Peer Review File

Manuscript Title: A viral biomolecular condensate coordinates assembly of progeny particles

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Charman et al., presents a very well organized manuscript with highly relevant key results. The authors show that the protein L1 52/55K encoded by adenovirus 5 phase separates. The authors also show that the region responsible for L1 52/55K phase separation lays in the first 119 amino acids of this protein. In addition, the authors demonstrate that virions lacking the 119 initial amino acids in L1 52K/55K protein assemble empty capsids devoid of packaged genomes. Hence, the authors conclude that being unable to phase separate leads to a measurable defect in the production of virions, which is producing capsids devoid of genomes. The novelty of this paper is exactly being able to demonstrate causation, or functional relevance of phase separation for a virus, which is indeed something that is missing in the field. Despite being very important observations, this reviewer advises caution with the interpretation of the data, as causation is not robustly established by these experiments. For example, it would be important to understand what happens with the temperature sensitive mutant L1 52/55K EL334-335GP at the nonpermissive temperature in terms of phase separation. The mutation is outside the zone reported in the present manuscript as important for phase separation - EL334-335GP – and the consequence for viral production is the same, the assembly of capsids devoid of genomes. In addition, the IVa2-null HAdV-C5 mutants was reported to also assemble empty capsids, containing 52/55K in amounts comparable to wild type. This reviewer would advise the authors to further support their observations by investigating how phase separation promotes genome packaging. Phase separation may be critical for concentrating material (all packaging related proteins and vDNA) but in the present manuscript this is not explored. It is known that L1 52/55K binds to packaging sequences indirectly and that together with the viral proteins (IVa2, L4 33K, L4 22K, and IIIa) and the cellular proteins (chicken ovalbumin upstream promoter transcription factor (COUP-TF), Oct-1 and CCAAT displacement protein (CDP)) assists the process of genome packaging.

• Originality and significance: The results are original and of higher significance, as the functional relevance of phase separation in the lifecycle of a virus has not been formally demonstrated for any of the virus reported to utilize this type of compartmentalization in their lifecycles. The consequences would be important for several disciplines including pharmacology, cell biology, immunology, evolution.

• Data & methodology: The approaches, methodology, presentation, quality of the figures are all suitable to address the scientific question asked. It should be extended to other mutants and further characterized in the context of how phase separation interferes with genome packaging.

• Conclusions: The causation that phase separation is important for Ad5 genome packaging stated by the authors is, in my opinion, not established. It would be important to address the following additional points:

1) What happens with the temperature sensitive mutant of L1 52/55K EL334-335GP at the nonpermissive temperature in terms of phase separation of the protein, as this mutant is only a 2 amino acid change rather than a deletion of 190 amino acids. The mutation is outside the zone reported in the present manuscript as important for phase separation - EL334-335GP – and the consequence for viral production is the same, the assembly of capsids devoid of genomes.

2) how does the IVa2-null HAdV-C5 mutant fail to assembles capsids with genomes?

3) By which mechanism does phase separation of L1 52/55K promote genome packaging, is it to concentrate material, perform specific reactions?

• Suggested improvements:
1) Does the mutant virus (EL334-335GP) L1 52/55K phase separate? This mutant produces a similar phenotype to that reported in this work and has the advantage of only changing 2 amino acids. The loss of ability to phase separate and the possibility to revert the phenotype could be explored to further establish causality.

2) If propensity to phase separate is the function of the first 190 amino acids of L1 52/55K granting genome packaging, would it be possible to add this 190 amino acids to another protein in the VLNBs to observe rescue of the phenotype?

3) The IVa2-null HAdV-C5 mutant assembles empty capsids that contain 52/55K in amounts comparable to wild type. How does that fit into the story?

4) Figure 2 – the types of the FRAP recovery curves are very different between the puncta and the peripheral. Would you like to comment?

5) given that it is possible to form in vitro empty viral pseudo particles from minimal constituent components, would it be possible to assemble packaged particles by adding viral DNA?

Minor:

Line 64 – the world diameter has a typo.

Figure 2 – what is the time post-infection of the analysis?

Figure 3 – The comment that “the condensates were prone to bunching, particularly at high concentrations, and formed amorphous bunched aggregates when incubated for a further 2 h” may relate to the system used to test in vitro, in which the equilibrium is reached and may say nothing

about the biology itself. Also, the sentence “this suggests that the highly dynamic behaviour of VLNBs observed in infected cells may require additional viral or cellular factors” it may require fluxes in an out of the nucleus as well, or a particular environment.

• Clarity and context: The text is extremely clear and well organized.

Referee #2 (Remarks to the Author):

This is a very elegant study where the authors investigate the phase separation properties of adenovirus protein 52K. This protein was previously known to be required for genome packaging, and proposed to act as a tether between capsid components and adenovirus cores during assembly. Using click-chemistry labeling of recently synthesized proteins, and immunolabeling directed towards structural proteins in adenovirus infected cells, the authors detect the presence of small condensations that they term Viral Late Nuclear Bodies (VLNB). VLNBs are not labeled for the adenovirus DNA binding protein, and so they seem to be different from the well characterized replication centers. Formation of VLNBs in both transfected or infected cells depends on the presence of protein 52K, and in particular the N-terminal region of the protein predicted to be intrinsically disordered and rich in Pro, Ala and Gln residues (PAQ region). In vitro, 52K forms condensates whose size is modulated by the presence of viral genomes, resulting in nano-condensates which, interestingly, have a size similar to that of the viral particle (~0.1 μ). In the absence of 52K or its PAQ region, VLNBs are not formed and only genome-less particles are assembled. These observations led the authors to conclude that phase separation and formation of condensates by 52K is required to coordinate capsid assembly and genome packaging in adenovirus.

The observations reported provide new, valuable information to understand assembly of complex viral particles in the cell. The manuscript is well written and the data are robust and clearly presented. After reading it, I am convinced that:

a. Protein 52K induces liquid-liquid phase separation and formation of condensates
b. Viral genomes modulate the size of 52K condensates
c. Protein 52K, and in particular its PAQ region, are required for condensate formation, as well as for adenovirus genome packaging

What is not so clear to me, and would benefit from some clarification, is what is the proposed role of VLNBs (or other 52K-induced condensates) in adenovirus assembly. I am not convinced, either, that one can conclude that the phase separation properties of 52K are directly involved in genome packaging.

1. VLNBs and their role in adenovirus assembly.

1.1. In the latest years, several nuclear bodies related to adenovirus replication and assembly have been described. Apart from the previously known replication centers and the PRZ (already mentioned in the manuscript), (Komatsu et al., 2016) described the Virus-induced Post-Replication

(ViPR) body, while (Pfitzner et al., 2020) reported a Late Virion Accumulation Compartment (LVAC). It would be helpful for the readers to briefly relate the VLNBs with these other compartments, if possible.

1.2. Apart from the PRZ, (Condezo and San Martín, 2017) mention "electron-dense, smooth inclusions" that were labeled by antibodies against 52K and fiber, but not core protein VII or viral genomes, and were therefore considered as accumulations of excess or misfolded proteins. Could the newly reported VLNBs correspond to these "smooth inclusions"? And if so, are they active players in assembly, or just waste storage sites?

1.3. Some statistics on the diameter of VLNBs would be useful, for comparison with condensates formed by 52K in vitro and with the previously reported bodies mentioned above.

1.4. VLNBs formed by 52K are distinct from replication centers, where new adenovirus genomes are synthesized. Would the authors propose that the viral genomes travel from the RC to the VLNB?

1.5. In fig. 1g, label for 52K is observed around the replication center (labeled with DBP). The authors indicate that these are "smaller foci". As above, some indication of the size of these foci would be useful for interpretation. Could these smaller foci correspond to the modulation in condensate size induced by the viral genomes in vitro? Taking into account that smaller foci appear in the PRZ, previously proposed to be the assembly site, is it possible that they are showing the actual location where 52K interacts with the viral genomes, as previously proposed (Condezo and San Martín, 2017)?

2. Causal relation between 52k-induced phase separation and genome packaging

In the absence of 52K, condensates are not formed, and in the absence of the PAQ region, some condensates are formed but their size is not modulated by the viral genome. In both cases, only empty particles are assembled. These two observations are correlated, but is there a causal relation? Does this mean that phase separation is used to promote successful genome packaging, or are there other activities of the protein critical for packaging? The need for the N-terminal region of 52K for packaging had previously been described, and other functions had been assigned, for example, interaction with other packaging factors (IVa2) (Perez-Romero et al., 2006). A Cys residue at the N-terminus of 52K has also been shown to promote homooligomerization of the protein (Hasson et al., 1992). If VLNBs and the "smooth inclusions" lacking viral genomes described in (Condezo and San Martín, 2017) are the same, as suggested above, it might well be that it is not phase separation and VLNB formation per se, but rather the modulation of phase separation carried out by the viral genomes, what is essential for packaging. In this case, unregulated phase separation might be deleterious for packaging.

3. Minor issues:

3.1. GFP-tagged 52K and N-terminal deletion constructs: the tag is presumably at the C-term, but this should be explicitly indicated

3.2. The legend of fig 1f states: "Co-localization of 52K, hexon trimers (hex), fiber trimers (fib), and cement protein IIIa". However, the bottom panels show colocalization of hexon with IIIa, not 52K with IIIa.

3.3. Discussion, 4th line from the end of 1st paragraph: "The accumulation of empty, non-infectious particles in the absence of VLNBs suggests that phase separation of structural proteins is not

required for assembly of the particle's protein shell. This is expected given that the in vitro formation of empty viral pseudo-particles from minimal constituent components in solution is well known." This last sentence is misleading; in vitro formation of VLPs is well known FOR SOME VIRUSES, but not for adenovirus, where no in vitro assembly system has been developed so far, to the best of my knowledge.
3.4. Discussion, the first two sentences of the second paragraph ("Viral membraneless… specific processes") are confusing. Could they be more clearly written, perhaps by specifying what "processes" are repeatedly referred to?
3.5. The authors may consider mentioning a recent publication proposing that phase separation mediated by DBP may also been involved in adenovirus RC formation (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8473285/).

Condezo, G.N. and San Martín, C. (2017) Localization of adenovirus morphogenesis players, together with visualization of assembly intermediates and failed products, favor a model where assembly and packaging occur concurrently at the periphery of the replication center. PLoS Pathog, 13, e1006320.
Hasson, T.B., Ornelles, D.A. and Shenk, T. (1992) Adenovirus L1 52- and 55-kilodalton proteins are present within assembling virions and colocalize with nuclear structures distinct from replication centers. J Virol, 66, 6133-6142.
Komatsu, T., Robinson, D.R., Hisaoka, M., Ueshima, S., Okuwaki, M., Nagata, K. and Wodrich, H. (2016) Tracking adenovirus genomes identifies morphologically distinct late DNA replication compartments. Traffic.
Perez-Romero, P., Gustin, K.E. and Imperiale, M.J. (2006) Dependence of the encapsidation function of the adenovirus L1 52/55-kilodalton protein on its ability to bind the packaging sequence. J Virol, 80, 1965-1971.
Pfitzner, S., Hofmann-Sieber, H., Bosse, J.B., Franken, L.E., Grunewald, K. and Dobner, T. (2020) Fluorescent protein tagging of adenoviral proteins pV and pIX reveals 'late virion accumulation compartment'. PLoS Pathog, 16, e1008588.

Referee #3 (Remarks to the Author):

In this work, the authors report findings from their studies of infecting cells with the adenovirus and querying a potential role for biomolecular condensates in the process of generating progeny particles. The key finding, as stated by the authors, is the observation that bodies, referred to as viral late nuclear bodies (VLNBs) form, driven by phase separation of the 52K protein. These bodies are proposed to be distinct from viral replication compartments. Further, the authors conclude that the N-terminal intrinsically disordered region of the 52K protein is "necessary and sufficient" for forming VLNBs. They zero in on a 47-residue stretch within the N-terminal IDR and conclude that the presence of this stretch is central to the formation of progeny particles with packaged genomes. Indeed, this result is striking. The question is if a persuasive case has been made for phase separation, for VLNBs in the context of infected cells being "liquids", and if a causal linkage between

phase separation, specifically the role of the so-called PAQ region and the generation of progeny particles has been made. Based on close reading of the current narrative, the case made is circumstantial. This, in and of itself, makes for very interesting reading. However, as it stands, the narrative is not persuasive. This can be remedied in one of two ways: Either by providing the definitive causal linkage through a series of key experiments or by altering the current narrative to be considerably more nuanced and significantly less decisive. A sampling of the key issues and points that came up during the reading is provided below. The hope is that this inventory will be of use in rethinking some of the assertions, reconsidering some of the analysis, and revisiting some of the experiments.

Overall assessment

A key issue pertains to the strong assertions regarding the connections between the physics of separation and the biology of AdV infections. Where these connections are articulated, the impression that gets conveyed is of a direct connection between equilibrium phase behavior of the 52K protein and biological functions. However, at every juncture, the description connects non-equilibrium aspects namely, dynamics of a process that may or may not be phase separation, to the functions being characterized. This, unfortunately, is going to end up inadvertently misleading those who are uninitiated while animating those in the know into potentially futile debates about whether a definitive case has been made for or against the functional import of condensates formed by phase separation.

Specific Issues:

1. As noted above, a key problem is the lack of distinction between phase separation as a manifestation of equilibrium thermodynamics vs. a description (for the most part) of the dynamics of forming so-called VLNBs. The dynamics will be different for different quench depths (expression levels or protein synthesis rates). Indeed, the confusion created by the totality of the findings is readily summarized in this sentence: “We conclude that VLNBs, which contain viral structural proteins and the 52K packaging protein, are formed as rounded droplet-like bodies that undergo dynamic changes in size and morphology coincident with progeny production and reorganization of the nucleus.” This results in a lack of definitive evidence in favor of phase separation, let alone LLPS, as the process underlying VLNB formation.

2. The content of Figure 1d is difficult to parse. And the figure caption is not helpful. What do the various numbers along the abscissa imply?

2. What is the basis for asserting that viral replication compartments are distinct from viral late nuclear bodies? This is unclear from Figure 1e. Of import and interest is the possibility that the lack of co-localization between “VRCs” and “VLNBs” is a manifestation of dynamical arrest that is being couched in parlance (albeit implicitly) as an equilibrium phenomenon? If these are indeed distinct bodies with non-overlapping locales and distinct components, the expectation would be that these bodies can form independently of one another.

3. In Figure 1f, there are four components listed in the caption, but only two colors shown in the figure.

4. What is the physical basis for grouping phenotypes into distinct classes – as shown in Figure 1h? Were these phenotypes chosen using an automated approach? If yes, please share details and please explain why there are precisely five phenotypes. If the classification of phenotypes was ad hoc, then how reliable and /or robust is the classification? Further, for a given expression level, are there truly five distinct phenotypes if all cells are imaged at the same, later time point > 28 min?

5. Figure 2 shows data for VLNBs formed in doxocycline inducible lung cell lines. These cells are distinct from the HBEC cells used for Figure 1. While one observes rapid recovery of fluorescence after photobleaching in Figure 2, it is not clear if a similar set of observations will result for the HBEC cell lines. This is relevant because the VLNBs are multicomponent bodies. Will all components of these bodies show similar FRAP traces? In all likelihood, the answer is no. If so, is it appropriate to characterize VLNBs as liquids and the process by which they form as LLPS? Again, the answer is no. Phase separation, yes, but in all likelihood these are, equilibrium, viscoelastic bodies, and in the context of the experiments the functionalities are being investigated of bodies that are out of equilibrium.

6. Figure 3 makes the case for the N-terminal IDR being necessary and sufficient. Has a persuasive case been made for this statement? Careful scrutiny suggests that this is not the case. There is no doubt that removal of the IDR shifts the apparent saturation concentration upward - by almost two orders of magnitude Figure 3h. This is very interesting, but this does not, on its own establish sufficiency. An easy remedy is to characterize the phase behavior of the IDR on its own.

7. The more serious issue pertains to the general pronouncements that lead to a focus on the 47-residue PAQ region. What role is this region playing? Again, Figure 3h suggests that deletion of the PAQ region does not fundamentally compromise the driving forces for phase separation. Instead, the key role of the PAQ region appears to be influencing the dynamics of condensates formed by 52K. And these dynamics seem to be very important for functions as well. The IDR itself is very interesting. The 47-aa residue stretch has no aromatic groups. Pro, Ala, Gln make up 49% of the sequence, whereas Arg, and Gly / Ser / Thr make up ca. 30%. Interestingly, the 60% of the sequence that is C-terminal to the PAQ region, is where this IDR encodes most of the interactions that drive phase separation. There are three large acidic blocks, interspersed by positively charged blocks (Arg-rich with very few Lys residues). The N-terminal PAQ region also has sizable Arg-containing blocks. The mechanism of phase separation, at least in vitro, likely involves complex coacervation adapted to polyampholytic sequences. Such systems readily form arrested phases because the electrostatic interactions between blocks that are oppositely charged residues can be amplified significantly, especially if those include Arg as the cationic residues. It is quite likely that the PAQ region, the Pro residues in particular, acts as a spacer that provides competition to the electrostatic interactions of the 48-119 region of the IDR while also enabling some degree of dynamical exchange (prosaically known as a fluidizing entity). Replacing some or all the Arg residues with Lys should dramatically alter the picture. Likewise, replacing Gln with Gly should enable a more potent spacer. All this is important, because the IDR sequence seems to enable slow dynamics of maturation whereby these dynamics are synchronized with the production of progeny particles. Presumably, an equilibrium structure obtained via classical phase separation may actually be disruptive for producing progeny particles. What the deletion of the PAQ region points to is the possibility that there needs to dynamical resonance. A process of phase separation that is too fast (with the wrong kind of IDR) or

too slow (the ΔPAQ) will either compromise progeny particle production or lead to genome-less particles as observed by the authors. The key seems to be in realizing a particular region along the dynamical (non-equilibrium) phase diagram as opposed to the equilibrium phase diagram. Such a result would be very exciting and seems to be a lot more in line with the data presented here, as opposed to the narrative presented to go with the data.

In conclusion, this is an important story with a potentially exciting storyline. However, in its current form, there appears to be a forced connection between the data and the equilibrium physics of phase separation. This can be remedied in one of two ways: Allow for an alternate interpretation and carry out a different set of experiments that can get help resolve the two plausible interpretations. Or, significantly downplay - almost eliminate - the connection to phase separation, except in the discussion, and just report the data without insisting on the connection to phase separation.

Author Rebuttals to Initial Comments:

Response to Referees' comments

Referee #1 (Remarks to the Author):

Charman et al., presents a very well organized manuscript with highly relevant key results. The authors show that the protein L1 52/55K encoded by adenovirus 5 phase separates. The authors also show that the region responsible for L1 52/55K phase separation lays in the first 119 amino acids of this protein. In addition, the authors demonstrate that virions lacking the 119 initial amino acids in L1 52K/55K protein assemble empty capsids devoid of packaged genomes. Hence, the authors conclude that being unable to phase separate leads to a measurable defect in the production of virions, which is producing capsids devoid of genomes. The novelty of this paper is exactly being able to demonstrate causation, or functional relevance of phase separation for a virus, which is indeed something that is missing in the field. Despite being very important observations, this reviewer advises caution with the interpretation of the data, as causation is not robustly established by these
experiments. For example, it would be important to understand what happens with the temperature sensitive mutant L1 52/55K EL334-335GP at the nonpermissive temperature in terms of phase separation. The mutation is outside the zone reported in the present manuscript as important for phase separation - EL334-335GP – and the consequence for viral production is the same, the assembly of capsids devoid of genomes. In addition, the IVa2-null HAdV-C5 mutants was reported to also assemble empty capsids, containing 52/55K in amounts comparable to wild type. This reviewer would advise the authors to further support their observations by investigating how phase separation promotes genome packaging. Phase separation may be critical for concentrating material (all packaging related proteins and vDNA) but in the present manuscript this is not explored. It is known that L1 52/55K binds to packaging sequences indirectly and that together with the viral proteins (IVa2, L4 33K, L4 22K, and IIIa)
and the cellular proteins (chicken ovalbumin upstream promoter transcription factor (COUP-TF), Oct-1 and CCAAT displacement protein (CDP)) assists the process of genome packaging.

• Originality and significance: The results are original and of higher significance, as the functional relevance of phase separation in the lifecycle of a virus has not been formally demonstrated for any of the virus reported to utilize this type of compartmentalization in their lifecycles. The consequences would be important for several disciplines including pharmacology, cell biology, immunology, evolution.

• Data & methodology: The approaches, methodology, presentation, quality of the figures are all suitable to address the scientific question asked. It should be extended to other mutants and further characterized in the context of how phase separation interferes with genome packaging.

• Conclusions: The causation that phase separation is important for Ad5 genome packaging stated by the authors is, in my opinion, not established. It would be important to address the following additional points:

1) What happens with the temperature sensitive mutant of L1 52/55K EL334-335GP at the nonpermissive temperature in terms of phase separation of the protein, as this mutant is only a 2 amino acid change rather than a deletion of 190 amino acids. The mutation is outside the zone reported in the present manuscript as important for phase separation - EL334-335GP – and the consequence for viral production is the same, the assembly of capsids devoid of genomes.

2) how does the IVa2-null HAdV-C5 mutant fail to assembles capsids with genomes?

3) By which mechanism does phase separation of L1 52/55K promote genome packaging, is it to concentrate material, perform specific reactions?

• Suggested improvements:

1) Does the mutant virus (EL334-335GP) L1 52/55K phase separate? This mutant produces a similar phenotype to that reported in this work and has the advantage of only changing 2 amino acids. The loss of ability to phase separate and the possibility to revert the phenotype could be explored to further establish causality.

This is a fantastic suggestion. We now show that temperature sensitive 52K forms biomolecular condensates at both the permissive (32.0°C) and non-permissive temperatures (39.5°C) during infection with the temperature sensitive mutant virus ts369 (**Figure 4j; Extended Data 8f**). However, the viral minor capsid protein IIIa (a key determinant of successful packaging) does not localize to condensates at the non-permissive temperature (**Figure 4j & k**). Interestingly, both 52K and IIIa are incorporated into incomplete particles at the non-permissive temperature, indicating that these proteins remain viable for assembly at both permissive and non-permissive temperatures (**Extended Data 8a**). This demonstrates that the assembly of complete and successfully packaged particles specifically, occurs only when IIIa is recruited into viral condensates. These revisions are covered in the main text (**lines 232-260**).

2) If propensity to phase separate is the function of the first 190 amino acids of L1 52/55K granting genome packaging, would it be possible to add this 190 amino acids to another protein in the VLNBs to observe rescue of the phenotype?

The short answer is that we do not expect this to be possible, since although critical, phase-separation of 52K is likely not its sole contribution to viral assembly. The 52K protein interacts directly or indirectly with several other different viral proteins involved in assembly/packaging, as well as the viral genome. In addition, 52K is incorporated into immature particles, suggesting that 52K continues to contribute to assembly downstream of the organization of 52K and capsid into biomolecular condensates. Presumably, even if inducing phase-separation of a capsid protein recapitulated the organization of capsid proteins into condensates, it would not recapitulate the full functionality of 52K.

We also note that we fused the IDR to GFP in an attempt to establish whether the IDR is alone sufficient to induce phase-separation of a protein. However, for reasons unknown this IDR-GFP fusion failed to express in either mammalian cells or bacteria (data not shown). This suggests that interrogating the above hypothesis by generating IDR-fusions may not be trivial.

In the revised manuscript we describe the proposed role of phase-separation in the context of adenovirus assembly in order to clearly communicate the contributions of 52K to viral assembly.

3) The IVa2-null HAdV-C5 mutant assembles empty capsids that contain 52/55K in amounts comparable to wild type. How does that fit into the story?

This is a very interesting question that we cannot answer fully at this junction. Although IVa2 is essential for packaging, the exact mechanistic function of IVa2 remains unknown.

We propose that phase-separation of 52K organizes capsid proteins such that capsid assembly is coordinated with the provision of viral genomes and genome-associated core proteins and packaging factors such that complete packaged particles are assembled (**Extended Data 10**). This model suggests that all proteins essential for capsid assembly or genome packaging likely integrate into the assembly process either via organization at biomolecular condensates or via association with viral genomes. Given the reported interaction of IVa2 with viral genomes (specifically the packaging sequence), perhaps the latter seems the more likely hypothesis. Unfortunately, our attempts to establish the localization of IVa2 were inconclusive, since immunostaining using either one of two different antibodies raised against IVa2 resulted in very high background signal that was not specific to IVa2 (data not shown).

4) Figure 2 – the types of the FRAP recovery curves are very different between the puncta and the peripheral. Would you like to comment?

Previously, we had not reliably established exactly how the later localization of 52K to the nuclear periphery relates to the earlier biomolecular condensates. For this reason, we had characterized both. Thanks to the reviewers' suggestions, experiments with the temperature sensitive mutant have helped clarify this relationship, indicating that 52K localizes to the nuclear periphery following particle assembly and removal of 52K from particles during maturation (**Extended Data 8a-c**). As such, punctate biomolecular condensates and peripheral accumulates represent the localization of 52K at different stage of the assembly process, and therefore likely differ in their composition and properties. We also note, that FRAP of punctate bodies vs peripheral accumulates interrogate different forms of recovery, namely recovery of whole bodies by exchange with the nucleoplasm, and recovery by internal exchange, which makes it difficult to cross-compare these data sets usefully. For this reason, we have elected to remove data pertaining to FRAP of peripheral accumulates, since these data do not contribute constructively to understanding the functionality of biomolecular condensates and are challenging to interpret.

5) given that it is possible to form in vitro empty viral pseudo particles from minimal constituent components, would it be possible to assemble packaged particles by adding viral DNA?

Although *in vitro* assembly of viral capsids is possible for a number of viruses, to the best of our knowledge, such a system has not been established for AdV. As such, there is no established AdV pseudo particle assembly assay to which DNA could be added in an attempt to generate packaged particles. Our intention was to communicate that generally speaking, capsid assembly from free constituent capsid proteins in solution is thought to occur readily. Thus, it is of little surprise that some (empty) capsids form in the absence of biomolecular condensates. In contrast, assembling packaged particles which amongst other things requires a genome be condensed to fit inside a capsid,

presumably requires a more active or nuanced process. It might be possible to engineer a system in which *in vitro* phase-separation of 52K in conjunction with all of the necessary factors could yield packaged particles. This would be very exciting, but it falls well outside the scope of this current manuscript. To avoid miscommunication, we have changed the language used in our discussion which now reads "Our experiments with Δ52K mutant adenovirus show that incomplete capsids form in the absence of viral BMCs, indicating that capsid assembly which is independent of genome-packaging does not require phase-separation. This is consistent with the self-assembly of free capsid proteins into capsids" (**lines 300-304**).

Minor:

Line 64 – the world diameter has a typo.

We thank the reviewer for pointing this out. This has now been corrected in the revised manuscript.

Figure 2 – what is the time post-infection of the analysis?

Live cell imaging was captured between 22-24 hours post-infection. This information has now been added to the corresponding figure legends (now **Figure 1h-j**) in the revised manuscript.

Figure 3 – The comment that "the condensates were prone to bunching, particularly at high concentrations, and formed amorphous bunched aggregates when incubated for a further 2 h" may relate to the system used to test in vitro, in which the equilibrium is reached and may say nothing about the biology itself. Also, the sentence "this suggests that the highly dynamic behaviour of VLNBs observed in infected cells may require additional viral or cellular factors" it may require fluxes in an out of the nucleus as well, or a particular environment.

We agree fully with the reviewer. Our intention was to dissuade the reader from over-extrapolating the ripening and aggregation observed *in vitro* since this could be very misleading, particularly given the absence of other relevant factors and constituent proteins of viral condensates. This should be less of an issue in the revised manuscript since discussion pertaining to ripening and aggregation of *in vitro* condensates has been removed in order to refocus and accommodate additional data.

Referee #2 (Remarks to the Author):

This is a very elegant study where the authors investigate the phase separation properties of adenovirus protein 52K. This protein was previously known to be required for genome packaging, and proposed to act as a tether between capsid components and adenovirus cores during assembly. Using click-chemistry labeling of recently synthesized proteins, and immunolabeling directed towards structural proteins in adenovirus infected cells, the authors detect the presence of small condensations that they term Viral Late Nuclear Bodies (VLNB). VLNBs are not labeled for the adenovirus DNA binding protein, and so they seem to be different from the well characterized replication centers. Formation of VLNBs in both transfected or infected cells depends on the presence of protein 52K, and in particular the N-terminal region of the protein predicted to be intrinsically disordered and rich in Pro, Ala and Gln residues (PAQ region). In vitro, 52K forms condensates whose size is modulated by the
presence of viral genomes, resulting in nano-condensates which, interestingly, have a size similar to that of the viral particle (~0.1 μ). In the absence of 52K or its PAQ region, VLNBs are not formed and only genome-less particles are assembled. These observations led the authors to conclude that phase separation and formation of condensates by 52K is required to coordinate capsid assembly and genome packaging in adenovirus.

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We agree with the reviewer that placing our finding in context with a number of very interesting discoveries made in recent years regarding the spatial organization of the adenovirus infected nucleus

will aid interpretation of our findings. To this end, we have revised the text to provide more context throughout.

We find that formation of biomolecular condensates precedes peripheral localization of 52K. The 52K protein localizes to nuclear periphery in cells in which the LVAC has formed, indicating that peripheral localization is a very late (post-assembly) phenotype. For context, the LVAC is now indicated in images showing peripheral localization of 52K (**Extended Data 7f**). This relationship is further clarified by additional data regarding the temperature sensitive mutant ts369 which indicate that localization of 52K to the nuclear periphery is maturation-dependent and thus must represent the localization of 52K following its incorporation into particles and subsequent removal during maturation (**Extended Data 8a-c**).

The formation of ViPR bodies is reported to occur co-incident with late-phase changes in viral replication compartment morphology. We observe the same changes in replication compartment morphology co-incident with organization of 52K and capsid proteins at biomolecular condensates (**Extended Data 7e & 7f**). We have also observed small ViPR bodies coincident with biomolecular condensates, and large ViPR bodies coincident with peripheral accumulates of 52K (not shown in this manuscript). This is very interesting, and could give further insight into ViPR body function, and requirements for genome-packaging. However, given that the function of ViPR bodies has not been established experimentally, we do not think it would be helpful or fair to the field to comment on the functional relationship between ViPR bodies and viral condensates at this time.

1.2. Apart from the PRZ, (Condezo and San Martín, 2017) mention "electron-dense, smooth inclusions" that were labeled by antibodies against 52K and fiber, but not core protein VII or viral genomes, and were therefore considered as accumulations of excess or misfolded proteins. Could the newly reported VLNBs correspond to these "smooth inclusions"? And if so, are they active players in assembly, or just waste storage sites?

This is a great question and one that we have thought about a lot. It is possible that "smooth inclusions" and our biomolecular condensates are one and the same. However, we believe that viral condensates are active players in the assembly process rather than waste storage sites, and that the organization of 52K and capsid proteins at biomolecular condensates is a prerequisite for coordinated assembly/packaging. Supporting observations are:

1. Viral biomolecular condensates form even at limiting MOI (0.01 PFU/cell), indicating that they do not form as a result of excessive protein accumulation generated by over-infecting the cells (**Extended Data 1c**).
2. Viral biomolecular condensates are likely not the sites of misfolded proteins/protein quality control. The heat shock chaperone Hsc70 binds to exposed hydrophobic patches on misfolded proteins and localizes to sites of misfolded protein/protein quality control during herpesvirus infection. We now demonstrate that Hsc70 is recruited to the nucleus and forms nuclear foci during adenovirus infection. However, by co-staining for Hsc70 and 52K we show that these putative sites of protein quality control are not the same as biomolecular condensates de-marked by 52K (**Extended Data 1d**).
3. Viral biomolecular condensates are dynamic, transient, and coincide with ongoing progeny production. This indicates that the viral biomolecular condensates do not result from arrested phase-separation/deleterious phase-transition. This has now been confirmed by live cell imaging (**Extended Data 7g; Supplementary Video 2**).

4. We have now demonstrated that at the non-permissive temperature, biomolecular condensates formed by ts369 fail to recruit IIIa, a key determinant of successful packaging (**Figure 4h-k**). This indicates that successful packaging occurs only when IIIa is localized at condensates.
5. We now show that reducing arginine content in the 52K protein IDR (mutant R/K) diminishes its propensity for phase-separation (**Figure 3c-e; Extended Data 3a & 3b**). Expression of this R/K mutant *in trans* fails to complement biomolecular condensate formation and progeny production of a 52K-negative virus (**Figure 4l-n**).

1.3. Some statistics on the diameter of VLNBs would be useful, for comparison with condensates formed by 52K in vitro and with the previously reported bodies mentioned above.

We agree that this information is important and should be included. During WT AdV infection without inhibition of viral genome replication, we observe biomolecular condensates of various sizes; ranging from approximately 1 µm downwards. However, we note that the size of smaller condensates must be interpreted with caution, as many of these smaller condensates are close to or below the limit of resolution. We have now included an analysis of condensate size with or without inhibition of DNA-replication by hydroxyurea (**Figure 4g**).

1.4. VLNBs formed by 52K are distinct from replication centers, where new adenovirus genomes are synthesized. Would the authors propose that the viral genomes travel from the RC to the VLNB?

Although viral biomolecular condensates are distinct from the DBP-demarked core of replication compartments, (aka. Single stranded DNA Accumulation Site or DAS), they are found in close proximity. The localization of smaller condensates immediately surrounding replication compartment cores suggests that there is a relationship between replication compartments and condensate size (**Figure 4c**). By visualizing nascent viral genomes using EdU labelling and click chemistry, we now show viral biomolecular condensates at or overlapping with sites of genome replication (the peripheral replicative zone – or PRZ) surrounding the DAS (**Figure 4d**). It is likely therefore, that very little if any translocation of genomes is necessary. This is consistent with packaging occurring immediately after genome-replication or coincident with genome replication, as previously reported. We also show that inhibiting viral genome replication by addition of hydroxyurea results in fewer smaller condensates, indicating that modulation of condensate size is dependent on the provision of viral genomes (**Figure 4d-g**).

1.5. In fig. 1g, label for 52K is observed around the replication center (labeled with DBP). The authors indicate that these are "smaller foci". As above, some indication of the size of these foci would be useful for interpretation. Could these smaller foci correspond to the modulation in condensate size induced by the viral genomes in vitro? Taking into account that smaller foci appear in the PRZ, previously proposed to be the assembly site, is it possible that they are showing the actual location where 52K interacts with the viral genomes, as previously proposed (Condezo and San Martín, 2017)?

Yes, we believe that the modulation of condensate size observed in cells corresponds to the modulation in condensate size induced by the viral genomes *in vitro*, although, we do not yet know the biophysical basis responsible for condensates of decreased size.

2. Causal relation between 52k-induced phase separation and genome packaging

In the absence of 52K, condensates are not formed, and in the absence of the PAQ region, some condensates are formed but their size is not modulated by the viral genome. In both cases, only empty particles are assembled. These two observations are correlated, but is there a causal relation? Does this mean that phase separation is used to promote successful genome packaging, or are there other activities of the protein critical for packaging? The need for the N-terminal region of 52K for packaging had previously been described, and other functions had been assigned, for example, interaction with other packaging factors (IVa2) (Perez-Romero et al., 2006). A Cys residue at the N-terminus of 52K has also been shown to promote homooligomerization of the protein (Hasson et al., 1992). If VLNBs and the "smooth inclusions" lacking viral genomes described in (Condezo and San Martín, 2017) are the same, as suggested above, it might well be that it is not phase separation and VLNB formation per se, but rather the modulation of phase separation carried out by the viral genomes, what is essential for packaging. In this case, unregulated phase separation might be deleterious for packaging.

These are all great points and are now incorporated into our model (**Extended Data 10**). Our revisions have allowed us to define better the contribution of phase-separation/biomolecular condensates to viral assembly. We have updated and improved our discussion accordingly. To summarize and answer some of the above questions:

- We propose that phase-separation alone is not sufficient to 'do' the packaging. We propose that phase-separation organizes capsid proteins in order to regulate and coordinate viral assembly such that capsid assembly and packaging are linked.
- We propose that it is modulation of condensates by viral genomes which is indicative of packaged particles being assembled.
- We propose that other known packaging factors, including those reported to interact with 52K (e.g., Iva2), likely function at the point of assembly when capsid proteins and genomes meet.
- Coordinated assembly also requires that 52K is incorporated into the particle downstream of its organization at biomolecular condensates and upstream of its processing and release during maturation.

3. Minor issues:

3.1. GFP-tagged 52K and N-terminal deletion constructs: the tag is presumably at the C-term, but this should be explicitly indicated.

We thank the reviewer; this has now been corrected in the revised manuscript which reads "To investigate viral NBs using live imaging, we engineered a lung cell line (A549) to express 52K tagged at its C-terminus with GFP" (**lines 96-97**).

3.2. The legend of fig 1f states: "Co-localization of 52K, hexon trimers (hex), fiber trimers (fib), and cement protein IIIa". However, the bottom panels show colocalization of hexon with IIIa, not 52K with IIIa.

This has been clarified in the revised legend of Figure 1 which now reads “Immunofluorescence labeling of the 52 kDa protein (52K) with hexon trimers (hex), 52K with fiber trimers (fib), or cement protein IIIa (IIIa) with hexon trimers”.

3.3. Discussion, 4th line from the end of 1st paragraph: “The accumulation of empty, non-infectious particles in the absence of VLNBs suggests that phase separation of structural proteins is not required for assembly of the particle’s protein shell. This is expected given that the in vitro formation of empty viral pseudo-particles from minimal constituent components in solution is well known.” This last sentence is misleading; in vitro formation of VLPs is well known FOR SOME VIRUSES, but not for adenovirus, where no in vitro assembly system has been developed so far, to the best of my knowledge.

We fully agree with the reviewer and apologize for any unintended miscommunication on our part. Our intention was to communicate that generally speaking, capsid assembly from free constituent capsid proteins in solution is believed to occur quite readily. Thus, it is of little surprise that some (empty) capsids form when capsid proteins are not organized at biomolecular condensates. In contrast, assembling packaged particles which require a genome be condensed to fit inside a capsid amongst other things, presumably requires a more active or nuanced process. To avoid miscommunication, we have changed the language used in our discussion which now reads “Our experiments with Δ52K mutant adenovirus show that incomplete capsids form in the absence of viral BMCs, indicating that capsid assembly which is independent of genome-packaging does not require phase-separation. This is consistent with the self-assembly of free capsid proteins into capsids” (**lines 300-304**).

3.4. Discussion, the first two sentences of the second paragraph (“Viral membraneless… specific processes”) are confusing. Could they be more clearly written, perhaps by specifying what “processes” are repeatedly referred to?

This should no longer be a problem since our discussion section has been re-written to focus on our revised model.

3.5. The authors may consider mentioning a recent publication proposing that phase separation mediated by DBP may also been involved in adenovirus RC formation (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8473285/).

Yes, we agree this should be included, although regrettably due to space constraints we are unable to discuss the authors specifics findings. We have added the citation as evidence in support of viral sub-compartments formed by phase-separation in general.

Referee #3 (Remarks to the Author):

In this work, the authors report findings from their studies of infecting cells with the adenovirus and querying a potential role for biomolecular condensates in the process of generating progeny particles. The key finding, as stated by the authors, is the observation that bodies, referred to as viral late nuclear bodies (VLNBs) form, driven by phase separation of the 52K protein. These bodies are proposed to be distinct from viral replication compartments. Further, the authors conclude that the N-terminal intrinsically disordered region of the 52K protein is "necessary and sufficient" for forming VLNBs. They zero in on a 47-residue stretch within the N-terminal IDR and conclude that the presence of this stretch is central to the formation of progeny particles with packaged genomes. Indeed, this result is striking. The question is if a persuasive case has been made for phase separation, for VLNBs in the context of infected cells being "liquids", and if a causal linkage between
phase separation, specifically the role of the so-called PAQ region and the generation of progeny particles has been made. Based on close reading of the current narrative, the case made is circumstantial. This, in and of itself, makes for very interesting reading. However, as it stands, the narrative is not persuasive. This can be remedied in one of two ways: Either by providing the definitive causal linkage through a series of key experiments or by altering the current narrative to be considerably more nuanced and significantly less decisive. A sampling of the key issues and points that came up during the reading is provided below. The hope is that this inventory will be of use in rethinking some of the assertions, reconsidering some of the analysis, and revisiting some of the experiments.

Overall assessment
A key issue pertains to the strong assertions regarding the connections between the physics of separation and the biology of AdV infections. Where these connections are articulated, the impression that gets conveyed is of a direct connection between equilibrium phase behavior of the 52K protein and biological functions. However, at every juncture, the description connects non-equilibrium aspects namely, dynamics of a process that may or may not be phase separation, to the functions being characterized. This, unfortunately, is going to end up inadvertently misleading those who are uninitiated while animating those in the know into potentially futile debates about whether a definitive case has been made for or against the functional import of condensates formed by phase separation.

Specific Issues:
1. As noted above, a key problem is the lack of distinction between phase separation as a manifestation of equilibrium thermodynamics vs. a description (for the most part) of the dynamics of forming so-called VLNBs. The dynamics will be different for different quench depths (expression levels or protein synthesis rates). Indeed, the confusion created by the totality of the findings is readily summarized in this sentence: “We conclude that VLNBs, which contain viral structural proteins and the 52K packaging protein, are formed as rounded droplet-like bodies that undergo dynamic changes in size and morphology coincident with progeny production and reorganization of the nucleus.” This results in a lack of definitive evidence in favor of phase separation, let alone LLPS, as the process underlying VLNB

We thank the reviewer for these thoughtful comments and distinctions. We agree that understanding and communicating how phase-separation (as a manifestation of equilibrium thermodynamics) contributes to viral assembly (which is not an equilibrium process) is crucial to understanding the contribution of viral biomolecular condensates to the assembly process. In part, the answer lies in understanding how changes in 52K localization phenotype correspond to the assembly process. Additional experiments summarized in the overview of our revisions clarify the situation, and results have allowed us to revise our interpretation accordingly. We propose that viral biomolecular condensates are transient, and that the localization of 52K to the nuclear periphery is a post-assembly phenotype. This indicates that the progression of localization phenotypes corresponds with different stages in viral assembly, rather than representing the progressive non-equilibrium evolution of condensates.

2. The content of Figure 1d is difficult to parse. And the figure caption is not helpful. What do the various numbers along the abscissa imply?

The numbers indicate the number of nuclei analyzed. This has now been removed as the individual data points are already plotted (**Figure 1d**).

2. What is the basis for asserting that viral replication compartments are distinct from viral late nuclear bodies? This is unclear from Figure 1e. Of import and interest is the possibility that the lack of co-localization between "VRCs" and "VLNBs" is a manifestation of dynamical arrest that is being couched in parlance (albeit implicitly) as an equilibrium phenomenon? If these are indeed distinct bodies with non-overlapping locales and distinct components, the expectation would be that these bodies can form independently of one another.

This is an interesting idea, but we are convinced that the viral condensates that organize capsid proteins are not generated from viral replication compartments either by dynamic arrest or any other phenomenon. Although not clear from Figure 1e alone, these compartments differ in their composition. This is evidenced throughout the revised manuscript, and is consistent with extensive investigation of factors localized to viral replication compartments reported in the literature:

1. Both DBP and E4orf6 localize to viral replication compartments but not to punctate viral condensates. This is shown in (**Figure 1g; Figure 4c & 4n; Extended Data 1a & 1b; Extended Data 7e**).
2. The 52K protein and capsid proteins localized at punctate viral condensates are not observed at viral replication compartments (**Figure 1f; Figure 4c & 4n; Extended Data 1a & 1b**).
3. The recruitment of cellular proteins to VRCs (e.g., USP7 and others) has been described extensively. For example, PMID: 31764999; PMID: 28972080; PMID: 28972080. To the best of our knowledge, none of these studies identify the same components at nuclear bodies.

We agree that VRCs and viral NBs should be able to form independently of one another. This is what we see, although with the caveat that VRC formation is required for expression of viral late genes (including 52K and capsid proteins):

1. Viral replication compartments form prior to viral nuclear bodies (**Extended data 7e**), this is to be expected, as viral genome replication is a prerequisite for the expression of viral late genes (which include 52K and capsid proteins).

2. Because viral genome replication is required for late gene expression, endogenous viral NBs cannot form independent of VRCs *per se*. However, 52K forms NBs that can recruit IIIa when expressed independent of infection (**Figure 2g & 2h**), demonstrating the ability of 52K to form NBs independent of VRCs.

3. In Figure 1f, there are four components listed in the caption, but only two colors shown in the figure.

Now that this has been pointed out, we agree that the caption is confusing. To clarify, because the available antibodies are raised in either mice or rabbits, we are unable to label with more than two antibodies at a time. For this reason, we assessed co-localization of these 4 components using complementary dual labelling. In each dual-labelling experiment, two different secondary antibodies are used, each presented in a different color (anti-rabbit in green, anti-mouse in magenta). We have updated the figure legend to minimize any future confusion. The legend now reads "Immunofluorescence labeling of the 52 kDa protein (52K) with hexon trimers (hex), 52K with fiber trimers (fib), or cement protein IIIa (IIIa) with hexon trimers".

4. What is the physical basis for grouping phenotypes into distinct classes – as shown in Figure 1h? Were these phenotypes chosen using an automated approach? If yes, please share details and please explain why there are precisely five phenotypes. If the classification of phenotypes was ad hoc, then how reliable and /or robust is the classification? Further, for a given expression level, are there truly five distinct phenotypes if all cells are imaged at the same, later time point > 28 min?

Since the progress of infection in a population of infected cells is highly heterogenous, our imaging will capture cells at multiple different stages of the infection. Because no two cells or the corresponding process of infection are exactly the same, there are an infinite number of subtly distinct phenotypes that can exist. However, by categorizing the phenotype observed in each cell into 5 broad ad-hoc categories we can reliably describe how the localization of 52K changes with the progress of infection. The analysis was performed in a blinded fashion. No cell phenotypes were identified during analysis that did not fit the 5 defined categories.

The percentage of cells corresponding to each category at the different time points suggests a progression of phenotypes whereby biomolecular condensates are transient. We propose that the non-punctate, non-diffuse localization of 52K precedes the formation of nuclear bodies, and that 52K localization culminates at the nuclear periphery. Additional experiments outlined in our summary of revisions (including live cell imaging) confirm this progression and have allowed us to designate peripheral localization as a post-assembly phenotype. We have also now added wide view images of cells at 32 hours post-infection demonstrating that this endpoint is typical of all infected cells (**Extended Data 7f**).

5. Figure 2 shows data for VLNBs formed in doxocycline inducible lung cell lines. These cells are distinct from the HBEC cells used for Figure 1. While one observes rapid recovery of fluorescence after photobleaching in Figure 2, it is not clear if a similar set of observations will result for the HBEC cell lines. This is relevant because the VLNBs are multicomponent bodies. Will all components of these bodies show similar FRAP traces? In all likelihood, the answer is no. If so, is it appropriate to characterize VLNBs as liquids and the process by which they form as LLPS? Again,

the answer is no. Phase separation, yes, but in all likelihood these are, equilibrium, viscoelastic bodies, and in the context of the experiments the functionalities are being investigated of bodies that are out of equilibrium.

Presumably, the reviewer's assertion that the bodies are out of equilibrium is based on the progression from punctate to peripheral localization of 52K and the assumption that a non-equilibrium process such as viral assembly cannot require phase-separation at or near equilibrium, as logic would dictate that phase-separation at or near equilibrium should be inhibitory to assembly. However, this assumes a direct transition of punctate biomolecular condensates into peripheral accumulates and a direct transition of condensates into particles. In both cases we de do not think that this is so. This is discussed in our revised model outlined in the overview of our revisions.

In support of our approach, it is important to point out that the system used to assess FRAP requires the integration and selection of a transgene into cells that enables expression of a fluorescent 52K-GFP fusion protein. The bronchial epithelial cell line used in this study already contains integrated trans genes under the control of puromycin and neomycin selectable markers, meaning that this line is not amenable to selection. This is why we used an immortal transformed lung cell line (A549). We generated this cell line to express very low levels of 52K-GFP. These very low levels of 52K-GFP are not sufficient to form nuclear bodies (**Extended Data 2b & 2c**). This was intentional to avoid analyzing nuclear bodies comprised only of 52K-GFP. The visualization of nuclear bodies requires virus infection and recruitment of 52K-GFP to multicomponent bodies, the formation of which is enabled by the much higher levels of viral proteins generated during infection. This is confirmed by co-localization of 52K-GFP and the viral protein IIIa (**Extended Data 2c**). We also note that this is the same system used to visualize the progression of 52K localization phenotypes in live cells (mentioned in the above comments). Interestingly, mono-component bodies generated by high levels of transgene expression independent of infection (for example by transient transfection) are not mobile enough to come into contact with each other and fuse (data not shown). Presumably, the mobility of multicomponent bodies is enabled by virus-induced reorganization of the host nuclear architecture. In short, the nuclear bodies analyzed are multicomponent viral condensates in the context of virus infection, and they recover fluorescence rapidly and fuse upon contact in a fluid-like manner.

We agree that analyzing FRAP of an additional capsid protein would greatly strengthen the current data set. Regrettably this has not been possible, as the generation of a stable cell line that expresses IIIa-GFP was not viable. This is very typical of viral structural proteins expressed *in trans*, which are often deleterious to cell viability, and exert a selective pressure even when basal expression levels are kept low by control of the doxycycline inducible promoter. In preliminary trials, transient expression of major capsid proteins proved even more toxic to cells than expression of IIIa.

6. Figure 3 makes the case for the N-terminal IDR being necessary and sufficient. Has a persuasive case been made for this statement? Careful scrutiny suggests that this is not the case. There is no doubt that removal of the IDR shifts the apparent saturation concentration upward - by almost two orders of magnitude Figure 3h. This is very interesting, but this does not, on its own establish sufficiency. An easy remedy is to characterize the phase behavior of the IDR on its own.

We have established that the N-terminal IDR of 52K plays a key role in phase-separation. However, as the reviewer rightly points out, we have not established that this region is alone sufficient for

phase-separation. We did not state that the IDR is sufficient, and it was not our intention to imply this. However, on reflection, we can see how the way this was described could confuse sufficiency of 52K for nuclear body formation (which we intended to communicate) with sufficiency of the IDR (not intended). We have carefully revised the manuscript to avoid any miscommunication that the N-terminal IDR is likely to be sufficient for phase-separation, as we do not believe this has been established, and it may well be that additional features of 52K contribute to its phase-separation.

7. The more serious issue pertains to the general pronouncements that lead to a focus on the 47-residue PAQ region. What role is this region playing? Again, Figure 3h suggests that deletion of the PAQ region does not fundamentally compromise the driving forces for phase separation. Instead, the key role of the PAQ region appears to be influencing the dynamics of condensates formed by 52K. And these dynamics seem to be very important for functions as well. The IDR itself is very interesting. The 47-aa residue stretch has no aromatic groups. Pro, Ala, Gln make up 49% of the sequence, whereas Arg, and Gly / Ser / Thr make up ca. 30%. Interestingly, the 60% of the sequence that is C-terminal to the PAQ region, is where this IDR encodes most of the interactions that drive phase separation. There are three large acidic blocks, interspersed by positively charged blocks (Arg-rich with very few Lys residues). The N-terminal PAQ region also has sizable Arg-containing blocks. The mechanism
of phase separation, at least in vitro, likely involves complex coacervation adapted to polyampholytic sequences. Such systems readily form arrested phases because the electrostatic interactions between blocks that are oppositely charged residues can be amplified significantly, especially if those include Arg as the cationic residues. It is quite likely that the PAQ region, the Pro residues in particular, acts as a spacer that provides competition to the electrostatic interactions of the 48-119 region of the IDR while also enabling some degree of dynamical exchange (prosaically known as a fluidizing entity). Replacing some or all the Arg residues with Lys should dramatically alter the picture. Likewise, replacing Gln with Gly should enable a more potent spacer. All this is important, because the IDR sequence seems to enable slow dynamics of maturation whereby these dynamics are synchronized with the production of progeny particles. Presumably, an equilibrium structure obtained via
classical phase separation may actually be disruptive for producing progeny particles. What the deletion of the PAQ region points to is the possibility that there needs to dynamical resonance. A process of phase separation that is too fast (with the wrong kind of IDR) or too slow (the ΔPAQ) will either compromise progeny particle production or lead to genome-less particles as observed by the authors. The key seems to be in realizing a particular region along the dynamical (non-equilibrium) phase diagram as opposed to the equilibrium phase diagram. Such a result would be very exciting and seems to be a lot more in line with the data presented here, as opposed to the narrative presented to go with the data.

We thank the reviewer for these insightful comments and helpful suggestions. We have now included a set of experiments that interrogate the role of the N-terminal IDR in progeny assembly. We have generated four 52K mutants in which amino acid residues in the IDR have been substituted or repositioned (**Extended Data 5d & 5e**). Experiments with these mutants support a role for phase-separation in the coordinated assembly of progeny particles. These revisions are covered in the main text (lines 261-291).

1. Positively charged arginine and lysine residues and negatively charged aspartic acid and glutamic acid residues that fall within the first 145 amino acids of the N-terminus have been repositioned to break up patches of charged amino acids in the IDR. This mutant requires higher concentrations to phase-separate *in vitro*, evidencing the contribution of charged patches to phase-separation of 52K (**Figure 3c; Extended Data 3b**). This mutant fails to form nuclear bodies when ectopically expressed in cells (**Figure 3d & 3e**) and fails to complement progeny production of a 52K-null virus (**Extended Data 9b**). However, we note that this mutant is also mislocalized to the cytoplasm, complicating interpretation of its loss of function (**Figure 3e**).
2. Arginine residues that fall within the first 145 amino acids of the N-terminus have been substituted with Lysine residues. This mutant requires higher concentrations to phase-separate *in vitro*, evidencing the contribution of arginine residues to phase-separation of 52K (**Figure 3c; Extended Data 3b**). This mutant fails to form nuclear bodies when ectopically expressed in cells, instead exhibiting a nuclear diffuse localization (**Figure 3d & 3e**). When a cell induced to express the R/K mutant is infected with a 52K-null virus, R/K remains nuclear diffuse and fails to complement progeny production of a 52K-null virus (**Figure 4m & n**). These data indicate that arginine residues in the IDR of 52K are critical for phase-separation and progeny production.
3. Glutamine within the first 30 amino acids of the N-terminus have been substituted with glycine. This was anticipated to enable a more potent spacer as suggested by the reviewer. These mutations had only limited impact on phase-separation *in vitro* (**Figure 3c; Extended Data 3b**), and there was little to no observable difference on the ability of this mutant to form nuclear bodies when ectopically expressed in cells (**Figure 3d & e**). Correspondingly, the ability of this mutant to rescue progeny production of a 52K-null virus was comparable to WT 52K (**Extended Data 9b**). This suggests that the mutations made were not significant enough to impact the biology of 52K.
4. Proline residues that fall within the putative spacer region of the 52K protein IDR have been substituted with alanine. These mutations had limited impact on phase-separation *in vitro* (**Figure 3c; Extended Data 3b**) and did not prevent formation of nuclear bodies when ectopically expressed in cells (**Figure 3d & e**). When a GFP tagged P/A mutant was ectopically expressed in cells, the bodies formed by the P/A mutant exhibited a slower recovery after photobleaching and lower total recovery compared to GFP-tagged WT 52K or a GFP-tagged Q/G mutant (**Figure 3g-i**). This suggests that removing the proline residues from the putative spacer does impacts dynamic IDR-IDR interactions. When a cell induced to express the P/A mutant is infected with a 52K-null virus, P/A formed nuclear bodies (**Figure 4n**). However, this mutant fails to complement progeny production of a 52K-null virus (**Figure 4m**). This indicates that the putative spacer is required for assembly of viral progeny downstream of the organization of 52K and capsid proteins at biomolecular condensates. We propose that the N-terminal proline-rich spacer region is required either to maintain phase-separated proteins in a state-conducive to downstream assembly or to directly facilitate assembly itself.

In conclusion, this is an important story with a potentially exciting storyline. However, in its current form, there appears to be a forced connection between the data and the equilibrium physics of phase separation. This can be remedied in one of two ways: Allow for an alternate interpretation and carry out a different set of experiments that can get help resolve the two plausible interpretations. Or, significantly downplay - almost eliminate - the connection to phase separation, except in the discussion, and just report the data without insisting on the connection to phase separation.

We thank the reviewer for their suggestions. We have carried out additional sets of experiments that elucidate the contribution of phase-separation to viral assembly. We agree that at some level, phase-separation at or near thermodynamic equilibrium seems contrary to viral assembly, which requires the assembly of components (viral structural proteins and the viral genome) into an ordered state. However, the transition does not have to be direct. We propose that viral capsid proteins are organized into dynamic condensates. As the reviewer rightly alludes to, it should be expected that maintaining dynamic interaction of these proteins acts in opposition to their assembly into ordered particles. We propose that 52K and viral capsid proteins are maintained in this dynamic state until the provision of viral genomes disrupts this status quo, enabling capsid assembly that is coordinated with genome packaging.

The reviewer suggests that progeny production may require IDR-IDR interactions in balance, and that the IDR sequence "seems to enable slow dynamics of maturation whereby these dynamics are synchronized with the production of progeny particles". This may very well be the case, when considered in the context of our proposed model of viral assembly. We find that the substitution of proline in the putative spacer results in (mono-component) nuclear bodies that are less dynamic (in terms of FRAP) than their WT counterparts (**Figure 3g-i**). This is consistent with unregulated IDR-IDR interaction in the absence of an effective spacer that provides competition to the interactions of the 48-119 region of the IDR. This may compromise progeny production by rendering these organized components inconducive to downstream assembly.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The novelty of this paper is the demonstration that phase separation has a role in viral assembly being functionally relevant. The biggest concern of this reviewer was the lack of causation to substantiate this claim. The experiments now performed by the authors with the ts mutant, elegantly show that although the tsmutant is able to phase separate, it is unable to recruit the minor capsid protein IIIa. As this protein is essential for viral packaging, the authors have concluded that phase separation behaves as a organizing centre of viral assembly by bringing IIIa to the liquid condensates.

The question of why the IIIa is not recruited, has not been addressed and may have to do with the binding capacity of 52K to IIIa independently of phase separation and thus IIIa behaves as a client of 52k in condensates. Therefore, this reviewer thinks that the authors have now established the causation needed for the paper to be published in Nature and that this paper contributes greatly to our understanding of the role that pahse separation could play in the viral lifecycles.

The authors have done more. They increased number of in silico experiments to understand the molecular grammar enabling the phase separation of the protein 52k. In doing so, they observe the recruitment of IIIa to cytosolic puncta under some conditions, reinforcing the client idea of IIIa.

The authors have thus revised their manuscript, established causation and functional relevance, transforming this paper into an important contribution to virology and to the field of biological phase separation.

Referee #2 (Remarks to the Author):

The manuscript has been extensively revised. Many new important data have been added regarding the dynamics of BMC formation, the interplay between proteins 52K and IIIa, and the role of different key residues in the function of 52K. The authors now make a much better case on the relation between condensate formation and mature virus progeny production. The work also highlights some open questions for future work, such as why do incomplete (presumably genome lacking) particles contain detectable amounts of core protein VII, or what kind of particles (if any) are produced in the absence of concurrent genome replication.

I have only one minor comment: in extended data 7f, it is difficult to see what the yellow arrows are labeling. The authors may consider adding a brief text to the figure caption indicating by which criteria the location of the virion accumulation compartment is determined, and/or add a zoomed-in panel.

I congratulate the authors on this important piece of work.

Referee #3 (Remarks to the Author):

The authors have revised their original MS to make substantial changes by adding brand new data. The revisions made, specifically the content of the new Figure 3, which were motivated in response to comment 7 raised in my original review, are riveting. One seldom feels gratified as a reviewer, but as it turns out, every prediction made held up to scrutiny. And I would venture to go one step further and add that the likely model is probably that one that was proposed in the review of the MS. The authors are to be congratulated for the impressive amount of work and for putting their findings on a footing that spans so many different length scales. I cannot think of a more clean illustration of the physiological relevance of the sticker-and-spacer formalism. It is also astonishing how well the internal dynamics of condensates, influenced by the spacers, contributes to the production of progeny particles. From a MS that maybe (at least in my biased view) had a nebulous connection between the production of progeny particles and phase separation, we now have a transition to a MS that makes the connection more definitively and decisively. A high bar was set for the authors, and it is humbling to see that they crossed this high bar with room to spare. I believe this MS checks every box one would set up for publication in Nature. I have no further revisions to request. It was a joy and a privilege to watch the trajectory of this work, and learn from the work.