Liquid-liquid Phase Separation Mediates the Formation of Herpesvirus Assembly Compartments

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February 23, 2022

Re: JCB manuscript #202201088

Dr. Hongyu Deng
Chinese Academy of Sciences
15# Datun Rd, Chaoyang District, Beijing
Beijing 100101
Chile

Dear Dr. Deng,

Thank you for submitting your manuscript entitled "Liquid-liquid Phase Separation Mediates the Formation of Herpesvirus Assembly Compartments". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers express enthusiasm that your study demonstrates a role for LLPS as being involved in herpesvirus cytoplasmic envelopment, and we agree that this seems of high interest for the readership of JCB. While they are overall positive regarding your data, they have provided constructive feedback which we hope you agree will further improve your study. In particular, in revising we find it essential you address the following:

Reviewer #1:

"1. A key characteristic of LLPS is their susceptibility to disruption by aliphatic alcohols like 1,6-hexanediol or propylene glycol. Evidence that the LLPS-like structures formed by ORF52 in transfected or infected cells can be disrupted by 1,6-hexanediol or propylene glycol treatment, and that they dynamically re-form once the alcohol is removed, would significantly strengthen this study.
2. The authors show that the mutations M1, M2 and M3 prevent the formation of ORF52 LLPS in transfected cells, and that virus production is reduced, but they do not directly show that these mutations prevent cVAC formation. The authors should stain cells transfected with these BACs using antibodies that recognise ORF33, ORF38 or ORF45 for visualisation by fluorescence microscopy, to demonstrate that cVAC formation (as defined by formation of cytoplasmic punctae containing ORF33, ORF38 and ORF45) is impaired. The paper would be further strengthened by doing the same for mutants M4, M5, M6 and M7.
3. The authors should make reference to the previous study by Metrick, Koenigsberg and Heldwein (mBio 11, e00810-20; 2020) that shows HAV-1 pUL11 to be nascently disordered protein that undergoes phase separation. In this paper the authors posit that herpesvirus (HSV-1) secondary envelopment is driven by LLPS. It would be instructive for the authors to compare the properties of MHV-68 ORF52 and HSV-1 pUL11.
4. The data in Figures 4C, 4D, 4E, S1D, S1E and S1F are presented with error bars but the authors do not describe what these errors represent (SEM or SD), nor do they state how many times these experiments were performed. Additionally, the statistical tests used for Figures S1E and S1F are not described."

- Addressing other minor points would also be helpful, but not required.

Reviewer #2: Major critiques 1-3.

Reviewer #3: Main points 1+2.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original

microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Billy Tsai, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

Zhou et al present in vitro and cell-based evidence to support the hypothesis that gammaherpesvirus assembly is mediated, at least in part, by liquid-liquid phase separation and that the viral protein ORF52 is required for this process. This is not the first time that LLPS as a mechanism for herpesvirus assembly has been proposed, but it's the first I am aware of where strong evidence to support this hypothesis has been provided. Overall the story is compelling and very likely to be correct. However, several of the experiments presented are not entirely convincing and the description of the experimental procedures falls below the standard expected for publication in a quality journal like JCB.
Comments:
- A key characteristic of LLPS is their susceptibility to disruption by aliphatic alcohols like 1,6-hexanediol or propylene glycol. Evidence that the LLPS-like structures formed by ORF52 in transfected or infected cells can be disrupted by 1,6-hexanediol or propylene glycol treatment, and that they dynamically re-form once the alcohol is removed, would significantly strengthen this study.
- The authors show that the mutations M1, M2 and M3 prevent the formation of ORF52 LLPS in transfected cells, and that virus production is reduced, but they do not directly show that these mutations prevent cVAC formation. The authors should stain cells transfected with these BACs using antibodies that recognise ORF33, ORF38 or ORF45 for visualisation by fluorescence microscopy, to demonstrate that cVAC formation (as defined by formation of cytoplasmic punctae containing ORF33, ORF38

and ORF45) is impaired. The paper would be further strengthened by doing the same for mutants M4, M5, M6 and M7.
- The authors should make reference to the previous study by Metrick, Koenigsberg and Heldwein (mBio 11, e00810-20; 2020) that shows HAV-1 pUL11 to be nascently disordered protein that undergoes phase separation. In this paper the authors posit that herpesvirus (HSV-1) secondary envelopment is driven by LLPS. It would be instructive for the authors to compare the properties of MHV-68 ORF52 and HSV-1 pUL11.
- The data in Figures 4C, 4D, 4E, S1D, S1E and S1F are presented with error bars but the authors do not describe what these errors represent (SEM or SD), nor do they state how many times these experiments were performed. Additionally, the statistical tests used for Figures S1E and S1F are not described.
- There are many experimental procedures that are not described adequately in the manuscript. Specifically, the authors don't describe how their cells were transfected, the source of their antibodies (either literature citation or manufacturer plus catalogue number), nor how the RNA EU incorporation and click chemistry for labelling was performed.
- The co-localisation data presented in Figures 1C, 1D, 3I, 3J and S4D are not completely convincing. Only a single field is shown, with no quantitation. How representative are these images? How many fields were imaged, across how many biological replicates. The authors should provide additional fields as a supplemental figure to support their conclusions. S4D and S4E are particularly problematic as the extend of co-localisation between KSHV-ORF52 and MHV-68 ORF33 is very low - panels S4D and S4E don't contribute much to the story and could probably just be removed.
- It would be helpful for the authors to state the ratio of absorbance at 260 and 280 nm for their purified ORF52 protein, to confirm that final purified protein did not contain any contaminating nucleic acids from the bacterial expression host
- How were the gold nanoparticles used for registration of the fluorescence and EM images in Figure 1B? Were the nanoparticles covered with a fluorescent coating? If so, please describe clearly in the methods. If not, how were these ~50-100 nm gold particles visible in the fluorescence images such that they could be used for image alignment?
- Figure S1D: The growth kinetics of the mEosEM virus clearly differs from the other two viruses. The authors should comment on this.
- Figure S3E. This figure isn't discussed at all in the "Results" section of the manuscript and doesn't add significantly to the manuscript - it should be removed. If it is to be retained it will need to be discussed adequately in the results and the methods should be expanded to include details of how the RNA FISH experiments were performed.

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

Comments on "Liquid-liquid Phase Separation Mediates the Formation of Herpesvirus Assembly Compartments" by Zhou et al.

For herpes viruses, the nucleocapsid is first assembled within the nucleus and translocated to the cytoplasm for subsequent maturation steps. To generate a mature virion, dozens of tegument proteins and glycoproteins need to be recruited and assembled in the cytoplasm. This is a challenging task and a powerful orchestration mechanism is required. In this paper, the authors study the function of ORF52 in the virus assembly stage of the life cycle of a murine Herpesvirus. They uncovered that the abundant tegument protein ORF52 compartmentalizes the components necessary for virion maturation via a liquid-liquid phase separation mechanism.

It is known in literature that both α-herpesvirus and β-herpesvirus form cytoplasmic virion assembly compartments (cVACs) for the virus assembly. Built on their own previous research, the authors showed that cVACs are also formed during γ-herpesvirus (MHV-68) infection, and interestingly cVACs of γ-herpesvirus have liquid properties. The most abundant viral protein ORF52 is required for the formation of cVACs. The morphology of cVAC, the lack of membrane encloser, and the dynamic properties of ORF52 suggest that cVACs might be formed via liquid-liquid phase separation (LLPS). However, as the most abundant protein, ORF52 apparently does not possess phase separation capacity by itself. Interestingly, nucleic acids including DNA and RNA robustly undergo phase separation with ORF52. The authors generated a battery of ORF52 truncation mutants and point mutants with varying propensities of LLPS. Using these reagents, the authors demonstrated that the LLPS properties of ORF52 are critical for cVACs formation and importantly virion production.

Overall the experiments are well-designed and executed. The data are in high quality and also well presented. The findings are novel and significant. I'd like to suggest its pulication in Journal of Cell Biology with minor revision. I will list my critiques and suggestions below.

Major critiques

1.LLPS is a concentration-dependent phenomenon. Can the appearance of cVAC be purely correlated with the concentration of ORF52? Can the authors test the time-dependent expression level of ORF52 post infection and see whether there is coincidence of high ORF52 level and the appearance of cVAC? Alternatively, although ORF52/nucleic acid can undergo LLPS in vitro, other components including virus proteome or host proteome might work synergistically with ORF52 to form cVAC in vivo. Any prior evidence to indicate this possibility? It is worth discussing a bit.

2.Please attach a multiple sequence alignment of representative homogs of ORF52 in a supplemental figure. It helps the readers to evaluate the potential functions and appreciate the roles of conserved structural elements and residues from

evolution.

3.Is there a Western blotting of mCherry of constructs in Figure 4B? Are the full length fusion protein, in stead of mCherry alone, actually produced? After all, M1, M2, and M3 are truncations of major secondary structure elements. It wouldn't be surprised that the remaining portion of ORF52 can't be well folded and somehow protelyzed or not translated at all in vivo. Nevertheless, these variants can at least be viewed as ORF52-null strains.

4.My major suggestion is actually beyond the scope of this study. In future studies, the authors shall try to replace the IDR of ORF52 with a variety of IDRs from other proteins or design IDRs based on knowledge acquired in this study and see what class(s) of IDRs can rescue defects due to the deletion of ORF52's IDR. Knowledge acquired in these efforts will solidify the causality between LLPS of ORF52 and its function.

Minor critiques
1.On lines 272-273, it says "IDRs are typically enriched with positively charged amino acids, such as lysine (K) and arginine (R) (Shin and Brangwynne, 2017)." This statement is not true and some IDRs are enriched with positively charged amino acids, but many others aren't. Please re-phrase to reflect this fact.
2.Figure 3E, 3H, the label of y-axis is in itallic. No need to do so.
3.Figure 4C, 4D, 4E, the labels of x-axis is in tilted itallic. There is no reason for this. Just do normal orientation and font.
4.Figure 4C, is "FL" supposed to be "WT"?
5.For consistency, Figure 4D needs ticks on X-axis as in Figure 4C, 4E.

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

The concept of liquid-liquid phase separation (LLPS) has emerged as an intriguing mechanism that it contributes to the spatial and functional segregation of molecular processes within the cell. Zhou et al. investigated the virion assembly of a γ-herpesvirus in the cytoplasm and found that the virus formed the cytoplasmic virion assembly compartments (cVACs) as membrane-less organelles with liquid properties. ORF52, an abundant tegument protein mediated the formation of cVACs, inducing LLPS. The authors showed that addition of nucleic acids, either DNA or RNA, promoted ORF52-induced LLPS and further mapped the critical domains/residues of ORF52 important for LLPS. Although the roles of LLPS were reported in virus replication factories, mostly for viral gene expression and genome replication, it has not been clearly demonstrated as a mechanism for the virion assembly and egress. Thus, this manuscript provides an interesting insight regarding a viral strategy usurping a cellular process to efficiently perform the virion assembly. Although most experiments were logically executed and the manuscript is clearly written, the authors should address the following points to consolidate the conclusions.

Main points:
1. Based on results from live-cell imaging and CLEM and FRAP experiments, the authors concluded that cVACs of MHV-68 had liquid properties. In addition, the formation of cytoplasmic puncta was used as an indication for LLPS throughout the study. However, whether these puncta were sensitive to LLPS disrupting agents has not been examined. The authors should further validate whether these cVAC puncta were formed via LLPS by treating the infected cells with LLPS disrupting agents such as 1,6-hexanediol.
2. Phase separation of ORF52 was induced by adding nucleic acids, either DNA or RNA. As the authors discussed, the presence of cytoplasmic naked DNA at this stage was not relevant for the virus assembly. However, although the authors claimed that cytosolic RNAs seemed to be the main nucleic acids for driving ORF52 phase separation in vitro and the formation of cVACs, the role of viral RNAs is not properly examined. Since MHV-68 virion contains diverse vt-RNAs, it will be intriguing to see whether virion-associated RNAs including vt-RNAs can induce LLPS of ORF52.

Minor points:
1. Fig S1D: Multiple step growth curves of recombinant viruses should be analyzed for virus titer rather than viral genome copy number. In addition, graph symbols are hard to distinguish.
2. Among the ORF52 mutants generated, M6 mutation did not show any distinct phenotype and behaved like WT. However, M7 containing both M5 and M6 mutations was a lot more defective in viral growth than M5 alone. What would be the explanation for M7 phenotype? The authors should discuss this point.
3. Fig 4E: Y-axis labels are confusing and should be changed to be more readable.

July 18, 2022

Dear Drs. Tsai and Marat,

Thank you for serving as the Editors for our manuscript *No. 202201088* ("Liquid-liquid Phase Separation Mediates the Formation of Herpesvirus Assembly Compartments"). We appreciate the opportunity to improve our manuscript and thank the Reviewers for their helpful comments and suggestions. We have performed new experiments and revised the text and figures according to the suggestions of all three reviewers. We believe that the revised manuscript has addressed not only the major concerns listed by the Editors (Reviewer #1, Comments 1-4) but all the concerns raised by three Reviewers.

Because Reports may have up to 3 supplemental figures, but our original submission had 4, we re-organized the supplemental data (including new data) into 3 figures. To facilitate the assessment of our revision, we have prepared a table, summarizing the changes to figures in the revised manuscript. A point-by-point response to the Reviewers' comments is also attached at the end of this cover letter. In addition, the revised manuscript (with all changes highlighted) and source data are also uploaded, as required.

Thank you again for your considerations. We look forward to hearing from you soon.

Sincerely yours,

Hongyu Deng, Ph. D.
CAS Key Laboratory of Infection and Immunity
Institute of Biophysics
Chinese Academy of Sciences
Email: hydeng@moon.ibp.ac.cn

Table: a summary of the changes to figures in the revision

Original manuscript	Revised manuscript
	Fig. S1 H (new data)
Fig. S2	Fig. S2 A
Fig. S3 A	Fig. S2 B
Fig. S3 B	Fig. S2 C
Fig. S3 C	Fig. S2 F
Fig. S3 D	Fig. S2 E (replaced with new data)
Fig. S3 E	Deleted
	Fig.S2 D (new data)
Fig. S4 A	Fig. S3 C
Fig. S4 B	Fig. S3 D
Fig. S4 C	Fig. S3 E
Fig. S4 D	Fig. S3 F
Fig. S4 E	Fig. S3 G
	Fig. S3 A (new data)
	Fig. S3 B (new data)

Point-by-point response to Reviewers' comments:

Reviewer #1:
Zhou et al present in vitro and cell-based evidence to support the hypothesis that gammaherpesvirus assembly is mediated, at least in part, by liquid-liquid phase separation and that the viral protein ORF52 is required for this process. This is not the first time that LLPS as a mechanism for herpesvirus assembly has been proposed, but it's the first I am aware of where strong evidence to support this hypothesis has been provided. Overall the story is compelling and very likely to be correct. However, several of the experiments presented are not entirely convincing and the description of the experimental procedures falls below the standard expected for publication in a quality journal like JCB.
Response: We thank the Reviewer for the general comment and for acknowledging that "the story is compelling and very likely to be correct". We address the Reviewer's specific comments as follows.

Specific comments:
1. *A key characteristic of LLPS is their susceptibility to disruption by aliphatic alcohols like 1, 6-hexanediol or propylene glycol. Evidence that the LLPS-like structures formed by ORF52 in transfected or infected cells can be disrupted by 1, 6-hexanediol or propylene glycol treatment, and that they dynamically re-form once the alcohol is removed, would significantly strengthen this study.*

Response: We thank the Reviewer for the insightful suggestion, which is related to

Comment #1 by Reviewer #3. As the Reviewers suggested, we infected cells with MHV-68 and treated cells with 5% 1, 6-hexanediol at 24 hpi. Time-lapse images showed that cVAC structures were disrupted by 1, 6-hexanediol treatment (new Fig S1H, as attached below), indicating that LLPS drives the formation of cVACs in infected cells. Intriguingly, almost all cytoplasmic ORF52 translocated to and stayed in the nucleus after 1, 6-hexanediol treatment. 1, 6-hexanediol has been reported as a potent agent to induce permeability of the nuclear pore (Shulga et al, *Mol Cell Biol*. 2003 Jan;23(2):534-42. doi: 10.1128/MCB.23.2.534-542.2003; Düster et al, *J Biol Chem*. 2021;296:100260. doi: 10.1016/j.jbc.2021.100260). Our previous study also showed that ORF52 has a high affinity with DNA (Xu et al, *Mol Cell*. 2021 Jul 1;81(13):2823-2837.e9. doi: 10.1016/j.molcel.2021.05.002). Therefore, after translocating to the nucleus, ORF52 was retained within the nucleus, most likely due to its strong binding with genomic DNA. As a result, upon removal of 1, 6-hexanediol, re-formation of LLPS structure was not observed, because of the incorrect localization of ORF52. Nonetheless, our new data showed that cVAC formation is sensitive to LLPS disrupting agents such as 1,6-hexanediol. We have added the new data to the revised manuscript (lines 172-180).

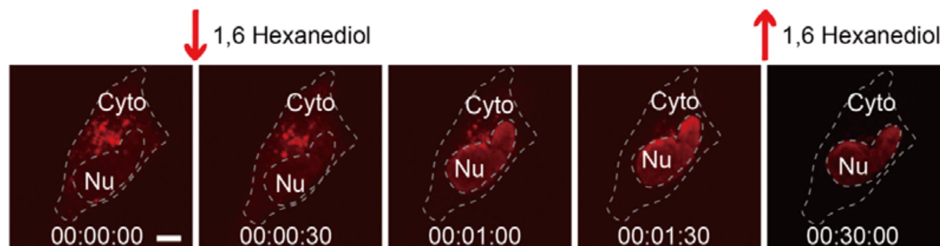

Fig. S1H. Fluorescence images of COS-7 cells infected with mCherry-ORF52 virus at a MOI=3 and treated with 1,6-hexanediol. Bar: 5 µm. These images are representative of at least three independent experiments.

***2.** The authors show that the mutations M1, M2 and M3 prevent the formation of ORF52 LLPS in transfected cells, and that virus production is reduced, but they do not directly show that these mutations prevent cVAC formation. The authors should stain cells transfected with these BACs using antibodies that recognise ORF33, ORF38 or ORF45 for visualisation by fluorescence microscopy, to demonstrate that cVAC formation (as defined by formation of cytoplasmic punctae containing ORF33, ORF38 and ORF45) is impaired. The paper would be further strengthened by doing the same for mutants M4, M5, M6 and M7.*

Response: We thank the Reviewer for the thoughtful comment. We followed the Reviewer's suggestion and transfected 293T cells with these mutant BACs (M1-M7) individually. We then stained the cells using antibodies against tegument proteins ORF33 or ORF45 for immuno-fluorescence assay. Our data showed that puncta was not formed in cells transfected with mutants M1, M2, M3, M4, M5 or M7. However, puncta formation was observed in cells transfected with mutant M6. Therefore, the

LLPS property of ORF52 correlates very well with cVAC formation and virus production. We have added the new data to the revised manuscript (lines 301-303, 305-306; new Fig. S3B, as attached below).

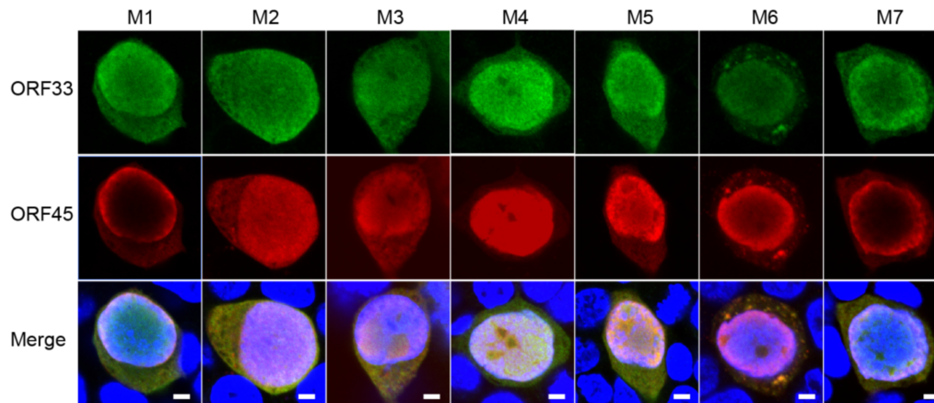

Fig. S3B. 293T cells were transfected with ORF52 mutant BAC, and detected by indirect immunofluorescence at 48 h post transfection. ORF33 was detected using a mouse anti-ORF33 monoclonal antibody, followed by an Alexa Fluor 488-conjugated secondary antibody (green channel). ORF45 was detected using a rabbit anti-ORF45 polyclonal antibody, followed by an Alexa Fluor 647-conjugated secondary antibody (red channel). Nuclei were stained with DAPI (blue channel). Bar: 5 µm.

3. *The authors should make reference to the previous study by Metrick, Koenigsberg and Heldwein (mBio 11, e00810-20; 2020) that shows HAV-1 pUL11 to be nascently disordered protein that undergoes phase separation. In this paper the authors posit that herpesvirus (HSV-1) secondary envelopment is driven by LLPS. It would be instructive for the authors to compare the properties of MHV-68 ORF52 and HSV-1 pUL11.*

Response: We thank the reviewer for raising this question. HSV-1 pUL11 is conserved among all herpesviruses. It's N-terminally myristoylated and palmitoylated, enabling its membrane-association and targeting to the Golgi. In Metrick's study, all the analyses were performed with UL11 expressed and purified from *E. coli*. As the authors themselves pointed out, "All experiments reported here used *E. coli*-expressed unlipidated UL11, whereas in HSV-1-infected cells or in uninfected cells overexpressing UL11, UL11 is both myristoylated and palmitoylated". Although the authors proposed that the recombinant, unlipidated HSV-1 UL11 is a conformationally dynamic, intrinsically disordered protein, there is a concern whether this characteristics of UL11 observed in vitro truthfully reflects its behavior in vivo.

The UL11 homologue in MHV-68 is ORF38. We previously showed that ORF38 localizes to trans-Golgi network (Shen et al, *Protein Cell*. 2014 Feb;5(2):141-50. doi: 10.1007/s13238-013-0005-0). As we demonstrated in this study, ORF38 does not possess LLPS property in mammalian cells (original Fig. S2A). Along the same line, Metrick's study predicted disorders in many other HSV-1 tegument proteins, one of

which is UL16. UL16 is also conserved in all herpesviruses and its homologue in MHV-68 is ORF33. As shown in our original Fig. S2A, ORF33 does not possess LLPS property either in mammalian cells. We would like to respectfully argue that data obtained from in vivo (the mammalian cells, in which HSV-1 or MHV-68 infection takes place) is likely to be more biologically relevant than data from in vitro (E. coli and test tube). Nonetheless, to confirm this and to better address the Reviewer's question, we have now expressed and purified MHV-68 ORF38 from *E. coli*. Intriguingly, ORF38 doesn't harbor LLPS property in vitro, even in the presence of RNA (please see Fig. R1 below).

Furthermore, MHV-68 ORF52 is a cytosolic protein, whereas HSV-1 UL11 (and MHV-68 ORF38) is membrane-associated. Therefore, we would like to respectfully propose that it is difficult to directly compare the properties of these two proteins.

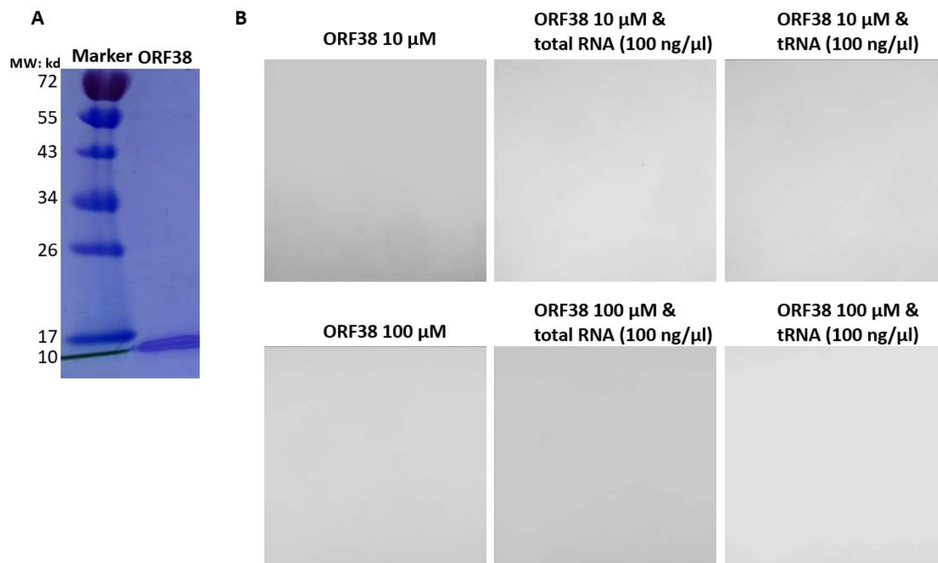

Fig. R1. (A) Purity of bacterially-expressed ORF38, analyzed by Coomassie blue staining. (B) In vitro phase separation assay of ORF38 alone or in the presence of RNA (total RNA isolated from COS-7 cells or yeast tRNA) at different concentrations.

4. *The data in Figures 4C, 4D, 4E, S1D, S1E and S1F are presented with error bars but the authors do not describe what these errors represent (SEM or SD), nor do they state how many times these experiments were performed. Additionally, the statistical tests used for Figures S1E and S1F are not described.*

Response: We thank the Reviewer for careful reading of our manuscript. We have added the information to the revised manuscript (lines 693, 697-698, 836, and 839-842).

Minor comments:

5. *There are many experimental procedures that are not described adequately in the manuscript. Specifically, the authors don't describe how their cells were transfected, the source of their antibodies (either literature citation or manufacturer plus catalogue number), nor how the RNA EU incorporation and click chemistry for labelling was performed.*

Response: We apologize for the negligence. We have added the related information to the revised manuscript (lines 450-451, and 453-464).

6. *The co-localisation data presented in Figures 1C, 1D, 3I, 3J and S4D are not completely convincing. Only a single field is shown, with no quantitation. How representative are these images? How many fields were imaged, across how many biological replicates. The authors should provide additional fields as a supplemental figure to support their conclusions. S4D and S4E are particularly problematic as the extend of co-localisation between KSHV-ORF52 and MHV-68 ORF33 is very low - panels S4D and S4E don't contribute much to the story and could probably just be removed.*

Response: We thank the Reviewer for raising these concerns. The co-localization data presented in Fig 1C, 1D, 3I, 3J and S4D are representative of at least three independent experiments. For each experiment, many cells were observed and at least 3 fields were imaged.

We previously showed that KSHV-ORF52 can substitute for the function of MHV-68-ORF52 in virus replication (Wang et al, J Virol. 2012 Feb;86(3):1348-57. doi: 10.1128/JVI.05497-11). Using the same complementation assay, we aimed to show that KSHV-ORF52 also possesses the ability to drive cVAC formation by phase separation (original Fig. S4D and S4E; Fig. S3G and S3H in the revision). We agree with the Reviewer that the co-localization between KSHV-ORF52 and MHV-68 ORF33 is low, as much of ORF33 is localized in the nucleus. This is also the case during WT MHV-68 infection (original Fig. 1C and 1D). However, quantitative analysis did show that more ORF33 was recruited to the ORF52 puncta which represented the formation of cVACs. In contrast, in the presence of KSHV-ORF52-4A mutant, no punctum was formed and ORF33 was distributed relatively evenly in the cytoplasm.

7. *It would be helpful for the authors to state the ratio of absorbance at 260 and 280 nm for their purified ORF52 protein, to confirm that final purified protein did not contain any contaminating nucleic acids from the bacterial expression host.*

Response: We thank the Reviewer for the suggestion. The ratio of absorbance at 260 and 280 is 0.56, indicating that the purified ORF52 is free of contaminating nucleic acids. We have added this information to the revised manuscript (lines 216-218).

8. *How were the gold nanoparticles used for registration of the fluorescence and EM images in Figure 1B? Were the nanoparticles covered with a fluorescent coating? If so, please describe clearly in the methods. If not, how were these ~50-100 nm gold particles visible in the fluorescence images such that they could be used for image alignment?*

Response: We thank the Reviewer for the question. The gold nanoparticles (50-100 nm) were not covered with a fluorescent coating, and we made reference to the original publication describing the method (Fu et al, Nat Methods. 2020 Jan;17(1):55-58. doi: 10.1038/s41592-019-0613-6). As shown as an example below, the gold nanoparticles were directly visible in green channel under 488 nm excitation light (Fig. R2 i-2; white arrowheads indicate gold particles). Under TEM, they appeared as black dots (Fig. R2 i-1; white arrowheads indicate gold particles). Therefore, these gold particles served as fiducial markers to align fluorescent images with EM images in CLEM (Fig. R2 i-3).

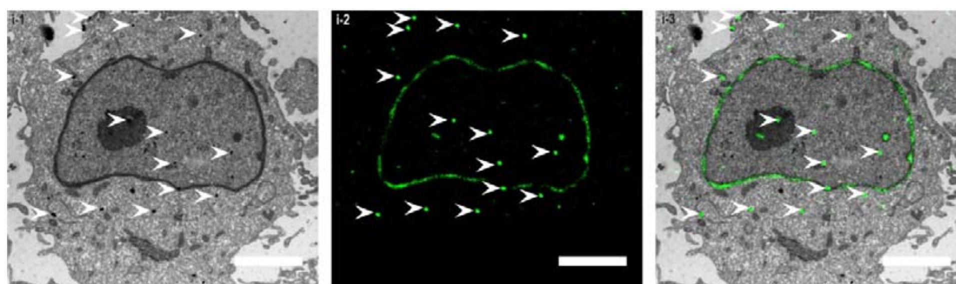

Fig. R2. SR-CLEM image of a 100 nm Epon section of a CHO cell expressing nuclear lamina A targeted by mEosEM. i-1, the EM image; i-2, single-molecule localization microscopy image; i-3, CLEM image. Scale bars, 4 μm. Arrowheads indicate gold nanoparticles.

9. *Figure S1D: The growth kinetics of the mEosEM virus clearly differs from the other two viruses. The authors should comment on this.*

Response: The Reviewer is correct to point out that the growth kinetics of mEosEM recombinant virus is slower than that of the WT virus. We hypothesize that the mEosEM tag (226 aa) associated with ORF52 (135 aa) may affect assembly of other tegument proteins and therefore slow down the production of progeny viruses.

10. *Figure S3E. This figure isn't discussed at all in the "Results" section of the manuscript and doesn't add significantly to the manuscript - it should be removed. If it is to be retained it will need to be discussed adequately in the results and the methods should be expanded to include details of how the RNA FISH experiments were performed.*

Response: We followed the Reviewer's suggestion and removed this panel.

Reviewer #2:

For herpes viruses, the nucleocapsid is first assembled within the nucleus and translocated to the cytoplasm for subsequent maturation steps. To generate a mature virion, dozens of tegument proteins and glycoproteins need to be recruited and assembled in the cytoplasm. This is a challenging task and a powerful orchestration mechanism is required. In this paper, the authors study the function of ORF52 in the virus assembly stage of the life cycle of a murine Herpesvirus. They uncovered that the abundant tegument protein ORF52 compartmentalizes the components necessary for virion maturation via a liquid-liquid phase separation mechanism.

It is known in literature that both α-herpesvirus and β-herpesvirus form cytoplasmic virion assembly compartments (cVACs) for the virus assembly. Built on their own previous research, the authors showed that cVACs are also formed during γ-herpesvirus (MHV-68) infection, and interestingly cVACs of γ-herpesvirus have liquid properties. The most abundant viral protein ORF52 is required for the formation of cVACs. The morphology of cVAC, the lack of membrane encloser, and the dynamic properties of ORF52 suggest that cVACs might be formed via liquid-liquid phase separation (LLPS). However, as the most abundant protein, ORF52 apparently does not possess phase separation capacity by itself. Interestingly, nucleic acids including DNA and RNA robustly undergo phase separation with ORF52. The authors generated a battery of ORF52 truncation mutants and point mutants with varying propensities of LLPS. Using these reagents, the authors demonstrated that the LLPS properties of ORF52 are critical for cVACs formation and importantly virion production.

Overall the experiments are well-designed and executed. The data are in high quality and also well presented. The findings are novel and significant. I'd like to suggest its pulication in Journal of Cell Biology with minor revision. I will list my critiques and suggestions below.

Response: We appreciate the Reviewer's summary of the key contributions of our work. We also thank the Reviewer for finding our experiments "well-designed and executed", data "in high quality and also well presented", and our work "novel and significant".

Major critiques:

1. *LLPS is a concentration-dependent phenomenon. Can the appearance of cVAC be purely correlated with the concentration of ORF52? Can the authors test the time-dependent expression level of ORF52 post infection and see whether there is coincidence of high ORF52 level and the appearance of cVAC? Alternatively, although ORF52/nucleic acid can undergo LLPS in vitro, other components including virus proteome or host proteome might work synergistically with ORF52 to form cVAC in vivo. Any prior evidence to indicate this possibility? It is worth discussing a*

bit.

Response: We thank the Reviewer for the insightful questions. As the Reviewer suggested, we examined the concentrations of ORF52 at different time points post infection by western blotting. ORF52 clearly manifested time-dependent expression, and its expression was not detected until 12 hpi (new Fig. S2D, as attached below), which coincides with the appearance of cVAC (Fig. 1A, in both original submission and revision; time-lapse images were collected from 12 hpi). By running different amounts of purified ORF52 protein on the same blot, we quantified the amount of ORF52 expressed (equivalent to 10^5 infected cells) and estimated the concentration of ORF52 in infected cells at different time points to be 0 μM (0 hpi), 0 μM (6 hpi), 1 μM (12 hpi), 6 μM (24 hpi) and 14 μM (36 hpi). These concentrations of ORF52 are sufficient to induce LLPS in the presence of nucleic acids, as we showed in original Fig. 3H. We have added the new results to the revised manuscript (lines 220-227; new Fig. S2D).

In addition, our work in progress has examined the effect of other viral proteins on ORF52-induced phase separation. Our preliminary data indicated that other viral protein may indeed promote ORF52-induced LLPS in vitro. We appreciate very much the Reviewer's insight and have added a bit of discussion to the revised manuscript (line 404).

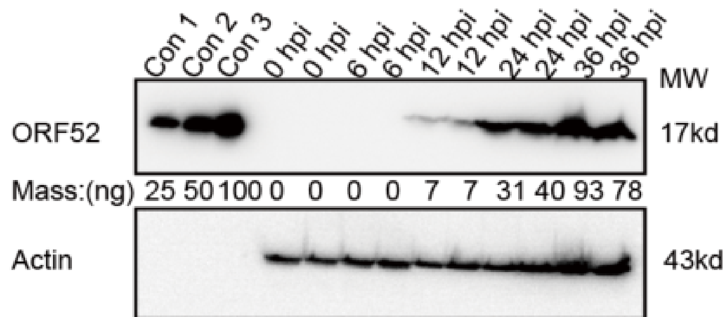

Fig. S2D. Expression levels of ORF52 in 293T cells after MHV-68 infection (MOI=3) at different hours post infection, as examined by western blotting. con1-3: Bacterially expressed and purified ORF52 proteins were loaded at the indicated amount to draw standard curve. Based on western blot result, we quantified the amount of ORF52 protein in infected cells (equivalent to extract from 10^5 cells). The volume of each cell is approximately 4×10^{-12} L. Thus, the cytoplasmic concentration equals mass (ng)$\times10^{-9}/(10^5\times15000\times4\times10^{-12})$ M. The concentration of ORF52 at each time points post infection was estimated to be 0 μM (0 hpi), 0μM (6 hpi), 1 μM (12 hpi), 6 μM (24 hpi) and 14 μM (36 hpi).

2. *Please attach a multiple sequence alignment of representative homogs of ORF52 in a supplemental figure. It helps the readers to evaluate the potential functions and*

appreciate the roles of conserved structural elements and residues from evolution.

Response: We agree with the Reviewer that providing a multiple sequence alignment of ORF52 homologues would help the readers to evaluate the potential functions and appreciate the roles of conserved structural elements and residues from evolution. However, we have previously published such information (J Biol Chem. 2007 Oct 26;282(43):31534-41. doi: 10.1074/jbc.M705637200, Figure 1, as attached below). Attaching a sequence alignment again would be completely redundant with our previous publication. Therefore, we politely request that the readers refer to the previous publication.

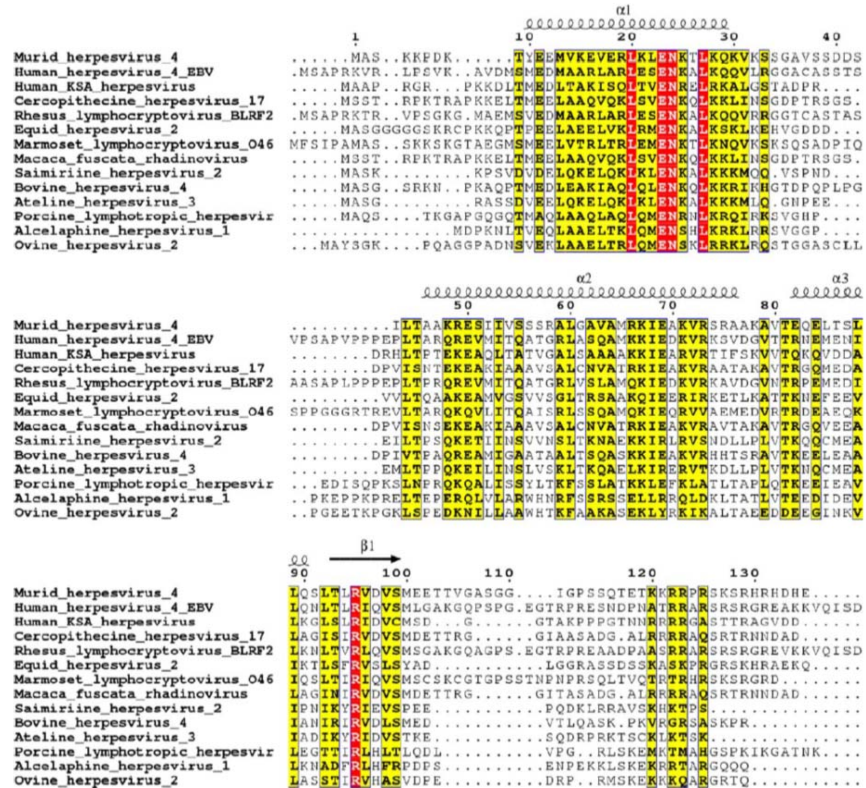

FIGURE 1. **Sequence alignment of herpesvirus ORF52 proteins.** Strictly conserved and conservatively substituted residues are colored with *red* and *yellow backgrounds*, respectively. The secondary structure elements of ORF52 are shown at the *top*, with coils representing α-helices and the *arrow* representing the β-strand. Produced with ESPript (42).

Fig. R3. Sequence alignment of herpesvirus ORF52 proteins.

***3.** Is there a Western blotting of mCherry of constructs in Figure 4B? Are the full length fusion protein, in stead of mCherry alone, actually produced? After all, M1, M2, and M3 are truncations of major secondary structure elements. It wouldn't be surprised that the remaining portion of ORF52 can't be well folded and somehow protelyzed or not translated at all in vivo. Nevertheless, these variants can at least be viewed as ORF52-null strains.*

Response: We thank the Reviewer for pointing out this issue. We examined the

expression of mCherry-ORF52 fusion proteins in 293T cells. Our result showed that although the expression level of M1 to M7 varied to some extent, all the fusion proteins were expressed (new Fig. S3A, as attached below; line 290). One thing we would like to point out is that a lower band was detected in all lanes (see Source data S3) due to the mCherry antibody we used. For reference, please find below a figure on the mCherry antibody from the manufacturer (Fig. R4).

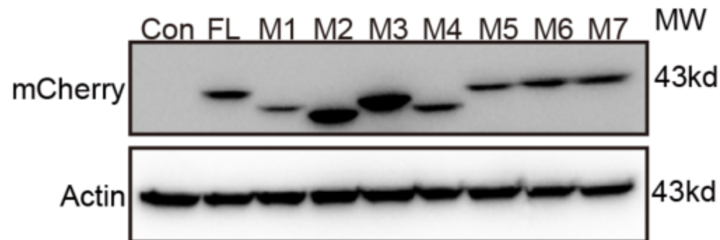

Fig. S3A. Empty vector or plasmids expressing mCherry-ORF52 or plasmids expressing mCherry-tagged mutants were individually transfected into 293T cells. Cells were collected for western blot at 48 hour post transfection.

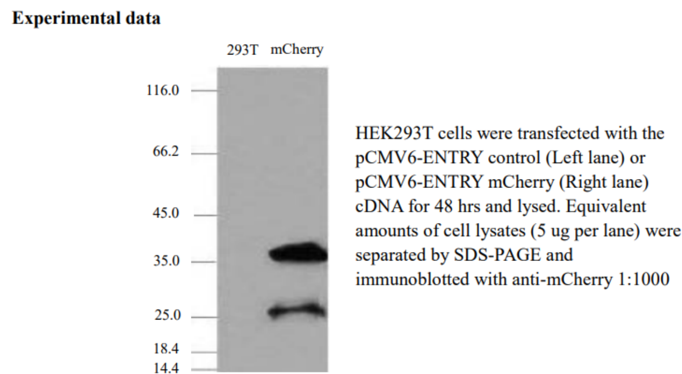

Fig. R4. Experimental data on the mCherry antibody from the manufacturer, showing an extra lower band detected on western blot.

4. *My major suggestion is actually beyond the scope of this study. In future studies, the authors shall try to replace the IDR of ORF52 with a variety of IDRs from other proteins or design IDRs based on knowledge acquired in this study and see what class(s) of IDRs can rescue defects due to the deletion of ORF52's IDR. Knowledge acquired in these efforts will solidify the causality between LLPS of ORF52 and its function.*

Response: We appreciate the Reviewer's insightful suggestion. Work is currently in progress to replace the IDR of ORF52 with IDRs from several cellular proteins, e.g. FUS and DDX4, and to examine whether they can rescue can rescue defects due to

the deletion of ORF52's IDR.

Minor critiques:

5. *On lines 272-273, it says "IDRs are typically enriched with positively charged amino acids, such as lysine (K) and arginine (R) (Shin and Brangwynne, 2017)." This statement is not true and some IDRs are enriched with positively charged amino acids, but many others aren't. Please re-phrase to reflect this fact.*

Response: Thank you. We have changed this sentence in the revised manuscript (line 285-286).

6. *Figure 3E, 3H, the label of y-axis is in itallic. No need to do so.*

Response: Thank you. We have made corrections.

7. *Figure 4C, 4D, 4E, the labels of x-axis is in tilted itallic. There is no reason for this. Just do normal orientation and font.*

Response: Thank you. We have made corrections.

8. *Figure 4C, is "FL" supposed to be "WT"?*

Response: We appreciate the Reviewer's careful reading of our manuscript. We used "FL" to indicate full-length protein, and "WT" to describe wild-type virus.

9. *For consistency, Figure 4D needs ticks on X-axis as in Figure 4C, 4E.*
Response: Thank you. We have corrected this error.

Reviewer #3:

The concept of liquid-liquid phase separation (LLPS) has emerged as an intriguing mechanism that it contributes to the spatial and functional segregation of molecular processes within the cell. Zhou et al. investigated the virion assembly of a γ-herpesvirus in the cytoplasm and found that the virus formed the cytoplasmic virion assembly compartments (cVACs) as membrane-less organelles with liquid properties. ORF52, an abundant tegument protein mediated the formation of cVACs, inducing LLPS. The authors showed that addition of nucleic acids, either DNA or RNA, promoted ORF52-induced LLPS and further mapped the critical domains/residues of ORF52 important for LLPS. Although the roles of LLPS were reported in virus replication factories, mostly for viral gene expression and genome replication, it has not been clearly demonstrated as a mechanism for the virion assembly and egress. Thus, this manuscript provides an interesting insight regarding a viral strategy usurping a cellular process to efficiently perform the virion assembly. Although most experiments were logically executed and the manuscript is clearly written, the authors

should address the following points to consolidate the conclusions.
Response: We appreciate the Reviewer's summary of the key contributions of our work. We also thank the Reviewer for finding our manuscript providing "an interesting insight" and "most experiments were logically executed and the manuscript is clearly written".

Main points:
1. *Based on results from live-cell imaging and CLEM and FRAP experiments, the authors concluded that cVACs of MHV-68 had liquid properties. In addition, the formation of cytoplasmic puncta was used as an indication for LLPS throughout the study. However, whether these puncta were sensitive to LLPS disrupting agents has not been examined. The authors should further validate whether these cVAC puncta were formed via LLPS by treating the infected cells with LLPS disrupting agents such as 1,6-hexanediol.*
Response: We thank the Reviewer for the insight suggestion, which is related to Comment #1 by Reviewer #1. We followed the Reviewer's suggestion and treated infected COS-7 cells with 5% 1, 6-hexanediol at 24 hpi. Time-lapse images showed that the puncta were disrupted by 1, 6-hexanediol, further confirming that the cVAC puncta were formed via LLPS (new Fig. S1H; lines 172-180).

2. *Phase separation of ORF52 was induced by adding nucleic acids, either DNA or RNA. As the authors discussed, the presence of cytoplasmic naked DNA at this stage was not relevant for the virus assembly. However, although the authors claimed that cytosolic RNAs seemed to be the main nucleic acids for driving ORF52 phase separation in vitro and the formation of cVACs, the role of viral RNAs is not properly examined. Since MHV-68 virion contains diverse vt-RNAs, it will be intriguing to see whether virion-associated RNAs including vt-RNAs can induce LLPS of ORF52.*
Response: We appreciate the Reviewer's critical comment. To test whether virion-associated RNAs especially vt-RNAs could induce ORF52 phase separation, we synthesized four vt-RNA with high abundance in MHV-68 virions (vtRNA1-4). Our in vitro phase separation assay showed that each vt-RNA efficiently induced LLPS of ORF52. We have included this new data in the revised manuscript (lines 252-253; new Fig. S2E, as attached below).

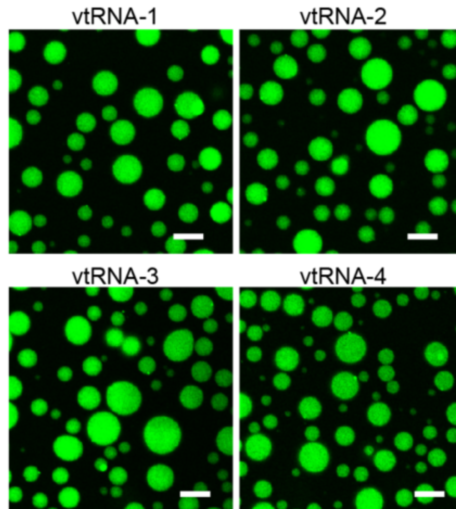

Fig. S2E. Phase separation assay of ORF52 with v-tRNA was performed in physiological buffer. 10 μM ORF52 protein (3% Alexa 488-labeled) was mixed with 100 ng/μl v-tRNA in 96-well plates coated with 20 mg/ml BSA. Mixtures were incubated and images were captured by confocal microscopy. Bar: 10 μm.

Minor points:

3. *Fig S1D: Multiple step growth curves of recombinant viruses should be analyzed for virus titer rather than viral genome copy number. In addition, graph symbols are hard to distinguish.*

Response: The Reviewer raised a legitimate concern that viral genome copy numbers may not correlate well with viral titers. However, based on our extensive experience with MHV-68 recombinant viruses, viral genome copy numbers usually have good correlations with virus titers. We have modified the graph symbols for better distinguishment.

4. *Among the ORF52 mutants generated, M6 mutation did not show any distinct phenotype and behaved like WT. However, M7 containing both M5 and M6 mutations was a lot more defective in viral growth than M5 alone. What would be the explanation for M7 phenotype? The authors should discuss this point.*

Response: Our hypothesis is that although M6 mutation did not show distinct phenotype in terms of the LLPS capability of ORF52, it may have effect on virus replication via other mechanism (e.g. specific protein-protein interaction), especially when co-mutated with amino acids 120/121 (M5).

5. *Fig 4E: Y-axis labels are confusing and should be changed to be more readable.*

Response: Thank you, we have changed the labels.

August 17, 2022

Re: JCB manuscript #202201088R

Dr. Hongyu Deng
Chinese Academy of Sciences
15# Datun Rd, Chaoyang District, Beijing
Beijing 100101
Chile

Dear Dr. Deng,

Thank you for submitting your revised manuscript entitled "Liquid-liquid Phase Separation Mediates the Formation of Herpesvirus Assembly Compartments". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

We agree that the final concerns of reviewer #3 need to be completely addressed prior to publication, in particular another reagent must be tested as described. Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Billy Tsai, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

Fig S1H: The authors presented the results of 5% 1,6-hexanediol treatment in disrupting LLPS induced by ORF52. However, the authors failed to show the reversibility of LLPS following the removal of 1,6-hexanediol, which the authors think may be due to unexpected nuclear localization of ORF52 with the increased permeability of nuclear pores by 1,6-hexanediol. It is not clear whether the disruption of LLPS is mainly due to ectopic localization of ORF52 in this case. How about lower % of 1,6-hexaneldiol, at the concentration of which may not affect the permeability of the nuclear pores? Unlike 1,6-HD, which can be detrimental to cell viability, propylene glycol (PG) has been used to dissolve liquid compartments in living cells because PG is known to be well tolerated by cultured cells at concentrations below 5%. The authors need to test the sensitivity of PG on cVAC formation.
Fig S1D: Now that labeling for two recombinant viruses became clear, it is obvious that the viral growth of both recombinant viruses was quite attenuated by more than 2 log, when the authors measured the viral genome copy number. Since viral genome copy numbers tend to be overestimated when compared to the actual infectivity, the authors should show the infectivity to characterize the viral growth phenotype in multiple step growth kinetics. In addition, the author should include the discussion regarding the growth phenotype as suggested by the reviewer #1 (point 9).

September 15, 2022

Dear Drs. Marat and Tsai ,

Thank you for giving us the opportunity to further improve our manuscript in a second round of revision. We have performed new experiments and revised the text and figures, according to the suggestions of Reviewer #3. We believe that our new revision has fully addressed the new questions raised by Reviewer #3.

Thank you again for your considerations. We look forward to hearing from you soon.

Sincerely yours,

Hongyu Deng, Ph. D.
CAS Key Laboratory of Infection and Immunity
Institute of Biophysics
Chinese Academy of Sciences
Email: hydeng@moon.ibp.ac.cn

Point-by-point response to Reviewers' comments:

Reviewers #1 & #2:
No further questions.

Reviewer #3:
Q1:
Fig S1H: The authors presented the results of 5% 1,6-hexanediol treatment in disrupting LLPS induced by ORF52. However, the authors failed to show the reversibility of LLPS following the removal of 1,6-hexanediol, which the authors think may be due to unexpected nuclear localization of ORF52 with the increased permeability of nuclear pores by 1,6-hexanediol. It is not clear whether the disruption of LLPS is mainly due to ectopic localization of ORF52 in this case. How about lower % of 1,6-hexaneldiol, at the concentration of which may not affect the permeability of the nuclear pores? Unlike 1,6-HD, which can be detrimental to cell viability, propylene glycol (PG) has been used to dissolve liquid compartments in living cells because PG is known to be well tolerated by cultured cells at concentrations below 5%. The authors need to test the sensitivity of PG on cVAC formation.

Response: We had previously tried lower % of 1,6-hexaneldiol, which still caused nuclear localization of ORF52. As the Reviewer suggested, we infected cells with MHV-68 and treated cells with 6% propylene glycol (PG) at 24 hpi. Time-lapse images showed that cVAC structures were disrupted by 6% PG treatment, but reformed gradually after removing 6% PG from the cell culture medium (new Fig S1H, as attached below), indicating that LLPS drives the formation of cVACs in infected cells. We have replaced the old Fig. S1H with the new data and revised the text accordingly (Page 6, lines 176-180; page 16, line 460; page 27, lines 843-845).

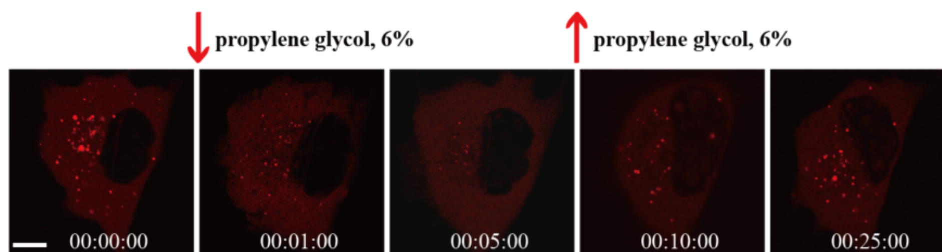

Fig. S1H. Fluorescent images of COS-7 cells infected with mCherry-ORF52 virus at an MOI=3 and treated with 6% PG at 24 hpi. Bar: 5 µm. These images are representative of three independent experiments.

Q2:
Fig S1D: Now that labeling for two recombinant viruses became clear, it is obvious

that the viral growth of both recombinant viruses was quite attenuated by more than 2 log, when the authors measured the viral genome copy number. Since viral genome copy numbers tend to be overestimated when compared to the actual infectivity, the authors should show the infectivity to characterize the viral growth phenotype in multiple step growth kinetics. In addition, the author should include the discussion regarding the growth phenotype as suggested by the reviewer #1 (point 9).

Response: As the Reviewer suggested, we performed plaque assays to examine the multi-step growth curves of the recombinant viruses, so as to show their infectivities and to characterize their growth phenotypes. As shown below, mCherry-ORF52 and mEosEM-ORF52 viruses exhibited almost the same growth curves. Although their titers were lower than the titer of WT virus (about 1 log lower at 96 hpi), the trends of their growth curves were very similar to that of the WT virus. We have replaced the old Fig. S1D with the new data (note that error bars are very small and almost invisible) and revised the text accordingly (Page 5, lines 134-139, line 150; page 27, lines 828-830).

Since the fluorescent protein mEosEM (226 aa) or mCherry (235 aa) is much bigger than ORF52 (135 aa), it is very likely that fusing these tags to ORF52 may affect the assembly of other tegument proteins and therefore slightly slow down the production of progeny viruses. However, as shown by the multi-step growth curves, the trends of their growth curves were very similar to that of the WT virus. We have added the discussion to the revised text (Page 5, lines 132-134).

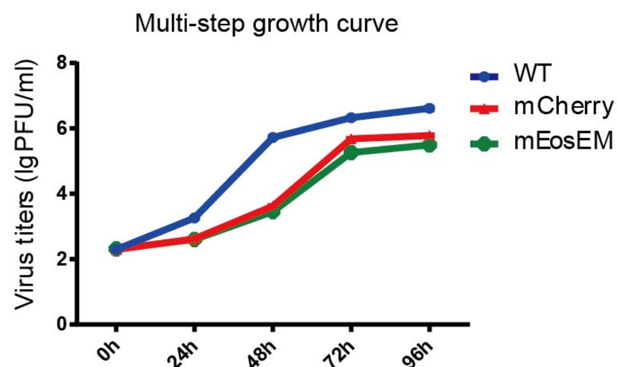

Fig. S1D. Multi-step growth curve of the recombinant viruses. BHK cells were infected with the recombinant or WT virus at an MOI= 0.05 and cultured for 4 days. Viral titers were examined at the indicated time points by plaque assays. Error bars indicate SD from triplicates.

September 21, 2022

RE: JCB Manuscript #202201088RR

Dr. Hongyu Deng
Chinese Academy of Sciences
15# Datun Rd, Chaoyang District, Beijing
Beijing 100101
Chile

Dear Dr. Deng:

Thank you for submitting your revised manuscript entitled "Liquid-liquid Phase Separation Mediates the Formation of Herpesvirus Assembly Compartments". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised.
Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.

1) Text limits: Character count for Reports is < 20,000, not including spaces. Count includes abstract, introduction, * combined results and discussion, and acknowledgments. Count does not include title page, figure legends, materials and methods, references, tables, or supplemental legends.

2) Figures limits: Reports may have up to 5 main text figures.

3) * Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications (you may alternatively indicate the diameter of the inset). Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. In order to accommodate readers with red-green color blindness, we ask that you please change the red/green color scheme used in the graphs in Figure S3.*

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes

f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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