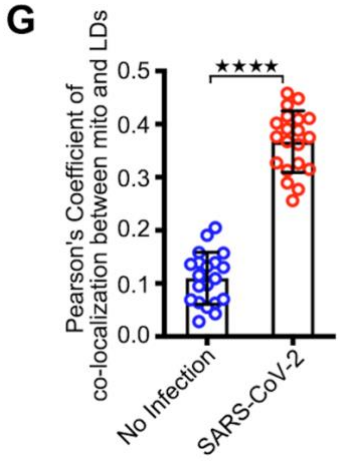
Response: Thank you for your helpful suggestions. We have added the quantification for -OA condition to compare next to +OA quants (**Revised Manuscript Fig 6D**).



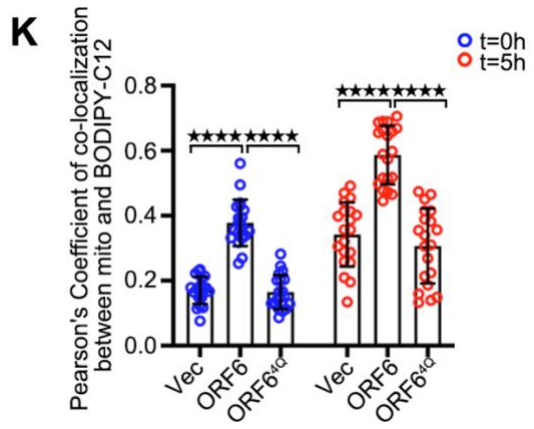
Revised Manuscript Fig 6D. The number of mitochondria-LD contacts per cell was counted from 20 cells and the number of mitochondria engaged in mito-LD contacts per LD was counted from 20 LDs. Two independent experiments. Two-tailed Unpaired Student's t-test, ****$p < 0.0001$.

6. Please quantify the differences between the +/- ORF6 of Fig 6G and +/- virus in Fig 6J since text states significantly higher/more.

Response: Thank you for your instructive suggestions. We added the quantifications of Original Manuscript Fig 6G and 6J by using ImageJ (JACoP, Pearson's Coefficient) (**Revised Manuscript Fig 6G and K**).



Revised Manuscript Fig 6G. Colocalization of LD and mitochondria (Pearson's Coefficient), n=20 cells, two independent experiments. Two-tailed Unpaired Student's t-test, ****p < 0.0001.



Revised Manuscript Fig 6K. Colocalization of BODIPY-C12 and mitochondria (Pearson's Coefficient), n=20 cells, three independent experiments. Two-tailed Unpaired Student's t-test, ****p < 0.0001.

Referee #2 (Report for Author)

This manuscript entitled "Viral Protein Links Lipid Droplets, the ER, and Mitochondria, and Modulates Lipid Flux for Virus Production" by M. Yue and collaborators describes the role of the ORF6 protein of SARS-CoV-2 in mediating endoplasmic reticulum (ER)-lipid droplet (LD) and LD-mitochondria contacts.
The authors first showed that amongst the proteins encoded by the genome of SARS-CoV-2, ORF6 is localized to LDs. Then, they characterized the molecular mechanism of the interaction of ORF6 with LDs and showed that ORF6 promotes the biogenesis of LDs by binding with DGAT1/2. Moreover, they showed that ORF6 promotes the formation of ER-LD contacts, promotes lipolysis by binding to ATGL, and link mitochondria to LDs, and finally peroxisome to LDs.
Although many of these results are interesting, the experiments described are not strong enough to support the authors' claims. Controls are missing and some data are over-interpreted. The authors should really add complementary experiments to make their point.

Response: Many thanks for your conclusion of our work. Your constructive suggestions help us significantly improve the quality of this manuscript.

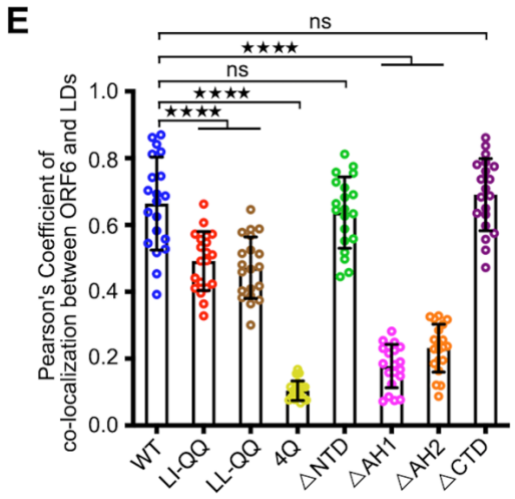
Below are some of the main issues with the manuscript:
Major points:
1. There are many experiments which are not associated with quantifications (for instance, Fig. 1G, 2G, 4E...); Co-localizations should be quantified (Pearson correlation for instance). Moreover, some quantifications are not properly done: statistics should be performed between independent samples, while here statistics (Fig. 3B, 3E, 3I etc...) are done considering individual cells as independent samples. The experiments should be repeated multiple times and statistics performed between experiments (using the mean of several cells quantified per experiment).

Response: We are grateful for the Reviewer's critical comments and suggestions. As we mentioned in our Method (lines 1050-1051), two or three independent experiments were analyzed. We have added this information in figure legends.

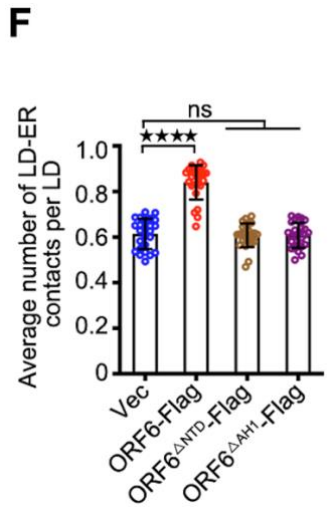
LD localizations of ORF6 (**Original Manuscript Fig 1G and 2G**) were quantified via ImageJ, Plugin named JACoP (Pearson's Coefficient) (**Revised Manuscript Fig EV1E**).

Original Manuscript Fig 4E were quantified via ImageJ, Plugin named co-localization. Contacts automatically identified by colocalization plugin with white pixels representing potential contact sites and were counted manually (contact number divided by LD number) (**Revised Manuscript Fig 4F**).

We also added the quantifications of LD numbers by using the mean of indicated cells from three independent experiments (**Revised Manuscript Fig 3B, 3E, 3I, 4I, 4L, and 5B**).



Revised Manuscript Fig EV1E. Colocalization of ORF6 and LDs (Pearson's Coefficient), n=20 cells, three independent experiments. Two-tailed Unpaired Student's t-test, ****p < 0.0001, ns means no significance.



Revised Manuscript Fig 4F. Quantification of average number of LD-ER contacts per LD. 25 cells from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ****p < 0.0001, ns means no significance.

2. Figure 1A, C: ORF6 is bound to LDs but some of the staining is not associated with LDs: what is the other localization of ORF6?

Response: Thank you for your comments. Beside the ER, peroxisomes and mitochondrial, our confocal data suggested that ORF6 may also partly localized on Golgi under steady state (**Response Letter Fig 2**).

Figures for referees not shown.

3. Figure 1G: the localization of the ORF6-4Q mutant should be determined: is it localized in the cytosol or is it associated with the endoplasmic reticulum or another organelle?

Response: Thank you for your comments. ORF6-4Q remained diffused, and no colocalization was found between ORF6-4Q and the ER, mito or Golgi, indicating that ORF6-4Q may be localized in the cytosol (**Response Letter Fig 3**).

Figures for referees not shown.

4. The role of the amphipathic helices AH1 and AH2 should be further investigated:
a. Are AH1 and AH2 alone able to recruit a protein at the surface of LDs? (could be answered using AH1- and AH2-GFP fusion proteins)

Response: We appreciate this helpful advice. We have generated ORF6-AH1+AH2 construct and found that this mutant still targets LDs by confocal microscopy (**Response Letter Fig 4**).

Figures for referees not shown.

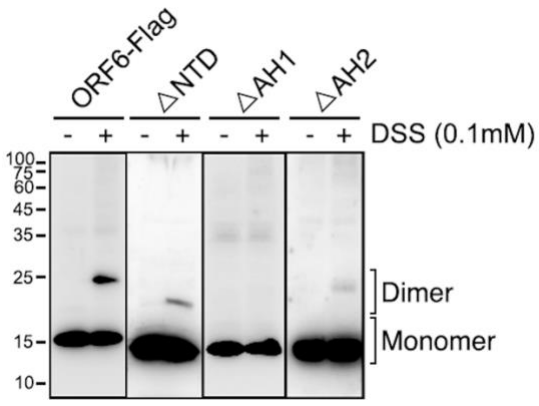
b. Are AH1 and AH2 sufficient to associate with the surface of LDs? This should be answered in an in vitro simplified system using synthetic peptides and artificial LDs. This kind of experiment could help understand the function of AH1 vs AH2.

Response: We appreciate this critical advice. Unfortunately, we are so sorry that we are not able to obtain the data based on the *in vitro* simplified system using synthetic peptides and artificial LDs.

c. The role of the dimerization of ORF6 remains unclear:
i. Figure 2: the data shown in this figure are over-interpreted: for instance, the authors claim that the AH1 helix alone is responsible for dimerization even if the level of dimerization of the deltaAH2 mutant is reduced by about 10-fold. The relative contribution of AH1 and AH2 really needs to be clarified.

Response: Thank you for your valuable suggestions. Using DSS assay, we further confirmed that ORF6$^{\triangle AH2}$ showed less homodimerization (**Revised Manuscript Fig 2F**).

We apologize for misleading elaboration. We have rephrased the sentence to classify the importance of AH2 domain in dimerization: "*Deletion of AH1 or AH2 significantly decreased the self-interaction (Fig 2D and 2E). ORF6△AH1 and ORF6△AH2 failed to dimerize via the DSS assay (Fig 2F). Intriguingly, ORF6△AH1 and ORF6△AH2 remained diffused and failed to associate with LDs (Fig 2G and EV1E), suggesting that ORF6 could be targeted to LDs via amphipathic helices mediated- homodimerization.*" (**Revised Manuscript Lines 147-151**).



Revised Manuscript Fig 2F. HEK293T cells were transfected with ORF6-Flag, or ΔNTD-Flag, or ΔAH1-Flag, or ΔAH2-Flag and were treated with 0.1 mM DSS for 30 min. Cell lysates were analyzed via WB.

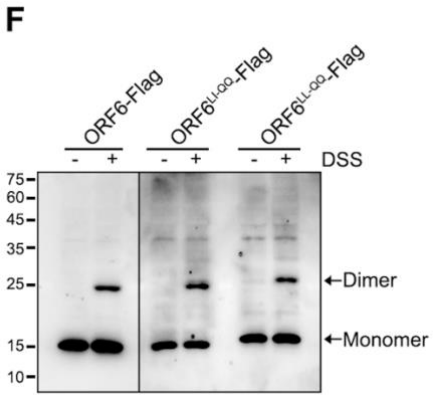
ii. the deltaAH2 mutant does not really associate with LDs. This is not analyzed in this way in the manuscript: only the role of AH1 is discussed.

Response: Thank you for your valuable suggestions. We apologize for misleading elaboration. We have rephrased the sentence to classify the importance of AH2 domain in LD targeting: "*Intriguingly, ORF6ΔAH1 and ORF6ΔAH2 remained diffused and failed to associate with LDs (Fig 2G and EV1E), suggesting that ORF6 could be targeted to LDs via amphipathic helices mediated- homodimerization.*" (**Revised Manuscript Lines 149-151**). We have deleted the model of original Fig 2H, and added the quantification of LD association of ORF6 truncations in Fig 2G (**Revised Manuscript Fig EV1E**).

iii. What is the effect of the mutations described in Figure 1 (LL-QQ and LI-QQ) on dimerization? Why did the authors only use the 4Q mutation in their analysis?

Response: Thank you for your insightful comments. We used ORF6-4Q as function loss mutant to confirm that dimerization is required but maybe not sufficient for LD localization.

We further found that both mutants LI-QQ and LL-QQ can dimerize (**Revised Manuscript Fig EV1F**).



Revised Manuscript Fig EV1F. HEK293T cells were transfected with ORF6-Flag or its mutants and treated or untreated with 0.1 mM DSS for 30 min. Cell lysates were analyzed via WB.

iv. The authors propose 2 models for the role of AH1 and AH2: in figure 1, AH1 and AH2 are both involved in binding lipid droplets, and in figure 2, AH1 is involved in ORF6 dimerization while AH2 is involved in binding to LDs. Are these models mutually exclusive and in that case which model is correct? If I understood correctly, the authors favor the second model. In that case, they should prove that this model is correct. Can AH1 be replaced by a dimerization domain? How does AH1 dimerize? The identification of point mutations disrupting the dimerization could be a valuable information. Can AH2 be replaced by another amphipathic helix known to interact with LDs?

Response: Thank you for your insightful comments. We have rephrased the sentence to classify the importance of AH2 domain in homodimerization and LD targeting (**Revised Manuscript Lines 147-151**).

Further structural analyses of the mutant should be performed. Unfortunately, we are so sorry that we are not able to achieve the structural mechanism by which ORF6 dimerize.

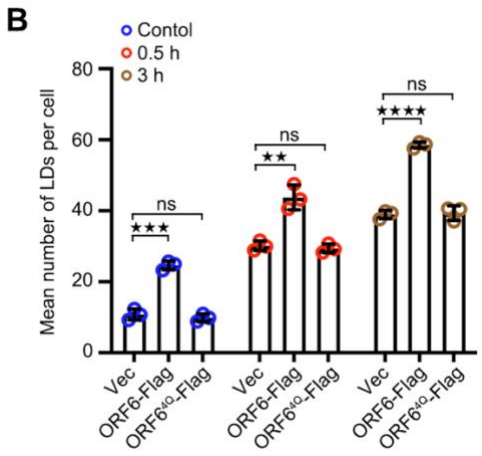
v. Figure 2E: the amount of ORF6-6His protein pulled-down by ORF6-GST is really higher than that of the mutants ORF6-GST. Could the authors comment this point.

Response: Thank you for your insightful comments. ORF6 is a small protein with 61 amino acids, any deletion on ORF6 may lead to decrease the ability of dimerization. Our data suggested that AH1 and AH2 are most critical domains for dimerization.

5. Figure 3: the authors claim that the ORF6 4Q mutant does not promote LD growth but there is no quantification shown. The same experiment as in Fig. 3A-B should be performed with this mutant.

Response: We appreciate this critical advice. Please refer Figure S2A for the data about ORF6 4Q mutant does not promote LD growth.

We have added the quantification for Figure S2A in the revised manuscript (**Revised Manuscript Fig 3B**).



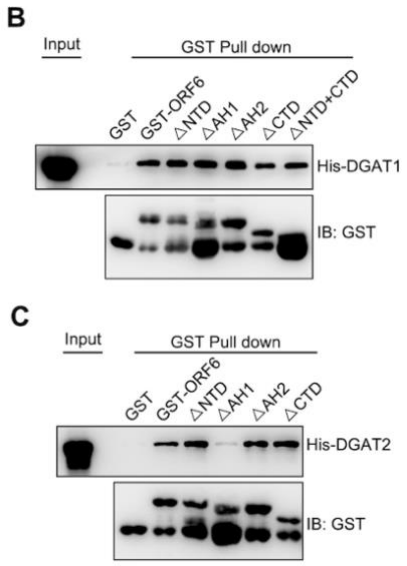
Revised Manuscript Fig 3B. Mean number of LDs in each cell was counted from 25 cells of three independent experiments. Two-tailed Unpaired Student's t-test, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns means no significance.

6. Figure 3F: why did the authors test the interaction of ORF6 with DGAT1/2? What is the mechanism of interaction between ORF6 and DGAT1/2? Could the authors identify point mutations disrupting the interaction? It would be useful to understand the role of the interaction in the activity of DGAT enzymes. Does ORF6 expression increase the level of DGAT1 and DGAT2? Is the effect transcriptional/post-traductional?

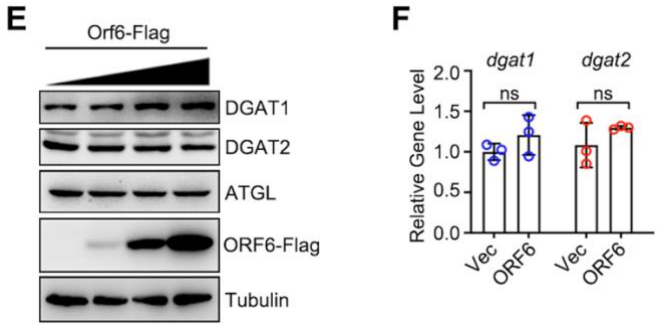
Response: LD biogenesis starts in the ER, where neutral lipid synthesis enzymes, including Diglyceride acyltransferase (DGAT), deposit TGs between the leaflets of the ER bilayer and bud out from the ER surface. We first found that ORF6 promotes LD biogenesis, then we asked whether ORF6 could associate with key enzymes such as DGAT1/2.

By *in vitro* GST pull-down, we found that ORF6$^{\triangle AH1}$ still binds DGAT1, but not DGAT2 (**Revised Manuscript Fig EV2B-C**). Unfortunately, we failed to obtain the truncation ORF6△AH1+AH2 due to too few amino acids. Based on our GST pull-down result, we proposed that ORF6 binds DGAT1 via AH1+AH2 domains because ORF6△AH1, ORF6△AH2, and ORF6△NTD+CTD all interact with DGAT1, binds DGAT2 via AH1 domain.

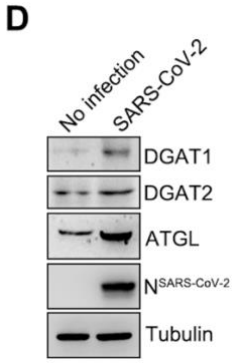
We found that ORF6 expression caused no obvious effect on the protein or RNA level of DGAT1 and DGAT2 (**Revised Manuscript Fig EV2E and EV2F)**. SARS-CoV-2 infection enhance the protein level of DGAT1 and DGAT2 (**Revised Manuscript Fig EV2D**), suggest that other viral proteins regulate DGAT1 and DGAT2.



Revised Manuscript Fig EV2B-C. The interactions of GST tagged ORF6 or the mutants with the His tagged DGAT1 and His tagged DGAT2 were analyzed by GST pull-down assay.



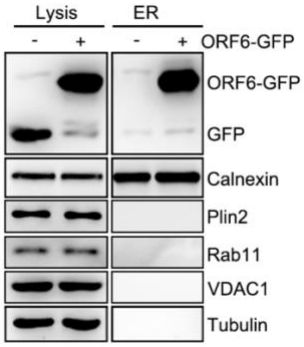
Revised Manuscript Fig EV2E. HeLa cells were transfected with gradient of ORF6-Flag for 24 h. Protein levels of endogenous DGAT1, DGAT2, and ATGL were analyzed via WB. **Revised Manuscript Fig EV2F.** HeLa cells were transfected with ORF6-Flag for 24 h. RNA levels of dgat1 and dgat2 were analyzed via QPCR.



Revised Manuscript Fig EV2D. Vero-E6 cells were infected or non-infected with SARS-CoV-2 for 24 h. Cell lysates were analyzed via WB.

7. Figure 4A: markers of other organelles (lipid droplets, mitochondria, endosomes...) should be added to control the purity of the fractions.

Response: Thank you for your insightful suggestions. We have added the makers of LDs, mitochondria, endosomes.



Revised Manuscript Fig 4A. Subcellular fractions were isolated from GFP or ORF6-GFP over-expressed cells. Calnexin represent the ER, Plin2 represent LDs, Rab11 represent endosomes, VDAC1 represent mitochondria, and Tubulin and cytosolic markers, respectively.

8. Figure 1 and Figure 4: is ORF6 present at the surface of LDs or in ER-LD contacts?

Response: Thank you for your insightful comments. According to our confocal data, ORF6 is present at the surface of LDs and also in ER-LD contacts.

9. Figure 4: is ORF4 promoting the formation of ER-LD contacts? ER-LD contacts should be quantified in control and ORF4 expressing cells.

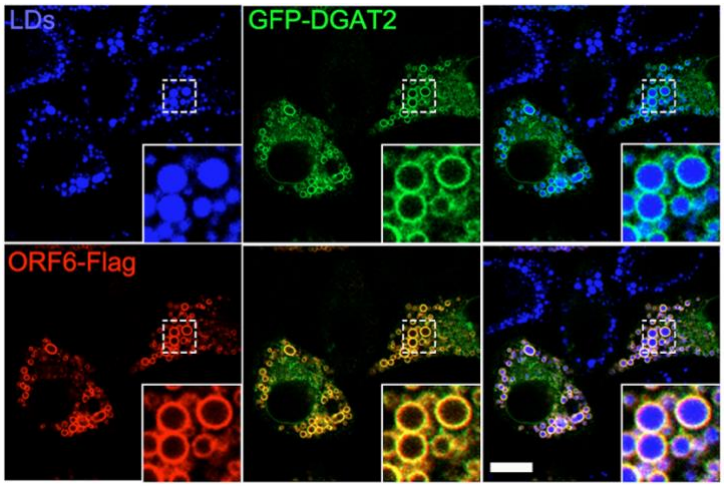
Response: Thank you for your helpful comments. There is no ORF4 among SARS-CoV-2 encoded viral proteins.

Our confocal data indicate that ORF6 may promote the formation of ER-LD contact. We have added the quantification of length of ER-LD contact via ImageJ (**Revised Manuscript Fig 4B)**.

10. Figure 4C: are DGAT proteins localized in the ER? Could the interaction with DGAT be involved in the formation of ER-LD contacts?

Response: Thank you for your helpful comments and suggestions. DGAT1 is ER-resident lipid synthesis enzyme. We found that ORF6△AH1 failed to promote link the ER to LDs (**Revised Manuscript Fig 4E-F**) but still binds DGAT1 (**Revised Manuscript Fig EV2B**), indicating that ORF6-DGAT1 interaction is not sufficient for the formation of ER-LD contacts.

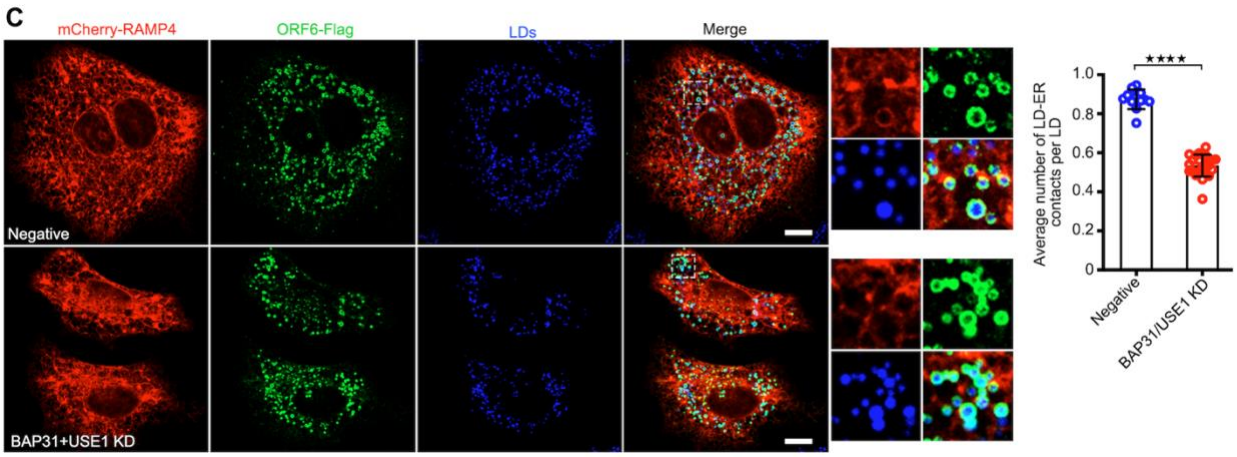
DGAT2 is LD-associated enzyme. We found that ORF6 co-localizes with DGAT2 on LDs, not on the ER (**Appendix Fig S1B**), indicating that the interaction with DGAT2 may not be involved in the formation of ORF6-induced ER-LD contacts.



Appendix Fig S1B. Cells were transfected with with GFP-DGAT2 with ORF6-Flag for 24 h and further were treated with 200 μM OA for 12 h. LDs were labeled with LipidTOX Deep Red (blue). Cells were fixed and then stained with anti-Flag (red). Cells were imaged by confocal microscopy. Scar bar represents 10 μm.

11. Figure 4: the analysis of the ORF4DNTD and ORF6DH1 mutant is not sufficient to conclude "that the interactions between ORF6-BAP31 and/or ORF6-USE1 were important for the formation of direct ER-LD contacts". The authors should silence BAP31 and USE1 and quantify ER-LD contacts induced by ORF6.

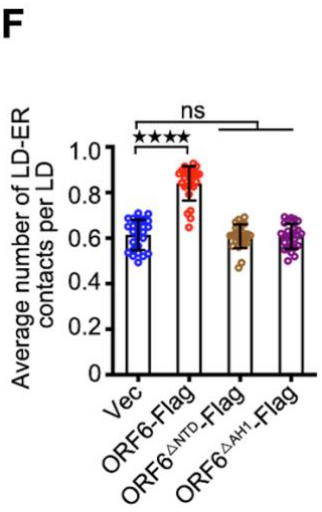
Response: Thank you for your helpful comments and suggestions. Please refer **Original Manuscript Fig EV3C** for the data about silence BAP31 and USE1 abolished the ER-LD contacts induced by ORF6. We apologize for missing the explanation in main text and have added the explanation and the quantification in the revised manuscript (**Revised Manuscript Fig EV3C**). Depletion of both BAP31 and USE1 abolished ORF6-induced ER-LD junctions.



Revised Manuscript Fig EV3C. Cells co-expressing the ORF6-Flag with mCheery tagged RAMP4 were transfected with BAP31 or USE1 siRNA for 36 h and treated with 200 μM OA for 12 h, then fixed and stained with anti-Flag (green). LDs were labeled with LipidTOX Deep Red (blue). Cells were imaged by confocal microscopy. Scar bar represents 10 μm. Quantification of average number of LD-ER contacts per LD (contact number divided by LD number). 12 cells (Negative) and 25 cells (BAP31/USE1 KD) from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ****p < 0.0001, ns means no significance.

12. Figure 4E: the colocalization should be quantified and/or EM experiments performed.

Response: We appreciate this critical advice. We have added the quantification of number of LD/ER contact. Colocalization-based analysis of the contact sites were performed using a plugin named colocalization in imageJ. Contacts automatically identified by colocalization plugin with white pixels representing potential contact sites and were counted manually (contact number divided by LD number) (**Revised Manuscript Fig 4F**). We have also added the quantification of EM experiments (**Revised Manuscript Fig 4B-C**)



Revised Manuscript Fig 4F. Quantification of average number of LD-ER contacts per LD. 25 cells from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ****p < 0.0001, ns means no significance.

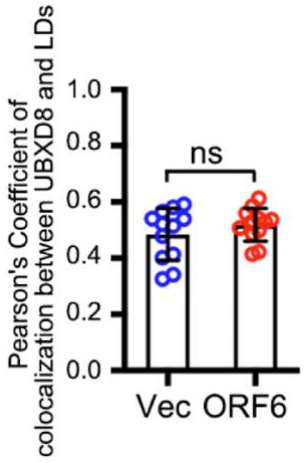
13. Are BAP31 and USE1 more localized in ER-LD contacts when ORF6 is over-expressed?

Response: Thank you for your helpful comments. The colocalization between ORF6 and BAP31 or USE1 was confirmed by confocal microscopy (**Original Manuscript Fig EV3B**). Given that ORF6 represents LD localization, from this data, we proposed that BAP31 and USE1 can be localized in ER-LD contact, but not more.

14. Figure S4C: the control images are missing. The modification of UBXD8 localization should be quantified.

Response: Thank you for your helpful comments. In Figure S4C, purple ROIs indicated the cell without ORF6 expression, and blue ROIs indicated the cell with ORF6 expression, we have added this information in figure legend in the revised manuscript.

We also added the quantification of UBXD8 localization by using ImageJ (Pearson's Coefficient) (**Revised Manuscript Fig EV4G**).



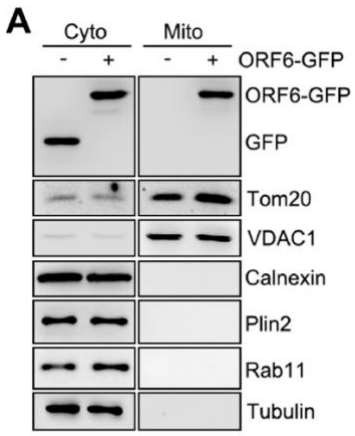
Revised Manuscript Fig EV4G. Colocalization of UBXD8 and LDs (Pearson's Coefficient), n=12 cells, two independent experiments. Two-tailed Unpaired Student's t-test, ns means no significance.

15. Page 14 line 292: "Furthermore, compared to non-infection, SARS-CoV-2 infection enhanced the interaction of ATGL with Plin2 and UBXD8". Is the interpretation of this experiment correct?

Response: We thank the reviewer for catching this mistake. We have rephrased the sentence "Furthermore, compared to non-infection, SARS-CoV-2 infection subverted the interaction of ATGL with Plin2 and UBXD8".

16. Figure 6A: markers of other organelles (lipid droplets, ER, endosomes...) should be added to control the purity of the fractions

Response: Thank you for your insightful suggestions. We have added the makers of LDs, ER, endosomes.



Revised Manuscript Fig 6A. Subcellular fractions were isolated from GFP or ORF6-GFP over-expressed cells. Tom20 and VDAC1 represent mitochondria markers, Calnexin represents the ER, Plin2 represents LDs, Rab11 represents endosomes, and Tubulin represents cytoplasm, respectively.

17. Figure 6: does ORF6 promote the association of LDs with mitochondria?

Response: Thank you for your comments. Our confocal and electron microscopy experiments support our hypotheses that ORF6 promotes LD-mito contact.

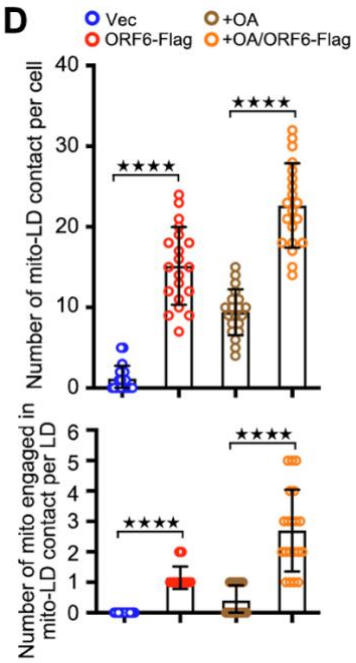
18. There are many different localizations of ORF6 that are described in this paper; what is the percentage of the protein present in each of these localizations?

Response: Thank you for your insightful comments. We found that approximately 6% of ORF6 present on mitochondria and 14% on ER (**Response Letter Fig 5)**.

Figures for referees not shown.

19. Figure 6C-D: it would be more appropriate to perform the quantification in cells not treated with OA: in cells treated with OA, LDs are huge and the interpretation of the date becomes difficult.

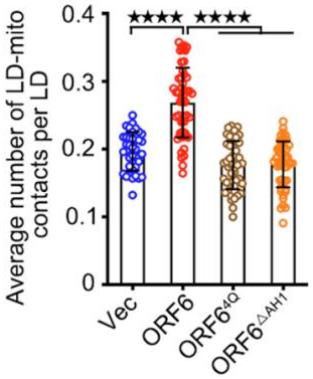
Response: Thanks for your insightful advice. We have added the quantification of number of LD/mito contact per LD for Original Manuscript Fig 6C and Original Manuscript Fig S5C (**Revised Manuscript Fig 6D**).



Revised Manuscript Fig 6D. The number of mitochondria-LD contacts per cell was counted from 20 cells. The number of mitochondrial engaged in mitochondrial-LD contact per LD was counted from 20 LDs. Two independent experiments. Two-tailed Unpaired Student's t-test, ****$p < 0.0001$.

20. Figure 6E and F: this experiment is biased by the effect of ORF6 on the number of LDs. At the resolution of fluorescence microscopy, the increased number of LDs will indeed increase the apparent number of LD-mito contacts even if the number of contacts is unchanged.

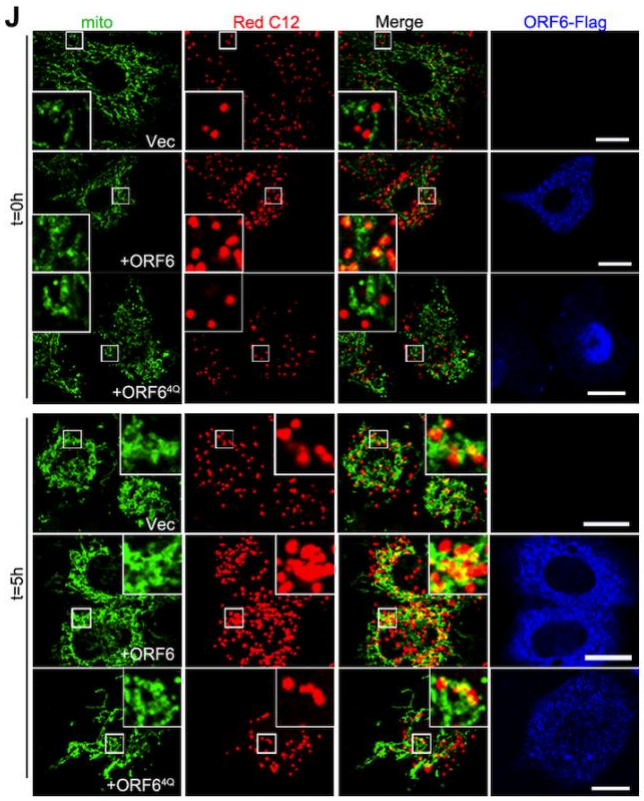
Response: Thanks for your insightful advice. We have added the quantification of number of LD/mito contact per LD (contact number divided by LD number) for Original Manuscript Fig 6E (**Revised Manuscript Fig 6I**).



Revised Manuscript Fig 6I. Quantification of average number of LD-mito contacts per LD (contact number divided by LD number). 37 cells (Vector), 50 cells (ORF6), 50 cells (ORF6^{4Q}), and 50 cells (ORF6△AH1) from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ****p < 0.0001.

21. Figure 6G: some staining on these images are over-saturated and make the interpretation of the data difficult.

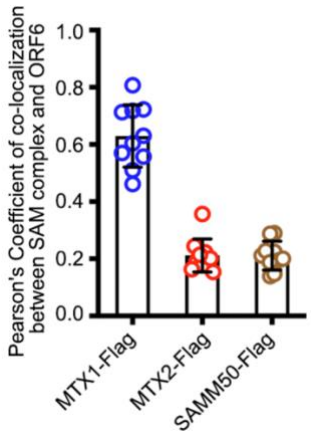
Response: Thank you for your insightful comments. We have replaced these over-saturated images (**Revised Manuscript Fig 6J**).



Revised Manuscript Fig 6J. Pulse-chase assays of vector or ORF6-Flag or ORF6^{4Q}-Flag stable cells with or without starvation treatment. HeLa cells were incubated in complete medium containing 1 μM BODIPY 558/568 C12 (red) for 16 h. Cells were washed with PBS three times and then chased or non-chased in EBSS for 5 h, and then were fixed and stained with anti-Flag (blue) and anti-Tom20 (green). Tom20 represents mitochondria marker. Cells were imaged by confocal microscopy. Scar bar represents 10 μm.

22. The co-localizations shown Figure S6A are not convincing: this should be quantified.

Response: Thanks for your insightful advice. We have added the quantification (**Appendix Fig S2B**).



Appendix Fig S2B. Colocalization of ORF6 and MTX1 or MTX2 or SAMM50 (Pearson's Coefficient), n=10 cells, two independent experiments.

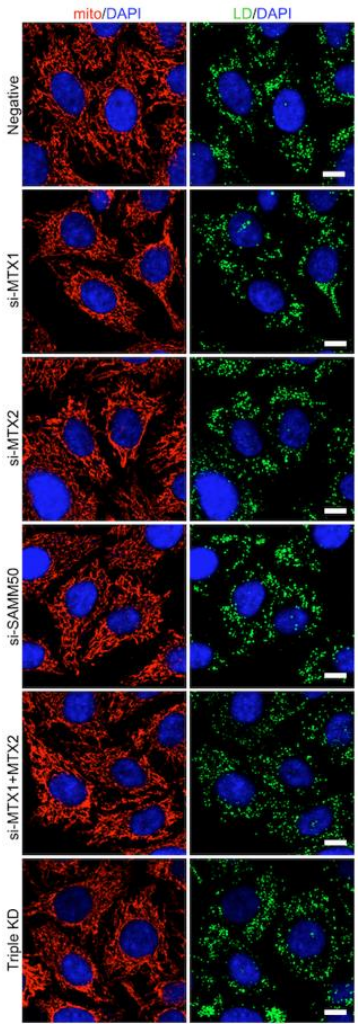
23. The interpretation of the experiment shown Fig. S6C is odd. The mutant lacking both AH1 and AH2 is missing.

Response: Thank you for your insightful comments. As we mentioned in the manuscript, ORF6$^{\triangle NTD}$, ORF6$^{\triangle AH1}$, ORF6$^{\triangle AH2}$, ORF6$^{\triangle CTD}$, and ORF6$^{\triangle NTD+CTD}$ all interact with MTX1 and MTX2 (**Original Manuscript Fig EV6C, Revised Manuscript Fig EV5I**), indicating that ORF6 binds to MTX1 and MTX2 via its two amphipathic helices. Unfortunately, we failed to obtain the truncation ORF6$^{\triangle AH1+AH2}$ due to too few amino acids (**Response Letter Fig 6**).

Figures for referees not shown.

24. Figure 7F: is there an effect of silencing MTX1, MTX2, and/or SAMM50 on the number of LD and the number of mitochondria? If so, this could bias the interpretation of this experiment.

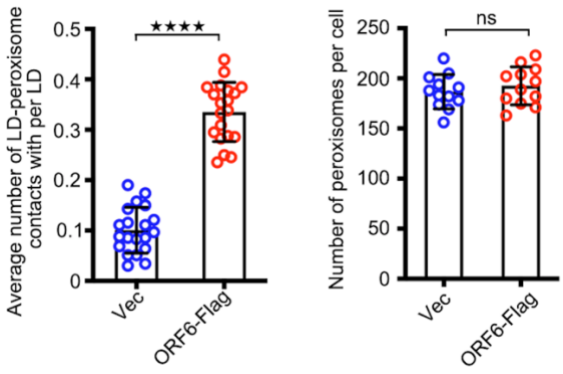
Response: We are grateful for the Reviewer's critical suggestions. We have checked the LD and mitochondrial number upon silencing MTX1, MTX2, and/or SAMM50. According to our confocal data, knockdown of MTX1, MTX2, and/or SAMM50 resulted in no effect on the number of LD and mitochondria (**Appendix Fig S2C**).



Appendix Fig S2C. Hela cells were transfected with siMTX1, and/or siMTX2, and/or siSAMM50 for 48 h and treated with 200 μM OA for another 12 h. Cells were fixed and stained with anti-Tom20 (red). LDs were labeled with BODIPY-493/503 (green). The nuclei were stained with DAPI. Cells were imaged by confocal microscopy. Scar bar represents 10 μm.

25. Figure S7: again the same controls are missing and no quantification is shown; is there an effect of ORF6 on the number of peroxisome? And the effect of ORF6 on the number of LDs affects the interpretation of the result.

Response: Thank you for your helpful suggestions. "Vec" means vector transfected cells, is the control group. We have added the quantification of average number of LD-peroxisome contacts per LD (contact number divided by LD number) and the number of peroxisomes upon ORF6 expression (**Appendix Fig S3B**). ORF6 expression has no effect on the number of peroxisomes.



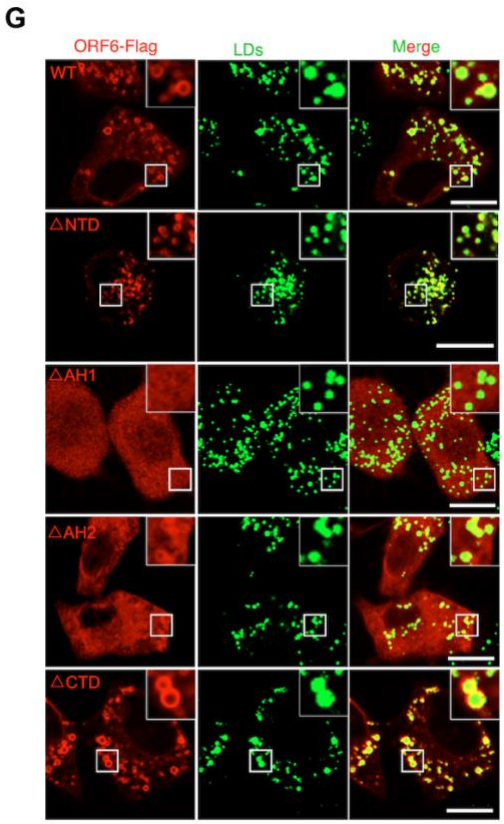
Appendix Fig S3B. Quantification of average number of LD-peroxisome contacts per LD. 20 cells from two independent experiments were calculated. Quantification of peroxisomes number, 12 cells from two independent experiments were calculated. Two-tailed Unpaired Student's t-test, ****$p < 0.0001$, ns means no significance.

Minor points:
1. The introduction could be shortened.

Response: We thank reviewer for this helpful suggestion. The introduction has be shortened.

2. Figure 2G: show the bodipy alone panels.

Response: We thank reviewer for this helpful suggestion. We have added the BODIPY alone panels (**Revised Manuscript Fig 2G**).



Revised Manuscript Fig 2G. HeLa cells were transfected with ORF6-Flag or indicated mutants and were treated with 200 μM OA for 12 h, and then fixed and stained with anti-Flag (red). LDs were labeled with BODIPY-493/503 (green). Cells were imaged by confocal microscopy. Scar bar represents 10 μm.

3. Figure 3 A: what is the meaning of the white and yellow ROIs? It is not described in the legend.

Response: We apologize for this missing information. White ROIs indicate cells expressing ORF6 and yellow ROIs indicate the cells without ORF6 expression. We have added this information in the figure legends.

4. Figure S2: there are 2 B panels.

Response: Thanks for pointing out our mistake. We have corrected it.

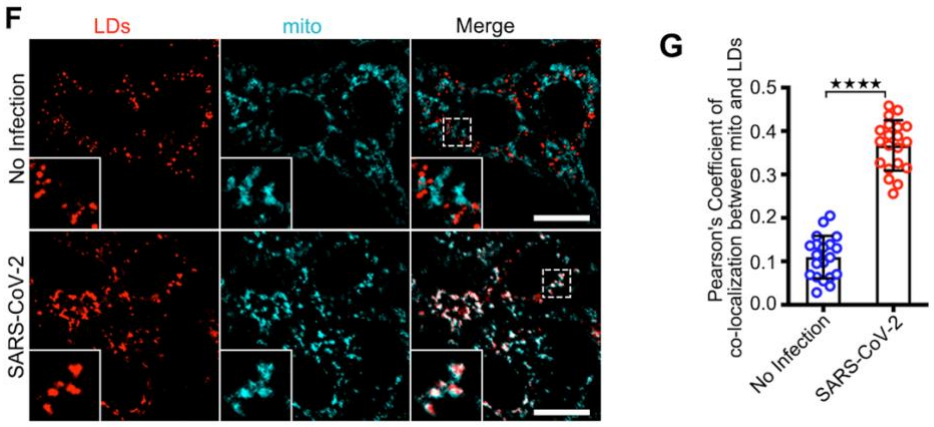
5. Figure S3C is never called. The KD of BAP31 and USE1 seems to affect the ER morphology. Could the authors comment.

Response: We apologize for this missing information, we have added the explanation in main text.

We found that ORF6 expression can partly change the ER morphology: ER is wrapping LD. USE1, a component of ER-associated Q-SNAREs (USE1, Syntaxin18, and BNIP1), plays a potential role in Golgi-ER retrograde vesicle trafficking and ER-LD interactions. BAP31 is the most abundant ER membrane protein, including in mitochondria-associated membrane, where it contributes to mitochondrial tethering by interacting with mitochondrial proteins Fis1 and TOM40. Knockdown of BAP31 and USE1 may affect the ER morphology upon ORF6 expression.

6. Figure J: change the LUTs.

Response: We guess you refer Figure 6J? We have changed the LUTs and added the quantification (**Revised Manuscript Fig 6F and G**).



Revised Manuscript Fig 6F and G. SARS-CoV-2-infected Vero-E6 cells were fixed and stained with anti-Tom20 (cyan). Tom20 represents mitochondria marker. LDs were labeled with LipidTOX Deep Red (red). Cells were imaged by confocal microscopy. Scar bar represents 10 µm. Colocalization of LDs and mitochondria (Pearson's Coefficient), n=20 cells, two independent experiments. Two-tailed Unpaired Student's t-test, ****p < 0.0001.

7. The materials and methods does not describe the methodologies used to quantify images (IF and EM)

Response: We apologize for this missing information, we have added quantification information in the methods.

Referee #3 (Report for Author)

In this manuscript, Yue and colleagues report the role of SARS-CoV-2 accessory gene ORF6 as an important factor to modulate LD biogenesis during viral infection. They identified two conserved amphipathic helices in ORF6 that are required for the association with the LD membrane. They also demonstrate through crosslinking and pull-downs that the AH1 is required for ORF6 dimerization, and that dimerization is essential for ORF6 recruitment to the LD. Furthermore, they show how ORF6 is involved in the connection between LD and both the ER and mitochondria through the

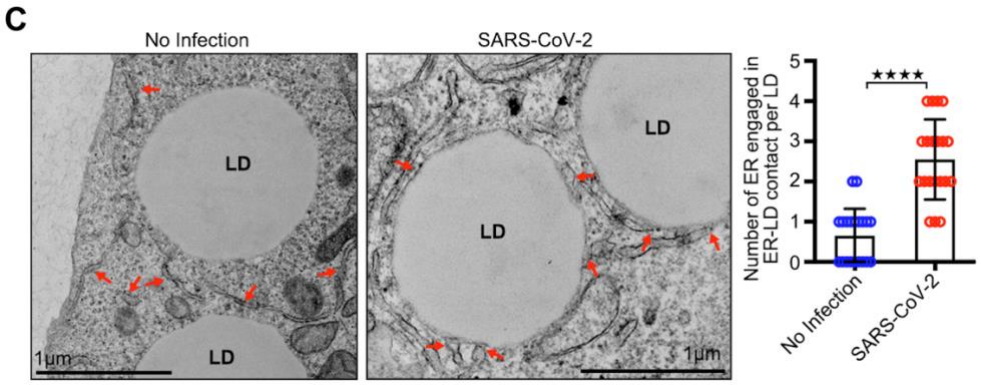
interaction with BAP31 and USE1 for the ER-LDs side and the mitochondrial SAMM complex to mediate the interaction with the mitochondria. Mechanistically, the authors show that ORF6 expression is able to activate lipogenesis through interaction with DGAT1 and DGAT2 and activate lipolysis of triacylglycerol and transfer of fatty acids from LD to mitochondria through activation of ATGL.
Overall, the data are well presented and appear for most aspect solid. Their mechanistic characterization of the role of overexpressed ORF6 is well performed, looking at many aspects performing interaction studies, mutational analysis, and imaging.

Response: Many thanks for your positive comments and conclusion of our work.

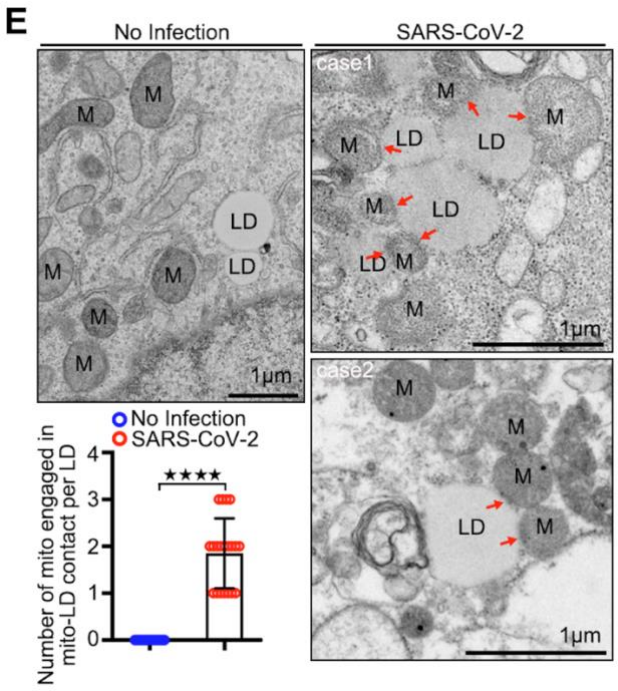
My main critique is that there are few experiments performed to validate their results in an infectious system, a limit that the authors themselves highlight in the manuscript discussion. Among the experiments conducted in the infection system, the authors show how viral infection alters the interactors of ATGL, mirroring their observations in transfected cells. In addition, they analyzed the viral titers upon knock-down or inhibition of the factors they identified as interactors (DGAT1 and DGAT2 and several others).
However, additional validation can be performed in the infectious system to prove that the observations made in the context of ORF6 overexpression can be translated in an infectious system. For instance, the authors never showed the localization of ORF6 in infected cells and considering that this is the main point of the manuscript, they should prove that during infection indeed ORF6 localizes clearly on LD and that mitochondria relocalize in their proximity (do ER/LD and LD/mitochondria contact sites increase also during infection?).

Response: We appreciate this critical advice. Unfortunately, we are so sorry that we are not able to show the LD localization of endogenous ORF6 in infected cells due to no working commercial ORF6 antibody for imaging. We have added this limitation in manuscript (**Revised Manuscript lines 497-498**).

As your suggested, we examined the relationship between mitochondria and LDs or ER and LDs in SARS-CoV-2 infected cells by using electron microscopy (**Revised Manuscript Fig 4C and 6E**).



Revised Manuscript Fig 4C. Representative transmission electron micrograph of non-infected or SARS-CoV-2 infected Vero-E6 cells. Red arrows mark the ER. LD, lipid droplets. Scar bar represents 1 μm. Quantification of number of ER engaged in ER-LD contact per LD. 20 LDs of two independent experiments were counted. Two-tailed Unpaired Student's t-test, ****$p < 0.0001$.



Revised Manuscript Fig 6E. Representative transmission electron micrograph of non-infected or SARS-CoV-2 infected Vero-E6 cells. Red arrows mark the mitochondria-LD contact. LD, lipid droplets. M, mitochondria. Scar bar represents 1 μm. Quantification of number of mitochondria engaged in mitochondria-LD contact per LD. 20 LDs of two independent experiments were counted. Two-tailed Unpaired Student's t-test, ****p < 0.0001.

Moreover, all the experiments performed in an infectious system require the analysis of the cell viability to prove that the perturbations applied (knock-down, inhibitors or chemicals such as OA) do not alter cell viability in absence of viral infection.

Response: We appreciate this critical advice. As your suggested, we have analyzed cell viability by using CCK8 kit (**Revised Manuscript Fig 3F, 3J, EV3D, 5M, 6N and 7H**).

Another important misconception that the author perpetrate throughout the manuscript is to refer to "viral replication" while measuring secreted infectivity (virus titers). This can be misleading since viral replication refers to a specific step (viral genome replication) of the virus infectious cycle.
The experiment they perform (virus titers) measures infectivity secreted from the cells and thus cannot rule out that the effect they observe is due to alteration in either entry, genome replication, virus assembly and/or virus release.
It is important that this point is clear, and the author have to either tune down their conclusion by stating that the exact step is still unknown and might not be "replication" or they need to perform additional experiments (such as entry assays with pseudoviruses to exclude entry and intracellular RNA quantification during viral infection to prove the reduction of viral replication) that unequivocally points toward an effect on viral replication.

Response: We thank reviewer for this critical comments and helpful suggestion. As suggested, we have tuned down our conclusion by stating the exact step is still unknown in the main text and changed "viral replication" to "viral production".

Point-by-point comments
Viability assays: Figure 5A and 5B: experiments with inhibitors require the analysis of cells viability and WB that shows that in all conditions the levels of ORF6 are equal.
All perturbations performed on infected cells require a viability control to prove that alterations of viral infectivity/replication are not due to cells death. Please include viability test for all treatments (inhibitors, chemicals or siRNA).

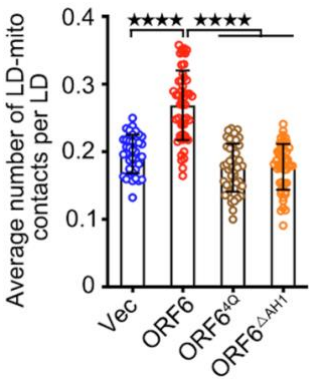
Response: We appreciate this critical advice. As your suggested, we have analyzed cell viability by using CCK8 kit (**Revised Manuscript Fig 3F, 3J, EV3D, 5M, 6N and 7H**).

>Figure 6D-F: The authors observe more mito-LD contact per cell upon ORF6 overexpression. However, they also see double the amount of LD in this condition. Using this kind of quantification, they cannot exclude that contact sites simply increase because more LD are presents. Number of LD/mito contact should be calculated on the single LD level (number of contacts for each LD), ideally including ORF6 positive and ORF6 negative droplets. Does the triple KD affect the number of LD? This could help solve this problem, since if KD does not affect LD number, the reduction in the number of LD-mito contact sites is indeed specific. In addition, there is no statement on how contact sites are calculated in the material and methods section. Please add a section explaining how this quantification has been performed.

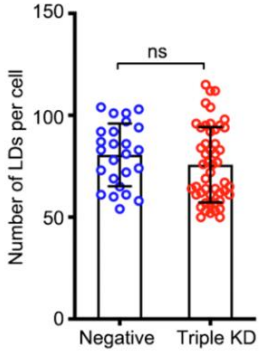
Response: We are grateful for the Reviewer's critical comments and suggestions. We have added the quantification of number of LD/mito contact per LD (contact number divided by LD number) for Original Manuscript Fig 6E (**Revised Manuscript Fig 6I**).

We found that Triple KD does not affect LD number (**Revised Manuscript Fig EV5K**).

Colocalization-based analysis of the contact sites of mitochondria-LDs and ER-LDs were performed using a plugin named colocalization in ImageJ. Contacts automatically identified by colocalization plugin with white pixels representing potential contact sites. We have added the image analysis in Methods.



Revised Manuscript Fig 6I. Quantification of average number of LD-mito contacts per LD (contact number divided by LD number). 37 cells (Vector), 50 cells (ORF6), 50 cells (ORF6^{4Q}), and 50 cells (ORF6△AH1) from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ****p < 0.0001.



Revised Manuscript Fig EV5K. Quantification of LD number. 25 cells (Negative) and 50 cells (Triple KD) from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ns means no significance.

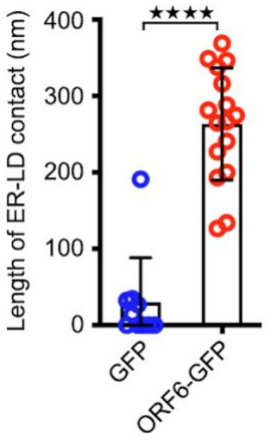
>Quantification of the IF is performed on 25 cells. However, the authors do not state how many biological replicates they used. Please ensure that at least two independent biological replicates are analyzed

Response: Thank you for your insightful comments. As we mentioned in our Method (lines 1050-1051), two or three independent experiments are analyzed. We have added the quantification information in figure legends.

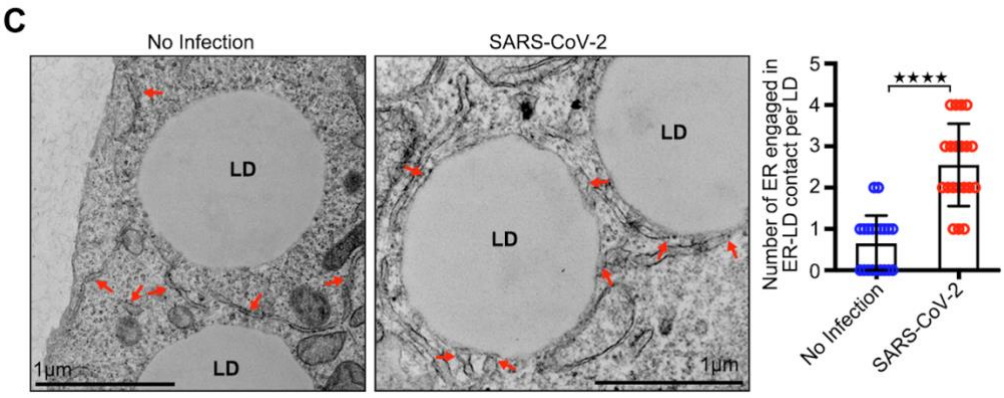
>I am not satisfied with the experiments showing the tethering of the ER to the LD. While the biochemical studies seem solid, the imaging approaches are not. ER membranes in the immunogold preparations are poorly visible and while extensive gold label is present, a single field of view without quantification is not enough to prove that ER is wrapping LD. The authors should provide high resolution TEM images of transfected and mock cells and quantify the number of LD wrapped or not by ER membranes. Similarly for the confocal studies, the authors should provide quantification of the colocalization observed in the context of the ORF6 and ORF6 mutants with the ER and LD.

Response: Many thanks for the critical comments and suggestions, these are indeed important points. We measured the approximate length of ER-LD contact based on the immunogold TEM (**Revised Manuscript Fig 4B**).

As your suggested, we examined the relationship between the ER and LDs in SARS-CoV-2 infected cells by using electron microscopy (**Revised Manuscript Fig 4C**).



Revised Manuscript Fig 4B. Quantification of length of ER-LD contact via ImageJ. 10 ER-LD contacts in GFP expressed cells and 16 ER-LD contacts in ORF6-GFP expressed cells of two independent experiments were calculated via ImageJ. Two-tailed Unpaired Student's t-test, ****p < 0.0001.



Revised Manuscript Fig 4C. Representative transmission electron micrograph of non-infected or SARS-CoV-2 infected Vero-E6 cells. Red arrows mark the ER. LD, lipid droplets. Scar bar represents 1 μm. Quantification of number of ER engaged in ER-LD contact per LD. 20 LDs of two independent experiments were counted. Two-tailed Unpaired Student's t-test, ****p < 0.0001.

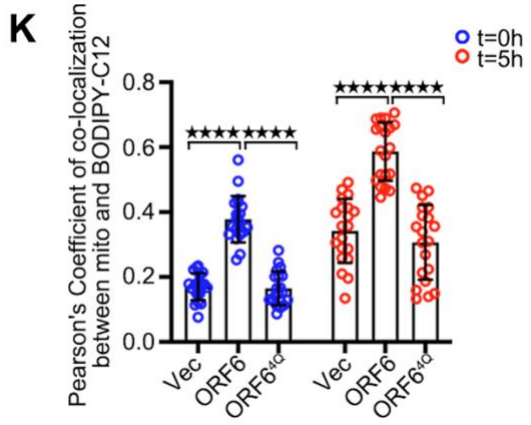
Minor points

>Induction of LD has been previously reported by https://www.nature.com/articles/s41467-022-31097-7 - Farley and co-workers showed that ORF6 induces LD and DGAT1 inhibitors are able to reduce viral titers in infected cells. Please cite this recent paper and discuss their results in light of your findings.

Response: We are grateful for the Reviewer's helpful suggestions. We have added this important reference in the revised manuscript (**Revised Manuscript lines 90-93**).

>Figure 6G: please include quantification (colocalization at the different time points)

Response: Thanks for your insightful advice. We have added the quantification (**Revised Manuscript Fig 6K**).



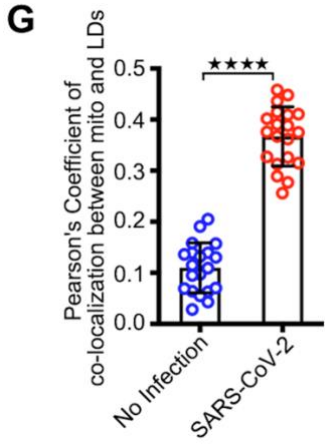
Revised Manuscript Fig 6K. Colocalization of BODIPY-C12 and mitochondria (Pearson's Coefficient), n=20 cells, three independent experiments. Two-tailed Unpaired Student's t-test, ****p < 0.0001.

Figure 6J: please include quantification. Also there seems to be bleed through between the two channels in the infected cells (BODIPY signal looks filamentous in the zoom panel of the infected cell).

Response: Thanks for your insightful advice. We have added the quantification.

For Figure 6J, BODIPY signal looks filamentous may due to fatty acid transfer to mitochondria through LD-mitochondria junctions.

We have added the quantification of Original Manuscript Fig 6J by using ImageJ (Pearson's Coefficient) (**Revised Manuscript Fig 6G**).



Revised Manuscript Fig 6G. Colocalization of LipidTox and mitochondria (Pearson's Coefficient), n=20 cells, two independent experiments.

>Sup Fig1E: Rig-1 should read Rig-I

Response: We apologize for this error. This has now been corrected.

Referee #4 (Report for Author)

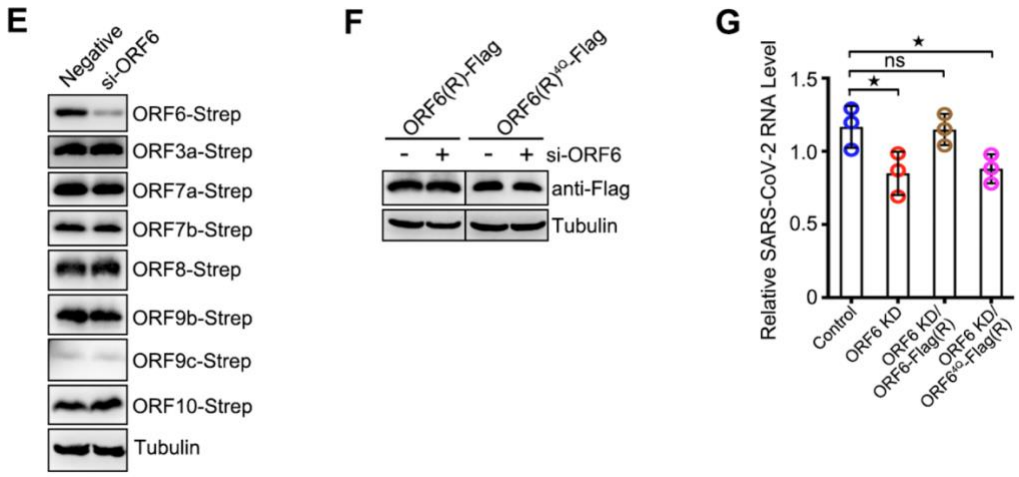
In the manuscript by Yue et al., the authors explore the functional role of the SARS-CoV-2 protein ORF6, which they find targets to the surface of lipid droplets through two putative amphipathic helices. The authors introduce several glutamine mutations into hydrophobic residues of the helices, causing a loss of lipid droplet targeting. The authors find overexpression of ORF6 in mammalian cells promotes larger lipid droplets and increased TAG storage, which they propose occurs via an interaction detected between ORF6 and DGAT1/2. Somewhat paradoxically, however, the expression of ORF6 also leads to increased TAG lipolysis, which they propose is mediated by interactions between ORF6 and ATGL. The authors also provide evidence that inhibiting lipid droplet formation or inhibiting lipolysis decreases viral titers. In addition to these data, the authors explore interacting partners of ORF6 based on published IP/MS datasets, identifying several ER and mitochondrial proteins that interact with ORF6 in their assays, including the mitochondrial SAM complex. The authors demonstrate that depleting a number of these interacting partners in host cells reduces viral infectivity.

While there are a number of interesting observations in the manuscript, the argument that ORF6 simultaneously promotes lipid droplet biogenesis and lipolysis is confusing and must be explored in greater detail. Further the conclusions of the manuscript would be greatly strengthened if effects on viral infectivity were tested by mutating ORF6 (i.e using the 4Q mutation) rather than knocking down host proteins, which in each case are known to have severe pleiotropic effects.

Response: Many thanks for your conclusion of our work. Your constructive suggestions help us significantly improve the quality of this manuscript.

Major points:
1. All experiments were done in the paper with either gain of function ORF6 expression or correlative experiments measuring viral titers in host cells with various treatments. However, loss of function experiments are necessary to strongly conclude that ORF6 and its targeting to lipid droplets is important for viral replication.

Response: We thank reviewer for this critical comments and helpful suggestion. As suggested, we used siRNA to knockdown ORF6 in SARS-CoV-2 infected cells and rescued ORF6 expression with RNAi resistant constructs ORF6(R)-Flag or ORF6(R)^{4Q}-Flag, and measure viral RNA level. Knockdown of ORF6 decreased SARS-CoV-2 production, and ORF6(R)-Flag expression in ORF6 KD cells rescued the viral production, but not ORF6(R)^{4Q}-Flag expression (**Revised Manuscript Fig EV5E-G**).



Revised Manuscript Fig EV5E. Cells were transfected with Negative or si-ORF6 for 24 h, and then further transfected with ORF6-Strep, or ORF3a-Strep, or ORF7a-Strep, or ORF7b-Strep, or ORF8-Strep, or ORF9b-Strep, or ORF9c-Strep, or ORF10-Strep for 24 h. Cell lysates were analyzed via WB. **Revised Manuscript Fig EV5F.** Cells were transfected with Negative or si-ORF6 for 24 h, and then further transfected with ORF6-Flag (RNAi resistant), or ORF6^{4Q}-Flag (RNAi resistant) for 24 h. Cell lysates were analyzed via WB. **Revised Manuscript Fig EV5G.** Vero-E6 cells were transfected with Negative or si-ORF6 for 24 h, and then further transfected with ORF6-Flag (RNAi resistant), or ORF6^{4Q}-Flag (RNAi resistant) for 12 h. Cells were further infected with SARS-CoV-2 for 24 h. Viral RNA level was determined by RT-qPCR, three independent replicates. Two-tailed Unpaired Student's t-test, *p < 0.05. ns means no significance.

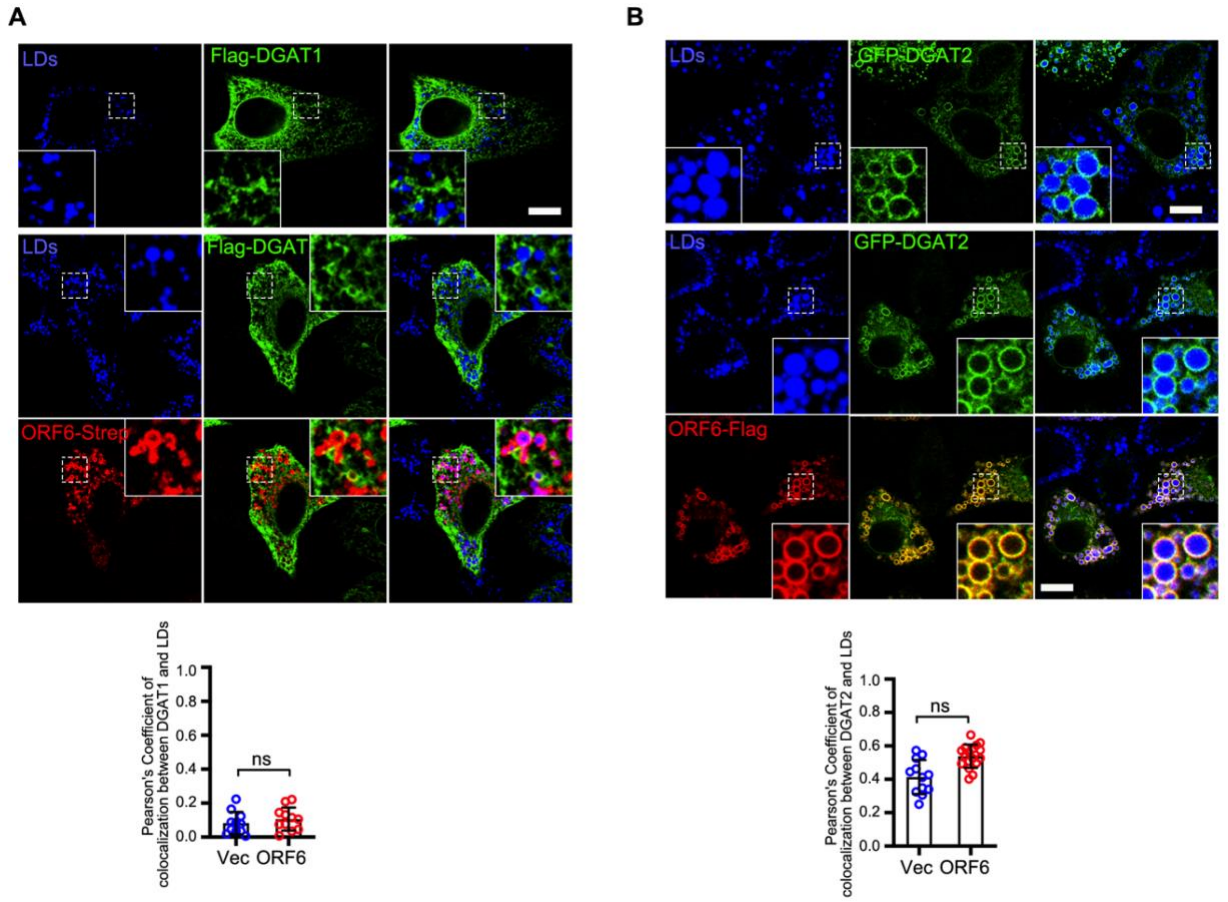
2. The ability of ORF6 to promote both biogenesis of lipid droplets and their lipolysis is confusing. ORF6 was shown throughout the manuscript to bind to DGATs, ATGL, BAP31, USE1, SAMM50, MTX1, and MTX2 in a manner dependent on a 7 amino acid stretch of protein. While the N-terminal deletion construct can be expressed in cells, is it possible this construct is improperly folded, leading to the loss of interactions?

Response: We appreciate this critical advice. Our co-IP and confocal data showed that ORF6$^{\triangle NTD}$ still dimerizes (self-interaction) and targets to LDs (**Revised Manuscript Fig 2E-H**), and ORF6$^{\triangle NTD}$ also interacts with DGAT1, DGAT2, MTX1 and MTX2 (**Revised Manuscript Fig EV2B, EV2C and EV5I**), suggesting that this mutant should be properly folded.

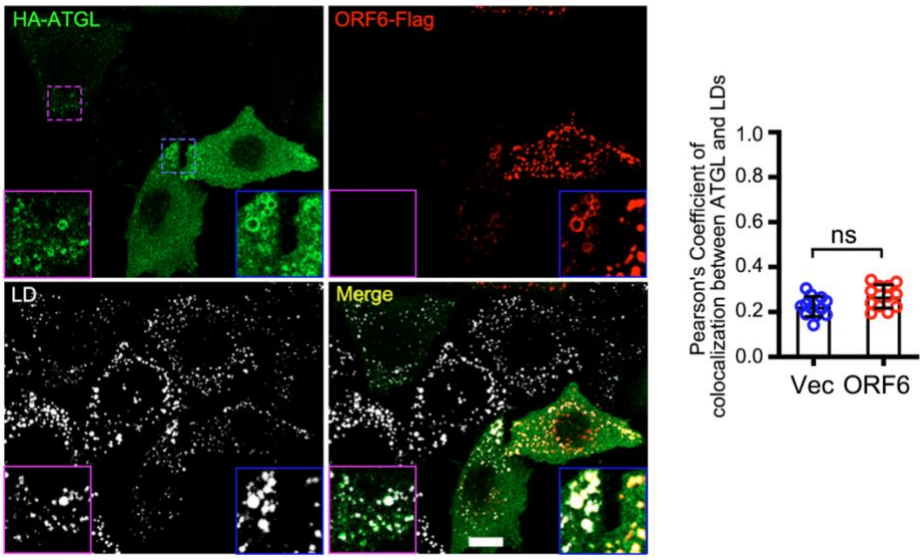
Further structural analyses of the mutant should be performed. Unfortunately, we are so sorry that we are not able to achieve this data.

3. Likewise, these interactions may not be indicative of functional roles. Do DGATs or ATGL become more targeted to LDs when ORF6 is overexpressed and does this depend on the N-terminus of ORF6?

Response: We appreciate this critical advice. DGAT1 is localized on the ER and DGAT2/ATGL target to LDs. Using confocal microscopy, we analyzed the localization of DGAT1, DGAT2 and ATGL on LDs upon ORF6 expression. ORF6 overexpression has no obvious effect on the association of DGAT1, or DGAT2, or ATGL with LDs (**Appendix Fig S1A, S1B, and S2A**).



Appendix Fig S1A. Cells were transfected with Flag-DGAT1 with or without ORF6-Strep for 24 h and further were treated with 200 μM OA for 12 h. LDs were labeled with LipidTOX Deep Red (blue). Cells were fixed and then stained with anti-Strep (red) and anti-Flag (green). Cells were imaged by confocal microscopy. Scar bar represents 10 μm. Colocalization of DGAT1 and LDs (Pearson's Coefficient), n=11 cells, two independent experiments. Two-tailed Unpaired Student's t-test, ns means no significance. **Appendix Fig S1B.** Cells were transfected with with GFP-DGAT2 with or without ORF6-Flag for 24 h and further were treated with 200 μM OA for 12 h. LDs were labeled with LipidTOX Deep Red (blue). Cells were fixed and then stained with anti-Flag (red). Cells were imaged by confocal microscopy. Scar bar represents 10 μm. Colocalization of DGAT2 and LDs (Pearson's Coefficient), n=12 cells (Vec) and n=17 cells (ORF6-Flag), two independent experiments. Two-tailed Unpaired Student's t-test, ns means no significance.



Appendix Fig S2A. HeLa cells were transfected with ORF6-Flag and HA-ATGL for 12 h and treated with 200 μM OA for 12 h, then fixed and stained with anti-Flag (red) and anti-HA (green). LDs were labeled with LipidTOX Deep Red (white). Cells were imaged by confocal microscopy. Scar bar represents 10 μm. Blue ROIs indicate cells expressing ORF6 and purple ROIs indicate the cells without ORF6 expression. Colocalization of ATGL and LDs (Pearson's Coefficient), n=12 cells, two independent experiments. Two-tailed Unpaired Student's t-test, ns means no significance.

4. The viral titer decrease in KD of BAP31/USE1 and in SAMM50/MTX1/MTX2 may be due to effects on ER or mitochondrial function, respectively, rather than loss of binding to ORF6. The SAM complex is required for mitochondrial morphology, cristae architecture, and proper respiration, for example. These conclusions would be stronger if the ORF6 interaction with each protein were specifically interrupted.

Response: Thank you for your instructive and critical suggestions. We agree with reviewer that the viral titer decrease in KD of BAP31/USE1 and in SAMM50/MTX1/MTX2 may be due to effects on ER or mitochondrial function. We have found that ORF6 NTD is responsible for ORF6-BAP31/USE1/SAM50 interactions, and ORF6 AH is responsible for ORF6-MTX1/MTX2 interactions. We are working on constructing interfering peptides based on ORF6 NTD and AH domains, to inhibit the binding of BAP31/USE1 and SAM complex, and evaluating the anti-viral effect of these peptides. This is another ongoing project.

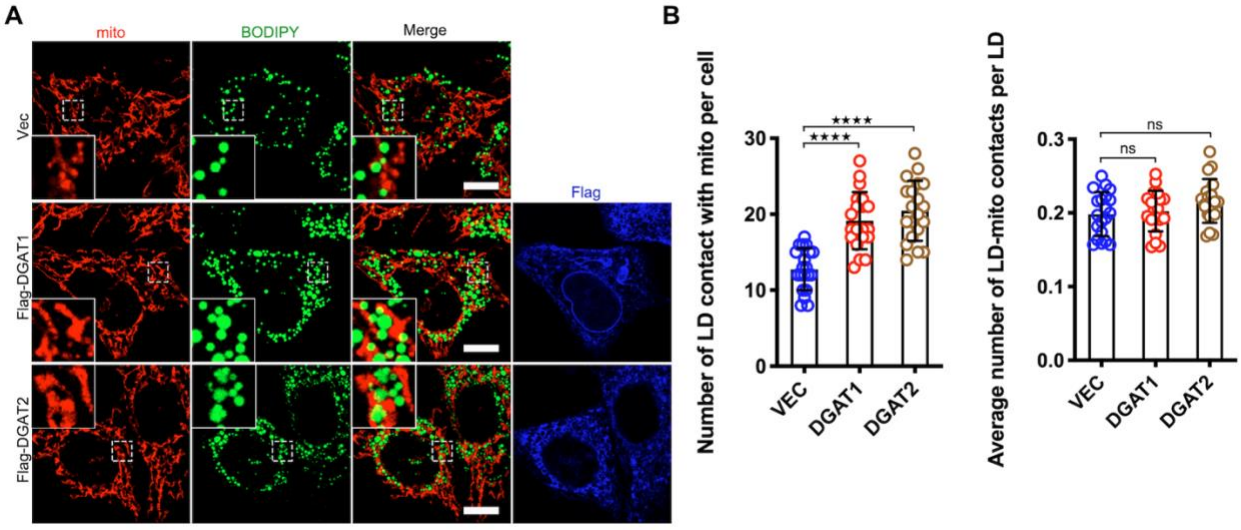
We have discussed these possibilities in the revised manuscript (**Revised Manuscript lines 498-503**).

5. The conclusion that ORF6 promotes lipid droplet-mitochondrial contact may be due to increased lipid droplet number in overexpression cells or increased TAG content, but not necessarily due to an interaction with the SAM complex. Does DGAT overexpression cause similar increases in contact between the organelles in their assay?

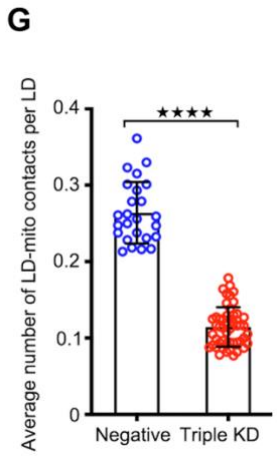
Response: We appreciate this critical advice. We have added the quantification of average number of LD-mito contact per LD in ORF6 overexpression cells (**Revised Manuscript Fig 6I**).

As you suggested, we examined the mito-LD contact in DGAT overexpression cells by confocal microscopy. We found that overexpression of DGAT1 or DGAT2 did increase the number of LD-mito contacts per cell, but have minor effect on the average number of LD-mito contacts per LD (contact number divided by LD number) (**Appendix Figure S5A-B**).

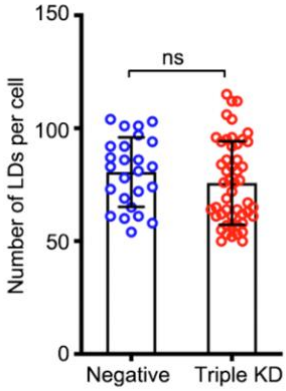
We found that knockdown of SAM complex (Triple KD) does not affect LD number, but decreased the average number of LD-mito contacts per LD (**Revised Manuscript Fig 7G and EV5K**), suggesting that the reduction in the number of LD-mito contact sites is indeed specific in ORF6 overexpression cells.



Appendix Figure S5A. HeLa cells were transfected with vector, or Flag-DGAT1, or Flag-DGAT2 for 12 h and further treated with 200 μM OA for 12 h, and then were fixed and stained with anti-Flag (blue) and anti-Tom20 (red). LDs were labeled with BODIPY-493/503 (green). **Appendix Figure S5B.** Number of LDs in each cell and average number of LD-mitochondria contacts per LD in (A) was counted from 20 cells of two independent experiments. Two-tailed Unpaired Student's t-test, ****$p < 0.0001$, ns means no significance.



Revised Manuscript Fig 7G. Quantification of average number of LD-mitochondria contacts per LD. 25 cells (Negative), 50 cells (Triple KD) from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ****$p < 0.0001$.



Revised Manuscript Fig EV5K. Quantification of LD number. 25 cells (Negative) and 50 cells (Triple KD) from three independent experiments were calculated. Two-tailed Unpaired Student's t-test, ns means no significance.

Dear Binbin,

Thank you for submitting a revised version of your manuscript. The study has now been seen by three of the original referees. While reviewer #3 is satisfied with the revision, reviewers #2 and #4 find that several of their initial points were not sufficiently addressed. Therefore, I would like to invite you to address the remaining referee comments and the following editorial issues:

1. It is important to address the point 1 from reviewer #2 in the revised manuscript by comparing data from individual experiments and quantifying non-transfected cells as a negative control. Please also address their point regarding GFP-BAP31 localisation and the minor points.
2. Please tone down the conclusions as indicated in the reports by reviewers #2 and #4.
3. Our publisher has done their pre-publication check on your manuscript. I have attached the file here. Please take a look at the word file and the comments regarding the figure legends and respond to the issues. Please also use this version when you resubmit the revised version.
4. Please make sure that the funding information in the manuscript and in our online submission system is complete. Currently, 2022KF006 is missing in our online system.
5. Please reduce the number of keywords to maximally five.
6. Please rename "Conflict of interest" section into "Disclosure and competing interests statement" (further info: https://www.embopress.org/page/journal/14602075/authorguide#conflictsofinterest).
7. We require a Data Availability Section at the end of Materials and Methods. As far as I can see, no data deposition in external databases is needed for this paper. If I am correct, then please state in this section: This study includes no data deposited in external repositories. Further information can be found at https://www.embopress.org/page/journal/14602075/authorguide#dataavailability
8. During our routine text plagiarism check, we noticed that several sentences show high similarity with published manuscripts for other groups (I have attached the screenshot). Please rephrase the text accordingly.
9. In our routine image check, our data analyst noted that some blots in Fig 5K and 5L, as well as 5H and 5I, appear to be derived from the same experiment. Please submit the source data for these panels (please see below for more information).
10. In figure EV1B, please add the boxes in the unmagnified image to indicate where the zoomed-in section is taken from.
11. In our routine image check, our data analyst noted that there is no image signal in Appendix figure S3B upper row, ORF6-Flag. We realise that this is a negative control, but some sort of background signal is expected. Please check and submit source data for this figure.
12. We generally encourage publication of source data for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need one file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as supplementary "Source Data". Please let me know if you have any questions about this policy.
13. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 3-4 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the image size is rather small and that text needs to be readable at the final size. Please send us this information together with the revised manuscript.

Please feel free to contact me if have any questions regarding this final revision. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the revised version.

With best regards,

Ieva

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Tel: +4962218891309
i.gailite@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the

work, we recommend a revision within 3 months (7th Jun 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #2:

This revised version of the manuscript entitled "Viral Protein Links Lipid Droplets, the ER, and Mitochondria, and Modulates Lipid Flux for Virus Production" by M. Yue and collaborators describes the role of the ORF6 protein of SARS-CoV-2 in mediating endoplasmic reticulum (ER)-lipid droplet (LD) and LD-mitochondria contacts. Many aspects of the manuscript have been improved, but some data are still over-interpreted and some image quantifications need to be improved.

Major comments:

With respect to quantification, for some experiments, the authors apparently did not change the way they conducted their statistical analysis.
In the previous review, I wrote in the first point "There are many experiments which are not associated with quantifications (for instance, Fig. 1G, 2G, 4E...); Co-localizations should be quantified (Pearson correlation for instance). Moreover, some quantifications are not properly done: statistics should be performed between independent samples, while here statistics (Fig. 3B, 3E, 3I etc...) are done considering individual cells as independent samples. The experiments should be repeated multiple times and statistics performed between experiments (using the mean of several cells quantified per experiment)."
For some experiments, statistical analyses are still comparing individual cells and not independent experiments. For instance, Figure 4F (and EV1E), and Figure 6G... Could the authors correct this?
Figure 3 A and B: in the legend, the authors wrote "White ROIs indicate cells expressing ORF6 and yellow ROIs indicate the cells without ORF6 expression.". Does it mean that the quantification was performed with cells from the same slide (comparing cells apparently not transfected to cells exhibiting an ORF6 labeling)? If it is the case, it might be dangerous as low expressing cells could still be positive but not be detected because of the sensitivity of the antibody used.

The data shown in the paragraph "SARS-CoV-2 ORF6 Homodimerization is Required for LD Association" do not support the conclusion. The data described only show that dimerization and LD binding are correlated (i.e. the WT protein as well as the LL-QQ and LI-QQ mutants dimerize and binds LDs; the DAH1 and DAH2 proteins do not dimerize and do not bind LDs) but do not show that homodimerization is required for LD binding.
For example, we could imagine that the same binding interface is involved in both dimerization and LD binding and that there are two pools of ORF6 in the cells: one consisting of a soluble dimer, and the other of a monomer bound to LDs. In this scenario, the authors would have obtained the same results, but however it cannot be said that homodimerization is required for LD fixation. If the authors want to conclude that dimerization is essential for LD association, they have to prove it. Otherwise, they need to change their conclusion.

In the previous review, I asked the following questions:

a. Are AH1 and AH2 alone able to recruit a protein at the surface of LDs? (could be answered using AH1- and AH2-GFP fusion proteins)
b. Are AH1 and AH2 sufficient to associate with the surface of LDs? This should be answered in an in vitro simplified system using synthetic peptides and artificial LDs. This kind of experiment could help understand the function of AH1 vs AH2.
The authors did not answer these questions: they made an AH1+AH2-GFP construct but they did not look at the ability of individual AHs to interact with LDs (AH1-GFP and AH2-GFP constructs). Moreover, the ability of these 3 constructs (AH1-GFP, AH2-GFP and AH1+AH2-GFP) to dimerize could be analyzed). This kind of analysis would have helped to differentiate the ability of the protein to dimerize and to bind LDs. Unless the authors want to change their conclusion, they should try this type of experiment.

Appendix Fig S1C: is GFP-BAP31 localized on the ER? The staining does not show a reticular pattern. Could the authors perform a colocalization experiment between GFP-BAP31 and an ER marker?

Minor comments:

The authors might consider deleting the paragraph about peroxisome-LD contacts. It does not add much to the manuscript and

is not associated with a molecular description of these contacts in ORF6 expressing cells. This part is too preliminary.

Figure 3G: the figure is only partially annotated.

English should be corrected throughout the manuscript.

Referee #3:

In the revised manuscript, the authors have addressed all the points raised and therefore I endorse the publication of the current version of the manuscript.

Referee #4:

The revised manuscript by Yue et al includes several additional control experiments and quantitative analyses that improve the work and address many of my concerns. Ultimately, due to the inability to generate clear separate function of mutants of ORF6, some of the findings regarding interacting proteins may not represent in vivo function of ORF6. The authors do a good job in the revised discussion to address limitations, but the abstract should also be toned down. The authors have not demonstrated that the interaction with the SAM complex "links mitochondria to LDs", for example. The authors also have not convincingly demonstrated a mechanistic role for interaction with DGAT1/2, merely a correlative one.

Specific points:
1. Given that the AHs are required for lipid droplet targeting and that ORF6 can be found in the mitochondrial fraction, why does the AH mutant fail to localize to mitochondria in cells? This raises the concern that the interaction with SAM/MTXs is not physiologically meaningful, and the connection between ORF6 and mitochondria remains a weak point of the manuscript. Effects on mitochondrial metabolism/beta-oxidation may be true independent of a physical interaction with ORF6.
2. The authors provide evidence that ORF6 interacts with DGAT and that increased TAG synthesis occurs as a result of ORF6 overexpression. However, the authors find that increased DGAT1/2 stability during viral infection is not due to ORF6. Ultimately, without mechanistic insights, the authors should tone down conclusions that the interaction with DGAT1/2 causes a rewiring of metabolism.

Point-by-point response to reviewers:

Referee #2:

This revised version of the manuscript entitled "Viral Protein Links Lipid Droplets, the ER, and Mitochondria, and Modulates Lipid Flux for Virus Production" by M. Yue and collaborators describes the role of the ORF6 protein of SARS-CoV-2 in mediating endoplasmic reticulum (ER)-lipid droplet (LD) and LD-mitochondria contacts. Many aspects of the manuscript have been improved, but some data are still over-interpreted and some image quantifications need to be improved.

Response: Many thanks for your valuable and positive comments and conclusion of our work.

Major comments:

With respect to quantification, for some experiments, the authors apparently did not change the way they conducted their statistical analysis.
In the previous review, I wrote in the first point "There are many experiments which are not associated with quantifications (for instance, Fig. 1G, 2G, 4E...); Co-localizations should be quantified (Pearson correlation for instance). Moreover, some quantifications are not properly done: statistics should be performed between independent samples, while here statistics (Fig. 3B, 3E, 3I etc...) are done considering individual cells as independent samples. The experiments should be repeated multiple times and statistics performed between experiments (using the mean of several cells quantified per experiment)."
For some experiments, statistical analyses are still comparing individual cells and not independent experiments. For instance, Figure 4F (and EV1E), and Figure 6G... Could the authors correct this?
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Response: Many thanks for your suggestion. We have corrected the quantification by comparing data from individual experiments.

For Fig 3A, Fig5A, FigEV2A, FigEV4G, and Appendix Fig S3A, the quantifications were performed with cells from different slides (comparing cells transfected with vector with ORF6). But for representative confocal images, we used the cells from the same slide in which some cells were with low or no ORF6 expression while some cells were with high ORF6 expression, a way that was frequently used for the representative imaging data.

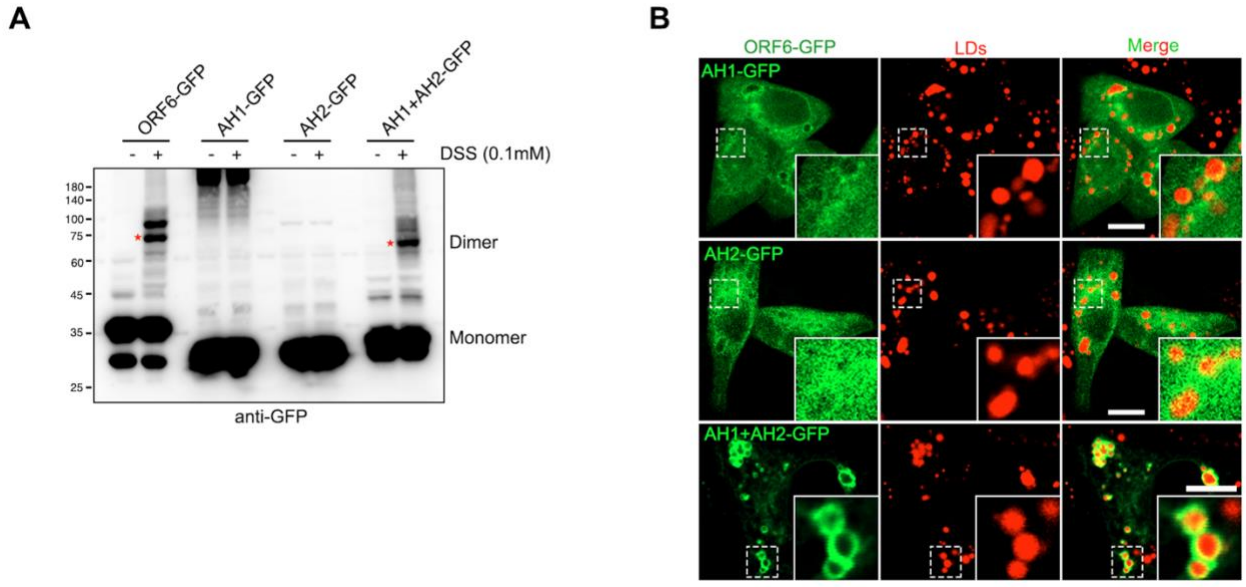
The data shown in the paragraph "SARS-CoV-2 ORF6 Homodimerization is Required for LD Association" do not support the conclusion. The data described only show that dimerization and LD binding are correlated (i.e. the WT protein as well as the LL-QQ and LI-QQ mutants dimerize and binds LDs; the DAH1 and DAH2 proteins do not dimerize and do not bind LDs) but do not show that homodimerization is required for LD binding.
For example, we could imagine that the same binding interface is involved in both dimerization and LD binding and that there are two pools of ORF6 in the cells: one consisting of a soluble dimer, and the other of a monomer bound to LDs. In this scenario, the authors would have obtained the same results, but however it cannot be said that homodimerization is required for LD fixation. If the authors want to conclude that dimerization is essential for LD association, they have to prove it. Otherwise,

they need to change their conclusion.

Response: Many thanks for your suggestion. We have toned down our conclusion by replacing "required" to "important".

In the previous review, I asked the following questions:
a. Are AH1 and AH2 alone able to recruit a protein at the surface of LDs? (could be answered using AH1- and AH2-GFP fusion proteins)
b. Are AH1 and AH2 sufficient to associate with the surface of LDs? This should be answered in an in vitro simplified system using synthetic peptides and artificial LDs. This kind of experiment could help understand the function of AH1 vs AH2.
The authors did not answer these questions: they made an AH1+AH2-GFP construct but they did not look at the ability of individual AHs to interact with LDs (AH1-GFP and AH2-GFP constructs). Moreover, the ability of these 3 constructs (AH1-GFP, AH2-GFP and AH1+AH2-GFP) to dimerize could be analyzed). This kind of analysis would have helped to differentiate the ability of the protein to dimerize and to bind LDs. Unless the authors want to change their conclusion, they should try this type of experiment.

Response: Thank you for your insightful comments and helpful suggestions. We have checked the localization and dimerization of AH1-GFP, AH2-GFP and AH1+AH2-GFP. Our data indicate that AH1-GFP and AH2-GFP failed to dimerize or localize on LDs while AH1+AH2-GFP can form dimerization and targets to LDs (**Revised Manuscript Appendix Fig 1**).



Revised Manuscript Appendix Fig 1. (A) HEK293T cells were transfected with ORF6-GFP or indicated mutants and were treated with 0.1 mM DSS for 30 min. Cell lysates were analyzed via WB. (B) HeLa cells were transfected with ORF6-GFP or indicated mutants and were treated with 200 μM OA for 12 h, and then fixed. LDs were labeled with LipidTOX Deep Red. Cells were imaged by confocal microscopy. Scar bar represents 10 μm.

Appendix Fig S1C: is GFP-BAP31 localized on the ER? The staining does not show a reticular pattern. Could the authors perform a colocalization experiment between GFP-BAP31 and an ER

marker?

Response: Thank you for your insightful comments. GFP-BAP31 showed a low expression, so we generated a new construct BAP31-GFP. As your suggestion, we have checked the colocalizations between BAP31-GFP and mCherry-RAMP4, BAP31-GFP and ORF6-Flag. We observed a reticular pattern and good colocalizations of BAP31-GFP with mCherry-RAMP4 and BAP31-GFP with ORF6-Flag.

Figures for referees not shown.

Figures for referees not shown.

Minor comments:

The authors might consider deleting the paragraph about peroxisome-LD contacts. It does not add much to the manuscript and is not associated with a molecular description of these contacts in ORF6 expressing cells. This part is too preliminary.

Response: Thank you for your insightful comments and helpful suggestions. We have deleted this part.

Figure 3G: the figure is only partially annotated.

Response: Thank you for your instructive suggestions. We have re-arranged the panels.

English should be corrected throughout the manuscript.

Response: Thank you for your instructive suggestions. We have improved our English.

Referee #3:

In the revised manuscript, the authors have addressed all the points raised and therefore I endorse the publication of the current version of the manuscript.

Response: Many thanks for your valuable and positive comments of our work.

Referee #4:

The revised manuscript by Yue et al includes several additional control experiments and quantitative analyses that improve the work and address many of my concerns. Ultimately, due to the inability to generate clear separate function of mutants of ORF6, some of the findings regarding interacting proteins may not represent in vivo function of ORF6. The authors do a good job in the revised discussion to address limitations, but the abstract should also be toned down. The authors have not demonstrated that the interaction with the SAM complex "links mitochondria to LDs", for example. The authors also have not convincingly demonstrated a mechanistic role for interaction with DGAT1/2, merely a correlative one.

Response: Many thanks for your valuable and positive comments and conclusion of our work.

Specific points:
1. Given that the AHs are required for lipid droplet targeting and that ORF6 can be found in the mitochondrial fraction, why does the AH mutant fail to localize to mitochondria in cells? This raises the concern that the interaction with SAM/MTXs is not physiologically meaningful, and the connection between ORF6 and mitochondria remains a weak point of the manuscript. Effects on mitochondrial metabolism/beta-oxidation may be true independent of a physical interaction with ORF6.

Response: Thank you for your important comments. Although no oblivious colocalization was observed between ORF6△AH1 and mitochondria in the imaging assay, by purifying mitochondria, we found that ORF6△AH1 was enriched in mitochondria fraction. This data indicates that ORF6△AH1 localizes to mitochondria.

Figures for referees not shown.

2. The authors provide evidence that ORF6 interacts with DGAT and that increased TAG synthesis occurs as a result of ORF6 overexpression. However, the authors find that increased DGAT1/2 stability during viral infection is not due to ORF6. Ultimately, without mechanistic insights, the authors should tone down conclusions that the interaction with DGAT1/2 causes a rewiring of metabolism.

Response: Thank you for your insightful comments and helpful suggestions. We have toned down our conclusion: "*ORF6 increases LD biogenesis dependent on DGAT1 and DGAT2*".

2nd Revision - Editorial Decision **8th May 2023**

Dear Binbin,

Thank you for addressing the final editorial issues. I am now pleased to inform you that your manuscript has been accepted for publication.

Before we can forward your manuscript to our publishers, I would like to propose a few changes in the article title, abstract and synopsis. I have also written a short blurb that will accompany the title of your manuscript in our online table of contents. Please take a look at the text below and in the attached manuscript text file and let me know if any corrections or adjustments are necessary.

Title:
SARS-CoV-2 ORF6 protein mediates inter-organelle contacts and modulates host cell lipid flux for virus production

Blurb:
A lipid droplet-localized coronaviral protein alters cellular metabolism by promoting their contact formation with the endoplasmic reticulum and mitochondria

Synopsis
Viruses, including SARS-CoV-2, target lipid droplets to enhance viral replication. This study identifies SARS-CoV-2 protein ORF6 as a regulator of lipid droplet interactions with the ER and mitochondria.
- ORF6 localizes to lipid droplets and inserts into the lipid monolayer via its two amphipathic helices
- ORF6 reprograms cellular lipid metabolism to enhance lipolysis
- ORF6 promotes ER-lipid droplet contact formation and lipid droplet biogenesis via an interaction with ER membrane proteins BAP31 and USE1
- ORF6 links lipid droplets to mitochondria by binding to the mitochondrial outer membrane SAM complex, leading to increased ATP production.

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Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

Finally, we would like to promote your manuscript among the Chinese readership. Therefore, we would like to invite you to prepare a short summary of the manuscript in Chinese (1500-2000 Chinese characters), which we will promote on the WeChat platform 'BioArt'.

If you are interested in this opportunity, we recommend covering the article very close to its online publication date. Thus, ideally we would very much appreciate if you could send us a draft by replying to this invitation email within the next 7 working days. Please let us know within 3 working days whether you would be interested in contributing such a short summary in Chinese.

I have included below some general guidelines on how to prepare a summary and a link to recent examples for your reference. Please let me know if you have any questions about this.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Best regards,

Ieva

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Senior Scientific Editor
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

General Guidelines

1. These summary articles are meant to be targeting general audience so please limit the use of specialized technical terms, acronyms and jargon.
2. A summary usually starts with brief background information of the reported work, which is followed by explaining the findings in some detail, and ends with a short review of the conclusions as well as the implications of the work and future directions for the research.
3. The summary should at least contain one graphical item, such as a scheme or a figure from the paper.
4. Please provide ONE SINGLE document containing all text and graphical materials, ideally as a Word.docx or .doc file. Please DO NOT provide the document as a .pdf file.
5. Please DO NOT publicly release the document before the paper is officially published online.

Summary Examples

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EMBO J | 王松灵院士团队揭示组织内应力调控大型哺乳动物乳恒牙替换的新机制

EMBO Press Author Checklist

Corresponding Author Name: Binbin Ding	**USEFUL LINKS FOR COMPLETING THIS FORM**
Journal Submitted to: THE EMBO JOURNAL	The EMBO Journal - Author Guidelines
Manuscript Number: EMBOJ-2022-112542R	EMBO Reports - Author Guidelines
	Molecular Systems Biology - Author Guidelines
	EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods, Table EV1

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
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Please detail **housing and husbandry conditions**.	Not Applicable	

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	ACKNOWLEDGMENTS

Design

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If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
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Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Not Applicable	

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figure legends

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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
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If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	