Review of: "Nucleocytoviricota Viral Factories Are Transient Organelles Made by Phase Separation"

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Potential competing interests: No potential competing interests to declare.

General considerations

The manuscript by Rigou and colleagues is well written, convincing, and intriguing. The results are clear and generally well explained in the text; their hypotheses are confirmed by their results. I appreciate the meticulousness of the figure legends and how the Methods are described.

Some passages in the text should be improved in terms of explanation of what the Authors are doing (the trans-complementation experiment, for instance) or what the Authors expect from their experiments (see my comments below).

I noticed a difference between the section about the identification of viral factory scaffold proteins, which is highly exhaustive and detailed, and the rest of the manuscript in terms of speculation about the results obtained and the rigorousness of the experimental approach. That said, I would enjoy the same “approach” for all sections, but this is just a personal opinion.

The Introduction and Discussion sections sound like a “list” of facts and evidence, as they lack a bit of speculation by the Authors and a bit of context. I think that the manuscript would improve a lot if the Authors would add some elaboration of why their results are important, for example, from a clinical point of view or for public health issues, and from a genuinely research point of view, how this manuscript could open the way to the interpretation of the mechanism of infection of other viruses, and so on.

Below are my comments and suggestions for the sections in which modifications are required, divided into Major and Minor.

Section: Introduction

Minor

- The Authors should name this section as “Introduction”.
- I suggest the Authors add a Glossary for all the abbreviations they use.
- A brief description of what Phase Separation is would be useful for the generic reader to better understand what comes next; the same could be said for 1,6-hexanediol; the Authors should add just a couple of words, for example: “1,6-hexanediol is an organic compound proven to interfere with condensate formation due to its ability to alter hydrophobic interactions.” Similarly, while the Authors clearly point out why they chose mimivirus for their experiments, they should add a brief description of what mimivirus actually are.

Section: *Nucleocytoviricota* viral factories are biomolecular condensates

Major

- It is not clear to me what the correlation is between the OL and the IL in terms of units for each VF: is each VF composed of a single OL and a single IL? This is because the Authors report that: “the outer layer (OL) of two independent VFs” when referring to the Outer Layer, while: “the number of IL of the VFs,” when referring to the Inner Layer, so it seems that the OL may encompass multiple IL, is that correct?
- In Figure 1B, the morphology of infected*A.castellanii* cells is very different between the untreated and 1.6-hex treated conditions, making it a little difficult to evaluate the disruption of VFs. How do the Authors comment on the effect of the compound on whole cell integrity?
The Authors should consider changing the pseudocolor of DAPI to emphasize the difference between treated and untreated samples.
- It is difficult to observe the fusion of two independent VFs through the images provided in Figure 1H due to resolution limits, minor focus change, and cell movements; it would be more reasonable to describe this event as juxtaposition and *possibly* fusion. If the Authors have other evidence (for instance, Super Resolution microscopy images or EM images), it is welcomed to change the panel.

Minor

- The Authors should report what GVs stands for in the first section.
- In Figure 1A, it could be helpful to add a lower magnification image of the whole infected cell to better contextualize mimivirus VF.
- In this section, the Authors state that: “we fluorescently labelled proteins enriched in a previous proteome of mimivirus VFs” without naming the proteins in the text while showing an experiment with OLS1 and ILS1 in the figure, and in the subsequent section, they introduce proteins R561 and R252, which are actually OLS1 and ILS1. I suggest introducing the proteins clearly in the text early on, making it easier for the reader to understand the figure.
- In Figure 1F, the Authors show the major capsid protein (MCP), and they report that mollivirus accumulates in an uncharacterized sub-compartment in infected cells before being incorporated into the viral particles.
From the images shown, MCP does not seem to be accumulated in control cells, but just juxtaposed to the periphery of VF; maybe a higher magnification image could help.

Section: At least two scaffold proteins play key roles in mimivirus viral factory’s phase separation

Major

- The Authors must provide a representative western blot image for the Co-IPs they mention in the text.
What do the Authors mean by “some degree of proximity with all these client proteins”? Proximity does not always mean (functional) physical interaction. Why did the Authors decide to use a crosslinking agent?
- Please be consistent with protein nomenclature in the figure, as OLS1 in some graphs is reported as OLS1 while in others as R561, generating confusion.

Minor

- I recommend that the Authors explain briefly how the trans-complementation experiment should work, as it may not be

immediately comprehensible for the generic reader.

Furthermore, they report that: “Using the trans-complementing line, we demonstrated that*ils1* is an essential gene (Figure 3I)”, and they should discuss this result (which is not trivial) a little more.

- The Authors should indicate in the caption of Figure 3H if the red signal is anti-HA.

Section: Identification of client proteins demonstrates sub compartmentalization of functions

Major

- As requested for the Co-IPs mentioned in Figure 2A, a representative western blot image must be presented for the immunoprecipitation discussed in this section.

Minor

- Be consistent with the nomenclature for the experiment performed: in this section, two immunoprecipitations (IPs) are discussed, not coimmunoprecipitations (Co-IPs). Please correct also the figure legend.
- The following sentence is confusing and should be reformulated: “Surprisingly, while proteins associated with replication and transcription are incorporated in the VFs, replication proteins localized to the OL of the VF while transcription proteins accumulated at the IL (Figure 5A and Figure 6A-B).”
- Please indicate what “EU” labelling stands for.
- Figure 6I is not cited in the text. I also recommend giving more emphasis to the graphical abstract, as it perfectly recapitulates the findings discussed in the text. The Authors could recall the figure in the Discussion section.

Section: Discussion

As I pointed out in my General Considerations, I think that the manuscript would improve a lot if the Authors discussed more the importance and relevance of their results. The recent coronavirus pandemic has stressed dramatically how the scientific community was unprepared to face a worldwide health issue; the understanding of SARS-CoV-2 biology has led researchers to develop instruments and knowledge to overcome one of the most impressive health issues of our time. Hence, it is my opinion that work like this can be of tremendous importance.

Section: Methods

Major

- Not all the statistical analyses shown are correct. In the Statistics and reproducibility section, the Authors report that: “The null hypothesis ($\alpha = 0.05$) was tested using unpaired two-tailed Student’s t-tests,” which is incorrect for some of the experiments they show.

In Figure 3F, for example, where they have two different groups of related variables (a wild-type and ols1KO for non-complementing and complementing cells), which, in principle, makes the t-test performable, samples are cross-analyzed (wild-type vs wild-type ols1KO vs ols1KO). The Authors must perform ANOVA followed by the appropriate multiple comparison post-hoc test. Figures that need correction are: 1C, 3F, S9. For Figures 1D, 3B, 3D, and 6G, on the contrary,

unpaired two-tailed Student's t-tests are correct.

Minor

- In the subsection: "Immunofluorescence and fluorescent microscopy," *fluorescent* should be replaced with *fluorescence*.

Section: supplementary material

Supplementary Tables are named in the manuscript but not visible in the file.